An analysis of dormancy, ABA responsiveness, after-ripening and pre-harvest sprouting in hexaploid wheat (*Triticum aestivum* L.) caryopses

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Abstract

Embryo and caryopsis dormancy, abscisic acid (ABA) responsiveness, after-ripening (AR), and the disorder pre-harvest sprouting (PHS) were investigated in six genetically related wheat varieties previously characterized as resistant, intermediate, or susceptible to PHS. Timing of caryopsis AR differed between varieties; AR occurred before harvest ripeness in the most PHS-susceptible, whereas AR was slowest in the most PHS-resistant. Whole caryopses of all varieties showed little ABA-responsiveness during AR; PHS-susceptible varieties were responsive at the beginning of the AR period whereas PHS-resistant showed some responsiveness throughout. Isolated embryos showed relatively little dormancy during grain-filling and most varieties exhibited a window of decreased ABA-responsiveness around the period of maximum dry matter accumulation (physiological maturity). Susceptibility to PHS was assessed by overhead misting of either isolated ears or whole plants during AR; varieties were clearly distinguished using both methods. These analyses allowed an investigation of the interactions between the different components of seed development, compartments, and environment for the six varieties. There was no direct relationship between speed of caryopsis AR and embryo dormancy or ABA-responsiveness during seed maturation. However, the velocity of AR of a variety was closely associated with the degree of susceptibility to PHS during AR suggesting that these characters are developmentally linked. Investigation of genetic components of AR may therefore aid breeding approaches to reduce susceptibility to PHS.

Key words: Abscisic acid, after-ripening, dormancy, pre-harvest sprouting, seed, wheat.

Introduction

Seed dormancy and germination are controlled by components of intrinsic hormonal and metabolic pathways, that are influenced by external environmental cues (Finch-Savage and Leubner-Metzger, 2006; Kucera *et al.*, 2005; Holdsworth *et al.*, 2008). Following maturation and shedding from the mother plant, seeds of many species exhibit primary dormancy, and do not germinate even if placed under optimal conditions. This capacity for dormancy in the imbibed seed is an active state, and recent transcriptome analysis has shown that dormancy in *Arabidopsis thaliana* (hereafter arabidopsis) is associated with specific patterns of gene expression that are very different from those in germinating seeds (Cadman *et al.*, 2006; Carrera *et al.*, 2007). One pathway whereby the capacity for dormancy is lost is through the process of after-ripening in the unimbibed state. Following after-ripening, a seed population that previously exhibited a high level of dormancy on imbibition, will subsequently show a high level of germination under the same assay conditions. Both temperature and moisture content influence the speed of after-ripening of unimbibed seeds (Gosling *et al.*, 1981; Donohue, 2002; Steadman *et al.*, 2003; Bair *et al.*, 2006). Interestingly, in addition to gene expression differences between dormant and germinating seeds, there is also evidence that some changes in the transcriptome may occur in ‘dry’ seeds (Bove *et al.*, 2005; Leubner-Metzger, 2005) and a link between
proteome modification via reactive oxygen species and after-ripening was proposed (Oracz et al., 2007). Genetic control of after-ripening was recently shown through the cloning of a Quantitative Trait Locus (QTL), Delay Of Germination (DOG-1), from arabidopsis (Bentsink et al., 2006), that promotes an increased after-ripening time. Following after-ripening, germination is achieved if the right environmental conditions are met and, in some species, that includes the availability of light. For example, in arabidopsis, light is required for the completion of germination by the degradation of the PIL5 transcription factor (Oh et al., 2004, 2006), whereas in barley, white light enhances dormancy and promotes expression of a gene influencing ABA synthesis (Gubler et al., 2008).

Many genetic studies in diverse species have shown that ABA is responsible for the induction of dormancy, and mutations representing ABA biosynthesis (Koornneef et al., 1982; Groot and Karssen, 1992; Tan et al., 1997) and signal transduction (Koornneef et al., 1984; Hattori et al., 1992) reduce the dormancy status of freshly harvested seeds. The presence of ABA is required for the maintenance of dormancy following imbibition; analysis of arabidopsis mutants defective in ABA catabolism have indicated that removal of active ABA by ABA 8'-hydroxylase (CYP707A1, CYP707A2) is one requirement for loss of dormancy (Okamoto et al., 2006; Matakaidis et al., 2009). In addition, the N-end rule pathway of protein degradation was recently identified as a key activator of the removal of ABA sensitivity in response to after-ripening (Holman et al., 2009). In hexaploid bread wheat (Triticum aestivum) both mutant (Kawakami et al., 1997) and QTL-mapping approaches (Noda et al., 2002) have shown that ABA sensitivity is important for the acquisition of dormancy and, in barley (Hordeum vulgare), transgenic manipulation of ABA catabolism increased dormancy (Gubler et al., 2008). Physiological analyses have shown that, although ABA synthesis capacity is required for embryo dormancy in wheat (Garelo and Le Page-Degivry, 1999), dormancy is not related to embryo ABA levels (Walker-Simmons, 1987; Morris et al., 1989; King, 1993). Wheat varieties showing enhanced dormancy or resistance to pre-harvest sprouting (PHS) showed increased sensitivity to exogenously applied ABA (Walker-Simmons, 1987; Morris et al., 1989; Corbeneau et al., 2000). However, in barley, ABA content was shown to be correlated with primary dormancy from physiological maturity onwards (Benech-Arnold et al., 1999), and both ABA content and sensitivity were positively correlated with secondary dormancy, where the genes HvNCED1 and HvNCED2 were suggested to play key roles in ABA biosynthesis (Leymarie et al., 2008).

Pre-harvest sprouting (PHS) is a major limitation to stability of wheat production in parts of the world where cool damp conditions prior to harvest are a possibility and, in the United Kingdom (UK), is the major cause of increased alpha-amylase hydrolytic enzyme activity in damaged seed lots (Lunn et al., 2001). There is, therefore, a need to breed for increased resistance. PHS can be combated in part through manipulation of grain colour via the Red grain (R) locus, that provides some resistance to sprouting (Metzger and Silbaugh, 1970; Bassoi and Flintham, 2005). However, an inability easily to screen for this trait has hampered progress towards stable uniform wheat seed quality. Many studies have analysed the genetic control of PHS. One promising approach has been based on the observation that high PHS susceptibility is inversely related to high seed dormancy at harvest (Flintham et al., 2002; Mares et al., 2002). Several QTL associated with PHS have been identified (Anderson et al., 1993; Zanetti et al., 2000; Flintham et al., 2002). Most notably, QTL on chromosome 4A that influence dormancy level at harvest and ABA sensitivity and PHS susceptibility have been found by several different researchers (Kato et al., 2001; Noda et al., 2002; Torada et al., 2004; Mares et al., 2005; Chen et al., 2008), although it is not yet known if these represent the same locus. Taking a comparative molecular approach, transgenic wheat plants containing a functional Viviparous-1 (Vp-1) transcription factor gene from Avena sativa were shown to exhibit reduced susceptibility to sprouting, indicating that this gene [a transcription factor previously shown to repress germination in maize (McCarty et al., 1991)], may provide a transgenic route for reduced PHS susceptibility (McKibbin et al., 2002).

Key questions associated with understanding the phenomenology of PHS are the relationship between dormancy loss, measured in intact ears, and the development of dormancy and ABA sensitivity within caryopses during grain development, and how after-ripening affects dormancy capacity within the embryo. The relationship between dormancy, after-ripening, and ABA responsiveness in wheat caryopses and isolated embryos is analysed here. It is shown that genetically-related varieties showing different susceptibilities to PHS demonstrate distinct kinetics of after-ripening, and that ABA responsiveness of embryos from these varieties is not directly related to after-ripening behaviour. A positive correlation between speed of after-ripening and susceptibility of either intact or isolated ears to PHS has been identified. These results suggest that, although ABA is a key determinant of dormancy induction during grain development, timing of after-ripening may be more closely associated with the capacity of ears to withstand adverse weather conditions and to maintain resistance to PHS.

Materials and methods

Plant material

Winter wheat (Triticum aestivum L., varieties Claire, Charger, Haven, Malacca, Option, and Solstice) were used in experiments, as representing UK germplasm over the last 20 years with defined grower requirements in relation to PHS susceptibility (NIAB sprouting score; see Supplementary Fig. 1A at JXB online). Plants were grown in soil (five plants per 22.5 × 18 cm pot containing JI No. 2 compost) to the unfolded leaf stage (Zadok growth stage 13) and vernalized for 10 weeks at +6 °C with a 12 h photoperiod [PAR 140 μmol s⁻¹ m⁻² at 20 cm (pot height)]. Plants were grown in a naturally lit greenhouse without temperature control until

598 | Gerjets et al.
Zadok growth stage 59 (emergence of inflorescence completed) (Tottman, 1987) and then transferred to controlled environment rooms with 16 h photoperiod [PAR 560 μmol s⁻¹ m⁻² at 70 cm (plant height)] and a day/night temperature regime of +18/+14 °C, using a randomized block design in each experiment. Wheat ears were tagged at emergence of first anthers. All experiments were carried out over three growth cycles, and representative results obtained from single cycles are presented.

**Physiological analyses of grains during ear development**

For grain growth analysis, two ears per variety were harvested from each growth room at approximately weekly intervals between 18–112 days post-anthesis (dpa). Twenty grains were removed from floret positions 1 and 2 of central spikelets of each ear, weighed fresh and again after drying at 80 °C for 48 h. From plots of grain water content and dry matter, maximum grain volume (water content), physiological maturity (maximum dry matter), and harvest ripeness (15% moisture content) were determined. Germination was scored as seed coat rupture over the embryo, after harvest, 25 whole caryopses (‘grains’) were carefully dissected from the central portion of main stem ears avoiding damage, and placed in Petri dishes with two layers of Whatman No. 1 filter paper, and 5 ml of sterile distilled water and incubated in darkness. Three replications of each assay were carried out per time point analysed. For experiments on abscisic acid (ABA, +/–ABA Sigma-Aldrich) responsiveness, grains were incubated in 0, 10, and 50 μM ABA. The influence of assay temperature on the germination potential was analysed by placing the seeds in controlled environment rooms (CER) at +13, +18, and +22 °C. Germination was scored as seed coat rupture over the embryo, every day over a period of 7 d (see Supplementary Fig. 2A at JXB online), and germinated grains were removed from the Petri dishes. A weighted germination index (GI) was calculated using the formula previously described by Walker-Simmons (1987) giving the highest weight to seeds germinating on the first days. GI=(7×n1+6×n2+5×n3+4×n4+3×n5+2×n6+1×n7)/(total days× total number of grains), where n1, n2, ... n7 are the number of grains (or embryos) that germinated on the first, second, and subsequent days until the 7th day, respectively; 7, 6,.... 1 are the weights given to the number germinated on the first, second, and subsequent days, respectively. The maximum GI is 1.0. For Fig. 1, data collected in CER experiments were analysed using Genstat 12 (Lawes Agricultural Trust, VSN International Ltd, Hemel Hempsted, UK). The mean values across the germination assays of three ears at each time point were subjected to ANOVA and treatment means were compared using the least significant difference of the means of Fisher, calculated from the s.e.d (standard error of the differences of the means), using appropriate degrees of freedom when the ANOVA indicated significant differences.

**Embryos:** Embryos were dissected from grains during early grain development, between 20 dpa and 60 dpa, which covers Zadok stages 71 [Caryopsis (kernel) water ripe] to 87 (hard dough). ABA was applied in the concentrations of 0, 10, and 50 μM, respectively. Germination was scored when the coleoptile and/or the coleorhiza had extended past the outer edge of the scutellum (see Supplementary Fig. 2A at JXB online).

**Induction and analysis of caryopsis sprouting within ears**

On whole plants: For the induction of pre-harvest sprouting on whole plants, a misting system was developed to apply over-misting to wheat plants in CERs. Four fine nozzles (0.6096×12.7 mm) sprayed for five times a day for 5 min at 00.30 h, 06.00 h, 10.00 h, 12.30 h, 14.00 h, and 19.00 h with respect to virtual dawn. Misting was initiated at physiological maturity (PM, defined as the maximum dry weight) and terminated at what would have been harvest ripeness in untreated plants (HR, defined when 15% moisture content of the grains). Ten ears per variety were randomly harvested once a week, and assessed for sprouting by cutting the ears into three equal parts and counting the number of sprouted grains. An average number of grains per ear was calculated based on 15 ears per variety, this value was then used to calculate the spraying percentage.

On isolated ears: 10 ears per variety were harvested at intervals of 10 dpa from 50 dpa to 110 dpa. The ears were transferred to a CER equipped with a misting system (see Supplementary Fig. 2C at JXB online). The misting regime was carried out for a period of 7 d, with five blasts of mist during the day at 00.30 h, 06.00 h, 10.00 h, 12.30 h, 14.00 h, and 19.00 h lasting for 5 min each time. The ears were harvested and stored at –20 °C until analysis. Ears were cut into three equal parts (top, middle, and bottom thirds), and the number of sprouted grains and the number of total grains per ear part determined.

Sprouting and after-ripening were directly compared using data obtained from the same growth cycle. Germination Index values for this comparison were obtained from curves fitted to mean values of after-ripening time-courses for each variety at 18 °C. Correlation coefficients (r values) using the mean values were obtained from the resulting data.

**Results**

**Analysis of after-ripening kinetics of intact wheat caryopses**

The kinetics of caryopsis after-ripening was analysed in six varieties of UK winter wheat. These varieties were chosen for their relative susceptibilities to pre-harvest sprouting. According to the categorization provided by the National Institute of Agricultural Botany (NIAB), two of the varieties were classified as PHS susceptible (Charger and Haven), compared with more recent introductions from breeding programmes, classified as more sprouting resistant (Solstice and Option), two others were classified as intermediate (Malacca and Claire) (see Supplementary Fig. 1A at JXB online). Two varieties are very closely related (derived from sibs in a breeding programme, Option and Solstice), whereas others are more distantly related (Claire and Malacca) (see Supplementary Fig. 1B at JXB online).

Plants were grown from anthesis onwards in controlled environment rooms to reduce variation resulting from environmental influences during maternal growth on subsequent caryopsis behaviour. Under standard growing conditions, grains of all six varieties reached maximum water content (grain volume) at approximately 54 dpa and maximum dry mass (physiological maturity; PM) about 8 d later, whereas harvest ripeness (HR; 15% moisture content) occurred around 104 dpa (Table 1). In general, over different experiments, Haven, Malacca, and Solstice produced slightly larger grains and Charger and Option the smallest. As expected, there were no obvious differences in the temporal pattern of grain growth between the PHS-susceptible and -resistant varieties (Table 1). Assays were carried out using whole caryopses isolated from ears from
Grains that complete germination earlier. Hence in relation to analysis of seed after-ripening status, not only is final germination percentage recorded, but also the time at which final percentage is achieved. Intact caryopses of all varieties showed a very low GI up to the point of PM at all assay temperatures (Fig. 1). Between PM and HR several varieties (including Claire, Charger, and Haven) showed a sustained increase in GI, whereas others (Option, Malacca, and Solstice) remained relatively dormant. Following harvest, seeds were maintained within ears at a constant temperature. After-ripening induced loss of dormancy most rapidly in Claire and Charger, with Option taking the longest time to achieve a high GI score. For all varieties the rate of dormancy loss was similar at the two warmer assay temperatures during the period of after-ripening, whereas the lower assay temperature did appear to hasten dormancy loss after HR, but delay dormancy loss towards the end of the after-ripening time-course.

Investigation of changes in ABA responsiveness during after-ripening

The degree of ABA responsiveness in intact seeds and isolated embryos has previously been associated with the capacity for dormancy in many species including wheat (Walker-Simmons, 1987; Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). However, it has recently been shown that in arabidopsis, processes of after-ripening (dormancy loss) and dormancy induction were not controlled by the same genetic loci, and should be considered separate developmental pathways (Carrera et al., 2008). To address this issue in wheat in the present study the relationships between changes in ABA responsiveness were investigated during maturation and post-harvest after-ripening in wheat caryopses and embryos. Initially, the responsiveness of whole caryopses were analysed for all six varieties during maturation (Fig. 2) and subsequently for up to 250 dpa (see Supplementary Fig. 3 at JXB online) using concentrations of ABA up to 50 μM, previously shown to be highly repressive to wheat embryo germination.
Developing intact caryopses of Option, Solstice, and Malacca showed very high levels of dormancy, even without the addition of ABA, whereas varieties Claire, Charger, and Haven all showed an initial high dormancy that was highly reduced by 100 dpa (Fig. 2). In these latter three varieties ABA was shown to have an inhibitory effect at high concentrations early during the time period investigated (i.e. up to 80 dpa), but not at later time points. This indicated that responsiveness to ABA for these varieties changed during grain dehydration. Following HR, during subsequent after-ripening only Malacca, Solstice, and Option demonstrated ABA responsiveness, although this was not pronounced (see Supplementary Fig. 3 at JXB online). To determine the contribution of the embryo, the responsiveness of isolated embryos was investigated during grain development, from 30–60 dpa [corresponding to the late milk, Zadok stage 77, to hard dough, Zadok stage 87 (Tottman, 1987)]. Due to the structure of mature grains, it was not possible to isolate intact embryos from caryopses beyond 60 dpa (see Supplementary Fig. 2D at JXB online). Isolated embryos of all varieties showed a much higher GI over the investigated time period than intact caryopses in the absence of applied ABA (Fig. 3). Several varieties showed a sustained high GI (Claire, Charger, Haven, and Solstice), whereas others (Option and Malacca) were initially more dormant and, subsequently, GI increased. Exogenous ABA had a striking effect on embryo behaviour of all varieties, at 50 μM severely reducing GI. In all cases a distinct change in sensitivity was observed over the time period analysed, such that, although GI of untreated embryos increased in all varieties (with the exception of Malacca) by the end of the time-course, application of ABA was more effective at reducing GI at the beginning and end, suggesting a window of reduced sensitivity during grain-filling, between 40 dpa and 60 dpa. Embryos of Malacca remained responsive to ABA throughout this time period.

![Fig. 2. ABA responsiveness of wheat caryopses of six varieties during grain development and after-ripening of mature seeds. Whole caryopses were assayed for ABA sensitivity of germination as described in the Materials and methods at increasing days post-anthesis from PM to HR. Germination is reported using GI. Time of physiological maturity (PM) and harvest ripeness (HR) in relation to dpa are indicated for each variety. GI for post-harvest after-ripening is shown in Supplementary Fig. 3 at JXB online. Data represent means ±SE of the mean.](image)

![Fig. 3. ABA responsiveness of isolated wheat embryos during grain development. Embryos were isolated from caryopses (see Supplementary Fig. 2D at JXB online) at increasing days post-anthesis and assayed for ABA sensitivity of germination as described in the Materials and methods. Germination is reported using GI. Time of physiological maturity (PM) in relation to days post-anthesis is indicated for each variety as a vertical dotted line. Data represent means ±SE of the mean.](image)
Investigation of the role of after-ripening in determining susceptibility to PHS

Previous work has indicated a negative relationship between susceptibility to the developmental disorder PHS and level of seed dormancy at harvest, such that wheat varieties exhibiting high dormancy are genetically less susceptible to PHS under inductive conditions (Mares, 1993; Biddulph et al., 2008; Chen et al., 2008). As dormancy level at harvest only represents one point of after-ripening, the relationship between GI and susceptibility to sprouting was analysed throughout the after-ripening time-course. Two methods of inducing PHS in controlled environment rooms were used, either with whole plants or isolated ears, using a misting system that delivered defined quantity and droplet size of water at specified times throughout the assay period (see Supplementary Fig. 2B, C at JXB online). The white wheat variety JBW was used to define wet conditions leading to sprouting within ears of intact plants (see Supplementary Fig. 4 at JXB online), although it was found that sprouting in wet conditions was as high in susceptible varieties carrying the R gene as it was for JBW. Initially, the effect of misting whole plants from 55 to 115 dpa on the characteristics of grain development (Table 2) was analysed. The main effect of misting plants was to delay the rate of grain drying such that moisture content was maintained at 37–41% at 100 dpa (Table 2) at a time when grains were harvest ripe under standard dry conditions (Table 1). Misting plants also delayed physiological maturity by about 10 d although there was no effect on final grain size, compared with the dry conditions (compare Tables 1 and 2). In all varieties sprouting increased from a low level at around 60 dpa (Fig. 4). In some varieties sprouting was observable soon after the initiation of misting (Haven and Claire), whereas for others sprouting was not induced until after 20 d misting. Seeds of two varieties, Solstice and Option, maintained a low level of sprouting throughout the misting period. In breeding programmes, tests for susceptibility to PHS are sometimes carried out by misting isolated ears and assaying sprouting, and QTL analyses have also been carried out using this methodology (Chen et al., 2008). An analysis was carried out using this approach to determine more precisely the influence of misting on susceptibility to PHS. Whole ears were removed from plants at increasing days post-anthesis, and incubated vertically with misting for 7 d under the same regime as that used for whole plants (see Supplementary Fig. 2C at JXB online). In this way it was possible to assess sprouting susceptibility at specific stages of grain development using a methodology (assay of germination performance after 7 d) similar to that used to assess isolated caryopses (Fig. 1). Germination of caryopses was analysed in different parts of the ear (top, middle, and bottom) in response to misting in order to determine if the within-ear position influenced the sprouting of caryopses, as ear architecture has previously been shown to contribute to sprouting susceptibility (King and Richards, 1984; Paterson et al., 1989). This analysis was carried out using representative varieties showing either

Table 2. Influence of misting whole plants of different wheat varieties on characteristics of grain development

Timing (days post anthesis, dpa) and weight (mg) of grains at maximum grain volume, physiological maturity (max dry matter), and moisture content at 100 dpa of grains from plants grown under standard controlled environment (18/14 °C day/night) with overhead misting from 55 dpa onwards. r=PHS resistant, s=PHS susceptible.

| Variety | Max water content (mg) | Max dry matter (mg) | Moisture content @ 100 dpa (%) |
|---------|------------------------|---------------------|--------------------------------|
| Claire (s) | 51 | 55 | 56 | 75 | 37 |
| Charger (s) | 45 | 57 | 52 | 70 | 38 |
| Haven (s) | 56 | 48 | 59 | 64 | 38 |
| Option (r) | 42 | 52 | 49 | 75 | 37 |
| Malacca (r) | 51 | 70 | 59 | 78 | 41 |
| Solstice (r) | 50 | 61 | 59 | 74 | 37 |

Fig. 4. Influence of misting whole plants of different wheat varieties on seed germination in-ear. Whole plants were subjected to misting treatment (see Supplementary Fig. 2B at JXB online) as described in Materials and methods. Germination of seeds in-ear (% sprouting) was assessed at increasing dpa for each variety as vertical lines. Time of physiological maturity (PM) in relation to days post-anthesis are indicated for each variety. Data represent means ± SE of the mean.
slow (Option) intermediate (Malacca) or rapid (Claire and Charger) after-ripening. At early stages of analysis (from 50–80 dpa) ears of all varieties showed little or no propensity to sprout (Fig. 5). Subsequently, as observed with whole plant misting, both Claire and Charger demonstrated an increase in responsiveness to misting, with Claire showing the greatest induction of sprouting by HR. Throughout the time period analysed Option did not show any appreciable sprouting. Analysis of grain responsiveness as a function of position in-ear for both Charger and Claire revealed that caryopses located in the middle of the ear were more susceptible to misting than those located either at the top or bottom. A direct comparison between GI at different stages of after-ripening and susceptibility to sprouting either on plants or in isolated ears was carried out (Fig. 6). This analysis clearly distinguished the six varieties into two groups (Malacca, Solstice, and Option in one, and Haven Charger, and Claire in the other), with Option and Claire showing the most extreme separation. The correlation coefficients between GI and sprouting on whole plants amongst the six varieties at the respective after-ripening sampling times indicated differing associations between the two characters (60 dpa $r=0.38$, 70 dpa $r=0.94$, 80 dpa $r=0.51$, 90 dpa $r=0.76$, 100 dpa $r=0.93$, df=4).

**Discussion**

A number of studies have analysed the relationships between dormancy, ABA sensitivity of caryopses and embryos, and susceptibility to the disorder pre-harvest sprouting (PHS) in wheat. In this study, these characteristics have been analysed within the developmental context of the after-ripening time-course of seeds, using six genetically related UK wheat varieties. The reported experiments allow probing of the relationships between different characteristics of seed development, seed compartments, environmental interactions, and outcomes associated with germination and susceptibility to PHS. Germination potential of wheat caryopses increases with time of dry storage (Mares, 1983; Morris *et al.*, 1989), and the kinetics of after-ripening are related to variety and environment (Mares, 1983; Hagemann and Ciha, 1987). In addition after-ripening was postulated to occur in the developing seed (Gosling *et al.*, 1981). Analysis of after-ripening time-courses demonstrated distinct differences in kinetics for different varieties (Figs 1, 2). Whereas some varieties had already undergone significant after-ripening by harvest ripeness (HR; e.g. Claire and Haven), others did not show appreciable germination (e.g. Option). Although expression of dormancy (measured as percentage germination) has been shown to be increased in wheat at higher temperatures

![Fig. 5](image1.png)  
**Fig. 5.** Influence of misting isolated ears of different wheat varieties on seed germination in ear. Vertically positioned isolated ears were subjected to misting treatment (see Supplementary Fig. 2C at JXB online) as described in the Materials and methods. Germination of seeds in-ear is indicated as % sprouting at increasing days post-anthesis for each variety. Sprouting was assessed in the top, middle or bottom third of the ear in each case. Data represent means ±SE of the mean.

![Fig. 6](image2.png)  
**Fig. 6.** Interaction of GI and susceptibility to sprouting during after-ripening of different wheat varieties. For six wheat varieties at specified dpa, GI is plotted against sprouting (%). (A) Sprouting data derived from whole plants, (B) sprouting data derived from isolated ears. Note different GI scales for (A) and (B). M, Malacca; Op, Option; Sol, Solstice; H, Haven; Ch, Charger; Cl Claire.
(Gosling et al., 1981; Corbineau et al., 2000), a systematic reduction in GI was observed in this study at the lower temperature investigated in all varieties. Whole caryopses demonstrated some responsiveness to applied ABA during grain development for varieties showing an enhanced GI during this period (Fig. 2; Claire, Charger, and Haven) as has also been shown in some studies (Walker-Simmons, 1987; Morris et al., 1989; Corbineau et al., 2000), but following harvest these varieties were insensitive. Conversely, those varieties not germinating during grain development (Option, Malacca, and Solstice) showed some (but not great) responsiveness during dry after-ripening (see Supplementary Fig. 3 at JXB online). Analysis of isolated embryos during the period including physiological maturity (PM) showed that most initially demonstrated some degree of dormancy (with the exception of Solstice) that was lost by the end of the time period analysed (60 dpa) (Fig. 3). There did not appear to be a straightforward relationship between the evolution of dormancy characteristics of isolated embryos and ABA responsiveness during the time period studied, in contrast to other studies that have shown that PHS-resistant varieties exhibited enhanced dormancy characteristics of isolated embryos and enhanced responsiveness to applied ABA (Walker-Simmons, 1987; Morris et al., 1989; Corbineau et al., 2000). For example, all varieties were responsive to applied ABA, whether they showed high initial dormancy (e.g. Malacca) or no dormancy during the time-course (e.g. Solstice), and varieties that showed similar GI curves for loss of dormancy (e.g. Claire and Haven) showed different responsiveness to ABA (Claire being more responsive). Interestingly, there appeared to be a window of reduced responsiveness to applied ABA between 40 dpa and 60 dpa, a time period during which sensitivity to wet environmental conditions was previously shown to lead to enhanced sprouting (King, 1993). Many previous studies have analysed the relationship between dormancy level at harvest ripeness (in this study taken to mean degree of after-ripening at this time point) and propensity of seeds in ears to show PHS. Conclusions from these studies are that the fresh weight of caryopses remains high in ears subjected to high moisture environment (King, 1993), the capacity for in-ear sprouting increases during development can be related to varietal dormancy levels (Mares, 1983, 1993; Corbineau et al., 2000), and that therefore dormancy at harvest and PHS susceptibility have been assumed to be linked phenomena (Flintham et al., 2002). One criticism of this single point approach (for example, in QTL discovery programmes) is that it does not take into account the dynamic nature of after-ripening both before and after harvest. Therefore, the change in susceptibility of caryopses within the ear to sprout under wet conditions was analysed using either intact plants or isolated ears (Figs 4, 5; see Supplementary Fig. 2B, C at JXB online). This comparison was carried out to assess the consequences of using different methodologies to induce sprouting. In general, sprouting observed from whole plants was greater than for isolated ears, although in both cases sprouting could be observed following PM, concomitant with the first observable increases in GI of isolated caryopses as a result of after-ripening (Figs 1, 2). This is most likely because ears in whole plant experiments experienced wet conditions continuously from PM, whereas isolated ears were sampled under wet conditions for only one week for each time point analysed. Varieties showed great differences in susceptibility to wet conditions, continuous wet conditions led to up to 40% sprouting in Haven, but did not greatly affect Option caryopses. These results mirror field results, where sprouting is observed normally towards the end of grain development (Lunn et al., 2001).

Analysis of several different characteristics associated with grain after-ripening and sprouting sensitivity has allowed an investigation of the interactions between the different components of the system under study. There appears to be little positive correlation between after-ripening of whole caryopses and the expression of embryo dormancy during grain filling. All varieties showed little or no dormancy by 60 dpa (Fig. 3), although grain dormancy was maintained (Figs 1, 2), suggesting that dormancy is therefore due to other caryopsis structures or components, as suggested in other studies (Corbineau et al., 2000), although it has also been shown that inhibitory substances (products of the $R$ locus) are not important for loss of embryo dormancy latterly during after-ripening (Warner et al., 2000). A weak link was observed between after-ripening and ABA responsiveness of caryopses, although this differed between varieties (those taking longer to after-ripen showed relatively greater ABA responsiveness for longer during after-ripening; see Supplementary Fig. 3 at JXB online), and there was no correlation between after-ripening of caryopses and ABA sensitivity of isolated embryos. It was previously reported (Carrera et al., 2008) that, in arabidopsis, after-ripening and dormancy were genetically separate pathways, and that ABA only contributes to the induction and maintenance of dormancy of imbibed seeds, not to after-ripening. Results presented here, analysing six wheat varieties, also indicate no straightforward relationship between ABA function and after-ripening in wheat. This is in contrast to other studies that directly compared ABA/dormancy in PHS-resistant and -susceptible lines and suggested that ABA responsiveness of wheat embryos is related to dormancy and, by inference, subsequent susceptibility to sprouting (Walker-Simmons, 1987). As the varieties used here were bred for UK field conditions it is possible that genetic determinants have been selected differently under different breeding regimes. Our analyses of interactions between sprouting susceptibility and ABA responsiveness of either caryopses or embryos did suggest that some varieties showing increased (although this is not great) caryopsis and embryo ABA responsiveness (e.g. Option and Malacca) also showed resistance to sprouting. As whole caryopsis ABA responsiveness was weak, even in those varieties that showed it in this study, a general conclusion should be taken with caution. Although no correlation was observed between sprouting propensity during after-ripening and release of embryo dormancy during grain filling, a clear link was found between speed of after-ripening of whole caryopses and susceptibility to sprout under wet conditions (Figs 4, 5). A direct comparison of GI and sprouting, for
both whole plant and isolated ears, during after-ripening [including the period prior to HR as also undergoing after-ripening (Gosling et al., 1981; Corbineau et al., 2000)] demonstrated a distinct bias in the behaviours of the six varieties studied (Fig. 6). The three varieties showing relatively longer after-ripened and reduced sprouting (Option, Solstice, and Malacca) cluster together, and away from others with a greater propensity to sprout and a more rapid after-ripening. The observed clustering is more striking using whole plant sprouting compared to isolated ears, an assay more comparable with field conditions as ears experience wet conditions for longer, and remain attached to plants. A clear distinction between these two sets of varieties based on a link relating after-ripening and sprouting susceptibility suggests that these characters are developmentally linked. Rapid after-ripening occurring during grain development may therefore lead to an inability of caryopses to suppress germination under wet conditions in the field, and, conversely, slower after-ripening may reduce the chance of PHS. In addition, analysis of data indicated that the correlation between after-ripening and susceptibility to sprouting was greatest between physiological maturity and harvest ripeness. The $R$ locus has been used in breeding programmes to reduce susceptibility to PHS, and may function to enhance dormancy during the earlier stages of after-ripening during grain-filling at high embryo moisture content (Corbineau et al., 2000). However, even $R$-type wheats show PHS (compare Fig. 4 and Supplementary Fig. 4 at JXB online), and this may be due to a reduced capacity of $R$-locus products to suppress sprouting following more advanced after-ripening (Warner et al., 2000). As after-ripening is a genetically determined character (Van Der Schaar et al., 1997; Alonso-Blanco et al., 2003) that is separable from dormancy of imbibed seeds (Carrera et al., 2008) it is possible that the observed clustering (Fig. 6) represents genetically-determined differences in after-ripening characteristics of the genetically related varieties tested (see Supplementary Fig. 1 at JXB online). Both after-ripening of dry seeds and dormancy in the imbibed state are considered key traits defining the potential for seed germination. Evidence is emerging that these processes represent distinct developmental pathways (Carrera et al., 2008) that has implications for the study of genetic mechanisms controlling dormancy and after-ripening, particularly in domesticated crops. The distinction is an important consideration in relation to strategies for manipulating dormancy and/or after-ripening behaviour of seeds by breeders (Gubler et al., 2005). In many cultivated species, the capacity for dormancy in freshly harvested seeds is much reduced as a result of domestication (Harlan, 1992). Selection for rapid germination and seedling establishment has led to an inability to repress germination whilst seeds are still attached to the mother plant. Previously, dormancy at harvest ripeness has been used to identify QTL that may also influence PHS susceptibility (Torada et al., 2004; Mares et al., 2005; Nakamura et al., 2007; Chen et al., 2008; Ogbonnaya et al., 2008). The recent cloning of DOG1 from arabidopsis (Bentsink et al., 2006) has provided the first molecular component to allow investigation of the processes underlying after-ripening. Investigation of genetic components for after-ripening *per se* in wheat may aid breeding approaches to reduce PHS by including genetic components in addition to those responsible for dormancy level at harvest. In addition, cloning of such QTLs would allow a comparative analysis of the biochemical determinants of after-ripening. The physiological analysis of after-ripening kinetics described in this paper provides information related to the differing behaviour of caryopses of different wheat varieties that can be used to dissect the genetic control of after-ripening in wheat, for example, by analysis of genetic populations derived from varieties showing rapid and slow after-ripening. This would represent an initial step towards isolating and cloning the associated genetic components regulating the observed after-ripening behaviour.

**Supplementary data**

Supplementary data can be found at *JXB* online.

**Supplementary Fig. 1.** Diagram illustrating the pedigree relationships between the six varieties of wheat used in this study.

**Supplementary Fig. 2.** Illustration of the criterion used for wheat seed germination, and the methodological set-up to analyse PHS.

**Supplementary Fig. 3.** ABA responsiveness of whole caryopses during post-harvest after-ripening in the six varieties of wheat used in this study.

**Supplementary Fig. 4.** Influence of misting whole plants of the white wheat variety JBW on seed germination in-ear.

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Wheat grain after-ripening, ABA responsiveness, and sprouting

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