September 2011
Cost £11.06

Project Report No. 480

An integrated approach to stabilising Hagberg Falling Number in wheat: screens, genes and understanding

by

J. E. Flintham[1], M. J. Holdsworth[2], P. L. Jack[3], P. S. Kettlewell[4] and A. L. Phillips[5]

[1]John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH

[2]University of Nottingham, Sutton Bonington Campus, Sutton Bonington, LE12 5RD

[3]RAGT Seeds, Grange Road, Ickleton, Essex, CB10 1TA

[4]Harper Adams University College, Newport, Shropshire, TF10 8NB

[5]Rothamsted Research, Harpenden, Herts, AL5 2JQ

This is the final report of a 54 month project (RD-2005-3196) which started in January 2006. The project was a Defra and BBSRC sponsored LINK project (SA326) costing £2.2 million, of which HGCA provided £160,000. Industry partners were Campden BRI, Biogemma, Elsoms, KWS, Limagrain, nabim, RAGT, SWRI and LM SW Seed.

CONTENTS

1. ABSTRACT

Hagberg Falling Number (HFN) is the wheat quality characteristic which is least amenable to agronomic or post-harvest manipulation and thus almost totally reliant on breeding for improvement. It is, however, difficult to breed for stable HFN and also difficult to assess HFN stability in the Recommended List variety trials due to the highly weather-dependent variation in HFN inductive conditions from year to year.

The main aim of this LINK project was to provide the information to the breeding companies to enable the technique of marker-assisted breeding to be implemented for HFN. A secondary aim was to establish the genetic and molecular mechanisms underlying the two principal causes of low HFN: pre-harvest sprouting (PHS) and pre-maturity amylase (PMA).

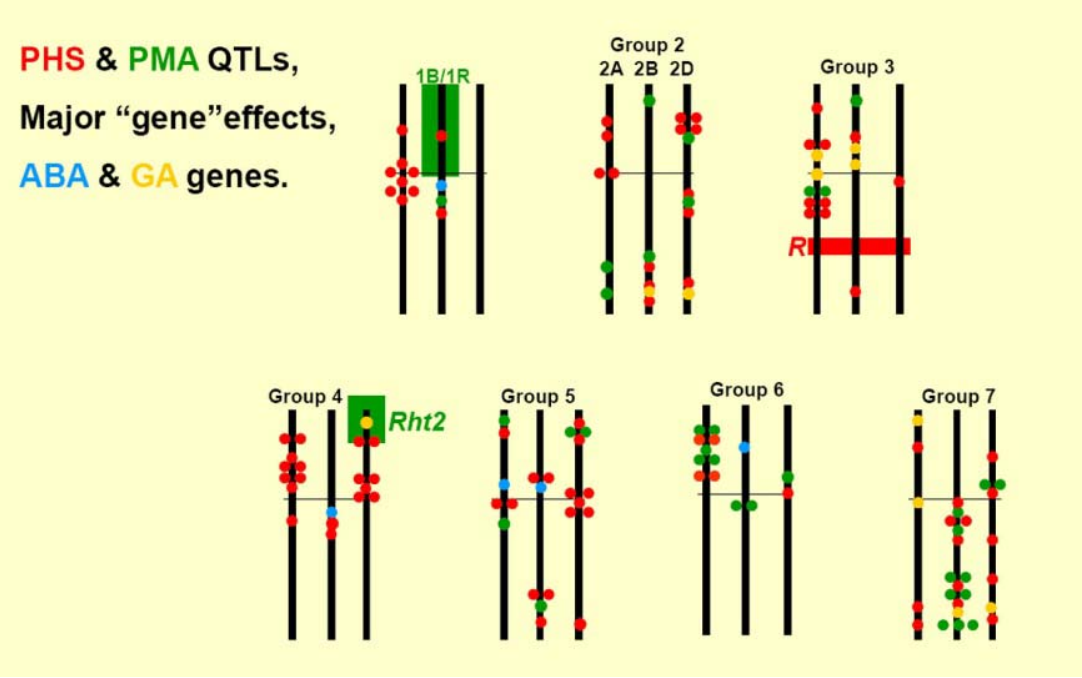
At the heart of the project was a series of field trials with mapping lines derived from several crosses between resistant parent varieties and susceptible parent varieties for PHS and for PMA. Some of these trials were overhead-irrigated to ensure PHS induction, and some were unirrigated to assess PMA if inductive weather occurred for PMA and not PHS. The position of genes controlling PHS and PMA (mainly quantitative trait loci [QTLs], each having a small effect) were located along the length of specific chromosomes by mathematical analysis following HFN measurement of grain from the mapping lines. The QTL locations were validated through the development of controlled environmental screening procedures for PHS and PMA and their use with selected mapping lines. The foundation for further developments in breeding for stable HFN was laid by identifying and mapping genes regulating hormones involved in amylase production in the grain. Understanding of PMA was enhanced by several cell biology and molecular techniques, which demonstrated that different varieties may develop PMA in different locations within the grain.

This project has provided, for the first time, a comprehensive understanding of the genetics of HFN in UK breeding material and this will enable varieties with more stable HFN to be commercially available within the next five to ten years.

2. SUMMARY

2.1. Introduction

Hagberg Falling Number (HFN) is a measure of the α-amylase content of flour made from harvested grain and is a major quality trait in wheat. HFN is sensitive to a number of environmental conditions that reduce the quality of grain, resulting in severe financial losses to growers and increased imports of grain for breadmaking. The increased unpredictability of weather conditions associated with global climate change suggests that low HFN will be an even greater problem in the future. The aim of this multidisciplinary project was to investigate the genetic and biochemical basis of the two major causes of low HFN in the UK: pre-harvest sprouting (PHS) and pre-maturity amylase (PMA). This very successful project has identified multiple genetic loci in UK bread wheat cultivars that confer resistance or susceptibility to PHS and PMA (Summary Figure 1), which will allow breeders to develop more resistant varieties. In addition, improved understanding of the molecular basis of PHS and PMA and characterisation of candidate genes will help to identify and validate these genetic loci. The project was divided into five workpackages as detailed below.



Summary Figure 1. Summary of QTL and candidate gene locations on the wheat genome.

2.2. Work Package 1: Physiological analysis of wheat seed dormancy and pre-harvest sprouting

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 is the major cause of increased α-amylase hydrolytic enzyme activity in damaged seed lots. 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. However, an inability to easily 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 susceptibly is inversely related to high seed dormancy at harvest. Several Quantitative Trait Loci (QTL) associated with PHS have been identified. Most notably QTL on chromosome 4A that influence dormancy level at harvest and abscisic acid (ABA) sensitivity and PHS susceptibility have been found by several different researchers.

Key questions associated with understanding the phenomenology of PHS are the relationship between seed dormancy loss, measured in intact ears and the development of dormancy and sensitivity to the phyto-hormone abscisic acid (ABA) within caryopses during grain development, and how after-ripening affects dormancy capacity within the embryo. In this project we analysed the relationship between dormancy, after-ripening and ABA responsiveness in wheat seeds and isolated embryos. We showed 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. We identified a positive correlation between speed of after-ripening and susceptibility of either intact or isolated ears to PHS. These resultssuggest that although ABA is a key determinant of dormancy induction during grain development, timing of after-ripening may be more closely associated with capacity of ears to withstand adverse weather conditions and maintain resistance to PHS.

A QTL approach was undertaken to identify genes that promote after-ripening using varieties identified with highly contrasting dormancy and after-ripening behaviour. This analysis identified a very strong QTL on chromosome 4AL, mapped to a region of only 4 centi-Morgans (cM). This QTL represents an exciting target for gene cloning in future work, to provide wheat breeders with a molecular marker to distinguish dormant and non-dormant wheat types.

2.3. Work Package 2: Pre-maturity alpha-amylase smart screen development

Pre-maturity alpha-amylase (PMA) is the most significant of a number of syndromes that result in high α-amylase levels in grain and low Hagberg Falling Number (HFN) in the absence of germination. PMA appears to be under different genetic and environmental control to pre-harvest sprouting (PHS). QTLs associated with variation in germination and PHS do not closely co-segregate with those few loci tentatively identified for PMA, and wheat varieties that have good resistance to PHS may be susceptible to PMA and *vice versa*. Similarly, the environmental conditions that give rise to PHS and PMA appear to be different. PHS is induced by a combination of warm temperatures late in grain development, producing low levels of dormancy, and rainfall around harvest time that promotes premature germination.In contrast, a range of environmental triggers have been shown to induce PMA-like symptoms: for example, transfer to low temperature, transient treatment with high temperature followed by low temperature at high humidity, and growth at lower temperature with high watering rates. The identification of reliable conditions for promoting and predicting PMA in susceptible lines was a key objective of Work Package 2.

In the first two years, a series of experiments was undertaken to compare published putative inductive conditions for PMA and the most appropriate was selected for use in a high throughput ‘smart’ screen. The screen was then used to phenotype mapping lines in the third year. The series of experiments was conducted in controlled environment cabinets and focused on testing published Australian work showing that transfers to a cool environment induced PMA. The experiments involved transferring plants of parents of mapping lines known to differ in susceptibility to PMA from a warm cabinet to either a cool or hot cabinet for a short period during grain growth, followed by α-amylase assay of grains using an antibody assay.

In the literature there is some evidence of a link between grain size and PMA, and this was evaluated by a statistical analysis of data on grain weight and HFN from UK Recommended List variety field trials obtained from Crop Evaluation Ltd. An experimental approach of manipulating grain size by degraining to artificially increase assimilate supply and grain size as a possible alternative PMA screening technique was also explored. Because ofthe volume of plants needed for the screen, four screens of Spark x Rialto and Option x Potent mapping lines were conducted in a glasshouse which included a purpose-built air-conditioned bay.

The results of the transfer experiments showed that both hot and cool transfers can induce PMA in UK germplasm, with one particular cool transfer regime found to be the most effective and repeatable. This cool transfer protocol was adopted for the smart screen and applied to mapping lines. The screens enabled a major gene affecting PMA to be identified, and data from the screens

has been used in Work Package 5 to map the location of PMA QTLs and to validate markers from field QTL phenotyping experiments.

Ultimately, this information will be used by breeders in the consortium to avoid taking to market varieties susceptible to PMA.

The grain weight analysis using variety trial data confirmed that a general link existed with heavier grains tending to have lower HFN both within and across varieties. The degraining experiment was successful in inducing larger grains, but PMA did not increase. This indicates that there may be genetic linkage between grain size and PMA, but not necessarily a mechanistic relationship.

In conclusion, Work Package 2 successfully achieved the aims of defining the inductive conditions for PMA in UK germplasm, and developed this into a high-throughput screen to augment field-based phenotyping of mapping lines.

2.4. Work Package 3: Molecular characterisation of pre-maturity alpha-amylase

This workpackage investigated the location of α-amylase in the grain in PMA-susceptible varieties, the role for gibberellic acid(GA) signalling in PMA and investigated the changes in gene expression during the induction process. As in the late-maturity amylase (LMA) syndrome identified in Australian varieties, PMA was shown to be an extremely stochastic process, with only a few grains in induced ears containing high levels of α-amylase. In Maris Huntsman, in which PMA appears to be constitutive, α-amylase was shown to be mainly associated with the transfer aleurone in the crease region of the grain. In Rialto, in which PMA is induced by cold temperature, the α-amylase enzyme is mainly found in the peripheral aleurone in the cheeks and dorsal surface of the grain. This suggests that different PMA syndromes exist in the two varieties. It was also shown that PMA symptoms can be reproduced in Cadenza by the overexpression of *GA20ox* in developing grain, presumably through increases in the levels of bioactive gibberellin; however, transcript analysis of candidate genes in the GA biosynthetic pathway in cold-induced PMA grain did not reveal any significant change in expression, nor were any changes in GA levels detected. Transcript profiling using microarrays identified differences in the cold response of PMA-susceptible (Rialto) and resistant (Spark) varieties. Further work will be required to examine whether such differences in the response to cold determine sensitivity to PMA.

2.5. Work Package 4: Identifying candidate genes for increased HFN stability in wheat using a post-genomics comparative approach

Candidate wheat genes with possible roles in regulating PHS/PMA were identified by investigation of model systems using sequence similarity and comparative transcriptome analyses. Possible roles for these candidates in wheat were defined via transgenesis, genetic mapping, analysis of novel variation in wheat and transcriptome analysis. Transgenic wheat lines containing suppression of candidate genes were produced and will be analysed within a follow-on project. Candidate genes from the GA (gibberellic acid) and ABA (abscisic acid) pathways were mapped onto the wheat genome, a small number co-locating with PHS/PMA resistance QTL. For the QTL on chromosome 4A identified in Work Package 1, TILLING (McCallum *et al.*, 2000) was used to identify mutants in a *GA20ox1* candidate gene from an EMS-induced population, and these are currently being back-crossed to remove excess mutations prior to characterisation for PHS responses.

2.6. Work Package 5: QTL Identification, validation and relation to candidate genes

Work Package 5 was designed to identify quantitative trait loci (QTLs) controlling HFN under conditions promoting PHS or PMA. PHS trials were conducted in winter-sown field plots which were irrigated from overhead during grain maturation, while PMA was investigated through its effect on HFN in non-irrigated plots.Experiments were repeated at different sites and across several years and on multiple mapping populations. A large number of resistance loci were identified, widely distributed across the wheat genome, indicating abundance of genetic variation. Transgressive segregation was observed more often than not, reflecting dispersal of resistance alleles among the mapping parents.

Resistance to PHS and PMA is determined by aggregate effects of major genes and multiple QTLs, but loci controlling PHS do not generally co-locate with PMA effects; the two syndromes are controlled by separate genetic mechanisms. It was found that both PHS and PMA exhibit moderate to high heritabilities, depending on seasonal variation in the severity of damage, but Genotype x Environment (GxE) effects were also prominent, affecting some loci more than others. Candidate genes are already known for the two strongest genetic effects controlling HFN (*Rht* and *R*). This study has furnished DNA markers for further investigation of other important QTL effects: for the development of novel marker technologies, for physiological characterization of their mechanisms, and for fine scale mapping and molecular analysis. There is, therefore, abundant potential for stabilising HFN in commercial wheat varieties for the future, using marker-assisted breeding to assemble novel PHS and PMA resistance gene combinations for more efficient deployment of existing elite germplasm.

3. TECHNICAL DETAIL

3.1. Introduction

Low Hagberg Falling Number (HFN) is an intermittent cause of loss of milling premium for growers. The two main causes of low HFN are pre-harvest sprouting (PHS) and pre-maturity amylase (PMA). The expression of both traits is strongly influenced by genetic and environmental factors. However, the fact that some UK wheats continue to perform reasonably well, even in the most stressful years, suggests that resistance genes do exist that could be exploited to stabilise HFN. The difficulty lies in being able to select for inherently complex traits when their physical expression, even in susceptible breeding material, cannot be guaranteed every year. This makes traditional breeding approaches extremely difficult and time consuming.

This project aimed to provide breeders with the locations on the chromosomes of the genes controlling HFN to enable breeding material susceptible to either PHS or PMA to be discarded at an early stage without the need for the appropriate weather to induce low HFN. Thus onlybreeding lines which are resistant to both PHS and PMA would be taken forward to commercialisation.

The project comprised five work packages. The first two aimed to devise controlled-environment screens for PHS and PMA, respectively, and use these to screen selected mapping lines to validate field results. The third work package aimed to increase understanding of PMA; the less well-understood of the two syndromes. The fourth work package used the approach of mapping the genes for hormones known to be involved in the mechanism of PHS and PMA formation to enable different approaches to breeding for low HFN in future. The fifth work package used field trials of mapping lines to determine the genetic map for PHS and PMA.

3.2. Work Package 1: Physiological analysis of wheat seed dormancy and pre harvest sprouting

Authors:M Holdsworth, J Foulkes, T Gerjets, D Scholefield (University of Nottingham), J Lenton(Consultant)

3.2.1. Aims

- To determine the developmental window of dormancy induction, maintenance and loss during grain maturation under standard environmental conditions
- To analyse varietal differences in degree of dormancy under defined conditions
- To define the relationship between depth of dormancy and susceptibility to PHS in a scalable way (from embryo to ear)
- To compare environmental conditions thatinduce PHS in controlled environments with those causing PHS in the field
- To develop a lab-based 'smart screen' for analysis of PHS in wheat lines

3.2.2. Outcomes

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 due to their relative susceptibilities to pre-harvest sprouting. According to the categorization provided by 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) and two others as intermediate (Malacca and Claire) (Table 1). Two varieties (Option and Solstice) are very closely related (derived from sibs in a breeding programme), whereas others (Claire and Malacca) are more distantly related.

Table 1. UK winter wheat varieties used in this study. Susceptibility (S) or Resistance (R) to pre-harvest sprouting and NIAB sprouting score are indicated.

Varieties	NIAB sprouting score
Claire (S)	4
Charger (S)	2
Haven (S)	2
Option (R)	8
Malacca (R)	6
Solstice (R)	8

Plants were grown from anthesis onwards in controlled-environment rooms to reduce variation resulting from environmental influences during maternal growth on subsequent caryopsis behaviour. Assays were carried out using whole caryopses isolated from ears from early during grain filling (15 days post-anthesis, Zadoks stage 75-77 medium milk stage (Tottman, 1987)) until the completion of after-ripening (at least six months after anthesis). Therefore all stages of maturation, desiccation and subsequent after-ripening of mature caryopses were included in the analyses. Isolated caryopses were assayed at three temperatures (+13°C, +18°C and +22°C) using a germination index (GI) to report germination potential over the seven days of assay, as has previously been used for analysis of wheat seed dormancy (Walker-Simmons, 1987). GI is a useful way to report germination, as it includes a positive weighting for 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 physiological maturity(PM) at all assay temperatures (Figure 1). Between PM and Harvest ripeness(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.

Investigation of changes in abscisic acid responsiveness during after-ripening

The degree of abscisic acid (ABA) responsiveness in intact seeds and isolated embryos has previously been associated with capacity for dormancy in many species including wheat (Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; Walker-Simmons, 1987). Initially the responsiveness of whole caryopses were analysed for all six varieties during maturation (Figure 2). Developing intact caryopses of Option, Solstice and Malacca showed very high levels of dormancy, even without addition of ABA, whereas varieties Claire, Charger and Haven all showed initial high dormancy that was highly reduced by 100 days post anthesisdays post-anthesis (Figure 2). In these latter three varieties ABA was shown to have an inhibitory effect at high concentration early during the time period investigated (i.e. up to 80 days post-anthesis), but not at later time points. This indicated that responsiveness to ABA for these varieties changed during grain dehydration.

To determine the contribution of the embryo, we investigated responsiveness of isolated embryos during grain development, from 30 to 60 days post-anthesis (corresponding to late milk Zadoks stage 77 to hard dough Zadoks stage 87 (Tottman, 1987)). Isolated embryos of all varieties showed a much higher Germination index (GI) over the investigated time period than intact caryopsis in the absence of applied ABA (Figure 3). Several varieties showed a sustained high GI (Claire, Charger, Haven and Solstice), whereas others (Option, Malacca) were initially more dormant, and subsequently GI increased. Exogenous ABA had a striking effect on embryo

behaviour of all varieties, at 50μM 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 and 60 days post-anthesis. Embryos of Malacca remained responsive to ABA throughout this time period.

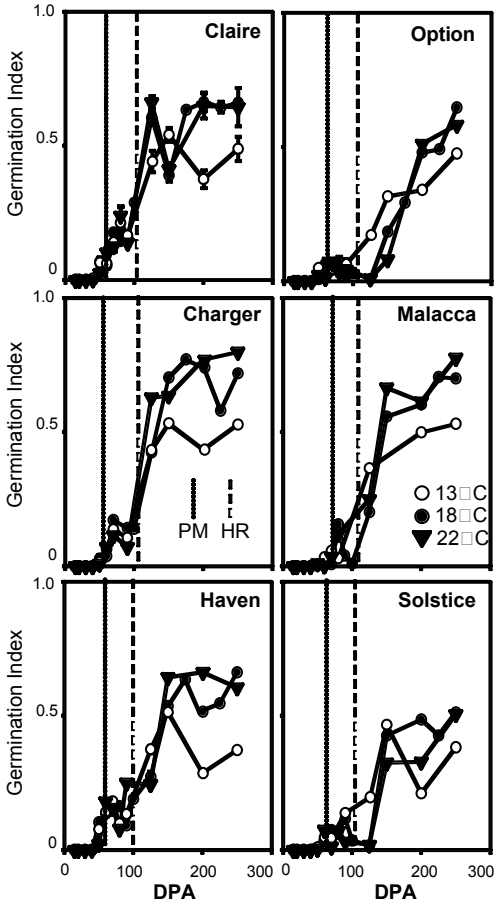


Figure 1. After-ripening of wheat caryopses of six varieties assayed at three temperatures.
Following removal from plants or storage of intact ears at constant temperature (18°C), isolated caryopses were assayed by incubation in water at three temperatures at the times indicated (days post-anthesis). Dates of physiological maturity (PM) and harvest ripeness (HR) are indicated for each variety. Germination response is reported as Germination Index (GI), giving an indication of germination timing, as earlier germination is more highly weighted in each assay. Germination assay method is described in the text. Vertical error bars equal the standard error of the differences of the means for all varieties (df= 5).

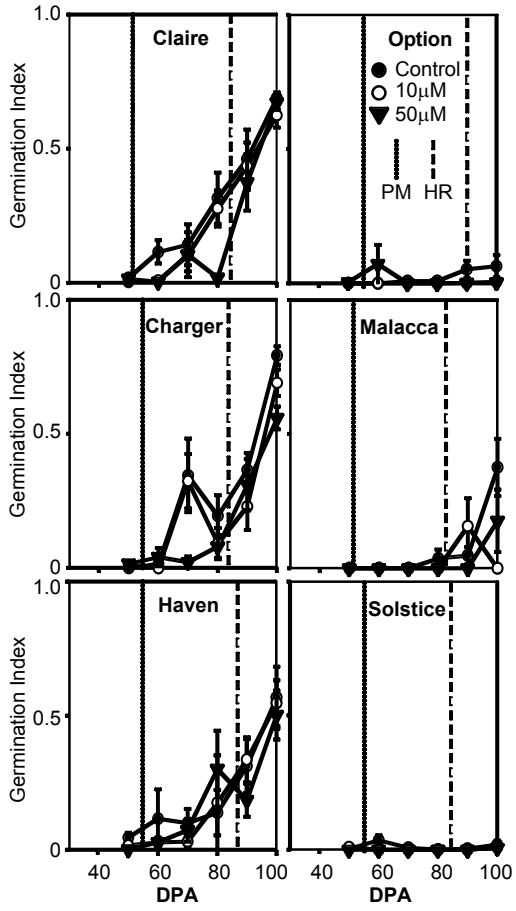


Figure 2. ABA responsiveness of wheat caryopses of six varieties during grain development and after-ripening of mature seeds.

Whole caryopses were assayed for ABA sensitivity of germination as described in the text 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 days post-anthesis are indicated for each variety. Data represent means +/- SE of the mean.

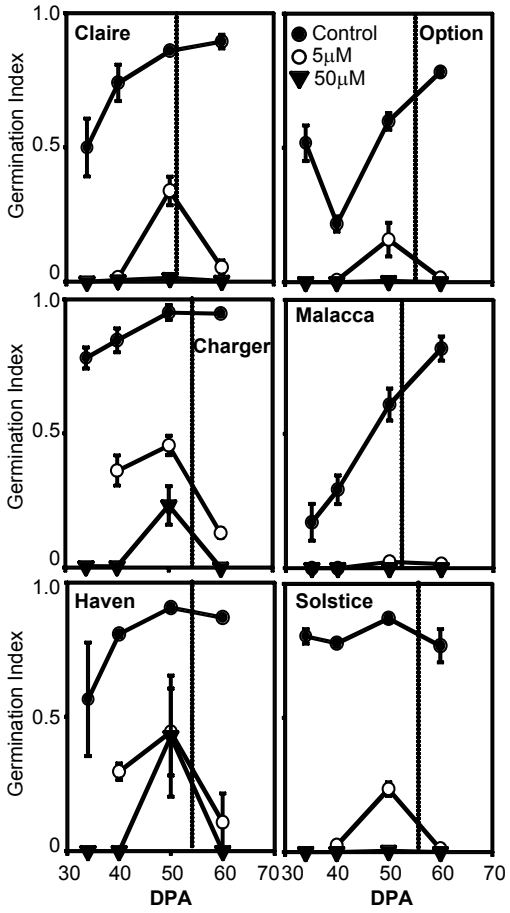


Figure 3. ABA responsiveness of isolated wheat embryos during grain development.

Embryos were isolated from caryopses at increasing days post-anthesis and assayed for ABA sensitivity of germination as described in the text. 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.

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 (Biddulph *et al.*, 2008; Chen *et al.*, 2008; Mares, 1993). The relationship between GI and susceptibility to sprouting was analysed throughout the after-ripening time-course. We used two methods of inducing PHS in controlled environment rooms, 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. Initially we analysed the effect of misting whole plants from PM up to HR on characteristics of grain development. In all varieties sprouting increased from a low level at around 60days post-anthesis (Figure 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 days misting. Seeds of two varieties, Solstice and Option, maintained a low level of sprouting throughout the misting period.

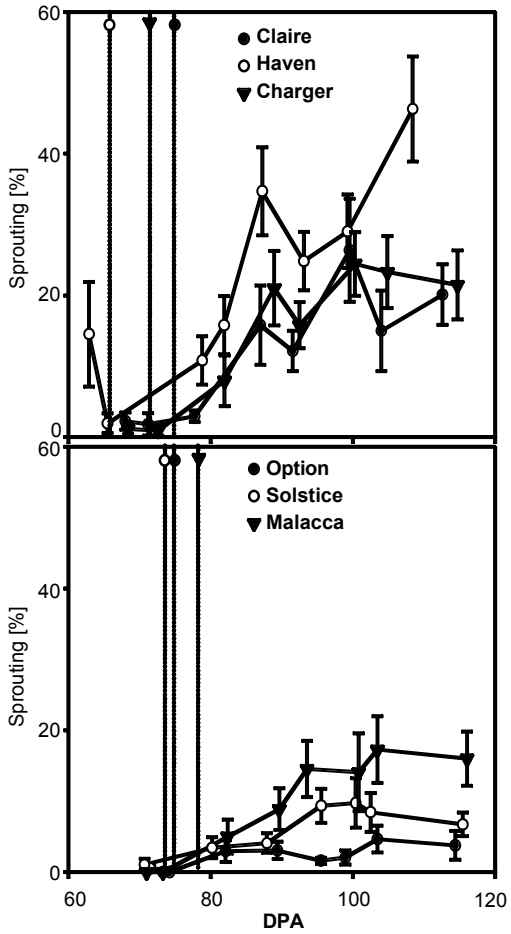


Figure 4. Influence of misting whole plants of different wheat varieties on seed germination in-ear. Whole plants were subjected to misting treatment as described in the text. Germination of seeds in-ear (% sprouting) was assessed at increasing days post-anthesis for each variety as vertical lines. Time of physiological maturity (PM) in relation to days post-anthesis is indicated for each variety. Data represent means ±SE of the mean.

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 (e.g. Chen *et al.*, 2008).We carried out an analysis 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 days under the same regime as that used for whole plants. 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 days) similar to that used to assess isolated caryopses (Figure 1). Germination of caryopses was analysed in different parts of the ear (top, middle, bottom) in response to misting in order to determine if within-ear position influenced 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 slow (Option) intermediate (Malacca) or rapid (Claire and Charger) after-ripening. At early stages of analysis (from 50-80days post-anthesis) ears of all varieties showed little or no propensity to sprout (Figure 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 (Figure 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 days post-anthesis r= 0.38, 70 days post-anthesis r=0.94, 80 days post-anthesis r= 0.51, 90 days post-anthesis r= 0.76, 100 r= 0.93, df= 4).

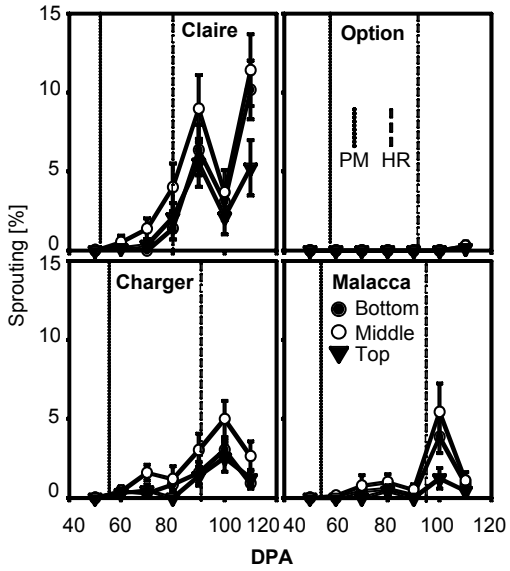


Figure 5. Influence of misting isolated ears of different wheat varieties on seed germination in ear.

Vertically positioned isolated ears were subjected to misting treatment as described in the text. 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.

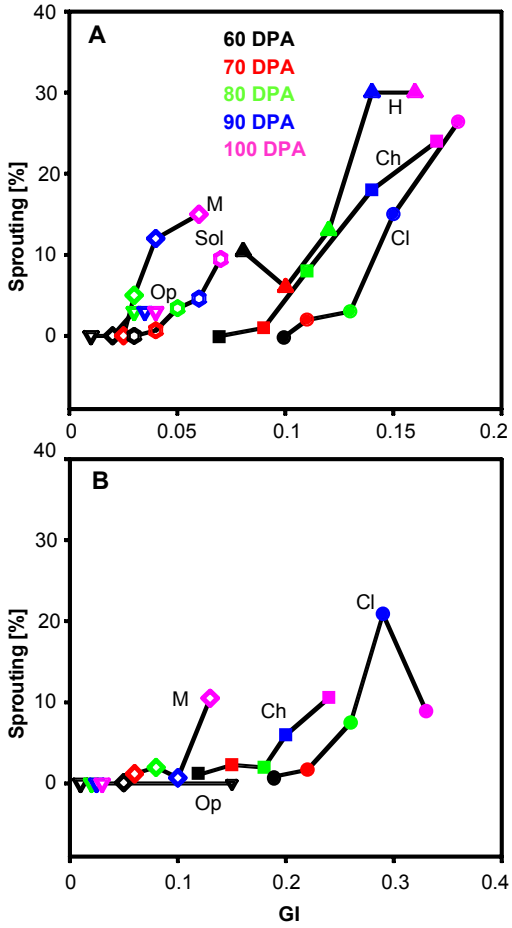


Figure 6. Interaction of GI and susceptibility to sprouting during after-ripening of different wheat varieties.

For six wheat varieties at specified days post-anthesis 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.

Genetic analysis of dormancy

Quantitative Trait Locus (QTL) mapping was carried out using a doubled haploid (DH) mapping population derived from Option and Claire screening for dormancy at harvest. QTL mapping indicated one major QTL on chromosome 4AL. The effect of this QTL is so large that the trait follows a bi-modal distribution indicating a single major gene locus that is closely associated with the markers Xgwm397, XBarc170, WMS0937 and WMS0902 that define a 3cM region. The same

dormancy/PHS QTL has been shown by others to be located between Xgwm397 and XBarc170 within the 4AL-13 deletion BIN, providing a highly defined chromosomal region for further analysis.

3.2.3. Perspectives

A number of previous 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 we have analysed these characteristics 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 (Hagemann and Ciha, 1987; Mares, 1983). Analysis of after-ripening time courses demonstrated distinct differences in kinetics for different varieties suggesting a genetic basis for this character (Figures 1 and 2).

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 (Figure 3), and there was no correlation between after-ripening of caryopses and ABA sensitivity of isolated embryos. We 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, analyzing 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 (Figures 4 and 5).

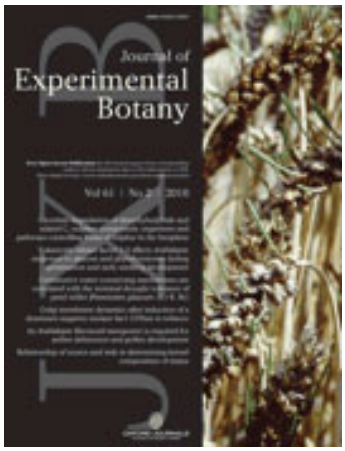
A direct comparison of GI and sprouting, for both whole plant and isolated ears, during after-ripening (including the period prior to harvest ripeness (HR) as also undergoing after-ripening (Corbineau *et al.*, 2000; Gosling *et al.*, 1981) demonstrated a distinct bias in the behaviours of the six varieties studied (Figure 6). The three varieties showing relatively longer after-ripening 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 correlationbetween after-ripeningand susceptibility to sprouting was greatest between physiological maturity and harvest ripeness.

As after-ripening is a genetically determined character (Alonso-Blanco *et al.*, 2003) that is separable from dormancy of imbibed seeds (Carrera *et al.*, 2008) it is possible that the observed clustering of after-ripening speed and sprouting propensity (Figure 6) represents genetically-determined differences in after-ripening characteristics of the varieties tested. 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 (e.g. Chen *et al.*, 2008). 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.

The physiological analysis of after-ripening kinetics described in this report 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. In addition, cloning of such QTLs would allow a comparative analysis of biochemical determinants of after-ripening. An initial analysis of genetic differences in after-ripening in two varieties studied here, Option and Claire, revealed the presence of a single major QTL for dormancy at harvest, located on chromosome 4AL. Future efforts will aim to clone the gene(s) corresponding to this QTL.

3.2.4. Refereed publication resulting from this work



Gerjets, T., Scholefield, D., Foulkes, M.J., Lenton J.R., Holdsworth, M.J. (2010) An analysis of dormancy, ABA responsiveness, after-ripening and pre-harvest sprouting in hexaploid wheat (*Triticum aestivum* L.) caryopses. ***Journal of Experimental Botany*** 61(2):597-607.

Our image of sprouting wheat featured on the cover issue of Journal of Experimental Botany.

3.3. Work Package 2: Pre-maturity alpha-amylase (PMA) smart screen development

Authors: A Farrell, P Kettlewell (Harper Adams University College)

3.3.1. Aims

- To define the conditions that induce PMA in UK susceptible varieties
- To establish a reproducible high-throughout 'smart' screen for PMA and assess the performance of the core set of genotypes
- To phenotype a number of mapping populations for PMA, leading to the identification of QTLs with linked genetic markers

3.3.2. Background

Pre-maturity alpha-amylase (PMA) is the most significant of a number of syndromes that result in high α-amylase levels in grain and low Hagberg Falling Number (HFN) in the absence of germination. PMA appears to be under different genetic and environmental control to pre-harvest sprouting (PHS). QTLs associated with variation in germination and PHS do not closely co-segregate with those few loci tentatively identified for PMA, and wheat varieties that have good resistance to PHS may be susceptible to PMA and *vice versa*. Similarly, the environmental conditions that give rise to PHS and PMA appear to be different. PHS is induced by a combination of warm temperatures late in grain development, producing low levels of dormancy, and rainfall around harvest time that promotes premature germination.In contrast, a range of environmental triggers have been shown to induce PMA-like symptoms: for example, transfer to low temperature, transient treatment with high temperature followed by low temperature at high humidity, and growth at lower temperature with high watering rates. The identification of reliable conditions for promoting and predicting PMA in susceptible lines was a key objective of this research.

In the first two years a series of experiments was undertaken to compare published putative inductive conditions for PMA and the most appropriate was selected for use in a high throughput 'smart' screen. The screen was then used to phenotype mapping lines in the third year.

3.3.3. Methodology

The series of experiments was conducted in controlled environment cabinets and focused on testing published work from Australian and UK groups, showing that transfers to a cool environment induced PMA. The experiments involved transferring plants of parents of mapping lines known to differ in susceptibility to PMA from a warm cabinet to either a cool or hot cabinet for a short period during grain growth, followed by α-amylase assay of mature grains using an

antibody assay. The full details of the methods of the main experiments were reported in a refereed paper (Farrell and Kettlewell, 2008).

In the literature there is some evidence of a link between grain size and PMA, and this was evaluated by a statistical analysis of data on grain weight and HFN from UK Recommended List variety field trials obtained from Crop Evaluation Ltd. Full details of the analysis are in a refereed paper (Farrell and Kettlewell, 2009). An experimental approach of manipulating grain size by degraining to artificially increase assimilate supply and grain size as a possible alternative PMA screening technique was also explored. Full details of the methods are published in Farrell and Kettlewell (2008).

Because of the volume of plants needed for the screen, a purpose built air-conditioned bay in a glasshouse was commissioned and this was used to conduct four screens of Spark x Rialto and Option x Potent mapping lines.

3.3.4. Results

The grain weight analysis using variety trial data confirmed that a general link existed with heavier grains tending to have lower HFN both within and across varieties (Figure 7). Full details of the results are in Farrell and Kettlewell (2009). The degraining experiment was successful in inducing larger grains, but PMA did not increase (Figure 8). This indicates that there may be a genetic linkage between grain size and PMA, but not necessarily a mechanistic relationship. Full details of the results are given in Farrell and Kettlewell (2008).

The results of the temperature transfer experiments showed that both hot and cool transfers can induce PMA in UK germplasm, with a cool transfer regime found to be the most effective and repeatable (Figure 9). Full details of the results of the main experiments are given in Farrell and Kettlewell (2008).

The cool transfer protocol was adopted for the smart screen and applied to mapping lines, with the parent varieties included as controls (Figure 10). Grain from the 2007 early harvest was analysed using a similar protocol to allow comparison between grain α-amylase content and Hagberg Falling Number (HFN) (Figure 11). Overall, the parent lines responded as expected although Spark did show some response to the cold-shock treatment and Charger had higher than expected α-amylase under both treatments.

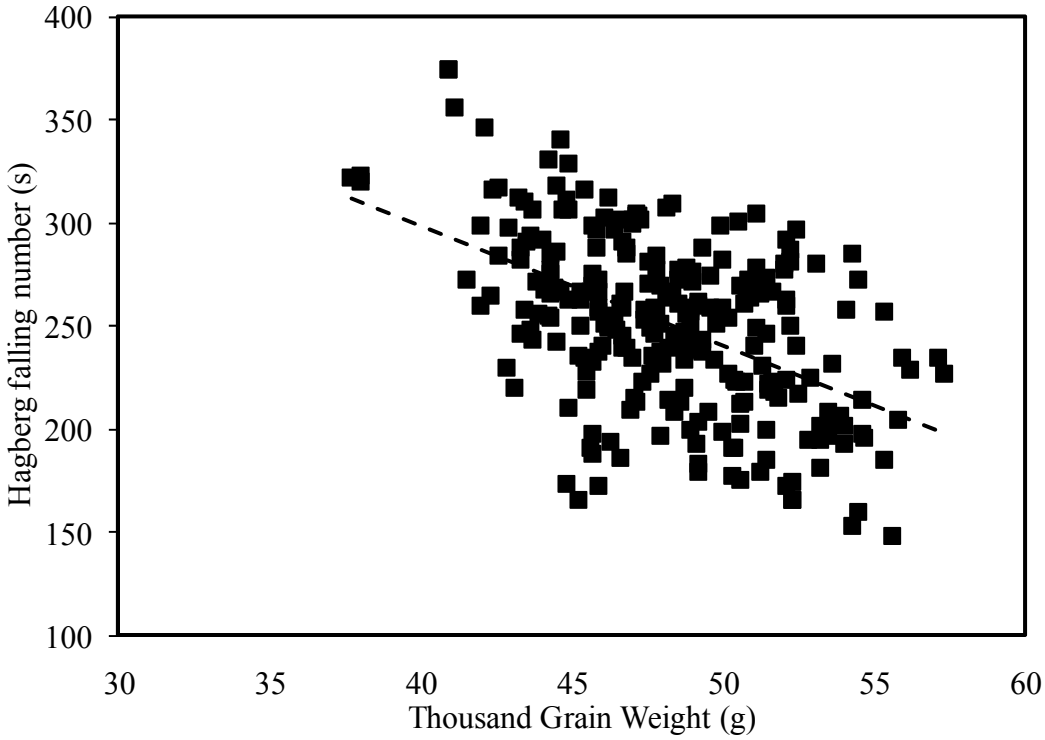


Figure 7.The relationship between Hagberg Falling Number and thousand grain weight for all Recommended List varieties from 1997-2007. Each data point represents one variety in one year measured over several sites and with values calculated using a running mean.

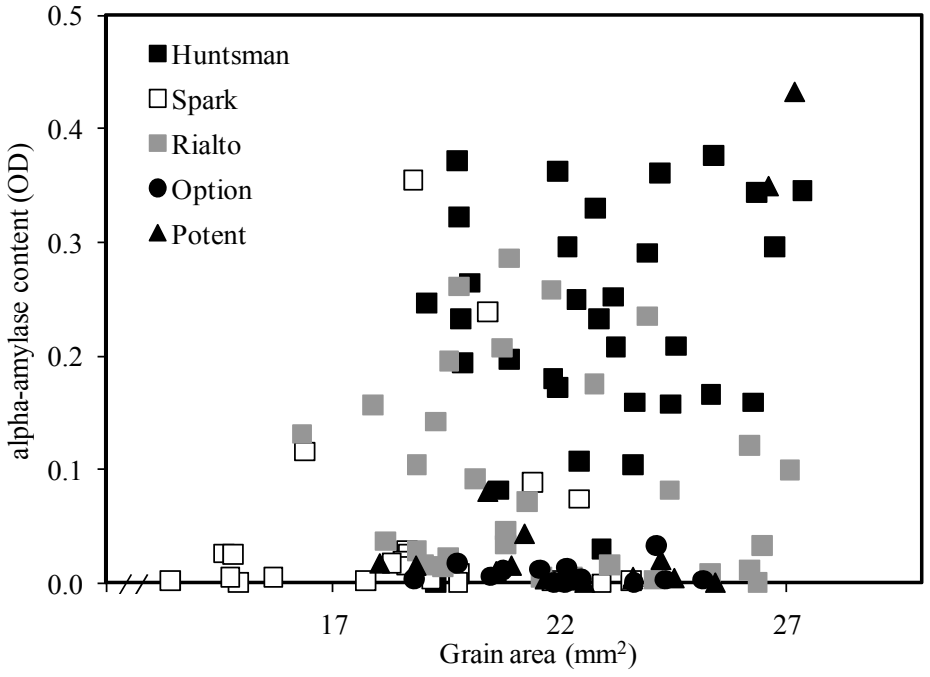


Figure 8.The relationship between α-amylase content and grain area in individual grains grown under contrasting thermal regimes with grain size manipulated through degraining.

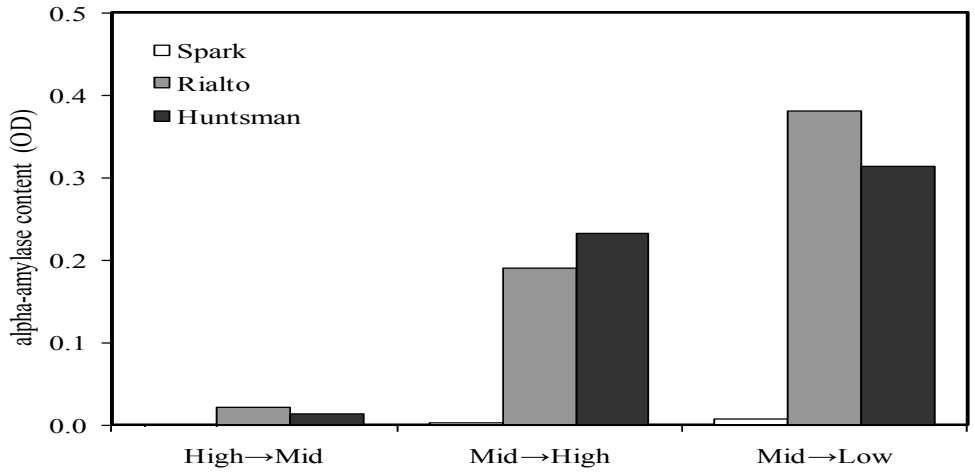


Figure 9.Grain α-amylase content of three wheat varieties grown under three thermal regimes (where low corresponds with cool summer weather, Mid corresponds with normal summer weather and High corresponds with hot summer weather, full detail in Farrell and Kettlewell, 2008). Data are means of 8 grains from 8 plants.

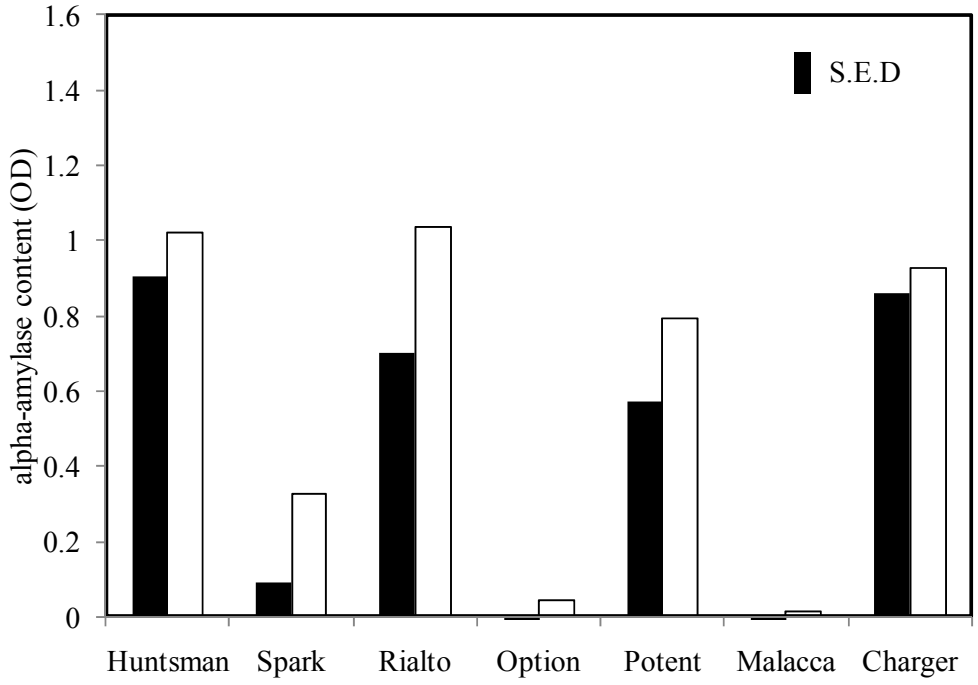


Figure 10.Grain α-amylase content of seven wheat varieties grown under control (open bars) or cold-shock (closed bars) conditions. Data are means following REML analysis with S.E.D for genotype x environment, n=32.

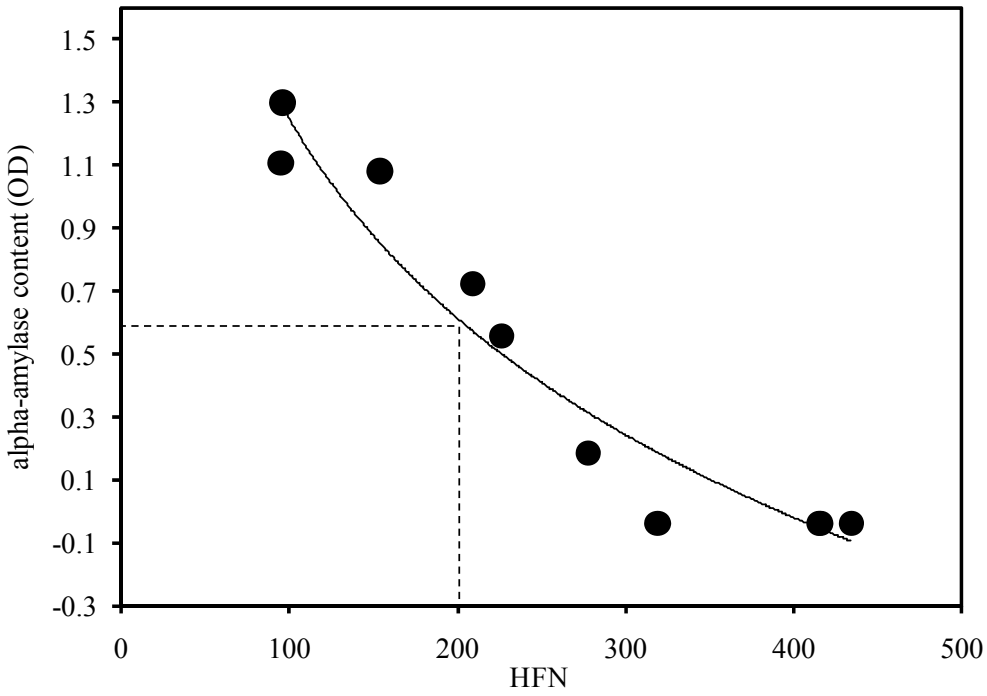


Figure 11.Grain α-amylase content and Hagberg Falling Number (HFN) of ten contrasting wheat varieties grown under field conditions (Work Package 5; 2007 early harvest). Broken lines show 200 HFN.

Data from the screens were used by John Innes Centre to map the location of PMA QTLs, a comparison was made with PMA QTLs detected in field-grown plants where the occurrence of PMA was weather dependent (see Work Package 5). Of the QTLs detected, the significant effect of the 1B/1R translocation on PMA was of particular interest. The screen data showed that in the Option x Potent mapping population under control conditions 63% of the lines with the 1RS translocation had PMA levels above the population median compared with 30% of lines without the translocation (Figure 12). When the cold-shock was applied the segregation was even more pronounced, with 71% of the lines with the 1RS translocation showing PMA levels above the population median compared with 19% for lines without the translocation. This has clear implications for the use of the 1B/1R translocation in bread wheat.

For Spark x Rialto a target population was screened with only those lines carrying 1BL and Rht2 (semi-dwarfing) markers included, as expected this reduced the occurrence of PMA bringing down the population median (Figure 13). Figure 7 also shows data for three lines carrying the 1RS and rht2 markers for comparison. The combination of the 1RS marker and the tall allele appears to result in a high occurrence of PMA under both control and cold-shock conditions. This is supported by the results from the field, where such lines have significantly lower HFN values than the rest of the population (data not shown; WP5).

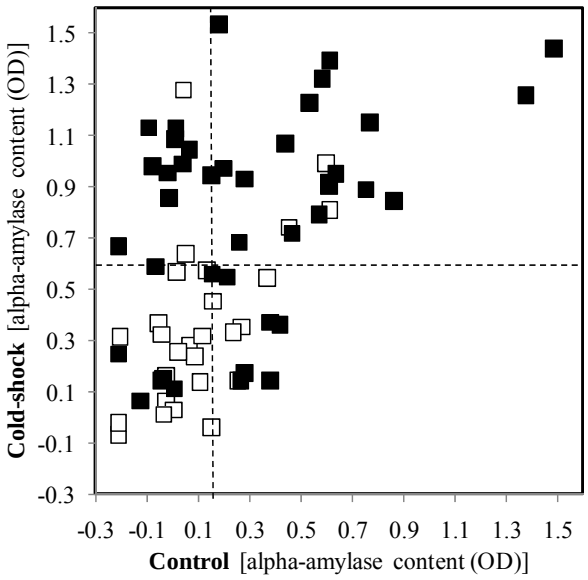


Figure12.Grain α-amylase content of Option x Potent mapping lines grown under control (x axis) or cold-shock (y axis) conditions. Lines carry the 1BS marker (open symbols) or 1RS marker (closed symbols). Broken lines show median level for the population. Data are means following REML analysis, n>8.

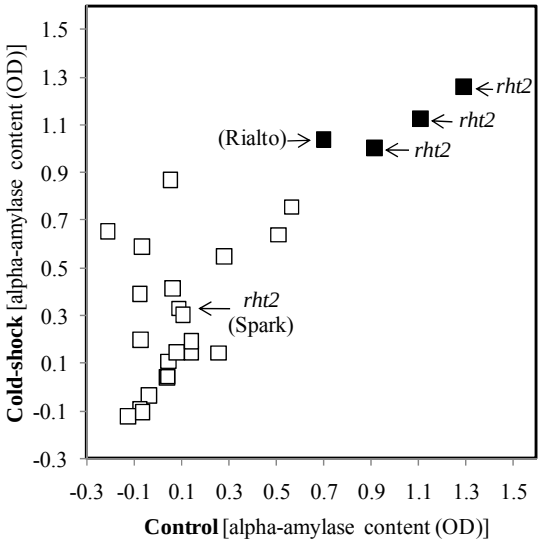


Figure 13.Grain α-amylase content of Rialto x Spark mapping lines grown under control (x axis) or cold-shock (y axis) conditions. Lines carry the 1BL marker (open symbols) or 1RS marker (closed symbols). Lines carrying rht2 are also indicated. Data are means following REML analysis, n>8.

3.3.5. Conclusions

This work has successfully achieved the aims of defining the inductive conditions for PMA in UK germplasm (a cool shock of around a 10°C drop in temperature at about 550 day degrees after anthesis for 8 days), and developing this into a high-throughput screen to augment field-based phenotyping of mapping lines and contribute to validation of markers for use by breeders.

3.4. Work Package 3: Molecular characterisation of pre-maturity alpha-amylase (PMA)

Authors: Y Wan, A Huttly, A Philips, P Hedden, P Shewry, A Phillips (Rothamsted Research)

3.4.1. Aims

- To discover if a single, common PMA syndrome exists in all susceptible varieties under the different inductive conditions that have been described in the literature
- To determine whether the PMA syndrome is confined to aleurone cells surrounding the endosperm cavity as defined by the abnormal expression of α-amylase genes in this region
- To test whether the genes expressed in aleurone cells during PMA are qualitatively the same as those observed during normal germination or PHS, and that PMA thus represents an abnormal activation of cavity aleurone responses
- To relate this abnormal activation of the cavity aleurone to gibberellin biosynthesis and/or signalling, and thus identify potential candidate genes that determine susceptibility to PMA

3.4.2. Establishment of methods and materials for PMA detection and analysis

At the start of the project in April 2006, the pre-maturity alpha-amylase (PMA) syndrome had been observed in UK wheats as a reduction in Hagberg Falling Number in the absence of sprouting. However, as the syndrome appears only irregularly in field-grown crops, pre-requisites for molecular characterisation were a controlled induction system and the molecular tools to investigate the timing and location of the production of α-amylase. The first task was the responsibility of Harper Adams University College and has been described in Work Package 2 above; this first part of the Work Package 3 report covers the development of analytical techniques to detect, quantify and locate α-amylase protein and mRNA in the developing wheat grain.

α-amylase assays

There are two possible approaches to measuring α-amylase in grain sample: activity assays using modified starch substrates and antibody-based methods. α-Amylases in wheat are divided into three classes on the basis of isoelectric pH and sequence diversity. While activity assays such as the Phadebas kit should detect all types of α-amylase present in grain, the prototype α-amylase ELISA kit developed by the Value Added Wheat CRC (Australia) Ltd used a monoclonal antibody directed against the alpha-Amy1 (high-pI) class that is prevalent in PMA. The latter assay has the advantage of specificity in that pericarp (green) α-amylase, which consists of α-Amy2 and α-Amy3 types, is not detected. However, we compared both assays for sensitivity and dynamic range.

At this time a PMA induction system had not yet been established, and field-grown material exhibiting PMA was in short supply. We therefore used transgenic wheat lines that we had developed within an earlier project. The ZmGlb1::AtGA20ox1 lines contain an Arabidopsis GA20ox1 cDNA under the control of the maize globulin-1 promoter in the cv. Cadenza, which in wheat directs expression in the embryo, scutellum and aleurone cells of developing grain. As GA20ox encodes a limiting step in gibberellin (GA) biosynthesis, overexpression should lead to increases in bioactive GA levels in developing grain. We had already shown that mature grain from these lines had increased levels in α-amylase in the absence of sprouting, and could therefore be considered to be ideal test material for the establishment of detection methods for α-amylase. Using extracts from individual mature grains of ZmGlb1:AtGA20ox1, we determined that the ELISA kit, while extremely sensitive, saturated at very low amylase levels; in contrast, the Phadebas assay, while somewhat lower in sensitivity, had a much greater dynamic range and thus was more suitable for quantification of widely varying α-amylase levels in individual grains or pools of grain and this assay was used for most subsequent experiments.

Quantitative real-time RT-PCR for α-amylase transcripts

The three classes of α-amylases in wheat are each encoded by a small multigene family: *Amy1* comprises 12-15 genes on group 6 chromosomes (estimated by Southern blotting and isozyme analysis of aneuploid lines); *Amy2* comprises 6-9 genes on group 7; *Amy3* comprises just three copies on group 5. Full-length sequences are available for only a 1-2 members of each gene family, but a combination of EST sequences and privately held genomic sequences enabled us to generate a multiple sequence lineup containing multiple members of most classes. This was used to design qrt-PCR primers that would discriminate between the three gene families but would amplify as many as possible of the family members. To establish and validate the qrt-PCR assay we used existing RNA samples of a grain development time course from cv. Hereward for which we had pre-existing microarray data (Wan *et al.*, 2008) including several α-amylase probesets covering members of each amylase class.

To further validate the assays we analysed a developmental time course of the transgenic line (ZmGlb:AtGA20ox1 in cv. Cadenza) using both α-amylase enzyme assays and real-time RT-PCR of transcripts (Figure 14). This showed that (i) the transgene, *AtGA20ox1*, was expressed 14 days post-anthesis and was particularly high late in grain development (42 days post-anthesis), (ii) *α-Amy3* was expressed only early in grain development (7-14 days post-anthesis), confirming earlier work (Baulcombe *et al.*, 1987), (iii) *alpha-Amy2* genes were expressed both early in grain development and also later (35-56days post-anthesis), the peak coinciding with that of the transgene, (iv) *α-Amy1* genes were expressed only at the later time points, again coinciding with the transgene and (v) α-amylase activity was high early in grain development (7-21 days post-anthesis) but with a second peak in mature grain. We therefore concluded that the high levels of α-

amylase in young mature grain was due to the presence of α-Amy2 and α-Amy3 isozymes, probably in the pericarp, while α-amylase in mature grain was due to induction of *α-Amy1* and *α-Amy2* genes late in grain development, in turn induced by high GA levels resulting from ectopic expression of the *GA20ox* transgene within the embryo and scutellum.

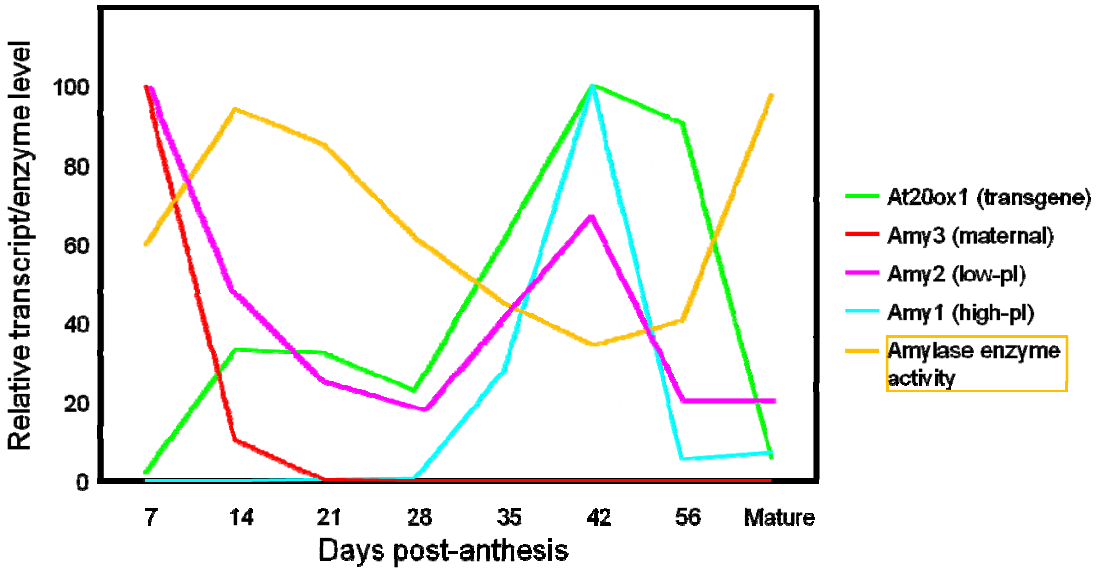


Figure 14. Developmental series of ZmGlb1::GA20ox transgenic lines, showing transcript and α-amylase activity profiles in grain.

Laser capture microdissection.

We originally intended to use laser capture microdissection (LCM) for isolation of specific cell types from immature and mature grain in order to identify the sites of expression of α-amylase and other genes and to isolate tissues for microarrays analysis. We successfully implemented LCM for some tissues of young developing grain but unfortunately we found that the thick cell walls of aleurone cells, a likely target of PMA effects, prevented the laser from excising the cells without substantial heating and tissue damage. We therefore investigated other means to identify the location of α-amylase in grain.

Tissue printing and immunolocalisation.

To identify the tissues of the grain in which α-amylase accumulates during PMA and related syndromes, we established a tissue printing technique to detect α-Amy1 protein in tissue sections. Mature and immature grains were sectioned while frozen using a Cryostat and the sections transferred to nitrocellulose-coated slides to allow protein transfer to take place. alpha-Amylase was detected using a monoclonal antibody to α-Amy1 obtained from SARDI, Adelaide, manufacturers of the α-amylase ELISA kit. The technique was established using germinated mature grain from cv. Cadenza and mature grain from the ZmGlb1:AtGA20ox1 transgenic line. In wild-type tissues of germinated grain after 3 days, α-Amy1 accumulated principally in the scutellum

tissue of the embryo and in the transfer aleurone cells (Figure15A). In ZmGlb1:AtGA20ox1 grains that accumulate α-amylase late in development, α-Amy1 is localised in the aleurone cells, particularly in the cheeks of the grain (Figure15B). This implies that in these transgenic lines, elevated levels of bioactive GA (although yet to be confirmed by analysis) results in premature induction of α-amylase in the aleurone cells, which are not normally active until induced by GA from the embryo and scutellum during germination.

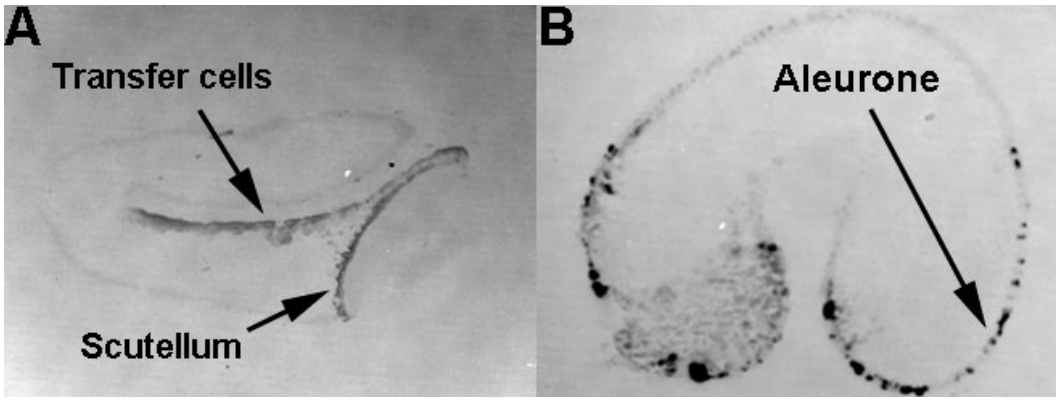


Figure 15. Immunolocalisation of α-Amy1 protein in tissue prints from mature grain. A: germinating Cadenza grain; B: mature grain of transgenic Cadenza carrying the ZmGlb1:At20ox1 transgene.

α-amylase reporter lines

Prior to the start of the project we had generated a small number of transgenic wheat lines containing an*α-Amy1* promoter-reporter construct consisting of the *α-Amy1-18* promoter coupled to the *GUS* gene encoding β-glucuronidase. These were to be used for detection of *α-Amy1* expression during PMA and had been generated by a student in the wheat transformation group. However, on testing of these lines we could find neither GUS activity in germinated grain nor any evidence for the presence of the transgene. We therefore decided to generate new transgenic reporter lines for both *α-Amy1* and *α-Amy2* classes in the PMA sensitive cultivar, Rialto.

Promoter-GFP constructs were prepared using 2kbp promoter fragments from the *α-Amy1-18* and *α-Amy2-54* genes (Huttly and Baulcombe, 1989) and these were introduced into immature embryos of cv. Rialto by microprojectile bombardment. Selection of transgenic shoots was through co-bombardment with a *Bar* herbicide resistance marker gene. A total of 18 *α-Amy1::GFP* and 26 *α-Amy2::GFP* lines were confirmed by PCR.

3.4.3. Induction of PMA in UK varieties

Comparisons between PMA (UK) and LMA (Australia)

Pre-maturity amylase in UK wheats may be synonymous with the syndrome of late-maturity amylase accumulation (LMA) identified in Australian wheat crops (Mares *et al.*, 1994). It is believed

that LMA in the field is induced by exposure to cool temperature during grain development as proposed by Mrva and Mares (2001). These authors developed an induction protocol for LMA using intact plants or detached tillers in temperature-glasshouses or growth rooms. To help assess whether PMA and LMA were identical syndromes we collaborated with Drs Mrva and Mares to test a small number of UK varieties under Australian induction conditions. We used the varieties Huntsman (constitutively high amylase), Rialto (inducible PMA) and Malacca (PMA-resistant) alongside the Australian control varieties Hartog (LMA-resistant control), Cranbrook (inducible LMA) and Spica (constitutive LMA). The induction experiment was carried out in quarantine glasshouses at SARDI, Adelaide using two pots each with two plants for each cultivar. Plants were grown at 25°C, spikes tagged at flowering and cool treatments (18°C) begun at 26 days after anthesis. Mature grains were harvested and tested using the α-Amy1 ELISA test. Amylase levels, averaged across 2 plants and 8 grains per spike, are shown in Figure 16.

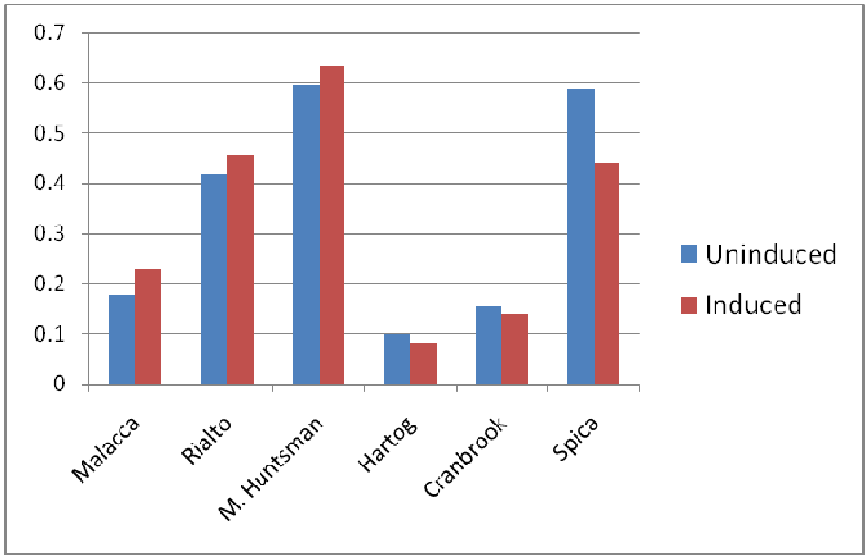


Figure 16. Amy1 protein detected by ELISA in mature grain of LMA-induced wheat varieties by Mrva and Mares (Adelaide)

This result was somewhat disappointing as although susceptible varieties accumulated more α-amylase than resistant varieties, it appeared that the inductive conditions used had little effect on α-amylase levels, even for Australian varieties. Thus while PMA/LMA-resistant varieties (Malacca and Hartog) had low levels of α-Amy1 protein and constitutive varieties (Spica and M. Huntsman) had high levels, there was no evidence of cold-induction of amylase in the susceptible varieties, Cranbrook and Rialto. This experiment did not, therefore, allow us to draw any conclusions about the inductive mechanisms in LMA or PMA.

Induction and assay of PMA

During the first 18 months of the project, a PMA induction protocol adapted for UK varieties was developed by Aiden Farrell and Peter Kettlewell at HAUC (see Work Package 2 above). Initially we analysed induced material supplied by HAUC and subsequently established the PMA induction protocol at Rothamsted Research. Mrva and Mares (2001) have reported that occurrence of LMA in sensitive cultivars is extremely stochastic, with variation in the presence and amount of α-amylase detected between ears and between individual grains. To investigate this in UK PMA-sensitive material we carried out a joint experiment with Harper Adams University College in which we measured the total α-amylase activity in individual grains of eight ears from induced (cold-treated) and uninduced plants, noting the position of each grain within the ear; the primary and secondary grains from the six central spikelets on one side of each ear were sampled. The Phadebas assay was used as this gave a greater dynamic range than the α-Amy1 ELISA test.

The raw data shows that treatment by cool temperature results in induction of a relatively small number of grains to high α-amylase levels, with a larger number of grains having more moderate increases in amylase. Thus, approximately one-third of grains were unaffected by cold induction, 40% had α-amylase levels increased by up to five-fold, while 25% of grains were induced by between five and one hundred fold over uninduced controls, mirroring the stochastic behaviour seen in LMA (Mrva and Mares, 2001). Plotting the distribution of PMA grains within the ear (Figure 17) suggests that upper and middle spikelet positions express higher levels of PMA than lower positions. However, given that only eight ears were sampled and that the data are heavily influenced by the small number of grains with extremely high α-amylase levels (as illustrated by the large error bars), any conclusion must be tentative.

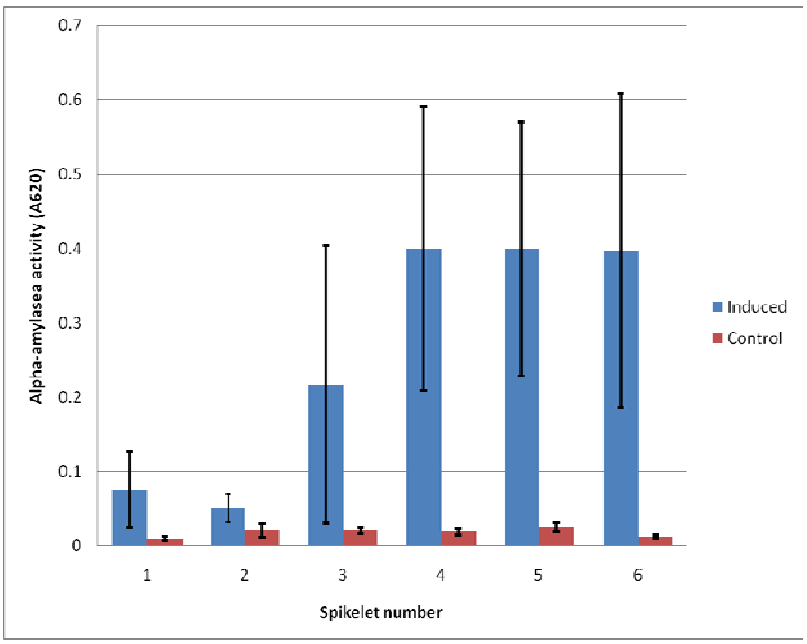


Figure 17. PMA induction in Rialto; α-amylase activity in different spikelet positions.

We next carried out two separate PMA induction experiments at Rothamsted Research using, in Experiment 1 (2008), cvs. Maris Huntsman (constitutive PMA) and Rialto (inducible PMA) and, in Experiment 2 (2009), cvs. Rialto and Spark (PMA resistant). Each experiment consisted of 30 plants of each cultivar in individual pots; the conditions used were as developed by Aidan Farrell at Harper Adams University College in Work Package 2 above, except that plants were vernalised, grown to anthesis in the glasshouse and then transferred to controlled environment at 23°C for PMA induction at 24 days post-anthesis (i.e. at 550 degree-days (Figure 18). As we also wanted to explore the timing of α-amylase accumulation, samples of developing grain were also collected at 32days post-anthesis and 40days post-anthesis, after the cold induction (28days post-anthesis and 36days post-anthesis for uninduced samples to allow for faster growth at 23°C). No sprouting was observed in any grain samples induced under these conditions.

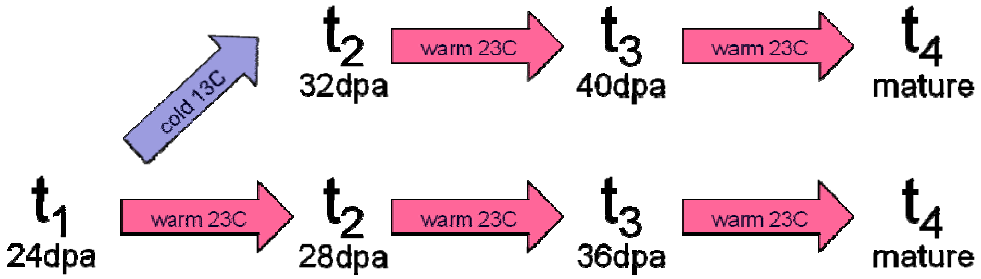


Figure 18. Induction of pre-maturity α-amylase (PMA) in controlled environment (sampling times: t_1=pre-induction; t_2=induction ; t_3=ripening; t_4=maturity).

All grain sampled were bisected and stored frozen as distal halves for α-amylase assay and proximal halves (with embryo) for RNA isolation or tissue printing. Both induction experiments were successful as indicated by α-amylase content of mature grain (Figure 19): M. Huntsman had very high levels of α-amylase even in the absence of induction, and this was further elevated by the cold treatment; Rialto had low α-amylase levels in control grain but higher levels in induced grain, while Spark had low levels and responded only weakly to cold treatment. The two experiments yielded somewhat different extents of PMA: although in both years α-amylase was induced in Rialto by the cold treatment, the level of enzyme activity observed in both control and induced was much higher in 2009 than in 2008, although the experiments had been carried out in exactly the same way and at approximately the same time of year. Similar variation in the extent of PMA induced has also been observed by Aidan Farrell and Peter Kettlewell at Harper Adams.

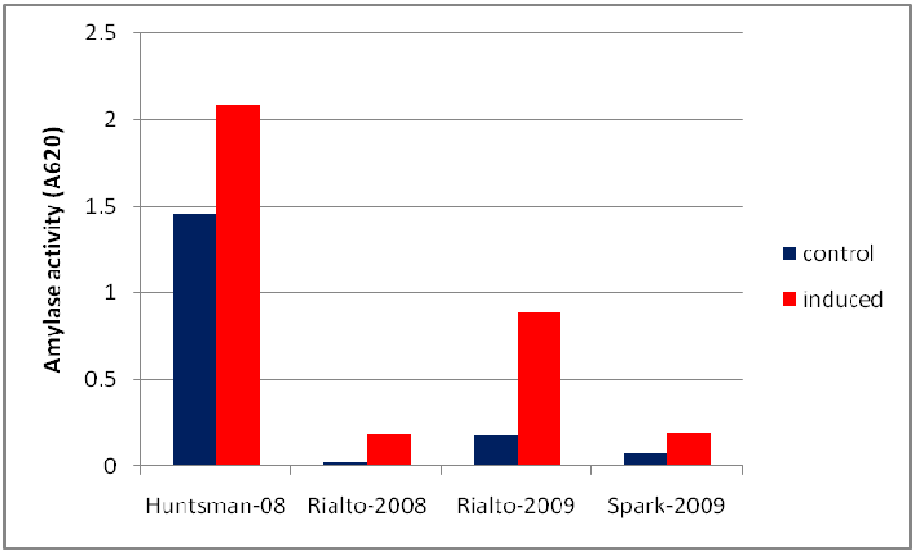


Figure 19. Pre-maturity amylase induction by cool temperatures at 24days post-anthesis in three varieties.

Again the stochastic nature of PMA is illustrated by the plot in Figure 20, which shows individual grains from Experiment 1 ranked for α-amylase content of the distal halves, with induced grain ranked from left to right and control from right to left. This random nature of PMA, and the relatively few grains with high levels of α-amylase activity, has implications for further work on the timing and pathway of α-amylase induction – as few grains appear to be involved, and these can only be identified at maturity, selecting immature grains for further study is difficult as effects in those grains responding to cold shock may be diluted by the majority that do not respond by producing α-amylase.

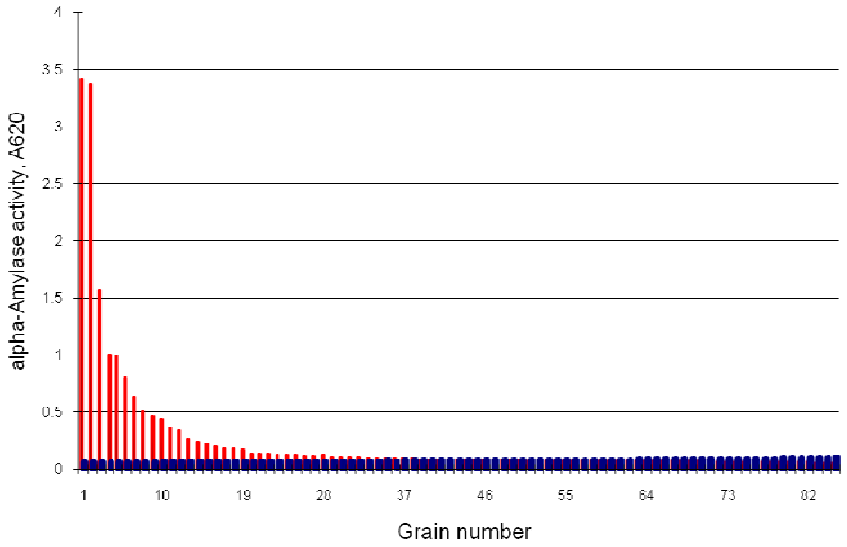


Figure 20.A-amylase activity of individualinduced (red) and control (blue) grains of Rialto (2008)

Location of α-amylase in PMA-affected grain

Having established the technique of tissue printing for immunolocalisation of α-Amy1 protein within transgenic high-amylase lines we used this to investigate the location of α-amylase in PMA-affected grain of different cultivars. We used the proximal halves of grain from the cold induction experiments described above, selecting grain with high levels of α-amylase in the corresponding distal half assayed above. Uninduced grain of Rialto and both induced and uninduced grain of Spark had uniformly low levels of amylase and no signals could be detected on tissue prints.

A large number of tissue prints to identify sites of α-amylase accumulation were carried out of which a representative set are shown in Figure 21. Uninduced grains of M. Huntsman contained high levels of α-amylase which was generally confined to the region of the crease aleurone cells surrounding the central cavity. There was no evidence of α-Amy1 protein in the peripheral aleurone cells of most sections. Tissue prints of high-amylase grain from M. Huntsman plants that had been induced by cold treatment also showed α-Amy1 protein in the crease region, but also extensive cross-reacting protein in the dorsal aleurone layers and often extending into the cheeks of the grain, presumably due to diffusion of secreted enzyme from the aleurone cells into the endosperm. These grains often had substantial damage to their internal structure, implying that cell wall hydrolases may also have been active. PMA-induced grains of cv. Rialto similarly contained cross-reacting α-Amy1 protein in the dorsal aleurone layer, in the adjacent endosperm tissue and also in the tissues around the central cavity. Grains from Experiment 2 in 2009 that had very high α-amylase levels also had tissue damage in the endosperm, particularly in the cheeks of the grain. A small sample of Rialto grain affected by PMA under field conditions was obtained from Peter Kettlewell at HAUC: α-Amy1 protein could be detected in only a few grains, and this was confined to the peripheral aleurone layer (Figure 21).

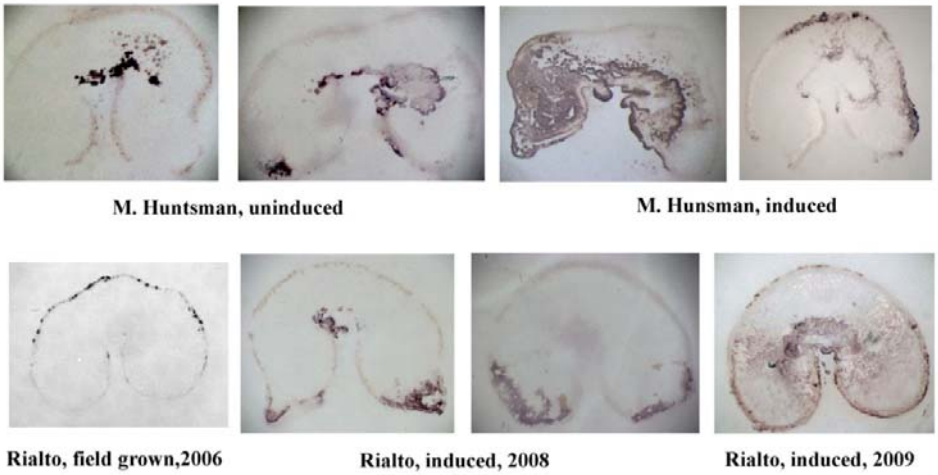


Figure21. Tissue prints of mature grain probed with antibodies to Amy1

We concluded from this that the constitutive high levels of α-amylase seen in cv. M. Huntsman is qualitatively different from that in induced PMA seen in Rialto and other cultivars, in that the α-Amy1 protein detected in uninduced M. Huntsman is confined to the region surrounding the cavity of the grain whereas that in PMA-affected grain is largely associated with the peripheral aleurone layer. This may explain contradictions between observations of LMA by Mrva and Mares (2006), who associated α-amylase production with cell death in the peripheral aleurone, and earlier UK studies carried out using grain printing on Phadebas starch gels (Greenwell *et al.*, 2001) which identified α-amylase activity only in the cavity region of Brigadier, a cultivar that contains Maris Huntsman in its pedigree.

Transcript changes following induction of PMA

The developing and mature grain collected in the two PMA induction experiments above were used to investigate changes in transcript abundance associated with α-amylase accumulation. Total RNA was extracted from the pools of 16 half-grain and use to generate probes for Affymetrix microarray hybridisation; the data were generated at Univ. Bristol and analysed at RRes in Genespring and dChip. Most analysis has been carried on Experiment 2, as this involved both sensitive (Rialto) and resistant (Spark) cultivars. Unsurprisingly, the strongest single effect was grain maturation, with more than 9000 transcripts affected when comparing immature (28 days post-anthesis) grain with mature grain. Particularly strong effects were also identified when comparing cold-induced and control grains in the Rialto background: for example, 391 transcripts were significantly ($p<0.05$) changed in abundance in grain immediately after the cold treatment – 301 up-regulated and 90 down-regulated. In the Spark cultivar, 251 genes were altered in expression by the cold treatment and the scale of induction or repression was generally lower than in Rialto. Similarly, induction of Rialto at 24days post-anthesis resulted in 67 genes changed in abundance in mature grain while cold treatment of Spark resulted in changes in 37 genes in mature grain.

Interactions between cultivar, treatment (+/- cold induction) and sampling time point were investigated using 3-way ANOVA. Having identified 153 probesets (genes) that showed significant interactions in the ANOVA analysis, these were ranked in terms of the fold change between the different samples in order to identify the major effects. There was a strong interaction between cold treatment and cultivar at timepoint 2 (immediately after induction) and at timepoint 3 (8 days later; see Figure 18). Many of the genes highlighted in this interaction encoded enzymes of carbon metabolism, including several plastid components. Plotting the data for all samples suggests that the main effect is a reduced expression of these components in control (uninduced) grains of Rialto (Figure 22); however, inspection of the equivalent data from Experiment 1 (for cv. Rialto only) which included a pre-induction time point (t_1, see Figure 18), revealed that cold treatment actually promotes increased expression of the genes while warm temperature reduces expression (not

shown). While the significance of the effect on plastid components is not immediately apparent, it illustrates the difference in response to cold treatment between Rialto and Spark, a recurrent theme in the data that suggests that PMA induction in Rialto may relate to the temperature sensitivity of this variety. Investigating this hypothesis will require further work on a wider range of PMA-sensitive and -resistant varieties.

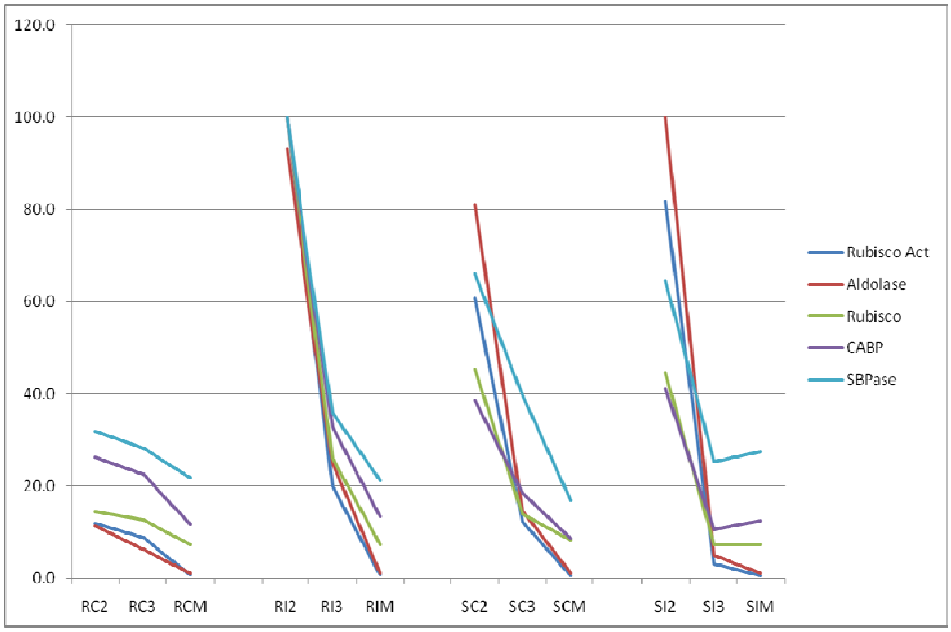


Figure 22. Differential response of Rialto and Spark to cold: plastid components. (R: Rialto, S: Spark, C: Control, I: Induced, 2: Timepoint2, 3: Timepoint3, M: Mature grain.

A second, highly significant set of genes highlighted by the ANOVA analysis of Experiment 2 encode α-amylases of the Amy1 class. These genes are more highly expressed in induced grains of Rialto at timepoint 2 (post-induction) than control grains – see Figure 23 (left panel). However, grain samples from Rialto also show high levels of α-Amy1 genes expression at Timepoint 2, whether induced or not. To investigate this we analysed further microarray data from Experiment 1 (Figure 23, right panel). In contrast to the 2008 experiment, the 2009 data showed an opposite effect, with low levels of *α-Amy1* transcript before and immediately after cold induction, and rising in mature grain. While it is possible that these different patterns of α-amylase gene expression are due to the stochastic behaviour of PMA, in which only a small proportion of grain are affected, this is unlikely as the experimental replicates (independent pools of 16 grain from multiple individual plants) showed good correlation. The difference in the behaviour of α-Amy genes in the two experiments therefore remains unexplained, although one hypothesis is that any differences in the watering regime between the two experiments could affect the PMA response, as water deficit has been previously shown to reduce amylase levels in grain (Gooding *et al.*, 2003).

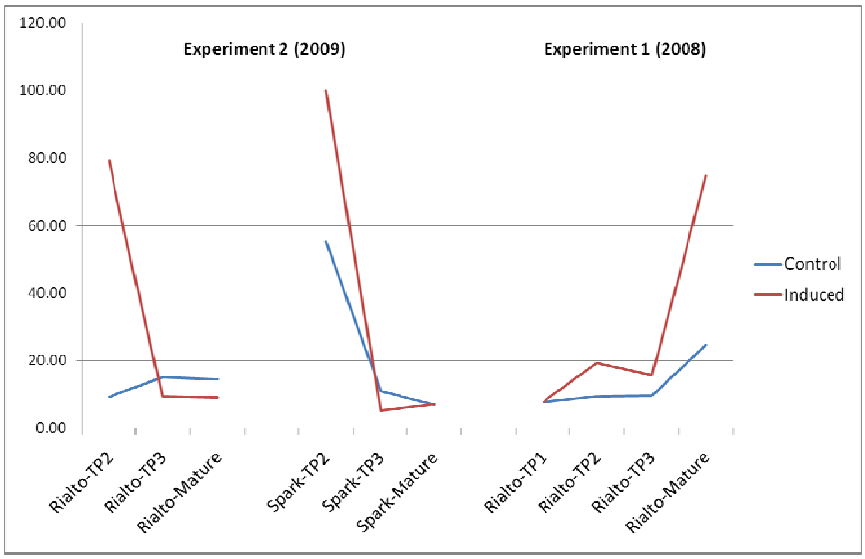


Figure 23. α-Amy1 gene expression in Rialto and Spark following cold induction. TP=timepoint.

We therefore are presented with two possible mechanisms for α-amylase accumulation in Rialto grains induced to under PMA. The data from 2008 suggests that cold treatment of plants at 24 days post-anthesis does not result in immediate increases in α-amylase message, but this accumulates later in development to result in high amylase levels in grain. In contrast, the data from 2009 suggests that α-Amy1 transcripts appear immediately after cold induction. As these high levels immediately decline, this implies α-Amy1 protein must persist through to mature grain. However, similar behaviour was observed in the Spark samples, which have little detectable α-amylase at maturity.

In order to resolve this conflict between the two sets of data, we have decided to repeat the induction experiment using the transgenic marker lines of Rialto generated within this project. These contain both α-Amy1 and α-Amy2 promoter-GFP constructs which will allow the induction and localisation of the α-amylase genes to be studied. As the same time, samples will be taken for QRT-PCR analysis of α-amylase transcripts. This work will be carried out within an HGCA-funded follow-on studentship between Harper Adams University College and Rothamsted Research.

PMA and hormone signalling

As PMA appears to involve inappropriate activation of aleurone responses, which are normally controlled by the GA and ABA hormones during germination, we investigated changes in these pathways through both transcript and hormone analysis. For the GA pathway, a total of nine genes encoding enzymes of GA biosynthesis, inactivation or signalling components were represented on the Affymetrix Genechip, and thirteen genes involved in ABA biosynthetic and inactivation. However, no significant differences were detected in any of these genes in response to PMA induction, in either Experiment 1 or Experiment 2. In a collaborative experiment with Aidan Farrell

at HAUC, GA and ABA hormone levels were also measured in grain immediately after cold induction, but again no consistent pattern of differences in hormone levels was observed.

These results suggest that induction of PMA does not involve large scale changes in GA or ABA metabolism in grain, but do not exclude the possibility of involvement of these hormones. As discussed above, only a small number of grains that undergo cold shock respond by developing high levels of α-amylase, so it is likely that changes in hormone levels, possibly localised to specific areas of the grain, are diluted by the majority of grains that fail to respond. Indeed, the observation that semi-dwarfing genes such at *Rht-D1b* provide protection against PMA (Mrva and Mares, 1996) suggests that GA signalling in the aleurone layer plays a key role in the accumulation of α-amylase.

α-Amylase promoter-GFP reporter lines

To identify suitable transgenic lines for the study of PMA, seeds of all lines were germinated and sections examined under a fluorescence microscope. Lines of α-Amy1-GFP and α-Amy2-GFP were identified in which GFP was clearly expressed in the scutellum and aleurone layer of germinating grain (Figure 24). These lines became available too late to include within the PMA induction experiments preformed within the project, but will be invaluable for the study of PMA induction. We aim to carry out induction experiments with these lines within the HGCA-funded HAUC-RRes joint studentship project that aims to study in more detail the role of GA and ABA in PMA.

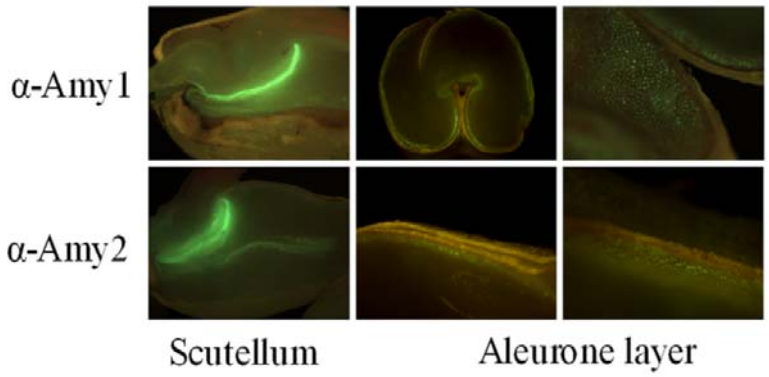


Figure 24. α-Amy1-GFP and α-Amy2-GFP reporter lines of wheat cv. Rialto showing expression during seed germination.

3.4.4. Conclusions

- PMA is characterised by the production of α-amylase enzyme in the aleurone layer of grain in the absence of germination.
- As in the LMA syndrome identified in Australian varieties, PMA is an extremely stochastic process, with a small number of grains in induced ears containing high levels of α-amylase.
- In Maris Huntsman, a variety with "constitutive" PMA, amylase is mainly associated with the transfer aleurone in the crease region of the grain. In Rialto, a variety with inducible PMA, the amylase enzyme is mainly found in the peripheral aleurone in the cheeks and dorsal surface of the grain. This suggests that different PMA syndromes exist in the two varieties.
- PMA symptoms can be reproduced in variety Cadenza by the overexpression of *GA20ox* in developing grain, presumably through increases in the levels of bioactive gibberellin.
- However, transcript analysis of candidate genes in the GA biosynthetic pathway did not reveal any change in expression during the induction and appearance of cold-induced PMA.
- Transcript profiling by microarrays identified differences in the cold response of PMA-susceptible (Rialto) and resistant (Spark) varieties. Further work will be required to examine whether such differences in response determine sensitivity to PMA.

3.5. Work Package 4: Identifying candidate genes for increased Hagberg Falling Number (HFN) stability in wheat using a post-genomics comparative approach.

Authors: M Holdsworth, S May, T Gerjets (NASC University of Nottingham), TBarsby,J Wilmer, F Torney (Biogemma), A Phillips,P Hedden, I Prosser, A Huttly(Rothamsted Research), J Snape, J Flintham, J Simmonds(John Innes Centre)

3.5.1. Aims of the work package

Candidate wheat genes with possible roles in regulating PHS/PMA will be identified from previous investigations of model systems using sequence similarity and comparative transcriptome analyses. Possible roles for these candidates will be defined via transgenesis, mapping through synteny with rice, analysis of existing and novel variation in wheat and transcriptome analysis. Definition of useful candidate genes will provide a valuable resource for plant breeders to develop marker-assisted breeding strategies to increase stability of HFN.

3.5.2. Hypotheses

- That information from previous molecular studies of model systems can be used to define a set of candidate genes with possible roles in regulating PHS/PMA.
- That the candidate gene list can be further refined using a variety of criteria related to functionality in wheat, to provide a core set.
- That co-alignment between candidate gene polymorphisms and QTLs can be used to develop gene-based diagnostics for direct selection of elite germplasm with stable HFN.

3.5.3. Hypothesis 1: Information from previous molecular studies of model systems can be used to define a set of candidate genes with possible roles in regulating PHS/PMA

Prior information was used to prioritise sets of candidate genes associated with ABA and GA biology, considered to have the highest probability of identifying useful markers for PMA and PHS (Table 2).

Table 2. Possible candidate genes for analysis in wheat.

Gene Name	Accession number	Other information	Activator or Repressor of Germination	Wheat similar sequence	Other notes
ABH1	AT2G13540	ABA signalling	Activator	TC238043	General effects on plant growth/development
ABI3	AT3G24650	ABA signalling	Repressor	vp1A etc	Holdsworth Group work on transgenics shows potential
ABI5	AT2G36270	ABA signalling	Repressor	AF519803 (TaABFA) and AF519804 (TaABFB)	Model to crop work shows potential
AtGA3ox1 (GA4)	AT1G15550	GA Biosynthesis	Activator	BQ805724	Wheat orthologue expressed during grain development and germination
AtGA3ox2	At1g80340	GA Biosynthesis	Activator		General effects on plant growth/ development
AtGA2ox3	At1g34500	GA Biosynthesis	Repressor	CN013015	Over-expression in dev. grain decreases amylase levels
AtGA20ox3	At5g07200	GA Biosynthesis	Activator	BE422495	Over-expression in dev. grain increases amylase levels
CTS	AT4G39850	Metabolism/Signalling	Activator	CA499624	Two genes in wheat
GAMYB	AY008692	GA signalling	Activator	AY615200	Detailed genetic/molecular work shows potential
CYP707A	At2g29090	ABA Catabolism	Activator	-	
OsWRKY71	BK005074	Amylase expression	Repressor	TC265823	
PKABA1	M94726	ABA signalling	Assumed repressor	M94726	
Red grain colour	Not yet available	Pericarp, Maternal Effect	Repressor	-	Partial wheat sequence from Japan (in press)
SAD1	AT5G48870	ABA signalling	Activator	TC237847	General effects on plant growth/development

3.5.4. Hypothesis 2: Candidate gene list can be further refined using a variety of criteria related to functionality in wheat, to provide a core set.

Analysis of candidate genes, definition of gene-specific primer sets: (UoN, RR, Biogemma)

Candidate genes associated with ABA biology

Using the rice genome sequence candidate gene, information from Arabidopsis was used to identify possible cereal sequelogues. This proved possible for only two candidate genes, AIP2 and ABH1 (Table 1). Other sequelogues were not found to be appropriate due to gene families or insufficient sequence homology. Rice gene structures were used to predict intron/exon boundaries in wheat for TaABH1 and TaAIP2, in combination with analysis of EST sequence data.This analysis allowed design of informative molecular markers.

Candidate genes associated with GA biology

At the start of the project we identified and prioritised candidate genes from the GA biosynthetic and signalling pathways. Previous work at RRes on the GA biosynthetic and signalling pathways in Arabidopsis and wheat had identified four key candidate genes in addition to *Rht*. From the biosynthetic pathway, GA20ox activity has been shown by overexpression to be limiting for bioactive GA levels in Arabidopsis and other species (Coles *et al.*, 1999; Vidal *et al.*, 2001), and is subject to an array of regulatory controls including feedback, developmental and environmental modulation of gene expression (Yamaguchi, 2008). GA20ox is encoded by at least four paralogous genes in wheat, but previous work has suggested that *GA20ox1* and *GA20ox3* were the principal forms expressed in developing grain (Evans, 2005). *GID1* encodes the GA receptor, and loss-of-function mutations in rice result in an extreme dwarf phenotype and in the production of seeds in which GA-dependent α-amylase production is completely abolished (Ueguchi-Tanaka *et al.*, 2005); we hypothesised that partial mutations would result in a semidwarf phenotype with reduced amylase production. G*AMyb* encodes a transcription factor that has been shown to be directly involved in GA induction of α-amylase in the barley aleurone layer (Gubler *et al.*, 1995; Zentella *et al.*, 2002); a loss-of-function mutation in the rice orthologue results in loss of α-amylase expression by the aleurone cells, and also impairs pollen development (Kaneko *et al.*, 2004).

These four candidate genes were targeted for further analysis and development of gene-specific primers sets for genetic mapping and expression analysis. However, after the start of the HFN project, RRes and JIC were awarded a grant from the BBSRC Crop Science Initiative, in which we proposed to map in wheat as many of the GA genes as possible in order to identify candidate genes controlling stature. The GA gene mapping objectives within the HFN and CSI projects were therefore merged to allow significantly greater progress than would otherwise have been possible.

Initial work on the four GA candidate genes described above was carried out by Biogemma. Briefly, BLAST searches were carried out using Arabidopsis and/or rice orthologues of the target genes to identify wheat candidates within EST databases. Further BLAST searches were carried out within other cereals to identify paralogous groups to enable identification of the likely wheat orthologue through a combination of phylogenetic analysis and comparison of likely syntenic mapping positions within the cereal genomes. Finally, candidate EST assemblies were aligned with the rice gene to identify the likely locations of introns and to allow the design of paralogue- specific primers. These primers were used to amplify homoeologous sequences from genomic DNA of wheat progenitors: *T. urartu* (A genome), *Ae. speltoides* (B genome) and *Ae. tauschii* (D genome); *T. turgidum durum* (A and B genomes) was also used in some cases where amplification from *Ae. speltoides* proved difficult. The amplified genomic fragments were sequenced and the primers and DNA sequences passed on to the consortium.

For identification of further genes from the GA biosynthetic pathway at RRes, a similar strategy was used except that *T. monococcum* was used as the source of A genome sequences. In additional to the four candidate genes described above, wheat sequences were also identified for *CPS*, *KS*, *KO*, *KAO*, *GA20ox2*, *GA20ox4*, *GA3ox1*, *GA3ox2*, *GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox5*, *GA2ox6*, *EUI* and *GID2*. All sequences will be made available to the project partners on the final project DVD.

Analysis of expression patterns (University of Nottingham, Rothamsted Research)

Global gene expression associated with wheat embryo dormancy and germination (University of Nottingham)

Transcriptome analysis was carried out on Affymetrix wheat arrays using RNA derived from dormant and germinating embryos of the varieties Option and Claire. RNA was extracted from three replicates for two time points, 8h and 20h imbibition, representing pre and post-germination samples in after-ripened seed. Samples were specifically taken using only embryo material (Figure 25).

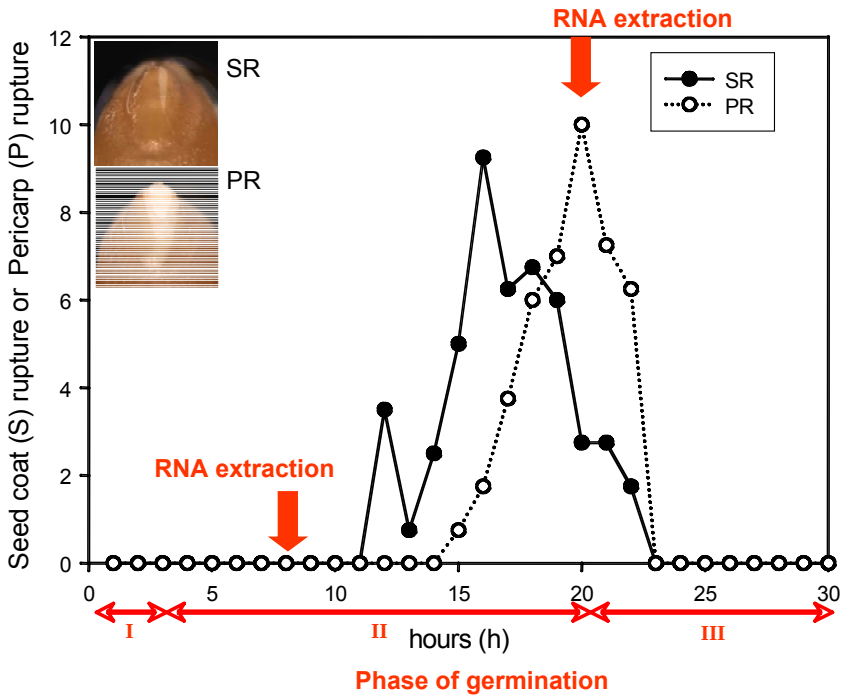


Figure 25.Germination time course for variety Option, indicating numbers of seed showing coat or pericarp rupture during imbibition, phase of germination and time points used for transcriptome analysis

Transcriptome analysis was carried out using the Affymetrix GeneChip® Wheat Genome Array. Transcriptome data was analysed using Genespring software. Principal Component Analysis (PCA) was carried out to analyse the relationships between genome expression at different time points and in different physiological states (dormant or after-ripened) (Figure 26). This analysis revealed that dormant transcriptomes at 8 and 20h imbibition are very similar, and similar to that of after-ripened embryos at 8h, but that the transcriptome of after-ripened seeds rapidly changes as germination proceeds. Analysis of the numbers of differentially-expressed genes during germination (Table 3) shows that the greatest change in genome expression occurs between 8 and 20h imbibition of after-ripened embryos, concomitant with the initiation of germination. Analysis of genome expression in dormant and after-ripened embryos at 8h imbibition demonstrates differential expression of several regulatory proteins (Table 4).

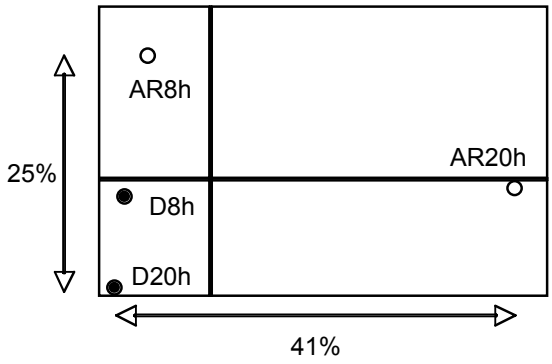


Figure 26. Principal Component Analysis (PCA) of variety Option transcriptome datasets derived from RNA extracted from imbibed embryos as indicated. AR, after-ripened; D, dormant freshly harvested. The first two components accounted for 66% of the variance (41% for the first and 25%, respectively).

Table 3. Numbers of differentially-expressed genes in comparisons of embryo samples from dormant or after-ripened seeds of variety Option. AR, after-ripened; D, dormant freshly harvested.

Comparison	Description:	Number of differentially UP-regulated genes
AR8h > D8h	Gene list showing transcripts >2 fold up-regulated in 8h imbibed AR seeds compared to 8h imbibed D seeds	1382
D8h > AR8h	Gene list showing transcripts >2 fold up-regulated in 8h imbibed D seeds compared to 8h imbibed AR seeds	995
AR8h > AR20h	Gene list showing transcripts >2 fold up-regulated in 8h imbibed AR seeds compared to 20h imbibed AR seeds	3329
AR20h > AR8h	Gene list showing transcripts >2 fold up-regulated in 20h imbibed AR seeds compared to 8h imbibed AR seeds	4968
D8h > D20h	Gene list showing transcripts >2 fold up-regulated in 8h imbibed D seeds compared to 20h imbibed D seeds	752
D20h > D8h	Gene list showing transcripts >2 fold up-regulated in 20h imbibed D seeds compared to 8h imbibed D seeds	834

Table 4. Example regulatory proteins differentially expressed in 8h imbibed dormant and after-ripened embryos of variety Option.

Rank	Fold change	Closest Arabidopsis gene	Arabidopsis description	Biological process
Genes Up-regulated in 8h imbibed after-ripened seed compared to 8h imbibed dormant seed				
4	21	AT5G51990	CBF4/DREB1D (C- REPEAT-BINDING FACTOR 4)	ABA
7	19	AT4G25420	GA5 (GA REQUIRING 5)	Gibberellin
15	15	AT5G24930	zinc finger (B-box type) family protein	
30	14	AT1G65680	ATEXPB2	Cell-wall modification
49	12	AT3G47450	ATNOS1/NOS1; nitric-oxide synthase	Stress
429	5	AT5G45340	CYP707A3 ABA degradation	ABA
532	4	AT1G47480	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G48700.1); similar to CXE carboxylesterase [Malus pumila]	Gibberellin
Genes Up-regulated in 8h imbibed dormant seed compared to 8h imbibed after-ripened seed				
45	9	AT1G55080	similar to PFT1 (PHYTOCHROME AND FLOWERING TIME 1)	Germination related
378	3	AT5G17230	PSY (PHYTOENE SYNTHASE)	ABA

It is likely that differential gene expression between embryos in Option and Claire results in the different phenotypes of these two varieties (dormant or non-dormant at harvest). Therefore in collaboration with Dr Gary Barker (University of Bristol), genes differentially expressed between the varieties Option and Claire were investigated by comparing transcriptome datasets for dormant and after-ripened embryos at both 8 and 20h imbibition. This analysis revealed a number of genes consistently differentially regulated between the two varieties at all stages of development (Table 5). For several of these genes it was also possible to allocate a chromosomal location from available wheat EST mapping data, and for some genes these locations correspond to known locations for QTL associated with seed dormancy and PHS. In these cases these genes may be useful candidates for further analysis in relation to development of PHS-related molecular markers.

Table 5. Example genes significantly (P value < 0.05) differentially regulated between varieties Option and Claire at 8 and 20 h imbibition. Chromosomal locations are indicated.

Gene:		Expression levels (Log_2 values):				Chromosomal Location:
		Variety Claire		Variety Option		
Affymetrix ID number:	Description:	8h	20h	8h	20h	
Ta.9397.1.S1_at		-0.23	-0.24	3.33	0.10	5BS
Ta.26080.2.A1_at		-0.25	-0.25	-0.03	0.34	1BL, 2DS, 2DL
Ta.2804.3.S1_s_at		0.00	-0.05	0.00	0.17	5AL,
Ta.7996.3.S1_x_at		0.06	-0.02	-0.03	0.11	5DL
Ta.10089.3.S1_a_at		-0.15	-0.16	-0.11	0.00	4AL, 6BS
TaAffx.46757.1.A1_at		-0.03	-0.07	0.09	0.27	2BL
Ta.2401.2.A1_at		-0.05	-0.14	-0.09	0.03	1AL, 1BS, 2BL

Analysis of the expression patterns of GA biosynthetic and signalling genes (Rothamsted Research)
A number of approaches have been used to investigate expression patterns of GA biosynthetic and signalling genes in order to prioritise candidates for mapping and for manipulation of expression in transgenic plants. Assembly of EST and genomic sequences has enabled us to develop paralogue-specific primers for real-time RT-PCR studies, and we have also used both Affymetrix microarrays and next-generation RNA sequencing to identify grain-, embryo- and endosperm-specific transcripts. Together, these approaches showed that *CPS*, *KS*, *KO*, *KAO*, *GA20ox1*, *GA20ox3*,*GA3ox1*, *GA3ox2*, *GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox5*, *GA2ox6*, *EUI*, *GID1*, *GID2* and *Rht* were all expressed in developing grain. Thus, of the candidate transcripts examined, only *GA20ox2* and *GA20ox4* have not been detected in grain.

Transgenics (Biogemma)
Biogemma, who provided the wheat transformation capability for the project, agreed to generate constructs and transgenic lines for two candidate genes for suppression via RNAi. For the GA pathway, the *GID1* gene was identified as a suitable target for as evidence suggests that it is encoded by a single homoeologous series in wheat (as *Rht*, whose semi-dwarfing alleles provide protection against PMA) and tissue specificity could be provided through the use of appropriate promoters. As with the ABA target, *ABH1*, the promoters used were rice Actin (constitutive) and Maize VP1; the latter is expressed in bothembryo and aleurone tissue in developing and germinating grain, the most likely sources of α-amylase production in both PHS and PMA. Both constructs were successfully produced by Biogemma.

Due to the closure of Biogemma's UK operations in Cambridge and the relocation of their wheat transformation capability, the production of transgenic wheat containing these constructs was substantially delayed. However, a total of 29 T_0 transgenic plants were produced with the OsActin::GID1 construct and transgene copy numbers determined. A single event was obtained with the ZmVP1::GID1 construct.

3.5.5. Hypothesis 3: Co-alignment between candidate gene polymorphisms and QTLs can be used to develop gene-based diagnostics for direct selection of elite germplasm with stable HFN

Molecular diversity of candidates in wheat (Rothamsted Research)
As described above, a combination of BLAST searches of EST databases and PCR from genomic DNA of wheat progenitor species identified a number of candidate orthologues of rice and Arabidopsis genes from the GA pathway. In some cases, these were further extended by RACE, genome walking and, since January 2010, by searching wheat genomic sequence data generated by the BBSRC-funded Wheat SNP Project. The number of paralogous genes identified encoding each biosynthetic enzyme or signalling component varied from one to five, with genes encoding 2-

oxoglutarate-dependent dioxygenases from the latter half of the GA biosynthetic pathway comprising the largest paralogous families. Comparison with the corresponding gene families in sequenced grass genomes such as rice (Sakamoto *et al.*, 2004; Lo *et al.*, 2008) and Brachypodium (Vogel *et al.*, 2010) suggested that for most components representative (if not necessarily full-length) sequence data had been identified for all paralogues. However, in the case of the GA2ox enzymes involved in GA turnover, only five of the 10-11 paralogues have been identified in wheat. Similarly, while in some cases (e.g.*GA20ox1*, *GA3ox2*, *GA2ox3*) sequence data for all three homoeologues has been obtained, while for others while there is polymorphism between aligned sequence reads it is not possible to assign these to particular homoeologous groups.

Definition of genome position using synteny and relationship to defined QTLs for dormancy/PHS (University of Nottinrham John Innes Centre, Biogemma)

Although information on gross synteny between rice and wheat allowed approximate locations for the above GA components to be identified, it was decided that more accurate mapping data would allow better comparisons between candidate gene locations and QTLs for PHS and PMA sensitivity. Conserved orthologous set (COS) markers are derived from conserved single copy genes and have been used to map orthologous genes across divergent grass species Commonly, COS markers are derived from introns as these are generally conserved in position within orthologous genes but polymorphic in sequence. COS markers were therefore designed from the consensus sequences of the GA biosynthetic and signalling genes described above, using conserved intron positions from rice where only EST data was available in wheat. Note that due to uncertainty in the identification of true orthologous genes between wheat and rice within multigene families, the COS markers were designed to distinguish between paralogous genes within wheat and no attempt was made to ensure amplification from other grass species.

Single-strand conformation polymorphism (SSCP), an electrophoretic technique that distinguishes between DNA fragments on the basis of both length and sequence polymorphism, was used to develop markers. Although gene-specific amplicons from hexaploid wheat contain up to three homoeologous sequences these can be successfully separated by SSCP. This was used initially to screen genomic DNA from parents of three mapping populations: Avalon x Cadenza, Spark x Rialto and Opata M85 x Synthetic W-7984 (ITMI reference population). A total of 48 primer pairs across 16 genes were tested; 33 pairs yielded products and 21 pairs gave detectable polymorphisms in one or more of the three pairs of parental lines. In some cases it was necessary to develop amplicons from multiple introns in order to identify polymorphisms, and for *GID2* and *CPS2* it was necessary to test additional parents.

Having identified suitable parental polymorphisms for most of the target genes, SSCP was used to screen doubled haploid or (for OxS) recombinant inbred mapping populations. SSCP results were

scored and genetic maps generated. In most cases only one of three homoeologues was mapped as the other two did not yield SSCP-detectable polymorphisms in the parental lines. The gene mapped successfully were *CPS*, *KS*, *GA20ox2*, *GA3ox1*, *GA3ox2*, *GA2ox1*, *GA2ox3*, *GA2ox6*, *GID1* and *GAMyb* (Figure 27); locations for *GA20ox1* and *KAO* have been reported elsewhere (Appleford *et al.*, 2006; Khlestkina *et al.*, 2010).All target genes except *CPS* agreed with published locations of rice orthologues (Sakamoto *et al.*, 2004; Lo *et al.*, 2008) and with rice-wheat synteny data (Singh *et al.*, 2007). A table of primers and flanking markers of mapped genomic products will be made available to all partners in the final project CD.

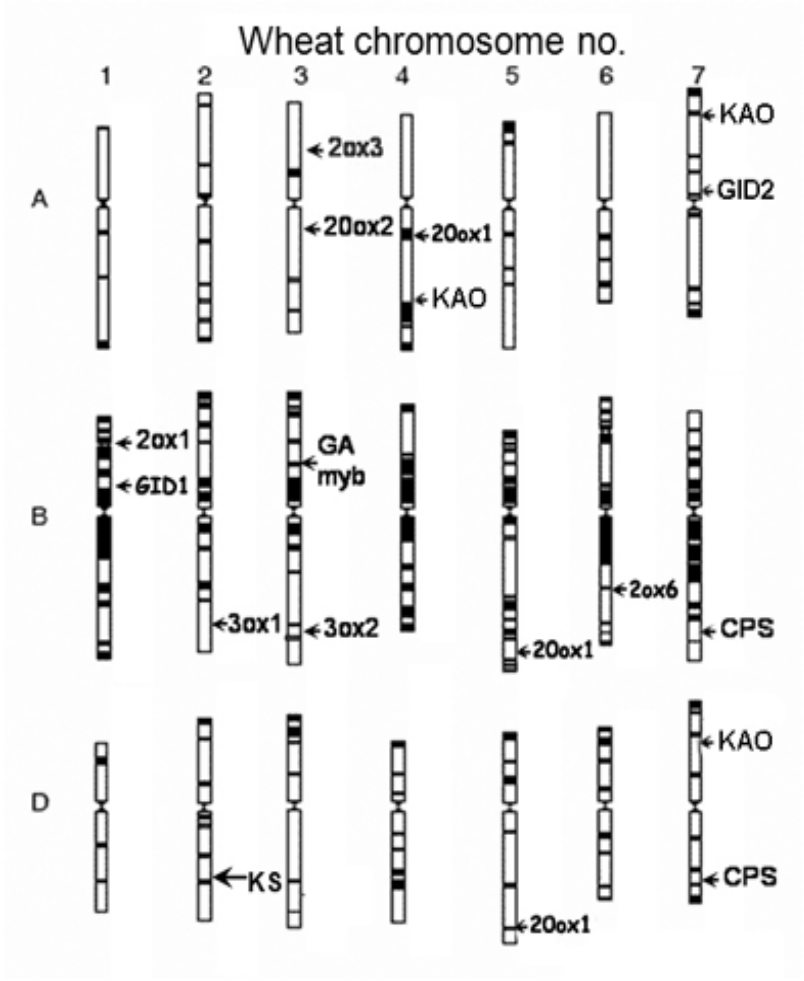


Figure 27: Location of GA biosynthetic and signalling genes in wheat

Relationship to mapping efforts at John Innes Centre (John Innes Centre)

Several GA genes underlie major PHS or PMA QTLs identified within workpackages 1,2 and 5. In addition to the previously reported linkage between QTLs for PHS and PMA with *GA20ox1* on chromosome 4A (Mares and Mrva, 2001) and Rht2 on chromosome 4B (Mrva and Mares, 1996), QTLs on the group 3 and group 7 chromosomes also were associated with GA pathway genes, but cannot be reported on here due to confidentiality issues. These linkages will be explored further in follow-on projects.

3.6. Work Package 5: QTL identification, validation and relation to candidate genes

Authors: J Flintham, J Simmonds, J Snape (John Innes Centre), P Jack (RAGT), P Werner (KWS), S Berry (Limagrain), T Henriksson (LM SW Seed), S Smith (Syngenta), J McKee (NIAB)

3.6.1. Aims

- To determine whether elite UK winter wheat germplasm exhibits adequate genetic variation for the improvement of resistance to PHS/PMA
- To discover if polymorphic DNA markers can locate QTLs controlling HFN on the genetic map of wheat
- To demonstrate that QTL markers can be used for marker-assisted breeding, to produce new genotypes with improved HFN
- To determine whether map alignments between QTLs and genes of the GA and ABA signalling pathways can identify potential candidate genes underlying QTL effects

3.6.2. Methods

Varieties

A set of thirty-two varieties was included in trials at John Innes Centre. Twenty-six of these were red-grained winter cultivars (sixteen parents of PHS/PMA mapping populations and ten additional check varieties), two were white-grained winter (JBW and Heraldo), three more white spring (BD159, Cranbrook and Spica) and one red spring (Chinese Spring). These varieties are listed in Appendix 1, with their Recommended List scores (where available) and scores from the JIC trials. The varieties spanned the ranges of resistance and susceptibility for both PHS and PMA among elite UK-adapted germplasm, plus extremely susceptible checks.

QTL Mapping Populations

Eleven populations of doubled haploid lines (DHLs), produced from ten parental cross combinations, were included in PHS and/or HFN field trials (Table 6). One of these was the WGIN (AC) mapping population (www.WGIN.org.uk). Other populations were supplied from existing collections, or specifically produced and genotyped for this project. They encompassed wide variation for PHS, PMA, and contemporary elite diversity.

One population (AR) was derived as a composite of F1- and reciprocal BC1-derived subpopulations, selected for single semidwarfing alleles at either *Rht-B1* or *Rht-D1* (i.e. tall and “double dwarf” genotypes were deselected). All other populations comprised random F1-derived DHLs.

Table 6. QTL Mapping Populations

Acronym	Parents	DHLs	Markers	Groups[1]	cM[2]	Chromosomes[3]
AC	AvalonxCadenza	107+9[4]	357	34	1861	All
LC	LynxxCadenza	39	89	21	2496	All
OC	OptionxClaire	122	162	21 (1)	3147	All
MC	MalaccaxCharger	98	274	44(25)	1696	No 4A
HS	HavenxSoleil	112	152	34(50)	827	No 5B, 6A, or 7D
OP	OptionxPotent	91	221	29(15)	918	No 3D, 4D, or 6D
SR	SparkxRialto	129	264	30	1471	All
CB	ChargerxBadger	93	114	32(24)	1216	No 1D or 6D
RS	SavannahxRialto	129	269	35(16)	1496	All
AR	[5]AlchemyxRobigus	126	112	(112)	n.a.	All
RA	RobigusxAlchemy	89	5	1	n.a.	[6]

[1]Groups = number of independent linkage groups (unlinked markers).

[2]cM = Total combined length of linkage groups in centiMorgans.

[3]All 21 chromosomes were covered, except as indicated.

[4]AC DHLs = 107 red plus 9 white grained (all other populations red).

[5]AR DHLs were selected F1- and BC1-derived (Methods).

[6] A single chromosome was targeted.

DNA polymorphisms (SSR and/or DArT markers – Akbari *et al.* 2006) were used to construct genetic maps in Joinmap (Stam, 1993) and common markers were aligned with a consensus wheat genomic map (Somers *et al*, 2004). QTL Cartographer (North CarolinaStateUniversity) was used for QTL analyses: single marker ANOVA for identifying linkage groups (or unlinked markers) of significant effect, followed by interval mapping with LOD threshold 2.5.

PHS Trials

PHS trials were conducted in winter-sown field plots which were irrigated from overhead during grain maturation, similar to the system used at NIAB for Recommended List ratings for resistance to sprouting (McVittie and Draper, 1982). Selected varieties were trialled at NIAB in 2006, varieties and QTL mapping populations at JIC in each year 2005-09 (Table 7). Each genotype was represented by at least two randomised plots per trial, minimum plot size was a single one-meter row.

Table 7. PHS and HFN Trials of QTL Mapping Populations.

Population	PHS Trials	HFN Trials
AC	2005, 06, 07, 08	JIC07
LC	2005	JIC06, 07, 08
OC	2006, 07, 08	NIX06, 07 SW06, 07
MC	2005, 06, 07	SW06, 07
HS	2005, 06, 07, 08	ELS06, 07, 08
OP	-	KWS06, 07, 08 JIC07, 08
SR	-	KWS06, 07, 08 JIC07, 08
CB	2006, 07, 08	-
RS	-	JIC06, 07, 08
AR	-	JIC07 ADV07
RA	2009	KWS07, 08

All PHS trials were conducted in JIC irrigated plots, HFN trials in non-irrigated plots at JIC and the indicated breeders, in the years indicated.

NILs for *QPhs-2D* were also trialled in PHS plots in 2009.

Irrigation at John Innes Centre commenced at physiological maturity, taken as the date at which approximately 50% of main shoot peduncles first showed senescence (yellowing). At least two x two-hour periods of run-off irrigation were applied each weekday, in addition to natural rainfall. Irrigation was maintained until susceptible (white-grained) check varieties showed extreme PHS, when twelve robust shoots per sample were dried, threshed and scored for percentage visibly sprouted grains. Standard arcsine transformations of percent data were used for all statistical and QTL analyses.

HFN Trials

HFN (Perten, 1964) was measured on wholemeal from a minimum of twelve ears per sample, from at least two randomised genotype replicates of winter-sown, non-irrigated plots (minimum one-meter row per plot). HFN plots were grown at NIAB in 2006 (varieties only), at JIC and breeders' trial sites in 2006-08 (varieties and QTL populations – Table 7). Replicate blocks were generally

harvested separately, the first as close as possible to harvest ripeness and the second after further natural weathering in the field.

Marker-Aided Breeding of Near-Isogenic Lines (NILs)

Linked SSR markers were used in recurrent selected backcrosses to generate Near-Isogenic Lines (NILs) for *QPhs-2D*. The recurrent parent of these NILs was a white-grained DHL from the AC population carrying the susceptibility allele for *QPhs-2D*, the donor of the resistance allele was a red grained AC DHL. Pairs of NILs homozygous for the resistance or susceptibility alleles were selected from BC2F2 progenies (nine white-grained pairs and three red pairs). These NILs were trialled in the 2009 PHS trial at JIC, in four randomised replicates.

3.6.3. Results and discussion

Phenotyping

Irrigation of PHS plots induced sprouting in all genotypes, in each year of trials. Confidence in the PHS testing protocol was supported by close correlation between JIC results and those obtained for the same varieties in the NIAB (Recommended List method) trial in 2006 (Figure 28). Coefficients of determination between replicates (r^2) varied from 12% to 58% across the PHS trials of mapping populations, indicating significant and moderate to strong heritability of genetic PHS resistance effects.

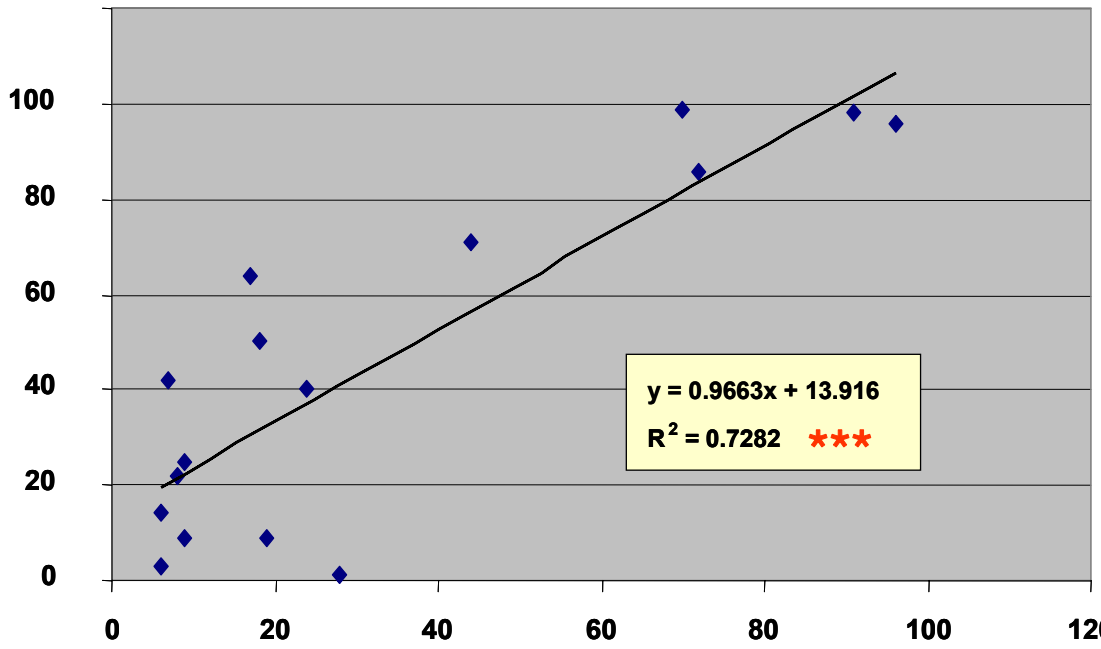


Figure 28. Correlation Between JIC and NIAB Scores for PHS Among Varieties in Irrigated Field Plots, 2006. Data show the mean percentage PHS across replicate samples from JIC (vertical axis) versus NIAB (horizontal) for seventeen common varieties.

In non-irrigated trials, HFN was more dependent upon seasonal effects, but significant QTL effects were detected in all of the HFN trials listed in Table 2. Variation for HFN in non-irrigated plots was attributed to PMA for early-harvested replicates (in the absence of evidence for PHS), or to PHS in late-harvested replicates in which HFN was significantly lower than in the early replicate (Lunn *et al.*2001). There were few cases where HFN confounded PHS with PMA leading to ambiguity in the interpretation of QTL effects.

Genomic Distribution of PHS and PMA QTLs

Figure 29 represents the alignments of significant *QPhs* and *QPma* effects on the consensus wheat genome map. Each circle on the map represents a single population trial in which the QTL was detected, and many of these loci were only detected in single trials. Such singular QTL detection events were not confirmed in any of the additional trials. Although these may represent real QTLs (which were suppressed elsewhere by interactions with local trial environments) it is equally possible that they may be type1 statistical artefacts.

At least two independent trials confirmed PHS QTLs on chromosomes 1A, 2D, 3A (2 loci), 3B, 3D, 4A (2 loci), 4D, 5D (2 loci) and 7B, and PMA QTLs on chromosomes1B, 4D, 5D, 6A, 7B and 7D. These QTLs for PHS and PMA located to separate loci in all cases (allowing for possible overlaps between *QPma* and *QPhs* loci on 4A and 5D).

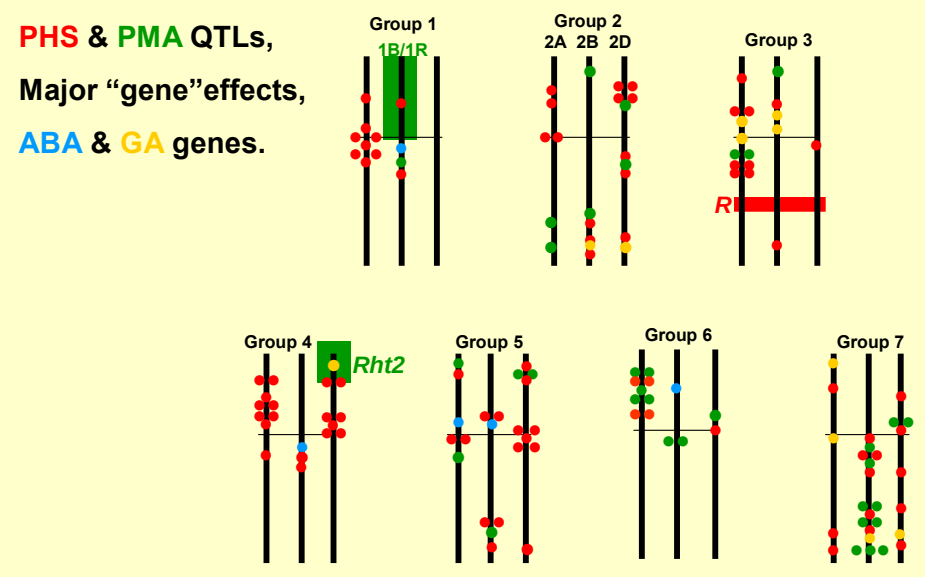


Figure 29. Genomic Distribution of HFN and Candidate Gene Loci.

Loci controlling PHS are coloured red (QTLs as circles, and the major *R* homoeoloci for red seed coat); effects on PMA are in green (QTL circles, the 1BS/1RL chromosome translocation and the *Rht2* dwarfing gene); candidate genes are shown in blue and yellow (abscisic acid and gibberellin pathways respectively). Each QTL circle denotes an independent observation of significant effect. The twenty-one wheat chromosomes are shown as vertical bars, with horizontal connecting centromeres within each homoeologous group.

The following five examples illustrate salient features of HFN genetics:

1Bs/1Rs chromosome translocation

Rye chromosome arm 1Rs had a consistently strong negative effect on HFN in the Spark x Rialto and Lynx x Cadenza mapping populations (Table 8). Similar results were obtained from SR lines in the PMA smart screen under controlled temperature shock treatment, and a negative 1Rs effect has also been observed in Australian germplasm (K. Mrva, 2008, pers. comm.). Neither the literature nor the present study yield any evidence for a 1B/1R effect on PHS, the PMA effect appears to be independent of embryo germination. Genetic recombination is suppressed in translocation heterozygotes, precluding interval mapping of such effects and candidate gene analysis. It is therefore not clear whether the PMA effect is due to a single gene, or how it is related to the well known "sticky dough" quality defect in 1Rs varieties.

Table 8. Effects of the 1Rs chromosome arm translocation on PMA in Lynx x Cadenza and Spark x Rialto mapping populations.

Population / Site	2006	2007	2008
LC / JIC	-61.2	-82.6	-99.4
SR / JIC	-	-109.6	-37.6
SR / KWS	-53.9	-29.4	-43.0

HFN effects given as (1Rs/1Bl) genotypes mean minus (1Bs/1Bl) genotypes mean, from non-irrigated HFN trials in the absence of visible sprouting. No data were available for SR at JIC in 2006.

Rht-D1 semidwarfism

The *Rht-D1* effect on HFN was of a similar magnitude to that of 1B/Rs, ranging from around 25 to 105 seconds across different trials (Table 9). Semidwarfs showed reduced PMA compared to tall siblings in the Spark x Rialto, Haven x Soleil and Lynx x Cadenza populations. A 4D effect was also detected close to the *Rht-D1* locus in PHS trials of Haven x Soleil, however the direction of the effect was reversed (semidwarfs were more resistant to PMA, but more susceptible to PHS). This suggests that Haven and Soleil differ at two closely linked loci, with resistance alleles in repulsion linkage.

Table 9. *Rht-D1b* effects on PMA in Spark x Rialto, Haven x Soleil and Lynx x Cadenza mapping populations.

Population / Site	2006	2007	2008
SR / JIC	NS	45.4	NS
SR / KWS	59.8	21.5	NS
HS / ELS	77.2	NS	75.4
?	98.3	67.9	NS

HFN effects are shown as semidwarf genotypes mean minus tall genotypes mean for each trial (NS = non significant effect).

Both *Rht-B1* and *Rht-D1* are known to be implicated in the expression of PMA (Gale *et al.* 1987). The dwarfing effects of these loci are caused by a lesion in DELLA/GA-mediated growth regulation (Peng *et al.* 1999). A-amylase genes are activated via GA signalling during germination, however amylase responses to exogenous gibberellin are normal in *Rht-B1/D1* semidwarfs (Gale and Marshall, 1975).

It therefore seems likely that the *Rht-D1* effect on PMA may be indirect, rather than an immediate consequence of defective GA signalling. One possible mechanism might be via the reduced grain size associated with *Rht* dwarfism (Greenwell *et al.* 2000). An indirect effect might also account for the variable size and significance of *Rht-D1* effects on PMA apparent in Table 9.

R grain colour genes

The Avalon x Cadenza population segregated nine white-grained and one hundred and seven red-grained DHLs; all other populations were entirely red-grained (evidently fixed for one or more dominant *R* genes). Segregation for grain colour was consistent with the parental genotypes *R-A1b / R-B1b / R-D1a* for Avalon and *R-A1a / R-B1a / R-D1b* for Cadenza. The nine white DHLs consistently had extremely high scores for PHS in irrigated trials, and were excluded from QTL analyses of the remaining red-grained lines in order to reduce error variance. The grain colour effect on PHS was also observed in PHS trials of varieties (Figure 30) and in NILs developed from the AC population (see *QPhs-2D* below).

The onset of sprouting under irrigation was one week earlier in white varieties, irrespective of their geographical origin or spring / winter flowering habit. The time course of sprouting in white and red varieties also illustrates the dynamic nature of genetic PHS resistance: at early stages both red and white genotypes are resistant, the difference in germination then increases to a maximum, followed by a progressive decrease until both red and white genotypes attain full germinability.

The *R-A1, R-B1* and *R-D1*homoeoloci encode triplicate MYB transcription factors which coordinate activation of the flavonoid biosynthetic pathway in pigmentation of the wheat grain testa (Himi and Noda 2005). Red pigmentation has a more or less direct effect on wheat dormancy (Flintham, 2000), however the mechanism remains obscure. It is interesting to speculate whether non-pigmented, dormant genotypes might be derived by specifically blocking flavonoid synthesis at a (pen)ultimate step and accumulating colourless pigment precursors in the testa. This would differ from control by the classic *R* loc (suppression of the entire flavonoid pathway), but would require the retention of inhibitory capacity by unpolymerized/colourless precursors.

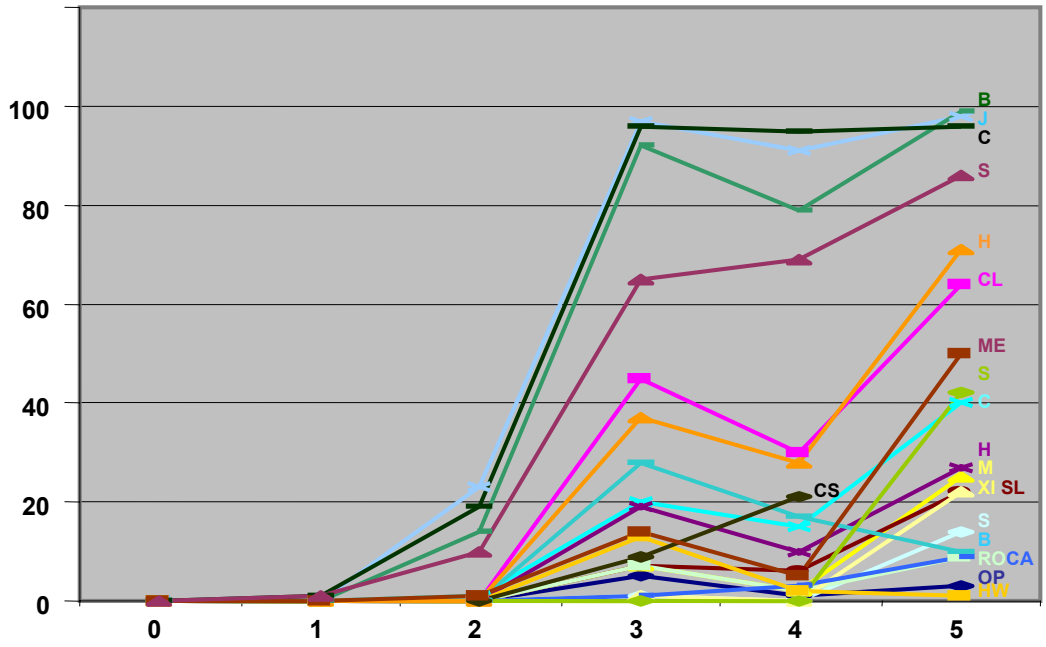


Figure 30. Onset of PHS in White and Red Varieties in JIC Irrigated Plots, 2006.

The vertical axis shows the percentage of visibly sprouted grains per sample, weeks of irrigation from physiological maturity on the horizontal axis.

White grained varieties (from top right): BD159 (B), JBW (J), Cranbrook (C), Spica (S).

Red varieties: Maris Huntsman (H), Claire (CL), Mercia (ME), Savannah (S), Charger (C), Haven (H), Malacca (M), Xi19 (XI), Soleil (SL), Spark (S), Badger (B), Rialto (RO), Cadenza (CA), Option (OP), Hereward (HW), and Chinese Spring (CS).

QPhs-2D

After grain colour, the strongest effect on PHS was associated with a major QTL on chromosome 2D, among Avalon x Cadenza DHLs. This effect was highly significant in four consecutive years of irrigated trials, and was confirmed in NILs derived from marker-aided backcrossing of a red, highly resistant DHL into a white, highly susceptible DHL.NILs (pairs of resistant / susceptible marker homozygotes multiplied from BC3F2 plants) also segregated for red / white grain colour and their performance under irrigation revealed both *R* and *QPhs-2D* effects (Figure 31).

QPhs-2D had no detectable effect in the white-grained NILs, whether because the resistance gene is ineffective in the absence of red pigment or because sprouting had already advanced to a degree which obscured any QTL effect. However, the resistance gene was effective for marker-aided resistance breeding in the red genotypes. New research will test the more general utility of *QPhs-2D* across a wider range of new germplasm.

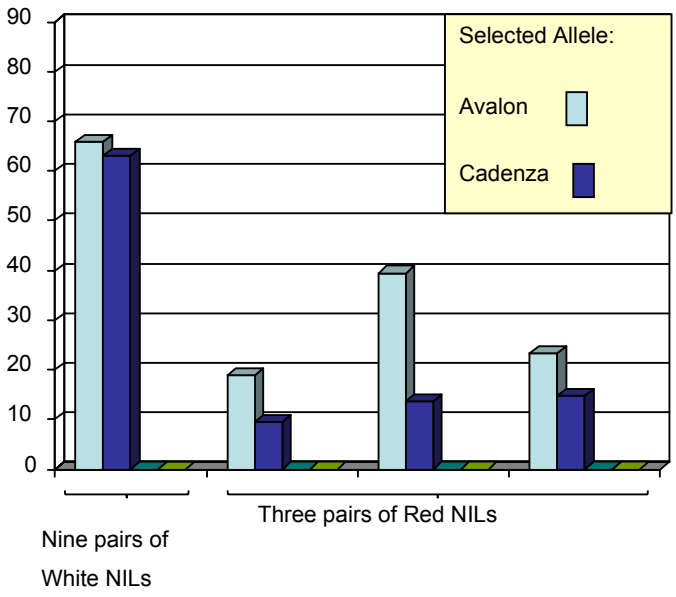


Figure 31. PHS in NILS for *QPhs-2D*.

Arcsine sprouting in samples from the 2009 PHS trial at JIC. No significant differences were detectable among the nine white lines, whereas the red lines carrying Cadenza alleles at *QPhs-2D* were significantly more resistant than their Avalon allelic NILs in all three cases (P<0.01).

Genotype x Environment interactions

Finally, PHS trials of the Option x Claire population illustrate the potential importance of GxE interactions in the expression of QTL effects (Table 10). Different QTLs had highly significant effects in each year of PHS trials, but none could be confirmed over more than one trial. QPhs-2Dii and QPhs-3A in particular commanded very high LOD scores and are therefore unlikely to be type 1 statistical artefacts. It would appear that some PHS resistance mechanisms are strongly dependent upon local weather variables. One implication of this is the necessity of multi-site/season validation for genetic analyses of HFN.

Table 10.*QPhs* effects in the Option x Claire mapping population: Genotype x Environment interactions under simulated rainfall.

***QPhs* locus**	**2006 trial**	**2007 trial**	**2008 trial**
QPhs-2Di*	NS	NS	-4.0 (3.0)
QPhs-2Dii*	NS	-11.6 (5.6)	NS
QPhs-3A	-6.6 (4.7)	NS	NS
QPhs-5B	NS	NS	-4.2 (2.9)
QPhs-7B	NS	NS	+4.2 (2.3)
QPhs-7D	NS	NS	-4.6 (3.0)

NS = no significant effect. Significant effects on arcsine percentage sprouting are shown as Option genotypes mean minus Claire genotypes mean (above their LOD scores from QTL interval mapping). *Two independent QTLs were indicated on chromosome 2D, separated by an interval of 70centiMorgan.

3.6.4. Summary

This study has revealed some important general features of genetic variation for HFN in UK elite germplasm:

- Both PHS and PMA exhibit moderate to high heritabilities, depending on seasonal variation in the severity of damage.
- Genotype x Environment (GxE) effects are also prominent, affecting some loci more than others (e.g. stable grain colour versus unstable *QPhs* effects on PHS).
- Resistance to PHS/PMA is determined by aggregate effects of major genes and multiple QTLs.
- Resistance factors are widely distributed across the wheat genome, indicating abundance of genetic variation.
- Transgressive segregation was observed more often than not, reflecting dispersal of resistance alleles among the mapping parents.
- Marker-assisted breeding can be used to control HFN, but may reveal novel epistatic (gene x gene) interactions.
- Loci controlling PHS do not generally co-locate with PMA effects; the two syndromes are controlled by separate genetic mechanisms.
- Candidate genes are already known for the two strongest genetic effects controlling HFN (*Rht* and *R*). This study has furnished DNA markers for further investigation of other important QTL effects: for the development of novel marker technologies, for physiological characterization of their mechanisms, and for fine scale mapping and molecular analysis.
- There is abundant potential for stabilising HFN in commercial wheat varieties for the future, using marker-assisted breeding to assemble novel PHS and PMA resistance gene combinations for more efficient deployment of existing elite germplasm.

3.7. References

Akbari, M. *et al.*2006. Diversity array technology (DArT) for high-throughput profiling of the hexaploid wheat genome. Theoretical & Applied Genetics**113**: 1409-1420.

Alonso-Blanco C, Bentsink L, Hanhart CJ, Vries HBE, Koornneef M. 2003. Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics **164:** 711-729.

Appleford, N.E.J., Evans, D.J., Lenton, J.R., Gaskin, P., Croker, S.J., Devos, K.M., Phillips, A.L., and Hedden, P. 2006. Function and transcript analysis of gibberellin-biosynthetic enzymes in wheat. Planta **223:**568-582.

Baulcombe, D.C., Huttly, A.K., Martienssen, R.A., Barker, R.F., and Jarvis, M.G.1987. A novel wheat alpha-amylase gene (alpha-Amy3). Mol. Gen. Genet. **209,** 33-40.

Biddulph TB, Plummer JA, Setter TL, Mares DJ. 2008. Seasonal conditions influence dormancy and preharvest sprouting tolerance of wheat (Triticum aestivum L.) in the field. Field Crops Research **107:** 116-128.

Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL, Holdsworth MJ. 2008. Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. Plant Journal **53:** 214-224.

Chen CX, Cai SB, Bai GH. 2008. A major QTL controlling seed dormancy and pre-harvest sprouting resistance on chromosome 4A in a Chinese wheat landrace. Molecular Breeding **21:** 351-358.

Coles, J.P., Phillips, A.L., Croker, S.J., Garcia-Lepe, R., Lewis, M.J., and Hedden, P.(1999. Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. Plant Journal **17:**547-556.

Corbineau F, Benamar A, Come D. 2000. Changes in sensitivity to abscisic acid of the developing and maturing embryo of two wheat cultivars with different sprouting susceptibility. Israel Journal of Plant Sciences **48:** 189-197.

Evans, D.J. 2005. Production and characterisation of transgenic wheat with altered gibberellin metabolism. Ph.D. Thesis: University of Bristol, Bristol, UK.

Farrell, AD and **Kettlewell, PS. 2008**. The effect of temperature-shock and grain morphology on alpha-amylase in developing wheat grain. Annals of Botany**102**: 287-293.

Farrell, AD and **Kettlewell, PS. 2009.** The relationship between grain weight and alpha-amylase in winter wheat: an analysis of UK Recommended List trials. Euphytica**168**: 395-402.

Finch-Savage WE, Leubner-Metzger G. 2006. Seed dormancy and the control of germination. New Phytologist **171:** 501-523.

Flintham, J.E.2000. Different genetic components control coat-imposed and embryo-imposed dormancy in wheat. Seed Science Research**10**: 43-50.

Gale, M.D. and **Marshall, G.A. 1975.** The nature and genetic control of gibberellin insensitivity in dwarf wheat grain. Heredity **35**, 55-65.

Gale, M.D., Salter. A.R. and Lenton, J.R. 1987. The induction of germination alpha-amylase during wheat grain development in unfavourable weather conditions. Pp. 273-282 in: Fourth International Symposium on Pre-Harvest Sprouting in Cereals(Ed. D.J. Mares), Westview Press.

Gooding, M.J., Ellis, R.H., Shewry, P.R., and Schofield, J.D.2003. Effects of restricted water availability and increased temperature on the grain filling, drying and quality of winter wheat. J. Cereal Sci. **37,** 295-309

Gosling PG, Butler RA, Black M, Chapman JM. 1981. The onset of germination ability in developing wheat. Journal of Experimental Botany **32:** 621-627.

Greenwell, P., Cauvin, S.P., Bhandari, D., Flintham, J., Gale, M., Briarty, G. and Evers, **A.D. 2000**. Prediction and prevention of excessive enzyme activity in cereals through investigation and manipulation of causal factors. HGCA Project Report No.247.

Greenwell, P., Cauvain, S.P., Bhandari, D., Flintham, J., Gale, M., and Briarty, G.2001. Prediction and prevention of excessive enzyme activity in cereals through investigation and manipulation of causal factors. HGCA Project Report No. 247.

Gubler, F., Kalla, R., Roberts, J.K., and Jacobsen, J.V.1995. Gibberellin-Regulated Expression of a Myb Gene in Barley Aleurone Cells - Evidence For Myb Transactivation of a High-Pl Alpha-Amylase Gene Promoter. Plant Cell **7:**1879-1891.

Gubler F, Millar AA, Jacobsen JV. 2005. Dormancy release, ABA and pre-harvest sprouting. Current Opinion In Plant Biology **8:** 183-187.

Hagemann MG, Ciha AJ. 1987. Environmental x genotype effects on seed dormancy and after-ripening in wheat. Agronomy Journal **79**: 192-196.

Harlan JR. 1992. Crops and Man. Madison: American Soc. Agronomy.

Himi, E. and Noda, K. 2005. Red grain colour gene (*R*) of wheat is a Myb-type transcription factor. Euphytica**143**: 239-242.

Holdsworth MJ, Soppe WJJ, Bentsink L. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist 179, 33-54.

Huttly, A.K., and Baulcombe, D.C.1989. A Wheat Alpha-Amy2 Promoter Is Regulated by Gibberellin in Transformed Oat Aleurone Protoplasts. EMBO Journal **8,** 1907-1913.

Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M., and Matsuoka, M.2004. Loss-of-function mutations of the rice GAMYB gene impair alpha- amylase expression in aleurone and flower development. Plant Cell **16:**33-44.

Khlestkina, E.K., Kumar, U., and Roder, M.S.2010. Ent-kaurenoic acid oxidase genes in wheat. Molecular Breeding **25:**251-258.

King RW, Richards RA. 1984. Water-uptake in relation to pre-harvest sprouting damage in wheat - Ear characteristics. Australian Journal Of Agricultural Research 35, 327-336.

Lo, S.F., Yang, S.Y., Chen, K.T., Hsing, Y.L., Zeevaart, J.A.D., Chen, L.J., and Yu, S.M.2008. A Novel Class of Gibberellin 2-Oxidases Control Semidwarfism, Tillering, and Root Development in Rice. Plant Cell **20:**2603-2618.

Lunn, G.D., Major, B.J., Kettlewell, P.S. and Scott, R.K. 2001. Mechanisms leading to excess *alpha*-amylase activity in wheat (*Triticum aestivum* L.) grain in the U.K.Journal of Cereal Science**33:** 313-329.

Mares DJ. 1983. Preservation of dormancy in freshly harvested wheat-grain. Australian Journal Of Agricultural Research 34, 33-38.

Mares DJ. 1993. Preharvest sprouting in wheat. 1. Influence of cultivar, rainfall and temperature during grain ripening. Australian Journal Of Agricultural Research 44, 1259-1272.

Mares, D.J., Mrva, K., and Panozzo, J.F.1994. Characterization of the High Alpha-Amylase Levels in Grain of the Wheat Cultivar Bd-159. Aust J. Agric. Sci. **45,** 1003-1011.

Mares, D.J., and Mrva, K.2001. Mapping quantitative trait loci associated with variation in grain dormancy in Australian wheat. Aust J. Agric. Sci. **52:**1257-1265.

McCallum, C. M., Comai, L., Greene, E. A. and S. Henikoff. 2000. Targeting Induced Local Lesions IN Genomes (TILLING) for Plant Functional Genomics.PlantPhysiology, June, 123, 439-442.

McVittie, J.A. and Draper, S.R. 1982. The irrigation of standing plots of winter wheat in order to assess varietal predisposition to pre-harvest sprouting. Journal of NIAB**16:** 45-49.

Morris CF, Moffatt JM, Sears RG, Paulsen GM. 1989. Seed dormancy and responses of caryopses, embryos, and calli to abscisic-acid in wheat. Plant Physiology 90, 643-647.

Mrva, K., and Mares, D.J.1996. Expression of late maturity alpha-amylase inwheat containing gibberellic acid insensitivity genes. Euphytica **88,** 69-76.

Mrva, K., and Mares, D.J.2001. Induction of late maturity alpha-amylase in wheat by cool temperature. Aust J. Agric. Sci. **52,** 477-484.

Mrva, K., Wallwork, M., and Mares, D.J.2006. alpha-Amylase and programmed cell death in aleurone of ripening wheat grains. J. Exp. Bot. **57,** 877-885.

Paterson AH, Sorrells ME, Obendorf RL. 1989. Methods of evaluation for preharvest sprouting resistance in wheat breeding programs. Canadian Journal of Plant Science 69, 681-689.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D. and Harberd, N.P. 1999. 'Green revolution' genes encode mutant gibberellin response modulators. Nature**400:** 256-261.

Perten, H. 1964. Application of the Falling Number method for evaluating alpha-amylase activity. Cereal Chemistry**41:** 127-140

Sakamoto, T., Miura, K., Itoh, H., *et al.*2004. An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol.**134:**1642-1653.

Salse, J.2009. Genomics in cereals: from genome-wide conserved orthologous set (COS) sequences to candidate genes for trait dissection. Functional & Integrative Genomics**9:**473-484.

Singh, N.K., Dalal, V., Batra, K., *et al.*2007. Single-copy genes define a conserved order between rice and wheat for understanding differences caused by duplication, deletion, and transposition of genes. Functional & Integrative Genomics**7:**17-35

Somers, D.J., Isaac, P. and Edwards, K. 2004. A high-density microsatellite consensus map for bread wheat. Theoretical & Applied Genetics**109:** 1105-1114.

STAM, P. (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. The Plant Journal**3:** 739-744.

Tottman DR. 1987. The decimal code for the growth-stages of cereals, with illustrations. Annals of Applied Biology 110, 441-454.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I.C., Kitano, H., Yamaguchi, I., and Matsuoka, M.2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature**437:**693-698

Vidal, A.M., Gisbert, C., Talon, M., Primo-Millo, E., Lopez-Diaz, I., and Garcia-Martinez, J.L.2001. The ectopic overexpression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. Physiol. Plant.**112:**251-260.

Vogel, J.P., Garvin, D.F., Mockler, T.C., *et al.* 2010. Genome sequencing and analysis of the model grass Brachypodium distachyon. Nature **463:**763-768.

Walker-Simmons MK. 1987. ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiology 84, 61-66.

Wan, Y.F., Poole, R.L., Huttly, A.K., Toscano-Underwood, C., Feeney, K., Welham, S., Gooding, M.J., Mills, C., Edwards, K.J., Shewry, P.R., and Mitchell, R.A.C.2008. Transcriptome analysis of grain development in hexaploid wheat. BMC Genomics **9**.

Yamaguchi, S.2008. Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol. **59:**225-251.

Zentella, R., Yamauchi, D., and Ho, T.H.D.2002. Molecular dissection of the gibberellin/abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. Plant Cell **14:**2289-2301.

APPENDIX 1. VARIETY SPROUTING AND HFN

Early HFN1	Early HFN2	Early Arc1	Early Arc2	Mid HFN	Mid Arc	Late HFN	Late Arc	Variety	Arc1.08	Arc2.08	HFN1	HFN2	ArcHFN1	ArcHFN2
357	328	0	0	394	0	203	8	Option	6	21	380	351	0	0
337	278	0	0	336	0	66	28	Claire	13	35	365	289	0	0
405	394	0	0	402	0	108	19	Malacca	10	24	434	258	0	0
297	279	0	0	281	6	62	36	Charger	23	33	340	224	0	12
267	188	0	0	221	8	75	22	Haven	41	52	251	258	0	8
359	351	0	0	406	0	255	6	Soleil	8	38	253	340	0	0
193	177	0	0	213	0	71	19	Potent	18	38	408	232	0	6
331	*	0	*	365	0	*	*	Solstice	8	37	400	387	0	0
320	*	0	*	294	0	*	*	Phlebas	16	29	304	326	0	0
286	260	0	0	376	6	184	8	Spark	10	40	391	354	0	6
266	248	10	0	246	8	163	10	Rialto	21	51	402	382	0	0
322	342	0	0	387	6	263	6	Xi19	10	21	387	377	0	0
303	201	6	10	214	12	62	53	JBW	48	66	185	115	20	21
317	361	0	0	343	6	62	36	Heraldo	43	64	323	193	0	10
252	238	0	0	258	0	163	17	Skotte	12	28	361	302	0	6
368	265	0	0	389	0	122	13	Avalon	15	38	384	277	0	0
343	348	0	0	364	0	113	13	Cadenza	0	31	393	394	0	0
243	238	0	0	222	0	64	17	Badger	24	39	262	265	0	0
299	257	0	6	281	6	178	8	Savannah	8	18	330	329	0	6
345	302	0	0	364	0	88	17	Hereward	23	47	377	268	0	0
*	180	*	0	*	*	62	34	Huntsman	29	57	235	215	0	0
*	102	*	8	*	*	68	24	Lynx	25	32	270	213	0	0
190	219	0	0	222	0	77	17	Napier	13	31	322	338	0	0
385	343	0	0	404	0	182	12	A32	10	24	392	353	0	0
200	201	0	0	235	0	109	14	Access	0	16	320	292	0	0
145	125	10	6	130	18	72	56	BD159	42	65	167	153	10	6
62	62	27	30	60	30	62	72	Cranbrook	59	69	63	62	27	24
380	399	0	0	273	0	168	8	Chinese S	6	*	251	284	0	0
338	314	0	0	366	6	78	22	Mercia	10	40	388	328	0	0
70	68	32	27	62	42	62	66	Spica	39	64	64	114	40	33
*	207	*	0		*	62	21	Robigus	16	32	344	331	0	0
*	279	*	0		*	138	15	Alchemy	13	29	356	360	0	0

APPENDIX 2. COMPARISON BETWEEN HAGBERG FALLING NUMBER (HFN) AND RAPID VISCO ANALYSIS (RVA) DATA

Introduction

Each year SWRI carry out assessment of soft winter wheat samples from CEL RL trial sites in the UK for alcohol yield and other distilling quality parameters. One of the features of the 2008 harvest was the presence of pre-germinated grains (pre-harvest sprouting), which had affected one of the sites that were to be used in the CEL assessment. Because of the sprouting issue, data from this site were not included in the final assessment of wheat varieties for CEL.

The 2008 harvest provided a unique opportunity for SWRI to look more closely at the sprouted samples in the context of The HFN Link Project. A set of samples was selected from the CEL Trials at similar nitrogen levels to those found on the sprouted site (ca 2 percent (dry)). These are listed in Table 1. Note that the sprouted samples all had slightly higher moisture levels.

Table 1. Samples that were used in the study, these were selected at similar nitrogen levels (approx 2 percent N) from 2008 CEL harvest trials

Sample ID	Moisture(%)	TN(%)
Istabraq (Advanta)	13.01	1.96
Istabraq (NIAB sprout)	14.34	1.98
Robigus (Trial Force)	11.89	1.98
Robigus (NIAB sprout)	13.41	1.95
Scout (Trial Force)	13.87	1.98
Scout (NIAB sprout)	14.44	1.99
Glasgow (Advanta)	13.17	2.06
Glasgow (NIAB sprout)	14.27	2.02
Viscount (Advanta)	12.82	2.02
Viscount (NIAB sprout)	14.21	1.98
Claire (Trial Force)	12.97	1.89
Claire (Advanta)	12.23	2.11
Claire (NIAB sprout)	14.53	1.94

The Rapid Visco Analyser (RVA) is a rotational, continuously recording viscometer, with heating, cooling and variable shear capabilities, and is specifically configured for the study of the viscosity

properties of starch-based materials. The equipment was originally developed for the rapid identification of sprouted wheat, and the purpose of the work reported here is to illustrate how the RVA properties can be related to the Hagberg Falling Number (HFN) data to build a more detailed picture of wheat sprouting.

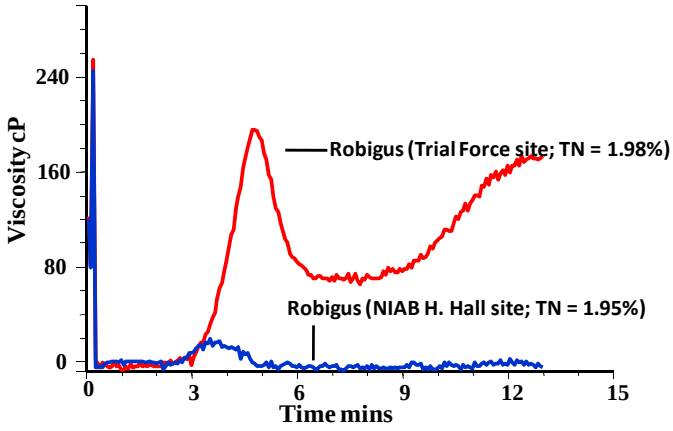
RVA analysis

Wheat samples were analysed by RVA using a standard programme for wheat and other unmalted cereals, as described in published work (Agu et al 2006). Samples were also sent to John Flintham at John Innes Centre for HFN analysis.

Results and discussion

Figure 1(a) and (b) shows typical RVA profiles for two commonly used distilling wheat varieties (Robigus and Istabraq). These have been selected from the full sample set as typical examples for illustrative purposes.

(a) Robigus



(b) Istabraq

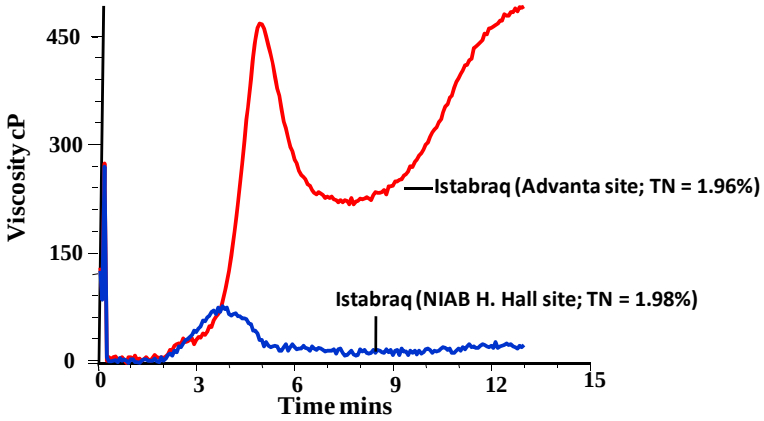


Figure 1. Typical RVA pasting characteristics for normal (red) and sprouted (blue) wheat (a) Robigus; (b) Istabraq

The 2 RVA charts show examples of each variety from a ‘normal’ and ‘sprouted’ site, and shows the strong contrasts between the normal and sprouted sample. The RVA profiles of the normal unsprouted samples show the peaks and troughs, relating to peak and final viscosity which are typical of cereal starch. In contrast the sprouted samples show a much lower, flatter, characteristic viscosity profile, which resembles that associated with malted barley. Another important feature is the disappearance of the final viscosity peak (loss of setback) that is characteristic of sprouted wheat.

Table 2 shows the RVA and HFN data for all13 samples used in the study. In all cases the sprouted samples gave much lower HFN, as well as very low RVA peak and final viscosities. Because of the way that the RVA instrument operates, the baseline is set automatically at the start of the run, so it is possible to generate negative values as the RVA run progresses, particularly when the sample viscosity is low, as they were for some of the sprouted samples.

Table 2. Comparison of Hagberg Falling Number (HFN) with RVAPeak and Final Viscosity data (HFN data provided by John Flintham (John Innes Centre))

Sample ID	HFN(s)	Peak Viscosity(cP)	Final Viscosity(cP)
Istabraq (Advanta)	188	467	491
Istabraq (NIAB sprout)	72	75	21
Robigus (Trial Force)	145	195	173
Robigus (NIAB sprout)	79	19	-5
Scout (Trial Force)	145	162	116
Scout (NIAB sprout)	86	11	-28
Glasgow (Advanta)	273	631	589
Glasgow(NIAB sprout)	89	287	155
Viscount	107	371	290

(Advanta)			
Viscount (NIAB sprout)	62	-29	-34
Claire (Trial Force)	244	153	126
Claire (Advanta)	273	390	450
Claire (NIAB sprout)	63	-59	-68

Table 2 shows that all the sprouted sites gave very low HFN values.This was generally supported by the RVA data, and these results show that both methods were effective at identifying sprouted material from the sprouted site. This is emphasised by the strong correlation between these parameters in Figure 2 which was very significant and confirms that both methods were well suited to identifying sprouted wheat.

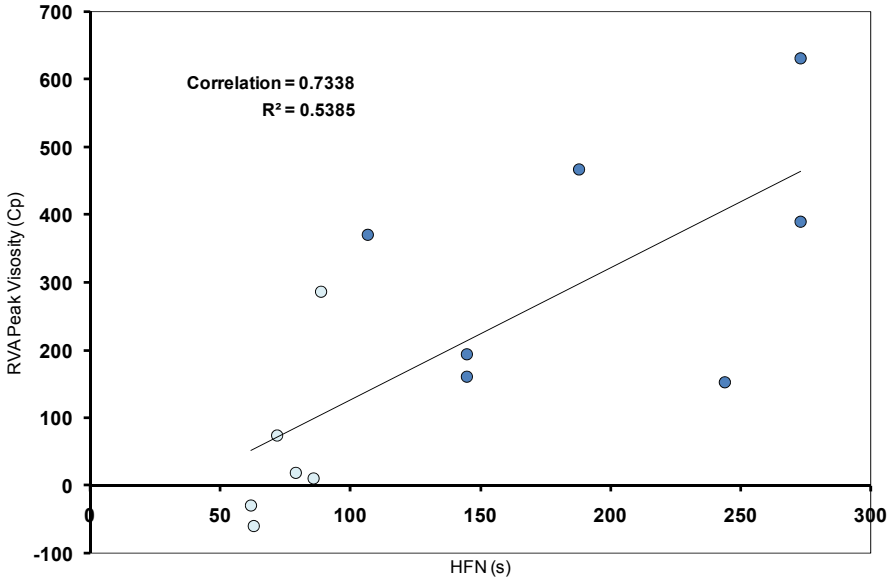


Figure 2. Correlation between HFN and RVA Peak Viscosity based on sample set (n=13) Sprouted samples are shown in light blue. Circled point shows 'sprouted' Glasgow sample

Figure 3 shows a stronger correlation between RVA final viscosity and HFN, with a much clearer differentiation between the sprouted and unsprouted samples. Unsurprisingly, this suggests that the RVA final viscosity reflects more closely the properties that are measured by the HFN.

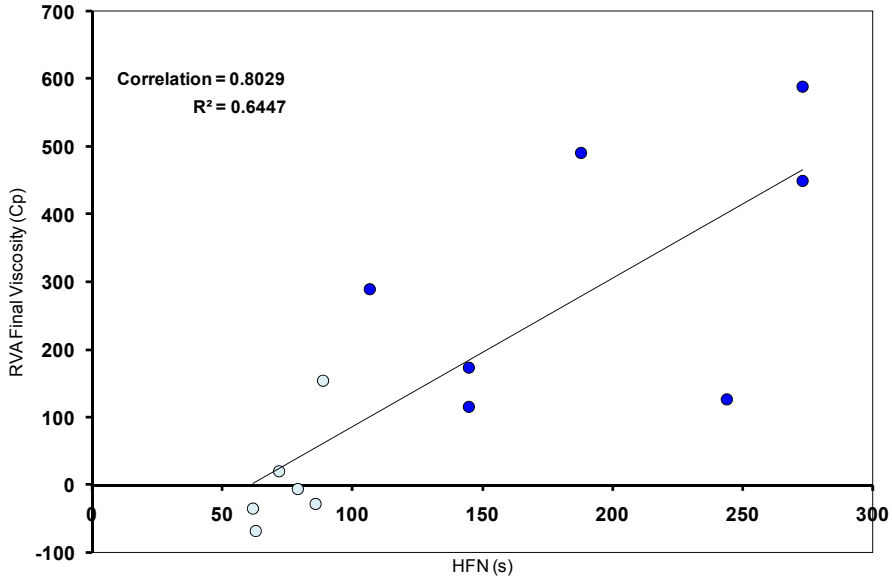


Figure 3. Correlation between HFN and RVA Final Viscosity based on sample set (n=13) Sprouted samples are shown in light blue. Circled point shows 'sprouted' Glasgow sample

It should be noted that although the correlations between the RVA peak and final viscosities and HFN were very significant, there was quite a large degree of variation between individual data points, within each set, blurring the division been the sprouted and unsprouted samples, potentially leading to some anomalous results at the borderline between these two categories.

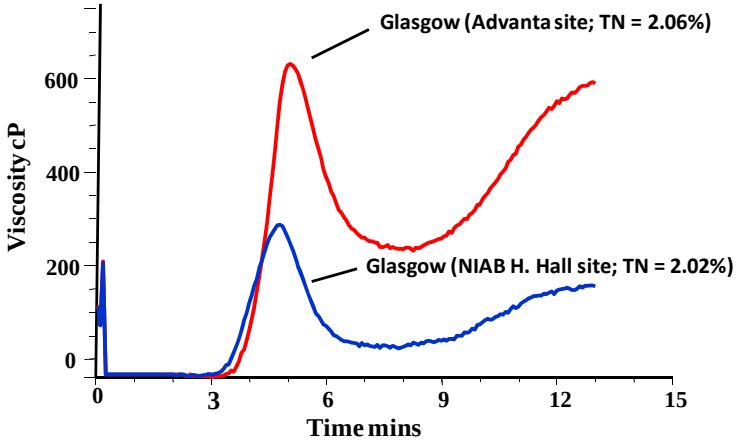


Figure 4. Typical RVA pasting characteristics for normal (red) and sprouted (blue) wheat (Glasgow)

A good example of this was the variety Glasgow, which gave particularly interesting results. This sample showed visible evidence of sprouting and was clearly identified by the HFN test as being sprouted. However, comparison of the RVA analysis of the sample from the sprouted site with the normal one (Figure 4) shows that although the overall viscosity parameters were lower, the RVA pasting profile of the sprouted sample was similar to a 'normal' sample profile. However, closer inspection of the RVA profiles for the two samples, clearly shows the loss of setback (reduction in

final viscosity), that would result from the action of α-amylase, generated during sprouting, on the cereal starch.

This indicates that different degrees of sprouting within a single site can give subtly different RVA profiles. It is not completely clear whether this would be reflected in genetic character of different varieties or, more likely, by differences in the growing conditions on the plots on which the samples were grown.However, currently we have little data relating reliably to the early stages of sprouting, and it is suspected that the anomalous Glasgow sample would fall into this category. It would be expected that there would be a limited effect on the viscous loading of a wheat sample if sprouting was minimal.

Conclusions

In general, the sprouted wheat samples gave RVA profiles that were characterised by much lower peak viscosities compared with normal samples, together with substantially reduced or minimal final (setback) viscosity. In contrast, samples that did not show any visible signs of sprouting gave profiles with typically high RVA peak viscosity and a substantial, well defined final viscosity (setback) peak.

Data generated from RVA analysis showed that on the whole, RVA data was consistent with the HFN method, and confirms that both methods are reliable indicators of spouting. It is useful therefore to consider both RVA and HFN data to give a more detailed picture of the patterns of sprouting in wheat. HFN gives a specific value that provides a numerical measure of sprouting, while the RVA profile provides more detailed, dynamic information about the behaviour of sprouting wheat.

Acknowledgement

Thanks to John Flintham (John Innes Centre) for providing HFN data on the wheat samples that were studied.

Reference

R.C. Agu, T.A. Bringhurst and J.M. Brosnan (2006):Production of Grain Whisky and Ethanol from Wheat, Maize and Other Cereals, *J. Inst. Brew*, 112(**4**), 314-323, 2006.