Role of the Seed Coat in the Dormancy of Wheat (*Triticum aestivum*) Grains

Submitted by

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Statement of Authorship

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference being made in the text of the thesis.

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Abstract

Pre-harvest sprouting (PHS) is an important economic problem which affects a significant proportion of the Australian wheat crop through quality downgrading. Grain dormancy is the most effective means of overcoming germination in the wheat spikelet at harvest maturity. It has been a consistent observation over a long period of time that dormant red-grained wheat genotypes are almost always more dormant than dormant white-grained genotypes. In white-grained wheat, there are two factors which contribute to dormancy, embryo sensitivity to abscisic acid (ABA) and an interacting and unknown seed coat factor. The proposed dormancy model is that complete dormancy can only be achieved with the coordinated expression of these two factors. This primary objective of this project was to determine the role of this putative seed coat factor in grain dormancy of white-grained wheat.

The physical effect of the seed coat on dormancy was investigated by inflicting damage to the seed coat of Hartog (non-dormant), QT7475 (intermediate-dormant) and SUN325 (dormant) and exposing grains to enriched oxygen conditions. Abrading, slicing and piercing the dorsal seed coat resulted in a loss of dormancy in SUN325 and QT7475, but the increase in germination was similar in both genotypes. The effect of physical damage to the seed coat was therefore not responsible for genotypic differences or the putative seed coat factor. Furthermore, in enriched oxygen conditions germination was also marginally increased in both QT7475 and SUN325, suggesting that the outer seedcoat of imbibed grains reduces the inflow of oxygen, but this increase in germination is not related to the putative seed coat factor.

Magnetic Resonance micro-Imaging (MRmI) was utilised to non-invasively visualise imbibition pathways, primarily to determine if dormant genotypes take up water differently and also to examine general imbibition pathways in the absence of germination. Water uptake followed a structured pathway in all of the genotypes, with the embryo and scutellum tissue hydrating rapidly during early imbibition while the seed coat hydrated more slowly. Water was only able to enter the embryo through the micropyle (a pore through the seed coat in the region of grain attachment to the spike) and there was no observed movement of water across the seed coat and into the endosperm. There was also no observed movement of water beyond the scutellum and into the endosperm in these

early stages of imbibition. The outer layers of the seed coat appeared to conduct water around the exterior of the grain and into the micropyle to hydrate the embryo. Grains of the dormant genotypes did not take up water differently to non-dormant genotypes.

The dormancy model in wheat was validated as a two-component system involving an embryo and an interacting seed coat factor, by performing F_1 reciprocal crosses between a dormant (SUN325), an intermediate-dormant (QT7475) and a non-dormant (Hartog) genotype. The dormancy phenotype was inherited as a recessive trait, with Hartog incurring a loss of dormancy when used as either the male or female parent. Complete dormancy was only achieved in the F_1 progeny when the dormant genotype (SUN325) as the female and QT7475 (intermediate-dormant) as the male parent, indicating that the seed coat from a dormant genotype has an effect on dormancy phenotype. A doubled haploid population of the cross SUN325 x QT7475 was also developed and screened in four different environments over two years, to locate the chromosomal region(s) associated with the seed coat factor. A significant QTL was detected close to the centromere on chromosome 3BL and in a similar region to the *R* genes. These genes are responsible for red seed coat colour.

As a result of the QTL associated with the putative dormant seed coat factor located near the *R* genes, RT-PCR to determine the expression of key genes in the flavonoid biosynthesis pathway was investigated in the seed coat developing wheat grains. A dormant red-grained genotype (R/W635) was also included in the expression analysis of chalcone synthase (*CHS*) and dihydroflavonol 4-reductase (*DFR*), in combination with the white-grained genotypes Hartog (non-dormant), QT7475 (intermediate-dormant) and SUN325 (dormant). At 10 dpa there was no difference in the expression of *CHS* and *DFR*, but at 15 dpa *CHS* was only expressed in the seed coat tissue of R/W635 and SUN325, those genotypes displaying complete dormancy phenotypes. There was no difference in the expression of *DFR* at 15 dpa in all of the genotypes. Subsequent High Performance Liquid Chromatography (HPLC) analysis of the alkaline extract from the seed coat from developing (10 – 30 dpa) and mature grains showed that there was a higher flavone-*C*-glycoside content in SUN325 compared to the other genotypes. This suggests that the longer activation of the flavonoid biosynthesis pathway in SUN325 may result in the production of flavone-*C*-glycosides, while the flavonoid compounds in R/W635 are incorporated into the red pigment, phlobaphene.

Alkaline extracts of the seed coat from seed coat of developing (10 – 30 dpa) and mature grains of white-grained genotypes Hartog (non-dormant), QT7475 (intermediate-dormant), SUN325 (dormant) and the red-grained genotype R/W635 (dormant) was analysed by HPLC to determine if there is a difference in the accumulation of types of flavonoid compounds. Catechin was found to be a minor component of the developing seed coat and present in even smaller quantities in the mature seed coat. When the seed coat extract from the mature grains was spiked with pure catechin and epicatechin compounds, the resultant catechin peak was greatly reduced, even absent, in the HPLC chromatograms from the spiked seed coat extracts of the dormant genotypes (SUN325 and R/W635). This indicates that the catechin/epicatechin compounds are somehow consumed or polymerised with compounds already present in the seed coat extract from mature dormant grains. The consumption of pure catechin/epicatechin compounds may be a clue to the action of the putative dormant seed coat factor.

General Introduction

Wheat is the most widely grown cereal crop in Australia, occupying 90% of the total cereal crop and contributes approximately $4 billion to the economy. An average of 17 million tons, or 83% of the total wheat production is exported annually, which equates to 8% of all world wheat exports (http://www.awb.com.au). Australian wheat is used internationally and domestically for a variety of foods, including bread, pasta and noodle production. Australian wheat exports are heavily reliant on a high quality product suitable for these end-uses, as well as to differentiate Australian wheat from that of other countries and to retain current markets. Therefore, it is important to continually produce high-quality wheat to preserve the worldwide value of Australian wheat.

Pre-harvest sprouting is the premature germination of wheat grains in the spike and is a phenomenon which occurs with rain on harvest mature wheat crops. The main result of pre-harvest sprouting is a severe downgrading in grain quality due to alpha-amylase activity (beginning of germination) degrading starches in the grain. The grain is subsequently only viable as animal feed and is no longer suitable for milling and baking, which results in an economic loss to the farmer and places pressure on market demands. Pre-harvest sprouting is a worldwide phenomenon and it is relatively common in Australia, in particular in northern NSW and Queensland due to the frequent occurrence of summer rainfall in these wheat-growing areas. The proportion of the Australian wheat crop affected by pre-harvest sprouting is in the region of 5 – 7% annually.

Both climatic conditions and agronomic practices can influence the frequency and occurrence of pre-harvest sprouting, although in general environmental conditions have a much greater effect. Aside from significant rainfall around harvest maturity, temperature and humidity during grain ripening have the next greatest effect on pre-harvest sprouting. In particular, hot and humid conditions during grain ripening increase the risk of premature germination after rainfall exposure, while cool conditions during the same period have the opposite effect. If the rainfall occurs in combination with low temperatures at harvest maturity, however, the likelihood of pre-harvest sprouting is also increased. Agronomic factors which reduce the incidence and severity of pre-harvest sprouting include adjusting sowing times to avoid harvest at times when rainfall is expected and utilising wheat varieties which reduce water transfer through the spike and into the grain. Several varieties

of wheat can influence water uptake through spike morphology, for example awnless and club ear types, while spike nodding angle may also effect wetting of the grains inside the spike. Genetic tolerance to pre-harvest sprouting or grain dormancy, where the grain fails to germinate at harvest maturity even in conducive conditions, is a much more effective mechanism to diminish damages incurred by rainfall during harvest.

In Australia, white-grained wheat is the only form of wheat grown due to the superior milling quality over red-grained wheats. Unfortunately, white-grained wheat is much more susceptible to pre-harvest sprouting than red-grained wheat, which means that the Australian wheat crop is highly susceptible to pre-harvest sprouting. Grain dormancy is present in some white-grained wheat genotypes, but the dormancy level never reaches that of dormant red-grained genotypes. It is therefore favourable to produce white-grained genotypes that expresses a dormancy level which allows protection against pre-harvest sprouting in Australian conditions. Understanding the physiological and genetic components of dormancy in white-grained genotypes is an important step in addressing the problem of pre-harvest sprouting for the Australian wheat industry.

Chapter 1: Review of Literature

1.1 Pre-Harvest Sprouting in Wheat

Pre-harvest sprouting, or the germination of grain in the spike, can occur prior to harvest maturity if the crop is exposed to wet, humid conditions. The majority of wheat-growing climates throughout the world experience rain during harvest, which can result in sprouted grain and a loss of quality premiums for the farmer. Although there are some management techniques and varietal differences that can reduce the risk in susceptibility, these are largely ineffective if environmental conditions persist.

1.2 Economic Impact

Pre-harvest sprouting is a common phenomenon in Australia, where wet or humid conditions prior to harvest cause germination of the grain in the spikelet. Pre-harvest sprouting affects around 15% of the crop annually in Queensland and Northern New South Wales where summer rainfall is prevalent (Ruan *et al.*, 1992), and occurs in approximately 10% of seasons in Southern Australia. Losses from sprouted grain has been up to $200 million in a wet harvest, but has a long-term average of around 5 – 7% of the crop annually. Australian wheat varieties are generally susceptible, or very susceptible, to pre-harvest sprouting and new low moisture standards may also contribute to greater levels of sprouted grain due to a delayed harvest.

Premature germination of grain in the spikelet before harvest causes significant losses in terms of decreased quality and yield reductions. Sprouted grain is unable to be used in baking which results in a financial penalty with downgraded grain. Yield can be affected by more than 10%, due to increased shattering and the presence of kernels with reduced biomass (Derera, 1979). There is also an adverse effect on test weight as a result of a decrease in specific gravity (Derera, 1989). The current tolerance level for sprouted grains in a commercial sample is up to 2%, providing the falling number is above a defined level. Levels above this can result in a loss of approximately $60 per ton if the classification is downgraded to feed (AWB Limited Receival Standards 2002/2003).

1.3 Environmental Conditions

The environmental influence on grain dormancy expression is well known but difficult to quantify. In general, warm to hot ripening conditions (20-30ºC) increase susceptibility to pre-harvest sprouting, while cool ripening (12-18ºC) environments promote tolerance (Gordon and Cross, 1998; Romagosa *et al.*, 2001). Walker-Simmons and Sesing (1990) also found that grain from plants grown at 25ºC was less dormant compared to 15ºC. These findings, combined with increased summer rainfall, correspond with the incidence of pre-harvest sprouting being greater in Northern Australia, where average temperatures during the growing season are relatively warm compared to Southern Australia.

Susceptibility to pre-harvest sprouting is also highly influenced by environmental conditions such as humidity. In particular, there is a strong correlation between humidity on germinability of grain at harvest maturity. During grain development, extended periods of high humidity can contribute to a decrease in drying rate of grains, which in turn leads to susceptibility to pre-harvest sprouting or premature germination in the spikelet (King, 1993). Rainfall during the final stages of grain development also contributes to a loss of dormancy, with an increase in germinability if rain occurs on ripened grains (Mares, 1993). The increase in germinability is possibly due to the grain becoming primed for germination through wetting and subsequent dehydration, causing rapid germination on further wetting (Mares, 1993).

Importantly, Torada and Amano (2002) discovered grain dormancy in white-grained wheats can be significantly reduced when grown in wet and humid conditions. This observation was in contrast to an earlier study conducted in Norway, where increased rainfall and humidity appeared to promote grain dormancy (Strand, 1989). Unfortunately this study had many problems, including threshing methods which may have resulted in damage to the seed coat and thus effected consistent dormancy expression, as well as cool temperature conditions which may negate the influence of high humidity during grain development. Exposure to dry conditions, by contrast, can result in grains with enhanced dormancy (Biddulph *et al.*, 2005).

Clearly environmental conditions highly influence grain dormancy, and it is therefore imperative to monitor these conditions when screening for this trait. By understanding the

effects of temperature, rainfall and humidity, it is possible to create growing conditions which decrease environmental effects and maximise actual genetic dormancy response.

1.4 Spike Morphology

The rate of water uptake by grains in the spikelet is significantly affected by water concentration in these vegetative structures (Bauer and Black, 1983). Depending on spike morphology, water is absorbed into the spikelet differently, with awned varieties taking up approximately 30% more water than awnless cultivars, thereby increasing sprouting risk (King and Richards, 1984a). In addition, club-type ears show greater water uptake and sprouting compared to less compact and awnless spikes (King and Richards, 1984a). There is also more recent evidence by King and von Wettstein-Knowles (2000), that the presence of epicuticular waxes on glumes delayed grain water uptake and hence delaying pre-harvest sprouting in barley. Another factor implicated in water uptake is the spike nodding angle. In barley, a more erect spike absorbs water more rapidly than a spike which is more than 90º from a vertical position (Brinkman and Luk, 1979). Wheat however is apparently different, with no difference in water loss of standing (erect) compared to windrowed (prostrate) wheat spikes, indicating that nodding angle would not improve water loss once rain has occurred (Clarke, 1982). There is also no difference in drying rate of awned and awnless spikes which demonstrates the lack of impact this trait has once rain saturates the spike (Clarke, 1982), despite the fact that glaucous lines were found to be more efficient in losing water after shaking than non-waxy types (King and von Wettstein-Knowles, 2000)

None of these studies, however, measured actual water content of grains in the spikelet after wetting treatment, which would have provided a more accurate indication of water movement through the spike. Gordon (1977) showed that grain moisture reaches saturation when the spike water concentration reaches approximately 150%, or after 20 hours of rain simulation. Therefore, although there is a difference in water uptake into the spike there is nothing to suggest this affects the water content of the grain after saturation despite the drying rate. Once the grain has reached critical water content, germination is likely to occur regardless of the spike water concentration at that time.

Attempts to reduce the incidence of pre-harvest sprouting through variation in spike morphology have yielded little advantage. King (1982) showed that there is a linear

relationship between germination of grains in the spike and isolated grains over 30 hours. This relationship means that grain dormancy is the main factor preventing pre-harvest sprouting, because germination of grains in the spike is merely delayed compared with germination of isolated grains. Moreover, the use of isolated grains removes the impact of spike structures (eg awns) on grain water uptake, where awnless types delay in-ear germination further (King and Chadim, 1982). Therefore although spike morphology does affect water uptake, this system has little actual impact on the incidence of pre-harvest sprouting.

1.5 Dormancy in Wheat

Grain dormancy is the failure of isolated seeds to germinate in optimum conditions of moisture, temperature and light (Belderok, 1968). Genetic dormancy exists in wheat and is a more reliable method of reducing the incidence of pre-harvest sprouting, due to the ability to withstand rainfall with reduced sprouting at harvest maturity. Dormancy in wheat is relatively easy to test for and is able to be introgressed into adapted cultivars, thereby making a genetic method more favourable for decreasing the incidence of pre-harvest sprouting.

In wheat, failure of the embryo to germinate in the presence of endogenous inhibitors has been identified as one of the major mechanisms involved in dormancy (Bradbeer, 1988). One of the most widely studied germination inhibitors is ABA, which has been shown to inhibit germination in wheat and is influenced by the germination promoter gibberellic acid (GA) (Bewley, 1997; Romagosa *et al.*, 2001). It is now accepted that ABA is the most important regulator of seed dormancy (Koornneef *et al.*, 2002). ABA levels increase slowly in initial seed development, then escalate in the mid-stages during the synthesis of storage reserves (primary dormancy), before decreasing slowly until harvest maturity (Bewley, 1997). There is little evidence that ABA is produced maternally, with dormancy imposed through synthesis in the embryo during development (Bewley, 1997).

Studies on ABA mutants, including ABA insensitive *Arabidopsis* (*aba*) and viviparous (v*p*) maize have shown that these genotypes are largely depleted of ABA and have little or no resistance to premature sprouting (Neill *et al.*, 1986; McCarty, 1995). The *vp1* maize mutants show an inability to respond to exogenously applied ABA (McCarty, 1995)

although *vp* lines also exhibit a higher expression of an embryo-specific gene (*Em*) with increasing ABA levels, indicating that ABA has an important function in embryo development (Paiva and Kriz, 1994). Therefore ABA not only has a function in preserving seed dormancy, it also significantly contributes to seed development and prevents fatal precocious germination observed in ABA mutant genotypes.

In wheat, it has been shown that there is little difference in the amount of ABA in dried grains of dormant and non-dormant genotypes, rather it is the sensitivity of the embryo to the hormone which dictates germinability (Walker-Simmons, 1987; Wang *et al.*, 1995; Himi *et al.*, 2002). This embryo sensitivity to ABA corresponds to an intermediate-dormant phenotype (Mares, 1999). The importance of ABA sensitivity in inducing dormancy is supported by 25% of F_2 half-grains, derived from a dormant x non-dormant cross, expressing a dormancy phenotype when ABA was applied to the germination medium. The remaining genotypes from this cross were not affected by the same exposure to exogenous ABA, indicating embryo sensitivity to ABA is a recessive genetic component of dormancy (Mares, 1996). Cutting grains in half destroys any inherent dormancy, leaving only embryo sensitivity to exogenously applied ABA. Embryo ABA levels in barley are also the same in dormant compared to after-ripened grains, however it is during imbibition that the ABA breaks down more rapidly into the inactive phaseic acid (PA), thereby allowing germination (Jacobsen *et al.*, 2002). These results have been mirrored in *Pinus monticola*, where an increase in a capacity to germinate during imbibition was correlated with high PA and other metabolites of ABA, while dormant genotypes maintained initial ABA levels and low levels of breakdown compounds (Feurtado *et al.*, 2004).

Genotypes are more dormant after growth in cooler compared to warmer temperatures, but it has been shown that sensitivity of grains to ABA is likely to be the driving factor in this observation, rather than differences in ABA content (Walker-Simmons and Sesing, 1990; Garello and Le Page-Degivry, 1999; Romagosa *et al.*, 2001). Despite this, imbibition at low temperatures (stratification) is an effective mechanism to break dormancy, with germination occurring slowly but more uniformly (Noda *et al.*, 1994). Differences in dormancy expression due to stratification and also dormancy-breaking compounds such as nitric oxide, appear to enhance the breakdown of ABA, while dormant grains maintain or even increase initial ABA levels (Bethke *et al.*, 2004; Gubler *et al.*, 2005). These observations indicate that although ABA content plays a role in germinability during

imbibition, the key to dormancy is not ABA content at maturity, but embryo sensitivity. The seed coat also plays an integral part in the dormancy and sensitivity to ABA of other species (eg tomatoes, *Arabidopsis*, wild oats), where the integrity of surrounding tissues or the seed coat are crucial in their dormancy expression (Corbineau *et al.*, 1991; Berry and Bewley, 1992; Debeaujon and Koornneef, 2000).

Hybridisation studies between dormant and non-dormant wheat genotypes also strongly suggested that another maternally inherited, a seed coat factor, was also involved in grain dormancy (Mares, 1999). This study showed that there are two main factors involved in wheat dormancy, the embryo sensitivity to ABA and another unknown and interacting seed coat component (Mares, 1999). Seed coat factors which have been implicated in dormancy in other species and are potentially active in wheat. Individually, the embryo factor provides intermediate dormancy but the seed coat factor alone provides little or no inhibition of germination, while the combination of both results in complete dormancy (Mares, 1998). Therefore there is much interest in identifying this putative seed coat factor as a natural and simple protection against pre-harvest sprouting. The possible role of the white-grained wheat seed coat factor in grain dormancy in relation to the red grain pigment and dormancy in other species will be discussed in the following paragraphs.

1.5.1 Dormancy in Red-Grained Wheats

Dormant red-grained wheat is observed to be almost always more dormant than dormant white-grained wheat (Flintham, 1992; McCaig and DePauw, 1992; Mares, 1993) and although dormancy in white-grained wheats has only been discovered relatively recently (Mares, 1987; DePauw and McCaig, 1990), it is possible that the dormancy mechanism may have similarities. In some instances, wild relatives show greater dormancy characteristics than domesticated bread wheat, indicating that selection has contributed to the susceptibility to pre-harvest sprouting in current varieties (Flintham, 1992; Lenton, 2001). Red colour pigments are located in the testa and are controlled by up to three genes located on chromosomes 3D, 3A and 3B and designated R-D*lb*, R-A*lb* and R-B*lb* respectively (McIntosh *et al.*, 1995). The red-grained genotype is dominant over white colour (Gale, 1989). The three red genes can be considered additive, with genotypes containing all three genes slightly more dormant than those with only one or two genes (Flintham, 2000), but the largest difference in dormancy is observed between white

cultivars with all *r* alleles, and red genotypes with at least one R gene (Freed *et al.*, 1976). Intensity of red colour is not associated with the number of red genes (McEwan, 1980).

The *R* genes responsible for the red pigment in red-grained wheat have been considered to be a phenotypic marker of dormancy, however mutagenesis and recombination studies have shown that the red pigment and dormancy can be separated (DePauw and McCaig, 1983), although the dormancy was reduced in the white-types compared to the red-grained parent (Mares, 1993; Warner *et al.*, 2000). The separation of the two factors suggests that red coat colour and dormancy may be only genetically linked on the same chromosome (Flintham, 2000; Warner *et al.*, 2000), but the high dormancy level associated with red wheats has never been transferred in its entirety into white genotypes (Mares, 1993; Hucl, 1995; Flintham *et al.*, 1999). This observation is supported, by the production of a white mutant line (EMS-AUS) from a dormant red line (AUS1490), where dormancy is reduced in the mutant line (Mares, 1998; Himi *et al.*, 2002). This mutant line is has been investigated in detail, and it was discovered that the only difference from the red dormant variety AUS1490 was a single amino-acid in the putative *R* gene, which may be responsible for the observed decline in dormancy (Himi and Noda, 2005).

In reciprocal crosses involving red x white genotypes, it is apparent that the interaction between the embryo and seed coat dormancy factor in red genotypes is more complex than the relationship in white grained wheats (Mares, 1993). Dormancy appears to be at least partially dominant in red grained genotypes in contrast to recessive in white grained lines (Mares and Ellison, 1990). A study by Mares (1993) using a red (dormant) x white (non-dormant), with the red genotype as the female parent, showed that the F_1 grains were intermediate-dormant. These results indicate that the red seed coat is partially effective in combination with a heterozygous embryo, which is not observed in similar crosses with white-grained genotypes. However further genetic studies have shown that the presence of the red seed coat without embryo dormancy does not result in a dormant phenotype (Flintham *et al.*, 1999; Mares, 1999). A good example of this is the red variety Chinese Spring, which is non-dormant and ABA insensitive (Noda *et al.*, 2002). Therefore, there appear to be two components involved in the dormancy of red-grained wheat, an embryo factor and an interacting seed coat factor related to the *R* gene or red-grained phenotype (Flintham, 2000; Warner *et al.*, 2000).

Although there have been recent investigations into the function of the *R* genes (Himi *et al.*, 2005), there has been very little biochemical characterisation of the red pigment. It has been suggested that dormancy is enhanced in red-grained genotypes by precursor compounds to the red pigment inhibiting germination (Stoy and Sundin, 1976; Mares, 1999). Some of these proposed precursor compounds have been shown to inhibit the germination of genetically susceptible white-grained genotypes (Stoy and Olsen, 1980). Other possible suggestions to the function of the red pigment in dormancy include as a barrier to oxygen or water diffusion and will be discussed in the following paragraphs.

1.5.2 Dormancy in White-Grained Wheats

Although red-grained wheats were determined to be more dormant than dormant white-grained wheats, there is a range of dormancy phenotypes discovered in white-grained wheat genotypes (DePauw and McCaig, 1983; Mares, 1987). Early genetic studies using dormant and non-dormant genotypes were unable to conclusively determine the mode and number of genes involved in dormancy in white-grained wheat, due to confounding factors such as the environment (Mares, 1996). However, it was apparent that with the addition of exogenous ABA, grains were more likely to show dormancy characteristics and a definitive segregation pattern (Noll *et al.*, 1982). There was also evidence of more than one recessive gene involved in complete dormancy expression through genetic studies on dormant and non-dormant genotypes (Mares, 1999). Embryo sensitivity to ABA of dormant grains is, as mentioned earlier, is a major component of dormancy, however this trait on its own only contributes to intermediate, not the complete dormancy observed in some white-grained lines (Mares and Ellison, 1990). Therefore, the presence of more than one factor is involved in the dormancy of white-grained wheat.

Reciprocal crosses between dormant and non-dormant white-grained genotypes generate progeny with no evidence of dormancy, in contrast to similar crosses using a red-grained dormant female parent. Comparison of dormancy phenotype in successive generation derived from crosses between dormant and non-dormant white-grained parents show segregation only occurred in the F_3 grain generation (Mares, 1993, 1996). This study determined the dormancy phenotype of F_1, F_2, and F_3 grain populations derived from crosses involving dormant white x non-dormant white and dormant red (single *R* gene) x non-dormant white. In both cases, two or more genes appeared to be involved and

dormancy comparable with the dormant parent was not recovered until F_3. One explanation for this observation is that at least one of the dormancy genes is expressed in the maternal seed coat tissue where segregation lags one generation behind the embryo and endosperm. In a later study the embryo sensitivity factor was shown to be controlled by at least one major gene and complete dormancy possibly involves an interaction between the embryo sensitivity to ABA (Walker-Simmons, 1987; Mares, 1996) and unknown factors, one of which is only expressed in the seed coat (Mares, 1996; Flintham *et al.*, 1999). More recent studies have also reported the expression of at least one dormancy gene in the seed coat of dormant white-grained genotypes (Mares, 1999; Warner *et al.*, 2000).

1.6 Possible Roles of the Seed Coat Factor in Dormant White-Grained Wheats

1.6.1 Structure, composition and function of the wheat seed coat

The wheat seed coat is made up of entirely of non-living cells and consists of six basic layers, the epidermis, hypodermis, cross cells, tube cells, testa and nucellar layer (Evers and Millar, 2002). This tissue is complex and therefore an understanding of its structure may provide clues to the nature and possible location of the seed coat dormancy factor. The layers of the seed coat are formed by the ripened ovary wall, and are tightly packed with relatively thick cell walls in comparison to the endospermic tissue (Fulcher and Duke, 2002). In cereals the testa, the fifth layer, is fused to the pericarp which is a combination of the four outermost layers (Evers and Bechtel, 1988). The pericarp is clear with the colour pigments located in the testa, while the cuticular layer (nucellar tissue) below contains waxy compounds (King, 1989). There has been suggestion that the testa itself contains waxy hydrophobic cutins and there are lipid compounds in the testa and nucellar layers (Butcher and Stenvert, 1973; Moss, 1973). Hinton (1955) also reported that the testa is relatively impermeable to water and Woodbury and Wiebe (1983) showed evidence that this is probably due to waxy compounds.

The grain is completely covered by these layers except for a small region near the embryo which contains a pore or channel through the seed coat and into the embryo (Bradbury *et al.*, 1956). The micropyle is the remnants of the pollen tube in many species (Tran and Cavanagh, 1984) and is present in wheat as a pore which is formed by an opening in the

testa (Bhatnagar and John, 1972). It is thought that the micropyle is involved in the distribution of nutrients to the developing grain and is located to the dorsal side of the area of attachment of the grain to the spike (Evers and Bechtel, 1988). The micropyle has been reported as a channel through heavily pigmented testa cells leading almost directly to the embryo, but possibly with a thin cuticular layer covering this channel (Bradbury *et al.*, 1956). Wheat grains also contain a crease region, which is a groove that runs across the ventral side of the seed. This region is responsible for transferring nutrients into the developing grain, but during grain ripening becomes pigmented and hardens into a corky and impermeable substance (Evers and Millar, 2002).

The seed coat itself is an important structure in germination and dormancy of many seed species (Bewley, 1997) and protects the embryo and germination energy source from biotic and abiotic stresses. Removal or damage to the seed coat reduces dormancy in wheat grains, regardless of the genotype (Mares, 1999). It appears likely that the seed coat factor involved in dormancy is superimposed on this requirement for physical integrity and that the seed coat of dormant white-grained wheat is somehow different from non-dormant red-grained and other white-grained genotypes (Mares, 1999).

1.6.2 Water uptake

Seed water uptake has been studied primarily in many species where the seed coat acts as a major barrier to imbibition through a waxy cuticle and lignified cells (Bewley and Black, 1982). It has also been suggested in early studies that species like *Pisum elatius*, containing high tannin or phenolic levels in the seed coat, are impermeable to water (Marbach and Mayer, 1974, 1975). A correlation between water uptake and tannin content was also detected in faba beans, however there was no difference in germination between the high and low tannin lines (Kantar *et al.*, 1996). In fact, the presence of tannins appeared to have a greater effect on seed water uptake than cracks in the testa. A study in *Arabidopsis* showed that penetration of the testa by tetrazolium (representing water) was not restricted in non-dormant mutants which lacked testa pigmentation, indicating that barriers in water uptake due to pigmentation may influence dormancy in this species (Debeaujon *et al.*, 2000). Therefore, it appears that the presence of phenolic compounds and tannins are influential in water uptake, but it is unclear whether these compounds evoke an increase or decrease in permeability.

It has been suggested that pigmentation in the seed coat may also hinder imbibition, with lighter seeds allowing greater water uptake than darker genotypes (Huang *et al.*, 1983; Debeaujon *et al.*, 2000). This has been shown in pea species, where a dark coat colour (*Pisium elatus*) is impermeable, while the light coated *Pisium sativum* is completely permeable to water (Marbach and Mayer, 1974). Huang *et al.*, (1983) also showed that there is a variation in the imbibition rate and penetration of water into red and white-grained wheats, which could then be related to differences in structural components of the seed coat. The activity of catechol oxidase in developing pea seed coats has been shown to be related to the seed coat colour, and the oxidation of phenolic compounds by this enzyme reduces permeability to water (Marbach and Mayer, 1974, 1975). Another study also demonstrated that the presence of catechin tannin, a proposed precursor to the pigment in red wheats, has a limited effect on water uptake (Clarke and DePauw, 1989; Jiang, 1998). These studies provide evidence that seed coat colour in wheat is not associated with imbibition rate, rather other layers of the seed coat may be responsible for dormancy characteristics (Kelly *et al.*, 1992). It is therefore likely that permeability of the testa to water differs between species in terms of both structural and chemical composition and these factors may be a component of dormancy expression in wheat grains (Yoshida *et al.*, 1996).

Studies of the structural composition and physical nature of the seed coat have shown that imbibition in species with thick and waxy seed coats is influenced by imperfections in the seed coat, for example maize (Ruan *et al.*, 1992) and soybean (McDonald Jr *et al.*, 1988). A failure to imbibe water, or 'hard seededness' is a good example of this, where water uptake will be severely restricted until there is some mechanical disturbance to the seed coat causing cracks (Briggs and MacDonald, 1982; Woodbury and Wiebe, 1983). Wheat has a relatively thin seed coat and it is unlikely that cracks through the seed coat are required for imbibition into the embryo as is observed in these species with hard and waxy seed coats.

It has been proposed, however, that cracks in the pericarp of wheat (in the brush section), can be caused by temperature and rate of drying during grain development as well as mechanical damage, thereby allowing greater water movement beneath the pericarp (Woodbury and Wiebe, 1983). Despite this, testa as well as pericarp thickness of maize seeds have not been found to be related to water uptake (Ruan *et al.*, 1992). Cracks in the

pericarp are also unlikely to change imbibition rate or pathway in wheat grains according to Woodbury and Weibe (1983) who found that a split in the pericarp did not allow increased infiltration of water into the embryo or endosperm. Briggs and McDonald (1983) also found that splits in the outer pericarp of barley grains increase water infiltration and thereby assist in water conductance between this tissue and the testa. The pericarp is the outermost layer of the wheat seed coat and for this reason it is unlikely that splits in this tissue will result in any changes in water uptake into the grain, rather increase water movement beneath the pericarp.

The presence of waxy layers and the microstructure of other layers of the seed coat can affect the movement of water through the layers, with these layers acting as pathways for water diffusion through wheat and barley grains (Briggs and MacDonald, 1982; Woodbury and Wiebe, 1983). It has also been observed that the pericarp prevents movement of dye (both Eosin and Trypan Blue) into the testa, with the outer pericarp layers staining readily, while the dye never penetrated into the inner seed coat layers (testa and nucellar) (Briggs and MacDonald, 1983; King, 1984b). The dye did however enter the embryo only a small time after exposure to the solution. Hinton (1955) showed that the testa has the greatest resistance to water permeation, indicating that there is little infiltration of water through the seed coat into the endosperm during imbibition. In addition to this, it was determined that the outer pericarp is a porous tissue which is able to conduct water around the surface of the seed (Briggs and MacDonald, 1983).

There has been numerous attempts to study water uptake by wheat grains during tempering (Stenvert and Kingswood, 1976), the effect of threshing (Clarke and DePauw, 1989) and differences in seed coat colour (Huang *et al.*, 1983), but until now most of the techniques have been crude and destructive and there has been little indication of actual imbibition pathways. Dyes have previously been a popular choice for visualising seed water uptake, for example in barley (Briggs and MacDonald, 1983; Allen *et al.*, 2000), but this has not provided high resolution of water distribution in grain structures and requires slicing of the grain, thereby destroying the grain structure. Stenvert and Kingswood (1976) used autoradiography to determine water determine in wheat, but this is also a destructive and invasive technique. Physically cutting the seed results in a restricted two-dimensional view, where only a few viewpoints can be observed and hence little information obtained.

In addition to this, dyes are likely to have different permeability through seed structures than water (Terskikh *et al.*, 2005).

Investigations of the uptake of water into seeds using Magnetic Resonance Imaging (MRI) showed that water is taken up via the micropyle rather than through the seed coat (Yoshida *et al.*, 1996; Manz *et al.*, 2005). MRI in its standard form does not provide the fine resolution required for plant structures. By contrast, MR micro-imaging (MRmI), or Nuclear Magnetic Resonance (NMR) microscopy, provides an excellent opportunity to visualise water distribution in plants *in vivo* (Kockenberger *et al.*, 2004). MRI is based on the principle that ^{1}H nuclei resonate when exposed to a strong static magnetic field and in combination with microscale imaging, provides a highly-resolved spatial representation of seed water distribution (Kockenberger, 2001). By far the greatest benefit of MRI is the ability to visualise water distribution non-destructively, non-invasively and three-dimensionally. Another advantage of MRI is an ability to measure changes over time (Kockenberger *et al.*, 2004). To achieve a three-dimensional perspective, images of a grain are taken sequentially in multiple two-dimensional slices, resulting in a complete image of water distribution (Song *et al.*, 1998). It is possible to produce images with cellular resolution in conducive plant material, but generally resolution around ten to several hundred micrometres is informative and typical with plant MRmI (Kockenberger, 2001).

MRmI in seeds is still not a particularly common technique because of the expense and availability of equipment, but has the potential to assist in knowledge of historically elusive biological processes like imbibition (Gruwel *et al.*, 2004). Recently, there have been high-resolution investigations into water uptake of tobacco (Manz *et al.*, 2005) and pine seeds (Terskikh *et al.*, 2005). In wheat, early MRmI technology was utilised for visualising water movement during grain drying (Jenner *et al.*, 1988) and more recently in mature wheat (Song *et al.*, 1998). There have been a very small number of MRmI studies on imbibition in wheat grains, but they have only reported poor quality images with low resolution and limited results (Yoshida *et al.*, 1995; Gruwel *et al.*, 2004).

Imbibition is the first important stage of dormancy expression, as the dormancy state of a grain cannot be determined without exposure to water. Water uptake by wheat seeds is triphasic, with an initial rapid uptake phase (0-10 hours of imbibition), a lag phase of water infiltration into intracellular spaces (10-21 hours of imbibition) and a final rapid hydration

phase, which is only reached in non-dormant or germinating seeds (Krishnan *et al.*, 2003; Terskikh *et al.*, 2005). This final phase involves a second rapid uptake of water associated with a large increase in seedling size (Manz *et al.*, 2005). The failure of dormant wheat grains to reach this final stage may be as a result of differences in seed coat permeability, water distribution through the grain or other possible dormancy factors, but there has been little detailed investigation of imbibition in wheat, especially in dormant and non-dormant genotypes.

Imbibition is a strictly physical process which has been demonstrated in peas, with dead seeds continuing to take up as much water as living seeds (Ohmura and Howell, 1960). As water uptake is the first process in germination, it has been proposed that dormant genotypes may imbibe water differently in comparison to non-dormant (Bewley and Black, 1982). The study by Yoshida *et al.,* (1995) using MRmI suggested there was a difference in imbibition between a sprouting susceptible and a tolerant wheat variety, however the main increases in water uptake occurred during germination (around 72 hours). This large increase does not relate to dormancy, rather it is determined by the high water content of the young seedling, and in fact there was no indication in this study if the grains were after-ripened (hence non-dormant). Therefore any differences in uptake cannot be assigned to a lack of dormancy, but rather a time of germination. It is possible that differences may occur with water movement through the seed coat or the depth of penetration as well as variations in the main storage areas of imbibed water for dormant and non-dormant genotypes. The use of MRmI to measure these differences in actual dormant lines may show if water uptake varies in comparison to non-dormant lines.

1.6.3 Oxygen permeability

Water is probably taken up into the seed through the micropyle before infiltrating the embryo and forming a moisture layer throughout the seed coat. Oxygen diffuses slowly into water (10^{-4} times the diffusion through air) and this moisture may create a barrier for oxygen to reach the embryo and stimulate growth of the seedling (Porter and Wareing, 1974; Cochrane and Duffus, 1983). However oxygen may also enter the seed via the micropylar region rather than through the waxy cuticular layers of the seed coat, which may act as a separate barrier (Marbach and Mayer, 1974). In species other than wheat, oxygen diffusion through the seed coat has been extensively studied: both in terms of

oxygen consumption by the embryo and to a lesser extent actual movement through seed coat layers (Navasero *et al.*, 1975; Lenoir *et al.*, 1986; Adkins and Simpson, 1988).

It is commonly accepted that the removal of some seed coat layers (eg the pericarp or testa) results in a total loss of dormancy in many species, including wheat (Sikder, 1967; Navasero *et al.*, 1975; Ueno, 1996). This loss of dormancy may be associated with increased permeability to oxygen, however there was no firm relationship established with increased availability of oxygen for the embryo, or any other factor (eg escape of germination inhibitors). Results of experiments addressing oxygen permeability in seeds have been relatively inconclusive. Some species having structures surrounding the embryo which prevent oxygen diffusion, for example beechnuts (Barthe *et al.*, 2000), while there are some reports that oxygen is consumed in the seed coat or surrounding structures, thereby acting as a barrier (Lenoir *et al.*, 1986). There was no evidence of the seed coat restricting oxygen uptake in *Vicia faba*, in a study using excised embryos and intact seeds (Durham and Wellington, 1961). It could be expected that a strong and thick seed coat would be able to restrict oxygen diffusion in legume species compare to cereals, however Navasero *et al.* (1975) also detected a difference in oxygen uptake between hulled and de-hulled rice grains. Rice has a much thinner seed coat than leguminous species, indicating that the seed species is probably more important in determining oxygen permeability of the seed coat or surrounding structures, rather than the physical properties of the seed coat.

In wheat, the permeability of the seed coat to oxygen has rarely been addressed. The testa, in combination with the waxy nucellar cuticle, are likely to be a region of limited permeability to water (Porter and Wareing, 1974), however there is little to suggest this relates also to oxygen permeability. If water enters the embryo mainly through the micropyle, it is reasonable to assume that oxygen may follow the same pathway. A study by Durham and Wellington (1961) used grains with the seed coat removed and demonstrated that varying oxygen and air mixtures has a mild effect on germination between red and white genotypes, but there was no significant difference between air and oxygen treatments within genotypes. Germination of embryos in low oxygen, even nitrogen, environments was also investigated (Bewley and Black, 1982). These results suggest that a lack of oxygen does not contribute to dormancy, due to the embryo having only a small oxygen requirement for germination. However all cells need oxygen for

growth, therefore the initial presence of oxygen in the embryo, or the removal of surrounding grain structures may be a confounding factor in these studies.

It has been suggested that oxygen consumption by the seed coat due to the oxidation of phenolic compounds in beechnut restricts diffusion into the embryo (Barthe *et al.*, 2000). Whilst oxygen consumption by structures surrounding the embryo may prevent germination, it is not clear if this is a function of dormancy (Marbach and Mayer, 1974), particularly in wheat. Another study in beechnut seeds found an increase in oxidative products in isolated embryos, with an increase also observed after exposure of the embryos to heightened oxygen pressures (Edwards, 1969). The oxidation of phenolic compounds by catechol oxidase has been shown to reduce permeability to water (Pollock and Kirsop, 1956). As this reaction is dependent on the presence of oxygen, it is possible that the oxidation of phenolic compounds may have an effect on the diffusion of oxygen to the embryo. In barley, the consumption of oxygen by polyphenol oxidase, or oxidation of phenolic compounds, enhances dormancy (Lenoir *et al.*, 1986). Despite this report, the oxidation of phenolic compounds is not a generally accepted mechanism of restricting oxygen diffusion to the embryo (Kruger and Reed, 1988).

Pigments in the wheat seed coat are phenolic compounds (Navasero *et al.*, 1975) and with peroxidase enzymes also located in these areas, the phenolic compounds may be responsible for oxygen reactions with these enzymes (Porter and Wareing, 1974). Navasero (1975) found that peroxidase activity was related to dormancy in rice, with a higher activity detected in grains which had failed to germinate. Although these results suggest that phenolic consumption of oxygen may influence dormancy, there was no evidence that oxygen was actually restricted from entering the grain. Peroxidase activity may be more likely to affect the rate of oxygen diffusion (Bhatt *et al.*, 1983), while Porter and Wareing (1974) proposed that it is the rate of reaction of oxygen with phenolic compounds, not a failure of diffusion. In their study on *Xanthium pennsylvanicum* Wallr., Porter and Wareing (1974) found that there appeared to be little hindrance of oxygen diffusion through the seed coat, but the reaction of oxygen with germination inhibitors (eg phenolic compounds) may render these compounds inert, thereby allowing germination. The slow reaction with oxygen will mean prolonging dormancy and when the reaction is complete, germination is able to occur. This hypothesis is not entirely convincing,

however, because the atmosphere provides an unlimited oxygen resource and therefore oxygen is not a limiting factor in the reaction of these compounds.

1.6.4 Chemical Inhibitors in the Seed Coat

Bioassays, using germination inhibitors extracted from the seed coat and other plant structures have been relatively successful. Phenolic compounds extracted from structures surrounding wild oat grains caused delayed germination in lettuce seeds (Chen *et al.*, 1982), while an extract from the bracts of *Triticum tauschii* contained compounds which inhibited germination of the grains of the same species (Gatford *et al.*, 2002). In both of these studies it was reported that vanillin was the inhibitory compound extracted from the hulls and bracts. Flavonoid compounds detected in the embryo tissue of other cereals (barley, rye, oat, triticale) have been linked to dormancy expression (Weidner *et al.*, 1996; Weidner *et al.*, 2002), while by contrast, active germination inhibitors isolated from wheat husks in other similar studies was found to be aromatic compounds (Kato *et al.*, 2002; Kato *et al.*, 2003).

Although there is strong evidence that phenolic compounds in the seed coat are involved in dormancy in other species, for example *Arabidopsis* (Debeaujon *et al.*, 2000), there is no definitive proof that similar compounds are involved in the dormancy of wheat. The study which reported inhibition of germination in the wild relatives of wheat from phenolic compounds in the bracts, showed that only the highly concentrated crude extract had a significant effect (Gatford *et al.*, 2002). Once this extract was slightly diluted there was no inhibition of germination, indicating that these compounds are unlikely to be active at actual physiological concentrations. Other studies also show phenolic compounds exposed to seeds above physiological concentration unable to reduce dormancy (Kato *et al.*, 2002; Weidner *et al.*, 2002). However, from the data presented it was impossible to determine if these compounds are active in biological conditions.

For many years in wheat it has been observed that red-grained are more dormant than white-grained wheats, demonstrating that the red pigment contributes, in some way, to dormancy expression. The red pigment is a polymerised flavonoid compound, suggesting that flavonoid precursors, which can behave as germination inhibitors (Winkel-Shirley, 1998), may be involved in dormancy of wheat. There have been very few investigations into the chemical nature and particularly the exact origin of the red pigment in the seed

coat. It is generally thought to be a proanthocyanidin or phlobaphene compound (Himi and Noda, 2004). In *Arabidopsis* where *transparent-testa* mutants were shown to lack anthocyanin and proanthocyanidin pigmentation which causes a decline in dormancy (Debeaujon and Koornneef, 2000; Kitamura *et al.*, 2004). However, seed coat pigments in other *Brassica* species, which has been characterised as proanthocyanidin (Marles and Gruber, 2004), or maize which is due to phlobaphene (Grotewold *et al.*, 1994), do not appear to be involved in dormancy these species.

Water extracts from wheat bran derived from grain of dormant red- and white-grained genotypes caused a delay in germination when applied to isolated wheat embryos, equivalent to 1 – 10 μM ABA (Himi *et al.*, 2002). Additionally the presence of bran extracts from dormant genotypes during germination enhances dormancy of intact and half grains (Mares, 1999). In both of these studies, there was no indication of the class of compound(s) extracted from the bran which caused inhibition of germination. Despite this, these studies provide some indication that part of grain dormancy in red-grained and dormant white-grained wheats is similar and possibly enhanced by a chemical inhibitor present in the seed coat.

Important research conducted by Stoy and Sundin (1976) showed that catechin tannins purified from green tea is capable of inhibiting germination in isolated wheat embryos. These catechin tannins have been shown to have a role in preventing germination in genetically receptive genotypes in a later study, with the dormancy response was controlled by a single semi-dominant gene (Stoy and Olsen, 1980). Catechin tannins as a dormancy factor has been tested further by Mares and Ellison (1990), who confirmed that there was an inhibitory effect of the compound. It is, however, not clear from this whether this genetic dormancy response to externally applied catechin tannins is distinct or merely a function of embryo sensitivity to ABA. Nevertheless, it is possible that catechin compounds that may be present in the wheat seed coat may be a function of dormancy, but there is no real evidence whether these compounds are present in high enough concentrations to induce dormancy. Furthermore, there is evidence that the seed coat of dormant wheat genotypes contain germination inhibitors, which may be flavonoid compounds.

1.6.4.1 Expression of flavonoid biosynthesis genes

Flavonoids have been implicated in a number of important plant functions, including UV-B protection, flower colour and disease resistance (Harborne and Williams, 2000). The flavonoid biosynthesis pathway has been studied in great detail in a number of different plant species, for example petunia (Holton and Cornish, 1995), *Arabidopsis* (Broun, 2005) and maize (Grotewold *et al.*, 1998). More recent studies have concentrated on transcription factors and regulation of the pathway, leading to analysis of specific gene expression in different plant tissues, eg (Debeaujon *et al.*, 2003). Highly polymerised flavonoid compounds, such as phlobaphene, as well as the regulatory genes involved in its synthesis have been characterised in the seed coat of maize (Winkel-Shirley, 2001). In *Arabidopsis*, the flavonoid pathway generating proanthocyanidins is responsible for the pigmentation of the seed coat and has been characterised by examining *transparent testa* (*tt*) mutants eg (Abrahams *et al.*, 2002; Pourcel *et al.*, 2005). In contrast to maize where the presence of phlobaphene has no effect on seed dormancy (Grotewold *et al.*, 1994), *Arabidopsis* mutants without the proanthocyanidin pigmentation show a dramatic reduction in dormancy (Debeaujon *et al.*, 2000; Kitamura *et al.*, 2004), unlike in Brassica species. A schematic representation of the flavonoid biosynthesis pathway is presented in Figure 1.1 which outlines branches and enzymes involved in the synthesis of major flavonoid compounds.

The red pigment in red-grained wheats has been suggested to be a polymerised flavonoid, phlobaphene, compound (Miyamoto and Everson, 1958; Himi and Noda, 2004). It has been well documented that the most dormant red-grained wheats are more dormant than the best white-grained wheats (Flintham, 2000; Warner *et al.*, 2000). In addition, removal of the red pigment by breeding or mutation invariably results in a reduction in dormancy phenotype (Mares, 1999; Warner *et al.*, 2000). Therefore, it is reasonable to assume that the red pigment may contribute biochemically to the high level of dormancy red-grained wheat. Furthermore gene expression may differ between white and red-grained wheats, thereby affecting the formation of important compounds the flavonoid pathway.

In contrast to plant species such as maize and *Arabidopsis*, the flavonoid biosynthesis pathway in wheat seed coat has not as yet been thoroughly investigated. Important enzyme, chalcone synthase (CHS) and dihydroflavonol-4-reductase (DFR), involved in the

production of major flavonoid groups through the pathway and relating to a number of plant species has recently been characterised in detail (Figure 1.1) (Winkel-Shirley, 2001). These major genes are expressed in maize (Mol *et al.*, 1998) and have also been found to be expressed in blue-grained wheat. Investigations in blue-grained wheat have found that different enzymes of the flavonoid pathway were upregulated during grain development (Yang *et al.*, 2004). *CHS*, encoding the first regulatory enzyme of the pathway was expressed more in the early stages of grain development, while there was a higher level of *DFR* expression, corresponding to an enzyme further down the pathway, in the later stages of grain development (Yang *et al.*, 2004). The blue pigment in blue-grained wheat, however, is located in the aleurone layer and is an anthocyanin compound, which is different to the pigment in the red seed coat.

Expression of genes encoding major enzymes in the flavonoid biosynthesis pathway, *CHS*, chalcone isomerase (*CHI*), flavanones 3-hydroxylase (*F3H*) and *DFR* have also been detected in the developing seed coat of red and white-grained wheats, with results suggesting that these genes are expressed at a much higher level in the red-grained genotypes (Himi *et al.*, 2005). By contrast, a biochemical analysis of CHI activity in the developing seed coat of red and white-grained wheats discovered that activity increased gradually in white-grained, but peaked at 20-25 days after ear emergence and then declined in red-grained genotypes (McCallum and Walker, 1990). In all of these studies, there was no investigations into the same gene expression or enzyme activity in the seed coat of dormant white-grained genotypes, or any attempt to measure differences in the gene or enzyme products. Despite this, it is evident that compounds associated with the red pigment, or the pigment compound itself, is associated with the increased dormancy of red-grained wheats.

The genes coding the DFR enzymes were also found to be located in a proximal region of the long arm of group 3 chromosomes, close to the *R* gene responsible for the red pigment and *Vp1* involved in embryo dormancy in maize. There has been a recent report that the *R* gene active in red-grained wheats is a transcription factor that upregulates the flavonoid biosynthesis pathway (Himi *et al.*, 2005). *Vp1* has also been implicated in upregulating anthocyanin synthesis in maize, but only in embryo and scutellum tissue (McCarty, 1995). Specific branches of the flavonoid pathway are upregulated by transcription factors in many species, for example maize, with the *P* gene responsible for phlobaphene production

also in maize (Grotewold *et al.*, 1994; Grotewold *et al.*, 1998), and proanthocyanidin synthesis in *Arabidopsis* with *transparent testa2* (*tt2*) and *tt8* (Debeaujon *et al.*, 2003). The study by Himi *et al.,* (2005) detected significant expression of genes in the flavonoid biosynthesis pathway in genetically dormant red-grained but not non-dormant white-grained genotypes, inferring that the pathway forming the red pigment is only upregulated in red-grained seed coats. Therefore, it is possible that the *R* gene upregulates a section of flavonoid biosynthesis pathway and this branch of the pathway is possibly inactive or blocked in white-grained wheat. However, neither non-dormant red-grained or dormant white-grained wheats were included in the investigation by Himi et al., (2005) and Himi and Noda (2004) and also there was apparently no attempt to measure gene products.

Dormancy in white-grained wheats is difficult to characterise, because of the lack of defining red pigment that usually confers dormancy, and the role of the red seed coat in dormancy is still unknown. In wheat there are two factors contributing to dormancy: embryo sensitivity to ABA, as well as an unknown interacting seed coat factor that causes dormancy levels greater than the embryo factor alone. Flavonoids can perform as germination inhibitors (Winkel-Shirley, 1998) and the seed coat factor in the dormant white-grained genotype may be a flavonoid compound synthesised in the seed coat and subsequently transferred to the embryo during imbibition, thereby delaying germination. By analogy, a precursor of the red pigment might accumulate in the seed coat of dormant white-grained genotypes where the absence of later steps in pigment polymerisation are not present.

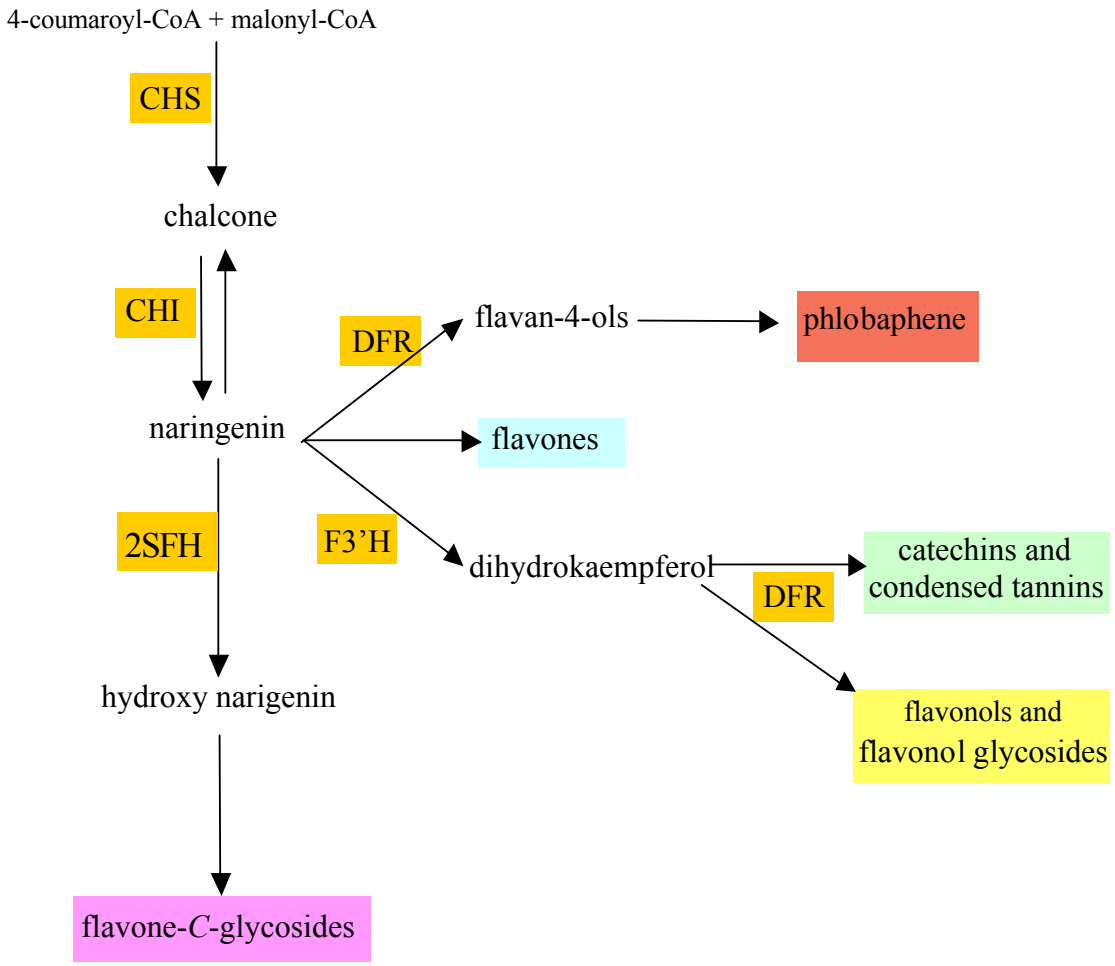


Figure 1.1: Schematic diagram of flavonoid biosynthesis pathway.

Major enzymes are boxed in orange and include CHS (chalcone synthase), CHI (chalcone isomerase), F3'H (flavonoid 3'hydroxylase), 2FSH (2S flavanone 2-hydroxylase) and DFR (dihydroflavonol 4-reductase). The coloured boxes highlight major subgroups of the pathway: phlobaphene (possible red grain pigment), flavones (eg apigenin), catechins, condensed tannins (eg proanthocyanidins), flavonols (eg quercetin) and flavonol glycosides. Flavone-*C*-glycosides are a lesser-known product of this pathway and are generated by 2SFH from naringenin in parsley (Britsch, 1990) and *Fagopyrum esculentum* (Kerscher and Franz, 1986) species, although so far not demonstrated in grasses. This pathway was adapted from Winkel-Shirley (2001) and Kerscher and Franz (1986).

1.6.5 Chromosomal regions associated with dormancy

Accurate characterisation of the phenotype of the parents and progeny in mapping populations is very important in detecting Quantitative Trait Loci (QTL) actually involved in grain dormancy and not other interacting factors, in particular the red seed coat colour (Flintham *et al.*, 2002). Populations derived from red-grained (dormant) x white-grained (non-dormant) genotypes have detected QTLs on the long arm of group 3 chromosomes, which is the location of the *R* gene (Groos *et al.*, 2002). The relationship between the red seed coat colour and dormancy is so strong that the QTL is a marker for dormancy, rather than any other regions associated with dormancy (Flintham and Gale, 1996). Therefore to avoid the confounding effect of the *R* gene, mapping population parents must have the same seed coat colour.

Most attempts to locate QTL regions associated with grain dormancy in wheat have concentrated on populations derived from crosses between dormant and non-dormant genotypes, eg (Kato *et al.*, 2001; Mares and Mrva, 2001; Groos *et al.*, 2002). A number of chromosomal regions associated with dormancy in wheat have been detected in these populations, with QTLs most commonly located on chromosome 4AL (Mares and Mrva, 2001; Mares *et al.*, 2005). Additional regions in dormant x non-dormant populations have also been detected on 2AL, 2DL and 3D (Mares, 1996; Mares and Mrva, 2001). The QTL located on chromosome 4AL is most likely related to embryo sensitivity to ABA (Himi *et al.*, 2002), or intermediate dormant phenotype and is consistent for a number of different populations (Mares *et al.*, 2005). Flintham (2002) also found a QTL on 4AL in a dormant red-grained population. The 4AL region therefore corresponds to a major component of dormancy, but other regions which contribute to a high level of dormancy expression requires carefully selected mapping parents.

Several investigations have attempted to locate dormancy QTL regions through populations derived from dormant and genotypes with some level of dormancy (Flintham *et al.*, 2002; Osa *et al.*, 2003; Mori *et al.*, 2005). These studies have also detected regions associated with the red seed coat colour, the *R* gene, which is also located on the group three chromosomes (Flintham, 2000; Groos *et al.*, 2002). QTLs have been reported on these group three chromosomes in studies using non-dormant x dormant genotypes in both

red-grained and white-grained populations (Mares, 1996; Osa *et al.*, 2003). The detection of weak QTLs on the group three chromosomes indicates that these regions are associated with dormancy, regardless of the presence of the active *R* gene, which supports the proposal that *R* genes are separate from other dormancy genes on chromosome three (Flintham, 2000; Warner *et al.*, 2000). QTLs not associated with the red seed coat colour have been detected on 3BL (Groos *et al.*, 2002), 3AL (Kulwal *et al.*, 2005), 3AS (Mori *et al.*, 2005) and weakly on 3D (Mares *et al.*, 2002). Despite this, it is still not known which regions of the genome are associated with the dormant seed coat factor and if it is related to the *R* gene, simply because the population selected for analysis do not differ for this specific factor.

1.7 Summary and Project Aims

The seed coat in some white-grained wheat genotypes contributes to a higher level of dormancy when this seed coat factor is expressed in combination with a major dormancy factor, namely embryo sensitivity to ABA (Mares, 1999). Although it has been documented that dormant red-grained genotypes are more dormant than white-grained genotypes, dormancy in white-grained wheat, in particular the seed coat factor, has not been characterised. The function of the *R* genes in dormancy, which cause pigmentation in the seed coat of red-grained genotypes, have been studied in greater detail. The red pigment has been suggested to interfere with water uptake (Huang *et al.*, 1983), react with oxygen and prevent diffusion into the embryo (Porter and Wareing, 1974) or generate compounds which inhibit germination (Stoy and Sundin, 1976). There has been no suggestion whether water uptake or oxygen diffusion through the seed coat is important in dormant white-grained genotypes. Furthermore, although the dormant white seed coat lacks the red pigments, it has been proposed that a build-up of flavonoid precursors to the red pigment may be responsible for inhibiting germination in dormant white-grained genotypes. Flavonoid compounds can behave as germination inhibitors (Winkel-Shirley, 1998; Rogozhin *et al.*, 2001). Dormancy genes separate from those corresponding to embryo sensitivity to ABA have been detected on the group three chromosomes, close to the position of the *R* genes (Flintham, 2000; Groos *et al.*, 2002).

The overall aim of this project was to investigate and possibly characterise the role of the seed coat in the dormancy of the white-grained wheat in the dormant genotype SUN325.

This aim was addressed by investigating the physical, biochemical and genetic aspects of the SUN325 seed coat. Included in this study was an intermediate-dormant white-grained genotype (QT7475), which did not appear to express the putative seed coat factor. The inclusion of this genotype, a dormant red-grained and a non-dormant white-grained genotype, enhanced comparison between the dormancy mechanisms of the different seed coats in wheat grains expressing various levels of dormancy.

The specific aims of the project were:

1) To locate chromosomal region associated with the dormant seed coat factor in the doubled-haploid population derived from SUN325 x QT7475 (dormant x intermediate-dormant).

2) To validate the two-component dormancy model in wheat, with dormancy consisting of an embryo and interacting seed coat factor.

3) To determine if the dormant seed coat acts as a barrier to oxygen diffusion or if there is greater resistance to physical damage.

4) To investigate imbibition pathways and the effect of the seed coat on water uptake or distribution in wheat grains of varying dormancy.

5) To determine if there are any differences in the expression of major genes in the flavonoid biosynthesis pathway, in the developing seed coat of dormant white- and red-grained genotypes.

6) To extract flavonoid compounds from the developing and mature seed coat and to compare the accumulation of compounds in dormant and non-dormant genotypes.

7) To develop a bioassay system to demonstrate the action of the seed coat in the expression of dormancy.

Chapter 2: General Introductory Methods

2.1 General Methods

Methods relating to specific work have been described in each relevant chapter. Outlined below are general materials and methods which have been used throughout this thesis and were not altered.

2.1.1 Plant Material

The following bread wheat (*Triticum aestivum L.*) germplasm, representing the range of genetic variation for grain dormancy in white-grained wheats and a red-grained control, was used in this research project:

Hartog – Australian, non-dormant (sprouting susceptible) white-grained cultivar.

Janz - Australian, non-dormant (sprouting susceptible) white-grained cultivar.

QT7475 – A white-grained breeding line with pedigree, AUS1408/Janz^{3}*//Cunningham, which has intermediate dormancy (intermediate tolerance to sprouting). The dormancy is derived from AUS1408, a landrace from the Transvaal region of South Africa (Mares, 1987). Cunningham is a non-dormant, locally adapted cultivar with similar pedigree to Janz.

SUN325B - A white-grained breeding line with pedigree, Hartog/Vasco//Aus1408/3/Hartog, which is dormant (sprouting resistant). The dormancy is derived from AUS1408, a landrace from the Transvaal region of South Africa (Mares, 1987). Vasco is a non-dormant, locally adapted cultivar.

R/W635 – A red-grained breeding line with pedigree, Hartog/AUS1490, which is dormant (sprouting resistant). The dormancy is derived from AUS1490, a tall red-grained genotype containing a single gene for seed coat colour on chromosome 3A (*R-A1b, R-B1a, R-D1a,* (McIntosh *et al.*, 1998)).

QT7475/SUN325B DH – a doubled haploid (DH) population developed using the wheat x maize (*Zea mays*) system (Kammholtz *et al.*, 2001).

2.1.2 Field and glasshouse growing conditions

Germplasm was grown in the field as short, 0.5 m, replicated rows and a glasshouse in at the Waite Campus in 2003 and 2004. Plants were maintained disease and pest free via the application of appropriate agricultural chemicals and irrigation was used as necessary to prevent moisture stress. A DH population consisting of 96 lines of the white-grained genotypes, QT7475 (intermediate-dormant) x SUN325 (dormant) was sown in four separate experiments. Each experiment was sown with the parental lines, a standard, Hartog, and in two replications, with one glasshouse and three field experiments. The field experiments were conducted at the Waite Campus, South Australia in 0.5 m single row plots in 2003 and 2004. In 2004, two field experiments were undertaken, with one experiment sown three weeks later to ensure exposure to warm ripening conditions. In all experiments, a non-dormant standard line, Hartog, was included as well as the parental lines at 50-plot intervals. Prior to physiological maturity, the experiments were covered with translucent white plastic for the remainder of grain ripening and harvest to prevent the confounding effect of rain on dormancy expression (Mares, 1993). The later sown experiment in 2004 was not covered because the risk of rainfall around grain ripening was low. The glasshouse experiment was also undertaken in 2004, with lines were grown in 7.5 cm tubes, one line per tube, and placed in plastic trays with a layer of soil. Care was taken to ensure the soil in the tubes remained moist and liquid fertilisation occurred weekly.

From approximately 35-40 days after anthesis, the progress of ripening in all trials was monitored visually at 2-day intervals taking note of the loss of chlorophyll from the leaves, stems, and spikes. Spikes were harvested from the primary tillers in each plot when all green colour had just disappeared from the leaves and stems of the plants and stored under cover at ambient temperature for 4 days, during which time the grain was recovered by gentle hand-threshing. Grain drying was completed by placing samples in a forced-air dryer for 24 hours at 35°C to ensure that the final moisture content was below 12% (harvest-ripeness). At 5 days after harvest, the grain was transferred to –20°C to preserve dormancy (Mares 1983) until all plots had been harvested and all the seed required for germination testing was available.

2.1.3 Germination testing

Plump grains with no obvious defects were counted into replicates of 50 and incubated at 20ºC in Petri dishes containing Whatman Filter paper moistened with 5 mL of reverse osmosis (RO) water. Germinated grains were counted and discarded daily so that newly germinated grains were recognised easily. Dormancy was expressed by either a cumulative percentage germination curve or weighted germination index. The germination index (GI) is weighted according to grains which germinate rapidly and is calculated from the following formula (Walker-Simmons, 1987):

$$GI = (7x_{n1} + 6x_{n2} + 5x_{n3} + \ldots\ldots\ldots 1x_{n7})/ \text{ total days of test x total no grains}$$

Where n1, n2, n3,….n7 are the number of grains germinated on day 1, day 2, day 3,….day 7. The maximum index is 1.0, while lower numbers represent increasing levels of grain dormancy.

2.2 Aleurone Samples and Preparation of Seed Coat Material from Developing and Ripe Grains

Purified aleurone tissue was kindly donated by Dr Bruce Stone, Latrobe University.

At harvest ripeness, grain was harvested from each of the genotypes and the seed coat removed with a Codema Laboratory Huller L505 (USA). Whole grain samples (100 g) were put into the machine for 2 minutes, yielding about 500 mg of seed coat (pericarp, testa and aleurone) material. Developing grains were dissected from the spikelet and the embryo removed and discarded. The seed coat was then sliced through the dorsal side, exposing the developing endosperm which was subsequently scraped away with a spatula. Three seed coats, about 0.09 g in sample weight, were collected in a 1.5 mL tube. The seed coat of developing grains contained more water and was substantially thicker than the mature seed coat.

An 85-95 mg sample of the seed coat isolated from mature grains was extracted overnight in a glass test-tube with 2.5 mL 0.1M NH_2OH. The sample was then centrifuged at 10,000 g for 3 minutes and the soluble extract removed from the solid seed coat material. Once removed, the extract was filtered with a disposable syringe filter and the pH adjusted to

neutral with the addition of dilute acetic acid. Extraction of the developing seed coat (10, 15, 20, 25 and 30 days post anthesis) involved the addition of 1 mL of 0.1M NH_2OH to approximately 90 mg seed coat (pericarp, testa and a small amount of aleurone) material (3 grains) in a glass test-tube and soaked overnight. The liquid extract was subsequently removed using a pipette, adjusted to neutral pH, dropwise using dilute acetic acid, filtered and 20 µL of the extract analysed by HPLC. The HPLC method was a 70 minute run and has been outlined in Chapter 6 (*Section 6.2.6*).

2.3 Results

2.3.1 Examination of seed coat preparation with fluorescent microscopy

A small amount of seed coat material that was removed by the Laboratory Dehuller was soaked in a 0.1% solution of calcafluor (Fluorescent Brightner 28, Sigma USA) for 5 minutes. The calcafluor solution was subsequently removed and the seed coat material was washed once with water and mounted on a microscopy slide. Slides were viewed on an Olympus BX51 light and fluorescence microscope with objective lens 10 (0.25NA) and 20x (0.5NA) magnification using filters for calcaflour: band pass filter with 330-395 nm excitation band and long pass emission filter 420LP.

Figure 2.1 shows the structure and fluorescence of a fragment of the isolated aleurone tissue. The image shows that the aleurone tissue has large structured cells with thick cell walls which fluoresce brightly. Compared to the removed seed coat material (Figure 2.2), this aleurone is clearly distinguishable, fluoresces much more brightly, and therefore was easily identified in the seed coat material. The microscopic images show that the different layers of the seed coat were removed by the de-hulling machine, with both fragments (*Panel A*) and large chunks (*Panel B*) of seed coat visible (Figure 2.2). It is also apparent that the different cell types of the seed coat fluoresce at varying levels (Figure 2.2, *Panel B*), but not at the level of the aleurone tissue. Figures 2.3 (*Panel A*) and 2.2 (*Panel B*) show the heterogeneity of the removed seed coat material, including dense cell masses (DC), trichomes (T) and transparent tissue which is probably the outer pericarp (OP). It is not possible with this low resolution to identify cells derived from specific layers of the seed coat, but these images show that there is clearly a heterogenous mix of seed coat material.

Figure 2.3 (*Panel B*) includes some contaminating aleurone tissue (Al) in the removed seed coat material. This tissue was rarely encountered in the slides mounted with seed coat, and it was predicted that this contaminating aleurone tissue comprised of approximately 1% of the total removed seed coat material.

2.3.2 HPLC analysis of pure aleurone

The isolated aleurone obtained from Dr Bruce Stone was extracted with the alkaline extraction method (*Section 2.3*) and analysed by HPLC. Figure 2.4 shows that the extract is dominated by two major compounds (peaks A and B), which have strong absorbances at 280 and 340 nm. The UV/Vis spectra in Figure 2.5 suggest that peak B (Figure 2.4, *Panel B*) is ferulic acid, a prevalent compound in the aleurone tissue (Parker *et al.*, 2005). The compound represented by peak A, with λ_{max} of 280 nm, is a simple phenolic compound (Figure 2.4, *Panel A*). Together these two compounds constitute the bulk of the compounds extracted from the aleurone, with the chromatograms showing only a few other very minor peaks.

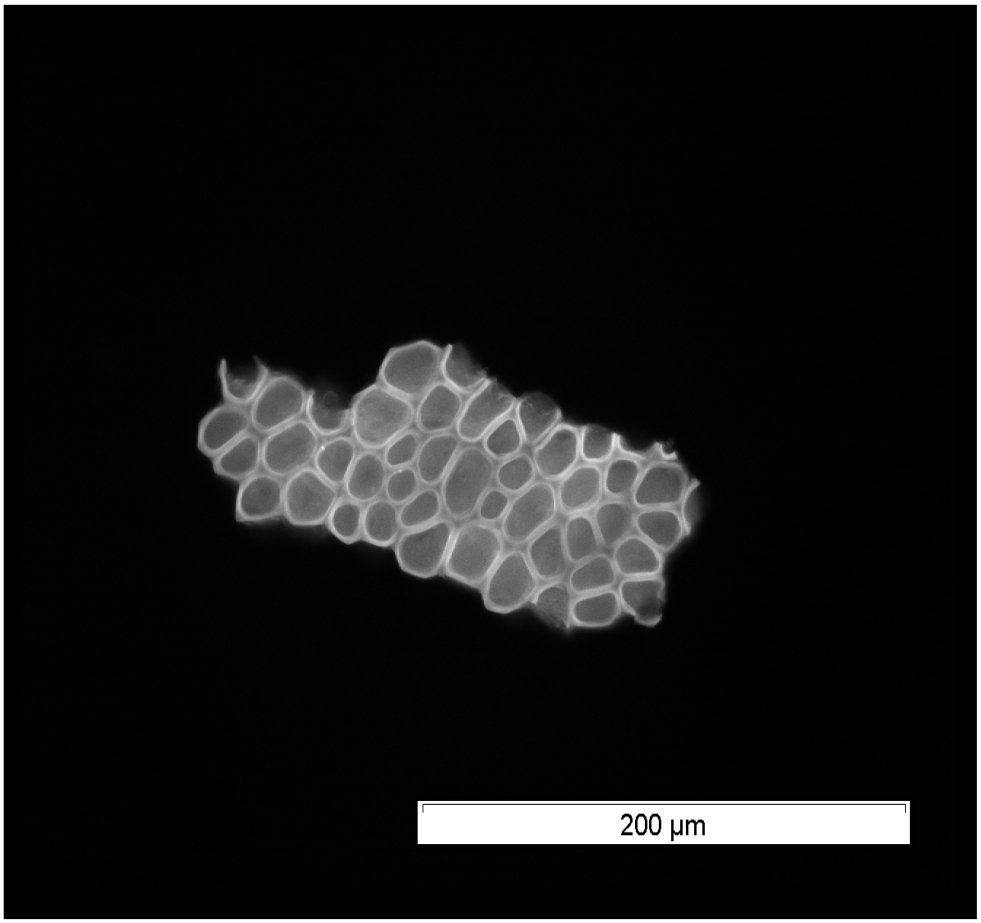


Figure 2.1: Fluorescent micrograph of isolated aleurone tissue (20x magnification)

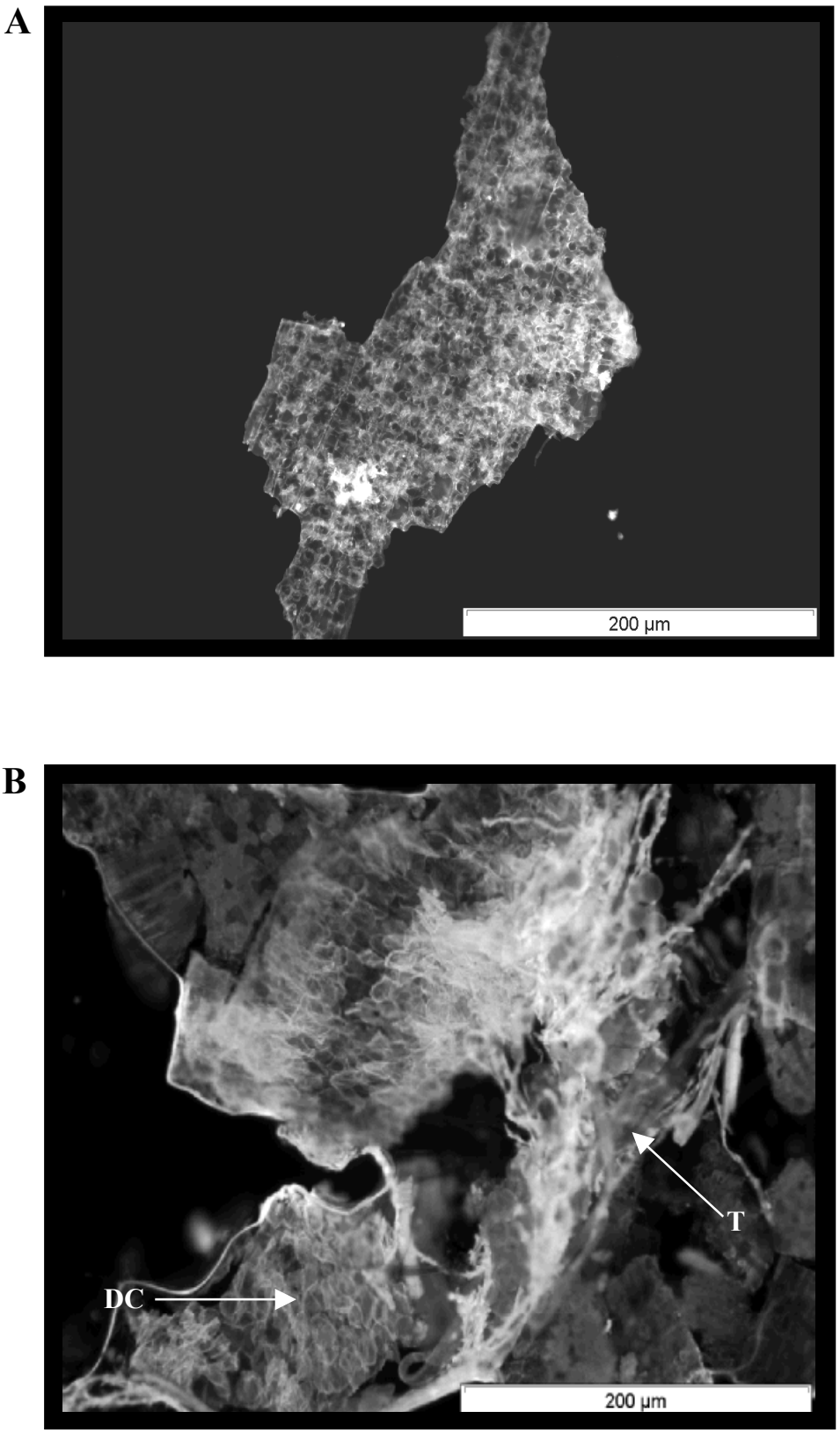


Figure 2.2: Fluorescent micrographs of seed coat material. Arrows indicate different cell types. Panel A: a thick fragment of seed coat, Panel B: heterogeneous seed coat material.

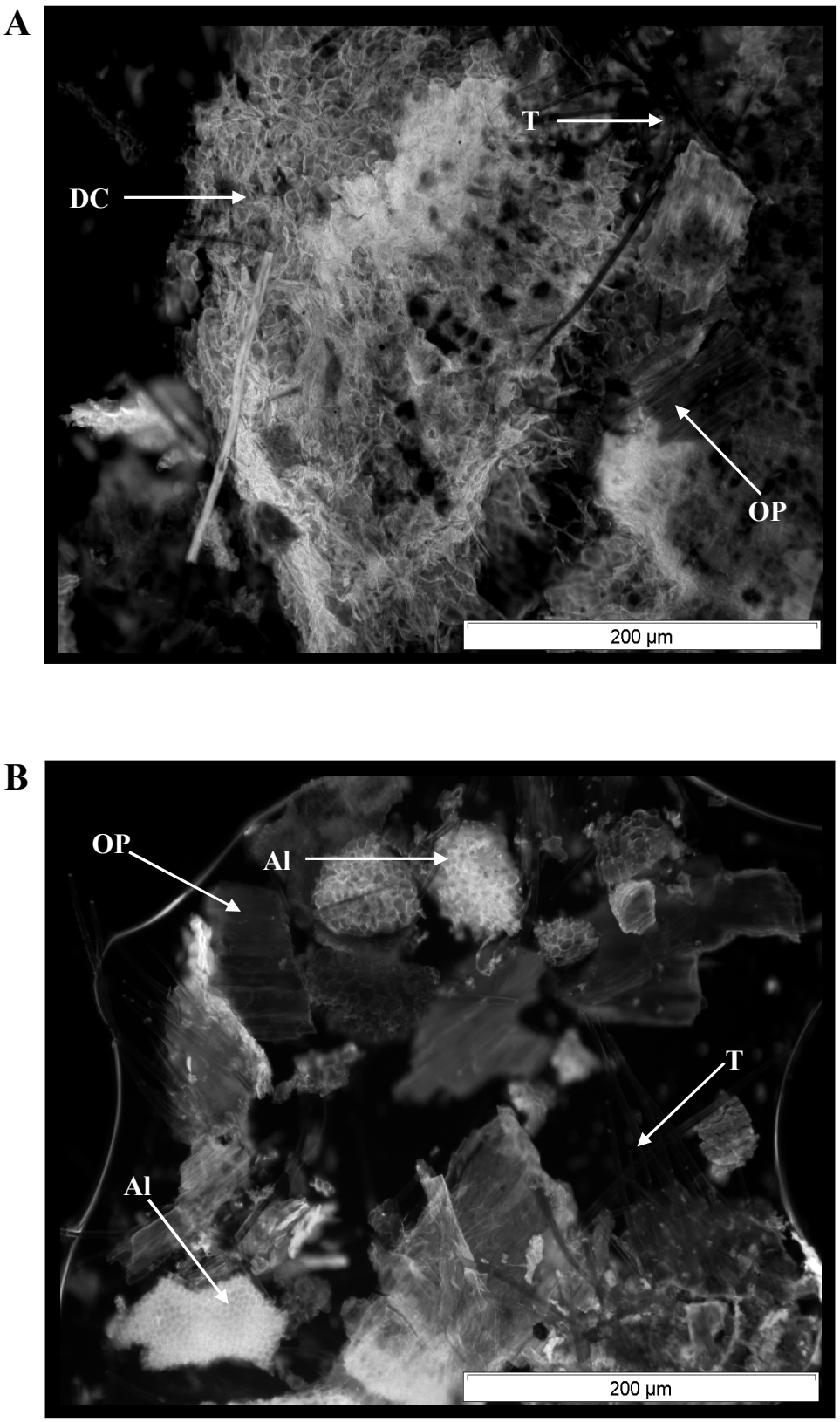


Figure 2.3: Fluorescence micrographs of seed coat material. Arrows indicate different seed coat cell types. Panel A: heterogenous seed coat material, Panel B: heterogenous seed coat material with aleurone contamination.

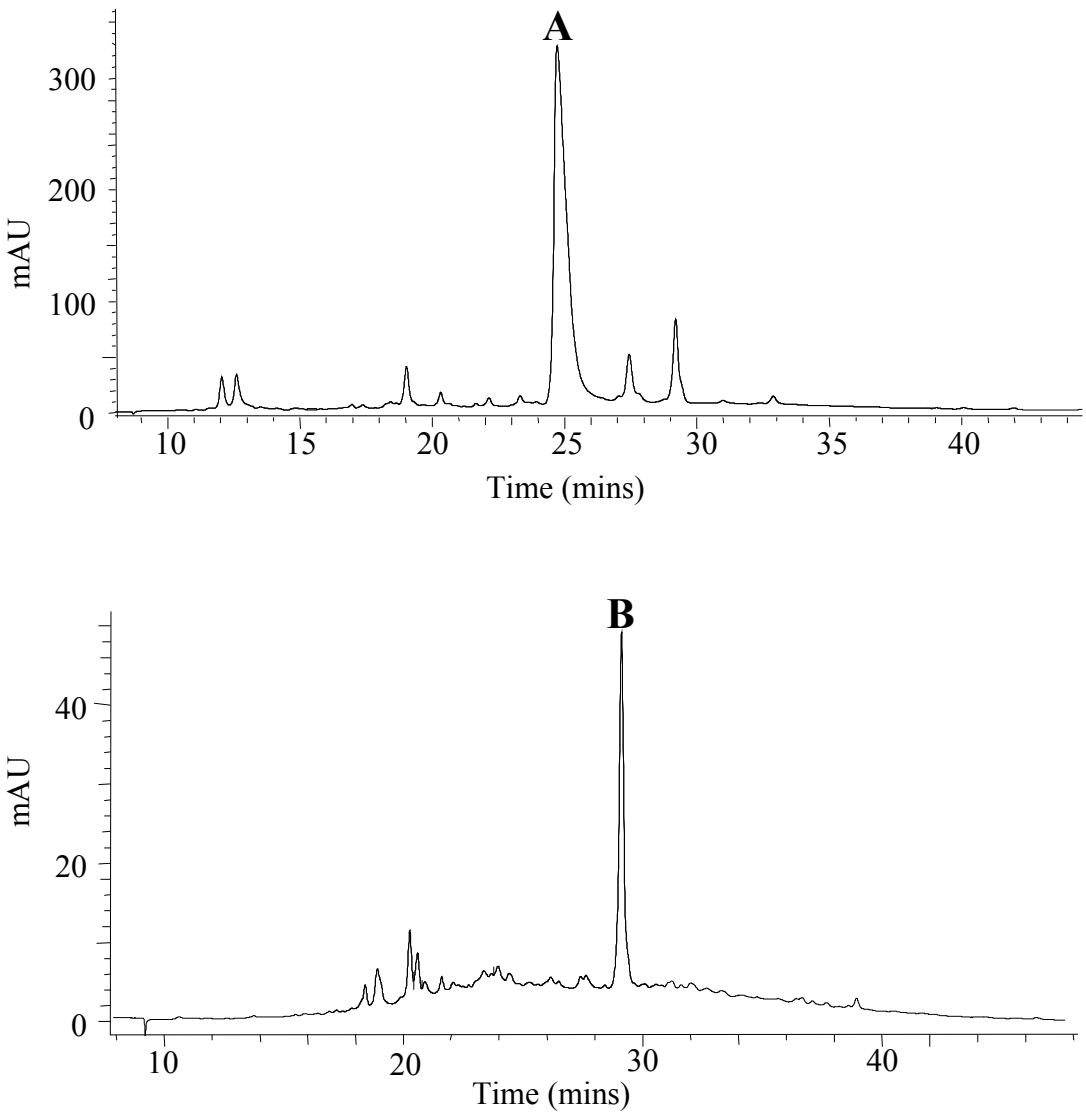


Figure 2.4: HPLC chromatograms of alkaline extract from pure aleurone tissue. *Panel A*: 280 nm and *Panel B*: 340 nm. Major peaks are labelled.

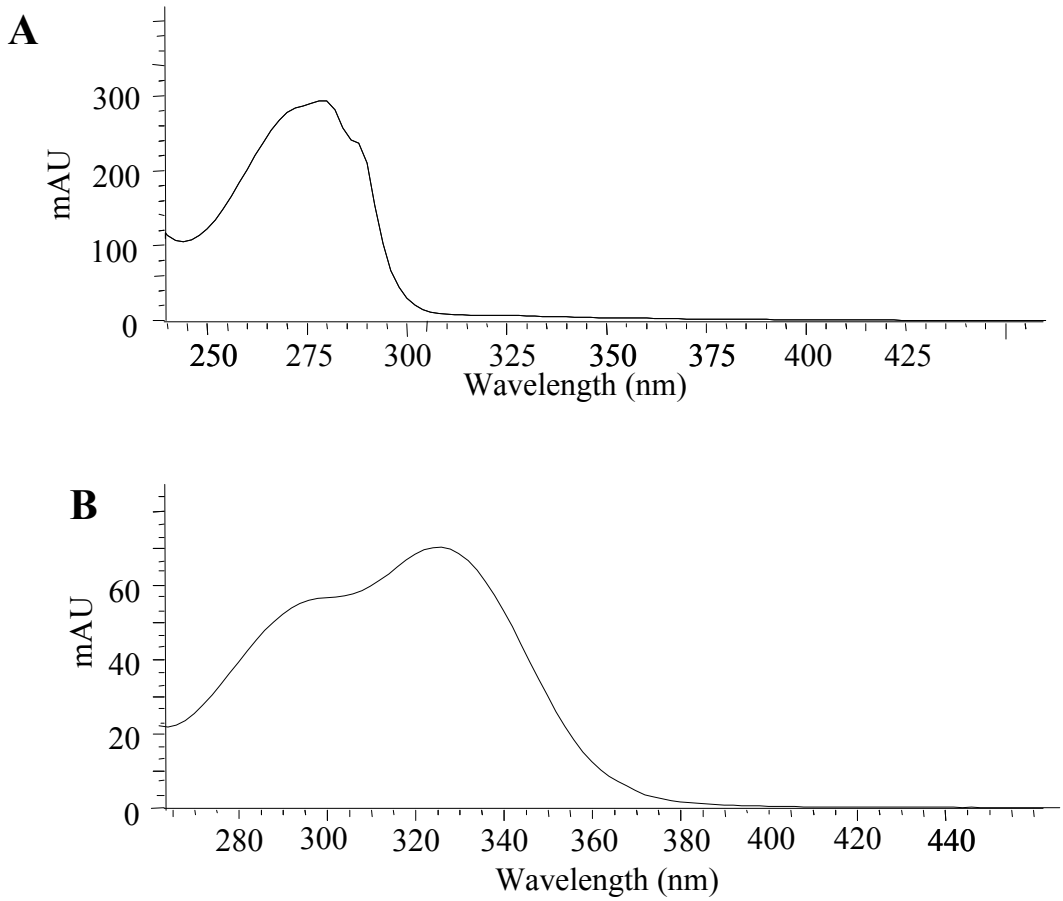


Figure 2.5: UV/Vis spectra of major peaks identified in the aleurone extract (Figure 2.4). Panel A: representing peak A has a λ_{max} of 285 nm and Panel B: representing peak B has a λ_{max} of 290 and 325 nm and is consistent with the spectrum of ferulic acid (Figure 7.

Chapter 3: Permeability to Oxygen and Effect of Physical Damage to the Seed Coat on Germination and Dormancy

3.1 Introduction

The effect of physical damage to the wheat seed coat on germination and dormancy has been documented in a number of studies (Woodbury and Wiebe, 1983; Clarke and DePauw, 1989; Ueno, 1996). Dormancy is severely reduced when the seed coat of wheat grains is removed or the grains cut in half (Durham and Wellington, 1961; Mares, 1992) and even slight abrasion of the outer layers of the wheat seed coat through mechanical threshing will affect dormancy expression (Mares, 1999). Based on these observations, it has been suggested that the seed coat is an important tissue in dormancy expression (Woodbury and Wiebe, 1983; Ueno, 1996). However it is not clear whether the putative dormant seed coat factor of SUN325 provides a physical restriction to germination, or if there is a difference in the effect of damaging the seed coat between dormant and non-dormant genotypes. There is also little information on the effects of damaging specific areas of the seed coat or whether damage increases germination irrespective of where it is inflicted. Diffusion of oxygen through the seed coat has been implicated in the dormancy of several cereal and dicot species (Stabell *et al.*, 1998; Barthe *et al.*, 2000) and it is possible that it also plays a role in wheat. For example, the seed coat of SUN325 may differ in permeability to oxygen diffusion or contain compounds that consume oxygen and prevent germination.

A number of different techniques have been employed to measure oxygen consumption by plant structures including direct measurements on the seed coat as a diffusion barrier (Sinclair, 1988) and oxygen uptake or consumption of the seed (de Visser *et al.*, 1990). Other studies have used enriched oxygen environments to determine if germination is increased when the oxygen diffusive pressure and hence permeation of oxygen through the seed coat is enhanced (Wellington, 1956; Grange *et al.*, 2003). These experiments have proven to be an effective and simple way of determining the role of oxygen diffusion through the seed coat on germination and dormancy and this method was adapted for the current investigation.

Chapter 3: Permeability to Oxygen and the Effect of Physical Damage to the Seed Coat on Germination and Dormancy

In this section, the impact of physical damage to the seed coat and exposure to oxygen-enriched atmospheres on germination and dormancy were investigated. The specific aim was to determine if these factors could be related to proposed genetic differences between the seed coats of dormant and non-dormant genotypes. The genotypes utilised in this series of experiments represent the range of dormancy phenotypes in white-grained wheat and have been described in Chapter 2.

3.2 Materials and Methods

3.2.1 Plant material

Non-dormant, Hartog, intermediate, QT7475, and dormant, SUN325, white-grained genotypes only were used in this study.

3.2.2 Germination test

All germination tests were conducted as outlined in Chapter 2.

3.2.3 Physical damage to seed coat

A total of six different treatments with combinations of different methods for damaging the seed coat were compared, to determine the major and minor effects of seed coat damage on germination. Firstly, the seed coat on the dorsal surface of grains that had been soaked in water for approximately 15 hours was either 1) pierced with the point of a scalpel blade, 2) cut through the seed coat or abraded with a fine emery board. Secondly, the dorsal seed coat or crease of grains that had been soaked in water for 5 hours was soaked and pierced with a fine syringe needle, once or twice (dorsal) and once (crease). Finally, the outer pericarp of grains that had been soaked in water for 15 minutes was removed by making a shallow incision with a scalpel, and removing the layer with fine tweezers.

All treatments were conducted in replicate.

3.2.4 Germination of grain in oxygen-enriched atmospheres

Holes, 8.5 mm in diameter, were drilled in two 10 L vacuum desiccators and close fitting hoses inserted to enable the desiccators to be filled with oxygen/nitrogen (40/60%). The sides of the desiccator were clamped, and the area around the hose sealed with masking tape to prevent any exchange of air with the exterior. Seeds, two replicates of 50 grains, were place in open Petri dishes in the desiccators and moist paper towels placed on the bottom and sides to maintain humidity. Once the desiccator was sealed, the gas was injected via the hose with the outlet tap open so that the oxygen-enriched air could flush out the normal atmospheric air. After 10 minutes, the gas flow was stopped and the outlet tap closed after a few seconds to ensure the environment inside the desiccator was of

atmospheric pressure. Seeds were viewed through the transparent lid of the desiccator and scored for germination daily. Every second day the germinated seeds that were too large were discarded and the desiccators were re-gassed in the same manner. Control grains were placed in vacuum desiccators containing normal air.

3.3 Results

All treatments that involved substantial (multiple piercings, slicing and abrading) damage to the seed coat resulted in similar significant increases in germination index of all the genotypes, such that there was no difference between the treatments (Figure 3.1, *Panel A*). Table 3.1 shows that there is a highly significant effect of seed coat damage on GI, but no interaction between seed coat damage and genotypes. Interestingly in this investigation, damage to the seed coat only resulted in a partial loss of dormancy, for example, the germination index of SUN325 (dormant) only increased to a value comparable to undamaged QT7475 (intermediate dormant), and none of the treatments changed the dormancy phenotype to the non-dormant phenotype typical of Hartog.

Since no differences were seen with these treatments, a gentler approach to seed coat damage was attempted. Minor damage, caused by piercing the seed coat with the point of a needle, gave inconsistent results (Figure 3.1, *Panel B*). SUN325 was the only genotype that showed a significant increase in GI when the dorsal seed coat and crease region was pierced. Only dorsal piercings were successful in significantly increasing the GI of Hartog and QT7475 (Figure 3.1, *Panel B*). Piercing the dorsal region of the seed coat in SUN325 resulted in a GI greater than the GI of the control QT7475. These observations were supported by the ANOVA presented in Table 3.1 which shows that there is no significant interaction between genotype and piercing treatment.

Removal of the outer pericarp, leaving the inner layers of the seed coat appeared to increase the error associated with measurement of germination (Figure 3.1, *Panel C*). As a consequence, even though there appeared to be a general increase in germination when the outer pericarp was removed (Figure 3.1, *Panel C*), the observed differences were not significantly different. Table 3.1 also shows that there is a significant difference between the peeling treatments, but no difference between the GI of genotypes and treatments between genotypes.

Exposure of germinating grains to an oxygen-enriched atmosphere resulted in a minimal increase in germination index in the case of Hartog (0.08) and SUN325 (0.03), but a more pronounced increase in QT7475 (0.17) (Figure 3.1, *Panel D*) although the differences were not statistically significant from the control (Table 3.1). The ANOVA of the germination

indices displayed in Table 3.1 indicate that there is a highly significant effect of the oxygen treatment and genotype on GI, but no effect of treatment between genotypes.

The physical damage treatments to the seed coat resulted in a larger increase the GI of QT7475 and SUN325 than Hartog, which is a genotype with no dormancy. The GI of QT7475 was increased significantly with all the treatments, but was never increased to the same level of the control value of Hartog. Furthermore, although the GI of SUN325 was also increased significantly with all the treatments to the seed coat, the GI value only reached that of the QT7475 control when the seed coat was pierced dorsally once and twice. Only QT7475 showed a noticeable increase in GI when exposed to the enriched oxygen environment.

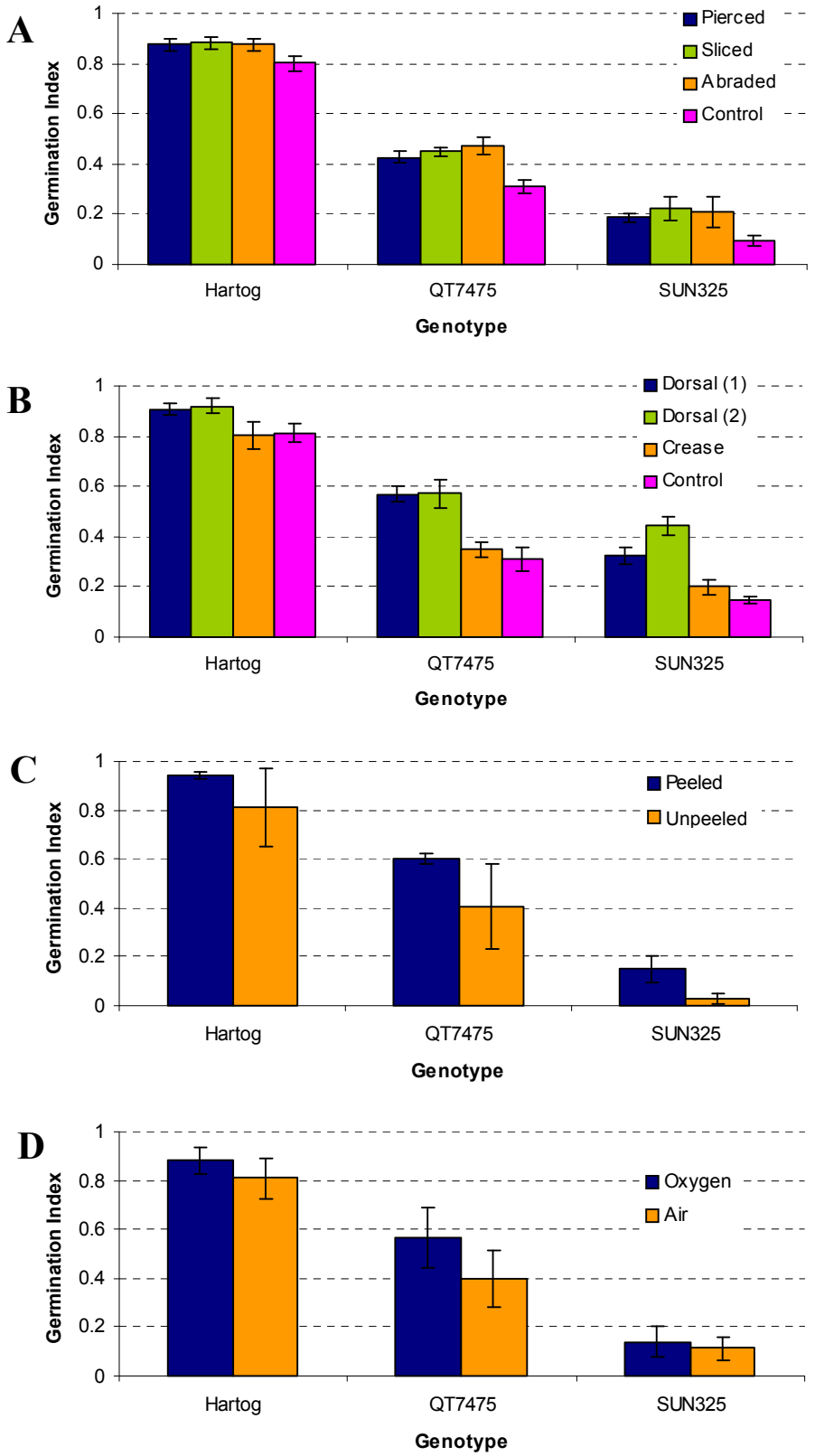


Figure 3.1: Effect of physical damage to the seed coat and enriched oxygen conditions on the GI of genotypes with differing dormancy phenotypes. *Panel A*: piercing, slicing and abrading the seed coat, *Panel B*: piercing the seed coat dorsally and through the crease region, *Panel C*: removing the outer pericarp tissue and *Panel D* enriched oxygen conditions.

Piercing, Slicing Abrading of the Seed Coat			
	d.f	**M.S**	**F.pr**
Treatment	3	0.045	<0.001***
Genotype	2	1.57	<0.001***
Treatment.Genotype	6	0.003	0.592
Residual	12	0.0036	
Piercing the Seed Coat Dorsally and in the Crease Region			
Treatment	3	0.03	<0.001***
Genotype	2	0.74	<0.001***
Treatment.Genotype	6	0.0077	0.134
Residual	12	0.0031	
Removal of Outer Pericarp Tissue (Peeled and Unpeeled)			
Treatment	1	0.066	0.118
Genotype	2	0.62	<0.001***
Treatment.Genotype	2	0.0017	0.919
Residual	6	0.02	
Enriched Oxygen Conditions			
Treatment	3	0.03	<0.001***
Genotype	2	0.74	0.002***
Treatment.Genotype	6	0.0067	0.122
Residual	12	0.0031	

Table 3.1: ANOVA of GI after physical damage to the seed coat and exposure to oxygen enriched conditions, with genotypes of differing dormancy phenotypes.

3.4 Discussion

The results presented in this chapter show that the seed coat has a physical effect on germination, but this is probably not related to grain dormancy expression. Seed coat treatments and exposure to oxygen-enriched atmospheres generally increased the germination index of all cultivars by a similar amount, although it was statistically non-significant. The treatments did not alter the ranking of genotypes for dormancy phenotype, or indeed have a significant impact on the differences between genotypes. Based on these observations it appears that the integrity of the seed coat, or at least the pericarp, represents a component of the dormancy phenotype of all cultivars but is not responsible for genotypic differences or the putative seed coat factor. In addition, the similar increases in germination resulting from both damage to the seed coat and exposure to oxygen-enriched atmospheres is consistent with the suggestion that the outer seedcoat of imbibed grains reduces the inflow of oxygen.

A study by Ueno (1996) found that removing the seed coat over the embryo increases germination to a non-dormant level, even if the genotypes are initially dormant. This is in contrast to the results presented in this study, where the GI was never increased to the non-dormant genotype, Hartog. Although physical damage to the seed coat enhances germination of the three wheat genotypes used, the relative increase was only small. The greatest impact of GI with damaging the seed coat is to increase the GI of SUN325 (0.175) to the level of untreated QT7475 (0.4) (Figure 3.1, *Panel B*). The other genotypes only showed a small increase in GI when the seed coat was damaged. In this current study the genotypes examined probably had a greater range of dormancy phenotypes, indicating that a similar effect on grain dormancy was probably observed in the study by Ueno (1996).

A germination response to the same extent, however, is not observed with peeling the outer pericarp. This is an important observation, because the seed coat factor, which causes the dormancy level of SUN325, does not appear to be completely lost with damage to the seed coat, rather only slightly affected. By contrast, the germination index of QT7475 and Hartog, which do not express the dormant seed coat factor also increased with physical damage to the seed coat. Therefore, the results suggest that the seed coat factor of SUN325 is not removed with physical damage to the seed coat, because if this was the case, it would be expected that the GI of SUN325 would increase to the level of QT7475 when the

seed coat was also damaged. The genotypic differences between SUN325 and QT7475 (ie the seed coat factor) was not removed with damage to the seed coat and therefore the seed coat appears to have a similar physical effect on germination in both genotypes.

Pericarp removal or seed coat damage appeared to have a similar effect in all cultivars and does not explain the proposed genetic differences in seed coat between SUN325 and the other genotypes. Although the pericarp was removed carefully, the germination index was increased more dramatically in QT7475 than the other genotypes, but the error was extremely large in these experiments. This error, and the germination response which deviates from the other types of physical damage is likely to be as a result of a variation in the germination of the controls, which may be due to the grain source. Despite this, the peeled grains still exhibited some dormancy, in particular the dormant genotype SUN325. It would be expected that pericarp removal and physical damage to the seed coat would eliminate genetic differences between the SUN325 and QT7475. This observation is in contrast to Ueno (1996) who found that removing the pericarp causes a complete loss of dormancy in all the tested genotypes. Another study did not detect any reduction of dormancy when the pericarp was cracked or split (Woodbury and Wiebe, 1983) and therefore obtained a results similar to that reported here. The results presented here, however, still provide little indication to the mechanism of the dormant seed coat factor of SUN325.

The results of the prior studies (Woodbury and Wiebe, 1983; Ueno, 1996), in comparison to the current investigation, indicate that the pericarp is an important seed coat structure in restricting germination, but absolute integrity of the outer layer is not required for dormancy expression. In addition to this, it was observed that some grains with the pericarp removed appeared to have a split in the testa above the embryo, which suggested that the grain was germinated, but this was not always the case. This artefact would tend to confound germination, because grains which were entire were counted as germinated, thereby contributing to the large error associated with this experiment and placing a larger emphasis on the outer pericarp on dormancy expression. It is also possible that different degrees of damage to underlying seed coat layers were incurred during removal of the outer pericarp by Ueno (1997) compared to this current study.

Woodbury and Wiebe (1983), however, have a more plausible hypothesis for the function of the pericarp in dormancy. Their investigation included the visualisation of dye and water movement beneath the outer pericarp, and the subsequent transfer of water from the distal end of the grain to the embryo through this mechanism. The theory devised to explain their observations and the delay in germination of a dormant grain exposed to water only at the distal end, was that an inhibitor in the seed coat is transferred to the embryo via capillary action beneath the outer pericarp (Woodbury and Wiebe, 1983). This hypothesis is in agreement with a loss of dormancy in SUN325, which contains a dormant seed coat factor, but, after damage to the seed coat, does not show an increase in germination to the level of QT7475 and Hartog. Despite this, it is likely that the integrity of the pericarp is important for maintaining normal germination because this tissue appears to have a role in dormancy expression.

There has been some speculation that physical damage to the seed coat allows better diffusion of oxygen into the embryo, thereby increasing germination (Clarke and DePauw, 1989), although this theory was not supported by an early study by Wellington (1956). A number of studies have concluded that the diffusion of oxygen through the seed coat does not represent a significant barrier to germination (Wellington, 1956; Porter and Wareing, 1974; de Visser *et al.*, 1990). Wheat has a relatively thin seed coat which lacks hardness and waxiness and is unlikely to restrict oxygen diffusion as does the hard waxy seed coats observed for other species like houndstoungue (Stabell *et al.*, 1998) and *Fagus sylvatica* (Barthe *et al.*, 2000). There is also some evidence that husks surrounding the seed are able to consume oxygen and hence inhibit germination, for example barley (Lenoir *et al.*, 1986) and rice (Navasero *et al.*, 1975), indicating that compounds in structures surrounding the seed are capable of restricting oxygen diffusion. The results in this current study indicate that that the seed coat may limit oxygen diffusion or availability, and hence germination, even in a non-dormant genotype such as Hartog. Despite this, there is no suggesting that the limitation of oxygen diffusion is involved in grain dormancy.

Germination of SUN325 was not significantly increased when exposed to high oxygen concentrations, whereas piercing of the seed coat in the crease region causes an increase in germination. All grains, once pierced, were placed with the crease in contact with the moist filter paper, a position probably not conducive to oxygen diffusion which is greater

through air than air to water. Thus the germination increase associated with the piercing the crease region did not appear to be consistent with the oxygen diffusion hypothesis.

The results presented in this investigation suggest that the seed coat has a physical effect on germination of the genotypes examined. Damage, in particular piercing the seed coat in the dorsal region, causes an increase in germination which may be as a result of a change in water uptake. Removing the outer pericarp would not significantly alter imbibition and therefore does not affect dormancy in SUN325 to the same extent as piercing. The observed increase in germination of the genotypes is probably due to the importance of the integrity of the seed coat to maintain normal germination, which is disrupted by piercing and removal of the outer pericarp. There is no indication that the seed coat is a formidable barrier to oxygen diffusion to the embryo, or that this is in any way involved in the dormancy of SUN325.

Chapter 4: Magnetic Resonance Micro-Imaging of Imbibing Wheat Grains

4.1 Introduction

Imbibing and maintaining grains under warm, moist conditions is the only mechanism to determine the dormancy or germinability of wheat grains. Movement of water into ripe, dry grains is therefore a critical step and potentially at least, variation in the rate or the pathway of water movement may affect measurement of the phenotype and might also be involved in the dormancy mechanism. Recent developments in Magnetic Resonance micro-Imaging (MRmI) (Kockenberger, 2001; Kockenberger *et al.*, 2004) now allow non-destructive real time visualisation of seed water distribution with high spatial resolution, and allow the determination of relative rates and pathways of water movement into living grain tissues. MRmI is a unique and powerful tool because unlike other approaches, it does not require destruction of grain integrity.

Dormancy can be affected by the differential permeability of the seed coat between dormant and non-dormant genotypes at least in some plant species (Bewley and Black, 1982). Differences in permeability could therefore be associated with dormancy in wheat and in one report (Huang *et al.*, 1983) the red seed coat of red-grained wheat has been implicated as a possible factor that influences water uptake. It is anticipated that these MRmI experiments would provide both a non-destructive and detailed picture of water uptake in a wheat grain and a clearer indication of whether dormant genotypes take up water differently to non-dormant genotypes. In addition, MRmI will indicate if the seed coat has an effect on imbibition in genotypes that express a putative seed coat factor.

The aim of this investigation is to investigate the imbibition of wheat grains using MR micro-imaging over a period of 18 hours and to visualise imbibition of grains in the absence of germination, even in the non-dormant genotype. This time period was selected to reduce the confounding effect of the high water content of growing roots and shoots. MRmI will assist in detecting water uptake pathways and distribution in the grain, identifying important stages during imbibition. In particular, the use of this technology will answer important questions about the permeability of wheat grains, and possible differences in water uptake in dormant and non-dormant genotypes.

4.2 Materials and Methods

4.2.1 Seed Water Uptake

Dormancy phenotype of grains was confirmed as outlined in Chapter 2 before commencing the following experiments. For the physical measurements of water uptake, 18 grains in four replicates were imbibed with RO water at 20ºC, samples of four grains were taken every hour for 18 hours and weighed to provide a whole grain weight. The average initial grain weight was subtracted from the average total fresh weight at each sampling time to determine the increase in grain weight during imbibition. The initial dry weight of the grains was determined by weighing each whole grain before exposure to water. Embryos from each grain were then excised from the endosperm (rest of the seed) with a scalpel and both components were weighed separately. Changes in the weight of the embryo and endosperm (rest of the seed) were calculated as: (weight – initial weight)*100/initial weight for the embryo and endosperm. The data was analysed by Genstat (Version 8.2.0158, Lawes Agricultural Trust) and subsequently fitted with a standard curve (exponential asymptopic $y = a + b^{(r*x)}$) for whole grains or simple linear regression ($y = ax + c$) for the endosperm data. The change in weight of the embryo tissue was also fitted with a standard curve (exponential asymptopic). This provided moisture content as a percentage of initial fresh weight of each grain component.

4.2.2 Imaging of Seed Water Uptake Using MRmI

All magnetic resonance experiments were performed at the Centre for Magnetic Resonance, University of Queensland, using a Bruker AMX300 (Germany) console interfaced to a Bruker 7T 15cm supercon vertical magnet. The longitudinal images were acquired with a 90° pulse with an echo time of 4.5 ms and a repetition time of 1.5s. The pulse length was 13 μs at 90° and 26 μs at 180° and had a 1.5x0.6x0.6 cm field of view with a slice thickness of 5mm. Acquisition time for the longitudinal images was 38 minutes. For the transverse images, the pulse length was 4 μs at 90° and 28μs at 180°. For both image orientations, the receiver gain was 500. The slice thickness was also 5 mm, with a field of view of 0.6x 0.6x0.8 and an acquisition time of 20 minutes. All the images colleted were 256x96 pixels in four (longitudinal) or eight (transverse) slices.

After soaking for a specific time, seeds were removed from the Petri dishes and the seed blotted dry on tissue. Teflon tape was used to completely cover the seeds to prevent any water loss. The seed was subsequently wrapped in tissue and suspended in a 60mm glass tube, which was then placed into the bore of the MRI machine. Images were collected in the transverse (horizontal) and longitudinal (vertical) orientation to provide a complete three-dimensional perspective of the grains.

Seeds from QT7475 were soaked in RO water and analysed at hourly intervals up to 18 hours. For comparison of water uptake between dormant, intermediate and non-dormant lines, grains were soaked for a total of 18 hours, with images taken within 3 hourly blocks of 1-3, 4-6, 7-9, 10-12, 13-15 and 16-18 hours. For imbibition experiments designed to investigate the effect of applying water to different parts of the grain, seeds were wrapped in Teflon tape with only the desired section (embryo/proximal or brush/distal end) uncovered. Seeds were then suspended from the Petri dish by the tape to ensure that only the exposed area was in contact with water.

The MRmI images obtained were compared to known grain structures. A diagram of transverse and longitudinal sections of a wheat grain with the major structures labelled is displayed in Figure 4.1 (*Panels* A and B). Most of these structures were identified in the MRmI images and the grain diagrams have been presented here to assist in the interpretation. Images representing a series of both transverse and dorso-ventral longitudinal virtual slices were collected to provide a complete three-dimensional representation of water distribution throughout the grains. In all images, red and bright yellow represents cellular tissue containing water, whilst tissue with higher water content is white. Scans (six transverse and four longitudinal) were taken every hour from the start of imbibition until 18 hours for the genotype QT7475, to illustrate in detail the general pattern of water uptake. Representative images from eight time points are presented here.

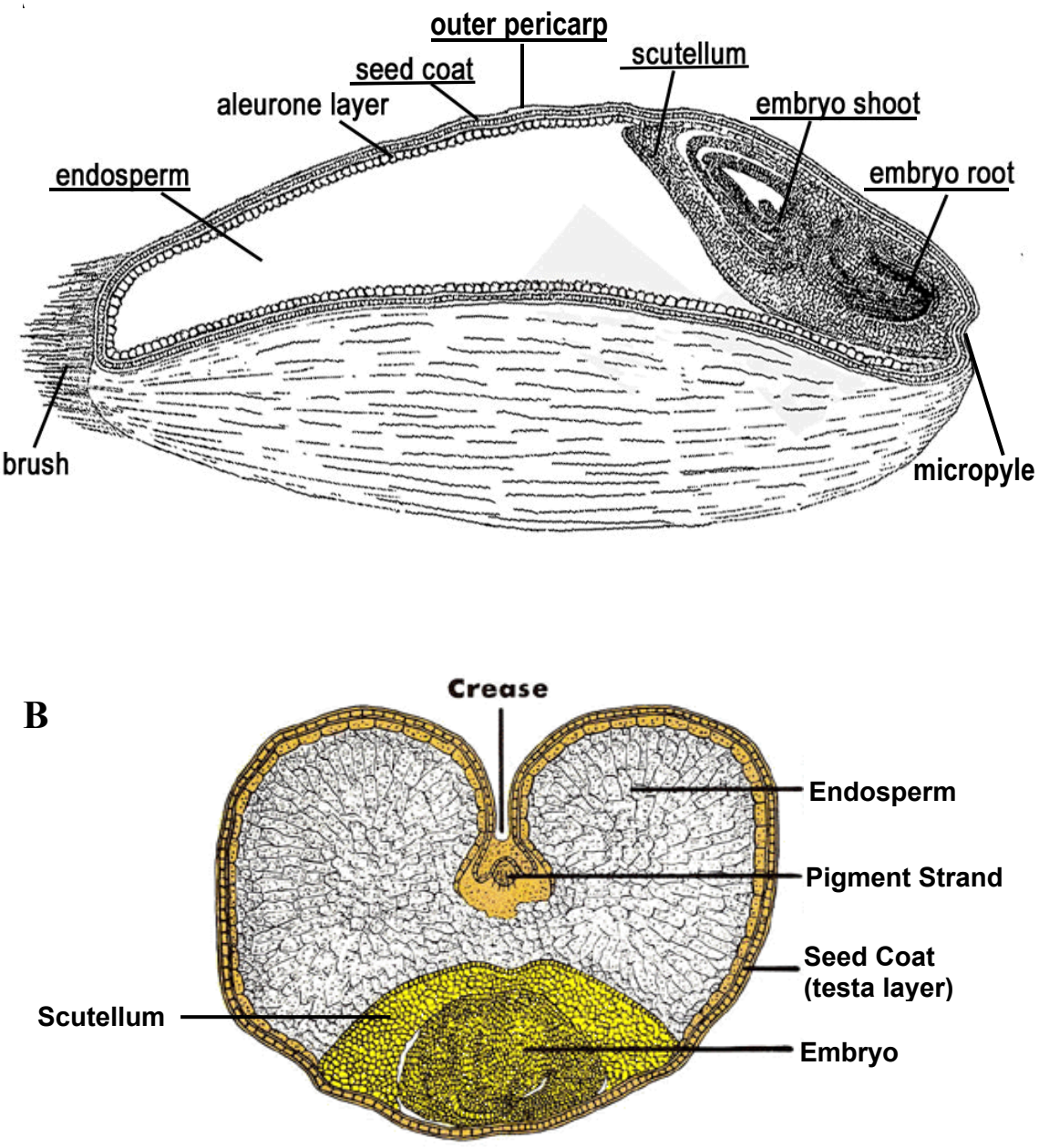


Figure 4.1: Diagram of a wheat grain showing major structures identifiable in the MRmI images (Figures 4.6 to 4.13). *Panel A*: longitudinal section (http://www.wheatbp.net), *Panel B*: transverse section (http://www.nawamillers.org/images/crosssectionviewofwheat.gif).

4.3 Results

4.3.1 Germination rate for genotypes used in the study

The germination rates of the genotypes were compared to determine the dormancy phenotypes. Hartog (white, non-dormant) germinated the most rapidly of all the lines, followed by the white intermediate-dormant line, QT7457. SUN325 (white, dormant) germinated slower than the other white lines, while R/W635 was the most dormant of all the lines and had the lowest germination percentage after seven days (Figure 4.2). These germination profiles show that the lines used in these experiments had maintained their dormancy phenotype from harvest maturity and subsequent storage at -20°C to preserve this dormancy (Mares, 1983).

4.3.2 Physical measurements of grain water uptake

Whole seeds were sampled and weighed at intervals during 18 hours of imbibition. Water uptake was initially rapid but slowed by about ten hours, based on the exponential asymptopic curve that provided a good fit, with a range of R^2 = 0.94 (Hartog), 0.96 (QT7475), 0.96 (SUN325) and 0.97 (R/W635) (Figure 4.3). All genotypes took up water at a comparable rate and the final grain water content, regardless of the initial grain weight, was also similar in all genotypes.

Figure 4.4 shows the increase in the embryo weight as a percentage of initial embryo weight, and therefore an estimate of the amount of water taken up. The data is fitted significantly with an exponential asymptopic curve, with R^2 = 0.76 (Hartog), 0.87 (QT7475), 0.79 (SUN325) and 0.94 (R/W635). It is apparent that water enters the embryo rapidly during imbibition, and by 10 hours the rate had decreased substantially (Figure 4.4).

Figure 4.5 shows the increase in weight of the endosperm, as a percentage of initial endosperm weight. The endosperm weight significantly (P>0.5 = 0.**) increased linearly in all genotypes, with values of R^2 = 0.22 (Hartog), 0.4 (QT7475), 0.48 (SUN325) and 0.52 (R/W635), although at a relatively moderate rate (Figure 4.5). The reduced rate of water uptake observed at 10 hours in the embryo sections (Figure 4.4) does not occur in the

endosperm. It also appears that the percentage endosperm weight increases in all genotypes throughout the 18 hours of imbibition. The final percentage in endosperm weight of the different genotypes after 18 hours imbibition is similar, with all genotypes showing an increase even though seed size does vary between the genotypes (Figure 4.5).

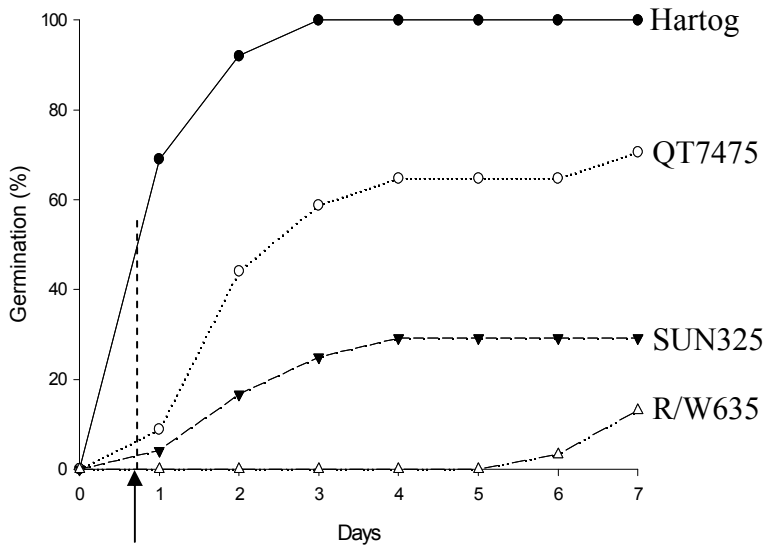


Figure 4.2: Germination (%) of a total of 50 grains over 7 days. Hartog (white, non-dormant), QT7475 (white, intermediate-dormant), SUN325 (white, dormant), R/W635 (red, dormant). Arrow and dashed line indicates earliest pericarp rupture in Hartog, the most non-dormant genotype.

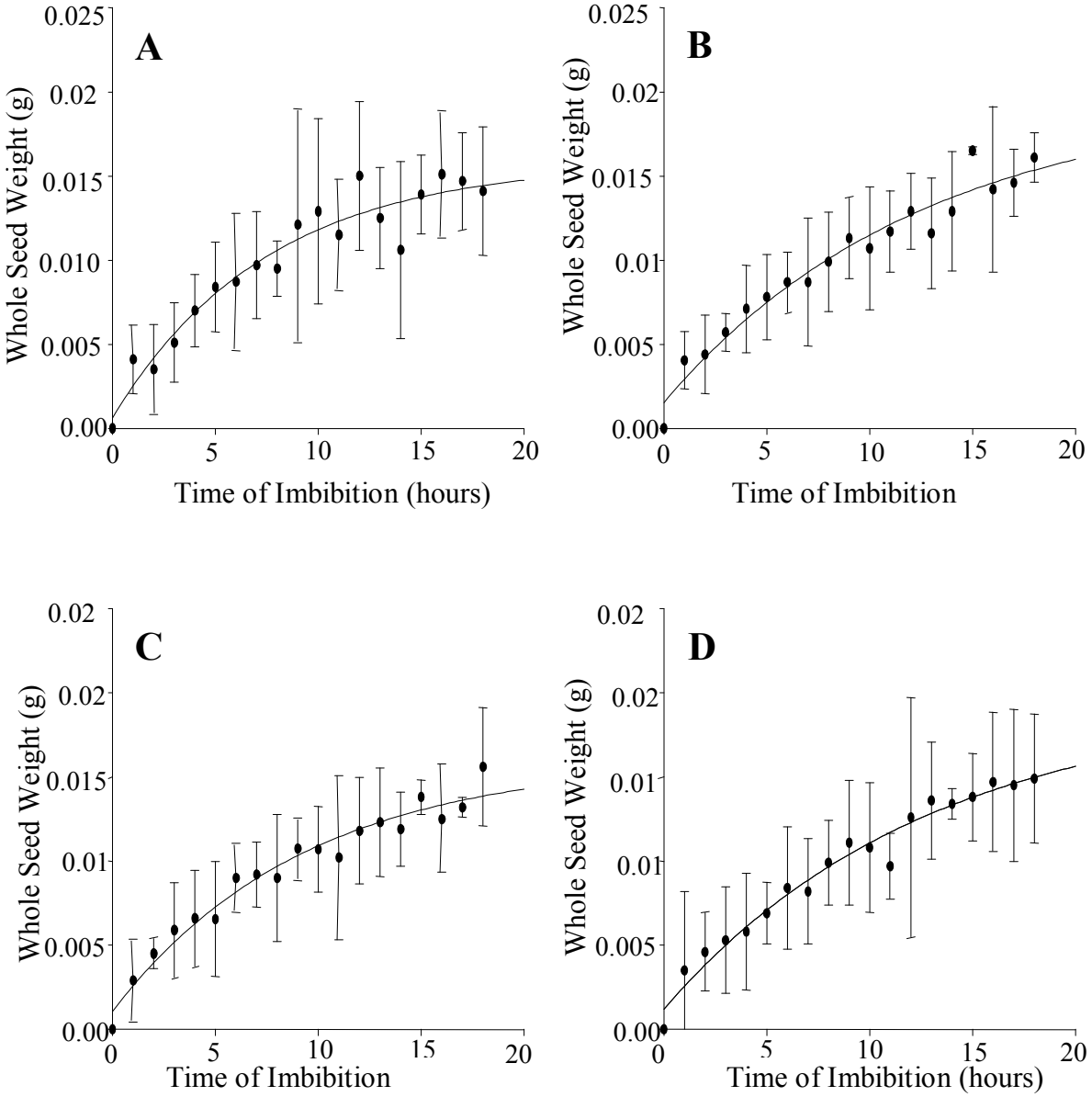


Figure 4.3: Water uptake as an increase in weight (g) of whole seeds over 18 hours of imbibition. *Panel A*: Hartog (R^2 = 0.94), *Panel B*: QT7475 (R^2 = 0.96), *Panel C*: SUN325, (R^2 = 0.96) *Panel D*: R/W635 (R^2 = 0.97). Data is fitted with an exponential asymptopic regression ($y = a + b^{(r*x)}$).

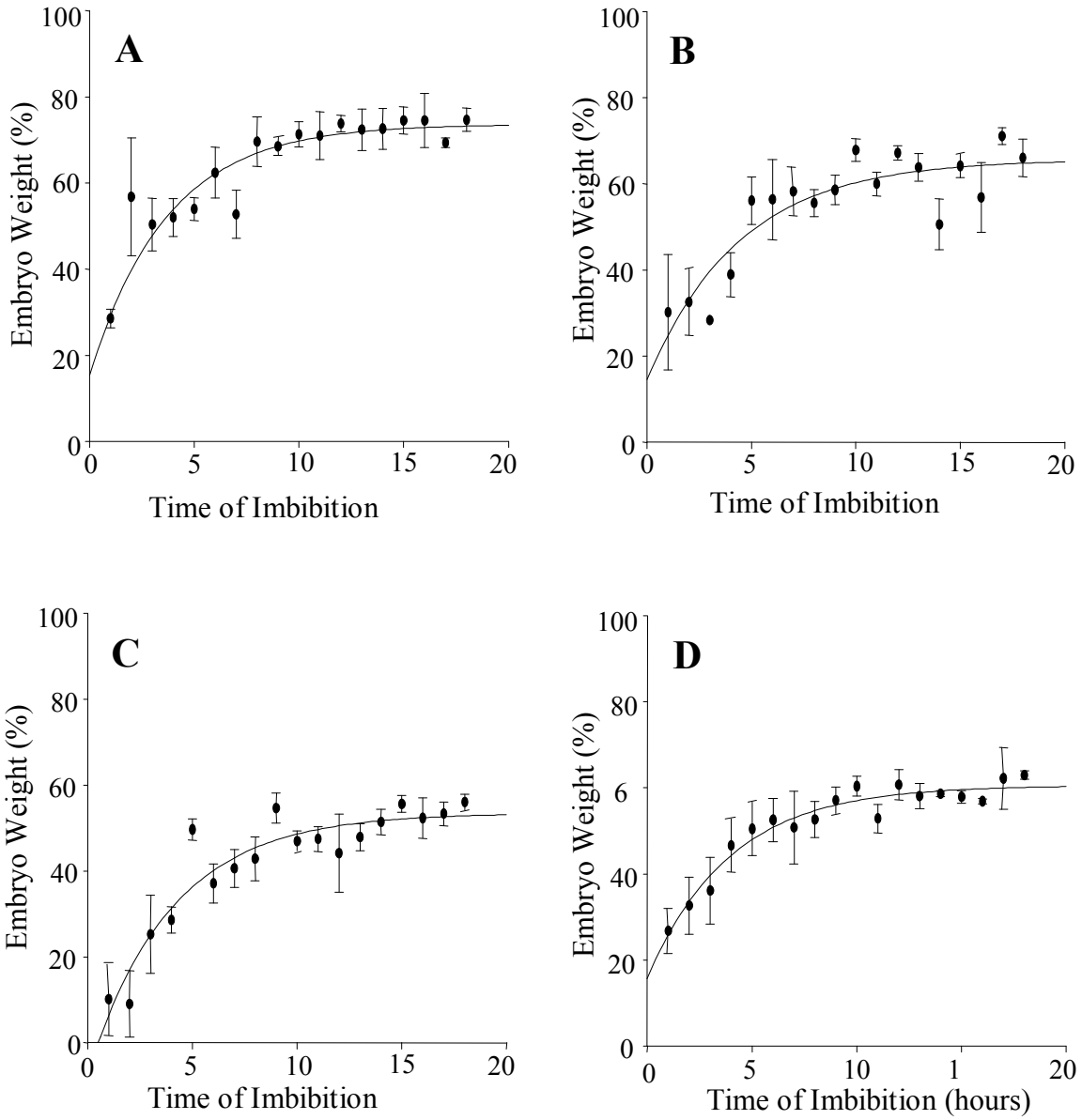


Figure 4.4: Change in percentage weight (g) of excised embryos during imbibition over 18 hours. *Panel A*: Hartog (R^2 = 0.76), *Panel B*: QT7475 (R^2 = 0.87), *Panel C*: SUN325 (R^2 = 0.79), *Panel D*: R/W635 (R^2 = 0.94). Data is fitted with an exponential asymptopic regression ($y = a + b^{(r*x)}$).

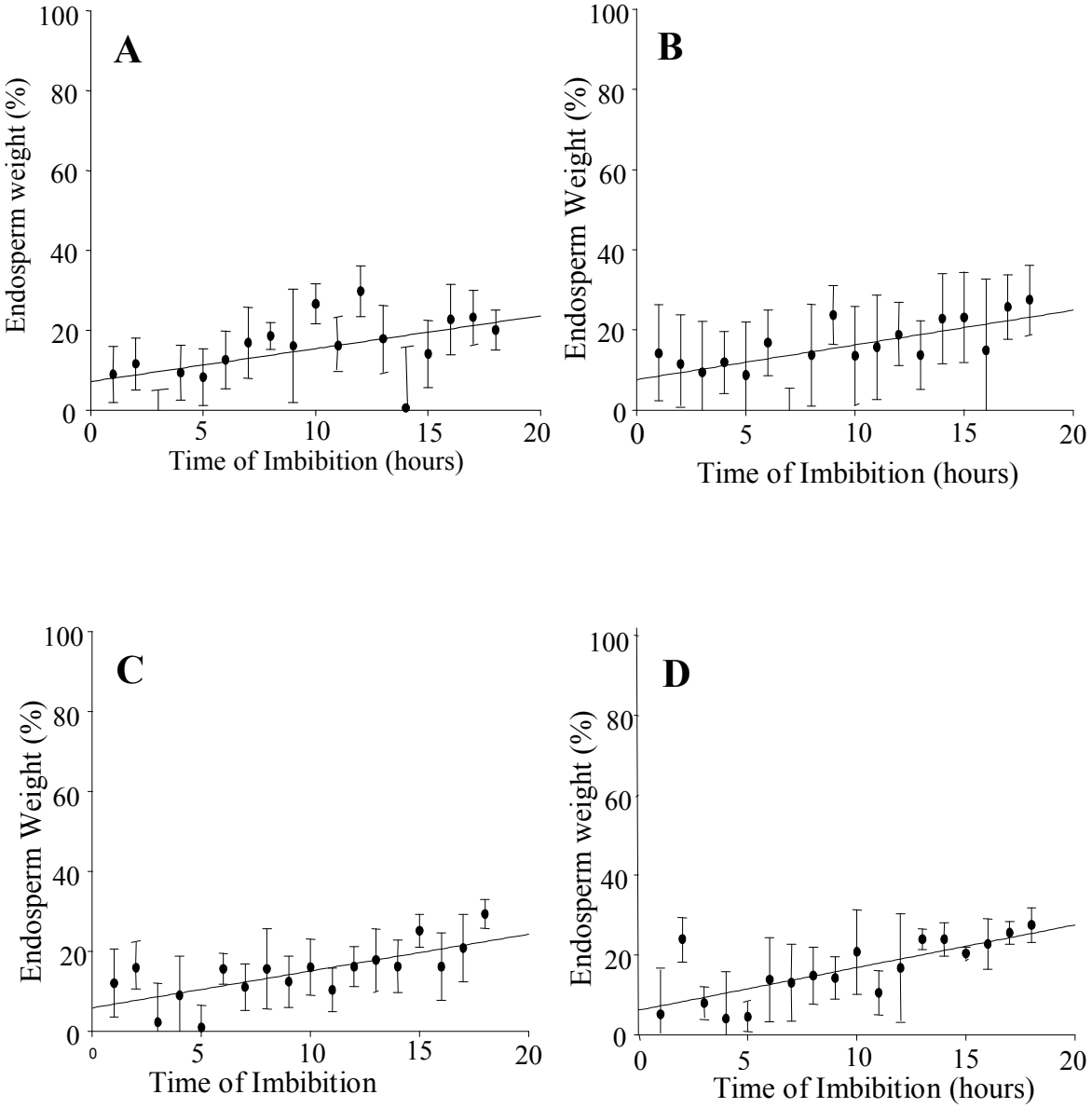


Figure 4.5: Change in percentage weight (g) of endosperm during imbibition over 18 hours. *Panel A*: Hartog (slope: 0.823, $R^2 = 0.22$); *Panel B*: QT7475 (slope: 0.863, $R^2 = 0.4$); *Panel C*: SUN325 (slope: 0.921, $R^2 = 0.48$), *Panel D*: R/W635 (slope: 1.067, $R^2 = 0.52$). Data is fitted with simple linear regression (y = ax + c).

4.3.3 MR Micro-Imaging of imbibing wheat grains

At two and three hours (Figure 4.6, *Panels A* and *B*), the grain was still dry and the image consequently very noisy and lacks detail. Nevertheless localised hydration was evident in the embryo (Figure 4.6, all *Panels*) and the scutellum was also hydrated, as identified in the longitudinal slice (*Panel B,* longitudinal slice 1) compared to other grain structures. An intense white spot, corresponding to an area of high water content, is apparent in the transverse sections in the embryo region. At six and eight hours imbibition (Figure 4.6, *Panels C* and *D*), there is a greater hydration than at the earlier time points, and in particular the seed coat appears to have taken up more water and is much more visible. In *Panel C*, embryo structures are apparent with the three-pronged radicle defined (transverse slice 4), and the micropyle also evident as a ring around a saturated (white) pixel (*Panel D*, transverse slice 4). Both of these structures are also clear in Figure 4.6 *Panel D* where the grain was imbibed for eight hours.

Water was also present, and uniformly distributed, within the seed coat in these earliest hours of imbibition (Figure 4.6, *Panels A* and *B*). Although water is present throughout the seed coat structure at these early stages, after six and eight hours the crease appears to have accumulated a greater amount of water than the dorsal surface, as indicated (*Panels C*, slices 1 and 2, and *Panel D*). There was no evidence in any of the images in Figure 4.6 that water had penetrated directly through the seed coat into the underlying starchy endosperm. In addition there does not appear to be any diffusion of water from the embryo, a region of extremely high hydration, into the surrounding tissues other than the scutellum. The water was also concentrated between the outer pericarp and testa (seed coat) tissue of the seed coat, which can be observed by close inspection of the transverse slices in Figures 4.6 and 4.7 (*all Panels*). There does not appear to be any movement of water across the testa layer and water is concentrated between the outer pericarp and the testa, as indicated in *Panel B* (transverse slice 2).

Images taken at 10, 12, 15 and 18 hours (Figure 4.7, *all Panels*) show only a small and gradual increase in hydration of the same grain tissues, embryo, scutellum and seed coat, in comparison with the period between two and eight hours (Figure 4.6), consistent with the observations from physical measurement of grain water uptake (Figure 4.3). In particular, the coleoptile is evident in Figure 4.7 (*Panel A*, transverse slice). The embryo continues to

hydrate between ten and 18 hours of imbibition, with the water distribution possibly becoming less localised and more widely distributed through the embryo region (Figure 4.7 *Panels A, B, C* and *D*). The vertical (longitudinal) slices of Figure 4.7 at all imbibition stages show that the seed coat contains more water than in the initial stages of imbibition (Figure 5.6). As in the earlier stages, Figure 4.7 shows there is still no evidence of water penetrating directly through the seed coat into the endosperm between two and 18 hours, or of water entering the embryo through any structure other than the micropyle.

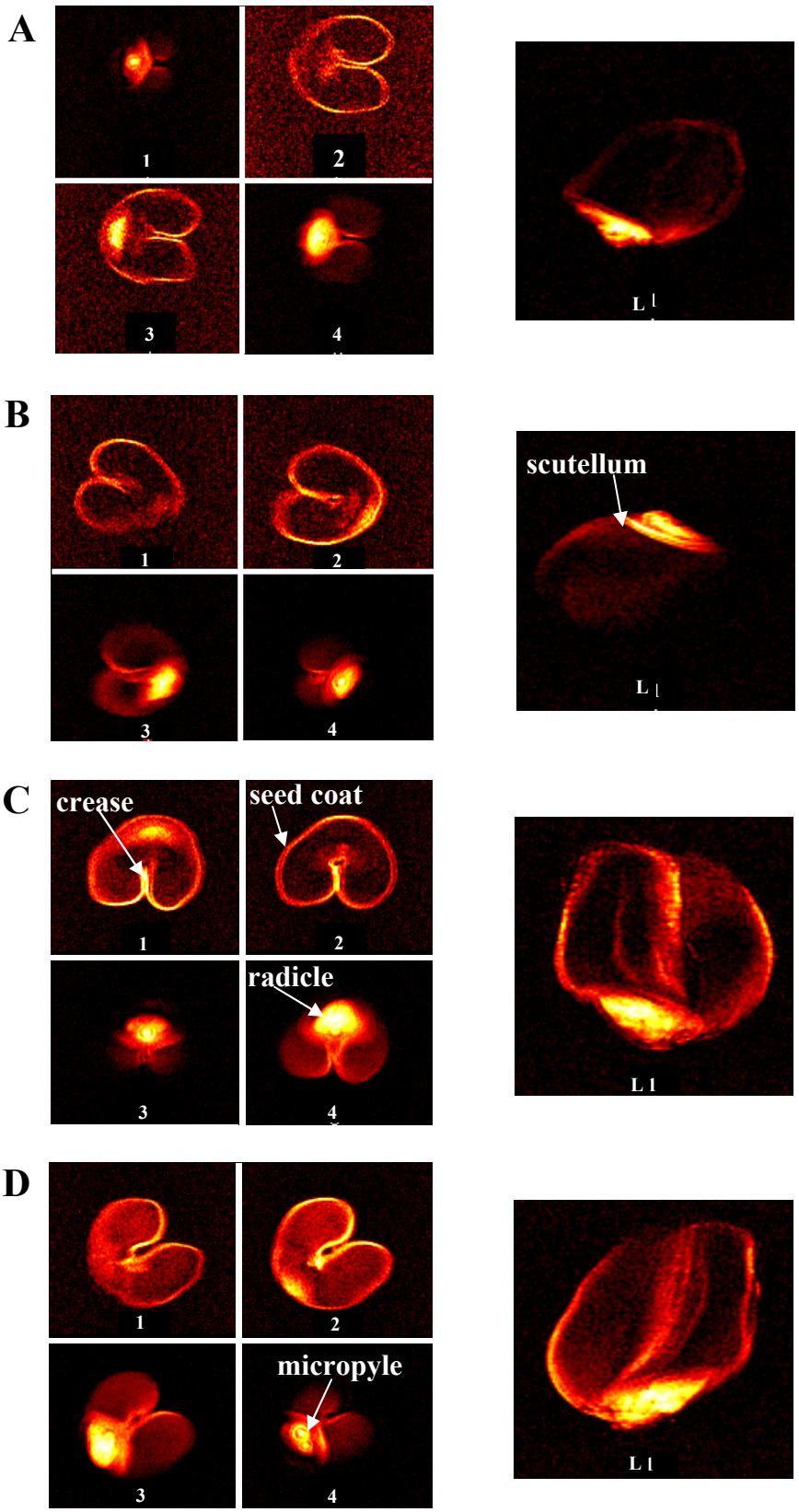


Figure 4.6: MRI images (transverse and longitudinal slices) of grain of the genotype QT7475 during imbibition. Panel A: 2 hours, Panel B: 3 hours, Panel C: 6 hours and Panel D: 8 hours. Grain structures are labelled.

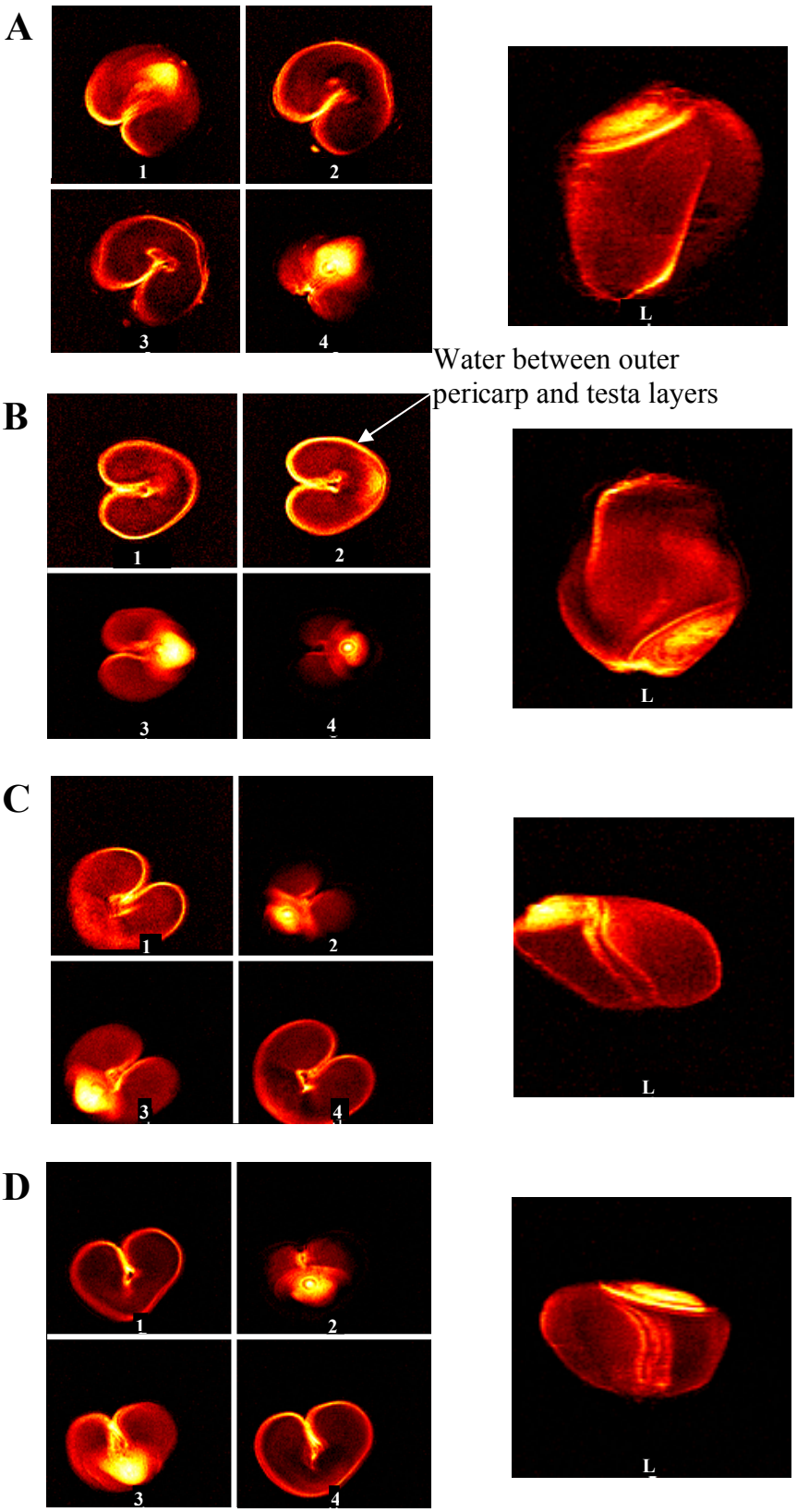


Figure 4.7: MRI images (transverse and longitudinal slices) of line QT7475 during imbibition. *Panel A*: 10 hours, *Panel B*: 12 hours, *Panel C*: 15 hours, *Panel D*: 18 hours of imbibition. Arrow indicates water concentrated between outer pericarp and testa (seed coat) tissue.

4.3.4 MR Micro-Imaging of imbibing wheat grains of differing dormancy

For this part of the investigation, images were taken within successive blocks of three hours from one to 18 hours, with a maximum of six scans per genotype. Only representative frames for each block are presented. At the first time point, water has entered the embryo, the seed coat is only barely hydrated and all of the grains are still relatively dry (Figure 4.8 *all Panels*). As before, water also appeared to hydrate scutellum tissue during early imbibition, as is demonstrated in the longitudinal slices in Figure 4.8 for Hartog (*Panel A*), R/W635 (*Panel D*) and to a less pronounced extent, QT7475 (*Panel B*). There was no discernable difference in the amount of water taken up or the distribution between any of the genotypes at this early stage during imbibition.

At the second time point (Figure 4.9), showing imbibition between three and six hours, all genotypes show a higher level of hydration in both the seed coat and embryo compared to pictures displayed in Figure 4.8. Hartog (*Panel A*), SUN325 (*Panel C*) and R/W625 (*Panel D*) in Figure 4.9 all had moderate level of hydration in the seed coat, and the entire seed coat structure is clearly visible. QT7475 (Figure 4.9, *Panel B*) did not show the same level of hydration in the seed coat in the transverse slices, but the longitudinal slice shows evidence of hydration which may not have been detected in the other perspective. Scutellar tissue was also highly hydrated in Hartog (Figure 4.9, *Panel A*), and what appeared to be the micropyle was clearly defined in all genotypes except R/W635 (*Panel D*). Figure 4.9 also shows discrete embryo tissue structures in both in the transverse and longitudinal sections of Hartog (*Panel A*), QT7475 (*Panel* B) and SUN325 (*Panel C*), but the same resolution is not as apparent in the red-grained genotype R/W635 (*Panel D*). Despite this, there was again no apparent difference in water content or distribution in any of the genotypes displayed in Figure 4.9.

Similarly, between six and nine hours of soaking (Figure 4.10) there was still no clear difference between the genotypes, with no evidence of water permeating directly through the seed coat into the starchy endosperm and the embryo and scutellum remained the most highly hydrated tissues. Whilst water was still localised within the embryo, the hydration has become more generalised throughout the tissue as water content increased in this region in all of the genotypes (Figure 4.10). The MRI produced highly resolved detail of

embryo structures in the transverse slices of Hartog (*Panel A*) and SUN325 (*Panel C*), showing the micropyle structure and radicle formation. Scutellum tissue is also visible in Hartog (*Panel A*) and SUN325 (*Panel C*). One sample of R/W635 (Figure 4.10, *Panel D* transverse slice 2*)* appeared to have a physical flaw in the seed coat, however, water still had not permeated directly through the damaged area to the endosperm, presumably because the flaw did not extend completely through the seed coat or testa layer.

The remaining Figures (4.11 to 4.13) show water uptake and distribution between nine and 18 hours imbibition, and indicate a gradual increase in hydration without a significant change in pattern of water distribution. Although the embryo is most likely still taking up water between 15 and 18 hours, it is at a slower rate and the images do not show an observable increase in hydration. This observation is consistent with the physical measurements of water uptake presented in Figure 4.3. In particular there has only been a slight increase in embryo hydration observable since nine to 12 hours imbibition. In the longitudinal section of SUN325 (Figure 4.13, *Panel C* longitudinal slice 1), the micropyle is present as a tube in front of the hydrated embryo tissue. All genotypes have a hydrated seed coat and the embryo region contains water surrounding the specific structures as well as in the micropylar area.

The seed coat is also highly hydrated in all of the genotypes, and with more water concentrated in the crease than other areas of the seed coat (Figure 4.11, 4.12, 4.13, *all Panels*). Despite this, none of the genotypes at any of the time points show any evidence of water moving through the seed coat and into the endosperm and the main entry of water into the grain is restricted to the micropyle. Water in the hydrated seed coat is located between the outer pericarp and testa layer. An excellent example of this is indicated by an arrow in Figure 4.13 *Panel C* in transverse slice two, but with again no observable movement of water through this testa layer.

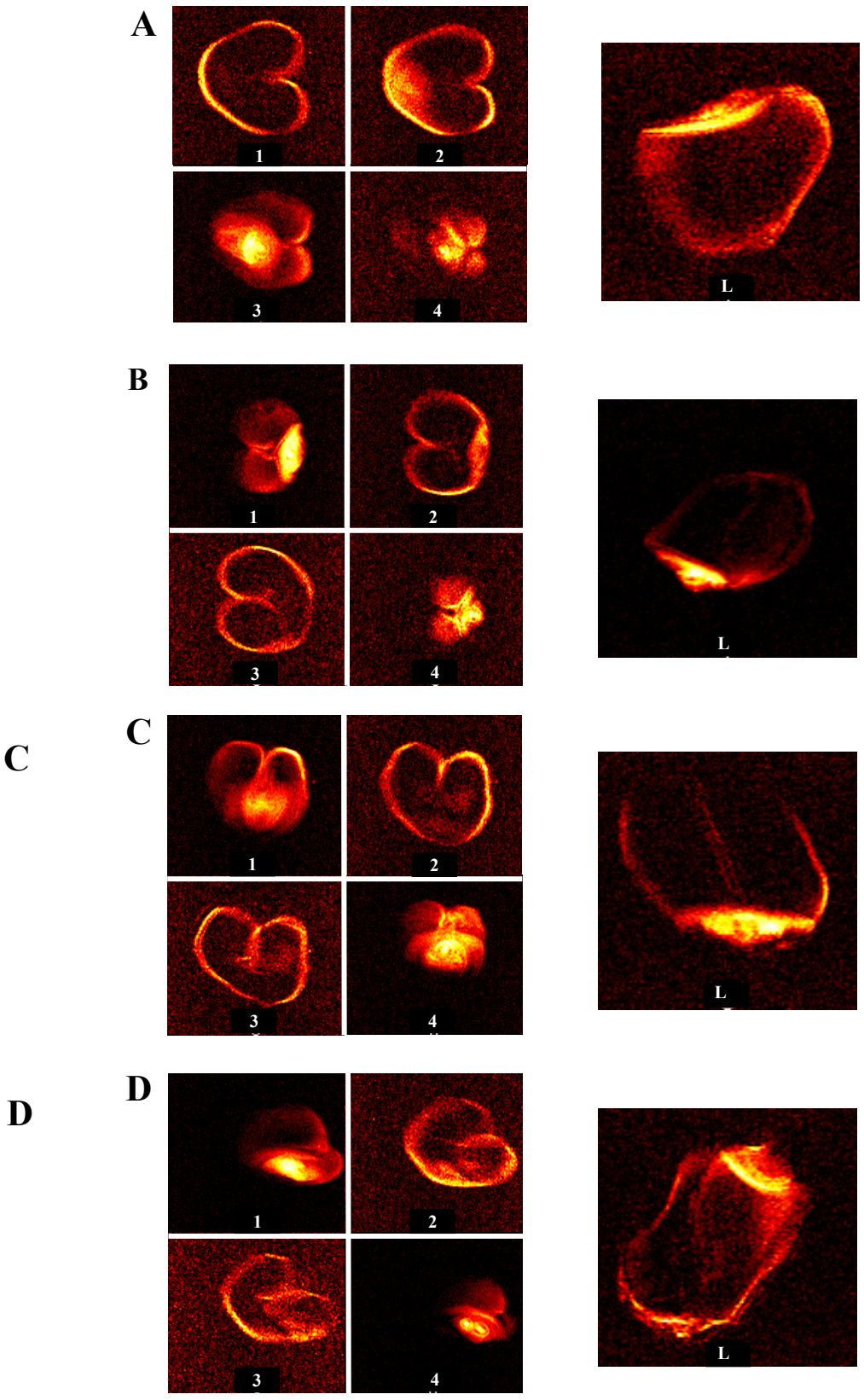


Figure 4.8: MRI images (transverse and longitudinal slices) of grains imbibed between 1 and 3 hours. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635

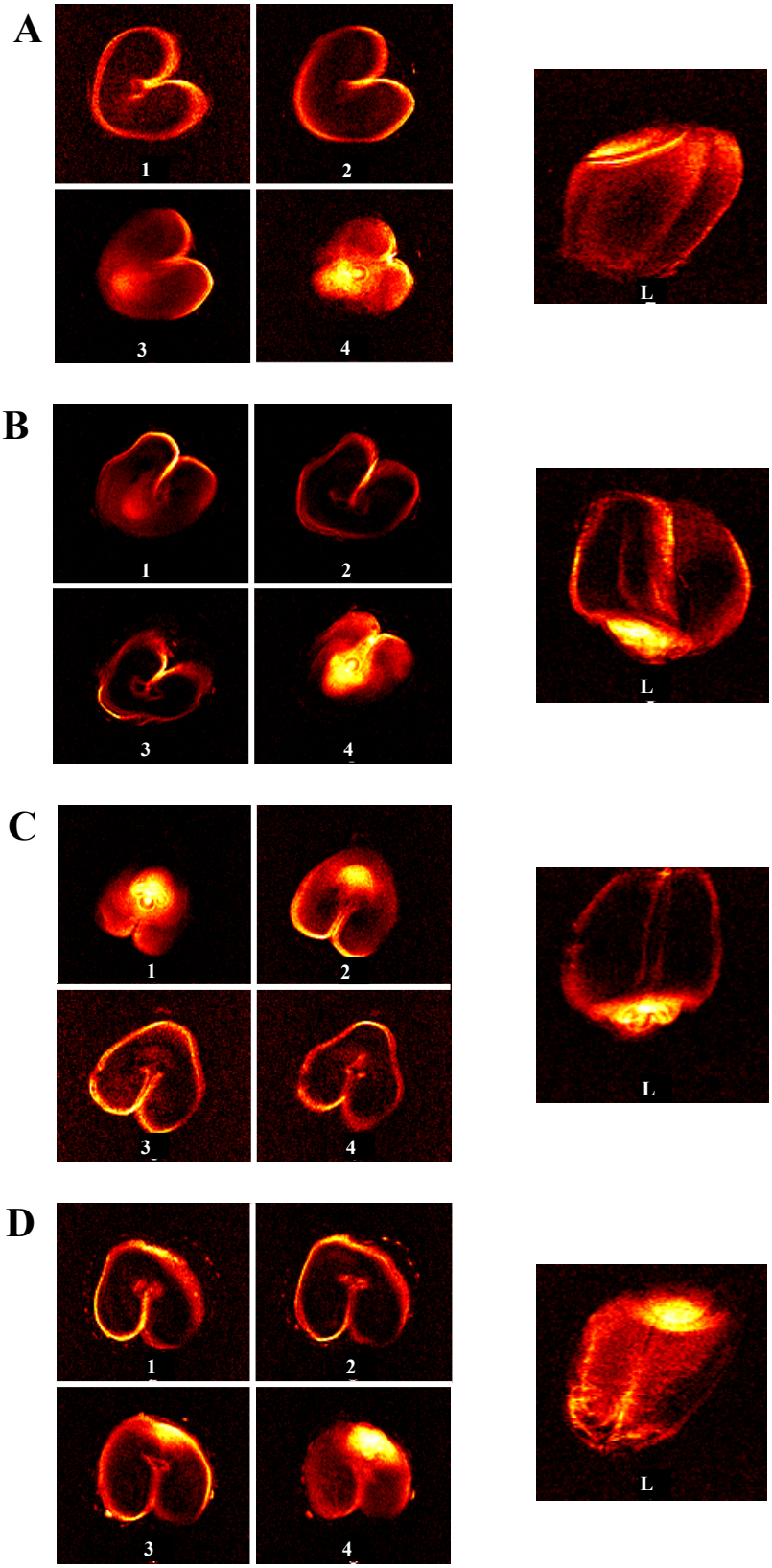


Figure 4.9: MRI images (transverse and longitudinal slices) of grains imbibed between 3 and 6 hours. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635

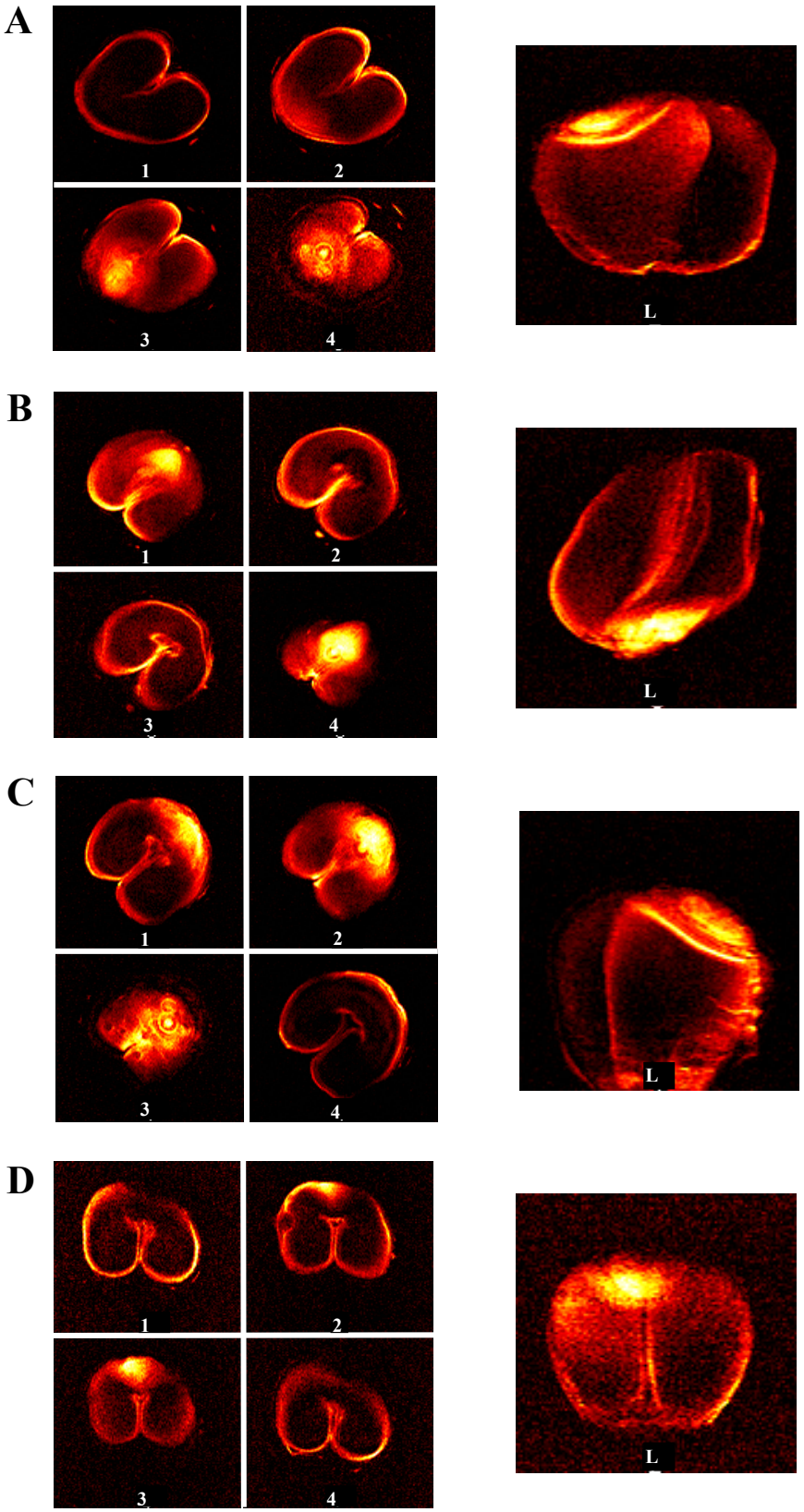


Figure 4.10: MRI images (transverse and longitudinal slices) of grains imbibed between 6 and 9 hours. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R\W635. Grain attributes are labelled.

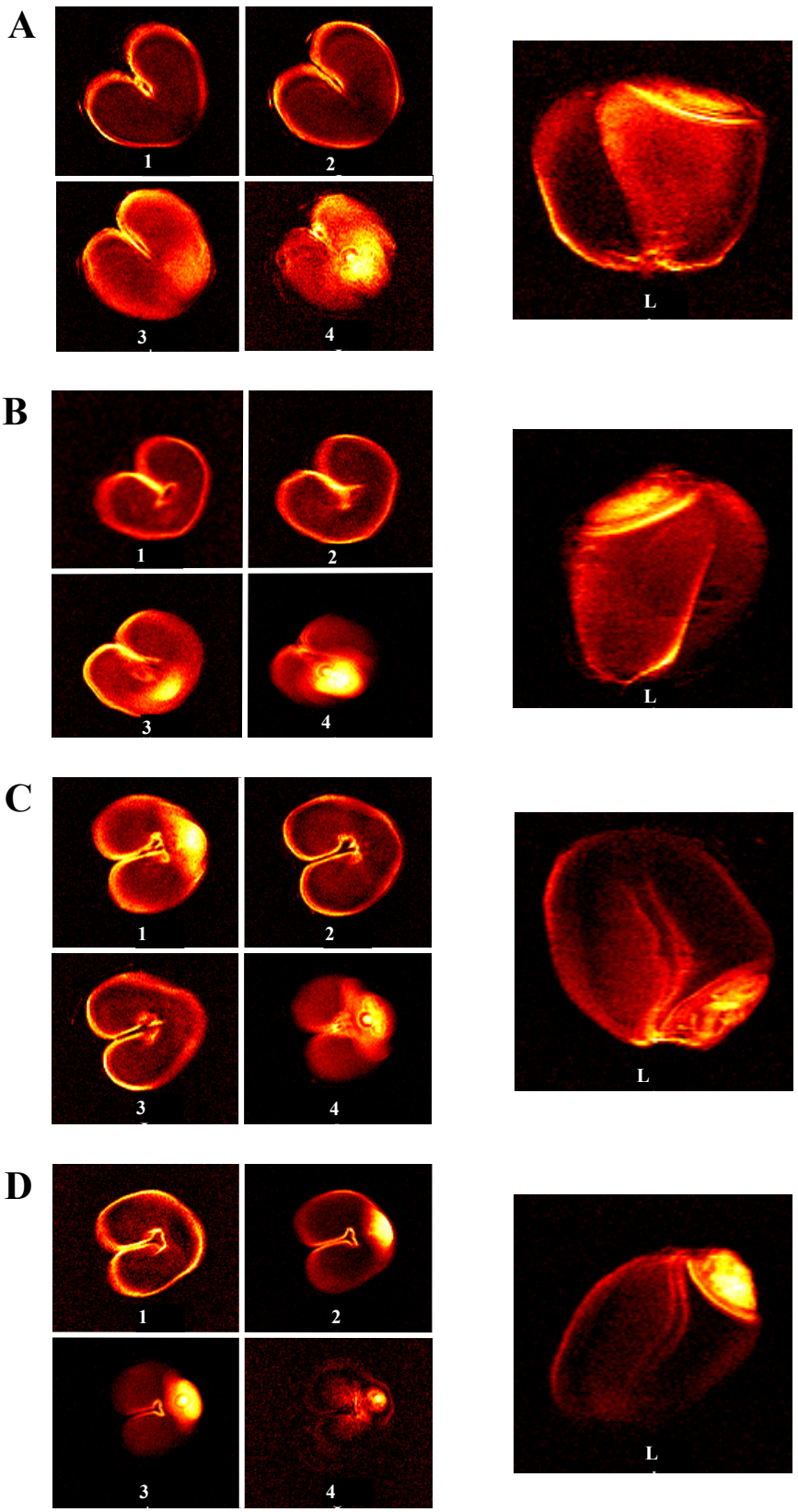


Figure 4.11: MRI images (transverse and longitudinal slices) of grains imbibed between 9 and 12 hours. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635

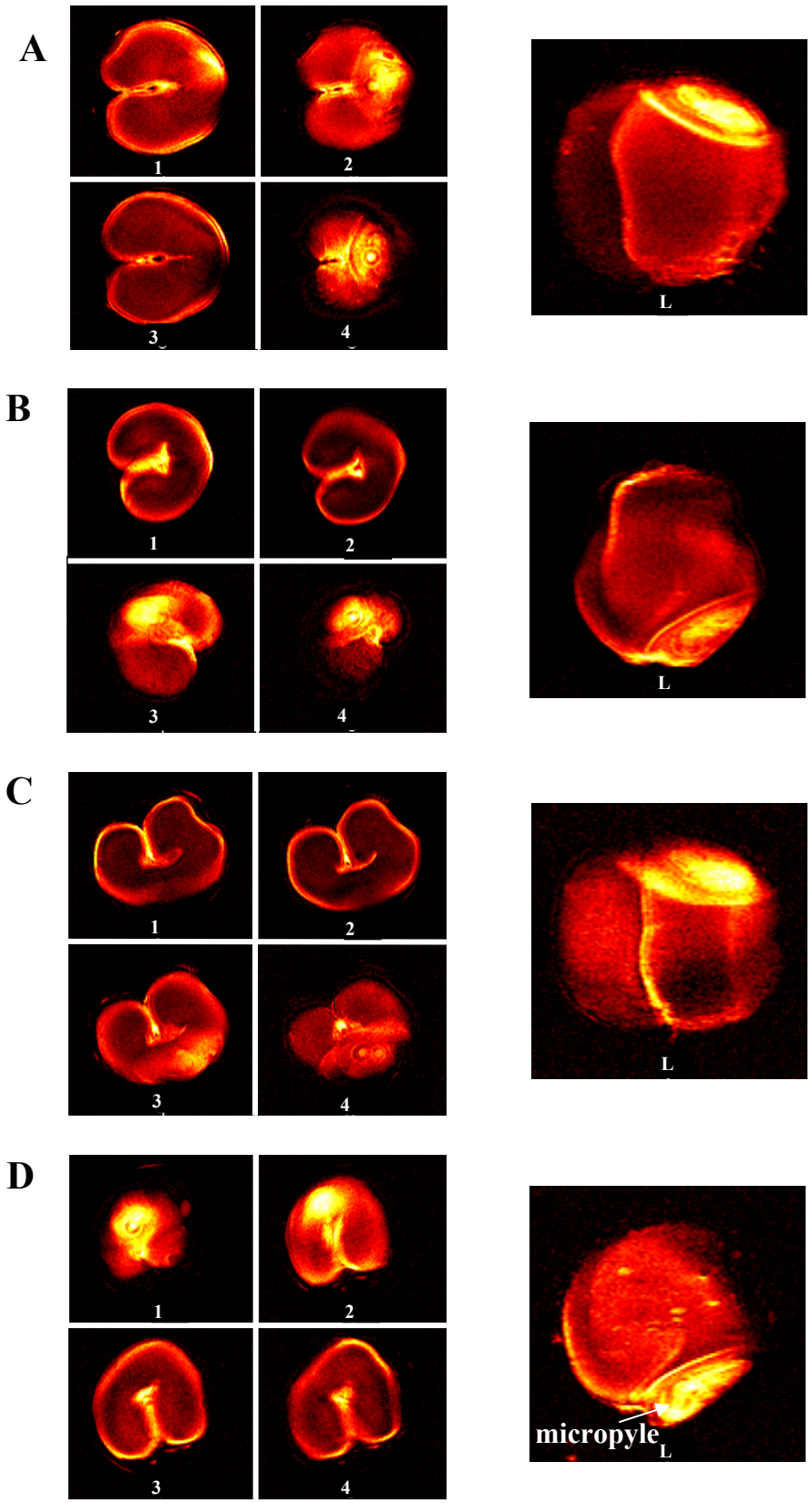


Figure 4.12: MRI images (transverse and longitudinal slices) of grains imbibed between 12 and 15 hours. *Panel A: Hartog, Panel B: QT7475, Panel C*: SUN325, *Panel D*: R/W635

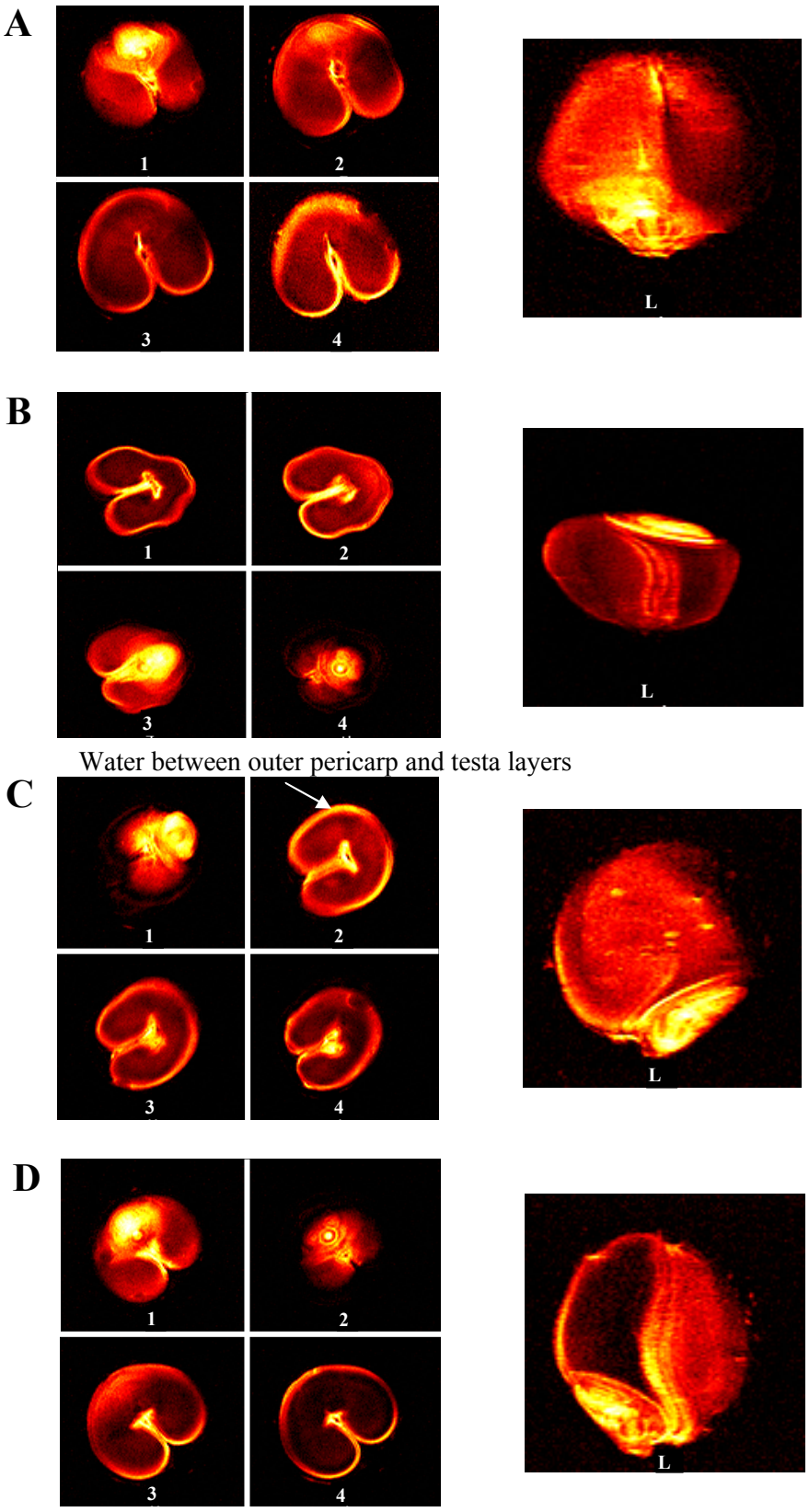


Figure 4.13: MRI images (transverse and longitudinal slices) of grains imbibed between 15 and 18 hours. *Panel A*: Hartog, *Panel* B: QT7475, *Panel C*: SUN325, *Panel D*: R/W635

4.3.5 MR Micro-Imaging of grains when access to water was restricted to either the proximal (embryo) or distal (brush) end of the grain

With only the embryo end of the grain exposed to water, the embryo appeared hydrated to a similar extent and pattern as grains imbibed normally on moist filter paper (Figure 4.14, *Panel A*). The seed coat was also hydrated, indicating that water is able to diffuse through the pericarp (*Panel A*), although less than for grain imbibed normally for 15 hours on moist filter paper where a much greater proportion of the grain surface was in contact with the water (Figure 4.11, *Panel B*). In addition, there was also more water in the crease region than the rest of the seed coat.

Figure 4.14 (*Panel B*) shows that restriction of water for imbibition to the distal (brush) region also does not significantly change hydration patterns of the grain. As with normal imbibition, the embryo clearly contained the highest water content, and it is also evident that flow of water into the embryo and scutellum only occurred through the micropyle (Figure 4.14, *Panel B*). This observation was supported by the micropyle structure that was apparent in the transverse slices. There was again no evidence of water permeating directly across the seed coat into the underlying endosperm (Figure 4.14), or entering the embryo by any other mechanism, in either of these two experiments (*Panels A and B*). In comparison to the grain imbibed only through the proximal end, the seed coat of the grain in *Panel B* appeared to have relatively greater water content, but there was no difference in a grain imbibed normally for 17 hours (Figure 4.13 *Panel B*).

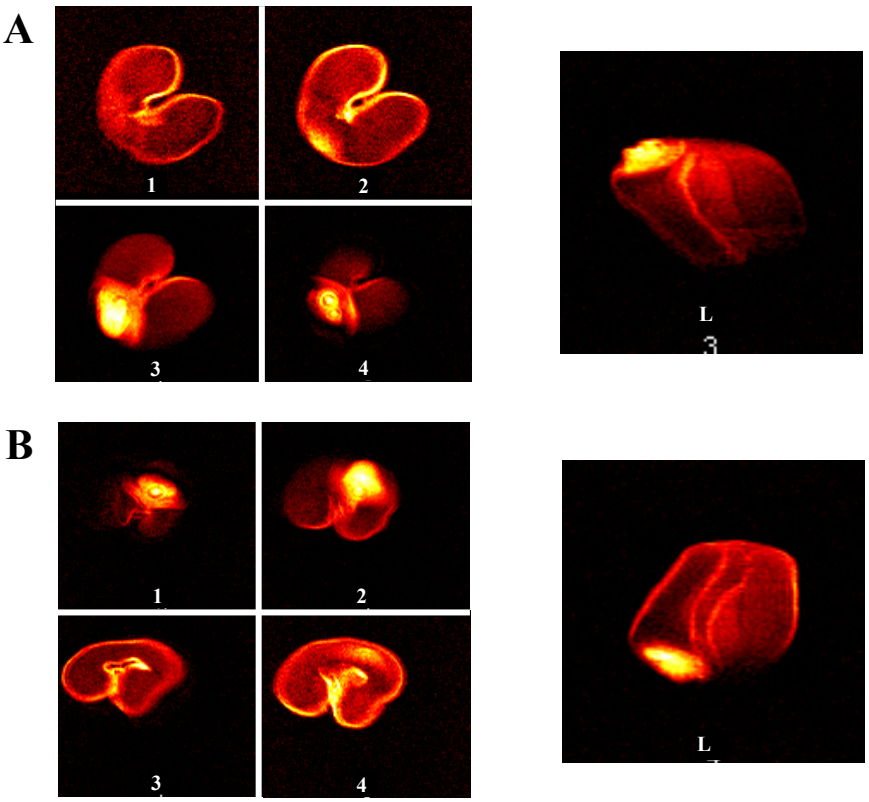


Figure 4.14: MRI images (transverse and longitudinal slices) of QT7475 when water was applied to only the proximal (embryo) or distal (brush) end of the grain. *Panel A*: Imbibition when water was applied to proximal end only, 17 hours, *Panel B*: Imbibition when water was applied to distal end only, 17 hours.

4.4 Discussion

Magnetic Resonance Micro-Imaging (MrmI) provides a unique opportunity to visualise the time course of water uptake by wheat grains without tissue destruction and with high resolution. MRmI provides an insight not only into mechanisms of water uptake, but also water distribution within the grain. Images taken at regular intervals over a period of 18 hours of imbibition clearly show that water entry into the embryo is through the micropyle. The first MRmI images were collected at two hours because the signal was too low before this stage of imbibition to generate a clear image, but even at this stage the embryo appears substantially more hydrated than any other component of the grain.

The reliance on the micropyle for water uptake into the embryo suggests that movement of water into the grain is an orderly and possibly regulated process and that there may be some control or barrier to the types of compounds which can enter this sensitive tissue. Micropylar tissue is the remnants of the pollen tube in many species (Tran and Cavanagh, 1984) and is present as a pore which is formed by an opening in the testa (Bhatnagar and John, 1972). In wheat, the micropyle is within, and to the dorsal side of the area of attachment of the grain to the spike (Evers and Bechtel, 1988). The micropylar region has not been studied for many years, but is reported to be a channel through heavily pigmented testa cells leading almost directly to the embryo, but possibly with a thin cuticular layer covering this channel (Bradbury *et al.*, 1956). It is possible therefore, that solutes dissolved in water could be restricted from accessing the embryo by this cuticle, and in turn it may influence water uptake into the critical tissues of the embryo and scutellum. Forcing water to enter through this tiny area is important for both germination and dormancy, because it may mean that compounds that inhibit germination or are toxic can be prevented from reaching the embryo. By contrast, water soluble compounds that may be involved in the dormancy mechanism, eg the catechin tannin-like compounds described by Stoy and Sundin (1976) and which prevent early germination, may be able to specifically penetrate this area.

Although the seed coat hydrates very rapidly during imbibition, the MRI images indicate that there is no direct permeation of water across the seed coat and into the endosperm. Earlier work reported evidence that the testa layer is resistant to water penetration (Hinton, 1955; Moss, 1973; Bewley and Black, 1982). Briggs and MacDonald (1983), found that

eosin dye was unable to penetrate the seed coat in barley unless it was physically damaged. Similarly, experiments involving imbibition with the I_2/KI stain for starch indicated that initial staining of endosperm starch was restricted to the area immediately adjacent to the scutellum (Mares, unpublished data). The testa contains waxy hydrophobic cutins and the presence of lipid compounds in the testa and nucellar layers contribute to a resistance to water diffusion across these tissues (Butcher and Stenvert, 1973; Moss, 1973; Woodbury and Wiebe, 1983). However, the use of dyes is unreliable because there is no accounting for differences in permeability through grain structures of dye compounds.

Other studies have suggested that water does eventually penetrate through the seed coat/testa (Butcher and Stenvert, 1973; Stenvert and Kingswood, 1976). Autoradiography studies with titrated water have suggested that penetration across seed coat of wheat is a possible pathway of hydration (Butcher and Stenvert, 1973; Stenvert and Kingswood, 1976). However this technique requires freezing and sectioning of grain tissue and is generally considered to have low resolution, particularly when dealing with small, mobile compounds such as water. The autoradiographs show grains with water apparently penetrating through the seed coat after just one hour of imbibition, whereas the MRI images indicate that there is no cross-seed coat movement of water at least in the first 18 hours of imbibition. Not only is evidence of seed coat permeability completely absent in the MRmI images, but also MRmI shows that the crease actually contains most of the moisture in the seed coat whilst most of the water permeation in the autoradiographs was located in the dorsal region. If water concentrates in the crease region, rather than the dorsal region which takes longer to hydrate, it is likely that the autoradiographs are not accurately representing imbibition. A possible explanation for the autoradiograph images is that the grain used in this study was machine threshed, and as a result there may have been some mechanical damage to the seed coat that allowed water infiltration directly into the endosperm. MRmI is able to actually show real-time, *in vivo* water distribution during water uptake through providing excellent non-destructive visualisation of the grain. MRmI thereby shows the mechanism of imbibition more completely and, importantly, correctly.

MRmI investigations of water movement into seeds of other species have also conflicting interpretations. In pine, Terskikh *et al.,* (2005)concluded that water penetrates the seed coat before hydrating the megagametophyte, cotyledons and finally the radicle. However, it is apparent from a re-examination of the MRmI images that the water may actually

hydrate the seed coat before diffusing into the root cap and cotyledons through the micropyle. The images from these authors do not appear to show conclusive evidence of water infiltrating across the seed coat. Another study involving tobacco showed that water only enters the embryo through the micropyle and there is no permeation across the seed coat (Manz *et al.*, 2005). Both studies provide evidence that the micropyle region is an important point of water entry, indicating that this pattern of water uptake is not species specific.

It is well documented that the outer layers of the seed coat have wick-like properties and due to the porous outer pericarp are able to conduct water around the surface of the seed (Hinton, 1955; Briggs and MacDonald, 1983). The MRmI images show that in grain where the only access to water was at the brush region, the water is able to flow around the grain through the capillary action of the pericarp and enter the seed through the micropyle. Furthermore, the MRmI images in Figure 5.12 (*Panel C*, slice 2) show evidence that the water in the seed coat is located between the outer pericarp and the underlying testa layer, indicating that this is the zone of water movement through the seed coat. The pericarp clearly conducts water to the micropyle, with the embryo still the most hydrated tissue even though it is not in contact with the water. The physical measurements of seed coat water uptake indicate a similar occurrence during normal imbibition, with the endosperm section of the grain showing a lower rate of water uptake compared to the embryo, which shows a rapid uptake of water during the early stages of imbibition. Therefore, during imbibition water can travel through the seed coat into the micropyle, as well as water entering directly through the micropyle.

The MRmI images provide insight into water distribution within the embryo and a high resolution picture of hydration of the structures in quiescent embryo. Embryo structures become visible after around three hours of imbibition, with the radicle showing relatively early hydration whilst the scutellum also became rapidly hydrated and continuing to hydrate as water uptake progressed. Water is restricted to within the scutellum and specific embryo structures during early imbibition, but becomes more generalised through the embryo as water uptake progresses. At no stage in the early process of imbibition, ie prior to 18 hours, does it appear that the water moves beyond the scutellum into the endosperm. The capacity of embryo tissue to absorb water increases during imbibition and has the characteristics of a purely physical process since it also occurs in heat-killed wheat seeds

(Studdert *et al.*, 1994). Terskikh *et al.*, (2005) reported a three-phase water uptake sequence in pine with propagation phase I (0-10 hours), related to the rapid uptake of water, and propagation phase II (10-21 hours) or the infiltration of intracellular spaces. The following phase of water uptake is defined as the 'saturation phase' which only occurs in dormant seeds, while phase III is the beginning of germination where there is a corresponding large increase in water uptake by the growing seedling. These defined phases are also observable in the water uptake of wheat grains.

The physical measurements of water uptake into the embryo over 18 hours are in agreement with that observed in the MRmI images. Even though the MRmI images show only slight differences in embryo hydration after 12 hours of imbibition, the physical measurements indicate that water uptake continues until 18 hours. It is likely that the initiation of germination results in endosperm hydration beyond the scutellum, but it is clear in this study that, prior to initiation of germination, water is restricted in the embryo scutellum region. This conclusion is supported by an earlier MRI study of wheat imbibition, where the endosperm only appears hydrated after germination has occurred (Yoshida *et al.*, 1995).

There are many species, for example A*rabidopsis*, where the prevention of water uptake is a factor in dormancy and where secondary cell walls in the seed coat prevent the exchange of moisture and gases with the environment (Haughn and Chaudhury, 2005). In wheat, however, it is clear from the present study that there is no difference in the amount of water uptake between dormant and non-dormant varieties. The failure to detect a difference between dormant and non-dormant wheat is not particularly surprising because species which rely on restricting water uptake for dormancy generally possess thick and waxy seed coats eg soybeans (McDonald Jr *et al.*, 1988). The wheat seed coat, as revealed by MRmI and microscopy studies eg (Bradbury *et al.*, 1956), is relatively thin in comparison to the rest of the seed, and there is no strong barrier to water infiltration into the embryo.

The testa is the layer of the seed coat which contains colour pigments and other compounds that may contribute to the impermeable nature of this tissue (Evers and Bechtel, 1988). Suggestions of differences in imbibition in red and white-grained varieties persisted through the early literature (Huang *et al.*, 1983), but appear to have been discounted by later studies that found no effect of grain colour on water uptake (King,

1984b; Clarke and DePauw, 1989). In *Pisum elatius*, there has been a suggestion that the oxidation of phenolic compounds, which constitute the compounds responsible for red seed coat colour, may affect water uptake into the seed coat (Marbach and Mayer, 1974). Although the red seed coat of red-grained wheats contains phenolic colour pigments, phlobaphenes, that are absent in white-grained wheat, this difference apparently has no effect on water uptake and most importantly water distribution in the grain. Although there is a lack of difference in imbibition and water distribution of red and white-grained wheat and dormant and non-dormant genotypes, it is possible that the dissolution of a compound(s) in the testa layer is a factor involved in dormancy. Water in the seed coat appeared to be concentrated, and possibly transferred to the embryo, in the seed coat layer between the outer pericarp and testa. This proposed dormancy mechanism might involve the movement of a compound(s) in testa through the capillary properties of the outer pericarp.

If compounds present in the testa influence dormancy, it may be expected that a difference in dormancy expression would occur depending on the mechanism of imbibition. For example if water could only taken up distally, then the grain would be more dormant through the concentration of the associated inhibitor in the water, moving through the seed coat and into the embryo. Woodbury and Wiebe (1983) conducted some preliminary work investigating a comparable theory, with dormant and non-dormant genotypes and found that dormant grains germinated significantly more slowly when the distal (brush), compared to the proximal (embryo) end was inserted in moist sand. This phenomenon could also be observed if access to the micropyle were blocked, as in intact spikes where the grain is still attached to the rachis. In both germination on moist filter paper and wetting of grain in spikes, a large area of the grain surface is in contact with water and could be anticipated to contribute to similar germination behaviour of grains. Therefore, the dormancy phenotype of genotypes could differ according to the region of the grain in contact with water and depending on the presence of a germination inhibitor in the seed coat. This will be further investigated in the following chapters.

Chapter 5: Genetic Analysis of a Putative Seed Coat Dormancy Factor

5.1 Introduction

Dormancy in white-grained wheats is relatively rare, nevertheless genotypes with dormancy ranging from intermediate to dormant have been reported (Mares, 1993). In white-grained wheats, dormancy appears to be controlled by two or more genes, one (at least) of which is expressed in the seed coat (Mares, 1999). A QTL located on chromosome 4A (Mares *et al.*, 2005) was associated with dormancy in white-grained wheats of diverse origin whilst chromosome 4A has also been reported to contain the gene controlling embryo sensitivity to ABA (Noda *et al.*, 2002). In dormant red-grained wheats, the red colour (*R*) genes contribute to dormancy (Flintham and Gale, 1996). The *R* genes are located on the distal end of the long arm of the homoeologous group three chromosomes and it has been suggested that the dormant phenotype is achieved through an interaction between the *R* gene(s) and other gene(s) located on these chromosomes or on chromosome 4A (Flintham, 2000). These other gene(s) on the group 3 chromosomes may be closely linked to the *R* genes, or alternatively the *R* genes may have a pleiotropic effect on dormancy (Flintham, 2000; Warner *et al.*, 2000).

This study examines a population derived from white-grained dormant (SUN325B) x intermediate-dormant (QT7475) genotypes. These genotypes are derived from similar genetic backgrounds and both contain the 4A QTL from their common ancestor, AUS1408, however QT7475 appears to lack the other dormancy gene(s) required to give the dormancy phenotype typical of AUS1408 and SUN325B and the gene controlling the putative seed coat factor. The aim of this investigation was to locate QTLs other than 4A, in particular QTL associated with the putative seed coat factor, and to determine if there is any relationship between the chromosomal location of the seed coat factor in white-grained wheats and the *R* genes in red-grained wheats.

5.2 Materials and Methods

5.2.1 Genetic material and growing conditions

96 doubled haploids derived from a cross between QT7475 (white-grained intermediate dormancy) x SUN325 (white-grained dormant), both of which contained AUS1408 alleles at the chromosome 4A dormancy QTL, were sown in four separate experiments. The three field experiments and glasshouse growing conditions, harvest, sample collection and germination tests have been described in Chapter 2.

5.2.2 Reciprocal crosses and dormancy phenotype of F_1 grains

Reciprocal crosses, with all possible combinations, involving Hartog (non-dormant), QT7475 (intermediate) and SUN325B (dormant) were prepared in three successive years under glasshouse conditions. Selfed parental spikes were used as controls by being emasculated, then fertilized with pollen from the same genotype and treated in exactly the same way as cross-pollinated spikes. F_1 grain was harvested when all green colour had just disappeared from the spike, leaves and stems of the maternal plants, dried for 5 days then stored at –20°C until required for germination tests. On average, spikes produced 15-20 fertile grains and spikes with less than 10 grains were not used in the analysis. Germination tests and calculation of germination indices were as previously described (Chapter 2).

5.2.3 DNA extraction and QTL analysis

The pre-harvest sprouting (PHS) trait was measured in 92 double haploid lines in Sun325B/QT7475 population. DNA was extracted from the parents and each of the DH lines using a DNA mini-prep method (Williams *et al.*, 2002). Previous literature reported the QTLs for pre-harvest sprouting (PHS) in wheat on chromosomes 4A, on the long arms of chromosomes 3A, 3B and 3D close to the genes controlling red-testa pigmentation (*R*) and on the short arm of chromosome 5A which is co-located with a grain colour QTL (Groos *et al.*, 2002).

SSR markers close to the location QTLs on chromosomes 3A (wmc173, wmc559, wmc169, barc270, barc25, gwm155, gwm314, gwm480, barc310), 3B (wmc418, wmc307, wmc527, gwm77, gwm66, gwm285, barc145, wmc48, barc164, wmc182, gwm107,

barc145, wmc48, barc164, wmc544, gwm131, wmc533, wmc1) and 3D (gwm3, barc6, gwm664, gdm99, gdm136, gwm52, barc125, barc68, gwm191, wmc505, gwm497) were selected and tested for the possible linkage with PHS in the DH lines of the Sun325B/QT7475. Only wmc418, wmc307, wmc527, gwm77, gwm66, gwm285, barc 323, gwm3, gwm314, gwm497 were polymorphic and segregated in the population. Primers labelled gwm, wmc and barc were selected from to SSR studies of (Roder *et al.*, 1998), the International Wheat Consortium and Cregan P., USDA-ARS, Beltsville, MD, USA respectively. Molecular and PHS data for each of the lines were entered into Map Manager QTXb19 (Manly *et al.*, 2001) for linkage and interval analyses. All mapping and molecular work was conducted by Judy Cheong, South Australian Research and Development Institute.

5.3 Results

5.3.1 Dormancy phenotype of F_1 grains derived from reciprocal crosses.

Reciprocal crosses were performed using parents with non-dormant (Hartog), intermediate-dormant (QT7475) and dormant (SUN325) phenotypes. The dormancy phenotype of F_1 grains was influenced by both maternal and paternal genotype but in all cases where one parent was the non-dormant cultivar, Hartog, the resultant F_1 grains were also non-dormant (Figure 5.1). In summary, Hartog x QT7475 or Hartog x SUN325B = Hartog self (non-dormant) = QT7475 x Hartog and SUN325B x Hartog, where the first named parent was the female. By contrast, QT7475 x SUN325B = or < QT7475 self whilst SUN325B x QT7475 was not significantly different from SUN325B self.

5.3.2 Germination characteristics of doubled-haploid lines

Doubled-haploid lines of the SUN325 x QT7475 population were grown in two replications and four different environments over two years. Within the experiments replicates were well correlated, with all correlation coefficients (*r*) greater than 0.8. Similarly, comparisons between the glasshouse data and the field experiments also showed significant correlations of r = 0.8, 0.65 and 0.78. The field experiments were also significantly correlated with r = 0.74, 0.82 and 0.72 (data not shown).

Mean germination index (GI) for the population varied between different environments and years (Table 5.1). The population was the most dormant in the glasshouse experiment, with the most dormant line showing no sign of germination in seven days, where the population mean was only 0.09 (Table 5.2 and Figure 5.1). Despite the low population mean, there was a line that had a GI of 0.9, which is extremely high for a line derived from parents with grain dormancy. The field experiment in 2003 also showed a high dormancy level with a population GI mean of 0.15 (Table 5.2 and Figure 5.2, *Panel B*), while the other two field experiments had mean GIs of 0.36 (covered) and 0.21 (uncovered) respectively (Table 5.2, Figure 5.2, *Panels C* and *D*).

The dormancy level of the population in the glasshouse experiment was high with the distribution skewed towards a low GI (Figure 5.2, *Panel A*), As indicated by the arrows,

the parental lines displayed a similar GI, with QT7475 having an average of 0.14 ± 0.01 and SUN325 0.049 ± 0.02. Only 10.8% and 17.5% of lines had a comparable GI to the parents. The majority of the doubled-haploid lines displayed a GI within the parental range (Figure 5.1).

The frequency distribution of GI of the population screened in the field in 2003 (Figure 5.2, *Panel B*) did not conform to a normal distribution but was skewed to the left, or a more dormant phenotype. The GI of parental lines again are very similar, with QT7475 having an average GI of 0.17 ± 0.033 and SUN325 0.13 ± 0.024, but with the majority of the lines still within this germination range.

The population distribution of the experiment in the field in 2004 which was covered with translucent white plastic during the late stages of grain ripening (Figure 5.2, *Panel C*) showed a distribution that appears bi-modal in comparison to the other experiments conducted in the field (2003 and 2004) and glasshouse. Furthermore, the parental lines QT7475 and SUN325 showed more distinct phenotypic separation, with a GI of 0.578 ± 0.006 (QT7475) and 0.189 ± 0.045 (SUN325), as indicated by the arrows in Figure 5.2 (*Panel C*). The majority of the doubled-haploid lines also had GIs well within this range displayed by the parent lines and there was little evidence of transgressive segregation.

The experiment which was late-sown in the field in 2004 and not covered during grain ripening (Figure 5.2, *Panel D*), showed an intermediate level of dormancy compared with the glasshouse or the 2003 field experiment, but there was still only a slight separation of the dormant and intermediate-dormant phenotype. Most of the population was located between the parents, but there was a number of lines which appeared to be more dormant than SUN325 (Figure 5.2, *Panel D*). The distribution did not have the bimodal shape of the uncovered field experiment (Figure 5.2, *Panel C*) and probably is the only distribution from this series of experiments which could be described as normal in a statistical sense.

An attempt was made to calculate the proportion of dormant and intermediate-dormant lines within the population, with lines classified as dormant or intermediate if they had GIs within one standard deviation of the respective parents (Table 5.1). The percentage of lines with phenotypes within the range of the parental lines varied between years and experiments. It is evident that in the glasshouse experiment and experiment one in the field

in 2004, most of the lines had GIs outside the parental range. Conversely, the phenotype of the lines grown in field experiment in 2003 and the covered field experiment in 2004 is similar to that of the parents. It is important to note that the proportion of lines with phenotypes comparable to QT7475 is either close to or well below the proportion of lines with dormant phenotypes. Although the frequency distribution of the uncovered field experiment appears bimodal, the proportion of lines within the parental range is below 50%. In addition, very few of the lines were within the range of the intermediate-dormant parent, QT7475.

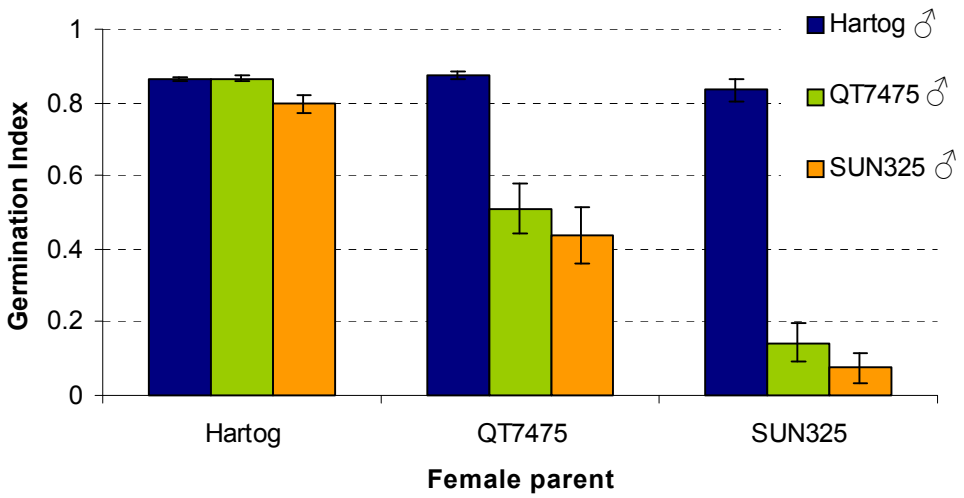


Figure 5.1: Germination Index of F_1 progeny derived from hybridisation of crosses between genotypes of differing dormancy.

Experiment	QT7475	SUN325	Total
Glasshouse	11%	10.7%	21.7%
Field 2003	48.8%	46%	94.8%
Covered	3.9%	30.9%	34.8%
Uncovered	21.9%	69.7%	90.6%

Table 5.1: Percentage of the population in each experiment with a dormancy phenotype within one standard deviation of the parents and the total percentage of the population that was not significantly different from the parents.

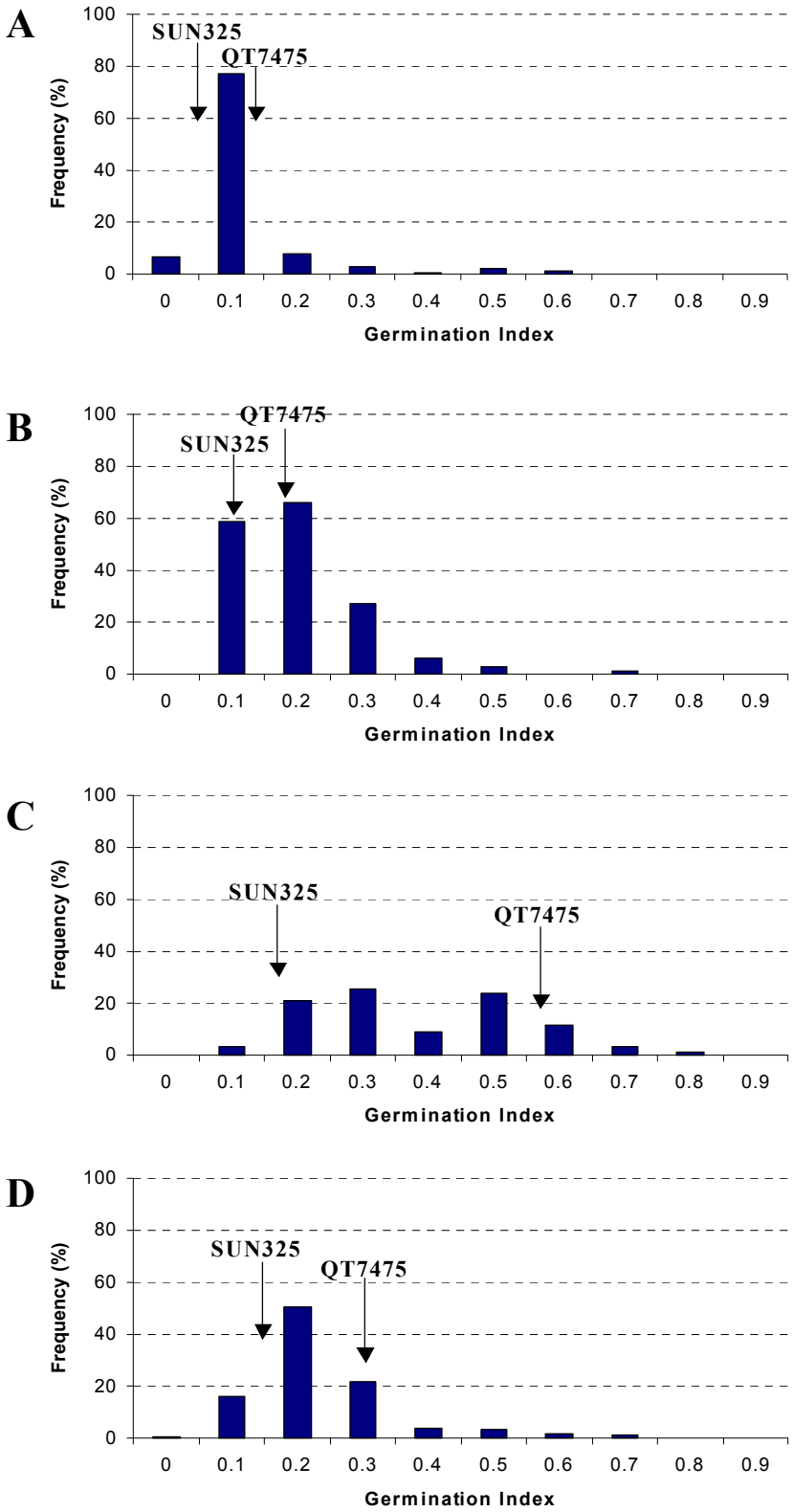


Figure 5.2: Frequency distribution for GI in the glasshouse experiment (2003). The arrows indicate the parental mean. *Panel A*: Glasshouse experiment (2004), *Panel B*: field experiment (2003), *Panel C*: field experiment covered (2004), *Panel D*: field experiment uncovered (2004).

Experiment	Average GI
Glasshouse	0.09 ± 0.001
Field 2003	0.15 ± 0.008
Covered	0.36 ± 0.013
Uncovered	0.21 ± 0.009

Table 5.2: The mean GI ± standard error of the population in each experiment.

5.3.3 QTL Analysis

Microsatellite markers targeting chromosomal regions such as the long arms of group 3, 5BL and 2A, previously reported to be associated with dormancy, were examined in detail for linkage to dormancy phenotype. Only the 3BL markers gave a LOD score > 3. Marker analysis of individual genotypes detected six polymorphic SSR markers, wmc418, wmc527, gwm77, gwm285, wmc307 and gwm66 located in a region of 78.6cM on the long arm of chromosome 3B. The QTL was consistently significant across all of the environments with maximum likelihood ratio statistic (LRS, (Manly *et al.*, 2001) values ranging from 11.7 (glasshouse 2003) to 17.7 (field 2003) and the averaged phenotypic data from the field experiments was used to generate the interval map (Fig 5.3). The SSR markers wmc527 and gwm77 flanked the highly significant QTL ($P<0.05$) and the marker gwm77 explained 17.2% of the trait variation. There was also some evidence of another QTL on chromosome 3D, but there was no polymorphisms in the SSR markers available for this region in this population.

When the lines were grouped accordingly to the presence of SUN325 or QT7475 alleles at wmc527 and gwm77 (Figure 5.3), the GI (means of the field experiments conducted in 2003 and 2004) frequency distribution separated into a bimodal pattern but with a significant overlap. There is evidence that there are lines which contain the SUN325 alleles but show comparable phenotypes to QT7475 (Figure 5.4).

The group three chromosomes in wheat are the location of other genes related to dormancy. Figure 5.7 shows the consensus map of chromosome 3B which includes a number of associated micro-satellite markers. Regions highlighted on this consensus map are the QTL detected in this study, the *R* gene controlling seed coat colour in red-grained wheat, mapped by Flintham and Gale (1996) and transferred to this map through the consensus map devised by Roder (1998) proposed location of viviparous-1, *taVp1*. The newly identified QTL on chromosome 3BL appears to be different from other known genes located in this chromosomal region.

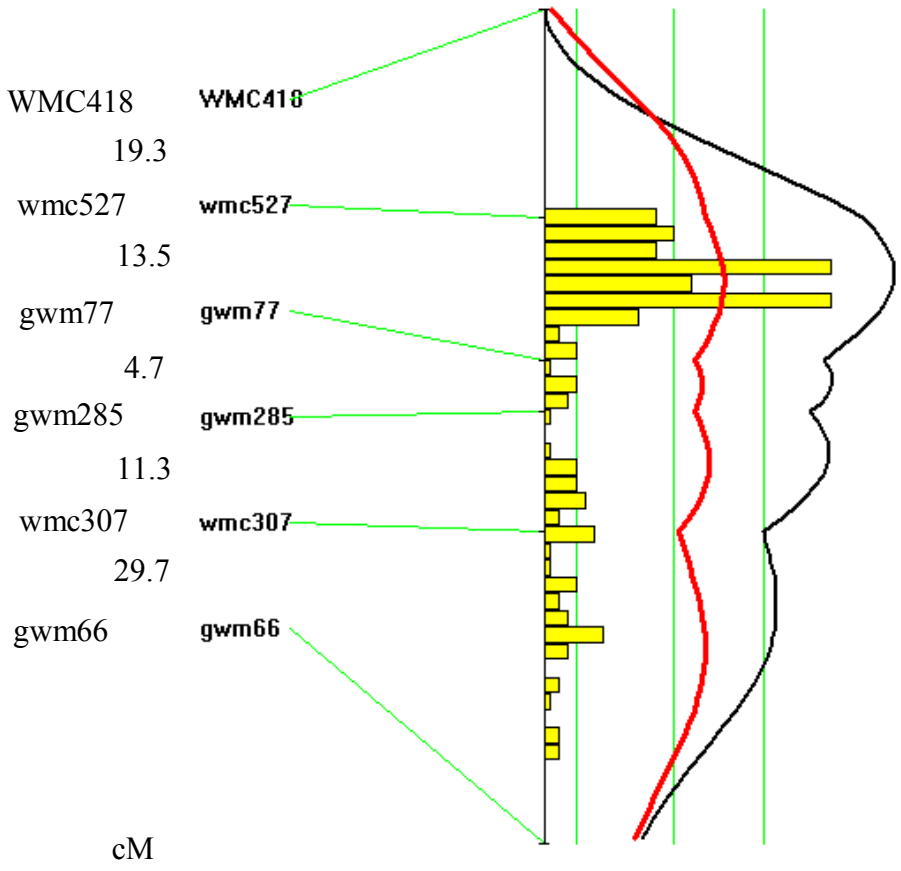


Figure 5.3: Partial interval map of a QTL associated with grain dormancy on chromosome 3BL. The vertical lines indicate suggestive (P = 0.63), significant (P = 0.05) and highly significant (P = 0.001) thresholds for the likelihood ratio statistic (LRS) (Manly *et al.*, 2001). The accompanying histogram under the LRS trace indicates the confidence interval of the QTL, as determined by bootstrap re-sampling. Red trace = field 2003, black trace = field 2004 (covered) and cM = centimorgan

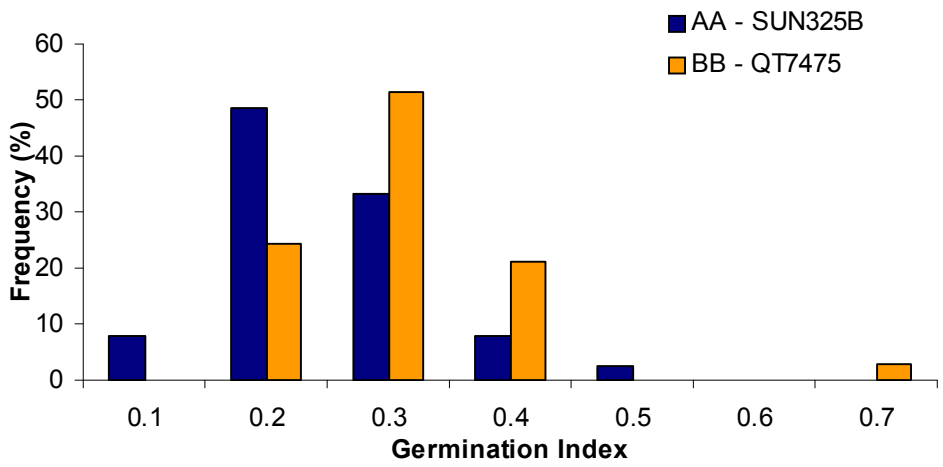


Figure 5.4: Frequency distribution for GI in subpopulations of lines grouped according to the presence or absence of the 3BL QTL flanked by the markers wmc527 and gwm77. AA (blue rectangle) corresponds to SUN325B alleles at this loci and BB (orange rectangle) is the presence of QT7475 alleles.

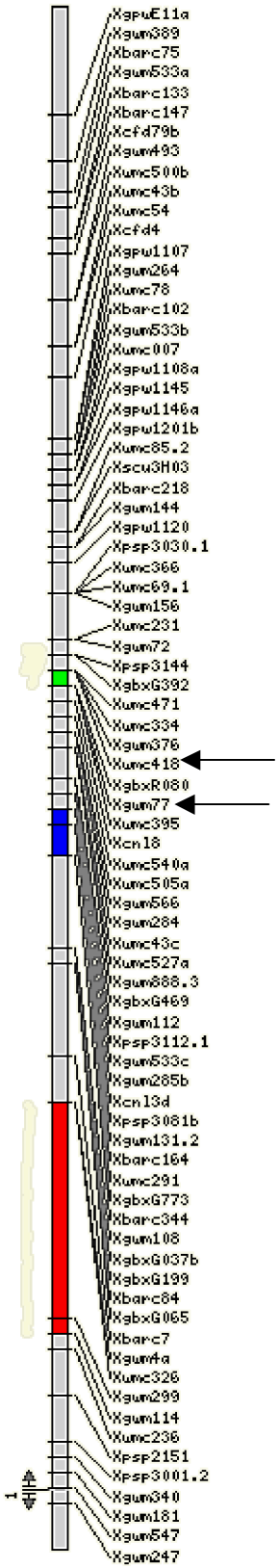


Figure 5.5: Consensus map of chromosome 3B. Highlighted regions include the proposed location of the *R* gene (red) (Flintham and Gale, 1996), *taVp1* (blue) (Bailey *et al.*, 1999) and the QTL detected in this study (green). Arrows indicate markers associated with the newly detected QTL.

5.4 Discussion

A model involving interaction between embryo sensitivity to ABA and a seed coat factor has been proposed to explain dormancy in white-grained wheat (Mares, 1999) . According to this model, complete dormancy only occurs with the expression of both of these factors, while the embryo factor on its own confers only an intermediate level of dormancy. The presence of dormancy genes expressed in the seed coat which interact with the embryo sensitivity factor has also been reported in red-grained wheats, but in this instance the seed coat effect is thought to be associated with the *R* genes controlling red seed coat colour (Flintham, 2000; Warner *et al.*, 2000). In this study, the dormancy phenotype of F_1 grains from reciprocal crosses between three different white-grained genotypes expressing a range of dormancy phenotypes was examined, in an attempt to validate both the model (Mares, 1999) and the presence of a seed coat factor thought to be involved in dormancy in white-grained wheat. In this material, the genotype of the seed coat and the embryo of the F_1 grains is determined by which parent was the female and which was the male. The F_1 progeny derived from these crosses showed dormancy phenotypes that were in agreement with the proposed model that there are both embryo and seed coat factors involved in grain dormancy expression (Figure 5.1). It was also evident that the embryo sensitivity factor was only effective when both the parental genotypes carried this trait (Figure 5.1). This indicates that embryo sensitivity to ABA is a recessive trait in white-grained wheats.

When SUN325 was the female parent, the F_1 grains had a SUN325 seed coat and if the pollen source was SUN325 or QT7475 (both containing embryo sensitivity) the dormancy phenotype was similar to the dormant parent, SUN325. By contrast, the GI of the F_1 derived from the reciprocal of this cross (QT7475 x SUN325) only showed intermediate-dormancy, presumably because the seed coat was derived from QT7475 and did not contain the seed coat factor. This observation supports the presence of a maternal seed coat factor provided by SUN325. It is evident that the seed coat factor is only effective in combination with the embryo sensitivity factor since the F_1 progeny generated from the cross between SUN325 x Hartog showed little to no dormancy (Figure 5.1), indicating that the dormant seed coat factor is only effective in combination with an embryo expressing sensitivity to ABA.

Based on consistent differences in dormancy phenotype observed over a number of experiments, and the same genotype at the 4A dormancy QTL, it was assumed that the white-grained intermediate-dormant x dormant population used in this study differed only for the expression of genes other than the one controlling embryo sensitivity to ABA, i.e. the seed coat factor involved in dormancy or a small number of genes including the seed coat factor. Dormancy expression is highly influenced by the environment and the population was screened in four of different environments over two years in order to ensure the results are due to genetic rather than environmental differences. Cool ripening temperatures were experienced for most of the experiments, which tended to increase the dormancy level of the population, which has also been observed in studies by Strand, (1989). This was most evident with the glasshouse experiment, but the frequency distribution was skewed towards low GI in all the field experiments with exception of experiment one in 2004, which gave the greatest discrimination between the parents and an apparent bimodal pattern (Figure 5.2, *Panel C*). The distribution between the dormant and intermediate-dormant phenotypes was continuous (except experiment 1 2004), which meant that separation into major groups was difficult. Although the frequency distribution of experiment one, 2004 (covered), appeared to separate into two distinct groups, the peaks in the distribution (at GI of 0.3 and 0.5) did not correspond with the mean values of the parents QT7475 (0.6) and SUN325 (0.2) suggesting that the distribution is probably not bimodal in reality.

The double haploid population of QT7475 x SUN325 was analysed for dormancy phenotype in a number of different environments over two years. In all of the experiments, the proportion of the population that has a comparable phenotype to SUN325, the dormant parent, varied considerably from 10.7% to 69.7% (average 39%). The most plausible explanation for this is that there may be more than one gene controlling variation in seed coat dormancy in this population. The average proportion of lines within the phenotypic range of QT7475 was only 21.4% (range 3.9-48.8%) and there were also lines outside the parental range. It would be expected if there was one gene controlling the difference in dormancy between the parents, that 50% of the lines would fall in each parental range. Due to the large variation with lines showing phenotypes corresponding to SUN325, it is difficult to determine how many genes are involved in this trait. The average proportion of lines showing dormancy equivalent to SUN325 is 39%, which is below the 50% expected

for a single gene, indicating that there may be more than one gene involved in the expression of the seed coat factor and therefore dormancy in this population.

Every line in this double haploid population is homozygous for the 4A QTL locus, associated with embryo sensitivity to ABA (Noda *et al.*, 2002) and grain dormancy in a number of mapping populations (Noda *et al.*, 2002; Mares *et al.*, 2005). The additional QTL on the long arm of chromosome 3B identified in this population (Figure 5.7) was consistent across all of the environments and explains a significant proportion (LRS of 17.7) of the trait variation in the population. The 3BL region is therefore a likely candidate for the dormant seed coat factor. QTL regions relating to dormancy have also been found on group three chromosomes in other studies, for example Groos *et al.,* (2002) who detected a region on 3BL, Mori *et al.,* (2005) on 3AS and Kulwal *et al.,* (2005) on 3AL. Mares *et al.,* (2005) also suggested that there are possible QTL regions on the group three chromosomes. Despite this, there are other genes on the group three chromosomes which are related to dormancy and may be interfering factors with these studies.

The *R* genes are located distally on the long arms of the group three chromosomes and it is generally accepted that the most dormant red-grained genotypes are more dormant than the most dormant white-grained genotypes (Flintham, 2000; Flintham *et al.*, 2002). In addition, the *Vp1* gene which is known to prevent premature germination, vivipary, in mutant maize genotypes (McCarty, 1995) is also located on the long arms of group three chromosomes (Bailey *et al.*, 1999). The exact role of *Vp1* in dormancy is still not entirely clear, although it is accepted that it is responsible for developmental control of ABA synthesis and has some involvement in the upregulation of the anthocyanin pathway (McCarty, 1995). Recent studies in wheat, however, have suggested an high degree of mis-splicing, with transcripts not capable of encoding a full-length protein (McKibbin *et al.*, 2002). The QTL detected on chromosome 3BL was compared to the proposed location of the *R* gene. The microsatellite map created by Somers *et al.,* (2004) shows that the micro-satellite markers associated with the QTL detected in this study, and those markers associated with the *R* gene reported by Groos *et al.,* (2002) and Bailey *et al.,* (1999), are separated by approximately 30cM. The *R* gene is located distally from the QTL detected in this study, which appears to be close to the centromere (Figure 5.3). This observation is consistent with the QTL data recorded by Groos *et al.,* (2002) which showed two regions associated with seed coat colour and dormancy on chromosome 3BL. The dormancy

region identified by (Groos *et al.*, 2002) was comparable to the QTL detected in this study, while the seed coat colour region was located approximately 30cM distal from their dormancy QTL (Groos *et al.*, 2002). It has been reported that *taVp1* has been reported to be located in this region (approximately 30cM proximal of *R* gene) (Bailey *et al.*, 1999), which may also be close to the QTL detected in this study (Figure 5.3).

The *R* genes have been identified on the long arm of the group 3 chromosomes and recently been suggested to be a transcription factor involved in the upregulation of the flavonoid biosynthesis pathway in the seed coat of red-grained genotypes (Himi *et al.*, 2005). In wheat, the *R* genes have a large effect on dormancy level and are likely to interfere with QTL analyses of populations with red-grained genotypes eg (Mori *et al.*, 2005). In these experiments there is no complicating red seed coat colour and therefore the QTL region can be considered independent of the seed coat colour. Flintham (2000) also suggested that genes other than the *R* genes on group three chromosomes are involved in dormancy and act either through a pleiotropic effect or are linked with the red seed coat (Warner *et al.*, 2000). The linkage theory has been discounted, however, because dormant red-grained wheats have been mutated to white-grained genotypes on two separate occasions with a resulting reduction in dormancy compared with the wild-type (Flintham, 2000; Warner *et al.*, 2000). Himi and Noda, (2005)also found that the failure to produce the red pigment was due to a single amino acid deletion. This present study identified a QTL region on 3BL that appeared to be proximal to the proposed location of the *R* gene. The seed coat component of dormancy in SUN325 is achieved through genes on group three chromosomes, but these genes alone do not confer a dormancy level as high as dormant red-grained genotypes, suggesting that the *R* gene possibly has a pleiotropic effect on dormancy.

The region detected in this study is comparable with a QTL located by Groos *et al.*, (2002), who used a red x white-grained population to find regions associated with grain colour and dormancy. They detected QTLs for grain colour on all of the group three chromosomes, however there was an additional region on chromosome 3BL which was associated with resistance to pre-harvest sprouting but not grain colour (Groos *et al.*, 2002). This region is close to the centromere and shares a common microsatellite marker (gwm77) with the QTL detected in this present study (Figure 5.7). Although Groos *et al.,* (2002) concluded that the region on 3BL was probably *taVP1*, it is now evident that this QTL is most likely

associated with a seed coat rather than an embryo factor in dormancy. A QTL was on 3B was also reported in other earlier dormancy studies (Anderson *et al.*, 1993; Zanetti *et al.*, 2000).

A recent investigation by Kulwal et al., (2005) found a QTL on 3AL which was believed to be associated with the red grain colour, while another study by Mori *et al.,* (2005) found a QTL on 3AS which is possibly related to a region also detected by Zanetti *et al.,* (2000) and Osa *et al.,* (2003). In addition to the 3B QTL found in this study, there was some evidence for the presence of another QTL on 3D, which may also be involved in dormancy. Unfortunately this QTL could not be validated due to limited polymorphism in the available 3D markers in this population, however 3D has previously been suggested to contain dormancy genes in a dormant white-grained genotype, AUS1408 (Mares *et al.*, 2002). Other mapping populations, or new markers near 3D could be developed to explore this further.

Chapter 6: Expression of Genes Involved in the Flavonoid Biosynthesis Pathway in the Seed Coat of Developing Wheat Grains

6.1 Introduction

The results reported in Chapter 5 indicated that a QTL located on chromosome 3BL was a potential candidate for the seed coat factor in dormant white-grained wheat genotypes. Furthermore this QTL was located in the same region as two genes associated with the red pigment in red-grained wheats and therefore has a putative role in grain dormancy. These genes are the *R* gene, a transcription factor of the flavonoid biosynthesis pathway, and DFR, an enzyme required for synthesis of the red pigment (Himi and Noda, 2004). Based on these similarities, it seems possible that the mechanism of the seed coat factor in dormant white-grained wheat genotypes might involve an accumulation, but not polymerisation, of the flavonoid precursors of the red pigment. Himi *et al*. (2005) have already shown that the genes controlling key enzymes required for synthesis of the red pigment are not expressed in the seed coat of non-dormant white wheats.

The aim of this component of the project, as stated in aims five and six of Chapter 1, was to compare the expression of key genes in the flavonoid biosynthesis pathway in the seed coat of dormant and non-dormant genotypes. *DFR* and *CHS* are major genes in the flavonoid biosynthesis pathway and the expression patterns of these genes was studied in the developing seed coats of four genotypes of differing dormancy. These genotypes included dormant, non-dormant and intermediate-dormant white-grained genotypes and a dormant red-grained genotype. It was observed by Himi and Noda (2004) that the synthesis of the red pigment occurs before 20 dpa, and expression of *DFR* and *CHS* in red-grained wheat is highest between 10 and 15 dpa. These time points were selected as the time points for this analysis.

In addition to gene expression analysis, direct evidence for the synthesis of flavonoids was examined using HPLC in mature and developing seed coat material. HPLC analysis was conducted in both red and white-grained wheat, with the red-grained wheat genotype serving as a positive control.

6.2 Materials and Methods

6.2.1 Plant material

The wheat genotypes, Hartog (white-grained, non-dormant), QT7475 (white-grained, intermediate-dormant), SUN325 (white-grained, dormant) and R/W635 (red-grained, dormant) were grown in a glasshouse (average temperatures 25/17°C day/night). At anthesis, spikes were tagged and single grains harvested from central primary and secondary florets at 10 and 15 dpa, corresponding to the development stage of highest expression of key genes involved in flavonoid biosynthesis (Himi and Noda, 2004). For detection of flavonoid compounds, grains were harvested at 10, 15, 20, 25, 30 dpa as well as at harvest maturity.

6.2.2 RNA isolation and cDNA synthesis

Developing grains were extracted and dissected as described in Chapter 2 (*Section 2.3*), frozen in liquid nitrogen and stored at -80ºC. RNA was extracted by grinding the seed coat samples in the same tube with a Teflon pestle in 500 µL of TRIZOL (Invitrogen, USA). After grinding the tissue as finely as possible, another 500 µL of TRIZOL was added and the solution incubated for five minutes at room temperature. To this solution, 200 µL of chloroform was added and shaken for 15 seconds, before incubating at room temperature (RT) for another 5 minutes. The separation of the aqueous phase was achieved by 4ºC centrifugation at 10,000 g for 15 minutes. The resulting aqueous phase was transferred carefully to a clean 1.5 mL tube and RNA was precipitated with 500 µL of isopropanol, before incubating at room temperature for 10 minutes. An RNA pellet was formed after centrifugation at 10,000 g at 4ºC for ten minutes, and the supernatant was removed and discarded without disturbing the pellet. The purified RNA was washed with 1 mL of 75% (v/v) ethanol and centrifuged at 4ºC at 10,000 g for 5 minutes. The supernatant was discarded again, and the pellet air dried for approximately five minutes ensuring it was not over dried. Finally, the dried RNA was suspended in 25 µL of sterile DECP-treated milli-Q water, and confirmed for quality by separation on a 1% TAE agarose gel. There was no evidence of DNA contamination in the RNA samples. The RNA concentration of each sample was determined by optical density (OD) (absorbance at 260 nm) of a 1:500 dilution using the following equation:

RNA conc. (µg/mL) = OD x dilution factor (500) x 40

First strand cDNA was synthesised with 2 µg of total RNA. RNA (2 µg) was added to 1 µL 50 µM oligo(dT), 1 µL 10 mM deoxynucleotide triphosphates (dNTP) and sterile DECP-treated milli-Q water to a final volume of 13 µL. This solution was incubated at 65ºC for 5 minutes after which the reaction mixture was placed on ice. To this mixture, 7 µL of a buffer solution (4 µL of 5x first strand buffer, 1 µL 14 mM DTT, 1 µL RNasIN Ribonuclease inhibitor (Promega, USA), 0.25 µL (50 units) Reverse Transcriptase (Superscript II Invitrogen, USA) and 1.75 µL sterile water) was added to make a final volume of 20 µL. The solution was incubated at 50ºC for 1 hour and adjusted to a final volume of 50 µL with the addition of 30 µL of DECP-treated sterile milli-Q water.

6.2.3 Bioinformatics and primer design

All nucleotide sequences were obtained from published wheat *DFR* and *CHS* sequences available from the NCBI database (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed using the ClustalW software (http://www.ebi.ac.uk/clustalw/). Primers were designed with the assistance of the computer applet program, NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html), with specific parameters listed below.

Well-conserved regions between the three published wheat genes for *DFR* and three *CHS* sequences of wheat, barley and rice were selected. Care was taken to ensure that primers were designed to a coding region of the gene and not to introns. Primer sequences were specifically designed to conserved regions of published wheat *DFR* and *CHS* sequences (Figures 6.1 and 6.2). The primers were analysed by NetPrimer, with parameters within this program used to design suitable primer pairs (Table 6.1): a Tm below 60ºC, hairpins with a ΔG no lower than –0.5 kcal/mol and cross-dimers no lower than –8 kcal/mol. Primer regions and amplified sequence of *DFR* and *CHS* are shown in Figures 6.1 and 6.2 respectively.

Primer Name	Nucleotide Sequence	PCR Product	Tm Values
DFR Forward	AGCGTCAACATCGAGGAG	178bp	53.37
DFR Reverse	GATGATGCTGATGAAGTCCA		53.28
CHS Forward	ATCACCCACCTTGTATTCTG	471bp	51.94
CHS Reverse	CTCGATGTTCTTGGAGATGA		52.82
GAPDH Forward	TTCAACATCATTCCAAGC	200bp	
GAPDH Reverse	CGTAACCCAAAATGCCCT		

Table 6.1: The nucleotide sequences of primers used to amplify gene-specific regions of DFR and CHS. Primer sequences for control gene GAPDH are also included.

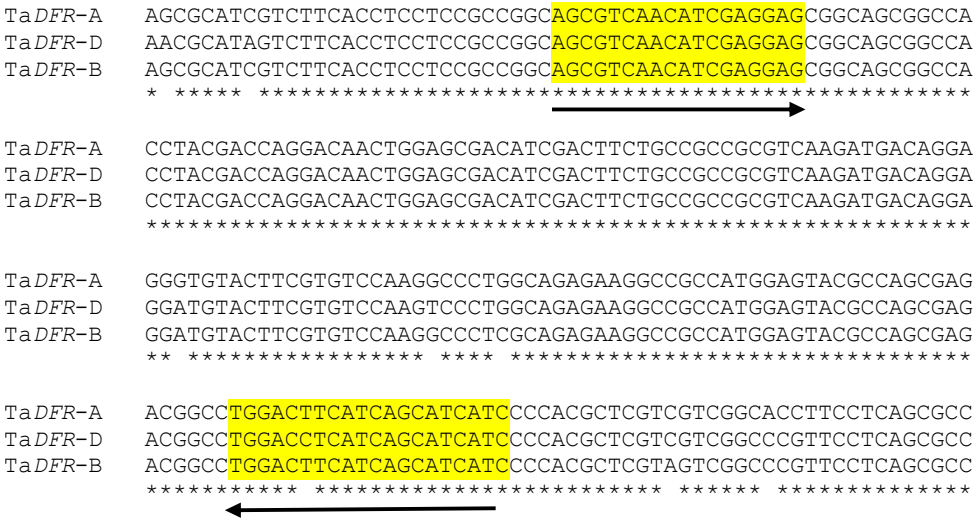


Figure 6.1: Partial DNA exon sequence of three wheat DFR genes. *TaDFR-A* (AB162138), *TaDFR-B* (AB162139) and *TaDFR-D* (AB162140), represent the different wheat genomes. This region of the gene was subsequently amplified by RT-PCR. Arrows show direction of DNA synthesis from primer.

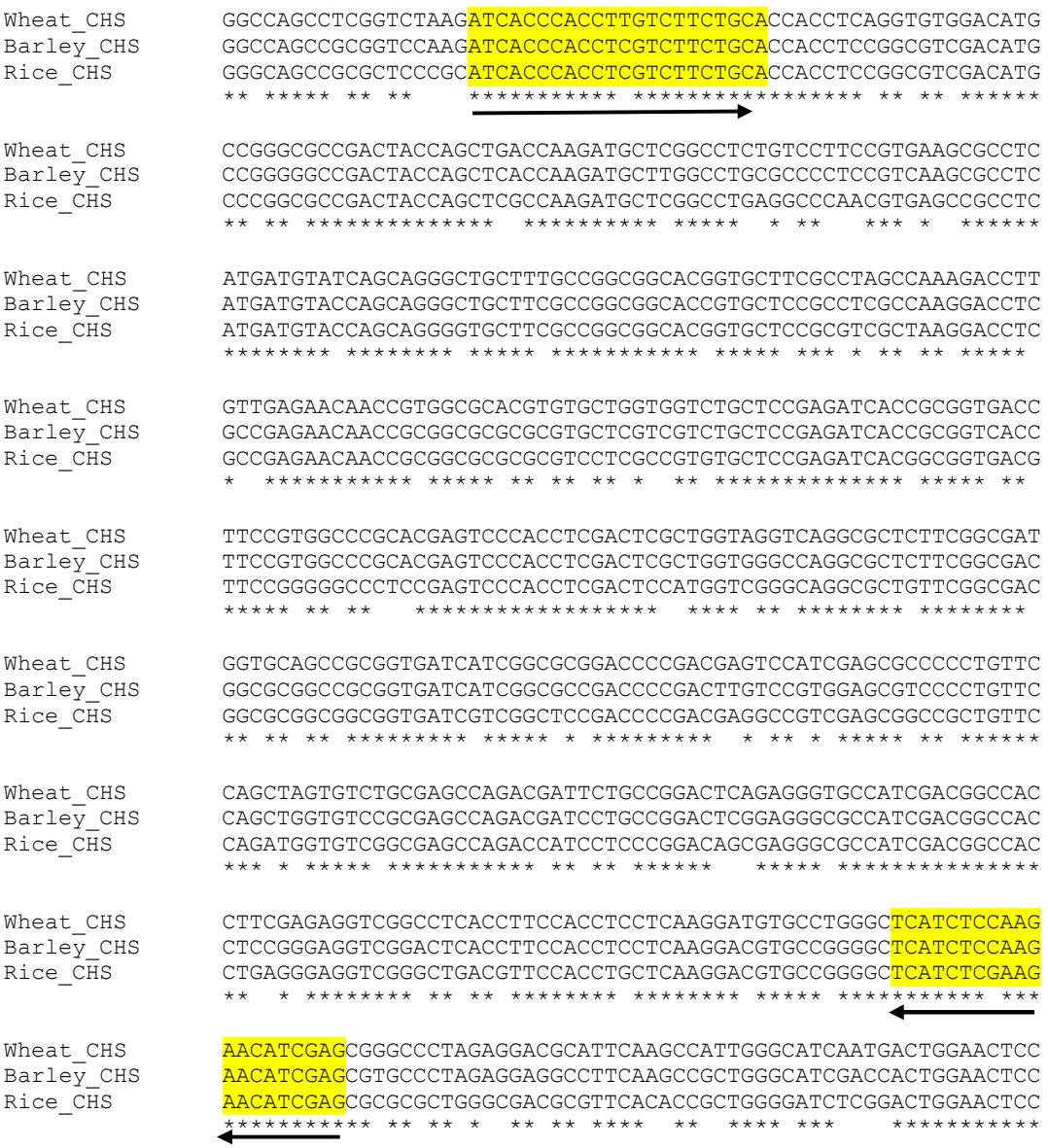


Figure 6.2: Partial DNA alignment of three published *CHS* genes. Primers were designed to conserved regions of *CHS* cDNA sequences of: wheat (AY286098), barley (X58339) and rice (X89859). This region of the gene was subsequently amplified by RT-PCR. Arrows show direction of DNA synthesis from primer.

6.2.4 Reverse Transcription (RT)-PCR

Polymerase Chain Reaction (PCR) was used to amplify cDNA fragments of *DFR* (155 bp), *CHS* (466 bp) and the control gene GAPDH. Primer pairs of *DFR* and *CHS* were ordered through Geneworks (Adelaide, Australia) and diluted to a concentration of 100 ng/µL with sterile water. PCR was performed in a 39 µL solution that contained 1 µL of cDNA and PCR mix per 100 µL containing: 10 µL 10x amplification buffer (100 mM Tris-HCI buffer, pH 9.0: containing 500 mM KCI, 10% Triton X-100), 4 µL 50 mM $MgCl_2$, 2 µL 10 mM dNTP, 2 µL of forward and reverse primer and 0.4 µL 1 unit Taq DNA polymerase. PCR thermal cycling involved an initial melting stage of 94ºC for 2 minutes, followed by 35 cycles of 20 seconds at 94ºC, annealing temperature of either 47ºC (*DFR*) or 54ºC (*CHS*) and an extension step of 72ºC for 1 minute.

Re-amplification of the PCR was necessary to increase the amount of product and visualise bands on the agarose gel. This was only performed to intensify bands for sequencing and to detect the *CHS* gene fragment. *DFR* was detected with only one round of PCR. To re-amplify bands, 1 µL of PCR product was used instead of cDNA in a (40 µL) reaction mixture and using the same cycling conditions.

6.2.5 DNA sequencing

6.2.5.1 Gel isolation

Gel bands were purified for sequencing using a MoBio Ultraclean Kit (MoBio, USA). Approximately 100 µL of each PCR reaction was combined and run on a gel with large wells. Bands were excised and the DNA purified according to Manufacturer's instructions. The concentration of the purified product was estimated from the gel with 3 µL of purified DNA on a 1% agarose gel.

6.2.5.2 PCR purification

QIAquick PCR Purification Kit (Qiagin, Denmark) of the PCR product was performed using a QIAquick spin column was used to clean the DNA sample. PRC reactions were combined to

increase DNA yield for purification and sequencing following Manufacturer's instructions. The purified DNA was eluted from the column in 30 µL and 3 µL was run on a 1% agarose gel to determine approximate DNA content of the purified product.

6.2.5.3 Direct sequencing of PCR product with BigDye sequencing reaction

BigDye® Terminator Version 3.1 Cycle Sequencing Kit was used to prepare the DNA for sequencing. The sequencing reaction was conducted as specified by Australian Genomic Research Facility (AGRF), with 5 – 10 ng (*DFR*) or 10 – 50 ng (*CHS*) purified PCR DNA template according to expected fragment size, in a total volume of 12 µL. This volume included 1 µL BigDye Terminator, 3 µL dilution buffer, 1 µL sequencing primer and the appropriate volume of purified DNA (4 µL or 6 µL). Sequencing was conducted with both the forward and reverse primers. The remaining volume was occupied by sterile water, if required. The PCR thermal cycling regime was an initial melting step of 30 seconds of 96ºC, followed by 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes.

6.2.5.4 Magnesium sulphate cleanup of PCR sequencing reaction

The protocol used for the cleanup of the sequencing reactions prior to capillary separation was provided by AGRF. A stock solution of $MgSO_4$, containing 30 mL sterile water, 70 mL absolute ethanol and 20 µL 1 M $MgSO_4$, was added in a volume of 75 µL to each PCR tube. The tubes were mixed by briefly vortexing and left to react at room temperature for a minimum of 15 minutes. The DNA was pelleted through centrifugation for 15 minutes at 3,000 g, and the tubes subsequently inverted over paper towels and tapped gently to remove excess supernatant. The pellet was dried further in a 37ºC oven for approximately 15 minutes, and samples submitted to AGRF for sequencing.

6.2.6 HPLC analysis of seed coat extracts

Seed coat from mature and developing grains was removed as described in Chapter 2 (*Section 2.3*). An alkaline extraction of the isolated seed coat was performed which has also been outlined in Chapter 2 (*Section 2.3*).

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis was conducted on a Hewlett Packard 1100 Series HPLC system equipped with a diode array detector, quaternary pump, solvent degasser, autosampler and a column compartment heated to 30ºC. Separation was achieved with a LiChroCART® 250-4 reverse phase C18 column (5 µm, 250 x 4 mm I.D.; Merck), which was protected by a guard column (LiChroCART® 4-4 RP-18 5 µm; Merck). The flow rate was 0.65 mL/min with a solvent system of: solution (A) water and 0.05% TFA (trifluoro-acetic acid) and solution (B) 80% acetonitrile, 15% water and 0.05% TFA (0 to 100% A in 60 minute then B until 70 minute). Products of the flavonoid biosynthetic pathway were detected at 250, 280, 340, 480 and 540 nm and absorbance at 340 nm used for estimation of flavone-*C*-glycosides. Peak areas were calculated with the accompanying HPLC software, HP ChemStation for LC© (Hewlett Packard).

6.3 Results

6.3.1 Expression of *DFR* and *CHS*

To determine any differences between dormant and non-dormant genotypes, RT-PCR was used to analyse the expression of *DFR* and *CHS* in developing seed coat tissue at 10 and 15 dpa, using gene-specific primer pairs of *DFR*, *CHS* and GAPDH (Table 6.1) at both 10 and 15 dpa. GAPDH was used as a positive control to ensure the quality of the extracted RNA and subsequently synthesised cDNA. The amplified DNA fragments of *DFR* (135 bp) and *CHS* (471 bp) were of expected size (Figure 6.3, *A* and *B*). Figure 6.3 also shows that there was no contamination of the no-DNA control reaction, in particular in the re-amplification of *CHS* (*Panel B*).

DFR was expressed at a detectable level at 10 dpa in all genotypes, as shown in Figure 6.4. No expression of *CHS* was detected after 35 cycles, with the initial PCR reaction failing to show any bands (data not shown) and therefore a re-amplification was performed. At 10 dpa bands for *CHS* were observed in all four genotypes after re-amplification.

At 15 dpa, *DFR* was expressed in all the genotypes (Figure 6.4) and there were no extra bands visible on this gel (data not shown). The initial PCR for *CHS* expression again failed to show any visible bands (data not shown). However, on re-amplification, *CHS* expression was detected but only in the dormant red-grained and dormant white-grained genotypes (Figure 6.4). There was no evidence of any other bands on this gel. The requirement for PCR re-amplification to visualise the *CHS* fragment on a gel indicates that expression is at a significantly lower level than *DFR* in all genotypes.

The PCR products from *DFR* amplifications were pooled on another gel, the band excised and the fragment purified for sequencing. Figure 6.5 shows the sequence alignment of the reaction product was of expected size (135 bp) and showed strong nucleotide identity with the *TaDFR5* (AY707920), except in the region close to the sequence primer (reverse primer) which is commonly an area of poor quality sequence. The *CHS* fragment was also purified and sequenced directly from the re-amplified PCR reaction. The sequenced *CHS* PCR product was

of expected size (471 bp) and had a sequence that was very similar to *CHS-1* (AY286098 (Figure 6.6). All amplified fragments were of expected size and confirmed the specificity of *DFR* and *CHS* primers.

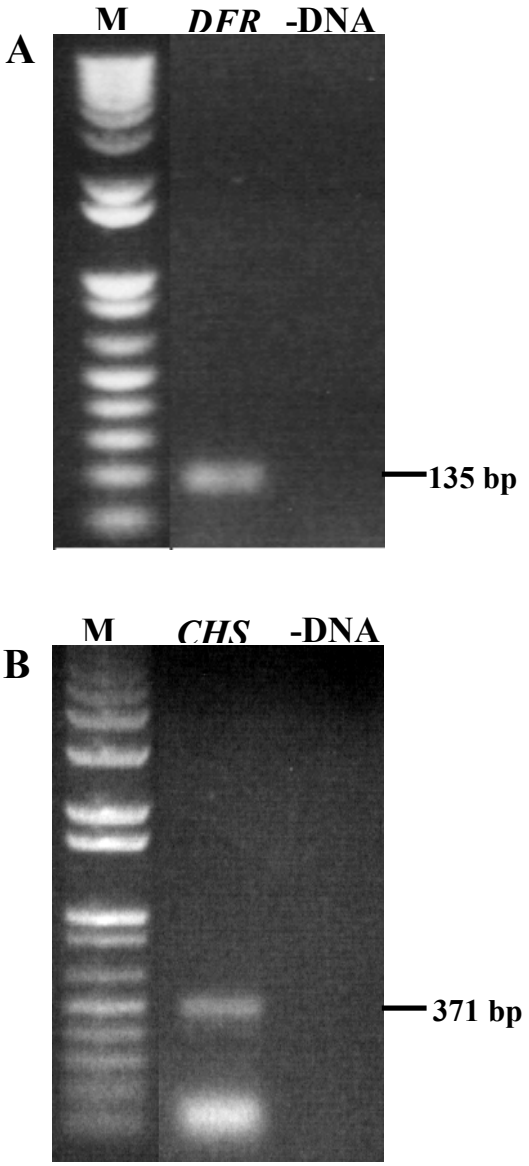


Figure 6.3: Amplified fragments of *DFR* and *CHS* from R/W635 showing fragment size and no-DNA control. Panel A: *DFR*, Panel B: *CHS*.

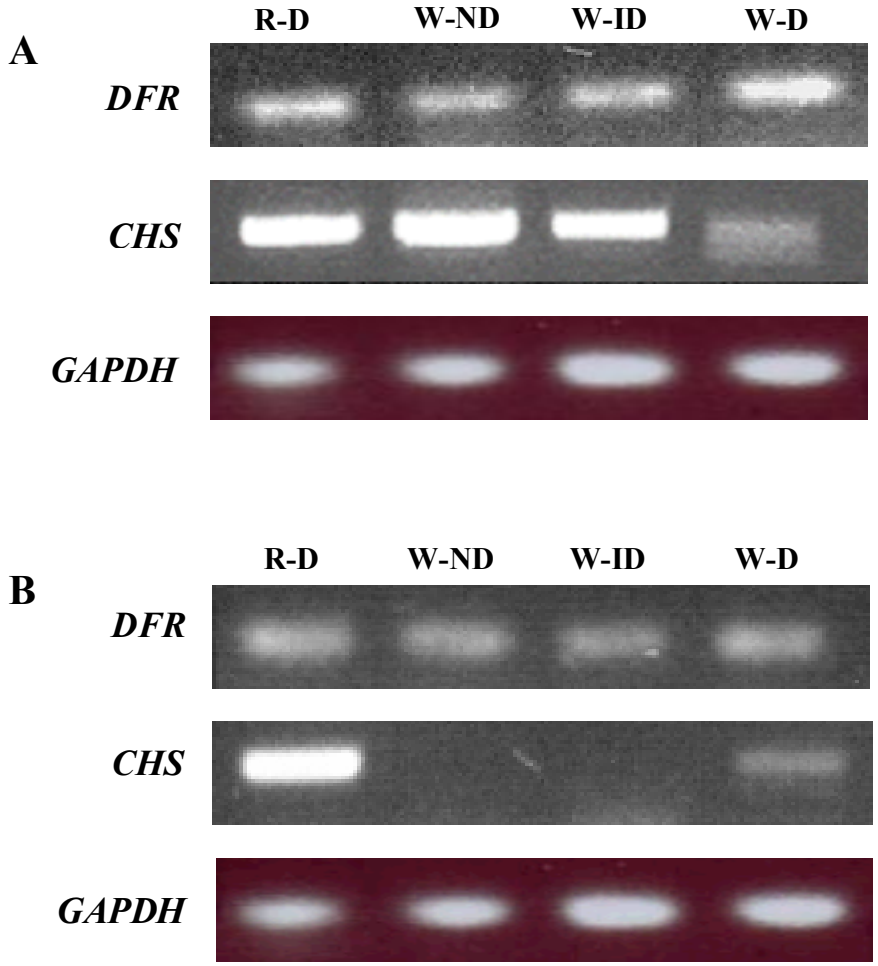


Figure 6.4: PCR amplified cDNA fragment, of *DFR* (178 bp) and *CHS* (471 bp) in seed coat tissue of genotypes at 10 dpa (*Panel A*) and 15 dpa (*Panel B*). GAPDH performed as a cDNA control. Lane 1: R-D (R/W635), Lane 2: W-ND (Hartog), Lane 3: W-ID (QT7475), Lane 4: W-D (SUN325).

```
TaDFR5          AGGCGCATCGTCTTCACCTCCTCCGCCGGCAGCGTCAACATCGAGGAGCGGCAGCGGCCA 420
DFR_seq_JR4     ----------------------------TAGCGTCAACATCGAGGAGCGGCAGCGGCCA 31
                                            ****************************

TaDFR5          GCCTACGACCAGGACAACTGGAGCGACATCGACTTCTGCCGCCGCGTCAAGATGACAGGA 480
DFR_seq_JR4     GCCTACGACCAGGACAACTGGAGCGACATCGACTTCTGCCGCCGCGTCAAGATGACAGGA 91
                ************************************************************

TaDFR5          TGGATGTACTTCGTGTCCAAGTCCCTCGCAGAGAAGGCCGCCATGGAGTACGCCAGCGAG 540
DFR_seq_JR4     TGGATGTACTTCGTGTCCAAGTCCCTCGCAGAGAAGGCCGCCATGAGACTAGCGGCTGAA 151
                ****************************************      **    **

TaDFR5          AACGGCCTGGACTTCATCAGCATCATCCCCACGCTCGTAGTCGGCCCGTTCCTCAGCGCC 600
DFR_seq_JR4     ACCC---------------------------------------------------------- 155
                * *
```

Figure 6.5: Alignment of nucleotide sequences of TaDFR5 (AY707920) and the sequenced PCR product. Region corresponding to forward and reverse primers is highlighted. Sequence was derived from direct sequencing of the PCR product with reverse *DFR* primer.

```
Wheat_CHS1        GGCCAGCCTCGGTCTAAGATCACCCACCTTGTCTTCTGCACCACCTCAGGTGTGGACATG 420
CHS_seq_JR11      ----------------------------------------TCTATAGAGTGTACA-G 16
                                                          **   * *** *** *

Wheat_CHS1        CCGGGCGCCGACTACCAGCTGACCAAGATGCTCGGCCTCTGTCCTTCC-GTGAAGCGCCT 479
CHS_seq_JR11      CCGGGCGCCGACTAC-AGCTGACCAAGATGCTCGGCCTCTGTCCTTCCCGTGAAGCGCCT 75
                  ************** ****************************** ***********

Wheat_CHS1        CATGATGTATCAGCAGGGCTGCTTTGCCGGCGGCACGGTGCTTCGCCTAGCCAAAGACCT 539
CHS_seq_JR11      CATGATGTATCAGCAGGGCTGCTTTGCCGGCGGCACGGTGCTTCGCCTAGCCAAAGACCT 135
                  ************************************************************

Wheat_CHS1        TGTTGAGAACAACCGTGGCGCACGTGTGCTGGTGGTCTGCTCCGAGATCACCGCGGTGAC 599
CHS_seq_JR11      TGTTGAGAACAACCGTGGCGCACGTGTGCTGGTGGTCTGCTCCGAGATCACCGCGGTGAC 195
                  ************************************************************

Wheat_CHS1        CTTCCGTGGCCCGCACGAGTCCCACCTCGACTCGCTGGTAGGTCAGGCGCTCTTCGGCGA 659
CHS_seq_JR11      CTTCCGTGGCCCGCACGAGTCCCACCTCGACTCGCTGGTAGGTCAGGCGCTCTTCGGCGA 255
                  ************************************************************

Wheat_CHS1        TGGTGCAGCCGCGGTGATCATCGGCGCGGACCCCGACGAGTCCATCGAGCGCCCCCTGTT 719
CHS_seq_JR11      TGGTGCAGCCGCGGTGATCATCGGCGCGGACCCCGACGAGTCCATCGAGCGCCCCCTGTT 315
                  ************************************************************

Wheat_CHS1        CCAGCTAGTGTCTGCGAGCCAGACGATTCTGCCGGACTCAGAGGGTGCCATCGACGGCCA 779
CHS_seq_JR11      CCAGCTAGTGTCTGCGAGCCAGACGATTCTGCCGGACTCAGAGGGTGCCATCGACGGCCA 375
                  ************************************************************

Wheat_CHS1        CCTTCGAGAGGTCGGCCTCACCTTCCACCTCCTCAAGGATGTGCCTGGGCTCATCTCCAA 839
CHS_seq_JR11      CCTTCGAGAGGTCGGCCTCACCTTCCACCTCCTCAAGGATGTGCCTGGGCTCATCTACAA 435
                  ********************************************************* ***

Wheat_CHS1        G-AACATCGAGCGGGCCCTAGAGGACGCATTCAAGCCATTGGGCATCAATGACTGGAACT 898
CHS_seq_JR11      ACAATATCAGG---------------------------------------------- 446
                    ** ***  *
```

Figure 6.6: Alignment of nucleotide sequences of CHS1 (AY286098) and the sequenced PCR product. Region corresponding to forward and reverse primers is highlighted. Sequence was derived from direct sequencing of the PCR product with forward *CHS* primer.

6.3.2 HPLC analysis of seed coat extracts

6.3.2.1 Seed coat from ripe grains

HPLC was used to analyse extracts of seed coats to determine if there are differences in the intermediates or products of the flavonoid biosynthetic pathway. The HPLC chromatograms (of one of three replicates) of seed coat extracts from ripe grains contained several significant peaks, labelled A, B, C and D, with retention times between 25 and 27 minutes (Figure 6.7). Peaks A, B and D are likely to be flavone-*C*-glycosides and have similar absorption maxima, while peak B is not a flavone-*C*-glycoside (Figure 6.7). Peak B is only present as a shoulder of peak C in QT7475 (*Panel B*), SUN325 (*Panel C*) and R/W635 (*Panel D*), but is relatively large in Hartog (Figure 6.7, *Panel A)* Figure 6.8 shows the UV/Vis spectra of the major peaks (Figure 6.7) with Peak A λ_{max} of 272 and 325 nm (*Panel A*), Peak C λ_{max} 275 and 315 nm (*Panel C*) and Peak D λ_{max} 275 and 325 nm (*Panel D*), that are typical of flavone-*C*-glycosides (Asenstorfer *et al.*, 2006). Peak B is the largest in Hartog and has a λ_{max} of 275 and 315 nm and does not have a UV/Vis spectra similar to flavone-*C*-glycosides (Figure 6.8, *Panel B*). The major peaks were clustered around a retention time of 24 to 27 minutes and were considered the same peaks in each genotype by observing and comparing the λ maxima of the UV/Vis spectra (Figure 6.7). Areas under the major peaks (Peaks A, C and D) appeared to be greater in the white-grained dormant genotype, SUN325, than the other genotypes. All HPLC analyses were conducted in three replications and the selected chromatograms are representative of the consistent differences and similarities in the proportions of the peaks between the genotypes. In these chromatograms, Hartog consistently appears to have the smallest (and most different) peaks compared to the other genotypes. The peaks of SUN325 and QT7475 are the most similar in proportions, but these major peaks (Peaks C and D) are larger in SUN325.

The white-grained dormant genotype SUN325 contained the greatest amount of flavone-*C*-glycosides in the mature seed coat, based on measurements of major peaks indicated in Figure 6.7 (Figure 6.9). Flavone-*C*-glycoside contents (mAU/mg) were significantly lower and similar in the red dormant genotype R/W635 and QT7475 (white-grained, intermediate-

dormant). The white-grained non-dormant Hartog was substantially lower than the other genotypes (Figure 6.9).

6.3.2.2 Seed coat from developing grains

During grain development until 30 dpa, there was a reduced amount and number of flavone-*C*-glycoside compounds in all the genotypes (Figure 6.10) compared to the mature seed coat (Figure 6.7). There is only one major flavone-*C*-glycoside peak in the developing seed coat extract, Peak A, which has a retention time of approximately 25 minutes, and is present in all of the genotypes (Figure 6.10). Peak A had absorption maxima (λ_{max}) of 276 and 317 nm (Figure 6.11). The chromatogram of SUN325 contained the highest amounts of peak A. The large peak, peak X which is evident in Hartog and QT7475 (Figure 6.9, *Panels A* and *B*), was observed in all of the genotypes and at all time points within the three replications and is possibly a result of aleurone contamination of the seed coat material (Chapter 2, Figure 2.4). The UV/Vis spectra of peak X, with a λ_{max} of 295 and 325 nm is highly similar to the major compound extracted from the aleurone tissue (Figure 6.10, *Panel B* and Figure 2.5, *Panel B*) and is probably the same compound. This peak is inconsistent throughout all the genotypes and replications, which is represented in Figure 6.13, and indicates that this compound is likely to be intermittent aleurone contamination. Furthermore, the early time points when aleurone tissue was less likely to contaminate the seed coat tissue sample (due to easier separation of the seed coat at early grain development), peak X is not as prevalent (Figure 6.13).

Figure 6.12 shows the amount of the compound represented by peak A (mAU/mg) in the chromatogram of the extract from the seed coat of developing grains. Statistical analysis of these peak area values from the three replications showed that there was a significant difference between the amount of the major flavone-*C*-glycoside peak (Peak A) in SUN325 and the other genotypes at each time point. At 10 dpa, SUN325 clearly contains the greatest amount of flavone-*C*-glycosides, while there is very little difference between the other genotypes. There is no statistical difference in the area of peak A in R/W635 and SUN325 at 15 dpa, but the area is significantly lower in QT7475 and lower again in the chromatogram of Hartog. The amount of flavone-*C*-glycosides, represented by the area of Peak A, at all the

other time points does not appear to increase relative to the values obtained at 15 dpa, although there is a slight observable increase in all the genotypes, excluding SUN325, between 10 and 15 dpa. At all of the time points except at 20 and 10 dpa, the amount of the compound represented by Peak A is significantly higher in R/W635 compared to Hartog and QT7475. The flavone-*C*-glycoside compound represented by Peak A in the chromatograms of SUN325 significantly exceeds that of the other genotypes, with only R/W635 at 15 dpa having a comparable area of Peak A.

The amount of the compounds represented by the major peaks in the mature seed coat extract is displayed in Figure 6.9 and is presented separately from the developing seed coat data because of the large discrepancy in the amount of flavone-*C*-glycosides. There was a much higher amount of flavone-*C*-glycosides, and phenolic compounds in general, detected in the mature seed coat. The mature seed coat was without the high water content associated with the developing seed coat, which probably served as a dilution factor and therefore there was a greater quantity of mature seed coat material used in flavone-*C*-glycoside extractions. Additionally, expression of the flavonoid biosynthesis genes precedes protein and product synthesis and the flavone-*C*-glycoside content in the seed coat will increase over time.

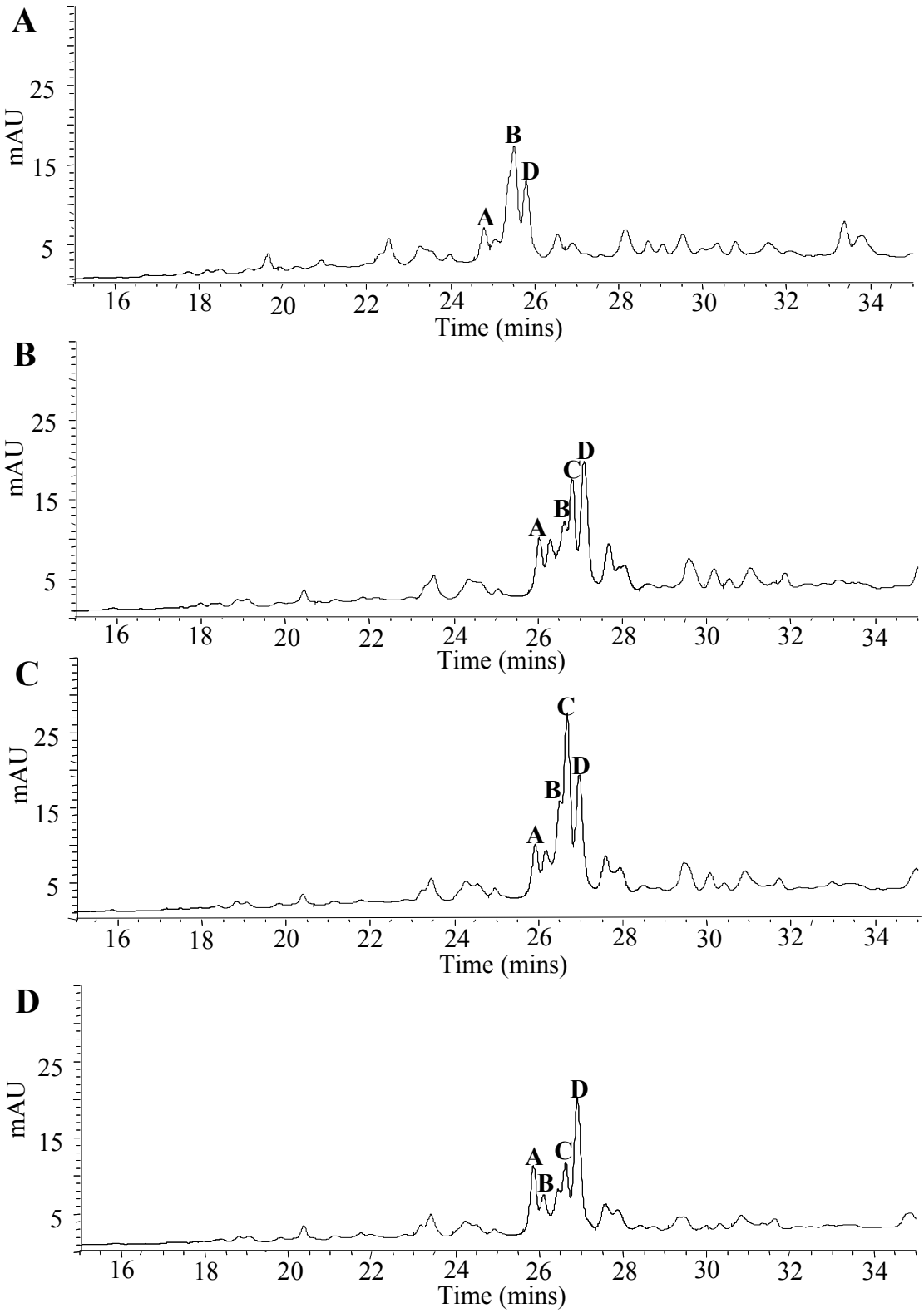


Figure 6.7: HPLC chromatograms monitored at 340 nm of flavone-*C*-glycosides extracted from mature seed coat *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635. Major flavone-*C*-glycoside peaks are labelled.

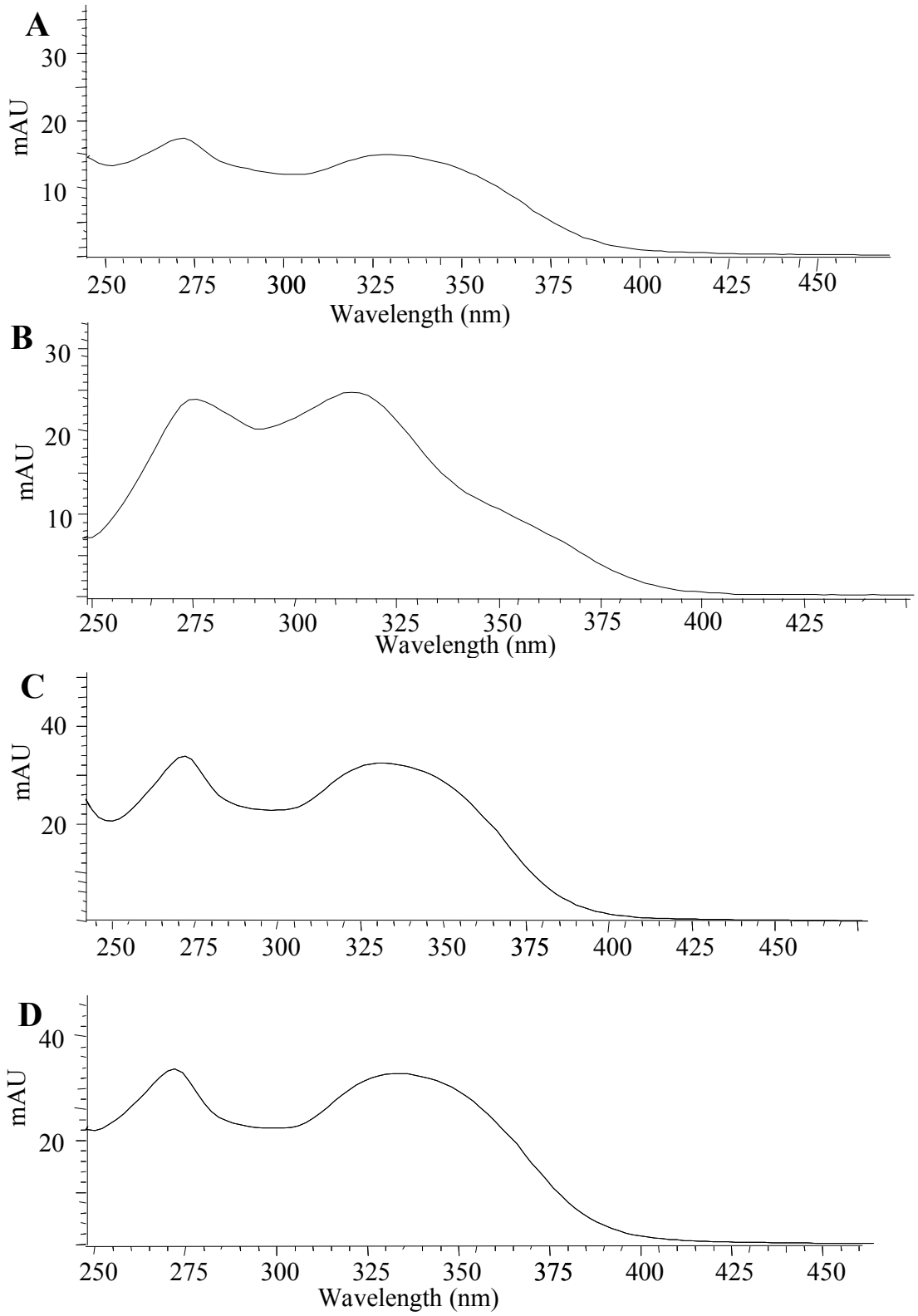


Figure 6.8: UV/Vis spectra of peaks A, B, C and D from Figure 6.7.

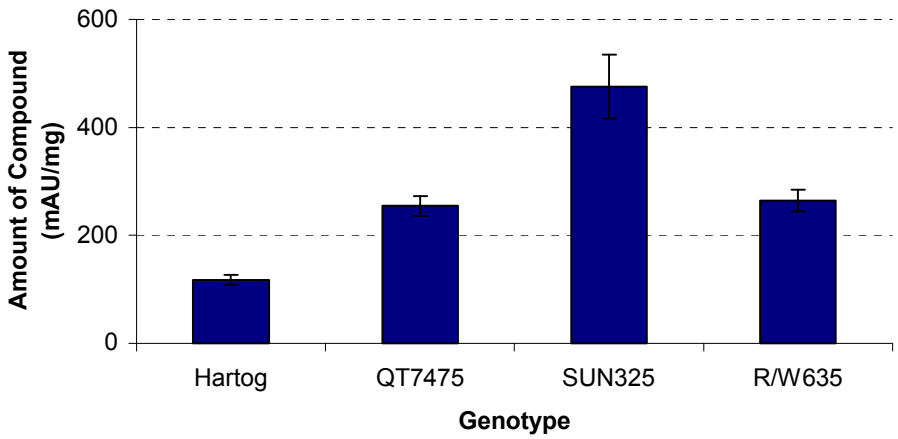


Figure 6.9: Flavone-*C*-glycoside content (mAU/mg) of seed coat from mature grains, determined by the amount of compounds represented by Peaks A, C and D (Figure 6.7) from the chromatograms.

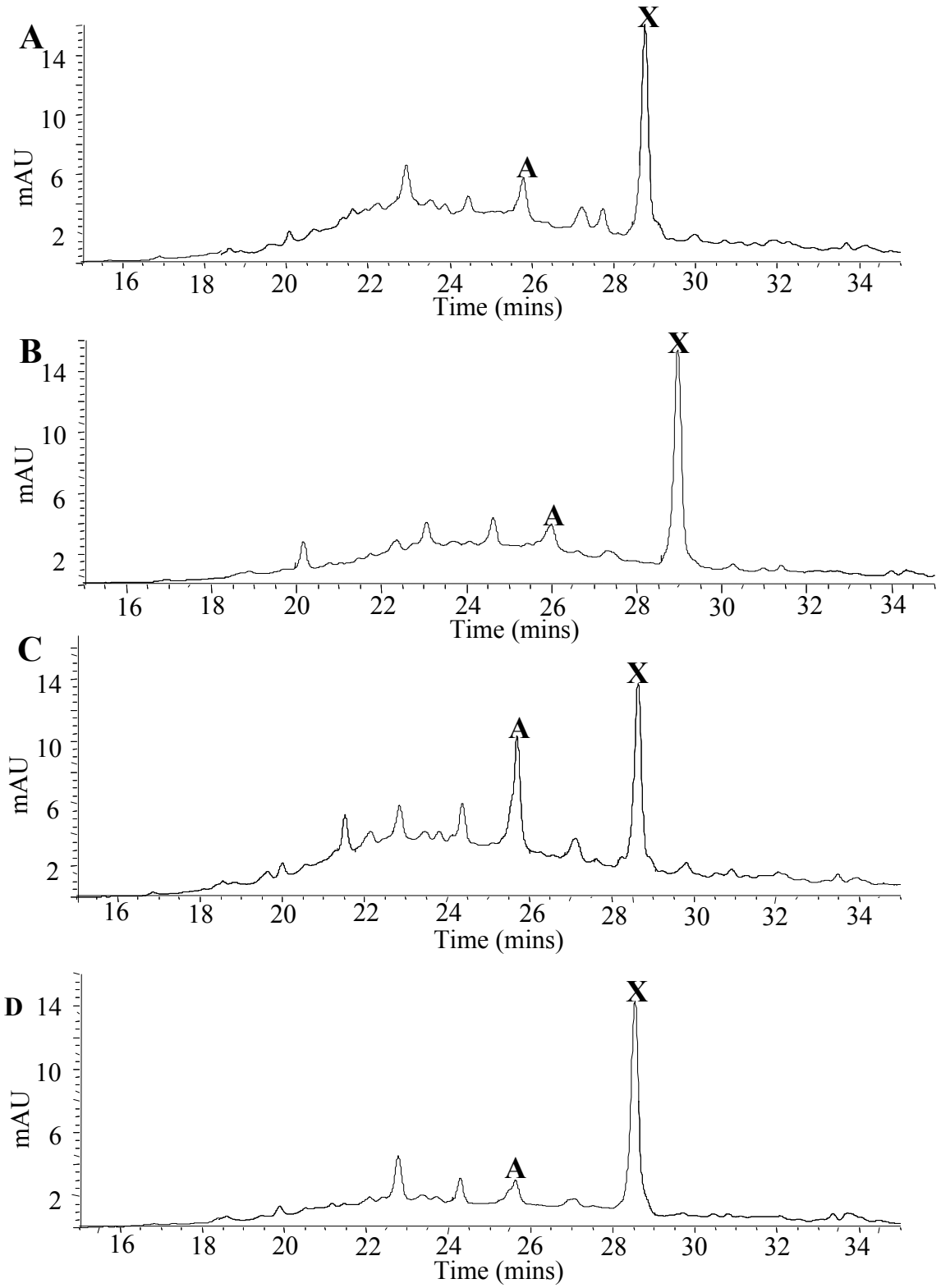


Figure 6.10: HPLC chromatograms monitored at 340 nm of developing seed coat extract at 30 dpa. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635. Peak A is a flavone-*C*-glycoside compound, peak B is another flavonoid compound.

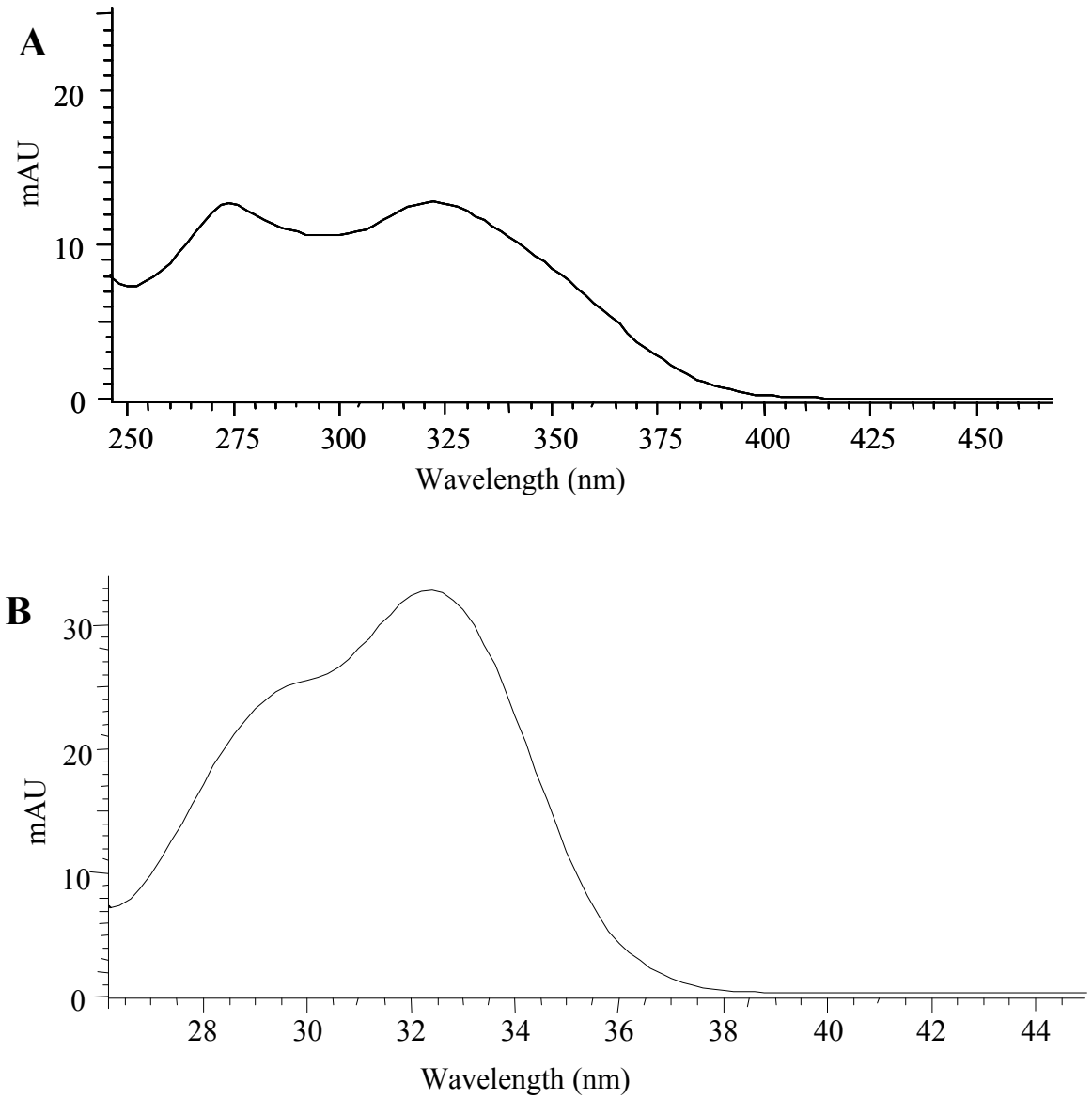


Figure 6.11: UV/Vis spectra of major peaks from Figure 6.10. *Panel A*: peak A (flavone-*C*- glycoside), *Panel B*: peak X (other phenolic compound).

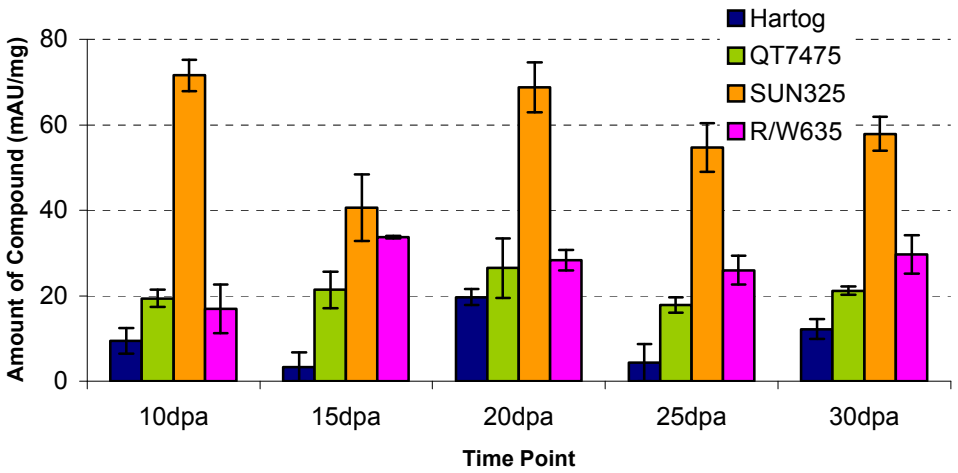


Figure 6.12: Flavone-*C*-glycoside content (mAU/mg) of the seed coat from developing grains, determined by the amount of the compound represented by Peak A (Figure 6.10) in HPLC chromatograms.

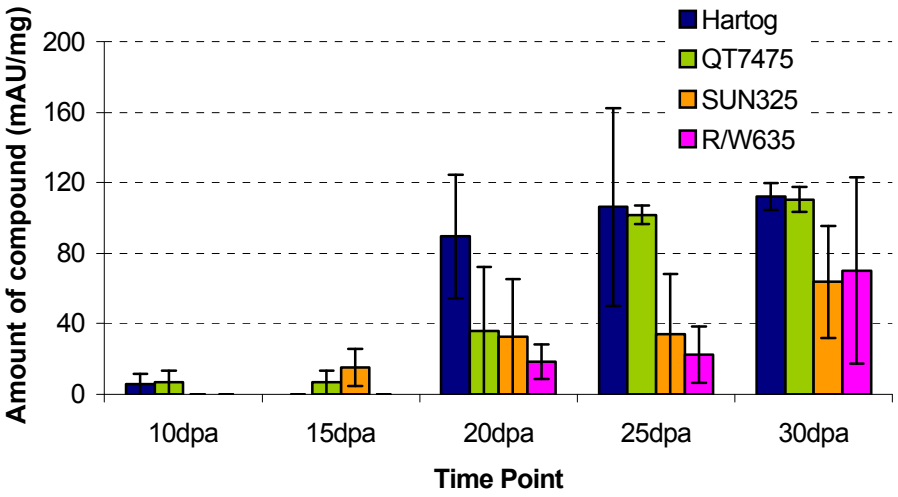


Figure 6.13: Amount of compound represented by peak X (Figure 6.10) in the HPLC chromatograms of the extract of the developing seed coat.

6.4 Discussion

CHS plays an integral role as the first step in the flavonoid biosynthetic pathway and generates important precursors and substrates for proceeding enzymes (Pelletier and Shirley, 1996), while *DFR* is important in producing the flavan-4-ol compounds which polymerise to form phlobaphene (Grotewold *et al.*, 1994). In this study, it was found that *DFR* was expressed in the seed coat of all genotypes at both 10 dpa and 15 dpa. By contrast *CHS* was expressed in all genotypes at 10 dpa but at 15 dpa was only present in the dormant genotypes, SUN325 and R/W635. Whilst these observations do not prove that the biosynthetic pathway is fully functional, except in R/W635 where there is accumulation of red pigment is an indicator of enzyme activity, they are nevertheless consistent with the possibility that specific compounds could accumulate in the seed coat of the white-grained genotypes and play a role in dormancy. A similar phenomenon has been observed in *Arabidopsis* where there was an accumulation of specific flavonoid compounds, epicatechin and catechin, which are precursors of the proanthocyanidin pigments responsible for seed coat colour in this species (Abrahams *et al.*, 2002). In this study there was an increased quantity of flavone-*C*-glycosides, most evident in the dormant white-grained genotype, SUN325. These compounds are not intermediates of the pathway leading to phlobaphene but rather are the products of an alternate branch in the pathway initiated with the common precursor, naringenin. This suggests that there may be enzymes further along the pathway from *DFR* which are inactive in SUN325, or that *DFR* is expressed but not functional. Furthermore, there was no evidence of accumulation of intermediate products of the branch of the flavonoid pathway leading to phlobaphene in any of the genotypes, red or white.

A demonstration of expression of *CHS*-like mRNA is not sufficient to prove a functional presence of CHS (Dixon and Steele, 1999; Springob *et al.*, 2003). Since *CHS* shows very high homology to other plant polyketide synthases (Springob *et al.*, 2003). It is possible that the *CHS* expression detected in this study could relate to a different gene with sequence homology to *CHS*. However, the sequence data derived from the RT-PCR was used for a Blast search on NCBI and showed strong sequence similarity with a Unigene *CHS* sequence (Accession number: Ta.28700) which has been validated with heterologous expression or genetic evidence of functionality (http://www.ncbi.nlm.nih.gov/).

Furthermore, the expression pattern in the red-grained control genotype was consistent with a previous study (Himi et al. 2005) and the deposition of red pigment. Is seems likely, therefore, that the *CHS* gene detected in the expression analysis is indeed the targeted gene.

DFR is an enzyme in the flavonoid biosynthesis that is involved in the branches of the pathway leading to formation of the precursors of phlobaphene (eg flavon-4-ols), catechins (proanthocyanidins) anthocyanins and flavonols. In this study, *DFR* was found to be expressed in both red and white-grained genotypes, which is in contrast to a similar expression analysis of developing red and white seed coats (Himi and Noda, 2004). These authors found that *DFR* is expressed in developing red-grained seed coats between five and 25 dpa, compared with only very low, or non-detectable expression in the seed coat of a white-grained cultivar (Himi and Noda, 2004). A further study concluded that *DFR* is not expressed in white-grained wheat and suggested that *DFR* expression is upregulated by the *R* gene (Himi *et al.*, 2005). Expression of both *CHS* and *DFR* was extremely low in the seed coat material used in this investigation. The transcript level of *CHS,* in particular, was so low that a second re-amplification step was required to visualise the amplified DNA fragment. Possible explanations for the variation from the observations reported by Himi and Noda (2004) and Himi *et al.,* (2005) could be the use of different genotypes or perhaps significantly different growth conditions during grain development and this may have affected transcript levels of these enzymes in the red seed coat. It has been reported that polymerisation of flavonoid (tannin) compounds, or pigmentation, in the seed coat of *Brassica carinata* is more extensive at cool temperatures (Marles *et al.*, 2003). This increased pigmentation was also reflected in the increased quantity of *DFR* transcript detected in the seedlings grown in cool temperatures (Marles *et al.*, 2003). The transcript levels of *DFR* and *CHS* in the red seed coat may therefore have been much higher in the study by Himi *et al.,* (2005) than this present study, due to the cooler growing temperatures in Japan compared to Australia. It is possible that *DFR* and *CHS* may have been expressed in the white-grained genotypes examined by Himi *et al.,* (2005), but given the that transcript levels may have been higher in the red-grained genotypes there would have been no need for the re-amplification step needed in the present study to detect expression.

The results obtained in this current investigation suggest that DFR is possibly not a key enzyme in regulation of phlobaphene synthesis. Whilst the *DFR* genes may be expressed in

white-grained wheat, there is as yet no evidence that there is a suitable substrate for the enzymes to synthesis the immediate precursors to the pigment compounds. It is also possible that *DFR* expression may be differentially controlled in red- and white-grained wheats, or that the pathway that generates the red pigment is blocked at a later step and that the presence of *DFR* and its substrate is not coupled to polymerisation to the red seed coat pigment.

Studies in *Arabidopsis* have also shown that there is differential regulation of genes, through regulatory genes or co-ordinated expression, for example between *CHS* and *F3H* which produce proanthocyanidin pigmentation in the seed coat (Pelletier and Shirley, 1996). There is also evidence of co-ordinated expression of flavonoid genes involved in the eventual synthesis of phlobaphene in maize, where the *P* gene activates both *CHS* and *DFR* in the pericarp (Grotewold *et al.*, 1998). The differential expression at 15 dpa of *CHS* observed between dormant and non-dormant genotypes in this study indicate that *DFR* and *CHS* are not co-ordinately expressed in the developing white wheat seed coat and the regulation of *CHS* is probably independent of *DFR*.

The *Arabidopsis transparent testa10* (*tt10*) mutant seeds are a lighter brown colour at harvest maturity and accumulate more soluble and less polymerised proanthocyanidins compared to wild-type seeds, even though DFR and CHS enzymes are still active (Pourcel *et al.*, 2005). Pourcel et al. (2005) reported that the wild-type *TT10* gene encodes a laccase-like enzyme that is required for oxidative polymerisation of flavonoids. After six months, however, the seed coat develops the dark brown of the wild type, probably as a result of non-enzymic oxidative polymerisation of the catechins. Darkening after harvest-ripeness has not been reported in the seed coats of white-grained wheat, indicating that flavonoid compounds are either not formed or if they are, they are relatively stable. By analogy with the *Arabidopsis tt10* mutant, it is possible that *CHS* is expressed in the non- and intermediate-dormant white-grained genotypes at 10 dpa and in the dormant white-grained genotype until at least 15 dpa, resulting in synthesis of compounds similar to the dormant red-grained wheat, R/W635. The failure of polymerisation to proceed in white-grained wheats may be because the pathway is only active for a short time, because the enzyme catalysing polymerisation is absent or because there is an interaction or sequestration step that removed the precursors. It has been reported that there is an accumulation of specific

intermediate enzymes and end products in *Arabidopsis,* when steps of the flavonoid pathway are blocked (Kitamura *et al.*, 2004; Pourcel *et al.*, 2005).

Flavone-*C*-glycosides are most common in cereals or grasses and the branch in the pathway at naringenin which produces these compounds is usually omitted from studies on flavonoid biosynthesis eg (Winkel-Shirley, 2001; Abrahams *et al.*, 2002). In wheat, flavone-*C*-glycosides are found in the bran and germ tissue (Feng *et al.*, 1988; Asenstorfer *et al.*, 2006). The enzyme involved in conversion of naringenin to hydroxy naringenin and flavone-*C*-glycosides through ring opening, is 2SFH (2S flavanone 2-hydroxylase) (Kerscher and Franz, 1986; Martens and Forkmann, 1999). As *CHS* is only expressed very early and only briefly during grain development in Hartog (non dormant) and QT7475 (intermediate dormant), there is no reason to suspect upregulation of any significant flavonoid biosynthesis pathways, despite the fact that *DFR* is still expressed in these genotypes. *DFR* may only be associated with, but not the determining factor for pigment accumulation in red-grained genotypes and contributes in a minor way to the flavonoid content in the seed coat. The HPLC chromatograms show that SUN325 contains more flavone-*C*-glycoside compounds than the other genotypes. The branch in the flavonoid biosynthesis pathway for flavone-*C*-glycosides occurs one enzymatic step after CHS generates naringenin (Chapter 1, Figure 1.1), which means that the expression of *CHS* is vital for the production of these compounds.

It has been reported in maize that the regulation of the anthocyanin and 3-deoxy flavonoid (eg phlobaphene) sections of the pathway are controlled by different transcription factors (Grotewold *et al.*, 1998). The *P* gene in maize is a transcription factor that upregulates phlobaphene and flavone-*C*-glycoside synthesis, or the 3-deoxy flavonoid branch of the pathway, while the *C1/R* transcription factors promote the accumulation of anthocyanin-like compounds (Grotewold *et al.*, 1998). Differences in transcription factors, their binding ability and functionality can contribute to regulation of different structural genes and hence diversity within a pathway (Schijlen *et al.*, 2004). Although Himi *et al.* (2005) have proposed that the *R* gene is a transcription factor for the flavonoid biosynthesis pathway similar to the maize *P* gene, the results presented here suggest a different situation. Furthermore, there has been a transcription factor recently discovered in rice, the *Rc* gene, which is responsible for upregulating the synthesis of red pigment (proanthocyanidin) in the seed coat, but only forms a brown pigment in the absence of an additional gene, *Rd*

(Sweeney *et al.*, 2006). The *Rd* gene is possibly homologous to the wheat *R* gene, but the *Rc* gene does not appear to be homologous with this region in the wheat genome (Sweeney *et al.*, 2006). This study by Sweeney, et al., (2006) suggests that there is a combination of transcription factors involved in red pigment production, and it seems likely that this would also be the case in wheat. The accumulation of flavone-*C*-glycosides indicates that the *R* gene is not a transcription factor upregulating the entire flavonoid pathway and may be responsible for only regulating the expression of a polymerisation enzyme to form the red pigment.

Regardless of the expression of both *CHS* and *DFR* genes at both 10 and 15 dpa, the level of flavone-*C*-glycosides in the dormant red-grained genotype, R/W635, is only similar to that of the non-dormant white-grained genotypes. It is likely that the reason for the lower flavone-*C*-glycoside content in the red seed coat is the removal of *DFR*-products by incorporation into large polymerised, phlobaphene compounds. SUN325 is different to other non-dormant white-grained genotypes because of the possession of a high concentration of flavone-*C*-glycosides in the seed coat extract. If the pathway to phlobaphene in white-grained wheats is blocked after *DFR*, as proposed earlier, it is possible that the pathway flows towards to the alternate flavone-*C*-glycoside branch. This could explain the increased amount of flavone-*C*-glycosides associated with the expression of both *CHS* and *DFR* genes. It appears unlikely that the flavone-*C*-glycosides represent the active 'seed coat' factor involved in dormancy since there is little or no accumulation in red-grained wheat relative to non-dormant white wheats. Thus if flavonoid precursors are involved, they were not detected in this study either because of low concentrations, poor extraction by the solvents used, or spectral characteristics significantly different from expected.

Highly polymerised flavonoids causing pigmentation in the seed coat may be synthesised to inhibit the growth of pathogenic microbes (Winkel-Shirley, 1998) or provide protection from heat or osmotic stress (Pourcel *et al.*, 2005). Flavonoids in general have been suggested to play a role in germination and dormancy, in particular with *transparent testa* mutants in *Arabidopsis* which lack pigmentation in the seed coat and germinated more quickly than wild type seeds (Koornneef *et al.*, 2002). In maize, flavone-*C*-glycosides are present in silks which are thought to have insecticidal activity (Grotewold *et al.*, 1998). Initially, it was proposed that white-grained wheats contained a limited ability to

synthesise flavonoid compounds because of a lack of the R gene which stimulates the pathway (Himi *et al.*, 2005). However, the results presented here indicate that there is expression of flavonoid biosynthesis genes at least in certain white-grained genotypes. Flavone-*C*-glycosides may be present in the seed coat to prevent microbial attack in the soil and are only at a high level in SUN325 because of the absence of enzymes to synthesise the red pigment, leading to the activation of the alternative flavonoid pathway. It is possible that flavone-*C*-glycosides are a component of the dormant seed coat factor, as they are highly water-soluble compounds and may be dissolved in the seed coat and transferred to the embryo during imbibition. Unfortunately, this hypothesis is not in agreement with the dormancy of red-grained wheat where a lower quantity of flavone-*C*-glycosides was detected, unless there is a compound of this type unique to both R/W635 and SUN325, which was not discovered in this study.

Chapter 7: Extraction of Flavonoid Compounds and Potential Germination Inhibitors from the Seed Coat of Dormant Wheat Genotypes

7.1 Introduction

In wheat, anecdotal evidence suggests that the most dormant red-grained are more dormant than the best white-grained wheats, suggesting that the red pigment contributes in some way to dormancy expression. Some rice genotypes have also been reported to express a red pigment in the seed coat which contributes to dormancy (Sweeney *et al.*, 2006). Similarly in *Arabidopsis,* where *transparent-testa* mutants were shown to lack anthocyanin and proanthocyanidin pigmentation, which was associated with a decline in dormancy (Debeaujon and Koornneef, 2000; Kitamura *et al.*, 2004). There have been very few investigations into the chemical composition of the seed coat of wheat grains, and in particular the exact origin of the red pigment, which is still unknown but it has been reported to be due to a proanthocyanidin (**1**) or phlobaphene (**2**) type flavonoid oligomer or polymer (Himi and Noda, 2004). Both proanthocyanidins and phlobaphenes are colourless and but may undergo oxidative rearrangement to give a coloured product. It is well known that proanthocyanidins (or condensed tannins) give a brown colour after oxidation (Marles *et al.*, 2003). Alternatively the red colour in red-grained wheat may originate from the dehydration of apiferol (**3**) or luteoferol (**4**), possible precursors of phlobaphene (**2**), to give a 3-deoxyanthocyanin, apigeninidin (**5**) or luteolinidin (**6**). These anthocyanins are quite stable and have a chromophore (for example, apigeninidin (**5**) has a λ_{max} of 468 nm (pH 2.0) and 530 nm (pH 11.0) (Baranac and Amic, 1990) which is consistent with a seed coat colour that turns red at high pH (Ram *et al.*, 2003).

One possible explanation of the role of the seed coat in dormancy of wheat grains is that precursors to the red pigment accumulate in red-grained wheat, and that these compounds are involved in the dormancy observed in these genotypes (Miyamoto and Everson, 1958). Winkel-Shirley (1998) suggested that one of the functions of flavonoid compounds could be as germination inhibitors. Phenolic acids, which biosynthesis is related the flavonoid pathway, have been shown to delay germination in species such as lettuce seeds (Chen *et al.*, 1982) and *Triticum tauschii* (Gatford *et al.*, 2002). Important research conducted by

Stoy and Sundin (1976) showed that putative catechins, extracted from green tea, are capable of inhibiting germination in isolated wheat embryos. Similarly, Himi *et al.* (2002) reported inhibition of germination of isolated wheat embryos by aqueous extracts of bran from dormant red-grained genotypes. These results appeared to be consistent with the presence of germination inhibitors in the seed coat of dormant wheat genotypes, but it remains to be demonstrated whether phenolic and flavonoid compounds isolated from grains or grain tissues are able to inhibit germination in their purified form.

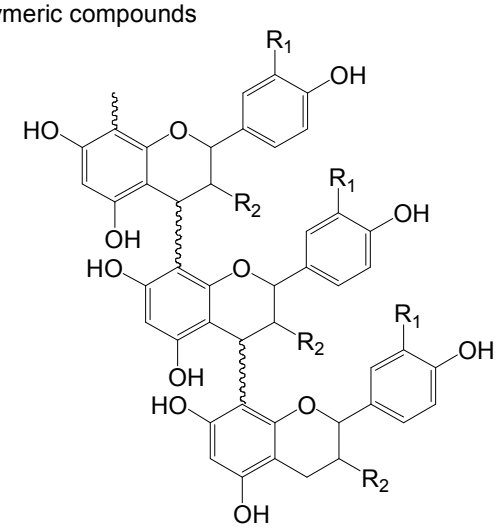
To date only a few flavonoid compounds have been detected in the seed coat of wheat grains. Miyamoto and Everson (1958) reported apparent differences in the tannin composition of dormant red- and non-dormant white-grained genotypes, with the white-grained genotypes having a lower tannin content than red-grained genotypes. Small amounts of flavone-*C*-glycosides (**7**) were found in the seed coat (Chapter 5, (Feng *et al.*, 1988; Feng and McDonald, 1989). It has already been determined that the flavonoid biosynthetic pathway is active in the seed coat of both red-grained and white-grained genotypes, but is upregulated for longer in red-grained and the dormant white-grained genotype SUN325 (Chapter 5). Whilst it seemed clear that there was a greater concentration of flavone-*C*-glycosides (**7**) in the seed coat of SUN325, it was not clear whether these compounds are related to dormancy. Several studies have reported ferulic acid (**8**) as the dominant compound (Ram *et al.*, 2003; Bunzel *et al.*, 2004; Parker *et al.*, 2005). In the majority of these studies, however, the seed coat material may have been contaminated by aleurone tissue, which is known to contain high levels of ferulic acid (**8**) (Parker *et al.*, 2005) (Chapter 2, Figure 2.4).

A standard detection method for these condensed tannins is the HCl/butanol assay. This method breaks bonds between polymerised tannins and the solution turns cherry red, due to the formation of cyanidin (**9**), if there are any of these compounds present in the extract (Porter *et al.*, 1986). Condensed tannins also release a small amount of catechin (**10**) or epicatechin (**11**) during this hydrolysis. It is expected that phlobaphene (**2**) should form apigeninidin (**5**) or luteolinidin (**6**) under these conditions to give a yellow colour to orange colour and a small amount of colourless apigeniflavan (**12**) or luteoliflavan (**13**).

The results obtained in Chapter 6 indicate that the flavonoid biosynthesis pathway is active in both red- and white-grained genotypes, but remains active for longer in the dormant genotypes. The aim of the experiments presented in this chapter was to extract biochemical

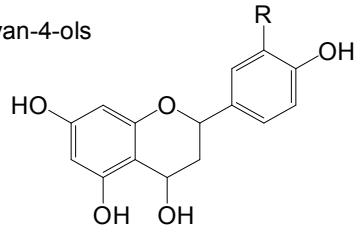
components from seed coats of red and white-grained genotypes (dormant and non-dormant) and determine if there is any evidence of accumulation of precursors for the proposed red pigment. A range of extraction methods attempting to optimise differences between red- and white-grained genotypes were investigated. Finally, whole grains were imbibed with seed coat extracts to determine if any of the extracts contained compounds with biological activity as germination inhibitors.

Polymeric compounds



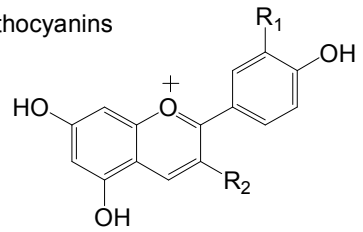
1 proanthocyanidin R_1=OH, R_2=OH
2 phlobaphene R_1=H or OH, R_2=H

Flavan-4-ols



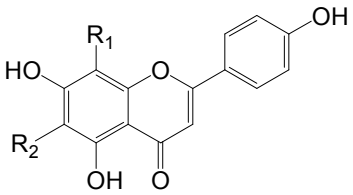
3 apiferol R=H
4 luteopherol R=OH

Anthocyanins



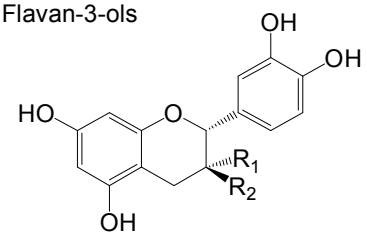
5 apigeninidin R_1=H, R_2=H
6 luteolinidin R_1=OH, R_2=H
9 cyanidin R_1=OH, R_2=OH

Flavones



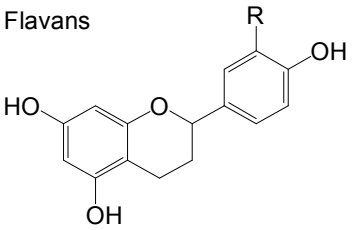
7 flavone-C-glycoside R_1=glu/gal , R_2=ara or R_1=ara , R_2=glu/gal
14 apigenin R_1=H, R_2=H

Flavan-3-ols



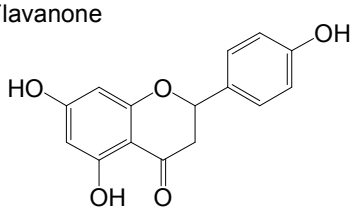
10 catechin R_1=H , R_2=H
11 epicatechin R_1=OH, R_2=H

Flavans



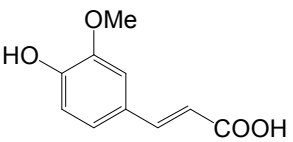
12 apigeninflavan R=H
13 luteoliflavan R=OH

Flavanone

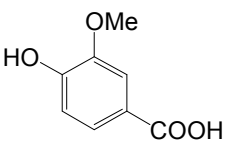


15 naringenin

Phenolic acids



8 ferulic acid



16 vanillic acid

Structures and names of major compounds in the wheat flavonoid biosynthesis pathway.

7.2 Materials and Methods

7.2.1 Plant material

Isolating of developing and mature seed coat material has been described in Chapter 2 (*Section 2.3*). In contrast to previous experiments, the red genotype used to extract of whole seeds and seed coat material for the bioassay and for initial methanol extraction of mature seed coat was AUS1490/Janz rather than R/W635 (both are derived from crosses between the red-grained, dormant genotype AUS1490 and locally adapted non-dormant white wheats).

7.2.2 Seed coat extraction and HPLC conditions

The alkaline extraction of the isolated seed coat material was the same as that stated in Chapter 2 (*Section 2.3*). HPLC conditions, 70 minute run, and elution solvents were also previously described in Chapter 6. For the methanol extracts, a shorter 50 minute HPLC program was employed, with the same solvents and flow rate as that described in Chapter 6. Standard compounds were dissolved in methanol to be comparable with methanol extracts of isolated seed coats. 10 mL of 70% aqueous methanol (v/v) was added to 500 mg of seed coat and shaken overnight. The extract was decanted from the seed coat debris and centrifuged at 10,000 g for 3 minutes. The extract was treated with 5 mL of hexane and shaken overnight to remove immiscible compounds and the hexane phase removed by pipette. The remaining methanol extract was dried, resuspended in 1 mL of 70% methanol, filtered and analysed by HPLC with the detector set at 280, 250 and 340 nm.

7.2.3 Standard flavonoid compounds

Flavonoid standards (excluding apiferol, **3**) were made up to a standard concentration according to extinction coefficient at wavelengths (λ_{max}) close to 280 nm (Table 7.1). Compounds representative of the major groups of flavonoids were selected: apigenin (**14**, flavone), catechin (**10**) and epicatechin (**11**, flavan-3-ol), ferulic acid (**8**, phenolic acid), apiferol (**3**, flavan-4-ol) and naringenin (**15**, flavanone). The powdered compounds were dissolved in 50 mL of 70% methanol in nano-pure water and filtered through a disposable filter prior to HPLC analysis. Effluent from the column was monitored at 280, 250 and 340 nm. Concentrations of the standards to analyse by HPLC were calculated to give similar

peak areas using published λ_{max} and extinction coefficient (ε) data (Table 7.1). For example with catechin:

$\varepsilon_{cat}/\varepsilon_{api}$ = 18,800/2,200 = 8.5

To achieve 20 µg/mL (apigenin equivalent):

20 x 8.5 = 169.3 µg/mL or 0.17 mg/mL

Flavonoid Compound (group)	Extinction coefficient	Absorbance (λ_{max})	Concentration (mg/mL)	Reference
Apiferol (**3**, flavan-4-ol)	2,200	276 nm	0.17	(Scott, 1964)
Apigenin (**14**, flavone)	18,800	269 nm	0.02	(Weast, 1976)
Catechin (**10**, flavan-3-ol)	2,200	276 nm	0.17	(Scott, 1964)
Ferulic Acid (**8**, phenolic acid)	12,302	296 nm	0.03	(Weast, 1976)
Naringenin (**15**, flavanone)	19,952	269 nm	0.02	(Scott, 1964)

Table 7.1: λ_{max} and extinction coefficients used to calculate the concentrations (mg/mL) of standard flavonoid and cinnamic acid compounds required to give similar peak areas

7.2.4 Synthesis of apiferol (3)

The synthesis of apiferol (**3**) was conducted according to the methods of Bate-Smith (1969), with minor modifications. 10 mg naringenin (**15**) was suspended in 2 mL of water in a glass test-tube. Naringenin was reduced with the addition of excess sodium borate at room temperature for one hour and subsequently transferred to a small conical flask with 10 mL of 6% acetic acid (pH 3.0). The excess sodium borate was removed by reaction with sodium chloride the solution stopped producing bubbles. *n*-Propanol was added and the extract filtered through Whatman 541 filter paper to remove the salt precipitate. The filtrate was evaporated leaving brownish crystals which were extracted further with methanol. The methanol extract was then evaporated to dryness.

Thin Layer Chromatography (TLC) was performed on Silica Gel 60 plates (Merck, USA) using methanol:chloroform (1:9, v/v) as the elution solvent was used to compare the synthesised apiferol (**3**) with standard naringenin (**15**). TLC showed that apiferol (**3**) was successfully synthesised from naringenin and subsequent exposure of the plate to HCl and

heating to 70°C resulted in the new compound turning red (data not shown). The TLC separation also showed that there was some naringenin still remaining in the sample.

7.2.5 HCl butanol Assay for tannins

The hydrochloric acid/butanol assay (Porter *et al.* (1986) was employed to detect the presence of tannins in the developing and mature seed coat. 60 mg (seed coat from two grains) of green seed coat tissue at 15 dpa was used or 50 mg of mature seed coat material. Reactions were conducted in 1.5 mL thick-walled, screw-top glass tubes with Teflon-lined lids. 1 mL of HCl/ butanol (5%:95%, v/v) solution and was added to the seed coat tissue and placed in a water bath at 95°C for one hour. After heating, the solution was cooled, filtered through a disposable cellulose acetate filter (13 μm diameter) and analysed by HPLC. Tannins were monitored at 280 nm and anthocyanidins at 480 nm and 540 nm. QT7475 was not available for tannin analysis in developing seed coat due to the limited amount of material, but all genotypes (Hartog, QT7475, SUN325, R/W635) were used in the analysis of mature seed coat. Extracts were spiked with catechin (**10**) and epicatechin (**11**) standards (described above) in a 1 in 10 dilution.

7.2.6 Spiking of alkaline extract of mature seed coat with authentic flavonoids

Isolated mature seed coat material was extracted with 0.1 M NH_3 as described in Chapter 6. The extract was adjusted to neutral pH, and 100 μL mixed with100 μL of a standard solution of catechin (**10**), epicatechin (**11**) or vanillic acid (**16**). The standard solutions were all made to a concentration of 0.2 mg/mL in water for consistency. The spiked samples were analysed by HPLC and monitored at 280, 250 and 340 nm.

7.2.6.1 Characterisation of extract components

A reverse-phase SPE column (60 mg Strata-X; Phenomenex, Lane Cove, NSW, Australia) was used to separate the seed coat extracts into hydrophobic or hydrophilic fractions. The column was initially activated with 5 mL methanol, rinsed with 5 mL of water and the sample (seed coat extract) loaded. A volume of 5 mL of water was added to elute the hydrophilic portion of the seed coat extract. The resulting solution was spiked in a 1:1 ratio with 2 mg/mL catechin. The hydrophobic phase of the seed coat extract was released from

the column by the addition of 1 mL methanol, which was also subsequently spiked with 1:1 catechin (**10**) and both solutions analysed by HPLC.

The activity of fractions was tested following oxidation by drying 100 μL of the seed coat extract in a speedivac for one hour. The dried extract was resuspended in a mixture containing 100 μL of water and 100 μL of 2 mg/mL catechin and analysed by HPLC.

Micro-centrifugation was used to separate fractions according to size 100 μL of the seed coat extract was centrifuged at maximum speed for 15 minutes. The bottom 50 μL of solution, was spiked with 50 μL of catechin while the top fraction was diluted to100 μL with water and spiked with 100 μL of catechin.

7.2.7 Bioassays for potential germination inhibitors in extracts of seed coat

7.2.6.2 Aqueous extract of whole seeds

The outer pericarp and embryo of SUN325 was removed from 300 (50 x 6 replicates) seeds to expose the underlying seed coat layers and each group of 50 seeds were shaken overnight in 6 mL water in 10 mL centrifuge tubes. The resulting extracts were transferred to clean tubes, centrifuged at 10,000 g for 2 minutes and the supernatant pipetted into clean 10 mL centrifuge tubes. 5 mL of water (control) or 3.5 water + 1.5 mL extract was to imbibe grains (50 grains per assay in 2 in duplicate) overnight on a shaker to enhance direct uptake into the grain. After shaking, grains were transferred to Petri dishes containing Whatman No.1 filter paper. Germinated grains were counted each day for seven days using the standard germination test procedure (Chapter 2, *Section 2.2*).

This experiment was repeated with QT7475.

7.2.6.3 Aqueous extract from isolated seed coat

A Laboratory Dehuller (L505) described in Chapter 2 was used to remove the seed coat from 500 g grain samples of genotypes Hartog, QT7475 and SUN325. 75 mL of water was added to 2.5 g of seed coat material in a beaker, the solution covered and shaken for two hours. 3 mL of the resultant solution was centrifuged at 10,000 g for three minutes. Duplicate samples of 25 grains of each genotype were imbibed in 2.5 mL of seed coat

extracts from each genotype, or 2.5 mL water (control) in 10 mL centrifuge tubes. These tubes were shaken overnight and transferred to 25 mm Petri dishes to complete the germination tests (Chapter 2).

7.2.6.4 Alkaline extraction of isolated seed coat

A standard alkaline extraction (Chapter 2, *Section 2.3*) was performed on 100 mg of seed coat removed from genotypes Hartog, QT7475, SUN325 and R/W635. The seed coat extract was adjusted to pH 6 with dilute acetic acid. Each genotype (50 grains in duplicate) was imbibed with seed coat extract of each genotypes and germination compared with a control containing NH_3, acetic acid and aqueous only.

7.3 Results

7.3.1 Analysis of seed coat extracts

Compounds representative of five flavonoid groups were dissolved in methanol and analysed by HPLC (50 minute method) and absorbance monitored at 280 nm. Retention times and peak areas of these compounds are shown in Table 7.2. Flavan-4-ol (apiferol **3**) and flavan-3-ol (catechin **10**/epicatechin **11**) were selected because they are direct precursors to proanthocyanidins (**1**) and phlobaphene (**2**) (Winkel-Shirley, 2001). These compounds gave peaks with similar retention times, 12.6 minutes (flavan-4-ol) and 12.5 minutes (catechin **10**) respectively. Ferulic acid (**8**), naringenin (**15**) and apigenin (**14**) have longer retention times of 18, 27 and 30 minutes. Peak areas of the standard compounds were similar except for apiferol (**3**), which had a low peak area. A HPLC program of 50 minutes was used to allowed direct comparison with the methanol extract of the mature seed coat.

The HPLC chromatograms (280 nm) of the methanol extracts of the mature seed coat of Hartog (Figure 7.2 *Panel A*), SUN325 (*Panel C*) and red dormant line AUS1490/Janz (*Panel D*) appeared to be very similar, with all peaks comparable in height and retention time. None of the peaks initially observed appeared to correspond with any of the standard compounds. It was noted however, that the methanol seed coat extracts clearly contained large amounts of unresolved material, especially noticeable in Hartog (Figure 7.2, *Panel A*) that gave a characteristic hump above the baseline. It is thought that this unresolved material is polymeric. The dormant red genotype used for this extraction has a lower amount of polymeric material than the white-grained genotypes (Figure 7.2, *Panel C*). Due to the 'hump' in the chromatograms, the peak area measurements are not necessarily a true indication of the relative amount of the compounds represented by the peaks. Polymeric and hydrophobic material dominated the chromatograms and there was little evidence of hydrophilic compounds with low retention times.

In each of the chromatograms, there were two major overlapping peaks at approximately 16 minutes (Figure 7.2, all *Panels*). The spectra of these peaks suggest that these major compounds are flavone-*C*-glycosides (λ_{max} values of 285 and 335 nm), part of a major group of these compounds in the seed coat that are extractable with methanol.

Compound (flavonoid group)	**Retention Time (mins)**
Apiferol (**3**, flavan-4-ol)	12.6
Apigenin (**14**, flavone)	30.1
Catechin (**10**, flavan-3-ol)	12.5
Ferulic acid (**8**, phenolic acid)	18.8
Naringenin (**15**, flavanone)	27.4

Table 7.2: Retention times and peak areas of the four selected standard compounds (50 minute HPLC run) monitored at 280 nm.

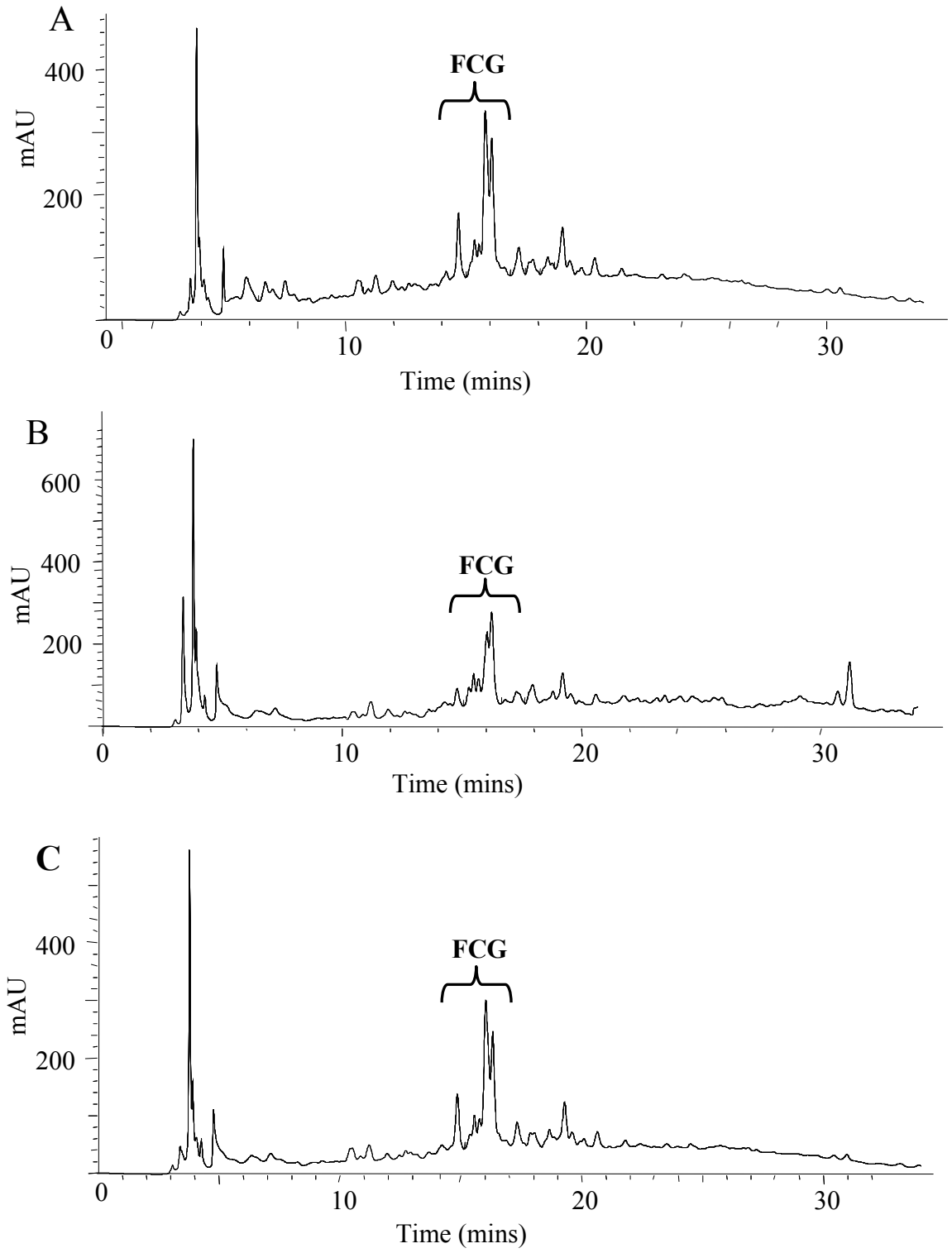


Figure 7.2: HPLC chromatograms (280 nm) of methanol extracts of mature seed coat. *Panel A*: Hartog, *Panel B*: SUN325, *Panel C*: AUS1490/Janz. Note the reduced polymeric material ('hump') extracted from AUS1490/Janz. Region of flavone-*C*-glycoside peaks (FCG) is marked.

7.3.2 Determination of tannins in the seed coat of developing and mature grain

The HCl/butanol extraction method used to detect tannins in the seed coat of mature and developing genotypes, was expected to produce a visible cherry-red or orange colour by acid hydrolysis of polymerised tannins (Porter *et al.*, 1986) or phlobaphenes. Extracts were analysed by both UV/Vis spectrophotometry and HPLC with the detector set at 280, 250, 340, 480 and 540 nm. There was either no evidence of formation of characteristic cherry-red colour (cyanidin **9**) or orange colour (apigeninidin **5**/luteolinidin **6**), or it was below the level of detection in all extracts. Thus no tannins or phlobaphenes were detected in the mature seed coat in all genotypes by spectrophotometric analysis. The cherry-red or orange colour was not observed when the HCl/butanol assay was performed on the isolated seed coat.

Figure 7.3 is a representative chromatogram of the HCl/butanol extract from the seed coat of developing grains (30 dpa). There was no observable difference between the chromatograms of the seed coat extracts from different genotypes and therefore only one is displayed. These HPLC chromatograms of HCl/butanol extracts contained much less polymeric material than the methanol extracts, with the chromatograms having few peaks and no 'hump' as was observed in Figure 7.2. Chromatograms (280 nm) of the genotypes contained one major peak at 14 minutes (peak A) and a smaller peak at 51 minutes (peak B) (Figure 7.3). Expansion of the absorption scale for the region of the chromatogram between peaks A and B reveals a number of other peaks (Figure 7.5 and Figure 7.3, inset). A relatively large peak was revealed at 23 minutes (Figure 7.5, peak A). The other peaks located in this region were minor and were not investigated further.

Peak A (Figure 7.3) had a λ_{max} of 284 nm (Figure 7.4 *Panel A*) for all genotypes which is slightly higher than the λ_{max} typical of catechin. Peak B (Figure 7.3) has a λ_{max} of 282 nm (Figure 7.4 *Panel B*) but does not have a spectrum similar to catechin (**10**) or epicatechin (**11**). Peak C (Figure 7.3) appeared to have an UV/Vis spectrum more synonymous to catechin, with a λ_{max} of 282 nm (Figure 7.4, *Panel C*). HCl/butanol extracts were spiked with catechin and analysed by HPLC (Figure 7.5). The putative catechin peak (Figure 7.3, peak C) was substantially larger in the spiked samples (Figure 7.5, Peak A). The UV/Vis

spectra of the peak enlarged by the addition of catechin is also consistent with the spectra of Peak A (Figure 7.5 and Figure 7.3, inset).

HPLC analysis of the mature seed coat for tannins was only performed on the genotypes Hartog and R/W635 (Figure 7.7), due to limited availability of mature seed coat material. The representative chromatogram presented in Figure 7.7 is similar to that of the green seed coat material (Figure 7.3), containing two dominant peaks (peaks A and B) on either side of a number of minor peaks (Figure 7.7) and had spectra that were identical to those shown in Figure 7.4 (*Panels A* and *B*). The minor peaks are displayed in the magnified insert (Figure 7.7). The peak at 23 minutes (peak C), which was observed in the extract of the seed coat of developing grains, is barely evident in the chromatogram of the mature seed coat. Therefore, there is only a small amount of catechin released during the HCl/butanol extraction in the mature seed coat in comparison to the developing seed coat.

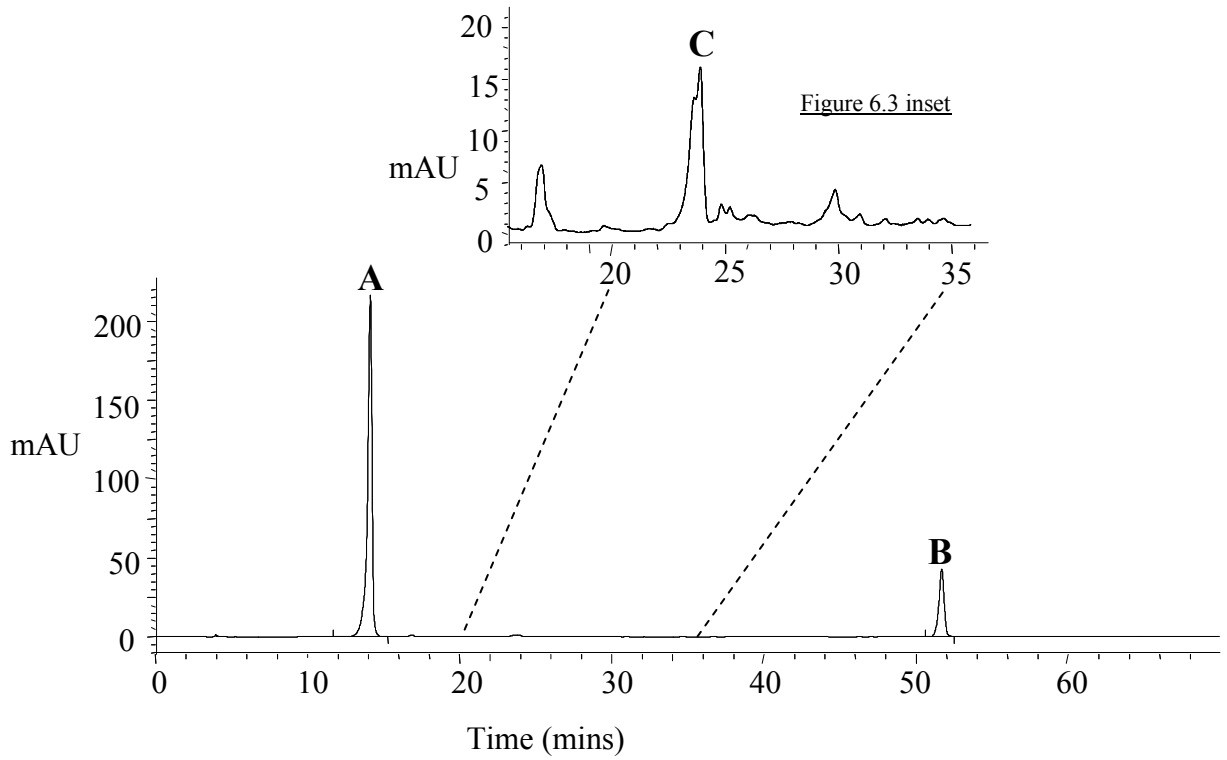


Figure 7.3: Representative HPLC chromatogram (280 nm) of HCl/butanol extracts of seed coat of developing grains (30 dpa), with major peaks labelled A and B. Inset is an enlarged region of the chromatogram between the two major peaks and showing the putative catechin peak (Figure 7.3 insert, peak C).

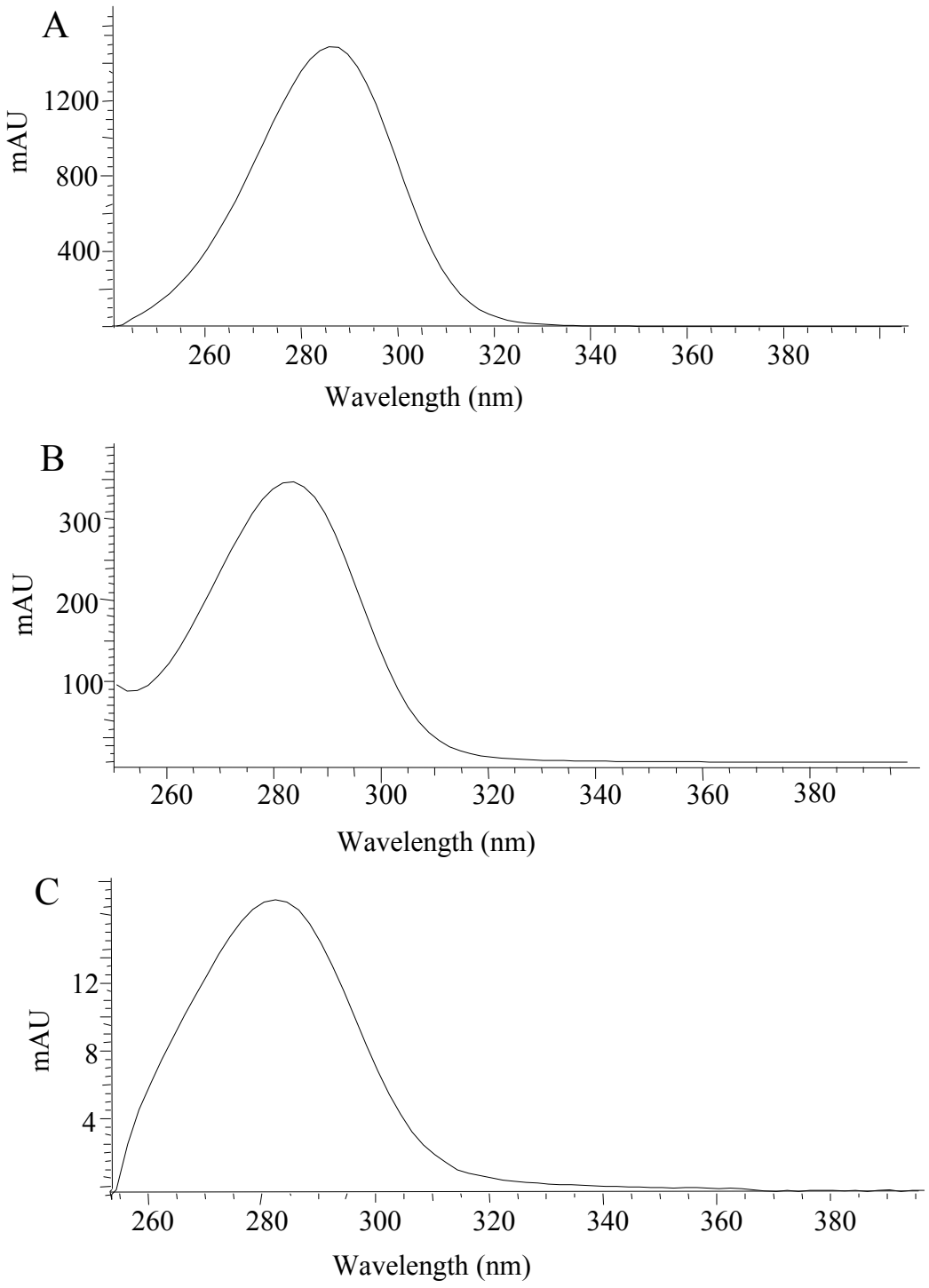


Figure 7.4: UV/Vis spectra of peaks A, B and C from Figure 7.3 and inset. *Panel A,* representing peak A, has a λ_{max} of 284 nm and *Panel B,* representing Peak B has a λ_{max} of 282 nm. Panel C represents Peak C from the inset of Figure 7.3, with a λ_{max} of 282 nm and is consistent with the spectrum of catechin.

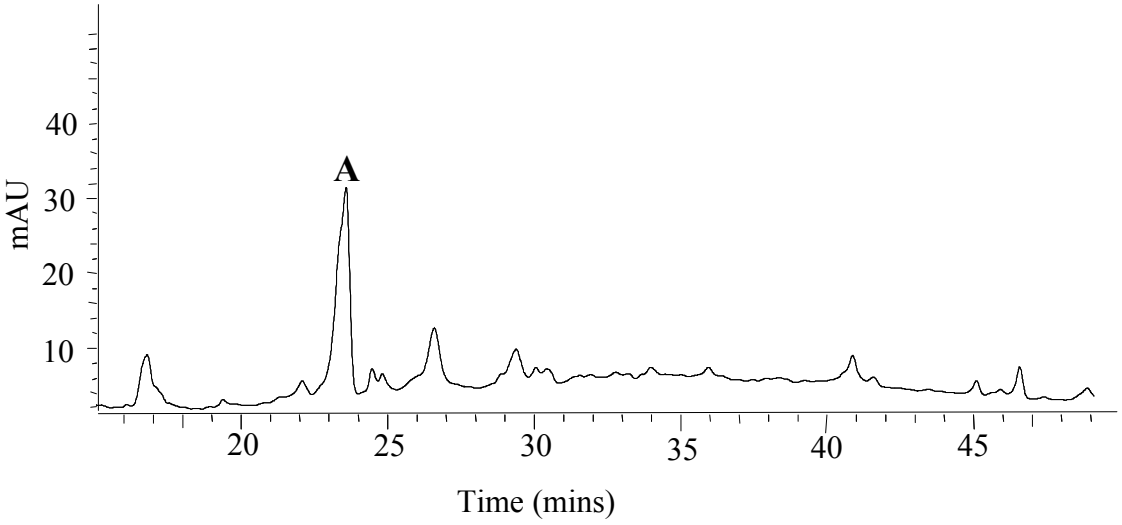


Figure 7.5: Representative segment of HPLC chromatograms (280 nm) of HCl/butanol extracts of developing seed coat (30 dpa) between major peaks (from Figure 7.3), following spiking of samples with catechin. Chromatogram is enlarged between 15 and 50 minutes.

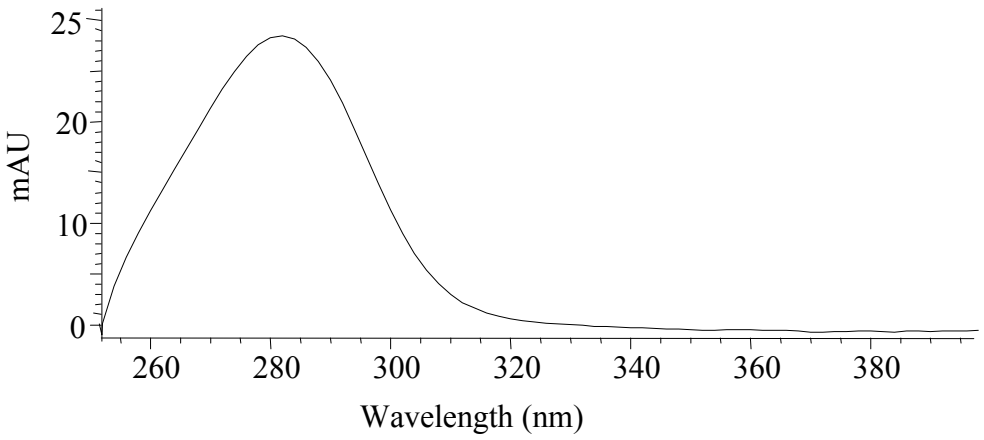


Figure 7.6: Spectrum of putative catechin peak from Figure 7.7, consistent with the proposed catechin peak spectra displayed in Figure 7.6. The λ_{max} is 282 nm.

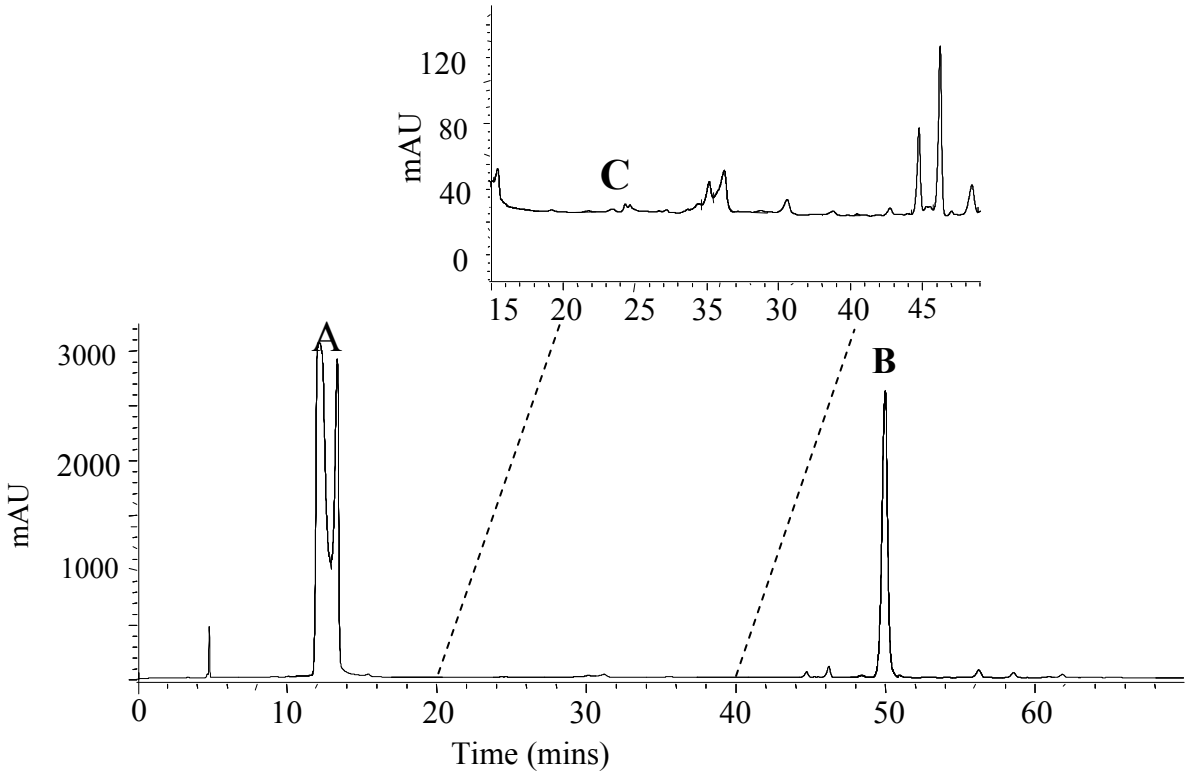


Figure 7.7: Representative HPLC chromatogram (280 nm) of HCl/butanol extracts of seed coat of mature grains. *Panel A*: Hartog, *Panel B*: R/W635. Inset is enlarged section between peaks A and B. Peak C is the putative catechin peak.

7.3.3 Alkaline extraction of seed coat

An alkaline extraction method was developed in an attempt to reduce the amount of polymeric material that masks peaks and potential differences between the genotypes. The majority of phenolic compounds have maximal absorbance at 280 nm, which is the wavelength that was examined in this series of experiments.

Figures 7.8 and 7.9 show the change in total phenolic content, as determined by the mAU/mg from the chromatogram measured at 280 nm, of the seedcoat through development and at maturity. In general, the phenolic and flavonoid content increased during the early stages of grain development, reaching a peak at 20 dpa in Hartog, 25 – 30 dpa in the case of QT7475 and SUN325, or 15 dpa in the red-grained genotype (Figure 7.8). At maturity (Figure 7.9) and 30 dpa (Figure 7.8), the red-grained genotype had a lower amount of total extractable phenolic compounds in comparison to the white-grained genotypes. SUN325 had a significantly higher flavonoid content at 10 dpa than the other white-grained genotypes, but a similar content to R/W635 (Figure 7.8). There were no further differences in the amount of phenolic extracted from the seed coat of the different genotypes at the various time points.

The HPLC chromatograms of the alkaline extracts of seed coat material from developing and mature grains are shown in Figures 7.10 and 7.13 respectively. The major peaks are labelled d (developing) or m (mature) A, B, C, D and E with all of these peaks present in each of the genotypes. The UV/Vis spectra of these peaks in developing grain (Figure 7.10) is presented in Figures 7.12 and indicates that peaks dA, and dB have a λ_{max} of 280 and 255 nm respectively and are probably simple phenolic compounds. Peaks dC and dE have more pronounced double peak, with λ_{max} of 285 and 315 nm (Peak dC) and 280 and 290 nm (Peak dD). The UV/Vis spectra of Peak dC appears similar to ferulic acid (**8**, Figure 7.12, *Panel C*) while Peak D has a λ_{max} of 280 nm and is likely to be a simple phenolic compound. These spectra indicate that these peaks C and E are probably complex phenolic compounds. Figure 7.15 shows the UV/Vis spectra of the major peaks in the chromatogram from the mature seed coat extract. The spectra of Peaks mA, mC and mD indicate that these peaks represent compounds with two excitation bands, and are conjugated phenolic compounds (Figure 7.15, *Panels* A, C and D). Peak mB is likely to be a simple phenolic compound (*Panel* B), while the UV/Vis spectra of Peak mD is clearly a

flavone-*C*-glycoside (*Panel* D). Peak mE appears similar to ferulic acid (Chapter 2, Figure 2.5).

It is apparent that the major compound in common between the extract from the seed coat at 30 dpa and the mature grain is peak B. The compounds represented by peaks mA, mC and mD and mE in Figure 7.15 are not evident as major compounds in the chromatogram presented in Figure 7.10. Peaks A, B and E had similar retention times, while the other major peaks that differed between the seed coat extract from the developing and mature grains, Peaks C and D, had slightly different retention times (Figures 7.10 and 7.13). Ferulic acid (**8**) (Peak mE) was not detected as a major compound in the extract from the developing seed coat.

Figure 7.11 and 7.14 show the relative amount (mAU/mg) of the compounds represented by the major peaks identified in each genotype, in the seed coat extracts from the developing and mature grains respectively. In the seed coat from the developing grain, peaks A and B are the lowest in R/W635 but there is no difference in the area of these peaks in the white-grained genotypes (Figure 7.11). The amount of the compound represented by peak D is also higher in R/W635 with this value comparable to SUN325 but significantly different from the other genotypes. Figure 7.14 shows that, in general, there is a greater amount of these compounds in the mature seed coat compared to the seed coat of developing grains. The amount of the compound represented by peak A shows a large increase in the chromatogram of the mature seed coat, rising the most dramatically in R/W635 (Figure 7.14). The amount of the compounds represented by peaks B and D, however, shows no difference between all of the genotypes. There is no difference in the amount of Peak C between QT7475 and R/W635, but this peak is lower in Hartog and significantly higher in SUN325 (Figure 7.14). The amount of the compound represented by peak E was lower in SUN325 and R/W635 at 30 dpa (Figure 7.11) compared to the mature seed coat, but remains similar in Hartog and R/W635 from 30 dpa to grain maturity (7.14).

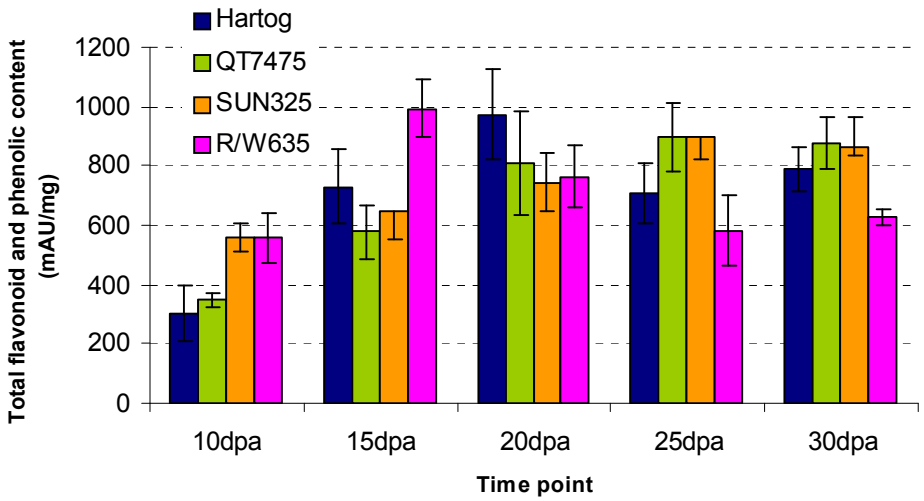


Figure 7.8: Total amount (mAU/mg) of phenolic and flavonoid compounds in the seed coat of developing grains (10 – 30 dpa), using an alkaline extraction method. The seed coat extract was analysed by HPLC and monitored at 280 nm.

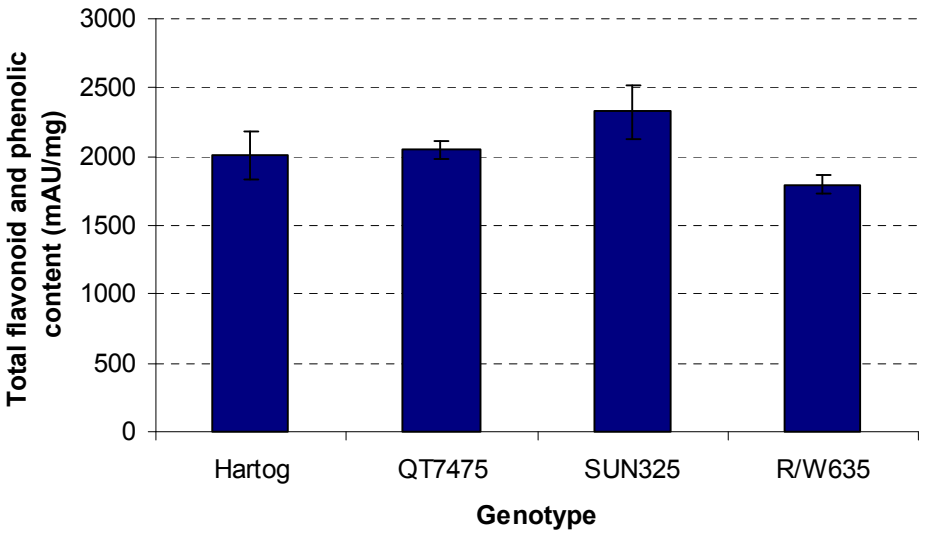


Figure 7.9: Total amount (mAU/mg) of phenolic and flavonoid compounds in the seed coat of mature grains, using an alkaline extraction method. The seed coat extract was analysed by HPLC and monitored at 280 nm.

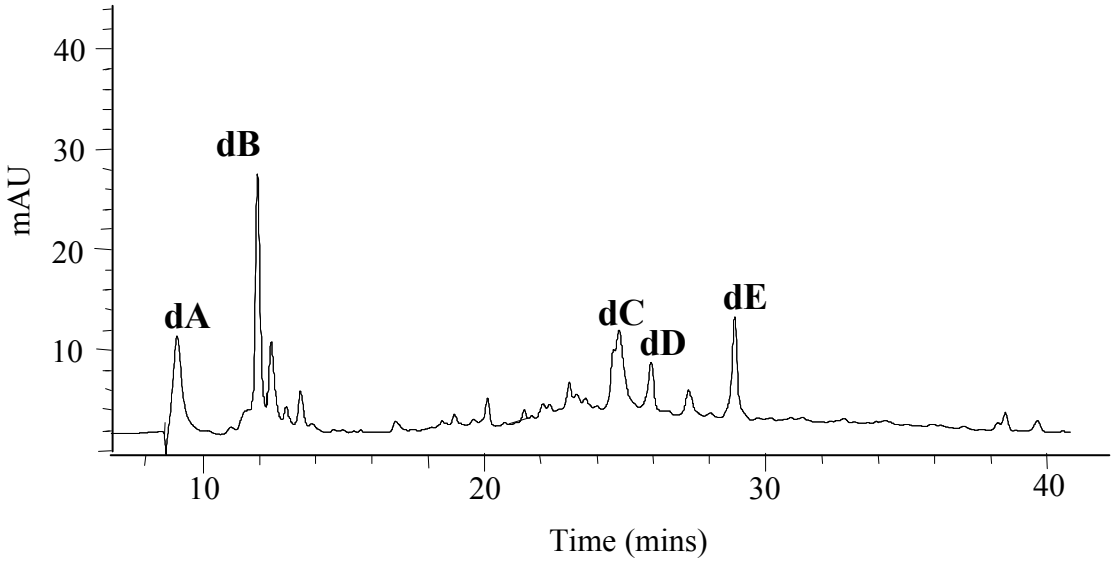


Figure 7.10: Representative HPLC chromatogram (280 nm) of alkaline extract of the seed coat of a developing grain (30 dpa). Major peaks are labelled. Chromatogram is enlarged between 6 and 40 minutes.

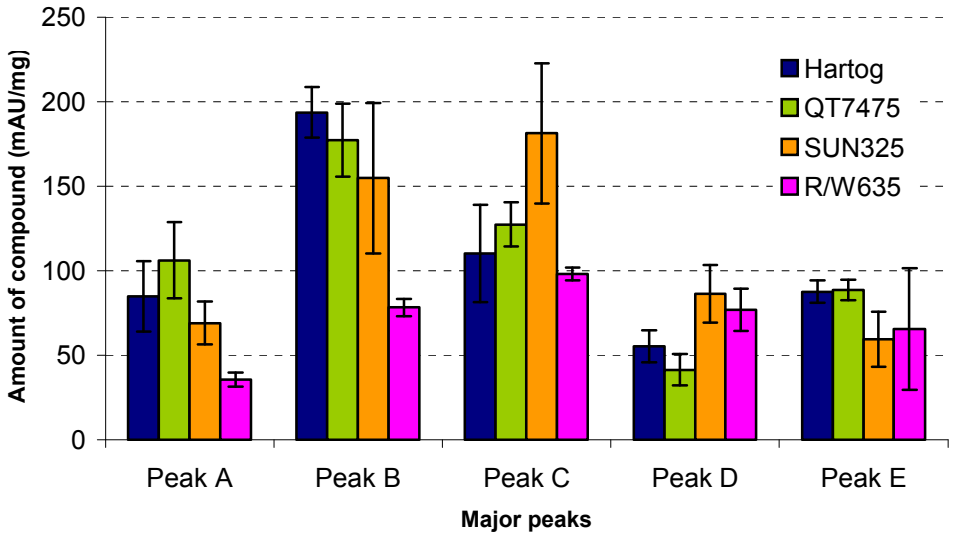


Figure 7.11: Total amount (mAU/mg) of the compounds represented by the major peaks identified from chromatograms generated by the alkaline extraction of the seed coat from developing grains (30 dpa). The seed coat extract was analysed by HPLC and monitored at 280 nm.

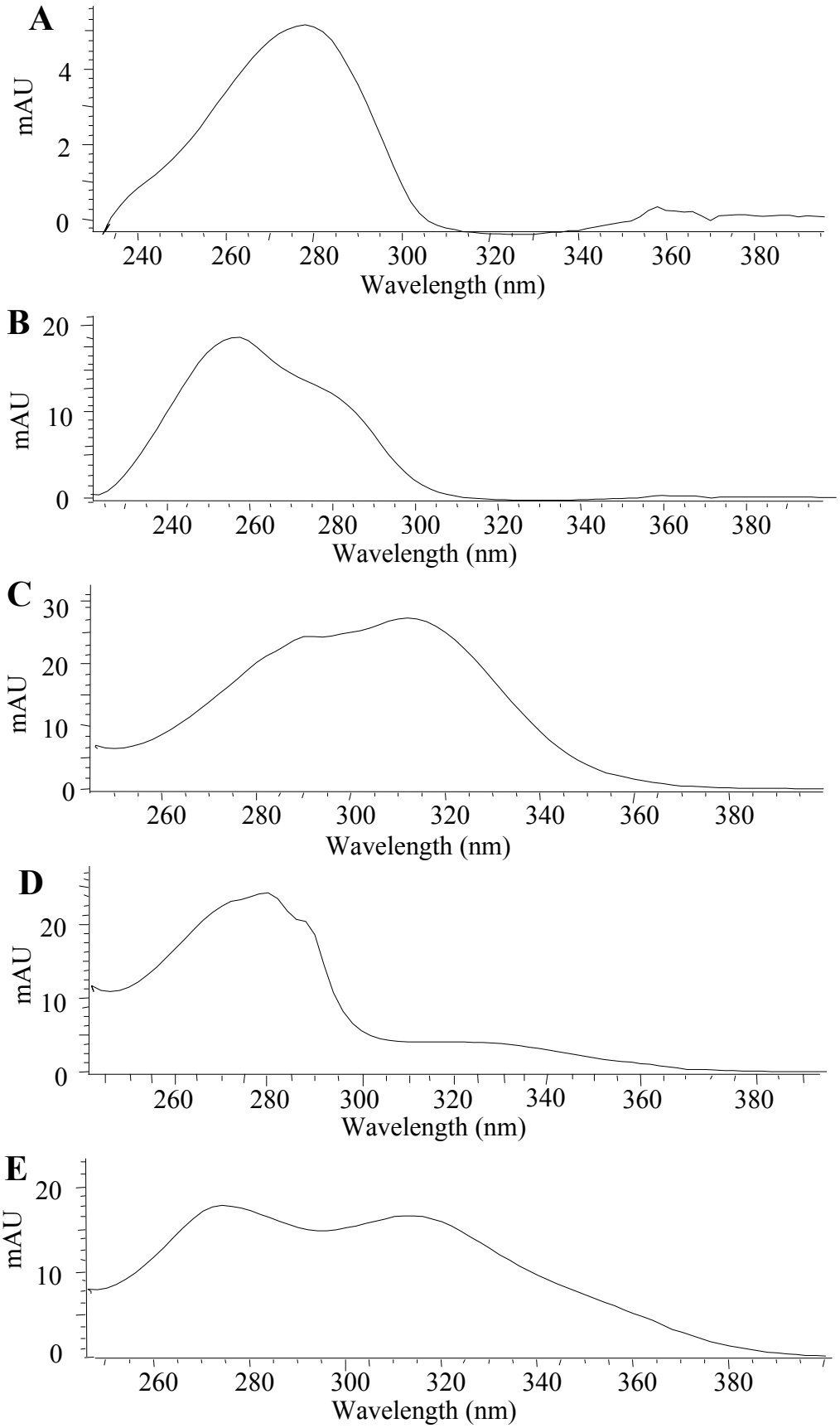


Figure 7.12: Spectra of peaks dA, dB, dC, dD from Figure 7.10. *Panel A*: λ_{max} 280, *Panel B*: λ_{max} 260 nm, *Panel C*: λ_{max} 285 and 315 nm, *Panel D*: λ_{max} 280 nm and Panel E λ_{max} 272 and 315 nm

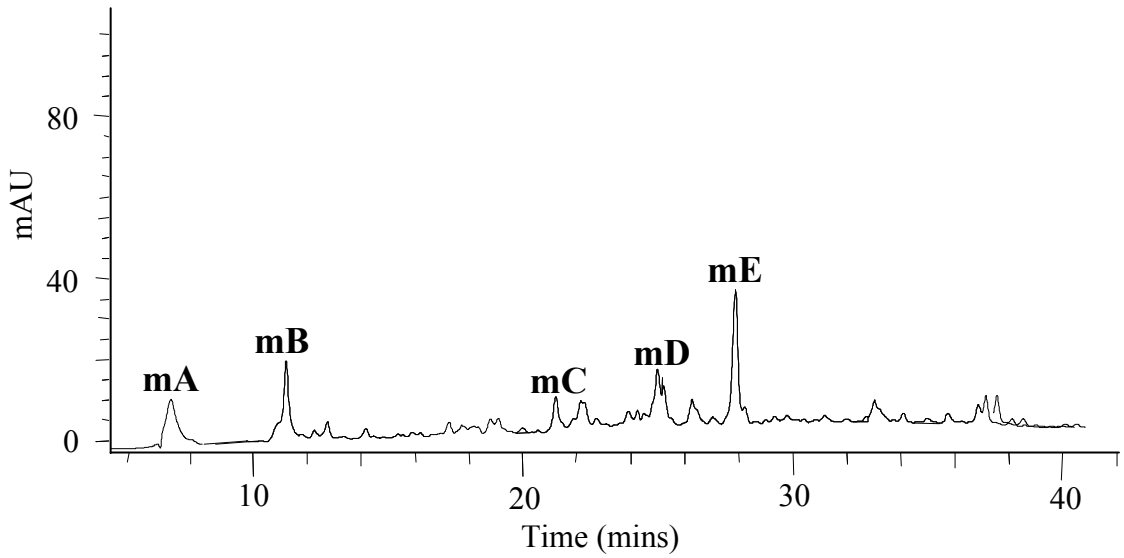


Figure 7.13: Representative HPLC chromatogram (280 nm) of alkaline extracts of seed coat material from mature grains. Major peaks are labelled A to E. Chromatogram is enlarged between 6 and 40 minutes.

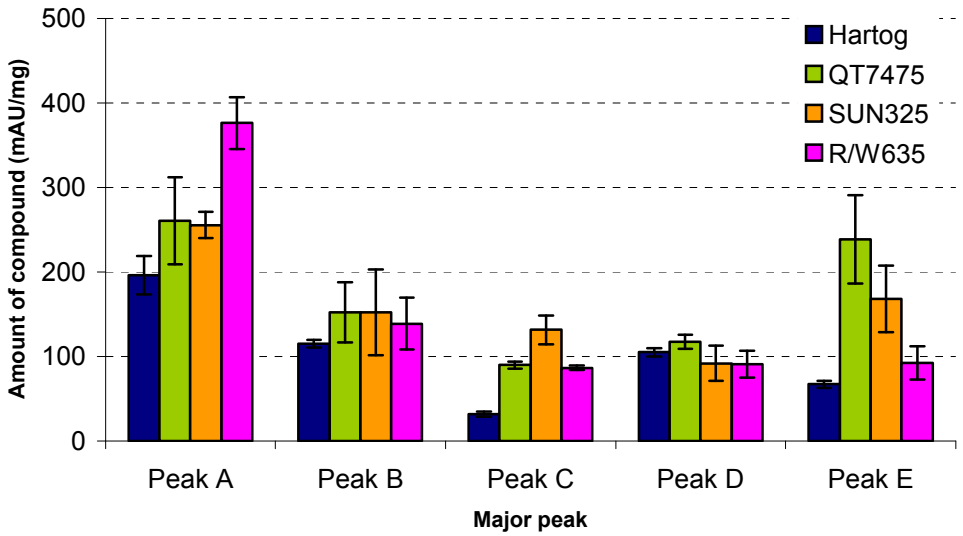


Figure 7.14: Peak areas of major peaks identified in the chromatograms generated from the alkaline extraction of the seed coat from mature grains. The seed coat extract was analysed by HPLC and monitored at 280 nm.

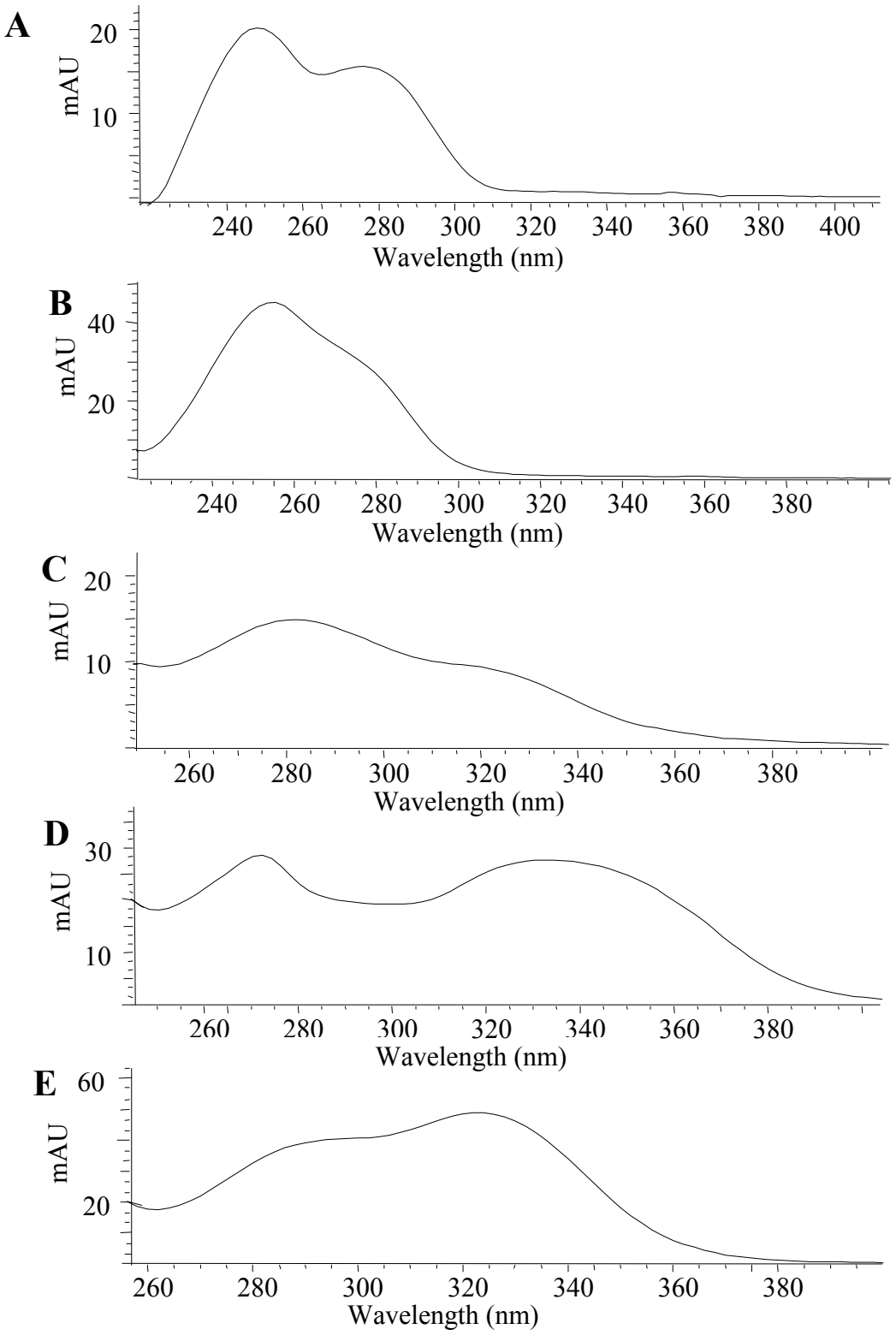


Figure 7.15: Spectra of peaks mA, mB, mC, mD, mE from Figure 7.13. *Panel A*: λ_{max} 245 and 280 nm, *Panel B*: λ_{max} 255 nm, *Panel C* λ_{max} 280 and 320 nm, *Panel D* λ_{max} 272 and 340 nm, *Panel E* λ_{max} 290 and 325 nm

7.3.4 Spiking of alkaline extracts of seed coat with authentic catechins

Although little catechin (**10**) was detected in the HCl/butanol assay, the HPLC chromatograms of all the genotypes contained minor peaks with retention time in the range 22-25 minutes, in similar to that expected for catechin (**10**) and epicatechin (**11**). Authentic catechin, epicatechin or vanillin, diluted in water, was added 1:1 to the alkaline extracts of the mature seed coat and the samples re-analysed by HPLC. The aims of this experiment were a) to attempt to identify the peaks in the chromatograms, and b) to determine whether there was a genotypic effect on relative recovery of added catechin similar to that observed with the HCl/butanol extracts.

Catechin had a retention time between 20 and 22 minutes, while the epicatechin peak was at 23 to 24 minutes and the vanillic acid (**16**) peak was at 22 minutes. Recovery of catechin and epicatechin, from extracts of mature seed coats spiked with authentic compounds, were substantially lower in the dormant genotypes, SUN325 and R/W635 (Table 7.3 and Figures 7.14 and 7.15, *Panel C* and *Panel D)* compared with Hartog and QT7475. By contrast, there was no such reduction in the peak area of vanillic acid (Figure 7.16), with chromatograms of all genotypes showing peaks of a similar height. The reduction in peak area of catechin in the dormant genotypes compared to the non-dormant genotypes is consistent with the observations in *Section 7.3.2*, using the HCl/butanol extraction method.

The concentration of flavone-*C*-glycosides (**7**) was shown to be higher in the seed coat of SUN325 than the other genotypes (Chapter 6) and these compounds were also monitored following the addition of catechin or epicatechin. The total peak area of flavone-*C*-glycosides (**7**) was reduced in seed coat extracts of SUN325 spiked with catechin, and to a lesser extent epicatechin and vanillin, compared to the pure extract (Table 7.3). There is no decrease in the peak area of flavone-*C*-glycosides in any of the other genotypes after the addition of catechin, epicatechin or vanillic acid.

7.3.4.1 Characterisation of factors in seed coat extracts responsible for losses of added catechin.

The reduction in recovered catechin from spiked seed coat extracts appeared to be a characteristic of the dormant genotypes, SUN325 and R/W635. This observation suggested that the seed coat of grains of these genotypes contained factors that could interact with, or degrade, catechin and epicatechin. The peak area of catechin, after spiking, was subsequently used in bio-assays of treated and fractionated seed coat extracts from each of the genotypes in an attempt to characterise this factor(s) involved. Extracts were dried in a Speedivac, or fractionated on a SPE column or by micro-centrifugation. It is apparent the factor in the seed coat that reacts with catechin in SUN325 and R/W635 was inactivated by drying (Table 7.5). The reactive factor also appears to be lost during size fractionation of the seed coat extract, with the catechin peak remaining the same in all the genotypes after micro-centrifugation. A slight decrease in the catechin peak area in R/W635 in the top or large particle size fraction of the micro-centrifuge tube was non-significant (Table 7.5).

The methanol fraction from the SPE column removed a large proportion of the polymeric material from the original extract (chromatogram not shown). The catechin peak in the aqueous fraction appeared to be slightly decreased in SUN325 compared to the other genotypes when the seed coat extract was separated using the reverse phase column, although the error was high and the difference was non-significant (Table 7.5).

Genotype	Catechin (10) peak mAU/mg	Epicatechin (11) peak mAU/mg	Vanillic acid (16) peak mAU/mg
Hartog	1698 ± 473[a]	1170 ± 431[a]	6174 ± 310[a]
QT7475	1723 ± 358[a]	1328 ± 270[a]	5819 ± 422[a]
SUN325	**520 ± 520[b]**	**179 ± 53[b]**	6135 ± 24[a]
R/W635	**451 ± 451[b]**	**256 ± 171[b]**	5682 ± 74[a]

Table 7.3: Area of catechin, epicatechin and vanillic acid peaks (measured at 280 nm) after spiking alkaline extracts of seed coat. Values labelled with a different letter (a or b) and in bold text are significantly different from each other.

Genotype	Pure extract mAU/mg	Catechin (10) spiked mAu/mg	Epicatechin (11) spiked mAU/mg	Vanillic acid (16) spiked mAU/mg
Hartog	312 ± 20[a]	338 ± 8[a]	408 ± 44[a]	318 ± 28[a]
QT7475	304 ± 4[a]	384 ± 74[a]	392 ± 94[a]	404 ± 70[a]
SUN325	**535 ± 66[b]**	**826 ± 236[b]**	**860 ± 312[b]**	**746 ± 122[b]**
R/W635	311 ± 24[a]	272 ± 162[a]	228 ± 32[a]	292 ± 172[a]

Table 7.4: Total area of flavone-*C*-glycoside peaks (measured at 340 nm) in pure and spiked alkaline extracts of seed coat. Values with different letters (a or b) and in bold text are significantly different.

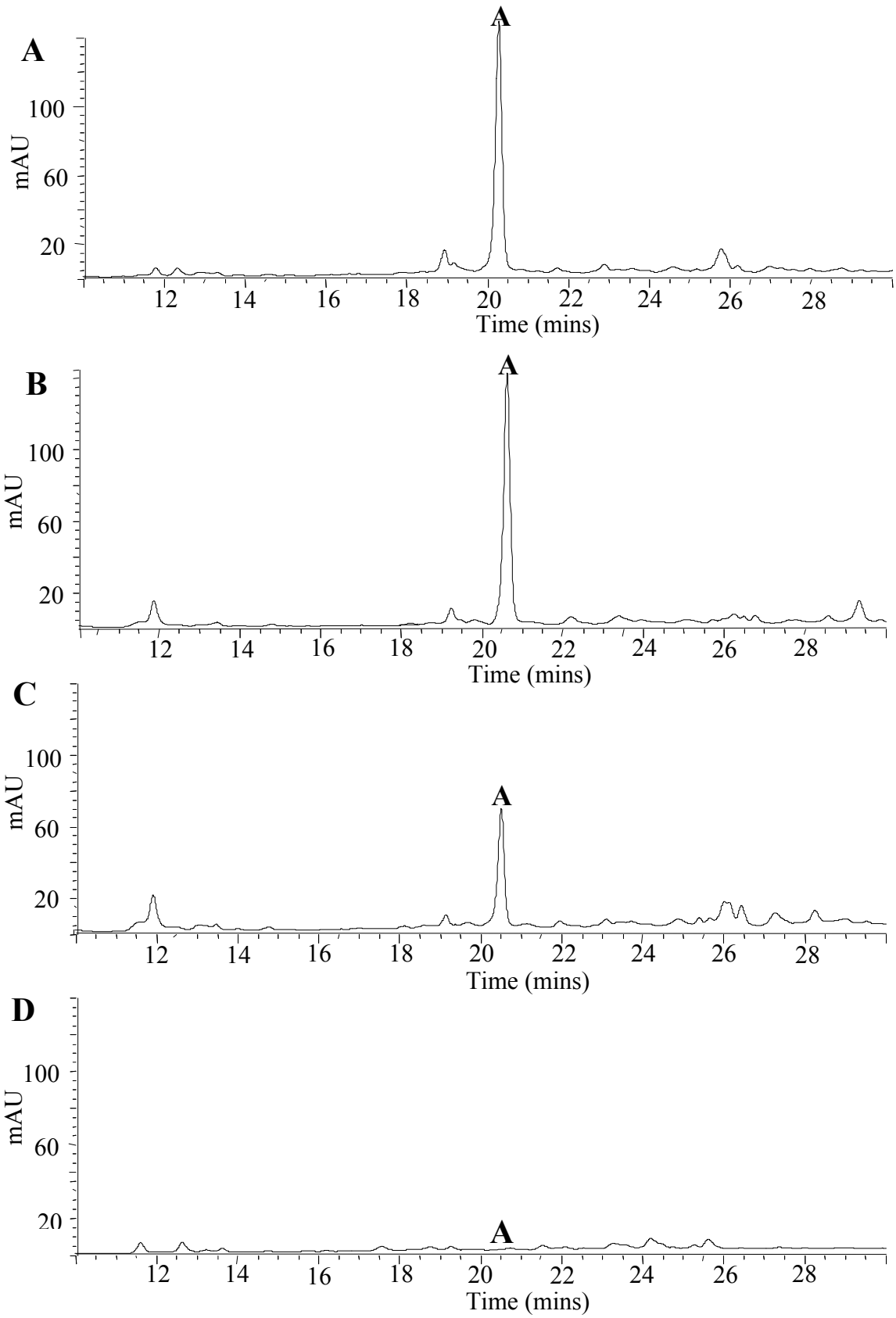


Figure 7.14: HPLC chromatograms (280 nm) of alkaline extracts of seed coat from mature grains spiked with catechin. Catechin corresponds to peak A. Chromatograms are enlarged between 6 and 30 minutes. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635.

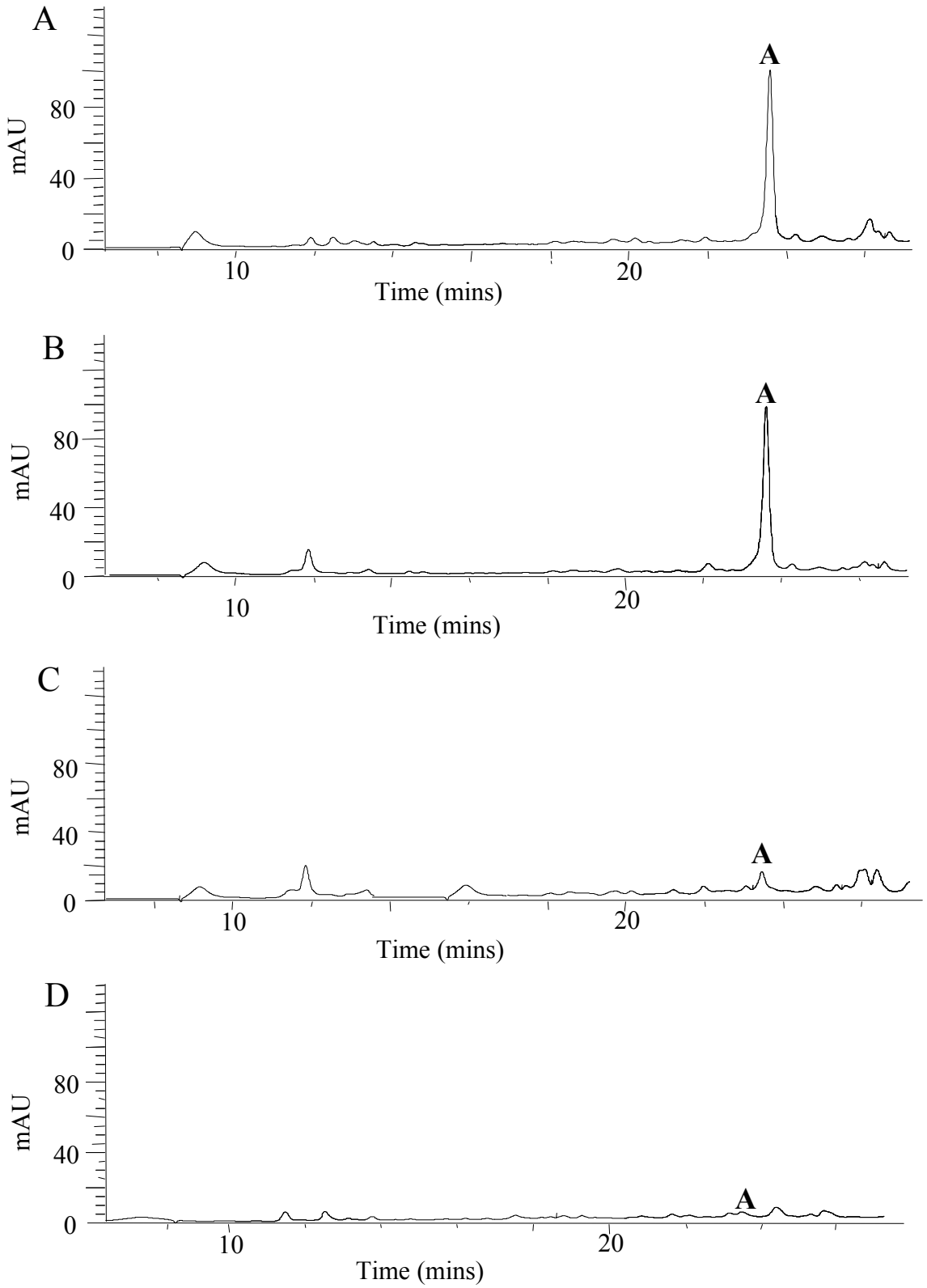


Figure 7.15: HPLC chromatograms (280 nm) of alkaline extracts of seed coat from mature grains spiked with epicatechin. Epicatechin corresponds to peak A. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635.

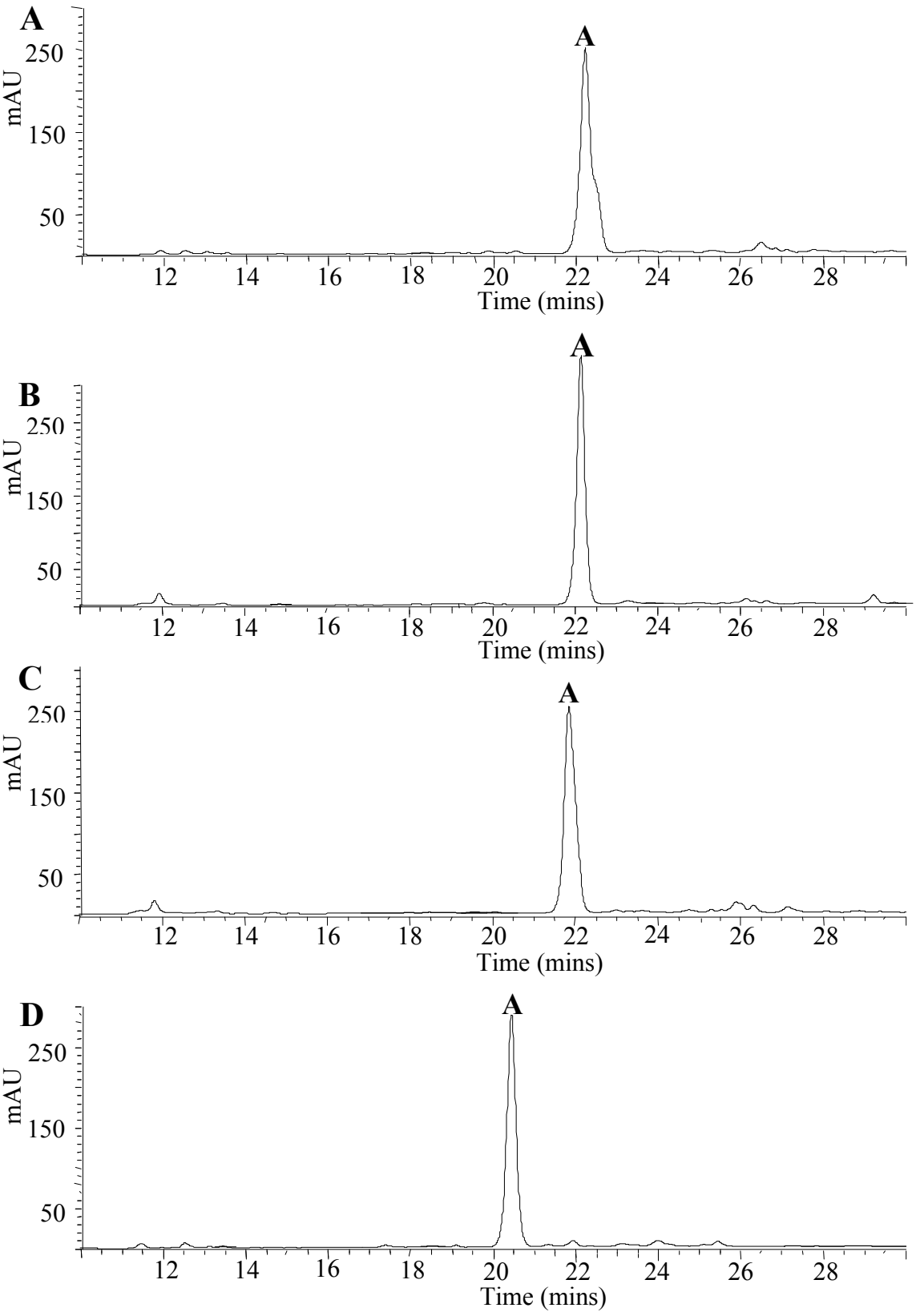


Figure 7.16: HPLC chromatograms (280 nm) of alkaline extracts of seed coat from mature grains spiked with vanillic acid. Vanillic acid corresponds to peak A. *Panel A*: Hartog, *Panel B*: QT7475, *Panel C*: SUN325, *Panel D*: R/W635.

Genotype	Extract dried mAU/mg	Micro-cent, top fraction mAU/mg	Micro-cent, bottom fraction mAU/mg	SPE water fraction mAU/mg	SPE MeOH fraction mAU/mg
Hartog	1034 ± 170[a]	696 ± 157[a]	974 ± 45[a]	2567 ± 449[a]	502 ± 201[a]
QT7475	1015 ± 159[a]	695 ± 210[a]	953 ± 179[a]	2694 ± 713[a]	647 ± 197[ab]
SUN325	970 ± 61[a]	712 ± 175[a]	861 ± 24[a]	1404 ± 835[a]	625 ± 189[ab]
R/W635	999 ± 138[a]	540 ± 177[a]	772 ± 137[a]	2752 ± 702[a]	904 ± 125[b]

Table 7.5: Area of catechin peak after spiking extracts that had been dried or fractionated according to size or hydrophobicity. Values labelled with a different letter (a or b) are significantly different.

7.3.5 Effects of seed coat extracts on germination

Several different methods were used in an attempt to extract potential germination inhibitor from the seed coat of the dormant genotype SUN325. The methods used were based on the assumption that if there is a compound in the seed coat that inhibits germination, it is likely to be highly soluble in water. The first method involved direct extraction of the whole seed of SUN325, with the outer pericarp and embryo removed. The resulting extract solution was subsequently used to imbibe fresh grains to determine if dormancy was affected by the extract solution.

The results of three separate experiments summarised in Figure 7.17 show that there is no significant difference in germination between the grains imbibed in water and those imbibed in the seed coat extract of SUN325. The data from the individual experiments was highly variable, resulting in the large standard error and no significant germination inhibition of grains.

In the second approach, seed coat material was removed from the grain and extracted with water and these extracts used in germination tests with water as control (Figure 7.18). As with the previous experiments (Figure 7.17), there was no significant difference between the germination of grains imbibed in seed coat extract and water (Figure 7.19). Another similarity with the previous experiments is the trend towards a lower germination index of QT7475 following imbibition with the seed coat extract from SUN325, however this because of the large errors associated with the experiment, the difference was not statistically significant.

In the final approach, the alkaline extraction method was employed. This involved performing an alkaline extraction of the removed seed coat, then neutralising this extract with dilute acetic acid prior to imbibition. Figure 7.19 displays the final experiment attempting to detect a germination inhibitor from the seed coats of dormant genotypes SUN325 and R/W635. It is clear that this method has failed to yield reliable data, and it is probable that the presence of acetic acid and ammonia (NH_3) in the imbibition solutions has contributed to this failure. It appears that the control solution may have promoted germination in SUN325, but inhibited germination in QT7475 (Figure 7.19). The

germination indices of Hartog treatments are also much lower than usual for this genotype, indicating this method has not provided reliable results.

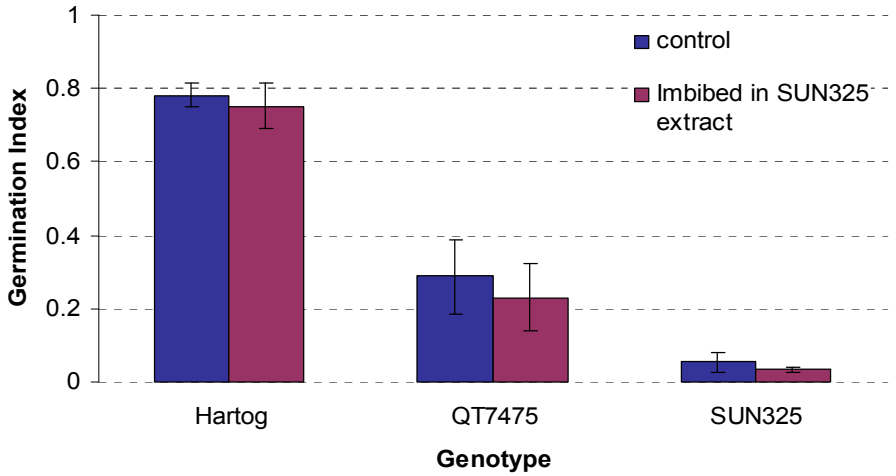


Figure 7.17: Germination of grains imbibed in water or an aqueous extract of whole grains of SUN325.

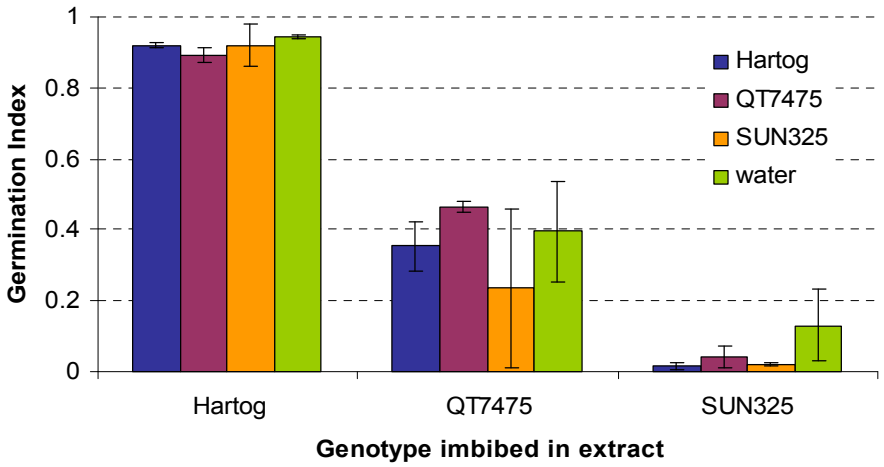


Figure 7.18: Germination of grains imbibed aqueous extracts of isolated seed coats.

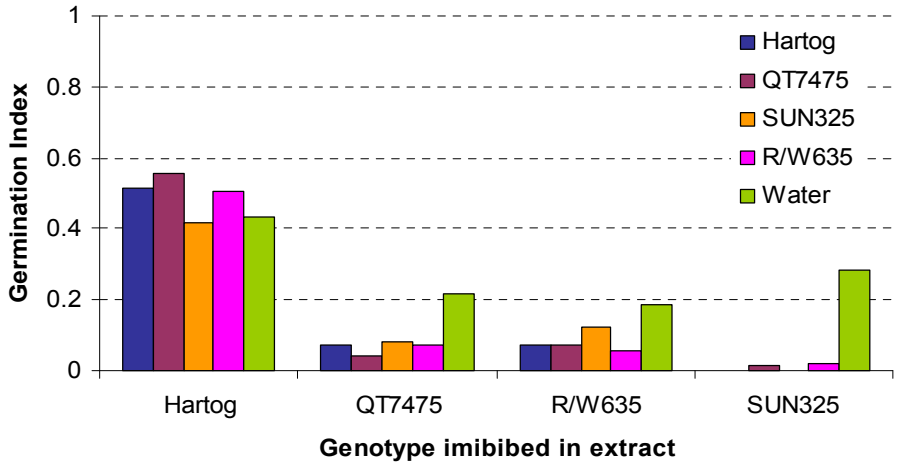


Figure 7.19: Germination of grains imbibed in alkaline extracts of isolated seed coats.

7.4 Discussion

The HPLC analysis of flavonoid peak areas in the developing seed coat extracted by alkaline method showed that the extractable phenolic content increases throughout development and culminates at maturity (Figures 7.8). It has been observed that traditional solvents of alcohol and water fail to effectively extract flavonoids and phenolic acids from wheat bran, compared to an alkaline solution (Feng *et al.*, 1988; Parker *et al.*, 2005). Feng and McDonald (1989) found that there was a greater amount of flavonoids in the white-grained compared to the red-grained genotypes, using an alkaline extraction method (pH11 NaOH). Although the methanol extract was in agreement with this observation, where there was a clearly larger 'hump' in the HPLC chromatograms of the white-grained genotypes (Figure 7.2), there was generally no significant difference in the amount of flavonoids between the different genotypes with the alkaline extraction (Figure 7.8) The alkaline extraction method also appeared to be much more efficient in extracting water-soluble phenolic compounds from the mature and developing seed coat, while reducing the confounding polymeric material extracted with 70% methanol.

Significant amounts of any compounds representing the major flavonoid groups were not detected in either the alkaline or the 70% methanol extracts of seed coat material. These compounds are mostly intermediates that pass directly to other compounds further along the pathway rather than accumulating. For example, naringenin (**15**) is a pivotal compound in the flavonoid biosynthesis pathway, but rarely accumulates because it is consumed for the synthesis of flavones, flavone-*C*-glycosides and other flavonoid compounds (Schijlen *et al.*, 2004). Similarly, free apigenin (**14**), with a retention time of 30 minutes, was also not evident in the seed coat extract (determined by UV/Vis spectra), which is consistent with accumulation in the wheat seed coat of apigenin-*C*-diglycosides (Chapter 5, (Feng and McDonald, 1989)

High concentrations of ferulic acid (**8**) have been detected in wheat bran in a number of studies, with relatively easy extraction from the outer layers of wheat grains through both aqueous and non-polar extractions (Fulcher and Duke, 2002) and wheat aleurone. Asenstorfer *et al.* (2006) reported only minor amounts of ferulic acid (**8**) in water or neutral hydroxylamine extracts of wheat meal but extracted large amounts with dilute alkali. Methanolic and alkaline extracts suggest that there is little free or bound ferulic acid

in the outer layers of the mature seed coat, although a compound with a comparable UV/Vis spectrum to ferulic acid (**8**) was detected in the extract from the seed coat of developing grains (30 dpa) (peak C Figures 7.10 and 7.12).

The developing seed coat extract showed a profile of total phenolic compounds similar to the mature seed coat, with the chromatograms at 30 dpa showing very little difference between the genotype (Figure 7.8). However R/W635 shows a large increase in total phenolic content at 15 dpa, which decrease until 25 dpa. This decrease may be related to the conversion of extractable flavonoid compounds into large non-extractable polymeric compounds such as proanthocyanidins (**1**) or phlobaphene (**2**), which is consistent with a slightly lower phenolic content in the mature seed coat tissue of R/W635 (Figure 7.9). Similar conclusions to the current work were reached by Feng and McDonald (2003) and Ram *et al.* (2003), who failed to detect a biochemical difference between red- and white-grained wheat by using various chromatographic methods and FT-Raman spectroscopy, respectively. It is possible that the biochemical differences between red- and white-grained wheats are substantially subtler than would be expected from the clear colour reaction of whole grains where red-grained wheats appear dark red while white-grained wheats turn straw yellow at high pH (Ram *et al.*, 2003).

An early study, Miyamoto and Everson (1958), reported an accumulation of catechin tannin in the seed coat of wheat grain with red pigmentation. This early study was subsequently supported by McCallum and Walker (Ram *et al.*, 2003), who extracted catechin (**10**) and proanthocyanidins (**1**) from wheat bran and suggested they may be involved in seed coat colour. Unfortunately these peaks had an absorbance that was too low to generate the UV/Vis absorption spectra required for identification. In wheat bran, the HCl/butanol assay and similar techniques have been used previously with success to detect proanthocyanidins (**1**) (Miyamoto and Everson, 1958; McCallum and Walker, 1990), although the yield of coloured compounds can be restricted due to interference by methoxyhydroquinone-glucosides (Asenstorfer *et al.*, 2006). This test was not successful with isolated developing seed coat material in this study for reasons which remain unclear. Possibly the amount of tannin was simple below the detection threshold and HPLC analysis was subsequently used as a more sensitive assay method.

The HCl/butanol extraction produced two major peaks and a minor peak in the HPLC chromatogram (Figures 7.5 and Figure 7.3 and inset), which were possibly related to the

extraction of tannin compounds. The minor peak, eluting at around 23 minutes, had identical spectrum and retention time as catechin (**10**). While the HCl/butanol extract yielded small amounts of catechin, but no coloured anthocyanidins were observed, which indicated that this catechin was not part of a proanthocyanidin (**1**) polymer. In the alkaline extraction where there were many clusters of small peaks which prevented identification of singular peaks. Importantly, there was very little extraction of tannin compounds in both the HCl/butanol and alkaline extraction which suggests that there is only a very small concentration of tannins in the seed coat of both developing and mature grains. This is in contrast to other reports of tannins in the seed coat of developing wheat grains, including white-grained genotypes (Miyamoto and Everson, 1958; McCallum and Walker, 1990).

The consistent loss of the catechin and epicatechin in the seed coat extracts of the dormant compared to the non-dormant genotypes was an unusual and unexpected result, but strongly suggests there is an active factor in the seed coat of these genotypes which is not present in the seed coat extract of the non-dormant genotypes. There has been no previous evidence that this kind of event has been described in other investigations, and there are only a few possible explanations which fit the loss of these compounds. This phenomenon may be a clue to the mechanism of dormancy in the seed coat of the genotypes SUN325 and R/W635.

One possibility is the presence of an enzyme or free radical species in the extract of the dormant seed coat that degrades the catechin. It is also possible that there is a compound that either polymerises with catechin or polymerises catechin to form a larger compound, which is not eluted during HPLC analysis as no new peaks were observed in the chromatogram.

It has already been determined that SUN325 contains more flavone-*C*-glycosides (**7**) than the other white-grained genotypes (Chapter 6), but Table 7.4 shows that the flavone-*C*-glycoside (**7**) content of SUN325 does not significantly decrease after the addition of catechin or epicatechin and are not involved in loss of catechin/epicatechin. At this stage it is impossible to conclude what mechanism by which catechin and epicatechin are lost, however a unique factor present in the seed coat extract of SUN325 and R/W635 may be an important component of seed coat dormancy.

To determine the qualities of the active factor, the seed coat extract was initially dried and resuspended in water before being spiked with catechin to determine if the compound was still active after drying. The catechin peak area (Table 7.5) was did not decline significantly after the seed coat extract of SUN325 and R/W635 was dried, indicating the active factor is sensitive to this treatment. This is an important observation in terms of dormancy, which is gradually lost over time and may be associated with the oxidation of a germination inhibitor in the seed coat.

The active factor was lost during Micro-centrifuge separation of compounds. Both top and bottom fractions, once spiked with catechin, showed little loss of catechin as the peak was high in all the genotypes. This loss of the active factor may also be due to oxidation. Similarly the reversed-phase separation of the seed coat extract did not give any conclusive results. More work is required to characterise this active factor in greater detail, in particular attempting to detect the compound through more refined extraction and HPLC analysis.

A bioassay system was employed to validate the ability of this active factor to behave as a germination inhibitor. Initially, the seed coat of the dormant genotype SUN325 was extracted with water, and the crude extract applied to whole grains of the genotypes used in this study. The genotype QT7475 was important in this bioassay, because it is intermediate dormant and expresses embryo sensitivity to ABA but contains no additional seed coat factor to facilitate complete dormancy. The use of water to release germination inhibitors from grain structures, with the resulting crude extract utilised as a germination media has been undertaken in another study and yielded a germination response (Weidner *et al.*, 1996; Gatford *et al.*, 2002; Kato *et al.*, 2002). Many other studies have used purified extracts or compounds to elicit a germination response from extractions of grain and plant structures (Himi *et al.*, 2002). One problem with previous studies using pure compounds to induce dormancy was that the concentrations of exogenous compounds applied to seeds have been inordinately high, and there is no strong evidence that the germination response is biologically significant.

Vanillic acid (**16**) was also included in this investigation into possible inhibitors in the seed coat, because there has been suggestion that this compound can act as a germination inhibitor in the bracts of *Triticum tauschii* (Gatford *et al.*, 2002). There is also some evidence that vanillic acid can decrease alpha-amylase activity in enzyme extracts of

whole wheat grains (Sharma *et al.*, 1986). Both of these studies required extremely large concentrations, of vanillic acid to induce a physiological response, which is unlikely to ever be encountered in natural circumstances. In combination with this, there was no effect of the addition of vanillic acid to the chromatogram (Figure 7.16), as the spiked peak of the authentic compound did not correspond to any peaks in the chromatogram, indicating that this compound is not an important component of the seed coat and not a possible dormancy factor.

In the present study, the bioassay clearly did not provide any indication that a germination inhibitor can be extracted from the seed coat of SUN325 and R/W635. This may result from the extraction and germination procedure, or there is no entry of the inhibitor into the embryo. It is possible that an inhibitor in the seed coat was extracted, but not in concentrations great enough to evoke a germination response. The amount of potentially inhibitory compounds present in the seed coat is probably lower in the seed coat than the in bracts, (Lenoir *et al.*, 1986). In addition to this, the alkaline extraction solution clearly affected germination because of the ammonia and acetic acid required for extraction and pH neutralisation. A further possibility is that the inhibitor is not taken up by the whole grains. The results presented in Chapter 4 indicate that the seed coat and micropyle may act as a filter limiting the entry or externally added compounds. Further research in this area may aim to isolate the active factor identified in the seed coat of SUN325, and apply it to whole grains to determine if it causes complete dormancy in a genotype like QT7475.

Another method of inhibitor bioassay that may provide useful results is the application of a purified compound to isolated embryos. Catechin tannins applied to embryos by Stoy and Sundin (1976) showed germination profiles analogous to the dormancy phenotype. Isolated embryos show no dormancy, but the addition of ABA (in appropriate concentration) can induce intermediate dormancy symptoms in susceptible genotypes, which suggests that complete dormancy could occur with the addition of ABA and the purified active factor, once it was identified. There has been inhibition of germination of isolated embryos of wheat grains exposed to an aqueous extract of wheat bran from dormant red- and white-grained genotypes, equivalent to 10 μL of ABA. The failure to extract a chemical germination inhibitor from the seed coat of the dormant genotypes does suggest this may not be the mechanism of the dormant seed coat factor. It is possible that the loss of

catechin in the alkaline extract of the seed coat may be a clue to determining the biochemical role of the seed coat in the dormancy in the dormant genotypes.

Chapter 8: General Discussion

This project has contributed to a better understanding of the role of the seed coat in wheat grains, both in terms of dormancy and as a protective tissue surrounding the embryo and grain.

Dormancy in white-grained wheats reputedly involves an interaction between traits expressed in the embryo and the maternal seed coat (Mares, 1999). Dormancy phenotypes of F_1 grain populations derived from reciprocal crosses involving dormant, intermediate and non-dormant genotypes were consistent with this model of dormancy. The results presented here confirm that the embryo and the seed coat of dormant and non-dormant genotypes are genetically different and that both traits in white-grained wheats are inherited as recessive characters. Furthermore, the results demonstrate that embryo sensitivity to ABA on its own provides an intermediate dormant phenotype, whilst the seed coat factor was only effective in conjunction with the embryo of a dormant genotype.

The research undertaken in this project was focussed on the seed coat component, and in particular the mechanisms that might be involved. Bearing in mind that the seed coat is different in dormant genotypes, the seed coat factor interacts or has an additive effect on the embryo trait and dormancy is lost with severe physical damage and after-ripening of the grain, the potential mechanisms were investigated. A number of possible functions that have been proposed in the literature for both wheat and grains of other species were either discounted or were consistent with the criteria and warranted more detailed research.

The seed coat and testa appears to be a barrier to water uptake and gaseous diffusion in many species, for example in *Arabidopsis* (Debeaujon *et al.*, 2000), and it has been suggested that this may also be the case in wheat (Marbach and Mayer, 1974). In this investigation, it was found that the wheat seed coat does not act as a barrier to oxygen diffusion, as evident by the lack of significant increase in germination in an enriched oxygen environment, and oxygen diffusion does not appear to be involved in the observed variation for the grain dormancy phenotype. Similar, there was little evidence for differential effects on water uptake or distribution during the critical few hours after the start of imbibition.

The MRmI images show that water is unable to penetrate directly through the seed coat into the grain at least during the first 18 hours of imbibition. The results clearly show that the inner layers of the seed coat, testa, or the outer surface of the aleurone act as an impermeable barrier to water, also limiting the penetration of pathogenic organisms into the nutrient-rich embryo and endosperm tissue. The MRmI images demonstrate that there is a structured and controlled imbibition pathway, with water only able to enter the grain through the micropyle and hence into the embryo. There was no difference between dormant and non-dormant genotypes, however, this does not mean that water uptake is not an important phase of dormancy expression since imbibition sets into motion the metabolic events that either permit or block germination. MRmI images of grain imbibed only through the distal (brush) end showed that the outer pericarp of the seed coat performs as a wick, conducting water around the outside of the grain, through the micropyle and into the embryo. The movement of water between the outer pericarp and testa layers means that a germination inhibitor(s), if present, could dissolve in the water and enter the embryo, preventing germination. Grains imbibed only from the distal end have prolonged dormancy, or take longer to germinate than grains imbibed normally (Woodbury and Wiebe, 1983). The MRmI images in this study only record the early stages of imbibition. Examination of later stages of imbibition and germination using MRmI studies could provide useful information on water movement into the aleurone and starchy endosperm.

Physical damage to the seed coat increased the germination rate in both dormant and non-dormant genotypes. It therefore appears unlikely that the physical characteristics of the seed coat are related to genotypic variation for dormancy. Nevertheless, it is clear from previous studies that severe damage to the seed coat like cutting the grain in half not only interferes with the interaction between the seed coat and embryo of a dormant grain, but also prevents the embryo from a dormant genotype to be expressed as an intermediate phenotype. The seed coat factor could be explained as either an interaction between the seed coat and embryo, or has an additive effect. It is not known if the seed coat trait disappears with the embryo factor during after-ripening, when dormancy is lost, or whether it is the embryo which is primarily involved in modulation of dormancy during this stage. It is probable that the seed coat has two components: one which restricts germination rate to some extent irrespective of genotype and another which is present only in dormant genotypes.

QTL analysis of the dormant x intermediate-dormant population, in which all lines were homozygous for the dormant parent allele at the 4A QTL (Mares *et al.*, 2005), reputed to confer embryo sensitivity to ABA (Noda *et al.*, 2002), indicated that the seed coat factor in dormant white-grained genotypes may be located in the same region of the long arm of the group three chromosomes as the *R* genes in red-grained wheats, in the case of this study, 3BL. The location of this QTL fits well with the substantial bank of evidence on the role of *R* genes in dormancy of red-grained wheats and the observed reduction in dormancy following mutation, or separation, from other dormancy factors in breeding populations. Based on the consensus maps, the *R* genes appear to be some genetic distance (approximately 30 cM) from the QTL detected in this project (Figure 5.7), however, this may be an error due to the use of different genetic materials and marker systems, the relatively small population size and lower recombination frequencies in the regions of chromosomes closer to the centromere. A population involving the dormant genotypes SUN325 and a single gene red-grained donor of *R-Blb* that could be phenotyped and genotyped for grain colour and genotyped for the 3B dormancy alleles could be developed to resolve this issue. Nevertheless, SSR markers identified in this study should be useful, in combination with the previously reported 4A dormancy QTL markers, for wheat breeders attempting to retain or select for dormancy in their wheat improvement programs.

The identification of the 3B QTL represented evidence of a clear differentiation between dormant and non-dormant genotypes and prompted further comparison with red-grained wheats. Recent investigations suggest that the *R* genes are transcription factors involved in the upregulation of the flavonoid biosynthesis pathway (Himi *et al.*, 2005) that produces the precursors of the red seed coat pigment. Expression analysis of flavonoid genes in the seed coat conducted in this project inferred that it is extremely unlikely that the *R* gene upregulates all of the flavonoid biosynthesis pathway, since major genes in this pathway were expressed in early grain development in both white- and red-grained genotypes. Therefore, although there is no functional *R* gene, the genes in the pathway are still expressed in white-grained wheat, albeit at low levels, and there appears to be a similar expression pattern of these major genes involved in flavonoid biosynthesis in red-grained wheats and the genotype SUN325. It is possible in a prior study by Himi *et al.,* (2005) may have failed to detect expression of these genes in the white seed coat, due to this low level of expression detected by re-amplification of the PCR product. Therefore, although the flavonoid pathway may be an important component of seed coat dormancy, there is no

indication from the results presented in this thesis that *CHS* and *DFR* are differentially expressed in dormant red- and white-grained genotypes. The accumulation of transcript, however, does not always relate to increased product, which is why it was important to employ HPLC to determine whether mRNA synthesis correlates with the appearance of flavonoids.

The presence of transcripts of key flavonoid pathway genes in SUN325 is consistent with the seed coat factor in this genotype being a germination inhibitor, possibly an accumulation of precursors of the red pigment, phlobaphene. However, the presence of transcripts was associated with an increase of flavone-*C*-glycosides, compounds that are not precursors of any known seed coat pigments. In addition, this accumulation was not replicated in the dormant red-grained genotype. One possibility is the *R* gene upregulates a gene or genes controlling polymerisation of precursors into pigment and that the accumulation of flavone-*C*-glycoside compounds in SUN325 is a reflection of the absence of a polymerisation enzyme, with precursors being diverted into an alternate pathway. In the event that a flavonoid compound is involved in the dormancy effects associated with the seed coats of SUN325 and R/W635, it was not detected by the HPLC methods employed in this investigation.

A comprehensive biochemical characterisation of the red pigment is imperative to properly understand dormancy in these genotypes. Unfortunately this work was outside the scope of this thesis, but knowledge of the red pigment would be extremely useful in dormancy studies in wheat.

The failure to devise a reliable and working bioassay prevents the discovery of an active seed coat dormancy factor and in particular causes difficulty in determining if flavone-*C*-glycosides are involved in dormancy. It would be advantageous to investigate the possibility of these compounds as germination inhibitors by applying a pure solution to grains. Extracting and purifying the flavone-*C*-glycosides from the red and white dormant seed coat would firstly require greatly enhanced HPLC techniques, but once separated, the purified flavone-*C*-glycosides can be dried and resuspended in water before applying to whole grains, in particular the intermediate-dormant genotype QT7475, at physiologically realistic concentrations.

One intriguing outcome of the research was the detection of a factor in the seed coat extracts of both dormant genotypes, SUN325 and R/W635, that reacted with exogenous catechin and epicatechin, reducing the spiked peak in the HPLC chromatogram. The mechanism underlying this effect remains unclear, however, on possibility is that a compound in the seed coat reacts with the catechin to form a polymeric compound that could not be detected by the current HPLC technique. In terms of dormancy, however, this hypothesis would not be consistent with an interaction between the embryo and a seed coat unless the polymeric compound was water soluble and mobile. Alternatively, the active factor in the seed coat could be transferred to the embryo where it could react with catechin and tannin-like compounds, that are known to be present in relatively high concentrations in the embryo. Another possibility is that the active factor in the seed coat extract is an enzyme that uses catechin as a substrate. However the active factor in the seed coat extract was lost with drying and resuspension in water, which would not be expected to affect the activity of an enzyme which can survive in the mature seed coat. It is possible that the severe oxidisation in the speedivac may have been effective in reducing the activity. The clear difference between dormant and non-dormant genotypes certainly warrants further investigation, particularly in the view of the earlier work by Stoy and Sundin (1976; 1980), suggesting that embryos of dormant wheat genotypes were sensitive to germination by catechin tannins. Further investigation of the active factor in the seed coat of dormant genotypes could not be pursued in this thesis, but a suggested approach could include different and more sensitive chromatographic techniques and improved extraction and purification techniques coupled with HPLC separation and LC-MS. Near isogenic genetic stocks or mutants, similar to those developed by Warner (2000) and Mares (1999), would facilitate this work significantly.

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