Effect of High Temperature Shock During Grain Maturation On Dormancy Of Wheat (*Triticum aestivum* L.) and Analysis of *TaDOG1*

Submitted by,

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Statement of authorship

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Abstract

Pre-harvest sprouting (PHS) is the germination of seed under wet environmental conditions whilst still on the mother plant prior to harvest. In wheat, PHS causes farmers substantial economic losses due to quality downgrading. A high level of dormancy is regarded as an important mechanism of resistance to PHS in cereal species, such as wheat (*Triticum aestivum* L.). Many dormancy quantitative trait loci (QTL) have been identified and the corresponding genes that impart tolerance to PHS are actively being sought. Besides genetic factors, environmental conditions during grain maturation have been shown to have profound effects on dormancy. These environmental factors include temperature, light, drought and nutrients.

This project aimed to determine the role of high temperature shock during wheat seed maturation on its dormancy at harvest-ripeness. The results of these experiments showed that seed of dormant or intermediate dormant wheat genotypes may have lower dormancy levels after experiencing a high temperature shock (5 days of 40°C). The highest sensitivity towards high temperature shock is limited to a short "window" of approximately five days starting around 25 dpa. The sensitivity increases and reaches a peak at 25 to 30 days post anthesis (dpa), causing an effective and quicker release of dormancy. The sensitivity later decreases and high temperature becomes less influential on dormancy. For the dormant genotype SUN325B, release from dormancy occurred 35 days earlier if treated with temperature shock. There was no significant correlation between the timing of the peak of sensitivity and factors such as daily temperatures before temperature shock, humidity or subsequent grain moisture losses. However, the stronger the intensity (40°C *versus* 35°C) and longer duration (5 days *versus* <5 days) of temperature shock seem to influence dormancy more significantly. Changes in embryonic and endospermic abscisic acid (ABA) concentration following temperature shock could not explain the change in dormancy levels in genotype SUN325B. Instead, the change in

dormancy levels following the temperature shock could potentially be ascribed to the loss of ABA sensitivity.

A comparison of the dormancy response to temperature shock of 23 genotypes that harbour different combinations of known dormancy QTL was performed. Strongly dormant genotypes, P07.683, 50213/Cunn798 and DM1073 #31, did not show an increased germination index at 65 dpa. Other genotypes, ranging from dormant to intermediate dormant phenotype under control conditions, i.e. displaying germination index less than 0.6 at 65 dpa, responded strongly to temperature shock. Dormancy levels significantly decreased, reflected by increases of germination index ranging from 0.4 to 0.9. It is highly likely that a temperature-shock-induced decrease in dormancy is a common response of these genotypes, and a higher number of combinations of QTL are required to resist dormancy changes.

The identification of *Delay of Germination 1* (*DOG1*) as the gene underlying a major dormancy QTL in *Arabidopsis thaliana* has led to stronger understanding of the mechanism of dormancy. Based on results of studies of the *AtDOG1* and *TaDOG1-like* genes in *Arabidopsis* and cereal species respectively, and their functional conservation and responsiveness towards temperature changes during seed development, it seems likely that the *TaDOG1-like* genes in wheat might play a role in temperature shock-regulated dormancy levels.

A search of publicly available bioinformatics databases revealed three expressed sequence tags (ESTs) with accession numbers *AK336217*, *X56782* and *D12921* that could be *TaDOG1-like* genes in wheat additional to the five previously reported by Ashikawa *et al*. (2010, 2014). Protein motif analysis showed that the orthologs of *DOG1* possess combinations of protein motifs specific to each clade. Promoter analysis revealed RY repeats (CATGCAT) and (ACGTG)-core-containing ABA-responsive elements-like sequences in the promoters of these genes. Comparative mapping analysis also showed that one *TaDOG1-like* gene (accession:

X56782, could reside in dormancy QTL regions in wheat and similarly, two rice putative orthologs (*Os01g0159000* and *Os05g0560200*) reside in dormancy QTL regions in rice.

In a reverse transcription quantitative PCR (RT-qPCR) analysis, no significant expression of two *TaDOG1-like* genes (accession: *AB555729* and *AK332921* or *TaDOG1-L1* and *TaDOG1-L2* respectively) were detected in either embryos or de-embryonated wheat grains of SUN325B at 45 and 60 dpa. However, both genes were detected to be expressed at 25 dpa and 30 dpa in the excised embryo tissues, signifying the mRNA to be short-lived. *TaDOG1-L1* showed a non-significant increase following temperature shock, hence failed to explain the increase in germination index. *TaDOG1-L2* was significantly down-regulated at 30 dpa following temperature shock, which could cause a lower protein accumulation towards 65 dpa. Further investigation could focus on the role of *TaDOG1-L2* in dormancy in white wheat genotypes to better explain the effect of temperature shock during the sensitive “window” on dormancy in general.

Literature Review

1.1 Dormancy and germination

Germination of a seed is defined as the protrusion of the radicle from surrounding structures following rehydration through imbibition, resumption of energy metabolism, cellular repair and protein synthesis (Nonogaki *et al.* 2010). Strictly defined, the germination process ends with the emergence of the radicle after which seedling growth occurs (Bewley *et al.* 2013). Under conditions favourable for germination, a lack of germinability of a hydrated/imbibed, intact and viable seed is indicative of dormancy. Plants use dormancy as a mechanism to regulate germination until suitable environmental conditions (which include sufficient amounts of light, heat, water and oxygen) are encountered. In agricultural crops, germination is important for the establishment of the next generation of plants and therefore rapid germination and growth of seedlings are desirable. In many cases such as in vegetable crops like lettuce, uniformity in germination and seedling growth is an important trait. Dormancy at harvest-ripeness is desirable for cereal crops such as wheat, barley, rice and sorghum to prevent germination when grains are exposed to moisture when it rains prior to harvest, also known as pre-harvest sprouting (PHS). On the other hand, excessive dormancy is undesirable due to poor emergence of plants.

1.2 Pre-harvest sprouting (PHS) and economic losses

Wheat is one of the most important food commodities in the world with global production reaching 700 million tonnes in 2011 (Statistics by *Food and Agriculture Organization of the United Nations*, http://www.fao.org/home/en/). Australia produced 27 million tonnes of wheat in 2011 which represented more than 3% of the global production. However Australia's share of global wheat trade was 15%, generating AUD 4 billion dollars and contributing significantly to the Australian economy.

Wheat cultivars that lack dormancy are vulnerable to PHS and dormancy is recognised as a major factor in resistance to PHS. During PHS, grains produce hydrolytic enzymes including α-amylase and endoprotease that can degrade starch and protein during processing. End products prepared from sprouted wheat grains are invariably lower in quality compared with products made from sound grain (Edwards *et al.* 1989). Problems include sticky dough as well as poor volume, crumb structure and colour.

According to Wheat Standards 2013/2014 released by Grain Trade Australia (GTA) (*http://www.graintrade.org.au/commodity_standards*), there is a zero tolerance policy towards sprouted grains during grade/varietal classification at receival, unless the falling number exceeds minimum standards. A downgrade to feed grade as a consequence of sprouting cause Australian farmers substantial economic losses. PHS is a problem in many wheat growing regions of the world, especially Australia where mainly white-grained wheat varieties (often low in dormancy levels) are grown. Historically, Australian farmers were encouraged to plant only white-grained varieties due to a penalty imposed if red grains were found in shipments to Great Britain. Subsequently this became mandatory for receival into milling grades due to the high demand of white-grained wheat flour. Australia has become one of the biggest suppliers of white-grained wheat to the international market, especially for the high-value, quality conscious Asian markets. Introgression of high levels of dormancy into white-grained cultivars suited to the Australian environment would improve resistance to PHS, and is consequently an important target trait for marker assisted selection by wheat breeders.

1.3 Structure, development and maturation of a wheat seed

Wheat (*Triticum aestivum L.*) is a monocot species that has seeds in the form of grains. The single seed consists of the embryo, scutellum, endosperm (starchy endosperm and aleurone), and testa which are enclosed by the inner and outer layers of the pericarp. The seed

can be hard or soft, red or white, forming different combinations (Hard Red [HR], Hard White [HW], Soft Red [SR] and Soft White [SW]) with different applications in the international market. The aleurone layer, a living tissue, produces and releases enzymes during germination for the mobilization of storage reserves in the endosperm. In an endospermic seed like wheat, the scutellum is reduced to a thin layer, different from a non-endospermic legume seed cotyledon where the massive cotyledon (also known as embryonic reserve) is the major site of storage reserve. The embryo, formed following the fertilization of the egg cell nucleus by the male pollen nucleus, is separated by the scutellum layer from the starchy endosperm. The seed then develops and matures into a quiescent state. At this stage, the seed has a low moisture content and metabolic activity. In the quiescent state, the seed awaits dispersal and exposure to moisture or imbibition for germination to establish the next generation of plants. In modern agriculture, farmers harvest wheat crops as soon as possible after grain maturation is completed or in some cases harvest early then complete grain drying by mechanical means.

1.4 The establishment and maintenance of dormancy

Wheat genotypes vary in terms of dormancy. Some genotypes establish dormancy during grain development and maintain it until or past harvest maturity, hence have less risk of suffering from PHS. Dormancy can be maintained in some highly dormant wheat genotypes for a long period of time and requires alleviation through after-ripening before germination can occur. On the other hand, genotypes that fail to establish or maintain dormancy are at risk of PHS. The complexity of the mechanisms involved in dormancy is not limited to genotypic factors. Variability in dormancy levels is often observed in many wheat genotypes at different planting seasons or when environmental conditions are manipulated (Lunn *et al.* 2002; Biddulph *et al.* 2007). This is also similar in the case of barley (Rodriguez *et al.* 2001). Although only limited molecular evidence have been presented in cereals, hormone and molecular changes have been shown to

occur in *Arabidopsis* when dormancy levels are affected by environmental events (Kendall and Penfield 2012).

Currently, the events involved in the establishment and the maintenance of dormancy still cannot be defined in molecular terms due to a lack of understanding of the mechanisms involved. This has made the quantification of dormancy by molecular means impossible. Therefore, we still rely to a large extent on physiological measurements of dormancy.

1.5 The measurement of seed dormancy in wheat

Currently, the most common and convenient measurement of dormancy in wheat is through a germination test (by measuring germinability). This germination test is based on visible germination (the emergence of the embryonic axis) through a germination time course. Typically, replicate samples of 50 sound and well-filled grains free from obvious defects, are incubated on moist filter paper in Petri dishes at 20°C. Germinated grains are counted at daily intervals and expressed as a cumulative percentage germination curve (germination time course) or as a weighted germination index (Walkersimmons 1988). Germination index gives maximum weight to grains that germinate rapidly and is calculated from the following formula:

Germination Index (GI) = (7 x *N1* + 6 x *N2* + 5 x *N3* + 4 x *N4* + 3 x *N5* + 2 x *N6* + 1 x *N7*)/ total days of test x total grains, where *N1, N2, N3, N4, N5, N6* and *N7* are the number of grains that have had germinated on day 1, day 2, day 3, day 4, day 5, day 6 and day 7 respectively. The maximum index is 1.0 if all grains germinate by day 1. Lower indices indicate higher grain dormancy and reduced germinability whereas higher indices reflect low grain dormancy. When GI is measured at intervals after anthesis, changes in GI follow a sigmoidal curve which reflects the establishment, maintenance and eventual release from dormancy.

Other terms such as “Germinating Power” or “Germination Energy” are basically similar, utilising the same concept of percentage germination over a period of time.

1.6 Complex mechanism of seed dormancy of wheat and other species

Pioneering research on wheat seed dormancy revolved around the analysis of hormones, physical attributes of a wheat seed, environmental factors and their effects on dormancy. The focus was then shifted towards mapping studies to identify QTL for dormancy and PHS. Due to the advances in mapping technology and genome sequencing, finer QTL maps have been generated in both the model species, *Arabidopsis thaliana* and in cereals such as rice, barley and wheat, leading in some instances to the identification of genes underlying the QTL of dormancy or putative candidate genes. Following decades of research aimed at understanding the mechanism of dormancy, although still far from being understood, a considerable amount of information and evidence has now been accumulated.

(a) Hormone (gibberellic acid and abscisic acid) regulation

The mechanisms of dormancy and germination are complex and current hypotheses involve a balance in amount and sensitivity towards hormones such as abscisic acid (ABA) in particular but possibly also gibberellic acid (GA) (Feurtado and Kermode 2007; Yamaguchi 2008).

In many plant species including wheat, GA plays a major role in germination through the increase in concentration and modification of the hormone itself, in parallel to the decrease in concentration and the deactivation of ABA (Nonogaki *et al.* 2010). GA biosynthesis mutants of *Arabidopsis thaliana* has been shown to lack capability to germinate, showing that GA is a germination-essential hormone (Koornneef and Vanderveen 1980). However, a lack of germination due to GA-deficiency under conditions favourable for germination is not regarded as dormancy. The role of GA on dormancy remains unclear. There are exceptions in dicot species such as, tomatoes and *Arabidopsis*, where GA is important to break physical dormancy (also see section 1.6e), imposed by the endosperm caps on embryos (Groot and Karssen 1987). The

endosperm caps provide a mechanical resistance to radicle protrusion, requiring GA to trigger the expression of endosperm-weakening enzymes in order to break the barrier (Bewley 1997; Finch-Savage and Leubner-Metzger 2006). However, evidence of the role of GA specifically on embryo dormancy is limited.

On the other hand, ABA is not only regarded as the key regulator of dormancy induction and maintenance, but also plays a major role in the deposition of storage reserve and stress tolerance acquisition, all significant events during seed formation (Kermode 2005; Nambara *et al.* 2010; Bewley 1997). Firstly, ABA has an antagonistic role on hormone GA, a mechanism that is unclear. ABA has been shown to promote dormancy possibly by suppressing biosynthesis of GA, and more recently it has been suggested that it limits the production of ascorbic acid, which is a substrate for many hormones such as GA and ethylene (Seo *et al.* 2006; Ye and Zhang 2012). In the past, rate-limiting genes involved in ABA metabolism and GA catabolism have also been shown to be antagonistic, providing a model for their roles in dormancy (Seo *et al.* 2009). In relation to physical dormancy, ABA has been shown to delay the onset of endosperm-weakening in *Arabidopsis* through regulation of GA/ABA ratio (Muller *et al.* 2006). This delays the breaking of physical dormancy, even though embryo dormancy has been lost but fails to germinate when imbibed under favourable conditions.

In relation to embryo dormancy, or seed dormancy which excludes physical dormancy, ABA is established and well-recognised as an important hormone in the induction and maintenance of dormancy (Bewley *et al.* 2013; Nambara *et al.* 2010; Finch-Savage and Leubner-Metzger 2006; Koornneef *et al.* 2002). Seeds differ in timing of dormancy induction, which can be ascribed to the role played by ABA (Koornneef *et al.* 2002). *Zeaxanthin epoxidases* (*ZEP*) and *9-cis-epoxycarotenoid dioxygenases genes* (*NCED*) are important genes in the ABA biosynthesis pathway for ABA expression whereas *ABA 8'-hydroxylase genes* and *ABA 9'hydroxylase genes* are involved in the catabolism of ABA, both mechanisms conserved across

many plant species (Lefebvre *et al.* 2006; Okamoto *et al.* 2006; Okamoto *et al.* 2011; Endo *et al.* 2011; Nambara *et al.* 2010; Saito *et al.* 2004). In plant species, such as *Arabidopsis*, the biosynthesis of ABA in the embryo is crucial for the dormancy mechanism (Karssen *et al.* 1983).

Under normal conditions, the levels of ABA in cereal grains increase during early grain development, reach a peak and ultimately decline prior to harvest maturity (Bewley 1997; Walker-Simmons 1987a; Benech-Arnold *et al.* 1999). In the cereal species, there is contrasting evidence that ABA concentration correlates to dormancy. In the past, embryonic ABA levels were found to be quite 'similar' in PHS susceptible wheat varieties and resistant varieties, displaying consistent lack of correlation whereas ABA sensitivity was always observed to be prolonged in dormant wheat seeds (Walker-Simmons 1987a, b; Morris *et al.* 1989). Variation in expression levels of biosynthesis and catabolism genes of ABA in barley were also observed in the field during different years, causing different levels of ABA expression that might be able to explain the observed differences in germinability (Chono *et al.* 2006). ABA sensitivity components which might be environmentally regulated were not included in the experiment.

It is normally observed that there are differences in ABA concentrations in dormant and non-dormant seeds following imbibition. In *Arabidopsis*, dry seeds of dormant and non-dormant genotypes were observed to have similar levels of ABA but decreased more rapidly in non-dormant seeds upon imbibition (Millar *et al.* 2006). Similarly, in barley, Jacobsen et al. (2002) reported similar ABA concentrations in freshly harvested and after-ripened barley grains but showed significant differences in the pattern of decrease following imbibition. Chono *et al*. (2006) also observed a lower ABA concentration in less dormant barley seeds following imbibition. ABA concentration decreases continuously following imbibition in less dormant seeds in comparison to a slight increase in ABA levels before the decrease in dormant seeds. In a wheat transcriptomic analysis, ABA metabolism genes have been shown to have no differential regulation between dormant and non-dormant seeds, whereas one *Zeaxanthin epoxidase* probeset was shown to

be upregulated in after-ripened (see section 1.7) wheat seeds following imbibition in comparison to dormant seeds, even though it did not alter ABA metabolism during imbibition (Liu *et al.* 2013a). Patterns of ABA concentration regulation following imbibition might shed light on the mechanism of dormancy but is still currently unclear.

Nevertheless, the main factor in resistance towards PHS requires a longer duration of dormancy maintenance. Even though high ABA levels are required for dormancy induction, ABA sensitivity appears more likely to be the mechanism of dormancy maintenance to resist PHS. The perception of this hormone ABA is important for the regulation of dormancy. As with other hormones, a receptor is required to recognise the specific structure of ABA prior to activation of downstream responses. The PYR1/PYL/RCAR1 family of ABA receptors in *Arabidopsis*, when activated by ABA, regulate members of the protein phosphatase 2C (PP2C) proteins (such as ABI1 and ABI2) (Ma *et al.* 2009; Park *et al.* 2009). Without ABA, PP2C proteins actively inhibit ABA responses by SNF1-related protein kinases (SnRK2) and transcription factors such as ABI3, ABI4 and ABI5 (Nambara *et al.* 2010). Downstream of the ABI transcription factors is the expression of bZIP (basic leucine zipper) transcription factors that induce ABA sensitivity, which theoretically translates into dormancy (Finkelstein *et al.* 2008). Recently, two ABA plasma membranes transporters have also been identified (Kang *et al.* 2010; Kuromori *et al.* 2010).

The research on ABA in *Arabidopsis* has been so extensive that the complete mechanism, from biosynthesis to perception, to signalling and to transcriptional output, is close to completion. ABA-related genes identified in *Arabidopsis* are highly conserved even in cereal species. In wheat, some of these ABA signalling components have also been identified, analysed and mapped (Nakamura *et al.* 2007; Bailey *et al.* 1999; McKibbin *et al.* 2002; Johnson *et al.* 2002; Nakamura and Toyama 2001).

Transcriptomic analysis in wheat has also shown that some ABA signalling components could be repressed during the transition from dormant state to a non-dormant state following

after-ripening, which revealed a possible mechanism to connect ABA sensitivity and dormancy (Liu *et al.* 2013a). Firstly, the authors observed upregulation of two *PP2C* probesets in imbibing dormant seeds whereas the same probesets remain unchanged in imbibing after-ripened seeds. Secondly, one *SnRK2* probeset was downregulated in imbibing after-ripened seeds whereas the same probeset did not change in imbibing dormant seeds. Thirdly, two *ABI5* probesets were also downregulated in imbibing after-ripened seeds. These changes clearly represent decay in ABA sensitivity when comparing dormant and after-ripened seeds. However, molecular changes leading to the decay and subsequently lower dormancy are unknown.

(b) QTL mapping, corresponding genes and candidate genes

Dormancy is a trait with high genetic and environmental variation. QTL analyses have identified various loci for dormancy and PHS distributed across nearly all chromosome groups (Kulwal *et al.* 2010; Gerjets *et al.* 2010; Munkvold *et al.* 2009; Kottearachchi *et al.* 2008; Anderson *et al.* 1993). Furthermore, the dormancy phenotype of populations in QTL studies often follows a normal distribution reflecting a highly complex regulation of dormancy in wheat that probably involves more than one gene. Among the dormancy loci that have been identified to date, QTL located on chromosomes 2B, 3A, 3B and 4A appear at highest frequency (Mares and Mrva 2001; Kato *et al.* 2001; Mares *et al.* 2009; Groos *et al.* 2002; Munkvold *et al.* 2009). It is worth noting that whilst the QTL on 3A, 3B and 4A have a significant effect on their own, they do not guarantee a high level of dormancy (Mares and Mrva 2001; Kottearachchi *et al.* 2006).

(i) *Mother of flowering time* (*MFT*) or *TaPHS1*

Recently, a wheat gene, *Mother of Flowering Time* (*MFT*), was identified as the gene underlying the QTL on 3AS via map-based cloning (Liu *et al.* 2013b). Two single nucleotide polymorphisms causing either mis-splicing or a truncated protein are

responsible for PHS susceptibility (Nakamura *et al.* 2011; Liu *et al.* 2013b). The identity of the underlying gene was further confirmed by the increased sprouting occurrence in RNAi knockdown mutants and was thus renamed *TaPHS1*. *TaPHS1* was first identified in a microarray study as a gene regulating dormancy under different temperature treatments (Nakamura *et al.* 2011). The seed-specific gene was shown to be upregulated at harvest maturity by a low temperature treatment during seed development (Chardon and Damerval 2005).

MFT encodes a phosphatidylethanolamine-binding protein in the embryo and can function as a negative regulator of ABA sensitivity in *Arabidopsis* by repressing the expression of ABI5 during imbibition (Xi *et al.* 2010). The gene is also transcriptionally regulated by ABI3, ABI5 and DELLA (repressor of GA signalling) proteins, suggesting a close connection towards the ABA and GA signalling pathways. In addition, ABA response elements were found in the promoter of *Arabidopsis MFT*, confirmed by immunoprecipitation assays (Xi *et al.* 2010).

However, very little evidence has been presented in wheat concerning its relationship to ABA. Nakamura *et al.,* 2011 overexpressed *TaPHS1* in wheat embryos and observed that immature naked wheat embryos that originally germinated precociously now appeared to be dormant. This is closely similar to the prolongation of dormancy by applying ABA on immature naked embryos.

(ii) ***Viviparous-1* (*Vp1*)**

One of the genes that is most often associated with dormancy in wheat is *Viviparous1* (*Vp1*). Orthologous to the *Arabidopsis ABI3 gene*, *Vp1* mutants in maize were first discovered to have embryos with reduced sensitivity towards hormone ABA and defective in ABA-regulated anthocyanin biosynthesis (Neill *et al.* 1986; Robichaud

et al. 1980). Due to the lack of ABA sensitivity of the embryo, precocious germination tends to occur (McCarty 1995, 1992; McCarty *et al.* 1989). Analysis of ancestral and modern wheat *Vp1* genes sequences showed common mis-splicing transcripts that probably originated before the evolution and domestication of wheat (McKibbin *et al.* 2002). ABA-induced *Vp1* expression occurs during wheat seed maturation and functions to repress GA-induction of *α-amylase genes* in the aleurone layer (Utsugi *et al.* 2008). A positive correlation was established between the levels of expression of *Vp1,* ABA sensitivity and seed dormancy (Nakamura and Toyama 2001). In rice, *Vp1* is responsible for the expression of *Sdr4* (corresponding gene for the QTL of dormancy in rice on chromosome 7), and in turn regulates the expression of *OsDOG1* (ortholog of the corresponding gene for the dormancy QTL in *Arabidopsis*).

When maize *Vp1 gene* was transformed into wheat, it greatly enhanced dormancy and resistance to PHS (Huang *et al.* 2012). Although mis-spliced non-functional *Vp1* is common in wheat genotypes, the function is conserved in other species such as maize. Ectopic expression studies suggest that the use of *Vp1* orthologs for functional compensation may be a promising strategy for breeding PHS resistant wheat cultivars.

It was originally suggested that the *Vp1* genes correspond to the 3AL QTL for dormancy in wheat. However, Osa *et al*. (2003) mapped *Vp1* to a region in 3A, which is 50 cM away from the QTL on 3AL indicating that *Vp1* is clearly a distinct gene from the QTL on 3AL.

(c) Red grain colour

Red wheat is often observed to be more dormant than white wheat (Flintham 2000; Himi *et al.* 2002). Red wheat has a wide variation of dormancy levels whereas finding a dormant white

wheat variety is relatively rare. The observation of red seeds with high dormancy is also common in other plant species such as red rice (Cohn and Hughes 1981). Even in model species *Arabidopsis thaliana*, the strength of the seed colour has been reported to influence the level of dormancy possibly by mechanical restriction of germination (Debeaujon *et al.* 2000; Debeaujon *et al.* 2001).

Wheat grain red colour is controlled by the "*R loci*" on homeologous chromosomes 3AL, 3BL and 3DL. The *R locus* has been demonstrated to enhance grain dormancy possibly via regulation of ABA sensitivity (Himi *et al.* 2002). In red-grained wheat populations, dormancy QTL on homeologous chromosomes 3, 3AL, 3BL and 3DL, appear to correspond to the *R genes* on the respective chromosomes (Groos *et al.* 2002; Osa *et al.* 2003). Three homeologous genes, *Tamyb-10*, which code for R2R3-type MYB domain proteins, are highly similar to *Arabidopsis* TT2 proteins that control PA synthesis and can be found within the *R locus* (Himi *et al.* 2011). These proteins could act as transcription factors to regulate the expressions of many enzymes in the flavonoid biosynthesis pathway (Himi *et al.* 2002; Himi and Noda 2004). The red pigments are composed of tannins such as catechin and proanthocyanin (PA) (a polymer of catechins), synthesized by the flavonoid biosynthesis pathway. It has been suggested that the catechins in red wheat grains act as germination inhibitors, hence conferring higher levels of dormancy in red wheat varieties (Kottearachchi *et al.* 2006; Miyamoto and Everson 1958). There is still a lack of evidence on the role of these pigments in dormancy, even though in barley, PA-free mutants have also been shown to have reduced levels of dormancy recently (Himi *et al.* 2012).

In rice, red colour is controlled by the *Rc* and *Rd* genes, coding for a basic helix-loop-helix (bHLH) transcription factor and a dehydroflavonol-4 reductase (DFR) respectively (Furukawa *et al.* 2007). In weedy red rice, there is an association between QTL for dormancy and pericarp colour on a specific cluster on chromosome 7 (Gu *et al.* 2005). The gene for red

colour within the cluster, *Rc* (non-orthologous to the wheat *R* gene), upregulates the expression of ABA biosynthesis genes (Gu *et al.* 2011). A similar interaction has not been reported in wheat.

Experiments on *Arabidopsis thaliana* showed that seed germination is influenced by red wavelengths during seed maturation (McCullough and Shropshire 1970). The perception of objects as red by the eye is due to the reflection of the red wavelengths of the spectrum. The presence of red pigment in the grain coat might act a natural filter of red wavelengths that affect dormancy. To my knowledge, this has never been specifically suggested in wheat. However, Fenner (1991) commented that seeds matured in an environment with low levels of red wavelengths due to filtration by the leaf canopy, might alter germination characteristics. However, this situation is only applicable if changes occur due to perception of light by the seed. Some studies have shown that dormancy of Ward's weed, fenugreek and thornapple seeds were affected even when fruits were covered from light, indicating that changes in light might be affecting the maternal plants directly (Gutterman 1980).

Some barley seeds can be dormant when imbibed under white light or blue light but be non-dormant in the dark. ABA levels were shown to change due to light-regulated changes of *NCED* genes in barley (Gubler *et al.* 2008). Red light and far red light seemed to have no effect on germination in barley but contrasting results were found in *Brachypodium and* wild oats (Barrero *et al.* 2012). However this does not rule out the original hypothesis as seed germination observed at harvest maturity when exposed to different wavelengths of light might be different from the effects of different light wavelengths exposure during seed maturation. For such a hypothesis, experimentation remains a challenge since whilst it is easy to irradiate plants with red wavelengths it is hard to exclude only red wavelengths.

In conclusion, despite considerable research, the direct relationship between the seed coat colour and dormancy is still unclear.

(d) Influence of light

Light quality during seed development affects dormancy (Finch-Savage and Leubner-Metzger 2006). For example, in *Arabidopsis*, a change in red to far-red ratio during seed development can alter light requirements during germination (Hayes and Klein 1974). When red to far-red ratio is high, sunlight is sufficient and is favourable for germination. On the contrary, when red to far-red light is low, conditions are not favourable for germination. Hence, *Arabidopsis* responds to this ratio during seed development in combination with temperature cues, to manipulate seed dormancy by regulating phytochromes (photoreceptors of red and far red light) (Dechaine *et al.* 2009). Recent analysis has also shown that temperature is important in determining which phytochrome predominates during seed development (Donohue *et al.* 2012).

In lettuce, seeds produced under different red to far red ratios have different ABA sensitivity and dormancy (Contreras *et al.* 2008). In wheat, altering the red and far red ratios before anthesis affects tillering, growth of leaf sheath, growth of spike-carrying internode and most interestingly, the level of *Abscisic Acid Insensitive 5* gene expression (Evers *et al.* 2007; Casal 1993). However to my knowledge, there have been no reports of variation in dormancy associated with manipulation of red to far-red ratio after anthesis.

Recent research on the influence of light has focused mainly on how dormancy can be terminated or germination manipulated. Phytochromes regulate many aspects of plant development including seed germination. Depending on species, hormone metabolism and perception are involved in the seed response towards light in combination with phytochromes (Seo *et al.* 2009). During *Arabidopsis* seed germination, phytochrome photoreceptors have been shown to be important regulators when subjected to different germination conditions such as light quantity and temperature (Heschel *et al.* 2008; Heschel *et al.* 2007). For example, phytochrome A (PHYA) proteins that mediate far red light-induced seed germination are low during initial stages of imbibition of *Arabidopsis* seeds, and require phytochrome B (PHYB) for a red light-

induced seed germination (Seo *et al.* 2009). Hence, environmental conditions during germination are important factors that regulate the precise timing of seed germination through manipulation of the functionally diversified family members of phytochromes. During seed germination, both GA and ABA can be regulated by light, which could explain how red light and far red light affect seed germination (Yamaguchi *et al.* 1998; Seo *et al.* 2006). In barley, blue light inhibits germination possibly via upregulation of ABA biosynthesis (which includes upregulation of a ABA biosynthesis gene and downregulation of a ABA catabolism gene) and ABA concentration, whereas red and far red light have no influence (Gubler *et al.* 2008; Barrero *et al.* 2014).

(e) Mechanical constraint imposed by the seed structures

In seeds of *Arabidopsis,* the embryo is enclosed by the seed coat and endosperm tissues and it has been suggested that the tissue imposing a mechanical constraint to germination is the endosperm (Nonogaki *et al.* 2010). Such a constraint requires strong growth potential of the embryo or GA-induced cell wall-weakening enzymes like endo-β-mannase for a successful protrusion of the radicle (Bewley 1997; Groot *et al.* 1988).

As a continuation to the argument that colour pigments contribute towards dormancy, the protein-binding pigment, proanthocyanidin, has been suggested to form a strong structural barrier impermeable to water (Debeaujon *et al.* 2001; Kermode 2005). However, this might not apply to wheat as no correlation of red colour and testa thickness with water uptake for the first 30 hours was reported in an analysis of 50 Australian red, white, hard and soft wheat varieties (King 1984). On the other hand, there is also contrasting evidence that water uptake differs during the early hours and at 24 hours after imbibition for red and white wheat varieties (Himi *et al.* 2002; Huang *et al.* 1983).

In the case of dormant and non-dormant white wheat seeds, water uptake, oxygen utilization and the resumption of metabolic events were of similar rates for the first few hours

after imbibition (Bewley *et al.* 2013; Bewley 1997). A Magnetic Resonance Micro-Imaging (MRMI) study has also shown that rates and patterns of water movement into the embryos of dormant and non-dormant wheat grains were similar for the first few hours and entry was restricted to the micropyle (Rathjen *et al.* 2009).

(f) Effects of the environment (from anthesis to seed maturity)

In many plant species, it has been reported that besides genetic differences, environmental conditions experienced by the maternal plant during seed maturation influence the level of germinability of seeds (Fenner 1991). Early observations showed that one genotype can display various degrees of dormancy after experiencing temperature variations during seed maturation. Among many environmental signals, temperature variation during seed maturation appears to be most influential in affecting the level of dormancy (Reddy *et al.* 1985). Low temperatures during seed maturation are generally associated with high levels of seed dormancy. Transcriptomic analysis of dry seeds matured at lower temperatures showed a higher expression of the *Delay of Germination 1* (*DOG1*), the causal gene for a QTL of dormancy in *Arabidopsis* (Kendall *et al.* 2011). ABA concentrations were also shown to be higher in the seeds.

In barley, the expression patterns of ABA biosynthesis genes have been shown to vary in different years due to environmental effects including temperature (Chono *et al.* 2006). In wheat, seed development and maturation under varying temperatures can affect ABA concentration and sensitivity providing a possible explanation for altered levels of dormancy (Walker-Simmons and Sesing 1990). Nakamura *et al*. (2011) has shown that *Mother of Flowering Time* (*MFT*) is upregulated in dry seeds matured at lower temperatures.

Besides changes in gene and hormone expression, one of the most interesting hypotheses regarding temperature and dormancy breaking is the influence of temperature on the structures of seed cell plasma membranes (Hilhorst 1998; Petel and Gendraud 1996). Based

on the fluid mosaic model, plasma membranes consist of a phospholipid bilayer which is fluid, forming a matrix permeable to many lipids and proteins. Changes in temperature were suggested by the authors to influence the membrane fluidity which in turn affects many signal transduction pathways.

1.7 Other mechanisms in cereals: after-ripening and nitric oxide

After-ripening and nitric oxide application are measures that can be taken to break dormancy of a seed.

(i) After-ripening

After-ripening is the natural dormancy-breaking process that occurs when dry seeds are stored under warm condition after maturation (Iglesias-Fernandez *et al.* 2011). In dry dormant cereal seeds, germinability may be inhibited or limited to a low and narrow temperature range depending on the occurrence of dormancy-breaking events even though suitable conditions for germination are met. Following after-ripening, dormancy can be alleviated and seeds not only progressively become germinable but the temperature range of which the seed germinates is also widened (Corbineau and Come 2000). During dry storage, seeds maintain moisture in order for transcriptional gene expressions to occur (Leubner-Metzger 2005).

During the transition from dormant to non-dormant in dry wheat seeds, transcriptional profiling studies have provided limited evidence of changes in ABA biosynthesis genes (Gao *et al.* 2012; Liu *et al.* 2013a). Liu *et al*. (2013a) also showed that following after-ripening, ABA levels did not differ unless seeds are imbibed. This is consistent with past studies that showed a lack of correlation between levels of ABA and dormancy of wheat seeds at harvest maturity (Walker-Simmons 1987a; Barrero *et al.* 2009). During this transition, wheat embryos slowly lose their sensitivity to ABA. Some

ABA signalling genes were found to be differentially regulated, suggesting a possible linkage to the loss of ABA sensitivity during after-ripening (Liu *et al.* 2013a). Liu et al. (2013) also reported differential regulation of ABA biosynthesis and GA biosynthesis during imbibition that would favour germination in wheat seeds whilst in barley, after-ripened seeds have a higher ABA catabolism gene expression during imbibition (Gubler *et al.* 2008).

Despite the evidence that regulation of ABA biosynthesis and signalling have a strong influence on seed dormancy and after-ripening, the exact mechanisms are still unknown.

(ii) Nitric oxide

Nitric oxide, produced naturally in plants, is involved in plant growth and development, germination, senescence and possibly also stress responses such as heat, salinity and drought (Beligni and Lamattina 2000, 2001; Durner and Klessig 1999). In *Arabidopsis*, nitric oxide can promote both breaking of dormancy and germination processes, although the mechanisms not fully understood (Arc *et al.* 2013a; Bethke *et al.* 2006). Nitric oxide, besides being a gaseous free radical that can easily diffuse through plasma membranes, is also a chemically reactive signal molecule that can target various proteins (Besson-Bard *et al.* 2009; Arc *et al.* 2013b). Nitric oxide accumulates in the aleurone layer of *Arabidopsis* seeds and transcriptionally enhances ABA catabolism during imbibition (Liu and Zhang 2009; Bethke *et al.* 2007). Furthermore, exogenous application of nitric oxide seems to affect ABA content during imbibition (Matakiadis *et al.* 2009). In wheat, nitric oxide was also shown to reduce ABA content and reverse the germination-inhibiting effect of blue light following imbibition (Jacobsen *et al.* 2013).

1.8 Dormancy genes in other plant species

(i) *Delay of germination 1* (*DOG1*) in *Arabidopsis* and orthologs in other species

There are altogether seven QTL for dormancy in *Arabidopsis*, known as the *Delay of Germination* (*DOG*) loci (Alonso-Blanco *et al.* 2003). Among the seven loci, four have additive effects, and in this respect are similar to the QTL for dormancy in wheat (Mares unpublished data). The gene corresponding to one of the major QTL, *AtDOG1*, was reported to cause high dormancy in the ecotype Cape Verde Island and is naturally variable (Bentsink *et al.* 2006). It was also shown recently that the *AtDOG1* is pleiotropic, influencing aspects not limited to dormancy such as flowering time, fitness and longevity (Chiang *et al.* 2013; Bentsink *et al.* 2006). The mechanism of *DOG1* on dormancy is unknown, but is possibly related to ABA pathway as the expression of *DOG1* for dormancy relies on the presence of ABA (Bentsink *et al.* 2006). It is expressed during early grain development, peaks and decreases towards the end of maturation. Protein levels on the other hand increase but remains stable until the end of maturation. Following after-ripening of *Arabidopsis* seeds, DOG1 protein levels remain high whereas following imbibition, both *DOG1* mRNA and protein quickly disappear (Bentsink *et al.* 2006; Nakabayashi *et al.* 2012). No strong evidence has been presented to suggest it has a role as a transcription factor despite its localisation in the nucleus (Nakabayashi *et al.* 2012). Target genes are also unknown. High levels of *DOG1* transcript and protein levels were also found in seeds which were matured in cooler temperatures and associated with higher levels of dormancy (Nakabayashi *et al.* 2012; Chiang *et al.* 2011). However, protein levels remained high after after-ripening when dormancy has been alleviated. A shift in isolectric point was observed suggesting possible roles of post-translational modifications in regulating dormancy (Nakabayashi *et al.* 2012).

Genes that are similar to the *AtDOG1* gene are present in plants such as *Lepidium sativum, Brassica rapa*, *Oryza sativa* and *Triticum aestivum* (Graeber *et al.* 2010; Rikiishi and

Maekawa 2010; Ashikawa *et al.* 2010). *DOG1* transcripts in *Lepidium sativum* increased when treated with ABA during early imbibition, suggesting an ABA-induction mechanism of the gene, possibly *via* ABI3 and ABI5 activity (Graeber *et al.* 2010). Analysis of the promoter of the *AtDOG1* and *BdDOG1* revealed possible RY repeats (CATGCATG) and ABRE (ABA Response Element) which are required for seed-specific gene expression mediated by ABI3 and ABI5 (Graeber *et al.* 2010; Bentsink *et al.* 2006).

There are four homologs of the *AtDOG1* gene, known as *AtDOG1-LIKE 1-4* (At4g18660, At4g18680, At4g18690, and At4g18650 respectively) with similarity to *AtDOG1* ranging from 23% to 54%, although not all of them have been shown to affect dormancy (Bentsink *et al.* 2006). In *Triticum aestivum*, a total of four *TaDOG1-like* genes have been identified so far, *TaDOG1L1* (AB555729), *TaDOG1L2* (AK332921), *TaDOG1L4* (AK330689) and *TaDOG1L5-1* (AK330559) (Ashikawa *et al.* 2013, 2010; Rikiishi and Maekawa 2010). Together with putative *DOG1-like* genes in *Brachypodium distachyon*, *Hordeum vulgare*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays*, their sequences can be differentiated into five distinct clades in a phylogeny analysis (Ashikawa *et al.* 2013).

Ectopic expression of *TaDOGL1-4* was performed in *Arabidopsis* and the genes were found to increase seed dormancy in *Arabidopsis* seeds (Ashikawa *et al.* 2010, 2013). More recently, transgenic wheat overexpressing *TaDOGL4* was generated and shown to have increased levels of seed dormancy whereas a knockdown of *TaDOGL4* was shown to have seeds with lower dormancy levels (Ashikawa *et al.* 2014). No evidence of dormancy function has been found in *TaDOG1L5-1.*

(ii) *Sdr4* and *qSD7-1* in rice

The QTL for dormancy in rice, *Sdr4,* was originally detected as one of the five major dormancy QTL in the cross between non-dormant *japonica* cultivar Nipponbare and the dormant *indica* cultivar Kasalath (Lin *et al.* 1998). The corresponding gene, *Sdr4,* was identified through map-based cloning and codes for a novel protein with unknown function (Sugimoto *et al.* 2010). Sugimoto et al. (2010) also demonstrated that the seed specific expression of *Sdr4* was restricted to the embryo and it affects expression of rice *DOG1-Like* genes. In addition, expression begins early during seed development and mRNA accumulates towards seed maturation. Analysis of the promoter region revealed RY repeats and ABA-responsive elements, suggesting seed-specific gene expression mediated by ABI3/VP1 and ABI5. In the same paper, the authors also showed that rice cultivars containing the *Sdr4* QTL have higher expression of *OsDOG1L1*. Hence, it is likely that the mechanism of dormancy conferred by QTL *Sdr4 i*s linked to the regulation of expression of the DOG1 protein.

In another experiment, map-based cloning identified *Rc* (see 1.6c) as the corresponding gene for dormancy QTL *qSD7-1* previously identified in weedy red rice and introgressed into cultivated rice for cloning and characterisation (Gu *et al.* 2005; Gu *et al.* 2011). *Rc* is not an ortholog of the wheat R gene and is pleiotropic affecting both grain colour and dormancy, the latter possibly by regulating ABA biosynthesis in the seed (regulation of *ZEP* and *NCED1*) (Gu *et al.* 2011). However, the understanding of its mechanism is still incomplete.

Thesis aims

In a field trial experiment in 2009, a hot spell with daily maximum temperatures in excess of 40°C lasted for more than a week. The occurrence of the hot spell coincided with the mid-point between anthesis and physiological maturity of an intermediate dormant wheat genotype (QT7475). As a result, it behaved in a non-dormant manner comparable to non-dormant genotypes (Mares unpublished data). This observation led to the suspicion that a high temperature shock during a critical stage during seed maturation might be affecting seed dormancy and increasing the risk of PHS. This however required further validation.

Observations of the influence of environmental effects on dormancy are common. ABA biosynthesis and signalling in the seed have been shown to be intensively regulated by environmental effects, such as temperature and drought in many plant species including wheat (Biddulph *et al.* 2007). ABA concentration and its signalling processes appear crucial for the establishment and maintenance of dormancy, whilst the concentration of ABA and the sensitivity of the embryo towards ABA have been shown to be regulated in studies in both *Arabidopsis* and wheat grown at different temperatures.

Since temperature effects on dormancy has been shown to involve regulation of dormancy-specific gene expression, such as the *DOG1 gene*, it is a good candidate to monitor expression in the seeds following changes in environment.

The aims of this project were to:

1) Investigate the effect of high temperature shock at various stages of grain development on dormancy of wheat grains,
2) Investigate the changes of ABA concentrations and sensitivity in response to high temperature shock,
3) Determine whether the dormancy effect is general or genotype-specific

4) Analyse the dormancy effect in genotypes with various QTL combinations towards temperature shock,
5) Investigate molecular changes of *TaDOGL1*, *TaDOGL2* and *TaDOGL4* genes following temperature shock,
6) Analyse the promoter and protein motifs of these genes and their putative chromosomal locations.

Materials and methods

Plant materials

Three bread wheat (*Triticum aestivum* L.) genotypes, SUN325B, B14 and Gladius (Table 1), selected to represent the observed range of genetic variation in white-grained wheat dormancy, were used for a series of experiments designed to determine the effect of a high temperature shock during grain development on the level of dormancy retained at harvest-ripeness. These genotypes were grown in a glasshouse or in a field environment in 2011, 2012 and 2013 in 5 separate experiments (parts 1 - 5). A larger set of related genotypes containing different dormancy QTL or combinations of QTL (Table 1), were used in a final experiment in 2013 (part 6).

Description of genotypes

SUN325B is a dormant genotype derived from Hartog/Vasco//AUS1408/3/Hartog, where Hartog and Vasco are non-dormant Australian white-grained wheat cultivars, and AUS1408 is a South African landrace with a dormant phenotype (Mares *et al.* 2005). One or more unidentified QTL appear to be present in the genotype in addition to the previously identified QTL on chromosomes 4A and 3B (Mares *et al.* 2009). B14, derived from a double haploid population SUN325B/QT7475 that was generated using the wheat x maize method (Kammholz *et al.* 2001), has intermediate levels of dormancy and QTL similar to QT7475. QT7475 is an intermediate dormant genotype derived from AUS1408/3/Janz//Cunningham, where Janz and Cunningham are non-dormant Australian white-grained wheat cultivars. QT7475 inherited the same QTL on 4A from AUS1408, but lacks the QTL on 3B (Mares *et al.* 2009) but possibly contains one or more unidentified minor QTL. An initial attempt to use Hartog, which appears in the pedigree of SUN325B, as the non-dormant control variety failed due to the extreme sensitivity of this variety

to high temperature and subsequently a less heat-sensitive variety, Gladius, a non-dormant Australian white-grained wheat cultivar was used.

A total of 23 additional wheat genotypes (SUN325B, 50213/Cunningham#799, P07.683, Yitpi, DM10.59.59, DM10.59.50, DM10.59.85, DM10.59.81, DM10.73.31, DM10.73.16, DM10.73.51, DM10.73.58, DM02.25.84, DM02.25.74, DM02.25.73, DM02.25.69, DM02.25.2, DM02.25.134, DM02.25.60, DM02.25.61. DM02.25.13, DM02.25.45, DM10.64.32) was selected to determine the effects of temperature shock on genotypes with different dormancy QTL (Table 1). 50213/Cunningham#799 is a doubled haploid line derived from SW95-50213/Cunningham where SW95-50213 is a white-grained, dormant landrace from China with dormancy similar to AUS1408 (Mares et al. 2005) and SUN325B. P07-683 is a doubled haploid line derived from DM6537*B8//SW95-50213/Cunningham#799, where DM5637*B8 is a dormant doubled haploid line derived from Sunco/SUN325B, with dormancy that exceeds that of SUN325B and SW95-50213. DM10.59 and DM10.64 lines are selections from Annuello//SW95-50213/Cunningham.A28 and Annuello//SW95-50213/Cunningham.B28 respectively where A28 and B28 are doubled haploid lines from SW95-50213/Cunningham that contain the 4A but not the 3A QTL. DM10.73 lines are selections from Annuello//SW95-50213/Cunningham.C52 where C52 is a doubled haploid line from SW95-50213/Cunningham that contains the 3A but not the 4A QTL of SW95-50213. DM02.25 lines are selections from an Annuello/DM5637*B8 doubled haploid population. Annuello and Yitpi are Australian white-grain, hard cultivars with no dormancy and intermediate dormancy respectively.

Table 1. Wheat genotypes used in temperature shock experiments together with their respective dormancy phenotype and QTL. Possible presence of unidentified QTL is represented with '+ Other(s)' (Mares unpublished data).

Genotype/Cultivar	Dormancy phenotype	Dormancy QTL
SUN325B	Dormant	4A + 3B + Other(s)
B14	Intermediate	4A + Other(s)
Gladius	Non-dormant	None
P07.683	Dormant	4A + 3AS + 3B + Other(s)
50213/Cunningham 799	Dormant	4A + 3AS + Other(s)
DM10.59.81		4A + Other(s)
DM10.59.85		4A + Other(s)
DM10.59.59		Other(s)
DM10.59.50		Other(s)
DM10.64.32		4A + Other(s)
DM10.73.31		3AS + Other(s)
DM10.73.16		3AS + Other(s)
DM10.73.51		Other(s)
DM10.73.58		Other(s)
DM02.25.84		4A + Other(s)
DM02.25.74		4A + Other(s)
DM02.25.45		4A + Other(s)
DM02.25.2		4A + 3B + Other(s)
DM02.25.73		4A + 3B + Other(s)
DM02.25.69		4A + 3B + Other(s)
DM02.25.61		3B + Other(s)
DM02.25.13		3B + Other(s)
DM02.25.134		Other(s)
DM02.25.60		Other(s)
Annuello	Non-dormant	None
Yitpi	Intermediate	4A

Plant culture

Seeds were pre-germinated on moist filter paper in petri dishes at 4°C on the first day and room temperature on two subsequent days. Those showing uniform germination were used in subsequent experiments. This precaution was taken to avoid non-viable seeds and to limit growth rate differences during subsequent growth in the glasshouse. Germinated seeds were then sown in pots (200 mm deep x 200 mm diameter) containing sterilised cocopeat soil mix. Four seedlings were sown 1 cm deep in the soil of each pot and watered daily. Temperature at spike height was recorded every hour. Glasshouse temperatures were set at 25°C/15°C however actual temperatures at spike height varied to some degree depending on ambient temperature and solar radiation. Plants were supplied with soluble/granular Nitrophoska fertiliser at regular intervals and disease and insect pests controlled by a pre-emptive spray regime. Individual spikes were tagged at anthesis with coloured tapes for accurate assessment of days post anthesis (dpa), allowing a maximum tolerance (+/-) of only two days.

Heat shock treatments

Plants were transferred to growth chamber PGC20 at 40°C/20°C day/night cycles (sine wave-like oscillation) (parts 1 - 4 and part 6) or 35°C/15°C (part 5) for up to 5 days before being returned to the glasshouse to complete ripening.

Plants were harvested at 60 dpa when chlorophyll had disappeared from all parts of the plants or at varying stages of after-ripening. Harvest was conducted consistently between 9-11 am to avoid diurnal effects, and grains recovered by gentle hand threshing for subsequent germination test and moisture content analysis.

Germination test

Replicate samples of 50 sound, well-filled grains free from obvious defects were incubated on moist filter paper (Toyo Roshi Kaisha Ltd, Tokyo, JPN) in Petri dishes in the dark at 20°C. Germinated grains were counted at daily intervals for a period of 7 days and expressed as a cumulative percentage germination curve or as a weighted germination index (Walkersimmons 1988). This index gives maximum weight to grains that germinate rapidly and is calculated from the following formula:

Germination Index (GI) = (7 x *N1* + 6 x *N2* + 5 x *N3* + 4 x *N4* + 3 x *N5* + 2 x *N6* + 1 x *N7*)/ total days of test x total grains, where *N1, N2, N3, N4, N5, N6* and *N7* are the number of grains that germinated on day 1, day 2, day 3, day 4, day 5, day 6 and day 7. The maximum index value is 1.0 if all grains germinate by day 1. Lower indices indicate higher grain dormancy or reduced germinability whereas higher indices reflect low grain dormancy. A germinated grain is defined as radical emergence of at least 1 mm in length.

For high temperature treatment analysis, standard deviations were calculated to determine significance.

Grain moisture content and weight calculations

Grain moisture content was collected at time points 30, 35, 40, 45, 50, 55, 60 and 65 dpa. Two replicates of twenty grains were weighed initially to obtain fresh weight (FW). These grains were then dried at 100°C in an oven for four days. The grains were then taken out and cooled to room temperature. Grains were weighed to obtain dry weight (DW). Grain moisture content, expressed as percent fresh weight, was calculated using the following formula:Moisture content (%) = [(FW–DW)/FW]*100.

Determination of ABA sensitivity

Twenty proximal half-grains were incubated, cut surface down, on filter paper moistened with 25 µM ABA (Sigma-Aldrich, Sydney, Australia) for a total of 7 days at 20°C. Twenty proximal half-grains incubated on filter paper moistened with water were used as a control. Germination index was calculated according to the formula described previously.

Temperature shock experiments

Experiment 1 - Control experiment without heat shock (glasshouse)

A total of a hundred and twenty pots, forty pots for each genotype, SUN325B, B14 and Gladius, were used. Spikes from three pots were harvested at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90 or 100 dpa for each genotype. Glasshouse temperatures covering the period from anthesis to ripeness are shown in Figure 1.

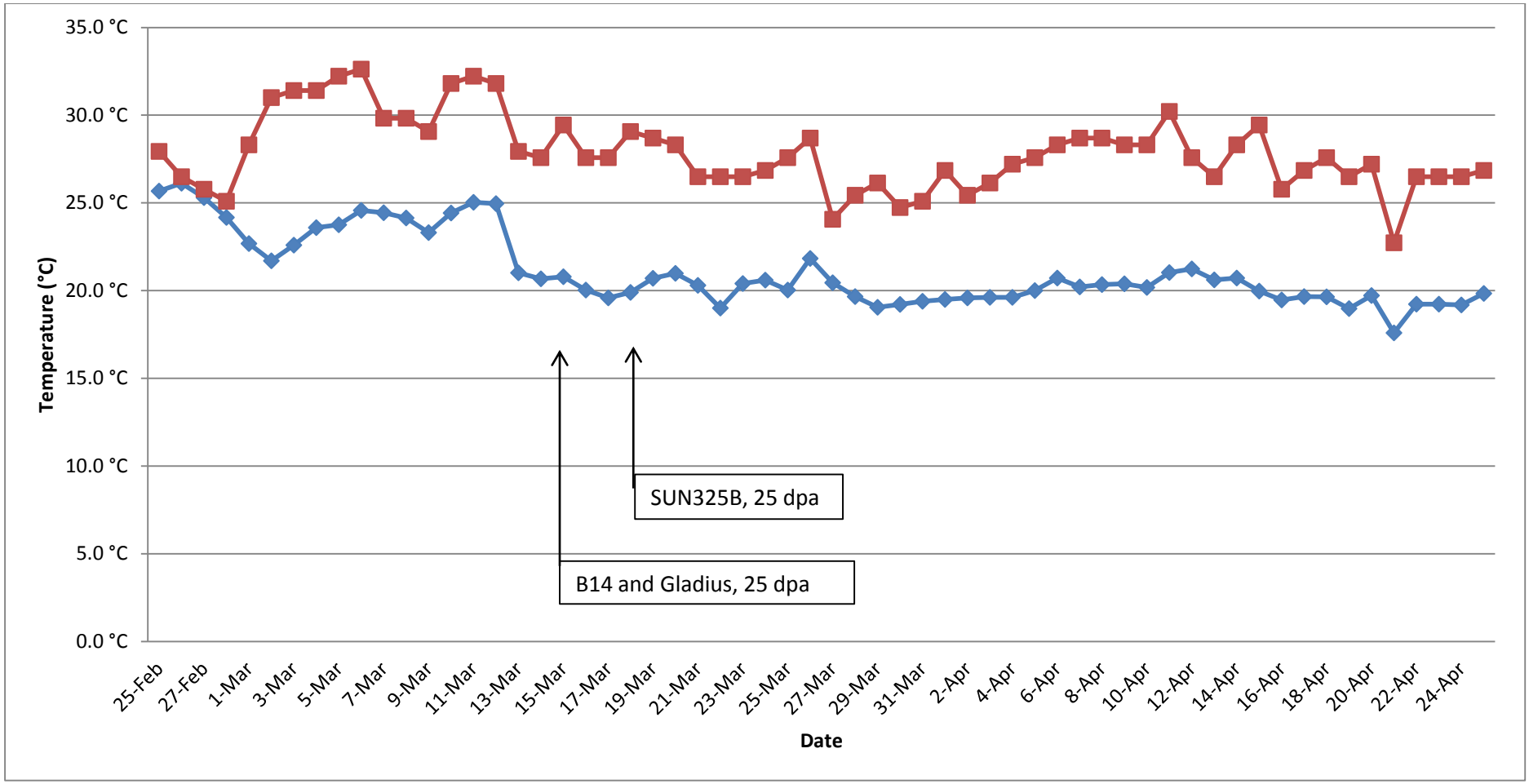


Figure 1. Glasshouse daily mean (blue) and maximum (red) temperatures between anthesis and ripening of SUN325B, B14 and Gladius for experiment 1.

Experiment 2 - Effect of a high temperature shock at different stages after anthesis

Thirty pots each of SUN325B, B14 and Gladius, were grown in the glasshouse. Glasshouse temperatures are shown in Figure 2.

Plants were subjected to a temperature shock of 40°C/20°C day/night (sine wave-like oscillation) at the specific stages after anthesis shown in Figure 3. The temperature shock was applied at 25, 30, 35, 40, 45, or 50 dpa, in each case the treatment lasted for five days. The growth chamber was set to a 14 hour/10 hour day/night cycle and plants were provided with sufficient water daily to prevent wilting due to high temperature shock. Plants were transferred to the growth chamber between 9-11 am and returned to the glasshouse at 9-11 am five days later. Spikes were harvested at 30, 35, 40, 45, 50, 55 and 60 dpa, and the grains removed for moisture content analysis. At 60 dpa, spikes from all treatments were harvested for germination tests. Germination tests and moisture content analysis were conducted as previously described.

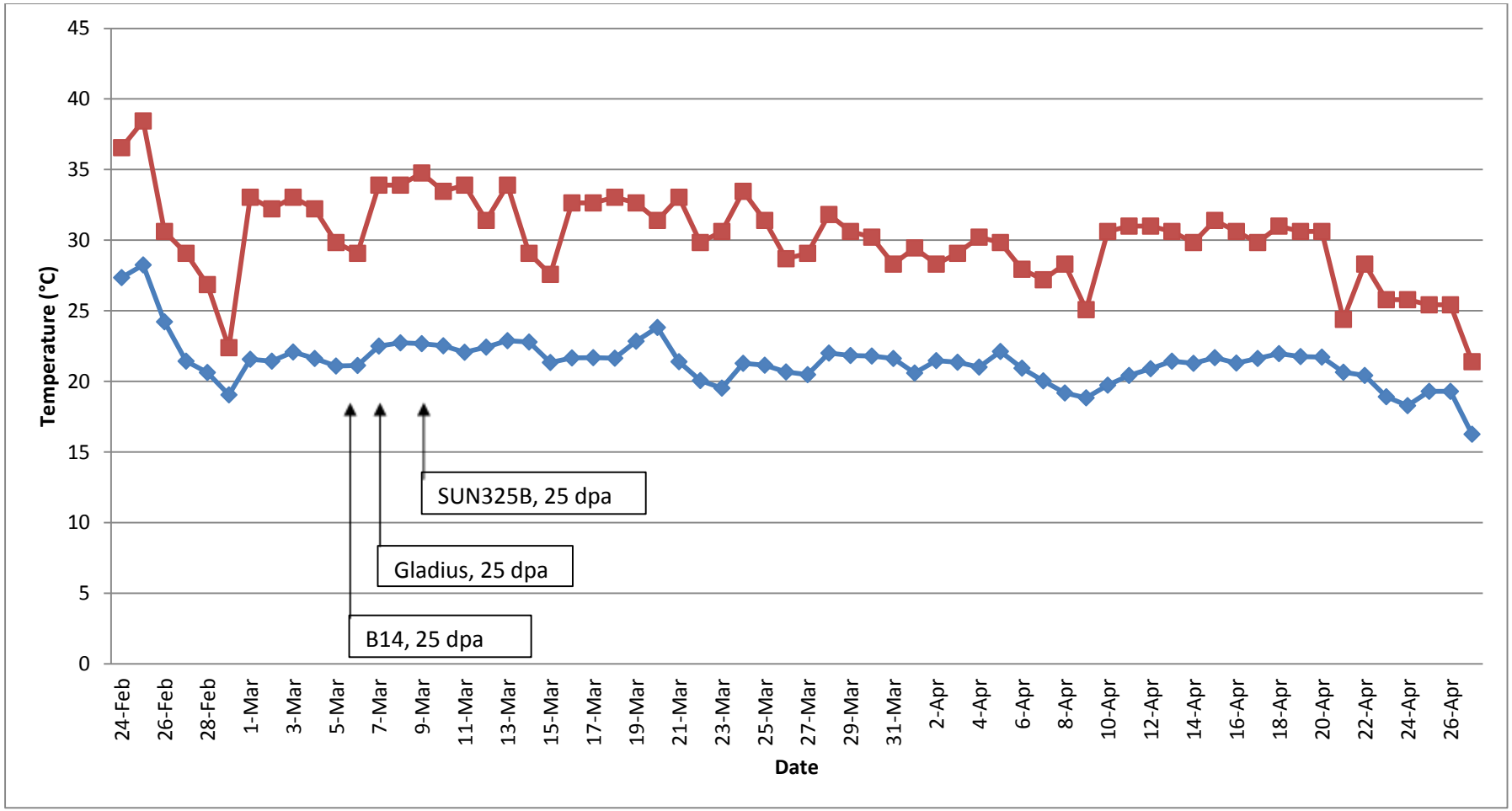


Figure 2. Glasshouse daily mean (blue) and maximum (red) temperature between anthesis and ripening of SUN325B, B14 and Gladius for experiment 2.

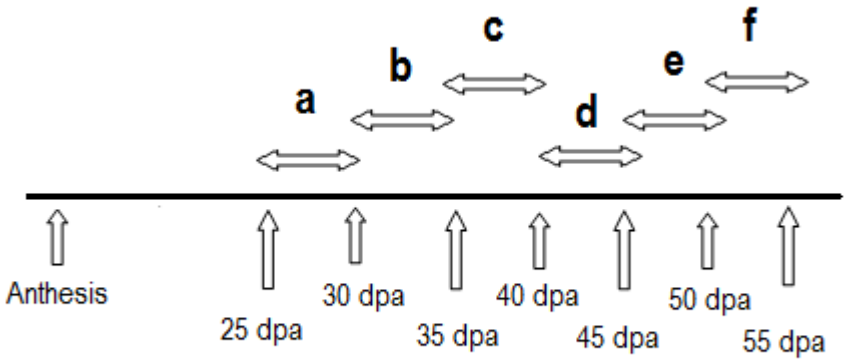


Figure 3. Application of temperature shock of 40°C/20°C day/night at (a) 25-30 dpa, (b) 30-35 dpa, (c) 35-40 dpa, (d) 40-45 dpa, (e) 45-50 dpa or (f) 50-55 dpa to genotypes, SUN325B, B14 and Gladius.

Experiment 3 - Effect of variation in timing and duration of a high temperature shock

Wheat genotype SUN325B was selected for a more detailed examination of the timing and duration of the temperature shock. Glasshouse temperatures are shown in Figure 4. A hundred and thirty pots were divided into fifteen temperature regimes and a control. Plants were subjected to a temperature shock of 40°C/20°C day/night for 2, 3, 4 or 5 days) at 25, 30 or 35 dpa. The growth chamber was set to a 14 hour/10 hour day/night cycle and plants were provided with sufficient water to prevent wilting due to the high temperature shock. Plants were transferred to the growth chamber between 9-11 am and returned to the glasshouse at 9-11 am after treatment. Spikes were harvested at 50, 55, 60, 65 and 70 dpa and grains were removed for subsequent germination index and grain moisture and weight measurements.

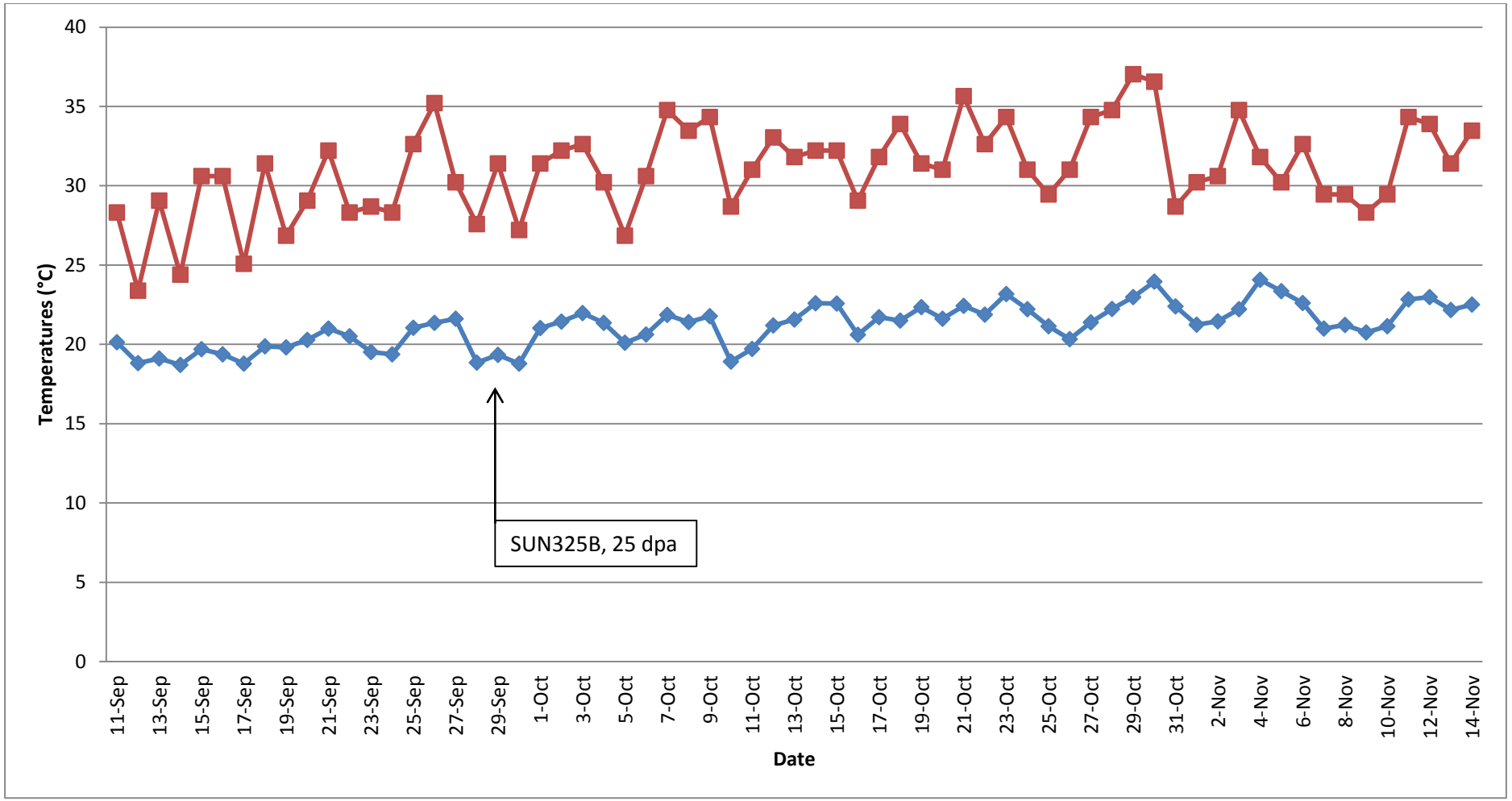


Figure 4. Glasshouse daily mean (blue) and maximum (red) temperature between anthesis and ripening of SUN325B for experiment 3.

Experiment 4 - Effect of a heat shock on plants grown in a field environment and the impact on grain abscisic acid (ABA) content

Wheat genotype SUN325B was grown in pots in the field, arranged on woven polypropylene weed matting to prevent weed growing in the surroundings of the pots. Temperature was monitored hourly and mean temperature, calculated (Figure 5). Plants were watered daily until being transferred to the growth chamber for a temperature shock. Plants from the field were subjected to a temperature shock of 40°C/20°C day/night cycles at 25, 30 or 35 dpa for five days in a growth chamber was set to 14 hour/10 hour day/night and plants provided with sufficient water to prevent wilting due to the high temperature shock. Plants were transferred to the growth chamber between 9-11 am and returned to the field at 9-11 am after treatment. Spikes were harvested at 50, 55, 60, 65, 70, 77, 84, 91 and 98 dpa. Grains were removed for subsequent germination index, ABA sensitivity test and grain moisture and weight measurements according to previously mentioned methods.

Hormone ABA Quantification

Spikes were also harvested at 25, 30, 35, 40, 45, 50 and 55 dpa for ABA quantification. Ten seeds from each sample with three biological replicates were collected and dissected on ice into embryo and embryo-less grains. The samples were separately wrapped in aluminium foil, frozen in liquid nitrogen and stored in a -80°C freezer. ABA was determined using a Phytodek® ABA Competitive ELISA test kit (Agdia, Indiana, USA). Tissue samples were homogenised in 80% HPLC grade methanol in plastic tubes with two ball bearings using FastPrep® Fp120 cell disruptor (Thermo Electro Corporation, USA). The homogenate was mixed overnight at 4°C and centrifuged at 3000 rpm to pellet plant debris. The supernatant was collected and the process repeated twice. Supernatants were combined and concentrated in a SpeedyVac vacuum concentrator to about 50 µL. 1 mL of Tris-buffered saline (25nm Trizma base, 100mM sodium

chloride, 1mM magnesium chloride, 3mM sodium azide, pH7.5) were added to dilute the extract. Standards provided in the test kit were run alongside samples in duplicates. A hormone standard strip was inserted into a microcentrifuge, 1 ml of 1X Tris-buffered saline added, incubated for five minutes and vortexed for 30 seconds. The 1000 picomoles/ml (1nM) ABA solution was then serially diluted (1:10 once and 1:5 five times) to ABA concentrations of 100, 20, 4, 0.8, 0.16 and 0.032 nM. 100 µL of standards and samples were added into testwells of the ELISA plate. ABA-tracer provided in the kit was mixed with 5 ml of 1X Tris-buffered saline and 100 µL of the diluted tracer was added into each test well. Test wells were mixed by gentle swirling and kept in a humid box in a refrigerator at 4°C for 3 hours. The box was then transferred to a 37°C incubator for 3 hours. Contents of the test wells were then expelled and 1X phosphate buffered saline with Tween®-20 wash buffer added for further washing twice. A *p*-nitrophenyl phosphate substrate tablet was dissolved in 5 mL of substrate diluent containing 0.02% of sodium azide. 200 µL of the substrate solution was added to each well, placed in the same humid box and incubate at 37°C in the incubator for 60 minutes. Absorbance value was then read at 405 nm. Well with 100% binding (B_0) was greater than 0.750 optical density (O.D.). % binding was calculated using the following formula:

% binding = (Standard or sample O.D. – 0% binding O.D.) / (B_0 O.D. – 0% binding O.D.) x 100%

Natural log values for each standard concentration were calculated and plotted on the x axis. Logit values were plotted on y-axis. Logit equation for standard and sample % binding values:

Logit = Ln[%binding/(100-(%binding))]

Sample concentration was calculated using the following formula:

[Sample concentration]=$e^{(\text{logit-(y-intercept)})/\text{slope}}$

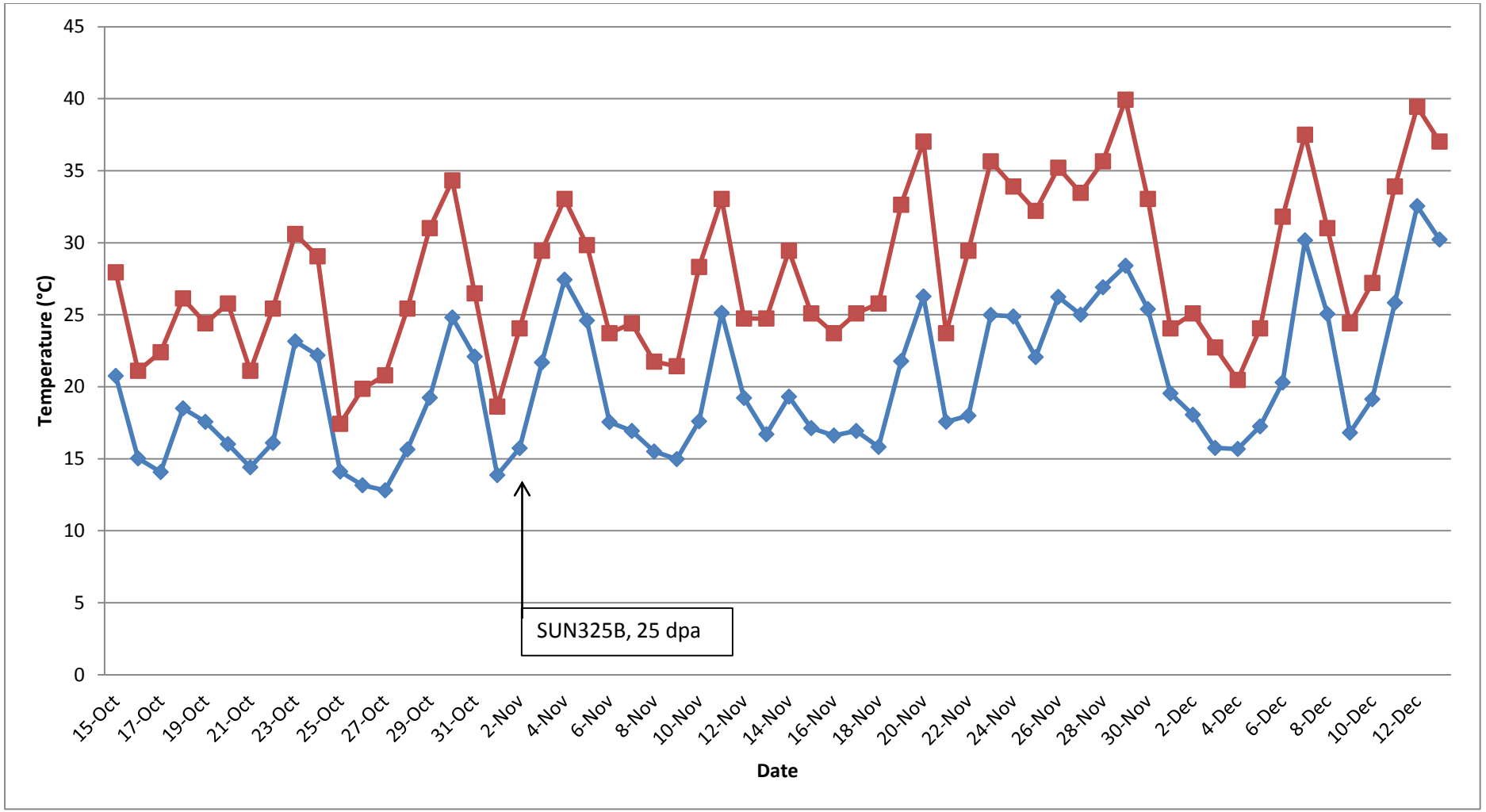


Figure 5. Field daily mean (blue) and maximum (red) temperature between anthesis and maturity of SUN325B for experiment 4.

Experiment 5 - Effect of reduction of high temperatures by 5˚C

Sixty pots of SUN325B were planted and subjected to the same glasshouse conditions and care. Plants were transferred to a growth chamber to be subjected to a temperature shock of 35°C/15°C day/night cycle (sine wave-like oscillation) at 25, 30 or 35 dpa for five days. Growth chamber was set to 14 hours/10 hours day/night cycles and plants were provided with sufficient water to prevent wilting due to high temperature shock. Plants were transferred to the growth chamber between 9-11 am and returned to the glasshouse at 9-11 am five days later until 65 dpa. Spikes were harvested at 50, 55, 60, 65, 70, 75, 80, 90 and 100 dpa and grains recovered for subsequent germination index and ABA sensitivity tests.

Experiment 6 - Effect of a heat shock on dormancy in genotypes with different combinations of dormancy QTL.

Seeds of 23 genotypes with different dormancy QTL and varying degrees of dormancy (see additional genotypes listed under 'Description of genotypes') were pre-germinated and planted in a total of a hundred and fifty pots in the glasshouse as in experiment 1. Spikes were tagged at anthesis for accurate assessment of days post anthesis (dpa), allowing a maximum tolerance (+/-) of only two days. Samples were harvested and tested for their germination rate at 45 dpa and 65 dpa following a 5 day temperature shock (40°C day/20°C) at 25 dpa.

Bioinformatics Analysis of *Delay of Germination 1*

Retrieval of *Arabidopsis Delay Of Germination 1* (*AtDOG1*) orthologous gene sequences

Arabidopsis thaliana Delay Of Germination 1 gene (*AtDOG1* or *At5g45830*) was used as a query sequence for a blast search against *Triticum* nucleotide sequences and expressed sequence tags (ESTs), in publicly available databases for sequences with a high degree of similarity to the *AtDOG1* gene. Blast searches (tBLASTx) were done against the NCBI (*National Center for Biotechnology Information*) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.* 1997), TriFLDB (*Triticeae Full-Length CDS Database*) (Mochida *et al.* 2009), Cereals DB 2.0 (Wilkinson *et al.* 2012) and the IPK CR-EST (*Crop EST*) Database v1.5. Similar sequences were selected with low stringency (E-value of <0.01) in the first round, but were later filtered with a stringent conserved domain search (see next section). These sequences were further used as queries for a BLAST (tBLASTx) search against other species: *Arabidopsis* thaliana, *Brachypodium distachyon*, *Hordeum vulgare, Oryza sativa* and *Zea mays*.

Delay of Germination1 conserved domain search (CD search)

A conserved domain search was done using the NCBI conserved domain search tool. Using an interface which utilizes RPS-Blast (Reverse Position Specific Blast), translated sequences were scanned against CDD v3.1 – 45736 PSSMs (*Pre-calculated Position-Specific Score Matrices*) database for 500 maximum matches with E value <0.01 and composition based statistics adjustment (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer *et al.* 2013). Additional sequences with the same protein conserved domains were retrieved and compiled from a conserved domain architecture search using the Conserved Domain Architecture Retrieval Tool (cDART) that searches NCBI *entrez* protein database using domain architecture instead of protein similarity (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) (Geer *et al.* 2002).

Multiple sequence alignment and phylogenetic analysis

Sequences with a similarity of >95% although with different accession numbers were eliminated. Multiple sequence alignments were done with Clustal Omega, an online tool which highlights areas of similarities between sequences (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al.* 2011). Sequences were aligned with mBed-like clustering guide-tree and iteration enabled, zero combined iterations, and maximum guide tree and hmm iterations set to default. The alignment result was sent to ClustalW2 Phylogeny for a phylogenetic tree generated using the neighbour-joining method, a distance method commonly used for preliminary phylogenetic analysis, with parameters 'distance correction', 'exclude gaps' and 'percent identity matrix' set to 'off' (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) (Larkin *et al.* 2007). Phylogenetic trees were constructed from the output of ClutalW2 Phylogeny using the Newick tree format of Treedyn 198.3 online phylogenetic tree construction tool (Dereeper *et al.* 2008).

Promoter elements and protein motifs identification

IWGSC (*International Wheat Genome Sequencing Consortium*) database (http://www.wheatgenome.org/) were utilised to obtain promoter sequences for *DOG1* and *DOGL1* sequences. The promoter sequences were then scanned for plant cis-acting regulatory DNA elements using online tool New Place (Higo *et al.* 1999). The results were compared for similarities with previously published ABA-related transcriptional elements identified in *DOG1* sequences in other species such as *Arabidopsis* and *Brachypodium (Graeber et al. 2010)*. The discovery of protein motifs in the DOG1 conserved domain-containing sequences was completed using online tool MEME (*Multiple Em for Motif Elicitation*) hosted in MEME suite web server with parameters: 6 amino acids minimum motif width, 50 amino acids maximum motif width, 15 maximum motifs and a cut-off of minimum occurrence in 10 sites and an E-value of <1 (Bailey *et al.* 2009). All significant motifs were submitted for a MAST (*Motif Alignment and Search Tool*)

search against the NCBI non-redundant protein database in the non-redundant and special databases category (Bailey and Gribskov 1998).

Comparative analysis

Flanking markers and adjacent markers for QTL of wheat dormancy were collected (http://wheat.pw.usda.gov/cgi-bin/cmap). Information regarding their positions in wheat deletion bins was then collected using the wheat bin map (Sourdille *et al.* 2004). Information regarding ESTs previously mapped to the deletion intervals was obtained from Grain Genes wEST-SQL resources (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi). These ESTs were then scanned for orthologs in rice using a tBLASTx search against the NCBI database. Information and sequences of flanking markers and nearest markers to rice dormancy QTL were obtained in GRAMENE Release #39 (http://archive.gramene.org/). The rice sequences were then used to scan genomic DNA sequences for adjacent genes in order to determine the positions of these markers and identify potential candidate genes flanked by, or situated near to the markers. Sequences of flanking and adjacent markers for dormancy QTL in wheat were obtained from Grain Genes 2.0 and searched for longer sequences. Sequences that span genes were obtained and used in a tBLASTx search to obtain rice orthologs. A comparative map was then generated to help identify candidate genes and potential chromosomal locations of *DOG1* orthologs in wheat and rice.

Gene expression

Plant materials

Plant materials were collected from experiment 3. Temperature shock of 40°C/15°C day/night cycles was applied on SUN325B during 25 dpa for a period of five days. Seeds were harvested according to previous experiments at 25, 30, 35, 45 and 65 dpa from both control and temperature shock-treated plants. Embryos were dissected from the grain and placed on dry ice, wrapped separately in aluminium foil and immediately frozen in liquid nitrogen before being stored in a -80°C freezer.

RNA extraction

Two separate RNA extraction methods were performed. Method 1 was used on embryo samples from 25, 30, 35, 45 and 65 dpa and de-embryonated grain samples from 25, 30 and 35 dpa. Method 2 was performed on de-embryonated grains from 45 and 65 dpa to avoid low RNA quality due to high starch content.

Method 1

Total cellular disruption of samples of (≈100mg) embryo and de-embryonated grains were performed using autoclaved mortars and pestles in 1 mL of RiboZol™. Homogenised samples were incubated in room temperature for ten minutes. 200 µL of chloroform was added and shaken vigorously for 15 seconds, then incubated at room temperature for three minutes. Samples were then centrifuged at 12000 x g for 15 minutes. 80% of RNA (upper aqueous phase) was transferred to a new tube for each sample. Re-extraction was performed on the remaining RNA and added to the new tubes. In order to precipitate the RNA, 0.25 mL of isopropanol and 0.25 mL pf 0.8 M sodium citrate/1.2 M NaCl were added and incubated for ten minutes at room temperature, followed by centrifugation at 12000 x g for eight minutes. The supernatant was

removed in each tubes and the remaining pellet washed with of 75% ethanol twice (each with 1 mL of ethanol, vortexed and centrifuged at 7500 x g for five minutes). Following the second wash, ethanol was removed and the pellet was air-dried. The pellet was then dissolved in 40 µL of water. RNA quality was determined to be between 1.6 and 1.8 (ratio of absorbance at $A_{260/}$ A_{280}).

Method 2

RNA was extracted from the embryo and de-embryonated seed samples using a modified silica-capture-based extraction procedure (Foissac *et al.* 2005). 0.1 g of samples were homogenized with 1.5 ml of lysis buffer (0.2 M Na Acetate pH 5.0, 25 mM Ethylenediaminetetraacetic acid [EDTA], 4 M Guanidine Hydrochloride, 2.5% Polyvinylpyrrolidone MW 40.000 or PVP-40) in a heavy-duty 150 µm thick plastic bag charged with 20mg Na-meta-bi-sulphite and PVP-40. 0.07 ml of 20% (weight/volume) sarkosyl (N-Lauroyl-Sarcosine) was added to the homogenate and incubated for ten minutes in 70°C. 0.4 ml chloroform/isoamyl alcohol (24:1 volume/volume) was added and mixed vigorously for thirty seconds, followed by a centrifugation step of fifteen minutes. 400 µL of upper phase supernatant was mixed with 25 µL Glass Milk mix and 380µL ethanol, followed by a centrifugation step of 8000 x g for 1 minute, a resuspension in 600µL Wash 1 (4 M Guanidine Hydrochloride, 0.2 M Na Acetate pH 5.0, 25 mM EDTA) and another centrifugation step of 8000 x g for 30 seconds. Pellet was washed twice with Wash 2 (Absolute ethanol:[10 mM Tris, pH 8.0, 1 mM EDTA and 100 mM NaCl], 8:2 ratio) with the same centrifugation steps with an additional 13000 x g centrifugation step for two minutes to remove traces of ethanol. Glass milk was dried at 65°C for thirty seconds. 70 µL of Tris buffer pH 8.5 was added, mixed, incubated at 65°C for one minute, remixed and centrifuged at 13000 x g for thirty minutes. RNA quality was determined using the same method.

cDNA synthesis

Synthesis of cDNA was performed using a cDNA synthesis kit (Bioline Pty. Ltd, New South Wales, Australia). First strand cDNA was synthesised from extracted RNA. A 10 µL solution consisting of RNA, 50 µM oligo $(dT)_{18}$, 10 mM deoxynucleotide triphosphate (dNTP) and sterile DECP-treated milli-Q water was incubated at 65°C for 5 minutes and placed on ice. 10 µL of solution (consisting of 4 µL of 5x first strand buffer, 1 µL of 10 u/µL RNase inhibitor, 0.25 µL of 200 u/µL reverse transcriptase and 4.75 µL of water) was added into the first solution, followed by incubation at 50°C for one hour.

Primer design

Primers were designed based on EST sequences (*AK330689, AB555729* and *AK332921*) using NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Parameters for primers were set at, <180 bp, 10 primers to return, melting temperatures (Tm) of 57-60°C with a maximum difference of 3°C between the Tm of the two primers, "primers must span exon-exon junction" selected and primer pair specificity checking parameters left at default. Primer sequences and corresponding product sizes are listed in Table 2 (also see Figure 6a, b and c).

Quantitative RT-PCR (qRT-PCR)

Three biological replicates of SUN325B proximal-half and distal-half grains were used for each time point and treatment. Actin and ADP-ribosylation factor Ta2291 were selected as a reference genes (Paolacci *et al.* 2009). qRT-PCR was performed using SensiMix™ SYBR Kit (Bioline Pty Ltd, New South Wales, Australia) and C1000 Touch™ thermal cycler (Bio-rad Laoratories Pty Ltd, New South Wales, Australia). A final volume of 25 µL was made up for each sample consisting of 12.5 µL of 2x SensiMix™ SYBR® (hot-start *Taq* polymerase, SYBR® Green 1 Dye, dNTP, stabilisers and enhancers), 2 x 0.25 µL of 25 µM for forward and reverse

primers, 2.5 µL of template (100 ng cDNA/reaction) and 9.5 µL of water. Thermal cycling conditions were set at 1 cycle of 95°C for ten minutes, followed by 40 cycles of 95°C for fifteen seconds, 60°C for fifteen seconds and 72°C for fifteen seconds. Only one peak was recorded for each amplification in the melting curve analysis. Quantitative PCR data was analysed using the delta-delta Ct method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen 2001).

Table 2: Primers used for quantitative RT-PCR to amplify *DOG1-like* genes in wheat and reference genes *Ta2291* and Actin.

Primer	5'-3' Sequence	Amplicon Size	Tm (°C)
*TaDOG1L1*_F	CGGAAAAGGAGGTGTGGACG	198 bp	60
*TaDOG1L1*_R	AACTTGTGCACGGCGAGGT		
*TaDOG1L2*_F	AGGTGTAGTTGGTTTGACTGGG	124 bp	60
*TaDOG1L2*_R	CCCAGCTACGACGAAACACT		
*TaDOG1L4*_F	GCGACGAGGGTTACCTGAC	185 bp	60
*TaDOG1L4*_R	GAGTCCTCCTGACGATTCTGC		
*Ta2291*_F	GCTCTCCAACAACATTGCCAAC	165 bp	55
*Ta2291*_R	GCTTCTGCCTGTCACATACGC		
Actin_F	GAATCCATGAGACCACCTAC	206 bp	55
Actin_R	AATCCAGACACTGTACTTCC		

GAGAGCCACTTGGATCACCATGACCGCCACCTCGCGGCCACAGCATCCCAACGGGAGCCT 60

AGCCCCGGCCTCCGACGGCGGCGAGTCCTTCGCCAAGTTCTTCGAGTGCTGGATCTTGGA 120

GCAGTCGCGTGACCTGGCCGCGCTCCGCGCCGCGGCCACGGCGCGGCCCGACGACGCTGA 180

CCTCCGGCGCCTCGTCGACCGTGTCCTCGGCCACTACGAGCACTACTACCGCGCCAAGTC 240

CGCGGCCGCCTCCGCCGACGTGCTCCCCATGTTCGCGCCCTCGTGGATCTCCGCCACCGA 300

GAGCCTCTACCTCTGGTGCGGCGGCTGGCGCCCCACCGCCGCGATCCAGCTGCTCTACTC 360

CAAGTCCGGCGTGCAGCTCGAGGCCAAGCTCCCGGCCTTCCTCGACGGCGGCAGCCTCGG 420

GGACGGCGACCTCGGCGGCCTCTCCGCCGAGCAGCTCCAGGCCGCGGACCAGCTGCAGCG 480

CCGCACCATCCGGGGGGAACGGGAGATCGAGGAGGCCGCCGCCGGCGCGCAGGAGTCGTT 540

GGCGACGACGAAGATGGTGGAGCTCGC**CGGAAAAGGAGGTGTGGACG**CGGCGGAGGGGAT 600

Forward

GGAGCGGGAGATGGATGCCAAGGCGGAGGCGATGAAGCGCGTGCTGGAGATGGCGGACGC 660

GCTGAGGCTGGAGACGCTGCGCGGCGTGGTGGGGCTGCTCCGGCCTGCGCAGGCCGTGCA 720

CTTCCTCGTGGCCGCCGCGGAGCTCC**ACCTCGCCGTGCACAAGTT**CGGCCGGCGCAAGGA 780

Reverse

CGGCGCCGCCGCGGCGGAGTGAGCCTGTAGGCTGCTGCGGCGCGCGCGCACGTGGTTGCT 840

CTGCTCCGCGCAACGCTCGTCGTCTAGCTAGCTCCGAGCTGCCTTCGTTTCGCCTGGTCA 900

ACGCGAGGTGTCGCGATAAGCTTTGACCATGATCCCCTTCTGCTTGGTCAACATGTGCGT 960

TCTGCCGCAGTGTCAAATCTGTGGTAAAACTTGTAC 996

Figure 6a. Forward and reverse primers designed to amplify *TaDOG1L1* in an expression analysis.

GACTGTACTGTACTACCCCCGCCTGGCCAGAAAAGGGAGAGGACCGGACGATGGAGCTGG 60

AGGCGGCGACGCGGCGCTTCCAACTCTGGCTCCGCGGGCTGCGGTCGCTGCGCCGCGACC 120

TCCGGACGGCGCGCTGGGCCGACGACCCGGCGCAGCTCGCCAAGCTGGTGGCCGGCTACG 180

TGTCCCACTTCGCCGACTACTGCGCGGCGCGGGCGGAGCTGGACCCGGTGTGGACGCTGG 240

CGGCGCCGTGGGCGAGCCCCGTGGAGCGTGGCGCGGCGCACTGGCTGGCCGGGTGGCGGC 300

CGACCACGCTGGTCCACCTGCTCTACACCGAGTCCGGTCGCCGCTTCGAGGCGCAGCTCC 360

CAGACCTCCTGCTCGGCGTGCGGTCGGGCAACCTCGGCGACCTCAGCCCGGCCCAGCTGG 420

CGCAGATCGACGAGCTGCAGCGCCGCACCGTGGCGCAGGAGGACGAGCTGTCGCGCGAGA 480

TGGCGCGGGTGCAGGAGGGCCACGGCGCGGTGGGCGCCGGCGGGGAGCTGGTGGACGTGG 540

GCGGGCTCGTCGGCCGCGTCGGCGCCGTGCTGGCCGGGGCGGACGCGCTGCGGCTGCGCA 600

CCATGAAGCGCGCCGTGGAGATCCTCGAGCCGGCGCAGGCGGCCGAGCTGCTCGTCGCCG 660

CGGCGGACATGGAGATCGGGTTCCGTGAGTTCGGGCTCAAGTACGACGGCGTCGGCGCCG 720

GCGGCTCGTAACGTCCAGCTAGGTAGATCGGTCACCATCGGCGCTCGGCAGGTCCAGCCC 780

GGTTTTTTTCC**AGGTGTAGTTGGTTTGACTGGG**ACAGGGAGGCTTTCCACTCTTGTAATC 840
Forward

TTGTTGCCGCGACGATTGTCCGCCAGATCGGTAGATCGACGGCTGTCGCCCGGTG**AGTGT** 900

TTCGTCGTAGCTGGGCCGTGTCAGGGCGTGCTGTTGACCGAGAGGCAAGTAATAAAATGC 960
Reverse

AGAGACACACTCTGCATGCTTTTTTGGAAAAAAAAAAAAAAAACGA 1006

Figure 6b. Forward and reverse primers designed to amplify *TaDOG1L2* in an expression analysis.

GGCTTACTCACAAGTCAGTCCCGTCACTCCCTGTCGCGCGCGAGAGACCAGATCGCCATG 60

GACATGGCACGCTACGTCGCGTTCCACCAGCAGTGGATCGCGGGCCAGCAGGCGGGCCTC 120

CGCGAGCTCGCGGAGGCGGCAGCCAATGCCGCCGCCGGGCGCGCCACGGACGCAGAGCTG 180

AGGGCCGTGGTTGAAAGGTGCATGCGCGGGTACCAGGAGTACGCCGCCAGCCGGCGTGCC 240

TTGGCGCGCGAGAACGGCGCGGCCTTCGTCGCCCCGCCCTGGTGCACGGCGTTTGAGAAC 300

TCCGTGCTCTGGCTCGGGGGCTGCCGCCCGTCTCTGGCCATCCGGCTTCTCTACTCAATC 360

TCCGGCGAGGGCTTGGAGGAGGACATCGAGGAGTTCGTCAGAGGCCGCGGGCGCGGCCTC 420

GCGGAGGGTATGGGCCTCGTCGGGATCACGGCCACGCAGCTGCAGCAGATCAACGACCTC 480

CACCGCTGCACGCTGC**GCGACGAGGGTTACCTGAC**GGAGCGTCTGGCGAGTCTGCAGGAG 540
Forward

AACATCGCCGACCGGCCGCTGCTTCCGATCGTTCGGGAGCGCGCCGCGGCGGCGGCAGCA 600

GCATTAGCCGGCCAGGACCGGAGCGCTAAGCGCGACGACATTCCCGGACGGCCCGTGGCA 660

GCAGAATCGTCAGGAGGACTCGCCGCCGAGGTGGACGCGGCGATGGAGAGCTACTCGGCC 720
Reverse

GGGCTGGCCAGGCTCCTGGAGGAGGCGGACGAGCTGCGCATGTCGACGGCCAGGGCGCTG 780

GCCACGGAGATCCTGACGCCGCGGCAGGCGGTGGAGATGCTGATGGCAGCCAAGCAGCTG 840

CACCTGGCGGTGCGCGACTGGAGCCGCCGGAAGGAGGAGGGCGCCCAGAACGCGCGTCTG 900

CCGCTCGCAGCCGCAGCGACGACCGCCCCCTCGGGCTCAAAACCATGACAGGCTCGCCAT 960

TCCTCAACTAGCTTGCTTTGCCGCCAACTTAAGTGTACAGAGTCTGAATAATCTGTTTAT 1020

TCTGGATTTCTAGATATTAATATCGTTGTTTCTCTGCCAAAAAAAAAAAAAAAACGA 1077

Figure 6c. Forward and reverse primers designed to amplify *TaDOG1L4* in an expression analysis.

4. Results

4.1 Temperature shock experiment

Experiment part 1

Germination index of proximal-half grain and whole grain of three wheat genotypes

Three wheat genotypes, Gladius, B14, SUN325B were grown in a glasshouse and samples harvested at 5 day intervals from 30 dpa until 80 dpa and then at 90 and 110 dpa. Average temperature and relative humidity during grain maturation were 20°C and 67.6% respectively. Gladius, a non-dormant genotype, exhibited the lowest degree of dormancy, early release from dormancy, as reflected by an earlier increase in germination index during grain maturation. By comparison, B14 (intermediate dormant) and SUN325B (dormant) exhibited higher levels of dormancy with release of dormancy delayed until approximately 75 dpa and 90 dpa, respectively (Figure 7). Compared to Gladius, B14 took at least 25 days longer to reach 50% germination whereas SUN325B took 35 days longer.

When grains were cut in half and the proximal halves incubated on moist filter paper, differentiation based on level of dormancy was still clearly apparent. The timing for release of dormancy was in the order, Gladius, B14 and SUN325B (Figure 8). The removal of distal half of the grain apparently did not alleviate dormancy completely. The difference between Gladius and SUN325B in time to 50% germination was approximately 10 days for proximal half grains compared with around 55 days for intact grains. However when the proximal-half grains of the three genotypes were incubated on 25 μM ABA, release from dormancy was prolonged (Figure 9a, b and c).

Contrary to expectation and previous experience the release from dormancy for half grains of Gladius in the presence of ABA was delayed compared with intact grains on water, reaching a GI of 0.5 approximately 20 days later (Figure 7 vs 9a). Nevertheless release from dormancy was still substantially earlier than that of B14 or SUN325B.

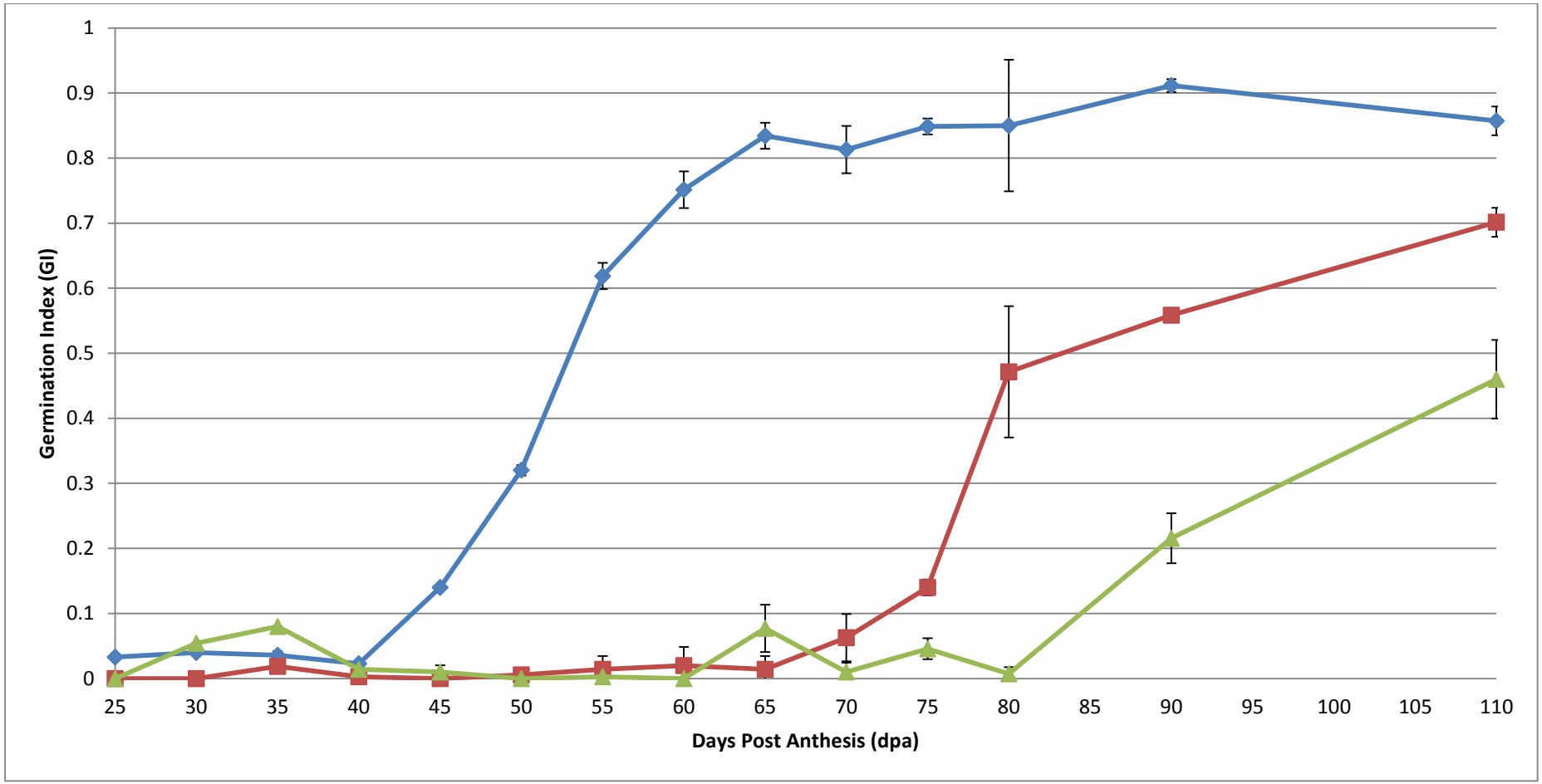


Figure 7. Changes in germination index of intact grains of three wheat genotypes, Gladius (blue), B14 (red) and SUN325B (green) between 25 to 110 dpa. All genotypes reached physiological maturity at 55 dpa (13% moisture content). Error bars represent standard deviation.

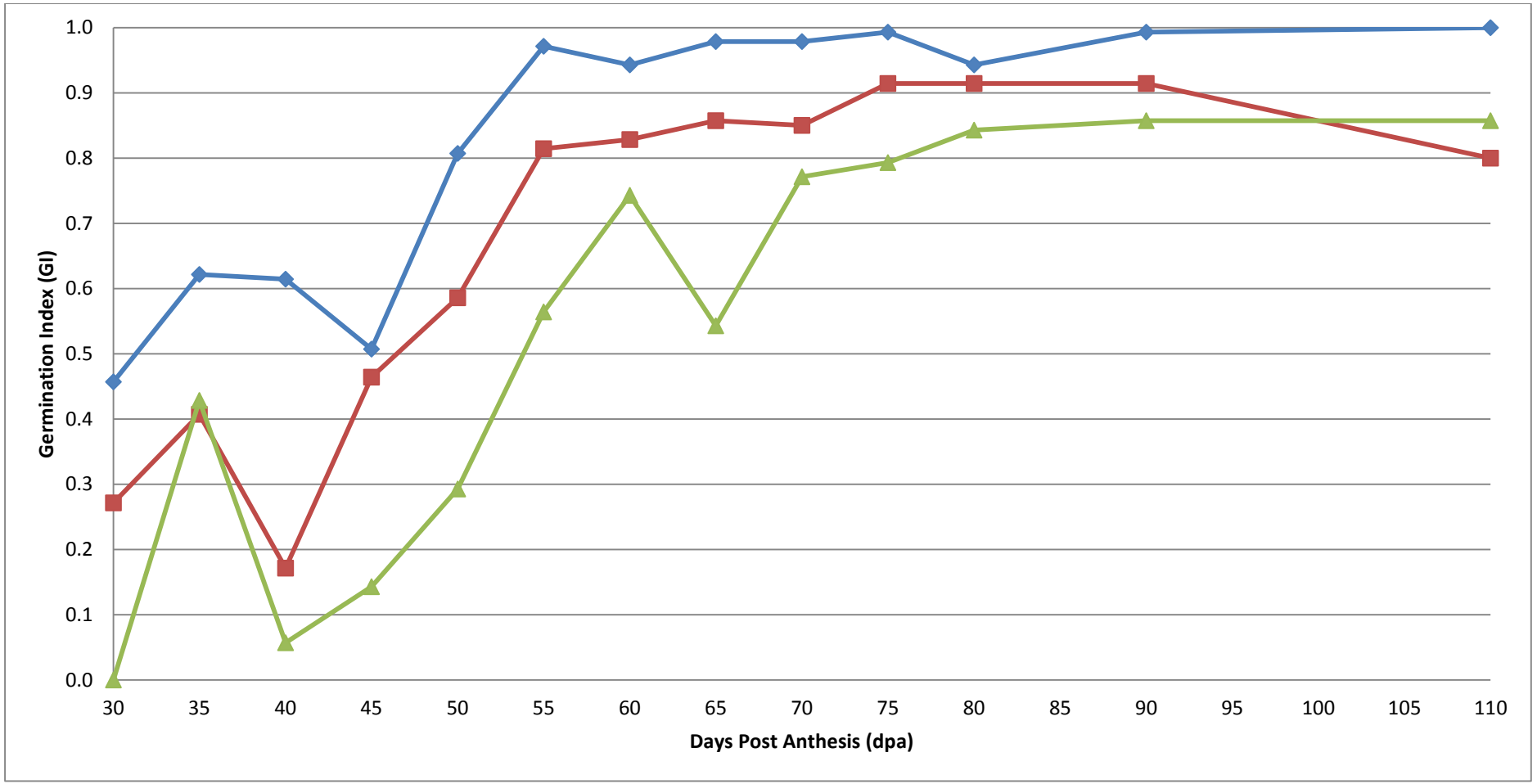


Figure 8. Changes in germination index of proximal-half grains of three wheat genotypes, Gladius (blue), B14 (red) and SUN325B (green) between 30 to 110 dpa.

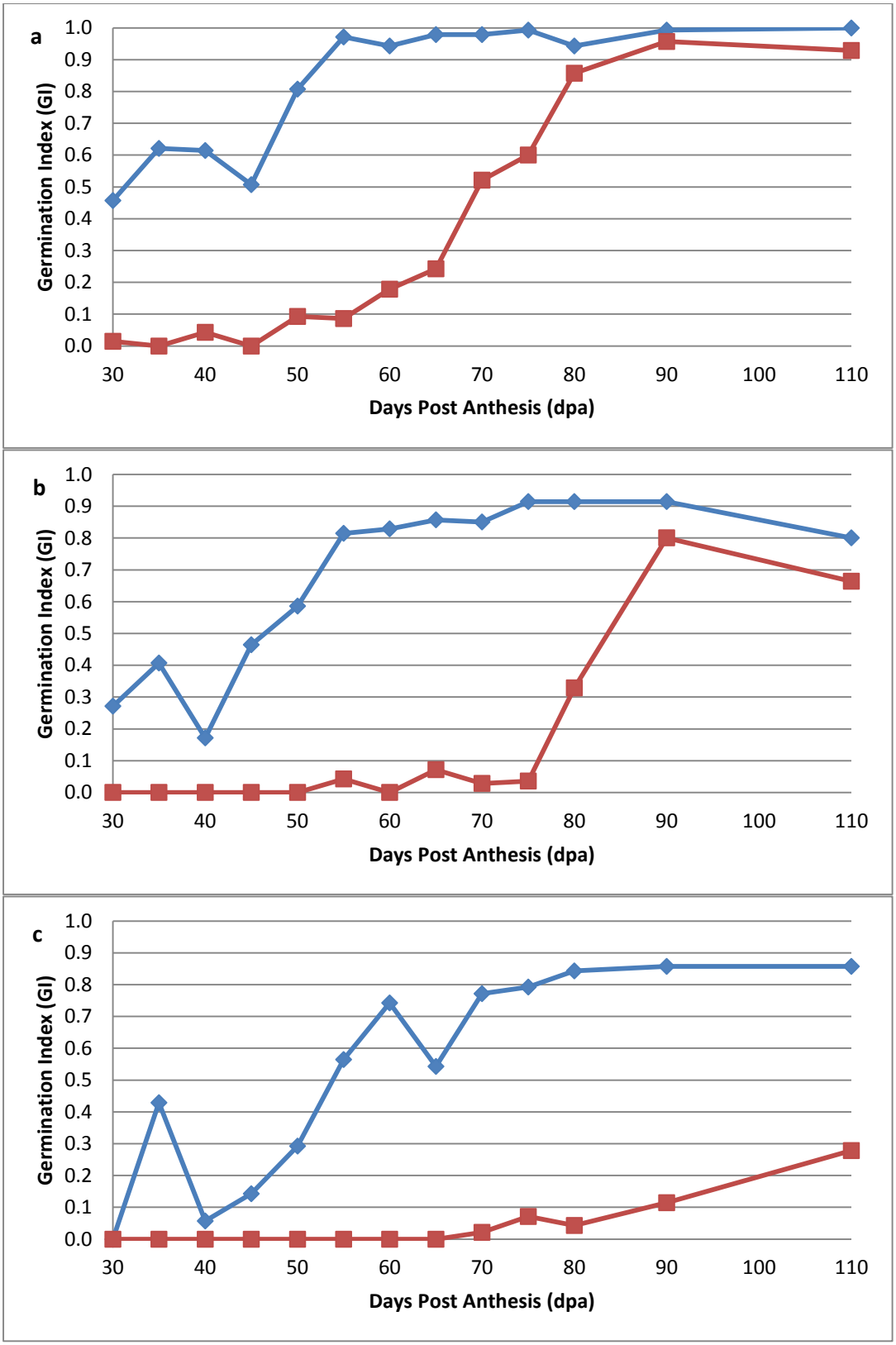


Figure 9. Changes in germination index of proximal-half grains incubated in water (blue) and 25 µM ABA (red) between 30 dpa and 110 dpa for wheat genotypes (a) Gladius, (b) B14 and (c) SUN325B.

Experiment part 2

Temperature shock application at different stages between 25 to 55 dpa

Plants of the three wheat genotypes, Gladius, B14 and SUN325B were subjected to a temperature shock of 40°C day/20°C night for a period of 5 days at 25, 30, 35, 40, 45, 50 or 55 dpa before returning the plants to the glasshouse to complete ripening. Seeds were harvested at 60 dpa for all plants and tested for germination index (GI). Figure 10 showed dormancy levels of Gladius (non-dormant genotype) remained low throughout for all control and treated samples, as reflected by the GI values in the range 0.80 and 0.92. Dormancy levels of B14 and SUN325B (intermediate dormant and dormant genotype, respectively) were shown to be highly responsive towards a temperature shock at 25 dpa and to a lesser extent, at 30 dpa. A temperature shock at 25 dpa or 30 dpa increased the GI of B14 at ripeness by at least 13-fold (0.81:0.06) and approximately 3-fold (0.18:0.06), respectively. Other temperature shock treatments at time between 35 dpa or 55 dpa did not affect dormancy levels of B14 at harvest ripeness. Similarly, temperature shock to SUN325B at 25 dpa and 30 dpa significantly reduced dormancy at harvest-ripeness with GI values of 0.58 and 0.17 respectively in comparison to controls that had zero germination. Again, shocks at later times were largely ineffective.

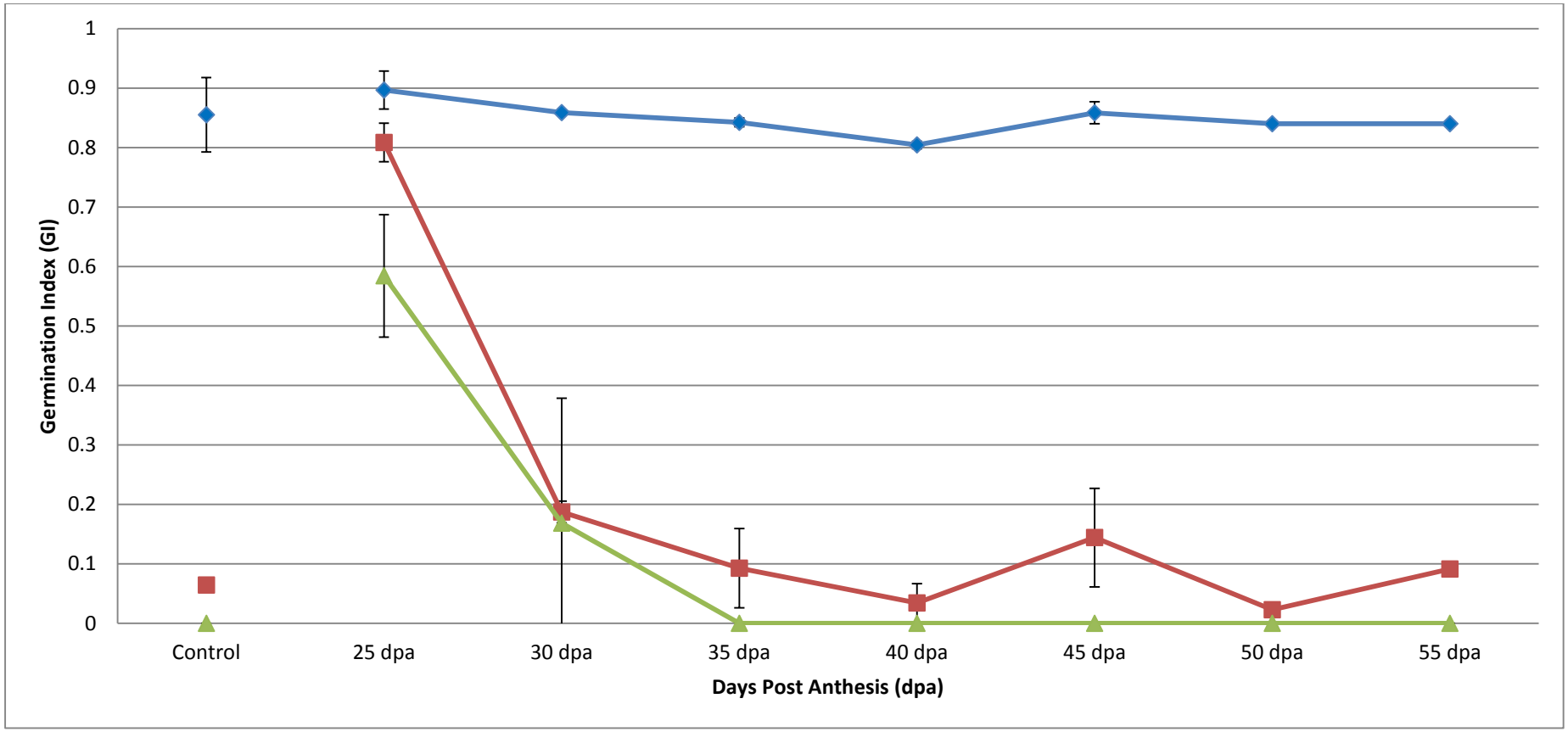


Figure 10. Germination index for genotypes, Gladius (blue), B14 (red) and SUN325B (green) harvested at 60 dpa following different temperature shock treatment at different stages between 25 to 55 dpa. Zero germination was recorded for control SUN325B and plants shocked at 35, 40, 45, 50 or 55 dpa. Error bars represent standard deviation.

Experiment part 3

Different durations of temperature shock

In experiment part 3, glasshouse-grown SUN325B plants were subjected to temperature shocks (40°C day/20°C night) of different duration (2, 3, 4 and 5 days) at 25 dpa, 30 dpa or 35 dpa. Average glasshouse temperature and relative humidity were 21.0°C and 60.9%. For plants that were subjected to 5 days temperature shock and harvested at 60 dpa, GI was highest if plants subjected to temperature shock at 30 dpa, which contrasted with results of experiment 2 (Figure 11). Dormancy release was the earliest in plants subjected to temperature shock at 30 dpa, followed by 35 and 25 dpa. The reduction in dormancy levels was clearly evident between 60 and 65 dpa for the temperature shock treated plants, in comparison to the control which remained highly dormant approaching 70 dpa (Figure 12a and b). Temperature shock of 2 days did not significantly affect dormancy levels but thereafter, each additional day of temperature shock increases germination index values by 0.1-0.2 units recorded at 60 dpa.

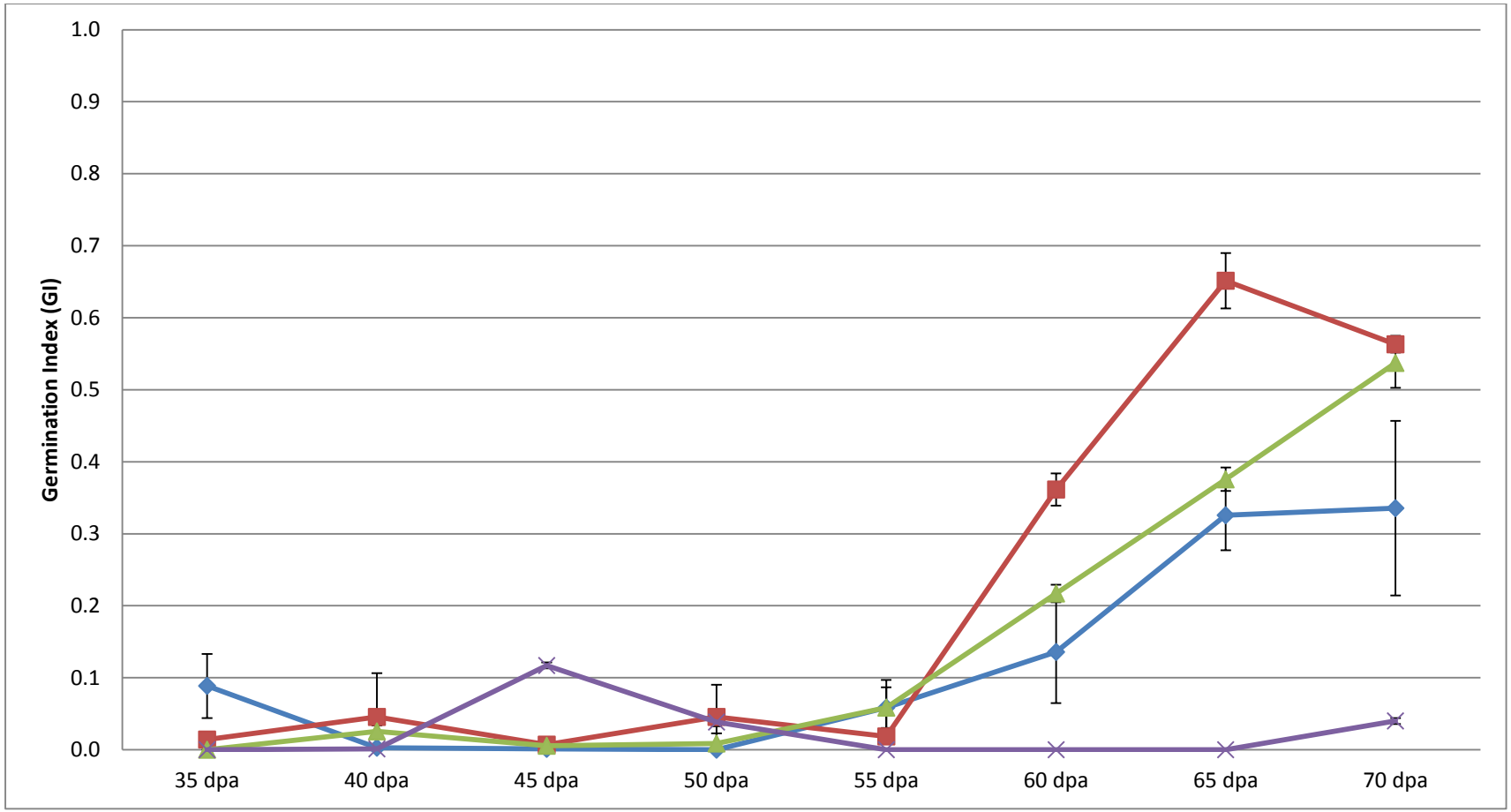


Figure 11. The release of dormancy of SUN325B following high temperature shock of 5 days at 25 (blue), 30 (red), 35 dpa (green) and control (purple). GI values for control at 35 dpa and 55-65 dpa were recorded as nil. Error bars represent standard deviation.

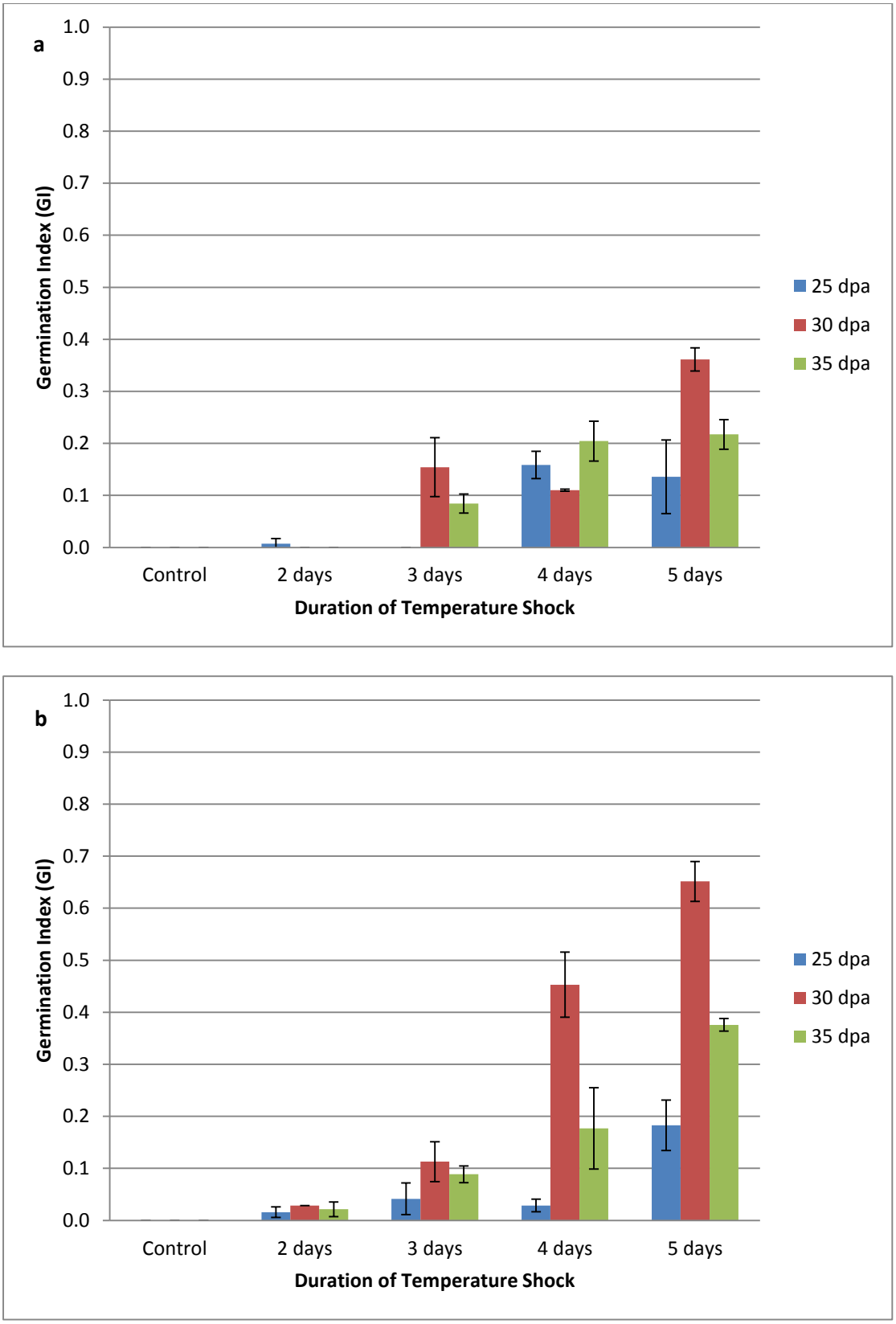


Figure 12. Germination index of high temperature shock-treated SUN325B at 60 dpa (a) and 65 dpa (b). GI values for control were recorded as nil. Error bars represent standard deviation

Experiment part 4

Comparison of glasshouse and field grown plants

In order to determine possible environmental variation in the most effective time point for a temperature shock, results from two planting seasons in the glasshouse (Part 2 and part 3) and one planting season in the field (Part 4) were compared. Temperature shock treatment of 40°C day/20°C night at 25 dpa or 30 dpa significantly increased the germination index of SUN325B at 60 dpa. However, SUN325B from glasshouse July 2012 were more responsive towards a temperature shock at 30 dpa. Field data revealed a lower mean temperature of 19.3°C during grain development and a relative humidity of 56.7%, in comparison to experiment part 2 and 3 (21.4°C, 67.8%; 21.0, 60.9% respectively).

Temperature values from anthesis until 25 dpa in all three experiments were recorded and added to obtain growth degree days (GDD) value. Higher GDD values represent higher daily mean temperature conditions and possibly faster rate of grain development. It is hypothesised that the reason the most effective time point to apply temperature shock shifted from 25 dpa to 30 dpa was due to slower rate of grain development. However, there was a lack of correlation between temperature conditions prior to the shock and the effective time point for the application of shock (Figure 13). In addition, there was also lack of evidence of correlation with mean environmental humidity.

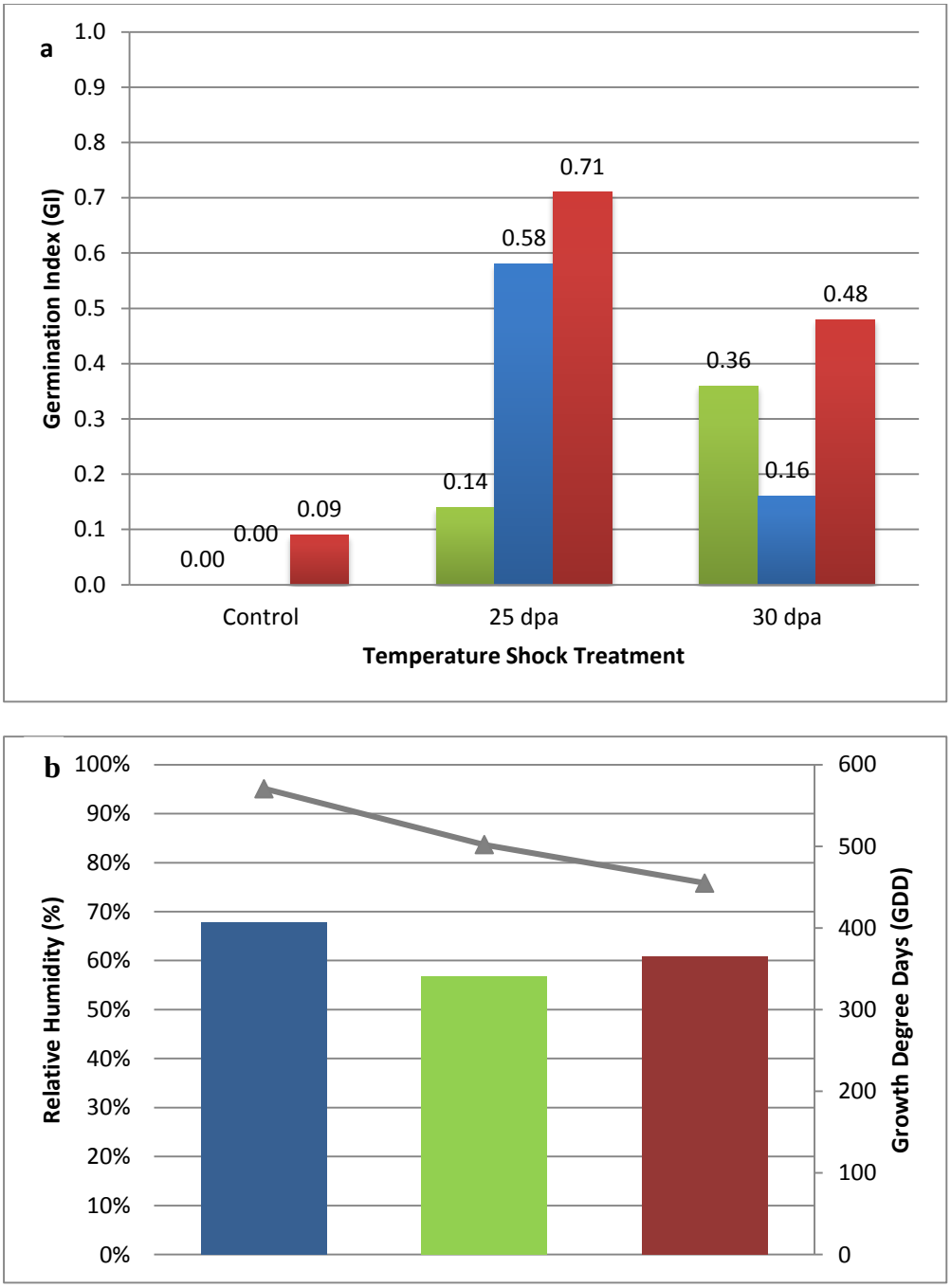


Figure 13. (a) Germination index comparisons of dormant genotype SUN325B for experiment 1 (glasshouse/blue), 2 (glasshouse/green) and 3 (field/red). (b) There was a lack of correlation between the temperature shock time point for maximum effect on dormancy and (i) temperature (GDD) and (ii) humidity (Bars), between anthesis and 25 dpa.

ABA Content

Grain samples of SUN325B (experiment 3) subjected to various temperature shock treatments in 2012 were collected at 5 day intervals from 25 to 50 dpa. Embryo ABA content was increased significantly at 30 dpa in response to the high temperature shock applied at 25 dpa. However, by 35 dpa, ABA concentration in the embryo for all treatments quickly declined to similar levels and is maintained until 50 dpa besides SUN325B which were shocked at 35 dpa that showed lower levels of ABA at 50 dpa (Figure 14). Following embryo removal, the remaining de-embryonated grain samples were analysed for ABA content accordingly. De-embryonated grain ABA concentrations were initially higher than in embryos, reaching a peak at 30 to 35 dpa, but then declined with the rate of decline being greater in the temperature shock treatments. Significant decrease of ABA was recorded at 45 dpa for controls but at 40 dpa for all temperature shock treatments. Similar to the changes of the embryo, ABA levels dropped to similar levels for all treatments by 50 to 55 dpa (Figure 15).

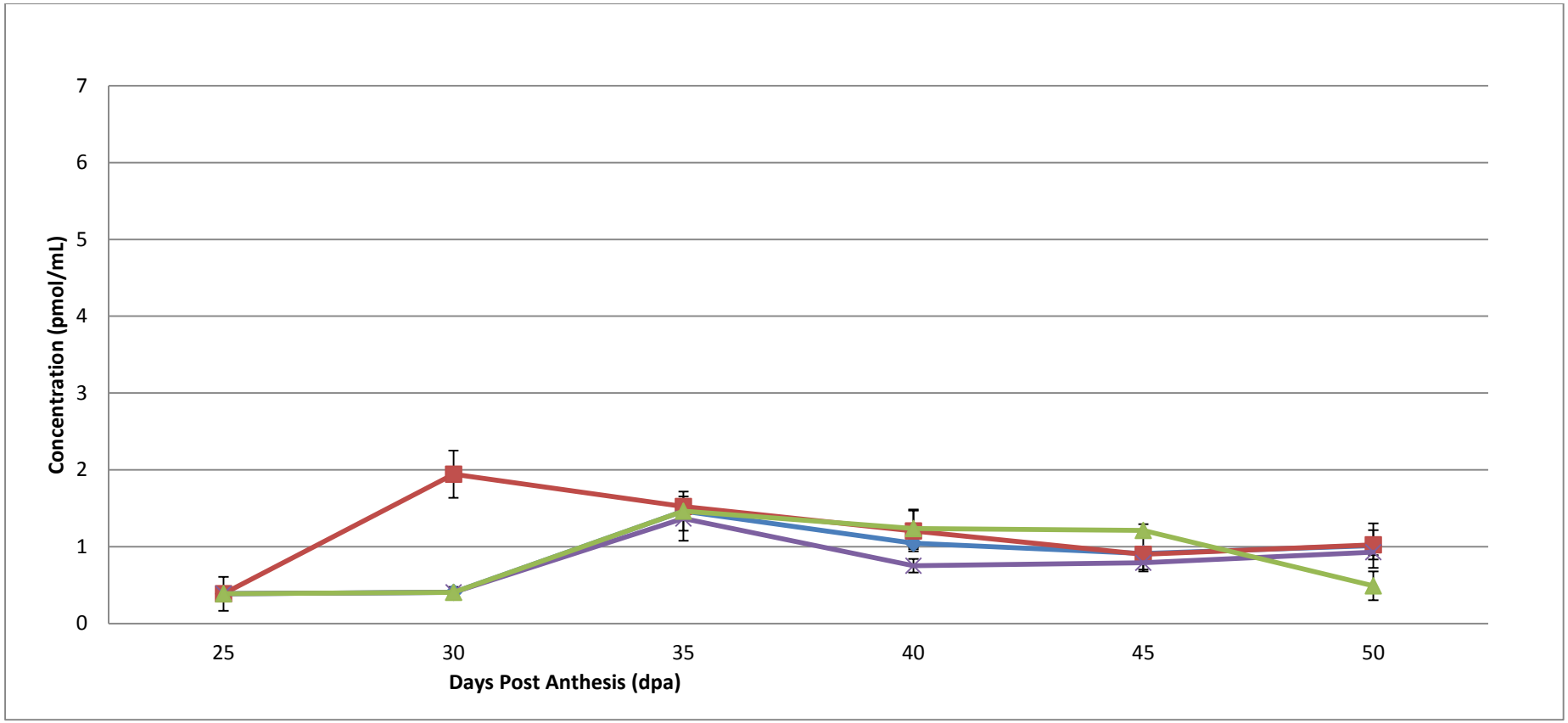


Figure 14. Abscisic acid concentration changes in embryo of glasshouse-grown SUN325B when treated with 5 days temperature shock in a growth chamber at 25 dpa (red), 30 dpa (purple), 35 dpa (green) and control (blue). All temperature-treated plants were returned to the glasshouse for maturation. Error bars represent standard deviation.

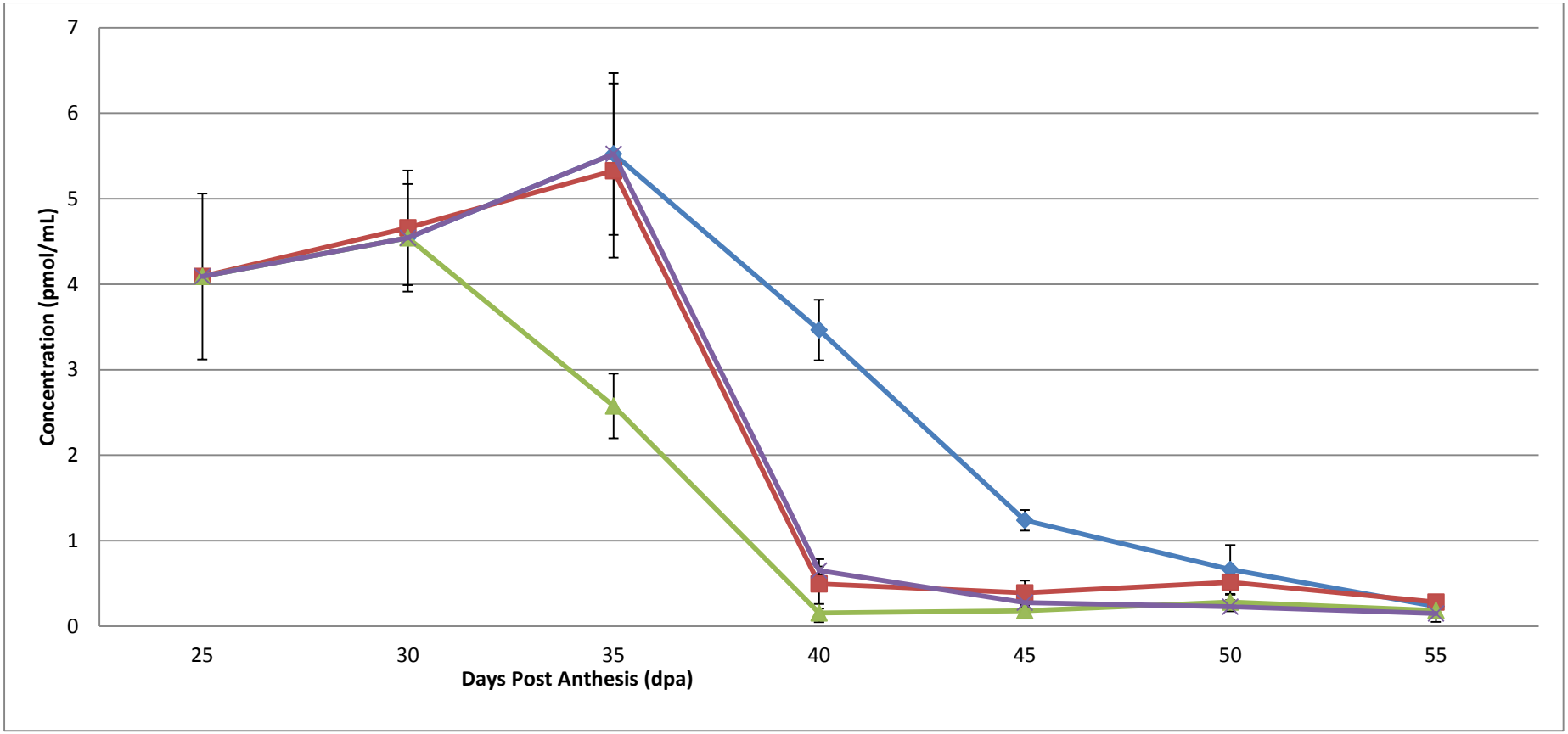


Figure 15 Abscisic acid concentration changes in de-embryonated grain of glasshouse-grown SUN325B when treated with 5 days temperature shock in the growth chamber at 25 dpa (red), 30 dpa (purple), 35 dpa (green) and control (blue). All temperature-treated plants were returned to the glasshouse for maturation. Error bars represent standard deviation.

Experiment part 5

Effect of a lower temperature shock

In experiment 5, SUN325B was grown in the glasshouse and subjected to a temperature shock of 35°C day/ 15°C night for 5 days at 25, 30 or 35 dpa. GI values were collected from 50 dpa to 70 dpa and compared to results from experiment 2. Compared with the 40°C shock, a temperature shock of 35°C caused only a slight increase in germination index that was apparent beginning 60 dpa and was more obvious at 65 and 70 dpa (Figure 16). This increase was slow and GI did not exceed 0.6 for any treatments even at 90 dpa (shocked at 25, 30, 35 dpa and control; GI values of 0.50, 0.60, 0.31 and 0.22 respectively) in comparison to GI values of experiment 3 involving temperature shock of 40°C where (shocked at 25, 30, 35 dpa and control: GI values of 0.86, 0.83, 0.74 and 0.63).

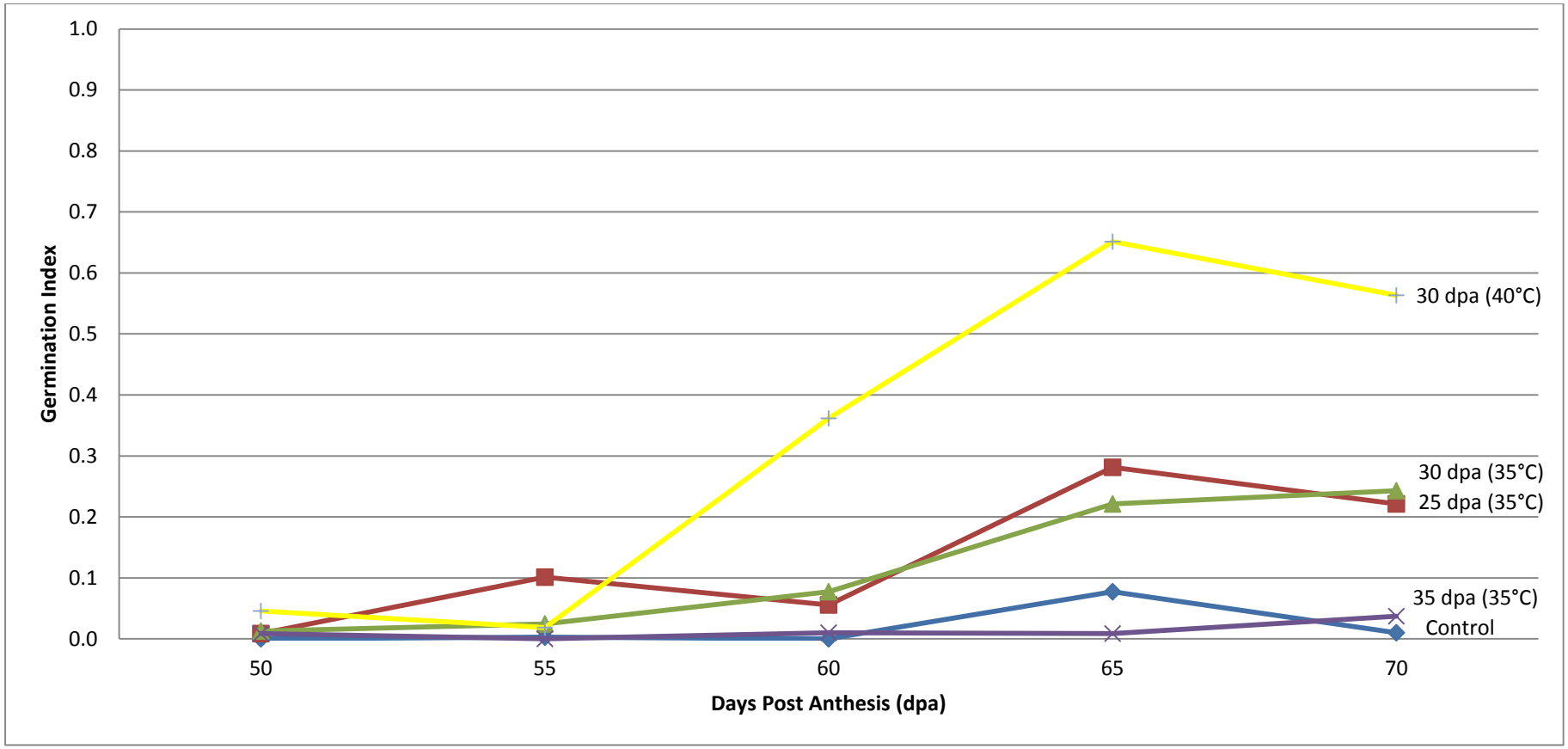


Figure 16. The release of dormancy of glasshouse-grown SUN325B following 5 days growth chamber high temperature shock treatments of 40°C (yellow), 35°C at 25 dpa (red), 30 dpa (green), 35 dpa (purple) and control (blue). All temperature-treated plants were returned to the glasshouse for maturation.

Experiment (general)

Effect of temperature shock on GI and ABA sensitivity of proximal-half grains

At 35 dpa, grains of SUN325B, B14 and Gladius which experienced a temperature shock of 40°C, along with controls, were harvested, cut in half, and the proximal half imbibed in water or ABA and tested for germination index. SUN325B showed the lowest GI, followed by B14 and Gladius (Figure 17). When ABA was applied, GI for SUN325B and B14 remained dormant with GI of zero. A temperature shock at 25 or 30 dpa increased germinability of proximal half grains for all three genotypes. SUN325B and B14 retained strong ABA sensitivity at 35 dpa despite the temperature shock, but Gladius showed an earlier loss of ABA sensitivity (Figure 17).

When SUN325B was temperature-shocked at 25, 30 or 35 dpa, the GI values of proximal halves of grains increased at rates that were similar to the pattern of rate of dormancy loss of intact grains (experiment 3), approaching a plateau near to 100% germination as the wheat grains reached 65 dpa (Figure 18a). When ABA was applied, the effect on ABA sensitivity was most apparent at 80 dpa for SUN325B when the greatest loss of ABA sensitivity occurred in plants treated at 25 dpa, followed by 30 dpa and 35 dpa (Figure 18b). A significant decrease in ABA sensitivity began at 60-65 dpa, coinciding with the time point of observable GI difference of whole grains treated with temperature shock at different time points.

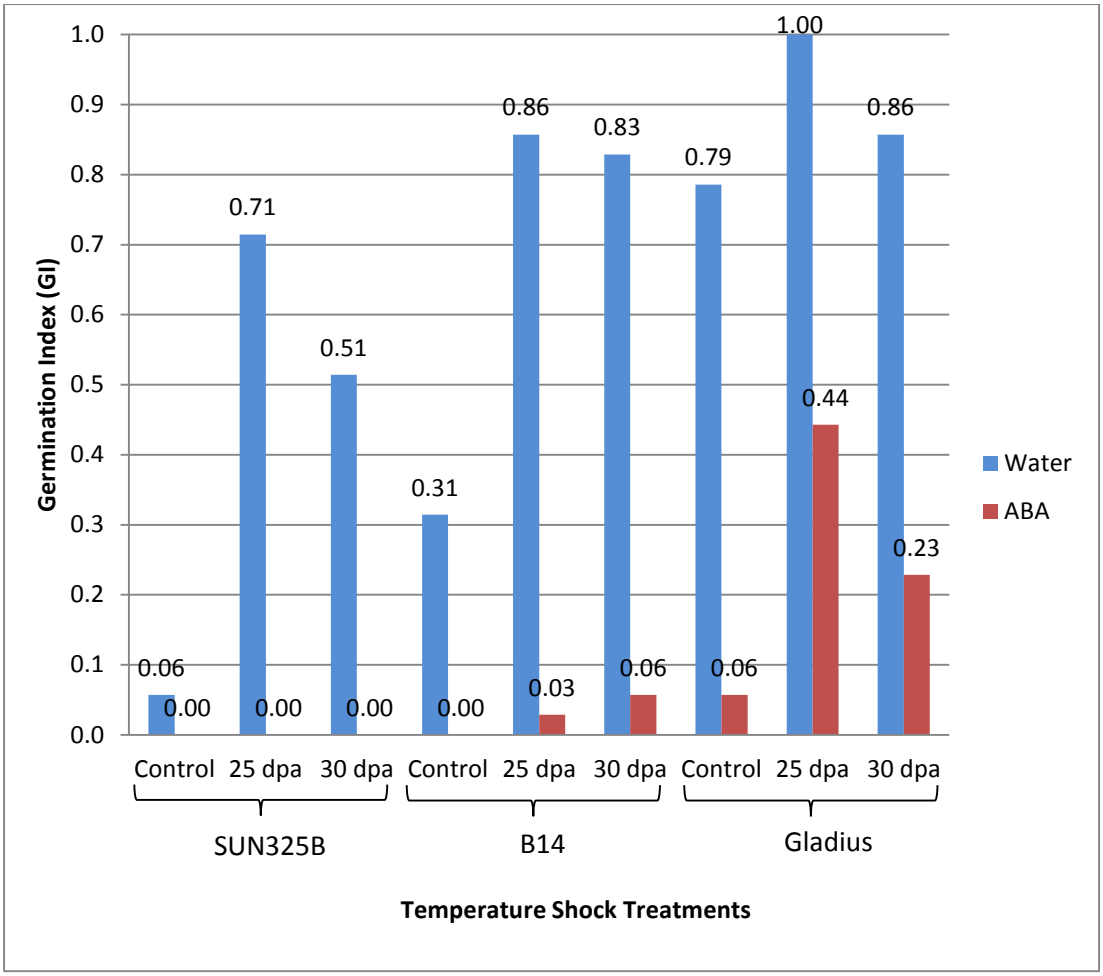


Figure 17. GI of proximal-half grains imbibed with water (blue) or 25 µM ABA (ABA sensitivity) (red) of SUN325B, B14 and Gladius following a temperature shock of 40°C at 25 or 30 dpa compared with non-treated controls.

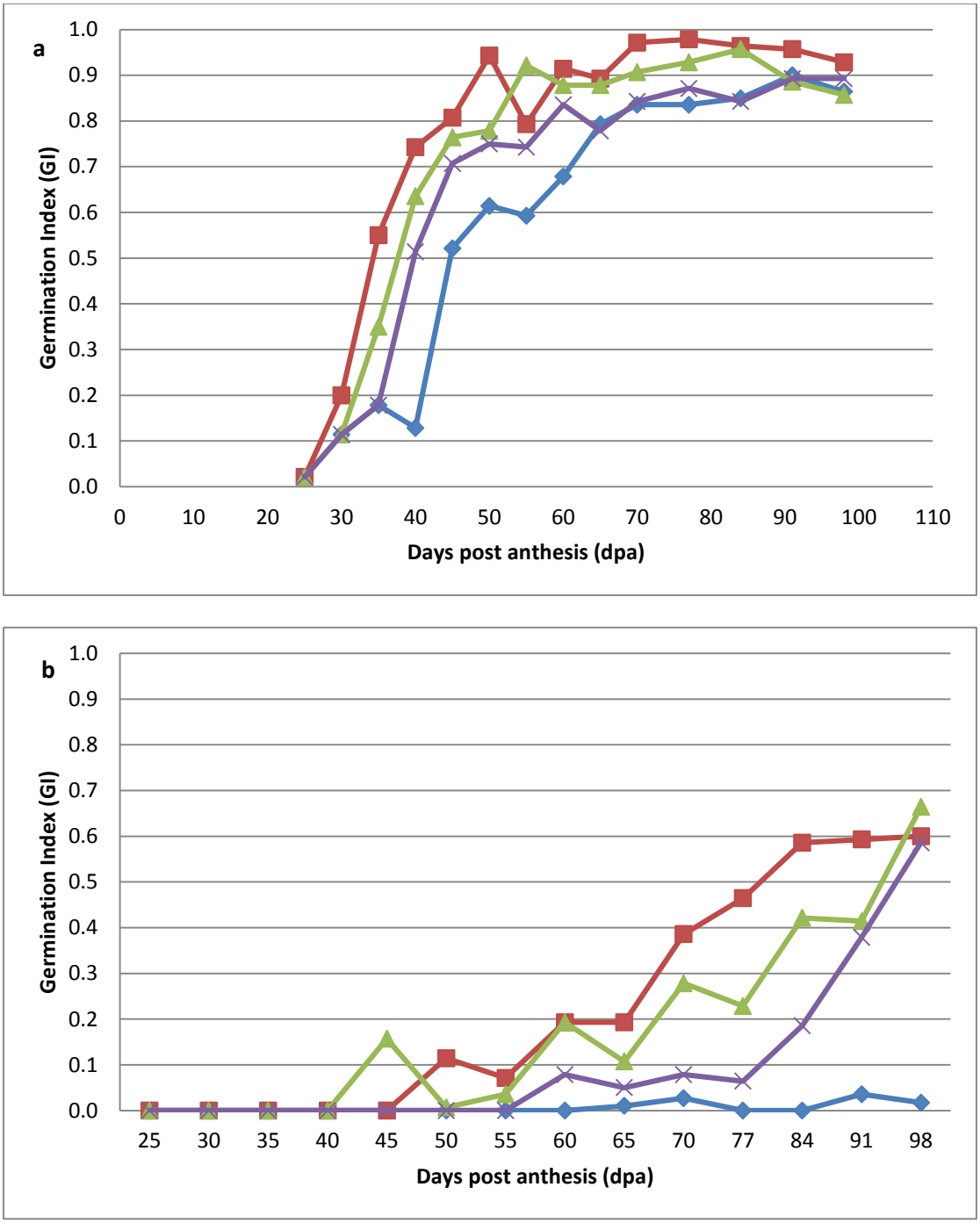
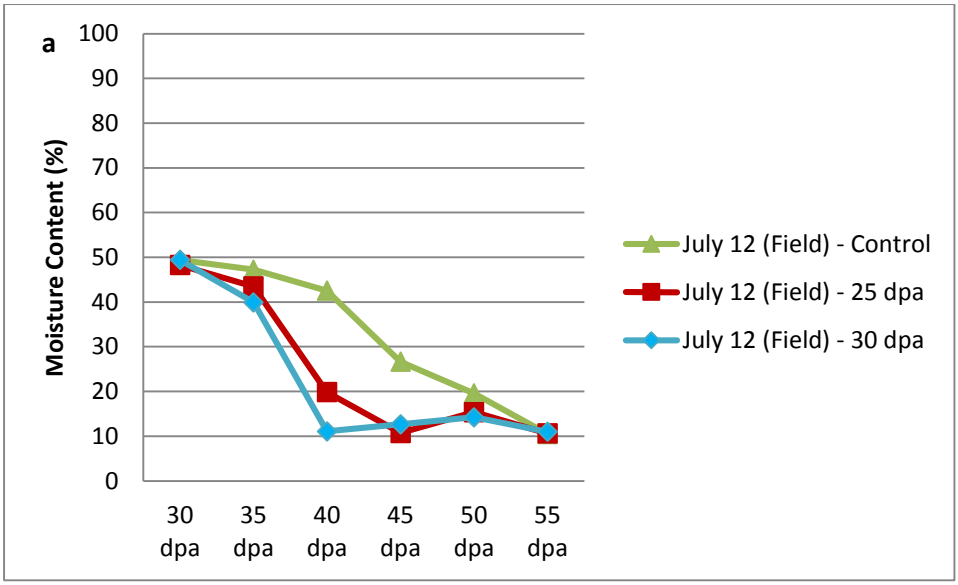


Figure 18 a and b. Change in GI of proximal-half grains imbibed in water (a) and ABA (b) of SUN325B following a temperature shock at 25 dpa (red), 30 dpa (green) or 35 dpa (purple) compared with a non-treated control (blue).

Grain moisture content and grain weight

Grain moisture content was determined throughout grain ripening. In two separate experiments (2 and 3), SUN325B subjected to temperature shock showed an increased rate of water loss (Figure 19a and b). By 55 dpa, most plants had already reached harvest ripeness (approximately 13% moisture content). Plants subjected to a temperature shock at 30 dpa lost grain moisture content slightly faster than those subjected to a temperature shock at 25 dpa (Figure 19a and b) even though the increases in GI values were more drastic in the glasshouse (experiment 2) after experiencing 5 days temperature shock at 25 dpa. This suggested a lack of correlation between rate of water loss and loss of dormancy due to temperature shock.

It also appeared that a temperature shock at 25 dpa caused the most significant reduction in grain weight in both field and greenhouse conditions. Plants shocked at 30 or 35 dpa showed a significant reduction of grain weight at harvest maturity in greenhouse conditions but not in field conditions (Figure 20). No significant reductions of grain weight were recorded when plants were shocked for shorter durations than 4 days at 25, 30 or 35 dpa.



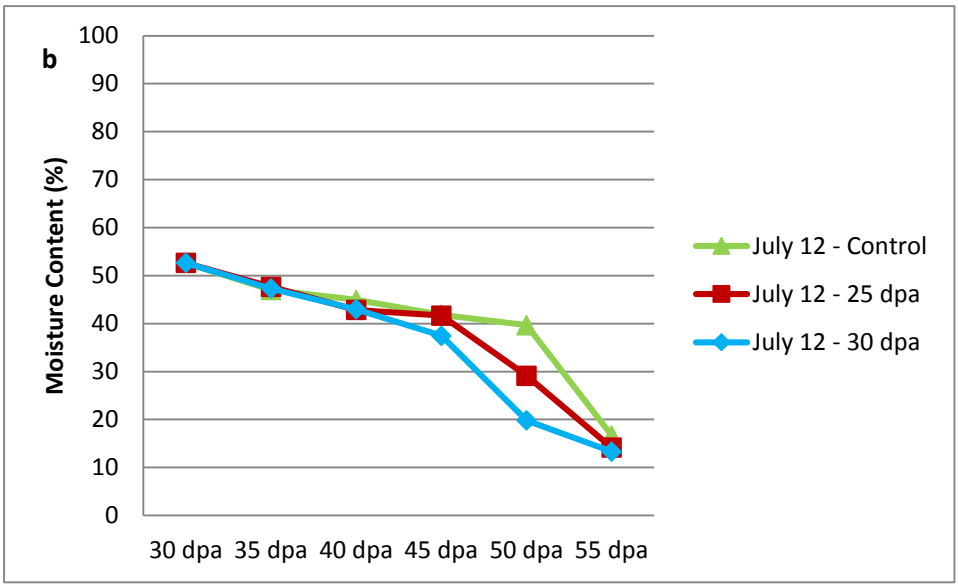
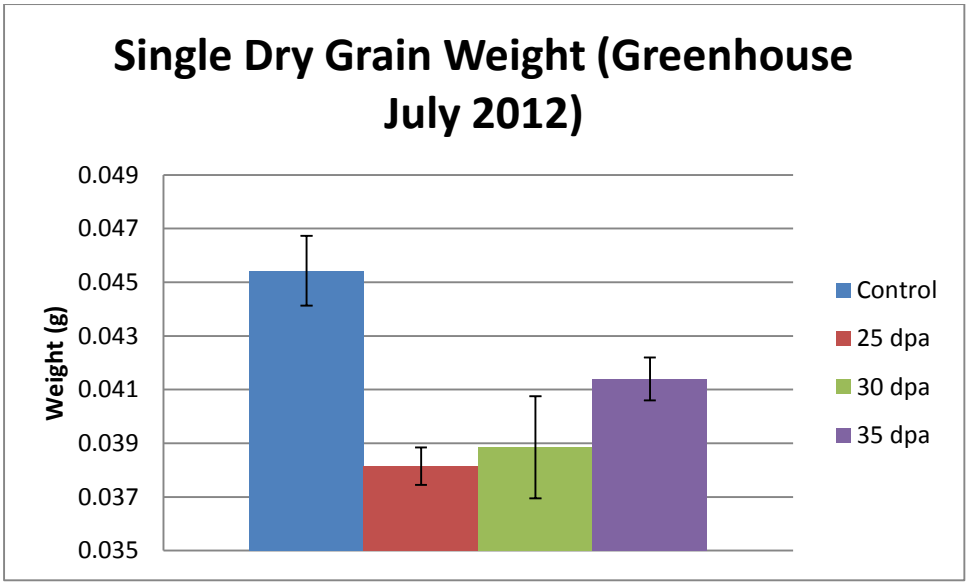


Figure 19 a and b. Rate of SUN325B grain moisture loss following high temperature shock at 25 dpa (red), 30 dpa (blue) or control (green) in the field (a) and in the glasshouse (b).



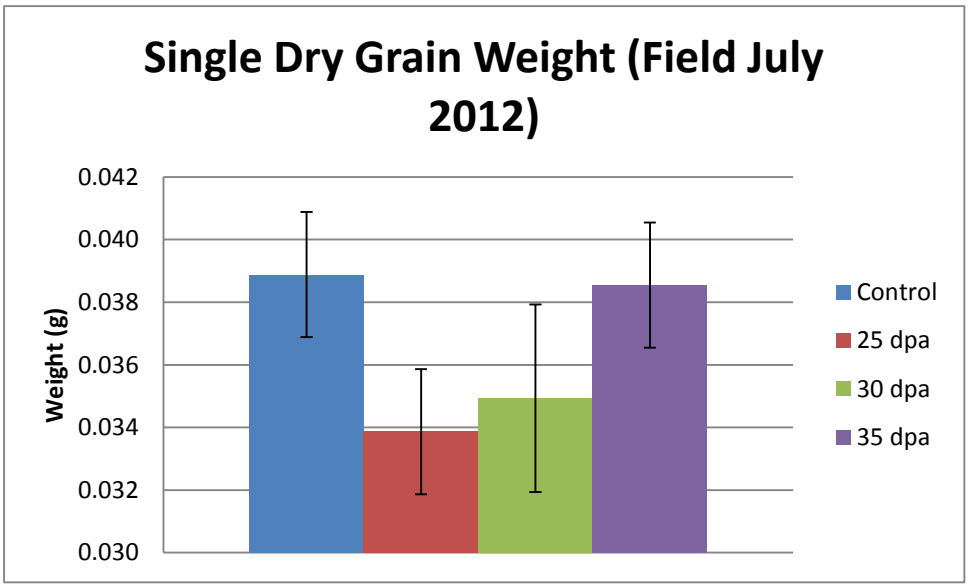


Figure 20 a and b. SUN325B grain weight at 60 dpa following high temperature shock at 25 dpa (red), 30 dpa (green), 35 dpa (purple) or control (blue) in the field (a) and in the glasshouse (b). Error bars represent standard deviation.

Experiment part 6

Genotypes with different dormancy QTL

Sets of genotypes with different dormancy QTL and varying degrees of dormancy were grown in the glasshouse and tested for their germination rate at 45 dpa and 65 dpa following a 5 day temperature shock (40°C day/20°C night) at 25 dpa. At 45 dpa, most of the genotypes with the exception besides P07.683, 50213/Cunn798, DM10.73 #31, DM10.59 #81, DM10.73 #16 showed a significant decrease of dormancy, surpassing GI value of 0.3 (Data not shown). At 65 dpa, 3 dormant non-treated genotypes, P07.683, DM10.73 #31 and 50213/Cunn798, displayed high levels of dormancy with GI values lower than 0.05. Following a temperature shock, GI values did not increase significantly, with GI values remaining below 0.15. At 65 dpa, 10 non-treated genotypes, DM10.59 #81, DM10.73 #16, DM02.25 #2, DM02.25 #45, DM02.25 #69, DM02.25 #74, DM02.25 #84, DM10.25 #73, DM10.64 #32 and SUN325B, displayed GI values lower than 0.1. Following a temperature shock, GI values increased significantly, surpassing 0.6 for all 10 genotypes except for DM10.73 #16 and DM02.25 #45. At 65 dpa, 9 non-treated genotypes, DM02.25 #13, DM02.25 #60, DM02.25 #61, DM02.25 #134, DM10.59 #50, DM10.59 #59, DM10.59 #85, DM10.73 #51 and Yitpi, had intermediate GI values ranging from 0.1-0.6. Following a temperature shock, GI values increased significantly, surpassing 0.7 (Figure 21).

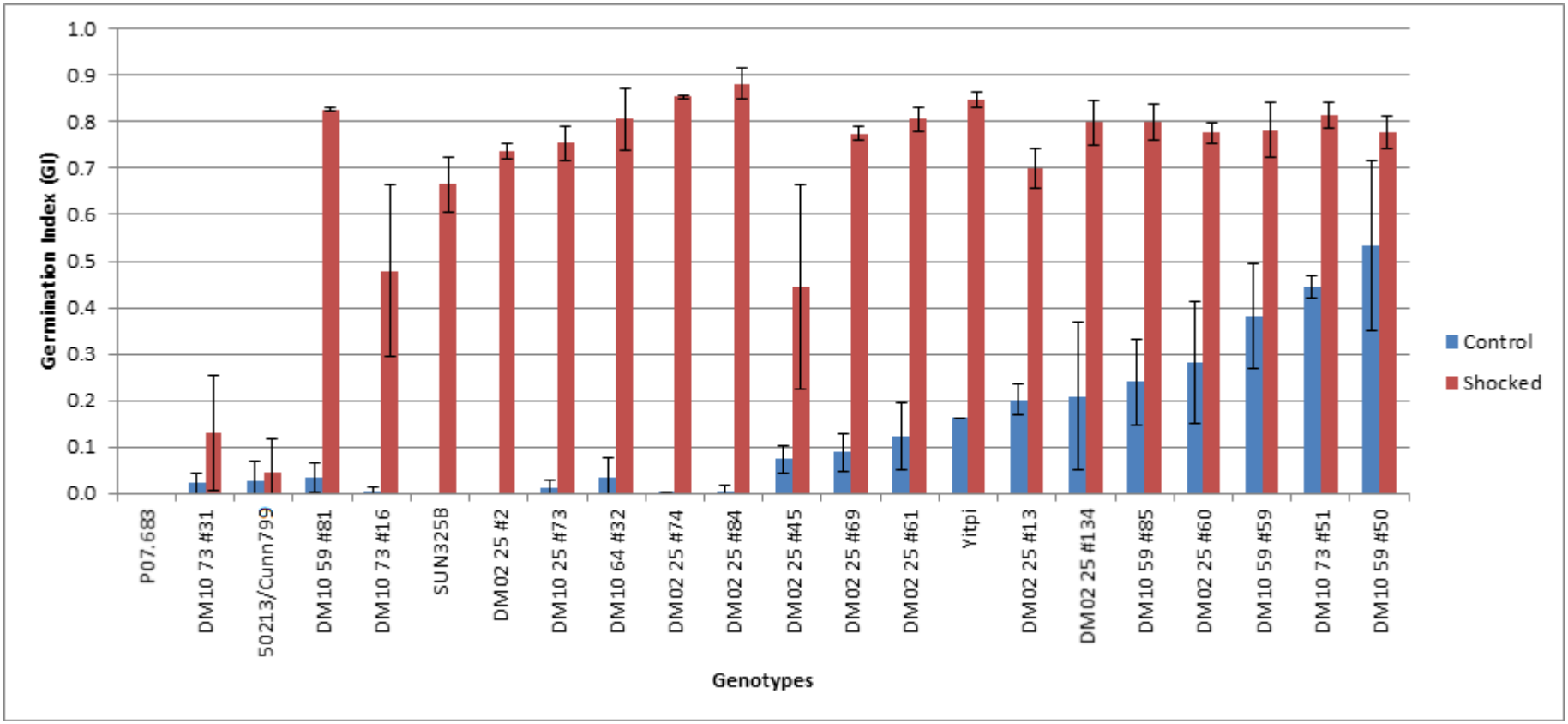


Figure 21. Analysis of dormancy of genotypes with various combinations of known and other (chromosomal position unknown) dormancy QTL in response to high temperature. Germination index was measured at 65 dpa following 5 days of 40°C temperature shock treatment at 25 dpa. Error bars represent standard deviation.

4.2 Sequence and expression analysis

Triticum aestivum Delay of Germination1-like genes (*TaDOG1L*) and orthologs in other species

Arabidopsis thaliana Delay of Germination 1 (*AtDOG1* or *At5g45830*) gene contains a Delay of Germination 1 (DOG1) conserved domain (pfam14144) when translated. This domain is representative of a family of plant proteins that appears to be highly specific in controlling seed dormancy (Bentsink *et al.* 2006). Besides *AtDOG1*, there are four other family gene members in *Arabidopsis*: *Delay of Germination-Like 1-4* (*DOGL1-4*) or *At4g18660*, *At4g18680*, *At4g18690*, and *At4g18650* respectively. In a conserved domain search, all of the family members contain the DOG1 conserved domain. However, the other members were previously shown to not affect dormancy (Bentsink *et al.* 2006).

tBlastx searches were performed using publicly available NCBI Blast tools against their respective databases for *Triticum* nucleotide and EST sequences similar to query *AtDOG1* and *AtDOGL1-4*. Seven sequences with accession numbers: *X56782*, *D12921*, *AK330559*, *AK336217*, *AK330689*, *AB555729* and *AK332921* were identified. AK330559, AK330689, *AB555729* and *AK332921* were previously identified as *TaDOG1L5-1*, *TaDOG1L4*, *TaDOG1L1* and *TaDOG1L2* respectively in a few studies and shown to contribute towards dormancy (Ashikawa *et al.* 2010; Ashikawa *et al.* 2014; Rikiishi and Maekawa 2010). When translated and scanned for conserved domains, all seven amino acid sequences were shown to contain conserved domain DOG1 (pfam14144) (Table 3).

Gene accession number *D12921* (also known as *HBP-1b(c1)*) which putatively code for a Histone Promoter-Binding Protein (HBP), and *AK336217* were translated and found to contain a DOG1 conserved domain with an addition of a basic region leucine zipper (smart00338) conserved domain on the same reading frame; *X56782* (also known as HBP-1b(c38)) which was also identified as a HBP, and *TaDOG1L5-1* were translated and found to contain a basic leucine

zipper DNA-binding and multimerization region of GCN4 domain (cd12193) on the same reading frame. Both domains are members of the B_ZIP1 superfamily (cl02576). The summary of the data is provided in table 3.

A search was also carried out to uncover other *DOG1-like* genes in other cereal species based on (i) similarity to wheat *DOG1L1-5* and (ii) conserved domain search (Table 4 and figure 22). A phylogenetic tree based on publicly available gene sequences that were translated and determined to contain the DOG1 conserved domain from species, *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum* and *Zea mays,* was constructed to determine the evolutionary relationships among the sequences.

Although redundant sequences were removed, the number of possibly unique genes exceeded the number of genes used in the phylogenetic tree by Ashikawa et al. (2013). However, there were four clades reported by Ashikawa et al. (2013) that were apparent in the phylogenetic tree in this study (Figure 22).

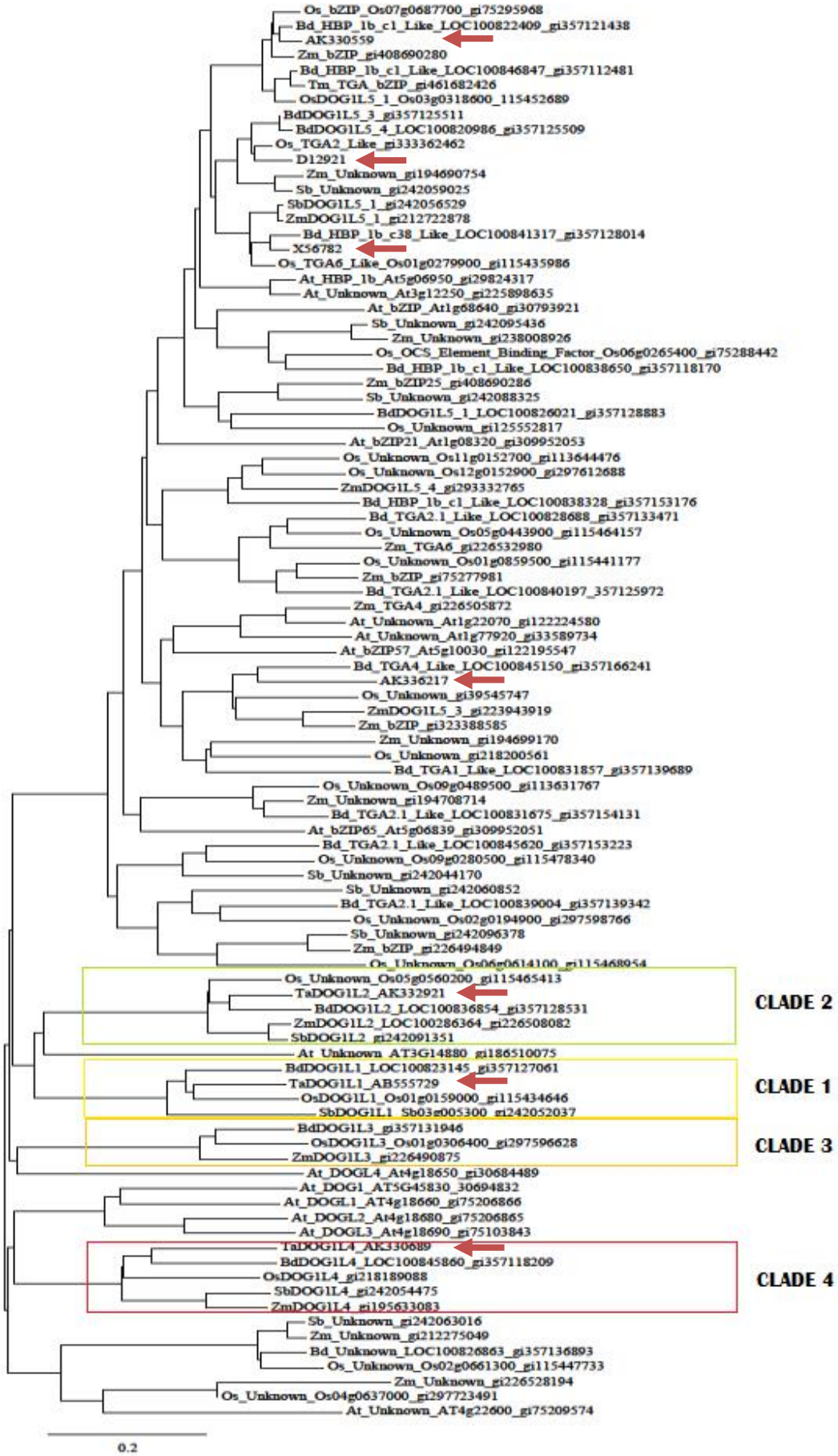
TaDOG1L4 was the closest relative of the AtDOG1 protein, followed by TaDOG1L1 and TaDOG1L2. Furthermore, functional analysis in two different studies had shown that *TaDOG1L1-4* could increase seed dormancy in *Arabidopsis* when ectopically expressed and was shown to be lowly expressed in a reduced seed dormancy mutant. Although sequence similarities of *TaDOG1L1-4* towards the *AtDOG1* are low, phylogenetic analysis and ectopic expression study showed close evolutionary relationship and functional conservation respectively.

Table 3. List of *Triticum aestivum* gene sequences that were translated and determined to contain conserved domains DOG1 (pfam14144), BRLZ (smart00338) and bZIP_GCN4 domain) cd12193, arranged from lowest to highest E-value.

Accession Number	pfam14144	E-Value	smart00338	E-Value	cd12193	E-Value
X56782	Present	1.70e-45	Not present	-	Present	5.12e-05
D12921	Present	1.07e-43	Present	1.71e-09	Not present	-
TaDOG1L5-1	Present	5.93e-40	Not present	-	Present	1.40e-03
AK336217	Present	7.30e-27	Present	1.19e-07	Not present	-
AK330689	Present	2.56e-24	Not present	-	Not present	-
AB555729	Present	8.05e-21	Not present	-	Not present	-
AK332921	Present	1.08e-10	Not present	-	Not present	-

Table 4. List of *Triticum aestivum* genes that were translated and determined to contain the DOG1 conserved domain and their best matched putative orthologs in *Hordeum vulgare*, *Brachypodium distachyon* and *Oryza sativa* based on a blastn search.

Sequence	*Hordeum vulgare*	Query Coverage, E-Value	*Brachypodium distachyon*	Query Coverage, E-Value	*Oryza sativa*	Query Coverage, E-Value
X56782	Unknown (*AK357239*)	100%, 0	*HBP-1b(c38)-like* (*LOC100841317*)	99%, 0	*TGA6-like* (*Os01g0279900*)	99%, 0
D12921	Unknown (*AK362993*)	100%, 0	*HBP-1b(c1)-like* (*LOC100820986*)	100%, 0	*HBP-1b(c1)-like* (*Os01g0808100*)	99%, 0
AK330559	Unknown (*AK363931*)	95%, 0	*HBP-1b(c1)-like* (*LOC100822409*)	61%, 0	*HBP-1b(C38)-like* (*Os07g0687700*)	64%, 0
AK336217	Unknown (*AK364746*)	93%, 0	*TGA4-like* (*LOC100845150*)	66%, 0	Unknown (*Os04g0637000*)	60%, 1e-131
AK330689	None		*TGA2-like* (*LOC100845860*)	79%, 0	None	
AB555729	*HvDOG1L1* (*AB555730*)	100%, 0	*HBP-1b(c1)-like* (*LOC100823145*)	100%, 0	Unknown (*Os01g0159000*)	100%, 0
AK332921	Unknown, (*AK248238*)	99%, 0	Unknown, (*LOC100836854*)	65%, 0	Unknown (*Os05g0560200*)	65%, 0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AK330559
Zm_bZIP_gi408690280
Bd_HBP_1b_c1_Like_LOC100846847_gi357112481
Tm_TGA_bZIP_gi461682426
OsDOG1L5_1_Os03g0318600_115452689
BdDOG1L5_3_gi357125511
BdDOG1L5_4_LOC100820986_gi357125509
Os_TGA2_Like_gi333362462
D12921
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Sb_Unknown_gi242059025
SbDOG1L5_1_gi242056529
ZmDOG1L5_1_gi212722878
Bd_HBP_1b_c38_Like_LOC100841317_gi357128014
X56782
Os_TGA6_Like_Os01g0279900_gi115435986
At_HBP_1b_At5g06950_gi29824317
At_Unknown_At3g12250_gi225898635
At_bZIP_At1g68640_gi30793921
Sb_Unknown_gi242095436
Zm_Unknown_gi238008926
Os_OCS_Element_Binding_Factor_Os06g0265400_gi75288442
Bd_HBP_1b_c1_Like_LOC100838650_gi357118170
Zm_bZIP25_gi408690286
Sb_Unknown_gi242088325
BdDOG1L5_1_LOC100826021_gi357128883
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At_bZIP21_At1g08320_gi30952053
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Os_Unknown_Os12g0152900_gi297612688
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Bd_HBP_1b_c1_Like_LOC100838328_gi357153176
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At_bZIP57_At5g10030_gi122195547
Bd_TGA4_Like_LOC100845150_gi357166241
AK336217
Os_Unknown_gi39545747
ZmDOG1L5_3_gi223943919
Zm_bZIP_gi323388585
Zm_Unknown_gi194699170
Os_Unknown_gi218200561
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Os_Unknown_Os09g0489500_gi113631767
Zm_Unknown_gi194708714
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Sb_Unknown_gi242096378
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Os_Unknown_Os05g0560200_gi115465413
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At_DOGL3_At4g18690_gi75103843
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Zm_Unknown_gi226528194
Os_Unknown_Os04g0637000_gi297723491
At_Unknown_AT4g22600_gi75209574
0.2
CLADE 2
CLADE 1
CLADE 3
CLADE 4

Figure 22. Unrooted phylogenetic tree constructed using translated genes containing the DOG1 conserved domain obtained from *Arabidopsis thaliana* (At), *Brachypodium distachyon* (Bd), *Oryza sativa* (Os), *Sorghum bicolor* (Sb) *Triticum aestivum* (Ta) and *Zea mays* (Zm). Three *Triticum aestivum* protein sequences of TaDOG1L1, TaDOG1L2 and TaDOG1L4 are closely related to the *Arabidopsis thaliana* DOG1 protein sequence. Red arrows point to *Triticum aestivum* translated seuqences. Clade 1 to 4 are clades identified in Ashikawa *et al*. (2013).

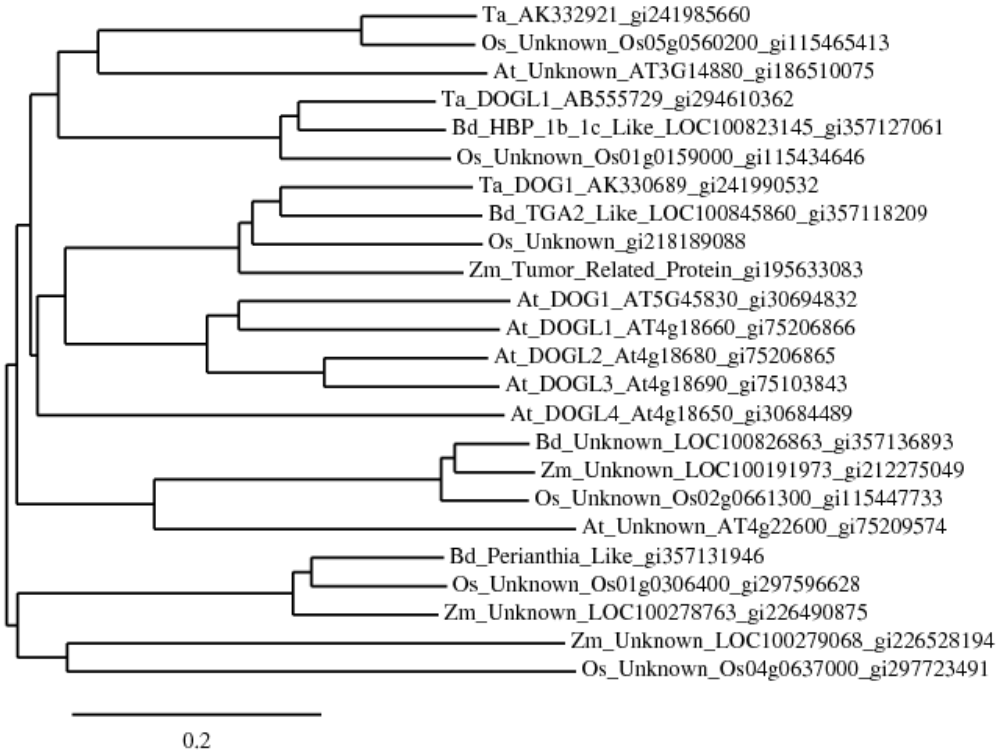


Figure 23. Unrooted phylogenetic tree of translated genes of *Brachypodium distachyon* (Bd), *Oryza sativa* (Os), *Triticum aestivum* (Ta) and *Zea mays* (Zm), most closely related to the AtDOG1 and AtDOG1-like proteins. Three *Triticum aestivum* protein sequences TaDOG1L1, TaDOG1L 2 and TaDOG1L 4 are closely related to the *Arabidopsis thaliana* DOG1 protein.

Promoter analysis

Analysis of genomic DNA databases revealed consistent occurrences of certain promoter motifs in *DOG1* sequences. For example, RY repeats (CATGCAT) and ABRE motifs (ACGC/TG core) occur in *AtDOG1* and *BdDOG1* promoters (Bentsink *et al.* 2006; Graeber *et al.* 2010). Promoters of *X56782*, *D12921*, *AK336217*, *TaDOGL1*, *TaDOGL2 TaDOGL4* and *TaDOGL5-1* were searched for the presence of RY and ABRE motifs using PLACE database of plant cis-acting regulatory DNA elements.

Promoter analysis on *X56782* found [ACGTG] core-containing ABRE-like motifs at approximately -1960, -2274 and -3082 from the ATG start site. Promoter scan on its putative ortholog (*Os01g279900*) found ABRE motifs that contain the [ACGTG] core at approximately -569 and -1975 from the ATG start site. RY motif was only found in the rice sequence at position -1028 from the ATG start site but not the wheat sequence.

A search on wheat *D12921* promoter revealed a possible RY motif in the promoter but this requires further validation due to the lack of sequence length and consistency in ATG start site information. In a promoter scan on its putative ortholog in rice (*Os01g0808100*), only one RY motif was identified approximately -397 from the ATG start site. ABRE motif containing the [ACGTG] core was found only in the rice sequence at approximately -3500 from the ATG start site.

Analysis of the promoter of *AK336217* showed the presence of a RY motif and [ACGTG] core-containing ABRE motifs, but the positions could not be confirmed due to lack of information of the ATG start site. Promoter of its ortholog (*Os04g0637000*) contains a RY motif at -1617 and an ABRE motif with the [ACGTG] core at -4401 from ATG start site.

One RY motif and two ABRE-like motifs with [ACGTG] core were found in the promoter of *TaDOG1L1*. The putative ortholog in rice (*Os01g159000*) contains an RY motif at

approximately -829 from the start site and three ABRE motifs with [ACGTG] core at -421, -599 and -1777 from the ATG start site.

Promoter sequence for *TaDOG1L2* was not found, but the promoter of its rice ortholog (*Os05g0560200*) contains two ABRE motifs with [ACGTG] core at positions -386 and -3198 from the ATG start site and a possible RY motif at -3222 from start site.

No putative ortholog for *TaDOG1L4* was identified in rice. A scan on the promoter revealed a possible RY motif and three ABRE motifs. All three ABRE motifs were of the [ACGCG] core.

Due to short sequence length of the promoter, analysis on *TaDOG1L5-1* could be performed but promoter of its putative ortholog (*Os07g0687700*) contains RY motif at approximately -1117 from the ATG start site. ABRE motifs with [ACGTG] core were also found at -319, -887 and -2068 from the ATG start site.

Protein motif analysis

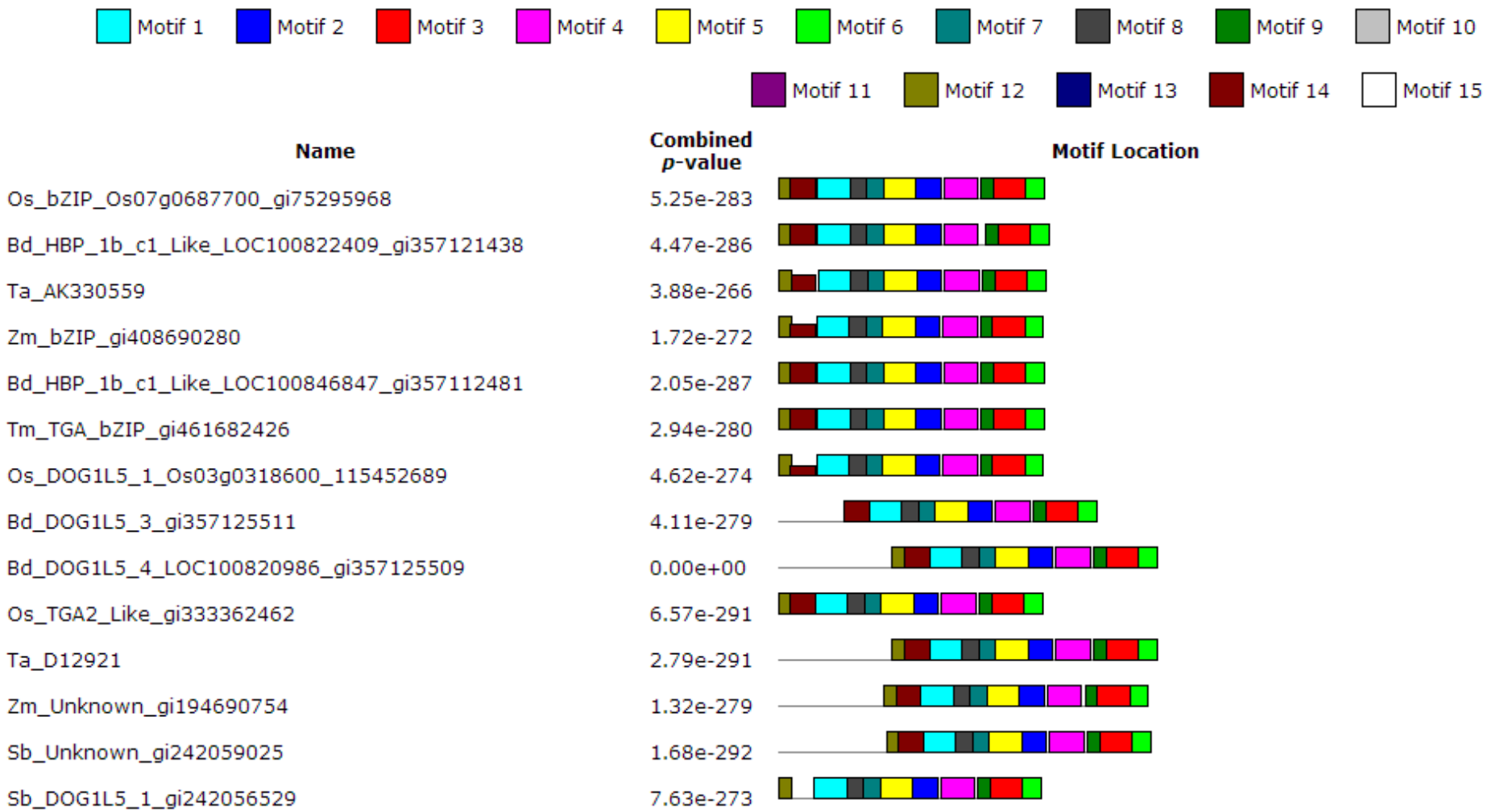
In order to further understand DOG1 and DOG1-like proteins, a motif scan was done on 93 sequences using the MEME (*Multiple Em for Motif Elicitation*) web server. The server uses an expectation maximisation algorithm to search for similar sequence patterns (or clusters of similar sub-sequences) in a dataset of sequences. 15 protein motifs were predicted from the scan (Table 5). Among the 15 motifs, motifs 2, 4 and 5 are the highest occurring motifs among the sequences.

Motifs 5, 2, 4, 13 and 10 (downstream the open reading frame) are unique to AtDOG1, AtDOG1L and TaDOG1L1-4 (clade 1, 2 and 4). Among the five motifs, only motif 5 was not predicted in AtDOG1L1 and TaDOG1L2. Interestingly, combination of motifs 5, 2, 4, 9, 3 and 6 is a unique characteristic of TaDOG1L5-1, AK336217, D12921 and X56782 (Figure 24).

The differences in motif distributions might indicate functional divergence due to evolution. In general, comparing the motif distributions of the 93 protein sequences, sequences that appear on figure 23, or the bottom half of figure 22 that includes clades 1, 2, 3 and 4, specifically contain at least 3 of the motifs of 5, 2, 4, 13 and 10, suggesting the possibility of functional conservation of these sequences in dormancy.

Table 5. Motifs predicted among the 93 DOG1 and DOG1-like protein sequences using MEME web server.

Motif	E-Value, No. of sites	Length	Consensus sequence
Motif 1	2.3e-2071, 63	41	KTLRRLAQNREAARKSRLRKKAYIQQLETSRIKLTQLEQEL
Motif 2	1.4e-1725, 87	29	DVFHVMSGMWKTPAERCFLWIGGFRPSEV
Motif 3	2.1e-1591, 62	41	AMAMGKLGTLENFVRQADNLRQQTLHQMHRILTTRQAARCF
Motif 4	1.4e-1502, 80	41	HLEPLTEQQLMGICNLQQSSQQAEDALSQGMEKLQQSLADT
Motif 5	7.0e-1202, 76	41	INELRTAVNAHIPDNDLRMIVDCCMAHYDEYFRLKGVAAKA
Motif 6	3.5e-848, 62	21	LAIHDYFHRLRALSSLWLARP
Motif 7	9.9e-727, 65	21	GNGAAMFDMEYARWLEEHNKH
Motif 8	4.6e-311, 28	21	QRARQQGIFISSSGDQTHSMS
Motif 9	9.2e-254, 38	15	PSGCSGNVANYMGQM
Motif 10	2.3e-236, 28	29	IVEILTPYQAVEFLVAAKRFHIGVHDWGR
Motif 11	2.0e-193, 24	15	QPPTLNIFPSWPMHH
Motif 12	5.6e-151, 24	15	MADASPRTDTSTDPD
Motif 13	1.1e-129, 28	29	EVDADVDKYVEGMKVVLVEADCLRMRTMK
Motif 14	7.1e-122, 73	29	TDEKNQMFEQGQVAAPTASDSSDKSKDKL
Motif 15	1.5e-111, 5	50	YCAARAELDPVWTLSAPWASPVERGAAYWLAGWRPTTLVHL LYTESGRRF


Motif 1
Motif 2
Motif 3
Motif 4
Motif 5
Motif 6
Motif 7
Motif 8
Motif 9
Motif 10
Motif 11
Motif 12
Motif 13
Motif 14
Motif 15
Name
Combined p-value
Motif Location
Os_bZIP_Os07g0687700_gi75295968 5.25e-283
Bd_HBP_1b_c1_Like_LOC100822409_gi357121438 4.47e-286
Ta_AK330559 3.88e-266
Zm_bZIP_gi408690280 1.72e-272
Bd_HBP_1b_c1_Like_LOC100846847_gi357112481 2.05e-287
Tm_TGA_bZIP_gi461682426 2.94e-280
Os_DOG1L5_1_Os03g0318600_115452689 4.62e-274
Bd_DOG1L5_3_gi357125511 4.11e-279
Bd_DOG1L5_4_LOC100820986_gi357125509 0.00e+00
Os_TGA2_Like_gi333362462 6.57e-291
Ta_D12921 2.79e-291
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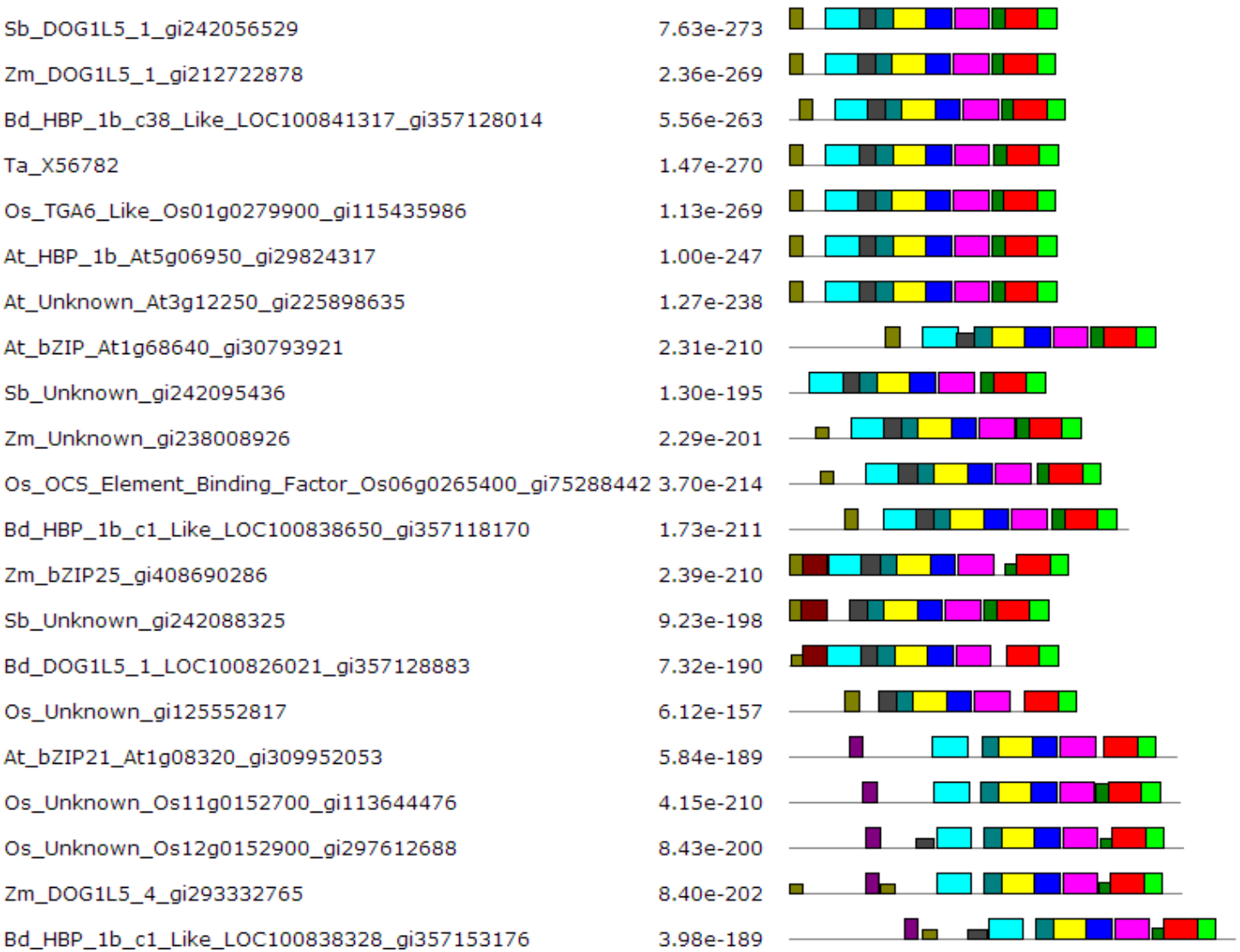
Sb_DOG1L5_1_gi242056529 7.63e-273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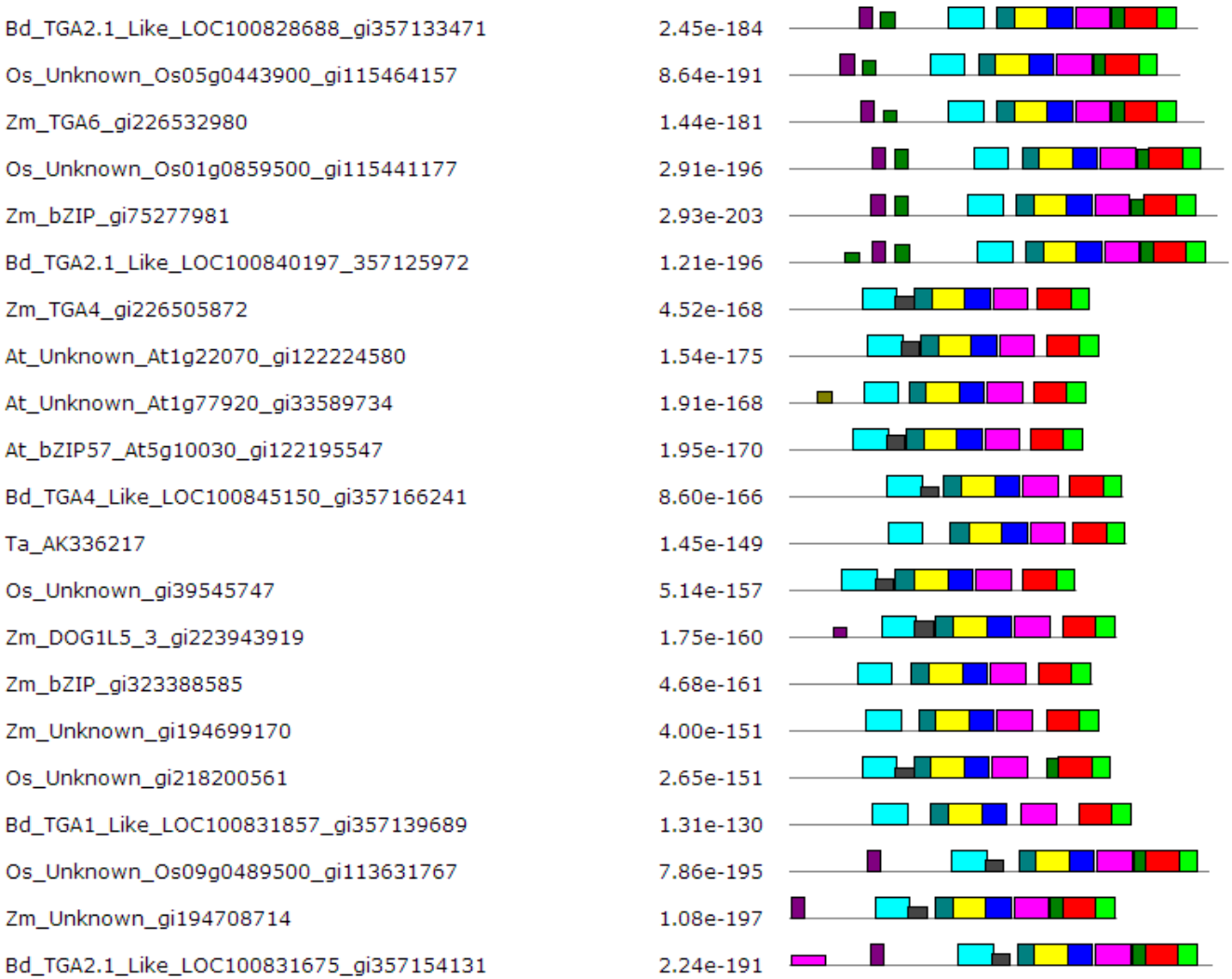
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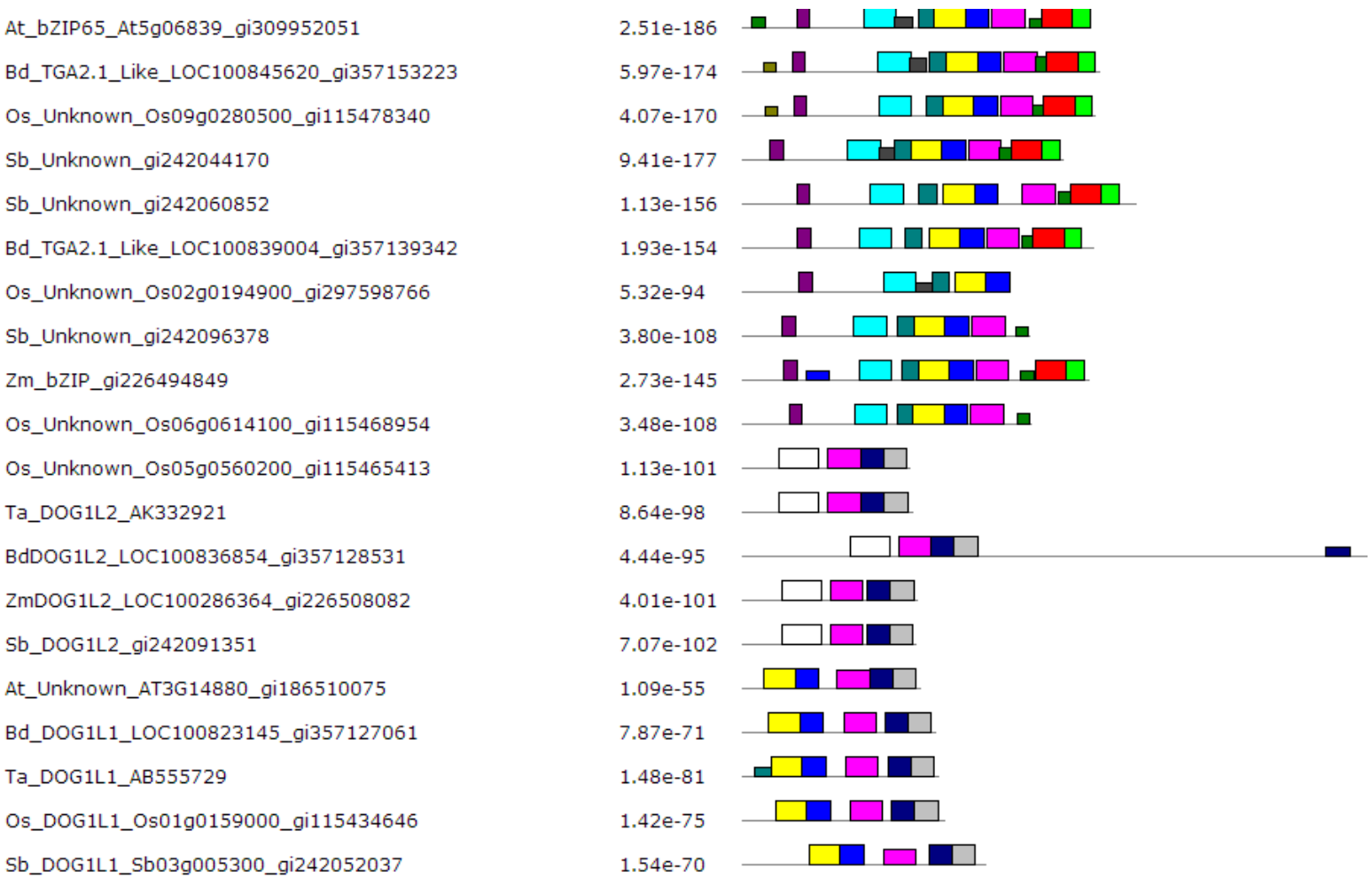
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Sb_Unknown_gi242060852
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Bd_TGA2.1_Like_LOC100839004_gi357139342
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Sb_Unknown_gi242096378
3.80e-108
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Os_Unknown_Os05g0560200_gi115465413
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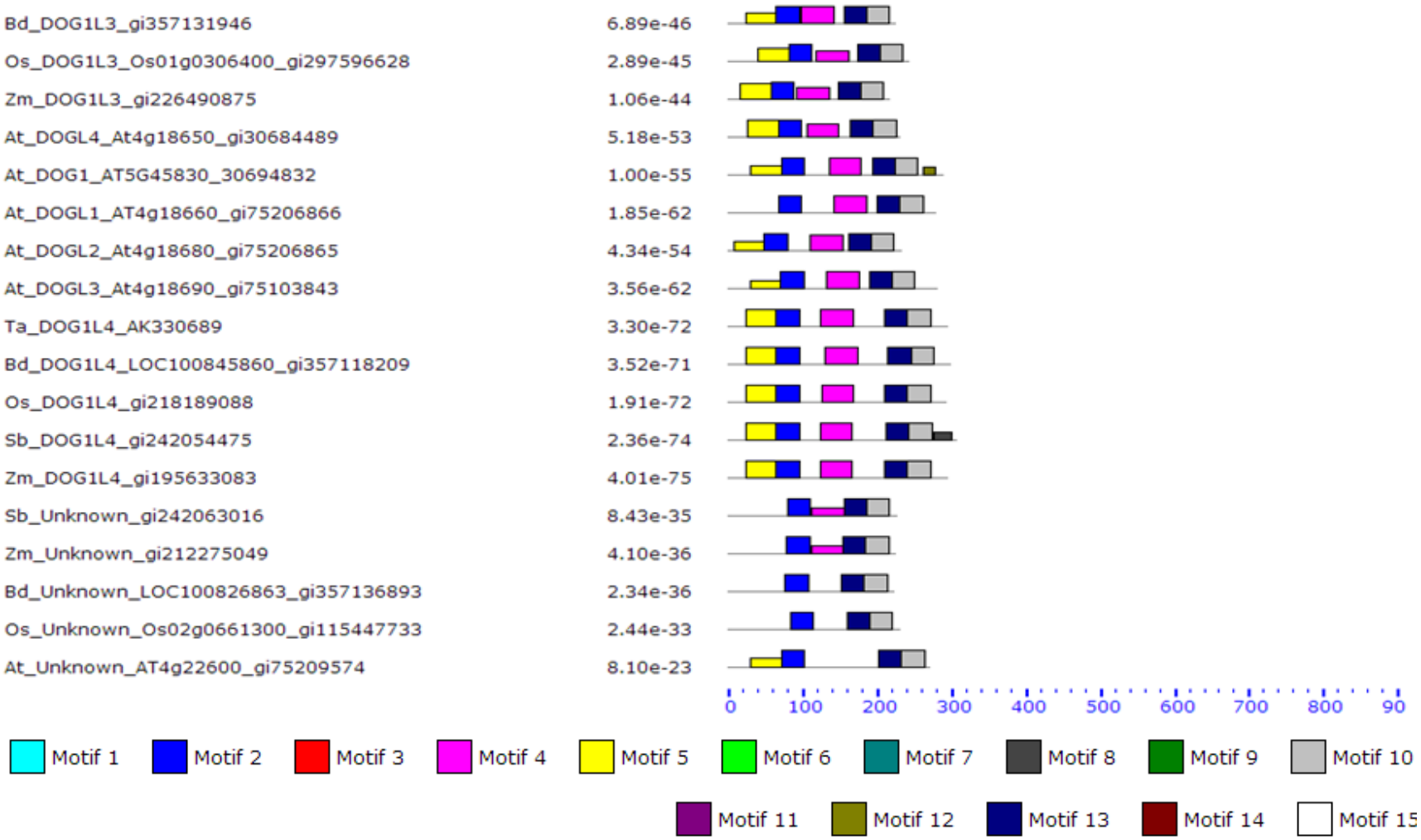

Figure 24. Motif distribution in the DOG1 and DOG1-like protein sequences predicted using MEME web server.

Comparative analysis and identification of candidate genes

A comparative analysis was then performed on the QTL for dormancy in wheat and rice to identify candidate genes for dormancy.

Wheat chromosome 3B *versus* rice chromosome 1

Dormancy QTL analysis reported in Mares et al. (2009) revealed a significant QTL on 3B flanked by markers *GWM77* and *WMC527*. This QTL might correspond to the QTL on 3B identified in Groos et al. (2002), which is positioned to the centromeric region of a QTL for grain colour. According to a comparison of the 3B consensus map and the deletion bin map, flanking markers *GWM77* and *WMC527* sit in two separate deletion bins, one proximal and the other distal to the location of the 3B centromere (C-3BS1-0.33 and C-3BL2-0.22) (Figure 25). Adjacent markers to the flanking markers have also been mapped to the two deletion bins. Analysis of the deletion bin-mapped wheat ESTs and their corresponding orthologs in rice revealed colinearity (Figure 26).

Flanking markers and nearest markers of the rice QTL for seed dormancy were collected and their sequences obtained. Scanning of the sequences against rice genomic DNA revealed adjacent genes to the markers. Through comparative analysis, the position of *X56782* was predicted to be located within the flanking markers of the 3B QTL identified in Mares et al. (2009) (Figure 27). Furthermore, the QTL on 3B could correspond to dormancy QTL identified in two studies in rice (Dong *et al.* 2003; Xie *et al.* 2011). Although the putative ortholog of *X56782* in rice wasn't located within any QTL regions in rice, the putative *TaDOG1L1* (*AB555729*) ortholog in rice (*Os01g0159000*) was situated within a rice dormancy QTL (Li *et al.* 2011). Gene *Os01g0142500* (*SLY1*/*Gibberellin Insensitive Dwarf 2*) was located within the same dormancy QTL. Wheat gene *D12921* did not seem to collocate within any QTL for dormancy. However, two rice genes with sequence similarity to protein phosphatase 2C, *HvABI1d* and *HvABI1e*, *Os01g0618200* and *Os01g0656200* could also correspond to the location of two markers for the

QTL of dormancy in rice (Dong *et al.* 2003; Seiler *et al.* 2011). Other genes such as *Os01g0583100* (sequence similarity to *Homology to ABI1*) collocate within the wheat dormancy QTL 3B. *Os01g232800*, a gene similar to ABI8, was also found to sit within the region of dormancy QTL in weedy rice identified in another study (Gu *et al.* 2005).

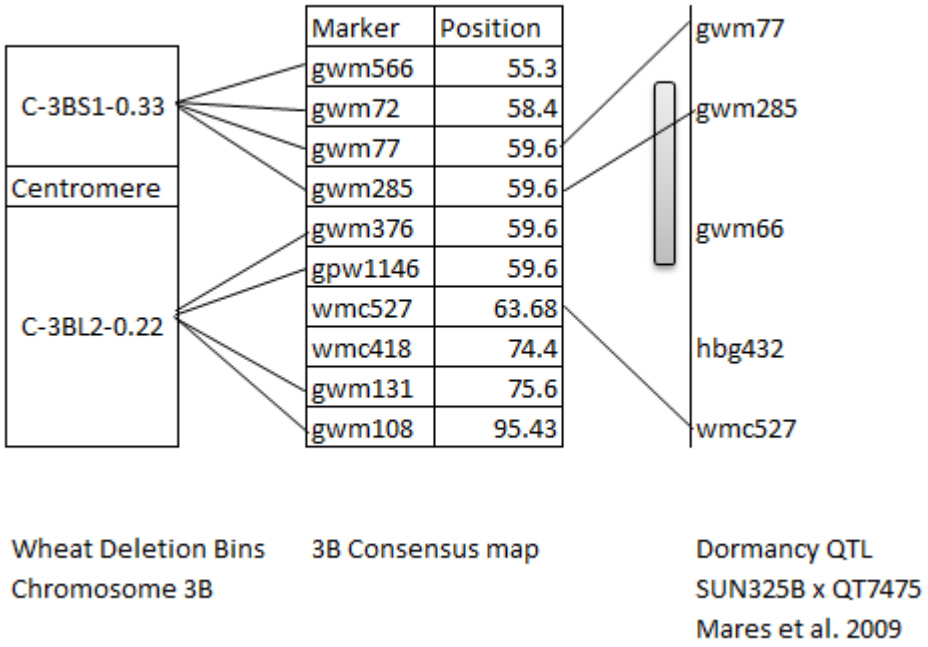

Figure 25. Flanking markers to the dormancy QTL on 3B in wheat and their adjacent markers sit on two different wheat deletion bins on chromosome 3B (Mares *et al.* 2009).

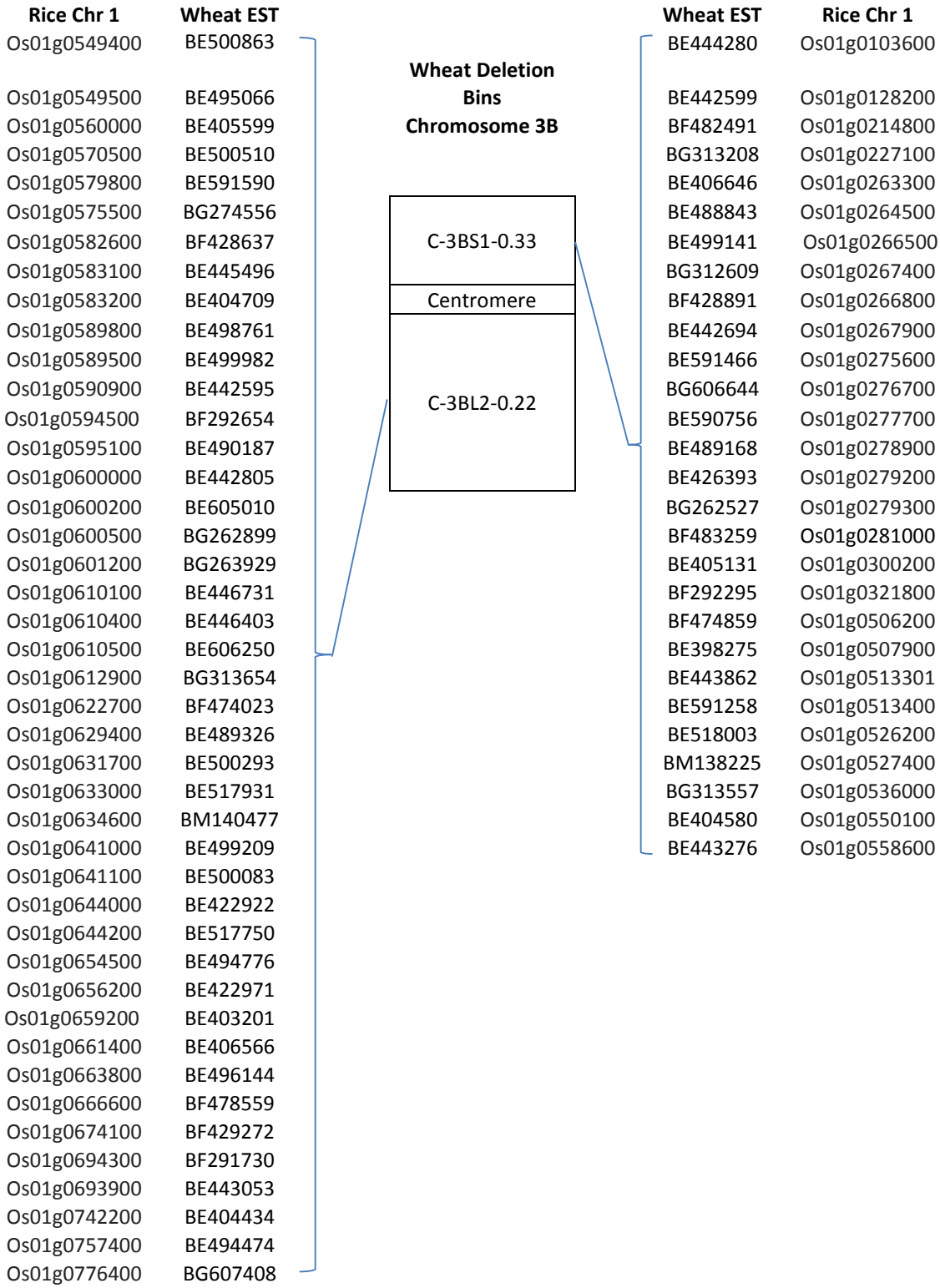

Figure 26. Colinearity shown between ESTs mapped in 3B deletion bins C-3BS1-0.33 and C-3BL2-0.22 with rice genes on chromosome 1 (Sorrells *et al.* 2003).

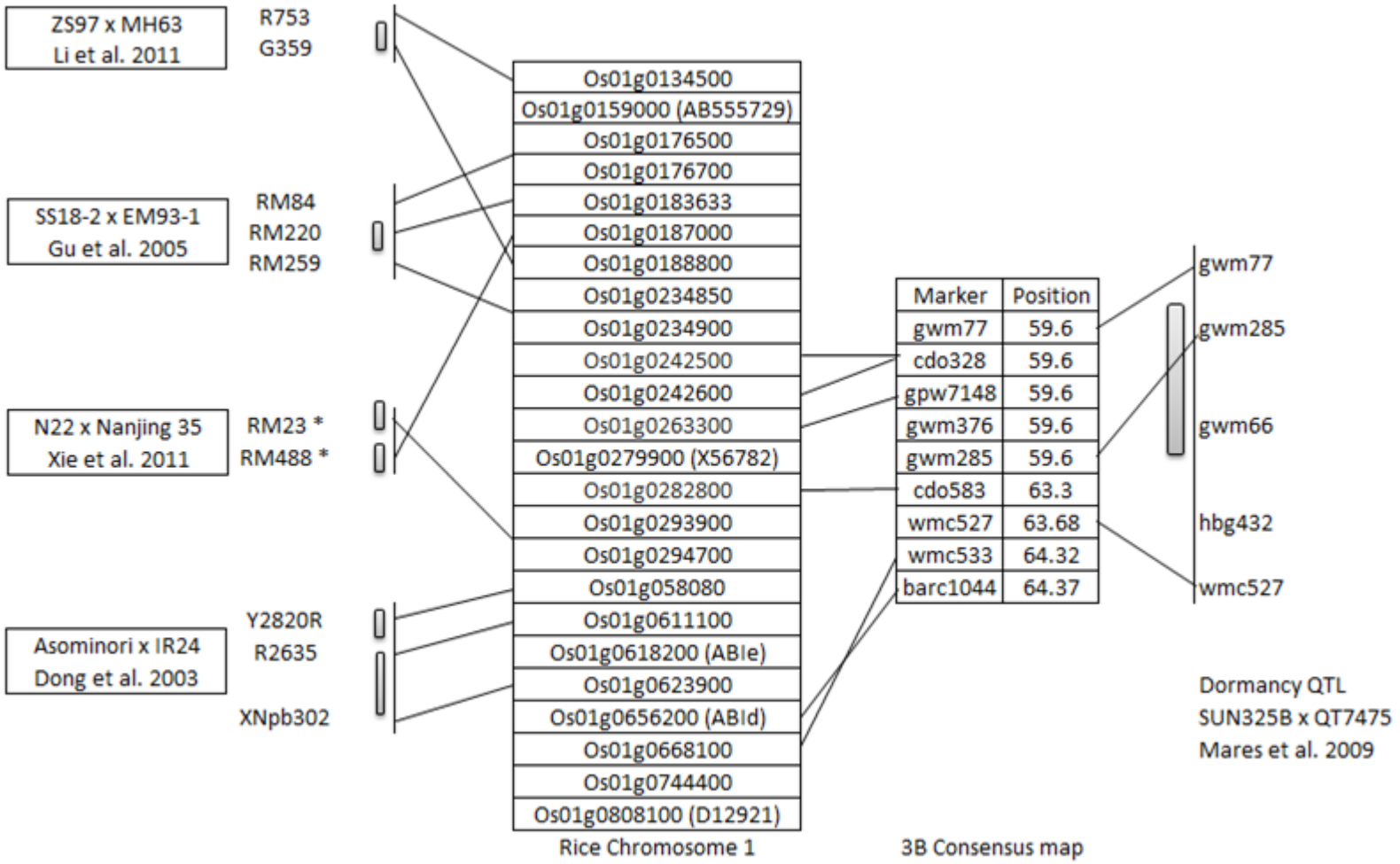

Figure 27. Comparative analysis between four reported rice dormancy QTL and a wheat dormancy QTL on 3B (Li *et al.* 2011; Gu *et al.* 2005; Xie *et al.* 2011; Dong *et al.* 2003; Mares *et al.* 2009).

Wheat chromosome 4A *versus* rice chromosome 3

Dormancy QTL analysis reported in Mares et al. (2005) revealed a significant QTL on wheat chromosome 4A flanked by markers *GWM269* and *BARC170*. These markers and their adjacent markers mapped in wheat deletion bin 4AL13-0.59-0.66 (Figure 28). Analysis of the deletion bin-mapped wheat ESTs and their corresponding genes in rice revealed a high degree of colinearity between the two regions (Figure 29).

However when compared, the wheat dormancy QTL on 4A did not seem to correspond to the rice QTL on chromosome 3 identified in cultivar Nipponbare of *japonica* rice (Hori *et al.* 2010). Analysing genes underlying the QTL region on 4A, only a transcription factor *TFIIS* (putative ortholog *Os03g81590*) appeared as a possible candidate gene. *Os03g015400* and *Os03g0153900*, genes similar to ABA biosynthesis genes in barley, *HvZEP3* and *HvZEP4* respectively, collocate with of rice dormancy QTL on chromosome 3 (Figure 30).

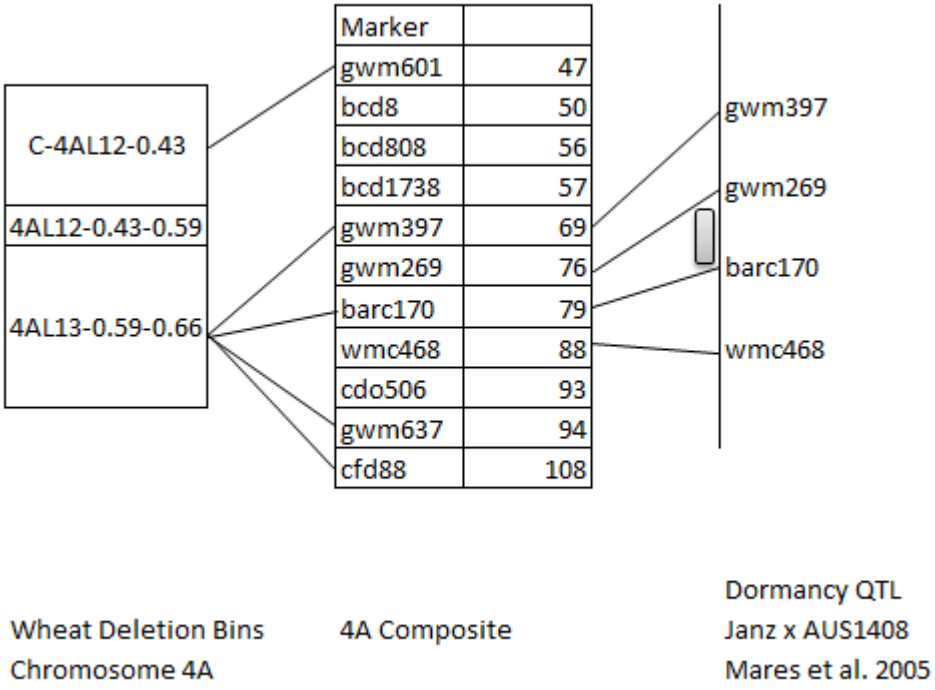

Figure 28. Flanking markers of the dormancy QTL on 4A in wheat and their adjacent markers mostly mapped on wheat deletion bins 4AL13-0.59-0.66 (Mares *et al.* 2005).

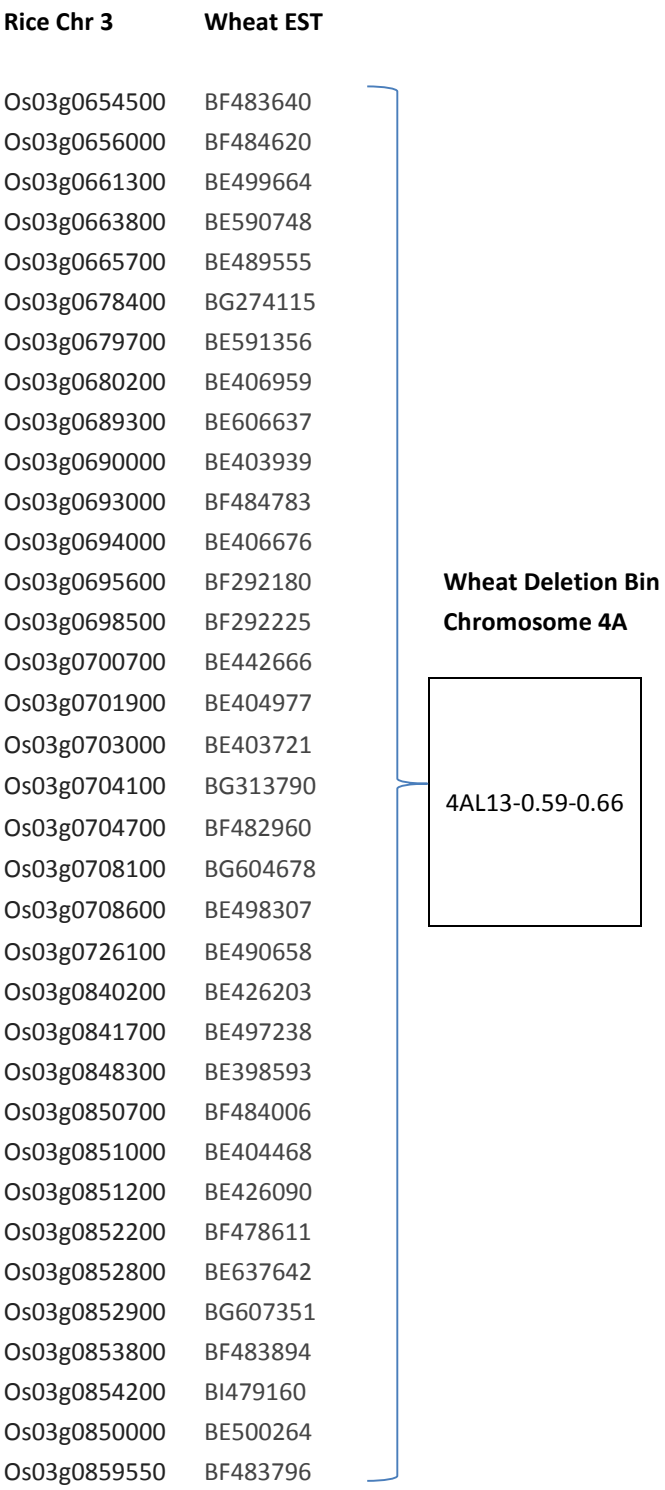

Figure 29. Colinearity shown between ESTs mapped in 4A deletion bin 4AL13-0.59-0.66 with rice genes on chromosome 3 (Sorrells *et al.* 2003).

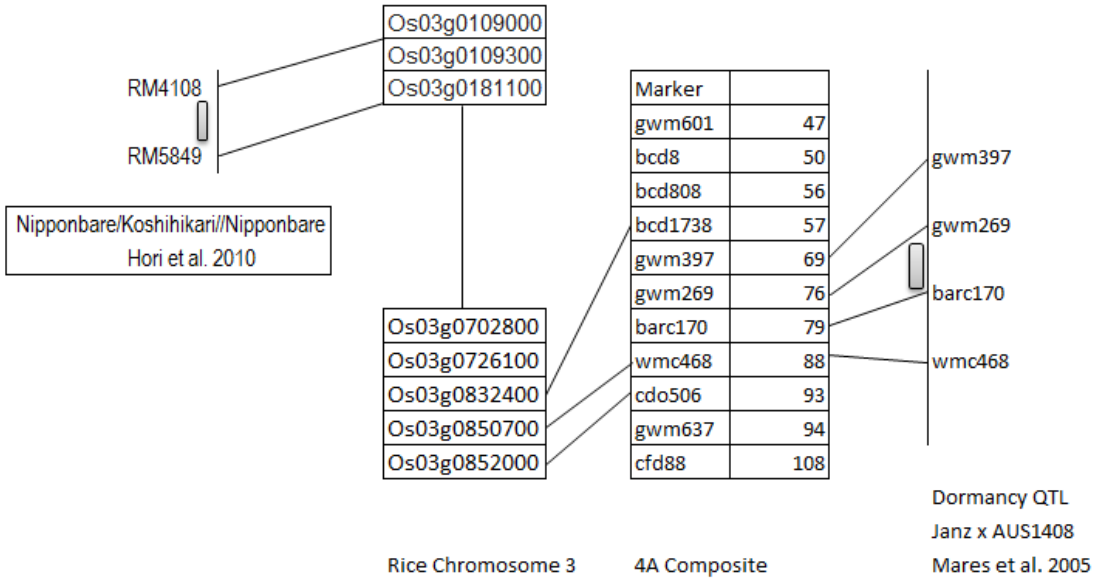

Figure 30. Comparative analysis showed rice dormancy QTL on chromosome 3 did not correspond to wheat dormancy QTL on chromosome 4A (Mares *et al.* 2005; Hori *et al.* 2010).

Wheat chromosome 2B *versus* rice chromosome 7

QTL analysis reported in Munkvold et al. (2009) revealed a significant QTL on 2B close to marker *WMC474*. The marker collocates with wheat deletion bin 2BS-0.53-0.75 (Figure 31). Analysis of the deletion bin-mapped wheat ESTs and their corresponding genes in rice revealed colinearity between the two regions (Somyong *et al.* 2011). *Os07g068770* (putative ortholog of *TaDOG1L5-1* or *AK330559*) was identified to sit very close to an EST (BE500206) within the same deletion bin as the marker *WMC474*. Further analysis also revealed that the rice dormancy QTL on chromosome 7 identified in rice might not correspond to the wheat QTL (Li *et al.* 2011).

Interestingly, comparative analysis revealed three barley genes, *HvSdr1*, *HvSdr2* and *HvSdr4* , which are putative orthologs of *Os07g0635000*, *Os07g0665000* and *Os07g0664400* in rice, identified in Seiler et al. (2011) as ABA biosynthesis genes to collocate in the same deletion bin, 2BS1-0.53-0.75. The three genes are adjacent to rice *ABAR/GUN5* gene (*Os07g0656500*), suggested as a candidate gene in Somyong et al. (2011). Not surprisingly, *Sdr4* (*Os07g0585700*) was identified as the corresponding gene for one identified QTL for dormancy in rice but is situated far towards the proximal end of marker *WMC474* if colinearity holds (Sugimoto *et al.* 2010; Lin *et al.* 1998).

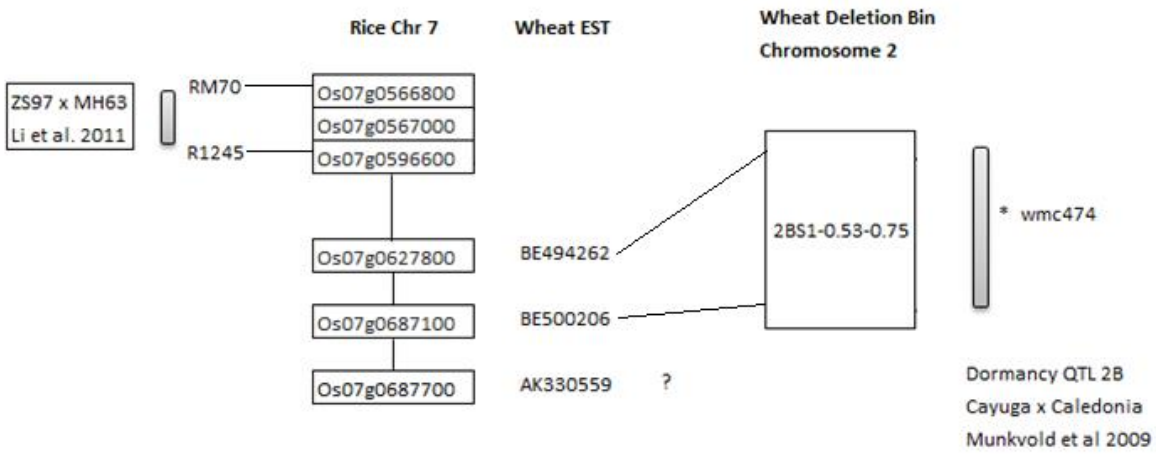

Figure 31. Nearest marker to the dormancy QTL on 2B in wheat, *WMC474* mapped on wheat deletion bin 2BS1-0.53-0.75 and a comparative analysis with rice dormancy QTL on chromosome 7 (Sorrells *et al.* 2003). Position of *TaDOG1L5-1* or *AK330559* was not verified in the deletion bin.

Wheat chromosome 1A *versus* rice chromosomes 5 and 10

QTL analysis in wheat reported a significant QTL on 1A close to marker *CFA2129* (Munkvold *et al.* 2009). The marker collocates with wheat deletion bin 1AL1-0.17-0.61. Analysis of the deletion bin-mapped wheat ESTs and their corresponding orthologs in rice revealed colinearity between the two regions (Figure 32). Interestingly, the rice dormancy QTL on chromosome 5 might correspond to the wheat dormancy QTL on chromosome 1. Through comparative analysis, *Os05g0560200* (putative ortholog of *AK332921*) was shown to collocate with the dormancy QTL of rice. Analysis of genes within the region also showed rice gene *Os05g0537400* (high sequence similarity to *HvABI1b*) to collocate with the dormancy QTL (Figure 33).

Wheat EST	Rice Chr 5
BE497600	Os05g0346200
BE498984	Os05g0346500
BE498861	Os05g0354400
BE499422	Os05g0355300
BE442559	Os05g0361500
BF483456	Os05g0363200
BE443068	Os05g0372100
BE404880	Os05g0377000
BE445834	Os05g0384800
BE406605	Os05g0386900
BF474819	Os05g0387200
BE442818	Os05g0388400
BE517991	Os05g0388500
BF484217	Os05g0389300
BM137729	Os05g0392700
BF292414	Os05g0393100
BF474634	Os05g0394200
BE496824	Os05g0399100
BE443930	Os05g0405000
BF428961	Os05g0430300
BE423345	Os05g0456200
BE442691	Os05g0458000
BE423193	Os05g0459900
BG606586	Os05g0474600
BF482683	Os05g0480600
BM137862	Os05g0481100
BG274294	Os05g0490200
BF483378	Os05g0490800
BE495028	Os05g0491100
BG314157	Os05g0495100
BE403885	Os05g0501700
BG604651	Os05g0510300
BE490233	Os05g0515500
BE591818	Os05g0518200
BF146193	Os05g0526400
BE406583	Os05g0532600
BF474284	Os05g0536200
BG263233	Os05g0540100
BE442940	Os05g0541200
BE637883	Os05g0543200
BE494527	Os05g0548900
BE490596	Os05g0553000
BF474340	Os05g0557700
BE399629	Os05g0565200

Figure 32. Colinearity shown between ESTs mapped in chromosome 1A deletion bin 1AL1-0.17-0.61 with rice genes on chromosome 5 and 10 (Sorrells *et al.* 2003).

1AL1-0.17-0.61

Wheat
Deletion Bin
Chromosome 1A

Wheat EST	Rice Chr 10
BE443622	Os10g0430900
BM134307	Os10g0432200
BE444527	Os10g0456200
BE443300	Os10g0481450
BE426097	Os10g0486600
BG607036	Os10g0480500
BG262947	Os10g0488100
BG607446	Os10g0488800
BG607906	Os10g0489200
BE637473	Os10g0490800
BG604953	Os10g0495300
BE494278	Os10g0498700
BG314205	Os10g0500600
BF200858	Os10g0500400
BG604744	Os10g0504600
BE494853	Os10g0505700
BE494376	Os10g0518200
BE442562	Os10g0521700
BE636978	Os10g0524500
BE638023	Os10g0542900
BE637209	Os10g0545300
BG608047	Os10g0554800
BE403956	Os10g0556100
BG314090	Os10g0556801
BE407013	Os10g0560450
BE352628	Os10g0561900
BE606632	Os10g0562100
BE497386	Os10g0567500
BE442716	Os10g0568200
BE497610	Os10g0571200
BF428897	Os10g0572300
BE498236	Os10g0572500
BG606995	Os10g0572700
BE500310	Os10g0574800
BE638005	Os10g0579600
BM134393	Os10g0579800

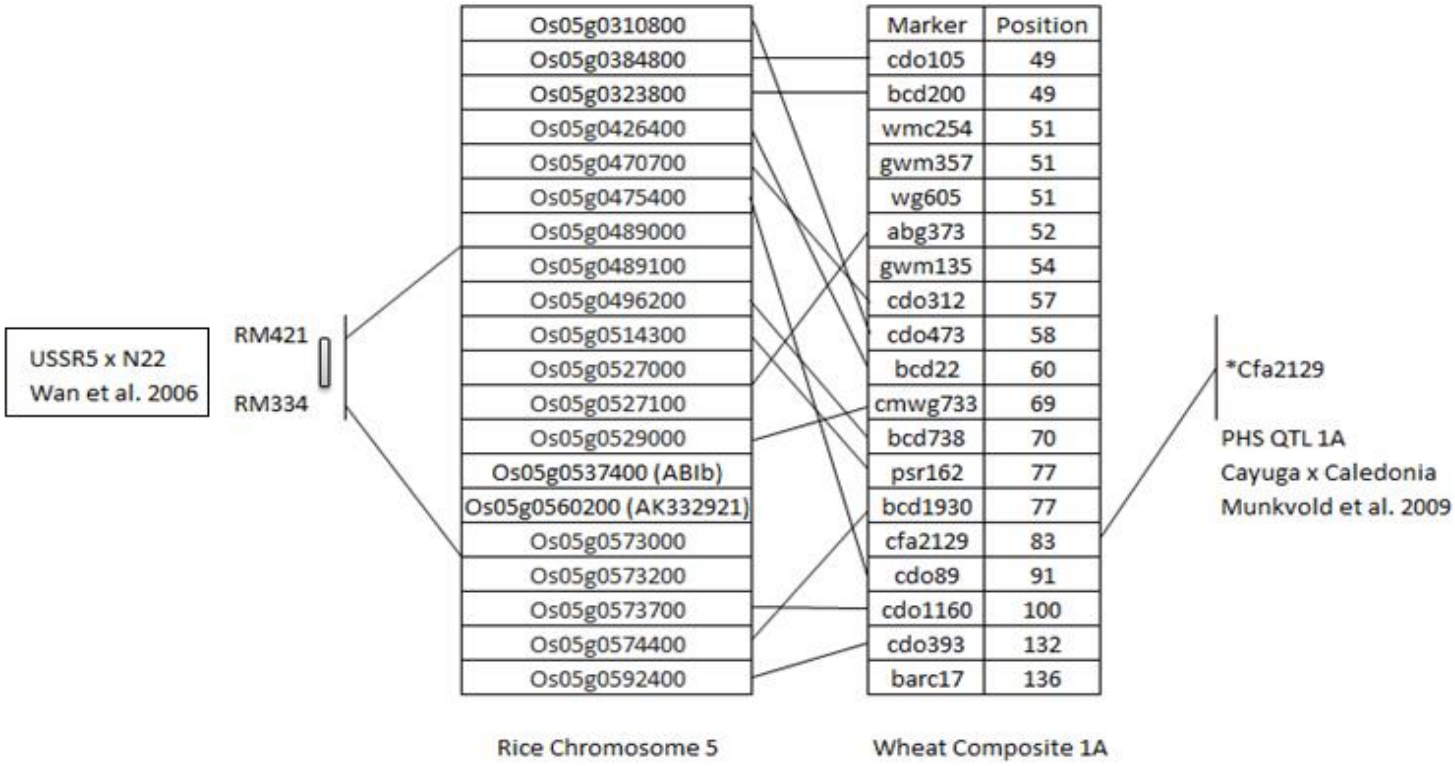

Figure 33. Nearest marker to the dormancy QTL on 1A in wheat, *CFA2129*, its adjacent markers and a comparative analysis with rice dormancy QTL on chromosome 5. *Os05g0537400* (putative ortholog of *HvABI1b*) and *Os05g0560200* (putative ortholog of *TaDOG1L2* or *AK332921*) seem to collocate with the dormancy QTL on 1A (Munkvold *et al.* 2009).

Quantitative RT-PCR expression analysis of *TaDOG1-like genes*

Expression levels of the *TaDOG1-like* genes were examined in SUN325B due to its severe change in dormancy levels.

Due to stronger bioinformatics evidence that suggests the dormancy functional conservation of *TaDOG1L1, TaDOG1L2* and *TaDOG1L4*, specific primers were designed to amplify these sequences in a quantitative RT-PCR analysis in dormant genotype SUN325B. RNA was extracted from previously stored embryo and de-embryonated grain samples collected at 30, 35, 45 and 65 dpa from control and temperature shock-treated plants (40°C for 5 days starting from 25 dpa to 30 dpa).

Significant expression levels of *TaDOG1L1* and *TaDOG1L2* were observed in embryo samples of control and temperature-shock treated grains at 30 dpa. Detectable expressions at later time points (30, 35, 45 and 65 dpa) were insignificant. There was also no expression of *TaDOG1-like* genes in the de-embryonated grain tissues.

Following temperature shock, there was no statistically significant differential regulation of *TaDOG1L1* expression (Figure 34). On the other hand, *TaDOG1L2* was shown to be downregulated almost 10-fold following temperature shock ($P < 0.01$) (Figure 35).

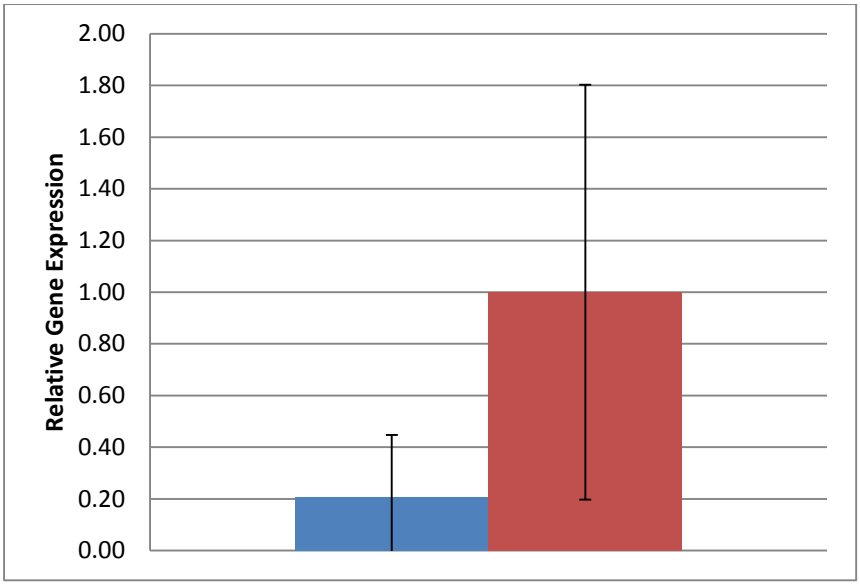

Figure 34. Expression levels of *TaDOG1L1* in embryo tissues of 5 days 40°C temperature-shocked (red) SUN325B at 30 dpa relative to control (blue) and. No detectable expression levels at later time points and also in the de-embryonated grain tissues. One way ANOVA was performed to determine statistical significance. P = 0.17.

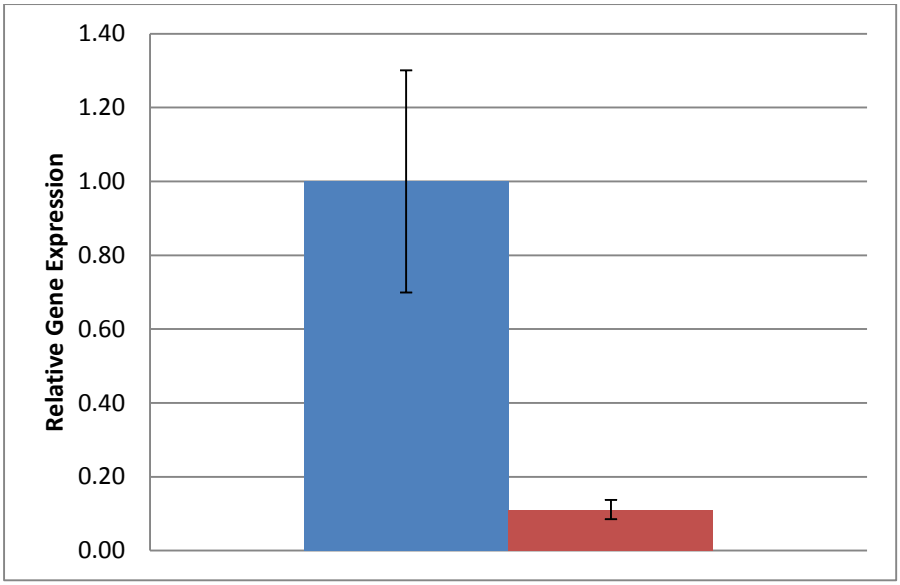

Figure 35. Expression levels of *TaDOG1L2* in embryo tissues of 5 days 40°C temperature-shocked (red) SUN325B at 30 dpa relative to embryo tissues of control (blue). No detectable expressions at later time points and also in the de-embryonated grain tissues. Temperature shock downregulates *TaDOG1L2* by 10-fold. One way ANOVA was performed to determine statistical significance. P = 0.007.

Discussion

Dormancy in white wheat

Dormancy is a complex mechanism which is influenced by genetic and environmental factors. Despite extensive research, our understanding of the regulation of dormancy is still incomplete. Dormancy, regarded as a major factor of resistance against PHS, is an important targeted trait for breeders. Introgression of high levels of dormancy into Australian white-grained wheat (*Triticum aestivum* L.), which generally exhibit low levels of dormancy, has been a difficult task due to the complexity of the dormancy mechanism. Historically, dormancy and PHS resistance have been associated with red grain colour linked to the "*R loci*", however, a relatively small number of dormant white-grained genotypes have been identified. This has led to the targeting of additional mechanisms underlying dormancy in white wheat (Mares *et al.* 2002; Flintham *et al.* 2002; Bassoi and Flintham 2005).

White wheat genotypes SUN325B (dormant), B14 (intermediate dormant) and Gladius (non-dormant) were analysed for their dormancy and response to high temperature shock in this study. This variation in dormancy was characterised by following the changes in grain germination index at intervals throughout ripening and after-ripening, giving rise to sigmoidal curves with significantly different times of release from dormancy. The differences in timing for the release of dormancy were reduced when proximal-half grains and were imbibed. In other words, seed tissues other than the embryo of white wheat grains contribute to the dormancy phenotype. This result is consistent with a previous experiment demonstrating that physical damage (such as slicing and abrading) results in partial losses of dormancy (Rathjen 2006).

Given such understanding, dormancy imposed by a seed coat might not solely be through mechanical restraint but possibly due to molecular interactions between the embryo and the endosperm or aleurone layer that involves ABA, GA and possibly other hormones. For example, ABA synthesised in the endosperm has been shown to be important for the induction

of dormancy possibly through the control of hormonal balance (Lefebvre *et al.* 2006). Expression of the ABA sensitivity component, *ABI4,* in *Arabidopsis* is embryo-specific but functions to assist the mobilisation of energy reserves in the endosperm for the germination process whereas *ABI5* was found to be expressed in the micropylar endosperm, suggesting important roles for both embryo and endosperm ABA signalling mechanism during seed germination (Penfield *et al.* 2006). Such interaction between the two different tissues can also be suggested in barley where differential expression of ABA signalling components (including *RCAR*, *ABI1* and *SnRK*) in both the embryo and endosperm were observed (Chen *et al.* 2013). In *Lepidium sativum*, although embryo signals are important for the stimulation of endosperm weakening during germination, endosperm GA biosynthesis is required for the remaining of the process (Mueller *et al.* 2006).

In this study, a prolongation of dormancy observed when a high concentration of ABA was applied during imbibition of proximal halves of grains suggests that the major source of ABA might be from the aleurone or endosperm fraction of the grain whereas ABA sensitivity components localised in the proximal-half of grains is sufficient for dormancy. Such high dormancy maintained by the application of ABA was shown to surpass the dormancy observed of whole grains in some instances. B14 and SUN325B which are more dormant, experience a lesser effect (dormancy release close to dormancy release of whole grains) when ABA was applied during imbibition in comparison to the profound effect observed in Gladius. This suggests that Gladius could contain components of ABA sensitivity that are longer lasting and capable of being responsive to ABA, but inhibited in normal imbibition conditions. This however requires further validation.

Observations of effects of extreme maternal temperature on dormancy in wheat and the presence of a sensitive window

To complicate the mechanism of dormancy, environmental changes play a major role in determining dormancy at harvest maturity. The effect of temperature changes during seed development on dormancy at harvest maturity have been reviewed (Fenner 1991; Donohue 2009; Kendall and Penfield 2012).

In a field trial experiment in 2009, an intermediate dormant wheat variety, QT7475 experienced maximum daily temperature conditions of 40°C or above for a period of 8 days during grain development between 25 to 35 days post anthesis (dpa) (Mares unpublished data). This hot spell coincided with the later stages of grain maturation of other fast growing genotypes in the same trial. Compared with data from previous trials, QT7475 seemed to have behaved in a non-dormant manner with higher germinability comparable to a non-dormant genotype, Hartog. This observation suggested that such a temperature shock could interact with a critical stage during grain development to significantly affect dormancy at harvest-ripeness.

This observation is consistent with reports that in barley, a "window" exists during grain maturation within which exposure to high temperature affects dormancy (Gualano and Benech-Arnold 2009). In the current study, five days of temperature shock treatment of 40°C day/25°C night was applied on various times after anthesis (25 dpa to 55 dpa). Treatments applied on 25 dpa, 30 dpa or 35 dpa had the most significant effect on dormancy. The treatment caused a dormant variety SUN325B to reach GI of 0.5 by as early as 65 dpa, whereas the GI of continuous glasshouse condition-grown controls remains below 0.5 surpassing 100 dpa. The intermediate dormant variety, B14 responded similarly to the treatment, exhibiting non-dormant behaviour at harvest maturity. Hence, this is highly similar to the case in barley where dormant genotypes were shown to remain dormant at harvest maturity, whereas many intermediate dormant varieties

were shown to become non-dormant by temperature changes (Gualano and Benech-Arnold 2009).

The sensitivity towards high temperature shock increases and reaches a peak around 25 to 30 days post anthesis (dpa), causing an effective and quick release of dormancy. The sensitivity is seemingly lost at 40 dpa and thereafter as we observe little or no effect on dormancy levels in the three genotypes. Hence, the molecular mechanism behind this effect is limited to a specific window, which due to factors unknown, and peaks at 25 to 30 dpa. This experiment attempted to determine whether the occurrence of the peak can be influenced by factors such as temperature or humidity before the treatment or the subsequent rate of moisture loss. Higher temperature during grain development in wheat has been shown to accelerate development of the grain (Nicolas *et al.* 1984). Whilst there seems to be a lack of correlation between the occurrence of the peak and these factors, there were other factors not included in this study such as water application, fertiliser application or light quality, which have all been implicated with the mechanism of dormancy. Therefore, the molecular mechanisms behind the effect of temperature shock on dormancy could be independent of wheat grain development.

Temperature shock affects seed coat and seed weight

In plant species such as *Stylosanthes hamate* and *Plantago lanceolata*, the thickness of the seed coat is positively correlated with the temperature during seed formation (Argel and Humphreys 1983; Lacey *et al.* 1997). In addition, plants species such as *Syringa vulgaris* develop strong mechanical resistance at high temperatures (Junttila 1973). In both cases strong mechanical resistance promotes dormancy. In *Lactuca sativa*, the pericarp layer of the seed coat was thicker when matured in higher temperatures (Drew and Brocklehurst 1990). Temperature following anthesis regulates the quality of the seed coat in many plant species which subsequently affects seed dormancy. Besides mechanical resistance, permeability towards

water or gasses might play a role in regulation of dormancy (Finch-Savage and Leubner-Metzger 2006). Such dormancy might exist in *Arabidopsis*, but temperature changes were shown to not affect seed coat morphology (Haughn and Chaudhury 2005; Kendall and Penfield 2012).

In the case of wheat, mechanical resistance of the seed coat is not well-reported. Furthermore, rate of water uptake was reported to be similar between non-dormant and dormant wheat grains (Rathjen *et al.* 2009). In this experiment, a significant reduction in seed weight was observed when temperature shock was applied at 25 dpa. This is a typical effect of temperature after anthesis on grain size (Chowdhury and Wardlaw 1978). However, the thickness of the seed coat was not analysed

Another interesting observation is that the temperature sensitive "windows" for effects on seed weight and dormancy may not be synchronous. Different from dormancy alleviation, seed weight was consistently, effectively and significantly reduced when temperature shock was applied at 25 dpa in the field and glasshouse, whereas the timing of effective temperature shock to reduce dormancy varied between 25 dpa and 30 dpa. This suggests that seed weight determination and dormancy reduction due to temperature shock involve independent mechanisms.

Dormancy affected by both temperature shock intensity and duration

Temperature stresses that influence plants processes can be separated according to the intensity and the duration of temperature imposed on the plant (Wahid *et al.* 2007). In this study, higher temperature shock that did not appear to physically damage the plants seemed to positively correlate with the effect on dormancy. Similarly with the duration of temperature shock, where longer temperature shock had stronger effect on dormancy.

Dormancy affected by temperature shock is related to ABA sensitivity but not ABA content

Fenner (1991) proposed the possibility of germination-inhibiting substances being produced when the maternal plant experienced lower temperatures whereas germination-promoting substances are being produced when experiencing higher temperatures. Up until now, the proposed "germination-inhibiting substances" remain unknown. Most explanations revolve around hormones GA and ABA in terms of regulation of concentration and sensitivity, the latter being more important in dormancy. However, often there is no clear relationship between the degree of dormancy and the concentration of hormones (Kermode 2005).

In barley, an earlier peak of ABA concentration in the embryo was reported when plants were matured in higher temperatures (3 weeks after pollination) but then concentrations fell back to be similar to non-treated controls (Goldbach and Michael 1976). In wheat, seeds consistently matured at a higher temperature experienced lower dormancy and an earlier peak of ABA synthesis in the embryo (Walker-Simmons and Sesing 1990).

Although we reported that the temperature shock at 25 dpa causes an early and higher increase of ABA in the embryo, ABA fell to a level similar to the control shortly thereafter. Furthermore, ABA detected in the endosperm showed a rapid fall in concentration in comparison to the control endosperm, which is not consistent with the role of ABA as a promoter of dormancy. Regardless, ABA levels in the endosperm-half of a dry grain were similar.

In this case, it is also possible that the determination of dormancy was not due to ABA content of dry grains but ABA content changes following imbibition. In barley, ABA content in the embryo of dry grain was reported to be similar for non-dormant and dormant genotypes but a more rapid decrease of ABA was found in the non-dormant genotype following imbibition (Millar *et al.* 2006). Dormancy breaking mechanisms such as after-ripening was also shown to cause a more rapid decrease of ABA content upon imbibition (Jacobsen *et al.* 2002).

Sensitivity towards hormone ABA is crucial to the maintenance of dormancy (Nambara *et al.* 2010). To date, many ABA signalling components have been identified as regulators of ABA sensitivity in *Arabidopsis*. ABA signalling components such as, *ABI1*, *ABI2*, *ABI4*, *ABI5, Enhanced Response to ABA 1* (*ERA1*) have been shown to be required for ABA sensitivity (Gosti *et al.* 1999; Finkelstein *et al.* 1998; Finkelstein and Lynch 2000).

A lack of ABA sensitivity has been reported to be a characteristic of lower dormancy. This suggests that either the temperature shock was not severe enough to alleviate dormancy in SUN325B by desensitising the seed towards ABA or the existence of additional resistance mechanisms of dormancy in SUN325B. Generally, the mechanism of temperature shock-affected dormancy seems to share some similarities with the mechanism of after-ripening in terms of hormone ABA. Similar patterns of ABA sensitivity reduction and regulation of signalling components were observed. On the other hand, ABA content of the dry dormant and after ripened seeds were always observed to be similar, but differences are observed when imbibed (Liu *et al.* 2013a; Barrero *et al.* 2009).

After 5-6 days of drought during seed development, a significant increase in *Sorghum bicolor* seed germinability was observed. Changes in both ABA content and sensitivity were similar to the effects of temperature shock. ABA content was found to spike when water stress was introduced but reduced to lower levels by the end of maturation (Benech Arnold *et al.* 1991).

Response of different genotypes towards temperature shock

Benech-Arnold et al, (2001) pointed out that barley cultivars with early dormancy release or long-lasting dormancy are not affected by environmental temperature. Non-dormant cultivars of barley will remain sprouting-susceptible whereas dormant cultivars will remain sprouting-resistant. Cultivars with intermediate dormancy levels may behave as sprouting-susceptible or

sprouting-resistant at harvest maturity depending on the environmental factors to which they are exposed.

This study showed that Gladius remained non-dormant whereas for SUN325B, although suffering a significant loss of dormancy, dormancy release was still 5 to 10 days after harvest maturity. Hence, they remain sprouting-susceptible and sprouting-resistant, respectively at harvest-ripeness. An analysis of a list of intermediate dormant to dormant genotypes with various dormancy QTL combinations showed most genotypes respond strongly to high temperature shock. GIs were observed to be significantly higher at 65 dpa compared with controls. The exceptions appeared to be the genotypes P07.683 and 50213/Cunn799, which displayed high levels of dormancy at 65 dpa even after temperature shock. Not surprisingly since 4A is a major QTL of dormancy, control genotypes with QTL 4A seem to have low GI values. Combination of 4A QTL and other QTL such as 3A and 3B seems to confer stronger dormancy at 65 dpa. However, following temperature shock, dormancy levels alter irrespective of the presence of any single QTL. The presence of combinations of QTL, in most cases 4A + (3A or 3B), appeared to be associated with stronger resistance towards temperature shock although it is acknowledged that these genotypes also contain other unidentified dormancy QTL. The expression of a dormancy QTL on chromosome 7L of barley for example is highly influenced by temperature (Gao *et al.* 2003). P07.683 and 50213/Cunn799 contain QTL 4A, 3AS and other unknown QTL.

Generally, this study suggests that higher levels of dormancy that can be maintained longer after harvest (by introgression of combinations of QTL for dormancy) is best to prevent PHS because intermediate dormant genotypes can be adversely affected by temperature shock to become susceptible to sprouting. Finally, understanding the relationship between dormancy QTL and maturation temperature/temperature shock would be advantageous for breeding programs into the future.

***TaDOG1-like genes* as candidates of molecular change following temperature shock**

DOG1, identified as the gene corresponding to the dormancy QTL in *Arabidopsis*, has been shown to accumulate during grain maturation. High levels of *DOG1* expression have been shown to be correlated to dormancy levels (Kendall and Penfield 2012; Bentsink *et al.* 2006). In addition, low seed maturation temperature caused an increase in *DOG1* expression levels (Nakabayashi *et al.* 2012; Kendall and Penfield 2012). Hence, it is highly likely that *DOG1* expression is regulated in wheat seeds when temperature shock is applied.

The Delay of Germination 1 (DOG1) conserved domain (pfam14144) is common to a family of plant proteins that appears to be specific in controlling dormancy in seeds (Bentsink *et al.* 2006) although little is known about the exact function of this domain. Furthermore, research on the orthologs of *AtDOG1* is still lacking even though there has been some progress in the identification and characterisation of putative orthologs of the gene in the member species of the Brassicaceae family such as, *Brassica rapa* and *Lepidium sativa,* and in the*Triticeae* family such as, *Hordeum vulgare* and *Triticum aestivum* (Ashikawa *et al.* 2010, 2013; Rikiishi and Maekawa 2010; Graeber *et al.* 2010).

In *Arabidopsis*, there are four *DOG1-like* genes with low sequence similarity ranging from (23.4% to 54.3%) and not surprisingly, mutations in these genes did not reduce dormancy (Bentsink *et al.* 2006). In an attempt to identify the wheat orthologs of *DOG1* and *DOG1-like*, a bioinformatics search was conducted and did not reveal sequences with high sequence similarity to *AtDOG1* or *AtDOG1L* genes. However, seven low similarity sequences were identified. Among the seven wheat sequences, four have been previously identified as *TaDOG1L1* (*AB555729*), *TaDOGL2* (*AK332921*), *TaDGO1L4* (*AK330689*) and *TaDOG1L5-1* (*AK330559*), whereas three (*AK33621*, *D12921* and *X56782*) have not previously been reported (Rikiishi and Maekawa 2010; Ashikawa *et al.* 2010, 2013). These genes show high sequence similarity to their orthologs in *Brachypodium*, rice and barley. In a phylogenetic analysis, the 5 clades described in Ashikawa

et al. (2013) were observed although the overall tree showed a higher density of sequences. *TaDOG1L1*, *TaDOG1L2* and *TaDOG1L4* were shown to be closest to the *AtDOG1* and the *AtDOG1-like* genes in terms of evolutionary distance in comparison to *TaDOG1L5-1*, *AK336217*, *D12921* and *X56782*. *TaDOG1L1*. *TaDOG1L2* and *TaDOG1L4* have been shown to be expressed in seeds, increase seed dormancy in *Arabidopsis* through an ectopic expression study (Ashikawa *et al.* 2010, 2013). More recently, *TaDOG1L4* was shown to increase seed dormancy in transgenic wheat (Ashikawa *et al.* 2014). However, the reason functional conservation was observed between *TaDOG1L1-4* and *AtDOG1* but not *AtDOG1L1-4* despite low sequence similarity to all *Arabidopsis* sequences remains unknown.

A conserved domain search revealed the presence of the cl02576 superfamily of domains in the reading frame of the *TaDOG1-like* genes which are not from Clade 1-4. The cl02576 domain consists of a basic region and heptad repeat of leucines (Jakoby *et al.* 2002). The basic region contains a nuclear localisation signal and a motif that binds to the DNA. Although the conserved domain scan did not reveal the same superfamily domain in the *Arabidopsis DOG1* sequence, it has been reported that the protein is localised in the nucleus (Nakabayashi *et al.* 2012). *AtDOG1* forms three isoforms through alternative splicing and the self-binding capability of all three isoforms is required for a proper function of the protein (Nakabayashi and Soppe 2013). Most of the cl02576 superfamily domain-containing proteins are transcription factors but no such evidence has been presented for *DOG1*.

When the promoter sequences of the *TaDOG1-like* genes were analysed, ABA-Responsive Elements (ABRE), [ACGTG] was consistently found in many of the putative orthologs, suggesting that ABREs might play a role in the regulation of these genes. Another element that is consistently present in the promoter sequences is the RY repeat [CATGCA]. Proteins such as ABI5 and ABI3 have been respectively shown to bind to these sites, indicating that these genes might be expressed as an ABA mediated response (Ezcurra *et al.* 2000; Choi

et al. 2000). This is consistent with analysis of promoters in *Arabidopsis*, *Brassica* and *Lepidium* although the position of the ABREs differs among different species (Bentsink *et al.* 2006; Graeber *et al.* 2010). Similarly, RY and ABRE motifs are also present in *SDR4* and *MFT* (causal genes for dormancy QTL in rice and wheat respectively), suggesting a highly conserved function of RY and ABRE promoter motifs-containing genes in dormancy (Sugimoto *et al.* 2010; Xi *et al.* 2010).

Protein function is predicted based on sequence similarity, often relying of BLAST methods (Altschul *et al.* 1997). However, protein motifs (short, highly conserved regions of proteins) that often correspond to catalytic sites and binding sites, can also be reliable in predicting protein function (Falquet *et al.* 2002). Protein motif analysis showed that among the pfam14144 domain-containing sequences, specific combinations of predicted motifs in the sequences differentiate them into specific clades. The combination of motifs 5, 2, 4, 13 and 10 (see table 5) is a sequence characteristic of AtDOG1, AtDOG1L1-4 and wheat members of Clade 1, 3 and 4 whereas other wheat sequences were found to contain a combination of motifs 5, 2, 4, 9, 3, 6 (see table 5). Hence, it is possible that functional conservation might be absent among these two groups of proteins. On the other hand, protein motif similarities sometimes do not signify functional similarity. For example, *AtDOG1L1-4* clearly have similar motif combination of 5, 2, 4, 13 and 10, but were shown to not affect *Arabidopsis* dormancy (Bentsink *et al.* 2006). Perhaps they have functional redundancies that allow the preservation of dormancy phenotype when singularly deleted, but further validation is required. With reservation, protein motifs combinations 5, 2, 4, 13 and 10 might be specific for the function of dormancy.

Comparisons of *DOG1* with *MFT* and *Sdr4* As Candidate Genes

DOG1, Sdr4 and *MFT* have been shown to be genes associated with some dormancy QTL in *Arabidopsis*, rice and wheat respectively (Liu *et al.* 2013b; Sugimoto *et al.* 2010). Promoter analysis suggests that *DOG1*, *Sdr4* and *MFT* expression could be regulated via the ABI3 and ABI5 protein mediated pathway. In addition, all three genes were localised in the

nucleus and expressed in the embryo, which suggests their role as transcriptional regulators in the embryo region.

However, only *MFT* and *DOG1* were shown to be regulated by temperature during seed maturation (Kendall and Penfield 2012; Nakamura *et al.* 2011). In addition, only *MFT* and *Sdr4* mutants were shown to exhibit a change in ABA sensitivity (Sugimoto *et al.* 2010; Xi *et al.* 2010).

The mechanism of action of *MFT* in the induction of dormancy is limited to the early stages of grain development. When *MFT* is introduced later during grain development, it may not contribute to dormancy levels at harvest maturity (Nakamura *et al.* 2011). This excludes *MFT* as a candidate responsive to the temperature shock.

Hence, due to various factors mentioned, the *TaDOG1L1*, *TaDOG1L2* and *TaDOG1L4* were chosen as candidate genes for expression analysis.

Comparative analysis reveals candidate genes

Given the progress in genomic sequencing in *Arabidopsis*, rice and wheat, comparative analysis has become easier. It is a useful tool to identify candidate genes or development of new markers for QTL analysis. Analysis of specific intervals consistently discovered specific gene order between closely related grass species, wheat and rice (Sorrells *et al.* 2003). Micro-colinearlity may vary and chromosomal rearrangements and discontinuities do occur between wheat and rice (Sorrells *et al.* 2003; Distelfeld *et al.* 2004). However, macro-colinearity could be expected and has been observed in many different studies (Devos and Gale 2000; Somyong *et al.* 2011; Paux *et al.* 2008; Liu *et al.* 2006).

Via comparative analysis, this study aimed to identify the possible chromosomal locations of *TaDOG1-like* genes and compare existing wheat QTL to corresponding QTL on rice. This also helped to identify potential candidate genes that could be regulated by temperature shock during grain development. Among the seven *TaDOG1-like* genes, only *X56782* has been placed within a dormancy QTL region of wheat on chromosome 3. Only two ortholog sequences

(*Os01g0159000* and *Os05g0560200*) with high sequence similarity towards the *DOG1* in rice were placed within the QTL regions of rice on chromosome 1 and 5 respectively. Interestingly, three genes with similarities to the barley *HvSdr1*, *HvSdr2* and *HvSdr4* sit inside a fairly small deletion bin on chromosome 2A together with the nearest marker to the PHS QTL. Rice *Sdr4* was identified by Sugimoto *et al*. (2011) as the causal gene for the rice dormancy QTL on chromosome 7 and its wheat ortholog might show a great potential as a candidate gene for the QTL on 2B.

ABI1 is a member of the protein phosphatase 2C family and is an important component of ABA signalling (Umezawa *et al.* 2009). Mutations of this gene confer loss of ABA sensitivity. One gene identified in rice that shares high sequence similarity to *ABI1b* of barley collocated with the dormancy QTL in rice on chromosome 5. Two other genes similar to *ABI1d* and *ABI1e* sit near to two nearest markers for two dormancy QTL on rice chromosome 1. In addition to ABA signalling, ABA biosynthesis genes were found to sit within a QTL region in rice on chromosome 3. Two genes with high sequence similarity to *HvZep3* and *HvZep4* were within the dormancy QTL on chromosome 3 in rice.

This study presents novel candidate genes that are closely related to ABA biosynthesis and ABA signalling that could correspond to dormancy QTL in wheat and rice. They represent new targets for future marker developments and fine mapping of dormancy QTL in wheat.

Gene transcription and dormancy

Transcription elongation factor, TFIIS is required for the efficient transcription by RNA Polymerase II (Grasser *et al.* 2009). Mutation analysis showed that *TFIIS* mutants show normal development besides earlier flowering, low dormancy and lower expression of *DOG1* (Mortensen and Grasser 2014). It seems that facilitation of gene transcription that involves *TFIIS* might be specific for the effect on dormancy. In response to ABA application on dormant wheat grains,

wheat transcriptomic analysis showed the repression of transcription genes related to chromatin assembly and cell wall modification (Liu *et al*. 2013a). In another study, *Histone Monoubiquitination 1* (*HUB1*) mutation was found to reduce seed dormancy, suggesting that changes in chromatin remodelling might be important for the transcriptional expression of genes important for the release of dormancy (Liu *et al.* 2007). *Histone deacetylase 6* and *histone deacetylase 19* are important chromatin remodelling factors in eukaryotes. Mutation analysis showed regulation of key embryogenesis genes such as *ABI3*, (Tanaka *et al.* 2008). All this evidence points to the regulation of dormancy.

Interestingly, two of the proposed *TaDOG1-like* genes in this study were identified to encode Histone promoter-binding proteins, members of the cl02576 protein family. These proteins form dimers and bind to specific promoter sequences, regulating gene expressions (Mikami *et al.* 1994; Shen and Gigot 1997). This is also consistent with the isoforms of *Arabidopsis DOG1* that forms dimers to be functional. Finally, the nuclear localisation of the three identified causal genes for dormancy QTL showed possible induction and maintenance of dormancy through a complicated yet specific manipulation of gene transcription.

Dormancy reduction of SUN325B following temperature shock could be regulated by *TaDOG1-L2*

Dormancy induction in *Arabidopsis* involves the hormone ABA and the regulation the expression of *AtDOG1* gene (Bentsink *et al.* 2006). *AtDOG1* expression is strongly influenced by the temperature of the maternal environment (Kendall and Penfield 2012). Although *TaDOG1-L1-4* genes showed low sequence similarity to *AtDOG1*, regulation of their expression influences dormancy of wheat (Ashikawa *et al.* 2014). Such functional conservation suggests the possibility of wheat possessing a similar mechanism of dormancy through the regulation of *TaDOG1-like* genes.

Indeed, the expression of *TaDOG1-L2*, was limited to the embryo region in the seed (mRNA detected at 25 and 30 dpa) where a significant down-regulation was detected following temperature shock, consistent with the observed increase germination index at 65 dpa. Messenger RNA of *TaDOG1-L1* and *TaDOG1-L2* was not detected at later times, 35, 45 and 65 dpa, signifying a short lived mRNA, which is also consistent with the pattern of expression of *AtDOG1* (Bentsink *et al.* 2006). Interestingly, *TaDOG1-L1* increased in expression following temperature shock but changes were not significant. Hence, it is possible that *TaDOG1-L1* is not functionally redundant to *TaDOG1-L2*. However, this requires further validation.

However, the down regulation should be interpreted with caution due to the extreme temperature affecting the stability of the reference genes. Although a flat gene expression was not detected for both reference genes, the difference of ct (PCR cycle) value was small. Given the nature of this extreme treatment, it is difficult that a completely stable reference gene could be found. Further research could be dedicated to the discovery of such reference genes. Given the common occurrence of such high temperature shocks in the natural environment of Australia, the need for a stable reference gene is required for future qPCR experiments.

This analysis only addresses one of the few environmental condition changes that alters dormancy levels in wheat. No evidence has yet to be presented to suggest that different environmental condition changes alters expression levels of different *TaDOG1* genes. However, the promoter analysis in this study could help shed light to the temperature control of gene expression. Furthermore, the functional conservation of all these genes has yet to be established.

Conclusion

Global climate change is causing unpredictable temperatures and rainfall patterns. Understanding seed dormancy is essential to aid the development of wheat cultivars that are PHS-resistant. This work has contributed to the understanding of wheat seed dormancy:

1) Wheat grain development is most sensitive to high temperature during a specific window that was captured between 25 dpa to 35 dpa. Exposure to a few days of high temperature during the window significantly affects the germinability of dormant and intermediate dormant wheat genotypes at harvest-ripeness.
2) The timing of an effective temperature shock might vary from season to season. However there was a lack of correlation with temperature, humidity and moisture loss during grain development.
3) This altered dormancy level does not seem to be ascribed to the changes in ABA content during grain development. However, it has not been ruled out that a more rapid ABA decrease might occur following inhibition, which involves ABA catabolism genes.
4) Temperature shock seems to cause a loss of ABA sensitivity. This was observed to occur in genotypes ranging from intermediate-dormant to dormant.
5) It seems that genotypes that are strongly dormant could contribute to a resistance to temperature shock but further validation is required.
6) *DOG1-like* genes in *Triticum aestivum*, *Brachypodium distachyon*, *Hordeum vulgare*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* form 5 specific clades, were similar to the ones identified in Ashikawa et al. (2013). Among the clades, protein sequences share specific protein motifs and the specific combination of motifs might contribute to their function.

7) Promoter analysis revealed RY and ABRE motifs that are reported to be regulated by *ABI3* and *ABI5*, consistent with the promoters in *Arabidopsis thaliana*, *Brassica rapa* and *Lepidium sativa* (Graeber *et al.* 2010).
8) Comparative analysis showed the potential locations of *TaDOG1-like* in wheat and rice, with *X56782* potentially coinciding with the QTL for dormancy on chromosome 3. Many candidate genes were proposed that could be useful in future marker development and fine mapping studies.
9) Temperature shock seems to cause a significant decrease of *TaDOG1-L2*, which could explain the lower germination index of genotype SUN325B at harvest-ripeness.
10) Both *TaDOG1-L1* and *TaDOG1-L2* mRNA are short lived and localised in the embryo region.

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