Investigating conditions that induce late maturity alpha-amylase (LMA) using Northwestern US spring wheat (*Triticum aestivum* L.)

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Technical Update

Abstract

The wheat industry rejects grain with unacceptably high α-amylase enzyme levels due to the risk of poor endproduct quality. There are two main causes of elevated grain α-amylase: (1) preharvest sprouting in response to rain before harvest and (2) late maturity α-amylase (LMA) induction in response to a cool temperature shock during late grain development. LMA induction was detected in a panel of 24 Northwestern US spring wheat lines. Thus, this problem previously described in Australian and U.K. varieties also exists in U.S. varieties. Because LMA induction results were highly variable using published methods, a characterization of LMA-inducing conditions was conducted in an LMA-susceptible soft white spring wheat line, WA8124. Problems with elevated α-amylase in untreated controls were reduced by raising the temperature, 25°C day/18°C night *versus* 20°C day/10°C night. LMA induction was not improved by colder temperatures (15°C day/4°C night) *versus* moderately cold temperatures (18°C day/7.5°C night or 10°C day/10°C night). While previous studies observed LMA induction by heat stress, it failed to induce LMA in WA8124. Thus, not all LMA-susceptible cultivars respond to heat. The timing of LMA susceptibility varied between two cultivars and within a single cultivar grown at slightly different temperatures. Thus, variability in LMA induction likely results from variability in the timing of the grain developmental stage during which cold shock induces LMA. Thus, it was concluded that the visual inspection of grain is needed to correctly identify LMA-sensitive spikes at the soft dough stage of grain development (Zadok's stage 85).

Introduction

The quality of wheat (*Triticum aestivum* L.) end-products can be compromised by the presence of α-amylase in grain and flour as a result of either preharvest sprouting (PHS) or the developmental problem called late maturity α-amylase (LMA) [reviewed by Mares and Mrva (2014)]. PHS is the initiation of grain germination on the mother plant in response to rainy conditions after physiological maturity but before harvest. The enzyme α-amylase is expressed during grain germination in order to breakdown starch as a fuel for seedling growth. α-amylase can also be induced by a cold or high temperature shock during grain filling without postmaturity rainfall. This LMA phenomenon, also called prematurity α-amylase (PMA), has been characterized in Australian (Mares and Mrva, 2008) and U.K. wheat (Farrell and Kettlewell, 2008; Farrell et al., 2013). This research detected LMA susceptibility in spring wheat from the Northwestern US and characterized the conditions that induce LMA to improve screening methods.

Grain with elevated α-amylase levels can have problems with poor endproduct quality, including cakes that fall and sticky bread or noodles (Ross and Bettge, 2009). α-amylase is synthesized during germination to fuel seedling growth by digesting starch into shorter polysaccharides. The induction of α-amylase can begin before grains visibly germinate. Thus, the wheat industry uses the Hagberg–Perten Falling Number (FN) method to detect α-amylase and starch digestion in wheat meal (Perten, 1964; Delwiche et al., 2015). The FN test measures increasing α-amylase activity based on the decreased gelling of a heated meal and water mixture. During the FN test, a stirrer is dropped through a heated meal and water mixture, and the time required for the stirrer to fall through the mixture is measured in seconds (s). The more starch digestion, the faster the stirrer falls. In the wheat industry, grain with an FN below 300 s is considered to have an elevated risk of poor end-use quality. Farmers receive steep discounts for wheat with an FN below 300 s; as much as 25 cents per bushel for every 25 s below 300 s in

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Washington state (Steber, 2017). Thus, it is important to select varieties with a decreased risk of low FN due to PHS or LMA.

LMA is defined as the inappropriate induction of the enzyme α-amylase in response to temperature fluctuations during late grain maturation, approximately the soft dough stage (Mares and Mrva, 2008, 2014; Barrero et al., 2013). α-amylase is highly expressed during early grain development to mobilize nutrients for pattern formation, and then decreases as the grain enters the maturation phase when sugar is converted to starch during endosperm development. Normally, α-amylase should not be induced again until grain germination. However, the high pI α-amylase *TaAmy1* mRNA can be expressed during late grain filling in LMA-susceptible wheat (Mares and Mrva, 2008; Barrero et al., 2013; Mieog et al., 2017).

Temperature fluctuations during grain maturation can induce LMA. Previous work showed a strong interaction between α-amylase expression, activity, and the environment/temperature shock (Gale et al., 1990; Mares et al., 1994). Australian cultivars responded to cool temperature shock when the grains were in the mid to late stages of grain maturation, soft (Zadoks stage 85) to hard dough stage (Zadoks et al., 1974; Barrero et al., 2013; Derkx and Mares, 2020). α-amylase was induced in developing grains when mother plants or their detached tillers were transferred from a warm glasshouse (i.e. 18–28°C) at 26–35 days past anthesis (dpa) to either a cool temperature chamber (18°C/12°C day/night) or to a water bath at 10°C for 7 days (Mrva and Mares, 2001; Mrva et al., 2006; Mares and Mrva, 2008). Plants or tillers were allowed to mature under the starting temperature conditions after the cold treatment. Thereafter, α-amylase can be detected in mature wheat spikes or individual grains based on either activity in an enzyme assay or protein accumulation in an ELISA assay (Mares et al., 1994; Verity et al., 1999; Farrell and Kettlewell, 2008; Kondhare et al., 2012; Derkx and Mares, 2020). While U.K. varieties induced LMA when moved from a mid (22°C) growing temperature to low (12°C) temperature shock, or from a mid (20°C day/10°C night) growing temperature to high (30°C day/20°C night) temperature shock, the cool temperature treatment (18/12°C) that induced LMA in Australian varieties failed to induce LMA in U.K. wheat (Farrell and Kettlewell, 2008). Given the variation between U.K. and Australian results, it appears that either there is insufficient information about the optimal LMA induction conditions or that these conditions vary for different germplasm.

LMA induction generally requires a low or high temperature shock during the late soft dough stage of grain development in semi-dwarf (*Rht-B1b* or *Rht-D1b*) wheat varieties but can occur without cold treatment in wheat with wild-type height alleles (*rht-B1a*, *rht-D1a*) (Farrell and Kettlewell, 2008; Mrva et al., 2009; Barrero et al., 2013; Farrell et al., 2013). The 1BL/1RS rye translocation into wheat was also associated with elevated LMA susceptibility.

Current evidence suggests that LMA and PHS differ in terms of underlying genetic mechanisms, the location of α-amylase expression, and the impact on end-product quality (Mares and Mrva, 2014; Ral et al., 2016; Kiszonas et al., 2018; Newberry et al., 2018). Mapping studies suggest that LMA and PHS are controlled by different loci. There are four families of α-amylase genes in wheat, *TaAmy1*, *TaAmy2*, *TaAmy3* and *TaAmy4*. *TaAmy1*, *TaAmy2* and *TaAmy4* appear to be induced with germination, and so are expressed during PHS (Mieog et al., 2017). However, only *TaAmy1* was induced during LMA of lines derived from 'Spica' wheat (Barrero et al., 2013). The FN method cannot differentiate between LMA and PHS since α-amylase is produced

in both cases. LMA and PHS can be differentiated based on the location of α-amylase activity in the grain (Mrva et al., 2006). During germination, gibberellin A (GA) hormone produced by the wheat embryo induces α-amylase expression in the single-cell aleurone layer that surrounds the wheat starchy endosperm. Thus, α-amylase enzyme levels are much higher at the embryo proximal than at the embryo distal end of sprouted grain (Bethke et al., 1999). In LMA, on the other hand, α-amylase is more evenly distributed between the embryo distal and proximal ends (Mrva and Mares, 1996). LMA was associated with randomly distributed patches of cell death in the aleurone layer (Mrva et al., 2006).

While PHS is well-studied, much remains to be learned about LMA especially in North American germplasm (Mares and Mrva, 2008, 2014). LMA was suspected in the U.S. western wheat because field-grown wheat experienced low FN without a sprout-inducing rainfall in California and in Washington state (Mares and Mrva, 2008; Sjoberg et al., 2020). A closer examination of weather data revealed that the suspected low FN in Washington state was associated with temperature fluctuations in the grain-filling period of development.

The goal of this study was to develop an LMA screening method for semi-dwarf U.S. Pacific Northwest spring wheat using the soft white spring breeding line WA8124 as an example. Initial screening experiments using the method of Mrva and Mares (2001) resulted in poor LMA induction, as well as the lack of consistency between biological replicates within experiments and between independent experiments. This may be due to the differences in climate and wheat germplasm. A developmental time course was used to optimize LMA induction. Research also examined whether altered temperature conditions could decrease α-amylase activity in untreated controls or improve the consistency of LMA induction.

Materials and methods

Germplasm

Samples were obtained from the 2013 Washington State University (WSU) soft white spring (SWS) variety trials conducted by the WSU Cereal Variety Testing programme and grown under controlled conditions for LMA testing. Spring club cultivar 'JD' (PI 656790) and soft white spring breeding line WA8124 (IDO599/S2K00095) were obtained from the WSU spring wheat breeding programme. The LMA-susceptible hard spring cultivar 'Kennedy' was obtained from the U.S. National Small Grains Collection, PI 420949. The propagule of wheat is a caryopsis that will be referred to as 'grain' in this article.

Greenhouse LMA induction in the spring wheat variety trial

The LMA induction experiments conducted were: (1) the two experiments conducted to characterize LMA induction in 24 soft white spring wheat cultivars (E1 and E2; Fig. 1); (2) the LMA time course experiments (Fig. 3); and (3) two temperature-shift experiments (Expt 1 and Expt 2). All LMA induction experiments were conducted beginning with plants grown under a basal growth condition until they were shifted to a cold or warm treatment chamber for 7 days to induce LMA, and then returned to the basal growth condition through the end of their life cycle.

The initial characterization of LMA in the soft white spring wheat cultivars and breeding lines from the 2013 WSU regional trials was conducted essentially as described for intact plants in
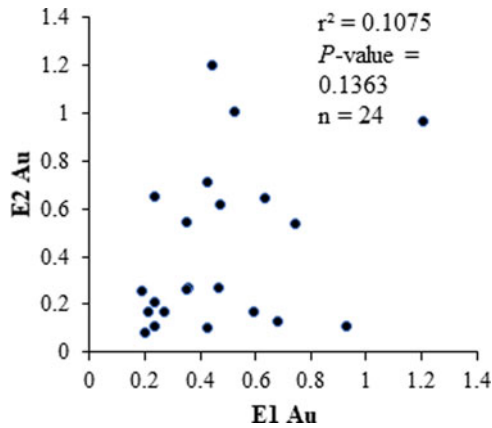
Fig. 1. Two-independent greenhouse LMA induction assays, E1 and E2, performed on 24 soft white spring lines failed to show a significant correlation. Results from treated plants that received a cold treatment at 26 dpa (18°C day/7.5°C night) are shown.

Mrva and Mares (2001) with the following differences: (1) the basal growth condition was a glasshouse where natural sunlight was supplemented with sodium lamps to obtain 300–400 µmol quanta m^{-2} s^{-1} light and a 16 h day/8 h night cycle and where the climate was controlled to obtain a 21–24°C day and 15–18°C night, as opposed to the warm greenhouse with a 12 h day reported by Mrva and Mares (2001) and (2) the 7-day cold treatment for LMA induction was performed in a Conviron™ growth chamber with an 18°C day/7.5°C night to be consistent with the cool nights typical of eastern Washington instead of the 18°C day/12°C night reported by Mrva and Mares (2001). There were 4 plants per genotype * treatment grown in 3.8-l pots. Spikes were tagged at anthesis and plants moved to the cold treatment chamber for 7 days at 26 dpa, and then returned to the basal growth conditions until fully senesced. Untreated controls remained under the basal growth conditions throughout the entire experiment. Comparisons were made using treated and untreated spikes that reached anthesis within 0–2 days of each other. The grains from one spike were ground as a single sample. There were four spikes per genotype * treatment. Enzyme assays were performed using the Phadebas® Amylase test in glass tubes (below). The experiment was repeated twice (E1 and E2).

Time course experiment

Time course experiments were conducted using the Washington state breeding line WA8124 and Kennedy to find the optimal time in grain development for LMA induction. The experiment was conducted essentially as in the soft white spring variety screen with minor modifications. The anthesis date was determined for the first three tillers of each plant based on the presence of yellow anthers within florets in the bottom third of the spike.

Two grains each were planted in 15 pots, and at least 3 spikes from 3 pots were treated per time point. All experiments were performed using a 16 h day/8 h night. The time course experiment was conducted three times, twice using a basal growth condition consisting of a greenhouse with a 21–24°C day/15–18°C night temperature, and once consisting of a Conviron™ growth chamber with a 25°C day/18°C night temperature. For LMA induction, plants were moved to a reach-in growth chamber with an 18°C day and 7.5°C night when spikes were between 20 and 34 dpa for 7 days of cold treatment. Derkx and Mares (2020) showed

that 7 days was the optimal incubation time for LMA induction. Samples consisting of 20 grains from the lower portion of each spike were ground for 30 s in a coffee grinder (Krups® F203). α-amylase enzyme assays were performed using the 96-well method. The number of growing degree days (GDD) past anthesis at each time point is calculated based on Miller et al. (2001) using the equation:

$$GDD \, (^{\circ}C) = \frac{(Max \, Temp + Min \, Temp)}{2}$$

where Max Temp and Min Temp are the daily maximum and minimum temperatures, respectively. GDD is used to improve the predictions of crop developmental stages by taking into account daily temperature fluctuations. Time points with significant α-amylase induction in cold-treated *versus* untreated samples were determined using Student's *t*-test.

Temperature-shift experiments

Experiments were performed to determine the optimal temperature conditions needed to induce LMA in WA8124 essentially using the method described for the time course experiments except that plants were treated at 21, 23 and 25 dpa using the indicated basal growth and treatment temperatures. In the first set of experiments (Expt 1), basal growth conditions were maintained in a glasshouse with a 21–24°C day/15–18°C night temperature, and in the second set of experiments in a Conviron™, GR96 growth chamber at the indicated temperature and relative humidity (Table 1). In the second set of experiments, the default basal temperature was 25°C day/18°C night, and plants were moved to a Conviron growth chamber (PGR15) at the treatment temperature at 21, 23 or 25 dpa, corresponding to 451, 494 and 537 GDD past anthesis (GDDpa). When the basal temperature of interest was different from the default, plants were moved to the treatment chamber at 451, 494 and 537 GDDpa. After 7 days of treatment, plants were returned to the basal growth chamber to mature for 3–4 weeks. Growth chamber humidity was maintained at 45–70% humidity using an EDV-4000 Rotary Desiccant Dehumidifier. Temperature and humidity were recorded either using the research greenhouse monitoring system (WSU Plant Growth Facilities) or using a Hobo datalogger UX100-003 (Onset®). Due to the practical constraint of using a growth chamber, wheat was planted weekly to obtain a cohort of plants ready for LMA treatment at different temperatures each week. At least 5 spikes from 3 plants were treated for each of the 3 time points, and a total of 12–35 spikes were examined for each treated and untreated condition. Individual spikes were hand-threshed, and 20 grains from the bottom of each spike were assayed for α-amylase enzyme activity using the 96-well method.

In an effort to reduce variation due to the fact that wheat spike florets do not reach anthesis synchronously, anthesis was scored based on the bottom third of the spike, and then α-amylase enzyme assays performed using 20 grains from the bottom third of the spike. Unfortunately, there was no obvious reduction in variation using the 20-grain method (Mean = 1.54, SD = 1.47, $n = 12$) compared with assays performed using the entire spike (A_{620} Mean = 1.00, SD = 0.73, $n = 12$) in WA8124 assays performed in the second set of experiments using a cold induction temperature of 18°C day and 7.5°C night. However, all time course and temperature-shift experiments reported here made use of the 20-grain method for consistency.

Table 1. The effect of basal and induction temperature on LMA induction in WA8124

Day/night temperature (°C)			A$_{620}$*Phadebas α-amylase						
Basal	Induction	Change	Untreated	Treated	T/U[a]	p*	T/Ū[b]	p*	N$_T$, N$_U$[c]
23/16[d]	18/7.5	−5/−8.5	0.3	1.54	5.1	0.008	5.9	<0.001	12, 12
23/16	15/4	−8/−12	0.34	0.52	1.5	0.256	2.0	<0.001	23, 14
23/16	10/10	−13/−6	0.26	2.27	8.7	<0.001	8.7	<0.001	14, 14
23/16	25/7.5	+2/−8.5	0.15	0.51	3.4	<0.001	2.0	<0.001	17, 17
23/16	32/25	+9/+9	0.24	0.19	0.8	0.11	0.8	0.11	17, 17
25/18	18/7.5	−7/−10.5	0.39	1.75	4.5	<0.001	4.7	<0.001	31, 26
25/18	15/4	−10/−14	0.80	2.37	3.0	<0.001	6.4	<0.001	30, 21
25/18	10/10	−15/−8	0.33	3.54	10.7	<0.001	9.6	<0.001	30, 19
25/18	25/7.5	0/−10.5	0.25	0.42	1.7	0.022	1.1	0.84	28, 18
25/18	18/18	−7/0	0.22	0.27	1.2	0.36	0.7	0.03	35, 21
25/18	32/25	+7/+7	0.16	0.15	0.9	0.60	0.9	0.60	29, 20
20/10	30/20	+10/+10	0.61	0.63	1.0	0.94	NA	NA	25, 16

[a]Fold induction, the average treated Au divided by untreated.
[b]The ratio between the average treated samples and untreated sample using an untreated value averaged across the entire experiment (Expt 1 = 0.26, Expt 2 = 0.37).
[c]Number of spikes treated (N$_T$) and untreated (N$_U$).
[d]The mean day/night temperature is shown. The actual temperature range during Expt 1 was 21–24°C day/15–18°C night.
*The significance of the fold induction.

Humidity experiment

WA8124 grains were planted in a glasshouse. Once the first three tillers were tagged at anthesis, the entire pot was moved from the glasshouse into two separate growth chambers (Conviron™ PGR15) at the basal growth temperature (25°C day/18°C night). One chamber was maintained at 40–60% day/60–70% night relative humidity with a dehumidifier and the other without a dehumidifier had a 45–65% day/85–95% night relative humidity. The effect of humidity control on grain that did not experience a cold treatment was examined by determining the α-amylase activity of 20 grains from each spike using the 96-well Phadebas enzyme assay.

Alpha-amylase enzyme assays

α-amylase enzyme activity was measured using the Phadebas® Amylase test either performed in glass tubes as described by the manufacturer Magle Life Sciences™ with modifications for wheat grain from Mares et al. (1994), or using a method adapted to 96-well plates (Kiszonas et al., 2018). For the glass tube method, 0.5 g of whole grain meal was mixed with 2 ml of extraction buffer (100 mM sodium maleate, pH 6.0, 5 mM calcium chloride) in a 13 × 100 mm glass culture tube. Samples were vortexed vigorously and incubated in a 50°C water bath for 10 min, then centrifuged for 10 min at 3000 rpm. 200 µl of the extract was transferred to a new culture tube, containing 1 ml of a Phadebas substrate suspension (2 g ground Phadebas tablets suspended in 40 ml extraction buffer). Samples were vortexed, and then incubated for 30 min at 50°C (as in Mares et al., 1994). Two technical replicates were performed for each 2 ml extraction. Reactions were stopped by the addition of 1 ml of 0.5 M NaOH, and then vortexed and centrifuged at 3000 rpm for 10 min. Finally, 300 µl of supernatant was transferred to a 96-well plate, and absorbance at 620 nm determined using a BioTek© SYNERGY 2 microplate reader (2006-2015 BioTek© Instruments, Inc.) running BioTek© Gen5 software (v. 2.06.10).

For the 96-well method, 1 ml of extraction buffer (as above) was added to 0.2 g of whole meal in a 2.0 ml low-protein-binding tube (Eppendorf®), vortexed, and then incubated for 10 min at 50°C. The sample was centrifuged for 10 min at 3000 rpm, and 40 µl of protein extract added to a 2-ml well containing 200 µl of Phadebas substrate suspension (as above). Every 96-well plate contained three standards of known FN and a blank consisting of the extraction buffer. Plates were incubated for 45 min at 50°C. The reaction was stopped by the addition of 200 µl 0.5 M NaOH. Plates were centrifuged for 5 min at 4000 rpm, and 0.2 ml of supernatant was transferred to a 96-well microtiter plate (Thermo Fisher Scientific, Waltham, MA) and absorbance at 620 nm determined in a Biotek™ Synergy™ 2 Microplate reader. A$_{620}$ absorbance units (Au) were used as a measure of enzyme activity. An ANOVA showed that plate was a significant factor ($P = 0.0021$) affecting enzyme activity. A linear regression based on the three controls of known FN and enzyme activity was used to calculate a corrected A$_{620}$ for each plate. Following correction, the plate was no longer a significant factor affecting Au.

Statistical analysis

The data were analysed using Students *t*-tests with the T-TEST procedure and using the PROC GLM procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC).

Results

LMA induction in Northwestern US spring wheat

Testing of the 2013 WSU Cereal Variety Trials revealed the presence of low falling numbers in some locations that did not receive rain at a time likely to induce PHS, raising the possibility that U.S. wheat varieties may have LMA susceptibility (Sjoberg et al., 2020). To examine whether soft white spring wheat from the

Northwestern US could induce LMA under conditions previously developed for Australian wheat, greenhouse LMA induction experiments were performed using lines from the 2013 WSU Cereal Variety Trials (Mrva and Mares, 2001; Mares and Mrva, 2008). When the results of the two independent experiments, E1 and E2, were compared, they were found to be uncorrelated (Fig. 1; $r^2 = 0.11$, $P = 0.14$). The lines that had higher α-amylase in the treated than in the untreated spikes in both experiments were 'WA8124' and 'Alturas' (Supplementary Fig. S1). Nine lines failed to induce LMA in both experiments, including 'JD', 'Babe', 'Louise', 'Zak', 'Whit', 'WB1035', 'WA8189', 'Diva' and 'Wakanz'. While LMA susceptibility was observed in U.S. wheat, the results were variable, indicating that further experiments were needed to optimize LMA induction conditions. The Washington state breeding line WA8124 was chosen for further experiments to optimize LMA induction conditions because it showed a consistent response with and without cold treatment.

Effect of temperature and relative humidity on untreated plants

In preliminary experiments, it was observed that when the glasshouse was used as the basal condition, untreated controls showed a high degree of variability in α-amylase enzyme levels (Supplementary Fig. S1). Thus, the effects of temperature and relative humidity on α-amylase levels without an LMA-inducing cold treatment were examined using WA8124 grown in a growth chamber. Plants were grown continuously in a growth chamber under four different temperature regimens, and then α-amylase activity was determined for individually harvested spikes. Incubation at the lower temperatures, 20°C day/10°C night and 23°C day/16°C night, resulted in higher and more variable α-amylase activity than incubation at the higher temperatures 25°C day/18°C night and 30°C day/10°C night (Fig. 2a). This difference in α-amylase activity was significant based on a pairwise t-test ($P < 0.05$). Interestingly, a warm daytime temperature of 30° C was able to maintain a lower α-amylase activity despite a cool 10°C night. In subsequent experiments, a controlled basal temperature of 25°C day/18°C night was maintained in a growth chamber instead of using a glasshouse. Next, the effect of differences in relative humidity on α-amylase activity was examined when plants were grown at 25°C day/18°C night. Lower relative humidity, 40–60% day/60–70% night, was associated with significantly lower α-amylase levels than uncontrolled relative humidity (40–65% day/85–95% night) (ANOVA: $P = 0.0003$; Levene's test: $P = 0.0043$; Fig. 2b). Variability in untreated controls can result from variation in basal temperature or humidity. Thus, using a dehumidifier to maintain lower relative humidity in the basal growth chamber can reduce variability in α-amylase levels.

Determining the developmental window for LMA induction

Previous work showed that LMA could be induced in Australian cultivar 'Kennedy' between 25 and 30 dpa (Mrva et al. 2006). Thus, an experiment was conducted to determine the developmental window for LMA induction in LMA-susceptible Northwest wheat line WA8124. WA8124 was selected for this experiment because it showed a reproducible LMA phenotype in E1 and E2. Plants grown under a 25°C day and 18°C night were transferred to the cold treatment chamber (18°C day/7.5°C night) at the indicated time point (Fig. 3a). WA8124 showed α-amylase induction with a 7-day cold shock treatment initiated between 20 and
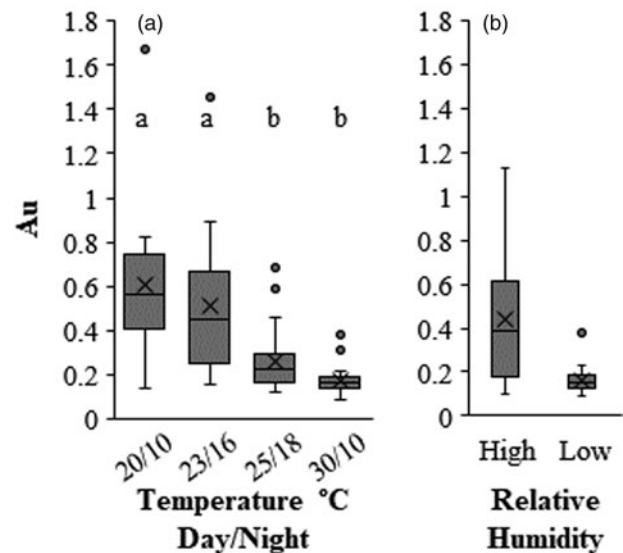

Fig. 2. Higher α-amylase activity and variation were observed under cooler and more humid conditions. The α-amylase activity of untreated WA8124 spikes grown in a Conviron™: (a) at the indicated basal temperatures and (b) at 25°C/18°C under high (45–60% day/85–95% night) and lower (40–60% day/60–70% night) relative humidity. (a and b) indicates statistically different groups as determined by t-test ($P < 0.05$). Without a dehumidifier, higher humidity was associated with both higher ($P = 0.0003$, ANOVA) and more variable α-amylase levels, $n = 21$.

24 dpa (Fig. 3a). When Kennedy was subjected to the same LMA induction conditions, α-amylase was induced between 26 and 29 dpa, consistent with previously published results (Supplementary Fig. S2; Mrva et al., 2006). Thus, WA8124 appeared to be susceptible to LMA induction earlier than Kennedy. The timing of LMA induction was later, between 25 and 27 dpa, when WA8124 plants were grown at a cooler basal temperature (21–24°C day/15–18°C night) prior to cold treatment (Fig. 3b). This suggested that the LMA induction window varies by cultivar and with the basal growth condition. Next, we examined whether calculating GDDpa could be used to improve the consistency of the LMA window when plants were grown at different basal temperatures. LMA was induced between 487 and 526 GGDpa at the cooler basal temperature (21–24°C day/15–18°C night) and between 430 and 516 GDDpa (20 to 24 dpa) at the warmer basal temperature (25°C day/18°C night). Thus, GDD can at least partially compensate for variability in the LMA induction window with variation in temperature after anthesis.

To estimate the LMA-susceptible stage of grain development, we examined the appearance of WA8124 grains at 20, 24 and 28 dpa during growth under the default basal temperature of 25°C day/18°C night (Supplementary Fig. S3a–h). WA8124 grains appeared to be at the soft dough stage of development, Zadoks stage 85, when LMA-susceptible (20 and 24 dpa), just as the grain lost its green coloration (Zadoks et al., 1974). By 28 dpa, WA8124 was no longer LMA-susceptible. At this point, the grain had lost its green coloration and the vascular bundle in the crease changed from green to yellow. These results agree with those of Barrero et al. (2013) and suggest that the physical appearance and texture of grain can be used to confirm the LMA-susceptible stage.

LMA induction under varying temperature conditions

Previous work has shown LMA induction by cold or high temperature shock (Mrva and Mares, 2001; Farrell and Kettlewell,
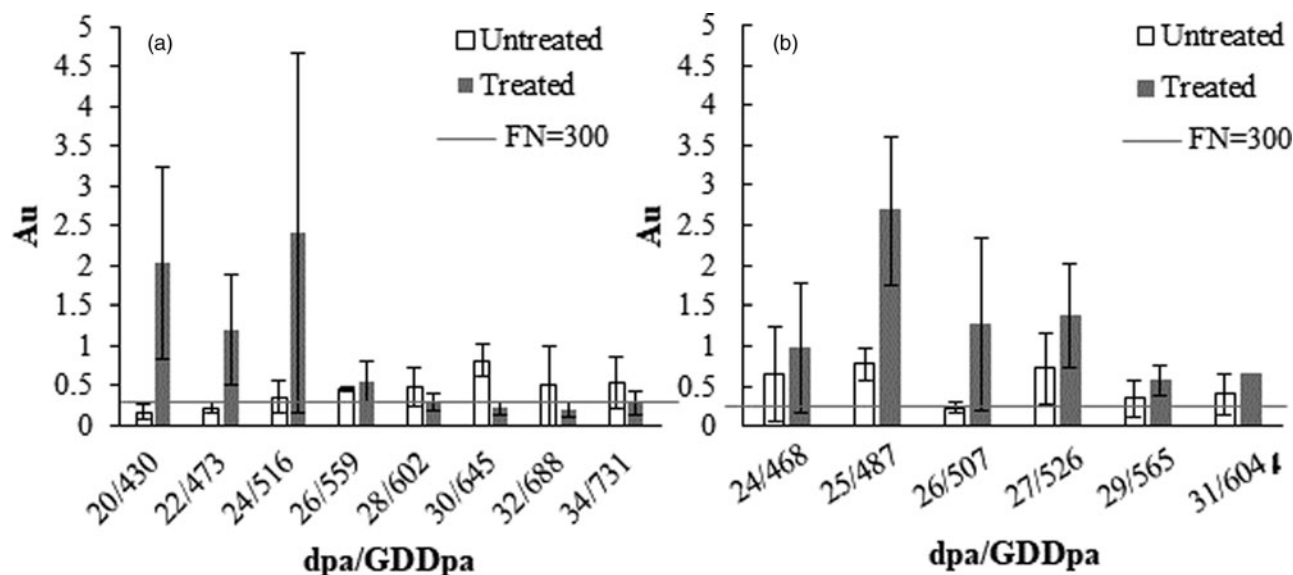
Fig. 3. LMA time course experiments performed on WA8124 with (a) a warmer basal day/night temperature of 25°C/18°C and a cold treatment of 18°C/7.5°C resulted in LMA between 20 and 24 dpa; while (b) a cooler basal day/night temperature regime of 21–24°C/15–18°C resulted in later LMA induction, between 25 and 27 dpa. Each bar represents the mean of 3 spikes ±SE.

2008). However, few controlled temperature regimes have been examined. We hypothesized that LMA induction would be more likely with a bigger temperature change or when both day and night temperatures changed. To address this, two experiments were conducted in WA8124 to look at the effects of different LMA induction temperatures (Table 1). Experiment 1 was conducted using a greenhouse as the basal growth condition with a temperature range of 21–24°C day/15–18°C night. Experiment 2 was conducted using a basal temperature of 25°C day/18°C night after experiments suggested that a higher basal temperature reduced variation in untreated controls (Fig. 2). Previous work showed LMA induction in Australian wheat with cold induction temperatures of either 18°C day/12°C night or 10°C day/10°C night, although the 10°C day/10°C night induction was performed by placing detached tillers in a 10°C water bath in either a warm or cool room (Mrva and Mares, 2001; Mrva et al., 2009; Emebiri et al., 2010; Tan et al., 2010). A cold shock temperature with a cooler night, 18°C day/7.5°C night, was chosen to be more consistent with the cooler night temperatures typical of the inland Northwestern US. The Student's *t*-test was used to evaluate whether α-amylase values were significantly higher in treated spikes than in untreated spikes from plants that remained in the basal chamber during the entire experiment. The 18°C day/ 7.5°C night treatment resulted in a 4.5- to 5.1-fold increase, the 10°C day/10°C night treatment an 8.7- to 10.7-fold increase and the 15°C day/4°C night treatment a 1.5- to 3.0-fold increase (Table 1). Induction at 15°C day/4°C night only had a significant effect in experiment 2. Thus, it does not appear that colder induction temperatures necessarily result in stronger LMA induction. Dropping the temperature only in the day (18°C day/18°C night) had no significant effect, whereas dropping the temperature only in the night (25°C day/7.5°C night) gave a significant induction of 1.7- to 3.4-fold, suggesting that cold nights are sufficient to induce mild LMA.

An analysis of variance (ANOVA) of the cold induction experiments showed that the temperature (used for cold treatment), treatment (treated *vs* untreated) and experiment (with two different

basal temperatures) were all significant factors (Table 2). The temp*expt also showed a significant interaction. However, when a Test of Hypotheses was performed using the Type III Mean Square with temp*expt as an error term, the effect of the cold induction temperature was not significant. This may be because the effect of temperature was not entirely consistent between experiments 1 and 2. It was clear that the colder 15°C day/4°C night gave neither stronger nor more reproducible LMA induction than the other two treatments. With the higher basal temp, both 18/7.5°C and 10/10°C induced LMA well, whereas 15/4°C did not.

One difficulty in interpreting this data was that the untreated controls were quite variable. This might have caused us to underestimate the effectiveness of some cold treatment temperatures in one experiment or the other. To examine this, we repeated the *t*-test using the mean of untreated values (Ū) over the entire run (Expt 1 of 0.26, and Expt 2 of 0.37) (Table 1). Based on this, the 15°C day/4°C night treatment significantly induced a low-level LMA in both experiments.

The effect of increasing temperatures on LMA was examined in WA8124 because previous work showed LMA induction by high temperatures in U.K. wheat (Farrell and Kettlewell, 2008). A high-temperature treatment of 32°C day/25°C night failed to induce LMA with both a 25°C day/18°C and 23°C day/16°C basal temperature (Table 1). The experiment was repeated using the same temperatures used by Farrell and Kettlewell (2008). Again, WA8124 failed to induce LMA when plants grown at a basal growth temperature of 20°C day/10°C night were treated for 7 days at a 30°C day/20°C night temperature. Thus, LMA-susceptible WA8124 does not induce LMA in response to high-temperature treatment.

Since WA8124 is known to be LMA-susceptible, the frequency of false negatives was examined comparing different basal and treatment temperatures (Table 3). In the present study, the average A_{620} of the FN 300 s control sample was 0.24 ($n = 11$). Based on a definition that a false negative is any treated A_{620} value below 0.24, we found that the false-negative rate was generally lower with the higher 25°C/18°C than the 23°C/16°C basal temperature.

Table 2. ANOVA of LMA induction experiments

Source	d.f.	Type III SS	Mean square	*F*-value	Pr > F
temp[a]	4	113.11	28.28	29.31	<0.001
treat[b]	1	77.22	77.22	80.05	<0.001
expt[c]	1	11.12	11.12	11.53	0.003
temp*expt	3	15.89	5.30	5.49	<0.001
treat*expt	1	0.97	0.97	1.01	0.316
Temp[d]	4	113.11	28.28	5.34	0.100

[a]Effect of the treatment temperature (temp).
[b]General effect of cold treatment.
[c]Effect of the basal temperature in the two experiments (expt).
[d]Tests of Hypotheses using the Type III MS for temp*expt as an Error Term shows that varying cold treatment temperatures had no significant effect.

Table 3. Percentage false-negative results (Au < 0.2) with different basal and treatment temperatures

Treatment temperature (°C day/°C night)	Basal temperature (°C day/°C night)	
	23/16	25/18
18/7.5	33%	0
10/10	0	13%
15/4	35%	0

The false-negative rate may be somewhat lower with the 10°C day/10°C night cold treatment temperature. However, this difference is not observed with a higher basal temperature. The higher 25°C/18°C basal temperature resulted in clearer differences between cold-treated and untreated temperatures (Fig. 4). While α-amylase activity was higher with a cold induction day/night temperature of 10°C/10°C than 18°C/7.5°C, it also appeared to be more variable.
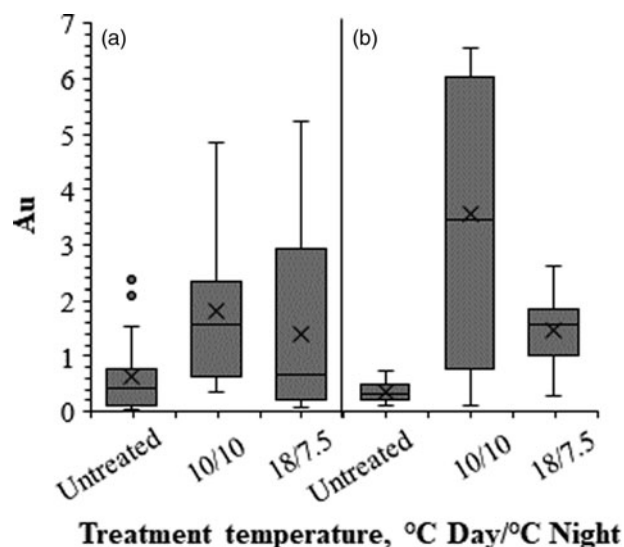
Discussion

The goal of this study was to improve LMA screening methods by refining our understanding of the developmental timing of LMA susceptibility as well as the temperature difference/shock needed to induce LMA using soft white spring WA8124 as an example. LMA is a notoriously variable trait such that a cold treatment during late grain maturation may or may not result in expression of the phenotype (reviewed by Mares and Mrva, 2008). This is partly explained by the fact that LMA susceptibility occurs within a relatively short developmental window (Mrva et al., 2006). Moreover, not all the grains on a spike induce LMA, likely because they reach anthesis and, therefore, the LMA window asynchronously (Mares et al., 1994). By trying to define the conditions that induced LMA in a single line of wheat, the strongest conclusion of this study was that it was not possible to define conditions that fully prevent LMA expression under 'untreated' conditions nor that always induce LMA under 'treated' conditions. It appears that genetic susceptibility to LMA results in variable α-amylase activity in mature grains and that cooler temperatures merely exacerbate this condition. In Fig. 4a, for example, the untreated and cold-treated samples largely overlapped. Raising the basal growth temperature reduced but did not eliminate this overlap in Fig. 4b.

Results in this study suggest that different varieties may become susceptible to cold-induced LMA at different times. The Australian cultivar Kennedy reached the 'window' of LMA susceptibility later than WA8124 (Fig. 3; Supplementary Fig. S2; Mrva et al., 2006). This may be due to the fact that the Kennedy developed more slowly than WA8124. For example, many Australian varieties are facultative spring wheats, whereas most northwest spring wheat varieties do not require vernalization (Porter and Gawith, 1999; Santra et al., 2009). Consistent with this notion, WA8124 reached the LMA window later when grown at a cooler basal temperature (Fig. 3b). Derkx and Mares (2020) found variation in the timing of LMA induction in four

Fig. 4. Distribution of α-amylase activity in untreated samples grown when the basal day/night temperature was (a) 21–24°C day/15–18°C night or (b) 25°C day/18°C night in untreated samples, and in samples cold-treated at 10°C day/10°C night or 18°C day/7.5°C night.

different varieties. This suggests that variation in developmental rate and the timing of the LMA window may be a major source of variation in LMA testing results, especially in cultivars that vary for loci controlling phenology and flowering time. Similar variation for the window of sensitivity has been observed in modelling the effect of temperature on PHS susceptibility (Rodríguez et al., 2001; Gualano and Benech-Arnold, 2009). Based on the present study, we recommend the use of GDDpa to estimate the correct time for LMA induction, followed by visual confirmation that a grain from the spike is at the late soft dough stage of grain development (Zadoks stage 85) (Fig. 3). Since the external appearance of the spike is also quite variable at Zadoks 85, it is important to inspect a grain rather than the spike when determining the time for LMA induction (Supplementary Fig. S3). Future work will need to test the efficacy of this approach in a larger population. However, even in the single variety, WA8124, there was a great deal of variation in the timing and amplitude of LMA induction over multiple experiments (Figs 2 and 4).

LMA induction experiments were conducted using different cold- and high-temperature treatments. Our initial hypothesis was that a colder temperature, like 15°C day/4°C night, might result in stronger LMA induction (Table 1). In fact, LMA was less consistently induced at 15°C day/4°C night than at 10°C day/10°C night and 18°C day/7.5°C night. The cool temperatures

that induced LMA in WA8124 were like those that induced LMA in Australian and U.K. germplasm (Mrva and Mares, 2001, 2002; Farrell and Kettlewell, 2008). Previous studies reported heat-induced LMA in a U.K. variety and in 14 isolates from a collection of landraces (Farrell and Kettlewell, 2008; Barrero et al., 2020). Interestingly, a high temperature shock of 32°C day/25°C night, as well as temperature conditions that matched those used in the Farrell and Kettlewell (2008) study, failed to induce LMA in WA8124 (Table 2). This indicates that not all varieties capable of inducing LMA in response to cold can also induce LMA in response to heat. The presence of varietal differences in whether high-temperature treatment can induce LMA suggests that there may be more than one genetic mechanism leading to this problem. Multiple loci affecting LMA have been identified (reviewed by Mares and Mrva, 2014). Previous work showed that the 1R translocation from rye to wheat chromosome 1B increased LMA susceptibility, while the presence of the GA-insensitive *Rht-B1b* or *Rht-D1b* dwarfing alleles decreased LMA susceptibility (Mrva et al., 2009; Farrell et al., 2013; Derkx and Mares, 2020).

It appeared that a higher basal temperature reduced the accumulation of α-amylase activity in the grain (Fig. 2). When the α-amylase activity was examined in plants grown continuously at warmer or cooler temperatures, it was observed that lower temperatures were associated with higher and more variable grain α-amylase levels than higher temperatures (Fig. 2a). This is consistent with the observation of Derkx and Mares (2020) that continuous growth in a cool season glasshouse resulted in LMA induction. This suggests that it is not so much the change in temperature as the presence of unusually cool temperatures during late maturation that result in elevated α-amylase levels. Based on examination of the single LMA-susceptible line WA8124, there was considerable phenotypic variability after cold temperature treatment for 1 week (Fig. 4). It appears, however, that cooler temperatures did result in the stronger expression of the WA8124 LMA phenotype given that (1) α-amylase activity was higher with cold induction than under continuous growth (untreated) at 20°C day/10°C night (Table 1) and (2) α-amylase activity was higher and less variable following continuous growth at a 20°C day/10°C night than at 23°C day/16°C night (Fig. 2a). Future work will need to explore if the elevated α-amylase activity at cooler temperatures results from gene induction or from loss of proper negative regulation.

While evidence suggests that LMA may not impact end-use quality as strongly as PHS, millers and bakers remain convinced that low FN grain poses a risk to baking quality regardless of the cause (Ral et al., 2016; Kiszonas et al., 2018; Newberry et al., 2018). As a result, farmers receive serious discounts for wheat with falling numbers below 300 s. This study suggests that there are varietal differences in the timing and conditions that induce LMA. We recommend that breeding programmes planning to initiate LMA testing begin with a cool temperature treatment that induces LMA in multiple breeding programmes, that they examine a range of time points between 20 and 28 dpa to identify the time most likely to induce LMA in their germplasm and that they examine grains from the spike as a secondary method to identify spikes at the LMA-susceptible (Zadoks 85) soft dough stage of grain development.

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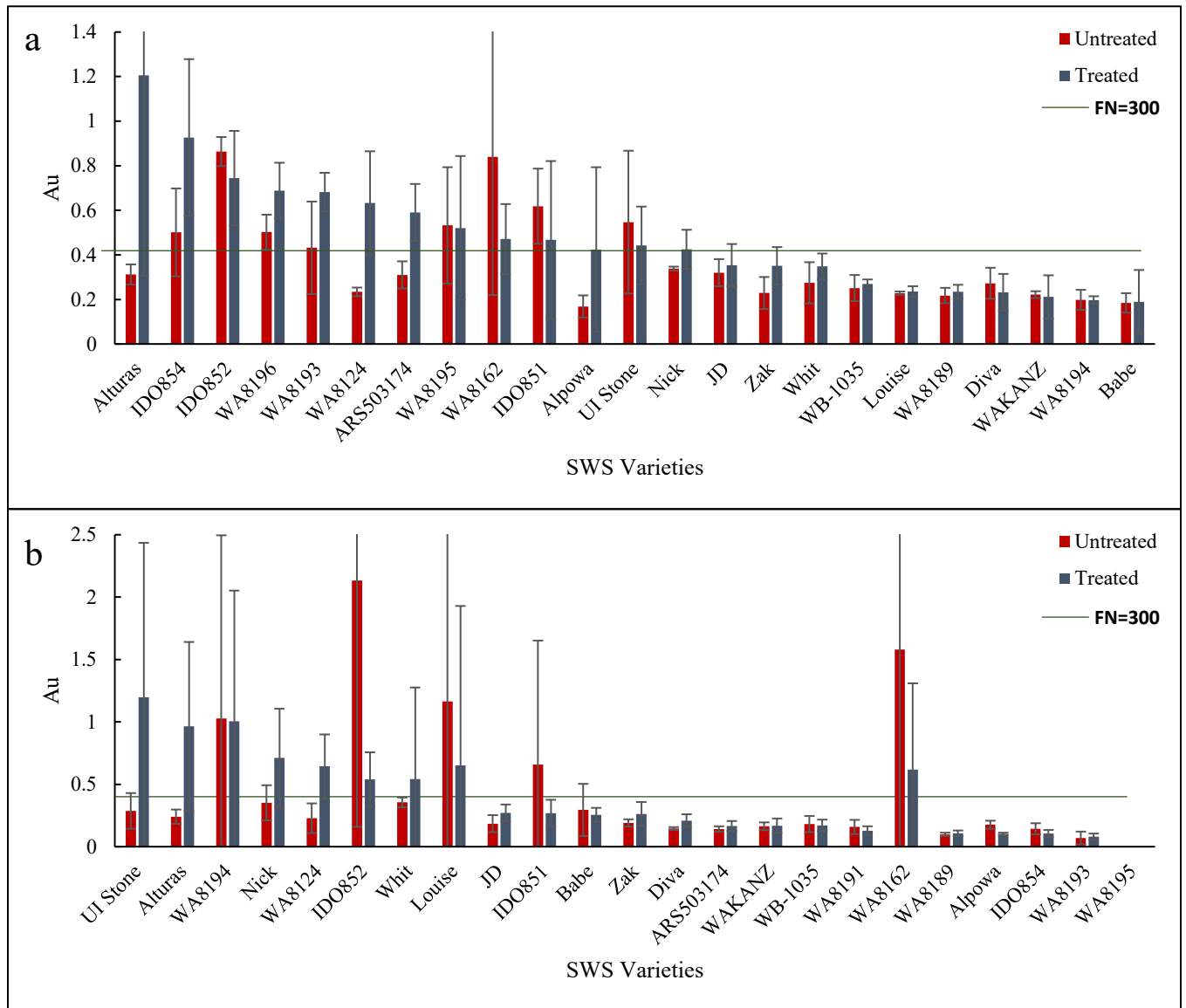
Fig. S1. Greenhouse LMA induction in soft white spring wheat in a) Environment 1 (E1) and b) Environment 2 (E2). Alpha-amylase activity is shown in A_{620} absorbance units (Au). 'Alturas' and 'WA8124' were successfully induced in both experiments. The green line represents the alpha-amylase value when FN = 300. Each bar represents 3 - 15 biological replicates ± standard deviation.
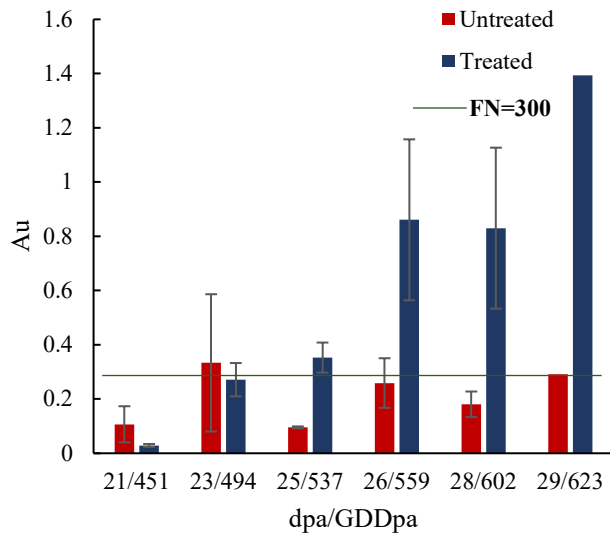
Fig. S2. LMA time course experiment on an Australian line, Kennedy: LMA was successfully induced in between 25 – 29 dpa. Each bar represents n = 1 – 5 spikes.

Fig. S3. The appearance of WA8124 grains and tillers: a-c) at 21 dpa, d-f) at 24 dpa, and g-h) at 28 dpa. Note that by the time the grains are no longer LMA susceptible, the vascular bundle has lost its green coloration.