Investigating the role of tetrapyrrole biosynthesis under drought stress in cereal transgenics

A thesis submitted in fulfilment of the requirement for the degree of

Doctor of Philosophy

By

Dilrukshi Shashikala Kumari Nagahatenna

(M.Phil, B.Sc University of Peradeniya, Sri Lanka)

Australian Centre for Plant Functional Genomics

School of Agriculture, Food & Wine

Faculty of Science

The University of Adelaide

Australia

January, 2015

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

Signature: _____ Date: _____

Acknowledgments

I extend my sincere gratitude to my supervisors Prof Peter Langridge and Dr. Ryan Whitford for their guidance, support and encouragement throughout my PhD candidature. I am extremely grateful for their advice, lengthy discussions and 'open door' policy which allowed me to meet them whenever I needed guidance. I especially thank my supervisors for their comments and suggestions on thesis and manuscripts. I thank my independent advisor Dr Julie Hayes for her support and guidance throughout my candidature as well as for her valuable comments on the thesis and manuscripts. I would also like to thank my postgraduate coordinators Associate Prof Ken Chalmers and Dr Cameron Grant for their support.

I wish to thank Dr Boris Parent for designing the drought experiment and for providing me with the guidance on gas exchange measurements. I am very thankful to Dr Everard Edwards for his advice and providing me the opportunity to use the LICOR 6400 and Mini-PAM and Dr Penny Tricker and Prof John Evans for their valuable support and comments on physiological analysis. I am very thankful to Mr John Toubia, Mr Patrick Laffy, Mr Juan Carlos Sanchez for helping me with the bioinformatics analysis. A big thank you goes to Dr Huwaida Rabie, Mr Julian Taylor and Mr Hamid Shirdelmoghanloo for their support in statistical analysis, Mrs. Yuan Li and Mrs Priyanka Kalambettu for qPCR analysis, Mrs Susanne Manning for providing me the technical support in RNA extraction and Mr. Raghuveeran Anbalagan for DNA extraction. I acknowledge Dr Ute Roessner, Dr Damien Lee Callahan and Mrs Alice Ng for conducting metabolite analysis, Dr Brent Kaiser and Dr Julie Dechorgnat for conducting leaf N analysis and Mrs Teresa Fowles and Mr Lyndon Palmer for conducting leaf Fe analysis. A special thank you to Dr Ursula Langridge, Mr Alex Kovalchuk and Mr Urey for

their support in growing transgenics in the glasshouse. I am very thankful to Dr Ainur Ismagul, Dr Nannan Yang and Dr Jingwen Tiong for their guidance on transient expression assay, Dr Agatha Labrinidis and Dr Gwen Mayo for helping me with the confocal microscopy work. I would also like to thank Dr Carl Simmons, Dr Ronald Smernik and Dr Robyn Groves for their constructive criticisms on the manuscripts. I wish to thank to Dr Monica Ogierman and Mrs Ruth Harris for their valuable guidance throughout my candidature. I wish to acknowledge the Dupont Pioneer, USA for funding this research project, the University of Adelaide, Australia and ACPFG for providing me the Adelaide Scholarship International (ASI) and ACPFG scholarships to conduct my PhD research. I also would like to extend my gratitude to the Grains Research and Development Corporation (GRDC), and Australian Society of Plant Scientist, (ASPS) Australia for providing me travel grants for attending conferences.

Last but not least, a very special thank to my beloved husband Buddhika Biyagama, my daughter Nethuni Biyagama, my mother Upamalika Nagahatenna and my father Priyantha Nagahatenna for their patience, understanding and encouragement during last 3 ½ years. I thank friends for supporting and encouraging me at all times.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

$^1O_2$	singlet oxygen
ABA	abscisic acid
ABCG2	ATP-binding cassette, subfamily G, member 2
ACTTAG	*Arabidopsis* activation tagging
ALA	aminolevulinic acid
AREB/ABF	ABA Responsive Element Binding protein/ABRE-binding factor
ATP	adenosine triphosphate
CAB	C-terminal chlorophyll a/b binding
CAPS	cleaved amplified polymorphic sequence
CDPK	calcium-dependent protein kinase
CE	carboxylation efficiency
Coprogen III	coproporphyrinogen III
CPO	coprogen III oxidase
FC	ferrochelatase
FLU	fluorescent protein
FLVCR	feline leukemia virus subgroup C cellular receptor
GluTR	glutamyl-tRNA-reductase
GluTRBP	GluTR binding protein
GP	golden promise
GPX	glutathione peroxidase
g_s	stomatal conductance
GSA	glutamate-1-semialdehyde aminotransferase
GUN4	genomes Uncoupled 4

H_2O_2	hydrogen peroxide
HAP	heme activated protein
HBP	heme binding protein
HEMA	*hemin deficient A*
HO	heme oxygenase
HO^-	hydroxyl radicals
hy1	*long hypocotyl*
Lhcb	light harvesting chlorophyll a/b binding
MEcPP	methylerythritol cyclodiphosphate
Mg-Proto IX	Mg-protoporphyrin IX
Mg-Proto IX ME	Mg-protoporphyrin IX monomethylester
NCBI	national center for biotechnology information
NF	norflurazon
NF-Y	nuclear factor Y
NOS	*nopaline synthase*
O_2^-	superoxide radicals
PAP – 3’	phosphoadenosine 5’-phosphate
Pchlide	protochlorophyllide
PGR7	proton gradient regulation7
PhANG	photosynthesis associated nuclear genes
PPO	protoporphyrinogen IX oxidoreductase
PQ	plastquinone
Proto IX	protoporphyrin IX
PSI and PSII	photosystems I and II

PYR/PYL/RCARs	pyrabactin Resistance 1/PYR1-Like/Regulatory Component of ABA Response 1
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	relative water content
sig2	sigma factor2
sig6	sigma factor6
SOD	superoxide dismutase
Sro9	suppressor of RHO3 protein 9
STN7	state transition 7
TSPO	tryptophan-rich sensory protein
UROD	urogen III decarboxylase
Urogen III	uroporphyrinogen III
WUE	water use efficiency
Ydj1	yeast dnaJ

Thesis Abstract

The tetrapyrrole biosynthesis pathway leads to chlorophyll and heme production and plays a key role in primary physiological processes such as photosynthesis and respiration. Recent studies have shed light on heme as a potential candidate molecule for triggering stress defence responses. However, detailed investigations are yet to be conducted to elucidate the potential role of heme in regulating responses to complex abiotic stress conditions such as drought. The terminal enzyme of heme biosynthesis is Ferrochelatase (FC), for which there are two isoforms encoded by separate genes (*FC1* and *FC2*). Previous studies propose that the two *FC*s synthesize two physiologically distinct heme pools with different cellular functions. The overall scientific goal of this thesis was to investigate the roles of the two *FC*s in photosynthesis, drought and oxidative stress tolerance. In this study, barley (*Hordeum vulgare*) was used as both a major cereal crop and also as a model plant for other commercially relevant rain-fed cereal crops. Two *FC*s in barley (*HvFC1* and *HvFC2*) were identified and their tissue-specific and stress-responsive expression patterns were investigated. These genes were cloned from the cultivar Golden Promise (GP) and transgenic lines ectopically overexpressing either *HvFC1* or *HvFC2* were generated. From 29 independent T_0 transgenic lines obtained for each *FC* construct, three single-copy transgenic lines ectopically overexpressing either *HvFC1* or *HvFC2* were evaluated for photosynthetic performance, oxidative and drought stress tolerance.

The two HvFC isoforms share a common catalytic FC domain, while HvFC2 additionally contains C-terminal chlorophyll a/b binding (CAB) domain. The two genes are differentially expressed in photosynthetic and non-photosynthetic tissues and have distinct stress responsive expression profiles, implying that they may have distinct roles. Transgenic plants

ectopically overexpressing either *HvFC1* or *HvFC2* exhibited significantly higher chlorophyll content, stomatal conductance (g_s), carboxylation efficiency (CE) and photosynthetic rate relative to controls under both non-stressed and drought stress conditions. Furthermore, these transgenics, showed wilting avoidance and maintained higher leaf water content and water use efficiency relative to control plants when subjected to drought stress. Overexpression of *HvFC*s significantly up-regulated nuclear genes associated with ROS detoxification under drought stress. It also reduced photo-oxidative damage caused by perturbation of tetrapyrrole biosynthesis in *tigrina*^{d12} mutants.

Taken together, this study indicates that both *HvFC*s play roles in photosynthesis and improving oxidative and drought stress tolerance. The results reported in this thesis suggest that both HvFC derived heme pools are likely to be involved in chloroplast-to-nuclear retrograde signaling to trigger drought and oxidative stress tolerance. This study also highlights the tetrapyrrole pathway as an important target for engineering improved crop performance in both non-stressed and stressed environments.

Keywords

Barley, Tetrapyrrole, Heme, Ferrochelatase, Chlorophyll, Drought stress, Photosynthesis, Photo-oxidation, Transcriptional regulation, Post-translational regulation, Stomatal conductance, Reactive oxygen species, Carboxylation efficiency

Outcomes arising from this thesis

The following is a list of Patent and publications that have been prepared in conjunction with this thesis.

Patent

Nagahatenna DSK, Whitford R (2015) Ferrochelatase compositions and methods to increase agronomic performance of plants United States Patent (In process)

Publications

Nagahatenna DSK, Langridge P, Whitford R (2015) Review-Tetrapyrrole-based drought stress signaling Plant Biotechnology Journal, 1-13

Nagahatenna DSK, Tiong J, Edwards EJ, Langridge P, Whitford R Altering tetrapyrrole biosynthesis by overexpressing *Ferrochelatases* (*FC1* and *FC2*), improves photosynthesis in transgenic barley Plant Molecular Biology (In preparation)

Nagahatenna DSK, Parent B, Edwards EJ, Langridge P, Whitford R Barley transgenics overexpressing *Ferrochelatase*s (*HvFC1* and *HvFC2*) maintain higher photosynthesis and reduce photo-oxidative damage under drought stress New Phytologist (In preparation)

List of Abstracts and Conference Presentations

Conference: 1

Name : International Association of Plant Biotechnology (2014)

Location : Melbourne, Australia

Authorship : Nagahatenna DSK, Langridge P, Whitford, R.

Abstract Title : Overexpression of barley *Ferrochelatase I* improves photosynthetic performance under drought stress conditions

Type : Oral presentation

Conference: 2

Name : ComBio (2014)

Location : Canberra, Australia

Authorship : Nagahatenna DSK, Langridge P, Whitford, R.

Abstract Title : Overexpression of barley *Ferrochelatase*s *I* and *II* improves photosynthetic performance under drought stress conditions

Type : Poster

Chapter 1: Introduction

Drought is a major abiotic stress factor, which adversely affects key plant physiological processes such as photosynthesis (Chaves 1991). Consequently, drought stress significantly reduces plant growth and crop yield (Boyer 1982). Improving the drought tolerance of major food crops such as cereals is a primary objective of plant breeding to secure future food production for the world's increasing population. Drought tolerance is a complex phenotype, and is under complex genetic control (McWilliam, 1989, Fleury et al., 2010). Drought stress responses are initiated by altering the expression of a multitude of genes necessary for 'reprogramming' of whole plant processes upon stress (Shinozaki et al., 2007). Understanding the genetic basis of drought tolerance as well as the underlying genes and biochemical pathways would greatly assist in developing superior genotypes.

The tetrapyrrole biosynthetic pathway generates chlorophyll and heme; key components of the photosynthetic machinery (Tanaka and Tanaka 2007). Tetrapyrroles possess a wide range of chemical properties and are implicated in a number of cellular processes. Chlorophyll acts as the major light-harvesting pigment for photosynthesis, while heme plays a key role in many different functions (Chen et al. 2010). It is an integral compound in photosynthetic and respiratory cytochromes, which are implicated in electron transport (Cramer et al. 1996; Kurisu et al. 2003). It also acts as a cofactor for the activation of several enzymes required for detoxifying reactive oxygen species (ROS) (del Rio 2011; Layer et al. 2010). Recently, heme was proposed to be the primary plastid signal, which modulates expression of nuclear genes during chloroplast biogenesis (Woodson et al., 2011; Woodson et al., 2013; Terry and Smith, 2013).

Heme biosynthesis is catalaysed by the enzyme ferrochelatase (FC). There are two FC isoforms, encoded by different *FC* genes. Based on their distinct expression profiles, protein structures and subcellular localization, it was suggested that the two FC isoforms may synthesise two physiologically distinct heme pools required for different cellular functions (Singh et al., 2002; Nagai et al., 2007; Scharfenberg et al., 2014). Studies have suggested that FC1-derived heme is implicated in stress defence response (Singh et al., 2002; Nagai et al., 2007) and chloroplast-to-nuclear retrograde signaling (Woodson et al., 2011; Woodson et al., 2013), whereas FC2-derived heme is important for photosynthesis (Singh et al., 2002; Scharfenberg et al., 2014). However, a detailed investigation of the distinct roles of FC1 and FC2 is yet to be conducted.

The overall scientific goal of the work reported in this thesis was to investigate whether there are two distinct functions for the two FC proteins in photosynthesis and in drought and oxidative stress tolerance in cereals. The specific objectives of this thesis were to:

1. Identify the number of *FC* genes in the genome of barley, a major global crop and a model for other commercially relevant rain-fed cereals, including wheat
2. Understand structure and subcellular localization of the FC protein, and the tissue-specific and stress-responsive expression profiles of the *FC* genes
3. Evaluate photosynthetic performance, and the oxidative and drought stress responses of barley transgenics ectopically overexpressing *HvFC1* and *HvFC2* relative to controls.

We currently know very little about whether only one or both FC-derived heme pools play roles in improving tolerance to abiotic stresses such as drought. However, heme

quantification in plants is challenging and imprecise heme analysis techniques are available. Therefore the knowledge obtained from *HvFC* overexpressing transgenics will allow us to infer potential involvement of the two heme pools in abiotic stress tolerance.

The thesis consists of six chapters, as follows:

Chapter 1 Introduction: General background to the research topic, briefly identifying the research gaps and stating the overall scientific goal as well as several specific objectives of the research.

Chapter 2 Literature review: A comprehensive literature review of the current knowledge of the tetrapyrrole biosynthesis pathway, highlighting research gaps in the literature related to tetrapyrrole biosynthesis and drought stress signaling. Based on the available evidence a model is proposed for how heme-mediated mechanisms could be targets for improving plant acclimation to drought stress.

Chapter 3 Altering tetrapyrrole biosynthesis by overexpressing *Ferrochelatases* (*FC1* and *FC2*), improves photosynthesis in transgenic barley: A report describing the identification and characterization of two barley *FC*s and their contribution to photosynthetic performance in non-stressed conditions.

Chapter 4 Barley transgenics overexpressing *Ferrochelatase*s (*HvFC1* and *HvFC2*) maintain higher photosynthesis and reduce photo-oxidative damage under drought stress: This chapter describes an investigation of the physiological roles of two types of barley *FC* in photosynthesis, anti-oxidation and wilting avoidance under drought stress.

Chapter 5 General discussion: A discussion of the significance of the research reported in this thesis, identification of areas of weakness and remaining questions, suggestions for improvements and future research directions.

Chapter 6 Contributions to knowledge: A summary of the significant contributions to scientific knowledge arising from this research.

This thesis also contains two appendices:

Appendix 1: Supplementary material for Chapter 3

Appendix 2: Supplementary material for Chapter 4

In the manuscript-style chapters (2, 3 and 4), minor changes have been made to provide a consistent format throughout the thesis. These include the renumbering of tables and figures and the consolidation of all references into a single list at the end of the thesis.

Chapter 2: Literature Review

Tetrapyrrole-based drought stress signaling

Dilrukshi S. K. Nagahatenna[1], Peter Langridge[1] and Ryan Whitford[1]*

[1] Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia, 5064, Australia

*Corresponding author:

Ryan Whitford

Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia, 5064, Australia

Tel: 61 88313 7171

Fax: 61 88313 7102

E-mail: ryan.whitford@acpfg.com.au

2.1 Statement of Authorship

Title of Paper	Tetrapyrrole-based drought stress signaling
Publication Status	☑**Published** ☐Accepted for Publication ☐Submitted for Publication ☐ Publication style
Publication Details	This manuscript was first submitted to the Plant Biotechnology Journal on the 28-1-2014. It was revised according to reviewer's comments and resubmitted to the journal on 2-10-2014 and 5-1-2015.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Dilrukshi Nagahatenna		
Contribution to the Paper	Critically examined relevant literature, wrote the manuscript and took primary responsibilty for manuscript revision.		
Signature		Date	22-01-15

Name of Co-Author	Peter Langridge		
Contribution to the Paper	Provided overall supervision and constructive critisisms on the manuscript.		
Signature		Date	22/1/2015

Name of Co-Author	Ryan Whitford		
Contribution to the Paper	Provided overall supervision and constructive critisisms on the manuscript.		
Signature		Date	22/1/15

2.2 Abstract

Tetrapyrroles such as chlorophyll and heme play a vital role in primary plant metabolic processes such as photosynthesis and respiration. Over the past decades, extensive genetic and molecular analyses have provided valuable insights into the complex regulatory network of the tetrapyrrole biosynthesis. However, tetrapyrroles are also implicated in abiotic stress tolerance, although the mechanisms are largely unknown. With recent reports demonstrating that modified tetrapyrrole biosynthesis in plants confers wilting avoidance, a component physiological trait to drought tolerance, it is now timely that this pathway be reviewed in the context of drought stress signaling. In this review, the significance of tetrapyrrole biosynthesis under drought stress is addressed, with particular emphasis on the inter-relationships with major stress signaling cascades driven by reactive oxygen species (ROS) and organellar retrograde signaling. We propose that unlike the chlorophyll branch, the heme branch of the pathway plays a key role in mediating intracellular drought stress signaling and stimulating ROS detoxification under drought stress. Determining how the tetrapyrrole biosynthetic pathway is involved in stress signaling provides an opportunity to identify gene targets for engineering drought-tolerant crops.

2.3 Introduction

Global food security in the face of a changing climate demands increasing agricultural production on finite arable land without increasing water use. With predicted population increase to around 9 billion by 2050, the World Food Summit on Food Security (2009) set a target of 70% increase in global food production. Rainfed agriculture will play a major role in meeting this demand since there is little opportunity for increasing irrigation schemes and many existing schemes are already under pressure. The single greatest abiotic stress factor

that limits worldwide rainfed agriculture is drought. The need to breed crops better adapted to drought stress is an issue of increasing urgency. Drought tolerance is a quantitative trait, under highly complex genetic control (Fleury et al. 2010; McWilliam 1989). In light of such complexities, the dissection and detailed understanding of individual pathways and processes that contribute to the various physiological mechanisms of drought tolerance is necessary.

2.4 Regulatory responses to drought stress

Plants have evolved complex signaling networks to sense and respond to drought stress. Such signaling cascades are composed of a suite of stress receptors, intercellular and intracellular signal transduction systems and transcriptional regulatory networks (Kuromori et al. 2014). These drought responsive signaling cascades can be triggered by diverse stimuli including osmotic shock, oxidative bursts, strong light, heat and wounding (Cruz de Carvalho 2008; Wang et al. 2003). Water deficit also leads to many cellular changes such as reduction in cell volume, disruption of inter- and intracellular water potential gradients, loss in cell turgor, disruption of membrane integrity, concentration of solutes, and denaturation of proteins (Bray 1997). Early recognition of these drought-induced cellular changes is the first step towards initiating plant acclimation responses. Abscisic acid (ABA), is a key stress-responsive phytohormone sensitive to these cellular changes, particularly to the loss of turgor (Schroeder et al. 2001). Water deficit first triggers ABA biosynthesis in roots, ABA is then distributed throughout the plant via the transpiration stream (Shinozaki and Yamaguchi-Shinozaki 2007). A series of recent genetic studies provide valuable insights into the molecular events from intercellular ABA-perception to ABA-induced gene transcription. Increased cellular ABA concentrations are first detected by receptors such as Pyrabactin

Resistance 1/PYR1-Like/Regulatory Component of ABA Response 1 (PYR/PYL/RCARs) (Ma et al. 2009; Park et al. 2009). Upon binding ABA, the receptor's conformation changes, leading to activation of an ABA Responsive Element Binding protein/ABRE-binding factor (AREB/ABF) (Shinozaki and Yamaguchi-Shinozaki 2007; Umezawa et al. 2010; Yamaguchi-Shinozaki and Shinozaki 1994). This master ABA responsive transcription factor regulates a diverse array of genes that coordinate cellular responses to the drought stress. Such cellular responses include stomatal closure, induction of stress proteins, and accumulation of various metabolites for the protection of cells against water deficit stress (Kuromori et al. 2014; Umezawa et al. 2010). This ABA-dependent pathway is considered as a major component of the drought stress signaling cascade. Drought stress signals can also be propagated through ABA-independent pathways. Often these are a result of early osmotic stress induced Ca^{2+} spiking/oscillation, which leads to calcium-dependent protein kinase (CDPK) activation and drought-responsive gene transcription. Additionally they can be a consequence of stress responsive selective proteolysis or phospholipid hydrolysis (Schulz et al. 2013; Zou et al. 2010).

Another trigger for drought stress signaling is via the accumulation of ROS. Under steady state conditions, major plant metabolic processes including photosynthesis and respiration generate highly toxic ROS (Tripathy and Oelmüller 2012). There are four types of ROS, namely singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) (Cruz de Carvalho 2008). In order to minimize potential cytotoxicity from ROS, plants have evolved efficient ROS detoxification mechanisms. When plants are exposed to stress, like drought and high light, the delicate equilibrium between ROS production and scavenging is perturbed (Cruz de Carvalho 2008; Van Breusegem and Dat

2006). ROS production is enhanced under drought stress due to limitations on CO_2 fixation and increased photorespiration. High concentrations of ROS are extremely deleterious and can cause severe photo-oxidative damage and cell death. However, low concentrations act as stress signals, triggering acclimation and defense mechanisms (Camp et al. 2003). Rapid increases in ROS production (oxidative burst) and ROS generated through stress induced metabolic imbalances have been shown to serve as stress signals (Mittler et al. 2004). It has been reported that ROS activates Ca^{2+} channels, induce protein kinases and the expression of a suite of nuclear genes (Pei et al. 2000; Pitzschke et al. 2009; Pitzschke and Hirt 2006). ROS is also implicated in inter-organelle communication (retrograde signaling), which in turn activates related signal transduction pathways (Laloi et al. 2007; Lee et al. 2007). For comprehensive reviews on molecular mechanisms underlying drought stress-signaling networks, refer to Bai et al. (2014), Baxter et al. (2014), Shinozaki et al. (2003) and Kuromori et al., (2014).

Although our knowledge of each signaling pathway is increasing, it is still difficult to develop a comprehensive picture of the multiple mechanisms governing drought stress signaling. Therefore, further investigations are required to discover how stress signaling pathways interconnect to form the major stress signaling cascades. The tetrapyrrole biosynthetic pathway has recently been implicated in wilting avoidance, a drought component trait (Allen et al. 2010; Thu-Ha et al. 2011). Based on detailed genetic and biochemical investigations, it has been proposed that tetrapyrrole biosynthesis is transcriptionally responsive to ROS mediated stress signaling (Nagai et al. 2007). An increasing body of evidence also suggests tetrapyrroles are involved in retrograde signaling. These signaling cascades work in concert to trigger stress-responsive gene expression. In this review we

outline the current knowledge linking tetrapyrrole biosynthesis to stress signaling since this may shed new light on molecular mechanisms important for enhancing drought tolerance.

2.5 Regulation of tetrapyrrole biosynthesis in plants

Tetrapyrrole biosynthesis is common to all higher plants and is responsible for the synthesis of chlorophyll, heme, siroheme and phytochromobilin which play vital roles in several primary metabolic processes (Tanaka and Tanaka 2007). Mg^{2+} containing chlorophyll, a cyclic tetrapyrrole, is the most abundant of plant tetrapyrroles. To date, five distinct chlorophylls, namely a, b, c, d and f have been identified in photosynthetic organisms. As the major light-harvesting compound, chlorophyll plays a key role in photosynthesis which converts light energy into chemical energy (Chen et al. 2010). Similar to chlorophyll, heme is a cyclic compound, which contains Fe^{2+} instead of Mg^{2+}. Although chlorophyll is confined to plastids, heme has widespread cellular distribution. It is an important co-factor for many enzymes involved in respiration and ROS detoxification within chloroplasts, mitochondria and peroxisomes (del Río 2011; Kirkman and Gaetani 1984; Layer et al. 2010). Siroheme, another Fe^{2+} containing tetrapyrrole, is a prosthetic group to nitrite and sulphite reductases, which are involved in nitrogen and sulphur assimilation, respectively. Phytochromobilin is a linear tetrapyrrole synthesized in plastids and serves as the functional precursor of the phytochrome chromophore, which is involved in a wide range of processes including perception of red and far-red light (Kohchi et al. 2001; Terry 1997).

The tetrapyrrole biosynthetic pathway has been well described (Cornah et al. 2003; Mochizuki et al. 2010; Tanaka et al. 2011; Tanaka and Tanaka 2007), and two strict control points have been identified, each responding to tetrapyrrole demand. These two major

regulatory points are: 1) aminolevulinic acid (ALA) synthesis and 2) at the branch point between chlorophyll and heme synthesis (**Fig 2-1**).

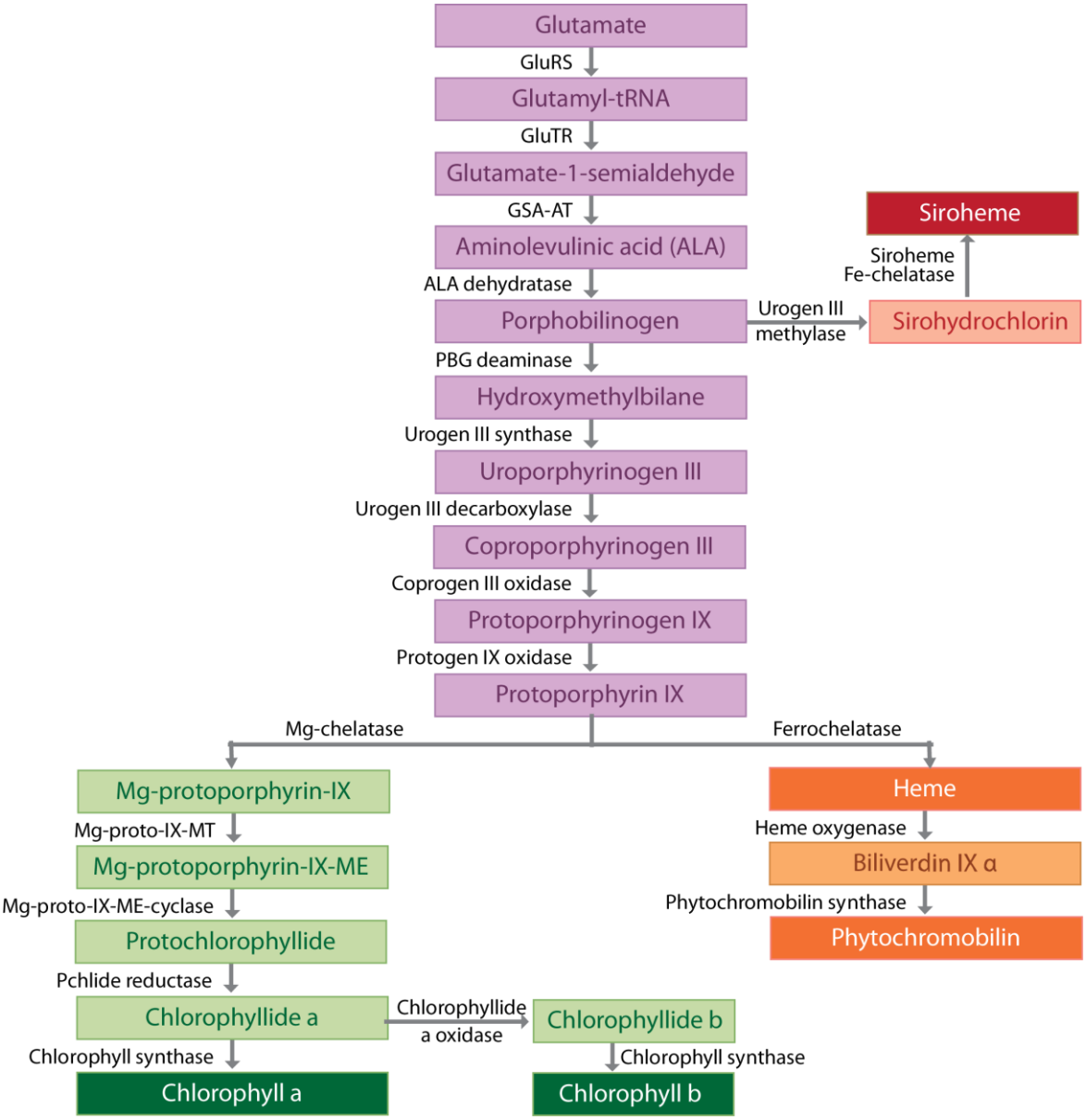


Fig 2-1. Tetrapyrrole biosynthetic pathway of higher plants, showing the major end products (white text in dark coloured boxes) and catalytic enzymes. The common enzymatic steps, chlorophyll, heme and siroheme branches of the tetrapyrrole biosynthesis pathway are represented in purple, green, orange and red, respectively. GluRS; Glutamyl-tRNA

synthetase, GluTR; Glutamyl-tRNA reductase, GSA-AT; Glutamate 1-semialdehyde aminotransferase, Mg-proto-IX-MT; Mg-Protoporphyrin IX monomethylester

ALA is the universal precursor necessary for the synthesis of all tetrapyrroles. Therefore, ALA synthesis is tightly regulated both transcriptionally and post-translationally. The main enzyme regulating ALA synthesis is glutamyl-tRNA-reductase (GluTR) (Tanaka et al. 2011). In *Arabidopsis*, GluTR is encoded by three *hemin deficient A* (*HEMA)* genes, which are differentially expressed across different tissues. They also each respond to distinct stimuli. For instance, *HEMA1* responds to a wide range of stimuli including cytokinins (Masuda et al. 1995), light (McCormac et al. 2001; McCormac and Terry 2002), circadian clock (Kruse et al. 1997), plastid derived signals (McCormac et al. 2001), and is highly expressed in photosynthetic tissues. In contrast, *HEMA2* expression is found exclusively in non-photosynthetic tissues and is not responsive to illumination (Kumar et al. 1996). The strong up regulation of *HEMA2* under oxidative stress induced by ozone application and ROS generating substances such as Paraquat and Rose Bengal, implies that *HEMA2* could play an important role in stress signaling and defense mechanisms (Nagai et al. 2007). The third member, *HEMA3*, is lowly expressed, and its role is as yet, not understood (Tanaka et al. 2011; Tanaka et al. 1997).

ALA synthesis is also regulated post-translationally by two important molecules, fluorescent (FLU) protein and heme. FLU is a nuclear-encoded plastid protein, which negatively regulates GluTR independently of heme, by binding to the C-terminal end of the enzyme. FLU specifically binds GluTR encoded by *HEMA1* (Meskauskiene and Apel 2002). This negative regulation of ALA synthesis via FLU, helps to prevent excessive accumulation of the

highly photo-oxidative chlorophyll branch intermediate protochlorophyllide (Pchlide). Interestingly, Meskauskiene et al., (2001) demonstrated that inactivation of FLU based negative regulation in the *flu* mutant, enhanced Pchlide content but did not affect heme content. Therefore, the proposed effect of FLU is more likely to be restricted to the chlorophyll branch of the pathway (Meskauskiene et al. 2001). Heme also exerts an inhibitory effect on GluTR activity by binding to its N-terminus. This was demonstrated by Vothknecht et al. (1998). Their study showed that a truncated GluTR, missing 30 amino acids at N-terminus, was highly resistant to feedback inhibition by heme *in vitro*. This was further supported in an *Arabidopsis long hypocotyl* (*hy1*) mutant, which showed deficiencies in heme oxygenase (HO) activity. HO is responsible for heme breakdown with *hy1* plants exhibiting a reduced rate of ALA synthesis and Pchlide content (Goslings et al. 2004). Moreover, it has been proposed that several soluble proteins may associate with heme in order to exert its inhibition on GluTR (Srivastava et al. 2005). However, the mode of action for both FLU and heme-regulated feedback are still not fully understood. How these negative regulatory mechanisms affect tetrapyrrole synthesis, particularly with regards to chlorophyll versus heme branch homeostasis under different physiological conditions, warrants future investigation.

At the branch point, protoporphyrin IX (Proto IX) serves as the common substrate for both chlorophyll and heme branches. Insertion of Mg^{2+} into Proto IX by the enzyme Mg-chelatase (MgCh) favors the chlorophyll branch of the pathway, whereas insertion of Fe^{2+} by Ferrochlelatase (FC) leads to heme biosynthesis. The MgCh enzyme consists of three subunits namely, CHLH, CHLI and CHLD with average molecular weights of 140, 40, and 70 kDa, respectively (Jensen et al. 1996). The other requirements for the activation of this

enzyme are an additional co-factor (Mg^{2+}), adenosine triphosphate (ATP) and a protein known as Genomes Uncoupled 4 (GUN4) (Davison et al. 2005; Larkin et al. 2003; Verdecia et al. 2005). In contrast, FC is a single-subunit enzyme, which does not require a cofactor or an external energy source for catalysis (Al-Karadaghi et al. 1997; Tanaka et al. 2011). Studies on higher plants provide evidence for two FC isoforms (FC1 and FC2) that each fulfill distinct cellular functions. For instance, *FC1* is abundantly expressed in roots relative to leaves and stems (Chow et al. 1998 2014; Singh et al. 2002; Suzuki et al. 2002). Transcriptional gene fusions to *β-glucuronidase* have demonstrated that *Arabidopsis FC1* (*AtFC1)* promoter is induced in response to wounding, oxidative stress and viral infection (Singh et al. 2002). Enhanced FC catalytic activity was also detected in chloroplasts of wounded leaves. This is further supported by subsequent studies, which demonstrated a marked induction of *AtFC1* expression in response to wounding, reagents generating ROS and drought stress (Nagai et al. 2007; Scharfenberg et al. 2014). In contrast, *AtFC2* was found to be expressed only within aerial parts of the plant and its expression is markedly down-regulated or unchanged under the same treatments.

Previous studies indicate that during daylight, saturation with the tetrapyrrole precursor, ALA causes a bias towards chlorophyll biosynthesis whereas under darkness there is a bias towards heme biosynthesis (Cornah et al. 2002). In the analysis of photodynamic changes in tobacco (*Nicotiana tobacum* L.), Papenbrock et al. (1999) demonstrated that ALA synthesis and MgCh activities increased during early light exposure, whereas FC activity was found to increase after a light to dark transition. This implies that cellular chlorophyll demand is higher during the day with a heme shift upon darkness. However, the extent of heme preference over chlorophyll biosynthesis, and vice versa depends upon the plant

developmental stage and its response to environmental stimuli. The dynamics of these changes in response to various physiological conditions, such as dehydration, is yet to be determined.

2.6 Tetrapyrrole biosynthesis activates ROS detoxification under stress conditions

Plants are constantly subjected to a wide range of environmental changes, which perturb cellular integrity and metabolism. Several studies have shown that tight regulation of tetrapyrrole biosynthesis becomes uncoupled upon exposure to stress conditions, leading to an over-accumulation of tetrapyrrole intermediates. Most tetrapyrrole intermediates including uroporphyrinogen III (Urogen III), coproporphyrinogen III (Coprogen III), Proto IX, Mg-protoporphyrin IX (Mg-Proto IX), Mg-protoporphyrin IX monomethylester (Mg-Proto IX ME) and Pchlide (**Fig 2-1**), act as strong photosensitizers (Cornah et al. 2003) and generate the extremely strong oxidizing agent $^1O_2$, upon illumination. Even though this free radical is highly hazardous, tetrapyrrole intermediate accumulation seems to concomitantly trigger cellular protection and defense mechanisms. For instance, Urogen III decarboxylase (UROD) and Coprogen III oxidase (CPO) antisense tobacco plants exhibiting excess levels of Urogen III & Coprogen III and showed enhanced resistance to viral infection (Mock et al. 1999). These plants also displayed increased activity of stress-responsive ROS detoxification enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) (Mock et al. 1998). It is interesting to note that, not only plastidal SOD, but both cytoplasmic and mitochondrial SOD activities are enhanced in these plants. As UROD and CPO are localized in plastids (Kruse et al. 1995; Mock et al. 1995; Smith et al. 1993) this indicates that increased tetrapyrrole intermediates in plastids are able to trigger anti-oxidative responses throughout the cell. Whether these tetrapyrrole compounds actually leak into the

cytoplasm and other subcellular compartments or whether they generate a rapidly transmissible intercellular signal to trigger this anti-oxidative response is unknown. Since Urogen III and Coprogen III have not yet been detected in the cytoplasm or any organelle except in chloroplasts, we can rule out the former possibility. However, available evidence has led us to speculate that oxidative stress generated by tetrapyrrole intermediate accumulation, is more likely to generate a rapid plastid signal that modulates nuclear gene expression implicated in antioxidative responses.

2.7 Enhanced tetrapyrrole biosynthesis is likely to confer drought tolerance via ROS detoxification

In recent years, the key tetrapyrrole precursor, ALA has been extensively used to improve plant growth and stress tolerance in many plant species. It has been reported that exogenous ALA application enhanced chlorophyll content (Al-Khateeb et al. 2006), photosynthetic rate (Wang et al. 2004), antioxidant capacity (Balestrasse et al. 2010), plant growth and yield (Al-Thabet 2006). Such observations have been consistently noted under various stress conditions (salinity, drought and high temperature), in a variety of plant species including barley, wheat, rice, potato, soybean, date palm, oilseed rape and cucumber (Li et al. 2011; Liu et al. 2011; Nishihara et al. 2003; Zhang et al. 2008). However, to date, only few studies have investigated the underlying molecular mechanisms for ALA promotion of dehydration tolerance. These few reports indicate that the application of 0.5-1 mgL-1 of ALA improved grain yield in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare*) under drought conditions (Al-Thabet 2006). ALA application at 0.1 and 1 mgL^{-1} concentrations also seems to promote chlorophyll biosynthesis, photosynthetic performance, biomass partitioning and ROS detoxification under water stress conditions (Li

et al. 2011). Strikingly, these plants exhibited low ROS production (H_2O_2 and O_2^-) when dehydrated, a likely consequence of increased activities of ROS scavenging enzyme such as Ascorbate peroxidase, catalase, GPX and SOD (Li et al. 2011). Significantly increased chlorophyll content upon exogenous ALA application suggests that ALA either increases tetrapyrrole biosynthesis or inhibits chlorophyll degradation. In the scenario where tetrapyrrole biosynthesis is increased, there are most likely increased amounts of Proto IX that can be utilized by FC to generate heme-derived antioxidant biomolecules for defense. This could explain the observed increased activity of antioxidative enzymes upon exogenous ALA application. The study by Thu-Ha et al. (2011) supports this conclusion since they demonstrated the significance of the branch point intermediate, Proto IX in dehydration tolerance. Transgenic rice plants overproducing Proto IX as a result of *Myxococcus xanthus PPO* overexpression appeared more tolerant to drought stress. These plants exhibited higher shoot water potential and leaf relative water content, less ROS production and higher ROS scavenging enzyme activity when compared to wild-type plants. Transgenics were able to maintain higher ALA synthesizing ability, through higher expression of *HEMA1* and glutamate-1-semialdehyde aminotransferase *(GSA)* upon dehydration and they also showed significantly higher heme content, FC activity and expression of *FC2, HO1* and *HO2* both in leaves and roots (Thu-Ha et al. 2011). These observations show that increased ALA synthesizing capacity and Proto IX levels lead to a bias towards the heme branch of the tetrapyrrole biosynthetic pathway. This proposed function of the heme branch in dehydration tolerance is further supported by experiments of Allen *et al.* (2010). By screening an *Arabidopsis* activation tagging (ACTTAG) population (100,000 lines) under water deficit conditions they demonstrated that both *AtFC1* and *AtFC2* overexpression confer wilting avoidance. The overexpression of these *Arabidopsis* genes in maize also

allowed plants to sustain yield upon water deficit, therefore further implicating the heme branch in drought stress signaling (Allen et al. 2010). A more recent study by Kim et al. (2014) also provides weight to the role of heme in stress perception. By using transgenic rice plants ectopically overexpressing *Bradyrhizobium japonicum FC*, this study demonstrated increased cytosolic FC activity, increased total heme content, resistance to polyethylene glycol induced osmotic stress as well as oxidative stress generated by peroxidizing herbicides.

Heme acts as an essential co-factor for ROS scavenging enzymes such as SOD and catalase (del Río 2011; Kirkman and Gaetani 1984; Zhang and Hach 1999). Not only heme, but also several other heme branch intermediates play important roles in ROS detoxification. It is well established that HO1 is a stress responsive protein, which protects plants against oxidative damage induced by UV-B radiation (Yannarelli et al. 2006) and H_2O_2 (Chen et al. 2009; Jin et al. 2013; Yannarelli et al. 2006). Several recent studies provide evidence that HO1 is involved in stomatal closure induction (Cao et al. 2007) as well as both lateral and adventitious root growth (Xu et al. 2011; Xuan et al. 2008). *HO1* is transcriptionally up-regulated in response to drought stress (Thu-Ha et al. 2011; Wang et al. 2014), implying that HO1 may play an important role in drought stress signaling. Furthermore, biliverdin IXα and carbon monoxide, products of heme breakdown by HO, also act as strong antioxidants (Barañano et al. 2002; Han et al. 2008; He and He 2014; Stocker et al. 1987).

Unlike the plastid-restricted tetrapyrroles, heme is capable of binding covalently and non-covalently to a large number of hemoproteins distributed across several cellular compartments (Espinas et al. 2012). In addition to the involvement to ROS detoxification, in

plastids, heme is an integral component of the cytochrome *b6f* complex, which is vital for electron transfer between photosystems I and II (PSI and PSII). In order to account for diverse functions outside plastids, either heme must be synthesized in different organelles, or transported to individual cellular compartments. Heme as well as heme biosynthetic enzymes, such as protoporphyrinogen IX oxidoreductase (PPO) and FC have been detected in purified fractions of chloroplasts and mitochondria of etiolated barley shoots (Jacobs and Jacobs 1987; Jacobs and Jacobs 1995). Interestingly, *in vitro* import assays have also shown that both FC1 and FC2 are localized to the stroma, thylakoid and envelope membranes of the chloroplast (Little and Jones 1976; Masuda et al. 2003; Papenbrock et al. 2001; Roper and Smith 1997) with FC1 additionally being imported into mitochondria (Chow et al. 1997; Chow et al. 1998; Suzuki et al. 2002). This may not reflect endogenous sub-cellular localization as subsequent *in vitro* import studies using purified pea and cucumber mitochondria exhibited undetectable FC1 activity (Lister et al. 2001; Masuda et al. 2003) whilst *in planta* analysis of FC1 reporter proteins showed strict localization to the chloroplast (Lister et al. 2001). To date, there is no *in planta* evidence showing FC1 localization to the mitochondria. These findings indicate heme biosynthesis is predominantly in the plastids. We can also infer that heme is transported throughout the cell, given that hemoproteins can be found in many subcellular compartments.

2.8 Potential role of tetrapyrrole biosynthesis in intracellular drought stress signaling

Plant survival under harsh environmental conditions is primarily determined by the ability to avoid, escape or tolerate stress conditions. At the very early stage of drought stress, drought avoidance or acclimation strategies allow plants to minimize transpiration water loss via stomatal closure, adjusting leaf architecture, reducing leaf growth and shedding older leaves

(Chaves et al. 2009). Plants can also avoid dehydration by maximizing water uptake through accelerated root growth (Mundree et al. 2002). Such adaptive alterations at the initial stages of water deficit stress can provide long-term protection from severe stress conditions. Some plants that exhibit developmental plasticity are able to escape drought by completing their life cycle before drought stress becomes lethal. Plants that contain increased levels of osmoprotectants such as proline, glycine, betaine and polyols are able to maintain turgor and protect cells from plasmolysis (Chaves et al. 2009). Similarly, plants with high levels of antioxidants in response to dehydration can mitigate against ROS damage (Cruz de Carvalho 2008). As outlined for ABA-dependent and ROS signaling, the induction of such drought adaptive strategies typically requires the perception of the dehydration stress, followed by inter- and intra- cellular stress signal transduction. Intra-cellular stress signaling cascades utilize secondary messengers for inter-organelle communication, leading to stress responsive gene transcription (Shinozaki and Yamaguchi-Shinozaki 2007).

Among different cellular organelles, chloroplasts are known to be remarkably dynamic and highly sensitive to environmental cues. Photosynthesis is predominantly regulated in the chloroplast and is considered a global stress sensor (Biswal et al. 2011). Light energy is the driving force for photosynthesis and changes in its intensity are rapidly perceived by the photosensitive PSII complex (Biswal and Pessarakli 2005; Biswal et al. 2003). Water deficiency dramatically affects CO_2 fixation as a result of stomatal closure, which limits CO_2 uptake. This also leads to over reduction of the electron transport system within PSII and therefore, problems with the dissipation of the absorbed light energy. This scenario ultimately causes significant redox imbalance and ROS generation, which consequently impairs the photosensor, PSII (Breusegem and Dat 2006). A series of genetic and

biochemical studies have revealed that these plastidal changes continuously signal to the nucleus to modulate gene expression via a process known as retrograde signaling. The existence of chloroplast-to-nucleus communication was first identified through a series of studies on chloroplast defective mutants as well as treatments with herbicides that affect chloroplast function such as norflurazon (NF). These studies revealed a marked reduction in nuclear gene expression of chloroplast-targeted proteins necessary for the assembly and functioning of the photosynthetic apparatus. This led Hess et al. (1998) to propose that functional chloroplasts are necessary for the expression of certain nuclear genes. This coordination process enables plastids to communicate chloroplastic demands, as a large number of plastidal proteins necessary for chloroplast biogenesis are encoded within the nuclear genome. These include nuclear-encoded polymerase, pentatricopeptide repeat proteins for RNA processing, photosynthesis associated enzymes, and importantly all tetrapyrrole biosynthetic enzymes (Hedtke et al. 2000; Pogson et al. 2008; Tanaka and Tanaka 2007).

Recent breakthroughs in understanding retrograde signaling have revealed novel pathways mediated under drought stress by 3'-phosphoadenosine 5'-phosphate (PAP) (Estavillo et al. 2011) and methylerythritol cyclodiphosphate (MEcPP) (Xiao et al. 2012). The identification of these compounds in plastidal signaling under stress conditions led the authors to speculate that plastids emit so-called 'operational signals' to the nucleus specifically upon stress, in order to prevent and repair ROS damage. To date, a series of studies have revealed a large number of chloroplast-derived signaling molecules. These signals are generated by changes to chloroplast redox status and ROS accumulation (Kleine et al. 2009). In the short term, cellular redox homeostasis is modulated by the plastquinone (PQ) pool. Redox signals

originating from imbalances of PQ abundance have been shown to regulate light harvesting chlorophyll a/b binding protein (Lhcb) expression as well as light-harvesting complex II (LHCII) protein content (Foyer and Noctor 2009; Yang et al. 2001). Recent studies with *Arabidopsis* mutants reveal that ascorbate and glutathione also play a key role in this redox homeostasis and signaling to the nucleus (Ball et al. 2004; Conklin and Barth 2004; Schlaeppi et al. 2008). However, the actual mechanisms for transferring the redox changes to PQ, glutathione and ascorbate pools remain elusive. Presently, the best candidate for a PQ derived redox signal is State Transition 7 (STN7), a thylakoid localized LHCII protein kinase (Pesaresi et al. 2009).

ROS has been implicated in operational signaling through studies with the *Arabidopsis* conditional *flu* mutant, which accumulates Pchlide upon darkness, a potent $^1O_2$ generator and photosensitizer (Camp et al. 2003; Laloi et al. 2007; Lee et al. 2007; Wagner et al. 2004). Affymetrix gene expression analysis by Camp et al. (2003) revealed etiolated *flu* seedlings, when exposed to light, rapidly activate the expression of 70 stress responsive nuclear genes. It has also been reported that excessive accumulation of $^1O_2$ in these seedlings suppresses photosynthesis-associated nuclear protein synthesis. Targets include small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBCS and RBCL), and LHCB2 (Khandal et al. 2009). Interestingly, thylakoid membrane localized EXECUTER1 and EXCECUTER2 proteins appear to mediate the $^1O_2$ induced signaling cascade between the chloroplast and nucleus (Kim and Apel 2013; Lee et al. 2007). Singlet oxygen itself is unlikely to serve as a long distance signaling molecule given its highly reactive nature and short half-life. It has been suggested that $^1O_2$ may interact with neighbouring plastid components to generate more stable lipid-based metabolites, which could potentially serve as signaling

molecules (Ramel et al. 2012; Ramel et al. 2013). H_2O_2 is proposed as a better signaling molecule because it is less toxic and has a longer half-life than $^1O_2$. Another candidate implicated in ROS derived plastid signaling is β-cyclocitral, a product of $^1O_2$-induced oxidation of carotenoids. Importantly, β-cyclocitral has the capacity to induce a significant portion of the $^1O_2$ responsive genes, which in turn activate defence responses (Ramel et al. 2012; Ramel et al. 2013).

2.8.1 Heme mediated chloroplast-to-nucleus signaling upon drought stress

It has been proposed that tetrapyrrole intermediates in both chlorophyll and heme branches are involved in chloroplast-to-nucleus communication during chloroplast biogenesis (Barajas-López et al. 2013; Chi et al. 2013; Kleine et al. 2009; Surpin et al. 2002; Terry and Smith 2013). Even though previous studies in *Chlamydomonas reinhardtii* (Johanningmeier 1988; Johanningmeier and Howell 1984), garden cress (*Lepidium sativum*) (Oster et al. 1996), *Arabidopsis* (Ankele et al. 2007; Strand et al. 2003) and barley (*Hordeum vulgare*) (La Rocca et al. 2001) provided support for Mg-Proto IX being a retrograde signal, this concept was disputed in subsequent studies (Mochizuki et al., 2008; Moulin et al., 2008).

The evidence for the involvement of Mg-Proto IX in plastid signaling originated from studies on genomes uncoupled mutants. The *gun* mutants which are deficient in heme oxygenase (*gun2)*, phytochromobilin synthase (*gun3)*, MgCh interacting protein (*gun4)* and CHLH (*gun5)* subunits, displayed continuous expression of *Lhcb*, even when chloroplast development is impaired by the herbicide NF (Mochizuki et al. 2001; Susek et al. 1993). In all *gun* mutants Mg-Proto IX content was drastically reduced and this was interpreted as

showing that this compound is an essential negative signal responsible for mediating nuclear gene expression. However, subsequent detailed analyses were unable to show a correlation between Mg-Proto IX content and degree of nuclear gene expression (*Lhcb*) in a range of *Arabidopsis* mutants grown under varying conditions (Mochizuki et al. 2008; Moulin et al. 2008). Furthermore, a Mg-Proto IX accumulating barley *xantha-l* mutant did not demonstrate a reduction in nuclear gene expression (Gadjieva et al. 2005).

Interestingly, in a detailed biochemical analysis, Voigt et al. (2010) demonstrated that in wild type plants as well as *gun1*, *gun2*, *gun4* and *gun5* mutants, unbound free heme content was significantly increased upon NF treatment. Subsequent studies revealed that unlike Mg-Proto IX, heme is more likely to be the primary tetrapyrrole-based plastidal signal that modulates nuclear gene expression. For instance, Woodson et al., (2011) demonstrated that an *Arabidopsis gun* (*gun6-1D*) mutant overexpressing *FC1* induces *photosynthesis associated nuclear* gene (PhANG) expression by increasing a specific heme sub-pool. Interestingly, overexpression of *FC2* is unable to enhance PhANG expression, implying that FC2-derived heme is less likely to be associated with retrograde signaling. This hypothesis was further confirmed by a recent study using *Arabidopsis sigma factor2* (*sig2*) and *sigma factor6* (*sig6*) (Woodson et al. 2013). SIG is responsible for chloroplast transcription and the recognition of a number of tRNA promoters by plastid-encoded RNA polymerase (PEP) (Kanamaru et al. 2001; Kanamaru and Tanaka 2004). Mutants lacking *SIG2* and *SIG6* are deficient in PEP-transcribed $tRNA^{Glu}$, which is a precursor for tetrapyrrole biosynthesis, and a substrate for GluTR. Consequently, these plants show a reduction in $tRNA^{Glu}$, GluTR, ALA and Pchlide levels (**Fig 2-1**), as well as PhANG expression. However, overexpression of *FC1* in *sig2* and *sig6* mutant backgrounds was shown to restore PhANG expression, implying that heme is

likely to be an important primary positive retrograde signal. Again, the overexpression of *FC2* in the *sig2* mutant background failed to increase PhANG expression (Woodson et al. 2013). This was further supported by the observation that long hypocotyl mutants, *hy1* and *hy2* which accumulate heme and biliverdin IX due to impairment of HO and phytochromobilin synthase, displayed elevated nuclear gene expression upon exposure to NF (Vinti et al. 2000). Even though, the involvement of tetrapyrrole biosynthesis in operational signaling has yet to be fully established, there is existing evidence that leads us to speculate that this pathway may transiently generate a positive heme-based stress signal necessary for modulating nuclear gene expression under adverse conditions.

2.8.2 A proposed model for heme action as a retrograde signal leading to stress-activated gene expression

The proposed role for heme as an operational signal in chloroplast-to-nuclear signaling can be broken down based on the timing of molecular events. In the first instance, tetrapyrrole biosynthesis may favor heme production upon stress. Persistence of the stress event in this case, would cause unbound free heme to accumulate and promote its efflux from the chloroplast. This would make more heme available for import to the nucleus. Once in the nucleus, heme may act to stabilize and activate specific transcription factor classes that bind to drought responsive promoters. Transcriptional activation of drought responsive genes would then lead to acclimation to the prevailing drought stress (**Fig 2-2**).

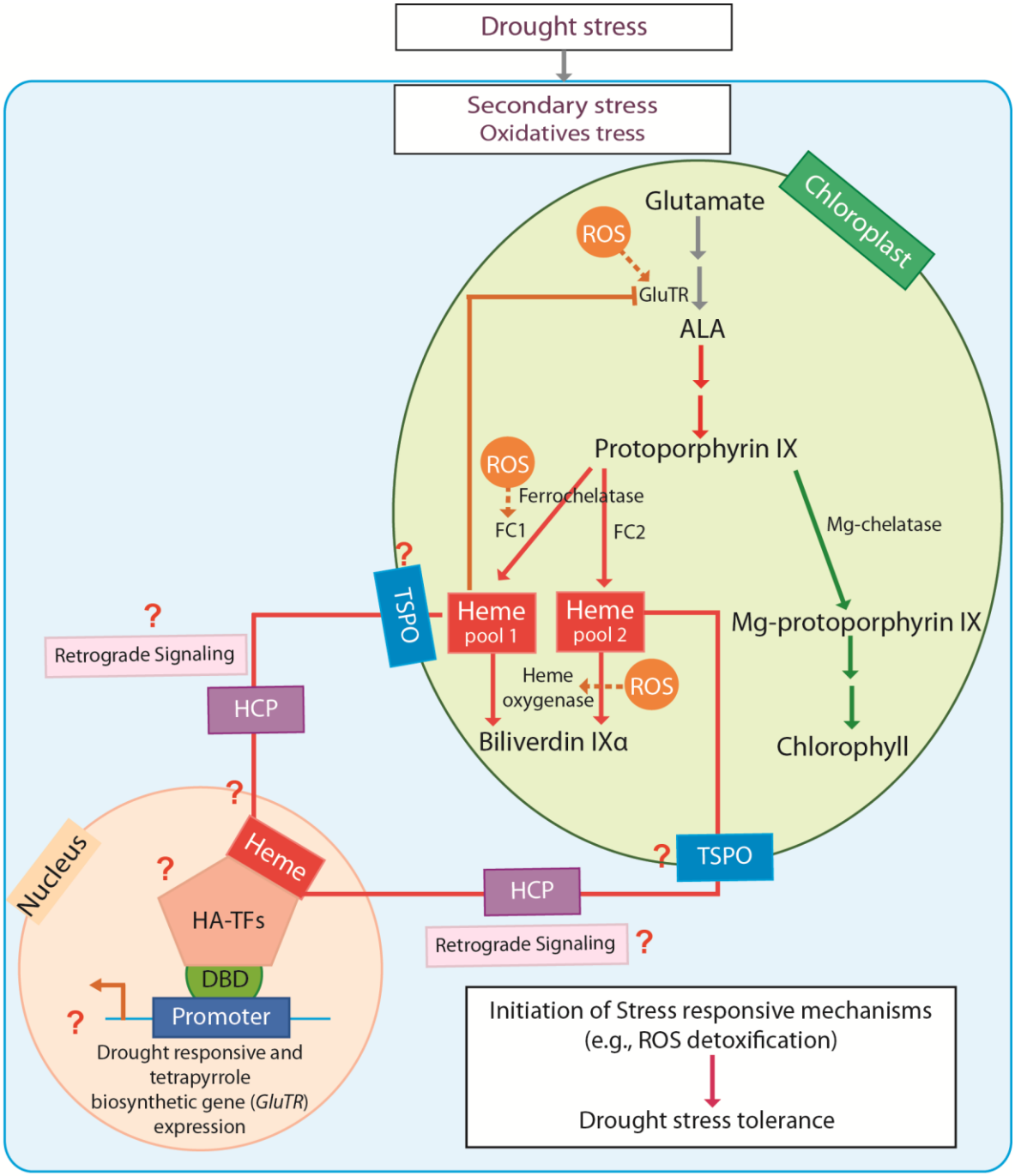


Fig 2-2. Proposed model based on current knowledge on the role of tetrapyrroles in drought stress signaling. Drought stress induces secondary stress events including chloroplast localised oxidative stress, which in turn favours heme production. This enhances accumulation of unbound free heme, the plastid signal, for chloroplast-to-nuclear communication. Because free heme is insoluble and cytotoxic, its mobility is likely to be

dependent upon both membrane and cytosolic localised heme carrier proteins (HCP) and transporters such as tryptophan-rich sensory protein (TSPO). Upon arrival in the nucleus, heme would post-translationally activate heme activated transcription factors (HA-TFs) including the nuclear factor Y (NF-Y). We propose GluTR, encoding the first rate-limiting enzyme of the tetrapyrrole pathway, along with a suite of drought responsive and reactive oxygen species (ROS) detoxification genes to be targets for this transcriptional activation. Heme-induced transcriptional activation would initiate, and re-inforce ROS detoxification, an important mechanism allowing plant's to adapt to the prevailing drought stress. Dashed arrows indicate ROS transcriptionally induce genes encoding tetrapyrrole enzymes. Red question marks denote mechanistic points warranting further investigations.

Under stress conditions, tetrapyrrole biosynthesis is perturbed leading to accumulation of intermediates (Mock and Grimm 1997; Strand et al. 2003). Given that this intermediate accumulation within the chloroplast, significantly improves ROS detoxification enzymatic activity throughout the cell (Mock et al. 1999; Mock et al. 1998), it is reasonable to assume that a stress signal might be transmitted from the chloroplast. Considering the literature available, we speculate that this stress signal is heme. For instance, it has been reported that, when tetrapyrrole flux is enhanced either by exogenous ALA application or by increasing Proto IX content, total heme content increases (Espinas et al. 2012) upon drought stress (Li et al. 2011; Thu-Ha et al. 2011). Plants with increased total heme content show enhanced resistance to drought and oxidative stress (Kim et al. 2014; Thu-Ha et al. 2011). Tetrapyrrole intermediate accumulation within the chloroplast (Breusegem and Dat 2006; Moulin et al. 2008; Mundree et al. 2002) might additionally be a direct source of oxidative stress, which may in turn reinforce channelling of heme precursors towards heme

production. Preferential channelling towards heme production is also supported by a study by Czarnecki et al. (2011) who showed that *Arabidopsis* GluTR binding protein (GluTRBP; previously called proton gradient regulation7 (PGR7)) when silenced, does not change ALA synthesizing capacity or chlorophyll content but does reduce heme content. Further investigations are necessary to elucidate the mechanistic trigger for this process under stress.

It has been proposed that only unbound free heme, present in very small amounts relative to total heme, is important in retrograde signaling (Terry and Smith 2013; Woodson et al. 2011). Free heme quantification techniques are imprecise; therefore little is known of the changes that occur in the free heme pool, particularly in response to stress. By combining different extraction techniques (Espinas et al. 2012), it was determined that free heme content increases in wild–type seedlings upon NF-induced oxidative stress (Espinas et al. 2012; Voigt et al. 2010). This contrasts with total heme content, which actually decreases upon NF treatment (Espinas et al. 2012; Woodson et al. 2011), implying that when chloroplasts experience oxidative cytotoxicity, a portion of the covalently bound heme may also be released to the free heme pool (Espinas et al. 2012). It is important to note that heme analysis in these various experiments was typically conducted a few days after the stress event and therefore rapid transient heme changes upon stress are currently unknown. In order to determine whether oxidative stress causes the accumulation of a transient free heme signal, during a complex event such as drought, precise time-resolved heme profiling will be needed. New approaches are necessary to elucidate the timing of tetrapyrrole changes following such stress events.

Heme is hydrophobic and it is exported from the chloroplast to the cytoplasm (Severance and Hamza 2009; Thomas and Weinstein 1990). However, free heme molecules are considered cytotoxic as they are able to react with oxygen to produce ROS (Kumar and Bandyopadhyay 2005). It has been proposed that due to low solubility of heme in aqueous solution, free heme is more likely to adhered non-specifically to heme trafficking proteins (Espinas et al. 2012; Thomas and Weinstein). A large number of heme transporters have been identified in mammalian cells, as compared to plants where only a few have been identified (Krishnamurthy et al. 2004; Quigley et al. 2004; Severance and Hamza 2009; Shayeghi et al. 2005). A candidate for heme transport in plants is the translocator protein known as tryptophan-rich sensory protein (TSPO) (Balsemão-Pires et al. 2011). In plants, TSPO is localized in the membranes of multiple organelles such as chloroplast, mitochondria, endoplasmic reticulum and the Golgi stacks (Balsemão-Pires et al. 2011; Guillaumot et al. 2009; Lindemann et al. 2004). TSPO has a high affinity to heme (Vanhee et al. 2011) and is translocated between sub-cellular compartments under abiotic stress conditions (Balsemão-Pires et al. 2011). Therefore, TSPO is considered a likely candidate for heme transport across organellar membranes as well as a transporter throughout the cytoplasm under stress (Balsemão-Pires et al. 2011; Taketani et al. 1995). In addition, *Arabidopsis* heme binding protein 5 (AtHBP5) has been identified as a chloroplast localized protein which contains hydrophobic heme-binding pockets (Lee et al. 2012). There are also a number of cytosolic localized heme carrier proteins, which transport heme between cellular organelles. In mammalian cells, a wide array of such proteins have been identified, including heme carrier protein 1 (HCP1), feline leukemia virus subgroup C cellular receptor (FLVCR) and ATP-binding cassette, subfamily G, member 2 (ABCG2) (Krishnamurthy et al. 2004; Quigley et al. 2004; Shayeghi et al. 2005). Recently, several studies have shown that cytosolic AtHBP,

homologous to mammalian heme binding proteins p22HBP/SOUL, bind cytosolic heme (Sato et al. 2004; Takahashi et al. 2008; Zylka and Reppert 1999). The presence of such a large number of heme carrier proteins supports the proposition that heme is more suitable as a signaling molecule than other tetrapyrroles.

It has been reported that in the nucleus, heme could post-translationally activate specific transcription factors that modulate a large number of genes necessary for stress acclimation. This proposition is based on studies conducted in yeast, where heme was shown to post-translationally activate the heme responsive transcriptional regulator, HAP1. HAP1 is a nuclear localized protein, which exists in a high-molecular weight complex in the absence of heme. This high molecular weight complex is composed of several heat shock proteins including HSP90, HSP70, Suppressor of RHO3 protein 9 (Sro9), and yeast dnaJ (Ydj1) (Hon et al. 2001; Hon et al. 2005). In the presence of heme, HAP1 binds to heme via a conserved heme responsive motif 7. This leads to the dissociation of Sro9 and Ydj1 from the complex resulting in complete activation of HAP1. The resulting stable dimeric HAP1 complex has a high binding affinity to the DNA *cis*-element CGGnnnTAnCGG (Zhang and Guarente 1994). The transcriptional activation of nuclear genes by the HSP70-HSP90-HAP1-Heme complex is important for controlling oxidative damage in yeast. So far, a similar HAP1 complex has not been identified in plants. However, it was recently determined that HSP90 is essential for modulating nuclear gene expression in *gun5* upon oxidative stress (Kindgren et al. 2012). *Arabidopsis* HSP90 is localized in the cytosol, chloroplast, mitochondria, endoplasmic reticulum and nucleus (Hubert et al. 2009; Krishna and Gloor 2001). If we suppose that the retrograde signal generated in *gun5* is heme, it would imply that heme-HSP90 interaction is necessary for activating nuclear gene expression. Interestingly, both

Arabidopsis HSP70 and HSP90 molecular chaperones were found to be important for stomatal closure under drought stress conditions (Clément et al. 2011). Taken together, it is probable that a similar mechanism in plants could initiate plant drought acclimation in response to oxidative stress.

In addition to HAP1, yeast contains another HAP2:3:4:5 transcriptional regulator complex which is post-translationally activated by heme. This complex triggers the transcription of a large number of genes through binding to CCAAT *cis*-elements (Maity and de Crombrugghe 1998; Mantovani 1998). *Arabidopsis* NF-YA:B:C complex members have been identified as orthologous of the yeast Hap2:3:4:5 complex (Stephenson et al. 2007). NF-Ys are heme-activated heterotrimeric complexes composed of NF-YA, NF-YB and NF-YC subunits (Stephenson et al. 2007). Importantly, the *cis* elements targeted by this complex are found in the promoters of several drought responsive genes (Li et al. 2008). A series of studies have demonstrated that *Arabidopsis* NF-Y is involved in drought tolerance via both ABA dependent and independent mechanisms (Nelson et al. 2007; Stephenson et al. 2007). For instance, *Arabidopsis* plants overexpressing *NF-YA5* were more resistant to drought stress due to prevention of water loss via ABA-induced stomatal closure (Li et al. 2008). Furthermore, transgenic *Arabidopsis* and maize plants over-expressing *AtNF-YB1* and *ZmNF-YB2,* respectively, exhibited drought tolerance phenotypes in an ABA independent manner. These plants were less wilted and maintained higher leaf water potential, chlorophyll content, stomatal conductance, photosynthesis rate and yield under water-limited field conditions (Nelson et al. 2007).

Several studies demonstrated that genes associated with tetrapyrrole biosynthesis (Stephenson et al. 2010) are also activated by NF-Y transcription factors. Direct evidence linking tetrapyrrole biosynthesis with transcriptional regulation by NF-Y's comes from wheat transgenics overexpressing *NF-YB3* which exhibited increases in the expression of *GluTR*, chlorophyll content and rate of photosynthesis under non-droughted conditions (Stephenson et al. 2011). Moreover, an Affymetrix genome array showed that wheat *NF-YC11* and *NF-YB3* transcription factor genes co-express with light-inducible tetrapyrrole genes encoding GluTR, CHLH subunit and UROD (Stephenson et al. 2010; Stephenson et al. 2011). Interestingly *GluTR,* among other light responsive genes, contain CCAAT-box motifs in their promoters (ie. within 500 bp of translation start site), which is typical for NF-Y binding *cis* elements (Stephenson et al. 2010). Since GluTR is the first rate-limiting enzyme for tetrapyrrole biosynthesis, such evidence would suggest that tetrapyrrole biosynthesis might be transcriptionally regulated by NF-Y (**Fig 2-2**). In depth analysis is necessary to elucidate how NF-Y mediated transcriptional regulation could impact on tetrapyrrole biosynthesis under non-stressed as well as drought stress conditions.

2.9 Concluding remarks and future perspectives

Research efforts have indicated that tetrapyrroles are implicated in drought stress tolerance via retrograde signaling and induction of drought responsive gene expression. It is evident that, the tetrapyrrole pathway is favoured towards heme production upon water deficit stress and this triggers acclimation mechanisms (**Fig 2-2**). Even though the primary regulatory points of this pathway are known, the full set of molecular mechanisms facilitating dehydration tolerance still need to be identified. Some fundamental questions remain unanswered: What triggers the channelling of tetrapyrroles towards heme branch

under stress? Is this in response to oxidative stress or do interacting proteins induce it? What influences heme efflux and its inter-organelle transport upon stress? Does heme activate nuclear gene expression via NF-Y transcription factors in plants?

It is important to note that recent studies have suggested the existence of two physiologically distinct heme pools, of which only one is involved in stress defence responses (Nagai et al. 2007; Scharfenberg et al. 2014; Singh et al. 2002; Woodson et al. 2011; Woodson et al. 2013). It has been proposed that the heme pool involved in stress defence is likely generated through the action of HEMA2 and FC1, given that the genes encoding these enzymes are each transcriptionally activated upon oxidative stress as opposed to the *HEMA1* and *FC2* genes which are transcriptionally repressed (Nagai et al. 2007; Singh et al. 2002). Supporting this notion is the finding that *Athema2* and *Atfc1* loss-of-function mutants produce significantly less total heme upon oxidative stress when compared to wild-type (Nagai et al. 2007). Such a clear distinction between heme sub-pools should be taken with caution given that Scharfenberg et al., (2014) recently demonstrated that *fc2* but not *fc1* improves salt and oxidative stress tolerance. However, the proposal that distinct functions exist for the two heme sub-pools is supported by the finding that only FC1-derived heme seems to be involved in retrograde signaling (Woodson et al. 2011; Woodson et al. 2013). Future investigations are necessary to dissect the role of these potential heme sub-pools and whether they contrast in their effect on stress defence responses.

Another important area of research will be development of sensitive assays to precisely quantify free heme. Even though new techniques for measuring free heme have emerged

(Espinas et al. 2012), they remain somewhat imprecise. Thorough time-resolved quantifications are necessary to elucidate changes in total vs free heme upon drought stress. Moreover, appropriate protocols are yet to be developed for quantifying other intermediates of the heme branch, such as biliverdin IXα and phytochromobilin. The presence of these important intermediates in relatively low quantities make their analysis extremely difficult.

Despite these current limitations, our understanding on the contribution of tetrapyrrole biosynthesis in drought stress signaling will be useful for directing future research aimed at unravelling gene targets for engineering drought tolerant crops.

2.10 Acknowledgement

This work was supported by grants from the Australian Research Council, the Grains Research and Development Corporation, the Government of South Australia and the University of Adelaide. We would like to thank Dr. Julie Hayes, Dr. Ronald Smernik and Dr. Carl Simmons for critical comments on the manuscript. The authors declare no conflict of interest.

Research questions

Over the past decade a number of molecular and biochemical studies conducted in the model plant, *Arabidopsis* have significantly broadened our understanding of the complex regulatory networks of the tetrapyrrole biosynthesis pathway. Recent studies propose that this pathway plays a pivotal role not only in the production of tetrapyrroles for key physiological processes, but also in stress signaling. Based on the literature reviewed, it appears that the heme branch of the pathway is implicated in drought stress signaling. It is timely to investigate whether the extensive knowledge gained from the model plant species, on teterapyrrole-mediated drought stress signaling can be applied to crop plants such as barley to improve their drought stress tolerance.

The overall objective of the work described in this thesis is to extend recent knowledge of the tetrapyrrole–based drought stress signaling to commercially relevant cereals. Thus, the overall research addresses the following scientific questions:

1. Does the tetrapyrrole biosynthetic pathway play a significant role in drought stress signaling in cereal crops?
2. Does modification of the heme branch of the pathway by ectopic overexpression of *FC*s, affect key physiological processes, in particular photosynthesis, in cereals?
3. Does enhanced flux through the heme branch of the pathway stimulate ROS detoxification thereby improving drought stress tolerance in cereals?
4. Do the two *FC*s have distinct roles in photosynthesis, oxidative and drought stress responses?
5. Could heme be a chloroplast operational signal, which modulates stress responsive nuclear genes upon drought stress?

Aims of this thesis

The primary aim of this PhD research is to improve our understanding of the significant contribution of tetrapyrrole biosynthesis in improving plant performance upon drought stress. This project also aims at investigating candidate genes of this pathway, which could potentially use as important targets in plant breeding to improve crop performance under water-limited conditions.

Chapter 3: Altering tetrapyrrole biosynthesis by overexpressing *Ferrochelatase*s (*FC1* and *FC2*), improves photosynthesis in transgenic barley

Dilrukshi S. K. Nagahatenna[1], Jingwen Tiong[1], Everard J. Edwards[2], Peter Langridge[1] and Ryan Whitford[1]*

[1] Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia 5064 Australia

[2] Agriculture Flagship, Commonwealth Scientific and Research Organisation, PMB 2, Glen Osmond, South Australia, 5064 Australia.

*Corresponding author:

Ryan Whitford

Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia 5064 Australia

Tel: 61 88313 7171

Fax: 61 88313 7102

E-mail: Ryan.Whitford@acpfg.com.au

3.1 Statement of Authorship

Title of Paper	Altering tetrapyrrole biosynthesis by overexpressing *Ferrochelatases* (*FC1* and *FC2*), improves photosynthesis in transgenic barley
Publication Status	☐Published ☐Accepted for Publication ☐ Submitted for Publication ☑ **Publication style**
Publication Details	This chapter was prepared as a manuscript for submission to the Plant Molecular Biology Journal.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Dilrukshi Nagahatenna		
Contribution to the Paper	Designed experiments, conducted bioinformatics analysis, gene expression analysis, transient expression assay and microscopy imaging, genotyping of transgenics, penotypic analysis of transgenics, gas exchange analysis, critically examined all genotypic and phenotypic data, conducted statistical analysis, interpreted the results and wrote the manuscript and took the primary responsibility for manuscript revision.		
Signature		Date	22-01-15

Name of Co-Author	Jingwen Tiong		
Contribution to the Paper	Contributed to trouble-shooting and performing the transient expression assay		
Signature		Date	20/1/2015

Name of Co-Author	Everard Edwards		
Contribution to the Paper	Provided constructive criticisms on the analysis of leaf level physiology of the trangenics and on the manuscript.		
Signature		Date	29/1/15

Name of Co-Author	Peter Langridge
Contribution to the Paper	Provided overall supervision of the research and constructive criticisms on the manuscript
Signature	Date 22/1/2015

Name of Principal Author (Candidate)	Ryan Whitford
Contribution to the Paper	Provided guidance in the design of the research, analysis of the data and writing of the manuscript.
Signature	Date 22/1/15

3.2 Abstract

Ferrochelatase (FC) is the terminal enzyme of heme biosynthesis. In photosynthetic organisms studied so far, there is evidence for two FC isoforms, which are encoded by two genes (*FC1* and *FC2*). Previous studies suggest that these two genes are required for the production of two physiologically distinct heme pools with only *FC2*-derived heme involved in photosynthesis. We characterized two *FC*s in barley (*Hordeum vulgare* L.). The two HvFC isoforms share a common catalytic domain, but HvFC2 additionally contains a C-terminal chlorophyll a/b binding (CAB) domain. Both *HvFC*s are highly expressed in photosynthetic tissues, with *HvFC1* transcripts also being abundant in non-photosynthetic tissues. To determine whether these isoforms differentially affect photosynthesis, transgenic barley ectopically overexpressing *HvFC1* and *HvFC2* were generated and evaluated for photosynthetic performance. In each case, transgenics exhibited improved chlorophyll content, photosynthetic rate, stomatal conductance (g_s) and carboxylation efficiency (CE), showing that both *FC1* and *FC2* play roles in photosynthetic performance. Our finding that modified tetrapyrrole biosynthesis improves photosynthesis opens opportunities to metabolically engineer improved crop performance.

3.3 Introduction

Production of the major cereal crops needs to improve to feed future food demands driven by population growth. This task will be challenged by production constraints due by increased climatic variability. Improving photosynthetic performance of rain-fed cereals may be a step towards achieving higher crop yields on limited arable land. As photosynthesis is a highly complex and regulated physiological process, the identification of genes and processes capable of enhancing photosynthetic efficiency is a high priority (Reynolds et al.

2009; Reynolds et al. 2000; Sharma-Natu and Ghildiyal 2005). Knowledge of these genes and processes will allow researchers and plant breeders to identify, track and ultimately deploy improved photosynthetic traits.

Tetrapyrroles are key components of photosynthesis. All higher plants synthesise two major tetrapyrroles, chlorophyll and heme (Tanaka and Tanaka 2007). In plastids, chlorophyll plays a vital role in the capture and conversion of light energy for photosynthesis (Chen et al. 2010), whilst heme is an integral component of the photosynthetic cytochrome *bf6* complex, necessary for photosynthetic electron transport (Cramer et al. 1996; Kurisu et al. 2003). Unlike chlorophyll, heme has a wide distribution within the cell and is required for a number of other cellular functions. For instance, in both the mitochondria and endoplasmic reticulum, heme is involved in electron transport through respiratory cytochromes, cytochrome b5 and P450s. In peroxisomes, it acts as a co-factor for activating ROS-detoxification enzymes, catalase and ascorbate peroxidase (Smith AG et al. 1999). Recently, it was proposed that heme serves as a plastid signal for modulating expression of a number of chloroplast biogenesis associated nuclear genes (retrograde signaling) (Terry and Smith 2013; Woodson et al. 2011; Woodson et al. 2013). Studies to date show that tetrapyrrole biosynthesis is modulated at two strict control points; aminolevulinic acid synthesis, and at the branch point between chlorophyll and heme synthesis (Cornah et al. 2003; Mochizuki et al. 2010; Tanaka et al. 2011; Tanaka and Tanaka 2007). At the branch point, Protoporphyrin IX (Proto IX) serves as the common substrate for tetrapyrrole biosynthesis. Insertion of Mg^{2+} into Proto IX by Mg-chelatase forms chlorophyll, whereas insertion of Fe^{2+} by Ferrochelatase (FC) is necessary for heme production (Moulin and Smith 2005).

In plants studied so far, there is evidence for two FC isoforms, which are each encoded by a single gene (*FC1* and *FC2*). Both FC isoforms exist as 36–42 kDa monomers (Smith et al. 1994), have similar catalytic properties, substrate affinity and specificity (Little and Jones 1976). However, the two *FC*s have distinct expression profiles. *FC1* is abundantly expressed in all plant tissues including roots, whereas *FC2* transcript levels are found only in aerial plant parts (Chow et al. 1998; Nagai et al. 2007; Scharfenberg et al. 2014; Singh et al. 2002; Smith et al. 1994). *In vitro* import assays indicate that both FC1 and FC2 are localized to the stroma, thylakoid and envelope membranes of the chloroplast (Little and Jones 1976; Papenbrock et al. 2001; Roper and Smith 1997), while FC1 is additionally imported into mitochondria (Chow et al. 1997; Chow et al. 1998; Papenbrock et al. 2001; Suzuki et al. 2002). These differences have led to the proposition that each FC has a distinct role in plant metabolism. Dual targeting of FC1 to both chloroplasts and mitochondria has been disputed in subsequent studies. For example, Lister et al. (2001) were unable to detect FC1 in *Arabidopsis* mitochondria, whilst pea mitochondria, in which previous import assays had been conducted, appeared to accept a variety of chloroplast-specific proteins in addition to *Arabidopsis* FC1 (Lister et al. 2001). Masuda et al. (2003) also found that FC1 and FC2 in cucumber are both solely targeted to chloroplasts.

FC2, but not *FC1,* has recently been demonstrated to positively co-express with light-responsive photosynthetic genes (Scharfenberg et al. 2014). *Arabidopsis fc2* knock-down mutants (*fc2-1*) exhibited a significant reduction in cytochrome *b6f*-bound heme and an impairment of photosynthetic electron transport and PSII efficiency (Scharfenberg et al. 2014). In comparison, *Arabidopsis fc1-1* knock-down mutants did not display obvious defects in photosynthetic development suggesting that only FC2 is directly required for

photosynthesis (Nagai et al. 2007; Scharfenberg et al. 2014). Taken together with disputed reports of *fc1* knock-out mutant lethality (Scharfenberg et al. 2014; Woodson et al. 2011), questions arise to whether *FC1* has a significant role in photosynthetic performance.

This study aimed to gain a deeper understanding of FC contributions to photosynthetic performance. For this purpose we used barley (*Hordeum vulgare* L.) as a model for commercially relevant rain-fed cereal crops. Two barley *FCs* (*HvFCs*) were identified and their tissue-specific expression patterns and subcellular protein localization were investigated. *HvFC1* and *HvFC2* were cloned from the cultivar Golden Promise. Transgenic lines ectopically overexpressing either *HvFC1* or *HvFC2* were generated and evaluated for photosynthetic performance. Our results show that the two *HvFC*s have differential tissue expression profiles, with HvFC1 localizing to plastid-like structures. Overexpression of either *HvFC1* or *HvFC2* improved chlorophyll content, stomatal conductance, carboxylation efficiency and photosynthetic rate in barley, demonstrating that both *FCs* affect photosynthetic performance.

3.4 Materials and Methods

3.4.1 Identification of two barley *FC* genes

Barley *FC* sequences were identified by comparison to FC sequences from a number of plant species including, *Arabidopsis*, cucumber (*Cucumis sativus*) and grass family members. These sequences were retrieved from the National Center for Biotechnology Information (NCBI) genomic database. Translated polypeptide sequences were used in a BLASTx search of barley derived genomic sequences from IPK, Gatersleben, Germany (http://webblast.ipk-gatersleben.de/barley). Protein motifs were identified by comparison to sequences in the

Pfam database (EMBL, Heidelberg, Germany) (http://pfam.xfam.org). All sequences were carefully evaluated for redundancy, splice forms and conserved catalytic domains.

3.4.2 Phylogenetic analysis

Retrieved FC1 and FC2 polypeptide sequences were aligned using the Muscle Alignment web server (http://www.ebi.ac.uk/Tools/muscle/index.html) and viewed in Jalview. N- and C-termini were trimmed from each protein sequence to demark the FC catalytic and CAB domains. Phylogenetic analysis was carried out using MEGA 5 software and the Maximum Likelihood method (www.megasoftware.net). The reliability of the tree was estimated by bootstrap analysis with 1000 replications (Hall 2013).

3.4.3 cDNA cloning and binary plasmid construction

Total RNA was extracted from whole *Hordeum vulgare* (cv. Golden Promise) seedlings 6 days post germination with RNeasy plant extraction kit (Qiagen). The cDNA was generated using SuperScript™ III RT (Invitrogen) and random primers. Full-length cDNA sequences from barley were PCR-amplified using either *HvFC1* (accession number AK251553) specific primers (forward, 5'-ATGGAGTGCGTCCGCTCGGG; reverse, 5'-TCACTGAAGAGTGTTCCGGAAAG) or *HvFC2* (accession number AK355192) specific primers (forward, 5'- ATGCTCCACGTTAGGCTC; reverse, 5'-TTAAGGGAGAGGTGGCAAGAT) by using Phusion® Hot Start high fidelity DNA polymerase (Finnzymes). The PCR amplification included a touch-down (A) and a classical (B) PCR as follows: 5 min at 94 °C, followed by 10 cycles (30 s at 94 °C, 45 s at 60 °C –1 °C/cycle, and 90 s at 72 °C), 20 cycles (30 s at 94 °C, 45 s at 50 °C, and 90 s at 72 °C), and a final 10-min extension step at 72 °C. The *HvFC1* (1455 bp) and *HvFC2* (1581 bp) PCR products were purified and cloned in the pCR8-TOPO vector

(Invitrogen) prior to sequencing. Sequence verified coding sequences were transferred into Gateway compatible pMBC32-based binary vectors (Curtis and Grossniklaus 2003) using LR-clonase (Invitrogen). Schematics of sequence verified binary vectors are described in supplementary **Fig S2**.

3.4.4 Barley transformation and analysis of transgenic plants

The pMDC32-HvFC1 and pMDC32–HvFC2 constructs (**Fig S2**) were transformed into barley (*Hordeum vulgare* L. cv. Golden Promise) using *Agrobacterium*-mediated transformation, as described by Tingay et al. (1997) and Matthews et al. (2001). Transgene integration was confirmed in independent T_0 lines by PCR using primer pairs for the hygromycin resistance gene (*Hyg*) and transgenes (**Table S2**).

HvFC1 and *HvFC2* transgene copy numbers were estimated in T_0 progeny using Southern blot hybridization as described by Sambrook and Russell (2001). Genomic DNA was digested with *HindIII* and *PvuIII* and the Southern blot was probed with the terminator sequence of the *nopaline synthase* (*NOS*) gene. Low-copy, independent transgenic lines were selected and total *HvFC1* and *HvFC2* expression levels were analysed by quantitative RT-PCR as described by Burton et al. (2004), using primers for coding regions of *HvFC* endogenes. mRNA copy number for each tested gene was normalized against four control genes (*GAPDH, HSP70, cyclophilin and tubulin*) as described by Burton et al. (2004). Descriptions of the probe and primer sequences used in these experiments are described in **Table S2**.

3.4.5 Transient expression of HvFC1-green fluorescent protein (GFP) fusion

For transient expression of GFP fusion constructs, N-terminal partial open reading frames including complete transit peptides of HvFC1 and HvFC2 were fused upstream and in-frame with *Spe*I and *Asc*I sites of the GFP fusion construct pMDC83 (https://www.unizh.ch/botinst/Devo-Website/curtisvector/), under the control of the 2 X cauliflower mosaic virus 35S (2X35S CaMV) promoter. For the N terminus GFP fusion, HvFC1 was amplified from the cDNA clone by PCR by using oligonucleotides that contained a *Spe*I site (ACTAGTATGGAGTGCGTCCGCTCG) and *Asc*I site (GGCGCGCCACTGAAGAGTGTTCCGGAAAG). HvFC2 was amplified by using oligonucleotides that contained a *Spe*I site (ACTAGTTATGCTCCACGTCAGGCT) and *Asc*I site (GGCGCGCCAAGGGAGAGGTGGCAAGATAC). N-terminus fusion of the small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase (SSU) was used as a control for plastid targeting protein. Onion (*Allium cepa* L.) epidermal cells were bombarded with vector DNA-coated gold particles (1,350 psi) using a Bio-Rad PDS-1000He Particle Delivery System according to the manufacturer's instructions. The samples were incubated at 27 °C in darkness, and GFP fluorescence in cells was detected by Nikon A1R confocal microscopy (Axioplan2 and Axiophoto2, Zeiss) after 24 hrs incubation.

3.4.6 Plant material and growth conditions

Wild-type barley (*Hordeum vulgare* L. cv. Golden promise), null segregants, T_1 and T_2 trangenic barley seeds were grown in pots containing coco-peat under controlled environmental conditions with 20-22 ^{0}C temperature, 50-60% relative humidity and a 12: 12 hr (light/dark) cycle. For phenotypic analysis, 3 to 4 week-old transgenics were evaluated for

plant development parameters including plant height, tiller number, number of leaves, shoot and root dry weights.

3.4.7 Photosynthetic measurements

In vivo gas exchange parameters were measured in developmentally equivalent fully expanded leaves from 4 to 6 week old plants using a LI-6400 portable photosynthesis system (Licor, USA). Measurement periods were from 9:00 am to 5:00 pm. The conditions of the IRGA chamber were set to light intensity of 2000 µmol m^{-2} s^{-1}, humidity of 50-60%, air of temperature 25°C, and reference air CO_2 concentration of 400 µmol mol^{-1}. Carboxylation efficiency (CE) = photosynthesis rate under saturated light (A_{sat})/intracellular CO_2 concentration.

3.4.8 Leaf N and Fe analysis

Total leaf N concentration was determined with an isotope ratio mass spectrometer (Seron, Crewe, Cheshire, UK) by Nitrogen analysis group at University of Adelaide according to Garnett et al. (2013). Total leaf Fe content was analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; Wheal et al. (2011) by Waite Analytical Services, University of Adelaide.

3.4.9 Chlorophyll content

Chlorophyll was extracted from leaf tissues using dimethyl sulfoxide (DMSO) and determined spectrophotometrically according to Hiscox & Israelstam (1979). Chlorophyll concentrations were calculated using the following equations. Chl*a* (g l^{-1}) = 0.0127 A_{663} –

0.00269 A_{645}; Chl*b* (g l^{-1}) = 0.0229 A_{645} – 0.00468 A_{663} (A_{663} and A_{645} are absorbances at 663 and 645nm).

3.4.10 Statistical analysis

One-way ANOVA was performed using GenStat software, and mean differences were analysed through LSD test. Differences were considered statistically significant when $P < 0.05$.

3.5 Results

3.5.1 Identification and sequence analysis of two types of *Ferrochelatases* in barley

Barley *FC* gene sequences were identified by comparison to publicly available plant *FC* sequences. As described in other plant species, we found two FC isoforms in barley, each encoded by a single gene. The two barley isoforms are 55.6% and 11.2% identical to each other at the amino acid and nucleotide levels, respectively. Similarity comparisons revealed that the two HvFCs share a high level of identity with their *Arabidopsis* orthologues (AtFC1 (62.3%) and AtFC2 (71.2%), respectively). As has been described for other plant FCs (Suzuki et al. 2002), multiple sequence alignment revealed that the HvFC1 and HvFC2 catalytic domains are highly conserved (**Fig S1**). Several proline and glycine residues, which play vital roles in hydrogen bonding, metal binding, and the stability of the protoporphyrin-interacting loop (Al-Karadaghi et al. 1997) are also highly conserved. FC2 contains an additional chlorophyll a/b binding (CAB) domain which has a light harvesting complex (LHC) motif (**Fig S1**). This domain is present in many photosynthesis-associated proteins.

The putative evolutionary relationship between HvFCs and those from other grass and dicot species was investigated by constructing a phylogenetic tree (Hall 2013). The resulting dendrogram demonstrated that the two FC isoforms in all plant species studied so far, belong to distinct clades (**Fig 3-1**).

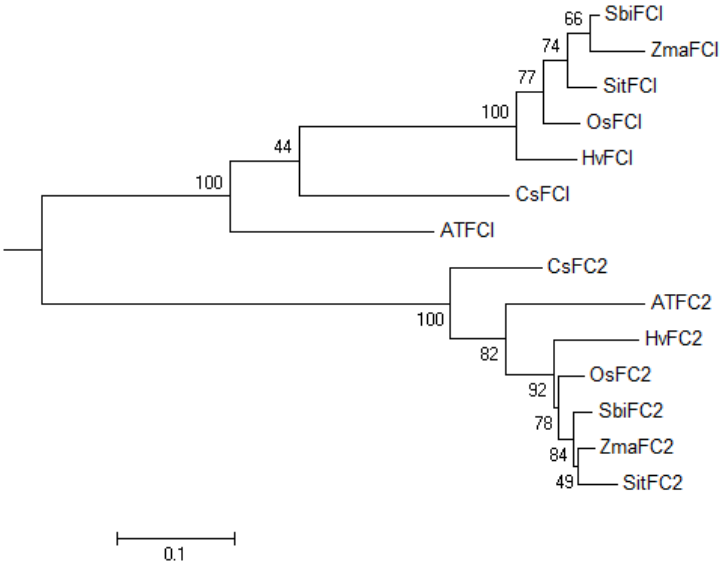


Fig 3-1. Phylogenetic relationship of HvFC1 and HvFC2 with other FC from grass and dicot species. At, *Arabidopsis* (*Arabidopsis thaliana*); Cs, cucumber (*Cucumis sativa*); Hv, barley (*Hordeum vulgare*); Os, rice (*Oryza sativa*); Sit, foxtail millet (*Setaria italica*); Sbi, Sorghum (*Sorghum bicolor*); Zma, Maize (*Zea maize*). The bootstrap percentages in which the associated taxa clustered together are shown above the branches.

3.5.2 Two types of barley *Ferrochelatases* have differential tissue specific expression patterns

To gain insight into the putative function of *HvFCs* during photosynthesis, we investigated *HvFC1* and *HvFC2* expression in photosynthetic versus non-photosynthetic tissues by quantitative RT-PCR. *HvFC2* expression was predominantly observed in leaves (photosynthetic tissues; **Fig 3-2**). Similar levels of leaf *HvFC1* expression were also observed,

but *HvFC1* transcript abundance was significantly higher in roots (non-photosynthetic tissues), suggesting a role for *HvFC1* outside photosynthesis.

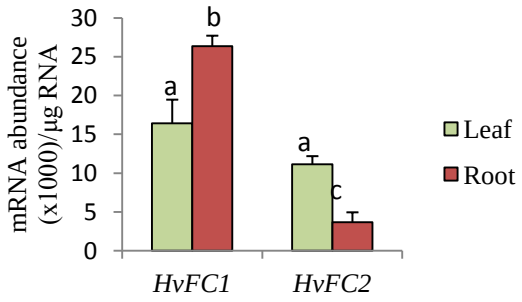


Fig 3-2. Differential expression profiles of *HvFC1* and *HvFC2* in photosynthetic and non-photosynthetic tissues. Data are presented as means ± standard error of three replicates. Means with the same letter are not significantly different at $P<0.05$, one-way ANOVA.

3.5.3 Barley FC1 is targeted to plastids

In order to investigate the subcellular localization of HvFC1, we employed a transient expression assay in onion epidermal cells (*Allium cepa* L.). HvFC1-GFP fusion proteins were detected in either irregular or oval shaped structures (**Fig 3-3**) consistent with the size and morphology of onion cell proplastids and associated stromules (Natesan et al. 2005). GFP fluorescence was not detected in small punctate structures, as expected if it were localised to mitochondria (Arimura and Tsutsumi 2002; Arimura et al. 2004).

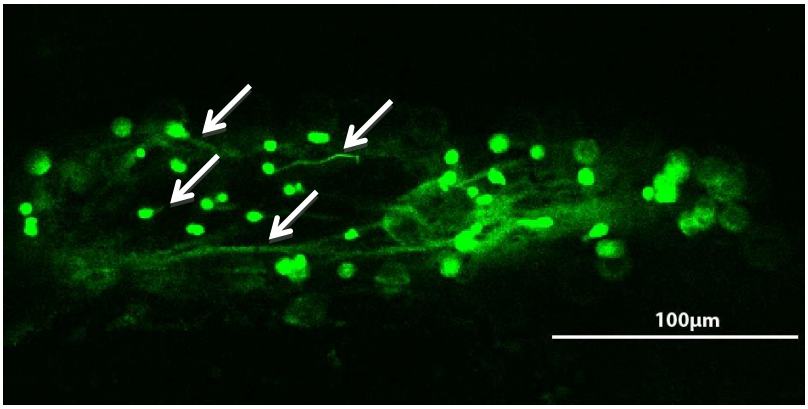


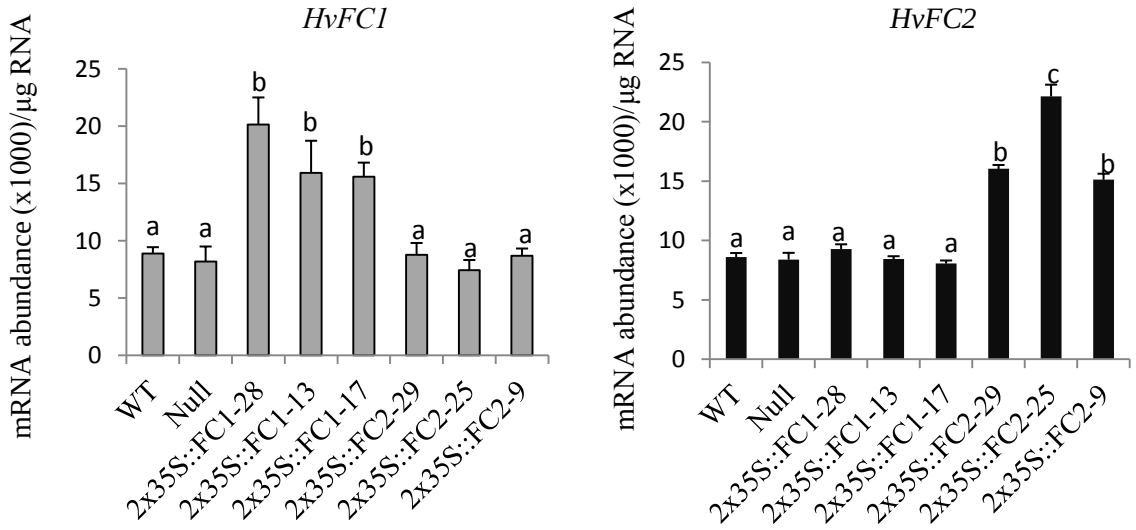
Fig 3-3. Fluorescence signals of HvFC1-GFP fusion protein in an onion epidermal cell. GFP fluorescence was located on either irregularly or oval shaped structures that are typical of onion cell proplastids and their associated stromules (arrows). Image was taken 24 hrs after bombardment. Bar 100 µm.

3.5.4 Increasing *HvFC* expression affects photosynthetic performance

To identify whether *HvFC1* and *HvFC2* have differential roles during photosynthesis, we generated transgenics (cv. Golden Promise) ectopically overexpressing either *HvFC1* or *HvFC2*. Coding regions of *FC* were cloned into the pMDC32 vector under the control of the 2x35SCaMV promoter (**Fig S2**). Twenty-nine independent T_0 transgenic lines were obtained for each *FC* construct, using *Agrobacterium*-mediated transformation. Southern blot hybridization showed that most T_0 transgenic lines had 2-5 copies of the transgene. Low copy number transgenic lines were selected and confirmed for transgene copy number by qPCR and subsequently analysed for *FC* expression by quantitative RT-PCR. Three single-copy transgenic lines, each ectopically overexpressing either *HvFC1* or *HvFC2*, were selected for further analysis (**Fig 3-4a**). T_2 transgenic plants were phenotypically evaluated under controlled conditions for growth and development. Untransformed plants and non-transgenic sibs (null segregants) were used as controls. PCR analysis with transgene-specific

primers confirmed the presence of *FC* transgenes in selected T_2 transgenic lines and their absence in wild-type and null segregants (**Fig 3-4b**).

a)



b)

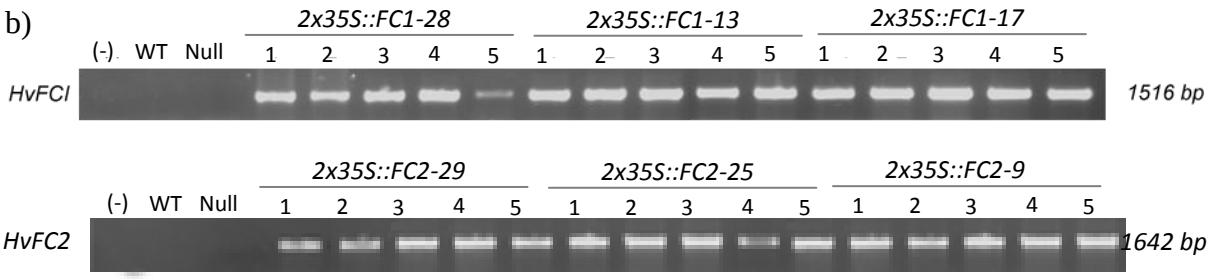


Fig 3-4. (a) Enhanced transcript levels of *HvFC1* and *HvFC2*, in three selected single-copy independent transformation events (T_1) relative to WT and null controls. Data are presented as means ± standard error for six replicates. Means with the same letter are not significantly different at P<0.05, one-way ANOVA. (b) Detection of the presence or absence of *HvFC1* and *HvFC2* transgenes using polymerase chain reaction (PCR) with transgene-specific primers. Lane (-) is a negative control. *2x35S::FC1-28*, *2x35S::FC1-13*, *2x35S::FC1-17* are three independent transformation events selected for *FC1* whereas *2x35S::FC2-29*, *2x35S::FC2-25*,

2x35S::FC2-9 are for *FC2*. 1-5, five biological replicates for each independent transformation event.

Molecular characterization of these transgenic lines confirmed that *HvFC1* and *HvFC2* were constitutively overexpressed and showed no obvious negative developmental defects relative to untransformed and null controls (**Table S1**). Three-week old T_2 transgenic plants (with the exception of line *2x35S::FC1-17*) did not show a significant difference in plant height, leaf number, tiller number and shoot or root biomass when compared to controls. However, all transgenic lines (with the exception of line *2x35S::FC1-13*), displayed a higher total chlorophyll content with no significant difference in chlorophyll a/b ratios relative to controls (one-way ANOVA, $P<0.05$) (**Fig 3-5a**). The A_{sat} was significantly higher in all transgenic lines relative to controls, however no significant differences (one-way ANOVA, $P<0.05$) were observed between *2x35S::FC1* and *2x35S::FC2* transgenics (**Fig 3-5b**). Stomatal conductance (g_s) relative to controls was higher in two of the three *2x35S::FC1* lines and only one of the *2x35S::FC2* lines (**Fig 3-5c**). CE was higher in all three *2x35S::FC1* lines and two of the three *2x35S::FC2* lines when compared to controls (**Fig 3-5d**). These findings show that both *FC* genes, when ectopically overexpressed, are able to improve the photosynthetic performance of barley, and therefore, both FC isoforms are likely to play important roles during photosynthesis.

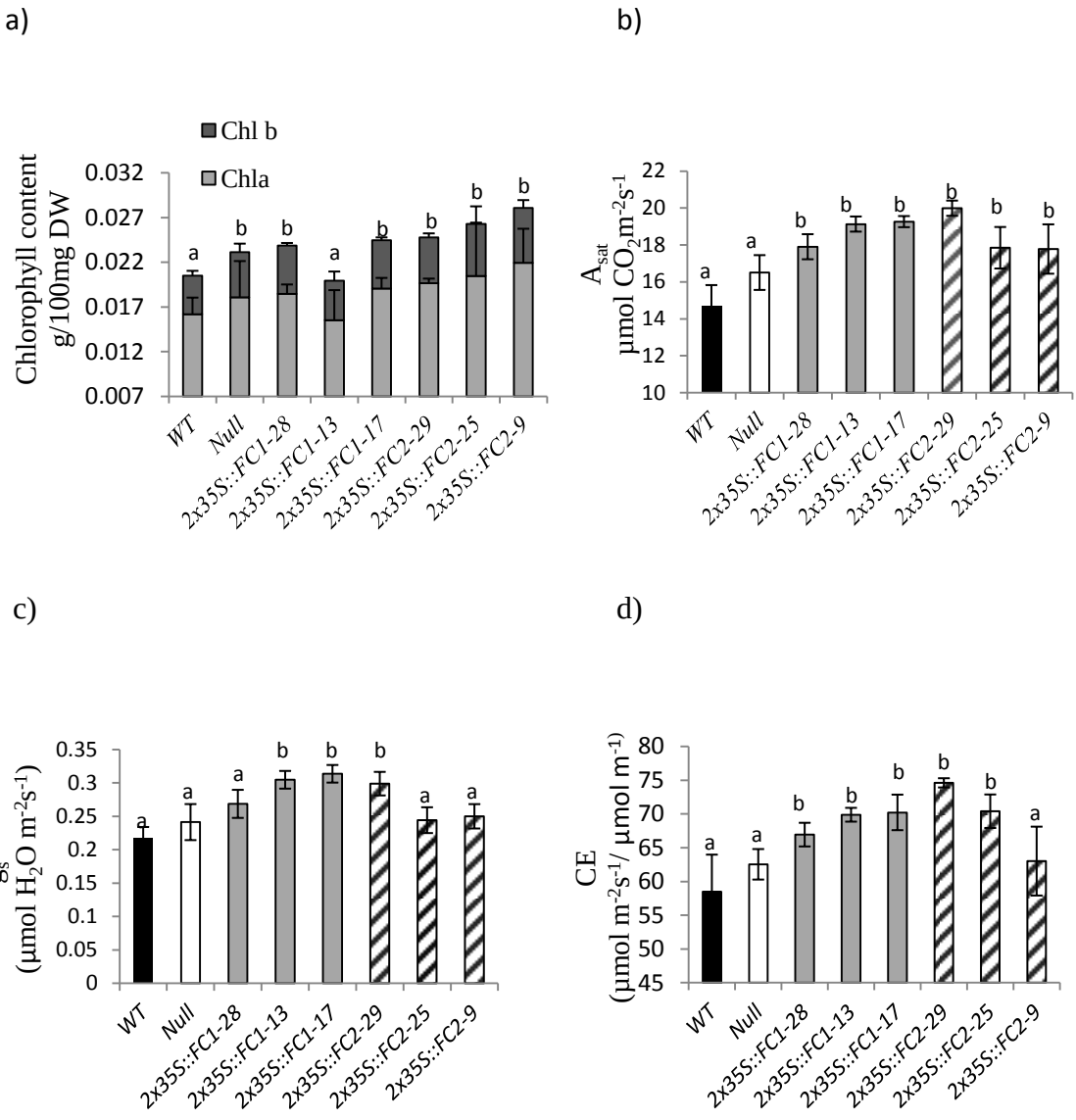


Fig 3-5. Photosynthetic performance of *HvFC* overexpressing transgenics relative to controls. (a) Chlorophyll *a* and *b* content, (b) Photosynthesis rate under saturated light, (c) Stomatal conductance (g_s), and (d) Carboxylation efficiency (CE) of three independent transformation events overexpressing either *HvFCl* or *HVFC2* relative to WT and null controls. *2x35S::FC1-28*, *2x35S::FC1-13*, *2x35S::FC1-17* are three independent transformation events selected for *FC1* whereas *2x35S::FC2-29*, *2x35S::FC2-25*, *2x35S::FC2-9* are for *FC2*. Data are shown as mean values ± standard error from 4 to 5 different plants. Means with the same letter are not significantly different at $P<0.05$, one-way ANOVA.

The observed improvement in CE suggested that these plants have either a higher Rubisco content, Increased Rubisco activation or a greater mesophyll conductance. Leaf nitrogen content, as a surrogate indicator for the amount of Rubisco (Field and Mooney 1986; Nijs et al. 1995), was measured in transgenic plants relative to untransformed controls and null segregants. Total leaf N concentration was not significantly different between transgenics and controls (one-way ANOVA, $P<0.05$), except for one line (*2x35S::FC2-29*) which showed a lower concentration (**Fig 3-6a**). These results indicate that the improved photosynthetic performance of the transgenic lines is unlikely to be a consequence of increased Rubisco content.

Because FCs catalyse the insertion of ferrous iron (Fe^{2+}) into protoporphyrin IX, it is possible that the observed photosynthetic differences may be a consequence of altered Fe homoeostasis. To test this, we measured total Fe concentration in photosynthesizing leaf tissue. No significant differences were observed between leaf Fe concentration of the transgenic and control lines. These results suggest that the observed phenotypic differences in photosynthetic performance are not likely to be the consequence of altered Fe acquisition and/or distribution (**Fig 3-6b**).

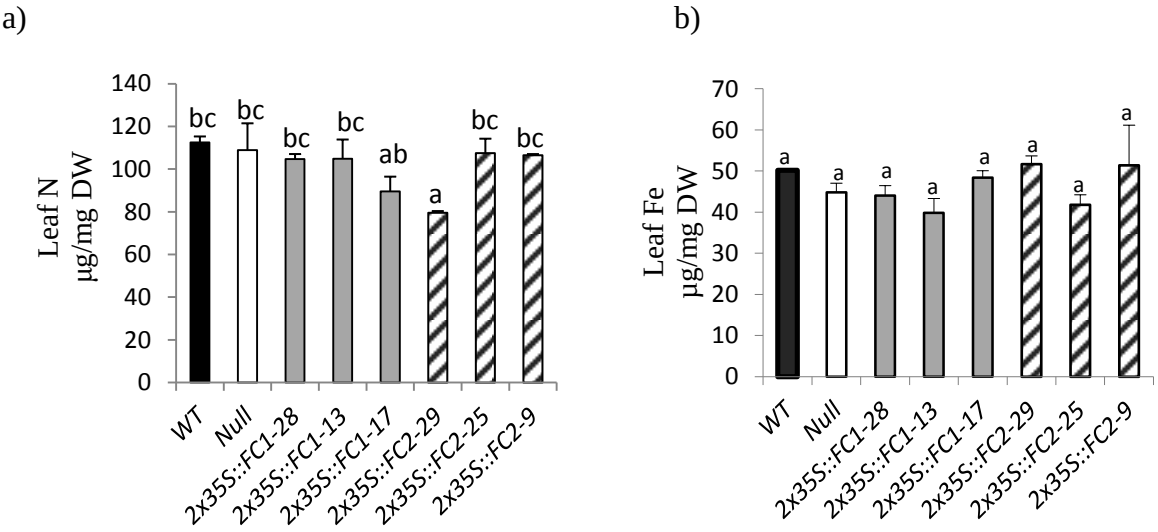


Fig 3-6. (a) Leaf N, and (b) leaf total Fe concentration of transgenic barley lines over-expressing either *HvFC1* or *HvFC2* relative to WT and null controls. *2x35S::FC1-28*, *2x35S::FC1-13*, *2x35S::FC1-17* are three independent transformation events selected for *FC1* whereas *2x35S::FC2-29*, *2x35S::FC2-25*, *2x35S::FC2-9* are for *FC2*. Data are shown as mean values ± standard error from three different plants. Means with the same letter are not significantly different at $P<0.05$, one-way ANOVA.

Collectively, our results suggest that although the two *HvFCs* have differential expression profiles and encode distinct isoforms, both play important roles in photosynthesis.

3.6 Discussion

3.6.1 Two barley FCs differ in structure and expression

The barley genome contains two genes encoding separate FC isoforms, which are 55.6% and 11.2% identical at the amino acid and nucleotide levels, respectively. Similarity comparisons demonstrate that the two HvFC proteins share conserved amino acids (proline and glycines) important for the tertiary structure in their catalytic domains (Al-Karadaghi et al. 1997). This similarity is common to all known plant FCs (**Fig S1**). High amino acid conservation in the

catalytic domains is suggestive of shared catalytic function for HvFC1 and HvFC2. FC catalyses the conversion of Proto IX into heme, a terminal step in the tetrapyrrole biosynthesis pathway.

Despite catalytic domain commonality, plant FC polypeptides form two distinct phylogenetic lineages (**Fig 3-1**). These two lineages are unlikely to have arisen from segmental duplication (Scharfenberg et al. 2014) and are separated by the presence of a characteristic C-terminal CAB domain containing a conserved LHC motif. HvFC2, as with other plant FC2 sequences, contains this domain (**Fig S1**) which is connected to the FC2 catalytic core by a proline-rich linker sequence (**Fig S1**) and is reported to be essential for enzymatic activity (Sobotka et al. 2011). The LHC motif is abundant in proteins associated with light harvesting complex and is important for anchoring the complex to the chloroplast membrane, binding chlorophyll and carotenoids, and facilitating interactions with other co-localised proteins (Takahashi et al. 2014). FC2 is reported not to be associated with the light harvesting complex of the photosystem, but regulates its own monomer-dimer transitions (Storm et al. 2013). However, the absence of a CAB domain in the only cyanobacterial (*Synechocystis* sp.) FC (an orthologue of plant FC2), leads to an aberrant accumulation of the chlorophyll precursor, chlorophyllide under high light stress (Sobotka et al., 2011). This suggests an indirect regulatory role for FC2 in controlling the balance of chlorophyll biosynthesis under stress.

In line with findings from *Arabidopsis* and cucumber (Chow et al. 1998; Scharfenberg et al. 2014; Singh et al. 2002; Suzuki et al. 2002), expression of *HvFC1* differs compared to *HvFC2*. *HvFC1* and *HvFC2* have similar transcript levels within photosynthetic tissues, but *HvFC1* is more highly expressed in non-photosynthetic tissues (**Fig 3-2**). Together with structural

divergence between the two isoforms these differential expression patterns indicate that HvFC1 and HvFC2 may have distinct roles in barley.

3.6.2 Both HvFC1 and HvFC2 are localized in chloroplast

FC2 has been shown to be targeted specifically to the chloroplast (Chow et al. 1998; Masuda et al. 2003; Suzuki et al. 2002). Although a number of studies suggest that FC1 is dual-targeted to both chloroplasts and mitochondria, other research indicates that FC1 is unlikely to be imported into mitochondria (Lister et al. 2001; Masuda et al. 2003). In order to investigate the localization of HvFC1 a transient expression assay was conducted. Our observations are suggestive of HvFC1 being localized to the chloroplast but not mitochondria (**Fig 3-3**), as GFP fluorescence was only detected in large irregular and oval shaped structures that are typical of onion cell proplastids and their associated stromules (Natesan et al., 2005), as opposed to smaller punctate structures typical of mitochondria Arimura & Tsutsumi, 2002; Arimura et al., 2004). This would indicate that in photosynthetic tissues the primary site of heme biosynthesis is the chloroplast. Given similar levels of *FC1* and *FC2* expression in photosynthetic tissues, and similar subcellular localization patterns (Masuda et al., 2003; Lister et al., 2001), it may be speculated that both isoforms of HvFC have similar functions in these tissues. However, it is possible that HvFC1 is targeted to mitochondria in non-photosynthetic tissues, such as the root where it is also expressed (**Fig 3-2**).

3.6.3 Both barley FC isoforms contribute to photosynthetic performance

To help determine whether *HvFCs* differentially affect photosynthesis, we generated transgenics ectopically overexpressing either *HvFC1* or *HvFC2* and measured plant growth

and development as well as various photosynthetic performance traits. *HvFC1* and *HvFC2* transgenics were developmentally equivalent relative to controls, with no obvious defects in plant height, leaf number, tillering, or shoot and root dry weights (**Table S1**). These findings are consistent across lines derived from different transformation events. This is in line with the findings of Kang et al. (2010) who demonstrated similar phenotypes for rice *FC1* and *FC2* overexpressing transgenics relative to wild-type.

Since increasing the concentration of heme has been reported to inhibit the activity of the first rate limiting enzyme of the tetrapyrrole pathway, GluTR *in vitro* (Vothknecht et al. 1998), we expected that overexpression of *FC*s would negatively regulate the pathway and lead to reduced chlorophyll accumulation. However, both *HvFC1* and *HvFC2* overexpressing transgenics exhibited higher total chlorophyll content relative to controls (**Fig 3-5a**). Although this finding was unexpected, an independent study conducted on rice transgenics overexpressing *FC1* and *FC2* also showed no reduction in total chlorophyll content (Kang et al. 2010). By contrast, *Arabidopsis FC1* and *FC2* overexpressing transgenics were found to have reduced chlorophyll content, even though heme content relative to controls was similar (Woodson et al. 2011). This indicates that mechanisms controlling tetrapyrrole biosynthesis are highly complex with further investigations necessary to elucidate the interactions between chlorophyll and heme branches. Increased total chlorophyll content in the *HvFC1* and *HvFC2* overexpressing barley lines in our study could result from preferential channelling of tetrapyrroles via the chlorophyll branch of the pathway, or from an overall increase in GluTR activity and consequent increased tetrapyrrole precursor availability. Whether the basis for this effect is transcriptional or post-translational is worthy of future investigation.

Our results found that overexpression of *HvFC1* and *HvFC2* each improve A_{sat}, g_s and CE (**Fig 3-5 b, c, d**), implying that both barley FC isoforms are directly involved in photosynthesis or regulation of its photosynthetic components. Photosynthesis is a highly complex and highly regulated process with the rate of carbon assimilation determined by a wide range of factors. For instance, greater g_s allows a greater rate of CO_2 diffusion into the leaf. This in turns improves photosynthetic capacity as indicated by the improved CE and carbon assimilation in both transgenics. Higher CE is unlikely to be a consequence of higher Rubisco content, as both *HvFC1* and *HvFC2* transgenics had similar leaf N concentrations relative to controls (**Fig 3-6a**). Further investigations are warranted to determine if altered Rubisco activity can explain the improved CE of these transgenics. Furthermore, we found that improved photosynthetic performance is not likely a result of altered Fe homeostasis (**Fig 3-6b**).

To date, there is no direct evidence supporting a role for FC or heme in photosynthesis. However, heme is a part of the cytochrome *b6f* complex, which has been demonstrated to be important for electron transport between PSI and PSII (Cramer et al. 1996; Kurisu et al. 2003). Therefore, one possible reason for improved carbon assimilation of HvFC overexpressing transgenics could be due to their higher electron transport capacity. Another likely reason for this may be related to the ability of heme to stimulate retrograde signaling. In plant cells, the majority of heme binds covalently and non-covalently to a large number of hemo-proteins such as nitrate reductase, NADPH oxidases, peroxidases, and catalases as well as b- and c-type cytochromes (Cornah et al. 2003; Mochizuki et al. 2010; Terry and Smith 2013). Additionally, a small proportion of the total heme content exists as unbound or free heme pool. It has been proposed that this free heme pool acts as a plastid signal for

modulating the expression of photosynthesis-associated nuclear genes (Terry and Smith 2013; Woodson et al. 2011; Woodson et al. 2013). By this mode of action, we can infer that *HvFC1* and *HvFC2* ectopic overexpressors may induce an increase in the free heme pool, which may, in turn, trigger nuclear gene expression for enzymes that affect carboxylation rate. We have attempted to evaluate the total and free heme pools in these barley transgenic lines by acid acetone extraction (Adrian Lutz *pers comm.*). However, analysis was confounded by difficulties in measuring free heme because it rapidly undergoes demetalation and is converted to Proto IX. In line with our observations, Espinas et al., (2012) reported that there is a substantial risk of losing heme when plant tissues are processed by acid acetone extraction. Therefore, our future investigations will focus on optimizing heme quantification assay. We also aim to determine which photosynthesis-associated nuclear genes are responsive to heme and how they may affect CE.

Even though previous evidence suggests that *FC1* and *FC2* are involved in distinct cellular functions, collectively our results indicate that both genes play similar roles in photosynthesis. This study highlights tetrapyrrole biosynthesis as a simple target for engineering photosynthetic yield potential, a trait considered as physiologically complex. The molecular identity of these gene sequences now allows beneficial expression alleles to be identified, tracked and ultimately deployed into cereal breeding programs.

3.7 Acknowledgement

This research was supported by the Australian Research Council, the Grains Research and Development Corporation, the Government of South Australia, the University of Adelaide and the Dupont Pioneer, USA. We thank Alison Hay, Anzu Okada for generating transgenic

vectors, Dr. Ainur Ismagul for barley transformation and Yuan Lee for quantitative RT-PCR analysis. We acknowledge Ute Roessner, Alice Ng and Adrian Lutz at Metabolomics Australia, Melbourne for heme quantification. We would also like to thank Dr. Julie Hayes, Dr. Penny Tricker and Dr. Robyn Grove for critical comments on the manuscript.

Chapter 4: Barley transgenics overexpressing *Ferrochelatases* (*HvFC1* and *HvFC2*) maintain higher photosynthesis and reduce photo-oxidative damage under drought stress

Dilrukshi S. K. Nagahatenna[1], Boris Parent[2], Everard Edwards[3], Peter Langridge[1] and Ryan Whitford[1]*

[1] Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia 5064 Australia

[2] INRA, Unité Mixte de Recherche 759 Laboratoire d'Ecophysiologie des Plantes sous Stress Environnementaux, F–34060 Montpellier, France.

[3] Agriculture Flagship, Commonwealth Scientific and Research Organisation, PMB 2, Glen Osmond, South Australia, 5064 Australia

*Corresponding author:

Ryan Whitford

Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, South Australia 5064 Australia

Tel: 61 88313 7171

Fax: 61 88313 7102

E-mail: Ryan.Whitford@acpfg.com.au

4.1 Statement of authorship

Title of Paper	Barley transgenics overexpressing *Ferrochelatases* (*HvFC1* and *HvFC2*) maintain higher photosynthesis and reduce photo-oxidative damage under drought stress
Publication Status	☐Published ☐Accepted for Publication ☐ Submitted for Publication ☑ **Publication style**
Publication Details	This chapter was prepared as a manuscript for submission to the journal of New Phytologist

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Dilrukshi Nagahatenna
Contribution to the Paper	Designed experiments, conducted drought assay, analysed plant water status, gas exchange parameters, gene expression, chlorophyll content and fluorescence, evaluated transgenics under herbicide-induced and tetrapyrrole-mediated oxidative stress, conducted statistical analysis, interpreted the results and wrote the manuscript and took the primary responsibility for manuscript revision.
Signature	Date: 22-01-2015

Name of Co-Author	Boris Parent
Contribution to the Paper	Designed drought assay and provided supervision on measurment of gas exchange parameters, provided constructive criticisms on data interpretation
Signature	Date: 16/01/2015

Name of Co-Author	Everard Edwards
Contribution to the Paper	Provided constructive critisisms on gas exchange parameter analysis of the trangenics and on the manuscript.
Signature	Date: 29/1/15

Name of Co-Author	Peter Langridge
Contribution to the Paper	Provided overall supervision of the research and writing the manuscript
Signature	Date 22/1/2015

Name of Principal Author (Candidate)	Ryan Whitford
Contribution to the Paper	Provided guidance in the design of the research, analysis of the data and writing of the manuscript.
Signature	Date 22/1/15

4.2 Abstract

We investigated the roles of two *Ferrochelatases (FC*s), which encode the terminal enzyme for heme biosynthesis, in drought and oxidative stress tolerance in the model cereal plant barley (*Hordeum vulgarae*). Three independent transgenic lines ectopically overexpressing either barley *FC1* or *FC2* were selected and evaluated under drought and oxidative stress. Both *HvFC1* and *HvFC2* transgenics showed delayed wilting and maintained higher photosynthetic performance relative to controls upon dehydration. In each case *HvFC* overexpression significantly up-regulated nuclear genes associated with ROS detoxification upon drought stress. Overexpression of *HvFC*s, also suppressed photo-oxidative damage induced by the deregulated tetrapyrrole biosynthesis mutant *tigrina*^{d12}. Previous studies suggest that only FC1 is implicated in stress defence responses, however our study demonstrates that both FC1 and FC2 affect drought stress tolerance. As FC-derived free heme has been proposed as a chloroplast-to-nuclear signal, heme could act as an important signal stimulating drought responsive nuclear gene expression. This study also highlights tetrapyrrole biosynthetic enzymes as targets for metabolic engineering towards improved crop performance under water-limited environments.

4.3 Introduction

Drought is one of the major abiotic stress factors which adversely affect plant growth and limit crop yield (Boyer 1982). Therefore, improving drought tolerance of major crops such as cereals is a primary objective of plant breeding. Improved crop performance under water-limited conditions will be necessary to satisfy food demands that are a consequence of a growing world population. The incidence and severity of drought events is likely to increase in the face of global climate change. Photosynthesis is one of the primary cellular processes

affected by drought (Chaves 1991). Drought stress significantly reduces photosynthetic rate by limiting CO_2 diffusion through the stomata and potentially inducing secondary effects such as oxidative stress that can damage the photosynthetic machinery (Chaves et al. 2009). Ultimately, this leads to substantial yield loss. However, drought tolerant C_3 plants have evolved efficient strategies to respond to drought stress. During drought stress, drought avoidance or acclimation mechanisms allow plants to minimize transpirational water loss. This can occur through stomatal closure, by adjusting leaf architecture, reducing leaf growth and by shedding older leaves (Chaves et al. 2009). Plants can also avoid dehydration by maximizing water uptake through accelerated root growth (Mundree et al. 2002). Plants exhibiting developmental plasticity can also escape drought by completing their life cycle before drought stress becomes lethal. Increased levels of osmoprotectants such as proline, glycine, betaine and polyols also allow plants to maintain turgor and protect cells from plasmolysis (Chaves et al. 2009). Similarly, high levels of antioxidants can mitigate ROS damage (Cruz de Carvalho 2008). Drought tolerance is a complex phenotype, which is under complex genetic control (Fleury et al. 2010; McWilliam 1989). It is apparent that stress responses are initiated by altering the expression of a multitude of genes necessary for 'reprogramming' the whole plant performance upon stress. Understanding the genetic basis of drought tolerance as well as underlying genes and biochemical mechanisms is a prerequisite for developing superior crop varieties.

The tetrapyrrole biosynthesis pathway supplies the essential compounds, chlorophyll and heme, for photosynthesis (Tanaka and Tanaka, 2007). Chlorophyll is the most abundant pigment in plants necessary for photosynthesis (Chen et al. 2010). Heme on the other hand, is an integral component of photosynthetic and respiratory cytochromes involved in

electron transport (Cramer et al. 1996; Kurisu et al. 2003). Unlike chlorophyll, heme is important for many cellular functions, including acting as a co-factor for enzymes able to detoxify reactive oxygen species (ROS) (del Río, 2011; Kirkman and Gaetani, 1984; Layer et al., 2010). Recently, it was proposed that a sub-pool of heme can serve as a retrograde signal triggering photosynthesis-associated nuclear gene expression (Woodson et al. 2011; Woodson et al. 2013). Both chlorophyll and heme are produced in the chloroplast. For their synthesis, 5-aminolevulinic acid (ALA), the initial common tetrapyrrole precursor is converted, through a series of reactions, into protoporphyrin IX (Proto IX). Insertion of Mg^{2+} into Proto IX through the action of Mg-chelatase leads to the production of chlorophyll, whereas the insertion of Fe^{2+} by Ferrochelatase (FC) results in the production of heme (Moulin and Smith 2005).

Several genetic and biochemical studies have proposed that an increased flux through the heme branch of the pathway improves tolerance to drought stress (Allen et al. 2010; Kim et al. 2014; Li et al. 2011; Thu-Ha et al. 2011; Nagahatenna et al., 2015a). In all plants investigated so far, FC, the terminal enzyme for heme biosynthesis, is only encoded by two genes. Therefore, this makes these genes ideal targets for engineering plants for drought stress tolerance. However, based on their differential stress responsive expression patterns, previous studies have suggested that only *FC1* is likely to be important for stress defence responses. For instance, transcriptional reporter gene fusions showed that *Arabidopsis FC1* is induced in response to wounding, norflurazon-induced oxidative stress and viral infection (Nagai et al. 2007; Scharfenberg et al. 2014; Singh et al. 2002). In contrast, *FC2* is repressed or remain unchanged under these stress conditions (Singh et al., 2002; Scharfenberg et al., 2014). These findings led the authors to propose that two physiologically distinct heme

pools are synthesized by each of these FCs with only *FC1*-derived heme being implicated in stress responses. A detailed investigation is yet to determine whether the two *FC*s contrast in drought and oxidative stress responses.

Towards understanding the roles for these genes in drought and oxidative stress responses, we used barley, a major global crop but also a model for wheat. Two *FC*s were identified and cloned from barley cultivar Golden Promise (GP). Transgenic lines ectopically overexpressing either *HvFC1* or *HvFC2* were generated with three independent lines selected for each FC isoform (Nagahatenna et al. 2015b). These were then evaluated under drought and oxidative stress. Oxidative stress was induced either by a herbicide (Paraquat) application or by exposing a tetrapyrrole biosynthesis deregulating mutant, *tigrina*^{d12} to a dark to light shift. Here, we report that ectopic overexpression of either *HvFC1* or *HvFC2,* improved drought stress tolerance and suppressed tetrapyrrole-induced photo-oxidative damage in *tigrina*^{d12} mutant.

4.4 Materials and Methods

4.4.1 Genetic materials

Barley transgenic lines (cv. Golden Promise (GP)) ectopically overexpressing either *HvFC1* or *HvFC2* were generated using *Agrobacterium*-mediated transformation. Twenty-nine T_0 transformants were screened for transgene copy number and expression by Southern hybridization and quantitative RT-PCR respectively. Three independent lines were selected for each FC isoform and evaluated for stress tolerance. For detailed information on the experimental procedure please refer to Nagahatenna et al., (2015b).

4.4.2 Plant growth and stress conditions

For *HvFC* gene expression analysis under oxidative stress, barley (*Hordeum vulgare* L.cv. Golden promise) plants were grown in pots containing coco-peat and field soil (50:50, v/v) under controlled conditions of 20-18 ^{0}C day/night temperature, 50-60% relative humidity and a 12:12 hrs photoperiod. In all drought assays, control plants were grown in pots containing a mixture of field soil and coco-peat (50:50, v/v) under the same environmental conditions as outlined above.

For evaluating performance of barley transgenics ectopically overexpressing *HvFC*s upon drought stress, one untransformed control, null segregant and transgenic seed was planted together in a single pot (25.5 cm in diameter and 23.5 cm in height), therefore exposing all plants to the same soil conditions. A total of five pot replicates were analysed per time point and per treatment. Each pot was lined with a polythene sheet to ensure no water added to the pot is lost due to drainage or air drying, so that all plants within the pot have access to the same soil water moisture. All plants were grown under growth conditions as outlined above.

For gene expression analysis, *tigrina*^{d12} mutants were grown with 24 hrs continuous light. To investigate the role of *HvFC* overexpression on teterapyrrole-mediated oxidative stress, control barley (*Hordeum vulgare* L.cv. Golden promise and cv. Bonus), transgenic lines (T_2), non-transgenic and transgenic *tigrina*^{d12} overexpressing either *HvFC1* or *HvFC2* were grown under controlled conditions outlined above, but with 24 hrs continuous light.

4.4.3 Drought assay

Each pot was watered equally to maintain similar pot weight for six weeks and then water was withheld. In order to identify variation of soil water potential in each pot upon drought stress, a calibration experiment was conducted concurrently. In the calibration experiment, control (cv. Golden Promise) plants were planted in similar polythene lined pots containing the same amount of soil and were grown under the same growth conditions outlined above. Predawn leaf water potential was measured daily, using a plant water status console (Model 3000, Soilmoisture Equipment Corp., P.O. Box 30025, Santa Barbara, CA 93105, USA) until plants wilted. This predawn leaf water potential was considered to be equivalent to the soil water potential in each pot. Furthermore, pot weight was measured daily to determine the soil moisture corresponding to the respective soil water potential. Based on the soil moisture and soil water potential, a water release curve of this soil mixture was constructed (**Fig S3**). In the drought assay, pot weights were monitored daily upon drought stress to identify corresponding soil water potential as indicated by the water release curve. A soil water potential of -0.6 MPa was maintained for 1 week and then plants were rewatered (**Fig 4-1**). Measurements were taken before stress, 2, 5, 8 and 15 days post water withholding as well as after rewatering (18 days post water withholding; **Fig 4-1**). These time points represent fully irrigated (0), -0.1, -0.3, -0.6, -0.6 MPa and fully rewatered soil water potentials.

4.4.4 Paraquat treatment

The second leaf from the top of the primary tiller of 3-week-old control and transgenic lines were dipped in a 20 µM Paraquat solution for 1 min under dark and re-exposed to 24 hrs continuous light. The level of necrosis in transgenics relative to controls was evaluated

following herbicide treatment. Transcriptional responses of *HvFC1* and *HvFC2* to Paraquat-induced oxidative stress was analysed in leaves of control plants by quantitative RT-PCR.

4.4.5 Screening and evaluating *tigrina^{d12}* mutants overexpressing *HvFC1* and *HvFC2* under tetrapyrrole-mediated oxidative stress

To investigate the role of the HvFC under tetrapyrrole-mediated oxidative stress, a representative transgenic line for *HvFC1* and *HvFC2* was crossed with *tigrina^{d12}*. Seeds from non-transgenic and transgenic *tigrina^{d12}* mutants (F_2), *HvFC* overexpressing lines (T_2) and control barley (*Hordeum vulgare* L.cv. Golden promise and cv. Bonus) were grown on a wet petri plate for 5 days in continuous darkness. Cotyledons were removed under safe green light and were illuminated under UV light to identify homozygous mutants in F_2 segregating population. Photographs were taken with a Canon 60D digital camera. Images were analysed for red fluorescence using ImageJ software.

Homozygous F_2 *tigrina^{d12}* mutant phenotypes were confirmed using a cleaved amplified polymorphic sequence (CAPS) marker, designed to the causative mutation in the *FLU* gene (Lee et al. 2003) (**Fig 4-5-1st and 2nd panel**). PCR analysis was conducted with *FLU* and transgene specific primers. For the analysis using CAPS markers, PCR product was digested with *Hae*III restriction endonuclease at 37 ^{0}C for 2 hours and 65 ^{0}C for 10 min. PCR products before and after digestion were analysed in 2% agarose gel and visualized by staining with ethidium bromide.

Mutant seedlings, which contain *HvFC* transgenes were identified by using a dominant transgene specific PCR marker (**Fig 4-5- 3rd panel**). Descriptions of the primer sequences

used are described in supplementary materials (**Table S3**). In order to investigate the effect of *HvFC* overexpression on tetrapyrrole-mediated oxidative stress, 3-week-old transgenic *tigrina*^{d12} mutants with all the above mentioned controls were grown under 24 hrs continuous light. Then the plants were subjected to 24 hrs dark period and re-illuminated.

4.4.6 Chlorophyll content

Chlorophyll was extracted from leaf tissues using dimethyl sulfoxide (DMSO) and determined spectrophotometrically according to Hiscox and Israelstam (1979). The total chlorophyll content was calculated using the following equation. Total chlorophyll (g l^{-1}) = 0.0202 A_{645} + 0.00802 A_{663} (A_{645} and A_{663} are absorbance at 645 and 663 nm).

4.4.7 Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured using a pulse-amplitude-modulated photosynthesis yield analyser (Mini-PAM, Walz, Effeltrich, Germany) with a dark leaf clip to ensure all measurements were taken at the same distance from the leaf. Maximum quantum yield of PSII photochemistry (calculated as ratio F_v/F_m = $(F_m–F_o)/F_m$) was determined by applying an 800 ms saturating light pulse to 30 min dark adapted leaves.

4.4.8 Measurements of Relative Water Content (RWC)

For leaf RWC measurement, leaves were excised between 09.00 hrs and 10.00 hrs, and their fresh weight was measured immediately. Rehydrated weight was determined by floating them in deionized water at 4^0C overnight. Leaf dry weight was measured by oven drying at 80^0C for 48 hrs. The RWC was calculated as follows: RWC (%) = (fresh weight – dry weight)/ (rehydrated weight – dry weight) X 100.

4.4.9 Photosynthetic measurements

In vivo gas exchange parameters were measured in developmentally equivalent fully expanded leaves from 4 to 6 week old plants using a LI-6400 portable photosynthesis system (Licor, USA). The conditions of the leaf cuvette were set to a light intensity of 2000 µmol m^{-2} s^{-1}, humidity of 50-60%, temperature 25 ^{0}C and reference air CO_2 concentration of 400 µmols^{-1}. Measurement period was from 09.00 hrs to 17.00 hrs. Instantaneous WUE and carboxylation efficiency (CE) were calculated based on gas exchange parameters. Instantaneous WUE = photosynthesis rate under saturated light (A_{sat})/ transpiration rate, CE = A_{sat}/ intracellular CO_2 concentration.

4.4.10 Gene expression analysis

Total RNA was extracted from leaf tissues of 3 week old control and transgenic plants before and during drought and oxidative stress using RNeasy plant extraction kit (Qiagen). cDNAs were prepared using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression was analysed by quantitative RT-PCR using primers from the coding regions of *HvFC1*, *HvFC2*, *Catalase* (*Cat*) and *Superoxide dismutase* (*Sod*) as described by Burton et al. (2004). mRNA copy number for each tested gene was normalized against four control genes (*GAPDH*, *HSP70*, *cyclophilin* and *tubulin*) as described by Burton et al. (2004). Descriptions of the probe and primer sequences used in these experiments are described in supplementary materials (**Table S3**). Relative expression was calculated using $2^{-\Delta\Delta CT}$ method as described by (Schmittgen and Livak 2008).

4.4.11 Statistical analysis

All data were statistically analysed by either one-way or two-way ANOVA using GenStat software, and mean differences were compared through LSD test. Differences were considered statistically significant when $P<0.05$.

4.5 Results

4.5.1 Overexpression of *HvFC1* and *HvFC2* maintained higher leaf water status and water use efficiency under drought stress, independently of stomatal closure

As an initial step to understand the role of *FC*s in drought stress responses, two *FC*s were identified in barley (Nagahatenna et al., 2015b). Barley transgenic lines (cv. GP) ectopically overexpressing *HvFC1* and *HvFC2* were generated by cloning coding regions of *FC* into the pMDC32 vector under the control of the 2x35SCaMV promoter (Nagahatenna et al., 2015b). Twenty-nine independent T_0 transgenic lines were screened for transgene copy number and expression. Three single copy, independent transgenic events each ectopically overexpressing either *HvFC1* or *HvFC2* were selected (Nagahatenna et al., 2015b) and evaluated upon drought stress. The gradual reduction in soil water potential over the period of the drought stress (**Fig 4-1**) was inferred using a standardised drying curve (**Fig S3**). This drying curve was previously determined to represent the relationship between pre-dawn leaf water potential, pot weight and the particular soil characteristics used in this experiment.

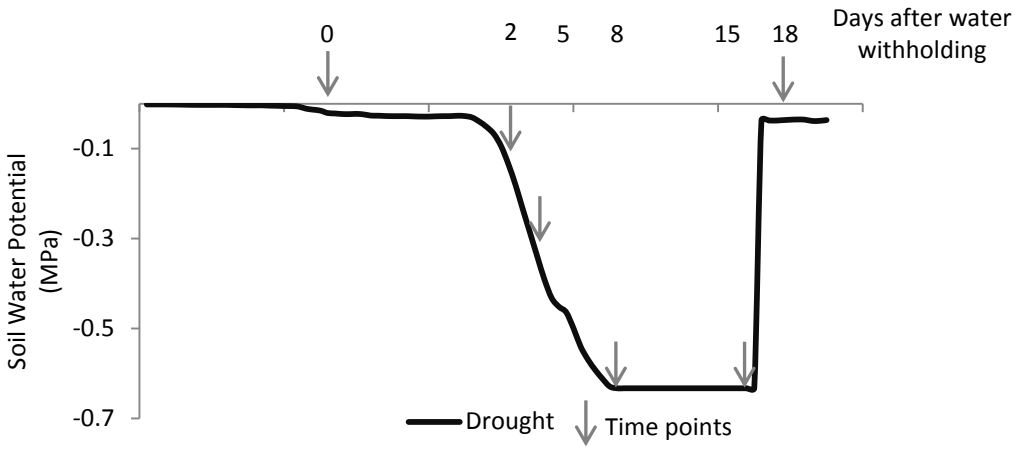
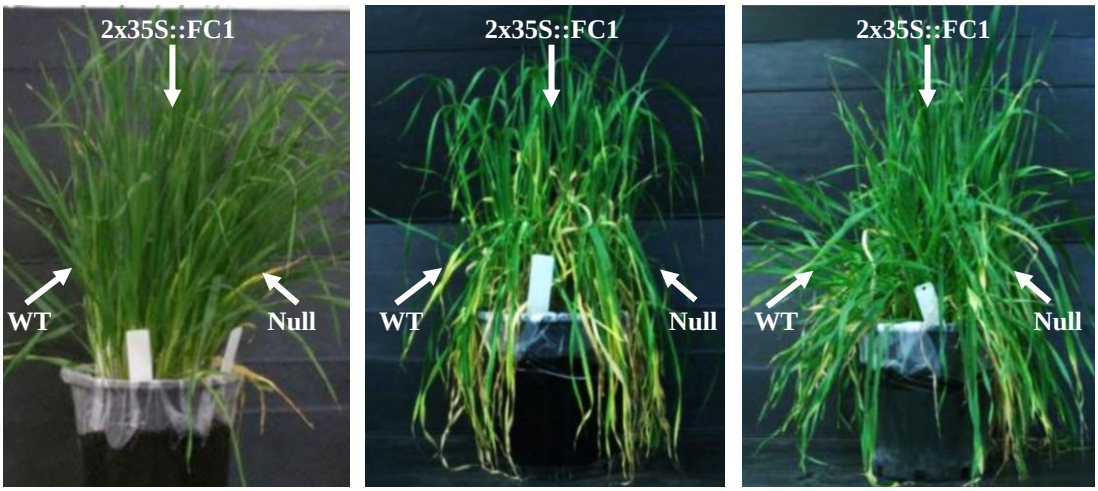
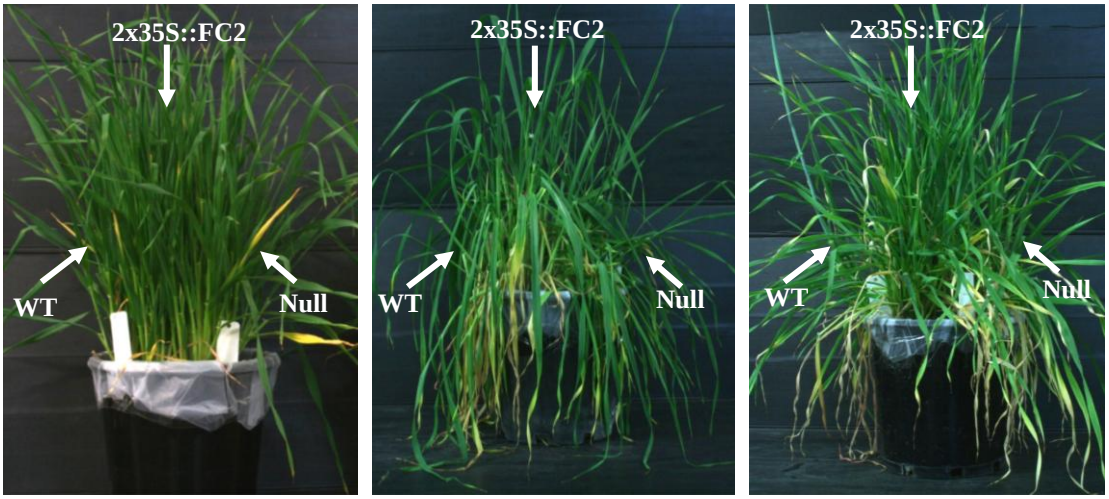


Fig 4-1. Variation of the soil water potential before, during and after drought stress. Six weeks after planting, watering was withheld. Minus 0.6 MPa soil water potential was maintained for a week and then plants were rewatered to initial soil water potential. Arrows indicate different time points where measurements were taken.

We first made qualitative observations on the relative time to wilting. Visual inspection revealed controls reversibly wilted at -0.6 MPa soil water potential (8 days post water withholding) whereas neither *HvFC1* nor *HvFC2* overexpressing transgenics exhibited wilting symptoms. This can be seen in **Fig 4-2** in the 8 day image. The plants at the rear of the pot (the transgenics) remain erect while those at the front are wilted (untransformed controls (WT) and null segregants).





Before stress | 8 days after withholding water | After rewatering

Fig 4-2. Phenotypes of 6 week old control plants and transgenic lines (T_2) grown under controlled environmental conditions in the absence of stress, 8 days post water withholding and after re-watering. The control plants were reversibly wilted 8 days after withholding water whereas both types of transgenics did not wilt.

Given that a plant's wilting point is in part governed by leaf water content, we measured leaf relative water content (RWC) before, during and after water deficit stress. Results revealed that both *HvFC1* and *HvFC2* transgenics (calculated as an average of data from three transgenic events per construct) had a higher leaf RWC during drought stress than controls (**Fig 4-3a**). At -0.6 MPa soil water potential (day 8), transgenics had on average 10-12% higher leaf RWC compared to controls. No significant difference in leaf RWC was observed before drought stress or after re-watering. Differences in leaf RWC also extended to observed differences in instantaneous Water Use Efficiency (WUE) in both types of transgenics when compared to the controls (**Fig 4-3b**). WUE for *HvFC1* transgenics (calculated as an average of data from three transgenic events per construct) were unchanged before and up to 5 days post water withholding, as well as after re-watering.

Here, only marginal increases in WUE were observed from 8 to 15 days post water withholding. In contrast, *HvFC2* transgenics exhibited significantly higher WUE 5 to 8 days post water withholding. These findings suggest that *HvFC1* and *HvFC2* differentially affect WUE.

Water is continually lost to the atmosphere via transpiration, therefore we investigated whether *HvFC* transgenics had higher leaf RWC and WUE as a consequence of lower stomatal conductance (g_s) relative to controls. However, *HvFC1* transgenics showed greater g_s before and during the early phases of drought stress (2 and 5 days post water withholding) relative to controls (**Fig 4-3c**). This observed difference ceased at a soil water potential of -0.6 MPa (8 days), and increases once more post rewatering. A similar trend, albeit not statistically significant, was observed between *HvFC2* transgenics and controls. These findings indicate that the higher leaf RWC and WUE is unlikely to be a consequence of reduced g_s for *HvFC1* and *HvFC2* overexpressing transgenics. Collectively these findings indicate that both *HvFC* transgenics maintain a positive leaf water status during drought stress, when compared to controls. The observed differences in WUE and g_s between *HvFC1* and *HvFC2* transgenics may be a consequence of alternate modes of action for FC isoforms.

4.5.2 *HvFC1* and *HvFC2* overexpressing transgenics maintained higher photosynthetic activity in well-watered condition and upon dehydration

Water and CO_2 are essential substrates for photosynthesis, therefore we investigated whether the observed positive water status and enhanced g_s in *HvFC* transgenics has the capacity to improve carboxylation and therefore ultimately carbon assimilation. Measurements of CE revealed that both *HvFC1* and *HvFC2* transgenics had higher CE relative

to wild-type controls before, during the early phases (2-5 days post water withholding) of drought stress and even 1 week at -0.6 MPa soil water potential (15 days post water withholding) (**Fig 4-3d**). The amount of photosynthetic pigment is important as they play a major role in light energy perception. Both *HvFC1* and *HvFC2* overexpressing transgenics exhibited significantly higher chlorophyll content before stress, -0.1 and -0.3 MPa soil water potentials (2 to 5 days after post water withholding) as well as after rewatering relative to controls (**Fig 4-3e**). This finding extends to photosynthesis rate under saturated light (A_{sat}), whereby both *HvFC1* and *HvFC2* transgenics have significantly higher A_{sat} before and 2 to 5 days post water withholding, relative to controls. The improvement in A_{sat} is between 3 and 4 µmol $m^{-2}s^{-1}$ in both transgenics relative to wild-type (**Fig 4-3f**). These results suggest that the overexpression of both *HvFC*'s have the capacity to maintain higher photosynthetic performance relative to controls under both well-watered and drought stress conditions.

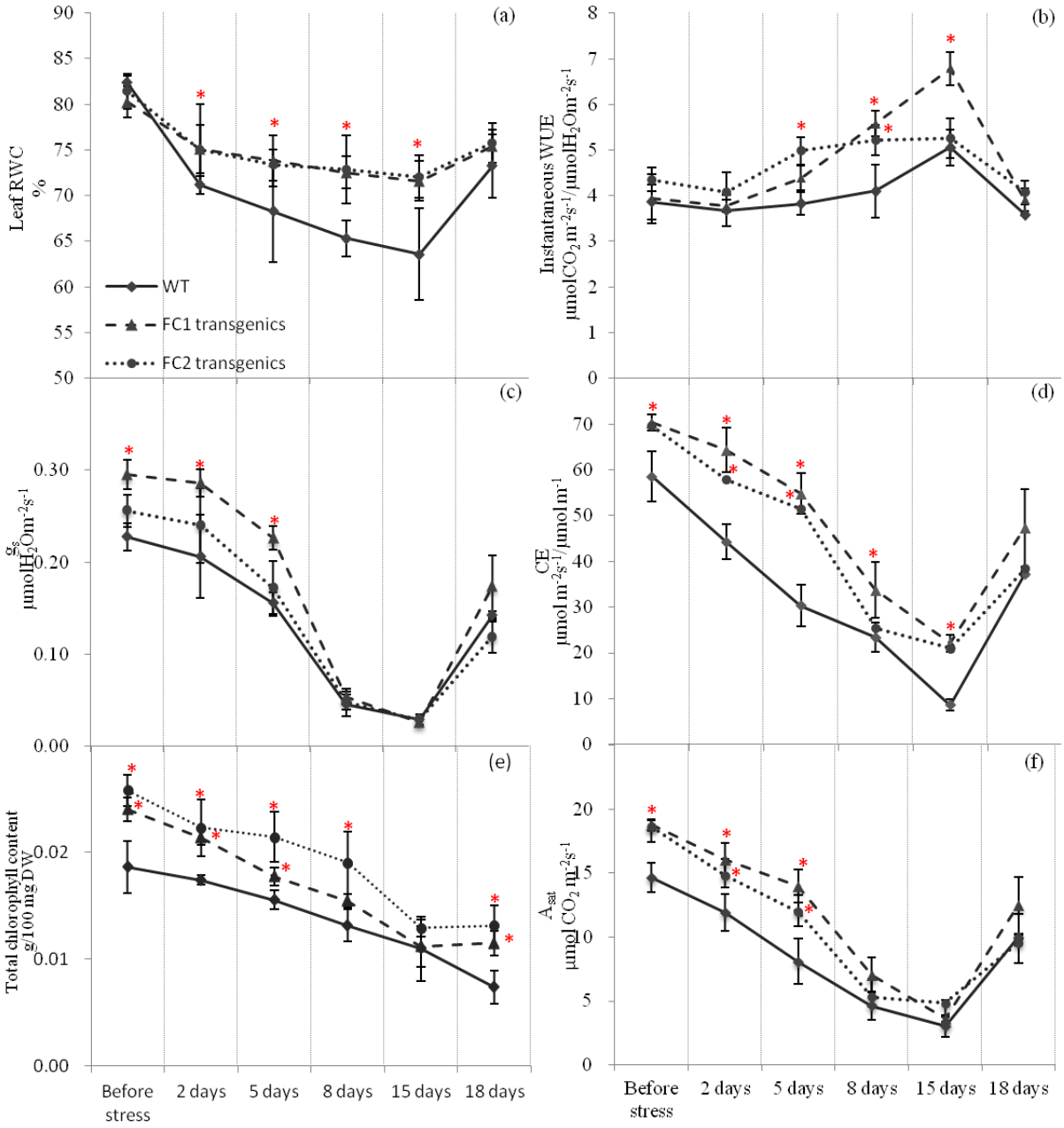


Fig 4-3. *HvFC* overexpressing transgenics maintained higher leaf water status and photosynthetic performance relative to controls upon drought. Physiological traits measured include: **(a)** Leaf relative water content (RWC), **(b)** Instantaneous water use efficiency (WUE), **(c)** Stomatal conductance (g_s), **(d)** Carboxylation efficiency (CE), **(e)** Total chlorophyll content and **(f)** Photosynthesis rate under saturated light (A_{sat}) relative to wild-type (WT) control plants upon drought stress. The data are shown as mean ± standard error (SE) of five plants for each of three independent transgenic lines per construct and WT.

Asterisks indicate a statistically significant difference between transgenics and controls, at *P*<0.05 based on two-way ANOVA.

4.5.3 Overexpression of *HvFCs* invokes expression of ROS detoxification markers

To investigate whether both types of transgenics have the ability to prevent drought-mediated oxidative stress, transcriptional responses of genes associated with ROS detoxification were analysed in controls versus a representative transgenic line for *HvFC1* and *HvFC2*, before, during and after water deficit stress. Transcripts targeted for analysis include *Cat* and *Sod*, as they have been previously shown to be transcriptionally responsive to drought and encode proteins important for ROS detoxification (Zhang and Kirkham 1994). *Cat* expression in *HvFC1* transgenics was significantly repressed (P<0.05) when compared to control plants both before stress and up to 8 days post exposure to drought (**Fig 4-4a**). As the stress progresses up to 15 days this trend reverses, whereby *Cat* is significantly up-regulated (P<0.05) in the transgenic relative to control. However, this observation was not significant when comparing plants overexpressing *HvFC2* to control plants both before stress and up to 8 days post exposure to drought (**Fig 4-4c**). Analysis of *Sod* mRNA levels in *HvFC1* transgenics revealed that *Sod* is transcriptionally up-regulated both at 8 and 15 days post water withholding, with no significant difference observed in transcriptional activity before the onset of stress (**Fig 4-4b**). This contrasts with *Sod* transcription in *HvFC2* transgenics which showed a significant down regulation (P<0.05) when compared to control plants before stress (**Fig 4-4c**). Similarly to *HvFC1* transgenics, *Sod* was transcriptionally up-regulated in *HvFC2* transgenics after 8 days exposure to drought stress. These findings show that both FC isoforms have the capacity to modulate nuclear encoded transcription of ROS detoxification enzymes upon drought stress.

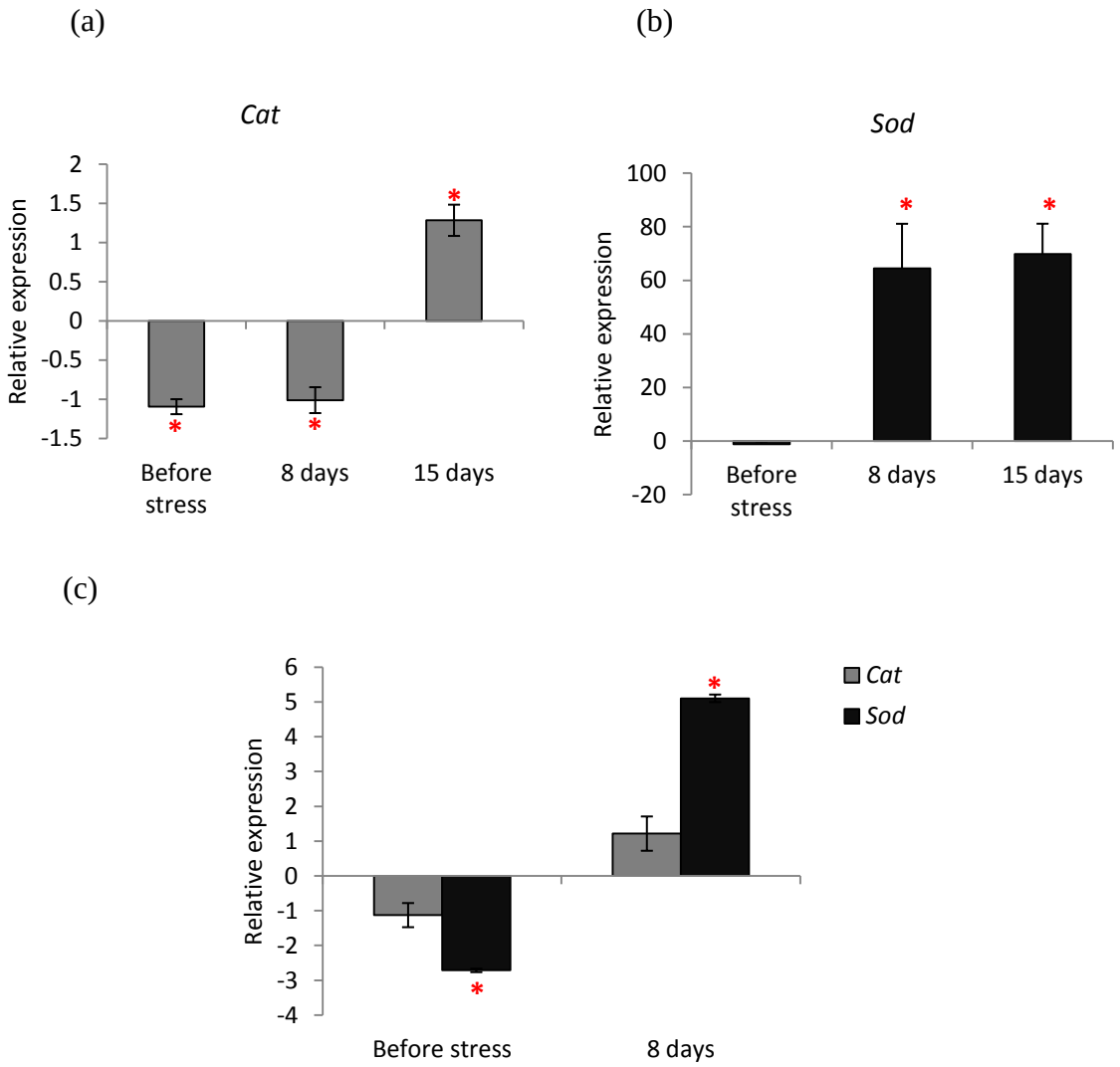


Fig 4-4. Transcriptional responses of ROS detoxification enzymes, catalase (Cat) and superoxide dismutase (Sod) in a representative transgenic line each ectopically overexpressing *HvFC1* (a, b) or *HvFC2* (c) under drought stress relative to WT control. The data are shown as mean ± standard error (SE) of 3 different plants. *Asterisks* indicate statistically significant expression difference between transgenics and WT control, at $P<0.05$ based on two-way ANOVA.

4.5.4 *HvFC* overexpression protects plants from tetrapyrrole-induced photo-oxidation

The apparent ability of both *HvFC* transgenics to improve ROS detoxification upon drought stress, prompted us to investigate whether ectopic overexpression of *HvFC*s improves

oxidative stress tolerance. This was tested by exposing *HvFC* transgenic leaves to 20 µM paraquat and visually assessing leaf photo-bleaching relative to wild-type and null controls. Qualitative observations of leaf photo-bleaching as a time-course post-paraquat treatment revealed no significant differences in leaf photo-bleaching between both *HvFC1* and *HvFC2* transgenics relative to their respective controls (data not shown). This would indicate that these transgenics do not improve tolerance to paraquat-induced oxidative stress, although visual differences between transgenics and controls may be observed if lower concentrations of paraquat were used.

In order to investigate whether ectopic overexpression of *HvFC*s, contribute to tetrapyrrole-induced oxidative stress tolerance, we used the *tigrina*^{d12} mutant. In *tigrina*^{d12} tetrapyrrole biosynthesis is deregulated and consequently these plants accumulate the highly photo-sensitizing chlorophyll branch intermediate, Pchlide under darkness. Etiolated mutant seedlings display strong red fluorescence at 655 nm by UV excitation due to Pchlide accumulation (Lee et al. 2003). When these plants were re-exposed to light, photosensitizing Pchlide generates $^1O_2$ and causes extensive photooxidative damage (Lee et al., 2003). A representative transgenic line for *HvFC1* and *HvFC2* was crossed with *tigrina*^{d12}. Homozygous *tigrina*^{d12} plants were detected within a segregating F_2 population using a CAPS marker, designed to the causative mutation in the *FLU* gene (Lee et al. 2003) (**Fig 4-5-1st and 2nd panel**). Lines containing *HvFC* transgenes were additionally identified by using a dominant transgene specific PCR marker (**Fig 4-5- 3rd panel**). Seedlings identified to be both homozygous for *tigrina*^{d12} and containing either *HvFC1* or *HvFC2* transgenes were compared to non-transgenic (cv. Golden Promise, cv. Bonus, *tigrina*^{d12}, Golden Promise x Bonus F_2 progenies) and transgenic controls (*2x35S::FC1*, *2x35S::FC2*) for both Pchlide accumulation

upon darkness and subsequent photo-oxidative damage induced by a continuous light treatment.

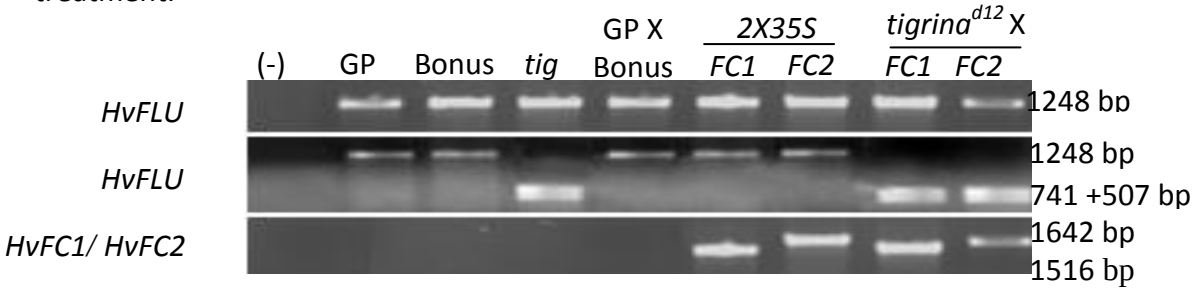


Fig 4-5. Molecular characterization of *tigrina*^{d12} mutants overexpressing *HvFC1* or *HvFC2* using a CAPS marker and transgene specific primers. PCR was conducted using *FLU* specific primers (top panel). The PCR amplicons were cleaved using *HaeIII* restriction enzyme and *tigrina*^{d12} mutants could be differentiated from other plants based on the cleaved fragment sizes (middle panel). The presence or absence of the transgenes was detected by using transgene specific primers (bottom panel). Lane (-) is the negative control.

Indeed, etiolated *tigrina*^{d12} seedlings displayed a strong red fluorescence (**Fig 4-6a**). However, *tigrina*^{d12} seedlings overexpressing either *HvFC1* or *HvFC2* exhibited significantly less red fluorescence at 655nm ($P<0.05$) when compared to non-transgenic *tigrina*^{d12} controls (**Fig 4-6a**). Fluorescence levels were similar between the non-transgenic controls (data not presented). These findings imply that ectopic overexpression of *HvFC1* and *HvFC2* can suppress Pchlide accumulation normally observed in etiolated *tigrina*^{d12} seedlings.

Potential photo-toxic effects were evaluated in these plants upon re-exposure to light by analysing total chlorophyll content and the chlorophyll fluorescence parameter, F_v/F_m. Total chlorophyll content was significantly reduced ($P<0.05$) in *tigrina*^{d12} mutants 24hrs post re-illumination (**Fig 4-6b**). However, chlorophyll content remained unchanged in transgenic *tigrina*^{d12} mutants overexpressing either *HvFC1* or *HvFC2*, suggesting that overexpression of

*HvFC*s suppress the potential photo-bleaching effects of *tigrina*^{d12} mutant. Similarly, *tigrina*^{d12} exhibited significant reduction in F_v/F_m 24hrs after re-illumination (**Fig 4-6c**). Such an effect was not observed in the *tigrina*^{d12} mutant overexpressing *HvFC1.* In the *tigrina*^{d12} mutant overexpressing *HvFC2*, chlorophyll fluorescence was reduced 24hrs after re-illumination relative to the before dark treatment, but it was not as strong as *tigrina*^{d12}. Taken together, these results indicate that both *HvFCI* and *HvFC2* have the capacity to suppress the photo-toxic effects caused by tetrapyrrole deregulation in *tigrina*^{d12}.

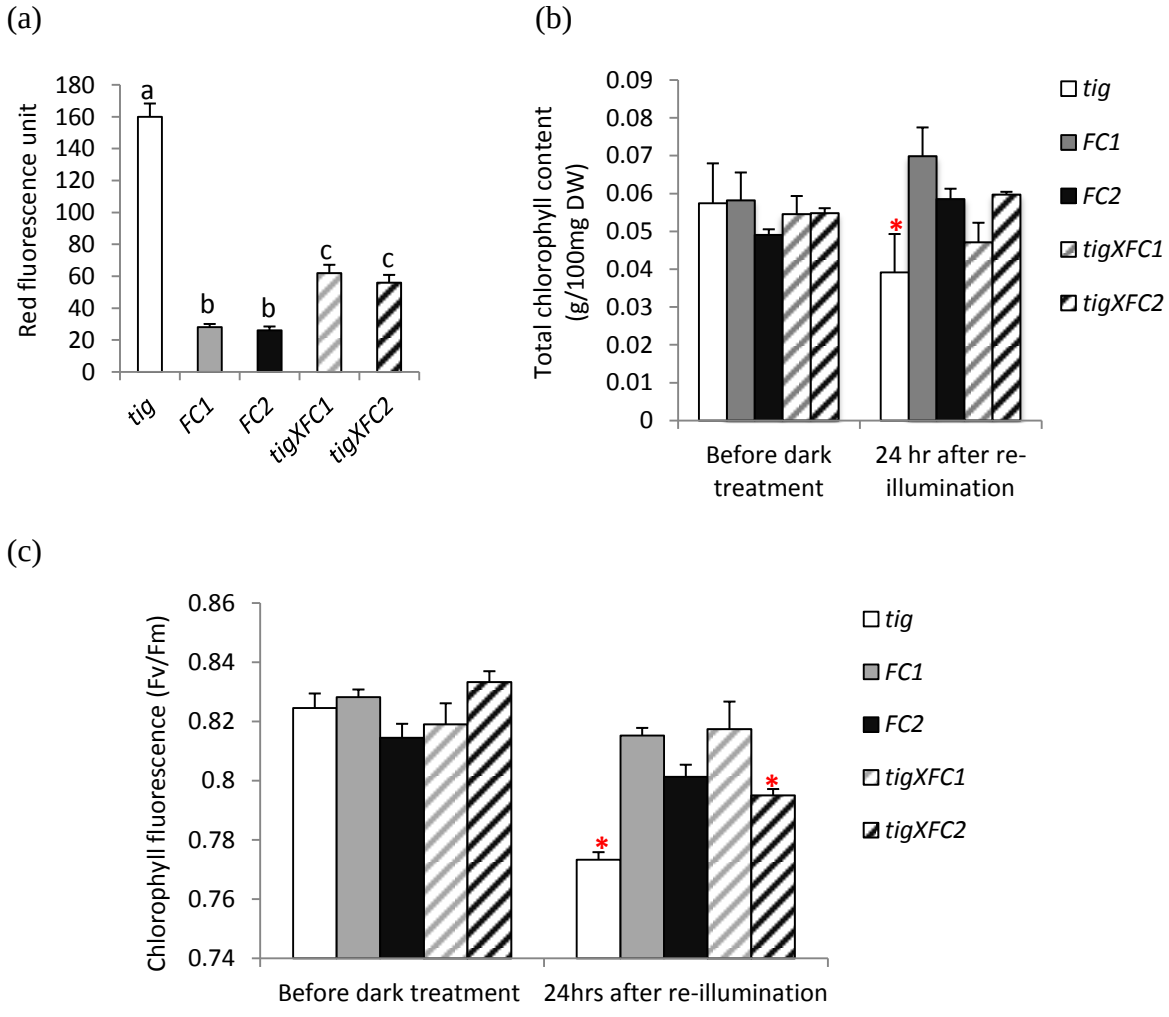


Fig 4-6. Ectopic overexpression of *HvFC1* and *HvFC2* suppresses *tigrina*^{d12} mutant phenotypes. (a) Red fluorescence of dark grown seedlings, which is an indication of the level

of Pchlide accumulation. The data are shown as mean ± standard error of 5 different plants. Means with the same letter are not significantly different at $P<0.05$ based on one-way ANOVA. (b) Total chlorophyll content and (c) Chlorophyll fluorescence (F_v/F_m) before dark treatment and upon 24 hrs after re-exposure to light. The data are shown as mean ± standard error of 5 different plants. *Asterisks* indicate a statistically significant difference relative to before treatment, at $P<0.05$ based on two-way ANOVA. *tig- tigrina*^{d12} mutant. *FC1, FC2*- representative transgenic lines ectopically overexpressing either *HvFC1* or *HvFC2*. *tig* X *FC1*or *tig* X *FC1- tigrina*^{d12} mutants ectopically overexpressing either *HvFC1* or *HvFC2*.

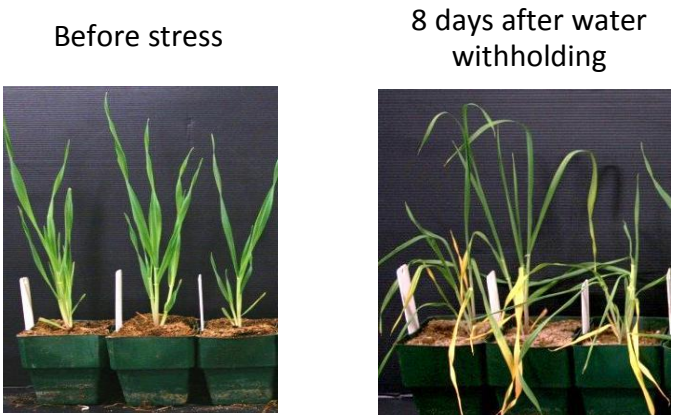
4.5.5 Barley *FC1* and *FC2* are differentially responsive to drought stress and oxidative stress

Our results indicate that both *HvFC1* and *HvFC2* play roles in drought and oxidative stress tolerance, while previous studies reported that only *FC1* is involved in stress defence responses (Nagai et al., 2007). Therefore, we investigated *HvFC*s stress responsive expression patterns. For this purpose, we compared well-watered WT control plants to plants under drought stress. Dehydrated plants were visually assessed for wilting and transcript abundance of drought responsive *Cat* and *Sod* were analysed to ensure that plants were successfully drought stressed. Expression of *HvFC1* and *HvFC2* was analysed in leaves of well-watered and drought stressed plants.

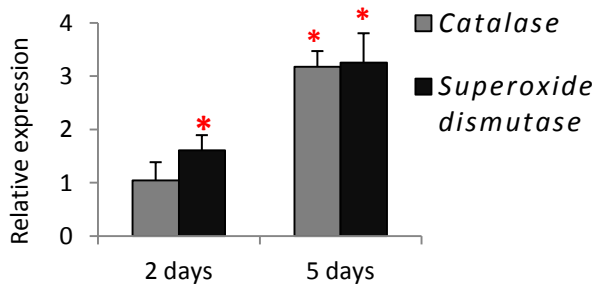
Control plants wilted 8 days post water withholding (**Fig 4-7a**). *Cat* expression was significantly up-regulated 5 days post water withholding relative to well-watered plants (**Fig 4-7b**). Transcript levels of *Sod* were also significantly increased 2 to 5 days post water withholding (**Fig 4-7b**). In-line with previous studies (Nagai et al., 2007; Singh et al., 2002;

Scharfenberg et al., 2014), *HvFC1* was significantly up-regulated, whereas *HvFC2* was down-regulated 2 to 5 days post water withholding relative to well-watered WT plants (**Fig 4-7c**).

(a)



(b)



(c)

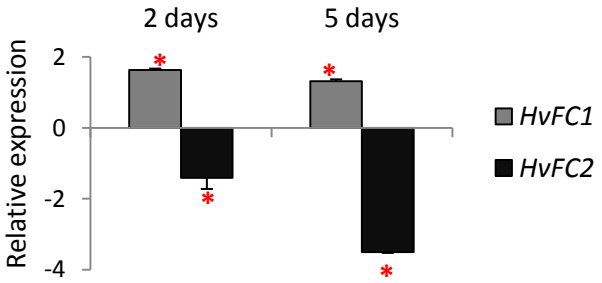
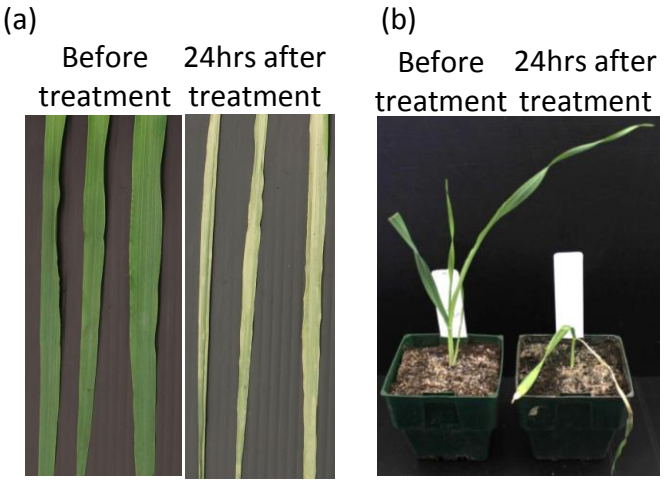


Fig 4-7. Transcript abundance of ROS detoxification markers (Cat and SOD) and HvFCs in control plants upon drought stress. **(a)** Phenotypes of control plants before stress and after exposure to drought stress. **(b)** Expression of *Cat* and *Sod* and, (c) *HvFC1* and *HvFC2* in drought treated plants relative to well-watered plants. The data are shown as mean ±

standard error (SE) of 3 different plants. *Asterisks* indicate a statistically significant expression difference relative to well-watered plants, at $P<0.05$ based on one-way ANOVA.

To investigate the transcriptional responses of *HvFC*s to oxidative stress, WT control plants were exposed to Paraquat-induced and tetrapyrrole-mediated oxidative stress. Paraquat treated leaves were severely photo-bleached 24 hrs after the treatment (**Fig 4-8a**). Even though, expression of *HvFC1* and *HvFC2* did not change 1.5 hrs post paraquat treatment, *HvFC1* was significantly up-regulated and *HvFC2* was markedly down-regulated 24 hrs after paraquat application (**Fig 4-8c**). When etiolated *tigrina*^{d12} mutants were illuminated, the leaves were severely photo-bleached (**Fig 4-8b**) in response to tetrapyrrole-induced oxidative stress. Transcript levels of *HvFC1* and *HvFC2* did not significantly change 1.5 hrs post illumination. However, both *HvFC1* and *HvFC2* were significantly down-regulated 24 hrs post-illumination (**Fig 4-8c**). It is important to note that, *HvFC1* expression was less affected than *HvFC2* by severe oxidative stress upon 24 hrs after re-illumination.



(c)

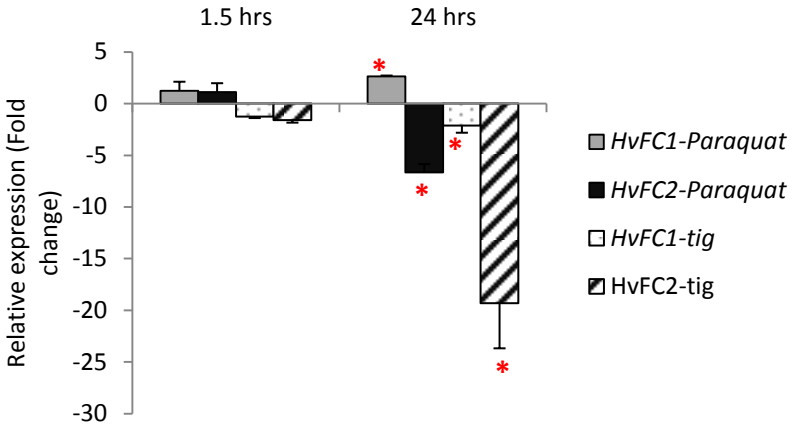


Fig 4-8. Phenotypes of WT control barley leaves and *HvFC* transcript abundance upon exposure to Paraquat-induced and tetrapyrrole-mediated oxidative stress. Leaves of control plants before and 24 hrs after exposed to (a) Paraquat-induced oxidative stress and, (b) tetrapyrrole-mediated oxidative stress in *tigrina*^{d12} mutants. (c) *HvFC1* and *HvFC2* transcript abundance upon oxidative stress relative to before stress. The data are shown as mean ± standard error (SE) of 3 different plants. *Asterisks* indicate a statistically significant expression difference relative to before treatment, at $P<0.05$ using one-way ANOVA.

Collectively, these results demonstrate that the two *HvFC*s differentially responsive to drought and oxidative stresses.

4.6 Discussion

4.6.1 Both *FC1* and *FC2* are implicated in maintaining higher leaf water status and photosynthetic activity upon drought stress

Transgenic plants ectopically overexpressing either *HvFC1* or *HvFC2* showed several favourable traits, which enable them to perform better under water-limited conditions. Even though control plants wilted at -0.6 MPa soil water potential (8 days post water

withholding), both types of transgenics did not show wilting symptoms (**Fig 4-2**). The leaf RWC of both types of transgenics was similar to controls prior to stress, but they were able to prevent depletion of leaf water content upon drought stress (**Fig 4-3a**). This finding is in agreement with Allen et al., (2010) who reported that *Arabidopsis* plants overexpressing either *FC1* or *FC2* are less wilted under terminal drought. As drought stress progresses, water uptake from the soil becomes more difficult because of the reducing soil water potential. This in turn causes a reduction in the intercellular plant water potential and therefore overall plant water status. One of the very early responses to water deficit is stomatal closure, which facilitates water retention by restricting evapotranspiration. Even though, we speculated that increased leaf RWC for both *HvFC* transgenics might be due to a lower g_s, *HvFC1* transgenics displayed significantly higher g_s whereas *HvFC2* transgenics showed non-significant increase in SC relative to the control (**Fig 4-3c**). Therefore, it is probable that *HvFC* transgenics are expressing a more efficient mechanism for water uptake. *HvFC* ectopically overexpressing transgenics are expected to have higher heme content. Several studies report that heme oxygenase (HO), which breaks down heme into an antioxidative compound, Biliverdin IX, is implicated in lateral root development (Chen et al. 2012; Xu et al. 2011; Xuan et al. 2008). Recently, Thu-Ha et al., (2011) reported that HO activity is significantly increased in root tissues during drought stress. They also reported that transgenic rice plants, which exhibited significantly higher FC activity, heme content and HO activity in roots, were able to maintain higher RWC upon drought stress relative to non-transgenic controls. This indicates that heme branch intermediates play important roles in roots upon dehydration. Therefore, it can be speculated that *HvFC* transgenics may have higher amount of heme and HO activity in roots and they might facilitate root development for more water acquisition upon drought stress.

Water is a vital component for cellular metabolism and lack of sufficient water leads to perturbation of key cellular processes such as photosynthesis. When either *HvFC1* or *HvFC2* were overexpressed, plants exhibited significantly higher A_{sat}, CE and instantaneous WUE under drought stress relative to WT controls (**Fig 4-3f, d, b**). A possible reason for the higher A_{sat} of the barley transgenics upon dehydration could be due to an expected increase in heme content, resulting from *FC* overexpression. Heme is an integral component of cytochrome *b6f* complex, which is implicated in photosynthetic electron transport. Lack of cytochrome *b6f*-bound heme in *fc2* knock out *Arabidopsis* mutants display impaired electron transport and PSII efficiency (Scharfenberg et al. 2014). Therefore, *HvFC2* overexpressing transgenics might contribute for improving photosynthetic electron transport capacity and PSII efficiency through increasing heme required for cytochrome *b6f* complex formation. In this context, we would expect that *FC* overexpressing transgenics to contain greater quantities of cytochrome *b6f*-bound heme than controls. *fc1* knock out *Arabidopsis* mutants on the other hand, do not display such a reduction in photosynthetic performance, therefore implying that FC1-derived heme may not necessarily be as important for photosynthesis (Scharfenberg et al. 2014). This contrasts with our previous report that both *HvFC1* and *HvFC2* overexpressing transgenics show improved photosynthetic rate and CE under non-stressed conditions (Nagahatenna et al., 2015b). We therefore expect that both FC-derived heme pools, are likely to contribute to photosynthetic performance as a consequence of improved electron transport and PSII efficiency. Since both types of *HvFC* transgenics significantly improve photosynthetic performance also under drought stress, this would support the proposal that both *FC*s play an important role in adapting photosynthesis to water stress.

Another possible explanation for the observed improvement in photosynthetic performance of barley transgenics upon dehydration could be related to the heme's ability to modulate expression of nuclear genes important for photosynthesis. Recent reports show that ectopic overexpression of *Arabidopsis FC1* transcriptionally up-regulates the expression of photosynthesis associated nuclear genes (PhANG). These include *light-harvesting complex b protein* (*LHCB*) and *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*Rubisco*). However these observations were based on assays in non-stressed plants (Woodson et al. 2011; Woodson et al. 2013). In contrast, *Arabidopsis* plants overexpressing *FC2*, failed to display transcriptional up-regulation of the same nuclear genes. These findings led the authors of these studies to propose that only the FC1-derived heme sub-pool acts as a plastid-to-nuclear signal. Furthermore, several other studies indicated that chloroplasts also transmit such signals under stress conditions, resulting in up-regulation of nuclear genes associated with stress acclimation mechanisms (Pfannschmidt 2010; Pogson et al. 2008). This signal has since been termed as an "operational signal" (Xiao et al., 2012; Woodson and Chory, 2012). Whether heme is the causal agent in such a signaling process on drought exposure is yet to be confirmed. Our study is supportive of such a role for heme given that overexpression of either *HvFC1* or *HvFC2* significantly up-regulates the expression of *Cat* and *Sod,* which encode proteins necessary for ROS detoxification upon dehydration (**Fig 4-4**). We therefore suggest that both FC-derived heme sub-pools could act as operational signals to protect plants from drought-induced oxidative damage. This proposed role for heme is further supported by a more recent study by Kim et al. (2014), who showed that ectopic overexpression of *Bradyrhizobium japonicum* cytosol targeted *FC* in rice, substantially increases FC activity, total heme content and tolerance to oxidative and polyethylene glycol-induced drought stress.

Based on this evidence, it is reasonable to assume that *HvFC* transgenics may also modulate expression of photosynthesis-associated nuclear genes via heme signal under drought stress to maintain higher photosynthetic capacity. Given that Rubisco is the primary enzyme necessary for CO_2 assimilation, improved photosynthetic activity of the transgenics could be due to increased Rubisco content as a result of its transcriptional up-regulation by heme. The gene encoding the large sub-unit of this enzyme is a major target for improving photosynthesis capacity (Galmés et al. 2014). Another such nuclear gene that may induce in *HvFC* transgenics could be LHCB, which encodes apoproteins required for binding major light harvesting pigments such as chlorophylls and xanthophylls in photosystem II (PSII) (Jansson 1994; Liu et al. 2012). These proteins also play important roles in modulating g_s under drought stress and preventing oxidative damage (de Bianchi et al. 2011; Xu et al. 2012). Whether *Rubisco* and *LHCB* are likely downstream targets of FC-derived operational signaling upon drought stress, is yet to be confirmed. If this is indeed the case, then enhanced expression of these genes could explain the observed improvement in photosynthetic performance while also providing additional protection against severe oxidative damage.

4.6.2 Both *FC1* and *FC2* prevent tetrapyrrole-mediated oxidative stress

Under stress, photosensitizing tetrapyrrole intermediates are accumulated leading to an oxidative burst (Mock et al., 1998; Mock et al., 1999). Previous study by Sobotka et al., (2010) indicates that FC2 plays an important role in preventing toxic intermediate accumulation. This evidence prompted us to investigate whether two *HvFC*s have distinct regulatory functions in preventing potential photo-oxidative damage upon stress. Here, we used *tigrina*^{d12} mutant, which is defective in FLU-based negative regulation of chlorophyll

biosynthesis. Consequently, the mutant plants accumulate a higher amount of Pchlide relative to wild-type, when grown in the dark (Lee et al., 2013). Indeed, the etiolated mutant displayed a strong red fluorescence, which is an indicator of Pchlide accumulation (**Fig 4-6a**). Notably, overexpression of either *HvFC1* or *HvFC2*, significantly reduced toxic intermediate accumulation (**Fig 4-6a**).

Pchlide acts as a strong photosensitizer. Therefore, when etiolated mutants are illuminated, they rapidly bleach and die, due to extensive photooxidative damage caused by $^1O_2$. Etiolated *tigrina*^{d12} mutant plants exhibited a severe photo-bleaching and significant damage to PSII efficiency upon illumination (**Fig 4-6b, c**). However, *tigrina*^{d12} ectopically overexpressing either *HvFC1* or *HvFC2*, substantially reduced these photooxidative damage. This suggests that both *FC1* and *FC2* play pivotal roles in preventing photo-oxidative damage caused by tetrapyrrole biosynthesis deregulation.

In line with our observations, *Arabidopsis flu* mutant, which is an ortholog of barley *tigrina*^{d12} also markedly, reduced Pchlide level, when crossed with heme accumulating *hy1* or *ulf3* mutants (Goslings et al., 2004). Therefore, the potential reason for the reduced intermediate levels in *HvFC* overexpressing *tigrina*^{d12} is more likely to be due to increased heme content. Heme serves as a negative regulator of the tetrapyrrole biosynthesis by inhibiting the activity of the first rate-limiting enzyme, glutamyl-tRNA reductase (GluTR) by binding to its C-terminal end (Vothknecht et al., 1998). Taken together, our results indicate that heme-based negative feedback mechanism protects plants from potential photo-oxidative damage under stress.

4.6.3 *FC1* and *FC2* are differentially responsive to drought stress and oxidative stress

Even though our study indicated that both *FC1* and *FC2* are good candidate genes for improving drought and oxidative stress tolerance, previous studies have proposed that only *FC1* is implicated in stress defence responses (Nagai et al. 2007; Scharfenberg et al. 2014). This was proposed solely based on *HvFC*'s differential transcriptional responses to distinct stress stimuli. In order to investigate whether two HvFC's have contrast stress responsive expression profiles, we analysed their transcriptional abundance in leaves of wild-type plants before and after exposure to drought and oxidative stress. In line with previous observations, *HvFC1* was significantly up-regulated upon drought stress whereas *HvFC2* was markedly down-regulated at the early stage of the drought stress (2 and 5 days post water withholding) (**Fig 4-7c**).

Similar differential expression profiles of *HvFC* were observed in response to Paraquat-induced oxidative stress (**Fig 4-8c**). Paraquat disrupts the electron transport system of PSI leading to generation of superoxide radical (O_2^-) which subsequently reduces into hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). In contrast, when etiolated *tigrina*^{d12} was illuminated, both genes were severely down-regulated (**Fig 4-8c**). Severe suppression of both *HvFC*s may be due to elevated toxicity of $^1O_2$ relative to H_2O_2 (Cruz de Carvalho 2008). However, it is important to note that *HvFC1* expression was less affected by photo-toxicity of $^1O_2$ compared to *HvFC2* (**Fig 4-8c**). Our results show that *FC1* and *FC2* are differentially responsive to drought and oxidative stress.

Collectively, our study highlights that despite the distinct stress responsive expression of *FC1* and *FC2*, increasing flux through heme branch of the pathway improves drought and

oxidative stress tolerance. Both *FC*s are good candidates as targets for metabolic engineering towards improved crop performance under water-limited environments. Both heme pools are likely to play important roles in triggering the regulatory machinery involved in drought and oxidative stress tolerance. Taken together, this study provides a significant contribution towards improving drought stress tolerance in cereals via manipulation of teterapyrrole biosynthesis.

4.7 Acknowledgement

This research was supported by the Australian Research Council, the Grains Research and Development Corporation, the Government of South Australia, the University of Adelaide and the Dupont Pioneer, USA. We thank Alison Hay for generating transgenic vectors, Dr. Ainur Ismagul for barley transformation and Yuan Lee for quantitative RT-PCR analysis. We would also like to thank Dr. Julie Hayes, Dr. Penny Tricker and Dr. Robyn Grove for critical comments on the manuscript.

Chapter 5: General Discussion and Future Directions

The tetrapyrrole biosynthetic pathway, which is a key component of primary plant metabolic processes such as photosynthesis and respiration, has been extensively studied in the model plant, *Arabidopsis* over the past decade. These studies propose that this pathway plays a vital role also in stress adaptation. A large body of evidence reviewed in this study, has implicated the heme branch of the pathway in drought stress signaling but there have been few studies on how this signaling process may function or if it is amenable to manipulation for enhanced stress tolerance. The research work reported in this thesis was conducted to explore the potential contribution of this pathway to drought stress signaling in cereals. In particular the potential to improve drought tolerance in barley was studied via the manipulation of heme biosynthesis and the potential of candidate genes of tetrapyrrole biosynthetic pathway, as effective targets for improving crop performance upon drought stress.

The results showed that modification of tetrapyrrole biosynthesis via ectopic overexpression of either *HvFC1* or *HvFC2*, positively influenced a number of favourable traits for stress adaptation, without causing deleterious pleiotropic effects. Barley transgenics exhibited higher photosynthetic performance when grown under non-stressed as well as drought stress conditions. Improved carbon assimilation rate of these barley transgenics, may contribute to increase biomass or grain yield. Future research should focus on elucidating whether these transgenics produce higher yield under field conditions.

Barley transgenics performed better than controls under water-limited conditions, and were less wilted, showed a significantly higher RWC, and WUE relative to control plants upon drought stress. Notably, *HvFC* ectopic overexpression significantly up-regulated nuclear genes associated with ROS detoxification upon drought stress. The overexpression of *HvFC*s also prevented accumulation of photo-sensitizing tetrapyrrole intermediates and subsequent photo-oxidation. These observations suggest that both *HvFC* can trigger physiological processes that improve photosynthesis, oxidative and drought stress tolerance. Collectively, this evidence indicates that both *FC*s can be used as targets for engineering cereals for improved performance under both non-stressed and stress conditions.

The exact mechanisms for enhanced performance through *HvFC* overexpression have not been resolved, although a few plausible mechanisms can be proposed. Ectopic overexpression of *HvFC* is expected to result in the synthesis of higher amounts of heme relative to control plants and the observed modified traits are more likely to be a consequence of increased heme content; for example, heme is important for photosynthetic electron transport (Cramer et al. 1996; Kurisu et al. 2003). It has been proposed that heme could act as a chloroplast signal to modulate nuclear gene expression associated with photosynthesis (Woodson et al., 2011; Woodson et al., 2013). Therefore, improved photosynthetic performance of *HvFC* overexpressing transgenics may be due to higher electron transport capacity and increased expression of photosynthesis associated nuclear genes. The proposed role of heme in chloroplast-to-nuclear retrograde signaling is further supported by the fact that *HvFC* overexpressing transgenics exhibited significant up-regulation of nuclear genes associated with ROS detoxification upon drought stress. This

contrasts with previous studies which propose that only the FC1-derived heme pool is involved in inter-organeller communication (Woodson et al., 2011; Woodson et al., 2013). The results presented here imply that both FC-derived heme pools could act as plastid signals under water-limited conditions to modulate nuclear genes associated with stress acclimation. Additionally, heme-based negative regulation of teterapyrrole biosynthesis appears to be a useful mechanism for preventing tetrapyrrole-mediated oxidative damage.

In order to understand the potential role of heme in improving drought stress tolerance, future work should address the following questions.

1. Does heme act as a chloroplast-to-nuclear operational signal?
2. What influences heme efflux from the chloroplast and its inter-cellular transport?
3. Does heme activate nuclear genes via heme-activating TFs, such as NF-Y?
4. What are the drought responsive nuclear genes triggered by heme?

To this end, the transgenic barley lines developed in this study will be a valuable resource to help answer these questions.

In plants, a higher proportion of the total heme pool is covalently or non-covalently bound to cytochrome complexes and hemoproteins such as cytochromes P450, nitrate reductase, NADPH oxidases, peroxidases, and catalases (Cornah et al., 2003; Mochizuki et al., 2010). In contrast, there is a relatively small amount of heme as free heme pool and it is believed to be implicated in inter-organellar communication (Thomas and Weinstein, 1990). Due to lack of precise heme quantification assays, very little is known about the physiological functions of these different heme pools under stress.

In this study, several attempts were made to quantify total and free heme amounts in *HvFC* transgenics and control plants using an acid acetone extraction method as described in Moulin et al., (2008). During the analysis, the heme signal was severely suppressed and it is assumed that heme may be either rapidly converted into another biological form or its ion is completely suppressed by co-eluting compounds. In line with our observations, Espinas et al., (2012) reported that there is a substantial risk of losing heme when plant tissues are processed by acetone extraction. Therefore, future investigations which focus on developing sensitive heme quantification assays would greatly help us to understand their potential roles under different physiological conditions.

Drought tolerance in plants is extremely complex, with a large suite of genes involved in initiating drought stress responses. The successful modification of complex physiological processes such as photosynthesis and abiotic stress tolerance by targeting a single gene, is a daunting prospect. Overexpression of a single gene is unlikely to lead to a significant impact on processes controlled by such a large arrays of genes, and where complex regulatory feedback mechanisms are often in place. However, targeting genes such as *FC*s, which may be involved in the production of a plastid signal, could offer an effective strategy. Increased production of plastid signal could modulate a multitude of nuclear genes associated with stress acclimation. Similarly, ectopic overexpression of a specific transcription factor also activates or represses a multitude of functional genes related to stress (Agarwal et al. 2010; Shinozaki and Yamaguchi-Shinozaki 2007). Even though TFs have been used as potential targets to improve stress tolerance, in some instances this causes undesirable pleiotropic phenotypes such as dwarfism and enhanced sensitivity to desiccation (Cominelli et al. 2008; Cominelli and Tonelli 2010; Ge et al. 2004). In this case, genes associated with the

production of plastid signals could be effective targets since their overexpression does not appear to be associated with undesirable pleiotropic phenotypes.

In conclusion, the research presented in this thesis indicates that manipulation of teterapyrrole biosynthetic pathway enhances photosynthesis and tolerance to oxidative and drought stress in barley. This study also demonstrates that both *FC*s can be used as candidate genes for metabolic engineering to improve crop performance in both non-stressed and water-limited environments.

Chapter 6: Contributions to knowledge

The significant contributions made by the research reported in this thesis to the advancement of scientific knowledge include:

1. Identification of the ability of two *FC* isoforms to improve photosynthetic performance when over-expressed in barley, without causing deleterious effects on plant growth or development
2. The finding that overexpression of either *HvFC1* or *HvFC2* provides protection from potential photo-oxidative damage and drought stress
3. Evidence that both heme pools (biosynthesis catalysed by FC1 and FC2, respectively) may play pivotal roles in photosynthesis, oxidative and drought stress tolerance
4. Data suggesting that the tetrapyrrole biosynthesis pathway is a potential target for metabolic engineering towards improved crop performance under both non-stressed and water-limited conditions.

References

Agarwal P, Agarwal PK, Joshi AJ, Sopory SK, Reddy MK (2010) Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes Molecular biology reports 37:1125-1135

Al-Karadaghi S, Hansson M, Nikonov S, Jönsson B, Hederstedt L (1997) Crystal structure of ferrochelatase: the terminal enzyme in heme biosynthesis Structure 5:1501-1510

Al-Khateeb A, Al-Khateeb S, Okawara R, Al-Abdoulhady I (2006) Promotive effects of 5-aminolevulinic acid (5-ALA) on fruit yield and quality of date palm cv Khalas J Biol Sci 6:1118-1121

Al-Thabet S (2006) Promotive effect of 5-aminolevulinic acid on growth and yield of wheat grown under dry conditions J Agron 5:45-49

Allen SM, Luck S, Mullen J, Sakai H, Sivasanker S, Tingey SV, Williams RW (2010) Drought tolerant plants and related constructs and methods involving genes encoding ferrochelatases United States Patent US 7,812,223 B2

Ankele E, Kindgren P, Pesquet E, Strand Å (2007) In vivo Visualization of Mg-ProtoporphyrinIX, a Coordinator of Photosynthetic Gene Expression in the Nucleus and the Chloroplast The Plant Cell 19:1964-1979

Arimura S-i, Tsutsumi N (2002) A dynamin-like protein (ADL2b), rather than FtsZ, is involved in Arabidopsis mitochondrial division Proceedings of the National Academy of Sciences 99:5727-5731

Arimura S-i, Yamamoto J, Aida GP, Nakazono M, Tsutsumi N (2004) Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution Proceedings of the National Academy of Sciences of the United States of America 101:7805-7808

Bai L, Wang P, Song C-P (2014) Reactive Oxygen Species (ROS) and ABA Signalling. In: Abscisic Acid: Metabolism, Transport and Signaling. Springer, pp 191-223

Balestrasse KB, Tomaro ML, Batlle A, Noriega GO (2010) The role of 5-aminolevulinic acid in the response to cold stress in soybean plants Phytochemistry 71:2038-2045

Ball L et al. (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis The Plant Cell Online 16:2448-2462

Balsemão-Pires E, Jaillais Y, Olson BJ, Andrade LR, Umen JG, Chory J, Sachetto-Martins G (2011) The Arabidopsis translocator protein (AtTSPO) is regulated at multiple levels

in response to salt stress and perturbations in tetrapyrrole metabolism BMC plant biology 11:108

Barajas-López JdD, Blanco NE, Strand Å (2013) Plastid-to-nucleus communication, signals controlling the running of the plant cell Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1833:425-437

Barañano DE, Rao M, Ferris CD, Snyder SH (2002) Biliverdin reductase: a major physiologic cytoprotectant Proceedings of the National Academy of Sciences 99:16093-16098

Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling Journal of experimental botany 65:1229-1240

Biswal B, Joshi P, Raval M, Biswal U (2011) Photosynthesis, a global sensor of environmental stress in green plants: stress signalling and adaptation Current Science(Bangalore) 101:47-56

Biswal B, Pessarakli M (2005) Photosynthetic response of green plants to environmental stress: Inhibition of photosynthesis and adaptational mechanisms Handbook of photosynthesis Taylor and Francis Group, Boca Raton, EE UU:739-750

Biswal UC, Biswal B, Raval MK (2003) Chloroplast biogenesis: from proplastid to gerontoplast. Netherland: Kluwer Academic Publishers.

Boyer JS (1982) Plant productivity and environment Science 218:443-448

Bray EA (1997) Plant responses to water deficit Trends in Plant Science 2:48-54

Breusegem FV, Dat JF (2006) Reactive Oxygen Species in Plant Cell Death Plant Physiology 141:384–390

Burton RA, Shirley NJ, King BJ, Harvey AJ, Fincher GB (2004) The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes Plant physiology 134:224-236

Camp ROD et al. (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis The plant cell 15:2320-2332

Cao Z-Y et al. (2007) Carbon Monoxide Promotes Lateral Root Formation in Rapeseed Journal of Integrative Plant Biology 49:1070-1079

Chaves M (1991) Effects of water deficits on carbon assimilation Journal of experimental Botany 42:1-16

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell Annals of botany 103:551-560

Chen M, Schliep M, Willows RD, Cai Z-L, Neilan BA, Scheer H (2010) A Red-Shifted Chlorophyll Science 329:1318-1319

Chen X-Y et al. (2009) Endogenous Hydrogen Peroxide Plays a Positive Role in the Upregulation of Heme Oxygenase and Acclimation to Oxidative Stress in Wheat Seedling Leaves Journal of Integrative Plant Biology 51:951-960

Chen Y-H, Chao Y-Y, Hsu YY, Hong C-Y, Kao CH (2012) Heme oxygenase is involved in nitric oxide-and auxin-induced lateral root formation in rice Plant cell reports 31:1085-1091

Chi W, Sun X, Zhang L (2013) Intracellular signaling from plastid to nucleus Annual review of plant biology 64:559-582

Chow K-S, Singh DP, Roper JM, Smith AG (1997) A Single Precursor Protein for Ferrochelatase-I fromArabidopsis Is Imported in Vitro into Both Chloroplasts and Mitochondria Journal of Biological Chemistry 272:27565-27571

Chow KS, Singh DP, Walker AR, Smith AG (1998) Two different genes encode ferrochelatase in Arabidopsis: mapping, expression and subcellular targeting of the precursor proteins Plant Journal 15:531-541

Clément M et al. (2011) The cytosolic/nuclear HSC70 and HSP90 molecular chaperones are important for stomatal closure and modulate abscisic acid-dependent physiological responses in Arabidopsis Plant physiology 156:1481-1492

Cominelli E, Sala T, Calvi D, Gusmaroli G, Tonelli C (2008) Over-expression of the Arabidopsis AtMYB41 gene alters cell expansion and leaf surface permeability The Plant Journal 53:53-64

Cominelli E, Tonelli C (2010) Transgenic crops coping with water scarcity New biotechnology 27:473-477

Conklin P, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence Plant, Cell & Environment 27:959-970

Cornah JE, Roper JM, Singh D, AG S (2002) Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and non-photosynthetic cells of pea (Pisum sativum L.) Journal of Biological Chemistry 362:423-432

Cornah JE, Terry MJ, Smith AG (2003) Green or red: what stops the traffic in the tetrapyrrole pathway? Trends in Plant Science 8:224-230

Cramer W, Soriano G, Ponomarev M, Huang D, Zhang H, Martinez S, Smith J (1996) Some new structural aspects and old controversies concerning the cytochrome b 6 f complex of oxygenic photosynthesis Annual Review of Plant Biology 47:477-508

Cruz de Carvalho MH (2008) Drought stress and reactive oxygen species: Production, scavenging and signaling Plant signaling & behavior 3:156-165

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta Plant physiology 133:462-469

Czarnecki O et al. (2011) An Arabidopsis GluTR binding protein mediates spatial separation of 5-aminolevulinic acid synthesis in chloroplasts Plant Cell 23:4476-4491

Davison PA, Schubert HL, Reid JD, Iorg CD, Heroux A, Hill CP, Hunter CN (2005) Structural and biochemical characterization of Gun4 suggests a mechanism for its role in chlorophyll biosynthesis Biochemistry 44:7603-7612

de Bianchi S, Betterle N, Kouril R, Cazzaniga S, Boekema E, Bassi R, Dall'Osto L (2011) Arabidopsis mutants deleted in the light-harvesting protein Lhcb4 have a disrupted photosystem II macrostructure and are defective in photoprotection The Plant Cell Online 23:2659-2679

del Río LA (2011) Peroxisomes as a cellular source of reactive nitrogen species signal molecules Archives of Biochemistry and Biophysics 506:1-11

Espinas NA, Kobayashi K, Takahashi S, Mochizuki N, Masuda T (2012) Evaluation of unbound free heme in plant cells by differential acetone extraction Plant and Cell Physiology 53:1344-1354

Estavillo GM et al. (2011) Evidence for a SAL1-PAP chloroplast retrograde pathway that functions in drought and high light signaling in Arabidopsis The Plant Cell Online 23:3992-4012

Field C, Mooney H photosynthesis--nitrogen relationship in wild plants. (1989) In: On the economy of plant form and function: proceedings of the Sixth Maria Moors Cabot Symposium," Evolutionary Constraints on Primary Productivity, Adaptive Patterns of Energy Capture in Plants," Harvard Forest

Fleury D, Jefferies S, Kuchel H, Langridge P (2010) Genetic and genomic tools to improve drought tolerance in wheat Journal of Experimental Botany 61:3211-3222

Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications Antioxidants & redox signaling 11:861-905

Gadjieva R, Axelsson E, Olsson U, Hansson M (2005) Analysis of gun phenotype in barley magnesium chelatase and Mg-protoporphyrin IX monomethyl ester cyclase mutants Plant Physiology and Biochemistry 43:901-908

Galmés J, Conesa M, Díaz-Espejo A, Mir A, Perdomo JA, Niinemets Ü, Flexas J (2014) Rubisco catalytic properties optimized for present and future climatic conditions Plant Science

Garnett T et al. (2013) The response of the maize nitrate transport system to nitrogen demand and supply across the lifecycle New Phytologist 198:82-94

Ge L et al. (2004) Overexpression of OsRAA1 causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity Plant Physiology 135:1502-1513

Goslings D, Meskauskiene R, Kim CH, Lee KP, Nater M, Apel K (2004) Concurrent interactions of heme and FLU with Glu tRNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants Plant Journal 40:957-967

Guillaumot D et al. (2009) The Arabidopsis TSPO-related protein is a stress and abscisic acid-regulated, endoplasmic reticulum–Golgi-localized membrane protein The Plant Journal 60:242-256

Hall BG (2013) Building phylogenetic trees from molecular data with MEGA Molecular biology and evolution 30:1229-1235

Han Y et al. (2008) Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa New Phytologist 177:155-166

He H, He L (2014) Heme oxygenase 1 and abiotic stresses in plants Acta Physiol Plant 36:581-588

Hedtke B, Börner T, Weihe A (2000) One RNA polymerase serving two genomes EMBO reports 1:435-440

Hess WR, Golz R, Börner T (1998) Analysis of randomly selected cDNAs reveals the expression of stress- and defence-related genes in the barley mutant albostrians Plant Science 133:191-201

Hiscox Jt, Israelstam G (1979) A method for the extraction of chlorophyll from leaf tissue without maceration Canadian Journal of Botany 57:1332-1334

Hon T et al. (2001) The Hsp70-Ydj1 molecular chaperone represses the activity of the heme activator protein Hap1 in the absence of heme Molecular and cellular biology 21:7923-7932

Hon T, Lee HC, Hu ZZ, Iyer VR, Zhang L (2005) The heme activator protein Hap1 represses transcription by a heme-independent mechanism in Saccharomyces cerevisiae Genetics 169:1343-1352

Hubert DA, He Y, McNulty BC, Tornero P, Dangl JL (2009) Specific Arabidopsis HSP90.2 alleles recapitulate RAR1 cochaperone function in plant NB-LRR disease resistance protein regulation Proceedings of the National Academy of Sciences 106:9556-9563

Jacobs JM, Jacobs NJ (1987) Oxidation of Protoporphyrinogen to Protoporphyrin, a step in chlorophyll and heme-biosynthesis – Purification and partial characterization of the enztme from barley organelles Biochemical Journal 244:219-224

Jacobs JM, Jacobs NJ (1995) Terminal Enzymes of Heme Biosynthesis in the Plant Plasma Membrane Archives of Biochemistry and Biophysics 323:274-278

Jansson S (1994) The light-harvesting chlorophyll ab-binding proteins Biochimica et Biophysica Acta (BBA) - Bioenergetics 1184:1-19

Jensen PE et al. (1996) Structural genes for Mg-chelatase subunits in barley: Xantha-f,-g and-h Molecular and General Genetics MGG 250:383-394

Jin Q, Zhu K, Cui W, Xie Y, Han B, Shen W (2013) Hydrogen gas acts as a novel bioactive molecule in enhancing plant tolerance to paraquat-induced oxidative stress via the modulation of heme oxygenase-1 signalling system Plant, cell & environment 36:956-969

Johanningmeier U (1988) Possible control of transcript levels by chlorophyll precursors in Chlamydomonas European Journal of Biochemistry 177:417-424

Johanningmeier U, Howell SH (1984) Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in Chlamydomonas reinhardi. Possible involvement of chlorophyll synthesis precursors Journal of Biological Chemistry 259:13541-13549

Kanamaru K et al. (2001) An Arabidopsis sigma factor (SIG2)-dependent expression of plastid-encoded tRNAs in chloroplasts Plant and Cell Physiology 42:1034-1043

Kanamaru K, Tanaka K (2004) Roles of chloroplast RNA polymerase sigma factors in chloroplast development and stress response in higher plants Bioscience, biotechnology, and biochemistry 68:2215-2223

Kang K, Lee K, Park S, Lee S, Kim Y, Back K (2010) Overexpression of Rice Ferrochelatase I and II Leads to Increased Susceptibility to Oxyfluorfen Herbicide in Transgenic Rice Journal of Plant Biology 53:291-296

Khandal D et al. (2009) Singlet Oxygen-Dependent Translational Control in the Tigrina-d. 12 Mutant of Barley Proceedings of the National Academy of Sciences of the United States of America 106:13112-13117

Kim C, Apel K (2013) 1O2-mediated and EXECUTER dependent retrograde plastid-to-nucleus signaling in norflurazon-treated seedlings of Arabidopsis thaliana Molecular Plant 6: 1580-1591

Kim J-G, Back K, Lee HY, Lee H-J, Phung T-H, Grimm B, Jung S (2014) Increased expression of Fe-chelatase leads to increased metabolic flux into heme and confers protection against photodynamically induced oxidative stress Plant Mol Biol 86:271-287

Kindgren P, Norén L, López JdDB, Shaikhali J, Strand Å (2012) Interplay between Heat Shock Protein 90 and HY5 controls PhANG expression in response to the GUN5 plastid signal Molecular plant 5:901-913

Kirkman HN, Gaetani GF (1984) Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH Proceedings of the National Academy of Sciences 81:4343-4347

Kleine T, Voigt C, Leister D (2009) Plastid signalling to the nucleus: messengers still lost in the mists? Trends in Genetics 25:185-192

Kohchi T, Mukougawa K, Frankenberg N, Masuda M, Yokota A, Lagarias JC (2001) The Arabidopsis HY2 Gene Encodes Phytochromobilin Synthase, a Ferredoxin-Dependent Biliverdin Reductase The Plant Cell 13:425-436

Krishna P, Gloor G (2001) The Hsp90 family of proteins in Arabidopsis thaliana Cell stress & chaperones 6:238

Krishnamurthy P et al. (2004) The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme Journal of Biological Chemistry 279:24218-24225

Kruse E, Grimm B, Beator J, Kloppstech K (1997) Developmental and circadian control of the capacity for δ-aminolevulinic acid synthesis in green barley Planta 202:235-241

Kruse E, Mock H-P, Grimm B (1995) Coproporphyrinogen III oxidase from barley and tobacco—sequence analysis and initial expression studies Planta 196:796-803

Kumar AM, Csankovszki G, Soll D (1996) A second and differentially expressed glutamyl-tRNA reductase gene from Arabidopsis thaliana Plant Mol Biol 30:419-426

Kumar S, Bandyopadhyay U (2005) Free heme toxicity and its detoxification systems in human Toxicology letters 157:175-188

Kurisu G, Zhang H, Smith JL, Cramer WA (2003) Structure of the cytochrome b6f complex of oxygenic photosynthesis: tuning the cavity Science 302:1009-1014

Kuromori T, Mizoi J, Umezawa T, Yamaguchi-Shinozaki K, Shinozaki K (2014) Drought Stress Signaling Network Molecular Biology:383-409

La Rocca N, Rascio N, Oster U, Rüdiger W (2001) Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of Lhc and RbcS genes Planta 213:101-108

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana Proceedings of the National Academy of Sciences 104:672-677

Larkin RM, Alonso JM, Ecker JR, Chory J (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling Science 299:902-906

Layer G, Reichelt J, Jahn D, Heinz DW (2010) Structure and function of enzymes in heme biosynthesis Protein Science 19:1137-1161

Lee H-J, Mochizuki N, Masuda T, Buckhout TJ (2012) Disrupting the bimolecular binding of the haem-binding protein 5 (AtHBP5) to haem oxygenase 1 (HY1) leads to oxidative stress in Arabidopsis Journal of experimental botany 63:5967-5978

Lee KP, Kim C, Landgraf F, Apel K (2007) EXECUTER1- and EXECUTER2-Dependent Transfer of Stress-Related Signals from the Plastid to the Nucleus of Arabidopsis thaliana Proceedings of the National Academy of Sciences of the United States of America 104:10270-10275

Lee KP, Kim C, Lee DW, Apel K (2003) TIGRINA d, required for regulating the biosynthesis of tetrapyrroles in barley, is an ortholog of the FLU gene of Arabidopsis thaliana Febs Letters 553:119-124

Li D-M, Zhang J, Sun W-J, Li Q, Dai A-H, Bai J-G (2011) 5-Aminolevulinic acid pretreatment mitigates drought stress of cucumber leaves through altering antioxidant enzyme activity Scientia Horticulturae 130:820-828

Li WX et al. (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance Plant Cell 20:2238-2251

Lindemann P, Koch A, Degenhardt B, Hause G, Grimm B, Papadopoulos V (2004) A Novel Arabidopsis thaliana Protein is a Functional Peripheral-Type Benzodiazepine Receptor Plant and Cell Physiology 45:723-733

Lister R, Chew O, Rudhe C, Lee M-N, Whelan J (2001) < i> Arabidopsis thaliana</i> ferrochelatase-I and-II are not imported into< i> Arabidopsis</i> mitochondria FEBS letters 506:291-295

Little HN, Jones OT (1976) The subcellular loclization and properties of the ferrochelatase of etiolated barley Biochem J 156:309-314

Liu D, Pei ZF, Naeem MS, Ming DF, Liu HB, Khan F, Zhou WJ (2011) 5-Aminolevulinic Acid Activates Antioxidative Defence System and Seedling Growth in Brassica napus L. under Water-Deficit Stress Journal of Agronomy & Crop Science 197:284-295

Liu X-G, Xu H, Zhang J-Y, Liang G-W, Liu Y-T, Guo A-G (2012) Effect of low temperature on chlorophyll biosynthesis in albinism line of wheat (Triticum aestivum) FA85 Physiologia Plantarum 145:384-394

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors Science 324:1064-1068

Maity SN, de Crombrugghe B (1998) Role of the CCAAT-binding protein CBF/NF-Y in transcription Trends in Biochemical Sciences 23:174-178

Mantovani R (1998) A survey of 178 NF-Y binding CCAAT boxes Nucleic acids research 26:1135-1143

Masuda T, Ohta H, Shioi Y, Tsuji H, Takamiya K-i (1995) Stimulation of Glutamyl-tRNA Reductase Activity by Benzyladenine in Greening Cucumber Cotyledons Plant and Cell Physiology 36:1237-1243

Masuda T, Suzuki T, Shimada H, Ohta H, Takamiya K (2003) Subcellular localization of two types of ferrochelatase in cucumber Planta 217:602-609

Matthews PR, Wang M-B, Waterhouse PM, Thornton S, Fieg SJ, Gubler F, Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with

adjacenttwin T-DNAs' on a standard Agrobacterium transformation vector Molecular Breeding 7:195-202

McCormac AC, Fischer A, Kumar AM, Söll D, Terry MJ (2001) Regulation of HEMA1 expression by phytochrome and a plastid signal during de-etiolation in Arabidopsis thaliana The Plant Journal 25:549-561

McCormac AC, Terry MJ (2002) Light-signalling pathways leading to the co-ordinated expression of HEMA1 and Lhcb during chloroplast development in Arabidopsis thaliana Plant Journal 32:549-559

McWilliam J (1989) The dimensions of drought In: F.W.G. Baker (ed.) Drought resistance in cereals. Wallingford, UK. CAB International 1-11.

Meskauskiene R, Apel K (2002) Interaction of FLU, a negative regulator of tetrapyrrole biosynthesis, with the glutamyl-tRNA reductase requires the tetratricopeptide repeat domain of FLU FEBS Letters 532:27-30

Meskauskiene R, Nater M, Goslings D, Kessler F, Camp Rod, Apel K (2001) FLU: A Negative Regulator of Chlorophyll Biosynthesis in Arabidopsis thaliana Proceedings of the National Academy of Sciences of the United States of America 98:12826-12831

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants Trends in plant science 9:490-498

Mochizuki N, Brusslan JA, Larkin R, Nagatani A, Chory J (2001) Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction Proceedings of the National Academy of Sciences 98:2053-2058

Mochizuki N et al. (2010) The cell biology of tetrapyrroles: a life and death struggle Trends in Plant Science 15:488-498

Mochizuki N, Tanaka R, Tanaka A, Masuda T, Nagatani A (2008) The Steady-State Level of Mg-Protoporphyrin IX Is Not a Determinant of Plastid-to-Nucleus Signaling in Arabidopsis Proceedings of the National Academy of Sciences of the United States of America 105:15184-15189

Mock H-P, Grimm B (1997) Reduction of uroporphyrinogen decarboxylase by antisense RNA expression affects activities of other enzymes involved in tetrapyrrole biosynthesis and leads to light-dependent necrosis Plant physiology 113:1101-1112

Mock H-P, Heller W, Molina A, Neubohn B, Sandermann H, Grimm B (1999) Expression of Uroporphyrinogen Decarboxylase or Coproporphyrinogen Oxidase Antisense RNA in Tobacco Induces Pathogen Defense Responses Conferring Increased Resistance to Tobacco Mosaic Virus Journal of Biological Chemistry 274:4231-4238

Mock H-P, Keetman U, Kruse E, Rank B, Grimm B (1998) Defense responses to tetrapyrrole-induced oxidative stress in transgenic plants with reduced uroporphyrinogen decarboxylase or coproporphyrinogen oxidase activity Plant Physiology 116:107-116

Mock H-P, Trainotti L, Kruse E, Grimm B (1995) Isolation, sequencing and expression of cDNA sequences encoding uroporphyrinogen decarboxylase from tobacco and barley Plant Mol Biol 28:245-256

Moulin M, McCormac AC, Terry MJ, Smith AG (2008) Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation Proceedings of the National Academy of Sciences of the United States of America 105:15178-15183

Moulin M, Smith A (2005) Regulation of tetrapyrrole biosynthesis in higher plants Biochemical Society Transactions 33:737-742

Mundree SG et al. (2002) Physiological and molecular insights into drought tolerance African Journal of Biotechnology 1:28-38

Nagahatenna DSK, Langridge P, Whitford, R (2015a) Tetrapyrrole-based drought stress signalling Plant Biotechnology J 1-13

Nagahatenna DSK, Tiong J, Edwards EJ, Langridge P, Whitford R (2015b) Altering tetrapyrrole biosynthesis by overexpressing Ferrochelatases (FC1 and FC2), improves photosynthesis in transgenic barley Plant Mol Biol in preparation

Nagai S et al. (2007) Induction of isoforms of tetrapyrrole biosynthetic enzymes, AtHEMA2 and AtFC1, under stress conditions and their physiological functions in Arabidopsis Plant Physiology 144:1039-1051

Nakashima K et al. (2009) Three Arabidopsis SnRK2 Protein Kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, Involved in ABA Signaling are Essential for the Control of Seed Development and Dormancy Plant and Cell Physiology 50:1345-1363

Natesan SKA, Sullivan JA, Gray JC (2005) Stromules: a characteristic cell-specific feature of plastid morphology Journal of experimental botany 56:787-797

Nelson DE et al. (2007) Plant Nuclear Factor Y (NF-Y) B Subunits Confer Drought Tolerance and Lead to Improved Corn Yields on Water-Limited Acres Proceedings of the National Academy of Sciences of the United States of America 104:16450-16455

Nijs I, Behaeghe T, Impens I (1995) Leaf nitrogen content as a predictor of photosynthetic capacity in ambient and global change conditions Journal of Biogeography:177-183

Nishihara E, Kondo K, Parvez MM, Takahashi K, Watanabe K, Tanaka K (2003) Role of 5-aminolevulinic acid (ALA) on active oxygen-scavenging system in NaCl-treated spinach (Spinacia oleracea) Journal of Plant Physiology 160:1085-1091

Oster U, Brunner H, Rüdiger W (1996) The greening process in cress seedlings. V. Possible interference of chlorophyll precursors, accumulated after thujaplicin treatment, with light-regulated expression of Lhc genes Journal of Photochemistry and Photobiology B: Biology 36:255-261

Papenbrock J et al. (2001) Impaired expression of the plastidic ferrochelatase by antisense RNA synthesis leads to a necrotic phenotype of transformed tobacco plants The Plant Journal 28:41-50

Papenbrock J, Mock H-P, Kruse E, Grimm B (1999) Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods Planta 208:264-273

Park S-Y et al. (2009) Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins Science 324:1068-1071

Pei Z-M et al. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells Nature 406:731-734

Pesaresi P et al. (2009) Arabidopsis STN7 kinase provides a link between short-and long-term photosynthetic acclimation The Plant Cell Online 21:2402-2423

Pfannschmidt T (2010) Plastidial retrograde signalling – a true “plastid factor” or just metabolite signatures? Trends in Plant Science 15:427-435

Pitzschke A, Djamei A, Bitton F, Hirt H (2009) A major role of the MEKK1–MKK1/2–MPK4 pathway in ROS signalling Molecular Plant 2:120-137

Pitzschke A, Hirt H (2006) Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants Plant Physiology 141:351-356

Pogson BJ, Woo NS, Förster B, Small ID (2008) Plastid signalling to the nucleus and beyond Trends in Plant Science 13:602-609

Quigley JG et al. (2004) Identification of a human heme exporter that is essential for erythropoiesis Cell 118:757-766

Ramel F, Birtic S, Ginies C, Soubigou-Taconnat L, Triantaphylidès C, Havaux M (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants Proceedings of the National Academy of Sciences 109:5535-5540

Ramel F et al. (2013) Light-Induced Acclimation of the Arabidopsis chlorina1 Mutant to Singlet Oxygen The Plant Cell Online 25:1445-1462

Reynolds M, Manes Y, Izanloo A, Langridge P (2009) Phenotyping approaches for physiological breeding and gene discovery in wheat Annals of Applied Biology 155:309-320

Reynolds MP, Van Ginkel M, Ribaut JM (2000) Avenues for genetic modification of radiation use efficiency in wheat Journal of Experimental Botany 51:459-473

Roper JM, Smith AG (1997) Molecular localisation of ferrochelatase in higher plant chloroplasts European Journal of Biochemistry 246:32-37

Sadok W, Naudin P, Boussuge B, Muller B, Welcker C, Tardieu F (2007) Leaf growth rate per unit thermal time follows QTL-dependent daily patterns in hundreds of maize lines under naturally fluctuating conditions Plant, Cell & Environment 30:135-146

Sambrook J, Russell DW (2001) Alkaline agarose gel electrophoresis Molecular cloning: a laboratory manual, 3rd ed Cold Spring Harbor, New York:5.36-35.39

Sato E, Sagami I, Uchida T, Sato A, Kitagawa T, Igarashi J, Shimizu T (2004) SOUL in mouse eyes is a new hexameric heme-binding protein with characteristic optical absorption, resonance Raman spectral, and heme-binding properties Biochemistry 43:14189-14198

Scharfenberg M, Mittermayr L, Von Roepenack-Lahaye E, Schlicke H, Grimm B, Leister D, Kleine T (2014) Functional characterization of the two ferrochelatases in Arabidopsis thaliana Plant, Cell & Environment 38: 280-298.

Schlaeppi K, Bodenhausen N, Buchala A, Mauch F, Reymond P (2008) The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore Spodoptera littoralis The Plant Journal 55:774-786

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method Nature protocols 3:1101-1108

Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants Nature 410:327-330

Schulz P, Herde M, Romeis T (2013) Calcium-dependent protein kinases: hubs in plant stress signaling and development Plant physiology 163:523-530

Severance S, Hamza I (2009) Trafficking of heme and porphyrins in metazoa Chemical reviews 109:4596-4616

Sharma-Natu P, Ghildiyal M (2005) Potential targets for improving photosynthesis and crop yield Current Science 88:1918-1928

Shayeghi M et al. (2005) Identification of an intestinal heme transporter Cell 122:789-801

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance Journal of Experimental Botany 58:221-227

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses Current Opinion in Plant Biology 6:410-417

Singh D, Cornah J, Hadingham S, Smith A (2002) Expression analysis of the two ferrochelatase genes in Arabidopsis in different tissues and under stress conditions reveals their different roles in haem biosynthesis Plant Mol Biol 50:773-788

Smith AG, Cornah JE, JM R (1999) Compartmentation of tetrapyrrole metabolism in higher plants Oxford: BIOS Scientific Publishers:281-294

Smith AG, Marsh O, Elder GH (1993) Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants Biochem J 292:503-508

Smith AG, Santana MA, Wallace-Cook AD, Roper JM, Labbe-Bois R (1994) Isolation of a cDNA encoding chloroplast ferrochelatase from Arabidopsis thaliana by functional complementation of a yeast mutant Journal of Biological Chemistry 269:13405-13413

Sobotka R, Tichy M, Wilde A, Hunter CN (2011) Functional assignments for the carboxyl-terminal domains of the ferrochelatase from Synechocystis PCC 6803: the CAB domain plays a regulatory role, and region II is essential for catalysis Plant physiology 155:1735-1747

Srivastava A, Lake V, Nogaj L, Mayer S, Willows R, Beale S (2005) The Chlamydomonas reinhardtii gtr Gene Encoding the Tetrapyrrole Biosynthetic Enzyme Glutamyl-tRNA Reductase: Structure of the Gene and Properties of the Expressed Enzyme Plant Mol Biol 58:643-658

Stephenson TJ, McIntyre CL, Collet C, Xue G-P (2007) Genome-wide identification and expression analysis of the NF-Y family of transcription factors in Triticum aestivum Plant Mol Biol 65:77-92

Stephenson TJ, McIntyre CL, Collet C, Xue G-P (2010) TaNF-YC11, one of the light-upregulated NF-YC members in Triticum aestivum, is co-regulated with photosynthesis-related genes Functional & Integrative Genomics 10:265-276

Stephenson TJ, McIntyre CL, Collet C, Xue G-P (2011) TaNF-YB3 is involved in the regulation of photosynthesis genes in Triticum aestivum Functional & Integrative Genomics 11:327-340

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance Science 235:1043-1046

Storm P, Tibiletti T, Hall M, Funk C (2013) Refolding and enzyme kinetic studies on the ferrochelatase of the cyanobacterium Synechocystis sp. PCC 6803 PloS one 8:e55569

Strand A, Asami T, Alonso J, Ecker JR, Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX Nature 421:79-83

Surpin M, Larkin RM, Chory J (2002) Signal transduction between the chloroplast and the nucleus The Plant Cell Online 14:S327-S338

Susek RE, Ausubel FM, Chory J (1993) Signal transduction mutants of arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development Cell 74:787-799

Suzuki T et al. (2002) Two Types of Ferrochelatase in Photosynthetic and Nonphotosynthetic Tissues of Cucumber: THEIR DIFFERENCE IN PHYLOGENY, GENE EXPRESSION, AND LOCALIZATION Journal of Biological Chemistry 277:4731-4737

Takahashi K, Takabayashi A, Tanaka A, Tanaka R (2014) Functional analysis of light-harvesting-like protein 3 (LIL3) and its light-harvesting chlorophyll-binding motif in Arabidopsis Journal of biological chemistry 289:987-999

Takahashi S, Ogawa T, Inoue K, Masuda T (2008) Characterization of cytosolic tetrapyrrole-binding proteins in Arabidopsis thaliana Photochemical & Photobiological Sciences 7:1216-1224

Taketani S, Kohno H, Furukawa T, Tokunaga R (1995) Involvement of peripheral-type benzodiazepine receptors in the intracellular transport of heme and porphyrins Journal of biochemistry 117:875-880

Tanaka R, Kobayashi K, Masuda T (2011) Tetrapyrrole metabolism in Arabidopsis thaliana Arabidopsis Book 9:e0145.

Tanaka R, Tanaka A (2007) Tetrapyrrole biosynthesis in higher plants Annual Review of Plant Biology 58:321-346

Tanaka R, Yoshida K, Nakayashiki T, Tsuji H, Inokuchi H, Okada K, Tanaka A (1997) The third member of the hemA gene family encoding glutamyl-tRNA reductase is primarily expressed in roots in Hordeum vulgare Photosynthesis research 53:161-171

Terry MJ (1997) Phytochrome chromophore-deficient mutants Plant, Cell & Environment 20:740-745

Terry MJ, Smith AG (2013) A model for tetrapyrrole synthesis as the primary mechanism for plastid-to-nucleus signaling during chloroplast biogenesis Frontiers in plant science 4: 14 10.3389/fpls. 2013.00014

Thomas J, Weinstein JD (1990) Measurement of heme efflux and heme content in isolated developing chloroplasts Plant physiology 94:1414-1423

Thu-Ha P, Jung H-i, Park J-H, Kim J-G, Back K, Jung S (2011) Porphyrin Biosynthesis Control under Water Stress: Sustained Porphyrin Status Correlates with Drought Tolerance in Transgenic Rice Plant Physiology 157:1746-1764

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) Agrobacterium tumefaciens-mediated barley transformation The Plant Journal 11:1369-1376

Tripathy BC, Oelmüller R (2012) Reactive oxygen species generation and signaling in plants Plant signaling & behavior 7: 1621-1633

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport Plant and cell physiology 51:1821-1839

Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death Plant Physiology 141:384-390

Vanhee C, Zapotoczny G, Masquelier D, Ghislain M, Batoko H (2011) The Arabidopsis multistress regulator TSPO is a heme binding membrane protein and a potential scavenger of porphyrins via an autophagy-dependent degradation mechanism Plant Cell 23:785-805

Verdecia MA, Larkin RM, Ferrer J-L, Riek R, Chory J, Noel JP (2005) Structure of the Mg-chelatase cofactor GUN4 reveals a novel hand-shaped fold for porphyrin binding PLoS biology 3:e151

Vinti G, Hills A, Campbell S, Bowyer JR, Mochizuki N, Chory J, López-Juez E (2000) Interactions between hy1 and gun mutants of Arabidopsis, and their implications for plastid/nuclear signalling The Plant Journal 24:883-894

Voigt C, Oster U, Börnke F, Jahns P, Dietz K-J, Leister D, Kleine T (2010) In-depth analysis of the distinctive effects of norflurazon implies that tetrapyrrole biosynthesis, organellar gene expression and ABA cooperate in the GUN-type of plastid signalling Physiologia Plantarum 138:503-519

Vothknecht UC, Kannangara CG, von Wettstein D (1998) Barley glutamyl tRNA(Glu) reductase: Mutations affecting haem inhibition and enzyme activity Phytochemistry 47:513-519

Wagner D et al. (2004) The Genetic Basis of Singlet Oxygen-Induced Stress Responses of Arabidopsis thaliana Science 306:1183-1185

Wang L, Ma F, Xu S, Zheng T, Wang R, Chen H, Shen W (2014) Cloning and characterization of a heme oxygenase-2 gene from rice (Oryza sativa L.), and its expression analysis in response to some abiotic stresses Acta Physiol Plant 36:893-902

Wang LJ, Jiang WB, Huang BJ (2004) Promotion of 5-aminolevulinic acid on photosynthesis of melon (Cucumis melo) seedlings under low light and chilling stress conditions Physiologia plantarum 121:258-264

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance Planta 218:1-14

Wheal MS, Fowles TO, Palmer LT (2011) A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements Analytical Methods 3:2854-2863

Woodson JD, Perez-Ruiz JM, Chory J (2011) Heme synthesis by plastid ferrochelatase i regulates nuclear gene expression in plants Current biology 21:897-903

Woodson JD, Perez-Ruiz JM, Schmitz RJ, Ecker JR, Chory J (2013) Sigma factor-mediated plastid retrograde signals control nuclear gene expression The Plant Journal 73:1-13

Xiao Y et al. (2012) Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes Cell 149:1525-1535

Xu S, Zhang B, Cao Z-Y, Ling T-F, Shen W-B (2011) Heme oxygenase is involved in cobalt chloride-induced lateral root development in tomato Biometals 24:181-191

Xu Y-H et al. (2012) Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in Arabidopsis Journal of experimental botany 63:1095-1106

Xuan W et al. (2008) The heme oxygenase/carbon monoxide system is involved in the auxin-induced cucumber adventitious rooting process Plant Physiology 148:881-893

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress The Plant Cell Online 6:251-264

Yang D-H, Andersson B, Aro E-M, Ohad I (2001) The redox state of the plastoquinone pool controls the level of the light-harvesting chlorophyll a/b binding protein complex II (LHC II) during photoacclimation Photosynthesis Research 68:163-174

Yannarelli G, Noriega G, Batlle A, Tomaro M (2006) Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species Planta 224:1154-1162

Zhang J, Kirkham M (1994) Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species Plant and Cell Physiology 35:785-791

Zhang L, Guarente L (1994) The yeast activator HAP1--a GAL4 family member--binds DNA in a directly repeated orientation Genes & development 8:2110-2119

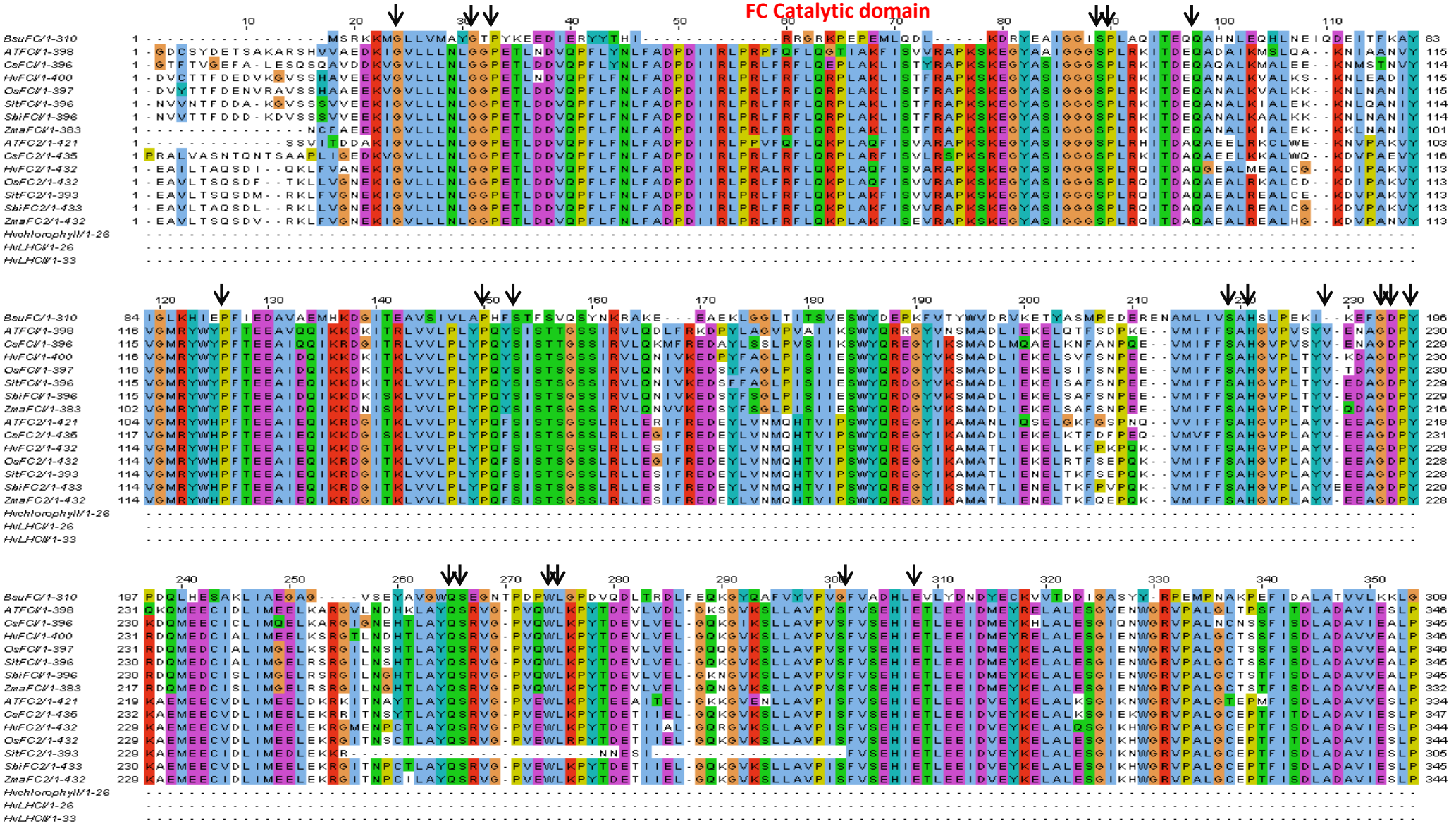
Zhang L, Hach A (1999) Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator CMLS, Cell Mol Life Sci 56:415-426

Zhang WF et al. (2008) Effects of 5-Aminolevulinic Acid on Oilseed Rape Seedling Growth under Herbicide Toxicity Stress J Plant Growth Regul 27:159-169

Zou J-J, Wei F-J, Wang C, Wu J-J, Ratnasekera D, Liu W-X, Wu W-H (2010) Arabidopsis calcium-dependent protein kinase CPK10 functions in abscisic acid-and Ca2+-mediated stomatal regulation in response to drought stress Plant Physiology 154:1232-1243

Zylka MJ, Reppert SM (1999) Discovery of a putative heme-binding protein family (SOUL/HBP) by two-tissue suppression subtractive hybridization and database searches Molecular brain research 74:175-181

Appendix 1: Supplementary data for Chapter 3



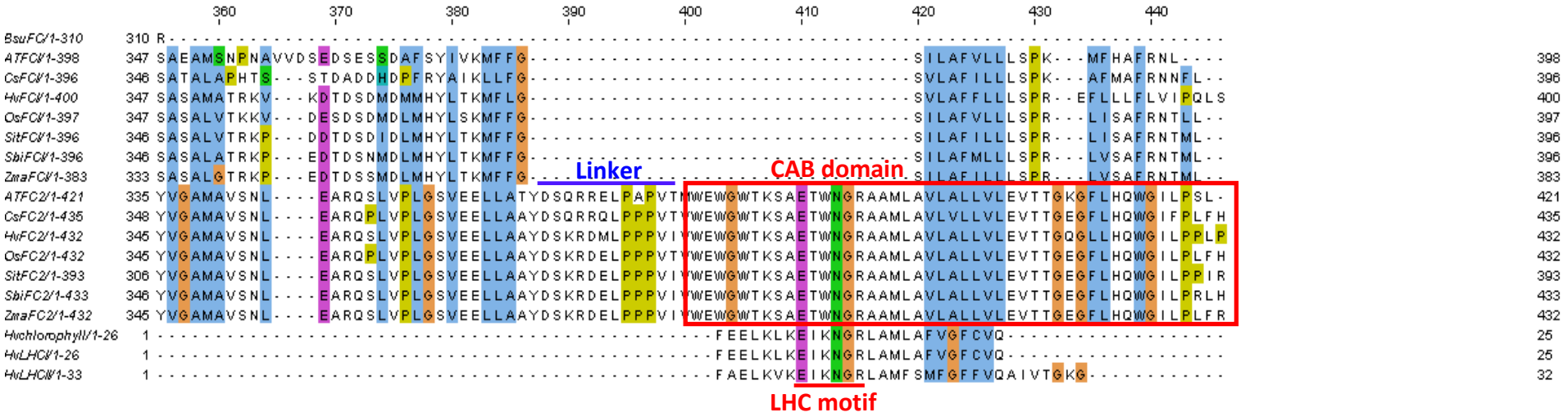


Fig S1. Similarity comparison of amino acid sequences of barley Ferrochelatase 1 (FC1) and barley Ferrochelatase 2 (FC2) to respective FC counterparts of other plant species: Bsu, Bacillus subtilis; At, *Arabidopsis* (*Arabidopsis thaliana*); Cs, cucumber (*Cucumis sativa*); Hv, barley (*Hordeum vulgare*); Os, rice (*Oryza sativa*); Sit, foxtail millet (*Setaria italica*); Sbi, Sorghum (*Sorghum bicolor*); Zma, Maize (*Zea maize*) and barley chlorophyll binding proteins (Hvchlorophyll binding protein, HvLHCI, HvLHCII) which contains C-terminal light harvesting complex (LHC) motif. The alignment was generated by using the programs MUSCLE and Jalview. Arrows indicate the conserved residues with deduced functions based on the biochemical studies or from the crystal structure of the B. subtilis enzyme (Al-Karadaghi et al., 1997). Red box indicates the chlorophyll a/b binding (CAB) domain which contains LHC motif, the characteristic feature of FC2. Blue line indicates the proline-rich linker sequence, which connects CAB domain to the FC catalytic core.

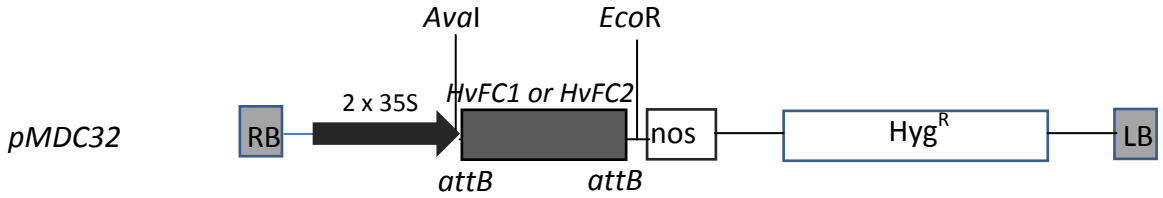


Fig S2. A schematic illustration of the pMDC32 constitutive expression vector used for barley transformation, which harbours a dual 35S promoter, and either *HvFC1* or *HvFC2*.

Table S1. Phenotypic characterization of transgenic lines ectopically overexpressing *HvFC1* and *HvFC2* relative to WT and null controls

Line	Plant height (cm)	Number of leaves	Tiller number	Shoot dry weight (mg)	Root dry weight (mg)
WT	5.7 ± 0.39 bc	4.2 ± 0.25 a	4 ± 0.48 a	45 ± 5.3 abc	14 ± 1.9 a
Null	5.4 ± 0.21 bc	4.0 ± 0.90 a	5 ± 0.63 a	50.2 ± 1.0 bc	18.6 ± 1.1 a
2x35S::FC1-28	5.7 ± 0.25 bc	4.0 ± 0.50 a	4 ± 0.31 a	61.5 ± 1.4 c	15.3 ± 0.6 a
2x35S::FC1-13	5.3 ± 0.47 bc	4.0 ± 0.26 a	4 ± 0.70 a	38.6 ± 1.8 ab	15.1 ± 1.8 a
2x35S::FC1-17	4.3 ± 0.14 a	3.5 ± 0.72 a	5 ± 0.65 a	29.4 ± 1.7 a	8.7 ± 2.9 a
2x35S::FC2-29	5.6 ± 0.07 bc	4.3 ± 0.34 a	4 ± 0.33 a	44 ± 6.4 abc	11.3 ± 1.2 a
2x35S::FC2-25	6.3 ± 0.3 c	4.1 ± 0.24 a	4 ± 0.29 a	48.8 ± 1.9 abc	16.6 ± 1.2 a
2x35S::FC2-9	4.9 ± 0.48 ab	3.7 ± 0.15 a	4 ± 0.21 a	53.6 ± 6.1 bc	22.2 ± 1.2 a

Data are presented as mean ± standard error of five replicates. Means with the same letter within a column are not significantly different at P<0.05, one-way ANOVA.

Table S2. Primers used in this study

Primers used for genotyping	Primer orientation	Sequence
Hygromycin	Fwd	CGCTCGTCTGGCTAAGATCG
	Rev	AGGGTGTCACGTTGCAAGAC
Transgene GOI	Fwd	CGAGGCGCGCCAAGCTATCAAA
	Rev	AATTCGAGCTCCACCGCGGT
qRT-PCR primer pairs		
HvFC1	Fwd	CGAGCATATTGAGAGACTGG
	Rev	TCACTGAAGAGTGTTCCGGA

HvFC2	Fwd	GGCCTGCACCGCGTAATTTA
	Rev	GCAGCAGAACGCCAATTTTC
GAPDH	Fwd	GTGAGGCTGGTGCTGATTACG
	Rev	TGGTGCAGCTAGCATTTGAGAC
HSP70	Fwd	CGACCAGGGCAACCGCACCAC
	Rev	ACGGTGTTGATGGGGTTCATG
Cyclophilin	Fwd	CCTGTCGTGTCGTCGGTCTAAA
	Rev	ACGCAGATCCAGCAGCCTAAAG
Tubulin	Fwd	AGTGTCCTGTCCACCCACTC
	Rev	AGCATGAAGTGGATCCTTGG

Appendix 2: Supplementary data for Chapter 4

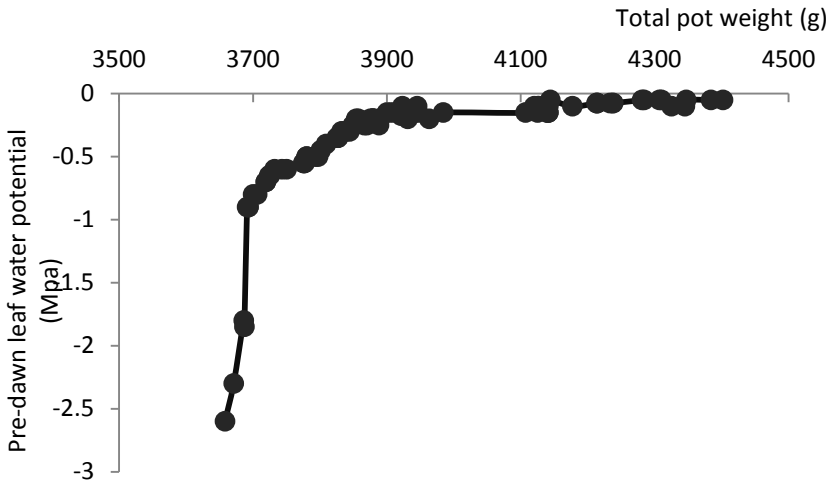


Fig S3. Standardized drying curve used in the drought assay for evaluating the physiological performance of transgenics and control plants.

Table S3. Primers used in this study

Primers used for genotyping	Primer orientation	Sequence
HvFC transgene GOI	Fwd	CGAGGCGCGCCAAGCTATCAAA
	Rev	AATTCGAGCTCCACCGCGGT
HvFLU	Fwd	ATGCAGGCGGCGGCCTCTTGT
	Rev	CAAGATTGGAGAATGACTGA
qRT-PCR primer pairs		
HvFC1	Fwd	CGAGCATATTGAGAGACTGG
	Rev	TCACTGAAGAGTGTTCCGGA
HvFC2	Fwd	GGCCTGCACCGCGTAATTTA
	Rev	GCAGCAGAACGCCAATTTTC
Catalase	Fwd	ATTTCAAGCAGGCTGGTGAG
	Rev	TCTGGATTTCATGGGTGACA
Superoxide dismutase	Fwd	CTTGAAGGACACCGACTTGC

	Rev	CTCAAAAAGCCAAATGACAGTG
GAPDH	Fwd	GTGAGGCTGGTGCTGATTACG
	Rev	TGGTGCAGCTAGCATTTGAGAC
HSP70	Fwd	CGACCAGGGCAACCGCACCAC
	Rev	ACGGTGTTGATGGGGTTCATG
cyclophilin	Fwd	CCTGTCGTGTCGTCGGTCTAAA
	Rev	ACGCAGATCCAGCAGCCTAAAG
tubulin	Fwd	AGTGTCCTGTCCACCCACTC
	Rev	AGCATGAAGTGGATCCTTGG