TRANSCRIPTION FACTORS INVOLVED IN COLD RESPONSE IN PLANTS

by

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A thesis submitted in fulfilment of the requirements
of the award for Doctor of Philosophy

The University of Adelaide
Faculty of Sciences
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August 2009

Declaration

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January 2009

Table of Contents

Declaration ..... **ii**
Table of Contents ..... **iii**
List of Figures and Tables ..... **vii**
Acknowledgements ..... **xi**
Abstract ..... **xii**
Abbreviations ..... **xiii**
Chapter 1. Literature Review **1**
1.1. Introduction 2
1.2. Cold and Abiotic Stresses 2
1.2.1. The Importance of Abiotic Stresses 2
1.2.2. The Importance of Cold Stress – Definition and Management 3
1.3. Effects of Cold and Abiotic Stresses on Plant Health 3
1.3.1. Effects of Abiotic Stresses 3
1.3.2. Effects of Cold Stress 4
1.4. Plant Response Mechanisms 4
1.5. Cold-induced Signalling Pathways 6
1.5.1. Signalling Crosstalk and Specificity 6
1.5.2. Cold Stress Signalling 6
1.5.2.1. ICE Family in Cold Stress Signalling 9
1.5.2.1.1. Structural Properties 10
1.5.2.1.2. Functions 13
1.5.2.1.3. ICE Family in Cereals 18
1.5.2.2. CBF Family in Cold Stress Signalling 20
1.5.2.2.1. CBF Structural Properties 20
1.5.2.2.2. Functions 22
1.5.2.2.3. Barley Genes Similar to the *AtCBF* Group 26
1.5.2.2.4. Project Aims and Scope 33
Chapter 2. Characterisation of *HvICE2* **36**
2.1. Introduction 37
2.2. Materials and Methods 38
2.2.1. Materials 38
2.2.2. Cloning of *HvICE*2 40
2.2.2.1. Bioinformatics 40
2.2.2.2. 5’ RACE of *HvICE*2 40
2.2.2.3. Cloning of PCR Fragments into pGEM-T Easy 42
2.2.2.4. Chemical Transformation of *E. coli* 42
2.2.2.5. Plasmid DNA Mini-preparations 43
2.2.2.6. Nucleotide Sequence Analysis and Manipulation 44
2.2.2.7. PCR Cloning of the Coding Region of *HvICE2* 44
2.2.2.8. TOPO® Cloning of *HvICE2* into pCR8 45
2.2.2.9. PCR Cloning of the Genomic Sequence of *HvICE2* 46
2.2.2.10. Cloning of the *HvICE2* Promoter by PCR-based Genomic Walking 47
2.2.2.11. PCR Amplification and Cloning of the *HvICE2* Promoter 48
2.2.3. Analysis of *HvICE2* 49
2.2.3.1. *HvICE2* Promoter Nucleotide Sequence Analysis 49
2.2.3.2. Production of Barley Cold Stress Treatment Series 49
2.2.3.2.1. Cultivation, Stress Treatment and Sampling of *Hordeum vulgare* L. cv. Golden Promise and Haruna Nijo 49
2.2.3.2.2. RNA Extraction 50

2.2.3.3. Production of Barley Salt Stress Treatment Series 51
2.2.3.4. Production of Barley ABA Treatment Series 53
2.2.3.5. Production of cDNA from the Barley Stress Series for qRT-PCR 54
2.2.3.6. Expression Analysis of *HvICE2 via* qRT-PCR 55
2.2.3.7. Expression Analysis of *HvICE2 via* Microarray Data Analysis 55
2.2.3.8. Expression Analysis of *HvICE2 via* Analysis of *HvICE2 Promoter:Reporter Gene* Transgenic Plants 56
2.2.3.8.1. Production of *GUS* and *GFP* Expression Clones Driven by the *HvICE2* Promoter 56
2.2.3.8.2. Barley Transformation and Growth of Transgenic Plants 57
2.2.3.8.3. Isolation of Genomic DNA from Transgenic Barley Plants 57
2.2.3.8.4. PCR Analysis of *HvICE2 Promoter*:*Reporter* Transgenic Barley Plants 58
2.2.3.8.5. Analysis of Reporter Gene Expression in Transgenic Barley Plants 58
2.2.4. Production and Analysis of Transgenic Barley Plants Over-expressing *HvICE2* 59
2.2.4.1. Production of an *HvICE2* Expression Clone using Gateway Technology 59
2.2.4.2. Barley Transformation and Growth of Transgenic Plants 60
2.2.4.3. Analysis of Transgene Expression by qRT-PCR in Barley Plants Over-expressing *HvICE2* 60
2.2.4.4. Southern Blot Analysis of Transgenic Barley Plants Over-expressing *HvICE2* 61
2.2.4.5. Analysis of Developmental Phenotypes of T_1 Barley Plants Over-expressing *HvICE2*. 64
2.2.4.6. Northern Blot Analysis of T_1 Barley Plants Over-expressing *HvICE2* 64
2.2.4.7. Freezing Treatment of Barley Over-expressing *HvICE2* 66
2.2.4.8. Analysis of Putative Downstream Gene Expression by qRT-PCR 69
2.3. Results 70
2.3.1. Identification, Isolation and Sequence Analysis of *HvICE2* 70
2.3.2. Analysis of *HvICE2* Expression 76
2.3.2.1. qRT-PCR Analysis of *HvICE2* Expression during Cold Stress 76
2.3.2.2. qRT-PCR Analysis of *HvICE2* Expression during Salinity Stress 79
2.3.2.3. qRT-PCR Analysis of *HvICE2* Expression during ABA Treatment 82
2.3.2.4. Analysis of *HvICE2* Expression using Microarray Tissue Series 82
2.3.2.5. Analysis of *HvICE2* Expression using *Promoter:Reporter Gene* Transgenic Plants 84
2.3.1. Analysis of Barley Plants Over-expressing *HvICE2* 89
2.3.1.1. Analysis of *HvICE2* Transgene mRNA Levels in Transgenic Barley Plants 89
2.3.1.2. Southern Analysis of Barley Plants Over-expressing *HvICE2* 90
2.3.1.3. Analysis of Developmental Phenotypes of Barley Plants Over-expressing *HvICE2* 91
2.3.1.4. Analysis of Seedling-stage Cold Stress Tolerance of Barley Plants Over-expressing *HvICE2* 96
2.3.1.5. Analysis of Cold Stress Response in Barley Plants Over-expressing *HvICE2* 96
2.4. Discussion 100
2.4.1. Isolation and Gene Analysis of *HvICE2* 100

2.4.2. Analysis of *HvICE2* Expression by qRT-PCR and Microarray. 102
2.4.3. Analysis of *HvICE2 Promoter*:*Reporter* Plants 104
2.4.4. Analysis of Barley Plants Over-expressing *HvICE2* 105
2.5. Conclusions 112
Chapter 3. Analysis of Arabidopsis *AtICE2* Transgenic Plants 114
3.1. Introduction 115
3.2. Materials and Methods 116
3.2.1. Materials 116
3.2.2. Production of *AtICE2* Over-expression and RNAi Constructs 116
3.2.3. Transformation of *A. tumefaciens* by Electroporation 117
3.2.4. *Agrobacterium tumefaciens*-mediated Transformation of Arabidopsis *via* Floral Dip Method 119
3.2.5. Growth and Selection of Transgenic Plants 119
3.2.6. Freezing Stress Treatment of Transgenic Plants with Over-expression or RNAi Knockdown of *AtICE2* 121
3.2.7. Expression Analysis of Putative Downstream Genes in Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2* 122
3.3. Results 124
3.3.1. Sequence Analysis of *AtICE2* 124
3.3.1. Production of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2* 124
3.3.2. Analysis of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2* 126
3.3.3. Development of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2* 129
3.3.4. Freezing Tolerance of *AtICE2* Over-expression or RNAi Knockdown Plants 131
3.3.5. Expression Analysis of Putative Downstream Genes in Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2* 131
3.4. Discussion 137
3.5. Conclusions 142
Chapter 4. Characterisation of Two Barley *CBFs* 143
4.1. Introduction 144
4.2. Materials and Methods 145
4.2.1. Materials 145
4.2.2. Growth of Plants, Cold Treatment and RNA Preparation 145
4.2.3. Production of Y1H Libraries 146
4.2.3.1. First- and Second-strand cDNA Synthesis 146
4.2.3.2. cDNA Purification 147
4.2.3.3. Preparation of Competent Yeast Cells 147
4.2.3.4. Yeast Transformation 148
4.2.4. Screening of cDNA Libraries using the Y1H System 150
4.2.5. Nucleotide Sequence Analysis and Manipulation 152
4.2.6. DNA Binding Analysis of Proteins 153
4.2.7. Production of Barley Drought Stress Treatment Series for qRT-PCR 154
4.2.8. Expression Analysis of *HvCBF16* and *HvCBF23* by qRT-PCR 156
4.2.9. Expression Analysis of *HvCBF23 via* Microarray Data Analysis 156
4.2.10. Genomic Mapping of *HvCBF16* and *HvCBF23* 156
4.3. Results 158
4.3.1. Production, Quality Analysis and Screening of cDNA Libraries 158
4.3.1. Characterisation of Barley *CBF* Genes from Y1H Clones 160
4.3.1.1. Sequence Analysis of Barley *CBF* Genes 160

4.3.1.2. DNA Binding Analysis of *HvCBF16* and *HvCBF23* 166
4.3.1.3. Expression Analysis of *HvCBF16 via* qRT-PCR 168
4.3.1.4. Expression Analysis of *HvCBF23 via* qRT-PCR 172
4.3.1.5. Expression Analysis of *HvCBF23* from Microarray Data Analysis 174
4.3.1.6. Mapping of *HvCBF16* 174
4.3.1.7. Mapping of *HvCBF23* 175
4.4. Discussion 177
4.5. Conclusions 185
Chapter 5. Characterisation of Barley Plants Constitutively Expressing *HvCBF2A* 187
5.1. Introduction 188
5.2. Materials and Methods 190
5.2.1. Materials 190
5.2.2. Bioinformatics 190
5.2.3. Production of Cold Stress Treatment Series using Barley Plants Over-expressing *HvCBF2A* 190
5.2.4. Production of cDNA for qRT-PCR 191
5.2.5. Northern Blot Analysis of Barley Plants Over-expressing *HvCBF2A* 192
5.2.6. Southern Blot Analysis of Barley Plants Over-expressing *HvCBF2A* 194
5.2.7. Analysis of Putative Downstream Gene Expression by qRT-PCR 195
5.2.8. Freezing Treatment of Barley Plants Over-expressing *HvCBF2A* 196
5.2.9. Plant Growth Conditions 196
5.3. Results 197
5.3.1. Sequence Analysis of *HvCBF2A* 197
5.3.2. Analysis of Transgene Copy Number and Segregation in Barley Plants Over-expressing *HvCBF2A* 198
5.3.3. Analysis of Developmental Phenotypes of Barley Plants Over-expressing *HvCBF2A* 199
5.3.4. Analysis of Seedling-stage Cold Stress Tolerance of Barley Plants Over-expressing *HvCBF2A* 204
5.3.5. Analysis of Cold Stress Response in Barley Plants Over-expressing *HvCBF2A* 205
5.4. Discussion 211
5.4.1. *HvCBF2A* Gene Analysis 211
5.4.2. Analysis of Barley Plants Over-expressing *HvCBF2A* 212
5.4.2.1. Analysis of Developmental Phenotypes 213
5.4.2.2. Freezing Tolerance 214
5.4.2.3. Expression Analysis of Putative Downstream Genes 215
5.4.2.4. General Discussion 220
5.5. Conclusions 223
Chapter 6. Concluding Remarks 225
Appendices 227
Appendix A.Oligonucleotides used in PCR 227
Appendix B.Contig Numbers and Alignment Scores and E Values from BLAST Analyses 229
Appendix C.Cold Stress Treatment Series Temperature Regime 231
Appendix D.Media Components 232
Appendix E.Sequence Alignments 233
Appendix F.DNA Sequences and Accession Numbers 238
References 241

List of Figures and Tables

Table 2.1 Primers for PCR amplification of *HvICE2* 5' region, full-length CDS and promoter. 41
Table 2.2 Primers used for qRT-PCR analysis of *HvICE2* expression in barley. 55
Table 2.3 Primers used for qRT-PCR analysis of transgenic plants over-expressing *HvICE2* 69
Table 3.1 Primers for PCR analysis of *AtICE2* constructs and transgenic plants. 118
Table 3.2 Primers used for qRT-PCR analysis of transgenic plants with over- or under-expression of *AtICE2* 123
Table 4.1 Bait and Prey constructs used in *HvCBF16* and *HvCBF23* DNA binding analysis. 154
Table 4.2 Genomic locations of *HvCBF16* and *HvCBF23* on barley chromosomes. 175
Table 5.1 Freezing treatment survival of wild type and transgenic barley plants over-expressing *HvCBF2A* 204

Figure 1.1 The stages of plant responses to abiotic stress. 5
Figure 1.2 Signalling pathways involved in cold responses in Arabidopsis. 8
Figure 1.3 Structural features of ICE family amino acid sequences. 11
Figure 1.4 Multiple sequence alignment of AtCBF1, AtCBF2 and AtCBF3 proteins. 21
Figure 1.5 Phylogenetic relationships of barley CBFs. 27
Figure 1.6 General domain structure of barley and monocot CBFs. 28
Figure 2.1 pGEM®-T Easy Vector map and sequence reference points. 43
Figure 2.2 Vector map of pCR8. 46
Figure 2.3 Schematic diagram of plant hydroponic layout. 52
Figure 2.4 Picture indicating sites that tissue samples were taken from. 53
Figure 2.5 Planting layout used to study freezing-tolerance of transgenic barley plants over-expressing *HvICE2* 67
Figure 2.6 Schematic diagram of plant freezing treatment layout. 68
Figure 2.7 Alignment of the translated nucleotide sequences of the coding regions of *AtICE1*, *AtICE2*, *HvICE1* and *HvICE2* 71
Figure 2.8 Photograph of agarose gel analyses of PCR products of the coding region and promoter of *HvICE2* 73
Figure 2.9 Schematic diagram of the genomic structure of *HvICE2*, *AtICE1* and *OsICE*. 74
Figure 2.10 Schematic diagram of the overlapping regions of *HvICE2* genomic walking fragments. 74
Figure 2.11 Schematic diagram illustrating the position of putative *cis*-elements within the promoter of *HvICE2* 75
Figure 2.12 Photographs of cold treated and untreated barley plants. 77
Figure 2.13 Graph of *HvICE2* expression during cold stress determined by qRT-PCR. 78
Figure 2.14 Photographs of salinity treated and untreated Golden Promise and Clipper x Sahara double haploid line 134 plants. 80
Figure 2.15 Graph of *HvICE2* expression during salinity stress determined by qRT-PCR. 81
Figure 2.16 Graph of *HvICE2* expression during ABA treatment determined by qRT-PCR. 83

Figure 2.17 Graph of *HvICE2* expression from Affymetrix microarray data. 84
Figure 2.18 pMDC107 and pMDC164 vectors containing the promoter of *HvICE2*.....85
Figure 2.19 Photograph of agarose gel analyses of restriction fragments of pMDC32/*maize Ubiquitin promoter:HvICE2 CDS*, pMDC164/ *HvICE2 promoter* and pMDC107/*HvICE2 promoter*.....86
Figure 2.20 Photographs of GUS staining on the seeds of cold-treated and untreated transgenic (*promoter:GUS reporter*) or wild type plants.....87
Figure 2.21 Photographs of representative cold treated and untreated transgenic (*HvICE2 promoter*:*GFP reporter*) and wild type plants.....88
Figure 2.22 Vector map of pMDC32 vector containing *HvICE2* coding sequence. 89
Figure 2.23 Graph of the *HvICE2* transgene expression in T_0 barley plants determined by qRT-PCR.....90
Figure 2.24 Image of autoradiograph of Southern blot of T_0 barley plants over-expressing *HvICE2*.....91
Figure 2.25 Graphic representation of the height, biomass and 1000 grain weight of barley plants over-expressing *HvICE2*. 92
Figure 2.26 Photographs of mature untreated wild type and transgenic barley plants over-expressing *HvICE2*.....93
Figure 2.27 Image of autoradiograph of northern blot of *HvICE2* expression in untreated wild type and transgenic barley plants over-expressing *HvICE2*.....94
Figure 2.28 Photographs of heads of wild type and transgenic barley plants over-expressing *HvICE2*.....95
Figure 2.29 Graphs of transgene and *CBF* expression during cold treatment in barley plants over-expressing *HvICE2* determined by qRT-PCR.....97
Figure 2.30 Graphs of *COR* gene expression during cold treatment in barley plants over-expressing *HvICE2* determined by qRT-PCR.....99
Figure 3.1 Vector maps of pTOOL2 and pJawohl8 transformation vectors containing *AtICE2* coding sequence. 125
Figure 3.2 Photograph of agarose gel analyses of restriction fragments of pTOOL2/*AtICE2* and pJawohl8/*AtICE2 RNAi fragment*.....126
Figure 3.3 Photograph of agarose gel analyses of PCR products amplified from genomic DNA from T_1 transgenic Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2*.....127
Figure 3.4 Photograph of agarose gel analyses of RT-PCR products amplified during analysis of Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2*.....128
Figure 3.5 Photographs of wild type and transgenic Arabidopsis plants with over-expression or RNAi knock down of *AtICE2* at flowering.....130
Figure 3.6 Photographs of treated wild type and transgenic Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* with a schematic diagram of the planting layout. 132
Figure 3.7 Graph of *AtICE2* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.....133
Figure 3.8 Graphs of *AtICE1* and *AtCBF3* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.....135
Figure 3.9 Graphs of *AtCOR47* and *AtCOR78* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.....136

Figure 4.1 Schematic diagram of the layout of the *cis*-elements present in the bait constructs used for yeast 1-hybrid screening. 151
Figure 4.2 Plant water status and sampling regime during the drought-stress treatment series..... 155
Figure 4.3 Schematic diagram illustrating the principle of Y1H screening. 158
Figure 4.4 Photograph of agarose gel analyses of PCR products amplified from clones from the Y1H cDNA library. 159
Figure 4.5 Photograph of agarose gel analyses of restriction mapping of PCR products amplified from clones isolated using a Y1H screen. 161
Figure 4.6 Graphs of the location and scores of predicted phosphorylation sites in HvCBF16 and HvCBF23. 162
Figure 4.7 Phylogenetic analysis of HvCBF16 and HvCBF23. 165
Figure 4.8 Alignment of the AP2-domain and conserved CBF signature motifs, from peptide sequences of selected CBF and AP2-domain containing proteins. 166
Figure 4.9 Photographs of yeast strains on selective agar plates during DNA binding analysis of *HvCBF16* and *HvCBF23*. 167
Figure 4.10 Graph of *HvCBF16* expression during cold stress treatment determined by qRT-PCR. 169
Figure 4.11 Graph of *HvCBF16* expression during salinity stress treatment determined by qRT-PCR. 170
Figure 4.12 Graph of *HvCBF16* expression during ABA treatment determined by qRT-PCR. 171
Figure 4.13 Graph of *HvCBF16* expression during cyclic drought treatments determined by qRT-PCR. 171
Figure 4.14 Graph of *HvCBF16* expression in various tissues determined by qRT-PCR. 172
Figure 4.15 Graph of *HvCBF23* expression during cold stress treatment determined qRT-PCR. 173
Figure 4.16 Graph of *HvCBF23* expression from Affymetrix microarray data. 174
Figure 4.17 Map location of *HvCBF16* and *HvCBF23* on *Hordeum vulgare* chromosome 5H. 176
Figure 4.18 Schematic diagram of a genetic map of the barley *CBF* gene cluster. ... 180
Figure 5.1 Graph of the location and scores of predicted phosphorylation sites in HvCBF2A. 197
Figure 5.2 Image of autoradiograph of Southern blot of T_1 and T_2 barley plants over-expressing *HvCBF2A*..... 199
Figure 5.3 Photographs and images of autoradiographs of northern blot of *HvCBF2* expression in untreated wild type and transgenic barley plants over-expressing *HvCBF2A*. 201
Figure 5.4 Graphic representation of the height, biomass and 1000 grain weight of barley plants over-expressing *HvCBF2A*. 202
Figure 5.5 Photographs of developmental phenotypes of heads of wild type and transgenic barley plants over-expressing *HvCBF2A*..... 203
Figure 5.6 Image of autoradiograph of northern blot of *HvCBF2*, three *COR* genes and *HvVRN-1* expression during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A*. 207
Figure 5.7 Graph of endogenous and transgene expression of *HvCBF2* during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR. 208
Figure 5.8 Graphs of *HvCor14b* or *HvDHN5* expression during cold treatment

in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR. 209
Figure 5.9 Graphs of *HvDHN8* or *HvVRN-1* expression during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR. 210
Figure 5.10 Schematic diagram of a hypothetical model of flowering initiation involving interactions between the *VRN* and *CBF* genes 219

Acknowledgements

For their invaluable support throughout this project, I am most grateful to the following people:

I am greatly indebted to my fantastic supervisors Dr. Andrew Jacobs, Dr. Sergiy Lopato and Professor Peter Langridge. I have heard that supervisors can make or break a PhD experience and I believe it, because I am certain that it is in no small part due to these three people that I have found it an exciting, stimulating and rewarding experience. I cannot thank you enough. Specifically, thank you to Andrew, for your unwavering patience, support, time, wisdom and guidance. Many times you have gone far beyond the call of duty and it is deeply appreciated. To Sergiy, thank you for your enthusiasm in the topic, your help and guidance and for always finding time. Thank you to Peter, for your help and direction for writing and particularly for encouraging me to pursue, helping me to organise, and approving my trip to Oregon during which I learnt so much; an invaluable gift.

I would also like to thank the members of this research group who have contributed to the success of this project by generously sharing their time, expertise and resources. Namely, these include Dr. Neil Shirley, for his time and expertise in the quantitative PCR section of this project, Dr. Ulrik John, for sharing his expertise in freezing stress and for performing the Arabidopsis freezing tolerance assays, and Dr. Rachel Burton, for the contribution of her cDNA populations and for reading draft manuscripts of this thesis. Thanks also to Ms. Margaret Pallotta, for her work in mapping the barley CBF genes and Ms. Alison Hayes for the contribution of their genomic walking libraries. In addition, thanks to Ms Natasha Bazanova, Dr. Juan Juttner, Dr. Ute Baumann, Ms. Jodie Kretschmer and Dr. Ursula Langridge, for their help advice and technical advice over the years. I would like to thank the members of the Langridge laboratory and ACPFG, for their support and technical advice throughout this project.

Thank you to Dr. Eric Stockinger, for sending me clones and for his enjoyable discussions about the pressing questions in the world of CBFs, and the world beyond.

Thank you to the group at Oregon State University, including Dr. Jeffrey Skinner, Mr Zoran Jeknic, Professor Tony Chen and Professor Patrick Hayes, for making the transgenic plants and inviting me into their research project. Thank you for your scientific advice, kindness, and for making me feel so welcome during my stay.

Thanks to Dr. Rebecca Cook-Johnson, for her critical reading of manuscripts.

I would like to thank Sarah Morran and Alexandra Smart, both for their help and their friendships; the laughs, the camaraderie and, when necessary, the commiseration - I couldn't have made it without you guys! Also to Fleur, Jenna, Wayne, Scott and all the other people at the Centre too numerous to mention, for their friendships and support.

Thank you to Michael Dow, for helping me in the critical moments and patiently getting me through.

Finally, to my family, Anne and Stuart, Elizabeth and Caroline, thank you for everything that you have done. For listening (or appearing to do so) even when you thought I was speaking gibberish, for caring in the rough times and celebrating in the good ones.

Abstract

Studies in Arabidopsis have shown cold stress tolerance can be enhanced by manipulation of the *CBF/DREB* and *ICE* transcription factor genes. To date, few studies have investigated *CBF* and *ICE* genes in crops species such as barley. Using a C-repeat element as bait, two *CBF* genes were isolated from a cold-stressed barley cDNA library, *HvCBF16* and *HvCBF23*. *HvCBF16* was induced by cold treatment but not by other abiotic stresses. *HvCBF23* was constitutively expressed and was not induced by cold treatment. The analysis of transgenic plants expressing these genes will determine their importance in cold tolerance.

Transgenic barley plants expressing the barley gene *HvCBF2A* were found to be more cold tolerant in controlled temperature trials, and hence were assayed to determine the basis of their acquired phenotype. Northern and qRT-PCR analysis showed that four genes known to be involved in cold tolerance were significantly upregulated. Importantly the increased expression was proportional to the level of transgene expression and levels were higher following cold treatment.

A homolog of the Arabidopsis *ICE* transcription factor was isolated from a freezing-tolerant barley variety (*Hordeum vulgare* L. cv. Haruna Nijo) and transcript analysis of *HvICE2* under various abiotic stresses showed that expression of *HvICE2* was induced at low temperatures, particularly in floral tissues. *HvICE2* was over-expressed using the maize *ubiquitin* constitutive promoter in transgenic barley. Expression analysis of putative downstream genes, including various *COR* genes, in the transgenic plants before and during cold treatment did not reveal any alteration in expression. This suggests *HvICE2* that the *COR* genes studied are not targets of *HvICE2* or that additional factors or conditions are required for effective function of HvICE2. Transgenic Arabidopsis plants were produced with over- or reduced-expression of the uncharacterised *ICE* gene, *AtICE2*. The cold tolerance of the *AtICE2* transgenic lines was not significantly different from wild type plants.

Abbreviations

AP2	Apetala 2
At	*Arabidopsis thaliana*
bp	Base pair
BSA	Bovine serum albumin
CBF	C-repeat binding factor
cDNA	Complementary DNA
COR	Cold-responsive
CRT	C-repeat
cv	Cultivar
DAP	Days after pollination
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRE	Dehydration response element
DREB	Dehydration response element binding (protein)
ERF	Ethylene responsive factor
EST	Expressed sequence tag
HPLC	High performance liquid chromatography
hrs	Hours
Hv	*Hordeum vulgare*
ICE	Inducer of CBF expression
Kb	Kilo base
L	Litre
LB	Luria-Bertani
M	Molar
min	Minutes
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
n	Nano
PCR	Polymerase Chain Reaction
QTL	Quantitative trait locus
RACE	Randomly amplified cDNA ends
RNA	Ribose Nucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
sec	Seconds
SD	Synthetic dropout
Ta	*Triticum aestivum*
Taq	*Thermus aquaticus*
Tm	*Triticum monococcum*
U	Enzymatic units
UV	Ultra violet
v/v	Volume per volume
V	Volts
w/v	Weight per volume
Y1H	Yeast 1-hybrid
YPD	Yeast potato dextrose

CHAPTER 1

Literature Review

1.1. Introduction

Abiotic stresses including low temperatures are a large problem throughout the world, significantly reducing crop yields. While classical breeding for resistance to these stresses has produced improved crop species, the limited genetic variability available in breeding populations and the time taken from first cross to the release of a new variety are major limitations of traditional breeding approaches. Consequently, there is substantial interest in using technologies such as molecular biology to improve stress tolerance. Recent experiments in Arabidopsis have found that cold stress tolerance can be enhanced by manipulation of the signalling pathways triggered by low temperature stress (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Qin *et al.*, 2004). Such pathways include the CBF (C-repeat binding factor) also known as DREB (dehydration responsive element binding)/ICE (inducer of CBF expression) signalling pathways from Arabidopsis and other plant species (Dubouzet *et al.*, 2003; Jaglo *et al.*, 2001; Qin *et al.*, 2004). This review will focus upon the importance of the CBF and ICE transcription factors and their functions in plants exposed to abiotic stresses, in particular, cold stress.

1.2. Cold and Abiotic Stresses

1.2.1. The Importance of Abiotic Stresses

Abiotic stress is the primary cause of crop losses worldwide, reducing average yields for most major crops by more than 50% (Boyer, 1982; Bray *et al.*, 2000). Abiotic stresses cause substantial losses to crops of barley, the second most important agricultural crop in Australia, with 4.5 million hectares planted for the year starting 2005, producing 9.6 million tonnes of grain, (Australian Bureau of Statistics, 2008). These stresses are environmental conditions such as extreme temperatures, low water availability (drought), high salt levels, mineral deficiencies/toxicities, wounding and UV irradiation all of which adversely affect plant health. In many cases multiple stresses challenge plants simultaneously. The development of plant varieties with enhanced ability to withstand abiotic stresses would have benefits for crop productivity worldwide (Australian Wheat Board, 2005).

1.2.2. The Importance of Cold Stress – Definition and Management

Freezing temperatures periodically account for significant losses in plant productivity and crop yield and are major factors limiting the geographical locations suitable for growing crops (Boyer, 1982; Thomashow, 1999). Two types of plant injury are associated with low temperatures; chilling and freezing, which occur when the temperature of the air at leaf height are respectively above and below the freezing point of water. In Australia and other regions of similar climate, the most significant effects of low temperatures on grain yield are caused by the effects of freezing stress (a 'frost' event) on floral tissues at anthesis (GRDC, 2005). Currently, the primary method of management involves delaying planting of grain crops in an attempt to avoid frost although this also reduces the yield due to poorer heading in warmer weather (GRDC, 2005).

Success in breeding for freezing tolerance has been limited, with the tolerance of wheat varieties today only marginally greater than it was in the early part of the last century (Thomashow, 1999). The continued adverse impact of frost on agriculture suggests that additional approaches, such as molecular biology techniques aimed at enhancing freezing tolerance of plants at the critical flowering stage, would be valuable. A GRDC media release (2005) states that an increase in frost tolerance of only 2°C would have a major effect on cereal crop productivity.

When tackling the problem of cold stress damage to crops, management and breeding for tolerance are best applied together for maximum success as the genotype of the variety establishes the crop potential while effective management allows the grower to optimise this potential.

1.3. Effects of Cold and Abiotic Stresses on Plant Health

1.3.1. Effects of Abiotic Stresses

Abiotic stresses cause a range of physiological effects in plants, many of which are common to multiple stresses. There is particularly large overlap between cold,

dehydration and salinity stresses. Common effects on plant health under these stresses include the production of secondary stresses such as osmotic or oxidative stresses, disruption of osmotic and ionic homeostasis and damage to proteins and membranes. In addition to these general effects, different types of stress affect plants in specific ways. Examples of some of the major effects of cold stress on plant health are described below.

1.3.2. Effects of Cold Stress

A number of studies indicate the primary cause of damage by freezing temperatures is membrane injury (Steponkus, 1984; Thomashow, 1999). The main source of this damage is the severe dehydration associated with removal of free water to form ice crystals, but damage may also be caused by the production of reactive oxygen species. Low temperatures may also cause protein denaturation and limit water uptake by the roots which further dehydrates the plant (Mittler, 2002; Thomashow, 1999; Viswanathan and Zhu, 2002).

1.4. Plant Response Mechanisms

Abiotic stresses trigger a multitude of physiological responses at the molecular level. The responses are complicated and highly regulated, resulting in activation of signalling pathways and genes encoding proteins that act directly in stress tolerance. The molecular responses can be divided into three steps (Figure 1.1):

1. Signal perception and transduction

This involves activation of osmosensors, phospholipid-cleaving enzymes, second messengers such as calcium ions and reactive oxygen species, kinases such as mitogen-activated protein (MAP) kinases and calcium-dependent protein kinases (CDPKs).

2. Transcriptional control

During this stage, many families of transcription factors are activated and act to induce the expression of target stress response genes to protect the plant. These include the *CBF*/*DREB* family, the *bZIP* family and the *MYC* and *MYB* families.

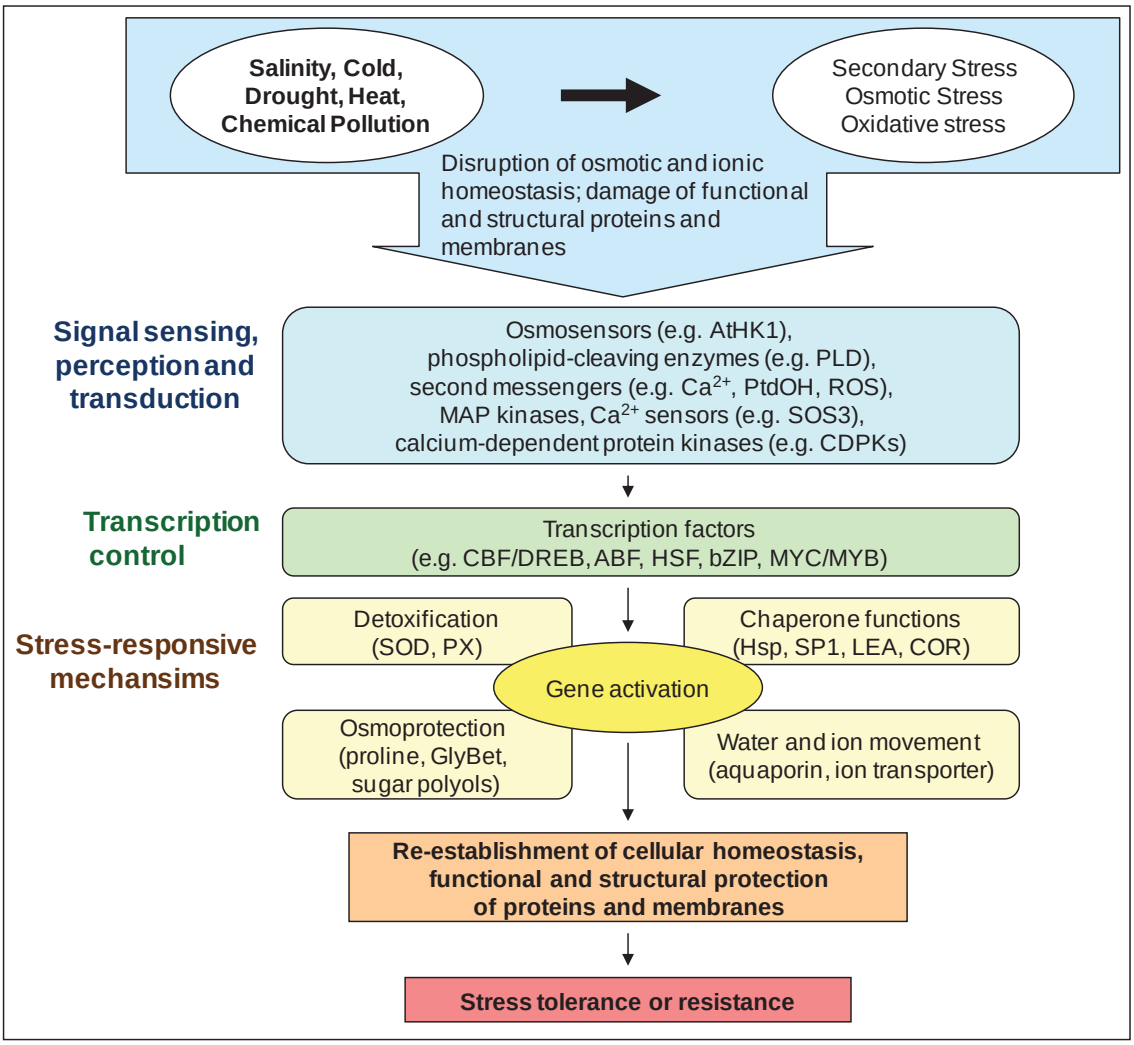


Figure 1.1 The stages of plant responses to abiotic stress.

Primary stresses, such as cold, high salinity, drought, heat and chemical pollution are often interconnected and cause cellular damage and secondary stresses such as osmotic and oxidative stress. The initial stress signals trigger downstream signalling processes and transcriptional controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Taken from Wang *et al*. (2003).

3. Stress response mechanisms

During the final stage, the proteins produced by the transcriptionally induced genes perform functions to enhance the resistance of the plant to the stress. This can be by acting as secondary messengers or in control, protection and restoration of cellular processes. The functions of activated proteins include detoxification by superoxide dismutases (SOD) and peroxidases (PX), osmoprotection *via* the production of proline,

glycine betaine and sugar polyols, maintenance and restoration of protein structure and function by heat shock proteins, late embryo abundance (LEA) and cold responsive (COR) group proteins and regulation of water and ion movement by aquaporins and ion transporter proteins.

1.5. Cold-induced Signalling Pathways

1.5.1. Signalling Crosstalk and Specificity

As discussed above, there are similarities between the effects of various abiotic stresses at the physiological level. There is also significant cross-talk between the signalling pathways activated by individual stresses. This is particularly evident in the activation of osmotic stress-response pathways as part of the cold, salinity and dehydration stress responses. The CRT/DRE signalling cascades play an important role in these pathways. The AtCBF (or DREB1) group of proteins and the pathways identified in Arabidopsis play a major role in cold responses although evidence suggests other signalling pathways are also involved (Chinnusamy *et al.*, 2007; Fowler and Thomashow, 2002). The closely-related AtDREB2 group proteins participate in plant responses to other abiotic stresses such as high salinity or dehydration, as do the MYB/MYC and bZIP families (Haake *et al.*, 2002).

1.5.2. Cold Stress Signalling

Many proteins which participate in cold signalling have been identified, however there are still significant gaps in the literature relating to the roles of each component in the responses. This section will describe some of the advances in knowledge for each stage of the cold stress responses. The first stage of cold signalling involves sensing of cold stress *via* sensors. The plasma membrane has been proposed to be a primary sensor of low temperature although nucleic acid and protein denaturation and/or metabolite concentration also play a role (Chinnusamy *et al.*, 2007; Viswanathan and Zhu, 2002). Low temperatures cause membranes to rigidify, resulting in cytoskeletal rearrangement and induction of stretch-sensitive Ca^{2+} channels which causes an influx of Ca^{2+} (Orvar *et al.*, 2000; Sangwan *et al.*, 2001; Thomashow, 1999). Specific fluctuations in cytoplasmic calcium levels (known as calcium signatures) are sensed by calcium sensor

groups of proteins such as calmodulin and CDPKs (calcium-dependent protein kinases) (Zielinski, 1998). Other secondary messengers involved in cold-responsive signal transduction are reactive oxygen species (ROS), and inositol trisphosphate (IP_3), which is negatively regulated by the phosphatase FRY1 (FIERY1) (Chinnusamy *et al.*, 2007; Viswanathan and Zhu, 2002; Xiong *et al.*, 2001).

Protein kinases and phosphatases are involved in cold signal transduction, including the recently identified Arabidopsis small Ca^{2+}-binding CBL proteins which act through protein kinases in cold response signalling. Various MAPK cascades including an ABP1 cascade involving AtMPK3 are also affected by low temperatures (Viswanathan and Zhu, 2002) although the identification of downstream components of this cascade will require further study.

Transcription of the APETALA2 (AP2) domain-containing *CBF/DREB1* transcription factor genes [specifically *AtCBF1* (*DREB1B*), *AtCBF2* (*DREB1C*) and *AtCBF3* (*DREB1A*)] are induced rapidly by exposure to cold stress. This suggests that the signalling component which triggers their expression, designated 'ICE' (inducer of CBF expression) is present at normal temperatures and is activated by cold treatment (Gilmour *et al.*, 1998). Recently, an *ICE* candidate gene, designated *AtICE1* was identified. This gene encodes a MYC-type transcription factor which becomes activated at low temperatures and induces expression of *AtCBF3* (Chinnusamy *et al.*, 2003). The AtCBF proteins play a prominent role in controlling gene expression and reconfiguring the metabolome in response to low temperature (Cook *et al.*, 2004; Usadel *et al.*, 2008). The CBF regulon of cold-responsive genes encode products which are effectors of stress resistance. The *COR* (cold responsive) genes include four subgroups: *RD* (responsive to dehydration), *ERD* (early dehydration-inducible), *KIN* (cold-inducible) and *LTI* (low temperature-induced) genes (Thomashow, 1999). The CRT/DRE-dependent regulation of genes through *AtCBF* genes will be discussed in more detail in a following section. A schematic diagram of the Arabidopsis cold-responsive transcriptional network is presented in Figure 1.2.

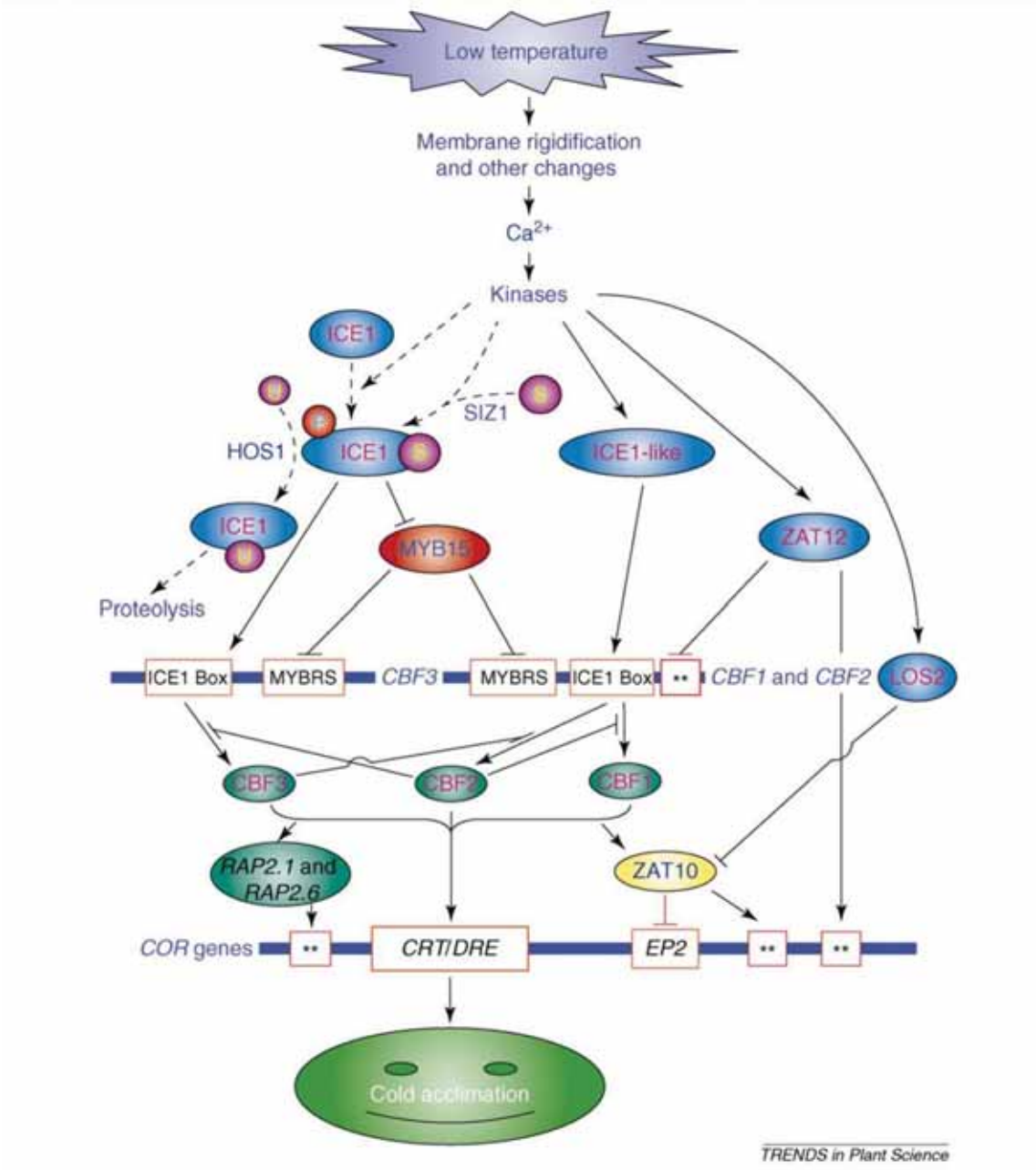


Figure 1.2 Signalling pathways involved in cold responses in Arabidopsis.
Cold stress triggers a cascade of events resulting in the activation and/or repression of a number of cold-responsive signalling molecules. Solid arrows indicate activation and lines ending with a bar show negative regulation. Broken arrows indicate post-translational regulation. ** indicate unknown *cis*-elements. Abbreviations: CBF; C-repeat binding factor, CRT; C-repeat elements, DRE; dehydration-responsive elements, HOS1; high expression of osmotically responsive genes 1, ICE1; inducer of CBF expression 1, LOS2; low expression of osmotically responsive genes 2, MYB; myeloblastosis, MYBRS; MYB transcription factor recognition sequence, SIZ1; SAP

and MiZ1, P; phosphorylation, S; SUMO, U; ubiquitin. Taken from Chinnusamy *et al.* (2007).

The role of ABA in cold stress responses is presently unclear. While firm conclusions cannot be drawn at this stage, evidence suggests that ABA and cold regulatory pathways have points at which they cross-talk but that ABA has a relatively minor role in cold responses and does not exert a great influence on the CBF/DREB1 pathway (Ishitani *et al.*, 1997; Thomashow, 1999). However, evidence such as the transient increase in ABA levels in response to cold, (although to a much lesser extent than during dehydration stress) and the reduced expression of some cold-regulated genes in ABA synthesis and signalling mutants support a role for ABA in cold stress response (Thomashow, 1999; Viswanathan and Zhu, 2002).

Genetic studies have also contributed significantly to knowledge about the responses of plants to low temperatures, identifying the *ESK* (eskimo1), *HOS1* (high expression of osmotically responsive genes1) genes and the *sfr* (sensitive to freezing), *cos* (constitutive expression of osmotically responsive genes) and *los* (low expression of osmotically responsive genes) mutants, as well as uncovering loci such as *vrn* and *Fr*, which are critical for freezing tolerance (Francia *et al.*, 2004; Ishitani *et al.*, 1998; Ishitani *et al.*, 1997; Reinheimer *et al.*, 2004; Snape *et al.*, 2001; Thomashow, 1999; Thorlby *et al.*, 1999; Vagujfalvi *et al.*, 2003; Xin and Browse, 1998). *Sfr6* was identified from a screen to identify mutants which were sensitive to freezing and was shown to affect the activation of the CBF regulon genes *via* the *CBF* genes (Boyce *et al.*, 2003). *hos1* mutants had high levels of expression of genes from the CBF regulon (*rd29A*, *cor47*, *cor15a*, *kin1* and *adh*) under cold stress. Further studies found that AtHOS1 is an E3 ubiquitin ligase which negatively regulates the AtCBF cold signal transduction pathway (Ishitani *et al.*, 1998; Ishitani *et al.*, 1997; Lee *et al.*, 2001).

1.5.2.1. ICE Family in Cold Stress Signalling

The MYC-like basic helix-loop-helix (bHLH) protein “AtICE1”, mentioned previously, was identified by Chinnusamy *et al.*, (2003) from a screen for mutations affecting

expression of the *AtCBF3* gene. Recently, a protein isolated from *A. thaliana* by S. Lopato and colleagues from a yeast 2-hybrid screen with the Arabidopsis Enhancer of zeste protein as bait was found to be the closest relative (by protein sequence identity) of the AtICE1 protein (Lopato and colleagues, unpublished results). Accordingly, the gene encoding this protein was designated *AtICE2*. Recently, a study reported the characterisation of SCRM2, a paralog of AtICE1 (Kanaoka *et al.*, 2008). This protein is AtICE2.

Presently, only a small number of studies have been published about homologs of *AtICE1* from other plant species, leaving much to be ascertained regarding the role of *AtICE1* homologs in cold responses. This review will present our understanding of the function and role of *AtICE1* in cold response and, where possible, information about the extent to which this is conserved in cereal plants.

1.5.2.1.1. Structural Properties

Members of the bHLH family of transcription factors, such as AtICE1, are characterised by an acidic domain in the NH_2-terminal region and a conserved bHLH DNA-binding and/or dimerisation domain near the COOH-terminus. These features are present in both the *AtICE1* and *AtICE2* genes. Figure 1.3 is an alignment of the deduced amino acid sequences of *AtICE1* and *AtICE2* with these domains and other structural features annotated.

bHLH DNA Binding Domain and MYC Element Specificity

The DNA binding domain of Arabidopsis ICE proteins has a basic helix-loop-helix structure which binds MYC domains in the promoters of target genes (Chinnusamy *et al.*, 2003). The N, E and R residues marked with asterisks in Figure 1.3 are core amino acid residues required for DNA binding (Kanaoka *et al.*, 2008). The amino acid sequences of these domains in the AtICE1 and AtICE2 proteins are identical (100% identity), suggesting these two proteins may have similar binding specificities (Chinnusamy *et al.*, 2003; Toledo-Ortiz *et al.*, 2003).

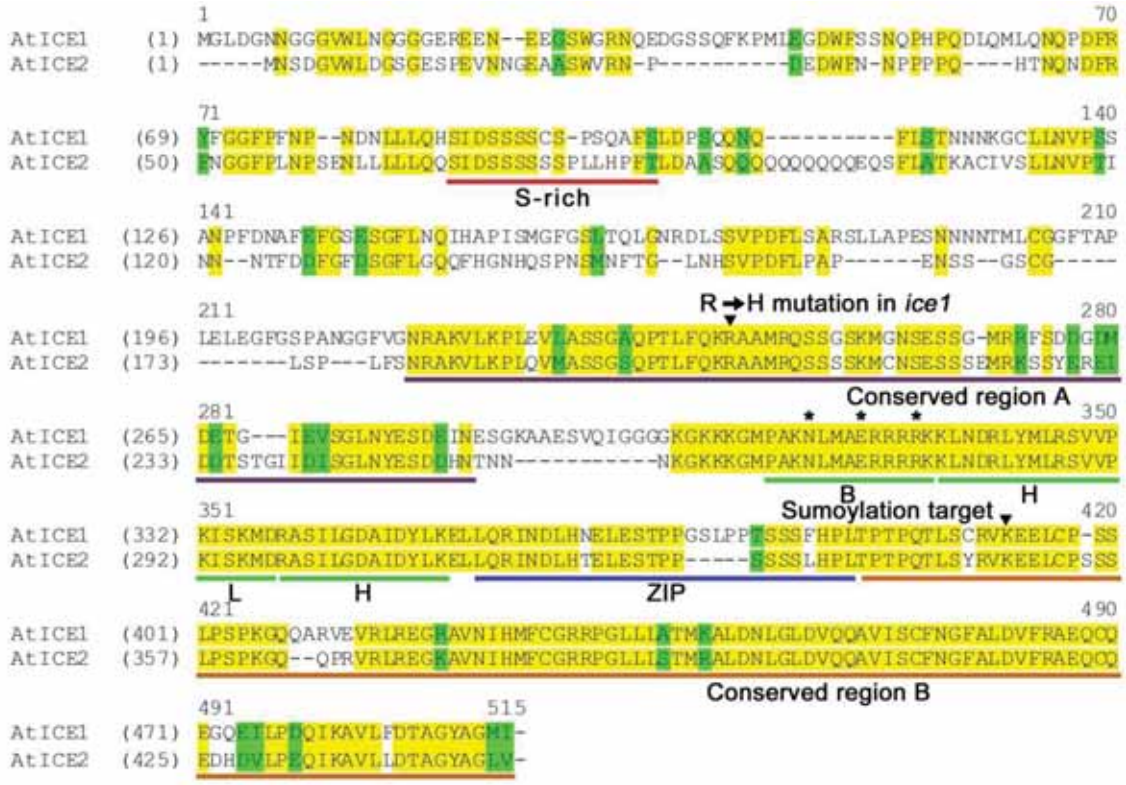


Figure 1.3 Structural features of ICE family amino acid sequences.

Yellow boxes represent identical residues, green boxes represent similar residues. Conserved regions are annotated with lines under the alignment labelled with domain names if known. The black triangles indicate the position of the mutation isolated by Chinnusamy *et al*. (2003) and Kanaoka *et al*. (2008) independently, and the residue targeted for sumoylation by SIZ1 (Miura *et al.*, 2007) as marked. Asterisks mark core residues for DNA binding (Kanaoka *et al.*, 2008).

Both the AtICE1 and AtICE2 proteins bind specifically to MYC-type regulatory *cis*-elements present in the *AtCBF3* promoter (Chinnusamy *et al.*, 2003; Lopato *et al.*, unpublished data; Zarka *et al.*, 2003). Multiple assays suggested that both AtICE1 and AtICE2 proteins bind to multiple MYC sequences in the promoter of the *AtCBF3* gene with the same specificity.

Recently, a study by Benedict *et al*. (2006) reported two novel *cis*-element sequences thought to be more logical candidates in terms of temporal induction of the AtICE and AtCBF regulon genes. They found the sequences *ICEr3* and *ICEr4* were enriched in

the promoters of AtICE-induced cold-responsive genes and correlated with early induction of gene expression (1-3 hours). Contrastingly, they also reported that the previously identified AtICE1 consensus binding sequence (Chinnusamy *et al.*, 2003; Zarka *et al.*, 2003) was not more commonly found in the promoters of stress-responsive than unresponsive genes and was temporally unlikely to be involved in early response to cold treatment (Benedict *et al.*, 2006).

Leucine Zipper Dimerisation Domain

Numerous MYC factors require additional co-transcription factors for transcriptional activation of target genes. They form dimer complexes with these factors through either their bHLH or Leucine Zipper domains (Baxevanis and Vinson, 1993; Murre *et al.*, 1989). Thus, as the bHLH domain of these proteins is required for DNA binding, it was suggested that the putative leucine zipper region of the AtICE1 and AtICE2 proteins may function as a dimerisation domain (Lopato *et al.*, unpublished data). Deletion mapping localised protein-protein interaction to this region and yeast hybrid assays found there is interaction between the AtICE1 and AtICE2 proteins, as well as between AtICE2 and Enhancer of zeste (Lopato *et al.*, unpublished data). However, bimolecular fluorescence complementation experiments by Kanaoka *et al*. (2008) found AtICE1 and AtICE2 had strong heterodimerisation with stomatal regulators MUTE, FAMA and SPCH bHLH proteins in nuclei but did not associate with one another. A different study reported that AtICE1 interacts with AtMYB15, a negative regulator of *AtCBF* expression (Agarwal *et al.*, 2006). Together, these results suggest that gene regulation by AtICE proteins can include specific hetero-dimer formation within the bHLH family and interaction with other transcription factors.

Conserved region A

There is a region of moderate sequence similarity between AtICE1 and AtICE2 in the N-terminal region of the protein, adjacent to the bHLH DNA binding domain. This region contains the KRAAM motif in which the *ice1* mutation lies. A mutation in the same residue in AtICE2 protein reproduced the phenotypes of the *ice1* mutant, suggesting that at least in some contexts (in this case, regulation of stomatal

differentiation), the KRAAM motif is important for AtICE function (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008).

Other Features

Deletion studies in yeast found that the NH_2-terminal acidic region of the AtICE2 acts as an activation domain (Lopato *et al.*, unpublished results). An acidic region is also present in the NH_2-terminus of AtICE1. There is however little similarity in this region of the AtICE1 and AtICE2 proteins (Figure 1.3). It has been speculated that phosphorylation or dephosphorylation may occur at a serine residue in a conserved serine-rich region of the AtICE proteins (Figure 1.3) (Chinnusamy *et al.*, 2003). The C-terminal regions of AtICE1 and AtICE2 are highly similar (Conserved region B; Figure 1.3) and contain the target site for sumoylation by SIZ1 (Miura *et al.*, 2007). No other function has been suggested for this region.

1.5.2.1.2. Functions

Regulation of *AtICE* group genes

Expression analysis has shown that *AtICE1* was constitutively expressed with greater expression in leaf and stem than root or floral tissues and was slightly upregulated by cold, salt and ABA treatments and unaffected by dehydration. Examination of the subcellular localisation of AtICE1 showed the protein was present in the nucleus at both warm and cold temperatures although activity studies found the AtICE1 protein was only active after cold treatment (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008). This suggests that the ability of AtICE1 to activate transcription is regulated by cold-induced post-translational modification of this protein or a transcriptional cofactor (Chinnusamy *et al.*, 2003). This theory is supported by the observation that despite the presence of AtICE1 protein in the nucleus in wild-type and *AtICE1* constitutive over-expression plants, cold treatment was required for expression of *AtCBF3* (Chinnusamy *et al.*, 2003). A schematic diagram of the ICE1 signalling pathway described above is presented as part of the cold-responsive signalling pathway in Figure 1.2.

Recently, a number of protein regulators of AtICE1 have been identified. AtSIZ1, a SUMO (small ubiquitin-related modifier) E3 ligase controls the activity of AtICE1 by sumoylation at K393, which activates or stabilises the AtICE1 protein. This activation is either caused by or in addition to the enhanced protein stability which results from sumoylation. AtICE1 activation or stabilisation triggers the cold-responsive gene expression cascade by facilitating expression of *AtCBF3* and repression of *AtMYB15* (a negative regulator of *AtCBF3*). Sumoylation of AtICE1 was moderately induced by cold, although at present it is not understood how this occurs (Miura *et al.*, 2007).

AtHOS1 (high expression of osmotically responsive genes) is a RING-type ubiquitin E3 ligase which negatively regulates AtICE1 function in cold response. Low temperatures trigger migration of AtHOS1 to the nucleus. At a late stage in the cold response, the activity of AtICE1 decreases as AtHOS1 ubiquitinates AtICE1 proteins, targeting them to the proteasome for degradation (Dong *et al.*, 2006; Lee *et al.*, 2001).

A recent study by Kanaoka *et al.* (2008) suggested that the stomatal-specific regulator SPCH acts immediately upstream of *AtICE1* and is required to induce the expression of *AtICE1* in the stomatal cell lineage. The significance of this in relation to the role of AtICE1 in cold response has not been determined.

There are presently few experimental results that provide information about the role of AtICE2 in any plant process. A recent study reported that AtICE2 accumulates in the nuclei of at least stomatal precursor cells and *AtICE2* expression was found to be largely overlapping with *AtICE1* in the tissues and developmental stages examined (stomatal cell lineages) (Kanaoka *et al.*, 2008). Expression analysis of *AtICE2* using an *AtICE2 promoter:GUS* construct found *GUS* expression was present in all organs examined except stigma, pollen and young seeds, with particularly high expression in young leaves and root tips (Lopato *et al.*, unpublished results). The expression of *AtICE2* during abiotic stresses has not been examined. To conclude, expression of *AtICE2* appears to be similar to that of *AtICE1*, although further studies are required to determine the extent of the similarity.

Targets of ICE Regulation

A screen for alteration in cold-response in Arabidopsis identified the *ice1* mutant (Chinnusamy *et al.*, 2003). Microarray data analysis of *ice1* plants revealed that in wild-type plants under cold stress, the AtICE1 protein triggers expression of a large regulon of genes encoding products involved in freezing tolerance processes by inducing expression of *AtCBF3*. These genes include many transcription factors, as well as known cold-response genes such as *rd29A* and *cor15a*. Conversely, cold-regulated expression of the other two *AtCBF* genes, *AtCBF1* and *AtCBF2*, was only slightly affected in comparison to wild-type plants, suggesting these genes are not direct targets of AtICE1 regulation (Chinnusamy *et al.*, 2003).

Lee and colleagues (2005) compared the cold-responsive transcriptomes of *ice1* and wild type Arabidopsis plants by microarray data analysis, determining that the expression of many cold-responsive genes was altered in *ice1* plants before and during cold treatment. It was hypothesised that these genes were directly or indirectly regulated by AtICE1. The authors suggested AtICE1 plays a critical role in maintaining the basal expression levels, as well as the rate of activation and deactivation of cold responsive genes. As AtICE1 was believed to be an early regulator of cold response, the expression of transcription factors was examined. These results indicated that AtICE1 preferentially targets early upstream transcription factors in cold-regulated gene expression. The expression of many transcription factors was altered, the major group being AP2 domain factors, including AtCBF3 and AtDREB2, followed by bZIP and WRKY proteins. The expression of ~38% of the cold-regulated genes involved in signal transduction, particularly Ca^{2+} signalling proteins, RLKs and lipid-signalling molecules were affected in *ice1* plants, suggesting these processes are regulated by AtICE1. In addition, a significant proportion of the genes from the AtCBF3 and AtCBF2 regulons were affected, in contrast to few genes from the regulon of the transcriptional repressor AtZAT12 (Lee *et al.*, 2005; Maruyama *et al.*, 2004; Vogel *et al.*, 2005), indicating AtICE1 plays a greater role in regulation of the regulons of AtCBFs, particularly AtCBF3, than that of AtZAT12.

A study by Benedict and colleagues (2006) found that the *AtICE1*-mediated transcription factor cascade likely includes transcription factors binding to the DRE, ABRE, and *cis*-elements in the promoters of *AtHos9* and *AtICE1*. They also concluded that the *ICE*/*CBF*-mediated cold signalling pathway contains positive and negative feedback loops. Interestingly, they found that light affected the transcriptional activity of *AtICE1* and *AtCBFs* with their corresponding *cis*-elements being less inducible at dusk. They propose that AtICE1 induces expression of *AtCBF1*, *AtCBF2* and *AtCBF3*, as well as *AtZAT12* and *AtNAC072* transcriptional repressors.

The only published study examining regulatory targets of AtICE2 is in the field of stomatal differentiation rather than cold response. Nonetheless, it was reported that AtICE1 and AtICE2 are required for expression of *SPCH*, a stomatal cell-specific bHLH transcription factor (Kanaoka *et al.*, 2008). As mentioned previously, AtICE2 is able to bind the same promoter elements of *AtCBF3* as the AtICE1 protein (Lopato *et al.*, unpublished results). This suggests that some of the gene targets of AtICE1, including AtCBF regulon genes, may also be activated by AtICE2 although experimental evidence is required to confirm this.

Role of ICE in Cold Tolerance

Studies of *AtICE1* transgenic plants have helped identify the role of AtICE1 in cold responses. No studies of the cold tolerance of *AtICE2* transgenic plants have been reported, although the results of *AtICE1* studies suggest similar studies may provide useful insights into the function of this gene.

The *ice1* plants contained a single mutation in *AtICE1* (Chinnusamy *et al.*, 2003). The mutation (Figure 1.3), changing Arginine 236 to Histidine, had a dominant negative effect, inactivating the protein. The mutation did not appear to affect the interaction between AtICE1 and the *AtCBF3* promoter or the function of the transcriptional activation domain, but was proposed to affect the activity of the protein by interfering with phosphorylation or dephosphorylation at a nearby Serine residue (Chinnusamy *et al.*, 2003). The *ice1* plants were defective in cold-regulated expression of the *AtCBF3*

and AtCBF target genes and consequently, both chilling and freezing tolerance was impaired. Interestingly, these phenotypes were not shared by T-DNA insertion mutants, which showed no obvious phenotypes. The lack of phenotype was interpreted as suggesting functional redundancy may exist within *AtICE* family of genes (Chinnusamy *et al.*, 2003). Indeed, a recent study drew the conclusion that there is functional redundancy between *AtICE1* and *AtICE2* in their roles in regulation of stomatal differentiation (Kanaoka *et al.*, 2008). Whether there is functional redundancy between the roles of *ICE* genes in regulation of cold tolerance remains to be determined.

Constitutive over-expression of *ICE1* did not induce expression of *AtCBF3* at normal temperatures. This is consistent with the lack of obvious growth or developmental abnormalities which were observed. Following cold treatment, accumulation of *CBF3* transcript and transcripts of *AtDREB* regulon genes was observed at levels far greater than those evoked in wild-type plants by cold treatment (Chinnusamy *et al.*, 2003). These results support the argument that post-translational modification of AtICE1 is required to *trans*activate *AtCBF3* expression. Importantly, plants over-expressing *AtICE1* showed improved tolerance to both freezing and chilling stresses which is consistent with the upregulation of the *AtCBF* regulon genes that was observed.

Other Roles of ICE1

As mentioned above, the first report has been presented describing a role for AtICE group proteins in stomatal differentiation (Kanaoka *et al.*, 2008). The group found that a gain of function mutation in *AtICE1* (named SCRM) caused constitutive stomatal differentiation and the loss of function of both *AtICE1* and *AtICE2* (named SCRM2) produced phenotypes resembling stomatal differentiation mutants. This indicates that dosage of the ICE genes determines progression through stomatal differentiation. Interestingly, the gain of function mutant identified in this study contained the same mutation in AtICE1, R236H, as was present in the *ice1* mutant, (Figure 1.3; Chinnusamy *et al.*, 2003) and *ice1* plants also had the constitutive stomatal differentiation phenotype (Kanaoka *et al.*, 2008).

Analysis of single and double T-DNA insertion mutants of the *ICE* genes suggested the roles of AtICE1 and AtICE2 are largely redundant in stomatal cell lineage differentiation (Kanaoka *et al.*, 2008). AtICE1 and AtICE2 partner with SPCH, MUTE and FAMA bHLH proteins to drive the three steps of stomatal differentiation. The SPCH, MUTE and FAMA proteins are present transiently within the specific stages of stomatal cell differentiation and function as heterodimers with broadly expressed AtICE proteins. At present, there has been no conjecture about the meaning of this link between cold tolerance and stomatal differentiation (Kanaoka *et al.*, 2008).

1.5.2.1.3. ICE Family in Cereals

The recent discovery of AtICE1 as an upstream regulator of the AtCBF3 cold response pathways represents an important step in elucidating the steps between stress detection and gene expression in the CBF/DREB response pathways. Numerous studies have shown that elements of the CBF/DREB cold response pathways are conserved in many species including cereals (Badawi *et al.*, 2007; Dubouzet *et al.*, 2003; Jaglo *et al.*, 2001; Qin *et al.*, 2004; Skinner *et al.*, 2005; Takumi *et al.*, 2008). Studies indicate a high degree of conservation in some areas but divergence in others.

Over-expression of a rice homolog of *AtICE1*, *OsbHLH2*, in Arabidopsis conferred increased salt stress tolerance and expression of *AtCBF3* and various *COR* genes. This suggests the role of these genes in stress tolerance is conserved. However, in contrast to the findings in Arabidopsis, the over-expression plants did not exhibit increased freezing tolerance and expression of the *AtCBF* regulon gene *AtKIN1* was not affected (Zhou *et al.*, 2009). The authors suggest that the rice *ICE* gene may function in stress response *via* different pathways to *AtICE1*. However, it is possible that lack of cold-response may be due to evolutionary divergence in elements of the cold-responsive *ICE1* pathway between Arabidopsis and rice. Whether different results would be obtained from over-expression of *OsbHLH2* in rice remains to be determined.

Two *ICE* genes were identified in wheat, *TaICE41* and *TaICE87*. These genes share several similarities with *AtICE1*: 1) both *ICE* genes are constitutively expressed and

expression is not affected by cold treatment, 2) the *ICE* genes transcriptionally activate the *CBF* gene *TaCBFIVd-B9* by binding (different) MYC elements in the promoter, 3) over expression of either *ICE* gene in Arabidopsis enhanced freezing tolerance and activated expression of *CBF3*, *CBF2* and several cold-regulated genes. In contrast to the Arabidopsis *ICE* genes, freezing tolerance was only enhanced in cold acclimated plants, suggesting other factors induced by low temperature are required for activity of wheat *ICE* genes (Badawi *et al.*, 2008).

Recently, the map locations of barley *ICE* genes have been reported. *HvICE1* is located on chromosome 7H and *HvICE2* is located on chromosome 3H. Unfortunately, neither of these genes co-localised with known quantitative trait loci (QTLs) for low temperature tolerance in Triticeae (Skinner *et al.*, 2006; Tondelli *et al.*, 2006). These results indicate that in the genotypes studied, allelic variation of *HvICE1* is not responsible for the trait assayed. This could mean that the function of *ICE* group genes in barley is different from their Arabidopsis counterparts and are not involved in stress response. Other possible explanations are that the role of cereal *ICE* genes in cold stress response is relatively minor, or that there is insufficient allelic variation at this locus in the genotypes studied.

As mentioned above, the *CBF* gene *AtCBF3* is a target of regulation by ICE transcription factors. However studies have shown that over-expression of *CBF* genes can increase freezing tolerance, making these genes obvious targets for direct study. The following section will introduce the CBF family and discuss the roles of these proteins in cold stress signalling and abiotic stress tolerance, first in Arabidopsis and then in cereals.

1.5.2.2. CBF Family in Cold Stress Signalling

A small closely related group of transcriptional activators were originally identified in Arabidopsis from a yeast 1-hybrid screen using the CRT element as bait (Stockinger *et al.*, 1997). These proteins were designated CBF1, CBF2 and CBF3 (collectively referred to here as CBF). Comprehensive expression analysis of the Arabidopsis *CBF/DREB* family suggested that while many members are responsive to other abiotic stresses such as dehydration or salinity, only the three *CBF* genes were transcriptionally activated by cold stress (Sakuma *et al.*, 2002). Numerous studies have confirmed the importance of the *CBF* genes in cold signalling (Chinnusamy *et al.*, 2004; Wang *et al.*, 2003; Xiong *et al.*, 2002). For these reasons, it was concluded only the three CBF factors play major roles in the cold responses (Sakuma *et al.*, 2002).

The first part of this section will refer solely to CBF proteins from the model plant Arabidopsis where the vast majority of research has been performed. The second part will describe information from barley and closely related plant species which may help predict how the *CBF/DREB* pathways relate in barley and Arabidopsis.

1.5.2.2.1. CBF Structural Properties

The *CBF/DREB* family genes are one of five subfamilies of the large EREBP/APETALA2 (AP2) transcription factor family that is unique to plants (Riechmann and Meyerowitz, 1998). This family contains 145 members in Arabidopsis, including the 14 *CBF/DREB* group genes (Sakuma *et al.*, 2002). As well as the highly conserved AP2 DNA binding domain common to all EREBP/AP2 members, CBF/DREB proteins contain nuclear localisation sequences and an acidic region which serves as an activation domain (Riechmann and Meyerowitz, 1998; Stockinger *et al.*, 1997; Thomashow, 1999).

AP2 DNA Binding Domain

The AP2 DNA binding motif is approximately 60 amino acids in length and contains two distinct regions: the YRG and RAYD elements (Riechmann and Meyerowitz,

1998). The YRG element contains an NH_2-terminal stretch of 20 amino acids which is rich in basic and hydrophilic residues and forms a three-stranded anti-parallel β-sheet. The β-sheet has a role in DNA sequence specificity and interactions (Allen *et al.*, 1998; Sakuma *et al.*, 2002). It has been suggested that two conserved amino acid residues, V14 and E19 in the YRG element may play important roles in recognition of target *cis*-elements by CBF/DREB proteins, with V14 being particularly important in CBFs (Liu *et al.*, 1998; Sakuma *et al.*, 2002). At the COOH-terminus the RAYD element forms an amphipathic alpha-helix packed approximately parallel to the β-sheet. It is thought that this element may contribute to the DNA binding interactions or mediate protein-protein associations (Allen *et al.*, 1998; Kizis *et al.*, 2001) (Figure 1.4).

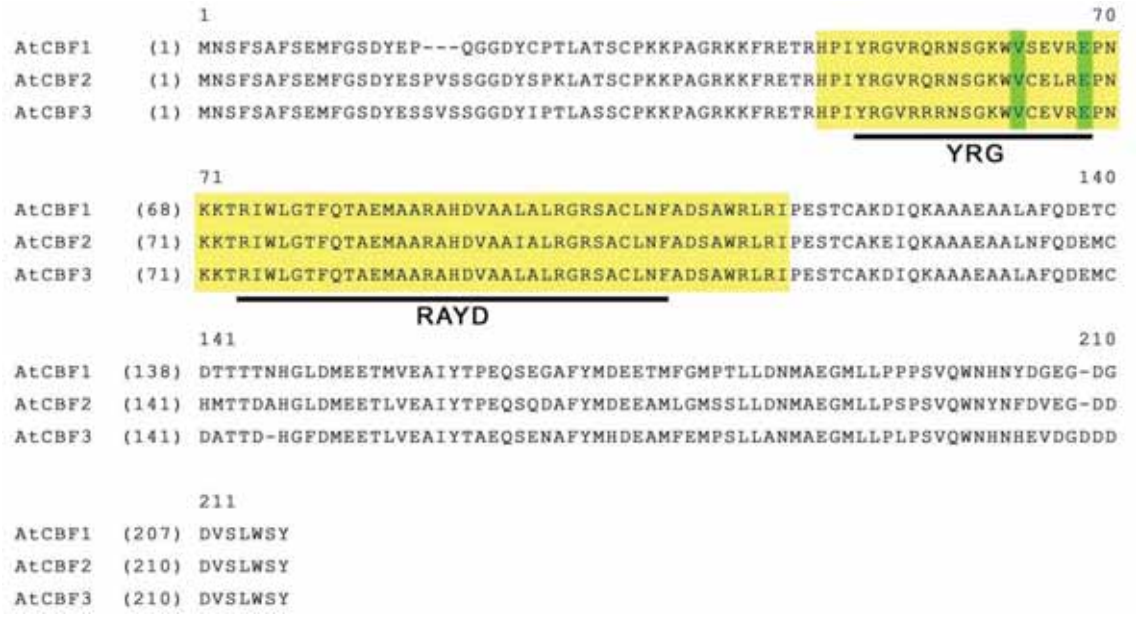


Figure 1.4 Multiple sequence alignment of AtCBF1, AtCBF2 and AtCBF3 proteins.

AtCBF1; Accession number NP_567721, AtCBF2; Accession number NP_567719, AtCBF3; Accession Number NP_567720. The AP2 domain is marked in yellow. Residues highlighted in green represent conserved residues important for target *cis*-element specificity. YRG and RAYD elements are indicated with solid lines.

Wang and colleagues (2005) found that the N-terminal region of AtCBF1, up to and including the AP2 domain (115 amino acids), are sufficient for promoter targeting and binding to CRT sequences. The remaining C-terminal portion (98 amino acids) is sufficient for gene *trans*activation.

The CRT element, otherwise known as the DRE or LTRE (low temperature response element) was first identified by Yamaguchi-Shinozaki and Shinozaki (1994) in the promoter of *rd29A* (*COR78*). This element is present in the promoters of many stress inducible genes and stimulates gene expression in response to cold, dehydration and high salinity but not in response to the presence of ABA (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 1994). The CBF proteins can *trans*activate gene expression through the CRT element (TACCGACAT) which contains a 6 bp core sequence A/GCCGAC (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Stockinger *et al.*, 1997). The underlined residues are essential for highly specific interactions with the AP2 domains of CBF/DREB proteins (Sakuma *et al.*, 2002).

Other Domains

The *CBF* genes also contain some common features of transcription factors: an acidic transcriptional activation domain near the COOH-terminus and a basic nuclear localisation sequence at the NH_2-terminus (Figure 1.4) (Liu *et al.*, 1998; Medina *et al.*, 1999; Riechmann and Meyerowitz, 1998; Stockinger *et al.*, 1997).

1.5.2.2.2. Functions

Regulation of *CBF* Genes

Cold-associated regulation of *CBF* genes appears to occur at least partially at the transcriptional level (Liu *et al.*, 1998; Shinwari *et al.*, 1998). Numerous studies reported that following cold treatment, expression of the Arabidopsis *CBF* genes is quickly induced (within approximately 15 minutes) but is transient, rapidly returning to almost pre-treatment levels shortly after 90 minutes (Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999; Novillo *et al.*, 2004).

Expression of individual *AtCBF* genes did not vary in leaf, stem or root tissues, nor was it notably affected by other abiotic stresses including dehydration or salinity stress, or by ABA (Liu *et al.*, 1998; Medina *et al.*, 1999; Sakuma *et al.*, 2002).

More recently, detailed expression data presented by Novillo and colleagues (2004) demonstrated that while expressions of *CBF1* and *CBF3* in response to cold treatment were very similar, *CBF2* transcripts accumulated at a slower rate and did not reach maximal levels until approximately two and a half hours after cold exposure. From these and other results, they proposed that *CBF2* negatively regulates the expression of *CBF1* and *CBF3* under normal conditions but upon cold stress, the repression is overridden and *CBF1* and *CBF3* are induced. The later increase in *CBF2* transcripts is proposed to reinforce repression of *CBF1* and *CBF3* and cause the decline in their transcript levels. This theory is supported by the presence of *CBF2* transcript at normal temperatures at levels over 5-fold greater than those of *CBF1* or *CBF3* (Chen *et al.*, 2002; Chinnusamy *et al.*, 2003; Fowler and Thomashow, 2002; Novillo *et al.*, 2004). In addition, the different expression profile of *AtCBF2* indicates that this gene is regulated during cold response *via* a different mechanism to *AtCBF1* and *AtCBF3*.

There is mounting information about the mechanisms by which the *CBF* genes are activated. As previously described, AtICE1 was found to participate in regulation of *CBF3* expression. The negative regulator ZAT12 acts independently of the ICE1 pathway to repress the *AtCBFs* and their downstream transcription factors, as well as its own regulon (Vogel *et al.*, 2005). The negative regulator *AtMYB15* is transcriptionally activated by cold stress. The product of this gene interacts with AtICE1 and represses expression of the *AtCBF* genes (Agarwal *et al.*, 2006). Other regulatory proteins affecting expression of *CBF* genes include negative regulators, HOS1 (described in Section 1.5.2.1.2) and FRY2 and the positive regulator LOS4 (Gong *et al.*, 2002; Lee *et al.*, 2001; Xiong *et al.*, 2002).

An interesting study by Zarka and colleagues (2003) determined that the cold sensing mechanism which activated the *CBF* genes appears to monitor absolute temperature.

Significantly, the maximum transcript levels observed were the same for gradual temperature downshifts as cold shock treatments. This represents an important finding because if expression had differed under the two treatments, the results of numerous studies utilising the cold shock treatment methods would be of little use to predict *CBF* expression in field scenarios. It was determined that 14°C was the threshold temperature at which accumulation of *CBF* transcripts became detectable (Zarka *et al.*, 2003).

Targets of CBF Regulation

Many targets of *CBF* genes have been identified from the presence/absence of CRT elements in gene promoters, and microarray experiments that examined gene expression profiles in wild-type and transgenic plants following cold stress (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Seki *et al.*, 2001; Vogel *et al.*, 2005). Induction of target genes in response to cold stress occurred slowly and gradually between two and ten hours post treatment and whilst some target genes were induced transiently, others maintained their cold response for the long-term (Fowler and Thomashow, 2002; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Seki *et al.*, 2002). CBF target genes are involved in a wide variety of processes which play important roles in low temperature responses. These include the transcriptional activators ZAT10, RAP2.1 and RAP2.6, as well as many other *COR* genes involved in processes such as phosphoinositide metabolism, osmolyte biosynthesis, ROS detoxification, hormone metabolism and membrane transport (Chinnusamy *et al.*, 2007; Fowler and Thomashow, 2002). Furthermore, Vogel *et al.* (2005) found that the majority of the highly cold-inducible genes were part of the CBF regulon. CBF1 has also been reported to be involved in the low temperature induced inhibition of plant growth by a gibberellin-mediated pathway which is independent of the CBF regulon (Achard *et al.*, 2008).

A recent study revealed that the *AtCBF1* and *AtCBF3* positively regulate cold acclimation by activating the same group of target genes. Neither *CBF* is involved in regulating other *CBF* genes. In addition, a subset of genes in the CBF regulon requires simultaneous expression of *AtCBF1* and *AtCBF3* to be properly induced. This indicates

that *AtCBF1* and *AtCBF3* have an additive effect in cold response to induce the whole CBF regulon (Novillo *et al.*, 2007).

Role of CBF Group in Cold Tolerance

Analysis of transgenic plants with altered expression of the *AtCBF* genes has highlighted the significance of their role in cold tolerance. Over-expression of each *CBF* transcription factor induced expression of very similar sets of genes and had similar effects on the biochemical composition, morphology and development of the transgenic plants (Gilmour *et al.*, 2004). Plants over-expressing *CBF1*, *CBF2* or *CBF3* showed high expression of downstream genes at normal temperatures and increased freezing tolerance (Gilmour *et al.*, 2004; Gilmour *et al.*, 2000; Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu *et al.*, 1998). These phenotypes show that transcript accumulation alone is sufficient to activate the *AtCBFs*. Growth and developmental abnormalities were associated with the constitutive over-expression of *CBF3* and with over-expression of *CBF1* and *CBF2* when examined by Gilmour *et al.*, (2004) although these effects were not seen in *CBF1* over-expression plants examined by Jaglo-Ottosen *et al.* (1998) (Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Liu *et al.*, 1998). These effects could be avoided in *CBF3* over-expression plants by the use of a stress-inducible promoter (Kasuga *et al.*, 1999).

The only *CBF* mutant which has been reported has a T-DNA insertion in *CBF2*. Interestingly, these plants had improved freezing tolerance and enhanced expression of *CBF1*, *CBF3* and CBF target genes. These results support the suggestion that AtCBF2 may be a negative regulator of *AtCBF1* and *AtCBF3* expression and cold responses (Novillo *et al.*, 2004). However the apparent contradiction of the results obtained from *CBF2* over-expression experiments suggests that much remains unknown about the function of this gene.

Recently, it has been discovered that DELLAs, a family of nuclear growth-repressing proteins, are components of CBF1-mediated cold response and contribute significantly towards CBF1-induced freezing tolerance by a separate mechanism from the CBF

regulon. Accumulation of DELLA proteins restrains plant growth at low temperatures and occurs when CBF1 reduces gibberellin content (Achard *et al.*, 2008).

1.5.2.2.3. Barley Genes Similar to the *AtCBF* Group

General and Structural Comparison

Only recently has research on *CBF* genes moved from Arabidopsis into crop plants. Although much of the detailed information known about the structure and modes of function of *CBF* genes is in Arabidopsis, studies have shown that many elements of the CBF response pathways are conserved in other species including wheat and barley. Therefore it is expected that a proportion of the findings from Arabidopsis summarised above may apply to barley plants.

The first barley *CBF* genes were discovered by Xue (2002a; 2003), and Choi and colleagues (2002) and were named *HvCBF1, 2* and *3*. These genes show a high degree of similarity to the Arabidopsis *CBF* genes in some regions while being less conserved in others.

Following this, genomic studies identified large families of *CBF* genes in wheat and barley, including some subgroups which are only found in grasses. Mapping studies have found a large cluster of these *CBFs* map to Group 5 chromosomes in wheat and barley which map at the peak of two overlapping QTLs associated with frost tolerance in Triticeae (Badawi *et al.*, 2007; Baga *et al.*, 2007; Francia *et al.*, 2007; Francia *et al.*, 2004; Miller *et al.*, 2006; Skinner *et al.*, 2006; Tondelli *et al.*, 2006; Toth *et al.*, 2003; Vagujfalvi *et al.*, 2005; Vagujfalvi *et al.*, 2003). These QTLs are: a) the frost survival *Fr-2* locus, known as *Fr-H2* in barley: one of two important QTLs for frost tolerance in *Triticeae*, and b) differential expression of the *COR* gene, *COR14b* (Vagujfalvi *et al.*, 2005; Vagujfalvi *et al.*, 2003). This suggests alleles for one or more of the *CBF* genes may be responsible for differential regulation of *cor14b* and frost tolerance traits, although it is not known whether the effects result from a single *CBF* gene, the combined effect of some or all of the CBFs, or are independent of the *CBF* genes.

In barley, a family of at least 20 *CBF* genes has been described with a similar degree of phylogenetic complexity to other monocots (Francia *et al.*, 2004; Skinner *et al.*, 2005). A phylogenetic tree of the barley *CBF* genes is presented in Figure 1.5. In cold-tolerant grasses such as wheat and barley, the *CBF4*-subgroup is more complex relative to that of the cold-sensitive rice (Skinner *et al.*, 2005).

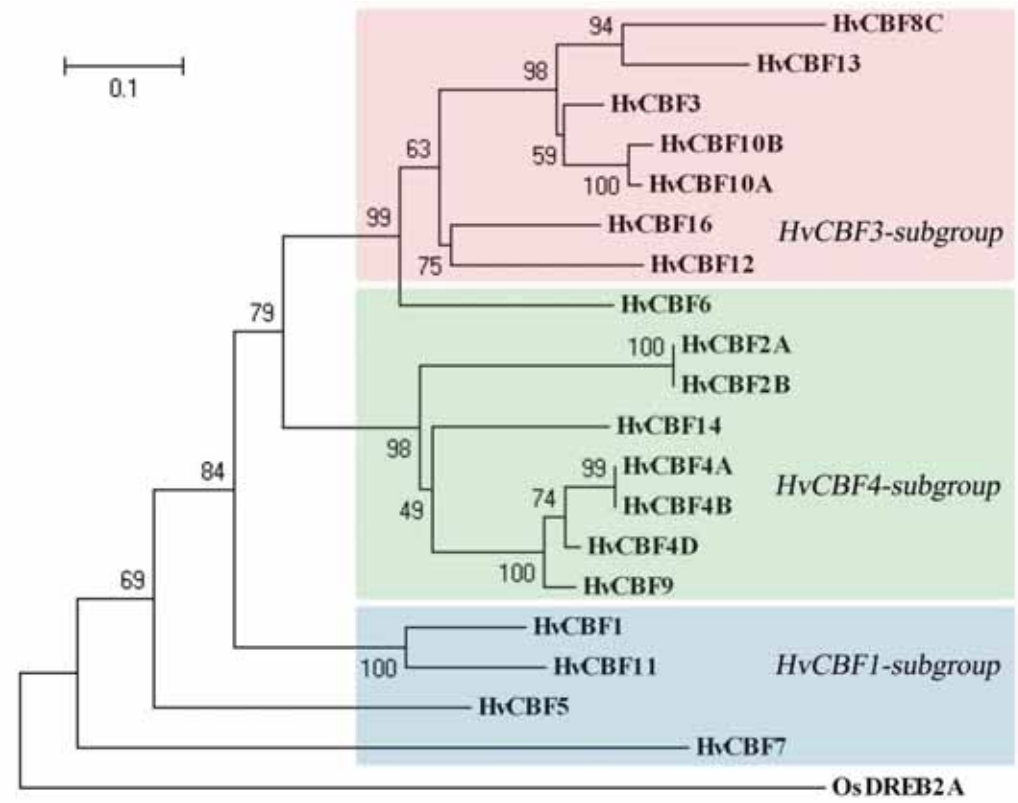


Figure 1.5 Phylogenetic relationships of barley CBFs.

A neighbour joining phylogenetic tree was derived from an alignment of published barley CBF polypeptides. For the pseudogene *HvCBF8C*, a theoretical polypeptide sequence was generated, accounting for frame shift mutations. OsDREB2A was used as an outlier as the AP2 domain-containing protein lacks the flanking CBF signature sequences. Coloured boxes denote members of the respective the HvCBF-subgroups.

Skinner and colleagues (2006) identified the map locations of 17 barley *CBF* genes, as well as other regulators of cold tolerance including *HvICE2* and *HvZAT12*. They found

that 12 *CBF* genes were located in two clusters on the long arm of chromosome 5H, coincident with the barley *Fr-2* frost tolerance QTLs mentioned above. Contrastingly, no QTLs for frost tolerance were present at the map locations of *ICE1*, *ZAT12*, or the remaining *CBF* genes, suggesting one or more of the *CBFs* at the *Fr-2* locus may be more important for frost tolerance and *Cor14b* expression (Skinner *et al.*, 2006).

Of late, research has focused on determining which of these *CBFs* may be most important in frost tolerance. Strategies have included examining the transcriptional profiles of the clustered *CBF* genes (Stockinger *et al.*, 2007; Vagujfalvi *et al.*, 2005) and fine mapping of the barley *CBF* cluster and the relationship of different subclusters of *CBFs* to frost tolerance (Francia *et al.*, 2007). The findings of these studies are presented in further detail below.

DNA-binding Domain and *Cis*-element Specificity

Major features of the CBF protein include a leader sequence of varying composition (15-40 amino acids), an AP2 domain flanked by the conserved CBF subfamily signature motifs and an acidic C-terminal domain postulated to act as an activation region (Skinner *et al.*, 2005) (Figure 1.6). In monocot CBFs, the AP2 domain and first signature motif are highly conserved (Skinner *et al.*, 2005). The sequence of a conserved motif in the Arabidopsis AP2 domain, DSAWR (Jaglo *et al.*, 2001) varies between subgroups. It was noted by Skinner *et al*. (2005) that the first signature motif displays all the characteristics of a nuclear localisation signal (Stockinger *et al.*, 1997).

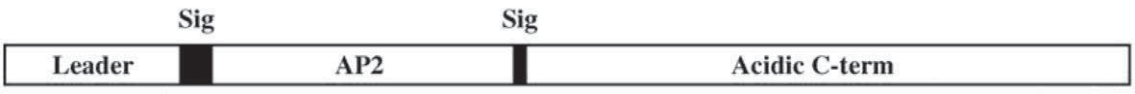


Figure 1.6 General domain structure of barley and monocot CBFs.
Taken from Skinner *et al*. (2005). The variable leader, AP2, and acidic C-terminal domains are noted. The AP2 domain-flanking CBF signature motif positions (Sig) are indicated as black blocks.

Like their Arabidopsis counterparts, barley CBFs have been shown to specifically bind CRT elements, derived from monocot or dicot promoters (Skinner *et al.*, 2005; Xue, 2002a; 2002b; 2003). The DNA binding properties of a representative subset of barley CBFs from each subgroup were examined by Skinner *et* al. (2005). Interestingly, while binding of HvCBF3- and HvCBF3-subgroup members occurred at warm and cold temperatures, binding of HvCBF4-subgroup members was cold-dependent. The barley CBFs also had different affinities for each of the three CRT *cis*-elements used, derived from *HvCor14b*, *HvDHN5* and *AtCor15a*, implying that sequences flanking the CRT core influence CBF binding (Skinner *et al.*, 2005).

Regulation of Barley CBFs

Recently, comprehensive expression analyses have provided a wealth of information about the expression of barley *CBFs*. Expression of many barley *CBFs* is induced by low temperatures and although some also respond to drought and/or salinity, none are affected by ABA treatment (Choi *et al.*, 2002; Skinner *et al.*, 2005; Stockinger *et al.*, 2007; Xue, 2002a). Comparison of the cold, salt and dehydration stress-responsive expression profiles of various barley *CBFs* in cold-tolerant (Dicktoo) and intolerant (Morex) genotypes revealed that the response level and duration differed between genotypes, rather than the ability to respond to a particular stress (Skinner *et al.*, 2005).

Expression of *HvCBF4*-subgroup members (*HvCBF2*, *4* and *9*) was greatly induced by low temperatures and mildly affected by dehydration and high salinity stresses. Expression was induced by cold within 1 to 4 hours with maximal response between 4 and 10 hours. Expression of *HvCBF2* and *HvCBF9* was more affected in the cold-tolerant Dicktoo and Nure varieties (compared to Morex and Tremois) and expression duration was greater for all three in Dicktoo (Skinner *et al.*, 2005; Stockinger *et al.*, 2007).

Most members of *HvCBF1*- and *HvCBF3*-subgroups displayed distinct expression profiles, differing in response to dehydration, salinity and/or cold stress types and response time. Unusually, and in contrast to the other barley *CBFs*, expression of

HvCBF8 was negatively regulated by all the stress treatments (Choi *et al.*, 2002; Skinner *et al.*, 2005; Stockinger *et al.*, 2007; Xue, 2002a).

A recent study found that the *VRN-H1/Fr-H1* locus is involved in regulation of barley *CBFs* by repressing or attenuating expression of multiple *CBFs* at *Fr-H2*. (Stockinger *et al.*, 2007). This finding represents a significant difference between the regulation of Arabidopsis and cereal *CBFs*. Plants harbouring the winter *VRN-H1* allele had significantly greater expression of cold-induced genes including *HvCBF2* and *HvCBF4*. In addition, robust expression of *Vrn-1* following vernalisation was associated with dampened *CBF* expression. (Stockinger *et al.*, 2007). Other regulatory factors in *CBF* expression include photoperiod and day length, with *CBF* transcript levels being higher in plants grown under short days. Individual CBFs vary in sensitivity towards the photoperiod effects. (Stockinger *et al.*, 2007)

In some cases, expression of various barley *CBFs* was sufficient to allow binding to CRT *cis*-elements and promoters and induces downstream gene expression. In others however, cold treatment was required to activate CBF binding to CRT elements (Skinner *et al.*, 2005; Xue, 2003). This suggests that in some cases, post-translational control is not required for stimulation of target gene expression, and in others, post-translational regulation occurs by an unknown mechanism in response to low temperatures.

In general, the larger CBF families in grass species have more complex transcriptional profiles than their Arabidopsis counterparts. There appear to be many similarities between the expression profiles of barley *CBF* genes and their homologous genes in other *Triticeae* species (Badawi *et al.*, 2007; Skinner *et al.*, 2005; Stockinger *et al.*, 2007; Vagujfalvi *et al.*, 2005).

Targets of Barley CBF Regulation

Studies have shown that barley CBF proteins can bind the promoters and *trans*activate expression of a set of *COR* effector genes. Two groups of barley CBFs were established: those which required low temperatures for DNA binding (members of the HvCBF4-subgroup) and those which did not (all other barley CBFs). Target genes include *HvCor14b*, *HvDHN5*, *HVA1s* and the Arabidopsis gene *AtCor15a* (Skinner *et al.*, 2005; Xue, 2002a; 2002b; 2003). This data supports the observation that due to the co-localisation of the *CBF* cluster with *Fr-H2*, and differential expression of *cor14b* QTLs, CBFs likely candidates for cold-responsive regulation of *cor14b* (Francia *et al.*, 2004; Tondelli *et al.*, 2006; Vagujfalvi *et al.*, 2000; Vagujfalvi *et al.*, 2003). In transgenic Arabidopsis plants with constitutive expression of various barley *CBFs*, two CBFs, HvCBF3 and HvCBF6, induced expression of all of the four tested *COR* genes from the CBF regulon (Skinner *et al.*, 2005). This suggests that barley homologs of these *COR* genes may also be targets of CBF regulation.

Other likely targets of barley CBFs include the barley homologs of CBF target genes in other cereals. These genes include *HvDHN8* and the barley homologs of tobacco genes *WDHN13* and *Wrab17* (James *et al.*, 2008; Takumi *et al.*, 2008). Analysis of transgenic rice plants over-expressing *OsDREB1A* or *HvCBF4* have identified many other stress-inducible target genes including numerous proteins with functions in stress tolerance (Ito *et al.*, 2006; Oh *et al.*, 2007). This suggests many of the undiscovered genes in the barley CBF regulon may function in providing protection for plants against abiotic stress.

Role of Barley CBFs in Cold Tolerance

There is compelling evidence suggesting barley CBFs play a significant role in cold tolerance. The *Fr-H1* and *Fr-H2* loci are the major QTLs for winter hardiness, contributing 60-80% of the phenotypic variation (Francia *et al.*, 2004). The mapping of a CBF cluster at the *Fr-H2* locus (Francia *et al.*, 2004; Tondelli *et al.*, 2006) represents an important link between frost tolerance and allelic variation in CBFs in *Triticeae*. Although the details of which genes are involved are not known at present, it is strongly

suggested that CBF(s) are responsible for the *Fr-H2* QTL (Miller *et al.*, 2006; Skinner *et al.*, 2006; Stockinger *et al.*, 2007). Stockinger and colleagues (2007) suggested that the *Fr-2* component of frost tolerance may be due to greater accumulation of *HvCBF2* and *HvCBF4* transcripts during normal growth and development. Studies in *Triticum monococcum* by Knox and colleagues (2008) found the greatest effect on frost tolerance was linked to *TmCBF12*, *TmCBF14* and *TmCBF15* and a smaller effect was mapped to a distal group of genes, including *TmCBF16*. They suggested allelic variation in these genes as a likely candidate to explain the *Fr-2* QTL in diploid wheat. In addition, the newly-discovered connection between *VRN-1/FR-H1* and *CBF* expression (Stockinger *et al.*, 2007) further emphasises the likely importance of one or multiple barley *CBFs* in frost tolerance. These results corroborate findings that a set of *COR* effector genes is activated by barley CBFs (Skinner *et al.*, 2005).

The role of cereal *CBFs* in stress tolerance has been confirmed by over-expression in rice, wheat and other grasses, resulting in improved stress responses (Ito *et al.*, 2006; James *et al.*, 2008; Oh *et al.*, 2007; Oh *et al.*, 2005; Pellegrineschi *et al.*, 2004). In addition, ectopic expression of barley *CBF* genes in Arabidopsis improved stress tolerance (Skinner *et al.*, 2005; Xue, 2002a). It should be noted however that, as with Arabidopsis, constitutive over-expression of cereal *CBFs* was associated with a reduction in growth (Skinner *et al.*, 2005). The use of a stress-inducible promoter alleviated the phenotypes (Ito *et al.*, 2006; Lee *et al.*, 2004; Shen *et al.*, 2003). Interestingly, over-expression of *HvCBF4* in rice did not cause growth stunting (Pellegrineschi *et al.*, 2004). This may be explained by the requirement of cold to activate DNA binding of the transcription factor, implying the downstream genes are not over-expressed at normal temperatures.

1.5.2.2.4. Project Aims and Scope

The broad objective of the work described in this thesis was to explore options for engineering cold and frost tolerance in wheat and barley by manipulating the expression of key genes regulating the cold response pathway. In the model species, *Arabidopsis thaliana*, the *ICE* and *CBF* genes have been shown to play important roles in stress response by activating the *CBF*/*COR* gene signalling pathway and thereby increasing stress tolerance. Previous studies have indicated that over-expression of *ICE* and/or *CBF* genes can increase cold-stress tolerance in transgenic Arabidopsis plants. In addition, several components of the Arabidopsis signalling pathway appear to be conserved in other species including crop species such as barley and wheat.

Therefore the aim of this project was to explore whether the ICE and CBF pathways, which have been well-characterised in a model species, can be exploited to engineer cold tolerance in the crop species barley. In addition to being an important crop species in its own right, the use of barley has a second practical purpose: it is a model for the genetically complex but economically important species bread wheat.

The specific aims of this project were as follows:

Firstly, investigate whether identification and manipulation of the barley homologs of the Arabidopsis *ICE* gene(s) would reproduce the results seen in Arabidopsis, by increasing the expression of *CBF* and *COR* genes and thereby increasing the cold tolerance of the plant. To achieve this aim, the role of *HvICE2* was characterised in cold stress response by sequence and expression analysis, and analysis of transgenic plants over-expressing *HvICE2*. Similarities or differences in protein sequence and/or gene expression were considered in relation to similarities or differences in the functions of the barley and Arabidopsis proteins. This work will be described in Chapter 2.

The next aim was to investigate how broad and robust the model Arabidopsis ICE/CBF pathway is by determining whether the manipulation of a closely related yet distinct

gene, *AtICE2*, was able to produce similar experimental results to those seen for *AtICE1*. This work addressed the question of whether *AtICE2* provides redundancy for *AtICE1* and therefore represents a second method of inducing the cold response pathway, or whether *AtICE2* is involved in a separate cold-response pathway or does not function in this process. These questions are important since they may provide clues to the existence of alternative regulatory pathways that may have application in our target species, wheat and barley. To this end, freezing tolerance and the expression of *CBF* and *COR* genes were analysed in transgenic plants with up- or down-regulation of *AtICE2*. This work will be described in Chapter 3.

The third section of work focussed further down the *ICE*/*CBF*/*COR* signalling pathway to see if manipulating the barley *CBF* genes directly would be a successful way of altering the pathway to increase freezing tolerance. To investigate this, we characterised barley *CBF* genes using two approaches. The first was to identify and clone *CBF* genes of interest using the yeast one-hybrid system to identify functional *CBFs* from a cDNA population prepared from cold-stressed barley tissue. The *CBF* genes were characterised by sequence and expression analysis, and genomic map location. It was, unfortunately, not possible within the time constraints of this project, to functionally characterise these proteins further by analysing the phenotypes of barley plants over-expressing these genes. This work will be described in Chapter 4.

Finally, the *CBF* gene *HvCBF2A* was characterised by analysis of transgenic plants over-expressing this gene. This was made possible by collaboration with Professor Tony Chen and Professor Patick Hayes (both of Oregon State University) who developed a series of transgenic plants over-expressing this gene. My contribution to this collaborative project included examining the expression of downstream *COR* genes and the development of the plants under normal conditions. The freezing tolerance of the transgenic plants was used to draw conclusions about the role of *HvCBF2A* in freezing tolerance and to explore the supposition that *HvCBF2A* is responsible for a component of the QTL associated with freezing tolerance, *Fr-2*, which co-localises to the genetic map position of *HvCBF2A*. This work will be described in Chapter 5.

Overall, this thesis describes work that investigated the degree of similarity between the *ICE* and *CBF* signalling pathways in barley and Arabidopsis, and whether this pathway can be manipulated to improve frost tolerance in barley and/or cereal crops.

CHAPTER 2

Characterisation of *HvICE2*

2.1. Introduction

The Arabidopsis *ICE* gene *AtICE1* plays an important role in cold stress response and stress tolerance by regulating the expression of *CBF* and *COR* genes and recent studies of wheat and rice *ICE* genes suggests the function of *ICE* genes has been conserved in cereals. To date however, no studies have described manipulation of cereal *ICE* genes in their native plant species, leaving many questions and unknown elements in our understanding of regulation of cold-induced responses and gene expression in *Triticeae*.

The aim of the work described in this chapter was to investigate whether the barley homolog of the cold stress-responsive transcription factor *AtICE1* plays a similar role in cold stress response in barley to that of *AtICE1* in Arabidopsis and could therefore be manipulated to improve plant cold tolerance. To achieve this, *HvICE2*, a barley homolog of AtICE1, was cloned and the gene structure analysed. The expression of *HvICE2* was measured after various abiotic stress treatments and in various tissues. Barley plants over-expressing *HvICE2* were examined for changes in cold-responsive signalling pathways and/or cold-tolerance.

2.2. Materials and Methods

2.2.1. Materials

Eppendorf microcentrifuges and liquid nitrogen were supplied by Adelab Scientific (SA, Australia). CP1000 automatic film processer was made by AGFA (Belgium). Xylene cyanol was supplied by Ajax Chemicals (NSW, Australia). Rotating test tube wheel was purchased from Analite (Australia). BigDye® Terminator v3.1 Cycle Sequencing Kit, formamide, RNAse Zap and RNase-free water were purchased from Applied Biosystems (CA, USA). Tryptone and agarose were supplied by Becton, Dickinson and Company (MD, USA). The Speed Vac SC110 (Savant) was purchased from Biolab (VIC, Australia). Immolase™ DNA Polymerase was supplied by Bioline (VIC, Australia). BD SMART™ RACE cDNA Amplification Kit and BD GenomeWalker™ Kit were purchased from BD Biosciences (NSW, Australia). DMSO, chloroform, glycerol, sodium acetate, NaH_2PO_4-H_2O, glacial acetic acid, formaldehyde, SDS, sodium hydroxide, triton X-100, glycerol and EDTA were supplied by BDH Laboratory Supplies (VIC, Australia).

Gene-Pulser apparatus and Gene Pulser® Cuvette were purchased from Bio-Rad Laboratories (CA, USA). Digital IXUS 70 camera was supplied by Canon (NSW, Australia). Sodium chloride was supplied by Chemsupply (SA, Australia). RG 2000 Rotor-Gene Real Time Thermal Cycler and Rotor-Gene V4.6 software were purchased from Corbett Research (NSW, Australia). Parafilm was supplied by Crown Scientific (SA, Australia). Sigma 2-5 plate centrifuge was purchased from DJB Labcare (UK). HR-T film and autoradiography cassettes were purchased from Fuji Medical Systems (SA, Australia). Sephadex G-100, Whatman filter paper, Hybond N+ membranes, Rediprime II Random Prime Labelling System kit and Agarose NA were from GE Life Sciences (NSW, Australia). DNA Engine TETRAD® 2 thermal cycler was purchased from GeneWorks (SA, Australia).

Vector NTI® software, Platinum *Taq* DNA Polymerase, AccuPrime™ Pfx DNA Polymerase, pCR8 vector, LR Clonase™ II enzyme mix, Superscript III Reverse Transcriptase kit, oligo$(dT)_{12-18}$ primer and *E. coli* RNase H was purchased from

Invitrogen (VIC, Australia). Snomax was made by Johnson Controls (CO, USA). Agar was supplied by Jomar Diagnostics (SA, Australia). LEICA MZFLIII fluorescence stereomicroscope was purchased from Leica Microsystems (VIC, Australia). Pots were supplied by Masrac Plastics (SA, Australia). EDTA disodium salt, glucose, isopropanol and ethanol were supplied by Merck Chemicals (VIC, Australia). HOBO data logger was made by Onset Computer Corporation (MA, USA). P^{32} radiolabelled dCTP was purchased from Perkin Elmer (AUS). Plasmid pGEM-T Easy, T4 DNA ligase and dNTPs were purchased from Promega (WI, USA).

SYBR Green PCR master mix, SYBR Green I dye, QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit were purchased from Qiagen (VIC, Australia). Dextran sulphate was purchased from Quantum Scientific (QLD, Australia). The vortex was purchased from Ratek Instruments (VIC, Australia). Restriction enzymes and buffers were supplied by Roche Applied Science (NSW, Australia). Macherey-Nagel NucleoSpin® Extract II Kit and Agarose (Molecular Biology Grade) were purchased from Scientifix (VIC, Australia). GENE FLASH transilluminator was from Syngene Bio Imaging (MD, USA). Chromas 2.23 software was supplied by Technelysium Pty. Ltd. (www.technelysium.com.au).

Custom oligonucleotides (including Oligo$(dT)_{18}$) X-gal, dATPs, tris base, bromophenol blue, ampicillin, spectinomycin, kanamycin, ethidium bromide, phenol (pH 4.3), guanidine thiocyanate, ammonium thiocyanate, ammonium acetate, MOPS, powdered ABA, DNA-sodium salt, ethidium bromide, X-glucuronide, potassium ferrocyanide, potassium ferricyanide, BSA, sodium phosphate, magnesium sulphate, tri-sodium citrate, calcium chloride, dimethylformamide, Ficoll 400, polyvinyl-pyrrolidone360, sodium hydroxide and reagents for liquid hydroponic media were supplied by Sigma-Aldrich (NSW, Australia).

NanoDrop™ ND-1000 Spectrophotometer was from Thermo Scientific (USA). Yeast extract was supplied by US Biological (MA, USA). The Cary 50 Bio UV-Visible spectrophotometer, Hewlett-Packard 1090 liquid chromatograph machine and Helix

DNA column were from Varian, Inc. (CA, USA). The 90L insulated cooler was made by Wild Country (Thailand).

2.2.2. Cloning of *HvICE2*

2.2.2.1. Bioinformatics

The sequence of *AtICE1* (Accession number: AY_195621) was used in a 'tblastn' similarity search against the barley EST database at the website of The Institute for Genomic Research (www.tigr.org/tdb/tgi/plant.shtml) to identify the homologous gene from barley. The Scores and E values of the closest match, contig TC134022 (*HvICE2*; Appendix F.1), were examined and determined to be acceptable for interspecies comparison (Appendix B.1). To determine whether *HvICE2* was more similar to *AtICE1* than any other Arabidopsis gene, the sequence of the contig TC134022 was compared (tblastx) against the non-redundant nucleotide database at the NCBI website (www.ncbi.nlm.nih.gov/).

2.2.2.2. 5' RACE of *HvICE2*

A 5' RACE cDNA library was prepared from equal quantities of RNA (700 ng total) extracted from vegetative and floral tissues from *H. vulgare* L. cv. Haruna Nijo using the BD SMART™ RACE cDNA Amplification Kit according to the manufacturer's protocol. 5' RACE PCR reactions were performed using Immolase™ DNA Polymerase enzyme according to manufacturer's directions. Briefly, 2 µl of 10x ImmoBuffer (supplied), 0.8 µl of 50 mM $MgCl_2$ (supplied), 3.2 µl of dNTPs (5 mM), 1 µl of DMSO, 1 U (0.2 µl) of Immolase™ DNA Polymerase enzyme (supplied), 1-2 µl of forward and reverse primer solutions (10 µmol), template cDNA library and sterile MQ water was added to 0.2 ml tube to a total volume of 20 µl. In the primary round of PCR, 3 µl of 5' RACE cDNA library was used as template. In the secondary round the template was 1 µl of a 1/30 dilution of the primary round of PCR. The gene-specific and generic primers used for each round of nested PCR are shown in Table 2.1. PCR reactions were performed in a DNA Engine TETRAD® 2 thermal cycler. Cycling parameters for the primary and secondary rounds of PCR were as follows: 95°C for 7 min, then 35 cycles

of 94°C for 10 sec, 60°C annealing for 30 sec followed by 68°C extension for 1 min and 40 sec, followed by one cycle of 72°C for 10 min.

Table 2.1 Primers for PCR amplification of *HvICE2* 5' region, full-length CDS and promoter.

Experiment	Round of PCR	Primer Pairs	Expected Size of Product	Sequencing Vector and Primers
HvICE2 5' RACE	Primary	Generic: UPM A mix Gene-specific: ICE_noTAG_R	Unknown	N/A
	Secondary	Generic: UNP Gene-specific: ICE_R7 or ICE_R3	Unknown	pGEM-T Easy: SP6 and T7 primers
HvICE2 full-length coding region	Primary	ICE_F0 : ICE_R0	1,512 bp	N/A
	Secondary	ICE_ATG_F : ICE_wTAG_R	1,140 bp	pCR8: GW1 and GW2 primers
HvICE2 genomic sequence	Primary	ICE_F0: ICE_R0	1,512 bp	pGEM-T Easy: SP6, T7 and ICE_R4 primers
HvICE2 Promoter Isolation: Genomic Walking Round I	Primary	AP1: ICE_R9	Unknown	N/A
	Secondary	AP2: ICE_R7	Unknown	pGEM-T Easy: SP6 and T7 primers
HvICE2 Promoter Isolation: Genomic Walking Round II	Primary	AP1: ICE_GW_R1	Unknown	N/A
	Secondary	AP2: ICE_GW_R2	Unknown	pGEM-T Easy: SP6 and T7 primers
HvICE2 Promoter Isolation: Genomic Walking Round III	Primary	AP1: ICE_GW_R4	Unknown	N/A
	Secondary	AP2: ICE_R6	Unknown	pGEM-T Easy: SP6 and T7 primers
HvICE2 Promoter Isolation: 3 kb PCR	Primary	ICE_PR_F1: ICE_R1	3,365 bp	N/A
	Secondary	ICE_PR_F2: ICE_PR_R1	3,215 bp	pCR8: GW1, GW2, ICE_PR_F4, ICE_PR_F3, ICE_GW_R3, ICE_GW_R4.

Primer names and expected sizes of the products for each round of PCR during amplification of the 5' region of the mRNA, the full-length coding region and the promoter of *HvICE2*. Primer sequences may be found in Appendix A.

Electrophoresis of PCR products was performed using agarose gels to separate the DNA according to its molecular weight. Prior to loading, 0.1 volume of loading dye (0.25% bromophenol blue w/v, 0.25% xylene cyanol, 30% glycerol) was added to the DNA. Agarose gels of 1-2% w/v were prepared in TAE buffer (1 mM EDTA buffer containing 4.9% Tris base w/v and 1.142% glacial acetic acid w/v) with the addition of 40 µg ml^{-1} of ethidium bromide. DNA was visualised under UV light using a transilluminator. Products of interest were cut from the gel and purified using a QIAquick Gel Extraction Kit according to the manufacturer's protocol. The concentration of DNA in the sample was quantified by spectrophotometry of neat samples at 260 nM using a NanoDrop™ ND-1000 spectrophotometer or examined by electrophoresis of 2 µl of the purified reaction product.

2.2.2.3. Cloning of PCR Fragments into pGEM-T Easy

Purified DNA fragments were ligated into the pGEM-T Easy vector for sequencing. Ligations were generally performed in a 10 µl volume reaction containing 2-3.5 µl of purified DNA fragment, 5 µl of 2X Rapid Ligation Buffer (supplied), 1 µl of pGEM-T Easy vector (10 ng $µl^{-1}$) (Figure 2.1), 0.5-1 µl of T4 DNA ligase and water (if required) to a total volume of 10 µl and ligations were incubated at room temperature for between 1.5 hrs and overnight.

2.2.2.4. Chemical Transformation of *E. coli*

E. coli cells were transformed by chemical transformation. Purified ligation reaction and 100 µl of frozen chemically competent DH5α cells were placed on ice to thaw. The ligation reaction was added to the cells and incubated on ice for 15-30 min. The mixture was placed in a water bath at 42°C for 1.5 min, on ice for 2 min and immediately resuspended in 1 ml of LB media (1% NaCl w/v, 1% tryptone w/v, 0.5% yeast e xtract w/v, pH 7.0) and was placed in a 37°C incubator with shaking for 1 hour. The cells were plated onto selective LB 1.5% w/v agar plates (1% NaCl w/v, 1% tryptone w/v, 0.5% yeast extract w/v, pH 7.0) containing 100 µg ml^{-1} ampicillin and 100 µl of 20 mg ml^{-1} X-gal and incubated at 37°C overnight.

Figure 2.1 pGEM®-T Easy Vector map and sequence reference points.
The PCR fragments were ligated between the 3' T overhangs to disrupt the *LacZ* gene (www.promega.com/vectors/t_vectors.htm).

2.2.2.5. Plasmid DNA Mini-preparations

White colonies were picked from overnight plates and transferred to 10 ml tubes containing 5 ml of LB media and ampicillin at 100 µg ml^{-1} and placed in a 37°C shaking incubator for 16 hrs. Plasmid DNA was isolated from 5 ml of overnight culture using a QIAprep Spin Miniprep Kit according to the manufacturer's protocol.

Restriction digests of plasmid DNA were generally carried out at 37 °C for 2 hrs in a volume of 20 µl, containing 5 U (0.5µl) of *Eco*RI, 2 µl of 10x Buffer H (supplied), 4 µl of plasmid DNA and 13.5 µl of sterile MQ water. Digestion products were separated according to their molecular weight in 1% w/v agarose gels containing ethidium bromide.

2.2.2.6. Nucleotide Sequence Analysis and Manipulation

Plasmid DNA sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit. Reactions were set up in 10 µl volumes containing 1 µl of Ready Reaction Premix (supplied), 1.5 µl of BigDye Sequencing Buffer (supplied), 0.5-1 µl of appropriate primer (10µM; Table 2.1), 1-2 µl of DNA template and water to 10 µl. Cycling parameters were as follows: 96°C for 30 sec, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. After cycling, sequencing reactions were precipitated using a magnesium sulphate/ethanol solution. Briefly, 75 µl of room temperature 0.2 mM $MgSO_4$ solution (70% v/v absolute ethanol and 0.02% v/v 1M $MgSO_4$) was added to the reaction, mixed by vortexing and incubated at room temperature for a minimum of 15 min to precipitate. The samples were pelleted by centrifugation at 13,000 rpm for 15 min and the supernatant was carefully discarded. The remaining pellet was dried at 37°C before being submitted for sequence reading to the Australian Genome Research Facility (SA, Australia).

DNA sequence analysis was performed using Chromas software (to visualise chromatograms) and further analyses, alignments and manipulation were performed using VectorNTI® software including ContigExpress® and AlignX® programs. The primer analysis functions from the Vector NTI® suite of programs were used to aid primer design. Database searches were performed using BLAST software (Altshul *et al*., 1990) accessed *via* the NCBI website (www.ncbi.nlm.nih.gov/BLAST/). Predicted domains were identified using the Conserved Domains Database during the course of protein BLAST searches at the NCBI website. Predicted protein molecular mass and pI were calculated using the pepstats software at the Mobyle portal (mobyle.pasteur.fr/cgi-bin/portal.py). Potential sumoylation sites were predicted using SUMOsp software (sumosp.biocuckoo.org/index.php).

2.2.2.7. PCR Cloning of the Coding Region of *HvICE2*

PCR primers were designed and used to amplify the complete coding region of *HvICE2* from Haruna Nijo cDNA populations produced from cold treated vegetative and floral tissues kindly supplied by Ms. Vanessa Richardson (The University of Adelaide). The

enzyme AccuPrime™ Pfx DNA Polymerase has proofreading capabilities and was used to ensure sequence integrity. Nested PCR was performed as described in the manufacturer's protocol. Briefly, the primary reactions using AccuPrime™ polymerase were performed in a total volume of 25 µl containing 2.5 µl of 10x AccuPrime™ Pfx Rxn Mix (supplied), 1 µl of 10 µmol forward and reverse primer solutions (Table 2.1), 0.25 µl of AccuPrime™ Pfx Polymerase (supplied) and sterile MQ water. PCR cycling parameters: 95°C for 2 min, 35 cycles of 95°C for 15 sec and annealing at 60°C for 30 sec and extension of 68°C for 2 min. The secondary reaction was performed as described above with a template of 1 µl of 30-fold dilution of the primary PCR reaction. The PCR products were electrophoresed and purified as described in Section 2.2.2.2.

2.2.2.8. TOPO® Cloning of *HvICE2* into pCR8

Using the Immolase™ DNA Polymerase enzyme, an adenine nucleotide was added to the 3' end of each strand of the purified DNA fragment to facilitate cloning into pCR8 vector (Figure 2.2) which has a single base pair thymine overhang on each end of the vector. To an 0.2 µl Eppendorf tube was added 7 µl of purified PCR product, 2 µl of 10x ImmoBuffer (supplied), 0.8 µl of 50 mM $MgCl_2$ (supplied), 3.2 µl of dATPs (1.25 mM), 5 U (1 µl) of Immolase™ DNA Polymerase enzyme (supplied) and sterile MQ water to a total volume of 20 µl. The reaction was incubated for 1.5 hrs at 5°C and the DNA was purified using a NucleoSpin® Extract II kit according to the manufacturer's protocol.

The purified PCR products were ligated by TOPO® Cloning into pCR8 according to manufacturer's instructions. Briefly, 1 µl of purified PCR product, 1 µl of salt solution (supplied), 0.5 µl of TOPO® Cloning vector pCR8 (supplied) and 3.5 µl of sterile MQ water were added to a 0.2 µl Eppendorf tube and incubated for 30 min at room temperature. The products were transformed into *E. coli via* chemical transformation and cells were spread onto selective LB agar plates containing spectinomycin at 100 µg ml^{-1} as described in Section 2.2.2.4. Plasmid DNA was isolated and sequenced as described in Sections 2.2.2.5 and 2.2.2.6 using vector-specific primers (presented in Table 2.1). Two sequencing reactions were performed with each primer and the results were compared to *HvICE2* by alignment as described in Section 2.2.2.6.

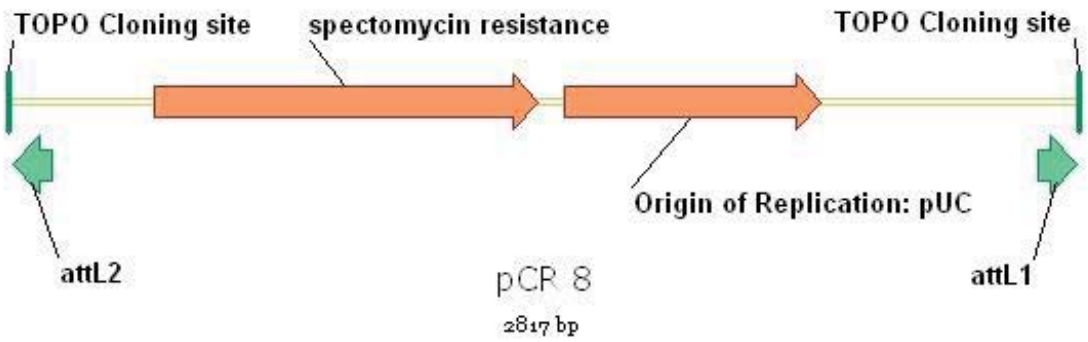


Figure 2.2 Vector map of pCR8.

attL1 and attL2 are recombination sites. The cloned fragment was ligated to each of the two TOPO cloning sites, circularising the vector.

2.2.2.9. PCR Cloning of the Genomic Sequence of *HvICE2*

PCR primers were designed to amplify the *HvICE2* coding region and included introns from genomic DNA. PCR was performed as described in Section 2.2.2.2 with the following modifications: One round of PCR was performed using 320 ng of *H. vulgare* L. cv. Haruna Nijo genomic DNA as template and 0.4 µl of 10 µmol forward and reverse primer stocks (Table 2.1). The annealing temperature of the PCR cycling parameters was modified to 58°C, and extension time to 3 min and 30 sec. DNA fragments were separated by electrophoresis, visualised on a transilluminator and fragments of expected size were excised, purified, ligated into pGEM-T Easy and transformed into *E. coli* as described in Sections 2.2.2.2, 2.2.2.3 and 2.2.2.4. Plasmid DNA was isolated and restriction digestion and sequencing using SP6 and T7 primers was performed (Appendix A) as described in Sections 2.2.2.5 and 2.2.2.6. Alignment of the *HvICE2* genomic nucleotide sequence with the sequences of *AtICE1* (Accession number: NC_003074 REGION: 9833926..9836484) and *OsICE1* (*bHLH116*) (Accession number: NC_008394 REGION: 40706573..40709471) was performed as described in Section 2.2.2.6.

2.2.2.10. Cloning of the *HvICE2* Promoter by PCR-based Genomic Walking

This method was based on that by Siebert *et al*. (1995). The genomic walking 'libraries' used in this study were kindly provided by Ms. Alison Hay (The University of Adelaide). These libraries consisted of restricted *H. vulgare* L. cv. Sahara genomic DNA fragments ligated to 'adaptor' DNA fragments. The libraries were restricted using the enzymes *Dra*I, *Eco*RV, *Nae*I, *Nru*I, *Pml*I, *Pvu*II, *Ssp*I and *Stu*I. AP1 and AP2 primers (Appendix A) (BD GenomeWalker™ Kits User Manual) were used in a nested PCR approach as forward primers. Gene specific reverse primers were designed at the 5' end of the target cDNA sequence using primer design and analysis programs from the Vector NTI® suite of as described in Section 2.2.2.6. The internal gene-specific primers were designed to ensure greater than 150 bp of sequence from the genomic walk would overlap with the known sequence.

Two rounds of nested PCR amplification was performed using Immolase™ enzyme mix according to the BD Genome Walker™ Kits User Manual. Briefly, primary PCR amplification was performed as described in Section 2.2.2.2 using 25 µl total reaction volumes and component volumes scaled up accordingly. Reactions contained 1 µl of forward primer (10 µmol) and 1 µl of gene-specific reverse primer (10 µmol) and 2 µl of DNA library as template. Primers used are presented in Table 2.1. Primary PCR reactions were diluted 50-fold in sterile water for use in the second round of PCR. Secondary PCR amplification was performed as described above with the use of 1 µl of a 50-fold dilution of the first round PCR products as template. PCR cycling parameters for the primary rounds of PCR were as follows: 7 cycles of 94°C for 25 sec and 72°C for 4 min, followed by 32 cycles of 94°C for 25 sec and 68°C for 4 min, followed by 68°C for 4 min. Cycling parameters for the secondary rounds of PCR were as follows: 5 cycles of 94°C for 25 sec and 72°C for 4 min, followed by 22 cycles of 94°C for 25 sec and 68°C for 4 min, followed by 68°C for 4 min. DNA fragments from each round were separated by electrophoresis, visualised on a transilluminator and fragments of >300 bp were excised, purified, ligated into pGEM-T Easy and transformed into *E. coli* as described in Sections 2.2.2.2, 2.2.2.3 and 2.2.2.4. Plasmid DNA was isolated and restriction digestion and sequencing using SP6 and T7 primers was performed (Appendix A) as described in Sections 2.2.2.5 and 2.2.2.6.

PCR, cloning and sequencing methods were repeated as above for second and third rounds of genomic walking using nested forward and gene-specific primers (Table 2.1) which were designed to the 5' region of the promoter obtained from the previous round of genomic walking. The sequences of the first, second and third rounds of genomic walking were assembled to make a continuous sequence as described in Section 2.2.2.6.

2.2.2.11. PCR Amplification and Cloning of the *HvICE2* Promoter

PCR amplification using nested primer sets was employed to amplify the promoter region of *HvICE2*. Nested PCR was performed with high fidelity Platinum *Taq* DNA polymerase enzyme according to the manufacturer's instructions. Briefly, primary reactions contained 2 µl of 10x PCR Buffer Minus Mg (supplied), 3.2 µl of dNTPs (5mM), 0.6 µl of 50 mM $MgCl_2$ (supplied), 0.5 µl of 10 µmol forward and reverse primers, 1 U (0.2 µl) of Platinum *Taq* DNA Polymerase, 320 ng of *H. vulgare* L. cv. Haruna Nijo genomic DNA as template and sterile MQ water to a final volume of 20 µl. The secondary PCRs were performed as above in 25 µl final volumes with the volumes of the components adjusted accordingly and 1µl of 50-fold diluted primary reaction as template. The primers used for the primary and secondary amplification of the promoter region of *HvICE2* are presented in Table 2.1.

PCR reactions were performed in a DNA Engine TETRAD® 2 thermal cycler. Cycling parameters for the primary and secondary rounds of PCR were as follows: 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 60°C annealing for 30 sec followed by 72°C extension for 4 min, followed by one cycle of 72°C for 10 min. The secondary reaction products were electrophoresed and fragments were purified from the gel and quantified as described in Section 2.2.2.2

The purified PCR products were ligated by TOPO® Cloning into pCR8 (Figure 2.2) as described in Section 2.2.2.8 using ~110 ng of purified PCR product. The products were transformed into *E. coli via* chemical transformation and cells were spread onto

selective LB Agar plates containing spectinomycin at 100 µg ml^{-1} as described in Section 2.2.2.4. Plasmid DNA was isolated and digested using *Mlu*I restriction enzyme as described in Section 2.2.2.5. Plasmid DNA sequencing and sequence analysis was performed as described in Section 2.2.2.6 using sequencing primers presented in Table 2.1. Two sequencing reactions were performed with each primer. To confirm the correct sequence had been cloned, the clone sequence was aligned with published sequence of *HvICE2* and the sequences of the products obtained in the first, second and third rounds of genomic walking (as described in Section 2.2.2.6).

2.2.3. Analysis of *HvICE2*

2.2.3.1. *HvICE2* Promoter Nucleotide Sequence Analysis

The sequence of the *HvICE2* 5' region, obtained from the assembled genomic walking sequences, (described in Section 2.2.2.10) was analysed to identify potential *cis*-elements by performing a PLACE Signal Scan search using the PLACE (Database of Plant *Cis*-acting Regulatory DNA Element) program (www.dna.affrc.go.jp/PLACE/). The annotations of the resulting elements were studied and elements which were reported as being involved in cold, salt, dehydration or ABA responses were recorded.

2.2.3.2. Production of Barley Cold Stress Treatment Series

2.2.3.2.1. Cultivation, Stress Treatment and Sampling of *Hordeum vulgare* L. cv. Golden Promise and Haruna Nijo

Barley plants of the frost-sensitive cultivar Golden Promise and comparatively cold-tolerant cultivar Haruna Nijo were germinated on filter paper in petri dishes and transplanted into six inch pots in coco peat potting mix with three plants per pot. Plants were grown to anthesis in Growth Chamber 1 at AGRF (SA, Australia) under a 10 hr light at 20°C/ 14 hr dark at 8°C cycle for the first four weeks; then a 12 hr light at 21°C/ 12 hr dark at 10°C cycle for the next four weeks; then a 14 hr light at 22°C/ 10 hr dark at 12°C cycle for the remainder of the experiment. Relative humidity was not controlled.

At anthesis, plants were exposed to subzero temperatures to simulate a radiative frost event in the AGRF frost chamber (SA, Australia). Temperature probes in various positions in the chamber were used to log the temperature throughout treatment. The timing of sampling was determined by the temperature readings from probes placed at floret height. Details of the temperature regime are presented in Appendix C. Flag leaves and whole spikes were sampled just prior to plants entering the frost chamber; when the temperature at floret height had dropped initially to 4°C; when the temperature had been held at the minimum treatment temperature of -5.5°C for 2 hrs; when the temperature increased to 4°C; and at 48 hrs after frost. Control plants remained in the growth room during treatment (under the conditions described above) and were harvested within 20 min of the treated samples being taken. Five plants were sampled per time point per treatment for each tissue type. This experiment was performed with the assistance of Ms. Alexandra Smart (ACPFG) and Dr. Juan Juttner (ACPFG).

2.2.3.2.2. RNA Extraction

Total RNA was isolated from control and cold-treated barley plants. The frozen tissues were ground to a powder under liquid nitrogen using a sterilised mortar and pestle or by vortexing the tissue in 10 ml plastic tubes containing four 5 mm ball bearings. Ground tissue (~100 mg) was mixed with 1 ml of Trizol-like reagent (38% v/v phenol (pH 4.3), 11.8% w/v guanidine thiocyanate, 7.6% w/v ammonium thiocyanate, 3.3% v/v 3 M sodium acetate (pH 5.0) and 5% v/v glycerol) and tubes were placed on a rotating wheel for 5 min at room temperature. The homogenate was centrifuged for 10 min at 11,000 x g at 4°C to pellet cellular debris, the supernatant was retained and 0.2 ml of chloroform was added. The samples were shaken vigorously for 15 sec and incubated for 3 min at room temperature before being centrifuged for 15 min at 11,000 x g. The aqueous phase was retained and 0.5 ml of isopropanol was added to the supernatant. The sample was incubated for 10 min at room temperature. After centrifugation for 10 min at 11,000 x g, the supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. Samples were centrifuged for 5 min at 7,400 x g at 4°C and the supernatant was removed. Samples were dried at 37°C until RNA pellets became transparent (~5 min) and were dissolved in 20 µl of double autoclaved sterile MQ water before being stored at -80°C.

The quality and concentration of the RNA was verified by electrophoresis on denaturing agarose gels containing formamide and formaldehyde to separate and visualise the integrity of the ribosomal RNA. The horizontal gel tank was sprayed with RNAse Zap and rinsed three times with MQ water. Gels were prepared by dissolving 1.85 g of agarose in 95 ml of sterile MQ water (autoclaved twice) and cooling to 55°C before adding 12.5 ml of 10x MOPS buffer (0.2 M MOPS buffer, pH 7.0 containing 50 mM sodium acetate and 10 mM EDTA) and 20 ml of formaldehyde (37%). Gels were pre-run for 30 min at 50 V in 1 x MOPS buffer. Prior to loading, 15 µl of loading mix (12% v/v 10x MOPS buffer, 21% v/v formaldehyde (37%), 60% v/v formamide, 1% v/v 10 mg ml^{-1} ethidium bromide, 6% v/v loading dye) was added to 0.75 µl RNA, the samples were incubated at 65°C for 10 min and transferred to ice. Gels were electrophoresed at 80 V for 2 hrs and the RNA was visualised under UV light using a transilluminator.

RNA extraction and electrophoresis was performed with the assistance of Ms. Alexandra Smart. The concentrations of the RNA were determined by spectroscopy of neat samples at 260 nm using a NanoDrop™ ND-1000 spectrophotometer.

2.2.3.3. Production of Barley Salt Stress Treatment Series

Clipper x Sahara double haploid line 134 from the Clipper x Sahara 3771 mapping population was used as the salt tolerant cultivar (Karakousis *et al.*, 2003). This line contains the chromosome 2H, 3H, 4H and 6H boron tolerance loci and the chromosome 1H salt exclusion locus. Golden promise plants were used as the salt sensitive control cultivar. Sterilised barley seeds were germinated on filter paper in Petri dishes and transplanted into a supported hydroponics system. Cylindrical plastic pipes (approximately 28 cm long and 4 cm diameter) were filled with plastic beads and were placed into pre-drilled holes in the lid of a black plastic tub as indicated in Figure 2.3. The pipes were sealed at the base with netting to allow nutrient solution to seep through to the roots. For the control and treated samples, 84 plants were grown in two tubs fed by 80 L of nutrient solution (Appendix D.1). One barley grain was planted in each tube

~2 cm deep. To provide aeration to the roots, a pump was set up with a timer to alternately drain and flood the plastic tub with nutrient solution at 20 min intervals over the entire course of plant growth. The nutrient solution was replaced fortnightly and the pH was adjusted to 6.5 using sodium hydroxide (1 M). Plants were grown in Growth Chamber 1 in the undercroft area at the University of Adelaide (SA, Australia) facilities under a 14 hr light cycle (700 µmol m s^{-2}) at 22°C and ten hr dark cycle at 16°C. Relative humidity was not controlled.

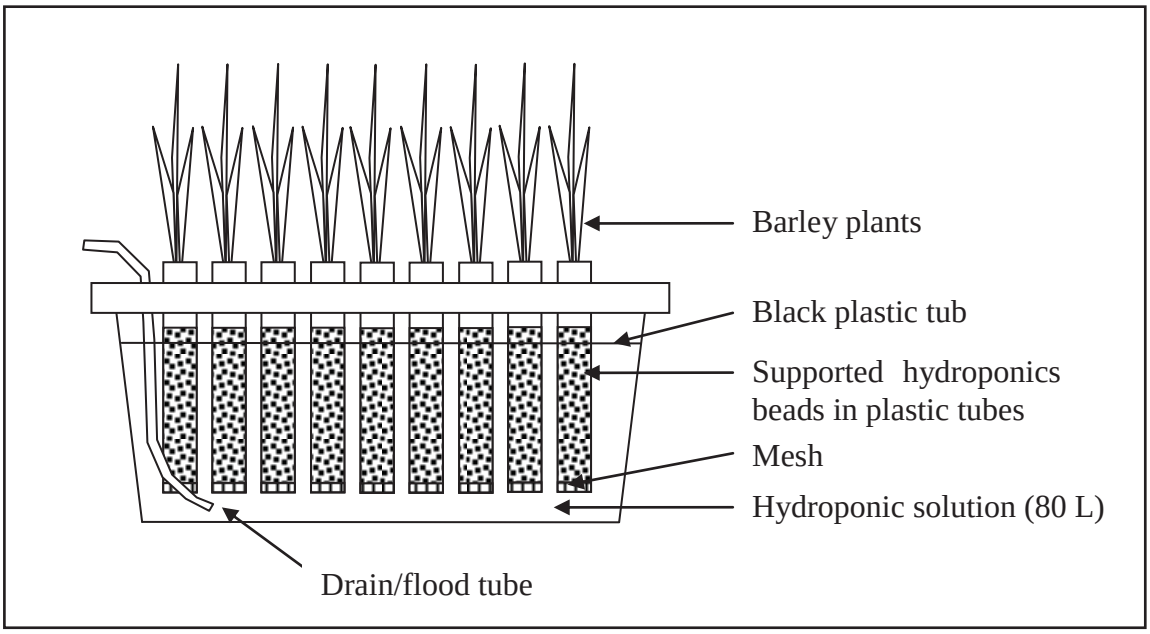


Figure 2.3 Schematic diagram of plant hydroponic layout.
Barley plants were grown as shown with 42 plants per tub.

Stress treatment and sampling commenced ten days after the seeds were placed into the hydroponics system. Salt treatment was performed by replacing the nutrient solution with fresh solution containing 25 mM NaCl (and the corresponding amount of calcium chloride) where the nutrient solution for the control plants was replaced with unaltered fresh solution. Subsequently, salt was added twice daily to the 'treated' plants at 10 AM and 4 PM to reach a final maximum concentration of 150 mM on the third day. At each treatment point where the new NaCl concentration was less than or equal to 100 mM, the $CaCl_2$ concentration was increased by 0.33 mM. For the final two additions of NaCl, the $CaCl_2$ concentration was increased by 0.25 mM.

Plants were grown for a further 11 days. The root and leaf tissue of three plants were sampled as shown in Figure 2.4 at 2 pm on each of 0, 1, 2, 3, 5, 7, 10 and 14 days after treatment. These tissues were immediately snap frozen in liquid nitrogen and stored at -80°C until required. Tissues were ground under liquid nitrogen and RNA was extracted and examined for quality and concentration by electrophoresis as described in Section 2.2.3.2.2. The salinity-stress treatment series was produced with the assistance of Ms. Alexandra Smart.

Figure 2.4 Picture indicating sites that tissue samples were taken from.
Leaf samples were taken from approximately 0.5 cm above the crown, as indicated. The lower tissue was taken as root samples.

2.2.3.4. Production of Barley ABA Treatment Series

The barley ABA treatment series was produced by Ms. Alexandra Smart (ACPFG). *Hordeum vulgare* L. cv. Golden Promise plants were grown in a supported hydroponics system as described in Section 2.2.3.3. Sterilised seeds were pre-germinated on filter paper in petri dishes, transplanted into the supported hydroponics system and grown for ten days. The ABA treatment was applied by replacing the nutrient solution with fresh solution containing dissolved powdered ABA to a final concentration of 10 µM. Root and leaf tissues were sampled from five plants at each of 0, 0.5, 1, 2, 4, 8, 12 and 24 hrs after the addition of ABA. Tissues were ground under liquid nitrogen and RNA was extracted as described in Section 2.2.3.2.2.

2.2.3.5. Production of cDNA from the Barley Stress Series for qRT-PCR

cDNA synthesis was performed using the RNA extracted from the cold- and salinity-stress treatment series described in Sections 2.2.3.2 and 2.2.3.3; and the ABA treatment series described in Section 2.2.3.4. The cold treatment cDNA synthesis and control PCR reactions were performed with the assistance of Ms. Alexandra Smart. The salinity and ABA treatment series cDNA synthesis and control PCR reactions were performed by Ms. Alexandra Smart.

cDNA synthesis reactions were performed using the SuperScript™ III Reverse Transcriptase kit in a 96 well plate as described in the manufacturer's instructions. Briefly, total RNA (2 µg) was combined with 500 ng of oligo$(dT)_{12\text{-}18}$ primer and 1 µl of 10 mM dNTPs (supplied) and RNase-free water to a total volume of 13 µl. The sample was incubated at 65°C for 5 min in a water bath and immediately placed on ice for at least 1 min. Reverse transcription master mix (7 µl) was added containing 4 µl of 5x first strand buffer (supplied), 1 µl of 0.1 M DTT (supplied), 0.5 µl RNaseOUT (supplied) and 0.35 µl (70 units) of SuperScript™ III RT. cDNA synthesis reactions were performed at 50°C for 1 hr and the reaction was terminated by incubation for 15 min at 70°C. Five cDNA syntheses were carried out per RNA sample and PCR of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used to determine the success of the cDNA synthesis (HvGAPDH_F and HvGAPDH_R primers; Appendix A). The reactions were performed as described in Section 2.2.2.2 in 10 µl total volumes with reaction component volumes adjusted accordingly. The primers used were HvGAPDH_F (forward) and HvGAPDH_R (reverse) primers (Appendix A) and 1 µl of neat cDNA as template. To screen for genomic DNA contamination, control *GAPDH* PCR reactions were performed as described above using 1 µl of 20-fold dilution of total RNA as template. Cycling parameters for all *GAPDH* PCRs were as follows: 95°C for 7 min, then 30 cycles of 95°C for 45 sec, 55°C annealing for 50 sec followed by 72°C extension for 40 sec, followed by one cycle of 72°C for 5 min. Five successful cDNA synthesis reactions were pooled and the cDNA was stored at -20°C.

2.2.3.6. Expression Analysis of *HvICE2 via* qRT-PCR

To analyse the transcript levels of *HvICE2*, qRT-PCR was performed as described in Burton *et al*. (2008), in consultation with Dr. Neil Shirley (The University of Adelaide). Prior to detailed investigation of the expression *HvICE2*, samples of the PCR product were purified by HPLC using a liquid chromatograph machine and a Helix DNA column, and sequenced (as described in Section 2.2.2.6), to confirm that the primers hybridised specifically. PCR products and primers of control genes (*HvCyclophilin*, *HvGAPDH*, *Hvα-tubulin*, *HvHeat shock protein 70* and *HvActin*) were kindly provided by Dr. Neil Shirley. The primers used to analyse the endogenous expression of *HvICE2* or the expression of the control genes are presented in Table 2.2 (Appendix A). A melt curve was obtained from the product following cycling by heating from 70°C to 99°C and was used to detect the presence of any non-specific sequences. Data was analysed using Rotor-Gene software.

Table 2.2 Primers used for qRT-PCR analysis of *HvICE2* expression in barley.

Gene	Forward Primer	Reverse Primer	Expected product size
Endogenous *HvICE2*	QPCR_ICE1_F2	QPCR_ICE1_R2	126 bp
HvICE2 transgene only	HvICE2_TX_F3	NOSTERM_R2	195 bp
HvCyclophilin	HvCyclophilin_F	HvCyclophilin_R	122 bp
HvGAPDH	HvGAPDH_F	HvGAPDH_R	198 bp
Hvα-tubulin	HvTubulin_F	HvTubulin_R	248 bp
HvHeat shock protein 70	HvHSP 70_F	HvHSP70_R	108 bp
HvActin	HvActin_F	HvActin_R	201 bp

Primer sequences are presented in Appendix A.

2.2.3.7. Expression Analysis of *HvICE2 via* Microarray Data Analysis

The barley EST contig sequence was analysed by performing a standard nucleotide-nucleotide BLAST search against the barley Affymetrix chip using the Barleybase suite of programs (‘Barley1’ database) (www.barleybase.org/). Analysis of the alignments, Scores and E values (Scores and E values presented in Appendix B.2) identified a single contig, Contig13678_at. The expression data for this contig was retrieved from the Affymetrix chip database and the information was presented graphically. Information

about the tissues sources of RNA used to probe the microarray was obtained from the website http://bioinf.scri.sari.ac.uk/affy/WEB_TISSUES/tissue_ types.htm.

2.2.3.8. Expression Analysis of *HvICE2 via* Analysis of *HvICE2 Promoter:Reporter Gene* Transgenic Plants

2.2.3.8.1. Production of *GUS* and *GFP* Expression Clones Driven by the *HvICE2* Promoter

Gateway® technology was used to transfer the *HvICE2* promoter from the pCR8 plasmid (Gateway® Entry vector) to the pMDC164 and pMDC107 plasmids (Gateway® Destination vectors) containing *GUS* and *GFP6* reporter genes respectively (Curtis and Grossniklaus, 2003). LR recombination reactions were performed to generate expression clones in which the *HvICE2* promoter was functionally linked to *GUS* or *GFP* and used to drive expression of the reporter gene *in planta*. The LR reactions were performed according to manufacturer's protocol. Briefly, 140 ng of pCR8/*HvICE2* promoter (Entry clone; Section 2.2.2.11) and 150 ng of pMDC164 or pMDC107 (Destination vector) and sterile 1x TE buffer (10 mM Tris-HCl buffer, pH 8.0 containing 1 mM EDTA) was added to a total volume of 8µl in an 0.2 ml Eppendorf tube and mixed. The LR Clonase™ II enzyme mix (supplied) was thawed on ice for ~2 min, vortexed briefly and 2 µl was added to the reaction. The reaction was vortexed briefly, centrifuged briefly and incubated at 25°C for 1 hr in a DNA Engine TETRAD® 2 thermal cycler. 1 µl of Proteinase K solution (supplied) was added, vortexed briefly and incubated at 37°C for 10 min to terminate the reaction.

A 1µl aliquot of the LR reaction was transformed into *E. coli* by chemical transformation and plated onto selective LB agar plates containing kanamycin at 50 µg ml^{-1} as described in Section 2.2.2.4. Plasmid DNA was isolated as described in Section 2.2.2.5. To confirm correct recombination had occurred, diagnostic digestion and electrophoresis of plasmid DNA was performed as described in Section 2.2.2.5 using 5 µl of pMDC164/*HvICE2 promoter:GUS* clone DNA, digested with *Mlu*I in Buffer H (supplied); and 5 µl of pMDC107/*HvICE2 promoter*:*GFP6* clone DNA, digested with *Acc*I in Buffer A (supplied).

2.2.3.8.2. Barley Transformation and Growth of Transgenic Plants

Agrobacterium tumefaciens-mediated barley transformation was performed by Dr. Rohan Singh and Ms. Konny Oldach using the procedure developed by Tingay *et al*. (1997) and modified by Matthews *et al*. (2001). The T_0 generation and control Golden Promise barley plants were grown in a glasshouse in six inch diameter pots with one plant per pot.

2.2.3.8.3. Isolation of Genomic DNA from Transgenic Barley Plants

Leaf tissue was harvested from T_0 and wild type Golden Promise plants and ground to powder under liquid nitrogen by adding two 6 mm and two 4 mm ball bearings to each plastic 10 ml tube and shaking vigorously in a paint shaker for 15-30 seconds. Samples were shaken 2-4 times and the tissue was kept frozen by immediately placing the tubes in liquid nitrogen between passes. Care was taken to ensure the tissue did not thaw at any stage in the process.

To extract genomic DNA from the barley tissue, ~10-50 mg of ground tissue was aliquotted into 1.1 ml strip tubes, allowed to warm to room temperature and 600 µl of extraction buffer (0.1 M Tris-HCl buffer, pH 7.5 containing 0.05 M EDTA and 1.25 % SDS w/v) was added. The tubes were sealed and shaken thoroughly to homogenise the tissue in the solution. The tubes were incubated at 65°C in an oven for 30 min and placed at -20°C for approximately 15 min. After the addition of 300 µl of 6 M ammonium acetate, the tubes were shaken well and incubated at 4°C for 15 min. The tubes were centrifuged in a Sigma 2-5 plate centrifuge for 15 min at 4000 rpm, the supernatant was retained and 360 µl of isopropanol was added. The samples were mixed and incubated at room temperature for 5 min to precipitate the DNA before pelleting the DNA by centrifuging for 15 min at 4000 rpm. The supernatant was carefully drained and the pellets were washed with 300 µl of 70% v/v ethanol. The tubes were centrifuged for 3-8 min at 7,400 x g, the ethanol was drained and the pellet was dried at 37°C (approx 30 min). The DNA pellet was resupended in 35 µl of Tris-EDTA + RNase (10 mM Tris-HCl buffer, pH 8.0 containing 5 µl of RNase H and 1 mM

EDTA (pH 8.0)) by incubation at 4°C overnight. Samples were mixed by gently flicking the tube prior to use.

2.2.3.8.4. PCR Analysis of *HvICE2 Promoter*:*Reporter* Transgenic Barley Plants

The insertion of the *HvICE2 promoter*:*reporter* constructs was confirmed by PCR on genomic DNA extracted from the T_0 barley plants. The PCR was performed using high fidelity Platinum *Taq* DNA polymerase enzyme according to the manufacturer's instructions. Briefly, reactions contained 5 µl of 10x PCR Buffer Minus Mg^{2+} (supplied), 8 µl of dNTPs (5mM), 1.5 µl of 50 mM $MgCl_2$ (supplied), 1 µl of 10 µmol forward and reverse primers, 1 U (0.2 µl) of Platinum *Taq* DNA Polymerase, 0.5 µl of neat genomic DNA as template and sterile MQ water to a final volume of 50 µl. The primers were GFPiF (forward) and GFPiR (reverse) for the transgenic plants containing the pMDC107/*HvICE2 promoter* construct and GUS_F (forward) and GUS_R (reverse) for plants containing the pMDC164/*HvICE2 promoter* construct. Cycling parameters were as follows: 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 55°C annealing for 30 sec followed by 72°C extension for 30 (pMDC107/*HvICE2 promoter* lines) or 60 sec (pMDC164/*HvICE2 promoter* lines), followed by one cycle of 72°C for 10 min. Control reactions were prepared containing 0.5 µl of a 50-fold dilution of the respective plant transformation construct plasmid DNA (isolated in Section 2.2.3.8.1; positive control), wild-type Golden Promise genomic DNA (negative control) or no template (negative control). The reaction products were electrophoresed as described in Section 2.2.2.2.

2.2.3.8.5. Analysis of Reporter Gene Expression in Transgenic Barley Plants

Transgenic barley plants in which the *HvICE2* promoter was functionally linked to the *GUS* or *GFP* gene were grown for analysis the expression of the reporter gene before and after cold treatment. Wild type and transgenic plants containing *GFP* as the reporter gene were germinated and grown on Petri dishes for 7 days. Cold-stress treatment was applied by placing the plants at 4°C for 48-52 hrs in the dark. The *GFP*

reporter gene plants were examined for expression of *GFP* before and immediately after treatment. For practical purposes, the plants were removed from the treatment in small batches to ensure expression of *GFP* was examined as quickly as possible after cold treatment. All plants were examined within one hour of being removed from cold treatment. The expression of *GFP* was determined using a LEICA MZFLIII fluorescence stereomicroscope. The settings were as follows: Filter: GFP2; Aperture: 2.5; Exposure: 7.1 for leaf images, 4.4 for root and seed images; Gain: 5.0.

Wild type and transgenic plants containing *GUS* as the reporter gene were germinated and grown on petri dishes for 7 days. Cold-stress treatment was performed by placing the plants at 4°C in the dark for 48 hrs. Untreated plants were kept in the dark as controls. Immediately following treatment, whole treated and untreated seedlings were immersed in freshly prepared staining solution (50 mM sodium phosphate buffer containing 10 mM EDTA, 0.1% v/v Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.5 mg ml^{-1} X-glucuronide (from 40 mg ml^{-1} stock in dimethylformamide)) to stain for GUS activity. The seedlings were vacuum infiltrated at 20-27 mm of Hg for 40 min and incubated at 37°C for 24 hrs. Throughout the staining, the tubes were wrapped in alfoil to ensure minimal exposure of the staining solution to light. The staining solution was removed and the seedlings were washed in a series of ethanol washes of increasing concentration (20%, 35%, 50% and 70%) for 2-3 hrs per washing step. The activity of GUS was determined visually and photographs were taken using a LEICA MZFLIII fluorescence stereomicroscope with white light and no filter.

2.2.4. Production and Analysis of Transgenic Barley Plants Over-expressing *HvICE2*

2.2.4.1. Production of an *HvICE2* Expression Clone using Gateway Technology

The Gateway®-compatible vector pMDC32 (Curtis and Grossniklaus, 2003) with 1.5 kb of the maize *ubiquitin* constitutive promoter (Christensen *et al.*, 1992) replacing the dual *35S CaMV* promoter was kindly provided by Dr. Klaus Oldach (Australian Centre

for Plant Functional Genomics). The replacement was performed by excision and ligation at the *Hind*III and *Kpn*I restriction sites; sequence of the maize *ubiquitin* promoter fragment in Appendix F.2. LR recombination reactions were performed to transfer the coding region of *HvICE2* from the pCR8 plasmid (Gateway® Entry vector) to the modified pMDC32 plasmid (Gateway® Destination vector). Reactions were prepared and transformed as described in Section 2.2.3.8.1 with the following modifications: 135 ng of pCR8/*HvICE2* (Section 2.2.2.8) was used as the Entry clone and 150 ng of pMDC32:*ubiquitin promoter* was used as the Destination vector in the LR reaction.

A 1µl aliquot of the LR reaction was transformed into *E. coli* by chemical transformation and plated onto selective LB agar plates containing kanamycin at 50 µg ml^{-1} as described in Section 2.2.2.4. Plasmid DNA was isolated as described in Section 2.2.2.5. To confirm correct recombination had occurred, diagnostic digestion and electrophoresis of plasmid DNA was performed as described in Section 2.2.2.5 using 5 U (0.5 µl) of each of *Dra*III and *Pst*I and 5 µl of plasmid DNA.

2.2.4.2. Barley Transformation and Growth of Transgenic Plants

Barley transformation and growth of T_0 plants and control Golden Promise plants was performed as described in Section 2.2.3.8.2.

2.2.4.3. Analysis of Transgene Expression by qRT-PCR in Barley Plants Over-expressing *HvICE2*

Leaf tissue harvested from T_0 plants and RNA was extracted as described in Section 2.2.3.2.2. Following quality analysis and quantification of the RNA by electrophoresis and spectrophotometry as described in Section 2.2.3.2.2, cDNA was synthesised and control *GAPDH* PCRs were performed as described in Section 2.2.3.5. The cDNA populations were analysed by qRT-PCR as described in Section 2.2.3.6 using a gene-specific forward primer (HvICE2_TX_F3) and a transgene-specific reverse primer (NOSTERM_R2) (Table 2.2) to ensure amplification of the transgene only.

2.2.4.4. Southern Blot Analysis of Transgenic Barley Plants Over-expressing *HvICE2*

Genomic DNA was extracted from leaf tissue of T_0 and Golden Promise plants as described in Section 2.2.3.8.3. To determine the genomic DNA concentration, 2 µl of neat genomic DNA was combined with 1 µl of sterile MQ water and electrophoresed in an agarose gel as described in Section 2.2.2.2. The strength of the bands observed was used to determine the volume of each sample to be used in the Southern blot. Neat genomic DNA (7-8.5 µl) of transgenic and wild type Golden promise plants was digested with *Eco*RV in 10 µl reaction volumes containing 0.75µl (30 U) of restriction enzyme and 1 µl of Buffer B (supplied). The samples were incubated at 37°C for 24 hrs before adding 2 µl of a digestion booster mix containing 0.5 µl (20 U) of restriction enzyme and 0.2 µl of Buffer B. The reactions were incubated for an additional 24 hrs at 37°C.

The restriction digests were electrophoresed as described in Section 2.2.2.2 on a 150 ml 0.9% w/v Agarose NA (high quality) gel. A 30 well comb was used with a 14.5 cm x 17.5 cm tray. Once cast, the gel was allowed to set for 20 min before the samples were loaded and electrophoresed at 33 V overnight. 400 ng of λ DNA (8 µl) cut with *Hind*III was loaded as a DNA marker. The DNA was visualised under UV light using a transilluminator.

To transfer the DNA to a nylon membrane, the gel was assembled into a Southern blot in a plastic tray from the bottom upward. In the order the components were added to the blot, the transfer comprised of a sponge, two sheets of Whatman filter paper, a plastic screen (to prevent the edges of the upper and lower components of the blot from touching), the agarose gel, the Hybond N+ membrane, two sheets of dry Whatman filter paper, a large stack of dry tissue and a weight. Before assembly, the sponge and filter paper were wetted with 0.4 M NaOH and the nylon membrane was soaked briefly in MQ water followed by 0.4 NaOH for 30 sec. Care was taken to ensure that once placed on the stack the membrane did not move and that no air bubbles remained between any

of the layers below the dry filter paper. Assembly of the blot was conducted quickly and the tray was filled with 0.4 M NaOH. The DNA was allowed to transfer for 10 hrs. The gel and membrane were visualised under UV light using a transilluminator to confirm complete transfer of the DNA to the membrane. The DNA was crosslinked to the membrane by exposure to UV light in a transilluminator for 30 sec. The membrane was rinsed for 1 min in 2x SSC (30mM tri-sodium citrate buffer containing 0.3M NaCl), blotted dry, sealed in cling wrap and stored at -20°C.

Pre-hybridisation of the Southern blot was conducted to block non-specific DNA binding sites. Pre-hybridisation solution was prepared containing 150 ml of 10x SSC, 105 ml of water, 30 ml of 50x Denhardt's III (2% w/v Ficoll 400, 2% w/v Polyvinyl-pyrrolidone360, 2% w/v BSA, 10% w/v SDS), 15 ml of DNA-sodium salt (5 mg ml^{-1}) and stored at -20°C until required. The Southern blot membrane was thawed and soaked in 5x SSC for 1 min and drained before being placed flat against the inside of a hybridisation bottle. Care was taken to ensure no air bubbles were present between the bottle and the membrane. The pre hybridisation solution was incubated at ~65°C before 6 ml was added to the hybridisation bottle. The bottle was incubated at 65°C overnight on a rotisserie in an oven.

The purified ~1.5 kb DNA fragment of the maize *ubiquitin* promoter (Appendix F.2) used as a probe was kindly provided by Ms. Natasha Bazanova (ACPFG). Probes were prepared for hybridisation using the Rediprime II Random Prime Labelling System kit according to the manufacturer's directions. Briefly, ~25 ng of purified DNA was diluted in sterile 1x TE buffer to 45 µl. The DNA was denatured by boiling for 5 min in a water bath and immediately cooled by placing on ice for 5 min. The DNA was added to the reaction tube (supplied) and 5 µl of P^{32} radiolabelled dCTP was added and mixed by pipetting. The reaction was incubated at 37°C for 30-60 min. The labelled probe was purified by passing through a Sephadex G-100 column using a Geiger counter (positioned ~3 cm from the column) to determine when the labelled DNA was present in the eluent. The solution eluted when the Geiger counter read greater than 500-1000 cps was retained and used as probe. DNA-sodium salt solution (5 mg ml^{-1}) (250 µl) was

added to the probe to inhibit non-specific binding of the probe to the membrane and the solution was denatured by boiling for 5 min and placed on ice for 5 min.

Hybridisation was performed by preheating hybridisation solution (30% v/v 5 x HSB (100 mM PIPES buffer pH 6.8, containing 3 M sodium chloride and 25 mM EDTA disodium salt), 30% v/v 50x Denhardt's III, 30% v/v of 25% w/v Dextran sulphate solution and 25 mg of DNA-sodium salt (5 mg ml^{-1})) at 65°C for 5 min. This solution was made fresh or thawed from frozen aliquots. The prehybridisation solution was drained from the hybridisation bottle containing the membrane and replaced with 6 ml of hybridisation solution. The hybridisation bottle was incubated at 65°C for at least 30 min before the labelled probe DNA was added. Care was taken to ensure the probe was pipetted into the hybridisation solution and not directly onto the hybridisation filter. The membrane was incubated in the hybridisation solution at 65°C overnight in a rotisserie oven.

The hybridisation solution was drained and membranes were washed in the hybridisation bottles in approximately 20 ml of Wash Solution 1 (2x SSC, 0.1% SDS) at 65°C for 20 min in a rotisserie oven. The membranes were removed and washed for 20 min in approximately 200 ml of preheated Wash Solution 2 (1x SSC, 0.1% SDS) in a plastic container in a 65°C water bath with shaking. The radiation level was determined using a Geiger counter and an additional wash was performed in Wash Solution 3 (0.5x SSC, 0.1% SDS) if necessary to reduce the radiation level of the majority of the membrane to or just above background (1-2 cps). The third wash was performed at 65°C for up to 20 min, depending on the radiation level. None of the membranes required further washing. The membranes were blotted dry with paper towel, placed between sheets of plastic used to expose HR-T film in an autoradiography cassette with two signal-intensifying screens. The cassette was placed at -80°C for between 6 hrs to 16 days, depending on signal intensity. The film was developed in a dark room under red safety lights using an AGFA CP1000 automatic film processer with standard solutions according to the manufacturer's directions. After use, membranes were stripped by incubating in 200 ml of boiling stripping solution (0.1% w/v SDS, 2 mM EDTA, pH 8.0) on a rocking platform until the solution reached room temperature.

This was repeated once. Membranes were sealed in cling wrap and stored in the dark at -20°C.

2.2.4.5. Analysis of Developmental Phenotypes of T_1 Barley Plants Over-expressing *HvICE2*.

Barley plants were grown as described in Section 2.2.3.8.2. The plants were inspected weekly and photographs were taken at various stages to record the physical appearance of the plants using a Digital IXUS 70 camera. Plant height measurements were taken using a ruler to measure the distance from the soil to the tip of the tallest leaf. Approximate 1000 grain weight was calculated by weighing 100 randomly selected threshed seed and multiplying the values by ten to reflect an approximation of 1000 grain weight. To calculate plant weight, heads and the aerial portion of each mature plant (cut at approximately 3 cm from the soil surface) were harvested separately. Prior to weighing, the plant tissue was dried at room temperature for four weeks and the seed was dried at 37°C for one week. The weights of the heads and remaining aerial biomass were added for each plant to calculate the total plant biomass.

2.2.4.6. Northern Blot Analysis of T_1 Barley Plants Over-expressing *HvICE2*

Northern blots were produced to analyse the *HvICE2* transgene expression levels in the wild type and *HvICE2* over-expressing barley plants used for developmental phenotype analysis. RNA was extracted from leaf tissue of T_1 plants as described in Section 2.2.3.2.2 and resuspended in 100 µl of sterile MQ water. The RNA was quantified by spectrophotometry as described in Section 2.2.3.2.2. 15 µg of RNA from each sample was concentrated prior to use by precipitation and resuspension as follows. 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% (w/v) ethanol were added to 100 µl (1 volume) of RNA and water solution. The tube was incubated at room temperature for at least 10 min, and 4°C for 3 hrs, centrifuged at 16,000 x g for at least 20 min and the supernatant was removed. The pellet was dried at 37°C for ~5 min and resuspend in 5 µl of sterile water.

The 15 µg of RNA was electrophoresed as described in Section 2.2.3.2.2 with the following modifications. A 200 ml 1.4 % w/v denaturing agarose gel containing 10% w/v 10x MOPS buffer and 10% w/v formaldehyde (37%) was prepared in a 20 x 15 cm tray and allowed to set for 1 hr at room temperature prior to use. The samples were electrophoresed at 55 V for 1 hr and 45 min, then 80 V for 1 hr and 10 min. Photographs of the rRNA bands under UV light were taken using a UV transilluminator, for use as a loading guide during interpretation of the northern blot results.

The gel was rinsed with sterile water briefly and assembled into a northern blot to transfer the RNA to a nylon membrane. The blot was assembled from the bottom upward on a glass plate placed across a plastic container. In the order the components were assembled on the plate, the transfer comprised of a wick (three wide strips of Whatman filter paper with both ends in the plastic container), the agarose gel, a sheet of Hybond N+ membrane, a parafilm dam separating the upper and lower components of the blot, two pre-wetted pieces of Whatman filter paper, two dry pieces of Whatman filter paper, a large stack of dry paper tissue and a weight (~1 kg). Before assembly, the wick was wetted with 20x SSC and the nylon membrane and two pieces of Whatman filter paper were wetted with 4x SSC. Care was taken to ensure that once placed on the stack the membrane did not move and that no air bubbles remained between any of the layers below the dry filter paper. Assembly of the blot was conducted quickly and the plastic container was filled with 20x SSC. The RNA was allowed to transfer overnight. The gel and membrane were visualised under UV light using a transilluminator to confirm complete transfer of the RNA to the membrane. The RNA was crosslinked to the membrane by exposure to UV light in the transilluminator for 30 sec. The membrane was blotted dry, sealed in cling wrap and stored at -20°C.

Prehybridisation of the northern blot membranes was conducted as described in Section 2.2.4.4 for Southern blot hybridisation with the following modifications. The pre-hybridisation solution (3 ml of 50x Denhardt's III, 5 ml of SSPE (3M NaCl, 0.2 M NaH_2PO_4-H_2O, 20 mM EDTA disodium salt, pH 7.4), 2 ml of 5 mg ml^{-1} DNA-sodium salt, 9 ml of formamide and 1 ml of water) was freshly prepared. 4x SSC was used to

soak the membrane rather than 5x SSC and the membrane was incubated at 42°C overnight rather than 65°C.

The DNA fragments of *HvICE2* used as probes were amplified by PCR amplification of plasmid DNA as using Platinum Taq DNA polymerase enzyme in 50 µl reaction volumes described in Section 2.2.2.11 with the following modifications. One round of PCR was performed. Amplification of the "Endogenous *HvICE2* only" probe DNA fragment used QPCR_ICE1_F1 (forward) and ICE_R0 (reverse) primers with an expected product size of 298 bp. The PCR template was 1 µl of sequenced plasmid DNA comprising *HvICE2* barley genomic DNA from the first round of genomic walking in pGEM-T Easy, described in Section 2.2.2.10. The annealing temperature and extension time used for PCR cycling were 58°C and 30 sec, respectively. The "Endogenous and transgene *HvICE2*" probe was amplified using ICE_ATG_F (forward) and ICE_RNAi_Rt (reverse) primers with an expected product size of 502 bp. The template was 45 ng of plasmid DNA of the coding region of *HvICE2* in pCR8, as described in Section 2.2.2.8. The annealing temperature and extension time used for PCR cycling were 63°C and 50 sec, respectively. All PCR products were electrophoresed and purified as described in Section 2.2.2.2. Probes were prepared, purified, DNA-sodium salt solution was added and the probe was denatured as described in Section 2.2.4.4.

Hybridisation, subsequent membrane washes, exposure of the films and membrane stripping was performed as described in Section 2.2.4.4 using northern blot hybridisation solution (0.5 ml of 50x Denhardt's, 1.25 ml of 20x SSPE, 0.25 ml of 5 mg ml^{-1} DNA-sodium salt, 2.25 ml of formamide, 0.5 ml of 25% w/v Dextran sulphate solution). The membranes were incubated in the hybridisation solution overnight at 42°C rather than 65°C.

2.2.4.7. Freezing Treatment of Barley Over-expressing *HvICE2*

Plants from transgenic Lines 3, 8, 10 and 11 and control Golden Promise barley plants were grown to examine their freezing tolerance. Four 48-pot thick plastic punnets were

planted with one plant per pot and grown in a growth room. A random design layout of the lines within the trays was used (Figure 2.5) and the trays were planted in pairs. The remaining holes filled with barley plants of the same age to avoid uneven chilling effects caused by irregular planting. The plants were grown in a growth room at 23°C for 12 hrs with light/18°C for 12 hrs in the dark for 3 weeks.

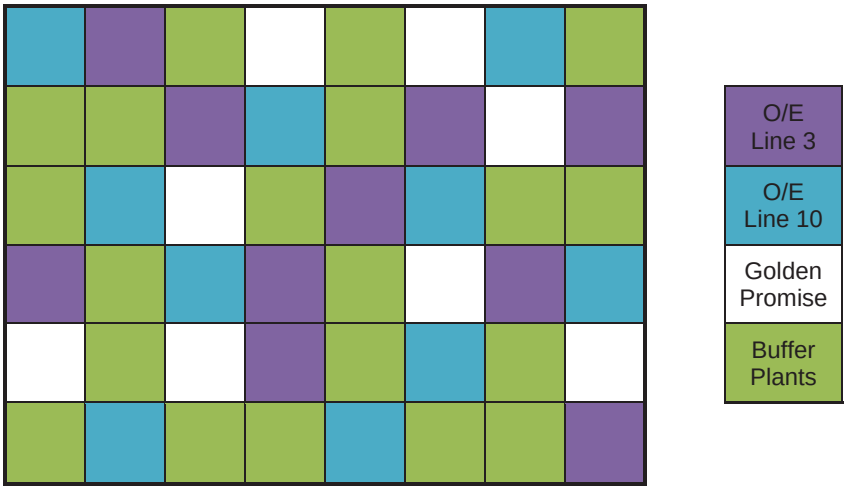


Figure 2.5 Planting layout used to study freezing-tolerance of transgenic barley plants over-expressing *HvICE2*.

A 90L insulated cooler was prepared containing a layer of approximately 2.5 cm thick sand and the plants were pre-chilled at 4°C for at 24 hrs. Treatment involved spraying the plants with Snomax, a solution to promote ice nucleation, and sealing the plants in the insulated cooler. A HOBO temperature data logger was sealed inside the cooler to accurately record the temperature at which the plants were treated and a temperature probe was positioned on the data logger with the display outside the cooler to allow the experimenter to determine when the desired minimum temperature of the treatment had been reached (Figure 2.6).

The sealed cooler was placed in a freezer at -20°C. This resulted in a gradual decline in the temperature inside the cooler at an appropriate rate to simulate the temperature drop in during field frost events. It was noted that the rate of temperature decline was directly related to and significantly affected by the amount of water in the system. The

plants were watered to capacity prior to treatment to ensure the rate of temperature change was as consistent as possible between different treatment batches. Following treatment, the plants allowed to thaw at room temperature before being returned to the growth chamber to recover. Survival was scored after three weeks.

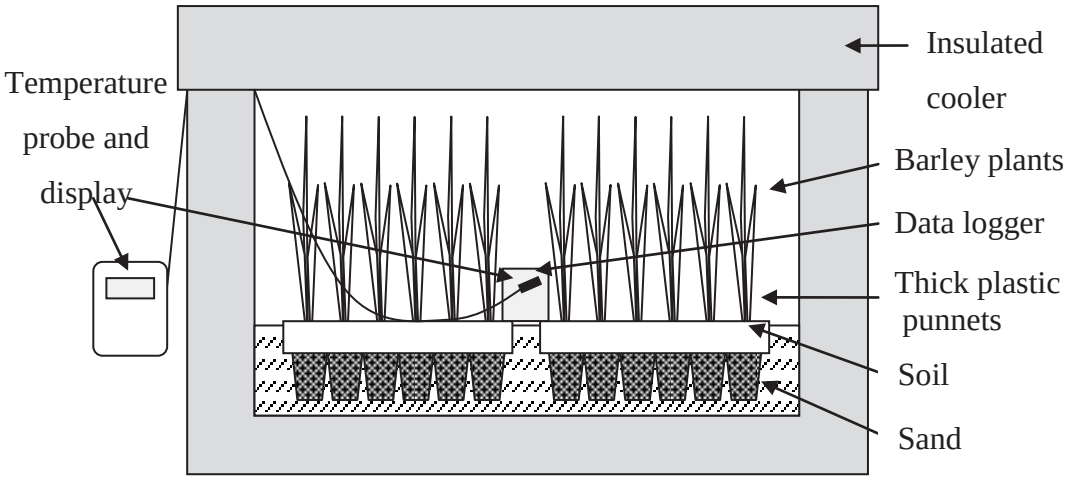


Figure 2.6 Schematic diagram of plant freezing treatment layout.
Two trays each containing 48 barley plants in individual pot holes were treated during one treatment.

Care was taken to ensure the insulated cooler was not still frozen from the previous treatment as this would affect the reproducibility of the results. Following treatment, the cooler was allowed to thaw at room temperature for at least 12 hrs and chilled at 4°C for at least 24 hrs before the next group of plants were treated.

Prior to treatment, samples were taken of each plant to confirm the presence of the transgene. Genomic DNA was extracted as described in Section 2.2.3.8.3 and PCR was performed as described in Section 2.2.3.8.4 with the following modifications: PCR was performed in 20 µl volumes using ICE_Tx_F2 (forward) and NOSTERM_R2 (reverse) primers. Transgenic plants were identified by visual comparison of the PCR product size with the positive control PCR product. For each transgenic line, survival was calculated using only plants producing a positive result in the genomic DNA PCR. All wild-type plants were included for the non-transgenic control calculations.

2.2.4.8. Analysis of Putative Downstream Gene Expression by qRT-PCR

Plants from each of transgenic Lines 3 and 11 were grown to examine the expression of putative downstream genes by qRT-PCR. For both experiments, seeds were planted in 48-pot thick plastic punnets with one plant per pot. A random design layout of the lines within the trays was used (Figure 2.5). The plants were grown in a growth room at 23°C for 12 hrs with light/18°C for 12 hrs in the dark for 3 weeks.

Plants were treated at 4°C and samples of treated and untreated plants were taken at 0 hrs, 6 hrs and 24 hrs after the commencement of treatment. Untreated plants were kept in the growth room under normal conditions. The initial sampling was performed just prior to dawn. Randomly chosen plants were sampled separately for each time point/treatment/line. Harvested leaf tissue was ground to a fine powder and genomic DNA was extracted as described in Section 2.2.3.8.3. To identify transgenic plants, PCR was performed as described in Section 2.2.3.8.4 using HvICE2_TX_F2 (forward) and NOSTERM_R2 (reverse) primers. RNA was extracted from three transgenic plants for each genotype at each time point as described in Section 2.2.3.2.2 and pooled. Synthesis of cDNA was performed as described in Section 2.2.3.5 and qRT-PCR was performed as described in Section 2.2.3.6 using the primers presented in Table 2.3.

Table 2.3 Primers used for qRT-PCR analysis of transgenic plants over-expressing *HvICE2*.

Gene	Forward primer	Reverse primer	Expected product size
HvICE2 transgene	HvICE2_TX_F2	NOSTERM_R1	175 bp
HvDHN8	HvDHN8_F	HvDHN8_R	278 bp
HvDHN5	HvDHN5_F	HvDHN5_R	106 bp
HvRD22	HvRD22_F	HvRD22_R	264 bp
HvCor14b	HvCOR14B_F	HvCOR14B_R	103 bp
HvCBF2	HvCBF2_F	HvCBF2_R	274 bp
HvCBF16	HvCBF16_F	HvCBF16_R	170 bp

Primer sequences are presented in Appendix A.

2.3. Results

2.3.1. Identification, Isolation and Sequence Analysis of *HvICE2*

The sequence of *AtICE1* (Accession number: AY_195621) was compared to the barley EST database at NCBI (www.ncbi.nlm.nih.gov) using the 'tblastn' program and the closest matches were analysed. Comparison of the length of the closest matched sequence (Accession number: TC134022; Sequence presented in Appendix F.1) and *AtICE1* lead to the conclusion that the EST was likely to contain the full-length coding region. Comparison of the TC134022 EST sequence to the sequences in the NCBI non-redundant database showed *AtICE1* and *AtICE2* were the Arabidopsis genes most similar to *HvICE2*, each with 35.3% amino acid identity with *HvICE2*. From this it was concluded that the EST contig TC134022 may encode a gene with a similar function to *AtICE1*; a barley homolog, the sequence of which was later published as *HvICE2* (Tondelli *et al.*, 2006). The results of the comparison also supported the premise that the EST sequence contained the full-length coding sequence of the gene, as no highly homologous sequences were found with additional sequence at the 5' or 3' ends.

HvICE2 encodes a putative protein of 379 amino acids with a calculated molecular mass of 39.5 (kDa) and a pI of 4.8. A potential sumoylation site was identified at the lysine residue of the GRIKEER motif (Figure 2.7) which aligns with the predicted sumoylation site in AtICE1 (Miura *et al.*, 2007). A sequence in the C-terminal region of HvICE2 (in Conserved region B) was found to be similar to the ACT domain superfamily. These domains are commonly involved in binding a small ligand such as an amino acid leading to regulation of the enzyme (ACT superfamily accession number: cl09141).

To determine whether *HvICE2* contains the functional motifs present in the Arabidopsis *ICE* genes, the translated nucleotide sequences of *AtICE1*, *AtICE2*, *HvICE1* (partial sequence) and *HvICE2* (Figure 2.7) were aligned. The HvICE2 bHLH DNA binding domain was identical to those of the Arabidopsis ICE proteins, including residues which are essential for DNA binding (Kanaoka *et al.*, 2008). The zipper domain was

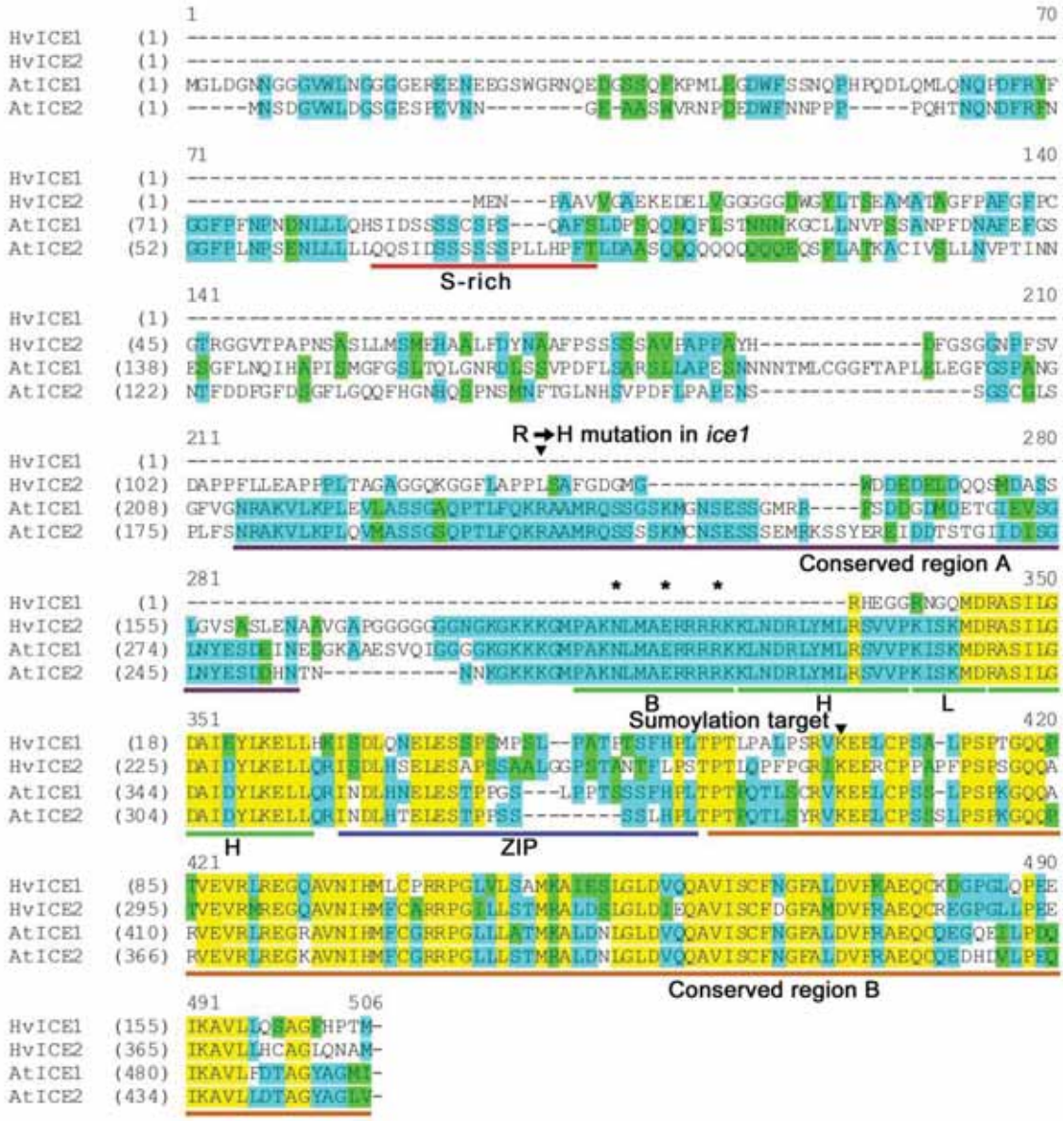


Figure 2.7 Alignment of the translated nucleotide sequences of the coding regions of *AtICE1*, *AtICE2*, *HvICE1* and *HvICE2*.

The barley and Arabidopsis *ICE* genes have high sequence similarity in some regions and poor similarity in others. Yellow, blue and green boxes indicate regions of complete conservation, regions of moderate conservation, and similar residues, respectively. The *HvICE1* sequence (TC143232, TIGR database, http://compbio.dfci.harvard.edu/tgi/) is truncated at the 5' end. The translated sequence of *HvICE2* cloned in this study from the barley cultivar Haruna Nijo was identical to the translated sequence of the *HvICE2* EST clone TC134022 (TIGR database). The translated sequence of *AtICE2* cloned in this study was identical to the published sequence of the Arabidopsis AtICE2 protein (At1g12860). Published features of

AtICE1 (AY195621) are annotated on this diagram as marked (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008; Miura *et al.*, 2007).

moderately conserved between HvICE2 and the Arabidopsis ICE proteins (Chinnusamy *et al.*, 2003) while Conserved region B was highly conserved. Contrastingly, the arginine residue which was altered in the *ice1* and *scrm* mutants (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008), and the serine rich region which was suggested to be the site of phosphorylation (Chinnusamy *et al.*, 2003), were not present in HvICE2. The N-terminal region, including Conserved region A, was also significantly different in all of the *ICE* genes examined here. In AtICE1, this region contains the activation domain.

To confirm that the *HvICE2* EST contig sequence was not truncated at the 5' end, 5' RACE PCR was performed. The fragments obtained from the second round of PCR were 657 bp and 359 bp for the products isolated using the ICE_R7 and ICE_R3 reverse gene-specific primers respectively. Sequence alignment of the sequences of the 5' RACE clones and the published *HvICE2* sequences (Appendix E.1) revealed fragments of the *HvICE2* gene had been successfully cloned and that the published sequence contained the entire *HvICE2* coding region. The 5' RACE clone sequences were almost the same length as the published contig sequence and did not contain any alternate start codons. The clone isolated using the ICE_R7 primer had four additional guanine residues on the 5' end of the contig sequence and the clone isolated using the ICE_R3 primer was missing the final nine resides.

The full-length coding region of *HvICE2* (1,140 bp) was amplified from Haruna Nijo barley cDNA by nested PCR (Figure 2.8A), TOPO® cloned into pCR8 and sequenced twice from either end of the clone. The identity and integrity of the clone was confirmed by alignment of these sequences with that of the published *HvICE2*.

The genomic sequence, spanning the coding region of *HvICE2*, including introns, was amplified from Haruna Nijo barley genomic DNA and cloned and nucleotide sequence analysis determined the sequence and position of the three *HvICE2* introns. Comparison of the intron/exon structure of the barley, Arabidopsis and rice *ICE*-type

genes revealed that all three contained the same number of introns, that the orthologous introns are of comparable relative size, and that each of the introns is situated at the same position in the gene (relative to the orthologous amino acid residues in the encoded proteins) (Figure 2.9).

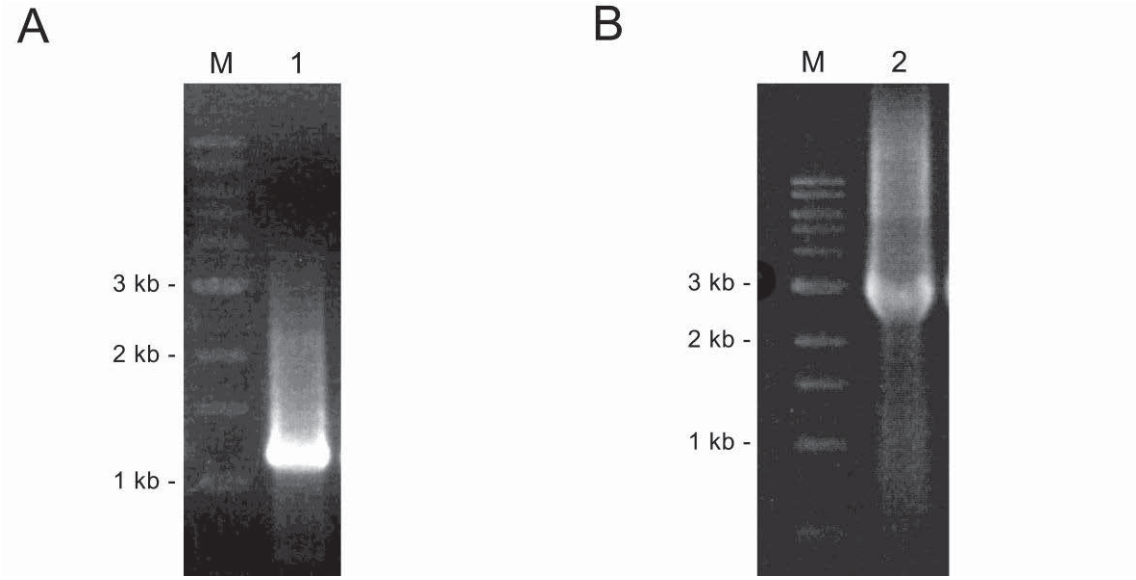


Figure 2.8 Photograph of agarose gel analyses of PCR products of the coding region and promoter of *HvICE2*.

(**A**) PCR products amplified from barley cDNA during cloning of the *HvICE2* coding region. Lane 1, fragment containing the *HvICE2* coding region (~1.2 kb). (**B**) PCR products amplified from barley genomic DNA during cloning of the *HvICE2* promoter. Lane 2, fragment of the *HvICE2* promoter (~3 kb). M, molecular weight marker.

The promoter sequence of *HvICE2* was obtained by genomic walking PCR. The sizes of the overlapping regions of the fragments isolated were 634 bp, 364 bp and 172 bp for the first, second and third rounds of genomic walking respectively (Figure 2.10). The products obtained from the first, second and third rounds of genomic walking enabled identification of 531 bp (product obtained from the library digested with *Ssp*I), 1,280 bp (product obtained from the library digested with *Dra*I) and 2,085 bp (product obtained from the library digested with *Nae*I) of new sequence respectively, totalling 3,896 bp upstream of the predicted start codon of *HvICE2*. The sequences were assembled to make a continuous sequence with the *HvICE2* coding region.

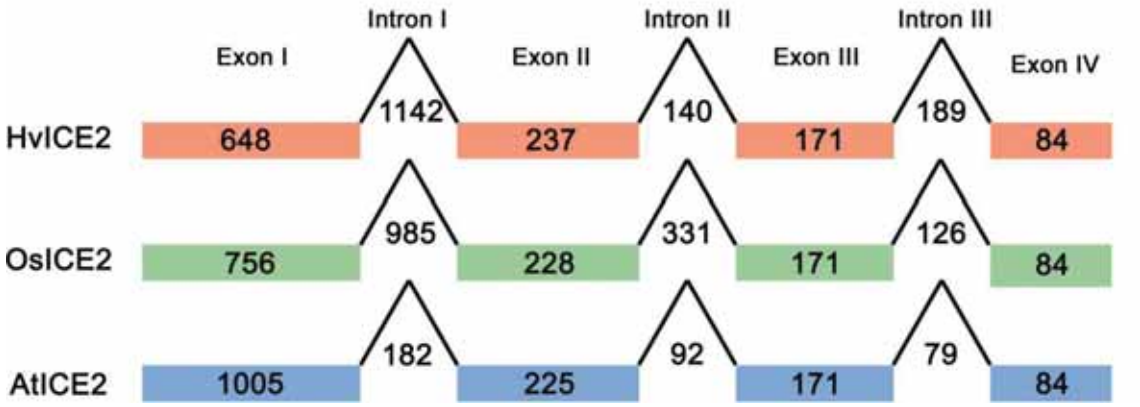


Figure 2.9 Schematic diagram of the genomic structure of *HvICE2*, *AtICE1* and *OsICE*.

The intron/exon structure of the *ICE* genes is conserved within monocots, and between monocots and dicots. Numbers represent respective intron or exon sizes in base pairs. Sequence accession numbers: *AtICE1* (AY195621) and *OsICE* (NP914885).

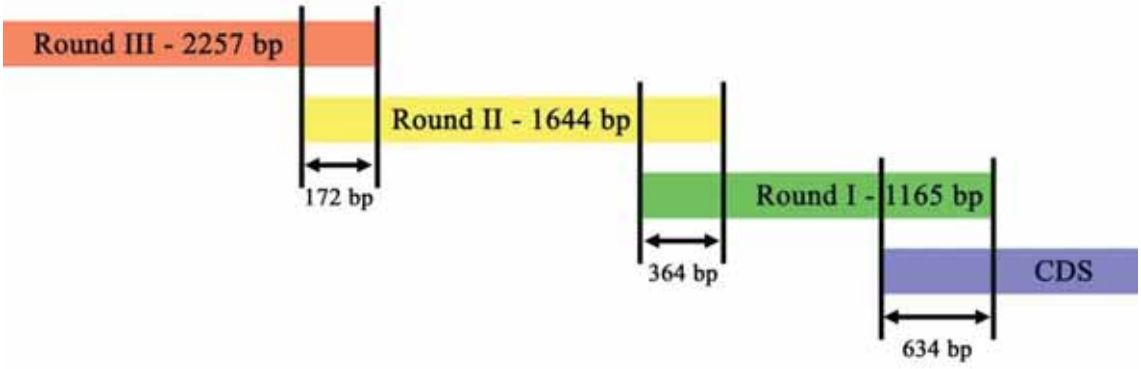


Figure 2.10 Schematic diagram of the overlapping regions of *HvICE2* genomic walking fragments.

The *HvICE2* coding region (CDS) and the fragments cloned during rounds I, II and III of genomic walking are annotated and are marked with their sizes. The sizes of overlapping regions are also marked. Diagram is not to scale.

The sequence of putative promoter region (1 kb region immediately upstream of the start codon) was examined using the PLACE database to identify the sequences of putative *cis*-elements, particularly those which have been shown to play a role in abiotic

stress responses (Figure 2.11). In the 500 bp of the promoter closest to the coding region, only four *cis*-elements were identified: MYC and MYB sites very close to the start site, and LTRE/CRT/DRE and bZIP sites at ~-250 bp from the start site. Further upstream, a number of putative *cis*-elements were identified for the common MYC, MYB and bZIP transcription factors (involved in many cellular processes), as well as PEATPRODH (involved in hypoosmolarity response) and GT-1 (involved in general transcription and salinity response). Only one ABRE element (ABA response) was identified, at ~-900 bp from the start site. One ARE1 element (oxidative stress response) was identified.

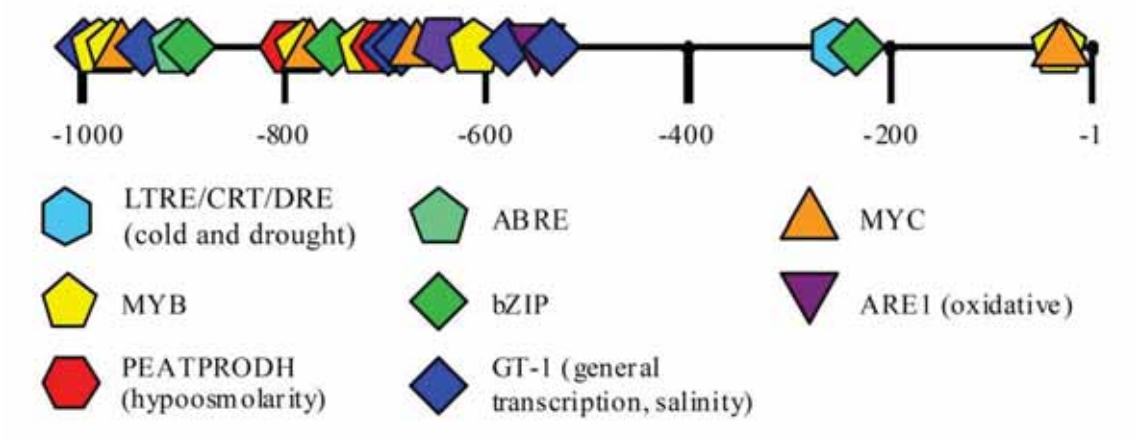


Figure 2.11 Schematic diagram illustrating the position of putative *cis*-elements within the promoter of *HvICE2*.

The black line represents the *HvICE2* promoter DNA sequence and the position of symbols (-1 to -1000 bp) represents the approximate location of putative *cis*-elements relative to the first base pair of the start codon at position 0. Diagram is not to scale. Putative *cis*-elements were identified by a PLACE database search. LTRE/CRT/DRE, Low temperature Response Element/C-Repeat element/Dehydration-Responsive Element; ABRE, ABA Responsive Element; MYC, Myc-type transcription factor binding site; MYB, Myb-type transcription factor binding site; bZIP, bZIP-type transcription factor binding site; ARE1, element involved in oxidative stress responses; PEATPRODH, element involved in hypoosmolarity responses; GT-1, element involved in general transcription and salinity. Comprehensive descriptions of the *cis*-elements may be found at www.dna.affrc.go.jp/PLACE/.

Nested PCR was performed to amplify 3,215 bp of the 5' region of *HvICE2*, hereafter referred to as the promoter, from Haruna Nijo barley genomic DNA (Figure 2.8B). The promoter was cloned using TOPO® pCR8 and sequenced twice along the entire length. Comparison of the promoter sequence to the *HvICE2* EST and the cloned sequences from genomic walking confirmed the identity of the promoter, verified that overall sequence integrity had been maintained and indicated that no sequence errors had been introduced in any putative elements of interest.

2.3.2. Analysis of *HvICE2* Expression

HvICE2 expression was examined during cold, ABA and salinity treatments and in various tissues by qRT-PCR, microarray data analysis and/or using *promoter*:*reporter gene* expression systems as described in the following sections.

2.3.2.1. qRT-PCR Analysis of *HvICE2* Expression during Cold Stress

Cold stress treatment was performed on Golden Promise (freezing sensitive) and Haruna Nijo (freezing tolerant) barley plants at anthesis. Details of the temperature regime used are presented in Appendix C. Prior to treatment, all plants appeared healthy and each genotype was physically uniform. Immediately following treatment, darker, duller patches were observed on the leaves of treated plants where cells had been ruptured by the formation of ice crystals within the leaf (Figure 2.12A). Four days after treatment, large sections of dead tissue were observed in the treated plants. The damage included necrosis of leaf and floral tissues with whole tillers being killed in many cases (Figure 2.12B). Freezing-induced sterility was observed with grain filling being poorer in treated plants relative to untreated plants. The degree of tissue damage and sterility observed was greater in plants exposed to longer treatment. The sterility and damage were also greater for the freezing-sensitive Golden Promise plants than for freezing-tolerant Haruna Nijo plants.

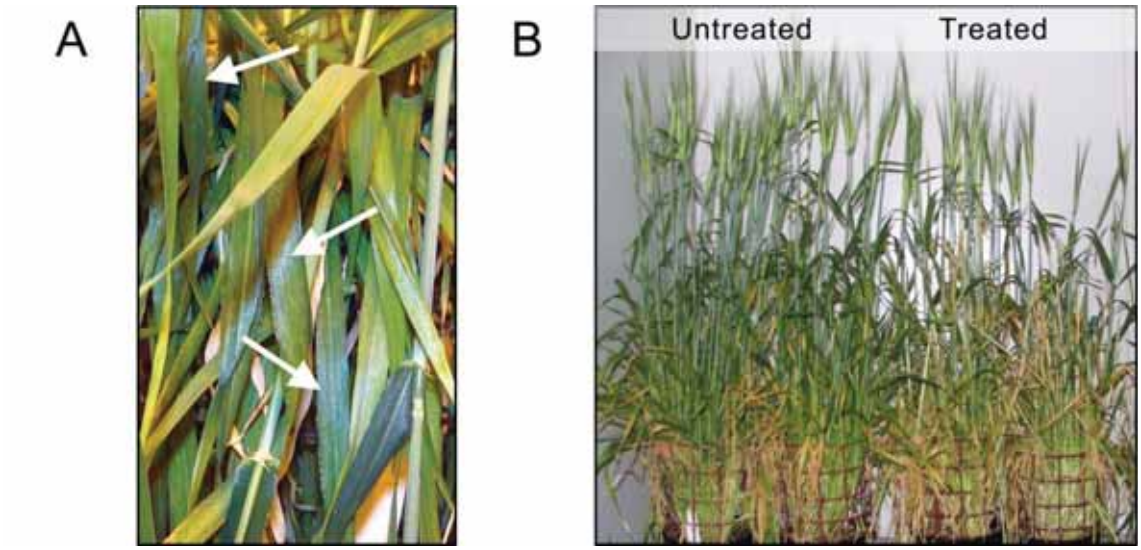


Figure 2.12 Photographs of cold treated and untreated barley plants.
(**A**) Damaged leaf tissues on cold-treated plants immediately after treatment. (**B**) Two untreated and two treated barley cv. Haruna Nijo plants four days after freezing treatment to a minimum of -5.5°C. Increased necrosis was present in leaf, stem and floral tissues of treated relative to untreated plants.

The mRNA levels of *HvICE2* were determined by qRT-PCR. In both Haruna Nijo and Golden Promise plants, mRNA levels of *HvICE2* were low in untreated plants in all tissues examined (Figure 2.13A & B). Expression of *HvICE2* was induced in Haruna Nijo and Golden Promise in both leaf and floral tissues with a greater response in the freezing-tolerant variety Haruna Nijo. Maximum expression observed during this experiment was approximately 62,000 copies μl^{-1} of cDNA in floral tissues of Haruna Nijo. This value is more than three-fold higher than the corresponding untreated values. Contrastingly, the maximum expression in Golden Promise was approximately 14,000 copies μl^{-1} of cDNA, approximately two-fold higher than the corresponding untreated levels (Figure 2.13A).

The expression patterns in floral and leaf tissues were generally similar for each genotype during exposure to low temperatures. Differences were observed between genotypes however: transcript levels increased more quickly following cold treatment in Haruna Nijo than Golden Promise, and levels in Haruna Nijo were consistently greater in floral than leaf tissues whereas levels were more similar in all tissues in Golden

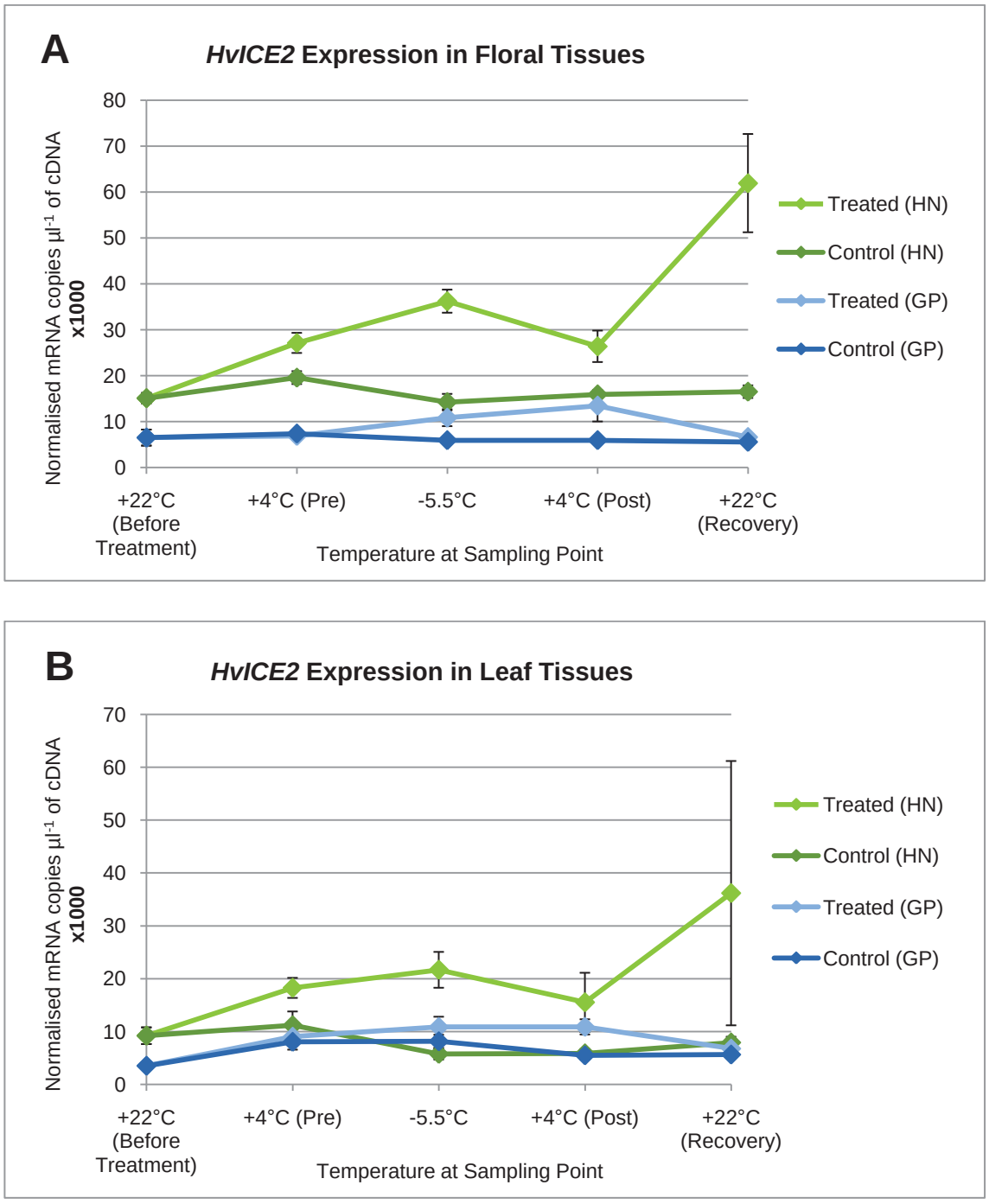


Figure 2.13 Graph of *HvICE2* expression during cold stress determined by qRT-PCR.

(**A**) *HvICE2* expression in floral tissues. (**B**) *HvICE2* expression in leaf tissues. *HvICE2* expression is upregulated in the leaf and floral tissues of plants during exposure to low temperatures. GP, Golden Promise; HN, Haruna Nijo. Error bars represent standard error.

Promise (Figure 2.13A & B). At four days post treatment (recovery) the transcript levels varied greatly in different treated Haruna Nijo tissue samples, particularly between leaf samples. This is evident from the size of the error bars in Figure 2.13A & B, which are a result of different leaf samples having distinctly high or low transcript levels, rather than variation in technical replicates.

2.3.2.2. qRT-PCR Analysis of *HvICE2* Expression during Salinity Stress

Clipper x Sahara double haploid line 134 and Golden Promise barley plants were treated with salt. Following treatment, necrosis was observed on leaves of treated plants, which was most prominent in leaf tips and older leaves (Figure 2.14A**)**. Growth of root and leaf tissues was visibly stunted in treated plants relative to controls (Figure 2.14B & C). This was greater in salt sensitive Golden Promise plants than in the comparatively salt tolerant Clipper x Sahara double haploid line 134 plants.

qRT-PCR was employed to determine the mRNA levels of *HvICE2*. At the majority of time points, mRNA levels of *HvICE2* were not significantly affected by the salt treatment in the tissues of either cultivar (Figure 2.15A & B). The greatest difference between the salt treated and untreated plants was in leaves after two days of treatment when the *HvICE2* mRNA level in treated Golden Promise plants was almost half that in control plants (Figure 2.15A). The basal transcript level of *HvICE2* in root tissue is extremely low (less than 1000 copies μl^{-1} of cDNA; Figure 2.15B) and is within the background range for qRT-PCR experiments.

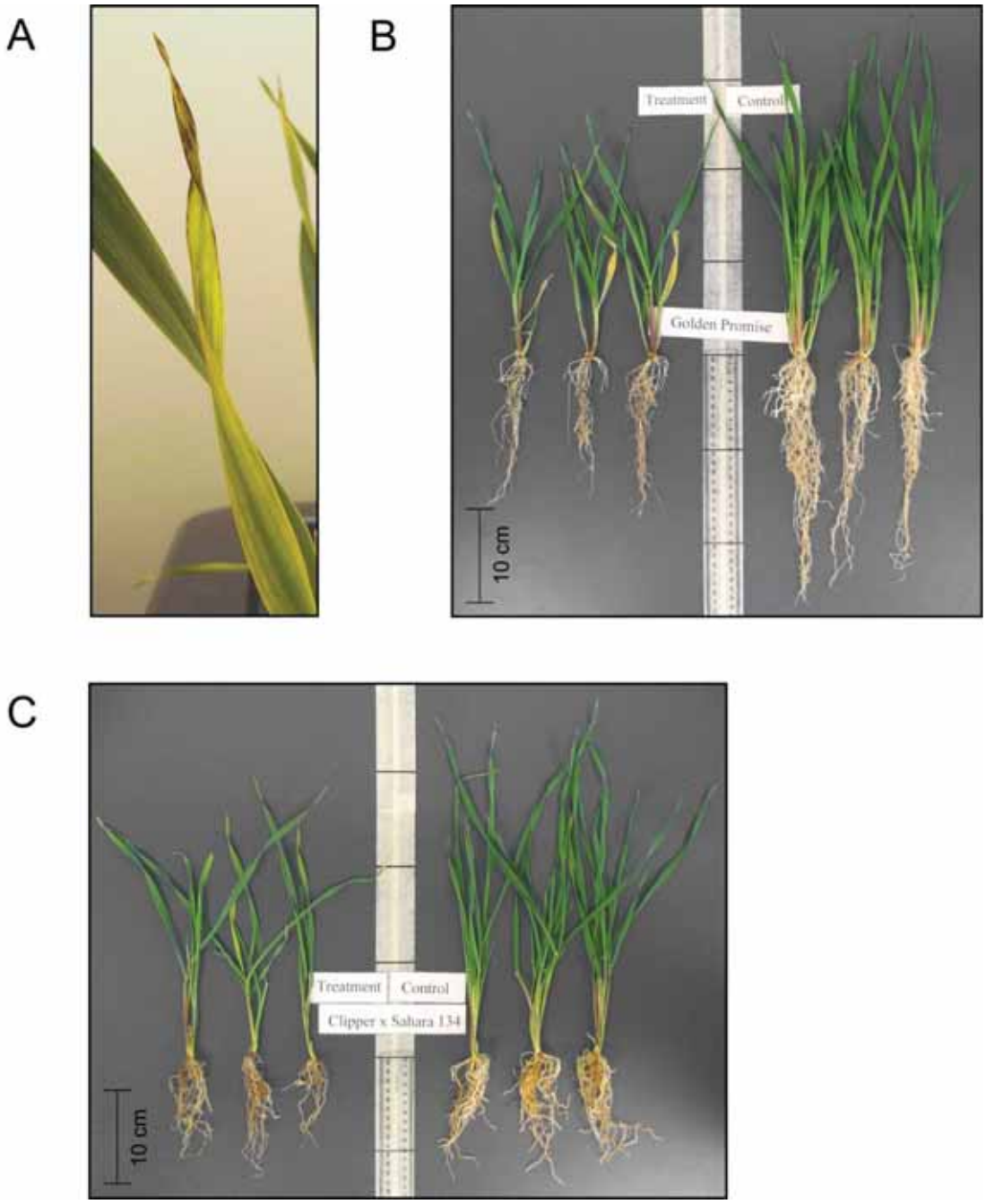


Figure 2.14 Photographs of salinity treated and untreated Golden Promise and Clipper x Sahara double haploid line 134 plants.

(**A**) Necrosis of a Golden Promise barley leaf after ten days of salt treatment. (**B**) and (**C**) Salinity-treated and control barley plants after ten days of salinity treatment, reaching a maximum concentration of 150 mM. Stunting of growth was observed in Golden Promise (**B**) and Clipper x Sahara double haploid line (**C**) treated plants relative to untreated (control) plants.

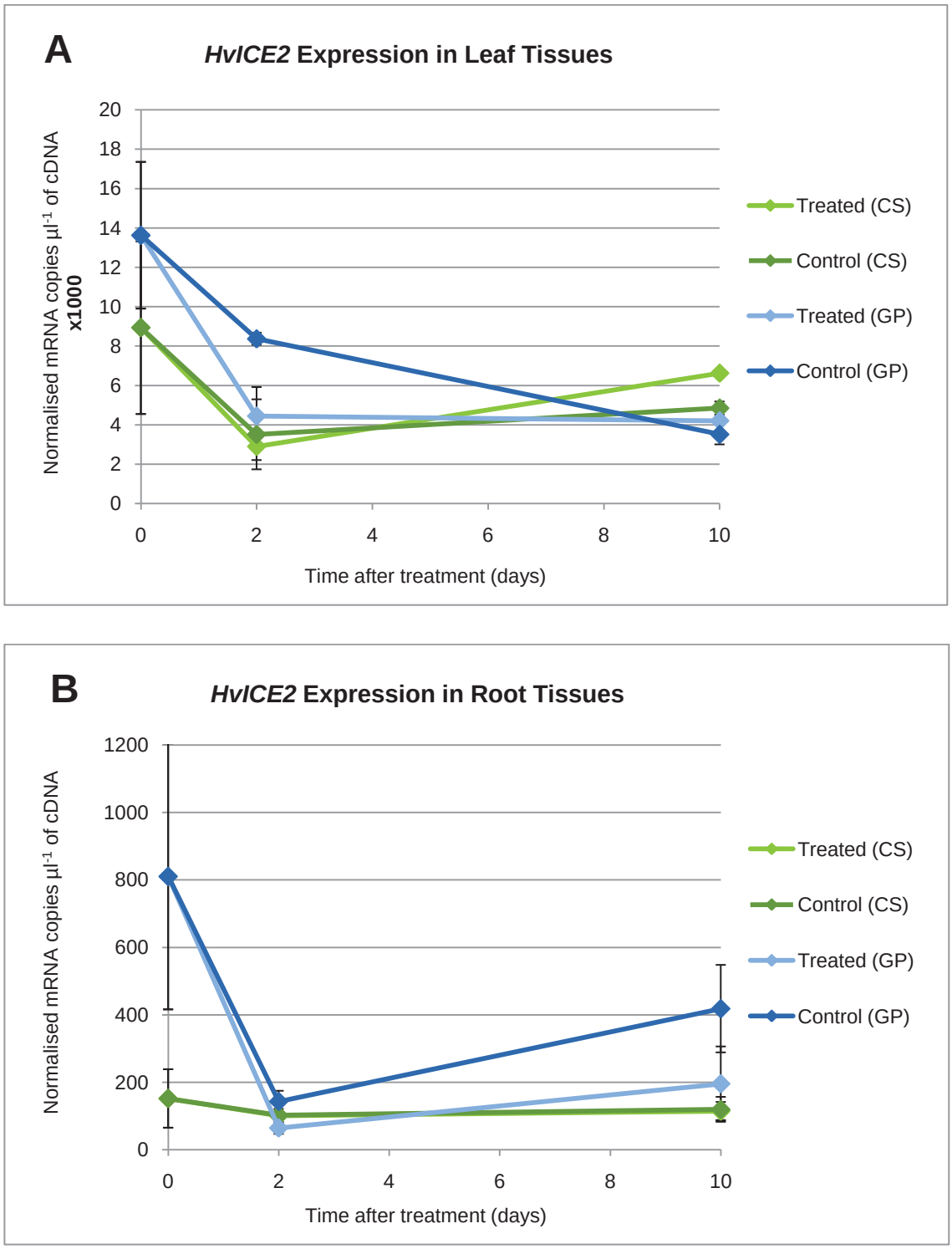


Figure 2.15 Graph of *HvICE2* expression during salinity stress determined by qRT-PCR.

(**A**) *HvICE2* expression in leaves. (**B**) *HvICE2* expression in roots. *HvICE2* expression was not affected by salt treatment (150 mM). GP, Golden Promise; CS, Clipper x Sahara double haploid line 134. Error bars represent standard error.

2.3.2.3. qRT-PCR Analysis of *HvICE2* Expression during ABA Treatment

Golden promise barley plants were treated with 10 µM ABA and the *HvICE2* mRNA levels was determined by qRT-PCR. Basal transcript levels of *HvICE2* in the roots were extremely low (Figure 2.16B) and within the background range for qRT-PCR. There is, however, notable upregulation of *HvICE2* gene expression (18-fold) in roots of treated samples after 12 hrs of ABA treatment and very low standard error values for these data points. A minor difference was observed between transcript levels of *HvICE2* in the leaves of ABA-treated and untreated plants at one hr after treatment commenced, when transcript levels in wild type plants were particularly low compared to other untreated time points (Figure 2.16A).

2.3.2.4. Analysis of *HvICE2* Expression using Microarray Tissue Series

To determine the expression of *HvICE2* in individual tissues, the Barleybase microarray database was searched for an EST corresponding to *HvICE2*. The search identified a contig corresponding to *HvICE2* with a very high degree of sequence similarity, Contig 13678_at (Appendix B.2). The tissue-specific expression data of this contig is presented as a graph in Figure 2.17. The Log_2 scale on the graph means that a difference in expression of one unit is equivalent to a two-fold increase in expression although the units cannot be correlated with absolute transcript levels. Details about the tissues tested on the microarray are available from the website http://bioinf.scri.sari.ac.uk/affy/WEB_TISSUES/tissue_types.htm.

Expression of *HvICE2* was at a similar level in the majority of tissues examined and where tissues were analysed for Golden Promise, the level of expression was similar to that in Morex (Figure 2.17). Greatest expression was observed in floral tissues at caryopsis (ten and 16 days after pollination) and in endosperm (22 days after pollination) tissues.

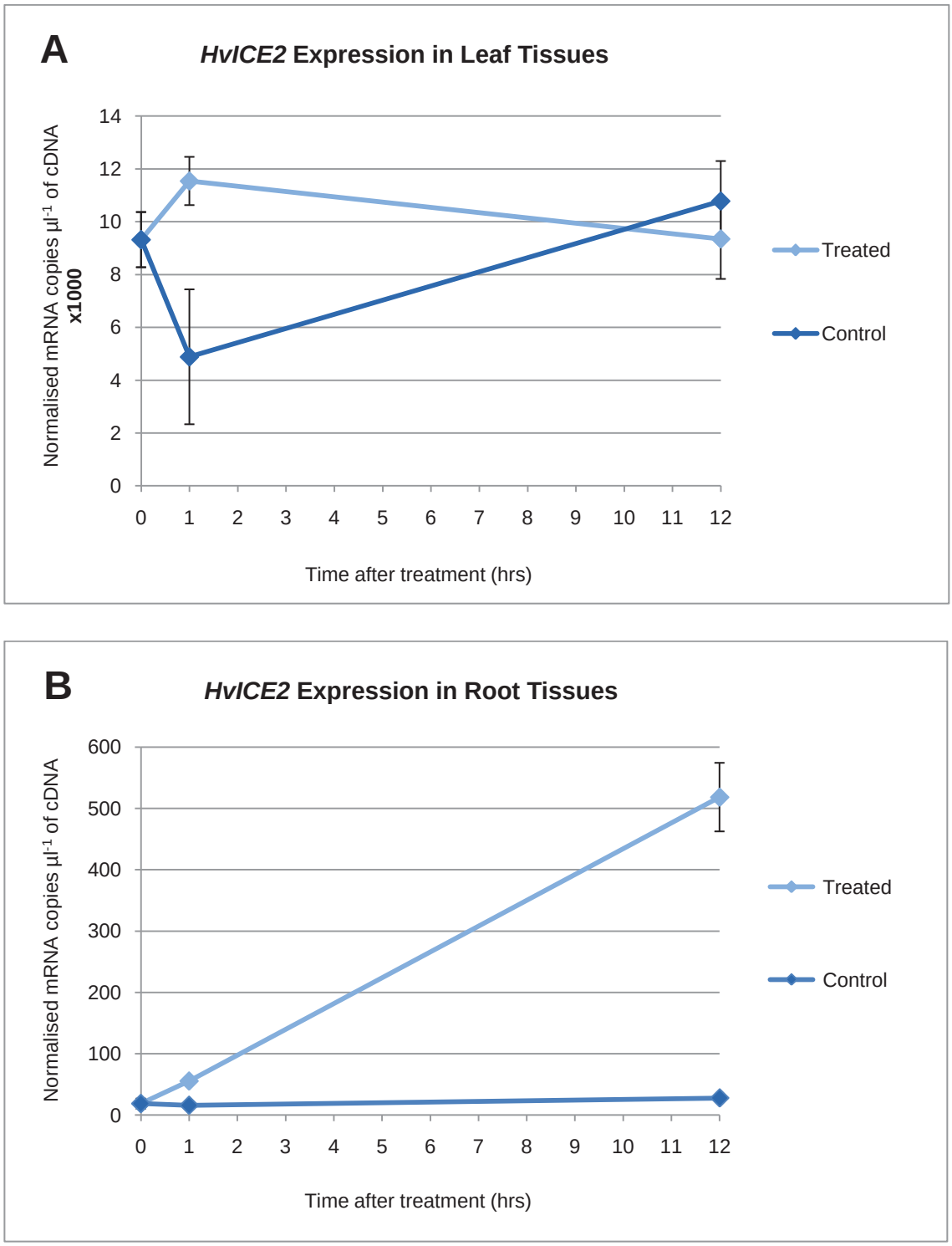


Figure 2.16 Graph of *HvICE2* expression during ABA treatment determined by qRT-PCR.

(**A**) *HvICE2* expression in leaves. (**B**) *HvICE2* expression in roots. ABA treatment (10 µM) did not induce high levels of expression of *HvICE2* in Golden Promise plants. Error bars represent standard error.

Figure 2.17 Graph of *HvICE2* expression from Affymetrix microarray data.
HvICE2 was expressed to a similar level in most of the tissues examined, with greater expression in flowering tissues at caryopsis and endosperm. An incomplete set of Golden Promise barley tissues were analysed (only coleoptile, crown, leaf and root). Information on the tissues studied is available from the website http://bioinf.scri.sari.ac.uk/affy/WEB_TISSUES/tissue_types.htm. DAP: days after pollination.

2.3.2.5. Analysis of *HvICE2* Expression using *Promoter:Reporter Gene* Transgenic Plants

In addition to qRT-PCR, the expression pattern of *HvICE2* was analysed using a *promoter:reporter gene* system to determine whether *HvICE2* was expressed in specific tissues or constitutively, both before and during cold treatment. Constructs for plant transformation were prepared (Figure 2.18) containing ~3 kb of the *HvICE2* promoter. Restriction digestion confirmed the *HvICE2* promoter had been correctly inserted upstream of the *GUS* and *GFP* reporter genes (Figure 2.19). The expected product sizes were 10,820, 2,504, 798 382 and 18 bp for the pMDC164/*HvICE2 promoter* construct, and 4,361, 3,988, 2,307, 1,519, 829 and 327 bp for the pMDC107/*HvICE2 promoter* construct. The constructs were used to transform barley.

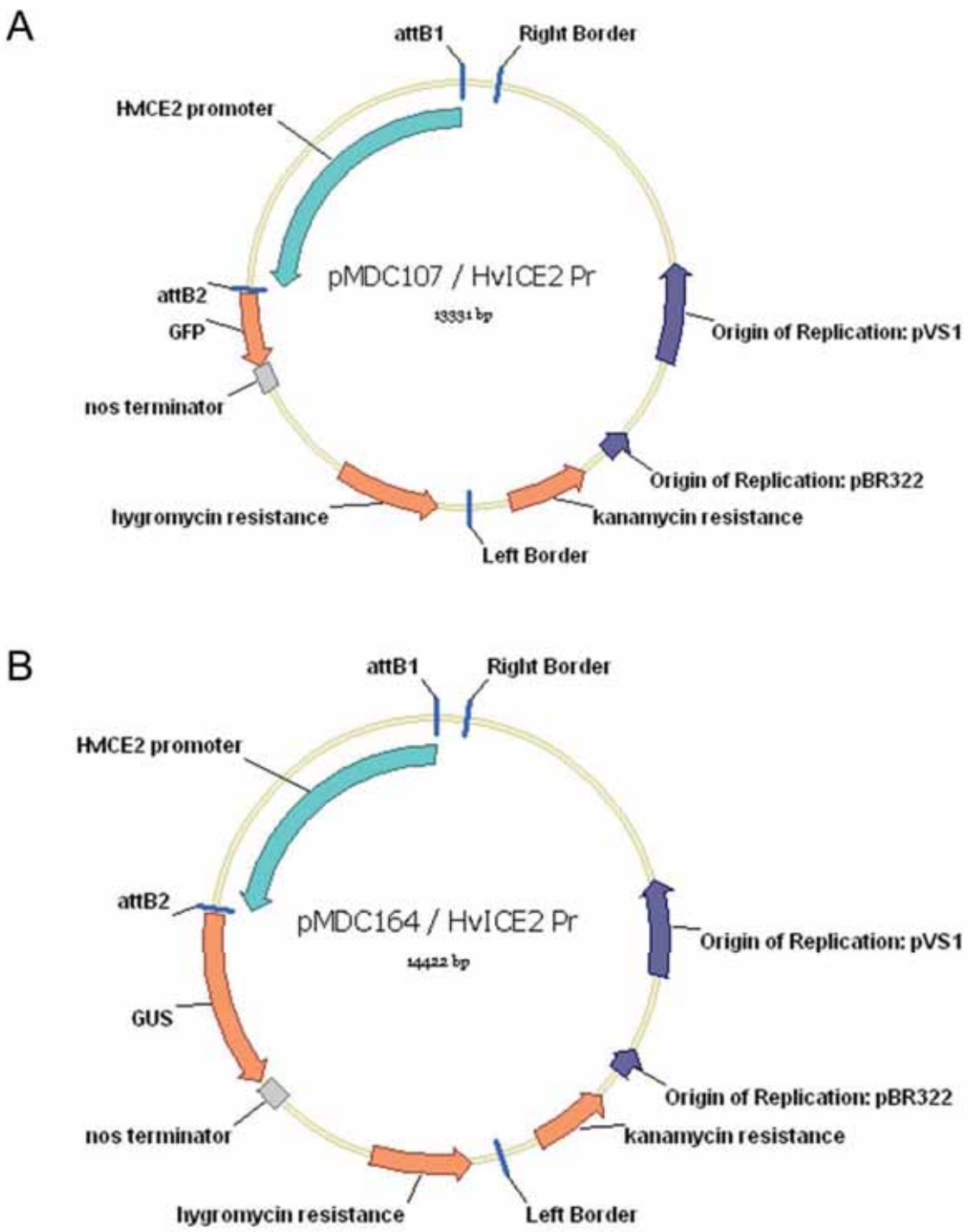


Figure 2.18 pMDC107 and pMDC164 vectors containing the promoter of *HvICE2*.

(**A**) ~3 kb of the *HvICE2* promoter in pMDC107. (**B**) ~3 kb of the *HvICE2* promoter in pMDC164. attB1 and attB2 are recombination sites.

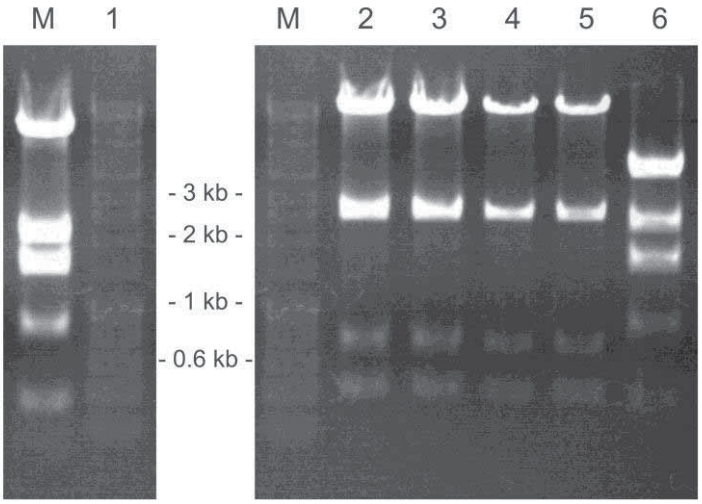


Figure 2.19 Photograph of agarose gel analyses of restriction fragments of pMDC32/*maize Ubiquitin promoter:HvICE2 CDS*, pMDC164/*HvICE2 promoter* and pMDC107/*HvICE2 promoter*.

Digestion of plant transformation constructs indicated correct assembly, producing fragments of expected sizes (refer to text). M, molecular weight marker; Lane 1, *HvICE2* over-expression construct (pMDC32/*Ubiquitin promoter:HvICE2 CDS*) digested with *Dra*III and *Pst*I; Lanes 2, 3, 4 and 5, *HvICE2* promoter driving expression of *GUS* reporter gene (pMDC164/*HvICE2 promoter*) digested with *Mlu*I; Lane 6, *HvICE2* promoter driving expression of *GFP* reporter gene (pMDC107/*HvICE2 promoter*) digested with *Acc*I.

The activity of the *HvICE2* promoter was analysed during cold treatment by examining reporter gene expression. Expression of *GUS* was examined by staining cold-treated and untreated transgenic and wild type plants for GUS activity. In general, little staining was observed in this experiment. No plants examined had GUS staining on shoots or roots, nor did the majority have staining on seeds. However some patches of staining were observed in ~30% of the transgenic seeds (n=30) (Figure 2.20) and, in general, were more commonly observed on cold-treated than untreated seed. The majority of GUS staining was on the upper surface of the seed (shown in Figure 2.20), with only small patches of weaker staining on the underside of a small proportion of the seed. Stained patches were not present on wild type seed.

Expression of *GFP* was examined by fluorescence microscopy before and within one hour following cold treatment in transgenic and wild type plants. Seed, root, leaf and sheath tissues were examined at various levels of magnification, however, *GFP* expression was not observed at any stage (Figure 2.21). The images in Figure 2.21 depict the same plants and approximate angle of view before and after treatment. The green colour observed in the seeds and roots of wild type and transgenic plants was due to autofluorescence, while the red colour in the leaf blades and sheath were due to chlorophyll fluorescence.

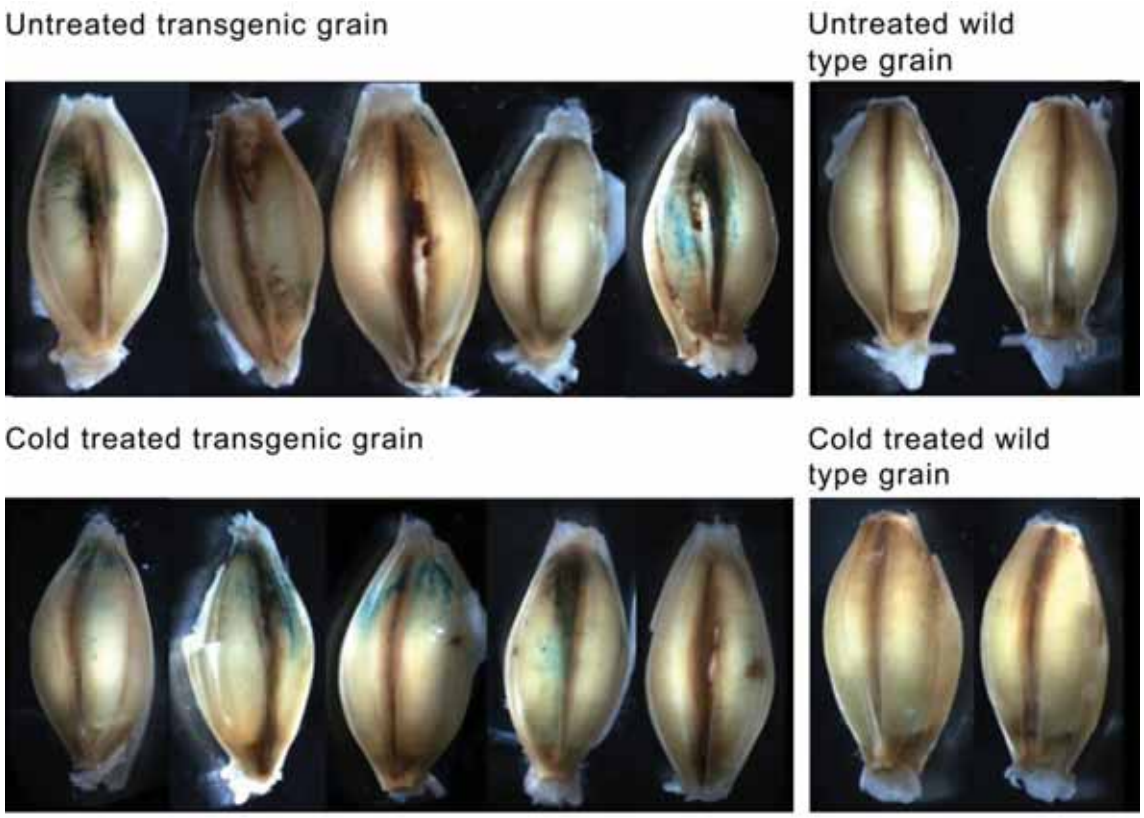


Figure 2.20 Photographs of GUS staining on the seeds of cold-treated and untreated transgenic (*promoter:GUS reporter*) or wild type plants.

Plants were examined following or without cold treatment (~48 hrs at 4°C). The presence of patches of GUS activity on seeds was inconsistent in treatment groups but where available, examples are preferentially pictured. Root and shoot tissues were excised from the seeds prior to photographing.

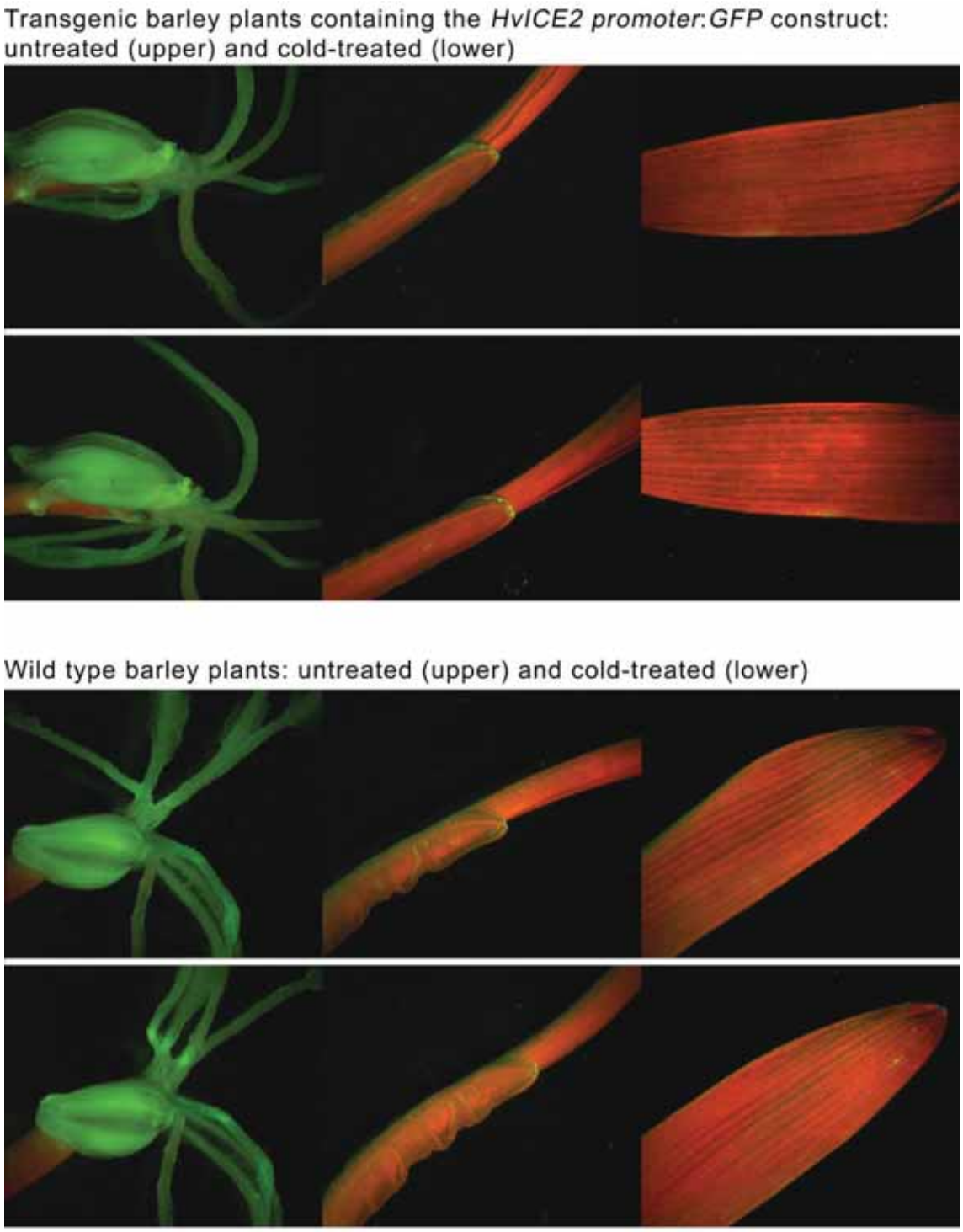


Figure 2.21 Photographs of representative cold treated and untreated transgenic (*HvICE2 promoter*:*GFP reporter*) and wild type plants.

Photographs were taken under UV light to detect *GFP* expression. No *GFP* expression of was detected in root, seed, sheath or leaf blade tissues, before or after cold treatment (~48 hrs at 4°C).

2.3.3. Analysis of Barley Plants Over-expressing *HvICE2*

To examine the effect of over-expression of *HvICE2* on plant cold tolerance and putative downstream expression, transgenic barley plants were produced with over-expression of *HvICE2* driven by the maize *ubiquitin* constitutive promoter. Constructs for plant transformation were prepared (Figure 2.22) and restriction digestion confirmed the *HvICE2* CDS had been correctly inserted (Figure 2.19). The expected product sizes were 7,276, 2,037, 1,501, 803, 293, 57 and 48 bp.

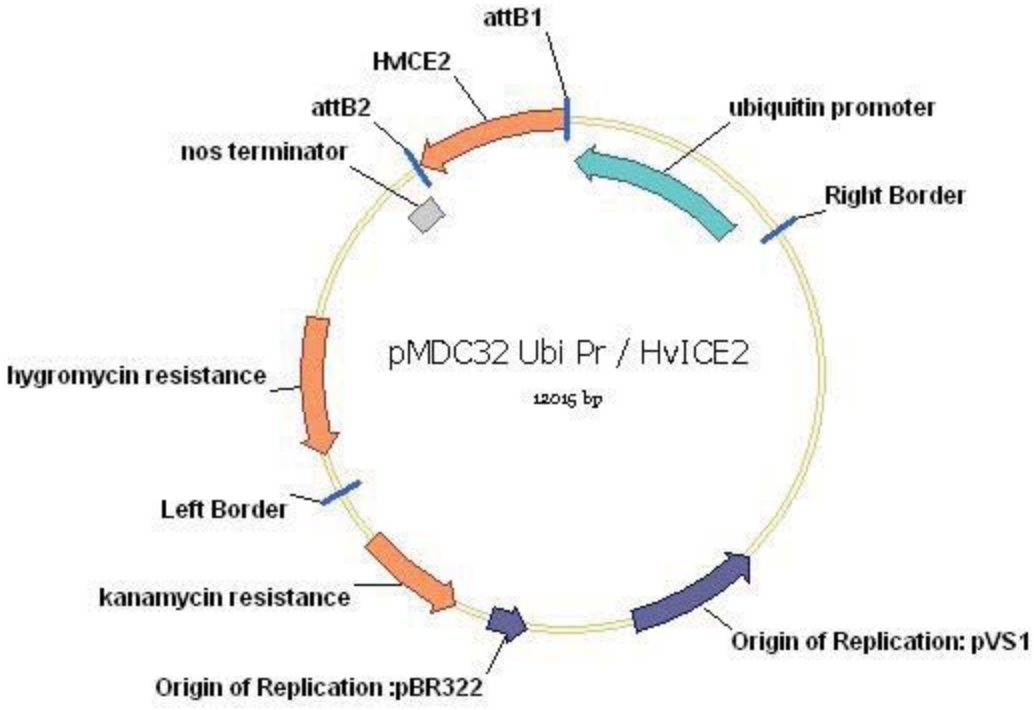


Figure 2.22 Vector map of pMDC32 vector containing *HvICE2* coding sequence. The maize *ubiquitin* promoter was used to drive expression of *HvICE2*. attB1 and attB2 are recombination sites.

2.3.3.1. Analysis of *HvICE2* Transgene mRNA Levels in Transgenic Barley Plants

The expression level of the *HvICE2* transgene in each T_0 plant was determined by qRT-PCR using primers specific for the transgene. Transgene mRNA levels varied between lines and was highest in Lines 11, 9 and 3, followed by Lines 1, 2 and 8 (Figure 2.23).

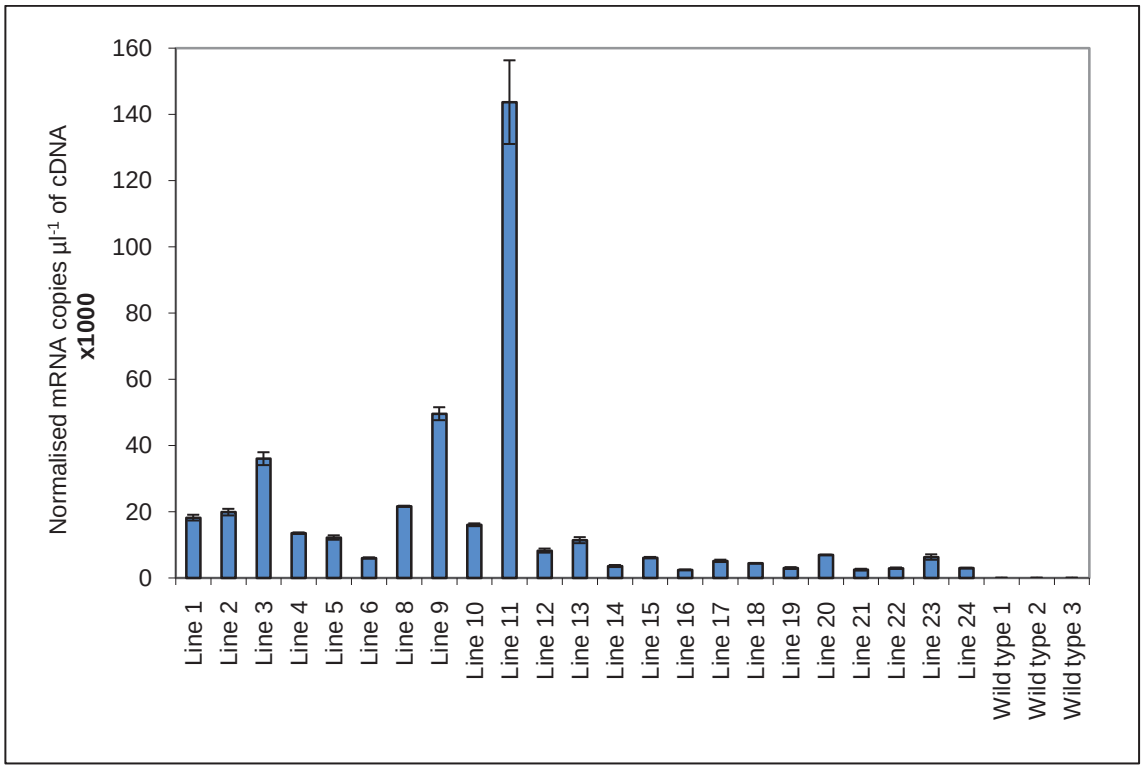


Figure 2.23 Graph of the *HvICE2* transgene expression in T_0 barley plants determined by qRT-PCR.

2.3.3.2. Southern Analysis of Barley Plants Over-expressing *HvICE2*

Southern analysis was used to determine the presence and number of copies of the transgene in each T_0 transgenic line. To avoid cross-hybridisation with the endogenous *HvICE2* gene, a probe was used which hybridised to a fragment of the maize *Ubiquitin* promoter, rather than *HvICE2*.

For many lines, bands were visible on the autoradiograph, allowing the presence and estimated number of copies of the *HvICE2* transgenic cassette to be determined (Figure 2.24). For some lines however, the resolution of the image obtained on the film was insufficient to enable exact determination of the copy number, despite optimisation of the Southern blot procedures. A number of the plants appeared to have single insertion events, including Lines 6, 8, 9, 14, 15 and 17, while other plants contained multiple copies, (commonly two or three copies). Darker bands may indicate the presence of tandem insertion events.

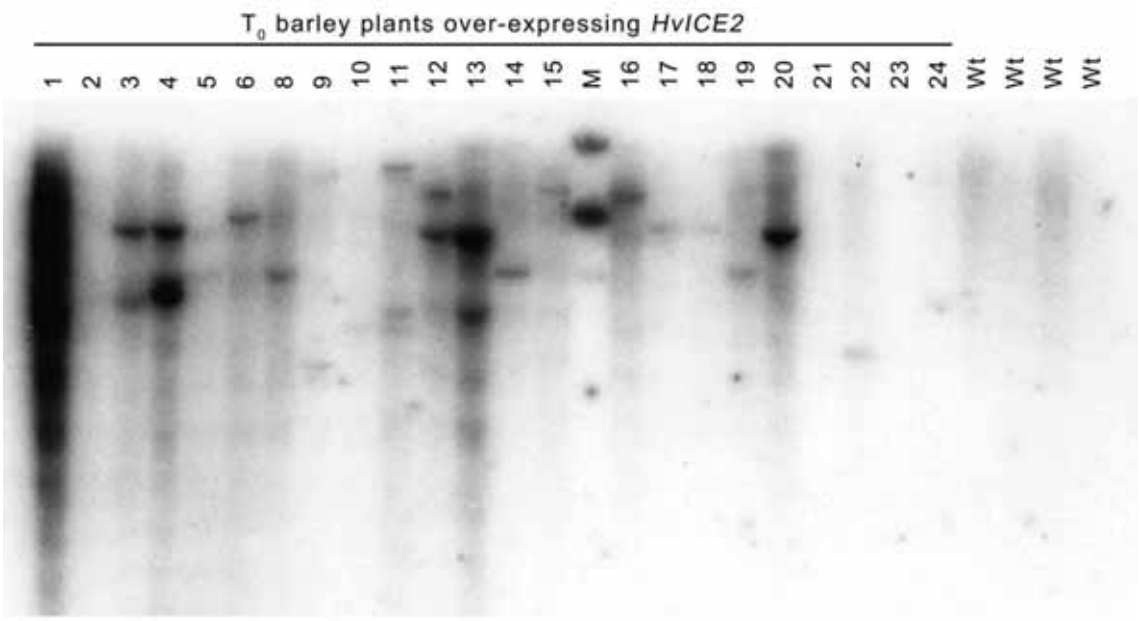


Figure 2.24 Image of autoradiograph of Southern blot of T_0 barley plants over-expressing *HvICE2*.

A probe was used hybridised to a section of the *Ubiquitin* promoter and was therefore specific to the T-DNA. The number of T-DNA insertion events varied between lines. M, Molecular weight marker; Numbers 1 to 24 (excluding 7), digested genomic DNA from T_0 barley plants; Wt, Wild type.

2.3.3.3. Analysis of Developmental Phenotypes of Barley Plants Over-expressing *HvICE2*

Analysis of developmental phenotypes was performed as part of the basic characterisation of the barley lines over-expressing *HvICE2*. T_1 plants were grown to maturity and monitored carefully over the life of the plants.

Growth, plant morphology and final plant height of lines over-expressing *HvICE2* were comparable to wild type throughout the life of the plants, with statistically insignificant variation within and between lines (Figure 2.25A; Figure 2.26). Biomass and approximate 1000 grain weight were examined and no statistically significant differences were observed in the transgenic plants relative to wild type (Figure 2.25B).

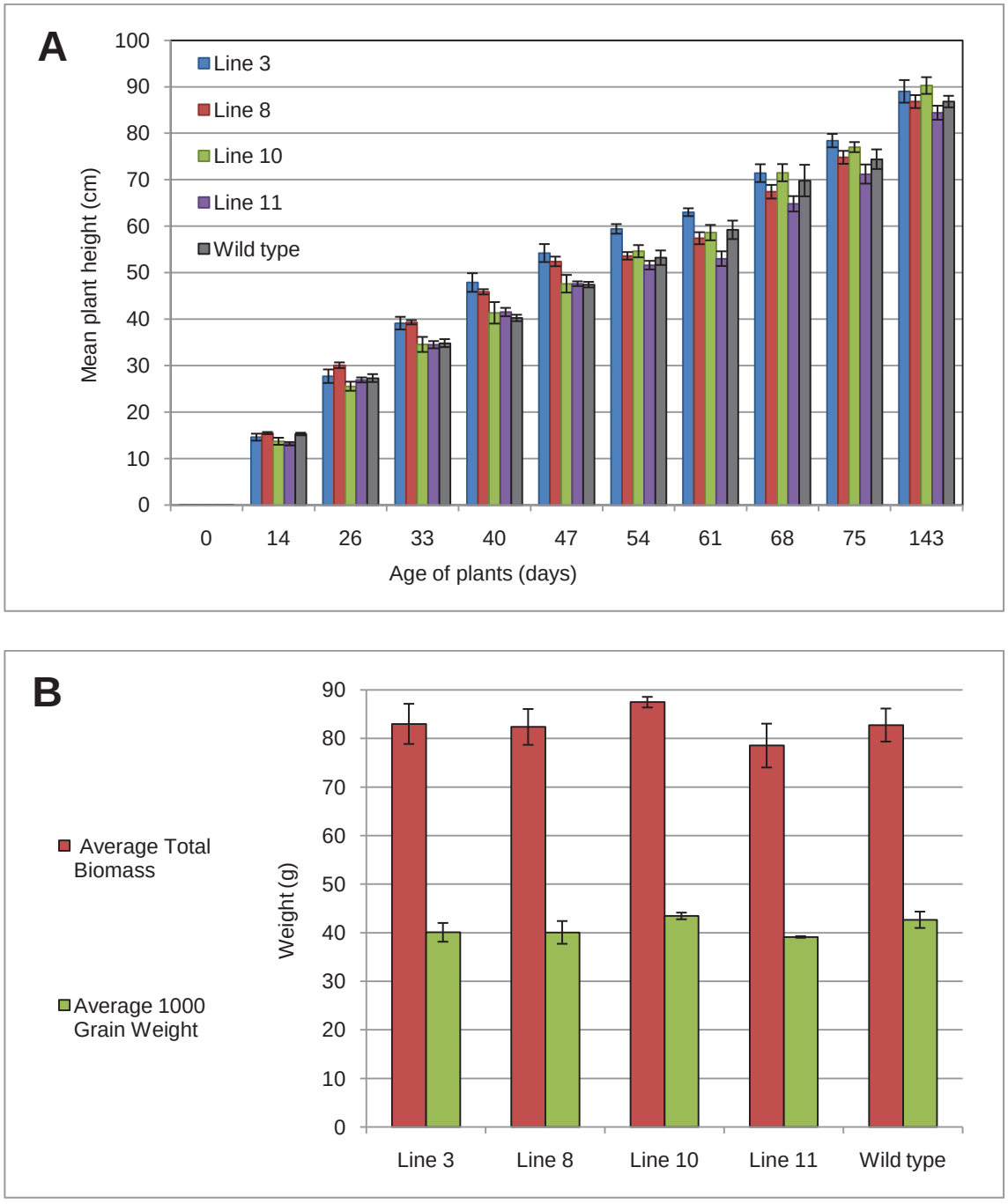


Figure 2.25 Graphic representation of the height, biomass and 1000 grain weight of barley plants over-expressing *HvICE2*.

(**A**) Plant height. (**B**) Average 1000 grain weight and total plant biomass. The weights of dried mature plants (aerial tissues only) were used to calculate total plant biomass. One-way ANOVA and grouped t-tests were performed for each trait. No significant differences were observed between wild type and transgenic plants in plant growth, total plant biomass or grain weight.

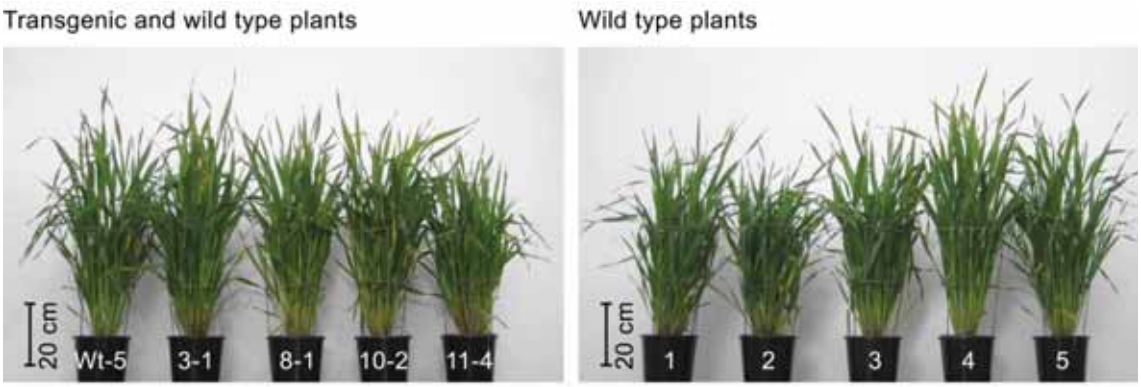


Figure 2.26 Photographs of mature untreated wild type and transgenic barley plants over-expressing *HvICE2*.

The plant height and variation seen within and between transgenic lines was similar to that between wild type plants. Note: Representative plants shown for each transgenic line. Photographs taken immediately prior to flowering. Wt, wild type.

Northern blots were produced to analyse the *HvICE2* transgene mRNA levels in the plants used for developmental phenotype analysis. As a probe could not be designed which would be specific to the transgene, the blots were probed with a) a probe which would hybridise to a section of the *HvICE2* 3' UTR and therefore only bind to endogenous *HvICE2* transcripts, and b) a probe which would hybridise to a section of the coding region of *HvICE2* and therefore bind both the endogenous and transgene *HvICE2* transcripts.

The probe designed to hybridise to both the endogenous and transgenic *HvICE2* transcripts was a 502 bp sequence which encoded the first 167 amino acids of *HvICE2*. This region was chosen as it is poorly conserved between the *ICE* genes from Arabidopsis (Figure 2.7), which indicated that it was likely to be gene-specific in barley. The *HvICE1* EST appears to be truncated at the 5' end and therefore the degree of similarity between the barley *ICE* genes cannot be determined (Figure 2.7). The probe designed to hybridise to only endogenous *HvICE2* transcripts was a portion of the 3' UTR. The conditions and stringency of the membrane hybridisation washes were comparable for both probes and the levels of radioactivity detected during probe purification were also similar for the two probes. The film exposure time differed for

the two blots however, being less than two days for the endogenous and transgene blot, and 16 days for the endogenous only blot.

The results of probing with the "endogenous only" probe showed the transcript levels of the endogenous *HvICE2* gene were very similar in all plants (Figure 2.27). The results of probing with the "endogenous and transgene" probe showed that two intensely dark bands were present in the transgenic samples, one of which (upper band) was not present in wild type samples and therefore probably corresponds to the level of transgene *HvICE2* mRNA (Figure 2.27). RNA degradation prevented analysis of Plant 4 from Line 11.

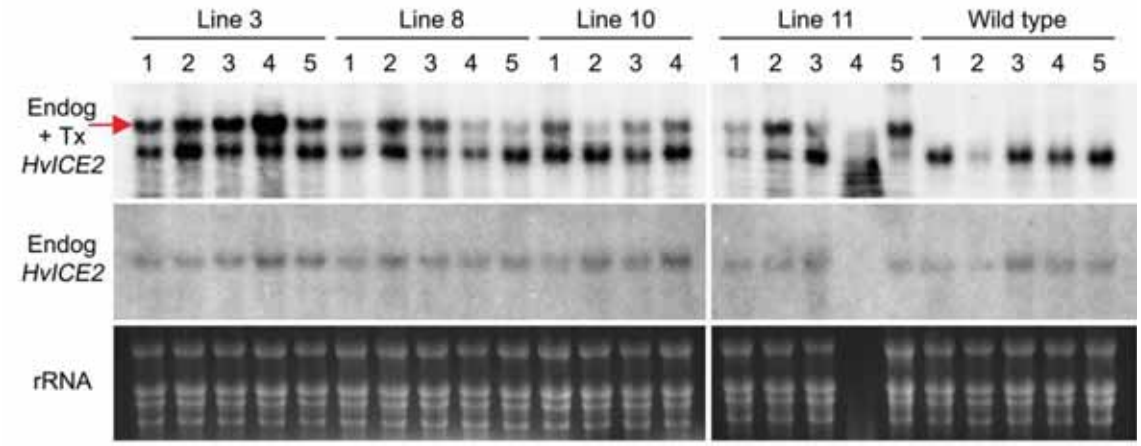


Figure 2.27 Image of autoradiograph of northern blot of *HvICE2* expression in untreated wild type and transgenic barley plants over-expressing *HvICE2*.

The results of hybridisation with probes for the endogenous (Endog) or endogenous and transgene (Endog + Tx) *HvICE2* are labelled above. A photograph of the rRNA is presented as a loading control. For reasons discussed in full in Section 2.4.4, it was concluded that signal strength of the upper band in the Endog + Tx blot was predominantly or entirely attributed to mRNA from the *HvICE2* transgene, as the level of endogenous *HvICE2* transcript was likely to be too low to be detected at this level of exposure. Accordingly, the lower band on this same blot was likely to be a result of cross-hybridisation with transcript from an unknown endogenous gene. Together, the results show that *HvICE2* transcript levels were high in transgenic plants relative to wild type plants.

Size and morphology of heads were examined and no significant differences were observed in the transgenic plants relative to wild type. Plants were also examined for unusual developmental phenotypes including approximate number of tillers and heads, time to maturity, leaf colour, and vigour and growth habit. No growth or developmental abnormalities were observed and transgenic plants appeared physically comparable to wild type plants (Figure 2.26; Figure 2.28).

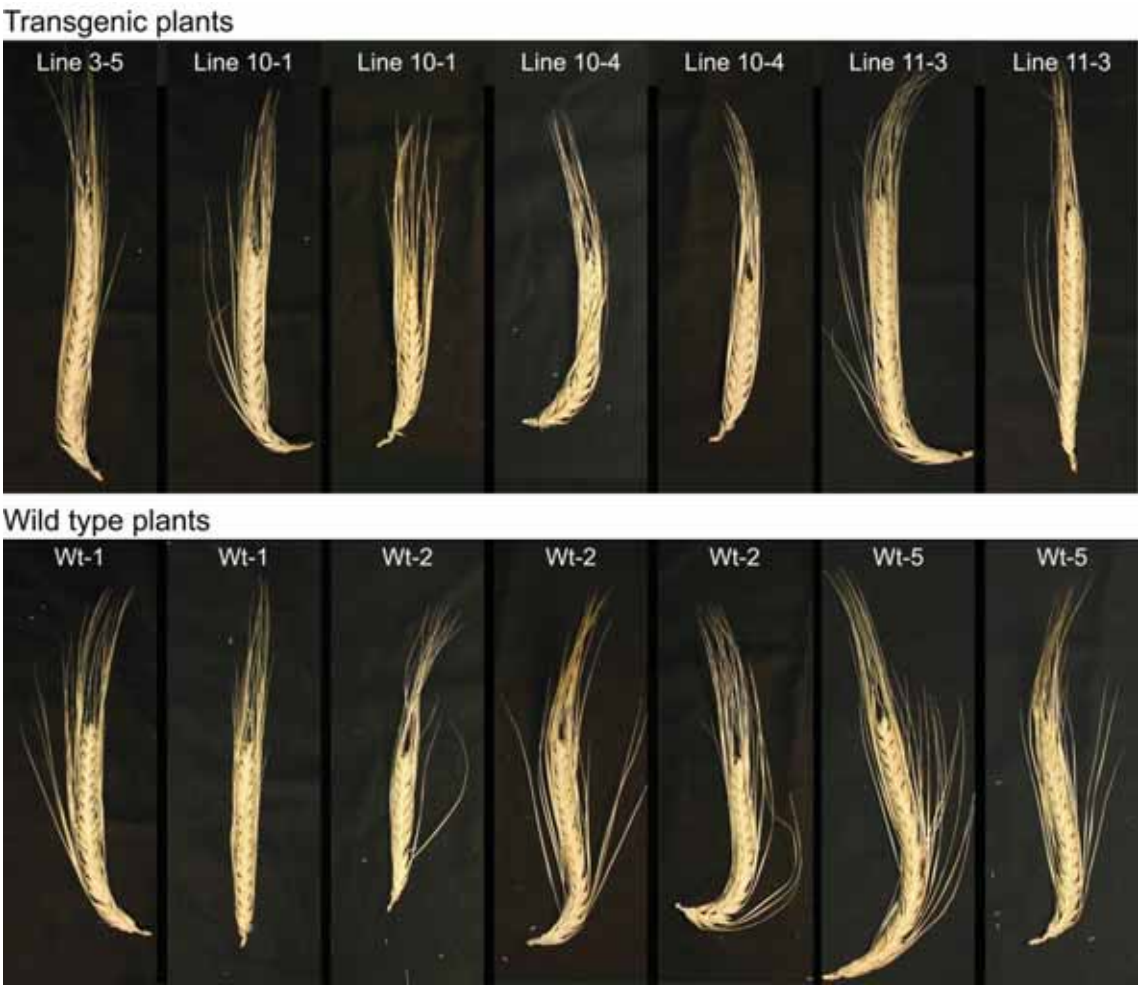


Figure 2.28 Photographs of heads of wild type and transgenic barley plants over-expressing *HvICE2*.

The level of variation in head size, filling and morphology was similar in transgenic and wild type plants. Wt, wild type.

2.3.3.4. Analysis of Seedling-stage Cold Stress Tolerance of Barley Plants Over-expressing *HvICE2*

Cold stress tolerance assays were conducted in a modified insulated cooler system designed to replicate environmental stresses. Unfortunately, the degree of variation produced in this assay system was too high to give meaningful results. Significant variation was observed both within and between genotypes and, in particular, the large spatial variation in the degree of plant damage both within a treatment and between treatments made interpretation difficult. For example, following one treatment, almost complete plant death (including controls) was observed in one region of the tray while survival of control and transgenic plants was high in another area. In addition, great difficulty was encountered when attempting to repeat the severity of a given treatment. For these reasons, data obtained from these experiments has not been presented.

2.3.3.5. Analysis of Cold Stress Response in Barley Plants Over-expressing *HvICE2*

qRT-PCR was used to determine whether the transcript levels of putative target genes of *HvICE2* (directly or indirectly) were upregulated in transgenic plants over-expressing *HvICE2*. The transcript levels of *HvICE2* and several putative downstream genes was compared in T_1 transgenic and wild type plants, prior to and during cold treatment. Transgenic Lines 3 and 11 were used as they possessed the highest levels of transgene transcript accumulation (excluding Line 9, as plants had atypical phenotypes).

Transgene-specific *HvICE2* primers were used to avoid cross-amplification of transcripts from the endogenous gene. *HvICE2* transcript levels were high in all of the transgenic lines although some variation was observed between plants within a line, which is evident in the size of the error bars shown (Figure 2.29A). Mean transcript levels of *HvICE2* in samples from Line 3 ranged from approximately 400,000 to nearly 600,000 copies μl^{-1} of cDNA and mean transcript levels in samples from Line 11 ranged from approximately 500,000 to 350,000 copies μl^{-1} of cDNA. As expected, the transcript levels were not significantly or consistently altered after cold treatment in the transgenic plants.

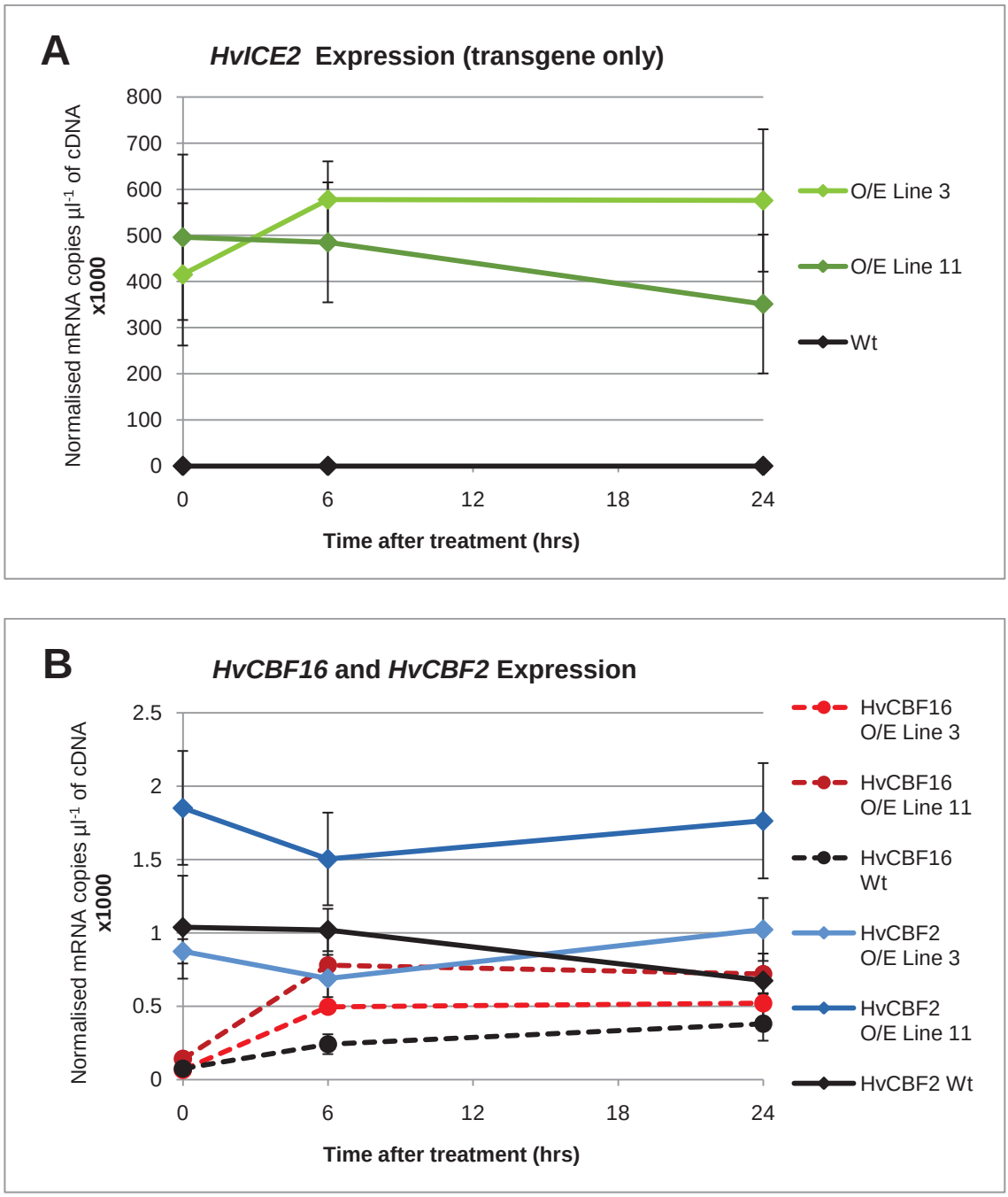


Figure 2.29 Graphs of transgene and *CBF* expression during cold treatment in barley plants over-expressing *HvICE2* determined by qRT-PCR.

(**A**) Transgene (*HvICE2*) expression. (**B**) Expression of *HvCBF16* and *HvCBF2*. Expression of *HvCBF16* was only slightly higher in transgenic plants relative to wild type plants and expression of *HvCBF2* was not altered in transgenic plants, before or during cold treatment. Wt, Wild type (untransformed) plants; O/E, Line over-expressing *HvICE2*. Error bars represent standard error.

Transcript levels of *HvCBF16* were slightly higher in the transgenic plants relative to wild type at six hrs of cold treatment (Figure 2.29B). The *HvCBF16* transcript levels were very low but the small standard error of the data points gives confidence the values are representative. Transcript levels of *HvCBF2* were not consistently higher in the transgenic lines relative to wild type (Figure 2.29B). In wild type plants, transcript levels of *HvCBF16* were extremely low prior to cold treatment, were higher after six hrs of cold treatment and remained constant until 24 hrs of treatment. Transcript levels of *HvCBF2* in wild type plants were not affected by the cold treatment.

mRNA levels of *HvDHN5* and *HvDHN8* were similar in transgenic and wild type plants, except after 24 hrs of cold treatment, when transcript levels were lower in the transgenic lines (Figure 2.30A). It should be noted that the standard error of the 24 hrs time point data is considerable. In wild type plants, expression of *HvDHN5* and *HvDHN8* was induced by cold treatment, particularly after 24 hrs of treatment.

mRNA levels of *HvCor14b* were comparable in transgenic and wild type lines for each time point (Figure 2.30B). mRNA levels of *HvRD22* were more variable in each of the transgenic lines but showed no clear difference when compared with wild type plants. In wild type plants, expression of *HvCor14b* and *HvRD22* was induced after 24 hrs of cold treatment.

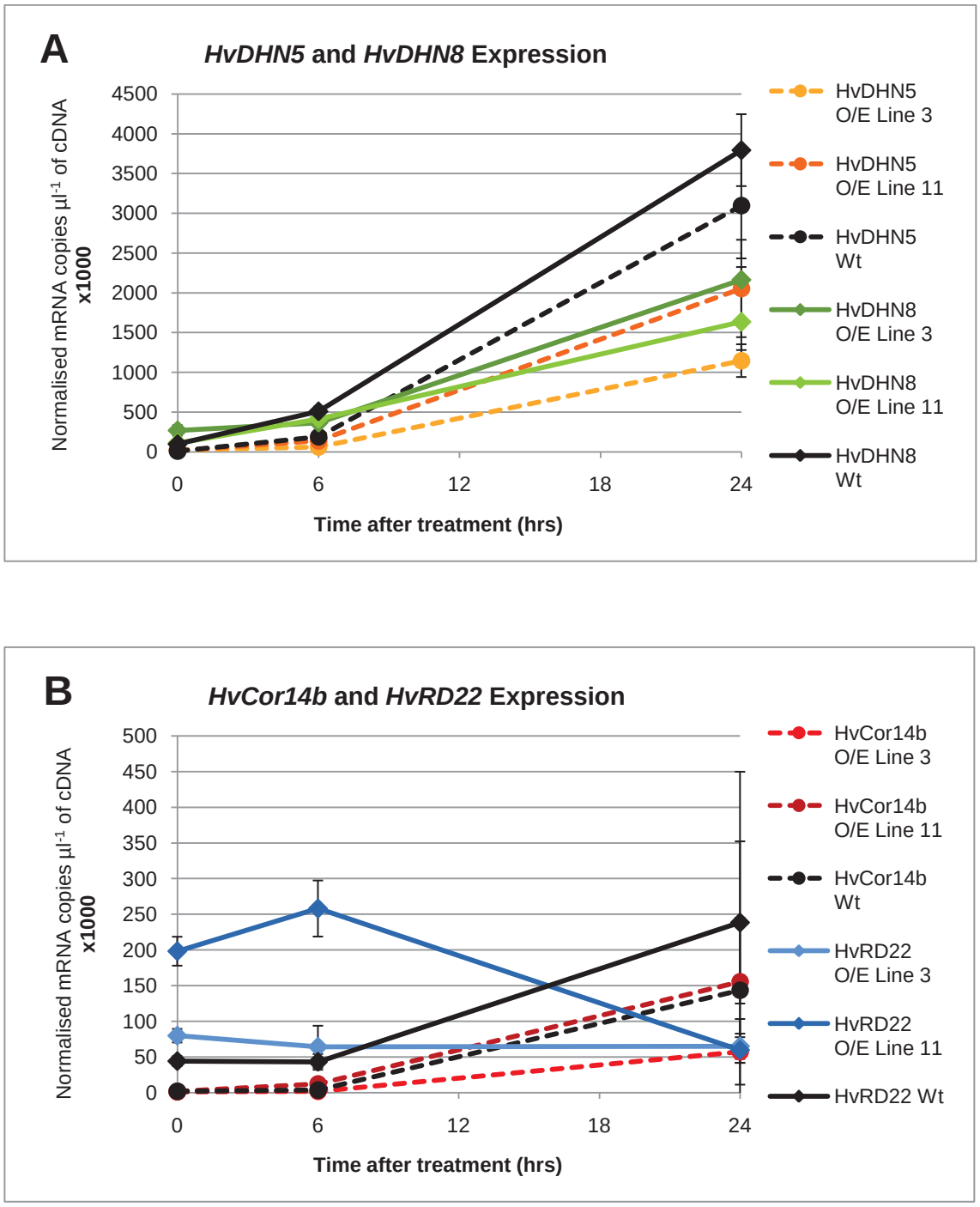


Figure 2.30 Graphs of *COR* gene expression during cold treatment in barley plants over-expressing *HvICE2* determined by qRT-PCR.

(**A**) Expression of *HvDHN5* and *HvDHN8*. (**B**) Expression of *HvCor14b* and *HvRD22*. Expression of the *COR* genes were not consistently induced in transgenic plants relative to wild type. Wt, Wild type (untransformed) plants; O/E, Line over-expressing *HvICE2*. Error bars represent standard error.

2.4. Discussion

2.4.1. Isolation and Gene Analysis of *HvICE2*

The barley *ICE*-like gene *HvICE2* was isolated because of its potential as regulator of *CBF* expression and cold tolerance in barley and progress was made towards characterisation of the gene. A ~1.1 kb cDNA of the coding region of *HvICE2* and genomic DNA fragments spanning the *HvICE2* coding region (~2.6 kb) and promoter (~3.2 kb) were amplified from barley cv. Haruna Nijo. Prior to amplification, genomic walking was used to discover the sequence of the promoter. The lengths of the *HvICE2* coding region and 5' untranslated region were confirmed by 5' RACE. It was revealed that three introns are present in *HvICE2*. In addition, the intron/exon structure of *HvICE2* is conserved in *ICE* genes from diverse plant species as the three introns are a) present in rice, Arabidopsis and barley b) of comparable relative size, and c) situated at the same position in the gene (relative to the homologous amino acid sequence in each of the genes) (Figure 2.9).

Previous studies have suggested that the two Arabidopsis ICE proteins share a significant degree of functional redundancy, likely due to their highly conserved DNA binding domains (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008). HvICE2 and the Arabidopsis ICEs had highly homologous bHLH DNA binding domains (Figure 2.7), which suggests they may be functionally alike and that target *cis*-element sequences may also be similar. This is consistent with recent findings that two wheat *ICE* genes are able to bind MYC elements in the promoter of a wheat *CBF* gene (Badawi *et al.*, 2008). Likewise, the sumoylation motif is conserved (Miura *et al.*, 2007), and was independently predicted in HvICE2 in this study. However, HvICE2 differs from Arabidopsis ICEs in the absence of two important functional motifs; the lysine rich region and KRAAM motif. Badawi and colleagues (2008) recently placed HvICE2 and a very close wheat homolog of HvICE2, TaICE41, in a clade of monocot ICE1-like proteins which are characterised by the absence of the KRAAM motif. They suggested that this difference may indicate these ICE proteins have distinct properties.

Numerous MYC transcription factors, including the Arabidopsis ICEs, form dimers with cofactors to activate transcription of target genes. The predicted dimerisation domain of the Arabidopsis ICE proteins (the leucine zipper domain) is moderately conserved in HvICE2 (Figure 2.7) and further experiments are required to determine whether HvICE2 is capable of dimerisation. The C-terminal region of HvICE2 contained a sequence similar to the ACT domain which are commonly involved regulation of proteins by binding ligands. A function for this region has not been suggested before now although this sequence was highly homologous with the corresponding regions of AtICE1 and AtICE2, which may indicate functional significance. Identification of this domain provides another possible mode of post-translational regulation of the ICE proteins which future studies could explore. The N-terminal region of the protein may contain the activation domain as, although the region is poorly conserved, it is recognised that this is not necessary for the function of an activation domain (Badawi *et al.*, 2007).

The promoter of *HvICE2* was also examined for possible functional motifs. Many putative *cis*-elements were identified (Figure 2.11). While this does not imply regulation by any particular transcription factor, it may be significant that few elements were found in the ~500 bp immediately upstream of the transcriptional start site as important *cis*-elements are often present in close proximity to the transcriptional start site (Berendzen *et al.*, 2006). Taking this approach, the LTRE/CRT/DRE and bZIP sites (at ~-250 bp) would make logical candidates for further analysis. However *AtICE1* is not regulated by CBFs in Arabidopsis, implying the LTRE/CRT/DRE motif (the binding site of CBF transcription factors) may not be an important regulatory motif for *HvICE2*. The scarcity of ABRE elements is also interesting, with only a single ABRE at -900 bp. The findings indicate that *HvICE2* is not greatly regulated by ABA levels (Figure 2.16A & B), which may be linked to the lack of ABRE elements in the promoter. The function of any of the putative elements identified in this work would need to be confirmed by further studies such as deletion mapping or mutagenesis.

2.4.2. Analysis of *HvICE2* Expression by qRT-PCR and Microarray.

The physical indicators of freezing stress which were present in the plants exposed to cold treatment, such as sterility, poor grain filling and damaged leaf and floral tissues, are all observed by farmers to a similar degree in barley crops after a radiation frost event at anthesis (Figure 2.12A & B). It was concluded that the freezing treatment was of comparable severity and effect to a natural radiation frost event, implying that results obtained from these samples may be applicable to field scenarios.

The expression of two wheat *ICE* genes was not affected by cold treatment (Badawi *et al.*, 2008) while the *AtICE1* expression is mildly upregulated by cold treatment in Arabidopsis (Chinnusamy *et al.*, 2003). Expression of the barley *ICE* gene *HvICE2* was moderately upregulated by cold (two- to three-fold) (Figure 2.13A & B). These increases in *HvICE2* expression represent a significant change, particularly as relatively minor changes in expression of transcription factors can have major effects on cellular processes. This suggests a role for *HvICE2* in cold stress response. The overall similarity between the cold-responsive expression profiles of *HvICE2* in Golden Promise and Haruna Nijo could indicate a similar mode of action in the two cultivars. However the earlier increase in expression in the cold-tolerant variety Haruna Nijo might indicate that an earlier detection and response to the cold treatment contributes to the increased cold stress tolerance of the variety. The large error bars for the Haruna Nijo samples at the recovery time point (four days after cold treatment) were caused by variation in the transcript level in different tissue samples, indicating that the expression of *HvICE2* differed between samples after cold treatment.

In the salinity stress experiment, necrosis was observed in the treated plants which was likely to be a manifestation of sodium accumulation within the oldest leaves. Plants manage high levels of salt by selectively transporting the Na^+ ions away from young growing tissues and into older leaves (Munns and Tester, 2008). Necrosis and stunted growth are common symptoms of salinity stress and the appearance and severity of these symptoms indicated that the salinity stress treatment was sufficiently but not unrealistically severe (Figure 2.14A, B & C).

Unlike *AtICE1*, which was slightly upregulated by salt treatment, *HvICE2* expression was not induced by salt treatment (Figure 2.15A & B; Chinnusamy *et al*., 2003). Presently, no information is available in the literature on the regulation of expression of cereal *ICEs* in response to salt stress, however over-expression of a rice *ICE* gene in Arabidopsis increased plant salinity tolerance (Zhou *et al.*, 2009). The expression profiles observed here suggest regulation of any role of *HvICE2* in salinity tolerance would occur at the post-transcriptional level.

ABA treatment did not induce large changes in the expression of *HvICE2* although weak and/or transient upregulation was observed in both tissues examined (Figure 2.16A & B). Although no studies have investigated the effect of ABA on expression of cereal *ICE* genes, the expression of *HvICE2* in response to ABA treatment was found to be generally similar to the mild upregulation of *AtICE1* (Chinnusamy *et al.*, 2003). In this case ABA was applied to the roots in the hydroponic solution. This method of treatment has been shown to trigger response pathways in other tissues of the plant as ABA is transported *via* the xylem vasculature (Hartung *et al.*, 2002; Sauter *et al.*, 2001; Wilkinson and Davies, 2002). However, it is possible that application of ABA directly to the leaves may trigger a more intensive response and this could be tested in further experiments. Although *HvICE2* expression was greatly upregulated in roots with a low standard error, it could not be concluded that there is a true response to ABA as the absolute expression levels of *HvICE2* in roots were at background levels for detection qRT-PCR.

Throughout the course of the experiments, the expression of *HvICE2* was examined in four barley varieties: Haruna Nijo, Golden Promise, Morex, Clipper x Sahara double haploid line 134 (Karakousis *et al.*, 2003). The *HvICE2* expression levels and overall profiles were similar in each of the genotypes with the only notable difference being slightly higher and more rapid induction of expression in the freezing-tolerant variety Haruna Nijo during the cold treatment. This is the first study in any species to determine expression of an *ICE* gene in different genotypes.

The microarray data suggest that *HvICE2* is constitutively expressed as expression is similar in most of the tissues and growth stages examined. Contrastingly, the qRT-PCR data showed more variation in the basal expression levels of *HvICE2* as expression was barely detectable in roots and only moderate in leaf and floral tissues (Figure 2.13A & B; Figure 2.15 A & B; Figure 2.16A & B). The transcript levels of *HvICE2* are low overall, which is common for many transcription factors (Figure 2.13A & B; Figure 2.15A & B; Figure 2.16A & B; Figure 2.17). *AtICE1* is also constitutively expressed although expression was slightly greater in leaf than root and floral tissues (Chinnusamy *et al.*, 2003).

Although the data suggested that there may be some variation in tissue-specific *HvICE2* expression, these differences must be interpreted with caution. The high overall metabolic activity of certain tissues (e.g. floral tissues and endosperm) may mean that elevated expression does not indicate a specific role for *HvICE2* in these tissues. Nevertheless, the highest levels of *HvICE2* expression were observed in the floral tissues of the freezing-tolerant variety Haruna Nijo during the cold treatment (Figure 2.13A). This could indicate *HvICE2* plays a role in floral tissues during cold stress response and as floral tissues are the primary site of damage during radiation frost events, *HvICE2* may make an interesting target for manipulation to improve freezing tolerance in crops.

2.4.3. Analysis of *HvICE2 Promoter*:*Reporter* Plants

Transgenic plants were produced with expression of *GUS* and *GFP* reporter genes driven by the *HvICE2* promoter. In contrast to the results from the qRT-PCR expression series, no constitutive or inducible expression of *GFP* was seen in any of the tissues studied (Figure 2.21). Likewise, in experiments using the *GUS* reporter system, GUS activity was not detected in any area except seeds, where it was inconsistent between samples (Figure 2.20). While patches of staining on the seeds may be indicative of *HvICE2* promoter activity, they could have been caused by fungal contamination of transgenic seeds which was uncommon on wild type seeds. However,

there were no visible signs of contamination. The lack of visible or significant reporter gene expression in the tissues studied (seed, root, sheath and leaf blade) may be due to the level of constitutive and inducible *HvICE2* expression (discussed above) being too low for detection using these systems. Another explanation could be that critical promoter elements lie beyond the ~3 kb of promoter cloned in this study.

2.4.4. Analysis of Barley Plants Over-expressing *HvICE2*

Over-expression of *AtICE1* in transgenic Arabidopsis plants enhanced freezing tolerance and induced transcription of downstream *CBF* and *COR* genes (Chinnusamy *et al.*, 2003). To determine whether *HvICE2* plays an analogous role in cold response in barley, the effect of over-expression of *HvICE2* on plant cold tolerance and putative downstream gene expression was examined. Plants with knocked down expression of *HvICE2* were not produced as analysis of Arabidopsis plants with knocked out expression of *AtICE1* did not prove fruitful (Chinnusamy *et al.*, 2003). Although no studies describe knock outs of cereal *ICE*, the mutation has not been suggested to be lethal. Transgenic Lines 3, 8, 10 and 11 were chosen for analysis, as these had high transgene expression and a minimum number of transgene insertion events (Figure 2.23; Figure 2.24). T_1 transgenic plants were examined for developmental phenotypes and the transgene expression level in these plants was determined by northern blot.

Two probes were used for northern blot analysis, one which hybridised to only the endogenous gene and one to both the endogenous gene and the transgene. Two bands were detected in the hybridisation using the 'endogenous and transgene' probe. From the combined results of the northern blot hybridisations, two conclusions were drawn: a) that it is likely that the upper band of this blot represents the transcript levels of the *HvICE2* transgene, and b) that the lower band is caused by cross-hybridisation with an unknown endogenous gene and not *HvICE2* endogenous transcript.

That the upper band represents the *HvICE2* transgene mRNA was concluded from the absence of the upper band in the wild type samples. That the lower band was not representative of *HvICE2* endogenous transcript was concluded from the observation

that transgene transcript levels were likely to be much higher than those of the endogenous *HvICE2* gene, as was shown by qRT-PCR (~50-fold difference was observed; Figure 2.15A and Figure 2.16A; Figure 2.29A), and was suggested by the longer in film exposure time required to visualise bands for the 'endogenous only' probe (16 days), relative to the 'endogenous and transgene' probe (<2 days). This suggests that signal corresponding to the endogenous *HvICE2* transcripts may be too weak to be detected at the level of exposure used for the 'endogenous and transgene' hybridisation, and therefore that the lower bands were likely to be caused by cross-hybridisation with a different endogenous gene. The generally high sequence similarity between *HvICE1* and *HvICE2* suggests *HvICE1* is a likely candidate for this, although this region corresponding to the *HvICE2* probe site is not present in the 5' truncated *HvICE1* EST sequence (Figure 2.7). In conclusion, it was determined that although transgene expression varied, there was high levels of constitutive over-expression of *HvICE2* in all the transgenic plants relative to wild type (Figure 2.27).

Constitutive over-expression of *HvICE2* did not affect the growth or development of transgenic plants (Figure 2.25; Figure 2.26; Figure 2.28). This is consistent with the lack of change in expression of putative downstream genes in the transgenic plants (discussed in detail below). Although over-expression of transcription factors is often associated with stunted growth or unusual developmental phenotypes, growth and development were also normal in Arabidopsis plants with constitutive expression of *AtICE1* or the rice *ICE* gene *OsbHLH2* (Chinnusamy *et al.*, 2003; Zhou *et al.*, 2009).

Preliminary freezing tolerance experiments were performed. However the variation in the system was too great to allow conclusions to be drawn about the freezing tolerance of transgenic barley plants over-expressing *HvICE2*. Future experiments could include freezing tolerance assays in a different system and published studies of wheat *ICE* genes suggest an extended, cold acclimation-style chilling period prior to freezing may improve the potential freezing tolerance of the transgenic plants (Badawi *et al.*, 2008).

In Arabidopsis, over-expression of *AtICE1* caused upregulated expression of *AtCBF3*, and thereby, indirectly upregulated expression of *Rd29A* and numerous other *COR* genes (Chinnusamy *et al.*, 2003). To determine whether over-expression of *HvICE2* upregulated barley *COR* and *CBF* genes, the expression of various *CBF* and *COR* genes in transgenic plants, under cold and normal conditions, was examined. This approach was validated by Badawi and colleagues (2008) recently, who suggested similar experiments would be required to evaluate the function of wheat *ICE* genes in cold response.

The two *CBF* genes examined, *HvCBF16* and *HvCBF2*, were chosen as their expressions are regulated by cold, they were considered in the literature to be particularly likely to be important for cold stress tolerance, and their roles in stress response were of particular interest to this project (Knox *et al.*, 2008; Stockinger *et al.*, 2007). The *HvDHN5*, *HvDHN8*, and *Cor14b COR* genes were chosen for analysis as their expression is considered a good indicator of overall plant cold tolerance (Knox *et al.*, 2008; Stockinger *et al.*, 2007; Tommasini *et al.*, 2008) and previous studies by this group and others have shown they are likely to be targets of *CBF* regulation (Skinner *et al*., 2005; Chapter 5 of this work). Expression of the barley homolog of the Arabidopsis stress-responsive *COR* gene *RD22* was also examined (Yamaguchi-Shinozaki and Shinozaki, 1993).

In general, the wild type expression of the *CBF* and *COR* genes studied here agree with our previous findings and those in the literature. Expression of *HvCBF16*, *HvDHN5*, *HvDHN8* and *HvCor14b* were all induced by cold treatment and exhibited induction kinetics as described in the literature (Figure 2.29B; Figure 2.30A & B; Skinner *et al*., 2005; Stockinger *et al*., 2007; Tommasini *et al*., 2008). The increase in expression of *HvRD22* observed under cold treatment in this study is consistent with findings from our previous work (Figure 2.30B; Jacobs and Pillman, unpublished results). In contrast to the findings of previous studies that *HvCBF2* expression was greatly induced by cold treatment (Skinner *et al*., 2005; Stockinger *et al*., 2007; Xue, 2003), the results in Figure 2.29B showed that *HvCBF2* expression was not upregulated during cold treatment. This difference is difficult to explain, as the conditions in each experiment were similar,

including treatment temperatures and sampling points and although the Golden Promise cultivar was not used, expression was examined comprehensively, in cold-tolerant, -intolerant, winter, facultative and spring varieties. One possible explanation may be derived from the findings of Stockinger and colleagues (2007), which showed that the level and temperature sensitivity of *HvCBF2* expression varied between cultivars and was sensitive to factors such as photoperiod and vernalisation. As no studies have yet examined *HvCBF2* expression in Golden Promise under any conditions, it is possible that the differences between our results and published findings were caused by varietal differences and/or environmental conditions.

Transgene expression was high and was at a similar level in both of the transgenic lines studied. Within each line however, transgene expression varied. This is likely due to segregation of transgene copies as the Southern blot results show that both of the transgenic lines examined had multiple transgene insertion events (Figure 2.24; Figure 2.29A).

Although a small increase in *HvCBF16* expression was seen between the transgenic plants over-expressing *HvICE2*, the level of expression at any stage was considerably lower than in the cold stress treatment series where a considerable induction was observed in wild type plants (Figure 4.10B; discussed in Chapter 4 of this work). In addition, the *HvCBF16* expression levels observed in the transgenic plants were within background levels of qRT-PCR at all time points. Together, these results suggest that expression of *HvCBF16* was not effectively induced in lines over-expressing *HvICE2* as the differences observed were not significant in the context of the maximum expression observed for this gene.

Over-expression of *HvICE2* and a short cold treatment was not sufficient to alter expression of *HvCBF2*, *HvCor14b* and *HvRD22* (Figure 2.29B; Figure 2.30B). Likewise, expression of *HvDHN5* and *HvDHN8* was at comparable levels in wild type and transgenic plants at most of the time points (Figure 2.30A). Although *HvDHN5* and *HvDHN8* transcript levels were lower in transgenic than wild type plants after 24 hrs of

cold treatment, caution should be used when considering this result as the standard error of the data points is high (particularly in the case of *HvDHN5*) relative to the difference in expression between genotypes.

Together, these results suggest that over-expression of *HvICE2* and cold treatment of up to 24 hrs was not sufficient to induce expression of any of the *CBF* or *COR* genes studied here. Although this result differs from that observed for over-expression of *AtICE1* in Arabidopsis (Chinnusamy *et al.*, 2003), these findings are consistent with the lack of associated QTLs for cold tolerance in *Triticeae* at the corresponding map location (Skinner *et al.*, 2006; Tondelli *et al.*, 2006) and indicate that *HvICE2* does not, alone, control the cold tolerance trait.

As mentioned in passing above, two studies have recently described the characterisation of cereal *ICE* genes: *OsbHLH2*, from rice (Zhou *et al.*, 2009), and *TaICE41* and *TaICE87*, from wheat (Badawi *et al.*, 2008). Of particular interest is TaICE41, which shares a very high degree of sequence identity with HvICE2 (95.8% amino acid identity) and is likely to be the wheat ortholog of HvICE2. These studies provide a useful context in which to consider the results presented here regarding *HvICE2*. Both studies report marked similarities between the functions of the rice and wheat *ICE* genes and their Arabidopsis counterpart, *AtICE1*; over-expression of these genes in Arabidopsis increased expression of several *AtICE1* target genes including *AtCBF3* and *COR* genes. In addition, the wheat *ICE* genes can bind the promoter and activate expression of a wheat *CBF* gene, *TaCBF1Vd-B9*. These results show that, despite a degree of amino acid divergence, wheat and rice *ICE* genes share common functionality with *AtICE1* in the heterologous Arabidopsis system and suggest that *ICE* genes from *Triticeae* may also function in activation of *CBF* genes in their native species, presumably triggering activation of the *CBF* transcriptional cascade. The wheat ICE target gene *TaCBF1Vd-B9* is a member of the same phylogenetic *CBF* subgroup (the *HvCBF4*-subgroup) as *HvCBF2*, the barley ICE putative target gene studied here. This suggests that the barley ortholog HvICE2 may likewise regulate barley *HvCBF4*-subgroup members, including *HvCBF2*, which is not consistent with our findings.

Though there are similarities between the functions of cereal and Arabidopsis ICEs, differences have been noted which may help to elucidate the reason our findings differ from those for *AtICE1*. In contrast to studies of *AtICE1*, over-expression of *OsbHLH2* in Arabidopsis did not alter *COR* gene expression during cold stress and although salt stress treatment induced differences in gene expression in the transgenic plants, only some AtICE1 target genes were affected (Zhou *et al.*, 2009). In addition, Arabidopsis plants with over-expression of wheat *ICE* genes required cold acclimation before enhanced freezing tolerance was observed (Badawi *et al.*, 2008). These results suggest that although cereal *ICE* genes may be capable of binding the promoters of *CBFs* and activating transcription *in vitro* and in heterologous systems, in a number of cases the effective function of cereal ICE proteins requires additional factors. Research has shown that in addition to AtICE1, the cold-response signalling pathway in Arabidopsis contains several regulatory components (including SIZ1, HOS1 and MYB15, etc.) and environmental conditions (e.g. low temperatures) which cooperatively control the ICE/CBF signalling pathway (Chinnusamy *et al.*, 2007). These or other factors may be the reason for the differences in function observed here and in the literature between cereal *ICEs* and *AtICE1*. Further, the question is raised of the degree to which the regulation and function of the *ICE* genes is conserved between plant species.

SIZ1 is a cold-regulated SUMO E3 ligase which positively regulates AtICE1 by stabilising and/or activating the protein (Miura *et al.*, 2007). A potential sumoylation site was found in HvICE2, and is conserved in ICE proteins from wheat and Arabidopsis, suggesting a SIZ1-like cofactor may regulate the activity of HvICE2. In support of this possibility, the cold-activation of SIZ1 may explain the need for cold treatment before increased cold tolerance was observed in Arabidopsis plants over-expressing wheat *ICE* genes (Badawi *et al.*, 2008). This scenario could also explain why over-expression of *HvICE2* was not sufficient to alter target gene expression, as *HvICE2* activity might not rely on absolute transcript levels but on stabilisation and/or activation of the HvICE2 protein caused by correct triggering of the SIZ1-like activator. Besides the sumoylation site, interaction with cofactors or regulators could also occur *via* the leucine zipper or bHLH dimerisation domains. The identification of the ACT domain in the conserved C-terminal region suggests that HvICE2 may be regulated by

ligand binding, providing another possible method of regulation of ICE proteins which has not been explored.

Alternate explanations for the results presented here could be that the genes selected as possible targets were not appropriate and/or the mechanism of action may be different to that of *AtICE1* and/or the rice and wheat *ICE* genes. In the case of *HvCBF16*, it is possible that *HvCBF16* is a target of *HvICE2* and the low expression observed is sufficient for gene function. Alternatively, *HvICE2* may not be involved in cold stress response in any way, as the results would tend to suggest.

As an additional note, although compelling evidence is presented to suggest wheat and rice *ICE* genes regulate *CBFs* genes, over-expression of the cereal *ICEs* has not yet been performed in their native species. These results therefore represent the first investigation of this type for cereal *ICE* genes.

Further experiments are required before conclusions can be drawn regarding the role of *HvICE2* in regulation of barley *CBF* and/or *COR* genes, or in cold response. In addition to cold tolerance assays, it would be useful to determine, *via* western blot analysis, whether over-expression of the *HvICE2* mRNA transcript resulted in increased protein content in the transgenic plants, and thereby investigate whether *HvICE2* was being regulated at the level of mRNA translation and/or protein stability. It would also be useful to determine whether HvICE2 shares the ability of its wheat ortholog to activate expression of *CBF* genes *in vitro*, or in a heterologous Arabidopsis system.

2.5. Conclusions

The aim of the work described in this chapter was to examine the similarities and differences between AtICE1 and its barley homolog, HvICE2, to determine whether HvICE2 plays a similar role in cold stress response and whether this gene could therefore be used to engineer cold tolerance by over-expression in barley plants. Comparison of the gene structures of *HvICE2* and *AtICE1* revealed that several important features are conserved, including the bHLH domain and sumoylation motif, however, two functional motifs, including the site of the *ice1* mutation, are missing or altered. Although conclusions cannot be drawn from sequence analysis alone, these similarities suggest certain aspects of the function of HvICE2, such as target sequence specificity and protein regulation, may be similar to that of AtICE1.

The *HvICE2* gene was characterised, including examining the intron/exon structure of the gene and confirming the full-length sequence by 5' RACE. Promoter analysis revealed the sequences of numerous putative *cis*-elements involved in stress response, whose function may be validated by further experiments such as promoter deletion mapping by transient/stable plant transformation.

HvICE2 expression is induced in floral and leaf tissues under low temperatures, such as in a radiation frost event, and mRNA levels are higher in the freezing-tolerant cultivar Haruna Nijo. This low temperature responsiveness suggests *HvICE2* may play a role in cold response. Contrastingly, *HvICE2* is either not involved in salinity or ABA responses or is regulated post-transcriptionally in response to these stresses, as *HvICE2* expression is relatively unaffected by salinity treatment or ABA treatment. *HvICE2* was constitutively expressed to a similar level in most tissues although expression was found to be higher than average in floral tissues and particularly low in root tissues.

In Arabidopsis, plants over-expressing *AtICE1* or *OsbHLH2* showed no growth or developmental abnormalities (Chinnusamy *et al*., 2003; Zhou *et al*., 2009). Likewise, transgenic barley plants over-expressing *HvICE2* were also developmentally normal. Although preliminary freezing tests were performed, the freezing tolerance of the

transgenic plants was not able to be determined. Expression of putative target genes of *HvICE2* was examined, including two barley *CBFs* (*HvCBF2* and *HvCBF16*), and four *COR* genes (*HvCor14b*, *HvDHN5*, *HvDHN8* and *HvRD22*). None of the *CBF* or *COR* genes were significantly upregulated in the transgenic plants relative to wild type plants. It was concluded that over-expression of *HvICE2*, combined with a short cold treatment, was not sufficient to alter the expression of these *CBF* and *COR* genes. Further experiments are required to determine whether this result is a product of additional conditions or cofactors being required for effective gene activation by *HvICE2*, or incorrect choice of target genes.

In conclusion, although several key functional motifs were conserved between HvICE2 and both Arabidopsis and wheat ICEs (Badawi *et al.*, 2008; Chinnusamy *et al.*, 2003), sequence divergence in some areas suggests HvICE2 may also have distinct properties from AtICE1. Although *HvICE2* expression is mildly to moderately induced by cold stress, there was nothing in the results to indicate that *HvICE2* plays a role in cold stress response by regulation of *CBF* and/or *COR* genes. Future work could include freezing assays using a more controlled system to determine whether over-expression of *HvICE2* in barley increases the freezing tolerance however the unaltered levels of *CBF* and *COR* gene expression in the plants casts doubt over whether these transgenic plants are likely to be more frost tolerant without additional unknown activating factor(s). Nevertheless, when the results are viewed in the context of studies of other cereal *ICE* genes (Badawi *et al.*, 2008; Zhou *et al.*, 2009) it is clear that further experiments should be performed to determine the role of *HvICE2* in abiotic stress responses.

Conclusions have not been drawn about whether barley *ICE* genes function in cold tolerance, the results do suggest that this component of the signalling pathway differs or is more complex than in Arabidopsis. It may prove fruitful to look ‘closer to home’ for new *ICE* genes to improve our understanding of the *ICE* component of the *ICE*/*CBF*/*COR* signalling pathway, and so ultimately determine new ways to improve cold tolerance in plants. Accordingly, characterisation of the closest relative of *AtICE1* in Arabidopsis, *AtICE2*, presents an attractive target for further study, and was explored in the work presented in the following chapter.

CHAPTER 3

Analysis of *AtICE2* Transgenic Arabidopsis Plants

3.1. Introduction

The study by Kanaoka *et al*. (2008) suggested the roles of *AtICE1* and *AtICE2* in stomatal cell differentiation were somewhat redundant and complementary. Currently, neither the expression of *AtICE2* under cold stress nor the effect of manipulating the *AtICE2* gene on cold tolerance has been studied. Characterisation of the role of *AtICE2* in cold response will also provide information about whether *ICE* genes other than *AtICE1* are able to trigger the signalling cascade and therefore how broad or robust the *ICE* section of the *ICE*/*CBF*/*COR* cold response pathway is.

This chapter describes an investigation of the role of *AtICE2* in cold stress signalling and comparison with the role of *AtICE1*. As stated in Chapter 1, AtICE1 induces expression of *CBF* and *COR* genes in response to cold treatment and thereby increases plant cold tolerance. The aim was to engineer constructs of *AtICE2* and using these, produce transgenic Arabidopsis plants that had either over-expression of *AtICE2* or reduced expression of endogenous *AtICE2*. The resultant transgenic plants would then be analysed for cold stress tolerance and gene expression levels of putative downstream target *COR* and *CBF* genes and the results would be compared to findings regarding *AtICE1*.

3.2. Materials and Methods

3.2.1. Materials

pENTR/D TOPO was purchased from Invitrogen (VIC, Australia). Murashige and Skoog Basal Medium, Murashige and Skoog Basal Salt 10 x Macronutrient, Murashige and Skoog Basal Salt 10 x Micronutrient Solutions, MES hydrate, carbenicillin disodium salt, rifampicin, glufosinate-ammonium, mannitol, dipotassium phosphate trihydrate, myo-inositol, nicotinic acid, pyridoxine.HCl, thiamine.HCl and glycine were purchased from Sigma-Aldrich (NSW, Australia). Vac-In-Stuff (Silwett L-77) was purchased from Lehle Seeds (Germany). Sucrose was purchased from Unilever (VIC, Australia) or Ajax Chemicals (NSW, Australia). Avanti® J-E Centrifuge System was produced by Beckman Coulter Inc. (NSW, Australia). Other materials were supplied as described in Section 2.2.1.

3.2.2. Production of *AtICE2* Over-expression and RNAi Constructs

To determine the levels of sequence similarity *AtICE2* shared with *AtICE1* relative to other Arabidopsis genes, the nucleotide sequence of *AtICE2* (NM_101157) was compared (blastn) to the non-redundant nucleotide database at the NCBI website (www.ncbi.nlm.nih.gov/).

Plasmid DNA of the *AtICE2* coding region and *AtICE2* cDNA fragment used for RNAi knock down, both in pENTR/D TOPO, were kindly provided by Ms. Natasha Bazanova (ACPFG) (sequences in Appendix F.3). The inserts were sequenced using M13F and M13R primers (Appendix A) as described in Section 2.2.2.6.

The *AtICE2* coding sequence and the *AtICE2* RNAi fragment were transferred to the destination vectors pJawohl8 (Genbank Accession Number AF408413) and pTOOL2 (kindly supplied by Dr. Andrew Jacobs) respectively by LR recombination reactions as described in Section 2.2.3.8.1 with incubation at 25°C overnight. A 1µl aliquot of each of the products was transformed into *E. coli* by chemical transformation and plated onto selective LB agar plates containing ampicillin at 100 µg ml^{-1} as described in Section

2.2.2.4. Plasmid DNA was isolated and diagnostic digestion and electrophoresis was performed to confirm recombination had occurred as described in Section 2.2.2.5 with the following modifications: 5 µl of plasmid DNA was used as template with *Sac*II (over-expression construct) or *Hind*III (RNAi construct) restriction enzymes.

3.2.3. Transformation of *A. tumefaciens* by Electroporation

A. tumefaciens (strain GV3101) cells were transformed by electroporation using a Gene-Pulser apparatus. A programmed setting was used for *A. tumefaciens* (cuvette size: 1 mm, Voltage: 2400 V, Capacitance 25 µF, Resistance: 200 Ω). Cuvettes (1 mm) and plasmid DNA was placed on ice for at least 1 hour prior to use. 40 µl of Agrobacterium cells (kindly supplied by Ms. Melissa Pickering, Australian Centre for Plant Functional Genomics) were thawed on ice for 5-10 min before chilled, purified mini plasmid DNA preparations (1 µl) were added. This mixture was transferred to a cuvette and pulsed. Transformed cells were immediately resuspended in 1 ml of YM media (0.01% w/v NaCl, 1% w/v mannitol, 0.04% w/v yeast extract, 0.02 w/v $MgSO_4.7H_2O$, 0.05 % w/v $K_2HPO_4.3H_2O$, pH 7.0). The sample was transferred to a 1.5 ml Eppendorf tube and placed in a 28°C water bath for 2 hrs. The cells were plated onto selective YM 0.8% w/v agar plates containing rifampicin at 25 µg ml^{-1}, kanamycin at 25 µg ml^{-1}, and carbenicillin disodium salt at 50 µg ml^{-1} and incubated at 28°C for 2 days. Colonies were picked from plates and transferred to 10 ml tubes containing 5 ml of selective YM media as described above and incubated at 28°C with shaking for 48 hrs. Glycerol stocks were prepared by mixing 1 ml of 50% v/v glycerol with 500 µl of Agrobacterium culture and snap freezing in liquid nitrogen.

To obtain plasmid DNA from the Agrobacterium, glycerol stocks were streaked onto selective LB plates and incubated at 28°C for two days to obtain single colonies. For each construct, a single colony was smeared onto a fresh plate to allow further growth before being used to inoculate 10 ml cultures of selective YM media. The cultures were incubated at 28°C for two days with shaking. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions including the optional Buffer PB washing step and eluted in 50 µl of water.

PCR was performed using the plasmid DNA isolated from the cultures as template, to confirm the transformation was successful. PCR was performed as described in Section 2.2.2.2 with the following modifications: 0.5 µl of 10 µM primer stocks were used (primers presented in Table 3.1) with 1 µl of plasmid DNA as template and only one round of PCR was performed. Positive control reactions were prepared containing 1 µl of a 10-fold dilution of the construct plasmid DNA used for Agrobacterium transformation. The cycling parameters were as described in Section 2.2.2.2 with an annealing temperature of 52°C and an extension time of 2 min and 10 sec.

Table 3.1 Primers for PCR analysis of *AtICE2* constructs and transgenic plants.

Experiment	Gene/ construct Target	Primer Pairs	Expected Size of Product
AtICE2 construct Agrobacterium confirmation PCRs	*AtICE2* over-expression	35S_Prom : ICE2_RNAir	678 bp
		35S_Prom : 35S_Term	1,657 bp
		35S_Prom : pTseq1	1,960 bp
	AtICE2 RNAi knockdown	35S_Prom : ICE2_RNAir	345 bp
		ICE2_RNAir : pTseq1	2,191 bp
AtICE2 transgenic plants genomic DNA PCRs	*AtICE2* over-expression	35S_Prom : 35S_Term	1,657 bp
	AtICE2 RNAi knockdown	35S_Prom : ICE2_RNAir	345 bp
AtICE2 transgenic plants semi-quantitative RT-PCRs	*AtICE2* over-expression or RNAi knockdown	ICE2_Ft : ICE2_R	1,347 bp
	AtActin (Control)	AtActin_F : AtActin_R	180 bp

Primer names and expected sizes of products during PCR analysis of *AtICE2* over-expression or RNAi knockdown plants. Primer sequences may be found in Appendix A.

3.2.4. *Agrobacterium tumefaciens*-mediated Transformation of Arabidopsis *via* Floral Dip Method

Cultures (2-10 ml) were prepared in 25 ml glass culture tubes as described above from freshly plated colonies either from transformation plates (RNAi construct) or plated glycerol stocks (over-expression construct). The cultures were incubated at 28°C with rotation at 120 rpm for two days. Each culture was used to seed a 50-150 ml culture and was incubated for approximately two days before being combined with fresh media and antibiotics to a final volume of 500 ml in a 1 L flask. The flask was incubated overnight. The cultures were centrifuged in four 250 ml flasks for 20 min at 4,000 rpm at 4°C in an Avanti® J-E centrifuge. The pellet was resuspended in 1 L of fresh 5% w/v sucrose solution and 500 µl of Silwet L-77 was added.

Columbia-0 Arabidopsis plants were grown on Arabidopsis Soil Mix (1 part white sand:1 part peat: 1 part perlite, containing 1 g L^{-1} $FeSO_4$, 3 g L^{-1} Osmocote plus, 1 g L^{-1} dolomite, 0.5 g L^{-1} gypsum and 0.5 g L^{-1} lime) at 21°C under 16/8 hr day/night conditions. The primary bolts were cut from Arabidopsis plants in advance of transformation and the floral dip was performed when the secondary bolts were ~2-10 cm long with few open flowers. The Arabidopsis flowers were gently swirled in the Agrobacterium/Silwet L-77 solution for 30 sec before the plants were placed in low light overnight in a plastic bag for 18-42 hrs. The plants were removed from the bag and placed in a growth room to complete seed set. The plants were watered sparingly for approximately three days after returning to normal growing conditions. Harvested seed was placed in paper bags at 37°C for one week and stored at room temperature or 4°C.

3.2.5. Growth and Selection of Transgenic Plants

Seed from the dipped (T_0) plants was vernalised for three days at 4°C in the dark, sown onto soil and grown for approximately three weeks prior to selection. The constructs transformed into the plants contained the *bar* selectable marker gene which allows plants to survive and grow in the presence of the herbicide glufosinate-ammonium.

Selection of T_1 transgenic plants involved spraying the seedlings with a BASTA solution (0.05% v/v glufosinate-ammonium solution containing 0.02% v/v Silwet L-77).

Tissue collected from mature plants for RNA/DNA analysis were ground in 2 ml tubes using two 4 mm ball bearings. If necessary, tissue was first crushed with a cold spatula to ensure even grinding. The tube was vortexed twice for 1 min with chilling in liquid nitrogen before and after passes.

Genomic DNA was extracted from the T_1 plants as described in Section 2.2.3.8.3 with the following modifications: the extraction was performed in 2 ml tubes rather than 1.1 ml strip tubes and the centrifugation steps were performed in a microcentrifuge rather than a plate centrifuge at 7,400 x g for 10 min, 11,000 x g for 6 min and 7,400 x g for 3 min in order of occurrence. PCRs were performed using Platinum *Taq* DNA polymerase enzyme according to the manufacturer's instructions. Briefly, reactions contained 5 µl of 10x PCR Buffer Minus Mg^{2+} (supplied), 8 µl of dNTPs (5mM), 1.5 µl of 50 mM $MgCl_2$ (supplied), 1 µl of 10 µmol forward and reverse primer stocks (primers displayed in Table 3.1), 1 U (0.2 µl) of Platinum *Taq* DNA Polymerase, 1 µl of neat genomic DNA as template and sterile MQ water to a final volume of 50 µl. The primers used and expected product sizes are displayed in Table 3.1. Positive control reactions were prepared using 1 µl of DNA from the Agrobacterium strains used for transformation as a template. Negative control reactions were prepared for experimental primer sets containing no template or genomic DNA from wild type plants. PCR reactions were performed in a DNA Engine TETRAD® 2 thermal cycler with cycling parameters: 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 55°C annealing for 30 sec followed by 72°C extension for 2 min, followed by one cycle of 72°C for 10 min.

RNA was extracted from the T_1 plants as described in Section 2.2.3.2.2. The concentration of the RNA was determined by spectroscopy using a NanoDrop™ ND-1000 spectrophotometer and confirmed by electrophoresis of 2 µg of RNA in a formaldehyde gel as described in Section 2.2.3.2.2. cDNA synthesis was performed as

described in Section 2.2.3.5 with modifications: 1 µg of total RNA was used as template and one reaction was performed per cDNA sample. Semi-quantitative RT-PCR was performed to examine the expression of *AtICE2* in the T_1 plants. Experimental PCRs were performed using Platinum *Taq* DNA polymerase as described above using 1 µl of neat cDNA as template and PCR primers as described in Table 3.1. Two reactions were prepared for each cDNA sample and submitted to either 25 or 35 rounds of temperature cycling using the cycling parameters described above for PCR on genomic DNA. Control reactions were prepared using primers designed to amplify the *AtActin* gene (Table 3.1). Positive control reactions containing 1 µl of the over-expression plasmid DNA as template were performed. Negative control reactions were performed containing either no template or cDNA prepared from wild type plants. The *AtActin* primers lay either side of an intron and were used to screen for genomic DNA contamination as an additional, larger PCR product would be amplified from a genomic DNA template. The *AtActin* positive control reaction contained 1 µl of purified *AtActin* PCR product kindly provided by Dr. Neil Shirley. The reaction products (25 µl) were electrophoresed as described in Section 2.2.2.2.

T_2 and T_3 plants were grown on soil, selected by BASTA spray and RT-PCR was performed as described above for T_1 plants. Over-expression and RNAi knockdown transgenic lines were chosen with the greatest up- or down-regulation of *AtICE2* respectively and general developmental phenotypes, freezing tolerance, and expression of putative downstream genes were analysed in T_3 plants. Photographs of plants were taken using a Digital IXUS 70 camera.

3.2.6. Freezing Stress Treatment of Transgenic Plants with Over-expression or RNAi Knockdown of *AtICE2*

Freezing treatment of the Arabidopsis lines was kindly performed by Dr. Ulrik John (Victorian Department of Primary Industries, VIC). The freezing tolerance assay methods were based upon the method of Xin and Browse (1998). Fifteen sterilised Arabidopsis seeds were sown in each quadrant of 100 x 20 mm Petri dishes containing modified 0.5x GM 0.8% (w/v) agar plates (10% 10 x macronutrients v/v, 2% 10 x micronutrients v/v, 3% sucrose w/v, 0.25 MES g l^{-1}, 0.2 myo-inositol g l^{-1}, 1 nicotinic

acid mg l^{-1}, 1 pyridoxine.HCl mg l^{-1}, 0.2 thiamine.HCl mg l^{-1}, 4 glycine mg l^{-1}, pH 5.5). Non-transgenic Columbia (Col-0) and *Cape Verde Island* (*Cvi-1*) ecotypes, and *eskimo1* (*esk-1*) mutant seed were sown as controls. Plates were incubated at 22°C under continuous light at 40 µmol$m^{-2}s^{-1}$ photosynthetic photon flux intensity for 14 days before being transferred to a programmable convective refrigeration chamber. The temperatures of the air in the chamber and of the surface of a Petri dish were monitored with thermocouples, linked to a data-logger programmed to take measurements at one min intervals. The temperature regime was as follows: the temperature was lowered from 22°C to -2°C over 30 min. Once the temperature fell below zero, plants were sprayed with a suspension of 1 mg/ml Snomax to nucleate ice formation. The temperature was held at -2°C for 16 h to achieve uniform freezing before being reduced at a rate of 1°C h^{-1} to -12°C. Upon the temperature reaching -10°C, -11°C, and -12°C plates were withdrawn, wrapped in parafilm and allowed to recover in the dark at 5°C for 24 hrs before being transferred to 22°C. Survival was scored after one week.

3.2.7. Expression Analysis of Putative Downstream Genes in Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2*

Herbicide-resistant Arabidopsis plants from two independent over-expression lines, two independent RNAi knockdown lines and wild type plants were grown on soil in a growth room. At five weeks of age, the plants were cold-treated at 4°C in the dark and samples of treated and untreated plants were taken at 0 hrs, 3 hrs and 24 hrs after treatment commenced. Treatment commenced just prior to the beginning of the 'dark' period of the light cycling and untreated plants were grown under normal conditions. Leaf tissue from at least five plants and bolts from at least two plants were sampled and pooled at each time point. RNA extraction and cDNA synthesis were performed on the samples as described in Sections 2.2.3.2.2 and 2.2.3.5, respectively. qRT-PCR was performed as described in Section 2.2.3.6 using the primers to experimental genes presented in Table 3.2, and PCR products and primers to *AtActin*, *AtCyclophilin*, *AtTubulin* and *AtGAPDH* which were kindly provided by Dr. Neil Shirley (primer sequences may be found in Appendix A).

Table 3.2 Primers used for qRT-PCR analysis of transgenic plants with over- or under-expression of *AtICE2*.

Gene	Forward primer	Reverse primer	Expected product size
AtICE2 (endogenous and transgene)	AtICE2_F2	AtICE2_R2	257 bp
AtICE1	AtICE1_F1	AtICE1_R1	264 bp
AtCBF3	AtCBF3_F1	AtCBF3_R1	137 bp
AtCOR78	AtCOR78_F1	AtCOR78_R1	296 bp
AtCOR47	AtCOR47_F1	AtCOR47_R1	219 bp
AtRAB18	AtRAB18_F1	AtRAB18_R1	280 bp

Primer sequences are presented in Appendix A.

3.3. Results

3.3.1. Sequence Analysis of *AtICE2*

Comparison of the nucleotide sequence of *AtICE2* (NM_101157) and the Arabidopsis EST database at NCBI (www.ncbi.nlm.nih.gov) using the 'blastn' program showed that *AtICE2* was highly homologous to *AtICE1* (score and E values of *AtICE1* from the search are presented in Appendix B.3). There are only two *ICE* genes in Arabidopsis, *AtICE1* and *AtICE2*, and they are phylogenetically well separated from their nearest relative, *AtbHLH061* (Badawi *et al.*, 2008). The similarity between AtICE1 and AtICE2 can be observed in the protein sequence alignment in Figure 1.3 and was discussed in Chapter 1. Briefly, the majority of the AtICE1 and AtICE2 protein sequences are highly conserved (60% identity over the whole proteins), including identical bHLH DNA binding domains. Other regions of high conservation include the leucine zipper domain, serine-rich region, sumoylation target motifs and KRAAM motif which was the site of the *ice1* mutation (Chinnusamy *et al.*, 2003). The main area of sequence variation is the N-terminal region which contains the activation domain (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008; Miura *et al.*, 2007).

3.3.1. Production of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2*

The 224 bp sequence used for RNAi silencing of *AtICE2* was positioned near the 5' end of the gene. This sequence had a low level of nucleotide identity (50.4%) with the *AtICE1* gene (alignment of the fragment used for RNAi silencing of *AtICE2* and the corresponding region of *AtICE1* in Appendix E.2). Sequencing confirmed the integrity of the *AtICE2* coding region and the *AtICE2* cDNA fragment used in the RNAi silencing (sequences in Appendix F.3). The cloned *AtICE2* sequences contained a small number of polymorphisms relative to the published *AtICE2* sequence which were attributed to ecotype variation. Constructs for plant transformation were prepared (Figure 3.1) and restriction digestion indicated the *AtICE2* fragments had been correctly inserted (Figure 3.2). The expected product sizes were 3,505, 1,670, 904 and 625 bp for the pTOOL2/*AtICE2* construct after digestion with *Sac*II and 5,373, 570 and 278 bp for the pJawohl8/*AtICE2 RNAi fragment* construct after digestion with *Hind*III. The two constructs were used to transform Arabidopsis by the floral dip method.

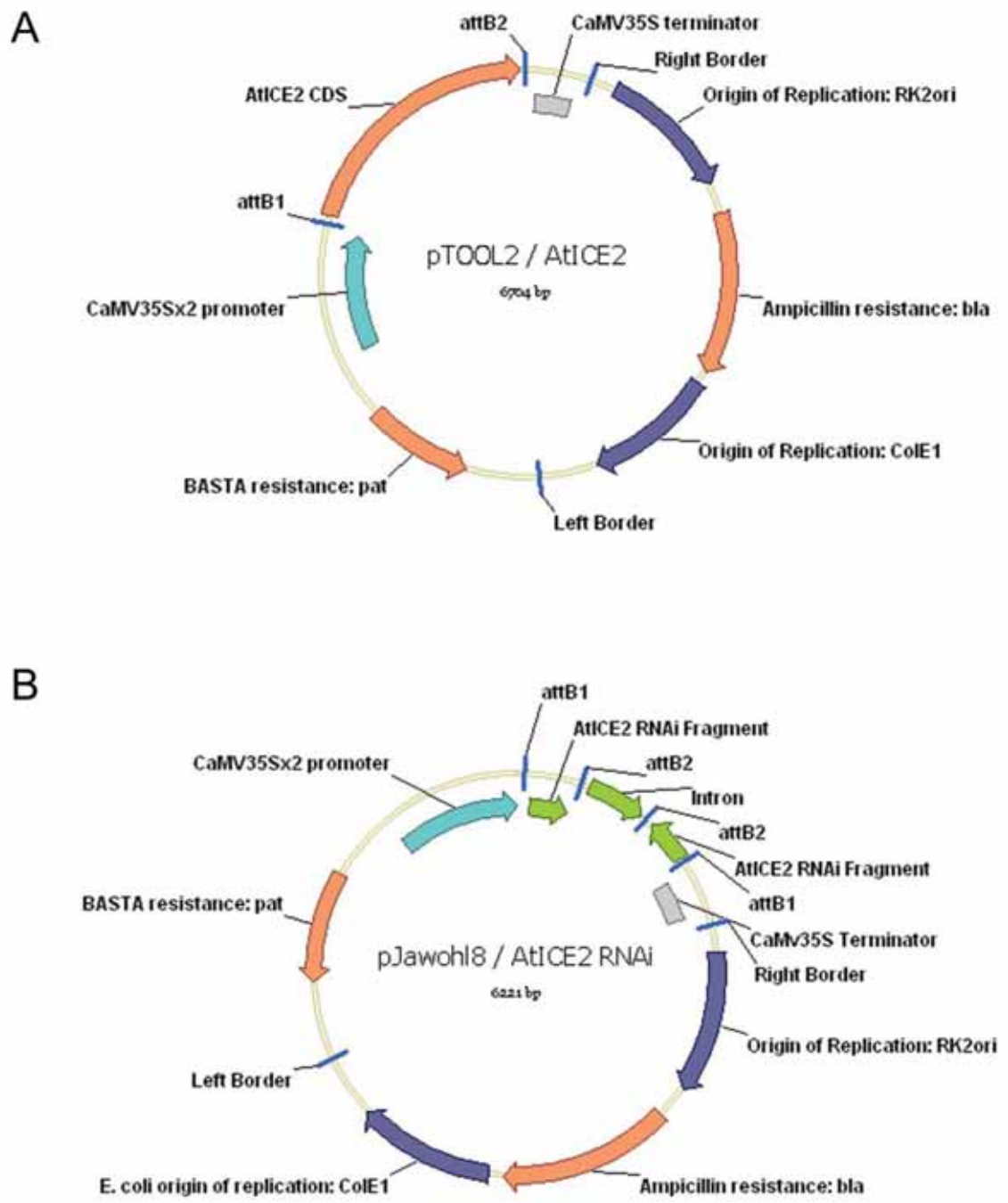


Figure 3.1 Vector maps of pTOOL2 and pJawohl8 transformation vectors containing *AtICE2* coding sequence.

(**A**) The predicted full length coding region of *AtICE2* in pTOOL2 (**B**) A 224 bp fragment of the 5' region of *AtICE2* in pJawohl8. attB1 and attB2 are recombination sites.

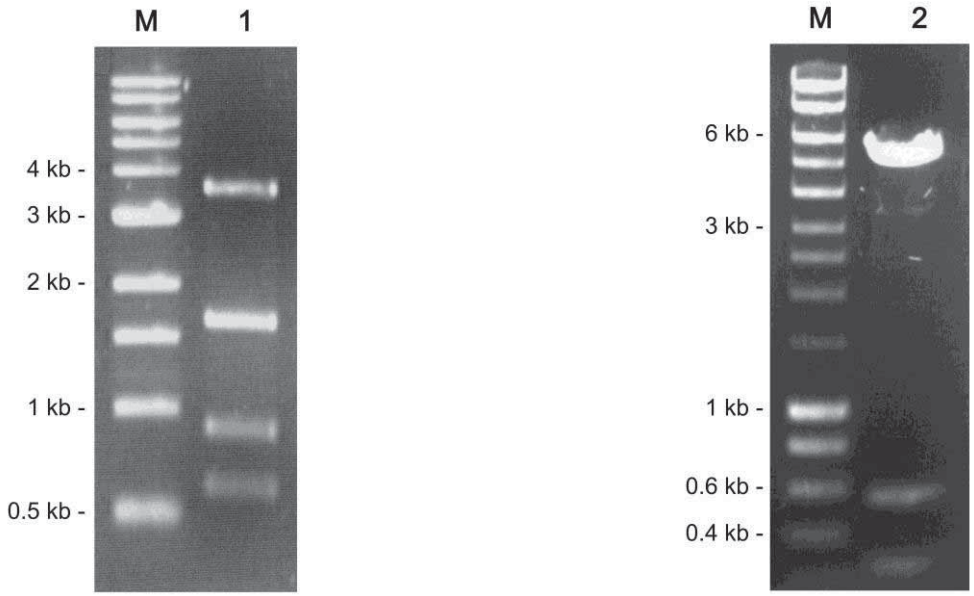


Figure 3.2 Photograph of agarose gel analyses of restriction fragments of pTOOL2/*AtICE2* and pJawohl8/*AtICE2 RNAi fragment.*

Digestion of plant transformation constructs indicated correct assembly, producing fragments of the expected sizes. M, molecular weight marker; Lane 1, *AtICE2* over-expression construct (pTOOL2/*AtICE2*) digested with *Sac*II; 2, *AtICE2* RNAi construct (pJawohl8/*AtICE2 RNAi fragment*) digested with *Hind*III.

3.3.2. Analysis of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2*

Transgenic plants (T_1) were selected by BASTA treatment of seedlings and PCR on genomic DNA confirmed the presence of the transgene (Figure 3.3). The expected product sizes were 1,656 bp for the *AtICE2* over-expression construct and 364 for the *AtICE2* RNAi silencing construct. In both cases, no product was amplified from wild type/non-transgenic genomic DNA. No 'escape' plants (non-transgenic plants that survived the selective treatment) were identified from over 50 putative transgenic plants tested.

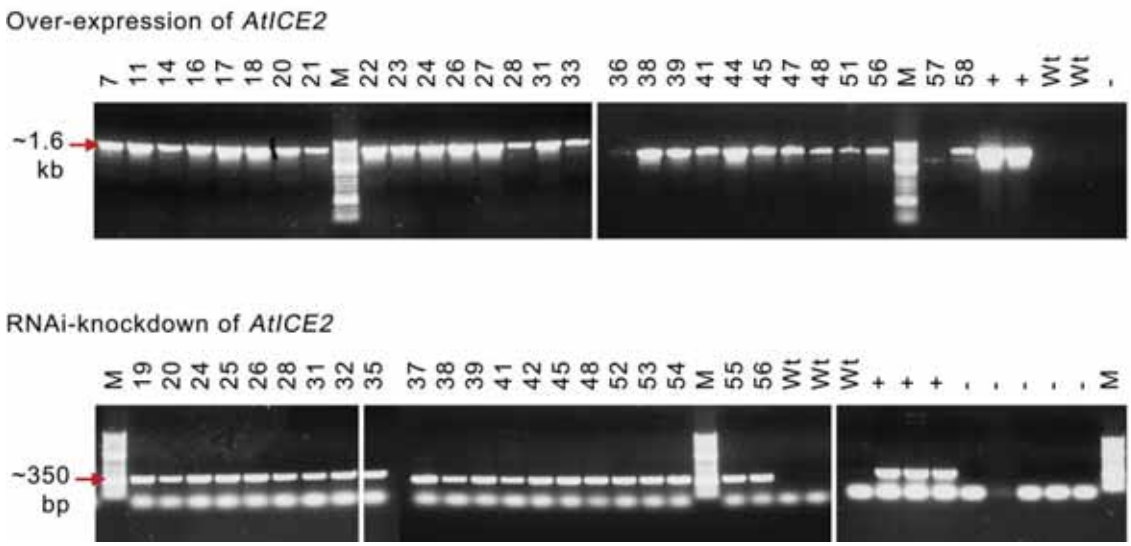


Figure 3.3 Photograph of agarose gel analyses of PCR products amplified from genomic DNA from T_1 transgenic Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2*.

Amplification of PCR products confirmed the presence of the T-DNA in the transgenic plants. The expected sizes of the PCR products were 1,656 bp and 364 bp for plants containing the *AtICE2* over-expression and RNAi silencing T-DNAs, respectively. Numbers above each lane represent the line number of the respective plants. M, molecular weight marker; Wt, wild type Arabidopsis plants; +, positive control: plasmid DNA of the transformation constructs as template; -, negative control: no template.

The mRNA levels of *AtICE2* in T_1, T_2 and T_3 plants were determined by RT-PCR with 25 or 35 PCR cycles to allow semi-quantitative amplification of high or low abundance transcripts. The primers used for RT-PCR analysis of the *AtICE2* over-expression or RNAi silenced plants amplified a 1,347 bp product (the entire coding sequence of AtICE2) and amplified products from both the endogenous and (in the case of the over-expression plants) transgene *AtICE2* mRNAs. Transcript levels of *AtICE2* in over-expression Lines O7 and O36 and corresponding T_2 sub-lines were high relative to wild type (Figure 3.4A). Transcript levels of *AtICE2* varied between T_3 lines, with plants from *AtICE2* over-expression Lines O7-7, O7-20, O36-8 and O36-9 having high levels of transcript accumulation while Lines O7-9 and O36-9 had transcript levels which were similar to wild type plants (Figure 3.4B).

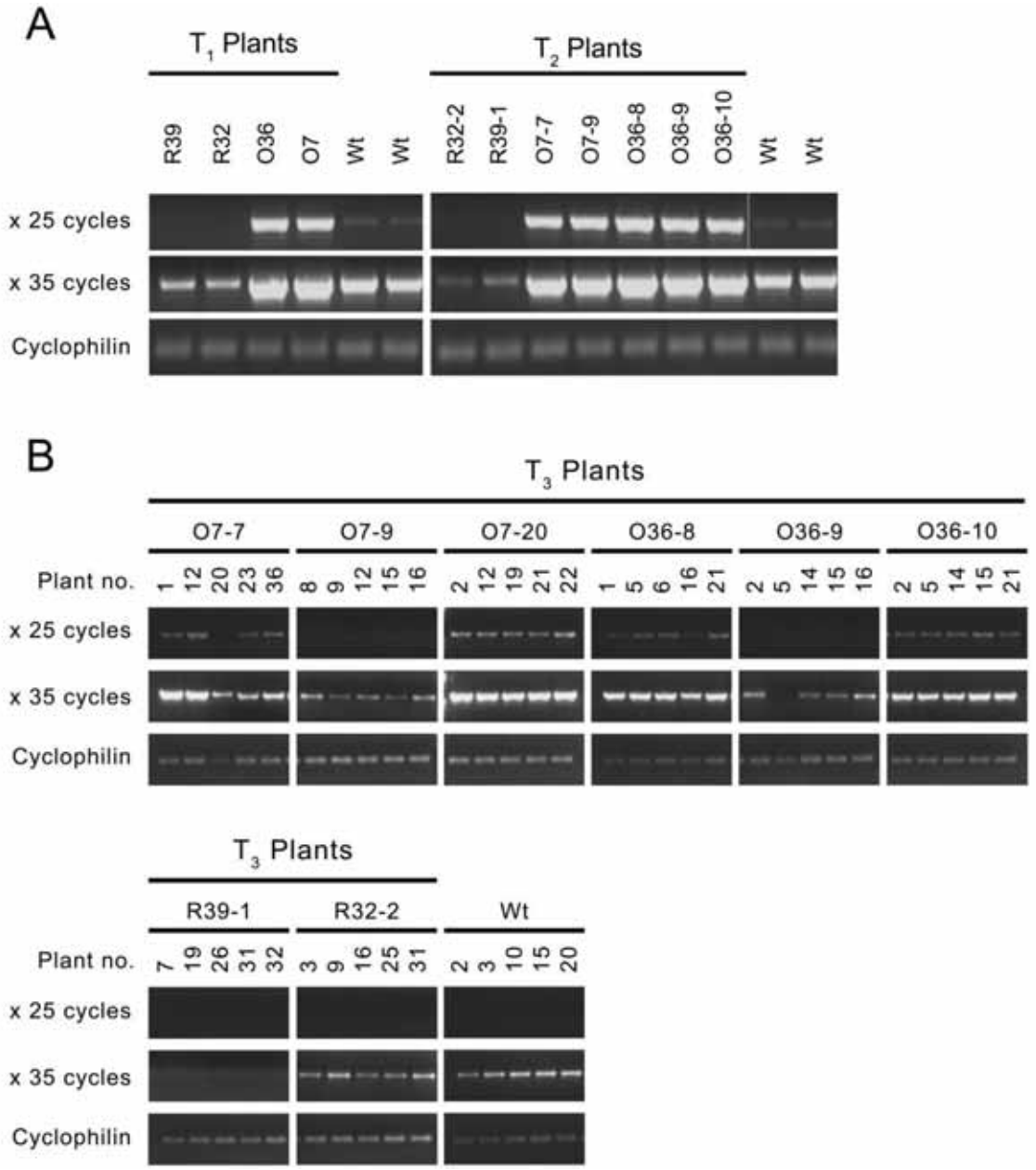


Figure 3.4 Photograph of agarose gel analyses of RT-PCR products amplified during analysis of Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2*.

(**A**) T_1 and T_2 plants (**B**) T_3 plants. The primers used for analysis of *AtICE2* transcript levels amplified a 1,347 bp fragment of the *AtICE2* coding region. *AtICE2* transcripts levels were higher or lower than wild type levels in the over-expression or RNAi plants respectively and were relatively consistent within each line. Five randomly chosen plants were analysed for each T_3 line. RT-PCR cycling conditions included 25 or 35 rounds of amplification. *Cyclophilin* products were amplified as loading controls with 35 rounds of amplification. Wt, wild type (untransformed) plants; O, plants with over-expression of *AtICE2* (e.g. O7); R, plants with RNAi knockdown of *AtICE2* (e.g. R39).

Transcript levels of *AtICE2* in RNAi Line R32 and R39 and corresponding T_2 sub-lines were reduced relative to wild type (Figure 3.4A). In T_3 plants from RNAi knockdown Line R39-1, *AtICE2* transcript levels were lower than wild type levels. However plants from the T_3 RNAi knockdown Line R32-2 appear to have similar *AtICE2* transcript levels to wild type plants (Figure 3.4B).

For each generation, over-expression and RNAi knockdown transgenic lines were chosen with the greatest up- or down-regulation of *AtICE2*, respectively. *AtICE2* over-expression Lines O7-7, O7-20, O36-8 O36-9, and O36-10; and RNAi Lines R32-2 and R39-1 were chosen for analysis of altered developmental phenotypes and cold stress tolerance. Although numerous T_1 lines were obtained containing the transgenic constructs (Figure 3.3), no other lines could be analysed as moisture in the seed storage facility rendered seed from the remaining lines infertile. Time constraints prevented a second round of plant transformations. Herbicide resistance segregation ratios were analysed in T_3 plants of the lines described above (n>40). *AtICE2* over-expression Lines O36-8 and O-7-7 were not segregating for herbicide resistance (100% survival rate) whereas Lines O36-9, O36-10, O7-9 and O36-20 each had a survival rate of approximately 88-93% following herbicide treatment. Both *AtICE2* RNAi lines were not segregating for herbicide resistance (100% survival rate).

3.3.3. Development of Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2*

The development and gross appearance of wild type Arabidopsis plants were compared with transgenic lines showing over-expression or RNAi knockdown of *AtICE2*. Plants were carefully monitored over their life-span and inspected for visible differences. No differences were observed between the populations of transgenic plants over-expressing or with RNAi knockdown of *AtICE2*, or wild type plants. Photographs of mature transgenic and wild type plants are presented in Figure 3.5.

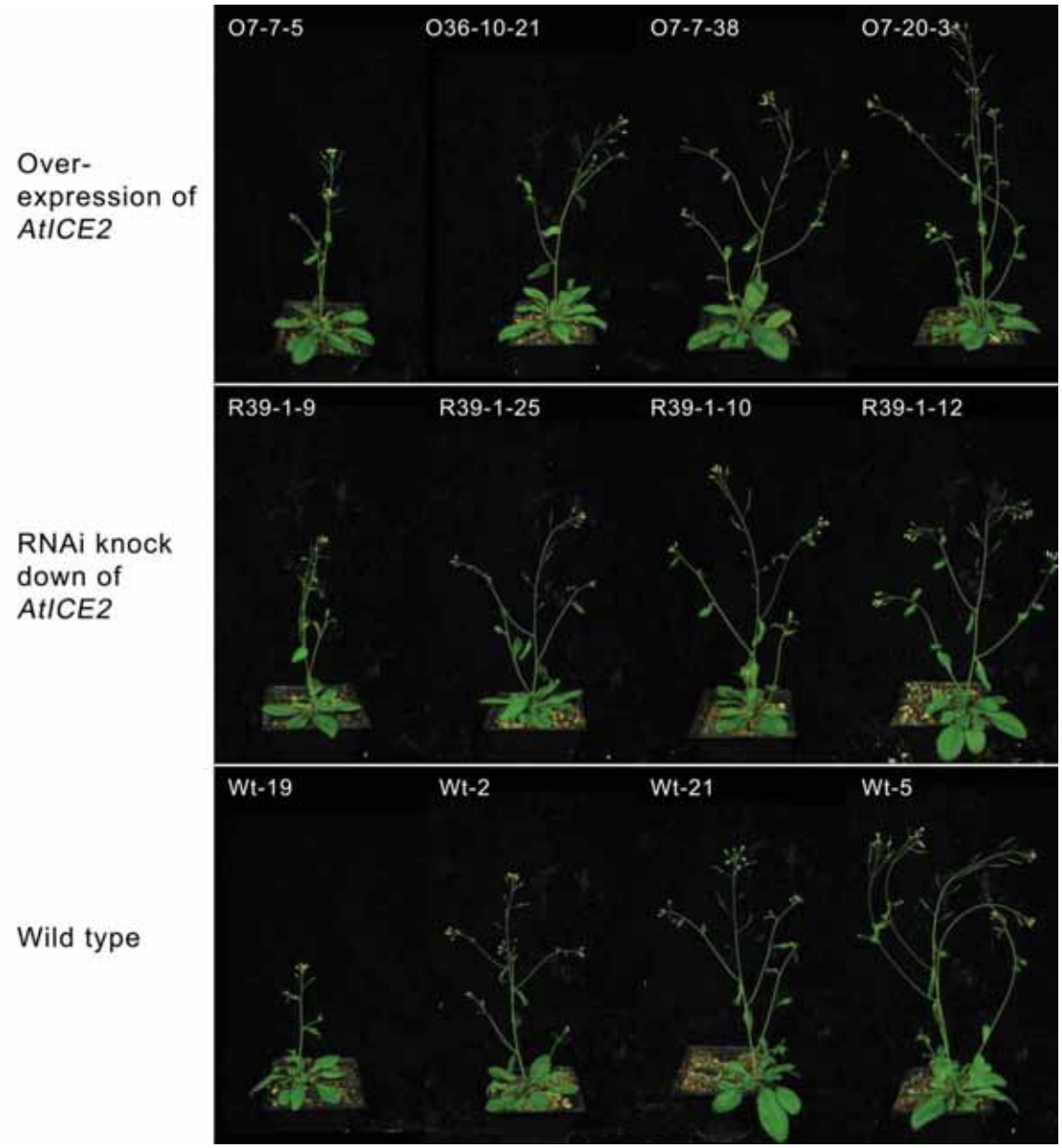


Figure 3.5 Photographs of wild type and transgenic Arabidopsis plants with over-expression or RNAi knock down of *AtICE2* at flowering.

The transgenic plants were comparable to wild type plants in every way examined. Photographs show the variation and general sizes of plants of the same age with one small, medium, large and extra large plant pictured from over-expression, RNAi or wild type genotypes. Wt, wild type (untransformed) plants; O, line over-expressing *AtICE2* (e.g. O7-7-5); R, line with RNAi knockdown of *AtICE2* (e.g. R39-1-9).

3.3.4. Freezing Tolerance of *AtICE2* Over-expression or RNAi Knockdown Plants

To test the hypothesis that up- or down-regulation of *AtICE2* expression would enhance or reduce plant cold tolerance, respectively, a plate-based freezing tolerance assay was performed by Dr. Ulrik John and colleagues (Victorian Department of Primary Industries, Melbourne). Fifteen plants were grown from each of five over-expression lines (Lines O7-7, O7-9, O36-8, O36-9, and O36-10), two RNAi lines (Lines R39-1 and R32-2) and wild type. On each plate, four different lines were planted in a quadrant format (Figure 3.6). A quadrant was included of *Cape Verdi Island* plants (negative control: freezing-sensitive cultivar (Cook *et al.*, 2004)) and *eskimo* (*ESK*) mutant plants (positive control: freezing-tolerant cultivar (Xin and Browse, 1998)).

Cold treatment reached minimum temperatures of -10°C, -11°C and -12°C. Prior to treatment there were equal numbers of plants on each quadrant per plate. Following treatment, no significant differences were observed in the survival frequency of any of the transgenic lines with altered expression of *AtICE2* compared with wild type. The frequency of survival was lower and higher in the plants from the *Cape Verdi Island* and *eskimo* plants respectively, compared to the Columbia ecotype which was used to produce the transgenic lines.

3.3.5. Expression Analysis of Putative Downstream Genes in Arabidopsis Plants with Over-expression or RNAi Knockdown of *AtICE2*

qRT-PCR was performed to determine whether the transcript levels of known target genes of *AtICE1* were altered in transgenic plants with up- or down-regulation of *AtICE2* expression (Chinnusamy *et al.*, 2003). The expression levels of *AtICE2*, *AtICE1*, *AtCBF3*, *AtCOR47*, *AtCOR17* and *AtRAB18* were compared in transgenic and wild type plants, prior to and during cold treatment. The primers used for analysis of *AtICE2* transcript levels were designed to a section of the coding region of *AtICE2* near the 3' end of the gene. The primers therefore amplify both the over-expressed and endogenous *AtICE2* mRNAs but not the RNAi fragment transcript, which was a section of the 5' end of the gene. The primer pairs used to amplify *AtICE2* or *AtICE1*

transcripts had low sequence similarity with the other *ICE* gene (<66% nucleotide identity).

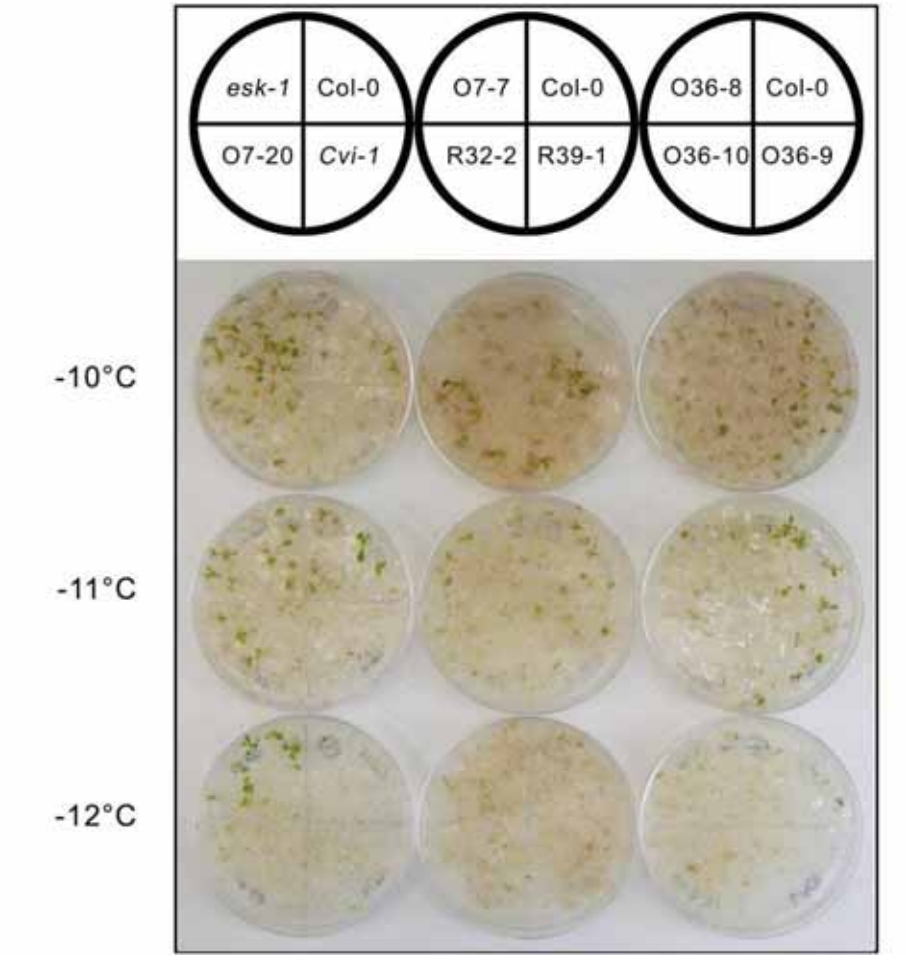


Figure 3.6 Photographs of treated wild type and transgenic Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* with a schematic diagram of the planting layout.

The freezing tolerance assay was performed by Dr. Ulrik John (Victorian Department of Primary Industries). No significant differences in survival following freezing treatment were observed between wild type plants and any of the over-expression or RNAi knockdown *AtICE2* transgenic lines. The minimum freezing temperatures reached were -10°C, -11°C, and -12°C and photographs were taken seven days after treatment. Col-0, wild type (untransformed) Columbia-0 plants; O, plants with over-expression of *AtICE2* (e.g. O7-7); R, plants with RNAi of *AtICE2* (e.g. RNAi39-1).

First, the transcript levels of *AtICE2* were determined to confirm up- or down-regulation of *AtICE2* in the transgenic lines relative to wild type plants. In wild type plants, *AtICE2* transcript levels were similar before and after cold treatment. However, in both transgenic lines over-expressing *AtICE2*, the transcript levels of *AtICE2* were significantly higher than wild type (P=0.010) and increased further after 24 hrs of cold treatment (Figure 3.7). *AtICE2* transcript levels were significantly lower in all RNAi samples relative to wild type levels (P<0.001) and cold treatment had no effect on the transcript levels (Figure 3.7).

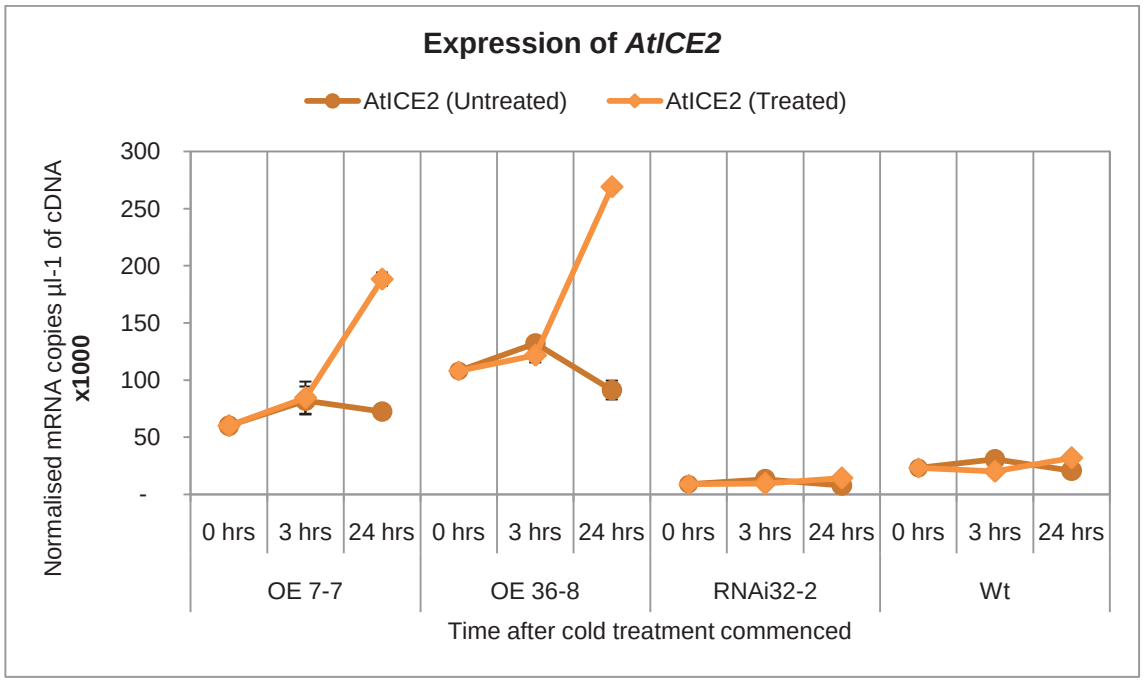


Figure 3.7 Graph of *AtICE2* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.

Relative to wild type, *AtICE2* transcript levels were high in transgenic lines over-expressing *AtICE2* and low in transgenic plants with RNAi silencing of *AtICE2*. Wt, wild type (untransformed) plants; O, line over-expressing *AtICE2* (e.g. O7-7); R, line with RNAi knockdown of *AtICE2* (e.g. R32-2). Error bars represent standard deviation.

The expression of *AtICE1* was investigated to determine whether *AtICE1* was co-ordinately regulated by *AtICE2* expression levels. Transcript levels of *AtICE1* were at similar levels in the untreated samples for all genotypes. After 24 hrs of cold treatment, transcript levels of *AtICE1* were higher in transgenic and wild type treated plants relative to untreated plants. However, the maximum transcript level reached was greater in *AtICE2* over-expressing Line O36-8 than in wild type, while that in Line O7-7 was lower than in wild type (Figure 3.8A).

As *AtCBF3*, *AtCOR47* and *AtCOR78* expression was shown to be regulated by *AtICE1* levels (Chinnusamy *et al.*, 2003; Lee *et al.*, 2005), it was investigated whether *AtICE2* was also able to regulate expression of these genes. In all the genotypes, *AtCBF3* transcript levels were higher in cold treated than untreated plants, with the greatest difference in expression occurring after 24 hrs of cold treatment (Figure 3.8B). When compared with wild type plants, a minor decrease in *AtCBF3* transcript levels was observed in the transgenic plants over-expressing *AtICE2*, while a minor increase in transcript levels was observed in transgenic plants with reduced expression of *AtICE2* (RNAi silencing).

The expression profiles of wild type plants were similar to those of the over-expression or RNAi lines. In all the plants, transcript levels of *AtCOR47* and *AtCOR78* were extremely low in untreated and cold treated plants after 3 hrs of treatment and the untreated plants did not differ over the time course. However after 24 hrs of cold treatment, *AtCOR47* and *AtCOR78* transcript levels were high (Figure 3.9A & B).

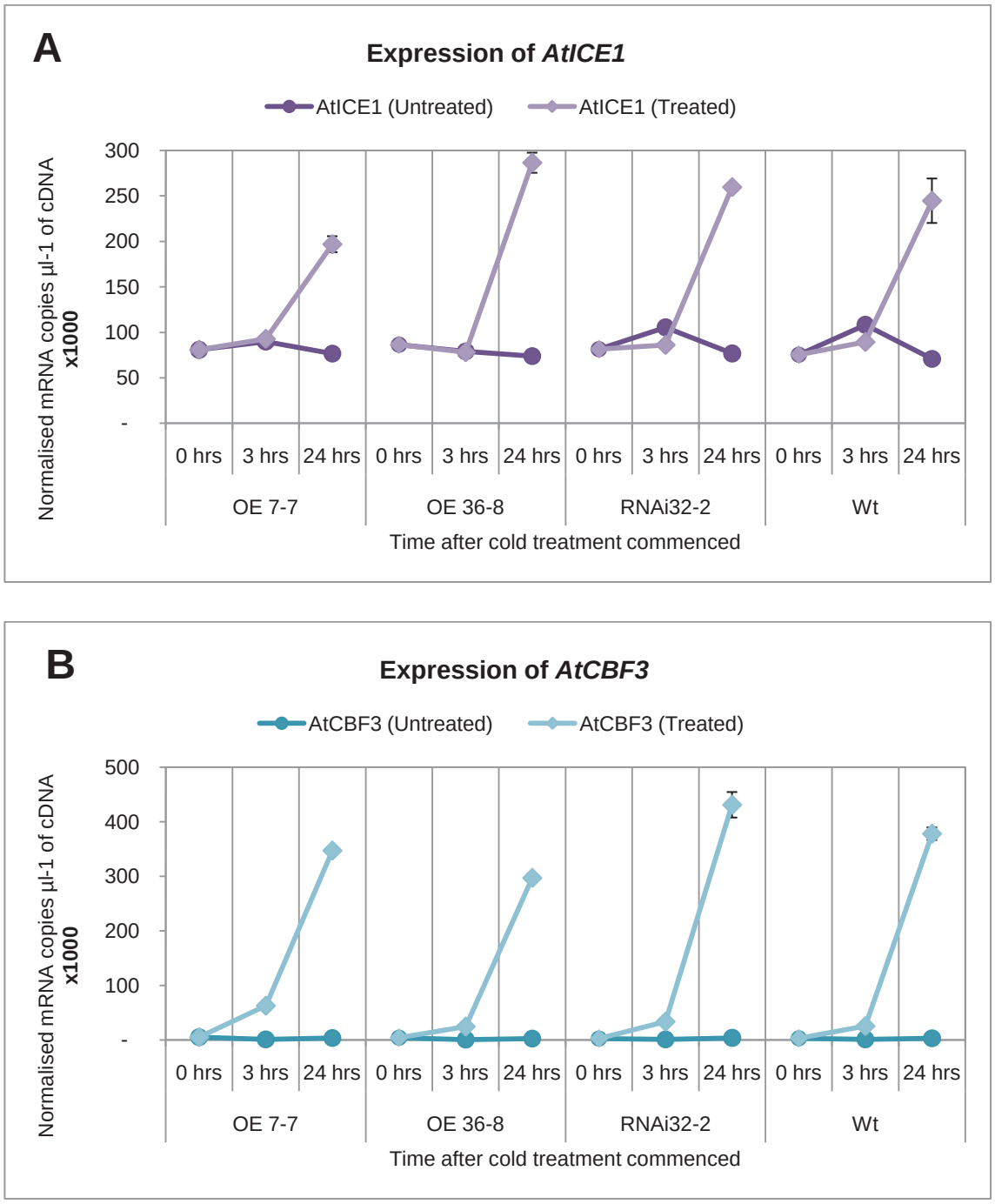


Figure 3.8 Graphs of *AtICE1* and *AtCBF3* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.

(**A**) Expression of *AtICE1*. (**B**) Expression of *AtCBF3*. Minor differences in *AtCBF3* expression were observed whilst *AtICE1* expression was alike in plants with over-expression or RNAi knockdown of *AtICE2* and wild type plants. Wt, wild type (untransformed) plants; O, line over-expressing *AtICE2* (e.g. O7-7); R, line with RNAi knockdown of *AtICE2* (e.g. R32-2). Error bars represent standard deviation.

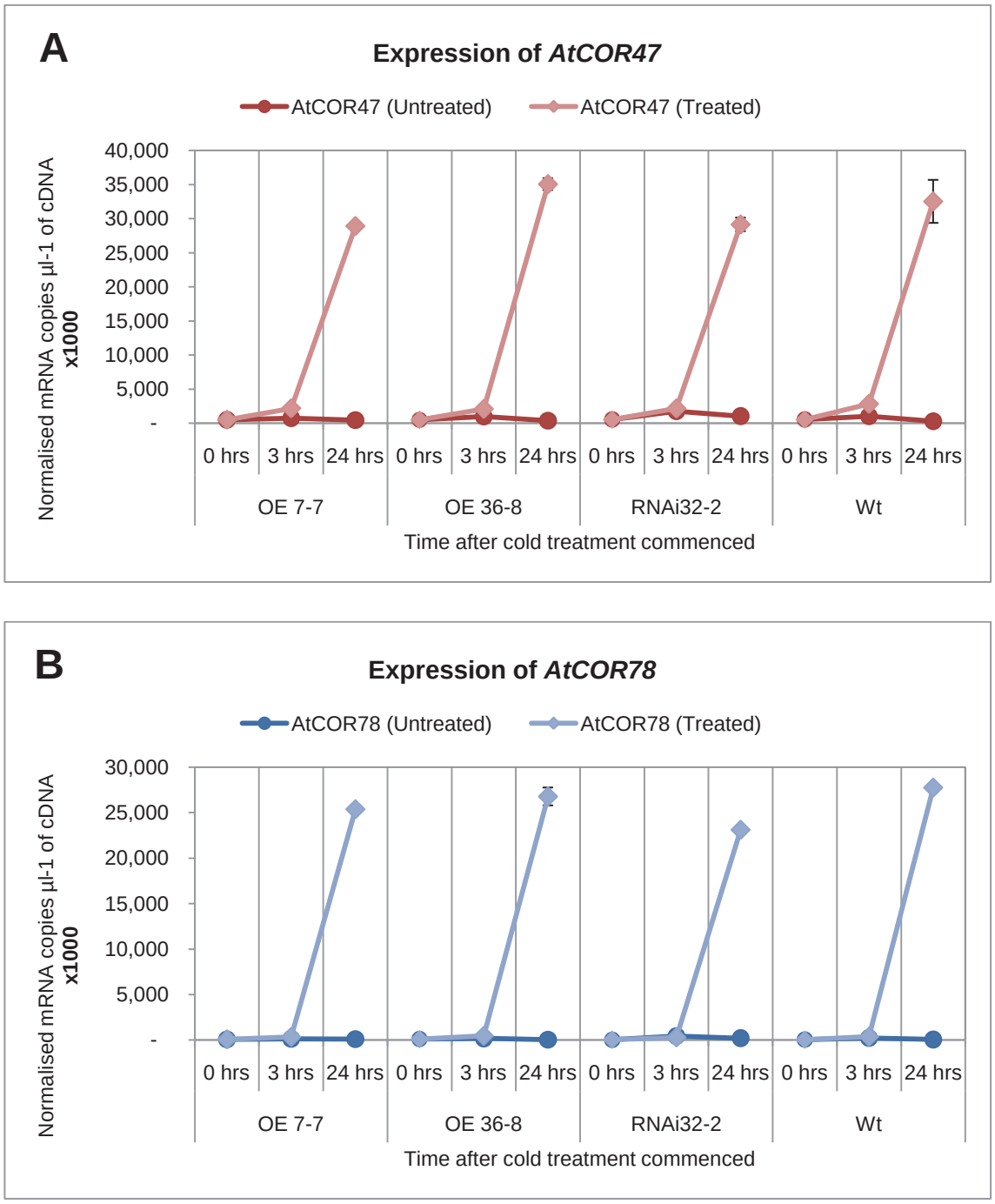


Figure 3.9 Graphs of *AtCOR47* and *AtCOR78* expression during cold treatment in Arabidopsis plants with over-expression or RNAi knockdown of *AtICE2* determined by qRT-PCR.

(**A**) Expression of *AtCOR47*. (**B**) Expression of *AtCOR78*. The expression profiles of *AtCOR47* and *AtCOR78* were similar in wild type plants and plants with over-expression or RNAi knock down of *AtICE2*. Wt, wild type (untransformed) plants; O, line over-expressing *AtICE2* (e.g. O7-7); R, line with RNAi knockdown of *AtICE2* (e.g. R32-2). Error bars represent standard deviation.

3.4. Discussion

The role of *AtICE2* in cold stress response was investigated to determine whether this gene played a similar role to *AtICE1*, and therefore represented an additional component of the *ICE*/*CBF*/*COR* signalling pathway which could be manipulated to improve plants cold tolerance. As mentioned previously, the ultimate goal of this work was to uncover information which may ultimately be able to be applied to crops species.

A high degree of sequence similarity is present between the AtICE1 and AtICE2 protein sequences, which includes the regions containing important functional motifs such as the bHLH DNA binding and leucine zipper dimerisation domains, putative sumoylation and phosphorylation target motifs and the region which contained the mutation in the *ice1* plants (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008; Miura *et al.*, 2007). This sequence similarity combined with functional similarities in previous studies has lead to the suggestion that the two *ICE* genes are functionally redundant (Chinnusamy *et al.*, 2003; Kanaoka *et al.*, 2008).

T-DNA insertion mutants of *AtICE2* were not available, therefore the role of *AtICE2* was investigated by producing transgenic lines either over-expressing this gene or with *AtICE2* silenced through RNAi knockdown. The region of *AtICE2* used for RNAi silencing shared a low level of sequence identity with *AtICE1* (~50%; Appendix E.2), so it is unlikely that mRNA levels of *AtICE1* would be directly affected by the RNAi silencing construct. PCR on genomic DNA confirmed that the T-DNA was present in the transformed plants (Figure 3.3). Herbicide resistance segregation ratios of T_3 plants indicated that over-expression Lines O7-7 and O36-8 and both RNAi lines were homozygous for the transgene, whereas over-expression Lines O7-9, O7-20, O36-9 and O36-10 were not.

Semi-quantitative PCR was used to examine *AtICE2* expression in the transgenic plants. In general, expression of *AtICE2* in the over-expression lines was greater than in wild type plants while expression of *AtICE2* in the RNAi knockdown plants was lower than in wild type plants (Figure 3.4A & B). There were exceptions in the T_3 lines where the

Lines 7-9 and 36-9 from the over-expression transformation and Line 32-2 from the RNAi knockdown transformation had similar *AtICE2* expression to wild type plants (Figure 3.4B). The reason for this change in expression between the T_2 and T_3 generations is not understood. However when examined by qRT-PCR, the expression of *AtICE2* in T_3 plants from RNAi Line 32-2 was found to be lower than that of wild type plants (Figure 3.7), and was at similar levels to the expression of T_3 plants from RNAi Line 39-1 (data not shown). Possible explanations for these results are that there are inconsistencies in the transcript accumulation of *AtICE2* (either transgene or endogenous) which could be caused by differences in expression or transcript stability, or that errors may have been introduced by the use of RT-PCR, a semi-quantitative method. Silencing of the transgene could also be occurring, by methylation of the T-DNA.

A plate-based freezing tolerance assay and qRT-PCR were performed to determine the effect of up- or down-regulating *AtICE2* expression. Freezing tolerance and the expression of target genes were already demonstrated to be affected by expression levels of *AtICE1* in Arabidopsis plants. In contrast to published results for over-expression of *AtICE1*, the transgenic lines with over-expression or RNAi silenced *AtICE2* (confirmed by RT-PCR of *AtICE2*: Figure 3.4B) had similar degrees of survival as wild type plants after freezing treatment (Figure 3.6). The freezing tolerance of the *eskimo* and *Cape Verdi Island* positive and negative control plants were consistent with published results which suggests the freezing assays were representative of those in the literature (Figure 3.6; Cook *et al*., 2004; Xin and Browse, 1998). The results presented here indicate that altering the expression of *AtICE2* was not sufficient to alter the freezing tolerance of transgenic Arabidopsis plants, and therefore that the freezing tolerance of Arabidopsis plants appears to be independent of *AtICE2* expression level.

The expression of *AtICE2* and the putative target genes *AtICE1, AtCBF3, AtCOR47* and *AtCOR78* were examined during cold treatment in wild type and transgenic plants with over-expression or RNAi silenced *AtICE2*. A constitutive promoter was used to drive transgene expression in the over-expressing lines. Despite this, transcript levels of *AtICE2* in transgenic lines were significantly higher after cold treatment. This was not

seen in wild type plants, where expression of *AtICE2* was unaffected by cold treatment (Figure 3.7). One explanation for this could be that expression of *AtICE2* is self regulated during cold stress and that the increased expression (as in the over-expressing lines) combined with the low temperatures triggers activation of the endogenous gene. Another explanation could be that there is decreased degradation of the *AtICE2* transcript at low temperatures, although this would need to be compensated for by decreased expression of the endogenous *AtICE2* gene to maintain consistent transcript levels in wild type plants. The transcription rate or transcript stability of the endogenous *AtICE2* gene has not been investigated.

In keeping with published findings in wild type plants, the expression of *AtICE1*, *AtCBF3*, *AtCOR47* and *AtCOR78* were upregulated following cold treatment (Figure 3.8A & B; Figure 3.9A & B; Lee *et al*., 2005; Maruyama *et al*., 2004; Seki *et al*., 2001). In the transgenic plants, the expression profiles of the putative target *CBF* and *COR* genes were comparable to wild type throughout the cold treatment (Figure 3.8A & B; Figure 3.9A & B). The greatest difference was a minor change in *AtCBF3* expression after 24 hrs of cold treatment which was lower in the over-expression plants and higher in the RNAi plants relative to wild type (Figure 3.8B). These results indicate that, unlike *AtICE1*, *AtICE2* does not appear to be involved in regulation of *CBF* or *COR* genes under cold stress.

As described above, no differences were observed in downstream gene expression or freezing tolerance between the plants with RNAi silencing of *AtICE2* and wild type plants. However, research has shown that there is a degree of functional redundancy between AtICE1 and AtICE2 and transgenic Arabidopsis plants containing a T-DNA knockout null allele *AtICE1* did not have the phenotype of the dominant negative *ice1* mutation (Chinnusamy *et al*., 2003; Kanaoka *et al*., 2008). It is possible that functional redundancy with the AtICE1 protein may have masked phenotypes caused by silencing of *AtICE2*.

Together, these results suggest that either *AtICE2* does not play a role in cold tolerance and/or regulation of *CBF* and *COR* genes, or that the role of *AtICE2* in cold tolerance differs from that of *AtICE1*. Another possible explanation could be that different genes are targeted from those studied here, or that additional cofactors or conditions may be required for the function of AtICE2. The activity of AtICE1 is controlled by environmental conditions, (i.e. low temperatures), and cofactors, such as SIZ1 and HOS1 (Chinnusamy *et al.*, 2003; Dong *et al.*, 2006; Miura *et al.*, 2007). In addition, expression of the AtICE1 target gene *SPCH* requires coactivation by another transcription factor, in this case AtICE2 (Kanaoka *et al.*, 2008). These findings and the presence of several regulatory motifs in the protein sequence of AtICE2 indicate that AtICE2 may also require post-translational regulation and/or cofactors for effective function. Badawi and colleagues (2008) suggested that a five amino acid deletion in the leucine zipper domain of AtICE2 relative to AtICE1 and cereal ICE1 proteins may modify the binding specificities of AtICE2 and could indicate functional differences between the AtICE1 and AtICE2 proteins. The function of AtICE2 may also have been altered by the sequence variations between the *AtICE2* transgene and published *AtICE2* sequences which were attributed to ecotype differences. However, these differences did not fall within any of the known important functional domains.

Quantification of cold tolerance is difficult due to the variable and sensitive nature of the trait. Ideally, *AtICE1* over-expression lines should be included in repeat assays of the freezing tolerance of the *AtICE2* transgenic lines. This would determine whether the increased tolerance described in the literature was apparent in the assay used here and hence, whether the lack of difference between the transgenic and wild type plants is related to a difference in *AtICE2* function from that of *AtICE1*, or is driven by differences in the specific assay and growth conditions used.

Constitutive over-expression or silencing of *AtICE2* did not significantly affect the growth or development of transgenic Arabidopsis plants in this study (Figure 3.5), which is consistent with the unaltered expression of downstream genes which was observed in the transgenic plants (Figure 3.8A & B; Figure 3.9A & B). T-DNA insertion mutants with a double knockout of *AtICE1* and *AtICE2* (*SCRM2*) had unusual

stomatal phenotypes but no unusual phenotypes were observed for knockouts of *AtICE2* alone either in previous studies (Oh *et al.*, 2007) or here. This indicates that knockout of a single *ICE* gene may not be sufficient and the analysis of cold tolerance and downstream *COR* gene expression in double gene knockouts in future experiments might prove informative.

3.5. Conclusions

The aim of this section of work was to investigate the role of *AtICE2* in cold response, to determine whether the *ICE* component of the *ICE*/*CBF*/*COR* signalling pathway included this closely related gene. In doing so, the robustness of the *ICE* section of the pathway would be determined, which may highlight ways in which this pathway may be applied most effectively to barley.

Although AtICE1 and AtICE2 are highly conserved at the protein sequence level, including regions spanning several AtICE1 functional domains, investigation of the function of *AtICE2 via* analysis of transgenic plants over-expressing or with reduced expression of *AtICE2* did not reveal any indications that these genes play similar roles in cold response. This was evident in the freezing tolerance and expression of target *COR* genes in the transgenic lines, which were similar to wild type in all cases. From these results, it was concluded that *AtICE2* does not function in cold response in the same manner as *AtICE1*, that different downstream genes are targeted or that the function of AtICE2 requires additional conditions or cofactors.

These results show that the *ICE* component of the *ICE*/*CBF*/*COR* signalling pathway has not yet been shown to extend to other *ICE* genes beyond *AtICE1*. This indicates that using our understanding of *AtICE1* to manipulate *ICE*-type genes in crop plants may not prove simple as these genes share even lower sequence similarity to *AtICE1*.

The results presented in Chapters 2 and 3 suggest that manipulation of *ICE* genes other than *AtICE1* to improve stress tolerance is a somewhat complicated task. Would it be more successful to travel further down the *ICE*/*CBF*/*COR* signalling pathway and manipulating barley *CBF* genes to improve cold tolerance? This question is investigated in the following chapter.

CHAPTER 4

Characterisation of Two Barley *CBFs*

4.1. Introduction

Over-expression of *CBFs* has successfully increased cold tolerance in Arabidopsis. Although there remains much to be discovered, the results of studies of barley *CBFs* have provided good evidence that the Arabidopsis and barley *CBFs* have similar roles in cold response, including data coming from over-expression of barley *CBFs* in Arabidopsis and rice, DNA binding assays, QTL mapping and expression analysis indicate this (Francia *et al.*, 2007; Oh *et al.*, 2007; Skinner *et al.*, 2006; Skinner *et al.*, 2005; Stockinger *et al.*, 2007; Tondelli *et al.*, 2006; Xue, 2002a; 2003).

Can *CBFs* be used to engineer cold tolerance in barley by identifying and cloning *CBF* genes from barley using the yeast 1-hybrid (Y1H) system and characterising these *CBFs*? The work described in this chapter addresses this question. To achieve this, barley plants were treated with freezing stress and floral tissues were harvested and used to produce a Y1H cDNA library. Y1H screens were performed using the CRT/DRE *cis*-elements from Arabidopsis and maize as bait, and identified *CBF* genes were characterised by genomic mapping and analysis of their expression under stress.

4.2. Materials and Methods

4.2.1. Materials

NEB Buffer 2 and *Hae*III were supplied by Genesearch (QLD, Australia). Cary 50 Bio UV-Visible spectrophotometer was from Varian, Inc. (CA, USA). The Hettich Rotanta 460R centrifuge was supplied by Adelab (SA, Australia). Perfection™ V700 Photo scanner was purchased from Epson (SA, Australia). Epicentre FailSafe 2x PCR PreMix G was purchased from Austral Scientific (NSW, Australia). BD Matchmaker™ Library Construction and Screening Kit and BD CHROMA SPIN™ TE-1000 Columns were purchased from BD Biosciences (NSW, Australia). Pierce Y-DER® Yeast DNA Extraction Reagent Kit was supplied by Quantum Scientific (QLD, Australia). Dynabeads® mRNA DIRECT™ Kit and magnet were purchased from Invitrogen (VIC, Australia). 3-amino-1,2,4-triazole and adenine hemisulphate were supplied by Sigma-Aldrich (NSW, Australia). Additional materials were supplied as described in Sections 2.2.1 and 3.2.1.

4.2.2. Growth of Plants, Cold Treatment and RNA Preparation

Samples of RNA from the plants used to produce the barley cold-stress treatment qRT-PCR series (Section 2.2.3.2) were also used to produce the Y1H cDNA library. RNA from treated floral tissues was pooled as follows: 30% of the total volume of RNA contained samples from first time point, 50% from the second time point and 20% from the third time point. The contribution from each time point was comprised of equal volumes of RNA from 12 individual heads. The concentration of the total RNA was ~1,700 ng μl^{-1}.

PolyA mRNA was extracted using a Dynabeads® mRNA DIRECT™ Kit according to the manufacturer's instructions. Briefly, RNA (100 µl; 170 µg) was heated at 65°C for 2 min and placed on ice. Resuspended Dynabeads (200µl; 1 mg) were pipetted into a 1.5 ml Eppendorf tube and the tip of the tube was placed on a magnet. After 30 sec, the supernatant was discarded and the tube was removed from the magnet. The Dynabeads were washed by resuspension in 100 µl of Binding Buffer (supplied) and the tube was returned to the magnet. After 30 sec, the supernatant was discarded and the tube was

removed from the magnet. Binding Buffer (100 µl) and the RNA were added to the Dynabeads suspension and mixed thoroughly by hand for 5 min at room temperature to allow the RNA to anneal to the Dynabeads. The tube was placed on the magnet for at least 30 sec and the supernatant was discarded. The Dynabeads were washed twice by removing the tube from the magnet, adding 200 µl of Washing Buffer B (supplied), replacing the tube on the magnet for at least 30 sec, and removing the supernatant. Care was taken to ensure all the supernatant was removed after the second wash. The RNA was eluted by adding 10 µl of 10 mM Tris-HCl (supplied), heating at 75°C for 2 min, immediately placing the tube on the magnet and pipetting out the eluted mRNA. The volume of the eluted RNA was 18µl.

4.2.3. Production of Y1H Libraries

4.2.3.1. First- and Second-strand cDNA Synthesis

cDNA synthesis was performed using components from the BD Matchmaker™ Library Construction and Screening Kit according to the manufacturer's instructions. First-strand cDNA was synthesised using the Oligo(dT) primer (Appendix A). Briefly, in a sterile 0.2 ml PCR tube, 2 µl of polyA RNA (Section 4.2.2), 1 µl of CDS III primer (supplied) and 1 µl of deionised water (supplied) were combined. The solution was mixed, centrifuged briefly, incubated at 72°C for 2 min, place on ice for 2 min and centrifuged briefly. The following components were added to the reaction tube: 2 µl of 5x First-Strand Buffer (supplied), 1 µl of 20 mM DTT (supplied), 1 µl of 10 mM dNTP mix (supplied) and 1 µl of MMLV Reverse Transcriptase (supplied). The reaction was mixed gently by tapping, centrifuged briefly and incubated at 42°C for 10 min. 1 µl of BD SMART III oligonucleotide (supplied) was added and the reaction was incubated at 42°C for 1 hr in a hot-lidded DNA Engine TETRAD® 2 thermal cycler. To terminate the reaction, the tube was placed at 75°C for 10 min. After cooling the reaction to room temperature, 1 µl of RNase H (supplied) was added and the reaction was incubated at 37°C for 20 min. First strand cDNA was stored at -20°C.

Second strand cDNA synthesis was performed by Long-Distance PCR. The following components were mixed in an 0.2 ml PCR tube: 2 µl of first-strand cDNA (synthesised

above), 70 µl of deionised water, 10 µl of 10x Advantage 2 PCR buffer (supplied), 2 µl of 50x dNTP mix (supplied), 2 µl of 5' PCR primer (supplied), 2 µl of 3' PCR primer (supplied), 10 µl of 10x GC-melt solution (supplied) and 2 µl of 50x Advantage 2 Polymerase mix (supplied). The solution was mixed gently by flicking, centrifuged briefly and placed in a thermal cycler pre-heated to 95°C. The lid temperature was set to track at 5°C above the reaction temperature during the following cycling conditions: 95°C for 30 sec, 30 cycles of 95°C for 10 sec and extension at 68°C for varying amounts of time, followed by 68°C for 5 min. The extension time was increased by 5 sec for each successive cycle, beginning at 6 min for the first cycle. The cDNA was stored at -20°C. As a quality control measure, one second-strand cDNA synthesis reaction was prepared initially and 5 µl of the reaction products were electrophoresed on a 1% agarose as described in Section 2.2.2.2 to examine the efficiency of the reaction. Upon obtaining desirable results, four replicate cDNA synthesis reactions were prepared and electrophoresed.

4.2.3.2. cDNA Purification

The cDNA from each reaction was purified using a BD CHROMA SPIN™ TE-1000 Column rather than the BD CHROMA SPIN™ TE-400 Columns supplied with the BD Matchmaker Kit as it had been found their use better enriched cDNA populations with rare longer transcripts without removing a detrimental proportion of the shorter transcripts (Dr. Sergiy Lopato, pers. comm.). Purification was performed according to the manufacturer's instructions and the resulting 20 µl of purified cDNA was stored at -20°C.

4.2.3.3. Preparation of Competent Yeast Cells

Yeast competent cells were prepared using the LiAc method as outlined in Appendix B of the BD Matchmaker™ Library Construction and Screening Kits user manual. Briefly, a YPDA 2.2% w/v agar plate (2% w/v tryptone, 1% w/v yeast extract, 2% w/v glucose, 0.003% w/v adenine hemisulphate) was streaked with frozen AH109 yeast (supplied) and incubated at 30°C for three days. YPDA medium (3 ml) was inoculated with one colony in a sterile 10 ml tube with the lid loosely attached. After incubation at 30°C

with shaking for eight hrs, 5 µl of the culture was transferred to a 250 ml flask containing 50 ml of YPDA medium. The flask was incubated at 30°C on a rotary shaker at ~230 rpm for 20 hrs, after which the cell density of the culture at OD_{600} was 0.219, as measured with a spectrophotometer. The cells were centrifuged in a Hettich Rotanta 460R bench-top centrifuge at 700 x g (2,170 rpm) for 5 min at room temperature (23°C). The supernatant was discarded, the cells were resuspended in 100 ml of YPDA and incubated at 30°C for five hrs, reaching a cell density of 0.351 (at OD_{600}). The cells were centrifuged at 700 x g for 5 min at room temperature, the supernatant was discarded and the cell pellet was resuspended in 60 ml of sterile, deionised water. The suspension was centrifuged at 700 x g for 5 min at room temperature, the supernatant was discarded and the cells were resuspended in 3 ml of freshly prepared 1.1x TE/LiAc Solution (1.1 ml of 10x TE (supplied), 1.1 ml of 1 M lithium acetate (supplied) and deionised water to 10 ml). The cells were centrifuged in two 1.5 ml tubes at 16,000 x g for 15 sec in a microcentrifuge, the supernatant was discarded and the pellets were resuspended and pooled in a total volume of 600 µl 1.1x TE/LiAc Solution. The competent yeast cells were transformed immediately as outlined below.

4.2.3.4. Yeast Transformation

Yeast transformation was performed using the BD Matchmaker™ Library Construction and Screening Kit according to the manufacturer's instructions for the MATCHMAKER Two-Hybrid System Kit. Briefly, denatured Herring Testes Carrier DNA (supplied) was denatured by twice heating at 100°C for 5 min and chilling on ice. The Herring Testes Carrier DNA (20µl) was combined in a pre-chilled, sterile 10 ml tube with 20 µl of purified double-stranded cDNA (Section 4.2.3.2) and 6 µl of 0.5 µg μl^{-1} pGADT7-Rec (supplied). The entire 600 µl of competent AH109 yeast cells (Section 4.2.3.3) was added to the cDNA solution on ice and mixed by vortexing gently before adding 2.5 ml of freshly prepared PEG/LiAc Solution (8 ml of 50% polyethylene glycol 3350 (supplied), 1 ml of 10x TE (supplied), 1 ml of 10x lithium acetate (supplied)). The suspension was vortexed briefly and incubated at 30°C for 45 min, mixing the cells at 15 min intervals. DMSO (160 µl) was added, mixed, and incubated at 42°C in a water bath for 20 min, mixing at 10 min intervals. After centrifuging the suspension at 700 x g for 5 min, the supernatant was discarded and the pellet was

resuspended in 3 ml of YPD Plus Liquid Medium (supplied). The mixture was incubated at 30°C with shaking for 60 min, centrifuged at 700 x g for 5 min, the supernatant was discarded and the pellet was resuspended in 30 ml of autoclaved 0.9% NaCl Solution. The solution was spread equally on 200 (150 mm diameter) selective SD 2.2% w/v agar plates (0.67% w/v yeast nitrogen base (supplied), 2% w/v glucose, 0.068% w/v drop out media (supplied)), lacking leucine and incubated at 30°C for seven days. After chilling the plates at 4°C for 3-4 hrs, the transformant colonies were pooled by washing plates with 5 ml of YPD medium containing 25% v/v glycerol. A glass spreader was used to dislodge cells and the suspension was poured onto the subsequent plate until the cells from five plates had been pooled. The resulting suspensions were pooled in a sterile flask. Each plate was washed twice to ensure the maximum number of cells was obtained in the minimum volume of medium.

The pooled liquid was mixed well and 1 ml room temperature aliquots were placed at -80°C for storage. To determine library clone insert size and diversity, dilutions of the library were spread onto selective SD 2.2% w/v agar plates lacking leucine, incubated at 30°C until colonies appeared. DNA was extracted directly from 24 single colonies using the Y-DER® Yeast DNA Extraction Reagent Kit according to the manufacturer's instructions with reaction volumes: 80 µl of Y-PER® Reagent, 64 µl of DNA Releasing Reagent A, 64 µl of DNA Releasing Reagent B, 32 µl of Protein Removal Reagent, 96 µl of isopropanol, 200 µl of 70% (v/v) ethanol and 15 µl of water. To aid DNA resuspension, the pellet was incubated in the water at 4°C overnight, vortexed briefly and incubated at 4°C for approximately 4 hrs.

PCR was performed to amplify the insert as described in Section 2.2.2.2 with the following modifications: only one round of PCR was performed in a total volume of 25 µl with reaction component volumes scaled accordingly. 1 µl of DMSO was used with 0.5 µl of 10 µM stock of 2HA_Rev and T7 primers. 0.5 µl of yeast DNA was used as template. The extension used in cycling was altered to 1 min and 30 sec. To determine the sizes of the library clones, the reaction products were electrophoresed in 1% w/v agarose gels as described in Section 2.2.2.2.

4.2.4. Screening of cDNA Libraries using the Y1H System

The libraries were screened based on the methods described in the BD Matchmaker™ Library Construction and Screening Kit user manual with modifications as described in Lopato *et al*. (2006).

The two yeast reporter strains used containing the Arabidopsis CRT/DRE or maize CRT/DRE 'bait' *cis*-elements integrated into the yeast genome as described in Meijer *et al*. (1998) and Lopato *et al*. (2006), using the pINT1-HIS3NB binary vector were kindly supplied by Dr. Pieter Ouwerkerk (Leiden University, The Netherlands). Briefly, this involved cloning the Arabidopsis and maize CRT/DRE *cis*-element sequences into the pINT1-HIS3NB vector (see Figure 4.1A & B for the oligonucleotides and corresponding constructs produced). The partially complementary oligonucleotides were annealed in a NaCl/TE buffer (10 mM Tris-HCl Buffer, pH 7.5 containing 100 mM NaCl and 1 mM EDTA) and the resulting overhangs on the products were used for directional cloning of the *cis*-element sequences into the *Spe*I and *Not*I sites of the binary vector pINT1-HIS3NB. The pUC29 sequence was excised by restriction digestion with *Nco*I and *Sac*I and double crossover was used to integrate the construct into the *PDC6* locus of yeast genomic DNA. Reporter strains, designated yCRT/DRE and yCRT/DRE-like were incubated overnight in rich YPDA media, mixed with glycerol to 25% final concentration and stored at -80°C in aliquots until use.

A modified Y1H method was used involving overnight yeast mating of a and α strains, one of which (reporter strain) contained bait DNA sequence integrated into the yeast genome, the other contained prey plasmids from the barley cDNA library; the mating efficiently brought together the bait and prey constructs. Aliquots of the yeast cDNA library and reporter strain cells were thawed in a room temperature water bath and 1 ml of one or the other bait strain and 500 µl of library cells were added to sterile 2 L conical flasks containing 25 ml of 2x YPDA containing kanamycin at 50 µg ml^{-1}. The flasks were swirled gently to mix and incubated at 30°C for 17 hrs with slow rotation at 37 rpm. The yeast cells were harvested by transferring the mixture to sterile 50 ml centrifuge tubes which were centrifuged at room temperature at 1,000 x g for 10 min, the supernatant was discarded. The mating flasks were rinsed twice with 200 ml of 1x

TE containing kanamycin at 50 mg ml^{-1}, which was retained and used to resuspend the corresponding cell pellet after the centrifugation step. The suspensions were centrifuged again for 10 min at 1,000 x g and the supernatants discarded.

The pellets were resuspended in 4 ml of 1x TE containing kanamycin at 50 mg ml^{-1} and for each mating, 200 µl of the suspension was spread on each of seven freshly prepared selective 2.2% w/v agar plates with selective SD media (-Leu, -His) containing 5 mM 3-amino-1,2,4-triazole (3AT) and seven plates with the same media containing 10 mM 3AT. 3AT was used to reduce possible leaky expression of the *HIS3* gene and hence slow non-specific growth on –His media. The plates were incubated at 30°C for five days.

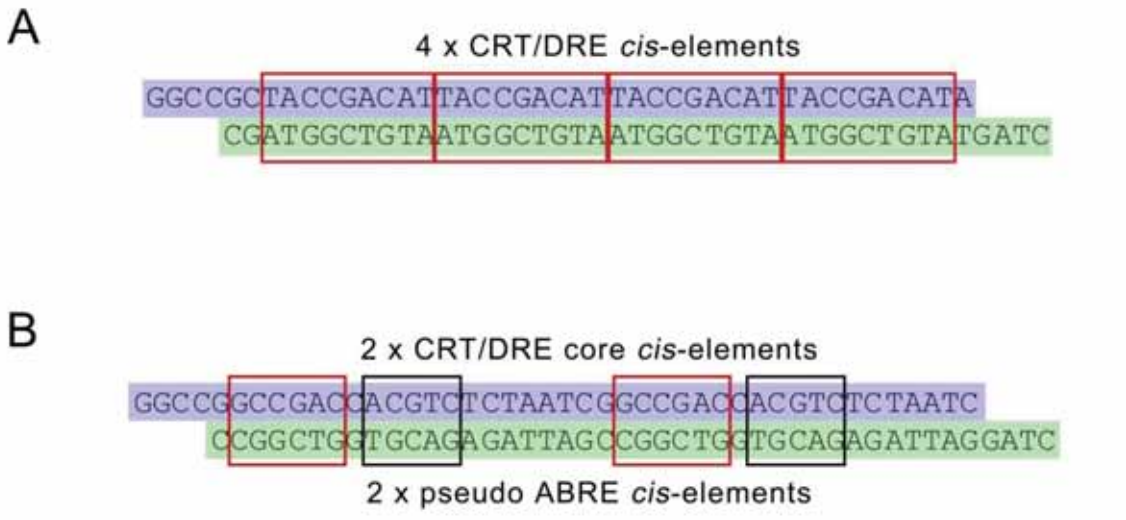


Figure 4.1 Schematic diagram of the layout of the *cis*-elements present in the bait constructs used for yeast 1-hybrid screening.

(**A**) Bait construct containing four repeated sequences of the Arabidopsis CRT/DRE, present in the yCRT/DRE bait strain. (**B**) Bait construct containing a maize CRT/DRE-like sequence, present in the yCRT/DRE2 bait strain. Y1H screening with CRT/DRE sequences as bait was used to identify barley *CBF* genes in the cDNA library prepared from cold-treated barley. The blue and green boxes represent different primers hybridised to form the bait fragment. CRT/DRE *cis*-elements are marked with red boxes. Two pseudo ABRE *cis*-elements are marked with black boxes.

DNA was extracted from single colonies and a reference plate of the yeast clones was prepared directly as described in Section 4.2.3.4. The reference plates contained more than 170 colonies from the screen using the Arabidopsis CRT/DRE and all the colonies (approximately 36) from the screen using the maize CRT/DRE. DNA was extracted from 72 clones obtained from the screen using the Arabidopsis CRT/DRE and 24 clones obtained from the screen using the maize CRT/DRE. To determine the length of the cloned inserts, PCR was performed using Epicentre FailSafe 2x PCR PreMix G and primers from the vector sequences adjacent to the insert. Reactions contained 12.5 µl of Buffer G, 0.5 µl of 50 µM ADLD_forward primer (forward), 0.5 µl of 50 µM ADLD_reverse primer (reverse), 1.5 µl of yeast DNA and 0.5 µl of Platinum *Taq* DNA polymerase in a total volume of 25 µl. A positive control reaction contained 1 µl of DNA from a clone obtained from a previous mating as template. A negative control reaction contained no template. Cycling parameters were as follows: 94°C for 2 min, then 31 cycles of 94°C for 30 sec and 68°C annealing/extension temperature for 3 min, followed by 68°C for 3 min. To confirm the plasmid DNA extraction and PCR amplification of the insert had been successful, 5 µl of the reaction products were electrophoresed on a 1% w/v agarose gel as described in Section 2.2.2.2.

To determine the diversity of the inserts with the same size PCR product, the products were digested with restriction enzymes. Digestion reactions were performed in 20 µl volumes containing 2 µl of Buffer 2, 3 U of *Hae*III (10 U μl^{-1}) and 5 µl of PCR reaction as template. Reactions were incubated at 37°C for 6 hrs and the enzyme was heat inactivated at 80°C for 20 min. Reaction products were electrophoresed as described in Section 2.2.2.2. The restriction patterns were analysed and clones with identical patterns were grouped.

4.2.5. Nucleotide Sequence Analysis and Manipulation

Plasmid DNA from clones from each group were transformed into *E. coli* as described in Section 2.2.2.4 and bacteria was spread onto LB plates containing ampicillin at 100 µg ml^{-1}. Where groups contained more than one member, DNA from at least three

clones were used in transformation. The *E. coli* clones were cultured and plasmid DNA was isolated as described in Sections 2.2.2.4 and 2.2.2.5. Sequencing was performed as described in Section 2.2.2.6, using T7 and 2HA_Rev primers (Appendix A).

Nucleotide sequence analysis was performed by database searches and sequence alignment as described in Section 2.2.2.6. The sequences of the inserts from different clones were compared by alignment to identify clones of the same gene. The consensus nucleotide sequences were used to search the NCBI sequence databases using BLAST software. The sequences were named *HvCBF16* and *HvCBF23*, employing the naming convention to apply the next unassigned sequential name. Peptide sequences were analysed to calculate the molecular weight, isoelectric point, and predict sumoylation sites using computer software as described in Section 2.2.2.6. The N-terminus of the mature proteins was predicted using TermiNator software and the presence and/or position of nuclear export signals, phosphorylation sites and signal peptide cleavage sites was predicted using NetNES, NetPhos, and SignalP software, respectively (accessed *via* http://au.expasy.org/tools/). Subcellular localisation was predicted using the MitProtII, PSORT, WoLFPSORT, ChloroP, Predotar and TargetP programs (http://au.expasy.org/tools/). Phylogenetic trees were constructed using the Neighbour Joining function of MEGA v 4.0.2 software (www.megasoftware.net).

4.2.6. DNA Binding Analysis of Proteins

Yeast transformation was performed as described in Section 4.2.3.4 transforming each bait strain with four prey constructs individually (Table 4.1). The resulting colonies were streaked in ~2 cm lines on selective SD (-Leu) 2.2% w/v agar plates, either containing 5 mM 3AT, grown overnight and replica-printed on the same plates and plates with –Leu and –His. The resultant plates were incubated at 30°C until growth was evident (~1-5 days). Images were taken of the plates using a Perfection™ V700 Photo scanner.

Table 4.1 Bait and Prey constructs used in *HvCBF16* and *HvCBF23* DNA binding analysis.

Component details		**Experimental design**	**DNA or strain origin**
Prey constructs	*HvCBF16* plasmid DNA	Experimental	*E. coli* mini-preparations (Section 4.2.5)
	HvCBF23 plasmid DNA	Experimental	*E. coli* mini-preparations (Section 4.2.5)
	TaDREB3 plasmid DNA	Positive Control - Known to bind the repeated Arabidopsis CBF/DRE *cis*-element construct. Negative Control - Known not to bind E2F *cis*-element construct.	Kindly provided by Ms. Sarah Morran
	Empty *pGADT7* vector DNA	Negative Control	Provided in BD Matchmaker™ Library Construction and Screening Kit
Reporter (bait) strains	yCRT/DRE (yeast strain containing repeated Arabidopsis CRT/DRE)	Experimental	Kindly provided by Dr. Sergiy Lopato
	yCRT/DRE2 (yeast strain containing maize CRT/DRE)	Experimental	Kindly provided by Dr. Sergiy Lopato
	yE2F strain (containing E2F *cis*-element)	Negative Control - Expected that *HvCBF16*, *HvCBF23* and *TaDREB3* will not bind this *cis*-element	Kindly provided by Dr. Sergiy Lopato

4.2.7. Production of Barley Drought Stress Treatment Series for qRT-PCR

The barley drought-stress treatment series was produced by Ms. Alexandra Smart. *Hordeum vulgare* L. cv. Sloop were used as it is an adapted South Australian malting cultivar and is therefore a more appropriate choice than the Golden Promise cultivar for the soil type and watering regime chosen for this experiment. Plants were grown in Growth Chamber 8 in the undercroft area at the University of Adelaide (SA, Australia) facilities under a 12 hr photoperiod with a 16°C day and 4°C night for the first four weeks, then a 17°C day and 6°C night for the next four weeks, followed by 23°C day and 10°C night for the remainder of the experiment. Relative humidity was maintained at 40-50% during the day and 80% during the night. Plants were grown in watertight bags containing six kilograms of 50% dried Roseworthy soil/50% Waikerie sand, with nutrients added (refer to Appendix D.2 for details).

A cyclic drought regime was employed to imitate the typical Southern Australian rainfall events (Figure 4.2). Plants were watered to field capacity by weight with tap water. Drought treatment was applied at emergence of the first flag leaf by gradually reducing the quantity of water added each day until plants showed visible wilting symptoms. Plants were re-watered to field capacity and left to dry without daily watering to wilting point. They were then rewatered. Control plants were watered daily. Leaf relative water content was measured over the entire experiment to monitor plant water status. Samples were taken at 2 PM at 3, 7 and 12 days after the first drought treatment was applied, then at 1 and 6 days after the second drought treatment was applied, as indicated by arrows in Figure 4.2. Leaf and whole spike tissues were sampled from five plants at each time point. Tissues were ground under liquid nitrogen and RNA was extracted as described in Section 2.2.3.2.2. cDNA synthesis was performed as described in Section 2.2.3.5.

Figure 4.2 Plant water status and sampling regime during the drought-stress treatment series.

The arrows indicate when samples were taken. The red line indicates the water status in the plant (between field capacity and wilting point) at various stages of the experiment. Figure was kindly provided by Dr. Thorsten Schnurbusch (ACPFG).

4.2.8. Expression Analysis of *HvCBF16* and *HvCBF23* by qRT-PCR

qRT-PCR was employed to examine the expression of *HvCBF16* and *HvCBF23*. The expression of *HvCBF16* was examined using the drought cDNA series described above, and/or the cold, salt and ABA treatment cDNA series used for analysis of *HvICE2* in Chapter 2. Preparation of the cDNA series is described in Sections 2.2.3.2, 2.2.3.3, 2.2.3.4, 2.2.3.5 and 4.2.7. Expression of *HvCBF16* was also examined in individual tissues using cDNA from a barley developmental tissue series kindly provided by Dr. Rachel Burton (Burton *et al.*, 2004). The expression of *HvCBF23* was examined during cold treatment, as described for *HvCBF16* above.

qRT-PCR was performed as described in Section 2.2.3.6 using the primers for *HvCBF16* presented in Table 2.3. *HvCBF23* was amplified using HvCBF23_F (forward) and HvCBF23_R (reverse) primers, with an expected product size of 204 bp.

4.2.9. Expression Analysis of *HvCBF23 via* Microarray Data Analysis

The barley Affymetrix chip was searched using the Barleybase suite of programs and data was analysed as described in Section 2.2.3.7 (Scores and E values are presented in Appendix B.2).

4.2.10. Genomic Mapping of *HvCBF16* and *HvCBF23*

Mapping of *HvCBF16* and *HvCBF23* was kindly performed by Ms. Margaret Pallotta (Australian Centre for Plant Functional Genomics). DNA fragments were PCR amplified from barley cv. Haruna Nijo genomic DNA using qRT-PCR primers of *HvCBF16* (Table 2.3) and *HvCBF23* (Section 4.2.8) and products were used as probes for RFLP analysis using *Eco*RI and *Dra*I respectively. Chromosome arm assignments were conducted using wheat-barley addition lines (Islam *et al.*, 1981) Genomic DNAs extracted from 146 lines of the Clipper x Sahara barley double haploid mapping population (Karakousis *et al.*, 2003) were used for linkage analysis. The “Find links”

function of Map Manager QTXb20 (Manly *et al.*, 2001) was used to position the loci. DNA extractions, Southern transfer and probe hybridisations were performed using standard methods (Rogowsky *et al.*, 1991; Sambrook *et al.*, 1989). Common markers were used to determine the relative locations on the Steptoe x Morex bin maps (Kleinhofs and Graner, 2001).

4.3. Results

4.3.1. Production, Quality Analysis and Screening of cDNA Libraries

Yeast one-hybrid (Y1H) screens were performed to identify *CBF* genes from barley which might be involved in cold tolerance. RNA was extracted from cold stressed barley and used to prepare a 'prey' cDNA library which was screened using CRT/DRE elements from Arabidopsis and maize as 'bait'. A diagram illustrating the general principle of Y1H screening is presented in Figure 4.3.

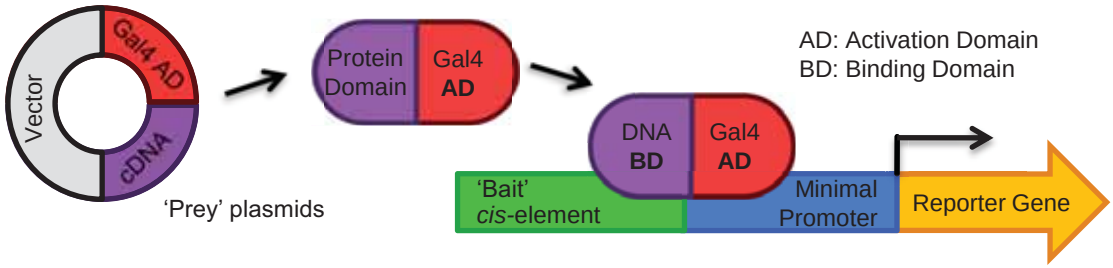


Figure 4.3 Schematic diagram illustrating the principle of Y1H screening.
Y1H screening was employed to identify *CBF* genes from barley floral tissues exposed to freezing stress. The diagram shows the components which were present in the yeast immediately following mating. In this study, the 'bait' *cis*-elements were CRT/DRE elements from Arabidopsis and maize and the 'prey' plasmids contained barley cDNA, functionally linked to DNA encoding the Gal4 activation domain. Fusion proteins transcribed and translated from the 'prey' plasmid had a Gal4 activation domain fused to a protein transcribed and translated from one of the cDNAs. If the 'prey' cDNA encoded a transcription factor with an appropriate DNA binding domain (in this case, an AP2 domain), the fusion protein was able to bind the 'bait' *cis*-element (in this case, the CRT/DRE element) in the promoter of the reporter gene. This would bring the Gal4 activation domain into proximity to the minimal promoter of the reporter gene and initiate transcription of the reporter gene. The reporter gene used in this study allowed growth on selective media lacking histidine.

The barley variety Haruna Nijo was used to prepare the cDNA library. This variety was selected since it has previously been shown to have a high level of tolerance to freezing

stress at flowering. Barley plants at anthesis were cold-treated to a minimum of -5.5°C in a freezing chamber to simulate a natural radiative frost event. A pooled sample of polyA RNA from treated floral tissues sampled at various time points during the treatment was used with the expression vector pGADT7-Rec to produce a yeast hybrid library. The mRNA and resulting cDNA was determined to be of good quality by gel electrophoresis of the second strand cDNA synthesis reactions. The clone insert size and diversity of the library was estimated using PCR to determine the approximate insert size in 24 independent clones (Figure 4.4). The largest insert identified was ~1.8 kb, the smallest was ~0.3 kb and the mean of the estimated clone sizes was ~820 bp. The standard deviation of the values was ~0.4 kb.

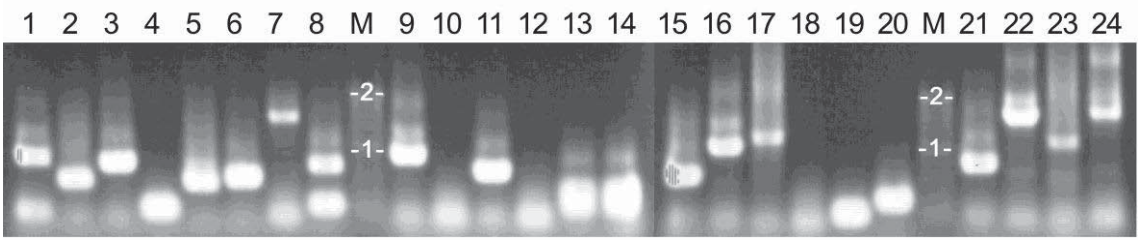


Figure 4.4 Photograph of agarose gel analyses of PCR products amplified from clones from the Y1H cDNA library.

PCR amplification of the inserts of a subset of cDNA clones was used to estimate the clone insert size and diversity of the Y1H cDNA library. The largest insert was ~1.8 kb, the smallest was ~0.3 kb and the mean of the estimated clone sizes was ~820 bp. Lanes 1 to 24: PCR products amplified from different barley cDNA clones; M: molecular weight marker, numbers fragment represent sizes in kilobases.

The yeast hybrid library was screened for interaction with either of two variations of the Arabidopsis CRT/DRE element by mating library cells and 'bait' yeast strains (yeast strains containing the *cis*-element in a minimal promoter, functionally linked to a reporter gene). Interaction between the *cis*-element and the library protein was detected by growth on media lacking histidine and leucine. 3-amino-1,2,4-triazole (3AT) was included in the media to suppress false positives by reducing possible leaky expression of the reporter gene *HIS3*. More than 170 colonies were obtained from the screen with

the Arabidopsis CRT/DRE bait and approximately 36 colonies were obtained from the screen with the maize CRT/DRE bait.

4.3.2. Characterisation of Barley *CBF* Genes from Y1H Clones

4.3.2.1. Sequence Analysis of Barley *CBF* Genes.

DNA was extracted from 72 and 24 colonies from the mating with the Arabidopsis or maize CRT/DRE bait, respectively. The diversity of the inserts was examined by restriction mapping of PCR fragments amplified from the cDNA clones. An example of the results of restriction mapping is presented in Figure 4.5, featuring clones from the Y1H screen using the Arabidopsis CRT/DRE bait. PCR fragments with identical restriction patterns were grouped, identifying at least six distinct groups of clones from each screen. From the grouped clones, ten representative clones were sequenced from the screen using the Arabidopsis CRT/DRE bait, and 19 representative clones from the screen using the maize CRT/DRE bait. Comparison of the nucleotide sequences of the clones with one another and published databases by BLAST analysis allowed a) copies of the same gene to be identified, and b) elucidation of the identity and/or putative function of the cloned genes by identifying homologous published sequences (scores and E values from BLAST searches are presented in Appendix B.4 & Appendix B.5). Clones encoding two distinct AP2 domain-containing proteins were identified.

At the time of discovery, both genes were uncharacterised and did not have homology with any published barley sequences. One gene had very high nucleotide sequence similarity to the *Triticum monococcum CBF* gene *TmCBF16*, including identical sequences over the N-terminal leader region which are usually highly variable between CBF proteins (Skinner *et al.*, 2005). No barley ortholog of *TmCBF16* had been published at this stage although this gene was subsequently published as *HvCBF16* by Stockinger and colleagues (2007). An alignment of the translated nucleotide sequences of the *HvCBF16* clones with that of *TmCBF16* is presented in Appendix E.3 (scores and E values of BLAST search in Appendix B.4). Seven clones encoding *HvCBF16* were identified from the yeast 1-hybrid screen using the Arabidopsis CRT/DRE as bait and a consensus sequence of *HvCBF16* from the clone sequences is presented in Appendix

F.4. Comparison of the sequences of the clones to that of the published *HvCBF16* sequence revealed that two conservative single nucleotide polymorphisms were present between Haruna Nijo and Tremois sequences, and that the genes encoded identical polypeptides.

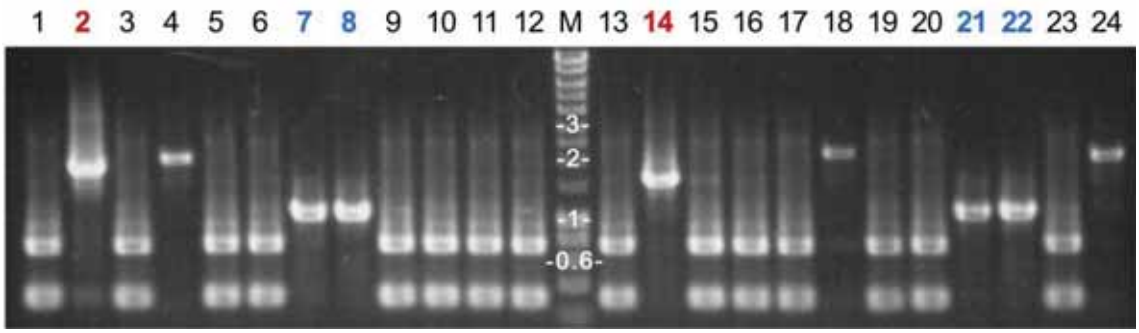


Figure 4.5 Photograph of agarose gel analyses of restriction mapping of PCR products amplified from clones isolated using a Y1H screen.

Restriction mapping was used to examine the diversity of the inserts isolated by Y1H screening of a barley cDNA library. The examples pictured were isolated using the Arabidopsis CRT/DRE *cis*-element as bait. Numbers 1 to 24: PCR products amplified from different barley cDNA clones, digested with *Hae*III; M: molecular weight marker, numbers fragment represent sizes in kilobases. Subsequent sequencing determined that clones 7, 8, 21 and 22 (blue) encoded HvCBF16, while clones 2 and 14 (red) encoded HvCBF23.

HvCBF16 encodes a putative protein of 227 amino acids, with a calculated molecular mass of 23.9 (kDa) and a pI of 4.73, not taking into account any post-translational modifications. Computer predictions using the TermiNator program indicated that the translation efficiency of the gene was very high (5/5) and the protein was highly stable (maximum possible). A number of serine residues were predicted to have high potential for phosphorylation, with a dense cluster at the N-terminal region and others scattered over the C-terminal region (Figure 4.6A). Phosphorylation was also predicted at two threonine residues and one tyrosine residue. No sumoylation sites or leucine rich nuclear export signals were predicted in HvCBF16 and it was suggested that the N-terminal end of the mature protein would be the start methionine (100% likelihood),

indicating no N-terminal peptide cleavage. Comparing the results of multiple prediction programs, moderately high scores were obtained for prediction of subcellular localisation of *HvCBF16* to the nucleus and chloroplast, while poor scores were obtained for the mitochondria or cytoplasm.

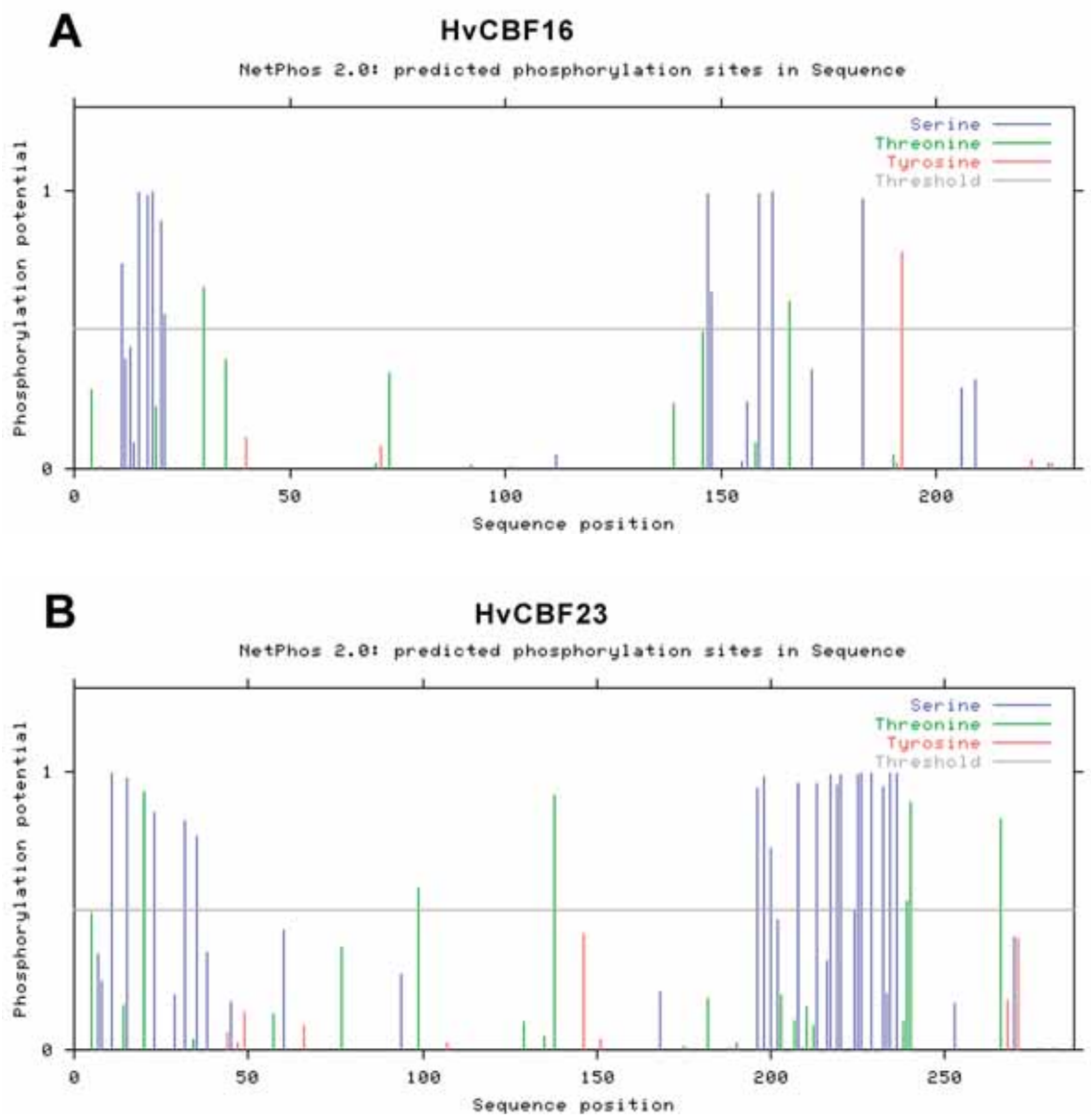


Figure 4.6 Graphs of the location and scores of predicted phosphorylation sites in HvCBF16 and HvCBF23.

(**A**) HvCBF16. (**B**) HvCBF23. Putative phosphorylation sites were predicted using NetPhos software (www.cbs.dtu.dk/services/NetPhos/).

BLAST analysis of the second AP2-domain-containing clone indicated that, beyond the AP2 domain, no significant similarity was observed with the published barley CBFs, or with any well-characterised proteins. Following the naming convention, the gene was named *HvCBF23*, as the next unassigned sequential name in the cereal *CBF* family. Although the names *HvCBF17-22* have not been assigned to sequences, these were passed by to avoid implying homology with wheat genes of the homologous names (Badawi *et al.*, 2007; Miller *et al.*, 2006). Four clones encoding HvCBF23 were identified from the yeast 1-hybrid screen using the Arabidopsis CRT/DRE as bait and the consensus sequence of the clone sequences is presented in Appendix F.5. An alignment of the translated nucleotide sequences of *HvCBF23* with the sequences of closely related AP2-domain-containing proteins *HvDRF1.3*, *TaDRFL2b*, *AtRAP2.4* and *AtERF060* is presented in Appendix E.4 (scores and E values of the BLAST search in Appendix B.5).

HvCBF23 encodes a putative protein of 282 amino acids with a calculated molecular mass of 30.4 kDa and a pI of 9.76, not taking into account any post-translational modification. Phosphorylation sites were predicted with a high level of confidence at many serine residues with a large cluster in the C-terminal region of the protein (Figure 4.6B). Four predicted threonine target sites were also identified. In contrast to *HvCBF16*, *HvCBF23* was predicted to have very low translation efficiency (1/5) and the program was not able to predict the half-life of the protein. Also, HvCBF23 was predicted with a high level of confidence (100% likelihood) to undergo protein processing in the form of N-terminal methionine excision, resulting in the N-terminus of the mature protein being the second amino acid in the sequence (proline). No signal peptide or leucine rich nuclear export regions were predicted. Moderately high but varying scores were obtained for localisation to the mitochondria, chloroplast or cytoplasm. Noticeably, no programs produced high scores for nuclear localisation of this transcription factor.

Phylogenetic analysis of the barley CBF family was published by Skinner *et al*. (2005) and was performed in this study using the sequences of published CBFs and other AP2-domain-containing proteins to determine the relationship between HvCBF16, HvCBF23

and other, better characterised proteins. A phylogenetic tree of selected representative barley, monocot and Arabidopsis CBFs and AP2-domain-containing proteins is presented in Figure 4.7, with the barley and Arabidopsis subgroups marked, as proposed by Skinner *et al*. (2005) and Sakuma *et al*. (2002). This tree was considered in combination with an alignment of the conserved CBF signature motifs and the AP2 DNA binding domain of HvCBF16, HvCBF23 and other CBFs and AP2-domain-containing proteins (Figure 4.8).

There is a high level of sequence conservation in the region surrounding the AP2 DNA binding domain between members of the HvCBF subgroups, including HvCBF16 (Figure 4.8). HvCBF16 is a member of the HvCBF3-subgroup and contains all five of the conserved residue blocks which are characteristic of this subgroup, as well as the two CBF signature motifs (Jaglo *et al.*, 2001; Skinner *et al.*, 2005). The positions of the conserved motifs are marked on a sequence alignment of HvCBF16 and several HvCBF3-subgroup members in Appendix E.5. Other members of the HvCBF3-subgroup include HvCBF3, HvCBF6, HvCBF10A and HvCBF12, as well as members of the *T. monococcum* CBF family for which homologs have not yet been identified in barley. One such protein, TmCBF15 (not shown on the phylogenetic tree), shares the greatest degree of sequence similarity with the HvCBF16 after TmCBF16.

The similarity between HvCBF23 and the barley CBFs was weak beyond the AP2 domain and HvCBF23 does not contain either of the flanking CBF signature motifs which are highly conserved in the barley and Arabidopsis CBFs (Figure 4.8). HvCBF23 was more similar to proteins in the distinct, diverged group of AP2 domain-containing proteins DREB subgroup A-6 than barley CBFs, and contains all four of the conserved motifs specific to this group (Nakano *et al.*, 2006; Sakuma *et al.*, 2002) (Figure 4.7). The positions of the conserved motifs are marked on a sequence alignment of HvCBF23 and several DREB subgroup A-6 members presented in Appendix E.4. HvCBF23 has a similar degree of sequence similarity with each of the other members of the DREB subgroup A-6 (Figure 4.7), which includes soybean and maize proteins (not shown), as well as wheat, barley and Arabidopsis members.

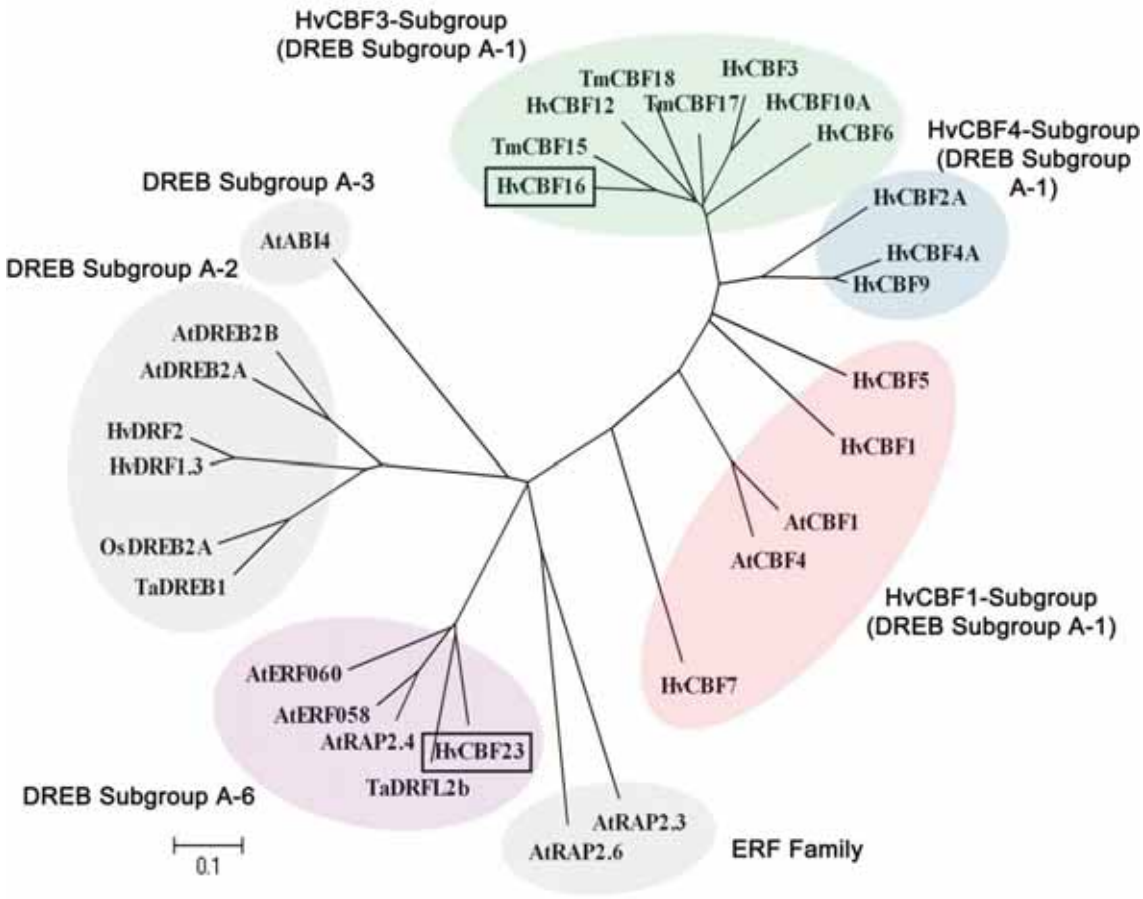


Figure 4.7 Phylogenetic analysis of HvCBF16 and HvCBF23.

The phylogenetic tree was derived from an alignment of the CBF polypeptides using the Neighbour Joining function of MEGA software (www.megasoftware.net). Scale indicates branch lengths. HvCBF16 and HvCBF23, the proteins identified in this work, are boxed. Ellipses denote different phylogenetic subgroups of CBF/DREB/AP2-domain-containing protein, with group names proposed by Skinner *et al*. (2005) and Sakuma *et al*. (2002).

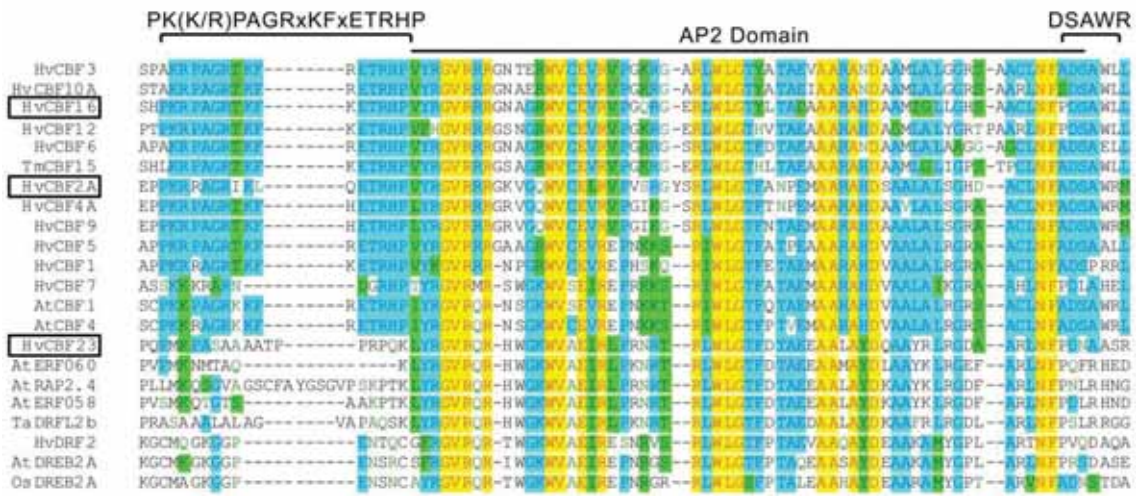


Figure 4.8 Alignment of the AP2-domain and conserved CBF signature motifs, from peptide sequences of selected CBF and AP2-domain containing proteins.

The flanking conserved CBF signature motifs (with respective consensus) and AP2 domain are marked (Jaglo *et al.*, 2001). There are several regions of conservation over all the proteins presented however, conservation is greatest within subgroups. There are high levels of conservation between the majority of barley CBFs, including HvCBF16. There are several conserved residues in *HvCBF23* and other members of the DREB subgroup A-6. Gene sequences were obtained from public databases under the given names (www.ncbi.nlm.nih.gov/), except that of *HvCBF23*, which is presented in Appendix F.5. Sequences were aligned using ClustalW software.

4.3.2.2. DNA Binding Analysis of *HvCBF16* and *HvCBF23*

To validate the results of the yeast one-hybrid screen, DNA binding analysis was performed by transforming each bait strain with each of four prey constructs (Table 4.1). The resulting strains were grown on two types of selective media: a) Media lacking leucine, to select for strains which were successfully transformed with the prey plasmid and b) media lacking leucine and histidine, to select for strains with interaction between the prey protein and the *cis*-element bait. Photographs of the results obtained in this experiment are presented in Figure 4.9.

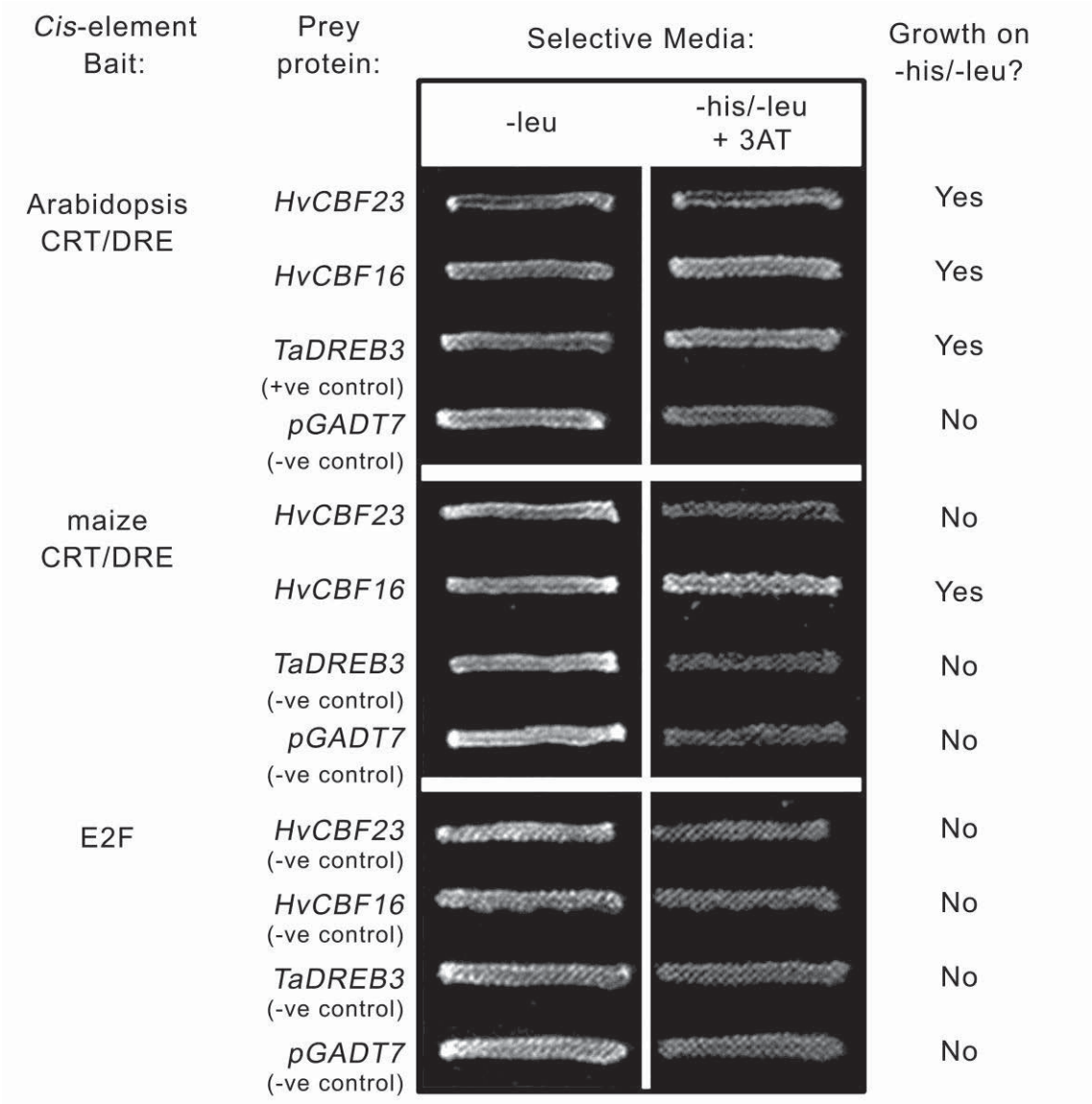


Figure 4.9 Photographs of yeast strains on selective agar plates during DNA binding analysis of *HvCBF16* and *HvCBF23*.

Growth on media lacking leucine indicates the presence of the prey plasmid in the bait yeast strain. Growth on media lacking leucine and histidine indicates interaction between the tested bait *cis*-element and prey protein. Both HvCBF23 and HvCBF16 are able to bind the Arabidopsis CRT/DRE *cis*-element but only HvCBF16 is able to bind the maize CRT/DRE *cis*-element. A *cis*-element which is bound by E2F type transcription factors and the empty prey vector (pGADT7) were used as negative controls. TaDREB3 is known to bind the CRT/DRE bait and was used as a positive control. The contrast of the images has been increased to improve visibility.

A *cis*-element which is bound by E2F-type transcription factors and the empty prey vector (pGADT7) were used as negative controls. TaDREB3 is known to bind the Arabidopsis CRT/DRE bait and was used as a positive control. All the strains grew on media lacking leucine. The strains containing Arabidopsis CRT/DRE bait, grew on media lacking leucine and histidine, when transformed with *AtCBF23*, *AtCBF16* or *TaDREB3* constructs, indicating all of these proteins can bind the Arabidopsis CRT/DRE element. Strains containing the maize CRT/DRE bait grew on media lacking leucine and histidine when transformed with the *HvCBF16* construct, indicating HvCBF16 can bind the maize CRT/DRE element. None of the strains containing the E2F bait grew on media lacking leucine and histidine, indicating none of these proteins can bind the E2F element. Likewise, none of the strains containing the empty prey vector grew on media lacking leucine and histidine, indicating the interaction was specific to the transcription factor genes present.

4.3.2.3. Expression Analysis of *HvCBF16 via* qRT-PCR

The expression of *HvCBF16* during cold, salinity and ABA treatments was determined by qRT-PCR using the cDNA series described in Chapter 2. In untreated plants, mRNA levels of *HvCBF16* were negligible in both the floral and leaf tissues (Figure 4.10A & B). During cold treatment, transcript levels of *HvCBF16* were greatly upregulated (40-fold to greater than 1,000-fold) and reached higher levels in leaf than floral tissues. The expression patterns of *HvCBF16* were similar in both Haruna Nijo and Golden Promise plants although transcript levels were greater in Golden Promise. The highest transcript levels were detected in the leaves of Golden Promise plants, where transcript levels at -5.5°C reached ~70,000 copies μl^{-1} of cDNA. The highest transcript levels in Haruna Nijo were also in leaf tissues at -5.5°C and were ~20,000 copies μl^{-1} of cDNA.

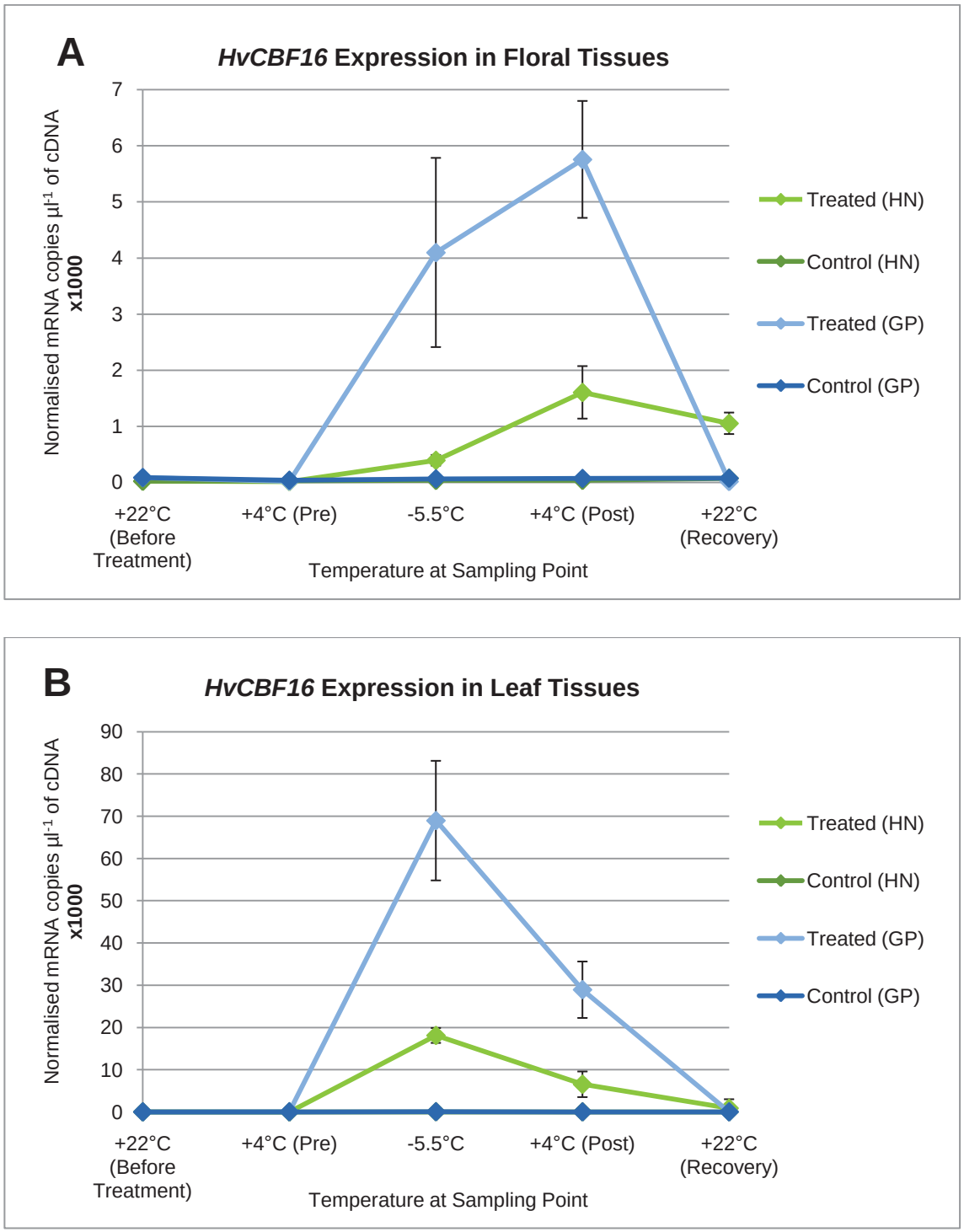


Figure 4.10 Graph of *HvCBF16* expression during cold stress treatment determined by qRT-PCR.

(**A**) *HvCBF16* expression in floral tissues. (**B**) *HvCBF16* expression in leaf tissues. *HvCBF16* was not expressed in untreated barley plants and gene expression was greatly upregulated in both the leaf and floral tissues of cold treated plants. GP, Golden Promise; HN, Haruna Nijo. Error bars represent standard error.

In both Golden Promise and Clipper x Sahara DH varieties, transcript levels of *HvCBF16* were negligible in untreated plants and remained constant throughout salinity treatment in both root and leaf tissues (Figure 4.11). Likewise, transcript levels of *HvCBF16* were negligible in Golden Promise plants in the ABA treatment series and Sloop plants in the drought treatment series, and were not affected by either ABA or drought treatment in any of the tissues examined (Figure 4.12; Figure 4.13). *HvCBF16* transcript levels were tested in 16 different tissues and although expression was greatest in anthers, floral tissues and peduncle, transcript levels were very low to negligible in all cases (Figure 4.14) and within the background range of detection for qRT-PCR. A search of the barley Affymetrix chip using the Barleybase suite of programs showed that none of the ESTs present corresponded to *HvCBF16*.

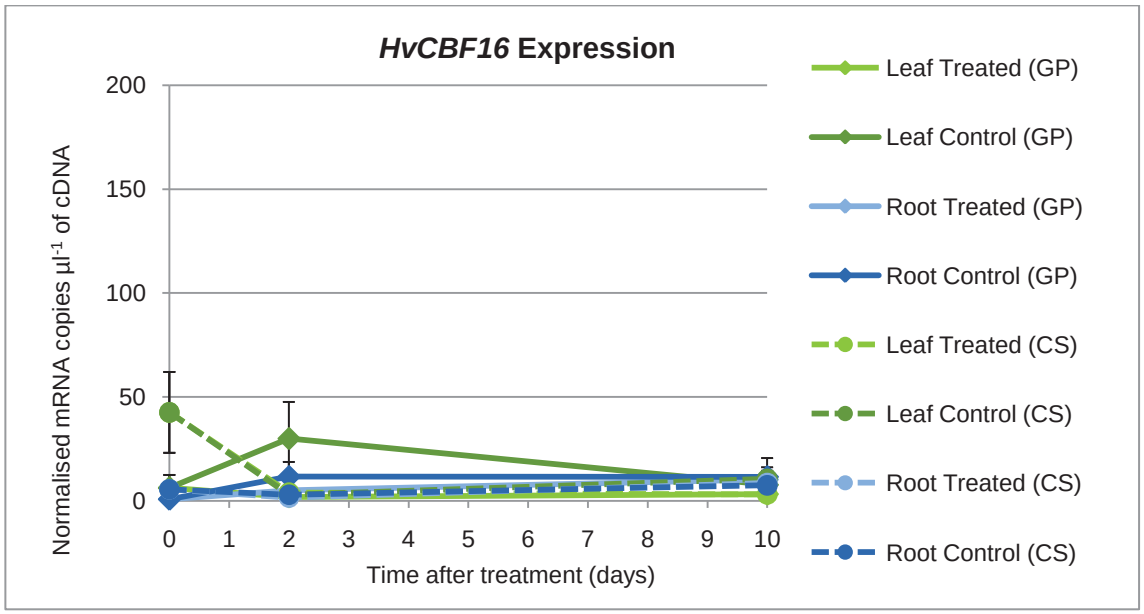


Figure 4.11 Graph of *HvCBF16* expression during salinity stress treatment determined by qRT-PCR.

HvCBF16 transcript levels were negligible in untreated barley plants and gene expression was not affected by salinity treatment (150 mM). GP, Golden Promise; CS, Clipper x Sahara double haploid line 134. Error bars represent standard error.

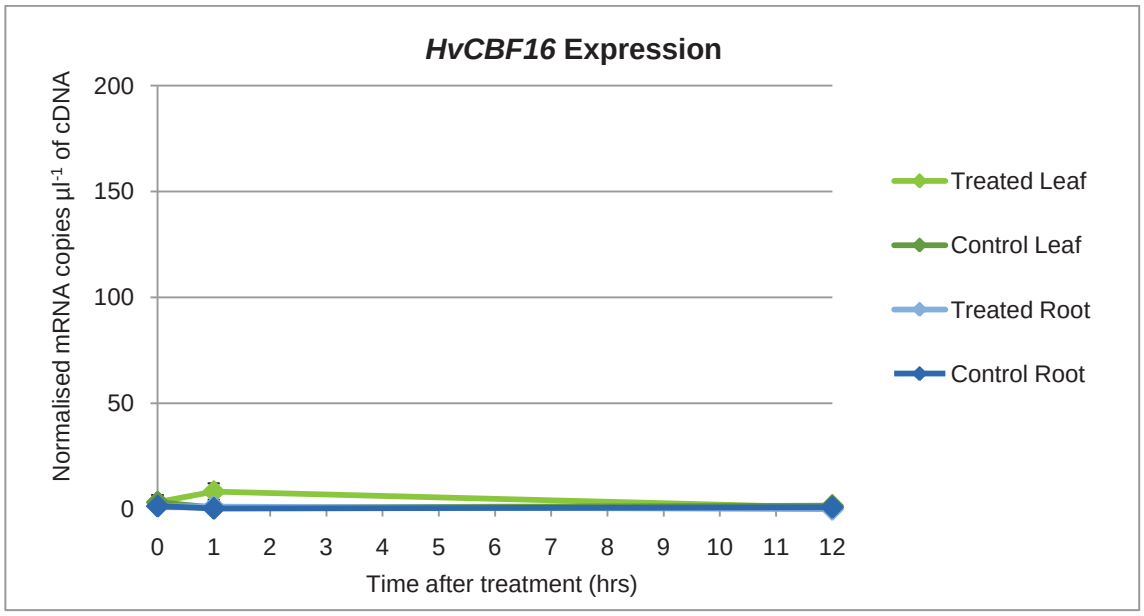


Figure 4.12 Graph of *HvCBF16* expression during ABA treatment determined by qRT-PCR.

HvCBF16 was not expressed in ABA-treated (10 µM) or untreated Golden Promise barley plants. Error bars represent standard error.

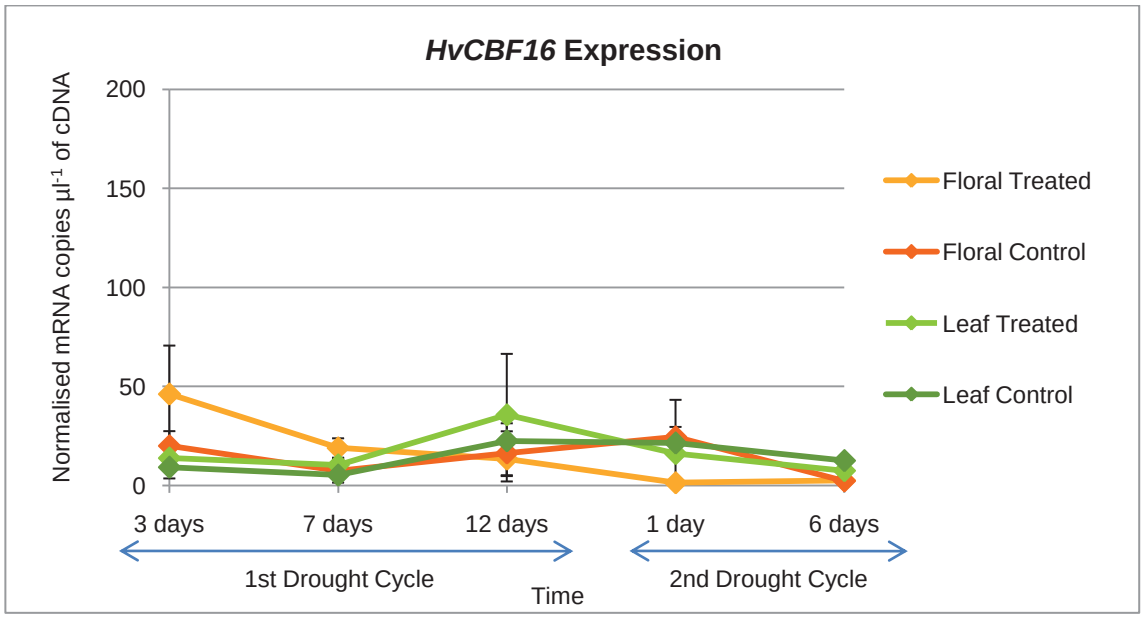


Figure 4.13 Graph of *HvCBF16* expression during cyclic drought treatments determined by qRT-PCR.

HvCBF16 transcript levels were negligible in Sloop barley plants and gene expression was not affected by cyclic drought treatments. Error bars represent standard error. Refer to Figure 4.2 for details of the treatment regime and sampling time points.

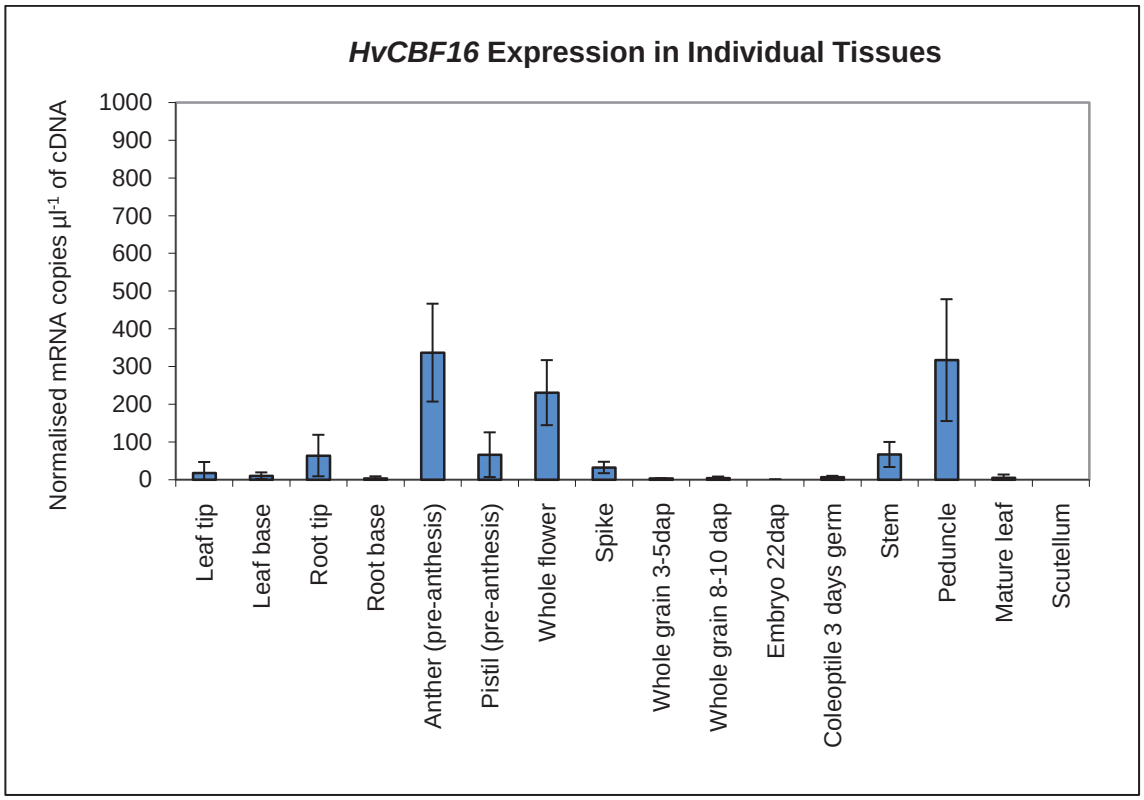


Figure 4.14 Graph of *HvCBF16* expression in various tissues determined by qRT-PCR.

HvCBF16 expression was extremely low in all tissues examined, with highest expression in anther, floral tissues and peduncle. Tissues obtained from Golden Promise barley plants. Error bars represent standard error.

4.3.2.4. Expression Analysis of *HvCBF23 via* qRT-PCR

The expression of *HvCBF23* was analysed during cold stress *via* qRT-PCR using the cold stress treatment cDNA series described in Chapter 2. The mRNA levels of *HvCBF23* in leaf and floral tissues from cold-treated and untreated Haruna Nijo or Golden Promise barley plants were high and varying, even within replicate plants for a single treatment (Figure 4.15A & B). No significant or consistent alteration in *HvCBF23* transcript levels was observed which could be related to the cold treatment. Transcript levels were slightly higher in floral than leaf tissues, with maximum levels being ~280,000 copies μl^{-1} of cDNA. The range of *HvCBF23* transcript levels was similar in Haruna Nijo and Golden Promise.

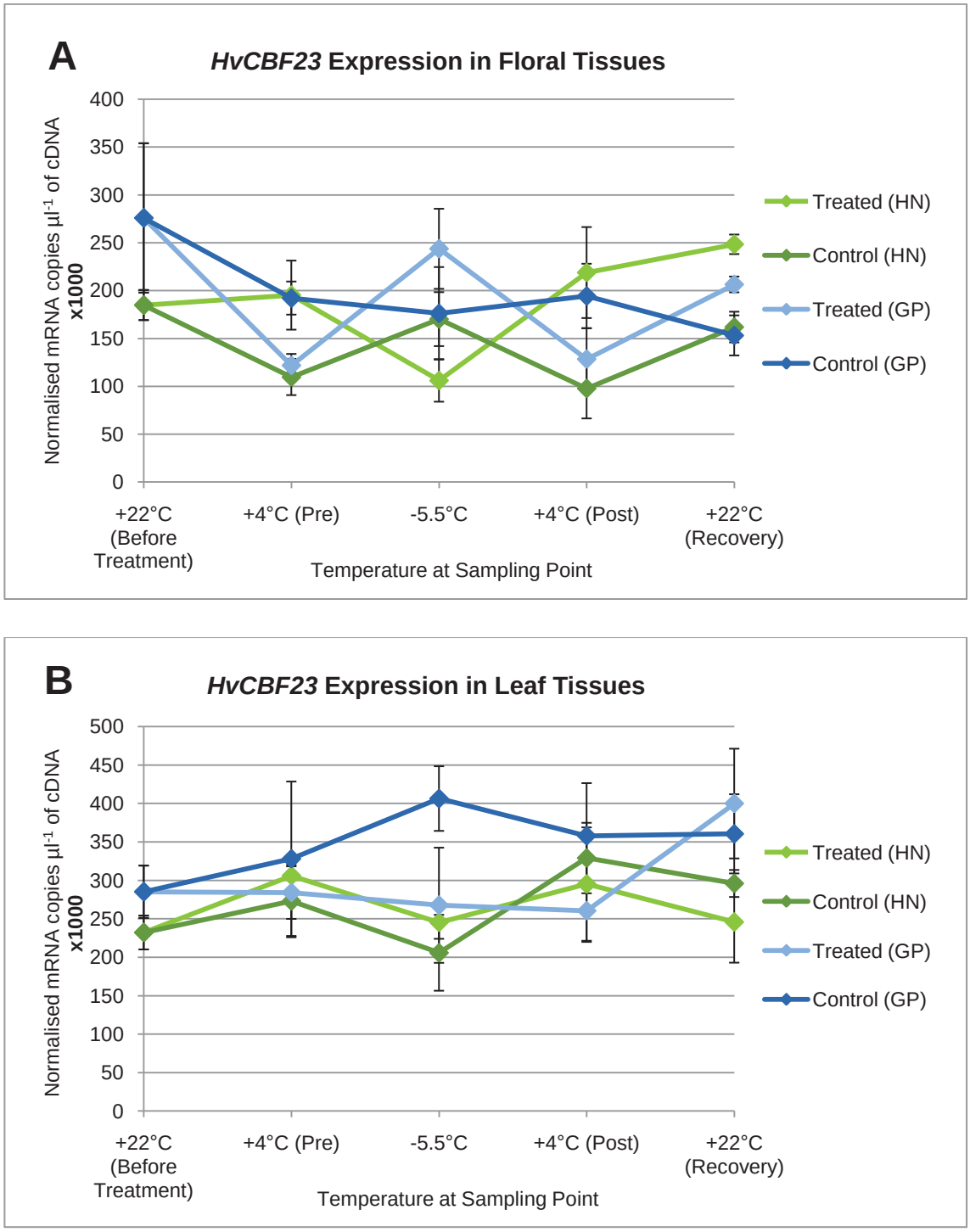


Figure 4.15 Graph of *HvCBF23* expression during cold stress treatment determined qRT-PCR.

(**A**) *HvCBF23* expression in floral tissues. (**B**) *HvCBF23* expression in leaf tissues. *HvCBF23* expression is high in both leaf and floral tissues and is not affected by cold treatment. GP, Golden Promise; HN, Haruna Nijo. Error bars represent standard error.

4.3.2.5. Expression Analysis of *HvCBF23* from Microarray Data Analysis

The expression of *HvCBF23* was examined on microarrays in a wide range of tissues and growth stages. *HvCBF23* transcript levels were high and at a similar level in all of the tissues, with no tissue having significantly higher or lower expression levels than any other (Figure 4.16). Where tissue was taken from both Morex and Golden Promise cultivars, the transcript levels in each genotype was very similar.

NOTE:
This figure is included on page 174 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.16 Graph of *HvCBF23* expression from Affymetrix microarray data.
HvCBF23 was expressed to a similar level in all of the tissues examined. An incomplete set of Golden Promise barley tissues were analysed (only coleoptile, crown, leaf and root). Information on the tissues studied is available from the website http://bioinf.scri.sari.ac.uk/affy/WEB_TISSUES/tissue_types.htm. DAP, days after pollination.

4.3.2.6. Mapping of *HvCBF16*

Genetic mapping was kindly performed by Ms. Margaret Pallotta (Australian Centre for Plant Functional Genomics). The results showed that the *HvCBF16* gene was located on the long arm of chromosome 5H in the Steptoe x Morex bin 10 (Table 4.2; Figure 4.17). Co-localised at this position are QTLs for traits involved in cold tolerance,

including LT_{50} (Hayes *et al.*, 1993), winter survival (Hayes *et al.*, 1993; Oziel *et al.*, 1996; Pan *et al.*, 1994), as well as salt tolerance in seedlings (Mano and Takeda, 1997).

4.3.2.7. Mapping of *HvCBF23*

As above, genetic mapping was kindly performed by Ms. Margaret Pallotta. *HvCBF23* was located near the centromere of chromosome 5H, likely on the long arm at the Steptoe x Morex bin 5/6 border (Table 4.2; Figure 4.17). This region is localised just outside (proximal) of the drought tolerance QTL described by Tondelli and colleagues (2006). A literature search did not reveal any QTLs of interest in this region.

Table 4.2 Genomic locations of *HvCBF16* and *HvCBF23* on barley chromosomes.

Genes	Chromosome	Steptoe x Morex Bin	Nearest north marker (cM from mapped fragment)	Nearest south marker (cM from mapped fragment)	Population mapped in
HvCBF16	5H	Bin 10	ksuA1 (5.1±1.9 cM)	HvPtr9 (1.7±1.2 cM)	CxS, W/B addition lines
HvCBF23	5H	Bin 5/6 border region	cdo749 (8.0±2.5 cM)	wg530 (1.5±1.1 cM)	CxS, W/B addition lines

These data were kindly provided by Ms. Margaret Pallotta (Australian Centre for Plant Functional Genomics). Genomic locations were determined using DNA fragments of *HvCBF16* and *HvCBF23* amplified by PCR with qRT-PCR primers. CxS: Clipper x Sahara 3771 DH; W/B: wheat-barley addition line.

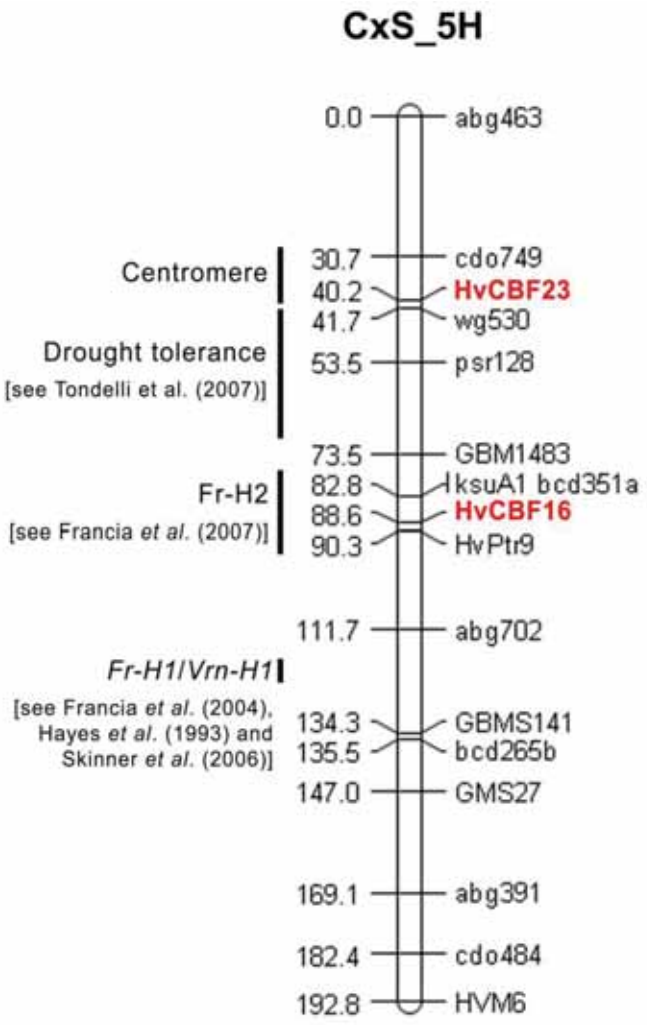


Figure 4.17 Map location of *HvCBF16* and *HvCBF23* on *Hordeum vulgare* chromosome 5H.

This figure was kindly provided by Ms. Margaret Pallotta (Australian Centre for Plant Functional Genomics). Genetic intervals (in cM) and the approximate position of the centromere are marked; presently, the precise position in relation to *HvCBF23* is not known. Data presented in the literature was used to infer the approximate QTL positions of *Fr-H1* (which colocalises with *HvVRN-1*), *Fr-H2* and drought tolerance.

4.4. Discussion

In Australia, the greatest financial losses to farmers due to freezing stress are caused by damage to floral tissues at anthesis. Accordingly, a cold treatment was performed on barley plants at anthesis from the freezing-tolerant cultivar Haruna Nijo to produce the yeast one-hybrid (Y1H) library. The Y1H method was used by Stockinger and colleagues (1997) to identify the first *CBF* gene from Arabidopsis using an Arabidopsis CRT element as bait. Here, the same method and core bait sequence was employed to isolate two uncharacterised barley CBFs, named HvCBF16 and HvCBF23, from this library.

PCR results indicated that the library contained large cDNA clones and was therefore of practical use. The yeast hybrid library was screened using two bait constructs which contained CRT/DRE *cis*-elements from Arabidopsis or maize (Figure 4.1A & B). cDNA clones of ~1.0 kb and ~1.2 kb were identified and were found to encode the barley CBF factors HvCBF16 and HvCBF23.

Although no sequence of *HvCBF16* was published at the time of identification, the sequence of *HvCBF16* from the Tremois cultivar was since published by Stockinger and colleagues (2007). The Tremois and Haruna Nijo *HvCBF16* alleles encode identical polypeptides and, as is typical of *CBFs*, *HvCBF16* does not contain introns. This indicates it is likely the two cultivars produce identical proteins, although the effect of the proteins in different cultivars could be altered by spatial or temporal differences in gene expression or factors related to the different cultivar backgrounds.

HvCBF16 was predicted to have high translation efficiency and protein stability using bioinformatic prediction software with proven success at predicting experimental results in *Arabidopsis thaliana* (Martinez *et al*., 2008). Many putative phosphorylation sites were identified including a dense cluster at serine residues in the N-terminal region of the protein (Figure 4.6A). This indicates that HvCBF16 may be regulated post-translationally by phosphorylation at one or more of these residues. No other post-

translational modifications were predicted, including sumoylation and N-terminal peptide processing.

HvCBF16 was predicted to be localised in the nucleus and/or chloroplast. In support of these predictions, several putative target genes of the barley CBFs are encoded in the nuclear genome, including *HvCor14b*, *HvDHN8* and *HvDHN5* (Cattivell *et al.*, 2002; Choi *et al.*, 1999). The significance of the localisation of HvCBF16 in the chloroplast, if real, is not clear.

HvCBF16 shares a very high degree of similarity with the *T. monococcum* protein TmCBF16. This includes identical sequences in the typically highly variable leader regions, which indicates HvCBF16 is likely to be the barley ortholog of TmCBF16 (refer to Figure 1.6 for general domain structure of CBFs) (Appendix E.5; Skinner *et al.*, 2005). Within barley, HvCBF16 is most similar to members of the HvCBF3-subgroup (Figure 4.7), and contains the conserved amino acid blocks which are characteristic of the subgroup, as well as in the AP2 domain and flanking CBF signature motifs (Appendix E.5; Figure 4.8; Jaglo *et al.*, 2001; Skinner *et al.*, 2005). The C-terminal activation domains were moderately conserved between HvCBF16 and other members of the HvCBF3-subgroup (Appendix E.5) but weakly with members of other subgroups (data not shown), which is consistent with studies in wheat (Badawi *et al.*, 2007). It has been shown that conservation in this region is important for the *trans*-activation properties of CBFs and was suggested by Badawi *et al.* (2007) that variation in the activation domain could alter functional properties including protein folding, interaction partners and *trans*-activation potential. Together, these findings suggest that HvCBF16 may be functionally similar to the other members of the HvCBF3-subgroup, targeting similar *cis*-elements and/or promoters due to their highly conserved functional domains and motifs, but may be functionally distinct from phylogenetically diverged CBFs from other subgroups.

Transcript levels were examined in many tissues and three different genotypes by qRT-PCR. In untreated tissues from all the genotypes studied, the transcript levels of

HvCBF16 were negligible or extremely low, easily within the background range of detection for qRT-PCR (Figure 4.10A & B; Figure 4.11; Figure 4.12; Figure 4.13; Figure 4.14). Expression of *HvCBF16* was greatly upregulated by cold treatment however the maximum transcript levels reached were still moderately low (Figure 4.10A & B). These results agree with published data which showed that *HvCBF16* mRNA was not detectable by northern blot in plants grown at normal temperatures and that expression was induced upon exposure to cold treatment (6°C) (Stockinger *et al.*, 2007). However, the treatments and chosen method of expression analysis differed from ours and therefore the timing, kinetics of induction and absolute transcript levels of *HvCBF16* during cold treatment in the two studies cannot be directly compared. During cold treatment, transcript levels of *HvCBF16* were higher in Golden Promise plants and/or leaf tissues however the expression profiles were similar in each of the cultivars examined (Figure 4.10A & B).

In contrast to the results with cold-stressed material, *HvCBF16* was not expressed in any of the tissues examined after drought, salinity and ABA treatments (Figure 4.11; Figure 4.12; Figure 4.13; Figure 4.14). Together, these results suggest that *HvCBF16* is important for cold-stress response but not for drought or salinity stress responses and acts independently of ABA. Further, the moderately low absolute level of expression after cold-activation may be compensated for by predicted efficient translation and high protein stability of HvCBF16. Very low basal expression levels and cold stress responsiveness has also been observed for other barley *CBF* genes (Skinner *et al.*, 2005; Stockinger *et al.*, 2007) and it was recently found that sequence conservation is particularly high in the promoters of cold-responsive tomato and potato *CBF* genes (Pennycooke *et al.*, 2008). These findings also suggest there is a high degree of selective pressure on *CBF* expression kinetics and supports the suggestion that cold responsiveness of *HvCBF16* is important for gene function, and perhaps ultimately for plant survival.

Like other members of the HvCBF3-subgroup, HvCBF16 was able to bind two CRT/DRE-derived motifs in Y1H analysis and yeast binding assays (Section 4.3.1; Figure 4.9) (Skinner *et al.*, 2005). Mapping showed that *HvCBF16* is located on the

long arm of chromosome 5H (Figure 4.17). This location is consistent with the results of fine mapping of the homologous gene in *T. monococcum*, which placed *TmCBF16* near the middle of the *CBF* cluster at *Fr-2*, between *TmCBF12* and *TmCBF13* (Knox *et al.*, 2008). The genomic structure of the region containing the *CBF* cluster in barley has been shown to be colinear with the corresponding region from diploid wheat (Francia *et al.*, 2007; Knox *et al.*, 2008). Considering these results collectively, it can be inferred that *HvCBF16* is likely to be positioned between *HvCBF12* and *HvCBF13*, as shown in Figure 4.18, modified from Francia *et al.* (2007). Several QTLs for abiotic stress tolerance are co-localised at the genomic map location of *HvCBF16* including the cereal frost tolerance locus *Fr-2* and winter survival (Hayes *et al.*, 1993; Oziel *et al.*, 1996; Pan *et al.*, 1994), LT_{50} (Hayes *et al.*, 1993), regulatory control of *Cor14b* expression (Francia *et al.*, 2004) and salinity tolerance (Mano and Takeda, 1997) QTLs. There is compelling evidence that the *CBF* genes clustered in this region are likely to be responsible for differences in *Cor14b* expression and freezing tolerance. This conclusion is reinforced by an absence of other candidate genes in this region (Stockinger *et al.*, 2007).

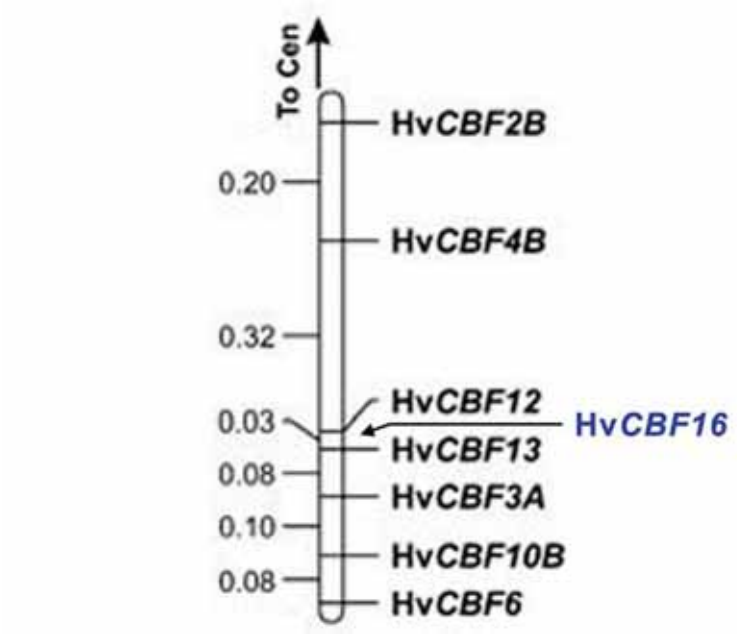


Figure 4.18 Schematic diagram of a genetic map of the barley *CBF* gene cluster. Modified from Francia *et al.* (2007). The putative position of *HvCBF16* within the cluster was inferred from data presented here and in the literature (Francia *et al.*, 2007; Knox *et al.*, 2008).

Studies investigating the relative importance of the *CBFs* at *Fr-2*, including *HvCBF16*, have not consistently settled upon the same genes. One study suggested greater expression of *HvCBF2* and *HvCBF4* may be important for cold tolerance (Stockinger *et al.*, 2007); another indicated that a mutant allele of *TmCBF12* and increased transcriptional sensitivity of *TmCBF12*, *TmCBF15*, and *TmCBF16* may be important (Knox *et al.*, 2008); a third found that the higher expression of *TaCBF1a*, *TaCBF1C* and *TaCBF7* was associated with freezing tolerance (Vagujfalvi *et al.*, 2005); and a fourth found that the expression of a different group of *CBFs* was upregulated in mutant wheat lines with increased freezing tolerance (Sutton *et al.*, 2009). The differences may be partly due to the different species studied (barley, diploid wheat or hexaploid wheat). An alternate interpretation is that there may not be one or a few 'master regulators' amongst the *CBF* genes but that the contribution of individual *CBFs* to freezing tolerance may vary in different genotypes depending on the alleles carried. If this were correct, it would suggest that the function of the *CBF* family as a group may be more important for cold tolerance than the role of *HvCBF16* or any other single *CBF*, and could provide opportunities to optimise the alleles of different *CBFs* from within and/or between species to maximise freezing tolerance. This scenario could be investigated by applying the method used by Knox *et al*. (2008) to barley to map freezing tolerance and *Cor14b* regulation to small subsets of *CBFs* if the appropriate genetic stock existed.

The second protein isolated here by yeast 1-hybrid analysis was shown to bind the CRT/DRE element and thereby fulfilled the functional requirements of a CBF. Naming was complicated as this protein was phylogenetically distinct from the barley CBF proteins and no clear naming convention has been developed for these transcription factors. The protein has been designated HvCBF23 until further characterisation may indicate a more appropriate name.

As with HvCBF16, many putative phosphorylation sites were predicted in HvCBF23 with a small cluster of serine residues near the N-terminus of the gene and a large cluster close to the C-terminus (Figure 4.6B). Several threonine targets were also predicted in similar locations. In contrast to *HvCBF16*, *HvCBF23* was predicted to

have very low translation efficiency. Also, HvCBF23 was predicted to undergo N-terminal methionine excision, a common and well-documented phenomenon in eukaryotes (Martinez *et al.*, 2008), which would result in the N-terminus of the mature peptide being the second amino acid in the protein sequence (proline). The impact of this putative modification on the function of the HvCBF23 protein is unknown.

Interestingly, while HvCBF23 was predicted to be localised to the mitochondria, chloroplast, and/or cytoplasm, none of the software packages suggested nuclear localisation. The significance of these predictions is not clear however they indicate that HvCBF23 may be involved in regulation of genes in the mitochondrial or chloroplast genomes, and may not regulate the usual target *COR* genes which are nuclear encoded. Alternatively, HvCBF23 may contain unrecognised nuclear targeting signals. Plastid or mitochondrial targeting sequences have been predicted in many AP2 domain-containing Arabidopsis and rice transcription factors, with at least one protein experimentally confirmed to be present in both organelles (Schwacke *et al.*, 2007). Despite this, review of the literature has not shed light on possible functions of *HvCBF23* in these organelles, with the primary link between *CBF* genes and chloroplast functions being protection of the chloroplast during stress, rather than regulation of organellar genes (Artus *et al.*, 1996; Savitch *et al.*, 2005; Shaikhali *et al.*, 2008).

HvCBF23 is a member of the small DREB subgroup A-6 (Sakuma *et al.*, 2002). As well as the AP2 DNA binding domain, HvCBF23 contains three of the four conserved motifs which are common to members of this subgroup (conserved motifs 1-3, refer to sequence alignment in Appendix E.4) (Nakano *et al.*, 2006; Sakuma *et al.*, 2002). The functions of the conserved regions are unknown. However they are highly conserved in diverse plant species which suggests they are functionally important.

The position of HvCBF23 in the phylogenetic tree (Figure 4.7) sheds little light on the role of this protein in plants as the functional information about proteins in DREB subgroup A-6 is sparse. Yet the high level of sequence similarity within this group (Appendix E.4) suggests that inter-species comparisons may be valid and informative. The limited studies suggest that members of this clade are primarily involved in abiotic

stress responses. The documented functions include roles in drought and freezing tolerance in alfalfa (Zhang *et al.*, 2007), salt and freezing tolerance in *Jatropha curcas* (a woody oil plant) (Tang *et al.*, 2007), transcription of the ABA responsive gene *rab17* in maize (Kizis and Pages, 2002), cold, drought and salinity tolerance in soybean (Chen *et al.*, 2009), and regulation of redox sensory function and stress in Arabidopsis (Shaikhali *et al.*, 2008). Preliminary results show that HvCBF23 effectively activates expression of the wheat cold stress-responsive gene *WCOR410* in transient expression assays (Sergiy Lopato, unpublished work), which indicates that *HvCBF23* may play a role in abiotic stress tolerance.

Binding assays confirmed the results of the Y1H screen, showing that HvCBF23 was able to bind the Arabidopsis CRT/DRE (Figure 4.9). In contrast, HvCBF16 was able to bind both Arabidopsis and maize CRT/DRE *cis*-elements, which establishes that there are functional differences in the binding specificities of these barley CBFs. This may result from the divergence in their AP2 domains (Figure 4.8) and may lead to differences in the sets of target genes regulated by HvCBF16 and HvCBF23.

HvCBF23 was constitutively expressed at a high level in all the tissues examined by microarray or qRT-PCR (Figure 4.15; Figure 4.16). *HvCBF23* transcript levels varied between samples during the cold treatment time course but the variation appeared to be independent of the cold treatment (Figure 4.15A & B). Although many unknown or poorly understood factors influence *CBF* expression, the variation in transcript levels observed may be the result of diurnal regulation, which has been linked to variations in *CBF* expression in wheat (Badawi *et al.*, 2007), rye (Campoli *et al.*, 2009) and Arabidopsis (Fowler *et al.*, 2005). The expression profile suggests that *HvCBF23* may not be regulated by cold stress at the transcriptional level but may be regulated post-translationally, for example, *via* phosphorylation at the predicted amino acid residues.

HvCBF23 was located near the centromere of chromosome 5H, likely on the long arm at the Steptoe x Morex bin 5/6 border (Table 4.2; Figure 4.17). This region is located just outside (proximal) of the drought tolerance QTL described by Tondelli and colleagues (2006). As a search of the literature did not reveal any QTLs of interest in

this region, the genetic location of *HvCBF23* does not provide any clues at present about the function of this gene.

The functions of HvCBF16 and HvCBF23 are not yet clear. Several characteristics of the HvCBF16 and HvCBF23 proteins were predicted using computer software, including stability, N-terminal processing and post-translational regulation by phosphorylation. These should be further investigated to determine whether predictions are correct and if so, whether these characteristics are important for a role in cold-responsive signalling. Other experiments could include independent confirmation of the binding abilities discovered here, *via* gel shift assays. Functional analysis of HvCBF16 and HvCBF23 would largely involve measurement of cold tolerance and *COR* gene expression in either plants over-expressing *HvCBF16* or *HvCBF23*, or barley genotypes with different alleles of these *CBFs*. It has been noted that the predicted high translation efficiency and protein stability of *HvCBF16* suggested that the use of a stress-inducible promoter may be advisable for over-expression of this gene, to avoid detrimental build-up of the over-expressed protein under normal conditions. This may help avoid accumulation of the protein in unstressed tissues but there may still be problems with protein persistence after the stress has been relieved.

Another key step in characterising these proteins will be a better understanding of their regulation. Factors already known to influence regulation of cereal *CBF* expression include photoperiod, temperature, diurnal changes and *Fr-1/VRN-1* and *Fr-2* alleles (Badawi *et al.*, 2007; Campoli *et al.*, 2009; Stockinger *et al.*, 2007). Future experiments controlling these variables may enable definition of the relative significance of each of these factors.

4.5. Conclusions

The aim of the work described in this chapter was to identify and characterise barley *CBF* genes with the long-term goal of using these genes to engineer plants with enhanced freezing tolerance. Two barley *CBF* genes were identified, *HvCBF16* and *HvCBF23* by yeast 1-hybrid analysis using the Arabidopsis and/or maize CRT/DRE *cis*-element as bait.

HvCBF16 is a member of the HvCBF3-subgroup while HvCBF23 is a member of the small, little-studied group of proteins in the DREB group A-6. Both proteins contain the conserved motifs specific to their respective subgroups, although the functional significance of these areas of similarity is not known. Both subgroups have been shown to be involved in the plant responses to cold and other abiotic stresses, with several cases of over-expression of these genes in various plant species resulting in increased cold, drought or salinity tolerance and/or increased expression of downstream *COR* genes. These findings support the suggestion that HvCBF16 and HvCBF23 may be involved in abiotic stress responses and may be useful candidates for generation of transgenic plants with improved stress tolerance.

Bioinformatic software predicted numerous interesting properties of these CBFs, many of which differed between *HvCBF16* and *HvCBF23*. While *HvCBF16* was predicted to be localised in the nucleus and/or chloroplast, modelling suggested HvCBF23 was localised to the mitochondria, chloroplast or cytoplasm. This implies different roles for these CBF proteins, however the significance of this is not clear at present. Clusters of putative phosphorylation sites were identified in the N- and C-terminal regions of HvCBF16 and HvCBF23 and further studies will determine whether these proteins are targets of phosphorylation.

HvCBF16 was not expressed under normal conditions, or during salinity, ABA or drought treatments, in any of the tissues studied. During a field-style cold treatment, *HvCBF16* transcript rapidly accumulated in both leaves and floral tissues. In contrast, *HvCBF23* had high constitutive expression in all tissues examined and has been shown

to activate expression of the wheat cold stress-responsive gene *WCOR410* in preliminary studies (Lopato, unpublished results). These results suggest that both *HvCBF16* and *HvCBF23* play a role in cold stress response, and that regulation of *HvCBF23* may occur post-translation.

HvCBF16 was mapped to the long arm of chromosome 5H, co-localising with important QTLs for cold tolerance including regulatory control over expression of *HvCor14b* and the frost-resistance *Fr-H2* locus. Although there are many other *CBFs* at this region and the function each is not clear at present, these results indicate that *HvCBF16* is a candidate to explain a component of these cold stress tolerance-related traits. On the other hand, *HvCBF23* was located near the centromere and was not coincident with any known QTLs for abiotic stress tolerance. Both CBF proteins were able to bind CRT/DRE elements in yeast binding assays, however the affinity of the proteins for different element variants differed, suggesting there may be differences between the sets of genes targeted by HvCBF16 and HvCBF23.

Together, these results suggest that *HvCBF16* and *HvCBF23* may play roles in abiotic stress response. The clear differences between HvCBF16 and HvCBF23 in gene expression and protein properties (both experimentally determined and predicted) suggest their roles in cold tress response may differ and further experiments are required to characterise these proteins.

A good first step towards understanding the individual and group role of barley *CBFs* in cold response would be analysis of transgenic barley plants over-expressing these genes. Although time constraints prevented analysis of *HvCBF16* and *HvCBF23* in this manner, collaboration with Professor Tony Chen and Professor Patrick Hayes (both of Oregon State University) enabled analysis of transgenic barley plants over-expressing *HvCBF2A*. Characterisation of the role of *HvCBF2A* in stress response by this method is described in the following chapter.

CHAPTER 5

Characterisation of Barley Plants Constitutively Expressing *HvCBF2A*

5.1. Introduction

The frost tolerance locus *Fr-2* is one of two major loci controlling frost tolerance in cereals and is coincident with QTLs for differential expression of *Cor14b* and *DHN5* (Francia *et al.*, 2004; Knox *et al.*, 2008; Stockinger *et al.*, 2007; Vagujfalvi *et al.*, 2000; Vagujfalvi *et al.*, 2003). The barley *Fr-H2* locus encompasses a cluster of at least 12 *CBF* genes, including *HvCBF2A* (Francia *et al.*, 2007; Francia *et al.*, 2004; Skinner *et al.*, 2006; Tondelli *et al.*, 2006). Recently, researchers have explored whether the *CBF* genes account for the effects of *Fr-2*. *HvCBF2* expression (the combined expression of the *HvCBF2* subfamily genes *HvCBF2A* and *HvCBF2B*) is induced by cold treatment. Further, freezing-tolerant cultivars contained greater low-temperature sensitivity and higher basal and cold-induced expression levels of *HvCBF2* than intolerant genotypes (Skinner *et al.*, 2005; Stockinger *et al.*, 2007). HvCBF2A is able to bind CRT *cis*-elements from *COR* gene promoters *in vitro* and transactivate expression in a low temperature-dependent manner (Skinner *et al.*, 2005). For these reasons, several studies have suggested *HvCBF2A* as a candidate for the phenotypic variation in freezing tolerance mapped to *Fr-H2*.

Transgenic plants over-expressing *CBF* genes from several species have shown enhanced freezing tolerance but this is often associated with dwarfing and developmental abnormalities due to constitutive over-expression of downstream target genes (Huang *et al.*, 2009; Ito *et al.*, 2006; Liu *et al.*, 1998; Zhao and Bughrara, 2008). However, over-expression of *AtICE1* enhanced freezing tolerance *via* activation of the *CBF* signalling pathways without causing growth abnormalities as the AtICE1 protein is inactive at normal temperatures (Chinnusamy *et al.*, 2003). As the activity of HvCBF2A is also dependent upon low temperatures, it was hypothesised that over-expression of *HvCBF2A* may increase freezing tolerance without detrimental effects. This hypothesis was based on the model of low target gene expression at normal temperatures followed by elevated expression at cold temperatures as HvCBF2A becomes active.

This section of work was performed as part of a collaboration with Professors Tony Chen and Patrick Hayes (Oregon State University), the aim of which was to investigate

whether *HvCBF2A* was responsible for a component of the freezing tolerance associated with the QTL at *Fr-2*. The aim of the work was to address the following questions: Is HvCBF2A capable of activating the expression of *COR* genes *in planta*, and does this increase freezing tolerance? Is any effect on *COR* gene expression temperature-dependent, and is over-expression of *HvCBF2A* associated with dwarfing or other abnormal developmental phenotypes? To achieve this, transgenic plants with constitutive over-expression of *HvCBF2A* were characterised by examining the expression of various *COR* genes at normal and low temperatures. The development of the transgenic plants under normal growing conditions was also examined and these results were compared with the outcomes of freezing tolerance experiments performed by Dr. Ottó Veisz and colleagues (Agricultural Research Institute of the Hungarian Academy of Sciences). Although previous studies have reported ectopic expression of cereal *CBF* genes in heterologous systems (Skinner *et al.*, 2005; Takumi *et al.*, 2008), this is the first study describing over-expression of a cereal *CBF* gene in its native species. It is anticipated that these results will provide information and an understanding of the role of HvCBF2A, and the wider group of cereal CBFs in freezing tolerance. Finally, the data may prove useful in the ultimate goal of engineering plants with increased resistance to freezing stress.

5.2. Materials and Methods

5.2.1. Materials

M-MLV Reverse Transcriptase enzyme and RQ1 RNase-Free DNase were supplied by Promega (USA). RNase Out Ribonuclease Inhibitor was supplied by Invitrogen (USA). DNeasy Plant Mini Kit was supplied by Qiagen (USA). Stratalinker UV Crosslinker was from Stratagene (USA). Additional materials were as described in Sections 2.2.1, 3.2.1 and 4.2.1.

5.2.2. Bioinformatics

Sequence analysis and prediction of functional motifs was performed using computer software as described in Sections 2.2.2.6 and 4.2.5.

5.2.3. Production of Cold Stress Treatment Series using Barley Plants Over-expressing *HvCBF2A*

Seed from T_1 and T_2 transgenic barley plants constitutively over-expressing *HvCBF2* (using the 35S promoter) was kindly provided by Professors Tony Chen and Patrick Hayes (Oregon State University). Barley plants from five transgenic lines and control, wild type Golden Promise and Dicktoo cultivars were grown in a growth room at 16°C under 8 hr light/16 hr dark.

As the vector used for preparation of the transformation construct contained a *GUS* reporter gene, GUS activity was analysed to differentiate between transgenic plants and null segregants. Small sections of leaf tissue (~5 mm x 5 mm) from two week old plants were stained for GUS activity. GUS staining solution was prepared as described in Section 2.2.3.8.5 and each leaf segment (with four parallel cuts to allow absorption of the staining solution) was immersed in 100 µl of staining solution in a 1.5 ml tube, wrapped in alfoil and incubated at 37°C overnight. The solution was removed using a vacuum trap and the leaf was washed in 1 ml of ~95% ethanol on a horizontal shaker for ~3 hrs. If necessary, the solution was replaced with fresh 95% ethanol and

incubated further. The plants were scored for GUS activity and null segregants were removed.

Once the plants were three weeks old, some were cold-treated at 4°C and samples of the entire aerial portion were taken from five cold-treated or untreated plants at 0, 8 and 96 hrs after the commencement of treatment. During treatment, the untreated plants remained in the growth room and the lighting in the cold and growth rooms were synchronised. The five plants sampled at each time point/treatment/line were pooled. Tissues were ground under liquid nitrogen using a mortar and pestle.

5.2.4. Production of cDNA for qRT-PCR

RNA was extracted as described in Section 2.2.3.2.2, with resuspension in 200 µl of sterile water. RNA was quantified by spectroscopy using a NanoDrop™ ND-1000. If necessary, RNA was concentrated prior to DNase treatment by precipitation and resuspension as described in Section 2.2.4.6 with the following modifications: The volume of RNA was 75 µl, the chilling incubation step was performed at -80°C for at least 30 min and the RNA pellets were dried at room temperature and resuspended in 20 µl of sterile water.

DNAse treatment of the RNAs was performed using RQ1 RNase-Free DNase according to the manufacturer's directions. Briefly, reactions contained 8 µg of RNA, 3 µl of RQ1 RNase-Free DNase 10x Reaction Buffer (supplied), 9 µl of RQ1 DNase Enzyme (supplied) and sterile water to a total volume of 30 µl. After incubation at 37°C for 30 min, the reactions were terminated by adding 3 µl of RQ1 DNase Stop Solution (supplied) and incubating for 10 min at 65°C.

cDNA was produced using OligodT_{20} primer and M-MLV Reverse Transcriptase according to the manufacturer's instructions. Briefly, reactions were performed in 0.5 ml tubes containing 7.3 µl of the DNase-treated RNA, 0.5 µg of OligodT_{20} and water to 10 µl. The RNA was heated at 70°C for 5 min and placed on ice. To each reaction was

added 5 µl of 5x M-MLV Buffer (supplied), 5 µl of dNTPs (10 µM each), 1 µl of RNase Out Ribonuclease Inhibitor, 1 µl of M-MLV Reverse Transcriptase enzyme (supplied) and water to 25 µl. The reactions were incubated at 70°C for 5 min, followed by 42°C for 1 hr.

PCR was performed using Platinum *Taq* DNA polymerase enzyme as described in Section 2.2.2.11 with the following modifications. The primers used were HvCyclophilin_F (forward) and HvCyclophilin_R (reverse) and 1 µl of a 10-fold dilution of cDNA was used as a template. Reactions containing cDNA derived from wild type Golden Promise plants or no template were used as negative controls. Positive control reactions were prepared using 1 µl of purified *cyclophilin* cDNA as a template, kindly provided by Dr. Neil Shirley.

5.2.5. Northern Blot Analysis of Barley Plants Over-expressing *HvCBF2A*

Northern blot membranes for analysis of the cold-stress treatment expression series were prepared using a different method from that described in Chapter 2. Membranes were produced in triplicate. 50 µg of RNA from each sample (Section 5.2.4) was concentrated by precipitation as described in Section 2.2.4.6 and resuspended to 6 µg μl^{-1}. For each lane, 15 µg (2.5 µl) of RNA was mixed with 7.5 µl of loading mix (described in Section 2.2.3.2.2). Gels were prepared by dissolving 1.92 g of agarose in 16 ml of 10x MOPS buffer and 136 ml of water and cooling to 55°C before adding 4.35 ml of formaldehyde (37%). Gels were poured immediately, allowed to set for one hour and pre-run for 30 min at 50 V in a clean electrophoresis tank containing 1x MOPS buffer. Prior to loading, the RNA was heated at 65°C for 25 min, cooled to room temperature and 1 µl of 1 mg ml^{-1} ethidium bromide solution was added. The samples were loaded and empty lanes were filled with 7 µl of loading buffer. The gels were electrophoresed at 70 V for 1 hr and 30 min and the RNA was visualised under UV light using a transilluminator.

The gels were rinsed twice in sterile water for 10 min each time and soaked in 250 ml of a solution of 50 mM NaOH and 10 mM NaCl for 40 min. They were then washed in

0.1M Tris buffer (pH 7.5) for 40 min and rinsed twice with water. The gels were soaked twice in 10x SSC at room temperature for 20 min each with gentle agitation. Nylon membranes were soaked briefly in RO water, followed by 10x SSC for 5 min. Four sheets of Whatman chromatography paper were prewetted in 10x SSC. The RNA was transferred to the nylon membrane in a downward transfer. From the bench upward was stacked: an 8 cm stack of paper towels, four sheets of dry Whatman chromatography paper, two sheets of the wet Whatman chromatography paper, the nylon membrane, the treated agarose gel, a parafilm dam separating the upper and lower components of the blot, two sheets of the wet Whatman chromatography paper, a Whatman chromatography paper wick, a sheet of cling wrap to prevent the wick drying out and a glass plate as a weight. The ends of the wick lay in reservoirs containing 10x SSC. Care was taken to ensure that once placed on the stack the gel did not move and that no air bubbles remained between any of the layers above the nylon membrane. The RNA was allowed to transfer overnight. The gel and membrane were visualised under UV light using a transilluminator to confirm complete transfer of the RNA to the membrane. The RNA was crosslinked to the membrane using a Stratalinker UV Crosslinker. The membrane was sealed in cling wrap and stored at -20°C.

Prehybridisation, hybridisation, subsequent membrane washes, exposure of the films and membrane stripping of the northern blot membranes were conducted as described in Section 2.2.4.6. The DNA fragments used as probes were obtained by restriction digestion of plasmid DNA of the coding region of *HvDHN5*, *HvDHN8*, *Hvcor14b*, *HvVN-1/HvBM5a* or *HvCBF2* in the vector pBluescriptK- (original clones kindly provided by Dr. Eric Stockinger (The Ohio State University, USA) and Dr. Jeffrey Skinner (Oregon State University, USA). Prior to digestion, the clones were sequenced to confirm their identity using T3 and M13F primers (Appendix A). Restriction digests were performed as described in Section 2.2.2.5 with the following modifications: 50 µl reactions were prepared containing 20 µl of plasmid of DNA, 5 µl of the appropriate buffer and 2 µl of the appropriate restriction enzyme. The restriction enzymes and buffers used for digestion of the clones were as follows: *Ava*I and Buffer B for *HvDHN5*, *Xho*I and *Eco*RI and Buffer H for *HvDHN8*, *Ava*I and Buffer B for *Hvcor14b*, *Xho*I and Buffer H for *HvVRN-1* and *Nhe*I and Buffer M for *HvCBF2*. Digestion products were electrophoresed and purified as described in Section 2.2.2.2.

Purified cDNA of the barley *cyclophilin* gene to use as a control gene probe was kindly provided by Dr. Neil Shirley (University of Adelaide). The probes were prepared, purified, DNA-sodium salt solution added and the probes denatured as described in Section 2.2.4.4.

Northern blots were also produced to analyse the *HvCBF2* transgene transcript levels in the wild type and barley plants over-expressing *HvCBF2A* used for analysis developmental phenotypes. RNA was extracted from the leaves of mature plants and a northern blot membrane was produced and analysed as described in Sections 2.2.3.2.2 and 2.2.4.6 using the *HvCBF2* probe described above.

5.2.6. Southern Blot Analysis of Barley Plants Over-expressing *HvCBF2A*

Powdered frozen tissue from five plants was pooled (untreated samples, 96 hrs time point) from each of the five transgenic lines and Golden Promise (Section 5.2.3). Two DNA extractions were performed per sample using the DNeasy Plant Mini Kit according to the manufacturer's instructions. Duplicate samples were pooled and the quality and concentration of the genomic DNA was analysed by electrophoresis of 3 µl of the samples as described in Section 2.2.2.2 using a standard of known concentration for visual comparison.

Aliquots of genomic DNA were digested with *Pst*I. Reactions contained 35 µl of Buffer H, 50 U (5 µl) of *Pst*I restriction enzyme and ~8.3 ng of genomic DNA in total volumes of 350 µl. Reactions were incubated at 37°C for 20 hrs with an additional 2 µl of restriction enzyme added after 12 hrs. The digested DNA was precipitated as described for precipitation of RNA in Section 5.2.4 and samples were resuspended in 15 µl of nuclease-free water. Loading Buffer (3 µl) was added and the samples loaded into a 0.8% w/v agarose gel containing 6 µl of 10 mg ml^{-1} ethidium bromide. 5 µl of loading buffer was loaded into empty wells to prevent curving of the DNA bands in the outer lanes. The samples were electrophoresed at 65V for 1 hr and 45 min. The gel was depurinated for 10 min in 0.2 M HCl and washed three times in RO water.

The gel was soaked with gentle agitation: twice in 250 ml of Southern Solution A (1.5 M NaCl, 0.5 M NaOH) for 30 min, and then twice in Southern Solution B (1M NH_4OAc, 20 mM NaOH). The nylon membrane was soaked briefly in RO water, followed by Southern Solution B for 5 min. The DNA was transferred to the nylon membrane in a downward transfer as described for a northern transfer above except Southern Solution B was used instead of 10x SSC. The gel and membrane were visualised under UV light to ensure transfer occurred and DNA was cross-linked to the membrane as described above for a northern transfer. The membrane was sealed in cling wrap and stored at -20°C.

Probes were synthesised and prehybridisation, hybridisation with probes, washing and exposure to film of the Southern membrane were performed as described in Section 2.2.4.4. Fragments of the *GUS* reporter gene and *HvCBF2* were used as probes. The *GUS* probe fragment was obtained by restriction digestion of plasmid DNA of a construct containing the pMDC164 vector (Curtis and Grossniklaus, 2003). The construct was digested in a total reaction volume of 50 µl containing 2 µl of *Nru*I restriction enzyme, 5 µl of Buffer B and 20 µl of plasmid DNA. The reaction was incubated at 37°C for 5 hrs and 65°C for 15 min to heat inactivate the enzyme. The reaction products were electrophoresed in an agarose gel, excised, purified and quantified as described in Section 2.2.2.2. Preparation of the *HvCBF2* probe is described in Section 5.2.5.

5.2.7. Analysis of Putative Downstream Gene Expression by qRT-PCR

qRT-PCR was performed using the cDNA populations from the barley cold-stress series described in Section 5.2.4. qRT-PCR analysis of the transcript levels of *HvCBF2*, *HvCor14b*, *HvDHN8*, *HvDHN5* and *HvVRN-1* was performed as described in Section 2.2.3.6. The primers and PCR product information for *HvCBF2*, *HvDHN8*, *HvDHN5*, *HvCor14b* are presented in Table 2.3. The *HvCBF2* primers were specific to the endogenous and transgene *HvCBF2* transcripts. In addition, expression of *HvVRN-1* was analysed using HvVRN1_F (forward) and HvVRN1_R (reverse) primers.

5.2.8. Freezing Treatment of Barley Plants Over-expressing *HvCBF2A*

The freezing stress tolerance assay was performed by Dr. Ottó Veisz and colleagues (Agricultural Research Institute of the Hungarian Academy of Sciences). Plants were grown for three weeks in a Conviron PGR-15 climatic chamber at 18°C with a 16 hr day/8 hr night cycle. Illumination was at 300 $\mu molm^{-2}s^{-1}$ PPFD. Freezing treatment was performed in a Conviron C-812 chamber with minimum freezing temperatures reached of –3°C and – 6°C. The freezing treatment was as follows: the temperature dropped by 1°C per hr to -1°C, was held at -1°C for 24 hrs, then dropped by 1°C per hr until the minimum temperature was reached (either -3°C or -6°C). This temperature was held for 24 hrs before the temperature was raised by 1°C per hr until it reached normal temperature (18°C). Following freezing treatment, plants were returned to the growth chamber at the original conditions for three weeks. Four replications of the experiment were performed with 20 plants per line per replicate. Survival was calculated as the number of plants still alive three weeks after the freezing treatment as a proportion of the number of plants treated.

5.2.9. Plant Growth Conditions

T_3 and T_4 plants and control Golden Promise barley plants were grown for analysis of developmental phenotypes in a glasshouse in six-inch diameter pots with one plant per pot.

5.3. Results

5.3.1. Sequence Analysis of *HvCBF2A*

HvCBF2A was predicted to be localised to the nucleus, with poor prediction scores for the chloroplast and mitochondria. The prediction models also suggested that *HvCBF2A* has a high translational efficiency and the protein stability score was the maximum value possible for the program. Putative phosphorylation sites were identified at three serine and three tyrosine resides (Figure 5.1). Comparison of the predicted phosphorylation sites in HvCBF2A to those in HvCBF4A and HvCBF9 identified three common sites which were conserved in all members of the HvCBF4-subgroup but not in other barley CBFs. In HvCBF2A, these residues and their respective phosphorylation prediction scores were S_{100} (0.99), S_{182} (0.84) and T_{214} (0.60). No sumoylation site, N-terminal cleavage site or nuclear export signal were identified.

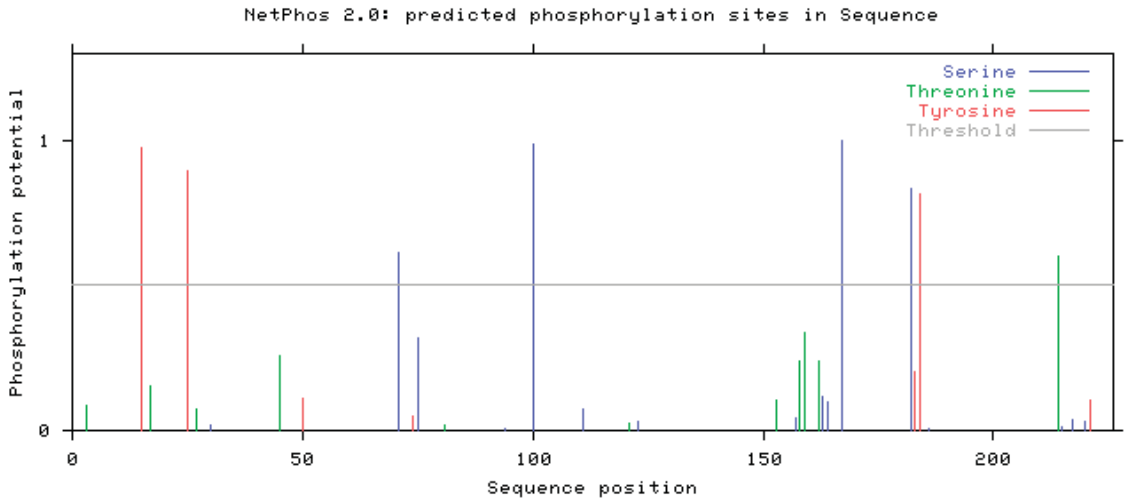


Figure 5.1 Graph of the location and scores of predicted phosphorylation sites in HvCBF2A.

Putative sites were predicted using NetPhos software (www.cbs.dtu.dk/services/NetPhos/).

5.3.2. Analysis of Transgene Copy Number and Segregation in Barley Plants Over-expressing *HvCBF2A*

Barley plants constitutively over-expressing *HvCBF2A* were analysed by Southern blot to determine the presence and number of copies of the transgene in each line. For each line, samples from five transgenic plants were pooled to use as a representation of the number of possible different insertion events present in the population. Using probes which hybridised to a section of the *GUS* reporter gene present in the T-DNA, T_2 plants were analysed from Lines 2-6, 3-6, 6-3 and 10-8 and T_3 plants were analysed from Line 13-3-3. In addition, segments of leaf from plants of these lines and two consecutive generations was analysed for GUS activity to confirm the presence of the T-DNA and whether it was segregating in the population. Together, these results enabled estimation of transgene copy number in the lines.

Bands were visible on the autoradiograph from the Southern blot for all the transgenic lines, enabling an estimation of the number of copies of the *HvCBF2A* T-DNA to be made (Figure 5.2). Single bands were present in Lines 2-6 and 13-3-3, indicating single insertion events. Lines 2-6 and 13-3-3 and corresponding sub-lines were not segregating for GUS activity and therefore may be homozygous. Southern blot analysis also showed that samples from Lines 3-6 and 10-8 contained one band and a second, less intense band, indicating either one or two insertion events. Line 10-8 and sub-lines were not segregating for GUS activity, indicating this line may be homozygous. Conversely, T_2 plants of line 3-6 were segregating for GUS activity. T_4 plants from the Sub-line 3-6-312-1 (n=14) were not segregating, indicating the T_4 line was likely to be homozygous.

Southern blot analysis of Line 6-3 produced three clear bands on the autoradiograph, indicating three insertion events. Plants from Line 6-3 (and all sub-lines) were not homozygous, as the populations were shown to be segregating by the presence or absence of GUS activity. Line 15 was not analysed by Southern blot but no homozygous lines were identified.

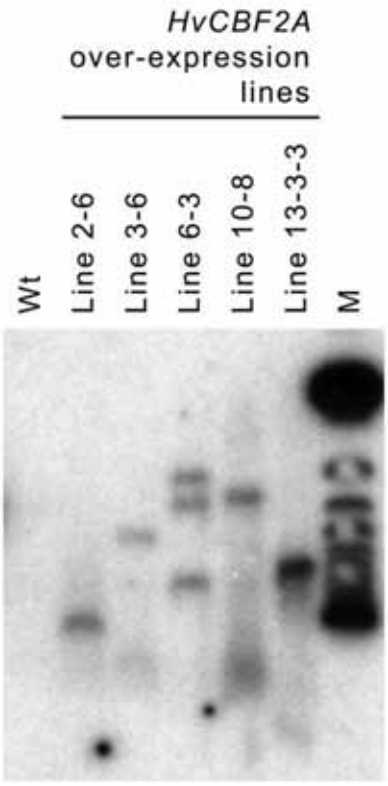


Figure 5.2 Image of autoradiograph of Southern blot of T_1 and T_2 barley plants over-expressing *HvCBF2A*.

Lines 2-6 and 13-3-3 may contain one copy, Line 6-3 may contain three copies, and Lines 3-6 and 10-8 may contain one or two copies of the *HvCBF2A* T-DNA. For each line, genomic DNA samples from five plants were pooled and digested with *Pst*I. The membrane was hybridised with a probe which was complementary to a section of the *GUS* gene. M, molecular weight marker; Wt, wild type (Golden Promise).

5.3.3. Analysis of Developmental Phenotypes of Barley Plants Over-expressing *HvCBF2A*

Analysis of developmental phenotypes was performed as part of the basic characterisation of the barley lines over-expressing *HvCBF2A*. Plants were grown to maturity and examined carefully over the life of the plants. Plants over-expressing *HvCBF2A* showed varying degrees of stunted growth and increased time to maturity with the average final plant height reached for each line significantly shorter than untransformed Golden Promise plants ($P<0.0001$) (Figure 5.3B; Figure 5.4A & B).

Figure 5.3B depicts selected transgenic plants and relative transcript levels of *HvCBF2*, illustrating the relationship between transgene mRNA level and the severity of the abnormal developmental phenotypes. The severity of the stunted growth, increased time to maturity and average total biomass phenotypes were directly proportional to transgene transcript level (Figure 5.3A & B; Figure 5.4A & B). The average total biomass of the mature plants was significantly less for the transgenic plants than the wild type plants ($P<0.0001$) (Figure 5.4B). In general, plants with the highest *HvCBF2* transcript levels were smaller and more petite, with thinner leaves, delayed flowering and maturation and ultimately produced fewer tillers.

Unusual phenotypes were also observed in the heads of transgenic plants. Grain filling was affected, the grains of the transgenic plants being less plump, resulting in significantly lower average 1000 grain weight compared with wild type plants ($P<0.0001$) (Figure 5.4B; Figure 5.5). For each transgenic line, the average total biomass, grain weight and final plant height were proportional, and lower values for each were present in the Lines 3-6-312-1, 6-3-1-1 and 15-5-8-1 which had the highest transcript levels of *HvCBF2* (Figure 5.3A; Figure 5.4B). Although there was significant variation in head sizes, shorter heads with fewer grains were more common in lines with high transcript levels of *HvCBF2*, compared to wild type plants (Figure 5.5). Dysmorphic heads were present in wild type plants, but were more common and more extreme in transgenic plants, especially but not solely in those with high transcript levels of *HvCBF2* (Figure 5.5). Infertility was also more common in the transgenic than control lines (Figure 5.5).

Northern blot membranes were produced to analyse the *HvCBF2A* transgene transcript levels in the plants used for analysis of developmental phenotypes. As a probe could not be designed specifically to the transgene, the blots were hybridised with a probe which was complementary to a section of the coding region of *HvCBF2A*, which would therefore hybridise to endogenous and transgene *HvCBF2A* transcripts. In addition, it was anticipated that the *HvCBF2A* probe would cross-hybridise to transcripts from the paralog *HvCBF2B*, thus detecting *HvCBF2* transcript levels (the combination of *HvCBF2A* and *HvCBF2B*). Bands corresponding to *HvCBF2* transcript were present in

all the samples from transgenic plants but were not detected in those from wild type plants (Figure 5.3A). The strength of the bands in the northern blot varied within and between transgenic lines with the least variation occurring between plants within lines 3-6-312-1 and 13-3-3-5-1 (Figure 5.3A).

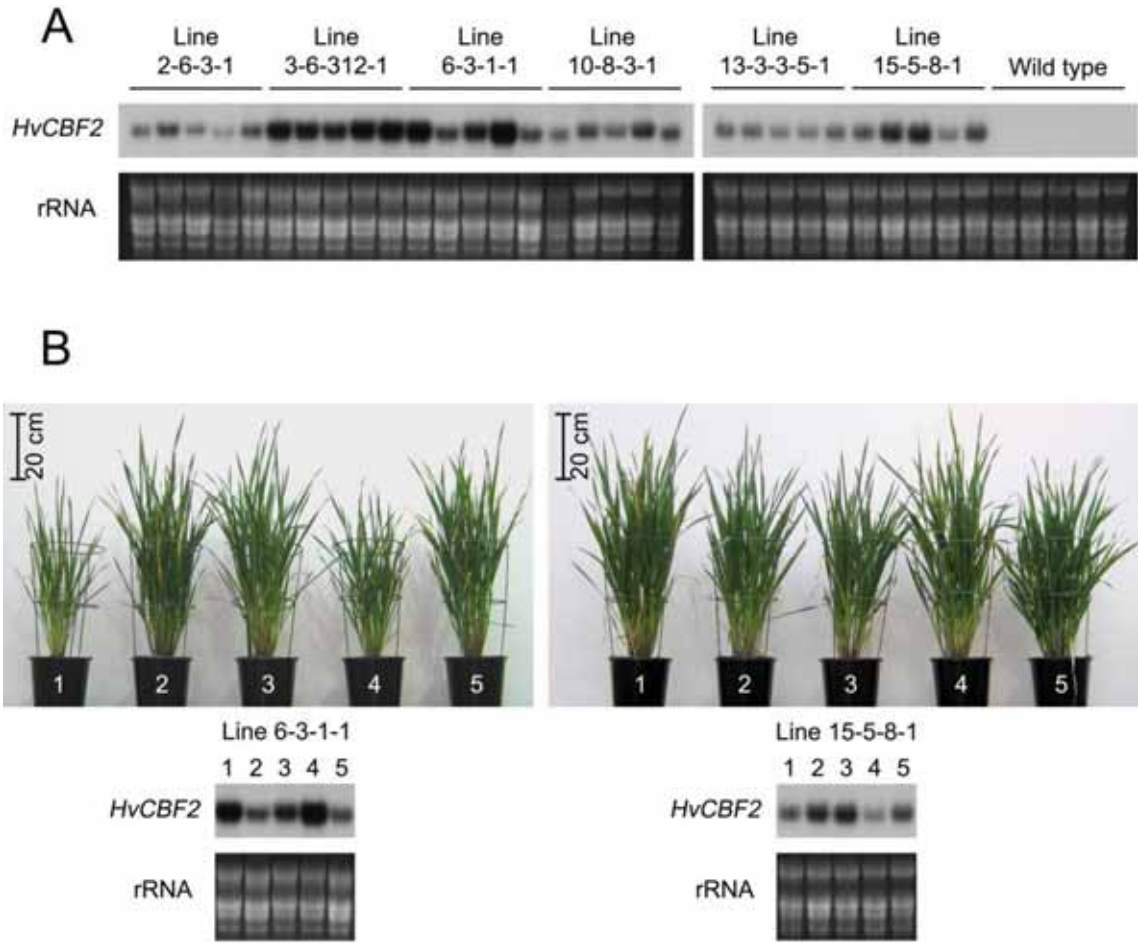


Figure 5.3 Photographs and images of autoradiographs of northern blot of *HvCBF2* expression in untreated wild type and transgenic barley plants over-expressing *HvCBF2A*.

(**A**) Northern blot analysis of *HvCBF2* endogenous and transgene expression. rRNA presented as a loading control. (**B**) Photographs of transgenic barley plants and corresponding northern blot analysis of *HvCBF2* transcript levels. Expression of *HvCBF2* was moderate to high in transgenic plants over-expressing *HvCBF2A*. Transgene expression varied between plants and higher transcript levels were associated with a greater degree of abnormal developmental phenotypes, including stunted growth and delayed flowering.

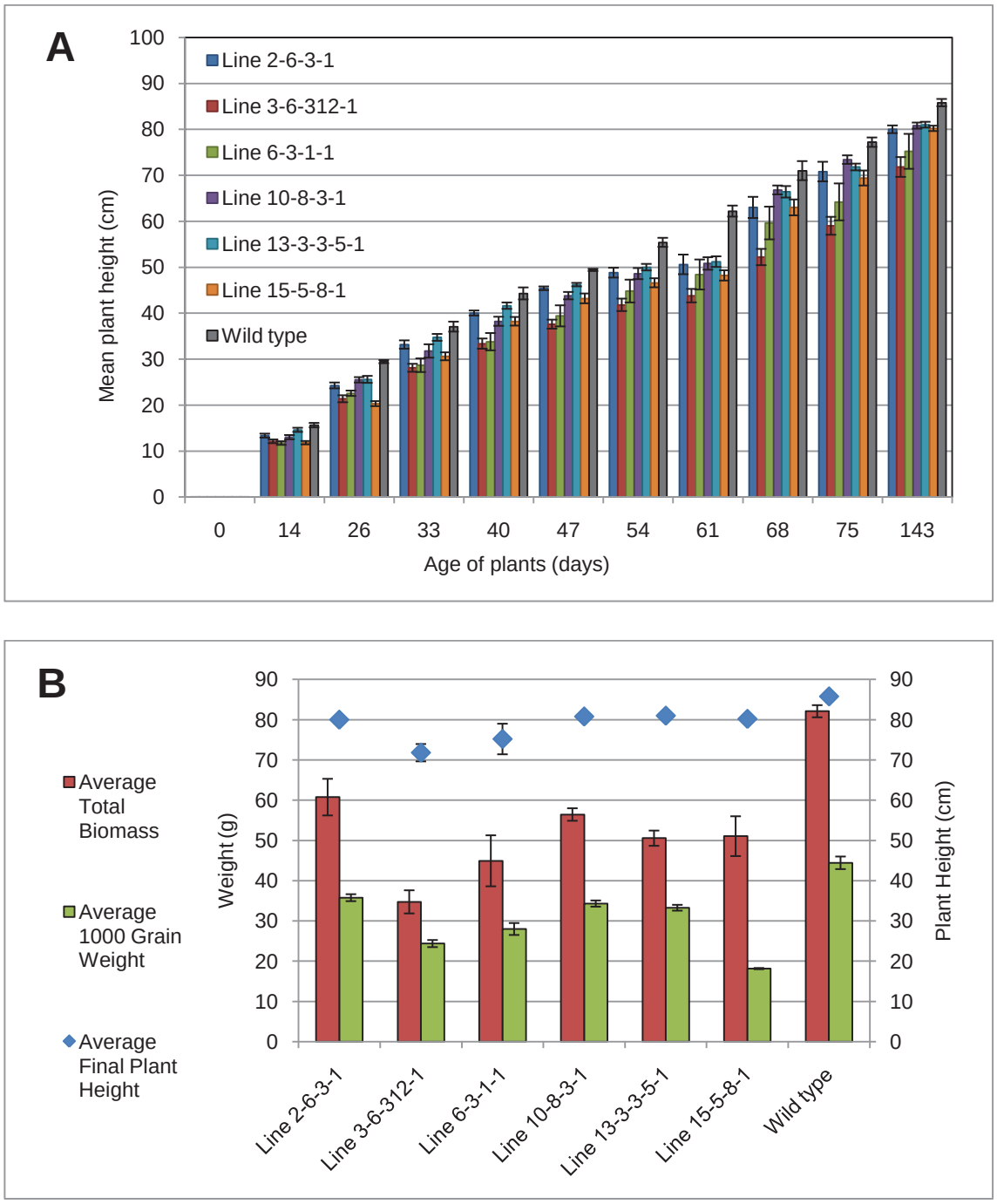


Figure 5.4 Graphic representation of the height, biomass and 1000 grain weight of barley plants over-expressing *HvCBF2A*.

(**A**) Plant height over the life of the plants. (**B**) Average final plant height, 1000 grain weight and total plant biomass. One-way ANOVA tests were performed for each trait. The average values of plant height, total biomass and 1000 grain weight were significantly lower ($P<0.0001$ for each trait) for the transgenic lines over-expressing *HvCBF2A* than wild type plants. The weights of dried mature plants (aerial tissues only) were used to calculate total plant biomass.

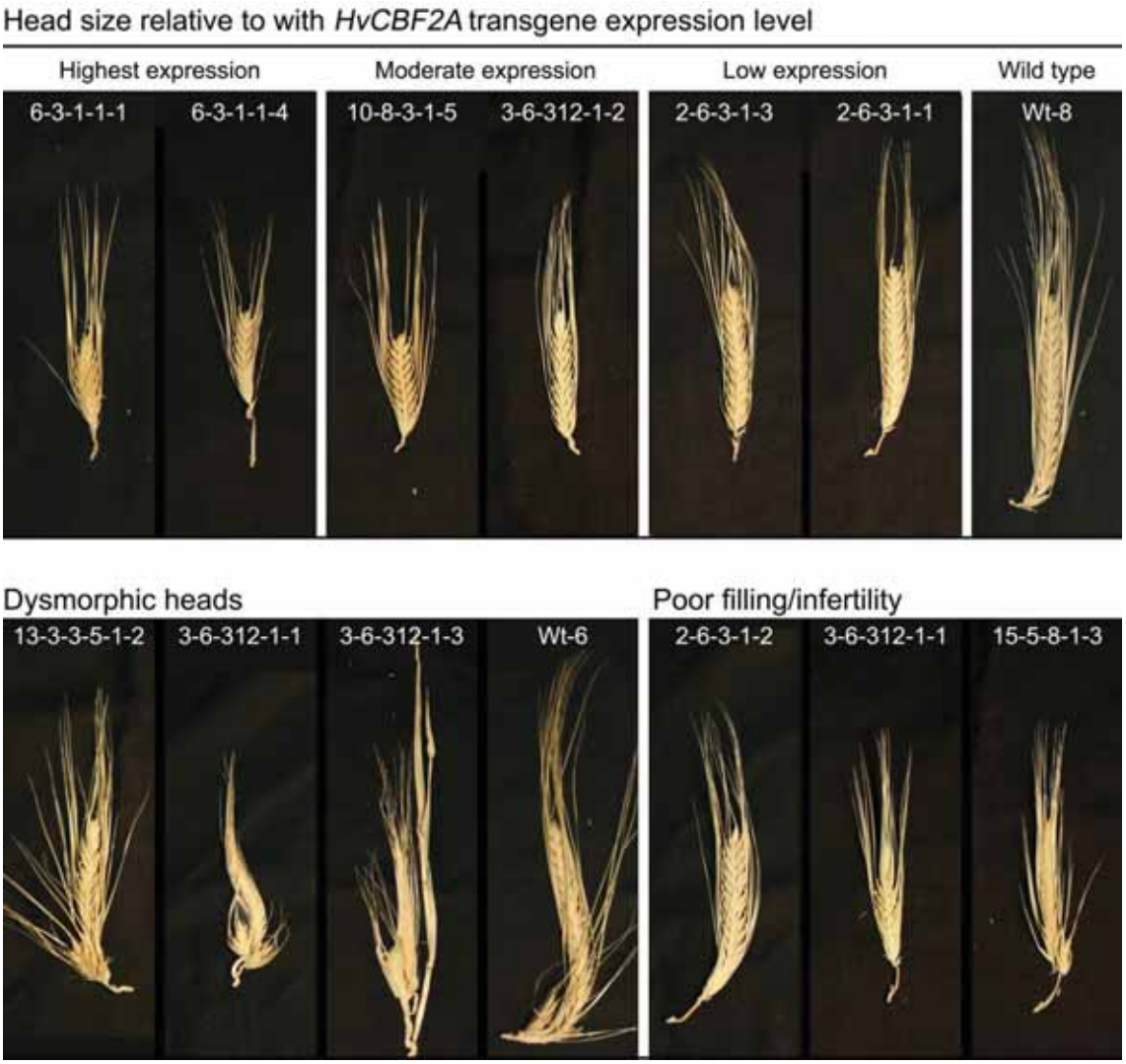


Figure 5.5 Photographs of developmental phenotypes of heads of wild type and transgenic barley plants over-expressing *HvCBF2A*.

Abnormal phenotypes were observed in the heads of barley plants over-expressing *HvCBF2A*, including poor filling and an increased incidence of short heads, infertility and/or dysmorphic morphologies. The incidence of smaller heads was approximately correlated with transgene mRNA levels however there was variation in transgenic plants and these phenotypes were not observed in all cases. Representative heads (with line numbers) are shown to illustrate observed trends. Wt, wild type.

5.3.4. Analysis of Seedling-stage Cold Stress Tolerance of Barley Plants Over-expressing *HvCBF2A*

A preliminary freezing assay was performed on barley plants over-expressing *HvCBF2A* by Dr. Ottó Veisz and colleagues (Agricultural Research Institute of the Hungarian Academy of Sciences). All wild type and transgenic plants died in all treatment trials with a minimum temperature of -6°C. Assays with a minimum temperature of -3°C indicated that the survival rates following freezing treatment were moderately greater for six out of seven of the transgenic lines over-expressing *HvCBF2A* when compared to wild type plants from the freezing-sensitive Golden Promise cultivar (Table 5.1). The seventh transgenic line had freezing tolerance similar to that of wild type Golden Promise plants. However, none of the transgenic lines had equivalent freezing survival to that of the freezing-tolerant cultivar Dicktoo. Presently, the transgene expression levels in the specific lines used for the freezing tolerance assay are not known, although transgene expression in some parent and daughter lines indicates there is variation in expression in the population (Figure 5.3A; Figure 5.6).

Table 5.1 Freezing treatment survival of wild type and transgenic barley plants over-expressing *HvCBF2A*.

NOTE:
This table is included on page 204 of the print copy of the thesis held in the University of Adelaide Library.

This data was kindly provided by Dr. Ottó Veisz (Agricultural Research Institute of the Hungarian Academy of Sciences). Three weeks after freezing treatment (-3°C), the majority of the transgenic lines tested had a greater rate of survival than the freezing-sensitive wild type Golden Promise cultivar. The rate of survival of the freezing-tolerant Dicktoo cultivar was greater than any of the transgenic lines.

5.3.5. Analysis of Cold Stress Response in Barley Plants Over-expressing *HvCBF2A*

To determine whether the expression of putative target genes was altered in transgenic barley plants over-expressing *HvCBF2A*, the mRNA levels of *HvCBF2* and several putative downstream genes were determined by northern blot and confirmed by qRT-PCR using cDNA from the same RNA populations. The transcript levels of *HvCBF2* (both endogenous and transgene transcripts), *HvCor14b*, *HvDHN5*, *HvDHN8* and *HvVRN-1* were compared in five transgenic lines, as well as wild type Golden Promise (freezing-sensitive) and Dicktoo (freezing-tolerant) plants, prior to and during cold treatment.

The results obtained from northern blot and qRT-PCR analysis were consistent (Figure 5.6; Figure 5.7; Figure 5.8A & B; Figure 5.9A & B). The mRNA levels of *HvCBF2* (both transgene and endogenous transcripts) varied between and within a transgenic line but were high in the transgenic plants compared to wild type Dicktoo and Golden Promise plants (Figure 5.6; Figure 5.7).

In untreated plants, transcript levels of *HvCor14b* were higher in the transgenic plants compared to Dicktoo and Golden Promise wild type plants (Figure 5.6; Figure 5.8A). During cold treatment, the expression of *HvCor14b* was further upregulated in the transgenic plants, reaching maximum transcript levels of ~40 x 10^6 copies μl^{-1} of cDNA after 96 hrs of treatment. Expression of *HvCor14b* was also upregulated in wild type Dicktoo and Golden Promise plants during cold treatment with the degree of upregulation being significantly greater in Dicktoo. The maximum transcript level reached in the transgenic plants was approximately two-fold greater than that of the freezing-sensitive Golden Promise plants but was slightly lower than the maximum *HvCor14b* transcript level reached in the freezing-tolerant Dicktoo cultivar.

In untreated plants, transcript levels of *HvDHN5* were high in transgenic barley plants over-expressing *HvCBF2A* but were very low in wild type Golden Promise and Dicktoo plants (Figure 5.6; Figure 5.8B). In both the transgenic and wild type plants, expression

of *HvDHN5* was greatly upregulated by cold treatment and the maximum transcript levels reached in Dicktoo plants were approximately two-fold greater than those reached in Golden Promise plants. Throughout the cold treatment, *HvDHN5* transcript levels remained significantly higher in the transgenic plants than in either Golden Promise or Dicktoo plants, reaching maximum transcript levels of ~25 x 10^6 copies μl^{-1} of cDNA, approximately 50% greater than Dicktoo and 200% greater than Golden Promise.

In untreated plants, transcript levels of *HvDHN8* were similar or higher in barley plants over-expressing *HvCBF2A* compared with wild type Dicktoo or Golden Promise plants (Figure 5.6; Figure 5.9A). Transcript levels in wild type and transgenic plants were higher after cold treatment, reaching similar levels in all genotypes after 8 hrs of treatment. However, after 96 hrs of cold treatment, transcript levels in Dicktoo and transgenic plants had continued to increase to approximately two-fold higher than Golden Promise where transcript levels remained constant. The maximum *HvDHN8* transcript level reached in the transgenic plants was ~1.2 x 10^6 copies μl^{-1} of cDNA and was slightly lower than the maximum level in Dicktoo.

In untreated plants, transcript levels of *HvVRN-1* were approximately two-fold higher in the transgenic plants (Figure 5.6; Figure 5.9B). After 96 hrs of cold treatment, transcript levels of *HvVRN-1* were upregulated in both Golden Promise and transgenic plants, resulting in a maximum transcript level in the transgenic plants of ~40,000 copies μl^{-1} of cDNA, which was approximately two-fold higher than the Golden Promise transcript levels. Throughout the experiment, transcript levels of *HvVRN-1* were extremely low in Dicktoo plants.

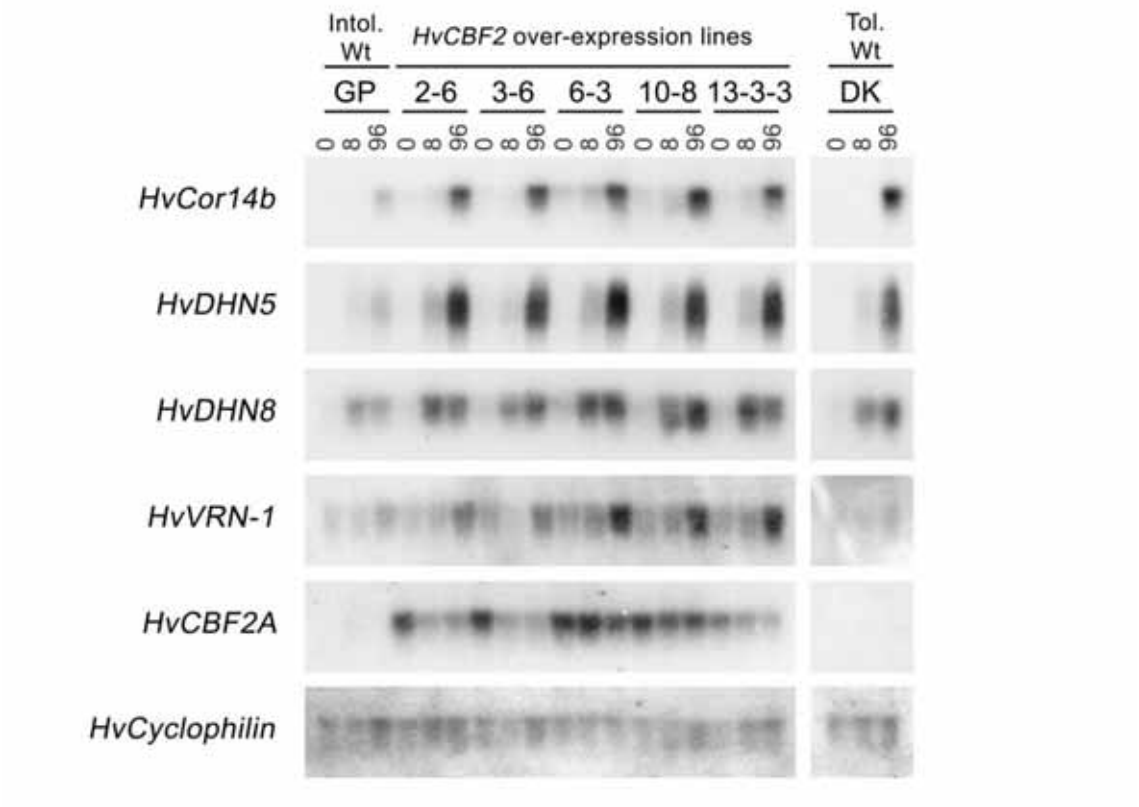


Figure 5.6 Image of autoradiograph of northern blot of *HvCBF2*, three *COR* genes and *HvVRN-1* expression during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A*.

Following cold treatment, expression of *HvCor14b*, *HvDHN5*, *HvDHN8* and *HvVRN-1* was upregulated in the transgenic plants relative to wild type Golden Promise plants. Plants were cold-treated and leaf samples were taken at 0, 8 and 96 hrs after the commencement of cold treatment (4°C). Five replicates were pooled to produce the RNA sample. The results of hybridisation with probes are labelled above, with *HvCyclophilin* shown as a loading control. GP: Golden Promise; DK: Dicktoo.

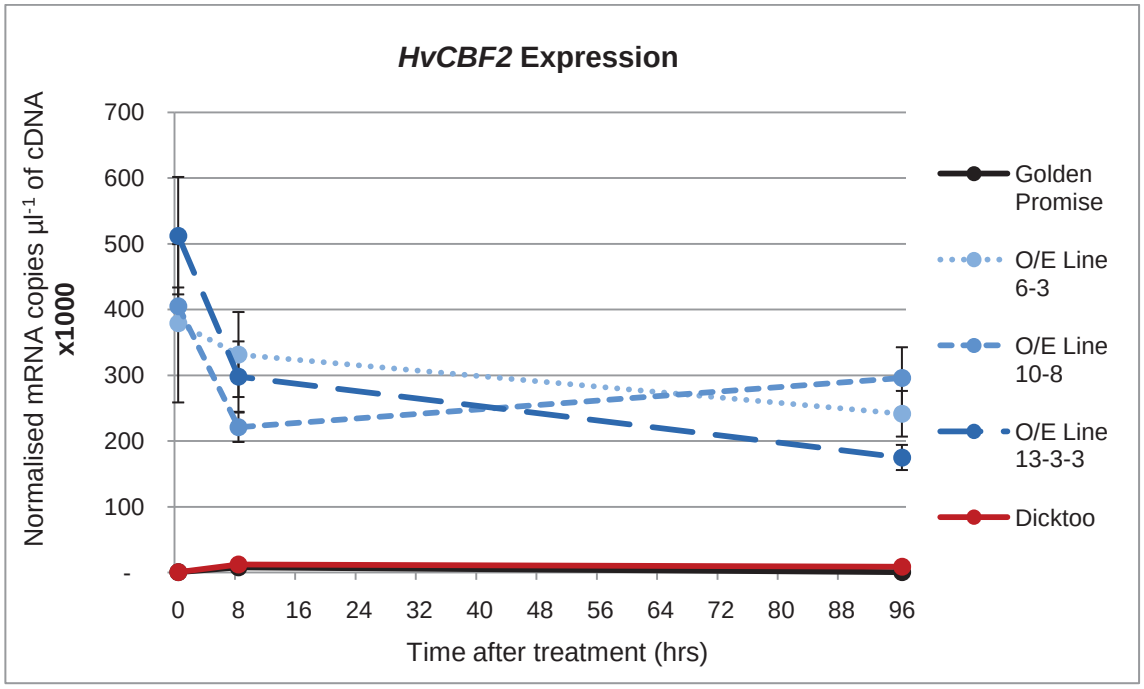


Figure 5.7 Graph of endogenous and transgene expression of *HvCBF2* during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR.

Expression of *HvCBF2* was high in the transgenic plants and extremely low in wild type Dicktoo and Golden Promise plants. O/E, Line over-expressing *HvCBF2A*. Error bars represent standard error.

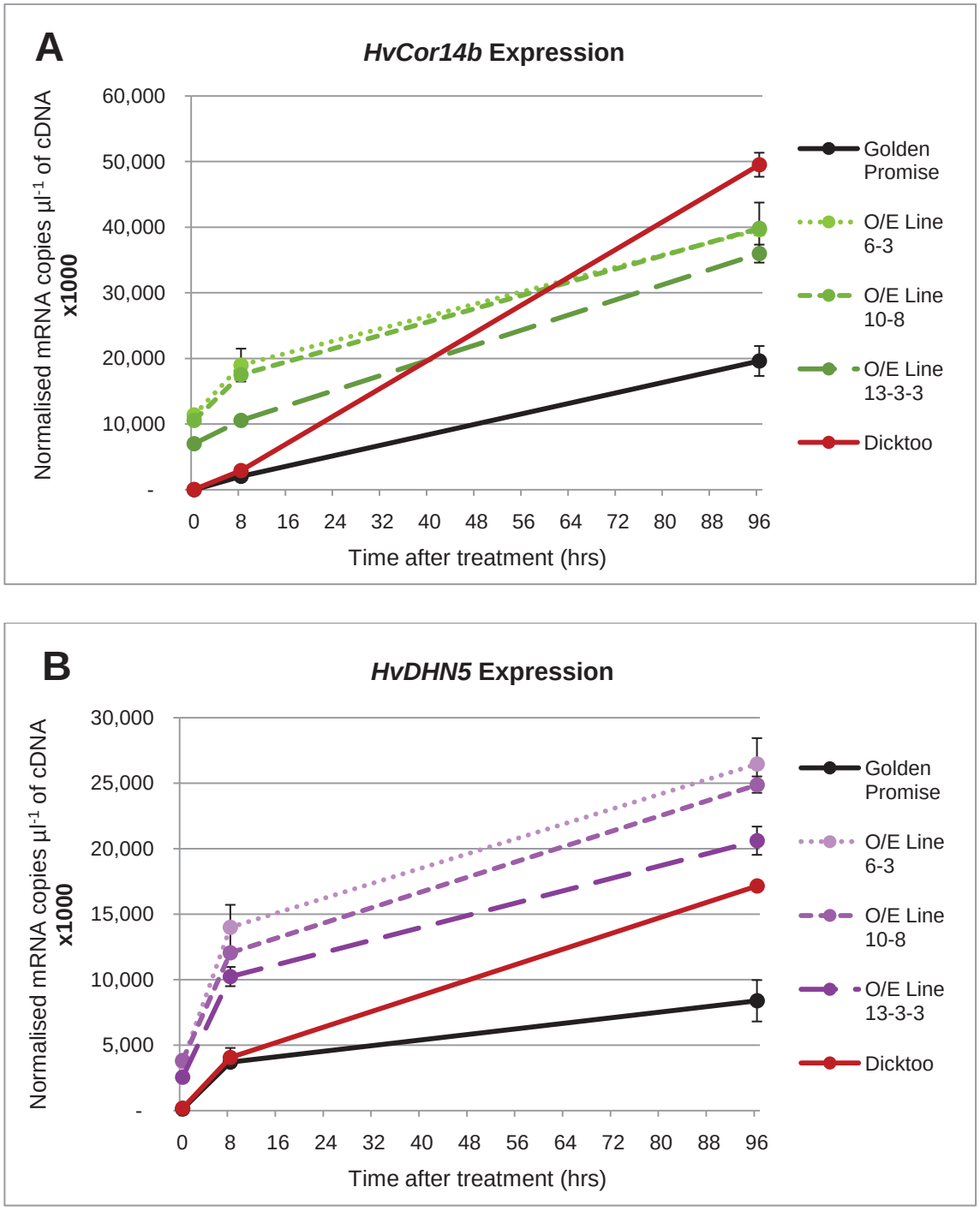


Figure 5.8 Graphs of *HvCor14b* or *HvDHN5* expression during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR.

(**A**) Expression of *HvCor14b*. (**B**) Expression of *HvDHN5*. Transgenic plants had high constitutive over-expression of *HvCor14b* and *HvDHN5*. Gene expression was further upregulated during cold treatment (4°C) and was greater than that observed in wild type Golden Promise plants. O/E, Line over-expressing *HvCBF2A*. Error bars represent standard error.

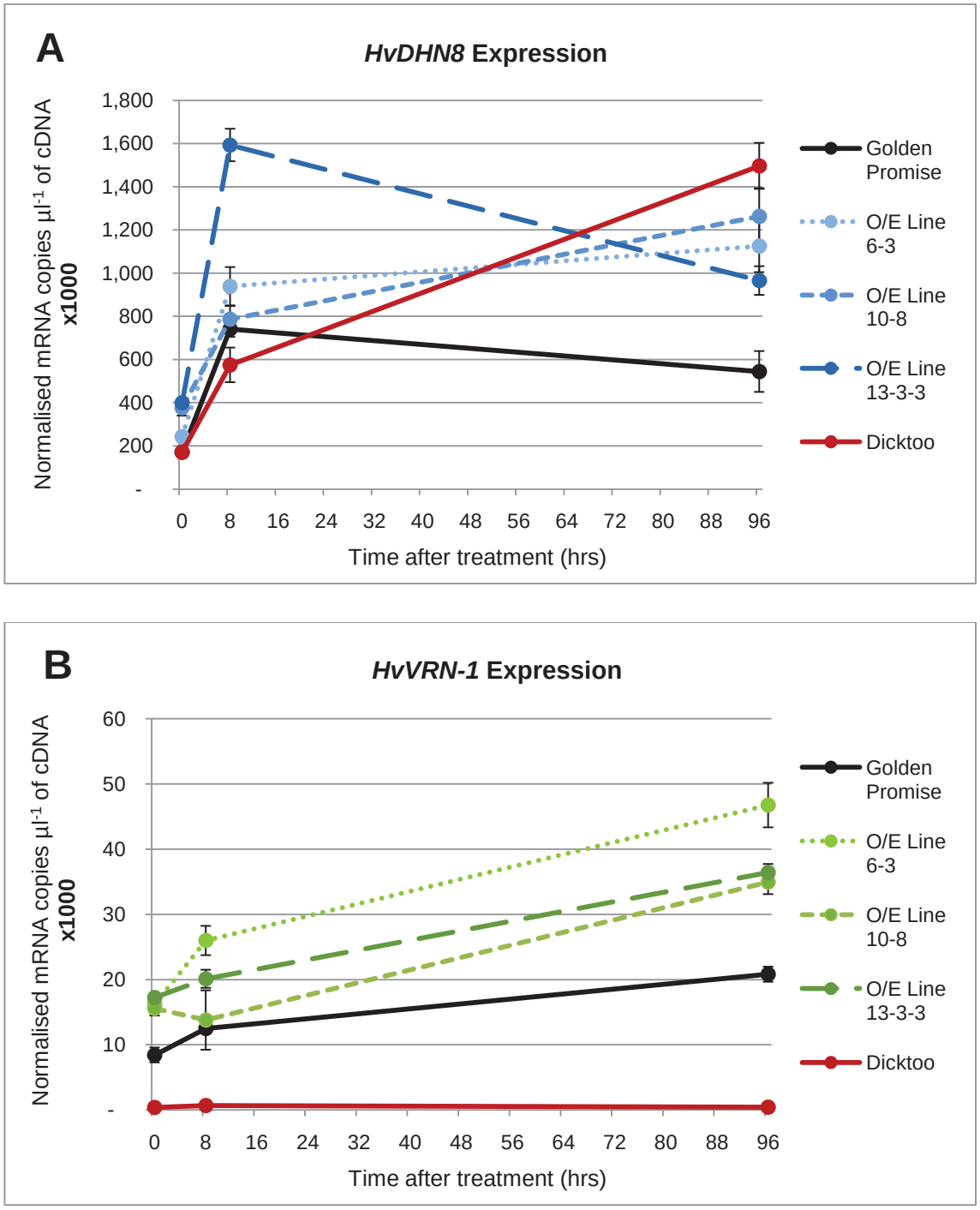


Figure 5.9 Graphs of *HvDHN8* or *HvVRN-1* expression during cold treatment in wild type and transgenic barley plants over-expressing *HvCBF2A* determined by qRT-PCR.

(**A**) Expression of *HvDHN8*. (**B**) Expression of *HvVRN-1*. Expression of *HvDHN8* was upregulated by cold treatment (4°C) in the transgenic lines, reaching higher transcript levels than in wild type Golden Promise. Expression of *HvVRN-1* was greater in the transgenic plants than wild type Golden Promise plants, before and during cold treatment. O/E, Line over-expressing *HvCBF2A*. Error bars represent standard error.

5.4. Discussion

5.4.1. *HvCBF2A* Gene Analysis

This chapter describes the characterisation of *HvCBF2A*, a candidate for the frost tolerance locus *Fr-2*. HvCBF2A is a member of the HvCBF4-subgroup, and contains the conserved sequences typical of this subgroup, as well as the classic conserved AP2 domain and flanking CBF signature motifs (phylogenetic tree of barley CBFs presented in Figure 4.7; sequence alignment of the barley HvCBF4-subgroup members in Appendix E.6 with conserved motifs annotated). As comprehensive phylogenetic and sequence analyses of HvCBF2A were performed previously by Skinner *et al*. (2005), similar studies have not been included in this work. As mentioned above, *HvCBF2A* and *HvCBF2B* are paralogs with extremely high levels of sequence identity (98.6% CDS nucleotide identity/98.2% amino acid identity). This property makes analysis of single gene transcript levels difficult, but suggests that the protein products of the two genes may have similar effects on cellular processes.

Multiple programs predicted HvCBF2A to be localised in the nucleus, with poor prediction scores for localisation in the chloroplast and mitochondria. Therefore HvCBF2A may play a role in regulating genes in the nuclear genome, and, in contrast to the predictions for HvCBF16 and HvCBF23 (discussed in Chapter 4), HvCBF2A is unlikely to be involved in regulation of the chloroplast or mitochondrial genomes. *HvCBF2A* was also predicted to have high translational efficiency and produce a highly stable protein.

DNA binding assays indicate that the DNA binding activity of HvCBF2A is post-translationally activated (Skinner *et al.*, 2005). Possible modes of post-translational regulation were explored by sequence analysis using prediction programs. No sumoylation sites, N-terminal cleavage sequences or nuclear export signals were predicted in HvCBF2A. In contrast to the results of analysis of HvCBF16 and HvCBF23 (Figure 4.6A & B), only a few residues were predicted to be targets of phosphorylation (Figure 5.1). However, three of these residues were conserved and predicted to be target sites in the two other members of the HvCBF4-subgroup,

HvCBF4A and HvCBF9, but were not conserved in any of the other barley *CBF* genes. The putative phosphorylation sites in several other AP2-domain-containing factors (AtCBF1, AtDREB2A, HvCBF3, HvCBF12 and TaDRF2Lb) were examined and found to be broadly dissimilar to those of HvCBF2A, HvCBF4A and HvCBF9 (data not shown). The predicted phosphorylation sites in HvCBF2A, HvCBF4A and HvCBF9 are marked on an alignment in Appendix E.6. Experimental evidence indicates that only members of the HvCBF4-subgroup are post-translationally regulated in response to low temperatures (Skinner *et al.*, 2005), suggesting that these highly conserved residues may be important for function. In addition, as no other methods of post-translational modification have yet been predicted in HvCBF2A, these highly conserved residues are of particular interest in the search for the method of cold-induced *trans*activation of HvCBF2A and the HvCBF4-subgroup members. Further work is required to determine whether these residues are important for regulation and, if so, the regulatory factors involved in their modification.

5.4.2. Analysis of Barley Plants Over-expressing *HvCBF2A*

To investigate whether *HvCBF2A* plays a role in cold response signalling by inducing the expression of downstream *COR* genes and increasing freezing tolerance, transgenic lines that constitutively expressing *HvCBF2A* were generated by Professors Tony Chen and Patrick Hayes (Oregon State University). Although studies have reported ectopic expression of cereal *CBF* genes in heterologous systems (Skinner *et al.*, 2005; Takumi *et al.*, 2008), this is the first study to analyse cereal plants with over-expression of a native *CBF* gene.

The analysis of Southern blot data and GUS activity in transgenic lines over-expressing *HvCBF2A* indicated that the populations of plants from each of Lines 2-6, 10-8 and 13-3-3 were likely to have one insertion event and may be homozygous. Line 3-6 appeared to contain at least two copies of the transgene (Figure 5.2) and plants from the T_4 Sub-line 3-6-312-1 appeared to be homozygous. The population of plants from Line 6-3 appeared to have three insertion events (Figure 5.2) and analysis of segregation ratios of T_2 plants and T_3 and T_4 sub-lines indicated the lines were segregating. Likewise, GUS activity segregation ratios in Line 15-5-8 and T_4 sub-lines indicated these lines were

also segregating. The Southern blot membranes were also analysed using a probe which hybridised to a section of *HvCBF2A* however, despite optimisation, cross-hybridisation with endogenous genes made the results difficult to interpret (data not shown). Southern blot analysis of the T_4 or T_5 lines would increase confidence in zygocity determination.

5.4.2.1. Analysis of Developmental Phenotypes

The developmental phenotypes of the transgenic lines over-expressing *HvCBF2A* were examined under glasshouse growth conditions. Northern blots were used to determine the level of transgene expression. The probe hybridised to transcripts from both the transgene and endogenous *HvCBF2A* genes and was expected to also cross-hybridise to transcripts from the paralog *HvCBF2B* (and possibly transcripts from other closely-related *CBF* genes). The results of the wild type samples in the northern blot indicate that the combined transcript levels of the endogenous *HvCBF2A* and any cross-hybridised endogenous gene(s) were too low to be detected (Figure 5.3A). This indicates that the transcript levels observed in the transgenic plants are primarily due to expression of the *HvCBF2A* transgene.

Although transcript levels varied between and within transgenic lines, there was constitutive over-expression of *HvCBF2A* in all the transgenic plants relative to wild type plants (Figure 5.3A). The greatest variation in transgene expression was in the transgenic Lines 6-3-1-1 and 15-5-8-1 (Figure 5.3A). Several developmental abnormalities were observed in the transgenic barley plants, including stunted growth and development, smaller final plant height and increased time to maturity. The severity of these phenotypes was correlated with transgene expression levels, with greater expression resulting in more severe abnormalities (Figure 5.3A & B; Figure 5.4). In addition, it was observed that high levels of *HvCBF2A* expression were commonly associated with reduced yield relative to wild type plants, caused by fewer tillers, shorter heads and/or poorer grain filling. Unusual head morphologies and infertility were observed in both wild type and transgenic plants, although the severity and frequency of these phenotypes was greater in the transgenic plants (Figure 5.3B; Figure 5.5). It was concluded that constitutive over-expression of *HvCBF2A* caused

growth and developmental abnormalities and that the degree of phenotypic effect was dependent upon the level of *HvCBF2A* transcript accumulation. The infertility phenotype suggests that some of the genes either directly or indirectly regulated by HvCBF2A are involved in fertility processes. These results are discussed in further detail below.

5.4.2.2. Freezing Tolerance

A freezing tolerance assay and qRT-PCR were performed to determine whether over-expression of *HvCBF2A* in transgenic barley plants altered the freezing sensitivity and the expression of *Cor14b* and *DHN5*; the traits mapped to *Fr-2*. The freezing tolerance assays were performed by Dr. Ottó Veisz and colleagues (Agricultural Research Institute of the Hungarian Academy of Sciences) and have been included in this thesis to allow interpretation of the results of the collaborative project as a whole. The freezing tolerance of the transgenic plants was compared to that of two reference barley cultivars: the freezing-sensitive cultivar Golden Promise (the background of the transgenic plants) and the well-characterised relatively freezing-tolerant Dicktoo (Skinner *et al.*, 2006).

For the majority of the transgenic lines over-expressing *HvCBF2A*, the survival following freezing treatment was greater than the untransformed plants from the frost-sensitive Golden Promise cultivar. This indicates that increased expression of *HvCBF2A* increased freezing tolerance in transgenic barley plants. These findings support the suggestion by Stockinger *et al*. (2007) that the increased cold tolerance of the Nure barley cultivar may be caused by the higher observed levels of constitutive and stress-induced expression of *HvCBF2* (and/or *HvCBF4*) relative to the cold-sensitive Tremois cultivar. High levels of constitutive expression of *HvCBF2A* was not sufficient to boost the freezing tolerance of the Golden Promise cultivar to or beyond the levels of the freezing-tolerant cultivar Dicktoo. The results of the freezing tolerance assay are discussed in the context of the *Fr-2* locus below.

The level of transgene expression was not analysed for the generation of transgenic plants used for the freezing tolerance assays. However expression analysis of the parental and daughter lines showed that in several cases, expression differed significantly in sibling plants of a single line (Figure 5.3A; Figure 5.6). This experiment could be repeated with analysis of transgene expression in individual plants to determine whether threshold levels of *HvCBF2A* transcript are required for function.

In addition, it would be interesting to determine whether over-expression of *HvCBF2A* was also associated with an increase in freezing tolerance at the reproductive stage of development. Mapping work has indicated that QTLs for vegetative and reproductive freezing tolerance overlap at the frost-resistance locus *Fr-1* which has, in turn, been associated with regulation of *HvCBF2A* expression (Francia *et al.*, 2004; Reinheimer *et al.*, 2004; Stockinger *et al.*, 2007).

5.4.2.3. Expression Analysis of Putative Downstream Genes

The next section of work focussed on whether the increased freezing tolerance observed in the transgenic plants over-expressing *HvCBF2A* could be related to earlier or greater activation of downstream stress-tolerance genes and also, whether the low temperature-induced DNA-binding activity of HvCBF2A, documented in the literature, resulted in different effects on target gene expression at normal and low temperatures. To this end, transcript levels of the *COR* genes *HvCor14b*, *HvDHN5* and *HvDHN8*, as well as *HvCBF2*, were measured in the transgenic barley plants, under unstressed conditions and at low temperatures (4°C). The expression of *HvVRN-1* was also examined in the transgenic plants. *VRN-1* (also known as *BM5a*) is the gene underlying the second major locus involved in freezing tolerance, *VRN-1*/*Fr-1*. *VRN-1* encodes a MADS box binding protein which is an important regulator of the vernalisation response and has recently been shown to regulate the expression of various *CBFs* at *Fr-2* including *HvCBF2* (Stockinger *et al.*, 2007). Examining the expression of *HvVRN-1* in the transgenic plants allowed the link between vernalisation and *CBF* signalling pathways to be investigated further. Golden Promise and Dicktoo cultivars were again used as controls for relative transcript levels of *COR* genes in freezing sensitive and tolerant

genotypes, respectively. The expression of *HvCBF2* has been determined by semi-quantitative methods in published work (Skinner *et al.*, 2005; Stockinger *et al.*, 2007).

Under unstressed conditions, transcript levels of *HvCBF2* (the combined transcript levels of *HvCBF2A* and *HvCBF2B*) were extremely low in both Golden Promise and Dicktoo wild type plants (Figure 5.6; Figure 5.7). During cold treatment, transcript levels of *HvCBF2* were elevated in both cultivars, with higher expression for a longer duration occurring in the freezing-tolerant cultivar Dicktoo (Figure 5.7). Published studies also showed that *HvCBF2* expression was quickly induced by cold treatment and that expression was greater and of longer duration in the cold-tolerant (Dicktoo and Nure) relative to -intolerant cultivars (Tremois and Morex) (Skinner *et al.*, 2005; Stockinger *et al.*, 2007).

Transcript levels of *HvCBF2* were consistently high in all of the transgenic lines relative to Golden Promise or Dicktoo, indicating high levels of transgene expression. For each transgenic line, transcript levels varied between time points (Figure 5.6; Figure 5.7). These differences are likely to be due to differences in expression in individual plants in a line may be caused by epigenetic effects such as transgene silencing.

Expression of COR genes

In wild type plants, the expression of the three *COR* genes examined, *HvCor14b*, *HvDHN5* and *HvDHN8*, were low prior to stress treatment and were induced after 8 and/or 96 hrs of cold treatment (Figure 5.6; Figure 5.8A & B; Figure 5.9A). These genes have been shown to be induced by cold treatment after 6 hrs of exposure (Stockinger *et al.*, 2007). The general expression profiles of each *COR* gene were similar in the two wild type cultivars however after extended cold treatment (96 hrs), expression of all three genes was greater in the freezing-tolerant cultivar Dicktoo than in Golden Promise (Figure 5.6; Figure 5.8A & B; Figure 5.9A).

Under unstressed conditions, transcript levels of all three *COR* genes were higher in plants over-expressing *HvCBF2A* relative to both wild type cultivars (Figure 5.6; Figure 5.8A & B; Figure 5.9A). After 8 hrs of cold treatment, the expression of all three *COR* genes remained higher in the transgenic plants than in wild type Golden Promise. In addition, the transcript levels reached after 8 hrs of cold treatment were similar to or greater than the maximum levels observed in untransformed plants (which occurred after 96 hrs of cold treatment). Transcript levels of the *COR* genes in the transgenic plants increased further as cold treatment extended and after 96 hrs of cold treatment, transcript levels of all three genes remained significantly greater than those in wild type Golden Promise.

These results indicate that over-expression of *HvCBF2A* induces the expression of *HvCor14b*, *HvDHN5* and *HvDHN8* under unstressed conditions and that cold treatment further induces expression of these genes to levels beyond those observed during cold stress in the background genotype. These results provide *in vivo* confirmation of the cold-induced DNA-binding activity of HvCBF2A (Skinner *et al.*, 2005) and agree with the results of ectopic over-expression of *CBF* genes in Arabidopsis where expression of *COR* genes was induced at normal and/or low temperatures (Gilmour *et al.*, 1998; Qin *et al.*, 2004; Skinner *et al.*, 2005; Zhou *et al.*, 2009).

The transcript levels of the three *COR* genes in the transgenic plants were greater than those detected in Dicktoo early during cold treatment (after 8 hrs) (Figure 5.6; Figure 5.8A & B; Figure 5.9A). As the cold treatment extended, the transcript levels of *HvDHN5* were consistently greater in transgenic plants than those in Dicktoo. In contrast, although the transcript levels of *HvCor14b* and *HvDHN8* continued to increase in transgenic plants, the highest levels were observed in Dicktoo after 96 hrs of treatment. Therefore, over-expression of *HvCBF2A* boosted the responsiveness and level of expression of the *COR* genes, but other components of the cold-induced freezing tolerance network are required to achieve the maximum induction of certain *COR* genes during longer exposures to low temperatures.

The basal levels of *COR* gene expression were upregulated in the transgenic plants relative to wild type, as measured by qRT-PCR, but the transcript levels remained below the level of detection for northern blot analysis (Figure 5.6; Figure 5.8A & B; Figure 5.9A). Northern blot expression analysis of *COR* genes in Arabidopsis plants over-expressing various barley and rye *HvCBF4* subgroup members also failed to detect *COR* gene expression in unstressed plants (Skinner *et al.*, 2005).

In conclusion, expression analysis of transgenic barley plants over-expressing *HvCBF2A* greatly suggests that HvCBF2A is involved in regulation of *COR* genes, including *HvCor14b*, *HvDHN5* and *HvDHN8*, during cold stress response, either directly *via* interaction with *cis*-elements in their promoters, or indirectly by regulating the expression or activity of other signalling molecules. CRT elements were found in the promoters of *HvDHN5* and *HvDHN8* (Choi *et al.*, 1999), suggesting these genes may be direct targets of HvCBF2A. These findings are consistent with the predicted nuclear localisation of HvCBF2A as all three of the *COR* genes studied here are in the nuclear genome (Cattivell *et al.*, 2002; Choi *et al.*, 1999).

Expression of HvVRN-1

VRN-1, an important regulator of vernalisation, is induced during vernalisation in winter genotypes and constitutively expressed in spring genotypes. It is a negative regulator of many *CBF* genes at *Fr-H2*, including *HvCBF2* (Stockinger *et al.*, 2007; von Zitzewitz *et al.*, 2005). A schematic diagram of the present understanding of the interaction between vernalisation, cold acclimation, photoperiod, reproductive competency and the *VRN* and *CBF* genes is presented in Figure 5.10. It is interesting then, that transcript levels of *HvVRN-1* were higher in plants over-expressing *HvCBF2A* than in wild type Golden Promise plants (Figure 5.9B), both before and after cold treatment. This suggests that HvCBF2A plays a role in the vernalisation response and that, in addition to regulation of *CBFs* by *HvVRN-1* expression, *HvVRN-1* may be positively regulated by *CBFs* in barley.

During vernalisation, many weeks of exposure to low temperatures are required to obtain maximum expression of *VRN-1*. By comparison, the cold treatment time points used in this experiment were relatively short. It was suggested previously that threshold levels of *VRN-1* activity may be required for attenuating *CBF* expression (Stockinger *et al.*, 2007). It would be worthwhile to determine the expression levels of *HvVRN-1* in the transgenic plants over longer periods at low temperatures. These results could be compared to the expression profiles of *HvVRN-1* in wild type plants throughout vernalisation, to determine whether or at what point the higher *HvVRN-1* expression present in these transgenic plants is likely to become functionally relevant.

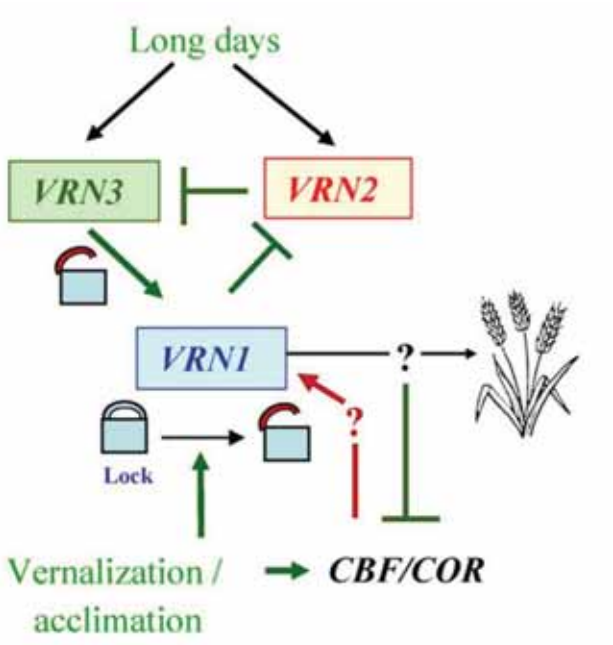


Figure 5.10 Schematic diagram of a hypothetical model of flowering initiation involving interactions between the *VRN* and *CBF* genes.

The lock represents the initially repressed state of reproductive competency. Vernalisation releases *VRN-1* repression and begins the transition from the vegetative to the reproductive stage. In photoperiod-sensitive genotypes, long days accelerate flowering by inducing *VRN-3* and thereby promoting *VRN-1* however, prior to vernalisation, this activity is repressed by high *VRN-2* transcript levels. Upon vernalisation however, *VRN-2* is down regulated, thereby releasing *VRN-3* which activates *VRN-1* and promotes flowering. It has been suggested that VRN-1 can directly or indirectly attenuate the *CBF*/*COR* pathway. At least one of the barley CBFs, HvCBF2A, appears to positively regulate the expression of *VRN-1*, particularly at low

temperatures. Red arrows have been added to the figure to indicate the position of this putative new link. This may or may not contribute to 'unlocking' the *VRN-1* locus in response to vernalisation. Modified from Galiba *et al*. (2009).

The northern blot results indicated that *HvVRN-1* was expressed at a low and similar level in Golden Promise and Dicktoo plants, with greatest expression after the longest period of cold treatment (96 hrs). In contrast, the results of qRT-PCR showed that expression in Dicktoo was significantly lower than in Golden Promise. This was not caused by primer hybridisation differences as the corresponding sequences were identical in each cultivar. A possible explanation is that there were differences in the hybridisation efficiency of the Dicktoo and Golden Promise samples which were analysed on different membranes, although the hybridisation was performed in parallel.

It has been suggested that the transition between vegetative and reproductive stages, which is caused by vernalisation, may increase freezing tolerance. In light of the findings presented here, using increased expression of *CBFs* may pose some difficulties in this regard if used in a winter cultivar background, as this condition appears to result in early induction of the expression of *VRN-1*. The complex interactions between the expression of barley *CBFs*, vernalisation genes, photoperiod and temperature induction make it difficult to predict the precise manner in which this new information will fit into the current model. Experiments examining the effect of over-expression of barley *CBFs* such as *HvCBF2A* on the vernalisation requirement in a winter background would provide valuable information about this system and whether upregulation of the expression of *CBFs* in the more cold-tolerant winter backgrounds may be a viable method of increasing stress tolerance. As an additional note, the delayed flowering phenotypes observed in the transgenic plants may be related to altered transition between vegetative and reproductive development due to over-expression of *HvVRN-1*. However, a spring background cultivar was used and the plants were grown over summer (long days), two conditions which indicate that higher expression of *HvVRN-1* may not be the primary cause of this phenotype.

5.4.2.4. General Discussion

When the results of the expression analyses, freezing tolerance assays and phenotypic analysis of plant development are considered together in the context of previous information, a comprehensive picture of the role of HvCBF2A in cold stress responses begins to appear which is consistent with *HvCBF2A* (and likely other co-localised *CBFs*) being responsible for the freezing tolerance QTL *Fr-2*. The role of *HvCBF2A* in cold stress response appears to be essentially analogous to that of the well-characterised Arabidopsis *CBF* genes. *HvCBF2A* is not transcribed under normal conditions but exposure to low temperatures quickly triggers gene expression. The transcripts are (predicted to be) transcribed with high efficiency, to produce partially inactive, probably stable HvCBF2A proteins. These proteins accumulate and are activated by an unknown factor during exposure to low temperatures. Following activation, the HvCBF2A proteins directly regulate their target genes by binding CRT elements in their promoters. Direct targets of HvCBF2A are likely to include *HvCor14b*, *HvDHN5* and *HvDHN8*, while it is less clear whether *HvVRN-1* is affected directly or indirectly by *HvCBF2A* expression. These and other target genes are induced, which enhances freezing tolerance.

This model accounts for many of the findings regarding *HvCBF2* function mentioned above, including expression, protein DNA binding, mapping, and over-expression developmental data, although the position of *HvVRN-1* in the model is not clear. Transcription of *HvVRN-1* and *HvCBF2A* is induced by low temperatures and HvCBF2A may assist in activating the low temperature-induced transcription of *HvVRN-1* (Figure 5.10). If this were correct, extension of the principle indicates that after the vernalisation requirement is satisfied, *HvCBF2A* expression would be repressed by HvVRN-1, resulting in the lower levels of *CBF* and *COR* gene expression which have been observed following vernalisation (Galiba *et al.*, 2009).

Although the HvCBF2A proteins appear to have been only partially active under normal growth conditions, this was sufficient to cause deleterious growth and developmental symptoms similar to those in transgenic plants constitutively over-expressing other *CBF* genes (Liu *et al.* 1998; Wang *et al.* 2005; Ito *et al.* 2006). A complementary

explanation could be that the activity of the HvCBF2A protein does not require cold treatment for binding to other targets, and that the constitutive over-expression of these other genes drains cellular resources, resulting in the developmental phenotypes observed. Future work could involve analysis of transgenic plants with stress-inducible over-expression of *HvCBF2A* as this approach alleviated similar phenotypes associated with constitutive over-expression of *CBF* genes in Arabidopsis (Kasuga *et al.*, 1999).

The findings here do not preclude other *CBFs* playing complementary, similar or contrasting roles in regulation of the *COR* genes but provide compelling evidence that *CBF* genes play an analogous role in cereal stress response signalling pathways and stress tolerance to that of the homologous *CBF* genes in Arabidopsis.

The major difference between the model for the action of *HvCBF2A* discussed here and that for Arabidopsis *CBF* genes is the requirement for post-translational modification to fully activate the HvCBF2A protein. Post-translational modification has been suggested to be required for the Arabidopsis DREB2-type proteins during drought stress response. *HvCBF2* was mildly induced by drought and salinity treatments (Skinner *et al.*, 2005) and many studies have shown that over-expression of *CBF* genes enhances tolerance to multiple abiotic stresses (Dubouzet *et al.*, 2003; Liu *et al.*, 1998; Qin *et al.*, 2004). It would be interesting to determine whether *HvCBF2A* is also involved in the activation of *COR* genes which occurs during drought and salinity stresses, and if over-expression of *HvCBF2A* enhances tolerance to multiple stresses.

Although the expression of *HvCBF2A* increased the expression of the *COR* genes studied here to levels similar to those in the freezing-tolerant cultivar Dicktoo, the overall freezing tolerance of the transgenic plants remained lower than Dicktoo. This indicates that suggests that *HvCBF2* plays an important role in stress response while supporting the accepted view that multiple QTLs and alleles contribute to cold stress tolerance (including *Fr-H1*/*HvVRN-1*) (Francia *et al.*, 2004; Reinheimer *et al.*, 2004). Therefore, these other components of plant stress tolerance must be identified and

optimised to improve on plant stress tolerance beyond the levels obtained by manipulation of *HvCBF2A*.

5.5. Conclusions

The barley *CBF* genes, including *HvCBF2A*, are the leading candidates to explain the freezing tolerance QTL mapped to *Fr-H2*. The role of *HvCBF2A* in cold stress response and freezing tolerance was investigated by characterising transgenic plants with over-expression of *HvCBF2A*. The results of this study may be useful to improve the current model for the role of barley *CBFs* in cold stress tolerance, as well as determining whether *CBF* genes, such as *HvCBF2A*, may be useful towards the goal of producing transgenic crop plants with enhanced tolerance to cold and other abiotic stresses.

Transgenic barley plants over-expressing *HvCBF2A* had greater freezing tolerance although this was accompanied by a dwarf phenotype when compared to wild type Golden Promise plants. Both these phenotypes may be a result of the upregulation of the expression of downstream *COR* genes, which was observed in the transgenic plants under normal conditions and to a greater degree at low temperatures. Thus, HvCBF2A appears to be involved in either direct or indirect regulation of *COR* genes during cold stress response. Analysis suggested that regulation of the activity of HvCBF2A by low temperatures is most likely to be by phosphorylation at one or more of three predicted phosphorylation sites which are completely conserved with other members of the cold-regulated HvCBF4-subgroup.

The increased expression of the *COR* genes in the transgenic lines was also associated with increased freezing tolerance. However, Dicktoo, the frost-tolerant control cultivar, had higher freezing tolerance, corresponding with similar, and in some cases higher levels of *COR* gene expression after extended exposure to low temperatures. The results indicate that over-expression of *HvCBF2A* was sufficient to boost the responsiveness and maximum expression of the *COR* genes, but that other components of the cold-induced freezing tolerance network are required to achieve the maximum induction of important cold-tolerance genes. These components will need to be identified in order to pursue the improvement of stress tolerance beyond the levels of natural variation by manipulation of *HvCBF2A*.

To investigate cross-signalling between vernalisation and cereal CBFs, the expression of *HvVRN-1*, an important regulator of the vernalisation response and *CBF* expression, was also examined in the transgenic plants. Expression analyses indicate that HvCBF2A may be involved in regulation of *HvVRN-1*. The significance of this result is not understood at present, however it is additional evidence of cross-signalling between the vernalisation response and CBF-signalling pathways. Further experiments should examine the stress tolerance of the transgenic plants to freezing at reproductive stages and under drought and salinity stresses.

The results presented here provide support for the common model which has been presented in the literature: that barley CBFs underlie the *Fr-2* locus, that the role of HvCBF2A involves cold-activated induction of *COR* gene expression which is associated with increasing the plant's resistance to freezing stress, and finally, that over-expression of cereal *CBFs* can increase stress tolerance in their native species.

Chapter 6. Concluding Remarks

The objective of this project was to explore whether the *ICE* and *CBF* pathways, which have been well-characterised in the model species Arabidopsis, could be used to engineer cold tolerance in crop species, such as barley. The results presented here indicate that Arabidopsis is a reasonable but imperfect model. The Arabidopsis information can be used with some success in barley, but that there are significant limitations to its application.

The upstream section of the pathway, including the *ICE* genes, appears to be more complex in barley than in Arabidopsis. Characterisation of *HvICE2* in barley showed significant differences from the Arabidopsis system. However, even in Arabidopsis where the *ICE* pathway is best characterised, the pathway proved more complicated than expected; characterisation of *AtICE2* also revealed significant differences from the function of *AtICE1*. It appears that the *ICE* section of the signalling pathway is not robust, and that the gaps in our understanding in both barley and Arabidopsis are significant. Additional work on the *ICE* pathway will be essential if these genes are to be of value in improving freezing tolerance in crop plants. For example, over-expression of *AtICE1* in cereals might prove to be informative in the investigation of whether the *ICE* pathway is functional in cereals and can be activated by this *ICE* gene.

In contrast, the results of characterisation of *CBFs*, both here and reported in the literature, have proved promising. Functional characterisation of various barley *CBF* genes suggests the *CBF*/*COR* section of the signalling pathway is similar in barley and Arabidopsis. The results presented here provide compelling evidence that cereal *CBF* genes such as *HvCBF2A* may be used to increase expression of stress tolerance genes and ultimately improve freezing tolerance. Further characterisation of *HvCBF16* and *HvCBF23* will determine whether similar results may be produced by over-expression of these genes.

Together, the results presented here suggest that the *CBF* genes are the most practical target to enhance freezing tolerance. Detailed analysis of the *CBFs* will be an important

step in determining the most effective genes and the best way to deploy them to enhance cold tolerance. However, the cereal *CBF* gene family is large and complex, making it difficult to determine the most useful way to characterise this family. A systematic approach could be used; characterising each gene in turn. Mapping freezing tolerance to different *CBF* genes in various genotypes may determine whether specific *CBF* genes are more important than others but this will require high resolution mapping to allow resolution of gene clusters. Functional characterisation of representative *CBF* genes from each *HvCBF* phylogenetic subgroup will also be important as this will indicate whether these groups perform different or functionally redundant roles in signalling.

The results presented in this thesis highlight many important questions which merit further investigation. One task remaining is to remove the detrimental developmental phenotypes without reducing the stress tolerance. Investigation of the tolerance to other abiotic stresses of transgenic plants over-expressing *HvCBF2A* or other cereal *CBFs* is also a priority. It would also be useful to determine what effect over-expression of *HvCBF2A* or other *CBF* genes would have in a stress-tolerant background cultivar, such as Dicktoo, to determine whether it is possible to use the *CBF* genes to increase stress tolerance of barley beyond the levels naturally occurring, or whether the variation present in the population already contains the optimal alleles of the genes in the *CBF* pathway.

Appendices

Appendix A. Oligonucleotides used in PCR

Primer	Sequence 5' → 3'
2HA_Rev	AGATGGTGCACGATGCACAG
35S_Prom	GACGCACAATCCCACTATCCTTCGC
35S_Term	CCCTTATCTGGGAACTACTCACAC
ADLD_forward	CTATTCGATGATGAAGATACCCCACCAAACCC
ADLD_reverse	GTGAACTTGCGGGGTTTTTCAGTATCTACGAT
AP1	GGATCCTAATACGACTCACTATAGGGC
AP2	AATAGGGCTCGAGCGGC
AtActin_F	GAGTTCTTCACGCGATACCTCCA
AtActin_R	GACCACCTTTATTAACCCCATTTACCA
AtCBF_R1	GAAGACTCGTAATCGGAGCCAAACA
AtCBF3_F1	TTTCAGCAAACCATACCAACAAAAA
AtCOR47_F1	CGGAGGAGAAGAAGGGGATTTTGGA
AtCOR47_R1	TCAAATGCAATCAACGAAAGCCACA
AtCOR78_F1	AACACACACCAGCAGCACCCAGAAG
AtCOR78_R1	TCGGAAGACACGACAGGAAACACCT
AtCyclophilin_F	TGGCGAACGCTGGTCCTAATACA
AtCyclophilin_R	CAAAAACTCCTCTGCCCCAATCAA
AtGAPDH_F	TGGTTGATCTCGTTGTGCAGGTCTC
AtGAPDH_R	GTCAGCCAAGTCAACAACTCTCTG
AtICE1_F1	CTTGTCTGCTCGGTCACTTCTTGCG
AtICE1_R1	AACCTCCTCATTCCCGAACTCTCCG
AtICE2_F2	CACCGAACTTGAATCTACTCCACCG
AtICE2_R2	CCGCTTGTTGAACATCCAATCCTAA
AtRAB18_F1	CCACTGACGAGTACGGAAACCCGAT
AtRAB18_R1	ATTCCTCCCAAGCCACCACCACTTT
AtTubulin_F	ATGTGGGTCAGGGTATGGAA
AtTubulin_R	CCGACAACCTTCTTAGTCTCCTCT
GFPiF	TCAAGGAGGACGGAAACATC
GFPiR	AAAGGGCAGATTGTGTGGAC
GUS_F	ATGTGGAGTGAAGAGTATCAGTGTGCAT
GUS_R	CGAAACGCAGCACGATACGCT
GW1	GTTGCAACAAATTGATGAGCAATGC
GW2	GTTGCAACAAATTGATGAGCAATTA
HvActin _F	GTCTTTCCCAGCATTGTAGG
HvActin_R	CGACACGGAGCTCATATAGAA
HvCBF16-2 F	ATGGACCTGGGCACGTACTAC
HvCBF16-2 R	TGCATAGAATCAAAGCAGCTG
HvCBF16L F	TACGCTAATTCCGAAGATGTG
HvCBF16L R	CTAGGTTGATGTCTTCTTC
HvCBF2_F	CCATCACCTCAAGCGACCTATCG
HvCBF2_R	GCCTGACGCCTGGTGGAAGAAC
HvCBF23_F	CTCTAATCCTTGTTCATTGTG
HvCBF23_R	CTGTTACAATCTGCAGAGCAG
HvCOR14B_F	TTGAGGATGTGAGCAAATGAG
HvCOR14B_R	TACATCGTCAATGACGAGACC
HvCyclophilin_F	CCTGTCGTGTCGTCGGTCTAAA
HvCyclophilin_R	ACGCAGATCCAGCAGCCTAAAG
HvDHN5_F	TGGCGAAGTTCCACCGTATGC

HvDHN5_R	ACGAAAACTGTTGCCACACTG
HvDHN8_F	GCTCCAGCTCCAGCTCGTCTA
HvDHN8_R	CTTCTCCTCCTCGGGCACTG
HvGAPDH_F	GTGAGGCTGGTGCTGATTACG
HvGAPDH_R	TGGTGCAGCTAGCATTTGAGAC
HvHSP_70_F	GCTGTTCTTTACTTCCCAGGAG
HvHSP_70_R	TCGCTAAGAACAAGACATCAC
HvICE2_TX_F2	GAAATTAAGGCGGTGCTCCTGC
HvICE2_TX_F3	CGGTCTCCAGAACGCGATGTAG
HvRD22_F	GCGTTCCAGGTGCTCAAGGT
HvRD22_R	CAAACGTGCCACTCCGTACAA
HvTubulin_F	AGTGTCCTGTCCACCCACTC
HvTubulin_R	AGCATGAAGTGGATCCTTGG
HvVRN1_F	AGAGGATGTGGCAGTGCAGCCTCAG
HvVRN1_R	CGCAACCGCATGATACACCAGGCTG
ICE_ATG_F	ATGGAGAACCCGGCGGCGGTGG
ICE_F0	AGTAGAAGTAACTGACCGTGCGAACTTG
ICE_GW_R1	ACCGTCACGATGCTAGGTGAAAACAGTC
ICE_GW_R2	ACCGCGATGAGTATGCCTCCTTAACACA
ICE_GW_R3	AAATTCCTCCCTGGTAAGCGCATTTTG
ICE_GW_R4	CACGATGGAATGGAATTATTGGAGAGAA
ICE_noTAG_R	CATCGCGTTCTGGAGACCGGCGC
ICE_PR_F1	CTACAGGTATGGATGACGGATCACGACAGTTCT
ICE_PR_F2	TTCGGTCTAATCAAATCTTGCTACCTGTA
ICE_PR_F3	TTAGACGTGGCAACCTTAGGCTGGGAACC
ICE_PR_F4	ATTCCCTTCCTCTCGCTTTACATTACAC
ICE_PR_R1	CACGCCCTCCTCTGTCCTCTCCCAC
ICE_R0	TGCATGGAGGACTGACTGACTGGA
ICE_R1	GCCATCGCCTCCGACGTGAGGTA
ICE_R3	CCGGACCCGAAGTCGTGGTATGC
ICE_R6	CAATCACCATCCTGGCGGTCTTGTCTCC
ICE_R7	CAGGTTCTTGGCCGGCATCCCCTTCTTC
ICE_R9	TTGCTGATCTTGGGCACCACGGAGCGCAG
ICE_RNAi_Rt	CACCCGCCGACCGCCGCATTCTCCA
ICE_wTAG_R	CTACATCGCGTTCTGGAGACCGGC
ICE2_Ft	CACCATGAACAGCGACGGTGTTTGGCTT
ICE2_R	TCAAACCAAACCAGCGTAACCTGCT
ICE2_RNAir	TGAGAACAGAGGACTCAATCCACATG
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
NOSTERM_R1	TTGCCAAATGTTTGAACGATC
NOSTERM_R2	TGATAATCATCGCAAGACCG
pTseq1	ACACATACAAATGGACGAACGGATAAACCTT
QPCR_ICE1_F1	GGAGACAAGACCGCCAGGATGGTGATT
QPCR_ICE1_F2	CTTGTGCTGTTCTTTGCTTTACCAGTTCC
QPCR_ICE1_R2	GTTCACGACGACCACAGAGAGGGAAGA
SP6	GATTTAGGTGACACTATAG
T3	ATTAACCCTCACTAAAGGGA
T7	GTAATACGACTCACTATAGGGC
UNP	AAGCAGTGGTATCAACGCAGAGT
UPM A mix	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT and CTAATACGACTCACTATAGGGC

Appendix B. Contig Numbers and Alignment Scores and E Values from BLAST Analyses

B.1 Scores and E Values of Comparison of *HvICE2* and TC134022 Nucleotide Sequences.

Results obtained from a nucleotide-nucleotide (blastn) search of the TIGR barley database (http://compbio.dfci.harvard.edu/tgi/plant.html).

Query gene name	HarvEST contig closest match	Score (bits)	E Value
HvICE2	TC134022	169	$2e^{-46}$

B.2 Contig Numbers of Affymetrix Barleybase Contigs Corresponding to *HvICE2* and *HvCBF23.*

Results obtained from a nucleotide-nucleotide (blastn) search of the Affymetrix Barley1 microarray database (www.plexdb.org/plex.php?database=Barley).

Query gene name	Affymetrix contig closest match (No. of sequences compiled)	Score (bits)	E Value
HvICE2	Barley1_13678 Contig13678 (4 members)	2052	0.0
HvCBF23	Barley1_04317 Contig4317 (52 members)	2288	0.0

B.3 Score and E Value for Similarity between *AtICE1* and *AtICE2* Nucleotide Sequences.

Results obtained from a nucleotide-nucleotide (blastn) search of the NCBI non-redundant database using *AtICE2* sequence, limited to *Arabidopsis thaliana*. After other *AtICE2* sequences, *AtICE1* was the closest match.

Query gene name	NCBI contig closest match	Score (bits)	E Value
AtICE2 (NM_101157)	Arabidopsis thaliana ICE1 mRNA (AY195621)	258	$7e^{-67}$

B.4 Score and E Values for Similarity between *HvCBF16** Nucleotide Sequence and Four Similar Genes.

* Isolated in this study during yeast 1-hybrid screening of a cold-treated barley cDNA library using a bait containing the Arabidopsis CRT/DRE.

Results obtained from a translated nucleotide-protein (blastx) search of the NCBI non-redundant database using *HvCBF16* sequence.

Hit #	NCBI contig closest match	Score (bits)	E Value
1	TmCBF16 [*Triticum monococcum*] (AAY32558)	319	$2e^{-85}$
2	LpCBF1a [*Lolium perenne*] (BAF36837)	264	$8e^{-69}$
5	TmCBF15 [*Triticum monococcum*] (AAY32556)	233	$2e^{-59}$
24	HvCBF3 [*Hordeum vulgare*] (AAX23694)	211	$5e^{-53}$

B.5 Score and E Values for Similarity Between *HvCBF23** Nucleotide Sequence and Four Similar Genes.

* Isolated in this study during yeast 1-hybrid screening of a cold-treated barley cDNA library using a bait containing the Arabidopsis CRT/DRE.

Results obtained from a translated nucleotide-protein (blastx) search of the NCBI non-redundant database using *HvCBF16* sequence.

Hit #	NCBI contig closest match	Score (bits)	E Value
1	Os09g0369000 [*Oryza sativa*] (NP_001063013)	181	$1e^{-43}$
2	DBF2 [*Oryza sativa*] (AAP70033)	180	$2e^{-43}$
6	DBF1 [*Zea mays*] (AAM80486)	132	$4e^{-29}$
27	RAP2.4 [*Arabidopsis thaliana*] (NP_177931)	104	$9e^{-21}$

Appendix C. Cold Stress Treatment Series Temperature Regime

Treatment commenced at the beginning of the ‘dark’ cycle of lighting.

The temperature was lowered from 20°C to 4°C at a rate of -5°C hr^{-1}.

The temperature was lowered from 4°C to -5.5°C at a rate of -1°C hr^{-1}.

The temperature was held at -5.5°C for 2 hrs.

The temperature was increased from -5.5°C to 4°C at a rate of +2°C hr^{-1}.

The temperature was increased from 4°C to 20°C at a rate of +5°C hr^{-1}.

The plants were removed from the freezing chamber and replaced into the growth room to recover under ‘normal’ conditions.

Appendix D. Media Components

D.1 Liquid Hydroponic Media: The ACPFG Cereal Growth Solution

Used as liquid hydroponic media in cultivation of barley plants. Concentrations represent those present in the final media.

Macronutrients	mM
NH_4NO_3	5.0
KNO_3	5.0
$Ca(NO_3)_2 \cdot 4H_2O$	2.0
$MgSO_4 \cdot 7H_2O$	2.0
KH_2PO_4	0.1
Na_2SiO_3	0.5
NaFe(III)EDTA	0.05

Micronutrients	µM
H_3BO_3	50.0
$MnCl_2 \cdot 4H_2O$	5.0
$ZnSO_4 \cdot 7H_2O$	10.0
$CuSO_4 \cdot 5H_2O$	0.5
Na_2MoO_3	0.1

D.2 Nutrients Added to Roseworthy Soil for Drought Stress Treatment of Barley Plants

	mg/kg soil
$CaCO_3$	5000
NH_4NO_3	350
$MgSO_4.7H_2O$	90
K_2SO_4	120
KH_2PO_4	150
$MnSO_4.H_2O$	7
$CuSO_4.5H_2O$	5
$ZnSO_4.7H_2O$	4.4
$NiSO_4.6H_2O$	0.15
H_3BO_3	0.5
$FeSO_4.7H_2O$	0.7

Appendix E. Sequence Alignments

E.1 Alignment of the Sequences of *HvICE2* and Two Clones Isolated by 5' RACE.

Only 696 bp of *HvICE2* (beginning at the 5' end) are shown. Clone sequences are labelled using the names of the gene-specific primers used to amplify them. Yellow and blue boxes indicate the positions of HvICE_R7 and HvICE_R3 primers, respectively.

```
>HvICE2-TC134022  ----GATCGAGTTGCAGAGGGATAAGTAGAAGTAACTGACCGTGCGAACTTGCCAAGAATCCGTGGGAGA
>ICE_R7 clone     GGGGGATCGAGTTGCAGAGGGATAAGTAGAAGTAACTGACCGTGCGAACTTGCCAAGAATCCGTGGGAGA
>ICE_R3 clone     ------------GGCAGAGGGATAAGTAGAAGTAACTGACCGTGCGAACTTGCCAAGAATCCGTGGGAGA

>HvICE2-TC134022  GGACAGAGGAGGGCGTGATGGAGAACCCGGCGGCGGTGGTGGGGGCGGAAAAGGAGGACGAGCTGGTGGG
>ICE_R7 clone     GGACAGAGGAGGGCGTGATGGAGAACCCGGCGGCGGTGGTGGGGGCGGAGAAGGAGGACGAGCTGGTGGG
>ICE_R3 clone     GGACAGAGGAGGGCGTGATGGAGAACCCGGCGGCGGTGGTGGGGGCGGAGAAGGAGGACGAGCTGGTGGG

>HvICE2-TC134022  CGGCGGCGGGGGCGACTGGGGGTACCTCACGTCGGAGGCGATGGCGACGGCCGGGTTCCCGGCGTTCGGG
>ICE_R7 clone     CGGCGGCGGGGGCGACTGGGGGTACCTCACGTCGGAGGCGATGGCGACGGCCGGGTTCCCGGCGTTCGGG
>ICE_R3 clone     CGGCGGCGGGGGCGACTGGGGGTACCTCACGTCGGAGGCGATGGCGACGGCCGGGTTCCCGGCGTTCGGG

>HvICE2-TC134022  TTCCCCTGCGGCACCAGGGGCGGCGTCACGCCCGCGCCGAACTCGGCGTCGCTGCTCATGTCCATGGAGC
>ICE_R7 clone     TTCCCCTGCGGCACCAGGGGCGGCGTCACGCCCGCGCCGAACTCGGCGTCGCTGCTCATGTCCATGGAGC
>ICE_R3 clone     TTCCCCTGCGGCACCAGGGGCGGCGTCACGCCCGCGCCGAACTCGGCGTCGCTGCTCATGTCCATGGAGC

>HvICE2-TC134022  ACGCCGCGCTGTTCGACTACAACGCCGCCTTCCCGTCGTCGTCGTCCTCCGCCGTCCCCGCGCCCCCGGC
>ICE_R7 clone     ACGCCGCGCTGTTCGACTACAACGCCGCCTTCCCGTCGTCGTCGTCCTCCGCCGTCCCCGCGCCCCCGGC
>ICE_R3 clone     ACGCCGCGCTGTTCGACTACAACGCCGCCTTCCCGTCGTCGTCGTCCTCCGCCGTCCCCGCGCCCCCGGC

>HvICE2-TC134022  ATACCACGACTTCGGGTCCGGCGGCAACCCCTTCAGCGTCGACGCCCCGCCGTTCCTTCTCGAGGCCCCG
>ICE_R7 clone     ATACCACGACTTCGGGTCCGGCGGCAACCCCTTCAGCGTCGACGCCCCGCCGTTCCTTCTCGAGGCCCCG
>ICE_R3 clone     ATACCACGACTTCGGGTCCGG----------------------------------------------------

>HvICE2-TC134022  CCGCCGCTGACGGCGGGGGCGGGAGGGCAGAAAGGGGGGTTCTTGGCGCCCCCGCTGTCGGCGTTCGGCG
>ICE_R7 clone     CCGCCGCTGACGGCGGGGGCGGGAGGGCAGAAAGGGGGGTTCTTGGCGCCCCCGCTGTCGGCCTTCGGCG
>ICE_R3 clone     ----------------------------------------------------------------------

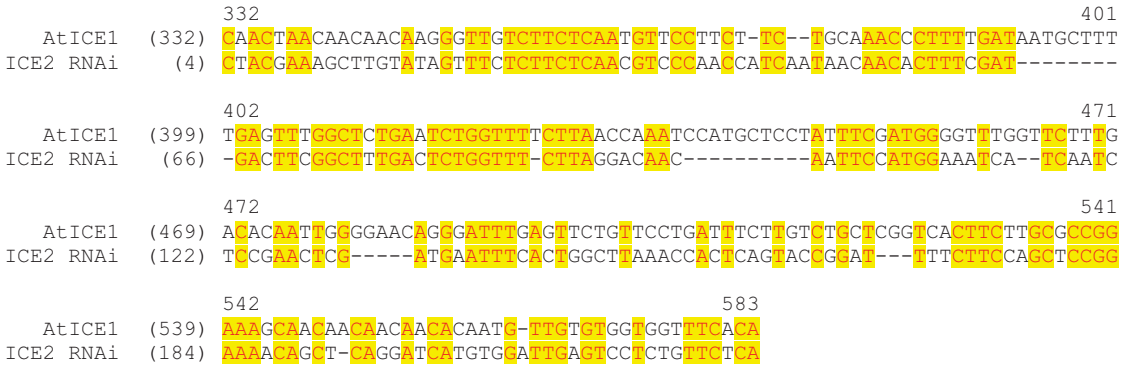
>HvICE2-TC134022  ACGGCATGGGGTGGGACGACGAGGACGAGCTGGATCAGCAGAGCATGGACGCCTCCTCCTTGGGGGTCTC
>ICE_R7 clone     ACGGCATGGGGTGGGACGACGAGGACGAGCTGGATCAGCAGAGCATGGACGCCTCCTCCTTGGGGGTCTC
>ICE_R3 clone     ----------------------------------------------------------------------

>HvICE2-TC134022  CGCCTCGCTGGAGAATGCGGCGGTCGGCGCGCCGGGGGGAGGTGGCGGCGGCGGCAACGGGAAGGGCAAG
>ICE_R7 clone     CGCCTCGCTGGAGAATGCGGCGGTCGGCGCCCCGGGGGGAGGTGGCGGCGGCGGCAACGGGAAGGGCAAG
>ICE_R3 clone     ----------------------------------------------------------------------

>HvICE2-TC134022  AAGAAGGGGATGCCGGCCAAGAACCTGATGGCGGAGCGGCGGCGCAGGAAGAAGCTCAACGACCGCCTCT
>ICE_R7 clone     AAGAAGGGGATGCCGGCCAAGAACCTG----------------------------------------------
>ICE_R3 clone     ----------------------------------------------------------------------
```

E.2 Alignment of the Nucleotide Sequences of the Fragment used for RNAi Silencing of *AtICE2* and *AtICE1* (AY195621).

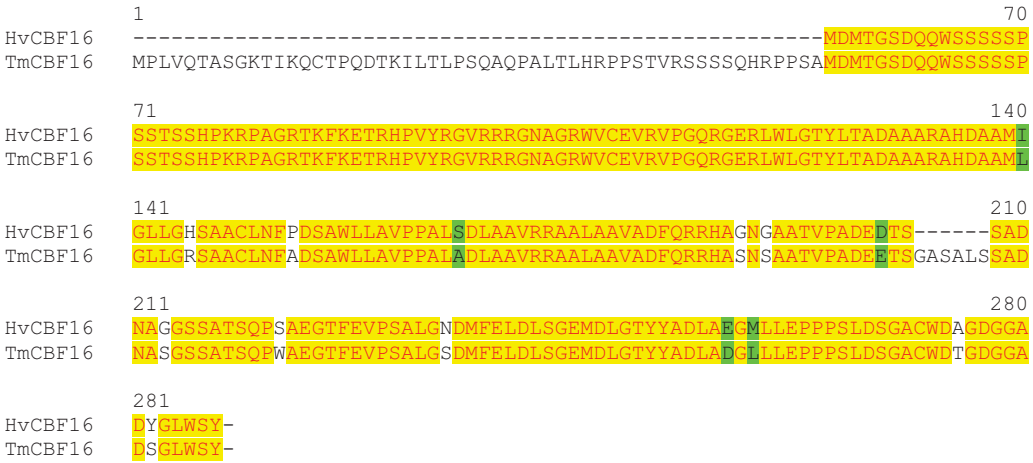
Yellow residues indicate sequence conservation.



E.3 Alignment of the Translated Nucleotide Sequences of the Coding Regions of *HvCBF16** and *TmCBF16* (EU076384).

* Isolated in this study during yeast 1-hybrid screening of a cold-treated barley cDNA library using a yeast reporter strain containing the Arabidopsis CRT/DRE as bait. The nucleotide sequence presented in this study was isolated from barley cv. Haruna Nijo and encodes an identical polypeptide sequence to the published HvCBF16 sequence from the Tremois cultivar.

Yellow indicates identical residues, green residues indicates similar residues.



E.4 Alignment of the Translated Nucleotide Sequences of the Coding Regions of *HvCBF23**, *TaDRFL2b* (ABC74512), *AtRAP2*.4 (NP_177931), *AtERF060* (NP_195688) and *HvDRF1*.3 (AAO38211).

* Isolated in this study during yeast 1-hybrid screening of a cold-treated barley cDNA library using a yeast reporter strain containing the Arabidopsis CRT/DRE as bait.

The positions of conserved motifs 1-4 which are specific to the A-6 subgroup/Group Ib ERFs (Nakano *et al.*, 2006) are marked with asterisks. The position of the AP2 domain is marked with carets.

Yellow indicates identical residues, blue indicates conserved residues, green indicates similar residues.

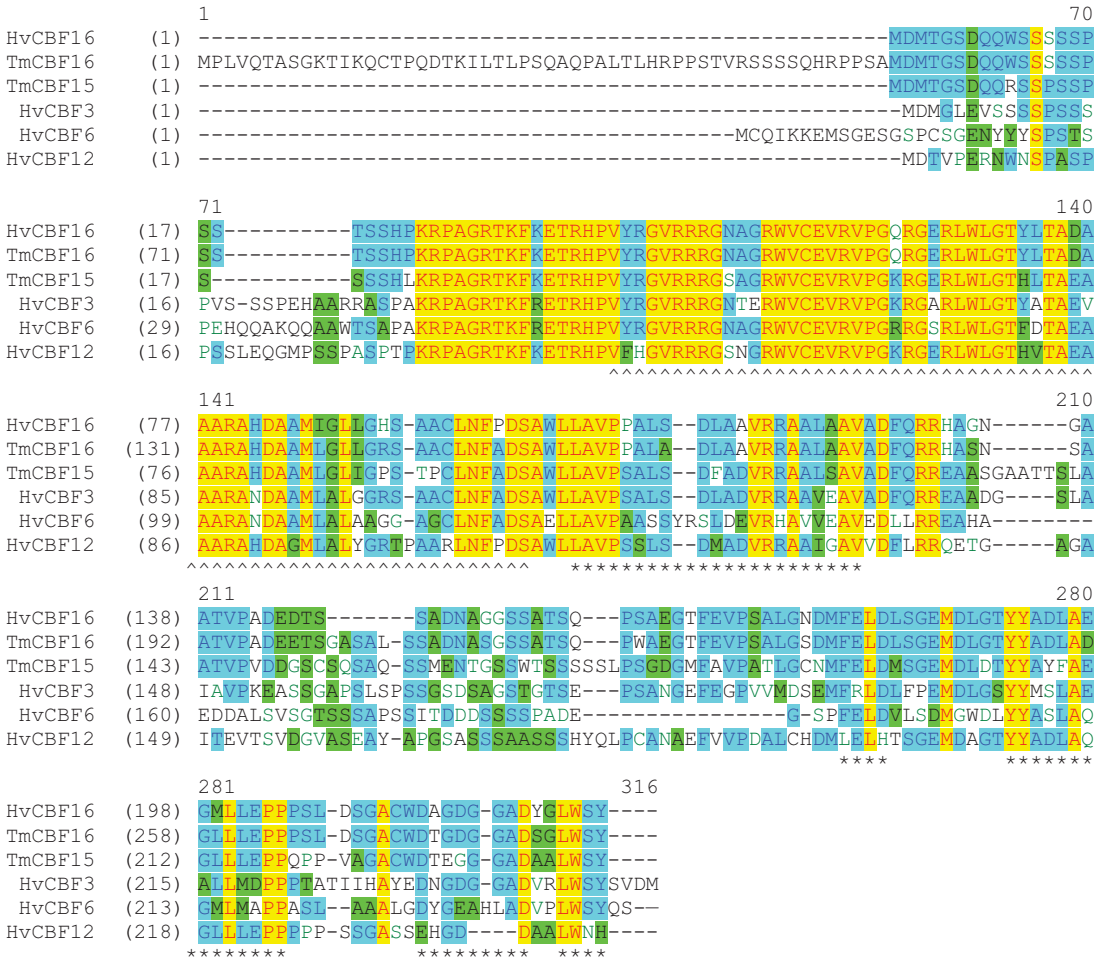
```
                 1                                                                   70
HvCBF23      (1) ----MPLLTPSSRP----SARTSPLPRTPRSPPPLHSHPSPTSP--------------------------
AtERF060     (1) -MAAIDMFNSNT---------DPFQEELMKALQPYTTNTDSSS---------------------------
TaDRFL2b     (1) MAAAIDMYKYNTSTHQIGSAASASDQELMKALEPFITIASSSSSHYPYQYYSSPSMTQNSYMATPSSSYA
AtRAP2.4     (1) MAAAMNLYTCSRS-------FQDSGGELMDALVPFIKSVSDSPS---------S-------SSAASASAF
HvDRF1.3     (1) -------MTVDRKG-------AQAAAAAAATSAPFEIPALQPGR--------------------------
                                         *****CM 4*****
                 71                                                                 140
HvCBF23     (37) --------------------FSFPHAAYSGYPYGVQAQAQTELSPAQMHYIQARLHLQRQTGQ-------
AtERF060    (34) ----------------------PTYSNTVFGFNQTTSLGLNQLTPYQIHQIQNQLNQRRN--------II
TaDRFL2b    (71) SSFAVSPLPTTAPASPSFSQLPPLYSSQYAASGMNGSMGLAQLGPAQIQQIQAQFFVQQQQQQRGL--AG
AtRAP2.4    (48) LHPSAFSLPPLPGYYPDSTFLTQPFSYGSDLQQTGSLIGLNNLSSSQIHQIQSQIHHPLPPTHHNNNNSF
HvDRF1.3    (31) -------------------KKRPRRSRDGPNSVSETIKRWKEVNQQLEHDPQGAKRARKP----------
                                                      ********CM 3*********
                 141                                                                210
HvCBF23     (80) PGHLGPRPQPMKPAS-----AAAATP-----PRPQKLYRGVRQRHWGKWVAEIRLPRNRTRLWLGTFDTA
AtERF060    (74) SPNLAPKPVPMKN------------------MTAQKLYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTA
TaDRFL2b   (139) GSFLGPRAQPMKQSGSPPRASAAALALAGVAPAQSKLYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTA
AtRAP2.4   (118) SNLLSPKPLLMKQSG---VAGSCFAYGSGVPSKPTKLYRGVRQRHWGKWVAEIRLPRNRTRLWLGTFDTA
HvDRF1.3    (72) PAKGSKKGCMQGK-G---------GP-----ENTQCGFRGVRQRTWGKWVAEIREPNRVSRLWLGTFPTA
                                                     ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^
                 211                                                                280
HvCBF23    (140) EEAALAYDQAAYRLRGDAARLNFPDNAAS----RG------PLHASVDAKLQTLCQNITASKNAKKSASV
AtERF060   (126) EEAAMAYDLAAYKLRGEFARLNFPQFRHEDGYYGGGS-CFNPLHSSVDAKLQEICQSLRKTEDIDLPC--
TaDRFL2b   (209) EDAALAYDKAAFRLRGDLARLNFPSLRRGGAHLAG------PLHASVDAKLTAICESLAAPS--------
AtRAP2.4   (185) EEAALAYDKAAYKLRGDFARLNFPNLRHNGSHIGGDFGEYKPLHSSVDAKLEAICKSMAETQKQDKST--
HvDRF1.3   (127) EVAAQAYDEAARAMYGPLARTNFPVQDAQAAPAVAVP----VATEGVVRGSSASCESTTTSNHSDVASSS
                 ^^^^^^^^^^^^^^^^^^^^^^^^^^                      *******CM 2*******
                 281                                                                350
HvCBF23    (200) SASTA--AATSSTPTSNCSSPSSDEASSSLESAESSSPSPTTTAAEVPEMQQLDFSEAPWDEAAG-----
AtERF060   (193) -------SETELFPPKTEYQESEYGFLRSDENSFSDESHVESSSPESGITTFLDFSDSGFDEIG------
TaDRFL2b   (265) ------------SK-NSEPESPKCSASTEGEDSASAGSPPPP-TPPVPEMEKLDFTEAPWDESE------
AtRAP2.4   (253) -------KSSK-KREKKVSSPDLSEKVKAEENSVSIGGSPPVTEFEESTAGSSPLSDLTFADPEEPPQWN
HvDRF1.3   (193) HNKQRQIQAPEISSRSDLLESTQSVEYSQQQSVPDAVSSIAMSTSEEDVYEPLEPISNLPDGEADCFDIE
                                                                              *********
                 351                                                                420
HvCBF23    (263) --FALTKYPSYEIDWDSLLATN------------------------------------------------
AtERF060   (250) -SFGLEKFPSVEIDWDAISKLSES----------------------------------------------
TaDRFL2b   (315) -TFHLRKYPSVEIDWDSILS--------------------------------------------------
AtRAP2.4   (315) ETFSLEKYPSYEIDWDSILA--------------------------------------------------
HvDRF1.3   (263) ELLKLMEADPVEVDPVTVGSWNEFQDAGANARGSWNEFQCARANTGVSWNANAGMEMGQQEPLYLDGLDQ
                *******CM 1********
                 421                                  460
HvCBF23    (283) ----------------------------------------
AtERF060   (273) ----------------------------------------
TaDRFL2b   (334) ----------------------------------------
AtRAP2.4   (335) ----------------------------------------
HvDRF1.3   (333) GMLEGMLHSDYPFPVWISEDRPMHNPAFHDAEMSEFFEGL
```

E.5 Alignment of the Translated Nucleotide Sequences of the Coding Regions of *HvCBF16*, TmCBF16* (EU076384), *TmCBF15* (EU076383), *HvCBF3* (AY785845), *HvCBF6* (AY785860) and *HvCBF12* (DQ095157).

* Isolated in this study during yeast 1-hybrid screening of a cold-treated barley cDNA library using a yeast reporter strain containing the Arabidopsis CRT/DRE as bait.

The positions of conserved motifs which are specific to the *HvCBF3*-subgroup (Skinner *et al.*, 2005) are marked with asterisks. The position of the AP2 domain is marked with carets.

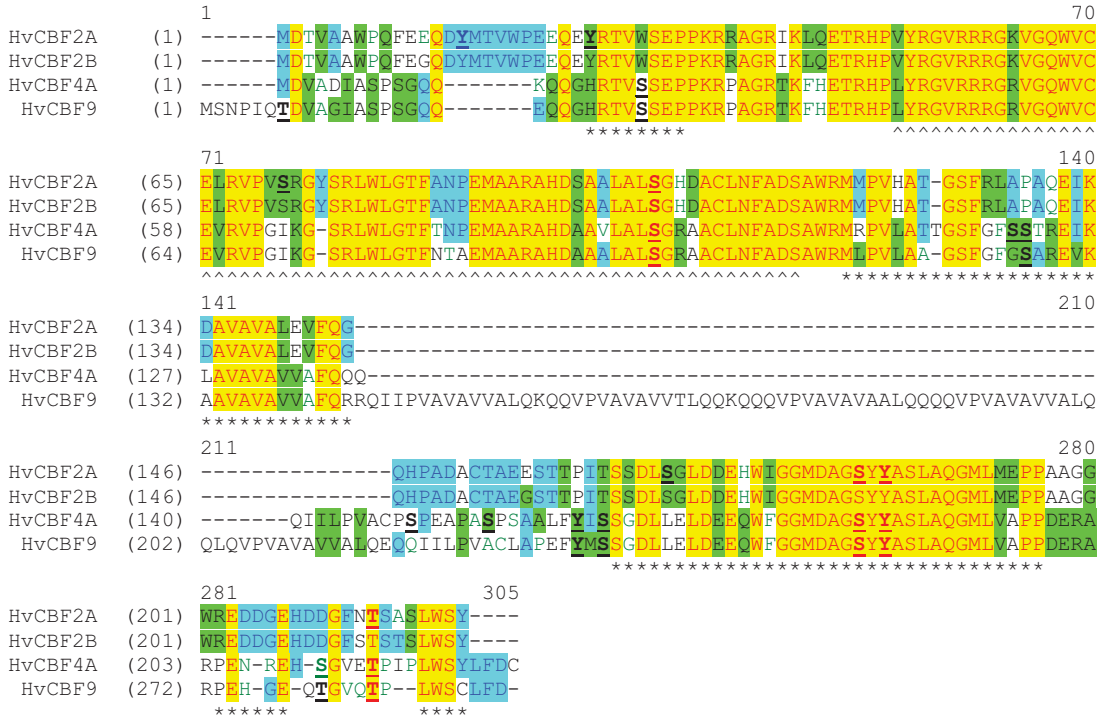
Yellow indicates identical residues, blue indicates conserved residues, green indicates similar residues.



E.6 Alignment of the Translated Nucleotide Sequences of the Coding Regions of *HvCBF2A*, *HvCBF2B* (DQ097684), *HvCBF4A* (AY785849) and *HvCBF9* (AY785878).

The positions of conserved motifs which are specific to the *HvCBF4*-subgroup (Skinner *et al.*, 2005) are marked with asterisks. The position of the AP2 domain is marked with carets. Predicted phosphorylation sites are marked as underlined bold residues on the HvCBF2A, HvCBF4A and HvCBF9 sequences.

Yellow indicates identical residues, blue indicates conserved residues, green indicates similar residues.



Appendix F. DNA Sequences and Accession Numbers

F.1 *HvICE2* Coding Sequence

Sequence from EST contig TC134022. Putative start codon marked in red.

```
   1 atggagaacc cggcggcggt ggtgggggcg gaaaaggagg acgagctggt gggcggcggc
  61 gggggcgact gggggtacct cacgtcggag gcgatggcga cggccgggtt cccggcgttc
 121 gggttcccct gcggcaccag gggcggcgtc acgcccgcgc cgaactcggc gtcgctgctc
 181 atgtccatgg agcacgccgc gctgttcgac tacaacgccg ccttcccgtc gtcgtcgtcc
 241 tccgccgtcc ccgcgccccc ggcataccac gacttcgggt ccggcggcaa ccccttcagc
 301 gtcgacgccc cgccgttcct tctcgaggcc ccgccgccgc tgacggcggg ggcgggaggg
 361 cagaaagggg ggttcttggc gcccccgctg tcggcgttcg gcgacggcat ggggtgggac
 421 gacgaggacg agctggatca gcagagcatg gacgcctcct ccttgggggt ctccgcctcg
 481 ctggagaatg cggcggtcgg cgcgccgggg ggaggtggcg gcggcggcaa cgggaagggc
 541 aagaagaagg ggatgccggc caagaacctg atggcggagc ggcggcgcag gaagaagctc
 601 aacgaccgcc tctacatgct gcgctccgtg gtgcccaaga tcagcaagat ggacagggct
 661 tcaatcctcg gtgacgcaat tgactacctg aaggagctcc tgcagaggat cagcgatctt
 721 cactccgagc tcgagtctgc tccaagctct gctgcactcg gtggaccatc gacggccaat
 781 accttcctgc cgtcgacgcc cactctgcag ccgttccccg gccgcatcaa ggaggagcgg
 841 tgcccgccgg ccccgttccc tagccccagc ggccagcagg cgacggttga ggtgaggatg
 901 agggaggggc aggcggtgaa catccacatg ttctgcgcgc gcaggccggg catcctgctg
 961 tccaccatga gggcgctgga cagcctcggc ctcgacatcg agcaggccgt catcagctgc
1021 ttcgacggct tcgccatgga cgtcttccgc gccgagcaat gcagggaggg ccctgggctg
1081 ctgccggagg aaattaaggc ggtgctcctg cactgcgccg gtctccagaa cgcgatgtag
```

F.2 Sequence of the Maize *Ubiquitin* Promoter

Fragment was ligated into pMDC32 to replace the dual 35S promoter using the *Hind*III (red) and *Kpn*I (blue) restriction sites. Sequence is presented 5' to 3', with the 3' end being positioned directly upstream of the gateway recombination sites for cloning (example of completed transformation construct in Figure 2.22).

```
   1 aagcttgcat gcctgcagtg cagcgtgacc cggtcgtgcc cctctctaga gataatgagc
  61 attgcatgtc taagttataa aaaattacca catatttttt ttgtcacact tgtttgaagt
 121 gcagtttatc tatctttata catatattta aactttactc tacgaataat ataatctata
 181 gtactacaat aatatcagtg ttttagagaa tcatataaat gaacagttag acatggtcta
 241 aaggacaatt gagtattttg acaacaggac tctacagttt tatcttttta gtgtgcatgt
 301 gttctccttt ttttttgcaa atagcttcac ctatataata cttcatccat tttattagta
 361 catccattta gggtttaggg ttaatggttt ttatagacta atttttttag tacatctatt
 421 ttattctatt ttagcctcta aattaagaaa actaaaactc tattttagtt tttttattta
 481 ataatttaga tataaaatag aataaaataa agtgactaaa aattaaacaa ataccttta
 541 agaaattaaa aaaactaagg aaacattttt cttgtttcga gtagataatg ccagcctgtt
 601 aaacgccgtc gatcgacgag tctaacggac accaaccagc gaaccagcag cgtcgcgtcg
 661 ggccaagcga agcagacggc acggcatctc tgtcgctgcc tctggacccc tctcgagagt
 721 tccgctccac cgttggactt gctccgctgt cggcatccag aaattgcgtg gcggagcggc
 781 agacgtgagc cggcacggca ggcggcctcc tcctcctctc acggcaccgg cagctacggg
 841 ggattccttt cccaccgctc cttcgctttc ccttcctcgc ccgccgtaat aaatagacac
 901 cccctccaca ccctctttcc ccaacctcgt gttgttcgga gcgcacacac acacaaccag
 961 atctcccccа aatccacccg tcggcacctc cgcttcaagg tacgccgctc gtcctccccc
1021 ccccccctc tctaccttct ctagatcggc gttccggtcc atggttaggg cccggtagtt
1081 ctacttctgt tcatgtttgt gttagatccg tgtttgtgtt agatccgtgc tgctagcgtt
1141 cgtacacgga tgcgacctgt acgtcagaca cgttctgatt gctaacttgc cagtgtttct
1201 ctttggggaa tcctgggatg gctctagccg ttccgcagac gggatcgatc taggataggt
1261 atacatgttg atgtgggttt tactgatgca tatacatgat ggcatatgca gcatctattc
1321 atatgctcta accttgagta cctatctatt ataataaaca agtatgtttt ataattattt
1381 tgatcttgat atacttggat gatggcatat gcagcagcta tatgtggatt ttttagccc
1441 tgccttcata cgctatttat ttgcttggta ctgtttcttt tgtcgatgct caccctgttg
1501 tttggtgtta cttctgcagg tcgactctag aggatccccg ggtacc
```

F.3 *AtICE2* (*Arabidopsis thaliana*, Columbia-0 Ecotype)*

The consensus sequence of the coding region of *AtICE2*, produced by alignment of sequencing of the *AtICE2* over-expression construct used for plant transformation.

*Section used RNAi silencing of *AtICE2* highlighted in red.

```
   1 atgaacagcg acggtgtttg gcttgacggc tccggtgaat ctccggaggt taataacggt
  61 gaagctgcgt cttgggtcag aaacccagat gaagactggt tcaataaccc accaccacca
 121 caacacacta atcaaaacga cttcagattc aatggtgggt ttcctttaaa cccctcagag
 181 aatctgcttc ttcttcttca gcaatcgatt gattcttctt cttcttctcc gttattacat
 241 cctttcacac tcaacgctac ttcacagcaa caacaacaac aggaacagtc tttcttagct
 301 acaaaagctt gtatagtttc tcttctcaac gtcccaacca tcaataacaa cactttcgat
 361 gacttcggct ttgactctgg tttcttagga caacaattcc atggaaatca tcaatctccg
 421 aactcgatga atttcactgg cttaaaccac tcagtaccgg attttcttcc agctccggaa
 481 aacagctctg gatcatgtgg attgagtcct ctgttctcga acagagcaaa ggttttaaaa
 541 ccgttacagg taatggcttc atctggctcg cagccaactc tgtttcagaa acgagctgca
 601 atgcgtcaga gttcgactag caaatcagag agttcttctg aaatgaggaa atcgagctac
 661 gagagagaga ttgacgatac tagtaccgga atcatcgata tctctggatt gaattacgaa
 721 tctgatgacc ataatactaa taacaacaaa ggtaagaaga aaggaatgcc tgcaaagaac
 781 cttatggctg agagaagaag aaggaagaag cttaatgata ggctttacat gcttagatca
 841 gttgttccca agatcagcaa aatggataga gcatcaatac ttggagatgc tattgattac
 901 ctcaaagagc ttttacaaag aatcaacgat cttcataccg aactcgaatc tactccaccg
 961 agttcttcaa gcttgcatcc gttaacaccg actccacaaa cgctgtctta ccgtgttaag
1021 gaagagttgt gtccatcttc ctccttgcca agccctaaag gccagcaacc aagagttgag
1081 gttagattaa gagaaggaaa ggcagtgaac attcacatgt tctgtggacg tagaccaggt
1141 cttttacttt ccaccatgag agctttggat aacctaggat tggatgttca acaagctgtg
1201 attagctgtt tcaacggttt tgctttggat gttttccgcg ctgagcaatg tcaagaagac
1261 catgacgtgt tacctgaaca aatcaaagca gtgcttttag atacagcagg ttacgctggt
1321 ttggtttga
```

F.4 *HvCBF16* (*Hordeum vulgare* L. cv. Haruna Nijo)

The consensus sequence of the coding region of *HvCBF16*, produced by alignment of the sequences of *HvCBF16* clones.

```
   1 atggacatga ccgggtcgga ccagcaatgg agctcctctt cctcgccgtc atcgacctcc
  61 tcgcacccga agcgccccgc cgggcgcacc aagttcaagg agacgcgcca cccggtgtac
 121 cgcggcgtgc ggcgccgggg caacgccggc cgctgggtgt gcgaggtgcg ggtccctggg
 181 cagcgcggcg agcggctttg gctcggcacg tacctcaccg ccgacgcggc cgcacgcgcg
 241 cacgatgccg ccatgatcgg cctgctcggc cactcagccg cgtgcctcaa cttccccgac
 301 tccgcgtggc tcctggccgt gccacccgcg ctctccgacc tcgcggccgt ccggcgcgcg
 361 gccctcgccg ccgtagcgga cttccagcgg cggcatgccg gcaacggcgc agccaccgtc
 421 cctgccgatg aggacacctc cagcgcggac aatgcgggcg gctcgtcggc gacgtctcag
 481 ccttcggccg aggggacgtt cgaagtgcca tccgcgctgg gcaacgacat gttcgagctg
 541 gacttgtctg gggagatgga cctgggcacg tactacgccg acctcgcgga ggggatgctc
 601 ctggagccgc cgccgtcgct ggacagcggg gcgtgctggg atgccggaga cggcggagct
 661 gactacgggc tatggagcta ctga
```

F.5 *HvCBF23* (*Hordeum vulgare* L. cv Haruna Nijo)

The consensus sequence of *HvCBF23*, produced by alignment of the sequences of *HvCBF23* clones. Putative start codon marked in red.

```
   1 cggcccgctc catgcctctg ttgacgccaa gctccagacc ctctgccaga acatcaccgc
  61 ttccaagaac gccaagaagc ccccctccgc tccactccca tcctagtccc acctcgccat
 121 tctccttccc ccacgccgcc tacagtggtt acccgtacgg ggtgcaggca caggcccaga
 181 ccgagctcag cccggcccag atgcactaca tccaggcacg cctccacctc cagcgccaga
 241 ccggccagcc gggccacctc ggcccgcggc cccagcccat gaagcccgct tcggcggcag
 301 cggccacacc gccgcggccg cagaagctct accgcggcgt tcggcagcgc cactggggca
 361 agtgggtggc ggagatccgc ctcccccgca accgcacccg cctctggctc ggcaccttcg
 421 acaccgccga ggaggcggct ctcgcctacg accaggccgc ctaccgcctc cgtggcgacg
 481 cagcgcgcct caacttcccc gacaacgccg cctcccgcgg cccgctccat gcctctgttg
 541 acgccaagct ccagaccctc tgccagaaca tcaccgcttc caagaacgcc aagaagtccg
 601 cctccgtctc cgcgtccacc gccgcagcca cgtcgtccac ccccaccagc aactgctcct
 661 cgccgtcctc cgacgaggcg tcgtcctcgc tcgagtccgc cgagtcgtcg tcaccatcac
 721 ccaccaccac cgcagcagag gttcctgaga tgcagcagct cgacttcagc gaggcaccat
 781 gggacgaggc agccggcttc gccctcacca agtacccgtc ctatgagatc gactgggact
 841 cgctcctcgc caccaattag cacccagttc accttcgtca gctactacta ccagtaccgt
 901 cttttagcgt gtcatgatgc taggttaatg ggtcgccgcg atgcagatgg cattttagac
 961 attctgcgcc ggcctttagc ggattagctc taagtctcta atccttgttc attgtgtaga
1021 cctatgattc gttctctttg tggtagggtt tggttagtcc ctcccggatg actataagcc
1081 ggcgtttttg tgcccggcgt ctccggtggt cggtcactgg tcagtgactc cggccggtga
1141 agtctgtcca ttgttctagc taggtgctgt tccttccgct gctctgcaga ttgtaacagt
1201 gggagacttg tcatgtaaaa tcagctcatc aaaaaatcgt gtaatgtgga aaa
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References

Australian Bureau of Statistics (2008) 2008 Year Book Australia. www.abs.gov.au/

Achard P, Gong F, Cheminant S, Alioua M, Hedden P and Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins *via* its effect on gibberellin metabolism. *Plant Cell,* **20**, 2117-29.

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X and Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of *CBF* genes and in acquired freezing tolerance. *J Biol Chem,* **281**, 37636-45.

Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M and Suzuki M (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO J,* **17**, 5484-96.

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C and Thomashow MF (1996) Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. *P Natl Acad Sci USA,* **93**, 13404-9.

Australian Wheat Board (2005) AWB website. www.awb.com.au

Badawi M, Danyluk J, Boucho B, Houde M and Sarhan F (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBFs*. *Mol Genet Genomics,* **277**, 533-54.

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F and Houde M (2008) Structure and functional analysis of wheat *ICE* (*inducer of CBF expression*) genes. *Plant Cell Physiol,* **49**, 1237-49.

Baga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB and Chibbar RN (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. *Funct Integr Genomic,* **7**, 53-68.

Baxevanis AD and Vinson CR (1993) Interactions of coiled coils in transcription factors: where is the specificity? *Curr Opin Genet Dev,* **3**, 278-85.

Benedict C, Geisler M, Trygg J, Huner N and Hurry V (2006) Consensus by democracy. Using meta-analyses of microarray and genomic data to model the cold acclimation signaling pathway in Arabidopsis. *Plant Physiol,* **141**, 1219-32.

Berendzen KW, Stuber K, Harter K and Wanke D (2006) *Cis*-motifs upstream of the transcription and translation initiation sites are effectively revealed by their positional disequilibrium in eukaryote genomes using frequency distribution curves. *BMC Bioinformatics,* **7**, 522.

Boyce JM, Knight H, Deyholos M, Openshaw MR, Galbraith DW, Warren G and Knight MR (2003) The *sfr6* mutant of Arabidopsis is defective in transcriptional activation *via* CBF/DREB1 and DREB2 and shows sensitivity to osmotic stress. *Plant J,* **34**, 395-406.

Boyer JS (1982) Plant productivity and environment. *Science,* **218**, 443-8.

Bray EA, Bailey-Serres J and Weretilnyk E (2000) Responses to abiotic stress. *Biochemistry and molecular biology of plants.* Rockville, M.D., American Society of Plant Physiologists.

Burton RA, Jobling SA, Harvey AJ, Shirley NJ, Mather DE, Bacic A and Fincher GB (2008) The genetics and transcriptional profiles of the cellulose synthase-like *HvCslF* gene family in barley. *Plant Physiol,* **146**, 1821-33.

Burton RA, Shirley NJ, King BJ, Harvey AJ and Fincher GB (2004) The *CesA* gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. *Plant Physiol,* **134**, 224-36.

Campoli C, Matus-Cadiz MA, Pozniak CJ, Cattivelli L and Fowler DB (2009) Comparative expression of *Cbf* genes in the *Triticeae* under different acclimation induction temperatures. *Mol Genet Genomics,* **2**, 141-52.

Cattivell L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchioni N and Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. *Plant Mol Biol,* **48**, 649-65.

Chen M, Xu Z, Xia L, Li L, Cheng X, Dong J, Wang Q and Ma Y (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, *GmDREB3*, in soybean (*Glycine max* L.). *J Exp Bot,* **60**, 121-35.

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X and Zhu T (2002) Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell,* **14**, 559-74.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M and Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev,* **17**, 1043-54.

Chinnusamy V, Schumaker K and Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J Exp Bot,* **55**, 225-36.

Chinnusamy V, Zhu J and Zhu JK (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci,* **12**, 444-51.

Choi DW, Rodriguez EM and Close TJ (2002) Barley *Cbf3* gene identification, expression pattern, and map location. *Plant Physiol,* **129**, 1781-7.

Choi DW, Zhu B and Close TJ (1999) The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv Dicktoo. *Theor Appl Genet,* **98**, 1234-47.

Christensen AH, Sharrock RA and Quail PH (1992) Maize *polyubiquitin* genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol Biol,* **18**, 675-89.

Cook D, Fowler S, Fiehn O and Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *P Natl Acad Sci USA,* **101**, 15243-8.

Curtis MD and Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol,* **133**, 462-9.

Dong CH, Agarwal M, Zhang Y, Xie Q and Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *P Natl Acad Sci USA,* **103**, 8281-6.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J,* **33**, 751-63.

Fowler S and Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the *CBF* cold response pathway. *Plant Cell,* **14**, 1675-90.

Fowler SG, Cook D and Thomashow MF (2005) Low temperature induction of Arabidopsis *CBF1, 2,* and *3* is gated by the circadian clock. *Plant Physiol,* **137**, 961-8.

Francia E, Barabaschi D, Tondelli A, Laido G, Rizza F, Stanca AM, Busconi M, Fogher C, Stockinger EJ and Pecchioni N (2007) Fine mapping of a *HvCBF* gene cluster at the frost resistance locus *Fr-H2* in barley. *Theor Appl Genet,* **115**, 1083-91.

Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS and Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) x 'Tremois' (spring) barley map. *Theor Appl Genet*, **108**, 670-80.

Galiba G, Vagujfalvi A, Li C, Soltész A and Dubcovsky J (2009) Regulatory genes involved in the determination of frost tolerance in temperature cereals. *Plant Sci,* **176**, 12-9.

Gilmour SJ, Fowler SG and Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol Biol,* **54**, 767-81.

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD and Thomashow MF (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol,* **124**, 1854-65.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM and Thomashow MF (1998) Low temperature regulation of the Arabidopsis *CBF* family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J,* **16**, 433-42.

Gong Z, Lee H, Xiong L, Jagendorf A, Stevenson B and Zhu JK (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *P Natl Acad Sci USA,* **99**, 11507-12.

Grains Research and Development Corporation (2005) Frost Risk Fact Sheet. *Grain zone: For growers,* www.grdc.com.au/growers

Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF and Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. *Plant Physiol*, **130**, 639-48.

Hartung W, Sauter A and Hose E (2002) Abscisic acid in the xylem: where does it come from, where does it go to? *J Exp Bot*, **53**, 27-32.

Hayes PM, Blake T, Chen TH, Tragoonrung S, Chen F, Pan A and Liu B (1993) Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness. *Genome,* **36**, 66-71.

Huang JG, Yang M, Liu P, Yang GD, Wu CA and Zheng CC (2009) *GhDREB1* enhances abiotic stress tolerance, delays GA-mediated development and represses cytokinin signalling in transgenic Arabidopsis. *Plant Cell Environ*.

Ishitani M, Xiong L, Lee H, Stevenson B and Zhu JK (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in Arabidopsis. *Plant Cell,* **10**, 1151-61.

Ishitani M, Xiong L, Stevenson B and Zhu JK (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell,* **9**, 1935-49.

Islam AKM, Shepherd KW and Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. *Heredity,* **46**, 161-74.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol*, **47**, 141-53.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O and Thomashow MF (1998) Arabidopsis *CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science,* **280**, 104-6.

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T and Thomashow MF (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol,* **127**, 910-7.

James VA, Neibaur I and Altpeter F (2008) Stress inducible expression of the *DREB1A* transcription factor from xeric, *Hordeum spontaneum* L. in turf and forage grass (*Paspalum notatum Flugge*) enhances abiotic stress tolerance. *Transgenic Res,* **17**, 93-104.

Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu JK and Torii KU (2008) SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to Arabidopsis stomatal differentiation. *Plant Cell,* **20**, 1775-85.

Karakousis A, Barr AR, Kretschmer JM, Manning S, Jefferies SP, Chalmers KJ, Islam AKM and Langridge P (2003) Mapping and QTL analysis of the barley population Clipper x Sahara. *Aust J Agr Res,* **54**, 1137-40.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol,* **17**, 287-91.

Kizis D, Lumbreras V and Pages M (2001) Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. *FEBS Lett,* **498**, 187-9.

Kizis D and Pages M (2002) Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway. *Plant J,* **30**, 679-89.

Kleinhofs A and Graner A (2001) An integrated map of the barley genome. IN Phillips RL & Vasil IK (Eds.) *DNA based markers in plants.* Dordrecht, Netherlands, Kluwer Academic Publishers.

Knox AK, Li C, Vagujfalvi A, Galiba G, Stockinger EJ and Dubcovsky J (2008) Identification of candidate *CBF* genes for the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. *Plant Mol Biol,* **67**, 257-70.

Lee BH, Henderson DA and Zhu JK (2005) The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. *Plant Cell,* **17**, 3155-75.

Lee H, Xiong L, Gong Z, Ishitani M, Stevenson B and Zhu JK (2001) The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. *Genes Dev,* **15**, 912-24.

Lee SC, Huh KW, An K, An G and Kim SR (2004) Ectopic expression of a cold-inducible transcription factor, *CBF1/DREB1b*, in transgenic rice (*Oryza sativa* L.). *Mol Cells,* **18**, 107-14.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell,* **10**, 1391-406.

Lopato S, Bazanova N, Morran S, Milligan AS, Shirley N and Langridge P (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Methods,* **2**, 3.

Manly KF, Cudmore RH, Jr. and Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm Genome,* **12**, 930-2.

Mano Y and Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). *Euphytica,* **94**, 263-72.

Martinez A, Traverso JA, Valot B, Ferro M, Espagne C, Ephritikhine G, Zivy M, Giglione C and Meinnel T (2008) Extent of N-terminal modifications in cytosolic proteins from eukaryotes. *Proteomics,* **8**, 2809-31.

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K and Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J,* **38**, 982-93.

Matthews PR, Wang M-B, Waterhouse PM, Thornton S, Fieg SJ, Gubler F and Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard *Agrobacterium* transformation vector. *Mol Breeding,* **7**, 195-202.

Medina J, Bargues M, Terol J, Perez-Alonso M and Salinas J (1999) The Arabidopsis *CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol,* **119**, 463-70.

Meijer AH, Ouwerkerk PB and Hoge JH (1998) Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. *Yeast,* **14**, 1407-15.

Miller AK, Galiba G and Dubcovsky J (2006) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. *Mol Genet Genomics,* **275**, 193-203.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci,* **7**, 405-10.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ and Hasegawa PM (2007) SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in Arabidopsis. *Plant Cell,* **19**, 1403-14.

Munns R and Tester M (2008) Mechanisms of salinity tolerance. *Annu Rev Plant Biol,* **59**, 651-81.

Murre C, McCaw PS and Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell,* **56**, 777-83.

Nakano T, Suzuki K, Fujimura T and Shinshi H (2006) Genome-wide analysis of the *ERF* gene family in Arabidopsis and rice. *Plant Physiol,* **140**, 411-32.

Novillo F, Alonso JM, Ecker JR and Salinas J (2004) CBF2/DREB1C is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in Arabidopsis. *P Natl Acad Sci USA,* **101**, 3985-90.

Novillo F, Medina J and Salinas J (2007) Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. *P Natl Acad Sci USA,* **104**, 21002-7.

Oh SJ, Kwon CW, Choi DW, Song SI and Kim JK (2007) Expression of barley *HvCBF4* enhances tolerance to abiotic stress in transgenic rice. *Plant Biotechnol J,* **5**, 646-56.

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH and Kim JK (2005) Arabidopsis *CBF3*/*DREB1A* and *ABF3* in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol,* **138**, 341-51.

Orvar BL, Sangwan V, Omann F and Dhindsa RS (2000) Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *Plant J,* **23**, 785-94.

Oziel A, Hayes PM, Chen FM and Jones B (1996) Application of quantitative trait locus mapping to the development of winter habit malting barley. *Plant Breeding,* **115**, 43-51.

Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I and Bedö Z (1994) Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare* L.). *Theor Appl Genet,* **89**, 900-10.

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K and Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana DREB1A* gene delays water stress symptoms under greenhouse conditions. *Genome,* **47**, 493-500.

Pennycooke JC, Cheng H, Roberts SM, Yang Q, Rhee SY and Stockinger EJ (2008) The low temperature-responsive, *Solanum CBF1* genes maintain high identity in their upstream regions in a genomic environment undergoing gene duplications, deletions, and rearrangements. *Plant Mol Biol,* **67**, 483-97.

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K and Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel *DREB1*/*CBF* transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol,* **45**, 1042-52.

Reinheimer JL, Barr AR and Eglinton JK (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). *Theor Appl Genet,* **109**, 1267-74.

Riechmann JL and Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. *Biol Chem,* **379**, 633-46.

Rogowsky PM, Guidet FLY, Langridge P, Shepherd KW and Koebner RMD (1991) Isolation and characterization of wheat-rye recombinants involving chromosome arm 1DS of wheat. *Theor Appl Genet,* **82**, 537-44.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K and Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Bioph Res Co,* **290**, 998-1009.

Sambrook J, Fritsch EF and Manniatis T (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.*, Cold Spring Harbor Laboratory Press.

Sangwan V, Foulds I, Singh J and Dhindsa RS (2001) Cold-activation of *Brassica napus BN115* promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx. *Plant J,* **27**, 1-12.

Sauter A, Davies WJ and Hartung W (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *J Exp Bot,* **52**, 1991-7.

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Huner NP, Shinozaki K and Singh J (2005) The effect of overexpression of two Brassica *CBF/DREB1*-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant Cell Physiol,* **46**, 1525-39.

Schwacke R, Fischer K, Ketelsen B, Krupinska K and Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice. *Mol Genet Genomics,* **277**, 631-46.

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y and Shinozaki K (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. *Funct Integr Genomic,* **2**, 282-91.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y and Shinozaki K (2001) Monitoring the expression pattern of

1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell,* **13**, 61-72.

Shaikhali J, Heiber I, Seidel T, Stroher E, Hiltscher H, Birkmann S, Dietz KJ and Baier M (2008) The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. *BMC Plant Biol,* **8**, 48.

Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q and Chen SY (2003) An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. *Theor Appl Genet,* **106**, 923-30.

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K and Shinozaki K (1998) An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem Bioph Res Co,* **250**, 161-70.

Skinner JS, Szucs P, von Zitzewitz J, Marquez-Cedillo L, Filichkin T, Stockinger EJ, Thomashow MF, Chen TH and Hayes PM (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in Arabidopsis. *Theor Appl Genet,* **112**, 832-42.

Skinner JS, von Zitzewitz J, Szucs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen TH and Hayes PM (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. *Plant Mol Biol,* **59**, 533-51.

Snape SW, Sarma R, Quarrie SA, Fish L, Galiba G and Sutka J (2001) Mapping genes for flowering time and frost tolerance in cereals using precise genetic stocks. *Euphytica,* **120**, 309-15.

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Ann Rev Plant Physio,* **35**, 543-84.

Stockinger EJ, Gilmour SJ and Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *P Natl Acad Sci USA,* **94**, 1035-40.

Stockinger EJ, Skinner JS, Gardner KG, Francia E and Pecchioni N (2007) Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*. *Plant J,* **51**, 308-21.

Sutton F, Chen DG, Ge X and Kenefick D (2009) *Cbf* genes of the *Fr-A2* allele are differentially regulated between long-term cold acclimated crown tissue of freeze-resistant and -susceptible, winter wheat mutant lines. *BMC Plant Biol,* **9**, 34.

Takumi S, Shimamura C and Kobayashi F (2008) Increased freezing tolerance through up-regulation of downstream genes *via* the wheat *CBF* gene in transgenic tobacco. *Plant Physiol Bioch,* **46**, 205-11.

Tang M, Sun J, Liu Y, Chen F and Shen S (2007) Isolation and functional characterization of the *JcERF* gene, a putative AP2/EREBP domain-containing transcription factor, in the woody oil plant *Jatropha curcas*. *Plant Mol Biol,* **63**, 419-28.

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Phys,* **50**, 571-99.

Thorlby G, Veale E, Butcher K and Warren G (1999) Map positions of *SFR* genes in relation to other freezing-related genes of *Arabidopsis thaliana*. *Plant J,* **17**, 445-52.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J,* **11**.

Toledo-Ortiz G, Huq E and Quail PH (2003) The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell*, **15**, 1749-70.

Tommasini L, Svensson JT, Rodriguez EM, Wahid A, Malatrasi M, Kato K, Wanamaker S, Resnik J and Close TJ (2008) *Dehydrin* gene expression provides an indicator of low temperature and drought stress: transcriptome-based analysis of barley (*Hordeum vulgare* L.). *Funct Integr Genomic*, **8**, 387-405.

Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger EJ, Stanca AM and Pecchioni N (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. *Theor Appl Genet*, **112**, 445-54.

Toth B, Galiba G, Feher E, Sutka J and Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. *Theor Appl Genet*, **107**, 509-14.

Usadel B, Blasing OE, Gibon Y, Poree F, Hohne M, Gunter M, Trethewey R, Kamlage B, Poorter H and Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. *Plant Cell Environ*, **31**, 518-47.

Vagujfalvi A, Aprile A, Miller A, Dubcovsky J, Delugu G, Galiba G and Cattivelli L (2005) The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat. *Mol Genet Genomics*, **274**, 506-14.

Vagujfalvi A, Crosatti C, Galiba G, Dubcovsky J and Cattivelli L (2000) Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost-tolerant and frost-sensitive genotypes. *Mol Gen Genet*, **263**, 194-200.

Vagujfalvi A, Galiba G, Cattivelli L and Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. *Mol Genet Genomics*, **269**, 60-7.

Viswanathan C and Zhu JK (2002) Molecular genetic analysis of cold-regulated gene transcription. *Philos T R Soc Lon B*, **357**, 877-86.

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG and Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. *Plant J*, **41**, 195-211.

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen TH, Hayes PM and Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. *Plant Mol Biol*, **59**, 449-67.

Wang W, Vinocur B and Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, **218**, 1-14.

Wang Z, Triezenberg SJ, Thomashow MF and Stockinger EJ (2005) Multiple hydrophobic motifs in Arabidopsis CBF1 COOH-terminus provide functional redundancy in trans-activation. *Plant Mol Biol*, **58**, 543-59.

Wilkinson S and Davies WJ (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant Cell Environ*, **25**, 195-210.

Xin Z and Browse J (1998) *Eskimo1* mutants of Arabidopsis are constitutively freezing-tolerant. *P Natl Acad Sci USA*, **95**, 7799-804.

Xiong L, Lee B, Ishitani M, Lee H, Zhang C and Zhu JK (2001) *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes Dev*, **15**, 1971-84.

Xiong L, Schumaker KS and Zhu JK (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell*, **14 Suppl**, S165-83.

Xue GP (2002a) An AP2 domain transcription factor HvCBF1 activates expression of cold-responsive genes in barley through interaction with a (G/a)(C/t)CGAC motif. *Biochim Biophys Acta*, **1577**, 63-72.

Xue GP (2002b) Characterisation of the DNA-binding profile of barley HvCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. *Nucleic Acids Res,* **30**, e77.

Xue GP (2003) The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. *Plant J,* **33**, 373-83.

Yamaguchi-Shinozaki K and Shinozaki K (1993) The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of *rd22*, a gene responsive to dehydration stress in *Arabidopsis thaliana*. *Mol Gen Genet,* **238**, 17-25.

Yamaguchi-Shinozaki K and Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell,* **6**, 251-64.

Zarka DG, Vogel JT, Cook D and Thomashow MF (2003) Cold induction of Arabidopsis *CBF* genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol,* **133**, 910-8.

Zhang JY, Broeckling CD, Sumner LW and Wang ZY (2007) Heterologous expression of two *Medicago truncatula* putative *ERF* transcription factor genes, *WXP1* and *WXP2*, in Arabidopsis led to increased leaf wax accumulation and improved drought tolerance, but differential response in freezing tolerance. *Plant Mol Biol,* **64**, 265-78.

Zhao H and Bughrara SS (2008) Isolation and characterization of cold-regulated transcriptional activator *LpCBF3* gene from perennial ryegrass (*Lolium perenne* L.). *Mol Genet Genomics,* **279**, 585-94.

Zhou J, Li F, Wang JL, Ma Y, Chong K and Xu YY (2009) Basic helix-loop-helix transcription factor from wild rice (*OrbHLH2*) improves tolerance to salt- and osmotic stress in Arabidopsis. *J Plant Physiol,* **166**, 1296-306.

Zielinski RE (1998) Calmodulin and calmodulin-binding proteins in plants. *Annu Rev Plant Phys,* **49**, 697-725.

Achard P, Gong F, Cheminant S, Alioua M, Hedden P and Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins *via* its effect on gibberellin metabolism. *Plant Cell,* **20**, 2117-29.

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X and Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of *CBF* genes and in acquired freezing tolerance. *J Biol Chem,* **281**, 37636-45.

Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M and Suzuki M (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO J,* **17**, 5484-96.

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C and Thomashow MF (1996) Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. *P Natl Acad Sci USA,* **93**, 13404-9.

Badawi M, Danyluk J, Boucho B, Houde M and Sarhan F (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBFs*. *Mol Genet Genomics,* **277**, 533-54.

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F and Houde M (2008) Structure and functional analysis of wheat *ICE* (*inducer of CBF expression*) genes. *Plant Cell Physiol,* **49**, 1237-49.

Baga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB and Chibbar RN (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. *Funct Integr Genomic,* **7**, 53-68.

Baxevanis AD and Vinson CR (1993) Interactions of coiled coils in transcription factors: where is the specificity? *Curr Opin Genet Dev,* **3**, 278-85.

Benedict C, Geisler M, Trygg J, Huner N and Hurry V (2006) Consensus by democracy. Using meta-analyses of microarray and genomic data to model the cold acclimation signaling pathway in Arabidopsis. *Plant Physiol,* **141**, 1219-32.

Berendzen KW, Stuber K, Harter K and Wanke D (2006) *Cis*-motifs upstream of the transcription and translation initiation sites are effectively revealed by their positional disequilibrium in eukaryote genomes using frequency distribution curves. *BMC Bioinformatics,* **7**, 522.

Boyce JM, Knight H, Deyholos M, Openshaw MR, Galbraith DW, Warren G and Knight MR (2003) The *sfr6* mutant of Arabidopsis is defective in transcriptional activation *via* CBF/DREB1 and DREB2 and shows sensitivity to osmotic stress. *Plant J,* **34**, 395-406.

Boyer JS (1982) Plant productivity and environment. *Science,* **218**, 443-8.

Bray EA, Bailey-Serres J and Weretilnyk E (2000) Responses to abiotic stress. *Biochemistry and molecular biology of plants.* Rockville, M.D., American Society of Plant Physiologists.

Burton RA, Jobling SA, Harvey AJ, Shirley NJ, Mather DE, Bacic A and Fincher GB (2008) The genetics and transcriptional profiles of the cellulose synthase-like *HvCslF* gene family in barley. *Plant Physiol,* **146**, 1821-33.

Burton RA, Shirley NJ, King BJ, Harvey AJ and Fincher GB (2004) The *CesA* gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. *Plant Physiol,* **134**, 224-36.

Campoli C, Matus-Cadiz MA, Pozniak CJ, Cattivelli L and Fowler DB (2009) Comparative expression of *Cbf* genes in the *Triticeae* under different acclimation induction temperatures. *Mol Genet Genomics,* **2**, 141-52.

Cattivell L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchioni N and Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. *Plant Mol Biol,* **48**, 649-65.

Chen M, Xu Z, Xia L, Li L, Cheng X, Dong J, Wang Q and Ma Y (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, *GmDREB3*, in soybean (*Glycine max* L.). *J Exp Bot,* **60**, 121-35.

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X and Zhu T (2002) Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell,* **14**, 559-74.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M and Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev,* **17**, 1043-54.

Chinnusamy V, Schumaker K and Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J Exp Bot,* **55**, 225-36.

Chinnusamy V, Zhu J and Zhu JK (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci,* **12**, 444-51.

Choi DW, Rodriguez EM and Close TJ (2002) Barley *Cbf3* gene identification, expression pattern, and map location. *Plant Physiol,* **129**, 1781-7.

Choi DW, Zhu B and Close TJ (1999) The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv Dicktoo. *Theor Appl Genet,* **98**, 1234-47.

Christensen AH, Sharrock RA and Quail PH (1992) Maize *polyubiquitin* genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol Biol,* **18**, 675-89.

Cook D, Fowler S, Fiehn O and Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *P Natl Acad Sci USA,* **101**, 15243-8.

Curtis MD and Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol,* **133**, 462-9.

Dong CH, Agarwal M, Zhang Y, Xie Q and Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *P Natl Acad Sci USA,* **103**, 8281-6.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J,* **33**, 751-63.

Fowler S and Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the *CBF* cold response pathway. *Plant Cell,* **14**, 1675-90.

Fowler SG, Cook D and Thomashow MF (2005) Low temperature induction of Arabidopsis *CBF1, 2,* and *3* is gated by the circadian clock. *Plant Physiol,* **137**, 961-8.

Francia E, Barabaschi D, Tondelli A, Laido G, Rizza F, Stanca AM, Busconi M, Fogher C, Stockinger EJ and Pecchioni N (2007) Fine mapping of a *HvCBF* gene cluster at the frost resistance locus *Fr-H2* in barley. *Theor Appl Genet*, **115**, 1083-91.

Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS and Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) x 'Tremois' (spring) barley map. *Theor Appl Genet*, **108**, 670-80.

Galiba G, Vagujfalvi A, Li C, Soltész A and Dubcovsky J (2009) Regulatory genes involved in the determination of frost tolerance in temperature cereals. *Plant Sci*, **176**, 12-9.

Gilmour SJ, Fowler SG and Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol Biol*, **54**, 767-81.

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD and Thomashow MF (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol*, **124**, 1854-65.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM and Thomashow MF (1998) Low temperature regulation of the Arabidopsis *CBF* family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J*, **16**, 433-42.

Gong Z, Lee H, Xiong L, Jagendorf A, Stevenson B and Zhu JK (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *P Natl Acad Sci USA*, **99**, 11507-12.

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Grain zone (accessed 15/07/05) www.grdc.com.au/growers

Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF and Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. *Plant Physiol*, **130**, 639-48.

Hartung W, Sauter A and Hose E (2002) Abscisic acid in the xylem: where does it come from, where does it go to? *J Exp Bot*, **53**, 27-32.

Hayes PM, Blake T, Chen TH, Tragoonrung S, Chen F, Pan A and Liu B (1993) Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness. *Genome*, **36**, 66-71.

Huang JG, Yang M, Liu P, Yang GD, Wu CA and Zheng CC (2009) *GhDREB1* enhances abiotic stress tolerance, delays GA-mediated development and represses cytokinin signalling in transgenic Arabidopsis. *Plant Cell Environ*.

Ishitani M, Xiong L, Lee H, Stevenson B and Zhu JK (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in Arabidopsis. *Plant Cell*, **10**, 1151-61.

Ishitani M, Xiong L, Stevenson B and Zhu JK (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell*, **9**, 1935-49.

Islam AKM, Shepherd KW and Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. *Heredity*, **46**, 161-74.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol*, **47**, 141-53.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O and Thomashow MF (1998) Arabidopsis *CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science,* **280**, 104-6.

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T and Thomashow MF (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol,* **127**, 910-7.

James VA, Neibaur I and Altpeter F (2008) Stress inducible expression of the *DREB1A* transcription factor from xeric, *Hordeum spontaneum* L. in turf and forage grass (*Paspalum notatum Flugge*) enhances abiotic stress tolerance. *Transgenic Res,* **17**, 93-104.

Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu JK and Torii KU (2008) SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to Arabidopsis stomatal differentiation. *Plant Cell,* **20**, 1775-85.

Karakousis A, Barr AR, Kretschmer JM, Manning S, Jefferies SP, Chalmers KJ, Islam AKM and Langridge P (2003) Mapping and QTL analysis of the barley population Clipper x Sahara. *Aust J Agr Res,* **54**, 1137-40.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol,* **17**, 287-91.

Kizis D, Lumbreras V and Pages M (2001) Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. *FEBS Lett,* **498**, 187-9.

Kizis D and Pages M (2002) Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway. *Plant J,* **30**, 679-89.

Kleinhofs A and Graner A (2001) An integrated map of the barley genome. IN Phillips RL & Vasil IK (Eds.) *DNA based markers in plants.* Dordrecht, Netherlands, Kluwer Academic Publishers.

Knox AK, Li C, Vagujfalvi A, Galiba G, Stockinger EJ and Dubcovsky J (2008) Identification of candidate *CBF* genes for the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. *Plant Mol Biol,* **67**, 257-70.

Lee BH, Henderson DA and Zhu JK (2005) The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. *Plant Cell,* **17**, 3155-75.

Lee H, Xiong L, Gong Z, Ishitani M, Stevenson B and Zhu JK (2001) The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. *Genes Dev,* **15**, 912-24.

Lee SC, Huh KW, An K, An G and Kim SR (2004) Ectopic expression of a cold-inducible transcription factor, *CBF1/DREB1b*, in transgenic rice (*Oryza sativa* L.). *Mol Cells,* **18**, 107-14.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K and Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell,* **10**, 1391-406.

Lopato S, Bazanova N, Morran S, Milligan AS, Shirley N and Langridge P (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Methods,* **2**, 3.

Lopato S, Li M and Basanova N (2005) IN Pillman KA (Ed.).

Manly KF, Cudmore RH, Jr. and Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm Genome,* **12**, 930-2.

Mano Y and Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). *Euphytica*, **94**, 263-72.

Martinez A, Traverso JA, Valot B, Ferro M, Espagne C, Ephritikhine G, Zivy M, Giglione C and Meinnel T (2008) Extent of N-terminal modifications in cytosolic proteins from eukaryotes. *Proteomics*, **8**, 2809-31.

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K and Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J*, **38**, 982-93.

Matthews PR, Wang M-B, Waterhouse PM, Thornton S, Fieg SJ, Gubler F and Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard *Agrobacterium* transformation vector. *Mol Breeding*, **7**, 195-202.

Medina J, Bargues M, Terol J, Perez-Alonso M and Salinas J (1999) The Arabidopsis *CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol*, **119**, 463-70.

Meijer AH, Ouwerkerk PB and Hoge JH (1998) Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. *Yeast*, **14**, 1407-15.

Miller AK, Galiba G and Dubcovsky J (2006) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. *Mol Genet Genomics*, **275**, 193-203.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*, **7**, 405-10.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ and Hasegawa PM (2007) SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in Arabidopsis. *Plant Cell*, **19**, 1403-14.

Munns R and Tester M (2008) Mechanisms of salinity tolerance. *Annu Rev Plant Biol*, **59**, 651-81.

Murre C, McCaw PS and Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell*, **56**, 777-83.

Nakano T, Suzuki K, Fujimura T and Shinshi H (2006) Genome-wide analysis of the *ERF* gene family in Arabidopsis and rice. *Plant Physiol*, **140**, 411-32.

Novillo F, Alonso JM, Ecker JR and Salinas J (2004) CBF2/DREB1C is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in Arabidopsis. *P Natl Acad Sci USA*, **101**, 3985-90.

Novillo F, Medina J and Salinas J (2007) Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. *P Natl Acad Sci USA*, **104**, 21002-7.

Oh SJ, Kwon CW, Choi DW, Song SI and Kim JK (2007) Expression of barley *HvCBF4* enhances tolerance to abiotic stress in transgenic rice. *Plant Biotechnol J*, **5**, 646-56.

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH and Kim JK (2005) Arabidopsis *CBF3*/*DREB1A* and *ABF3* in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol*, **138**, 341-51.

Orvar BL, Sangwan V, Omann F and Dhindsa RS (2000) Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *Plant J*, **23**, 785-94.

Oziel A, Hayes PM, Chen FM and Jones B (1996) Application of quantitative trait locus mapping to the development of winter habit malting barley. *Plant Breeding,* **115**, 43-51.

Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I and Bedö Z (1994) Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare* L.). *Theor Appl Genet,* **89**, 900-10.

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K and Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana DREB1A* gene delays water stress symptoms under greenhouse conditions. *Genome,* **47**, 493-500.

Pennycooke JC, Cheng H, Roberts SM, Yang Q, Rhee SY and Stockinger EJ (2008) The low temperature-responsive, *Solanum CBF1* genes maintain high identity in their upstream regions in a genomic environment undergoing gene duplications, deletions, and rearrangements. *Plant Mol Biol,* **67**, 483-97.

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K and Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel *DREB1*/*CBF* transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol,* **45**, 1042-52.

Reinheimer JL, Barr AR and Eglinton JK (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). *Theor Appl Genet,* **109**, 1267-74.

Riechmann JL and Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. *Biol Chem,* **379**, 633-46.

Rogowsky PM, Guidet FLY, Langridge P, Shepherd KW and Koebner RMD (1991) Isolation and characterization of wheat-rye recombinants involving chromosome arm 1DS of wheat. *Theor Appl Genet,* **82**, 537-44.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K and Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Bioph Res Co,* **290**, 998-1009.

Sambrook J, Fritsch EF and Manniatis T (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.*, Cold Spring Harbor Laboratory Press.

Sangwan V, Foulds I, Singh J and Dhindsa RS (2001) Cold-activation of *Brassica napus BN115* promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx. *Plant J,* **27**, 1-12.

Sauter A, Davies WJ and Hartung W (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *J Exp Bot,* **52**, 1991-7.

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Huner NP, Shinozaki K and Singh J (2005) The effect of overexpression of two Brassica *CBF/DREB1*-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant Cell Physiol,* **46**, 1525-39.

Schwacke R, Fischer K, Ketelsen B, Krupinska K and Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice. *Mol Genet Genomics,* **277**, 631-46.

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y and Shinozaki K (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. *Funct Integr Genomic,* **2**, 282-91.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y and Shinozaki K (2001) Monitoring the expression pattern of

1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell,* **13**, 61-72.

Shaikhali J, Heiber I, Seidel T, Stroher E, Hiltscher H, Birkmann S, Dietz KJ and Baier M (2008) The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. *BMC Plant Biol,* **8**, 48.

Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q and Chen SY (2003) An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. *Theor Appl Genet,* **106**, 923-30.

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K and Shinozaki K (1998) An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochem Bioph Res Co,* **250**, 161-70.

Skinner JS, Szucs P, von Zitzewitz J, Marquez-Cedillo L, Filichkin T, Stockinger EJ, Thomashow MF, Chen TH and Hayes PM (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in Arabidopsis. *Theor Appl Genet,* **112**, 832-42.

Skinner JS, von Zitzewitz J, Szucs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen TH and Hayes PM (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. *Plant Mol Biol,* **59**, 533-51.

Snape SW, Sarma R, Quarrie SA, Fish L, Galiba G and Sutka J (2001) Mapping genes for flowering time and frost tolerance in cereals using precise genetic stocks. *Euphytica,* **120**, 309-15.

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Ann Rev Plant Physio,* **35**, 543-84.

Stockinger EJ, Gilmour SJ and Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *P Natl Acad Sci USA,* **94**, 1035-40.

Stockinger EJ, Skinner JS, Gardner KG, Francia E and Pecchioni N (2007) Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*. *Plant J,* **51**, 308-21.

Sutton F, Chen DG, Ge X and Kenefick D (2009) *Cbf* genes of the *Fr-A2* allele are differentially regulated between long-term cold acclimated crown tissue of freeze-resistant and -susceptible, winter wheat mutant lines. *BMC Plant Biol,* **9**, 34.

Takumi S, Shimamura C and Kobayashi F (2008) Increased freezing tolerance through up-regulation of downstream genes *via* the wheat *CBF* gene in transgenic tobacco. *Plant Physiol Bioch,* **46**, 205-11.

Tang M, Sun J, Liu Y, Chen F and Shen S (2007) Isolation and functional characterization of the *JcERF* gene, a putative AP2/EREBP domain-containing transcription factor, in the woody oil plant *Jatropha curcas*. *Plant Mol Biol,* **63**, 419-28.

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Phys,* **50**, 571-99.

Thorlby G, Veale E, Butcher K and Warren G (1999) Map positions of *SFR* genes in relation to other freezing-related genes of *Arabidopsis thaliana*. *Plant J,* **17**, 445-52.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J,* **11**.

Toledo-Ortiz G, Huq E and Quail PH (2003) The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell*, **15**, 1749-70.

Tommasini L, Svensson JT, Rodriguez EM, Wahid A, Malatrasi M, Kato K, Wanamaker S, Resnik J and Close TJ (2008) *Dehydrin* gene expression provides an indicator of low temperature and drought stress: transcriptome-based analysis of barley (*Hordeum vulgare* L.). *Funct Integr Genomic*, **8**, 387-405.

Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger EJ, Stanca AM and Pecchioni N (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. *Theor Appl Genet*, **112**, 445-54.

Toth B, Galiba G, Feher E, Sutka J and Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. *Theor Appl Genet*, **107**, 509-14.

Usadel B, Blasing OE, Gibon Y, Poree F, Hohne M, Gunter M, Trethewey R, Kamlage B, Poorter H and Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. *Plant Cell Environ*, **31**, 518-47.

Vagujfalvi A, Aprile A, Miller A, Dubcovsky J, Delugu G, Galiba G and Cattivelli L (2005) The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat. *Mol Genet Genomics*, **274**, 506-14.

Vagujfalvi A, Crosatti C, Galiba G, Dubcovsky J and Cattivelli L (2000) Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost-tolerant and frost-sensitive genotypes. *Mol Gen Genet*, **263**, 194-200.

Vagujfalvi A, Galiba G, Cattivelli L and Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. *Mol Genet Genomics*, **269**, 60-7.

Viswanathan C and Zhu JK (2002) Molecular genetic analysis of cold-regulated gene transcription. *Philos T R Soc Lon B*, **357**, 877-86.

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG and Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. *Plant J*, **41**, 195-211.

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen TH, Hayes PM and Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. *Plant Mol Biol*, **59**, 449-67.

Wang W, Vinocur B and Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, **218**, 1-14.

Wang Z, Triezenberg SJ, Thomashow MF and Stockinger EJ (2005) Multiple hydrophobic motifs in Arabidopsis CBF1 COOH-terminus provide functional redundancy in trans-activation. *Plant Mol Biol*, **58**, 543-59.

Wilkinson S and Davies WJ (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant Cell Environ*, **25**, 195-210.

Xin Z and Browse J (1998) *Eskimo1* mutants of Arabidopsis are constitutively freezing-tolerant. *P Natl Acad Sci USA*, **95**, 7799-804.

Xiong L, Lee B, Ishitani M, Lee H, Zhang C and Zhu JK (2001) *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes Dev*, **15**, 1971-84.

Xiong L, Schumaker KS and Zhu JK (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell*, **14 Suppl**, S165-83.

Xue GP (2002a) An AP2 domain transcription factor HvCBF1 activates expression of cold-responsive genes in barley through interaction with a (G/a)(C/t)CGAC motif. *Biochim Biophys Acta*, **1577**, 63-72.

Xue GP (2002b) Characterisation of the DNA-binding profile of barley HvCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. *Nucleic Acids Res,* **30**, e77.

Xue GP (2003) The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. *Plant J,* **33**, 373-83.

Yamaguchi-Shinozaki K and Shinozaki K (1993) The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of *rd22*, a gene responsive to dehydration stress in *Arabidopsis thaliana*. *Mol Gen Genet,* **238**, 17-25.

Yamaguchi-Shinozaki K and Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell,* **6**, 251-64.

Zarka DG, Vogel JT, Cook D and Thomashow MF (2003) Cold induction of Arabidopsis *CBF* genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol,* **133**, 910-8.

Zhang JY, Broeckling CD, Sumner LW and Wang ZY (2007) Heterologous expression of two *Medicago truncatula* putative *ERF* transcription factor genes, *WXP1* and *WXP2*, in Arabidopsis led to increased leaf wax accumulation and improved drought tolerance, but differential response in freezing tolerance. *Plant Mol Biol,* **64**, 265-78.

Zhao H and Bughrara SS (2008) Isolation and characterization of cold-regulated transcriptional activator *LpCBF3* gene from perennial ryegrass (*Lolium perenne* L.). *Mol Genet Genomics,* **279**, 585-94.

Zhou J, Li F, Wang JL, Ma Y, Chong K and Xu YY (2009) Basic helix-loop-helix transcription factor from wild rice (*OrbHLH2*) improves tolerance to salt- and osmotic stress in Arabidopsis. *J Plant Physiol,* **166**, 1296-306.

Zielinski RE (1998) Calmodulin and calmodulin-binding proteins in plants. *Annu Rev Plant Phys,* **49**, 697-725.