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Understanding and regulating pre-maturity α-amylase activity in wheat grains to maintain high Hagberg Falling Number

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1. Abstract

The occurrence of pre-maturity α-amylase (PMA) in wheat grains is a major problem in the UK and elsewhere. Flour produced from PMA induced grains has poor bread making potential (low Hagberg Falling Number [HFN]). Several factors such as genotype, agronomy and environmental conditions are responsible for PMA induction. Out of which, cool and wet weather around mid-grain development is the major stimulus for PMA induction. This report presents four glasshouse experiments conducted to understand the role of the hormones abscisic acid (ABA) and gibberellins (GAs) in the mechanism of PMA induction. The ultimate aim of this project is to identify ways in which plant breeders can develop varieties with low PMA and more reliable HFN.

The hypothesis studied in this project was that there is a change in ABA and/or GA sensitivity or in their levels in wheat grains during PMA induction by a cool-temperature shock. *In situ* (Experiment 1) and *in vitro* (Experiment 3) experiments tested the first part of the hypothesis. These experiments studied ABA and GA sensitivity of intact grains with embryos or detached grains without embryos under cool-temperature shock-inducing and non-inducing conditions. Overall, these two experiments concluded that PMA is related to increased GA sensitivity in the susceptible variety Rialto (Experiments 1 and 3). The change in sensitivity occurs at about 36 day after anthesis (DAA) and is limited to the aleurone (not the embryo) (Experiment 3). There was little evidence for a change in ABA sensitivity (Experiments 1 and 3). In experiment 2, a cool-temperature shock significantly increased GA sensitivity in both *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf)genotypes with or without the 1B1R (wheat-rye) chromosome translocation. These results suggested that the risk of PMA induction is very high and represents a serious problem to wheat breeders and growers.

Experiment 4 tested the second option using an alternative approach. In this experiment, induced grains produced significantly low ABA levels compared to non-induced grains at 29/31 DAA. This offers one possible mechanism through which cool-temperature can alter GA sensitivity. A significant increase in α-amylase in fluridone-treated (ABA biosynthesis inhibitor) grains under non-inducing conditions was associated with a significant increase in GAs. A significant decrease in α-amylase for paclobutrazol-treated (GA biosynthesis inhibitor) grains under inducing conditions was associated with a significant decrease in GAs. These results show that GA levels also play a role in α-amylase formation in developing grains, in addition to GA sensitivity.

The results from this project suggested the strategy to develop a new wheat variety with better bread making quality, which involves three major steps: A. Screening PMA-inducible varieties through the cool-temperature shock screening method, B. Constructing a transgene containing GA hypo-sensitiveness (either through the GID1 [GA] receptor or the DELLA protein) with a promoter, which governs the gene expression specific only in the embryo or the aleurone of developing grains, and transform the transgene into a PMA-inducible variety and, C. Marker selection for GA hypo-sensitivity. Alternative strategy is to look for the available molecular markers (QTL), which govern the GA hypo-sensitivity in wheat genome and use them to develop breeding strategies to improve HFN stability e.g. use of high throughput approaches such as Marker-Assisted Selection and Targeting Induced Local Lesions in Genomes.

2. Introduction

Hagberg Falling Number (HFN) of harvested wheat grains is determined by the presence of a hydrolytic enzyme: α-amylase. Starch is the major component of wheat flour. α-amylase, which is a starch degrading enzyme, is normally produced in low levels throughout grain development, and occasionally, in excess levels especially during the later stages of wheat grain development. The interaction of α-amylase with the endosperm starch, measured as the HFN, plays an important role in determining not only the processing properties of grain, but also the quality of many end products. A negative relationship exists between the HFN and α-amylase activity in wheat grains (Barnes and Blakeney, 1974; Allen *et al*., 2005; Flintham *et al*., 2011). Flours with a low HFN (i.e. high α-amylase) have poor bread making potential (Lunn *et al.*, 2001a). Wheat grains with high levels of α-amylase render them unsuitable for commercial applications and unattractive to potential buyers resulting in a lower price (Mares and Mrva, 2008). This may occur as a result of unfavourable weather conditions, especially rain, prior to harvest or a complex interaction of genotype and temperature in the later stages of grain development. The first case is known as pre-harvest sprouting (PHS) and is accompanied by germination. The second case is known as pre-maturity α-amylase (PMA), where α-amylase is present in sound grains without any visible germination. PHS is the major cause of a low HFN, whereas PMA is the second major cause of a low HFN (Lunn *et al*., 2001a). The occurrence of PMAis independent of PHS, and represents a serious threat to the wheat industry.

During the occurrence of PHS and in germinating grains, plant hormones such as abscisic acid (ABA) and gibberellins (GAs) influence α-amylase synthesis, where ABA inhibits and GAs stimulate α-amylase synthesis in the embryo and aleurone (Chandler *et al*., 1984; Gold 1991; Hader *et al*., 2003). A similar role for ABA and GAs would, therefore, be anticipated in intact, developing grains during PMA induction. Thus, the role of embryo-derived ABA and GAs in α-amylase synthesis is well known in germinating grains, but it is less clear in developing grains.

During normal grain development and maturation in wheat, peak levels of endogenous GAs and ABA occur between 15–20 DAA and 25–40 DAA, respectively (McWha, 1975; Slominski *et al.,* 1979; Gutam *et al.,* 2008). The embryo synthesises low isoelectric point (pI; pH at which the molecule has no net charge) α-amylases (i.e. *α-AMY2*) in early stages of grain development, but their level decreases with a progress in grain development. In contrast, high pI α-amylases (i.e. *α-AMY1*) are produced in small amounts by the embryo (or by the aleurone) during the later stages of grain development. The occurrence of an ABA peak and low GA levels in the later stages of grain maturation suggests that ABA and GAs may play an antagonistic role in controlling α-amylase synthesis in PMA induction, as seen in germinating grains. Thus, mature grains generally have a very low level of α-amylase.

PMA induction is a phenomenon where an excess level of high pIα-amylase is synthesised in the later stages of grain development, and is mostly retained in mature grains (Mares and Mrva, 2008). Several studies across the UK (e.g. Farrell and Kettlewell, 2008) and Australia (e.g. Mares and Mrva, 2008) have shown that PMA induction involves the genotype x environment interaction, and a cool-temperature shock occurring at mid-grain development for a short time (i.e. 12 °C for 8 days) can also act as the inducing factor for PMA in susceptible varieties.

The surprise observation of PMA in susceptible genotypes under field and glasshouse conditions (Mares and Mrva, 2008) prompted further research on the physiological and molecular mechanisms associated with PMA induction in the UK, Japan, China, California, Mexico, South Africa and Australia (Mares *et al*., 2006). PMA induction is a major concern in UK winter wheat varieties, where cool and wet periods during the summer are common. Moreover, PMA is all the more damaging as it is difficult to detect in trials even in inductive conditions due to its stochastic behaviour in grains (Flintham *et al*., 2011).

Cornford *et al*. (1987) predicted that PMA induction was due to the overlapping of the curves that describe the decline in GAs and the increase in ABA with the time when aleurone cells becomes sensitive to GAs. However, several studies by Radley (1976), Gale and Lenton (1987) and Mares and Mrva (2008) were unable to show that PMA is due to an increase in GA levels. A high level of PMA was produced in PMA genotypes under inductive condition when there was no change in GA levels in grain, whereas the presence of GA-insensitive (GAi) dwarfing alleles such as *Rht-B1b*, *Rht-D1b* and *Rht-B1c* (formerly known as *Rht1*, *Rht2* and *Rht3*, respectively) (Flintham *et al*.,1997a) in wheat plants significantly reduced grain PMA in the UK wheat variety: Maris Huntsman (Gold and Duffus, 1993) and Australian wheat varieties: Spica and Lerma 52 (Mrva and Mares, 1996). These findings suggested the involvement of GAs in PMA induction (Mrva and Mares, 1995; Mares and Mrva, 2008). The combined effect of ABA/GA levels in grain (i.e. biosynthesis and degradation [turnover]) and the sensitivity of the grain embryo or the aleurone to ABA and GAs (i.e. signal transduction) at different stages of grain development could be important factors in PMA induction.

At present, PMA-screening methods have been developed successfully, and the susceptibility of wheat genotypes (high, moderate and resistant) has been identified in the U.K., Australia, Canada, Japan, China, South Africa, Mexico (CIMMYT; The International Maize and Wheat Improvement Center) and the United States of America(USA; California) (Mares *et al*., 2006). Now, considerable efforts are underway worldwide to eliminate PMA-susceptible wheat genotypes from the germplasm list. However, these genotypes have a number of desirable agronomic and quality attributes (Mares and Mrva, 1993). Moreover, the PMA syndrome is more widespread than previously thought as one of the original sources of the PMA-genotype (e.g. Maris Huntsman or

Mardler or Norman) has been extensively used by an international wheat breeding institute: CIMMYT to develop wheat germplasm, which later spread around the world (Gale *et al*., 1987; Mares and Mrva, 1993). Thus, it has become really difficult to eliminate PMA genotypes completely from the breeding programmes. Therefore, the ideal way to overcome the problem of PMA induction in future is to develop new wheat varieties, which have PMA-resistance genotypes or molecular markers and to couple this with the use of PMA-screening methods to test all cultivars for PMA to help to eliminate PMA-susceptible genotypes from the germplasm list. However, at present sufficient information about the molecular mechanism governing PMA induction is not understood enough to develop new molecular markers and cultivars.

Overall, the key aim of this project was to obtain a better understanding of the biochemistry and physiology of PMA induction and the involvement of ABA and GA turnover and signalling in PMA induction. Therefore, the hypothesis, which was previously suggested by Farrell and Kettlewell (2008) and Mares and Mrva (2008), that 'a change in either the sensitivity of the aleurone to ABA and/or GAs or a change in ABA and/or GA levels of intact, developing wheat grains will alter PMA under cool-temperature shock-inducing conditions' was examined under glasshouse studies. In this project, four glasshouse experiments were designed and conducted with the help of the literature reviewed to study the above hypothesis. Experiments 1, 2 and 3 test the first possibility of the hypothesis, whereas experiment 4 is focused on the second option. This project ultimately gave valuable information about the role of ABA and GAs in the mechanism of PMA induction in wheat grains and also, clues to the molecular markers that can be used to develop new wheat varieties with better bread making quality. The main objectives of experiments conducted in this project are listed below.

1. To study the effects of *in situ* applied ABA and GA_3 (gibberellic acid; active GA produced by one branch of the biosynthetic pathway used for endogenous applications) at mid-grain development on wheat grain α-amylase at maturity under cool-temperature shock-inducing and non-inducing conditions.
2. To investigate the effects of *in situ* applied ABA and GA_3 at mid-grain development on wheat grain α-amylase at maturity under cool-temperature shock-inducing and non-inducing conditions in mapping genotypes fixed for a non-1B1R or 1B1R translocation, but having either the *Rht-D1a* (tall) or the *Rht-D1b* (semi-dwarf) allele.
3. To investigate the effects of *in vitro* applied ABA and GA_3 on wheat grain α-amylase at several time points in the later stages of ripening under inducing and non-inducing conditions.
4. To study the effects of *in situ* applied FD (ABA biosynthesis inhibitor) and PB (GA biosynthesis inhibitor) to developing grains at 24, 25 and 26 DAA on α-amylase, ABA and GA levels of grains at 29/31 DAA under non-inducing and inducing conditions, respectively.

3. Experiment 1: ABA and GA sensitivity of intact, developing grains under non-inducing and inducing conditions (*in situ* study)

In this experiment, the *in situ* response of developing grains to applied ABA and GA_3 under cool-temperature shock-inducing and non-inducing conditions, measured as α-amylase activity, was studied using two UK winter wheat varieties, Spark (less PMA-susceptible genotype) and Rialto (highly PMA-susceptible genotype). The hypothesis examined in this experiment was, if a cool-temperature shock applied mid-way through grain development to induce PMA will alter ABA and/or GA sensitivity of the aleurone of intact, developing wheat grains. To test this hypothesis, a glasshouse experiment involving the *in situ* study was carried out with Spark and Rialto. This *in situ* experiment investigated the effect of four treatments (10% ethanol [mock treated], ABA [100 µM], GA_3 [50 µM] and ABA+GA_3 [100+50 µM]) applied to intact, developing grains at mid-grain development on α-amylase at maturity under cool-temperature shock-inducing and non-inducing conditions.

3.1. Materials and methods

Seeds of Spark and Rialto (Farrell and Kettlewell, 2008; Flintham *et al*., 2011) were obtained from the John Innes Centre (Norwich, UK). In the 1990s, both Spark and Rialto were recommended for bread making purposes in the UK (Anon., 1997). Spark has performed well and produced reasonably high HFN and good quality bread. However, Rialto did not perform well under cool and wet summer conditions of the UK, and often produced a low HFN, which contributed to poor quality bread. Later, it was found in field trials that Rialto was more susceptible to PMA under inductive conditions. In the last decade, these two varieties have been used in the UK for PMA related studies, where Spark served experimentally as a non-inducible genotype and Rialto as an inducible genotype (Farrell and Kettlewell, 2008; Flintham *et al*., 2011). These two varieties differ in their *Rht-D1* genotype. Spark has a GA-sensitive *Rht-D1a* (tall) allele, whereas Rialto has a GA-insensitive *Rht-D1b* (semi-dwarf) allele (Srinivasachary *et al*., 2009).

Seeds were planted on 5th October 2009 in a glasshouse in trays filled with John Innes No. 2 Compost (Keith Singleton's Seaview Nurseries, Cumbria, UK). Each variety was grown separately on glasshouse benches. Following germination and emergence, plants were vernalised at 4°C for eight weeks in a cold room and then transferred to 1.3 L pots containing John Innes No. 2 Compost (one plant/pot, pot dimensions: 11 x 12 x 18 cm) in a glasshouse. Plants were grown at minimum day/night temperatures of 15/5°C for one month. This was mainly to prevent plants from becoming devernalised by the warm temperatures used later (i.e. 25/15°C) and also to simulate field conditions. After one month, minimum temperatures were then gradually increased over five weeks by 2°C per week to 25/15°C. Plants were watered automatically with capillary matting wetted three times a day (for 10 min every time). Supplementary light (high pressure sodium, 400

W) provided a minimum of 16 h day length throughout the experiment. The protocol for growing plants is shown in Figure 1 (Farrell and Kettlewell, 2008). Excess side tillers were removed periodically to leave the main shoot plus four secondary shoots. Fertilisers and pesticides were applied to all plants, approximately with the same rate, at various stages of development as mentioned in appendix 1. The main spike on each plant was tagged with a coloured tape at early anthesis, i.e. ZGS61 (Zadoks *et al*., 1974). No fertilisers and fungicides were applied following ZGS71. The experimental design was completely randomised, where each variety was grown on separate benches in a glasshouse. Two temperature conditions used in this experiment were: cool-temperature shock-inducing and non-inducing. Hormone treatments applied were the four combinations as shown in Table 1. Therefore, the four combinations of hormonal treatments were arranged in a 2 x 2 factorial ANOVA with the two hormones (ABA and GA_3) as the two factors and the two concentrations as the two levels of each factor. As ABA and GA_3 were both dissolved in 10% ethanol, the 0 µM ABA plus 0 µM GA_3 treatments involved the application of 10% ethanol. Data from inducing and non-inducing conditions were analysed separately for each variety. There were ten replicate plants for each hormone treatment.

Table 1. Treatment design for experiment 1.

		Factor 1	
		ABA treatment	
		Level 1	**Level 2**
		Not applied	Applied
	Level 1	No ABA	100 µM ABA
Factor 2	Not applied	No GA_3	No GA_3
GA_3 treatment			
	Level 2	No ABA	100 µM ABA
	Applied	50 µM GA_3	50 µM GA_3

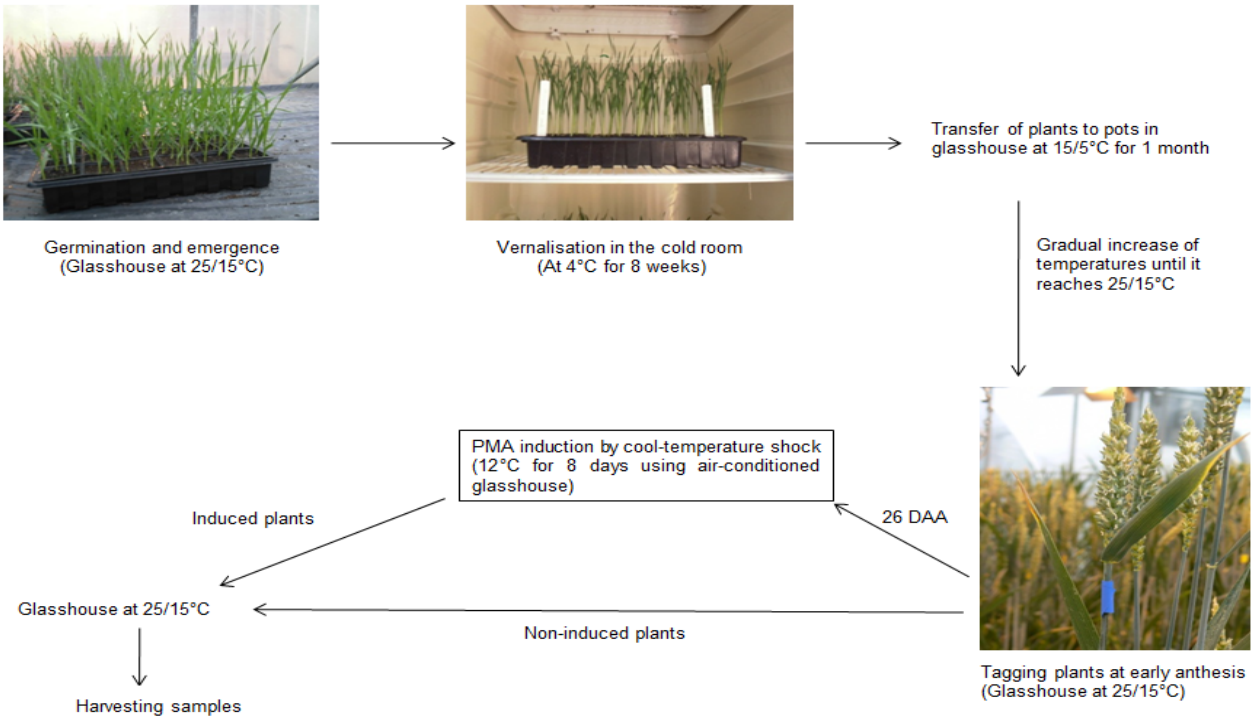

Figure 1. The schematic representation of the PMA induction protocol for winter wheat varieties. Experiment 1.

3.1.1. The cool-temperature shock-induction

The cool-temperature shock was given at 26 DAA by transferring plants to an air-conditioned glasshouse cooled to provide a constant temperature of 12°C for 8 days (Farrell and Kettlewell, 2008).It was assumed that the change to a cool-temperature was the main environmental difference experienced by the plants since other changes to the environment such as the light, water and nutrient application to plants were kept the same as far as possible. The cool-temperature shock-induction was conducted during mid-March to early April 2010. Average temperature in the warm and air-conditioned glasshouse for induced and non-induced plants during the induction period is shown in appendix 2.

3.1.2. The *in situ* hormone application to developing wheat grains

ABA or GA_3 solution (10 µl) was applied directly by a pipette onto the crease region of the two outermost grains (spikelet 7 or 9; counting acropetally) of the main shoot on each plant by gently pulling back the lemma with forceps in cool-temperature shock-induced and non-induced plants (Figure 2). Hormone application was carried out in three doses (1^{st} dose– 26 DAA, 2^{nd} dose– 29 DAA and 3^{rd} dose– 32 DAA) during the published window of sensitivity (Mares and Mrva, 2008), which corresponded to growth stages 75–77 (i.e. ZGS75–77) (Zadoks *et al.*, 1974).

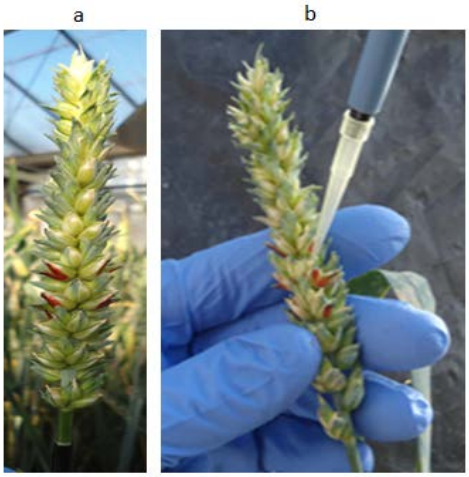

Figure 2. The image shows a. the two outermost grains from spikelet 7 and 9 marked by a marker pen and b. the application of hormone solution onto the grains with a pipette. Experiment 1.

The cool-temperature shock-induced plants were returned to a warm glasshouse heated to 25/15°C, where they remained until maturity. Plants were then harvested depending upon treatments given (i.e. induced plants were harvested at 64 DAA whereas non-induced plants were harvested at 60 DAA). The different time point for harvesting induced and non-induced plants was calculated from the difference in the degree days accumulated during the cool-temperature shock-induction. Expected design of degree DAA relative to DAA is shown in appendix 3 for cool-temperature shock-induced and non-induced plants.

3.1.3. Measurement of α-amylase activity by a modified Phadebasassay

Hormone-treated grains (the two outermost grains on spikelet 7 or 9 per replicate plant) were removed from harvested spikes. Distal half-grains were obtained by excising the embryos. Distal half-grains, i.e. the apical brush end half, were ground (one per well) against the force exerted by the stainless steel balls (one ball per well) in a 96-well block using a TissueLyser II at 2x30 Hz for 4 min. Following grinding, the stainless steel balls were removed and the 96-well block with flour was incubated in a 60°C water bath for 30 min. Buffer A (sodium maleate [100 mM, pH 6.0] plus calcium chloride [5 mM] and sodium azide [0.02%]) (pre-heated to 60°C) was added to each well (250 µl) and the contents mixed by pipetting up and down five times. The extraction was continued for exactly 5 min at 60°C. The amylazyme substrate solution (50 µl of one amylazyme tablet dissolved in 1 ml of buffer A; Megazyme International Ireland Ltd., Ireland) was added to each well and the reaction continued for a further 5 min at 60°C. Samples were removed from the water bath and immediately 300 µl of STOP solution (2% w/v tris Base, pH 9.5) was added to terminate the reaction. The contents of the block were mixed by shaking the block several times by hand and were held at room temperature for 5 min. Samples were centrifuged at 1233 g (3500 rpm) for 10 min. Supernatant (200 µl) was transferred to a clear flat-bottomed 96-well assay plate. Absorbance readings were taken at 590 nm using a microplate reader (BioRad [Benchmark], UK). These

absorbance readings were considered as the measurement of α-amylase activity and expressed as OD units/distal half-grain.

3.1.4. Statistical analysis and presentation

Alpha-amylase activity data were analysed statistically by a 2 x 2 factorial ANOVA with the two hormones (ABA and GA_3) as the two factors and the two concentrations as the two levels of each factor at $P<0.05$ with Genstat 13th edition. Analysed α-amylase activity data represents the mean of α-amylase activity measured from the two outermost grains (spikelet 7 or 9; counting acropetally) of the main spike per ten replicate plants (i.e. n=10). Data from inducing and non-inducing conditions were analysed separately for each variety. Plants were transferred as a group to another environment (i.e. air-conditioned glasshouse) for the induction. There was no true replication for the induction treatment, only subsamples. The way to truly replicate the induction treatment would have been to use four glasshouses, two for induction (12/12°C) and two for warm temperatures (25/15°C). Thus, the induction treatment is confounded with glasshouse effects that would occur normally from one glasshouse to another (e.g. lighting differences or some other environmental differences). Since the induction treatment data were not replicated, it was important to analyse the treatments under inducing and non-inducing conditions separately.

Each variety was also analysed separately for both experiments. There were two reasons behind analysing each variety separately. Firstly, each variety was grown on separate benches in a glasshouse. Secondly, there was a considerable variation in coefficient of variation (CV) values for α-amylase activity after hormonal treatments between the two varieties used. In order to reduce variance heterogeneity, α-amylase activity data were transformed using a power (i.e. x^{-2}) transformation prior to ANOVA. Variance heterogeneity and normality of the data were satisfactory after transformation. For the simplicity of understanding and to avoid the distortion of data caused by a power transformation, means of back-transformed treatments were used for presenting the results (Table 1, Figure 3).

3.2. Results

3.2.1. Under non-inducing conditions

The overall effect of applied ABA (100 µM) on α-amylase activity at maturity was not significant in either variety: Spark (P=0.414) or Rialto (P=0.207). Overall, applied GA_3 (50 µM) resulted in a significant increase in α-amylase activity at maturity in Rialto (P=0.007), but not in Spark (P=0.125). The two-way interaction between ABA and GA_3 was not significant in either variety Spark (P=0.299) or Rialto (P=0.106) (Figure 3).

3.2.2. Under cool-temperature shock-inducing conditions

In general, applied ABA produced no significant effect on grain α-amylase activity at maturity in Spark (P=0.572), but there was a marginally significant decrease in Rialto (P=0.061). Overall, applied GA_3 resulted in a significant increase in grain α-amylase at maturity in Rialto (P=0.005), but not in Spark (P=0.219).The ABA x GA_3 interaction was not significant in either variety: Spark (P=0.761) and Rialto (P=0.385) (Figure 3).

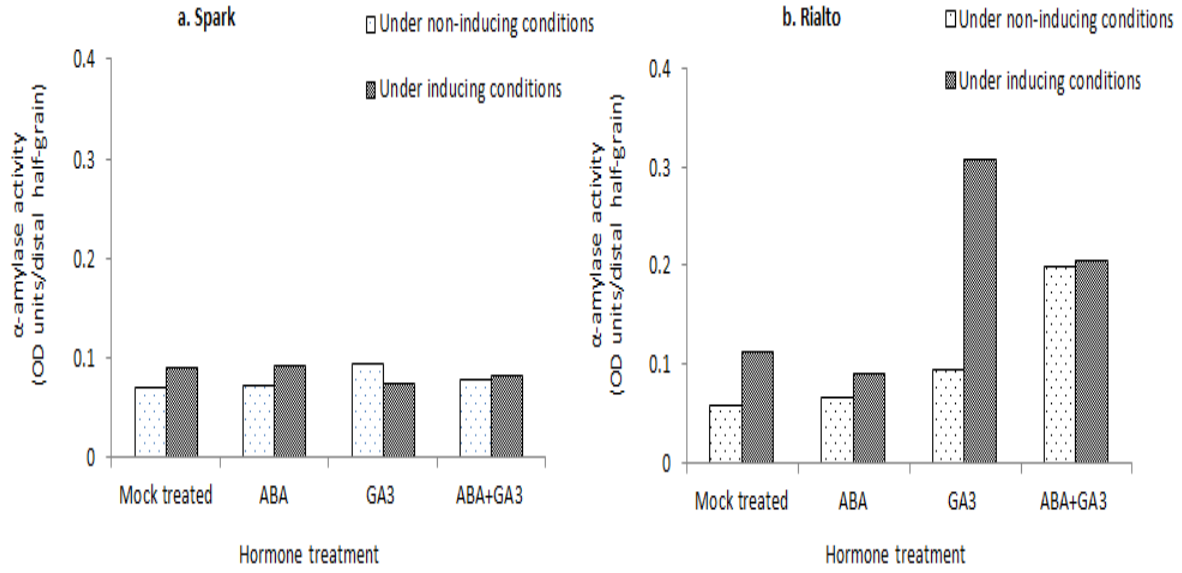

Figure 3. Effects of *in situ* applied hormones (mock treated, ABA [100 μM], GA_3 [50 μM] and ABA+GA_3 [100+50 μM]) at mid-grain development on grain α-amylase at maturity under cool-temperature shock-inducing and non-inducing conditions in two winter wheat varieties, a. Spark and b. Rialto. SEM values were back-transformed to OD units. Spark: Non-inducing conditions- SEM=0.028, d.f.=36 and CV=35.9%; Inducing conditions– SEM=0.032, d.f.=36 and CV=38.5%. Rialto: Non-inducing conditions– SEM=0.097, d.f.=36 and CV=51.4%; Inducing conditions– SEM=0.159, d.f.=36 and CV=85.6%. Experiment 1.

4. Experiment 2: ABA and GAsensitivity of intact, developing grains in a mapping population fixed for a non-1B1R or 1B1R translocation, but having either the *Rht-D1a* (tall) or the *Rht-D1b* (semi-dwarf) allele under non-inducing and inducing conditions(*in situ* study)

4.1. Spark x Rialto mapping genotypes

Genotyping of the population for simple sequence repeat (SSR) markers was carried out by John Innes Centre (Norwich, UK) and RAGT Seeds Ltd. (Saffron Walden, Cambridgeshire, UK) as described in Snape *et al*. (2007), as well as cytogenetic analysis according to Lukaszewski (2000). Experiment 2 will help to understand the role of the GA-insensitive *Rht-D1b* semi-dwarfingallele contributed by Rialto during PMA induction by a cool-temperature shock in the non-1B1R and 1B1R backgrounds.

Spark and Rialto were used as parental lines to produce mapping population. These parental lines have a recombinant double haploid genotype. Spark carries a distinct marker for the *Rht-D1a* (tall) allele and for Glu-B from wheat (i.e. does not contain the 1BS/1RS translocation), whereas Rialto carries a distinct marker for the *Rht-D1b* (semi-dwarf)allele and for Glu-B from rye (i.e. contains the 1BS/1RS translocation). Genotypes with the 1BS/1RS translocation were referred to as '1B1R genotypes', whereas those genotypes without the 1BS/1RS translocation were referred to as 'non-1B1R genotypes'. Seeds of S x R mapping genotypes were provided by John Flintham (John Innes Centre, Norwich, UK). Mapping genotypes used in this study were classified into four groups, partly based on their genetic background such as 'non-1B1R *Rht-D1a* (tall)genotypes' (SR2, SR3, SR5, SR7, SR8), 'non-1B1R *Rht-D1b* (semi-dwarf)genotypes' (SR36, SR67, SR77, SR87, SR92), '1B1R *Rht-D1a* (tall)genotypes' (SR16, SR21, SR23, SR38, SR58) and '1B1R *Rht-D1b* (semi-dwarf)genotypes' (SR4, SR24, SR34, SR85, SR119). Mapping genotypes in the non-1B1R *Rht-D1a* (tall)group or the non-1B1R *Rht-D1b* (semi-dwarf)group carry the wheat marker for Glu-B (i.e. the 1B1S arm), whereas mapping genotypes in the 1B1R *Rht-D1a* (tall)group or the 1B1R *Rht-D1b* (semi-dwarf)group carry the rye marker for Glu-B (i.e. the 1B1R arm). Additionally, the non-1B1R *Rht-D1a* (tall) group or the 1B1R *Rht-D1a* (tall) group contained a 'wild type' marker of *Rht-D1* (i.e. *Rht-D1a*), whereas the non-1B1R *Rht-D1b* (semi-dwarf)group or the 1B1R *Rht-D1b* (semi-dwarf)group contained a 'mutant' marker of *Rht-D1* (i.e. *Rht-D1b*).

4.2. Materials

In this experiment, seeds of 20 mapping genotypes mentioned above were sown on 10th October 2011. The main spike per replicate plant was tagged at early anthesis with coloured tapes. After the completion of anthesis, plant height (cm) was measured from the base of the plant to the tip of the main spike with a measuring tape. A cool-temperature shock-induction was applied between mid-March and early April 2012.

Plants were grown according to the PMA induction protocol (as described in experiment 1). ABA and GA_3 solutions were applied separately to intact, developing grains according to the method described in experiment 1. Samples from induced plants were harvested at 64 DAA, whereas those from non-induced plants were harvested at 60 DAA. α-amylase activity was measured using distal half-grains, i.e. the apical brush end half, by the Megazyme assay (details are given in Kondhare, 2012) and expressed as OD units/distal half-grain.

4.3. Statistical analysis

Square-root transformed α-amylase activity data, representing the mean activity measured from the two outermost hormone-treated grains (spikelet 7 or 9; counting acropetally) of the main spike

per ten replicate plants (i.e. n=10), were analysed by a two way ANOVA with Tukey's multiple comparison test at P<0.05 using Genstat 14th edition. Alpha-amylase data from 20 genotypes were analysed to test the effect of the *Rht-D1a* (tall) or the *Rht-D1b* (semi-dwarf) allele on grain PMA in the non-1B1R or the 1B1R background. Moreover, data from non-inducing and inducing conditions and also the data for the non-1B1R and the 1B1R background were analysed separately.

4.4. Results

4.4.1. Comparison of plant heights across four groups of mapping genotypes

In the non-1B1R background, plant height was not significantly different in *Rht-D1b* genotypes compared to *Rht-D1a* genotypes. In contrast, in the 1B1R background, *Rht-D1b* genotypes showed significantly reduced plant height (P<0.001) compared to *Rht-D1a* genotypes (Figure 4).

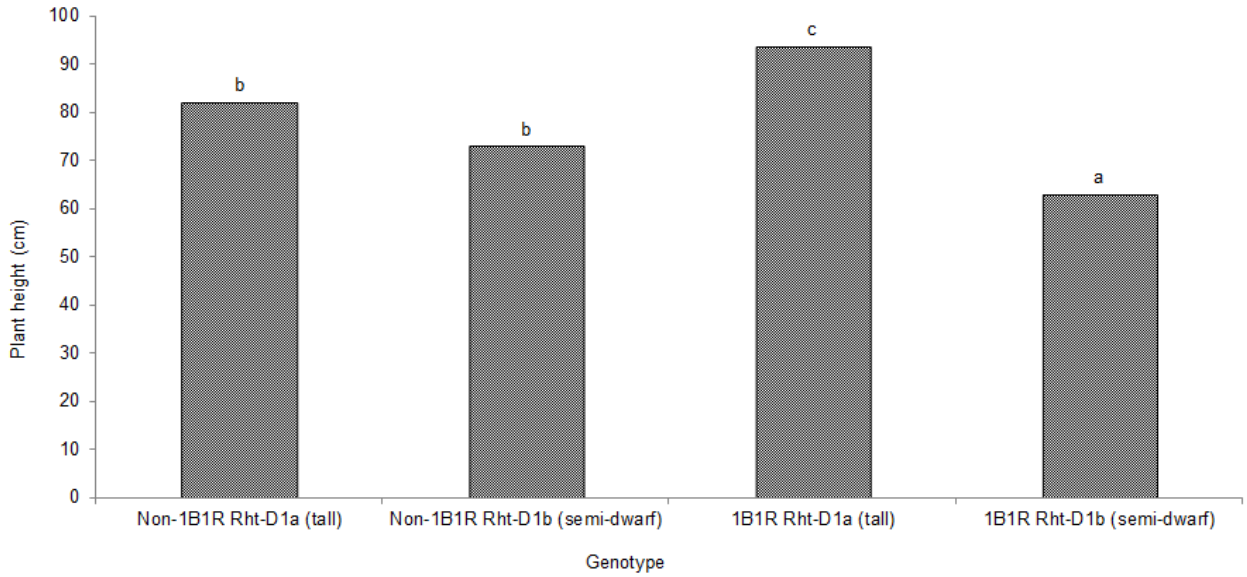

Figure 4. Comparison of wheat plant heights across four groups of mapping genotypes: non-1B1R *Rht-D1a* (tall), non-1B1R *Rht-D1b* (semi-dwarf), 1B1R *Rht-D1a* (tall) and 1B1R *Rht-D1b* (semi-dwarf) (SEM=5.32, d.f.=16 and CV=6.9%). Values are the means of ten replicate plants per genotype (n=10), where the height of only the main shoot was considered. Experiment 2.

4.4.2. Comparison of α-amylase levels across four groups of mapping genotypes

Under mock treated non-inducing conditions

In the non-1B1R background, there was no significant difference in grain α-amylase at maturity between *Rht-D1b* (semi-dwarf) genotypes and *Rht-D1a* (tall) genotypes. In the 1B1R background, *Rht-D1b* (semi-dwarf) genotypes showed significantly less grain α-amylase at maturity compared to *Rht-D1a* (tall) genotypes (Figure 5a).

Under mock treated inducing conditions

In the non-1B1R background, there was no significant difference in grain α-amylase at maturity between *Rht-D1b* (semi-dwarf) genotypes and *Rht-D1a* (tall) genotypes. In the 1B1R background, *Rht-D1b* (semi-dwarf) genotypes showed significantly less grain α-amylase at maturity compared to *Rht-D1a* (tall) genotypes (Figure 5b). Thus, an increase in the level of grain α-amylase was observed in untreated grains for all four groups of mapping genotypes under inducing conditions compared to non-inducing conditions.

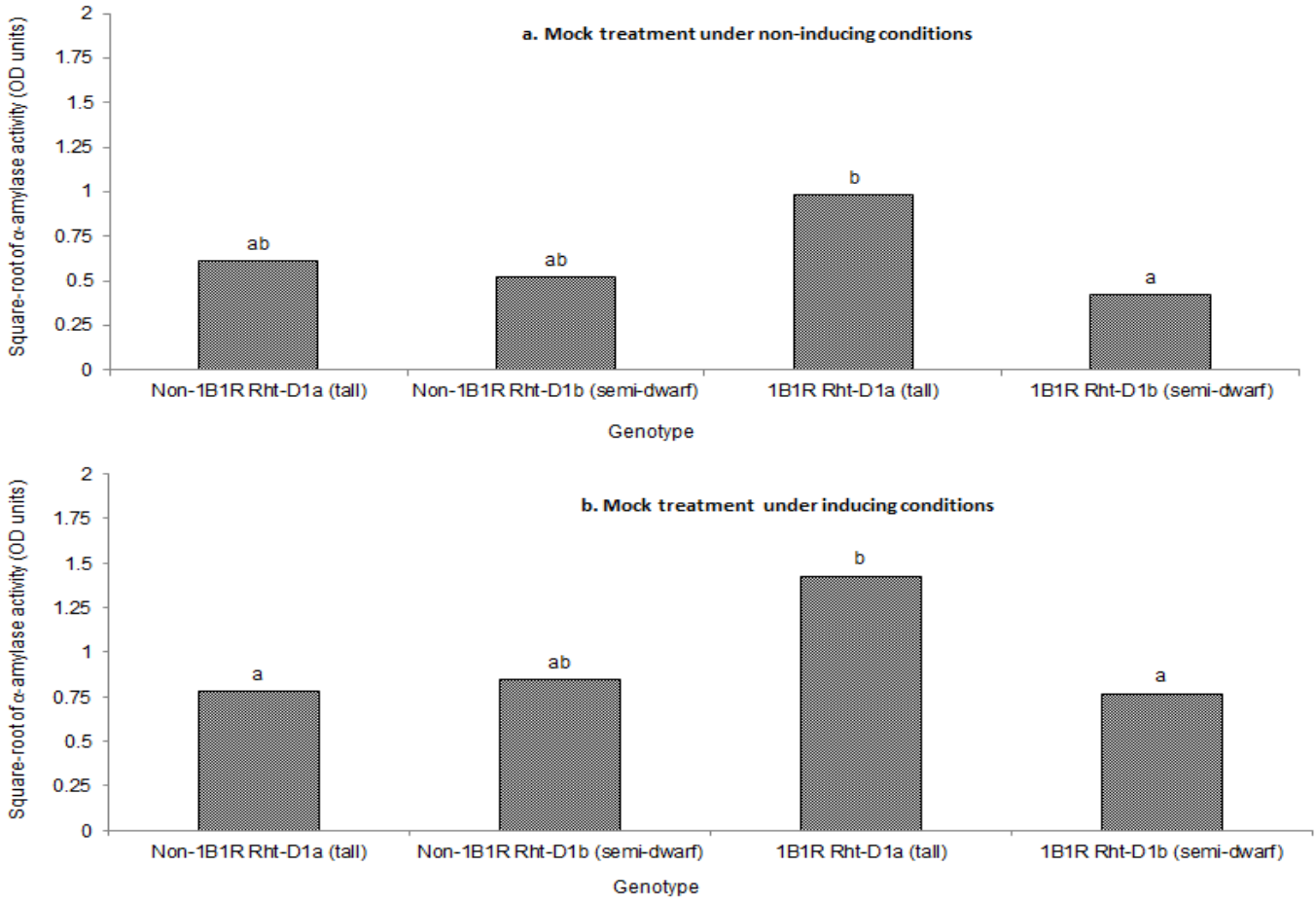

Figure 5. Comparison of α-amylase levels in mature grains across four groups of mapping genotypes: non-1B1R *Rht-D1a* (tall), non-1B1R *Rht-D1b* (semi-dwarf), 1B1R *Rht-D1a* (tall) and 1B1R *Rht-D1b* (semi-dwarf) for mock treatment under a. non-inducing conditions (P=0.033, SEM=0.20, d.f.=16, CV=35.7%) and b. inducing conditions (P=0.02, SEM=0.34, d.f.=16, CV=35.4%). Values are the means of grain PMA from five individual genotypes in each group, with the two outermost mock treated grains taken from spikelet 7 or 9 of 10 replicate plants for each genotype (n=5). Experiment 2.

4.4.3. Effect of a semi-dwarfing allele *Rht-D1b* on grain α-amylase

GA_3 treatment

In the non-1B1Rbackground, the grains of *Rht-D1b* (semi-dwarf) genotypes showed significantly less α-amylase for applied GA_3 compared to the grains of *Rht-D1a* (tall) genotypes under non-inducing conditions (Figure 6a). There was no significant difference in grain α-amylase for applied

GA_3 between *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes under inducing conditions, with both genotypes showing a high level of grain α-amylase (Figure 6b).

In the 1B1Rbackground, *Rht-D1b* (semi-dwarf) genotypes showed significantly less grain α-amylase for applied GA_3 compared to *Rht-D1a* (tall) genotypes under non-inducing conditions (Figure 6c). No significant differences in the grain α-amylase level for applied GA_3 was observed under inducing conditions between *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes, with both genotypes showing a high level of grain α-amylase (Figure 6d).

ABA treatment

In the non-1B1R background under non-inducing conditions, there was no significant effect of applied ABA on grain α-amylase in *Rht-D1a* (tall) genotypes (Figure 6a) compared to *Rht-D1b* (semi-dwarf) genotypes. Under inducing conditions, the grains of *Rht-D1b* (semi-dwarf) genotypes showed a significant reduction in grain α-amylase in response to applied ABA compared to *Rht-D1a* (tall) genotypes, for which the effect of ABA treatment was not significant (Figure 6b).

In the 1B1R background under non-inducing conditions, applied ABA showed a significant increase in grain α-amylase in *Rht-D1a* (tall) genotypes compared to *Rht-D1b* (semi-dwarf) genotypes, however this effect in *Rht-D1a* (tall) genotypes was not significantly different from the respective mock treated grains (Figure 6c). Similar results were observed for applied ABA towards grain α-amylase in *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes under inducing conditions in the 1B1R background, with a further small increase in grain α-amylase under inducing conditions compared to non-inducing conditions (Figure 6d).

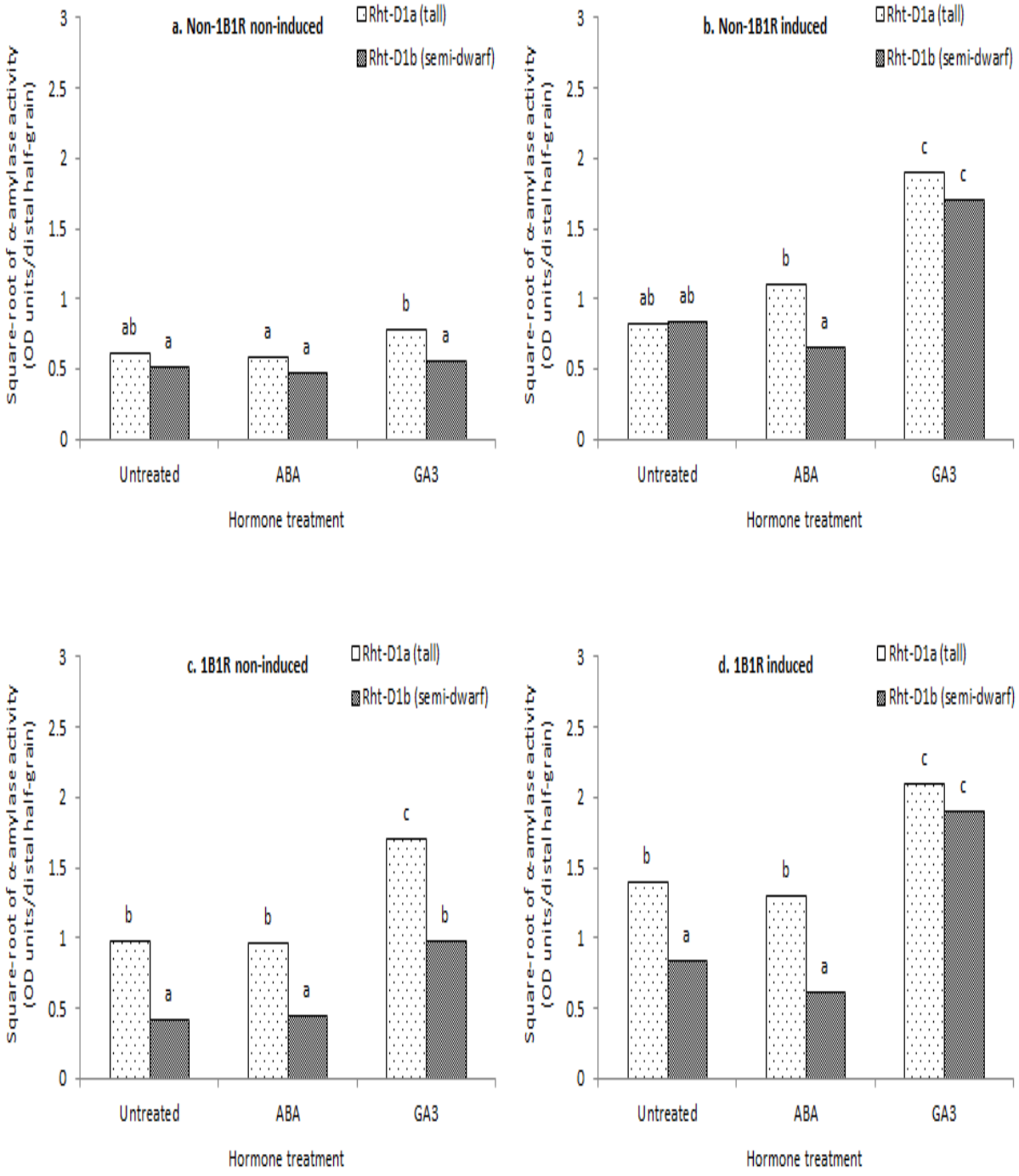

Figure 6. Effects of *in situ* applied ABA and GA_3 at mid-grain development on grain α-amylase at maturity in mapping genotypes fixed for a non-1B1R translocation, but having either the *Rht-D1a* (tall) or the *Rht-D1b* (semi-dwarf) allele a. under non-inducing conditions (SEM=0.31, d.f.=294, CV=54.3%); b. under inducing conditions (SEM=0.39, d.f.=294, CV=34%) and also, mapping genotypes fixed for a 1B1R translocation, but having either the *Rht-D1a* (tall) or the *Rht-D1b* (semi-dwarf) allele; c. under non-inducing conditions (SEM=0.49, d.f.=294, CV=54.2%) and, d. under inducing conditions (SEM=0.42, d.f.=294, CV=30.8%). Experiment 2.

5. Experiment 3: ABA and GAsensitivity of detached, developing grains under non-inducing and inducing conditions at three

different time points: 29/31, 32/36 and 36/40 DAA, respectively (*In vitro* study)

The key objective of experiment 3 was to define the time point at which GA sensitivity is increased in developing grains of induced plants in Rialto. Spark was also included in this experiment to confirm the earlier results from experiment 2. The hypothesis tested in this experiment was: a cool-temperature shock applied mid-way through grain development to induce PMA will alter ABA and/or GA sensitivity of the aleurone of developing wheat grains at 29/31 or 32/36 or 36/40 DAA in Spark and Rialto.

5.1. Materials and methods

Seeds of Spark and Rialto were planted on 28 February 2010 in a glasshouse at Harper Adams University College. Plants were grown according to the PMA induction protocol (as described for experiment 1). Each variety was grown separately on glasshouse benches. Plants were tagged at early anthesis (i.e. ZGS61) (Zodaks *et al*., 1974) and subjected to two temperature conditions: cool-temperature shock-inducing and non-inducing. The cool-temperature shock-induction was conducted during mid-grain development (i.e. ZGS75–77), which occurred in late July 2010. Samples from induced and non-induced plants (only the main spike per replicate plant) were harvested at three different time points: 29/31 DAA, 32/36 DAA and 36/40 DAA, respectively (Figure 7). In each case, the first temperature refers to the harvest time for the non-induced plants and the second temperature to the harvest time for plants receiving a cool-temperature shock.Spikes from 20 replicate plants were harvested at the time point: 29/31 DAA (n=20), whereas spikes from 10 replicate plants were harvested at the two time points: 32/36 DAA (n=10) and 36/40 DAA (n=10). The different time point for harvesting induced and non-induced plants was due to the difference in the degree DAA.

Isolating aleurone layers from developing grains is a very time-consuming and tedious process. Therefore, to save time and also due to the fact that the aleurone is the only source for α-amylase synthesis in embryoless, distal half-grain, it was decided to use distal half-grains to study the ABA and GA sensitivity of the aleurone from detached, developing wheat grains. Ten grains were pooled from the middle region of harvested spikes (only the main spike per replicate plant); surface sterilised with 70% ethanol for 1 min, followed by 5% sodium hypochlorite (available chlorine 10–15%, Sigma-Aldrich, UK) for 30 min and then rinsed five times with distilled water. After 1 hr soaking, grains were cut into equal halves using a scalpel (sterilised with 70% ethanol) and embryo halves were discarded. Distal half-grains were then incubated in 50 µl of hormone solutions (i.e. 10% ethanol [mock treated], ABA [100 µM] and GA_3 [50 µM]) with 950 µl of incubation medium (citric acid-sodium citrate buffer, 10 mM, pH 5 + $CaCl_2$, 0.2 M) for 72 hr at 25°C in the dark (Hader *et al*., 2003). Following incubation, the remaining incubation buffer was removed and samples were

freeze-dried (Edwards Modulyo F101, UK) for 4 days. Subsequently, dried samples were ground with one distal half-grain per well in a 96-well block using a TissueLyser II. Alpha-amylase activity in flour was measured by the Megazyme assay (as described for experiment 1) and expressed as OD units/distal half-grain. Square-root transformed α-amylase activity data were analysed by a one way ANOVA with Tukey's multiple comparison test ($P<0.05$). α-amylase activity data from inducing and non-inducing conditions and also data from each variety were analysed separately. Moreover, each time point was analysed separately.

Figure 7. Visual appearance of wheat spikes and grains harvested from Rialto plants at three time points during grain development (i.e. 29/31, 32/36 and 36/40 DAA). Experiment 3.

5.2. Results

5.2.1. Spark

1. 29/31 DAA (1st time point; a time point half-way through the cool-temperature shock-induction):Under non-inducing conditions in Spark, the *in vitro* applied ABA and GA_3 to distal half-grains produced no significant effect ($P=0.255$) on α-amylase compared to mock treated distal half-grains (Figure 8a). Similarly, under inducing conditions in Spark, the *in vitro* incubation of distal half-grains in ABA and GA_3 solutions resulted in no significant difference ($P=0.432$) in α-amylase compared to distal half-grains incubated in 10% ethanol (mock treated distal-half grains) (Figure 8a).

2. 32/36 DAA (2nd time point; two days after the cool-temperature shock-induction):Similar to the 29/31 DAA time point, the *in vitro* applied ABA and GA_3 to distal half-grains did not have significant effect on α-amylase compared to respective mock treated distal half-grains under both non-inducing (Figure 8a) and inducing conditions in Spark (Figure 8a).

3. 36/40 DAA (3rd time point; six days after the cool-temperature shock-induction): Under non-inducing conditions, distal half-grains treated *in vitro* with ABA and GA_3 solution produced no significant difference (P=0.499) in α-amylase compared to mock treated distal half-grains (Figure 8a). In contrast, under inducing conditions, the *in vitro* incubation of distal half-grains in GA_3 solution resulted in a significant (P<0.001) increase in α-amylase compared to mock treated distal half-grains, whereas the incubation with ABA solution had no significant effect on α-amylase (Figure 8a).

5.2.2. Rialto

1. 29/31 DAA: Under both non-inducing (Figure 8b) and inducing (Figure 8b) conditions in Rialto, the *in vitro* applied ABA and GA_3 to distal half-grains produced no significant effect on α-amylase compared to respective mock treated distal half-grains.

2. 32/36 DAA: Under both non-inducing (Figure 8b) and inducing (Figure 8b) conditions in Rialto, GA_3 applied *in vitro* to distal half-grains produced a significant increase in α-amylase compared to mock treated distal half-grains. However, this effect was more pronounced under inducing conditions than under non-inducing conditions. The *in vitro* applied ABA to distal half-grains produced a small, but not significant, increase in α-amylase compared to mock treated distal half-grains.

3. 36/40 DAA: Results similar to the 32/36 DAA time point were observed at the 36/40 DAA time point in Rialto for the *in vitro* applied ABA and GA_3 on α-amylase under both non-inducing (Figure 8b) and inducing (Figure 8b) conditions. However, there was further increase in grain PMA at the 36/40 DAA time point from the GA_3 treatment under both non-inducing and inducing conditions compared to the respective GA_3 treatment at the 32/36 DAA time point.

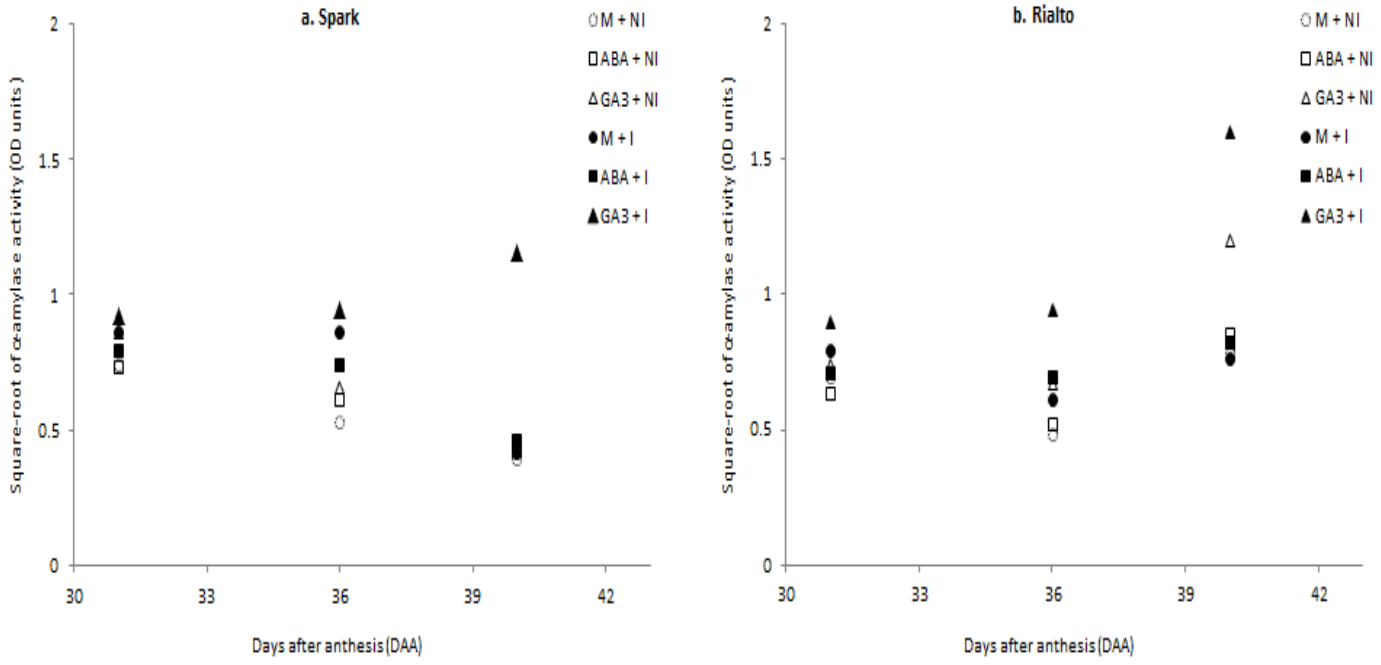

Figure 8. The *in vitro* ABA and GA_3 sensitivity of the aleurone of developing grains at 29/31, 32/36 and 36/40 DAA under non-inducing and inducing conditions, respectively, is shown as a timeline in a. Spark and b. Rialto. Abbreviations used in the graph: 'M'-mock treated [10% methanol treated]; 'ABA'-ABA [100 µM]; 'GA_3'- GA_3 [50 µM]; 'I'- cool-temperature shock-inducing conditions and 'NI'- non-inducing conditions. Spark- (1st time point [29/31 DAA]: non-inducing conditions [SEM=0.28, d.f.=57, CV=37.1%] and inducing conditions [SEM=0.30, d.f.=57, CV=36%], 2nd time point [32/36 DAA]: non-inducing conditions [SEM=0.28, d.f.=27, CV=37.1%] and inducing conditions [SEM=0.35, d.f.=27, CV=42.4%], 3rd time point [36/40 DAA]: non-inducing conditions [SEM=0.09, d.f.=27, CV=22.4%] and inducing conditions [SEM=0.36, d.f.=27, CV=54.2%]. Rialto- 1st time point [29/31 DAA]: non-inducing conditions [SEM=0.25, d.f.=57, CV=34.5%] and inducing conditions [SEM=0.39, d.f.=57, CV=49.8%], 2nd time point [32/36 DAA]: non-inducing conditions [SEM=0.15, d.f.=27, CV=27.1%] and inducing conditions [SEM=0.22, d.f.=27, CV=30.9%], 3rd time point [36/40 DAA]: non-inducing conditions [SEM=0.37, d.f.=27, CV=42.2%] and inducing conditions [d.f.=27, CV=36%, SEM=0.38]. Experiment 3.

6. Experiment 4: Effects of altered hormone levels on PMA induction

6.1. ABA and GA biosynthesis inhibitors

Paclobutrazol (α-tert-Butyl-β-(4-chlorobenzyl)-1H-1,2,4-triazole-1-ethanol), commonly abbreviated as PB, is a triazol type plant hormone biosynthesis inhibitor, which blocks biosynthesis of active GAs and therefore, decreases plant growth and development (Hedden and Graebe, 1985; Mehauchi *et al*., 1996). PB is xylem-mobile (Garcia-Martinez *et al*., 1987) and is generally used in agronomic and horticulture crops to reduce undesirable longitudinal shoot growth without affecting plant productivity (Hedden and Graebe, 1985; Rademacher, 2000). PB action inhibits the activity of *ent*-kaurene oxidase, which is an enzyme in the GA biosynthesis pathway that catalyses the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 2000). Fluridone (1-methyl-3-phenyl-

5-[3-trifluoromethyl) phenyl]-4-(IH)-pyridinone), commonly abbreviated as FD, is an ABA biosynthesis inhibitor (reduces endogenous ABA levels). It has a high competence of penetration and inhibitory effect compared to other chemical inhibitors of ABA biosynthesis in plants (Yamaguchi *et al*., 2007). FD inhibits the activity of phytoenedesaturase I, which catalyses the conversion of phytoene to phytofluene (Bartels and Watson, 1978; Fong *et al*., 1983). Carotenoids are the precursors for ABA biosynthesis in plants (Zeevaart and Creelman, 1988). Thus, FD action blocks ABA biosynthesis by inhibiting one of the enzymes of the carotenoid biosynthesis pathway.

This experiment studied the effects of *in situ* applied FD (20 µM) and PB (20 µM) to developing grains at 24, 25 and 26 DAA on α-amylase, ABA and GA levels of grains at 29/31 DAA under non-inducing and inducing conditions, respectively, in Rialto. Mainly, the second possibility proposed in the hypothesis was tested in this chapter alternatively using a chemical inhibitor of ABA biosynthesis- FD and a GA biosynthesis inhibitor- PB. Therefore, the use of FD and PB would give a hint that PMA induction is an abnormal response to endogenous hormone. The other objective of this experiment was to assess the potential of PB as an agent to control PMA.

6.1.1. Hypotheses

1. The *in situ* applied FD (20 µM) will decrease the ABA level of developing grains and applied PB (20 µM) will decrease GA levels of developing grains at 29/31 DAA under non-inducing and inducing conditions, respectively, in Rialto.

2. The *in situ* applied FD (20 µM) will increase, whereas applied PB (20 µM) will reduce, grain α-amylase at 29/31 DAA non-inducing and inducing conditions, respectively, in Rialto.

6.2. Materials and methods

In experiment 4, Rialto seeds were sown on 10th October 2011. Plants were grown according to the PMA induction protocol (as described in experiment 1). Plants (only the main spike per replicate plant) were tagged at early anthesis (i.e. ZGS61) (Zodaks *et al*., 1974). A cool-temperature shock-induction was applied between mid-March and early April 2012.

In this experiment, the crease regions of developing grains within the main spike (per replicate plant) were made visible by opening the lemma with forceps. All intact grains within the main spike were sprayed uniformly with FD (20 µM) or PB (20 µM) solutions using a hand-sprayer (0.5 Litre, HozelockSprayMister, UK) at the rate of about 5 ml per spike (Figure 9). A similar method for FD application (i.e. spraying the top of spikes) was used earlier by Yang *et al*. (2004) in wheat and by Tang *et al*. (2009) in rice to study the role of ABA in various aspects of grain filling. In this experiment, solutions were sprayed on three subsequent days starting at 24 DAA, which was just before a cool-temperature shock-induction was carried out. Mock treated plants were sprayed with

the same volume of 10% methanol. The main spike of each replicate plant was sprayed between 5 and 7 pm as stomata on grains were expected to be open, which would facilitate the uptake of these hormone inhibitors by intact, developing grains. Subsequently, plants with inhibitor-treated grains were subjected from 26 to 34 DAA to two temperature conditions: cool-temperature shock-inducing and non-inducing. Spikes from three replicate plants (only the main spike per replicate plant) were harvested at 29/31 DAA into liquid nitrogen and stored at -70°C. Subsequently, samples were then freeze-dried over four days. All inhibitor-treated grains from the harvested spike were pooled and ground to a fine powder using the tissue miller at Rothamsted Research (Harpenden, UK).

Figure 9. The image shows the *in situ* application of inhibitors to developing wheat grains in the spike using a hand-sprayer.

Extraction and analysis of ABA and individual GAs from wheat flour was performed by the Gas Chromatography- Mass Spectrometry (GC-MS) system according to the protocol described previously by Croker *et al*. (1990), with the guidance of Prof. Peter Hedden (Rothamsted Research, Harpenden, UK). The details are also given in Kondhare (2013; PhD Thesis; Harper Adams University). Aliquots of samples (i.e. 25 mg flour) were taken for measurement of α-amylase activity by the Megazyme assay (as described in experiment 1) and expressed as OD units. For α-amylase activity, data were analysed by a one way ANOVA with Tukey's multiple comparison test at $P<0.05$ with Genstat 14th edition. Square-root transformed α-amylase activity data from non-inducing and inducing conditions were analysed separately.

6.3. Results

6.3.1. Effects of *in situ* applied FD and PB on ABA and GA levels of grains

This part of the experiment determined the effects of *in situ* applied inhibitors (FD [20 µM] and PB [20 µM]) to developing grains at 24, 25 and 26 DAA on ABA and GA levels of grains at mid-way through the window of sensitivity (i.e. 29/31 DAA) under non-inducing and inducing conditions, respectively.

The ABA and individual GA levels were analysed in inhibitor-treated grains under inducing and non-inducing conditions using two way ANOVA with Tukey's multiple comparison test at $P<0.05$ (Table 2). Under both non-inducing and inducing conditions, *in situ* FD and PB treated developing grains contained no significant difference in ABA levels compared to respective mock treated grains. For mock treated grains, the cool-temperature conditions applied at mid-grain development significantly ($P<0.001$) reduced ABA levels of developing grains compared to non-inducing conditions (Table 2).

Under non-inducing conditions, *in situ* FD-treated grains produced a significant increase in GA_{20}, GA_{29}, GA_{44} and GA_{54} levels and a small, but not significant, increase in the GA_{19} level compared to mock treated grains. Under inducing conditions, FD-treated grains produced a significant decrease in GA_8, GA_{19} and GA_{20} levels and a small, but not significant, increase in GA_{29} and GA_{44} levels compared to mock treated grains. Under non-inducing conditions, *in situ* PB-treated grains produced a significant decrease in the GA_{19} level, whereas a significant increase in the GA_{29} level compared to mock treated grains. Moreover, *in situ* PB-treated grains produced a small, but not significant, decrease in the GA_8, $GA_{20,}$ GA_{44} and GA_{54} levels compared to mock treated grains. Under inducing conditions, PB-treated grains produced a significant decrease in GA_8, GA_{19} and GA_{20} levels and a small, but not significant, decrease in the GA_{44} level compared to mock treated grains (Table 2).

6.3.2. Effects of *in situ* applied FD and PB on grain α-amylase

Under non-inducing conditions, FD-treated intact grains showed a significant ($P=0.001$) increase in α-amylase compared to mock treated grains. However, PB applied to intact grains produced no significant effect on α-amylase compared to mock treated grains (Figure 10a). Under inducing conditions, grains treated *in situ* with FD and PB produced significantly ($P=0.001$) less grain α-amylase compared to mock treated grains (Figure 10b).

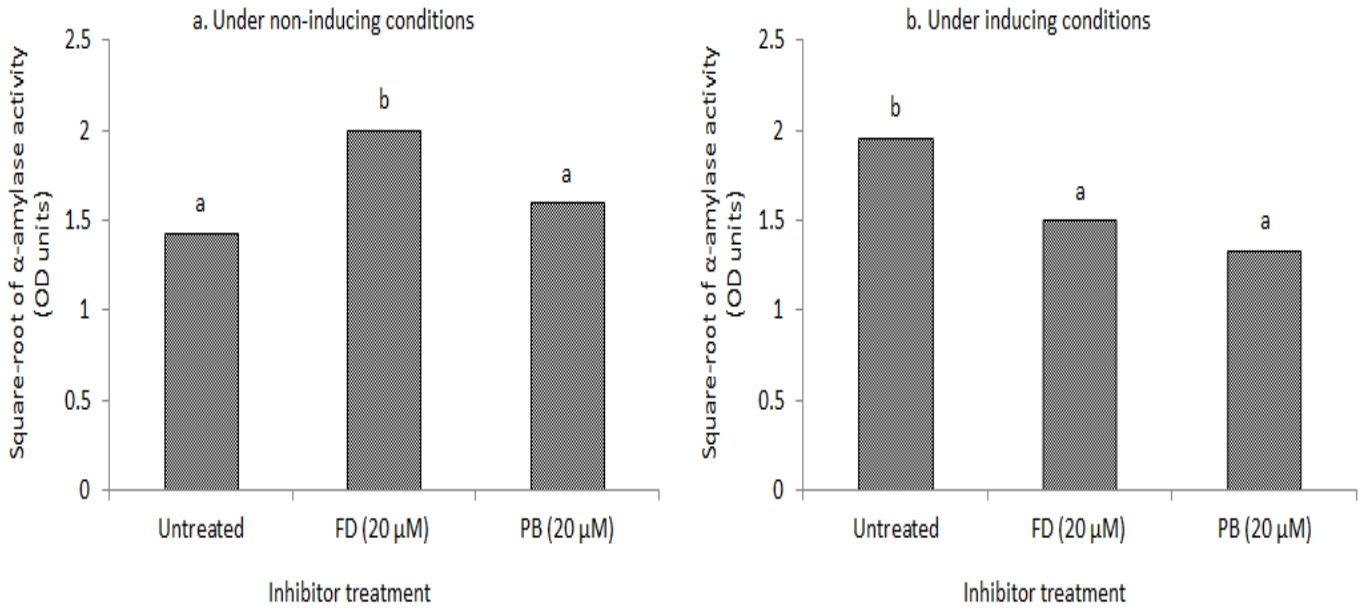

Figure 10. Effects of *in situ* applied FD (20 µM) and PB (20 µM) to developing grains at 24, 25 and 26 DAA on grain α-amylase at the 29/31 DAA time point a. under non-inducing conditions (n=3, SEM=0.10, d.f.=6, CV=6.2%) and b. under inducing conditions (n=3, SEM=0.11, d.f.=6, CV=7.2%), respectively. Experiment 4.

Table 2. ANOVA comparison for ABA and individual GA levels in developing Rialto grains treated *in situ* with FD (20 µM) or PB (20 µM) solution under cool-temperature shock-inducing and non-inducing conditions. n=3. CS=Cool temperature shock. Experiment 4.

		ABA and GA levels (ng/g dry weight)										
	Treatment	**ABA**	**GA_1**	**GA_{29}**	**GA_3**	**GA_8**	**GA_{20}**	**GA_{54}**	**GA_{44}**	**GA_{19}**	**GA_{53}**	
Non-inducing conditions	Mock treated	45.40 (bc)	0.75 (a)	1.16 (abc)	1.11 (a)	1.35 (ab)	0.43 (a)	25.51 (ab)	2.58 (a)	3.69 (ab)	0.84 (a)	
	FD (20 µM)	40.46 (abc)	0.63 (a)	1.98 (c)	3.12 (a)	1.51 (ab)	0.82 (b)	81.45 (b)	7.64 (b)	4.69 (ab)	0.87 (a)	
	PB (20 µM)	56.13 (c)	0.49 (a)	1.78 (bc)	3.06 (a)	0.92 (ab)	0.32 (a)	12.05 (a)	1.08 (a)	1.95 (a)	0.57 (a)	
Inducing conditions	Mock treated	28.90 (ab)	0.57 (a)	1.06 (abc)	3.67 (a)	2.68 (b)	0.61 (ab)	2.17 (a)	1.60 (a)	11.6 (b)	0.51 (a)	
	FD (20 µM)	24.26 (a)	0.45 (a)	0.70 (a)	2.03 (a)	0.50 (a)	0.30 (a)	0.35 (a)	0.56 (a)	7.51 (bc)	0.49 (a)	
	PB (20 µM)	30.53 (ab)	0.41 (a)	0.86 (ab)	1.39 (a)	0.72 (ab)	0.30 (a)	1.08 (a)	0.24 (a)	5.83 (ab)	0.48 (a)	
CS (P value)		<0.001	0.170	0.001	0.959		0.901	0.092	0.005	0.004	<0.001	0.003
Inhibitors (P value)		0.080	0.240	0.541	0.974		0.044	0.018	0.062	0.020	0.004	0.155
CS x Inhibitors (P value)		0.498	0.897	0.058	0.356		0.066	0.002	0.053	0.017	0.034	0.239
d.f		12	12	12	12		12	12	12	12	12	12
SEM		7.61	0.37	0.38	2.60		0.78	0.13	23.53	1.80	1.54	0.25
CV		20.2%	36.8%	30.7%	110.2%		61.0%	29.0%	115.1%	78.9%	26.2%	23.5%

7. Summary and discussion

α-amylase activity in induced grains is highly variable in a PMA susceptible variety, Rialto, and the level of α-amylase activity in induced grains varied not only between the spikelets in the central region of a spike, but also between the two outermost grains of the same spikelet (Figure 11). Stochastic behaviour of PMA induction in wheat grains was earlier observed by Gale *et al*. (1987), Mrva and Mares (2001a) and Flintham *et al.* (2011).

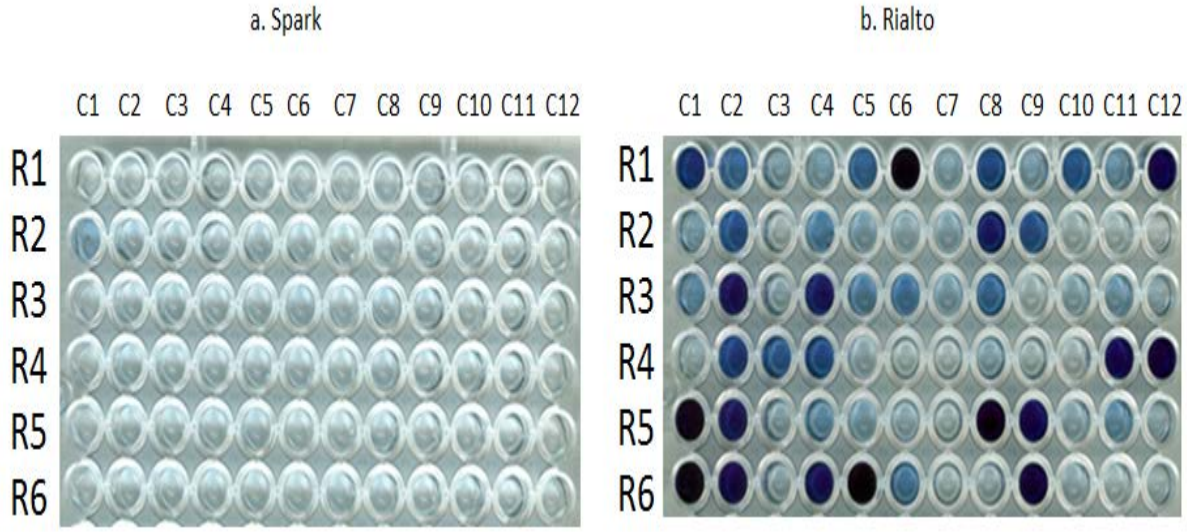

Figure 11. The image shows the variation in α-amylase activity of cool-temperature shock-induced grains in a. Spark (less PMA-susceptible genotype) and b. Rialto (highly PMA-susceptible genotype). Two outermost grains from the central region of the spikelet (i.e. spikelets 7, 9 and 11; counting acropetally) were selected from 20 replicate plants (only the main spike per replicate plant), which were subjected to the cool-temperature shock-induction at 26 DAA for 8 subsequent days. α-amylase activity in individual grain was measured using embryoless, distal half-grain at 60 DAA by the Megazyme assay in a 96-well plate using a microplate reader (as described in experiment 1). C=Column and R=Row. C1R1- Spike 1, spikelet 7, 1st outermost grain; C1R2- Spike 1, spikelet 7, 2nd outermost grain.C1R3- Spike 1, spikelet 9, 1st outermost grain; C1R4- Spike 1, spikelet 9, 2nd outermost grain.C1R5- Spike 1, spikelet 11, 1st outermost grain; C1R6- Spike 1, spikelet 11, 2nd outermost grain and so on.

- PMA is related to increased GA sensitivity and there was little evidence for a change in ABA sensitivity

- An approximate 3-fold increase in grain α-amylase after GA_3 treatment compared to the mock treated grain was observed under inductive conditions in experiment 1 in Rialto, which is consistent with the cool-temperature shock-induction being due to a change in GA sensitivity rather than an increase in GA levels.

- GA sensitivity was not induced in a PMA-susceptible genotype Rialto during the cool-temperature shock-induction (i.e. 31 DAA), but it was induced just after the induction when plants were returned back to a warm glasshouse (i.e. 36 DAA).

- Moreover, a cool-temperature shock significantly increased GA sensitivity in both *Rht-D1a* and *Rht-D1b* genotypes with or without the presence of the 1B1R translocation.

  Similar patterns of results were observed for Spark (low PMA-susceptible variety) and Rialto between the two sensitivity experiments, i.e. *in situ* application to intact grains with embryos (experiment 1) and *in vitro* application to half grains without embryos (experiment 3), with the exception of Spark in the *in situ* experiment 1. This confirms the fact that PMA was mainly induced in the aleurone tissue. This finding is consistent with Singh and Paleg (1984b) and Mrva *et al*. (2006), who observed that the PMA induction was an aleurone-dependent process (and independent of the embryo).

- As expected, *Rht-D1b* (semi-dwarf) genotypes with or without the presence of the 1B1R translocation showed significantly less grain α-amylase for applied GA_3 compared to the respective *Rht-D1a* (tall) genotypes under non-inducing conditions.

  Overall, the findings of experiment 2 with that of experiment 1 provide strong evidence that PMA is related to GA sensitivity of the aleurone. Under non-inducing conditions, the *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes showed increased GA sensitivity in the 1B1R background, but not in the non-1B1R background compared to the respective mock treatment (Figure 18c). This finding suggests that in the 1B1R genotypes, there might be a transfer of GA transduction (possibly, GA receptor) genes with the short 1R arm from rye to the wheat chromosome. However, there is not any supportive evidence yet available in the literature for this.

  Experiment 2 showed that a cool-temperature shock significantly increased GA sensitivity in *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes under both the non-1B1R and 1B1R backgrounds. These results suggested that the risk of PMA is very high as GA sensitivity was highly increased under inducing conditions irrespective of the presence of the *Rht-D1a* (tall) allele, the *Rht-D1b* (semi-dwarf) allele, with or without the 1B1R translocation in all genotypes and. hence, represents a serious problem to wheat breeders and growers.

  There was no change in ABA sensitivity as a result of the cool-temperature shock-induction in *Rht-D1a* (tall) and *Rht-D1b* (semi-dwarf) genotypes under both the non-1B1R and 1B1R backgrounds. The effect of a cool-temperature shock-induction on grain α-amylase in *Rht-D1a* (tall) plus 1B1R genotypes is not published elsewhere.

- In experiment 3, there was no significant increase in GA sensitivity across three time points (29, 32 and 36 DAA) for Spark under non-inducing conditions, whereas a significant increase in GA sensitivity at the latter two time points (i.e. 32 and 36 DAA) in Rialto was observed. A cool-temperature shock-induction increased GA sensitivity in grains of Spark only at 40 DAA, whereas it was induced earlier (i.e. 36 as well as 40 DAA) in Rialto. These results show that GA sensitivity was not induced in a PMA-susceptible genotype during the cool-temperature shock-induction, but it was induced just after the induction when plants were returned back to a warm glasshouse.

- Overall, the results from experiment 4 indicated that reducing ABA levels can stimulate PMA, possibly by contributing to an increase in GA sensitivity. In contrast, reducing GA levels can prevent PMA. Thus, it can be assumed that ABA or GA levels also play a role during PMA induction in wheat, in addition to GA sensitivity.

7.1. Predictions governing the induced GA sensitivity

Possible causes of the GA sensitivity induced by a cool-temperature shock in the *in situ* (experiments 1 and 2) and *in vitro* (experiment 3) studies in Rialto are an increase in the number of the GA receptors in the aleurone cells (Singh and Paleg, 1984a) e.g. GID1 receptor or an increase in the expression of GA signalling genes (Jiang *et al*., 1997) e.g. GAMYB proteins or an increase in GA sensitivity by reducing ABA content. This last possibility is supported by the results from experiment 4, in which induced grains showed significantly reduced ABA levels compared to non-induced grains overall at 29/31 DAA. To confirm the contribution of the first two possibilities, it is important to study the expression of the GA receptors genes (e.g. GID1 receptor) and genes of GA signaling downstream to the GID1, which includes the DELLA and GAMYB, in developing grains of PMA genotypes following a cool-temperature shock-induction.

7.2. Farmers' perspective

In order to control lodging, winter wheat, barley and oats are routinely sprayed in the UK with the growth retardant chlormequat chloride (Spink *et al*., 2004), which inhibits GA biosynthesis (Rademacher, 2000). Previous surveys and a literature review have shown that in chlormequat-treated cereal crops, the chlormequat residues can be detected in harvested cereal grains and grain-containing products. Even though the level of residues found in cereal grains were below the maximum residue limit (MRL), there is more pressure from the Food Standard Agency to minimise chlormequat chloride residues further in food products (Spink *et al*., 2004).

Similarly, PB is the plant growth retardant used in this project as the GA biosynthesis inhibitor to reduce GA levels in developing wheat grains. It was found that the *in situ* applied PB to developing wheat grains around mid-grain development significantly reduced grain α-amylase under cool-

temperature shock-inducing conditions, suggesting that PB can be used as an agent to overcome the problem of PMA occurrence in wheat grains. However, PB residues are persistent in the soil for a long time and there is more risk to human health from its residues being consumed when developing wheat grains are sprayed with the PB solution (Hedden, Pers. Comm.). If this approach is to be pursued, it will be important to use an alternative GA biosynthesis inhibitor, which is not persistent in the soil or in plant tissues and which has minimum adverse effects on human health. Thus, farmers could spray their wheat in the field around mid-grain development with that alternative GA biosynthesis inhibitor to reduce the risk of PMA occurrence. Currently, a GA biosynthesis inhibitor with the above properties is not available.

All findings reported in this project were from controlled-environment experiments and provide a useful insight about the role of ABA and GAs in the mechanism of PMA induction. However, they may not be extrapolated under natural field conditions. Therefore, similar experimentation under natural field conditions is recommended in future to confirm the findings of this project.

7.3. Strategy to develop a new wheat variety with better bread-making quality

I. Screening: Select a PMA-inducible variety through the cool-temperature shock screening method (Farrell and Kettlewell, 2008; Kondhare *et al*., 2012). PMA-inducible varieties already confirmed in the UK using this screen are Rialto, Cadenza, Potent, Maris Huntsman etc. (Farrell and Kettlewell, 2008; Flintham *et al*., 2011).

II. Transgenic for GA hypo-sensitivity: Construct a transgene containing GA hypo-sensitiveness (either through the GID1 [GA] receptor or the DELLA protein) with a promoter, which governs the gene expression specific only in the embryo or the aleurone of developing grains, and transform the transgene into a PMA-inducible variety.

III. Marker selection for GA hypo-sensitivity: Subject intact plants in a glasshouse experiment (Mrva and Mares, 2001a; Farrell and Kettlewell, 2008; Kondhare *et al*., 2012), or detached tillers in a field experiment, (Mrva and Mares, 2001a) to the cool-temperature shock based PMA induction screen protocol. Look for the effect of an introduced GA-hyposensitive transgene on PMA in the later stages of grain ripening under cool-temperature shock-inducing and non-inducing conditions.

An alternative strategy is to look for the available molecular markers (QTL), which govern the GA hypo-sensitivity in wheat genome and use them to develop breeding strategies to improve HFN stability e.g. use of high throughput approaches such as MAS (Marker-Assisted Selection) (Barkley and Wang, 2008) and TILLING (Targeting Induced Local Lesions in Genomes) (Parry *et al*., 2009; Uauy *et al*., 2009; Collard and Mackill, 2008).

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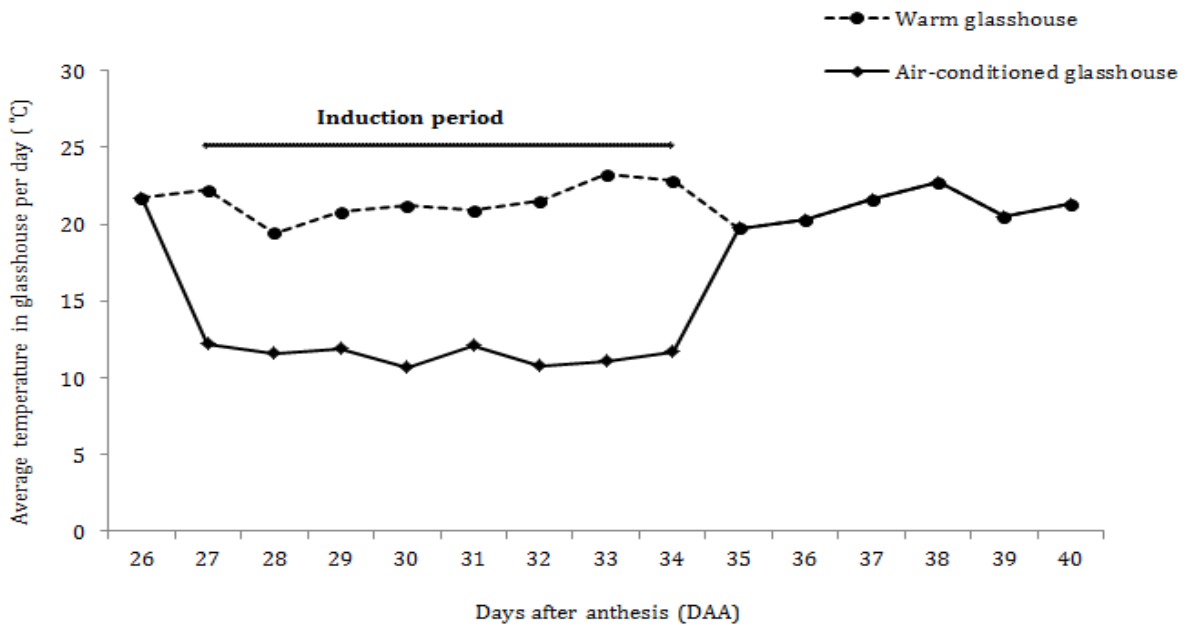
9. APPENDICES

Week	Development	ZGS	Fungicides	Fertilisers	Aphids
Week 0	Germination	ZGS 00	-	-	-
Week 1	Vernalisation	ZGS 10-12	Flexity + Eclipse	-	-
Week 9	Vernalisation	ZGS 13-14	-	-	-
Week 10	Seedling growth	ZGS 15-19	Fortress	-	-
Week 14	Stem elongation	ZGS 31-39	Flexity + Eclipse or Corbel + Envoy	NPK fertilizer	Nicotine smoke or Gazelle SG or Movento
Week 18	At spike emergence	ZGS 50-52	Fortress	-	Nicotine smoke or Gazelle SG or Movento
Week 22	Just before anthesis	ZGS 59-60	Flexity + Eclipse	NPK fertilizer	Nicotine smoke or Gazelle SG or Movento
Week 26	Milk development	ZGS 70-73	-	-	Nicotine smoke or Gazelle SG or Movento
Week 31-32	Ripening	ZGS 93-94	-	-	-

Appendix1. Timeline for fertiliser and pesticide application with the developmental growth stages. NPK fertiliser was used to provide additional nutrients. Several pesticides were sprayed to control powdery mildew and aphids.

*** Application rates-**

1. NPK fertiliser (Chempak Products, UK): Crystals (10 g) were dissolved in 5 L water and the solution was applied at the rate of 50 ml per pot.
2. Aphids: Gazelle SG (8 g in 2 L water; Certis, Solutions for Crop protection, UK) or Movento (1.16 ml in 2 L water; Bayer Crop Science, UK). Gazelle SG and Movento solutions were sprayed uniformly onto the leaves.
3. Flexity (1.16 ml in 2 L water; BASF The Chemical Company, UK), Eclipse (1.16 ml in 2 L water; BASF The Chemical Company, UK), Corbel (4.69 ml in 2 L water; BASF The Chemical Company, UK), Envoy (9.49 ml in 2 L water; BASF The Chemical Company, UK) and Fortress (1.16 ml in 2 L water; Dow AgroSciences Limited, UK). All solutions were sprayed uniformly onto the leaves.



Appendix 2. Average temperature in the warm and air-conditioned glasshouse for induced and non-induced plants during the induction period in experiment 2. In this experiment, the induction started on 15th March 2010 (i.e. 26 DAA) and ended on 23rd March 2010 (i.e. 34 DAA) for the first set of plants.

DAA	Non-inducing conditions		Inducing conditions	
	Average temperature	Degree DAA	Average temperature	Degree DAA
26	20	520	20	520
27	20	540	12	532
28	20	560	12	544
29	20	580	12	556
30	20	600	12	568
31	20	620	12	580
32	20	640	12	592
33	20	660	12	604
34	20	680	12	616
35	20	700	20	636
36	20	720	20	656
37	20	740	20	676
38	20	760	20	696
39	20	780	20	716
40	20	800	20	736

Appendix 3. Expected design of degree DAA relative to DAA for cool-temperature shock-induced and non-induced plants in experiment 2. During the cool-temperature shock-induction (i.e. 26-34 DAA), non-induced plants were grown at 25/15°C (day/night basis) whereas induced plants were grown at a constant temperature, which was 12°C.