LATE MATURITY ALPHA-AMYLASE IN NORTH AMERICAN SPRING WHEAT (*TRITICUM AESTIVUM L*.)

By

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Abstract

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Late maturity alpha-amylase (LMA) is considered a genetic defect in wheat (*Triticum aestivum L.*), resulting in the induction of alpha-amylase enzyme in response to a low or high temperature shock during late grain filling. The wheat industry uses the Hagberg-Perten Falling Number (FN) method to detect loss of gelling capacity due to the presence of alpha-amylase in meal or flour. Low FN is associated with higher risk of poor end-product quality, such as cakes that fall and sticky bread or noodles. To improve selection for LMA resistance, LMA testing methods were optimized and then used to characterize the LMA susceptibility in North American wheat. Preliminary LMA testing results were highly variable in cold-treated and in untreated controls. Warmer (25°C day/ 18°C night) and drier (~55-65% relative humidity) conditions reduced alpha-amylase levels in untreated controls. Colder LMA-induction experiments did not result in stronger or more consistent LMA-induction in spring wheat variety, WA8124. The most significant cause of LMA phenotypic variability appeared to be variability in the developmental window during which a 7-day low temperature shock triggered LMA. This window varied with environmental conditions prior to grain development and by genotype, such that WA8124

induced LMA at 20-24 days past anthesis (dpa) whereas Australian ‘Kennedy’ induced at 25-29 dpa. Only 21% of the 256 North American breeding lines characterized showed LMA resistance, suggesting that improved selection for LMA resistance is needed. In this panel, the tall rht-B1a rht-D1a genotype was associated with higher LMA in both cold-treated and untreated experiments, suggesting the presence of a constitutive LMA phenotype that did not require cold treatment. However, some rht-B1a rht-D1a lines required cold induction whereas some semi-dwarf lines had constitutive LMA, suggesting that the constitutive LMA phenotype is genetically complex. A preliminary genome-wide association study identified six significant marker-trait associations on chromosomes 2B, 3A, 3B, 5A, 7B, and 7D. The QLMA.wsu.7B locus detected in this study co-localized a QTL detected in four previous studies of Australian and CIMMYT germplasm. Future work will determine if these molecular markers are effective in selecting LMA resistance in U.S. wheat.

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Dedication

This thesis is dedicated to my mother and father for their unconditional love and the support.

CHAPTER ONE: INTRODUCTION

This dissertation discusses late maturity alpha-amylase (LMA) as a cause of problems with elevated alpha-amylase levels in grains of wheat. Elevated alpha-amylase levels can cause problems with poor end-use quality, and can result from susceptibility to two problems, preharvest sprouting and LMA (reviewed by Mares and Mrva, 2014). Preharvest sprouting is the initiation of grain germination on the mother plant when rain occurs before harvest of mature grain. LMA was first characterized when it was observed that elevated alpha-amylase could occur without sprout-inducing rainfall. LMA can be triggered by temperature fluctuations during grain maturation. Here we review the literature on LMA, preharvest sprouting, and expression of alpha-amylase to serve as an extended background to a thesis about LMA.

Allohexaploid wheat and its importance in agriculture

Bread wheat (*Triticum aestivum L.)* is an allohexaploid crop species that carries three genomes (A, B, and D), each comprised of 7 homoeologous chromosome groups (Shewry, 2009). During meiosis, A genome chromosomes pair only with the other A genome chromosomes, B with B, and D with D. These three genomes were evolutionarily derived from 3 diploid progenitors. The most recent hybridization of the diploid *Aegilops tauschii* and tetraploid *Triticum turgidum* progenitors gave rise to the hexaploid. Cultivation of diploid and tetraploid relatives suggests that wheat originated in the Middle East. The cultivation of hexaploid was introduced to east Asia around 2000BC from the Near East. Wheat is one of the most widely cultivated crops worldwide due in part to high yield potential and in part to the wide range of food products that can be made from wheat flour. The state of Washington is the 3rd largest wheat producing state in the US and produces the highest quality wheat in the world (Washington Grain Commission). 80% of the production is winter wheat, and 20% is spring

wheat. There are six wheat classes in the US, and each of them has its own end-product functions (Table 1.1). The starch properties and gluten protein from wheat grain provide lead to good viscoelasticity, which is essential for products such as noodles, bread, and cakes. The high percentage of starch content in wheat grain makes wheat an essential source of calories for both human and livestock nutrition.

Table1.1 Summary table of wheat classes and their end-product functions (Washington Grain Commission)

Wheat class	End product
Soft White (SW)	Pastries, cookies, cakes, cereals, flat breads, and crackers
Hard Red Spring (HRS)	Pan breads and artisan breads
Hard Red Winter (HRW)	Asian noodles, hard rolls, flat breads, and general purpose flour
Durum	Pasta, couscous, and Mediterranean breads
Hard White Winter (HWW)	Asian noodles, breads, and whole grain products
Soft Red Winter (SRW)	Pastries, crackers, and pancakes

What is wheat alpha-amylase?

The quality of wheat end-use products can be compromised by the presence of the starch-digesting enzyme alpha-amylase in the wheat grain. Alpha-amylase (EC 3.2.1.1) is well known as an amylolytic enzyme, which digests in alpha-1,4- glucosidic bonds in polysaccharides and in starch (Janecek et al., 2014). While alpha-amylases are found in four enzyme families in the Carbohydrate-Active enzymes (CAZy) system, most alpha-amylases belong to the glycoside hydrolayse (GH) 13 family. GH13 alpha-amylases have three domains, the main catalytic (beta/alpha)8-barrel domain A, the small domain B, and domain C. The enzymes within the same class share the same catalytic machinery and reaction mechanism for their catalytic domains to digest carbohydrates.

Perten-Hagberg Falling Number (FN) method has been used to predict wheat quality worldwide to measure total starch digestion (Hagberg, 1960; Delwiche, 2015). About 85% of the wheat that is grown in the state of Washington is exported to Asia (National Association of Wheat Growers). Wheat with a FN of less than 300 seconds (sec) can be discounted 25 US cents for every 25 sec below 300 sec and can cause end-product quality issues, such as mushy noodles and cakes that fall. Sound grains contains little alpha-amylase, and its starch, polysaccharides, remain undigested until mobilized to provide energy for germination (Clarke et al., 1984). However, when the LMA prone wheat varieties exhibit the phenotype, the FN can reach as low as 60 sec (Steber, 2017). Either LMA or PHS can cause low FN. Work has been done to investigate the relationships among PHS, LMA, low FN, and end-use quality.

There are four families of alpha-amylase genes in wheat, *TaAmy1*, *TaAmy2*, *TaAmy3*, and *TaAmy4*, which appear to be present in multiple copies in the wheat genome (Cheng et al., 2014;

Mieog et al., 2017; Ju et al., 2019). *TaAmy1*, *TaAmy2*, and *TaAmy3* transcript levels are all expressed during seed development, but decrease in mature, dry seeds. *TaAmy3* is highly expressed in between 10-30 dpa, the grain developmental stages, but it is not expressed germination. It is possible to have elevated alpha-amylase due to failure to down-regulate alpha-amylase produced during development (Lunn et al., 2001). While the precise mechanisms involved in the downregulation of developmental alpha-amylase are unknown, it is known that wheat needs to dry down for at least two weeks after physiological maturity in order to decrease the levels of the "pericarp alpha-amylase". *TaAmy1*, *TaAmy2*, and *TaAmy4* all appear to be induced during seed germination, suggesting that they are likely present during preharvest sprouting of wheat. Only *TaAmy1* is induced during LMA (Barrero et al., 2013).

Various enzyme assays are available for research purposes on the market. 1) High pI-specific ELISA is often used in research institutes, such as Wheat CRC and University of Adelaide, in Australia (Skerritt and Heywood, 2000; CSIRO). High pI TaAMY1 antibody specifically targets high pI *TaAMY1* protein (unit), which is highly induced by LMA. 2) Alpha-amylase assay kit Ceralpha method is manufactured by Megazyme, and it is available worldwide (McCleary et al., 2002; Megazyme). Wheat grain meal can be used as assay material. The reagent, Blocked p-nitrophenyl maltoheptaoside (BPNPG7), reacts with present alpha-amylase that was extracted from the flour sample in the aliquot. With additional digestion by alpha-glucosidase, part of the substrate, freed p-nitrophenyl reacts with tri-Sodium Phosphate and develops yellow color. The intensity of the yellow color directly reflects the level of total alpha-amylase in the sample. 3) Phadebas® Amylase Test, manufactured by Magle AB, is also a color matric assay (Phadebas®). The substrate is a blue dye that is color-insoluble. When the alpha-amylase that is extracted from the flour sample hydrolyses the substrate, water-soluble blue

fragments form. Again, the abundance of the blue solution is a function of the alpha-amylase activity in the flour sample. 4) Amylase assay SD method, also manufactured by Megazyme, has p-nitrophenyl maltosaccharide substrate for alpha-amylase and thermostable alpha-glucosidase to cleave and releases glucose and p-nitrophenyl (Megazyme). The phenolate ion develops color with the addition of stopping reagents. A ChemWell®-T auto-analyser fitted with a 405 nm filter is recommended.

Late maturity alpha-amylase can cause of low falling numbers without preharvest sprouting.

Late maturity alpha-amylase (LMA) is another cause of low falling numbers (FN) besides preharvest sprouting (PHS). High alpha-amylase generally reflects low FN. Mature grains exhibits low FN and quality when exposed to rain/ moisture during harvest time due to germination, in the case of PHS. LMA has been defined that the synthesis of high pI alpha-amylase during the middle stages of grain development with the absence of rain, and the alpha-amylase will be retained through the ripening (Mares and Mrva, 1994; Mares and Mrva, 2014). Studies have been done to characterize LMA in Australian and UK wheat germplasms (Mrva and Mares, 2001a; Ferrell and kettlewell, 2008). The low FN and financial losses due to LMA have made it become one of the most important traits in the breeding programs in Australia and Europe.

Programed cell death and its pattern were observed in LMA-affected wheat grains (Mrva et al., 2006). Due to the secretion of growth hormone gibberellic acid (GA) as well as an assortment of hydrolytic enzymes, such as alpha-amylase, turning the stored energy in the starchy endosperm into germination fuel, the one-layer aleurone is programmed to die proceeding from the embryo end to distal end (Kuo et al., 1996; Fath et al., 2000). LMA-affected

grains develop high pI alpha-amylase isozymes (*a-amy-1*), which is also present in germinating grains, as a result of cold temperature shock as ripening (Gale et al., 1990; Mares et al., 1994). Research has been done comparing the dying cells in endosperm of germinated wheat grains and those in LMA-affected grains. The aleurone layer was stained with Calcein (2 ll ml1 in 20 mM CaCl2; Molecular Probes) followed by N-(3-triethylammoniumpropyl)-4-f6-[4-(diethylamino)-phenyl]-hexatrienylgpyridinium dibromide (FM 4–64; 20 lM in 20 mM CaCl2, Molecular Probes), possibly representing cell death or calcium signaling in aleurone layer (Bethke et al., 1999). Patches of cell death were observed in the aleurone layer of both LMA-affected and germinated grains using confocal microscopy and fluorescent probes that were specific for identifying living and dead cells. The dead cell pockets were rather scattered throughout the LMA-affected grains; whereas, the pockets were seen only near the embryo end of germinated grain. Therefore, half-seed assay is commonly used to differ LMA-affected grains and germinated grains, where the embryo-half grain displays higher alpha-amylase in LMA-affected grains than in germinated grains. Meanwhile, *TaAMY1* specific ELISA detected the presence of high-pI alpha-amylase both cases.

LMA-affected grains retain excessive amount of alpha-amylase through ripeness. Alpha-amylase digests starch, polysaccharides, into smaller sugar and reduces gelling capacity of the wheat meal (Hagberg, 1960; Delwiche, 2015). During the FN test, water is heated to 100°C in a water bath to increase the temperature of the water flour mixture, which is measured for its viscosity after the mixture has been stirred for 60 sec. FN machine measures the amount of the time that the stirrer takes to fall through the gravy. It takes minimum of 300 sec, with the altitude correction, for the stirrer to fall through sound flour water mixture. On the other hand, FN scores

of damaged flour can be as low as 60 sec. Therefore, LMA affected grains can be differed from sound grains by their FN.

Known causes of LMA

When LMA was first observed in the breeding programs in Australia, it seemed to be a simple grain alpha-amylase heritance in generations, with no rain in the record (Bingham and Whitmore, 1966). Later low FN was seen in a breeding line in Pauw and McCaig (1983)'s work. Some early LMA characterization studies indicated the appearance of the trait, LMA, is highly associated with a cool temperature shock during grain developmental stages (Mares and Gale, 1990; Mares and Mrva, 2001a). In the US Northwest, wheat grains was severely discounted because of low FN that was caused by LMA in 2016 (Washington Grain Commission). Low FN (<250s) has continuously been observed in the WSU variety trials testings in a few locations since early 2010s, with no rain in the record.

Both constitutive and LMA-prone varieties have frequently appeared in studies investigating LMA. Low FN and high alpha-amylase activity are expected from LMA constitutive and successfully LMA induced wheat samples. The expression pattern of germination-like TaAMY1 varies among cultivars. For example, Spica and Lerma, Australian LMA constitutive lines, exhibit LMA phenotype in all environments, but not all the grains of each spike display elevated alpha-amylase (Mares and Gale, 1990). Whereas the expression of LMA in BD159, an Australian LMA-prone line, is dependent on specific environmental conditions, such as cool temperatures (Mares and Gale, 1990; Mares et al., 1994). Australian wheat that express LMA in either case does not have expression patterns where grains in certain position within each spike are more likely to have elevated alpha-amylase. However, Gale and

Lenton (1987) discovered the grains in the lower central region of the spike, especially in floret 2, are most likely display LMA phenotype in Huntsman, a UK LMA-prone cultivar.

Cold temperature shock is known to be associated with LMA induction in Australian LMA-prone wheat, and it has to be applied within the mid grain maturation stages. Mrva et al. (2006) examined the LMA sensitive window by performing time course experiments among BD159 and Kennedy, LMA-prone lines, and LMA constitutive and resistant lines as controls. The alpha-amylase activity was tested with high pI-specific ELISA in single grains. In Kennedy, high pI alpha-amylase activity that was associated with LMA could be detected starting from 25 day post anthesis (dpa) to 35 dpa.

Successful LMA inducing conditions vary among locations and/or germplasm in LMA-prone cultivars. Australian LMA constitutive lines exhibit higher alpha-amylase activity under cool temperatures during ripening stages when the temperatures are normally higher, and, likewise, the expression of LMA is more favorable when cooler temperatures taking place when the normal growing temperatures are warmer (Mares and Gale, 1990). Therefore, Mares and Mrva (2001a), Mrva and Mares (2002), and Mrva et al. (2006) examine whether certain lines/offsprings are susceptible to LMA by exposing wheat spikes to 13-18°C for 7-10 days in a 10°C water bath starting from 26 dpa or the mid grain developmental stages. After the cold treatment, the spikes were returned to a similar water bath in the warmer glasshouse to finish ripening. To shorten the experiments, immediate freeze drying or air drying the grains after the treatment is also valid to determine the amount of alpha-amylase (Mares and Mrva, 2008).

On the other hand, UK LMA-prone varieties are more likely to have higher alpha-amylase when the temperatures become higher during (Buchanan and Nicholas, 1980). Farrell and Kettlewell (2008) performed a series of temperature regimes on five UK varieties, which

required 8 weeks of vernalization, with various degrees of LMA susceptibilities. The inductions lasted 8 days starting 550 GDDpa. In other words, depending on the temperature setting of the starting growth chamber, the actual amount of days post anthesis varied. The detached tiller method was imitated from Mrva and Mares (2001a). LMA susceptible "Huntsman" was successfully induced by either a heat (20/10°C -> 20/30°C, "Mid"-> "High") or a cold shock(22/22°C -> 12/12°C, "Mid"-> "Low"), but the Australian induction conditions, transferring detached tillers from 30/15°C to 18/12°C, which was considered as "High -> Mid" did not induce LMA in "Huntsman".

Loci associated with LMA susceptibility

Several loci that are associated with elevated risk of LMA have been detected in a few studies (reviewed in Mares and Mrva, 2014, Table 1.2). Two major loci were mapped to the centromeric region of chromosome 3B and to the distal end of the long arm of chromosome 7B in multiple studies (Mrva and Mares, 2001b; Emebiri et al., 2010 ; McNeil et al., 2009). Other loci have been found on chromosomes 2DL, 3A, 3B, 3D, 4B, 4D, 5DS and 5BL (Tan et al., 2010).

There is an association between the *rht-D1a rht-B1a* genotype and elevated risk of LMA phenotype (Mrva et al., 2009). Cold-treated extreme tall synthetic wheat had significantly higher alpha-amylase activity than tall wheat, Spica, which is an LMA constitutive line (Mrva and Mares, 2001a). Semi-dwarf (*rht-D1b rht-B1a* or *rhtD1a rht-B1b*) wheat, such as Cranbrook and RAC655, require cold treatment during mid grain maturation stage to display LMA phenotype. Strategies such as artificial ranking certain percentage of grains within each tiller and rare data transformation used in Butler et al. (2009) are used to reduce inconsistency and to normalize

extremely skewed alpha-amylase activity dataset (Mrva and Mares, 2001b). It is interesting to see the trend where the cold induction required LMA-affected wheat lead to noisy data.

Table 1.2. Previously mapped LMA QTL.

Chr	Markers or QTL	Population	Reference
2D	barc159-gwm320	WW1842 x Whistler	Tan et al., 2010
3A	rPt-9057-wPt-4077	WW1842 x Whistler	Tan et al., 2010
3B	gwm264, wmc505, gwm566 wmc334, wmc540, gwm72, gwm131, gwm274 rPt-7228-barc147	Halberd x Cranbrook Halberd x Cranbrook WW1842 x Whistle	McNeil et al., 2009 Mrva and Mares., 2001 Tan et al., 2010
3D	wPt-0732-wPt-6262	WW1842 x Whistler	Tan et al., 2010
4B	barc20-gwm113	WW1842 x Whistler	Tan et al., 2010
4D	wPt-0710-wPt-4572, wPt-0472 wPt-0710	WW1842 x Whistler	Tan et al., 2010
5B	wPt-4791-barc232	WW1842 x Whistler	Tan et al., 2010
5D	barc143-wPt-6225	WW1842 x Whistler	Tan et al., 2010
6B	wPt-4858, wPt-7576, wPt-3309, wPt-5333, wPt-1730, wPt-5176, wPt-1541	AMpanel_CIMMYT	Emebiri et al., 2010
7B	wmc557, wmc70, wmc276, wmc581, gwm611, gwm577, wmc581, gwm146, gwm577, gwm344, wmc526, wmc276, 435m3c wmc276, gwm146, wmc273, gwm344, gwm611, gwm577, wmc500, wmc166, barc10, wmc581, wmc311, wmc517, gwm274, barc94, barc123, barc182, wmc70, wmc526, wmc557, wmc613, wmc10, gwm68 wPt-3723, wPt-0884, wPt-7108, wPt-7413 Xgwm577, Xwmc276, Xwmc273	Halberd x Cranbrook Halberd x Cranbrook AMpanel_CIMMYT Spica x Maringa	McNeil et al., 2009 Mrva and Mares., 2001 Emebiri et al., 2010 Tan et al., 2004

The relative impact of LMA and PHS on end-product quality

FN less than 300 sec caused by PHS usually yield poor end-use quality (Clarke et al., 1984); alpha-amylase is synthesized to mobilize starch and to fuel germination signaled by GA. Meanwhile, wheat has been severely discounted for its low FN and its potential poor end-product quality (Washington Grain Commission), including wheat with no signs of sprouting. Therefore, researchers have put efforts into investigating whether LMA affected grains can also result poor end-use quality (Ral et al., 2016; Kiszonas et al., 2018; Newberry et al., 2018). The general conclusion of the studies was that the correlation between FN and baking quality of LMA affected wheat was weak, yet Ral et al. (2016) found the alpha-amylase remained in the grains enhancing bread loaf volume and shelf life (Steffolani et al., 2012). The small scaled, 10g, baking trials concluded that the bread bake volumes of *AMY*3 overexpression lines (A_3OE) (Whan et al., 2014) wheat flour were just as good as they were of sound flour with additional baking improvers. Similar results were produced in Kiszonas et al. (2018); the loss of sponge cake volumes was not significantly reduced when the FN was less than 100 sec in SWW. Instead, the variety and location of the grains can affect the milling and baking quality more than whether the grains were affected by LMA.

There are limitations of these studies. A_3OE wheat has *TaAMY*3 over expressed, which has different characteristics than *TaAMY1*. In another word, A_3OE wheat does not accurately represent LMA affected wheat. With ELISA, Newberry et al. (2018) was able to measure the amount of *TaAMY1* in each grain sample. This study discovered that there is a weak correlation between the total alpha-amylase activity and *TaAMY1* expression level in LMA affected grains that has low FN, which suggested that other enzymes are involved. Alpha-amylase along is not sufficient to cause problems with the quality traits mentioned above, but it does not mean that

alpha-amylase does not contribute to the problems in the presence of other hydrolytic enzymes in sprouted flour. The small-scale bread baking quality test provides information about how the wheat grains perform as products, but usually the actual products appear to be different. Few information is available about the bread slicing performance when the flour has low FN. Near isogenic lines (NIL) would help researchers learning about the cake baking quality when the only difference is the presence of LMA phenotype. Wheat with constitutive LMA phenotype can assist researcher creating baking quality profile for LMA wheat and potentially a blending formula to protect growers.

Objectives

Due to the high financial loss in the wheat market caused by LMA-affected wheat and low FN in the US, LMA is becoming a more important trait in North American breeding programs. Mrva and Mares (2001a) described the methodology inducing LMA in Australian wheat varieties, but we suspect that some modifications are needed to work in the US wheat germplasm. So far, no studies have evaluated LMA susceptibility in North American wheat and, furthermore, attempted detecting significant loci that are associated with elevated risk of LMA phenotype in an association mapping panel. The following objectives will be investigated in this MS. dissertation:

1. Investigating the LMA induction window, basal and induction conditions in US Northwest wheat
2. Characterizing LMA susceptibility in North American hard red spring wheat association mapping panel using improved LMA screening methodology and genome-wide association approach (GWAS)
3. Developing near isogenic LMA constitutive lines using *rht-B1a rhtD1a* genotype

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CHAPTER TWO: INVESTIGATING CONDITIONS THAT INDUCE LMA IN U.S. NORTHWEST SPRING WHEAT (*TRITICUM AESTIVUM L.*)

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CONTRIBUTIONS: CMS, CCL, and MOP designed the experiments. KMT performed the experiment in Figure 1. The remaining experiments were performed by CCL. KGC and MOP provided guidance on statistical analyses. Statistical analyses were performed by CCL. The manuscript was written by CMS and CCL.

ABSTRACT

The wheat industry uses the Hagberg-Perten Falling Number (FN) method to detect unacceptably high alpha-amylase levels in wheat grain. Farmers receive discounts for an FN below 300 sec because this is associated with a high risk of poor end-product quality. There are two main causes of elevated grain alpha-amylase, (1) preharvest sprouting in response to rain before harvest, and (2) late maturity alpha-amylase (LMA) induction in response to a cold shock during late grain maturation. U.S. grain from Washington state experienced widespread problems with low FN due to LMA in 2016. A detailed characterization LMA-inducting condition was conducted in Washington soft white spring wheat line (WA8124) because

greenhouse LMA testing using published methods showed a high degree of variability. Problems with elevated alpha-amylase in untreated controls were reduced when the basal temperature was higher, 25°C day/18°C night versus 20°C day/10°C night. Variability in LMA induction may partly result from variability in the developmental window during which cold shock induces LMA since time course experiments revealed earlier LMA induction in WA8124 (20-24 days past anthesis (dpa)) than in cultivar 'Kennedy' (25-29 dpa). 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). Finally, investigation of LMA induction temperatures revealed that a heat stress treated previously reported to induce LMA in U.K. wheat failed to induce LMA in WA8124. Thus, not all LMA susceptible wheat respond to heat.

Keywords: wheat, late maturity alpha-amylase, LMA, end-use quality, falling number

INTRODUCTION

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

Grain with elevated alpha-amylase levels can have problems with poor end-product quality, including cakes that fall and sticky bread or noodles. The induction of alpha-amylase can begin before seeds visibly germinate. Alpha-amylase is synthesized to fuel seedling growth, and, thus, digests long starch chains into shorter chains. The wheat industry uses the Hagberg-Perten Falling Number (FN) method to detect starch digestion in wheat meal (Perten, 1964; Delwiche et al., 2015). The enzyme alpha-amylase digests starch, resulting in shorter starch chains with reduced gelling capacity, which in turn increases the risk of poor end-product quality. The FN test measures increasing alpha-amylase activity based on the decreasing viscosity of a heated flour meal and water mixture. During the FN test, the flour meal and water mixture is heated to 100°C in a glass tube while being stirred for 60 sec, then the stirrer is dropped and the machine measures the time required for a stirrer to fall through the mixture in seconds. The more starch digestion, the faster the stirrer falls. The minimum FN is 60 sec, the time the mixture is stirred. In the wheat industry grain with an FN below 300 seconds has higher risk of causing problems with poor end-use quality. Farmers receive steep discounts for FN below 300 sec, as much as 25 cents per bushel for every 25 sec below 300 sec (Steber, 2017). Thus, it is important to select varieties with a decreased risk of low FN due to preharvest sprouting or LMA.

LMA is defined as the inappropriate induction of the enzyme alpha-amylase when the wheat experiences unexpected temperature fluctuations in the late grain maturation stage (reviewed in Mrva and Mares, 2008; 2014). Alpha-amylase is highly expressed during early seed development to mobilize nutrients for pattern formation, then decreases as the grain enters the maturation phase of development when sugar is converted to starch during endosperm development. Normally, alpha-amylase should not be induced again until grain germination.

However, research has shown the induction of the high pI alpha-amylase *TaAmy1* mRNA levels during late grain filling in LMA susceptible wheat (Mares and Mrva, 2001; Barrero et al., 2013; Mieog et al., 2017).

Temperature fluctuations during grain maturation can induce LMA in wheat. Previous work showed a strong interaction between alpha-amylase expression, activity, and the environment/temperature shock (Mares and Gale, 1990; Mares et al., 1994). Australian cultivars responded to cool temperature shock well when the grains are in the middle of grain maturation. Alpha-amylase was induced when plants or detached tillers were transferred from a warm glasshouse (temperature range) at 26-35 days past anthesis (dpa) to either a cool temperature (18/12°C) or to a water bath at 10°C for 7 days (Mares et al., 2001; Mrva et al., 2006; Mares et al., 2008). While U.K. varieties induced LMA with a Mid (22°C) to Low (12°C) or a Mid (20/10°C) to High (30/20°C) 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 methods, it appears that either there is insufficient information about the optimal conditions for LMA induction 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 et al., 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. The presence of alpha-amylase protein can be detected both based on enzyme activity and using an ELISA assay

to detect protein accumulation (Farrell and Kettlewell, 2008; Verity et al., 1999; Derkx and Mares, in press).

Current evidence suggests that LMA and preharvest sprouting differ in terms of underlying genetic mechanisms, the location of alpha-amylase expression, and impact on end-product quality (Mares and Mrva, 2014; Newberry 2018). Mapping studies suggest that LMA and PHS are controlled by different loci. There are four families of alpha-amylase genes in wheat, TaAmy1, TaAmy2, TaAmy3, and TaAmy4. TaAmy1, TaAmy2, and TaAmy 4 appear to be induced with germination, and so are likely expressed during preharvest sprouting (Mieog et al., 2017). However, only TaAmy1 appears to be induced with LMA (Barrero et al., 2013). The FN method cannot differentiate between LMA and preharvest sprouting since alpha-amylase is produced in both cases. LMA and PHS can be differentiated based on the location of alpha-amylase activity in the grain (reviewed by Mares and Mrva, 2014). During germination, gibberellin A (GA) hormone produced by the wheat embryo induces alpha-amylase expression in the single-cell aleurone layer that surrounds the wheat starchy endosperm. Thus, alpha-amylase enzyme levels are much higher at the embryo proximal than embryo distal end of sprouted grain. In LMA, on the other hand, alpha-amyalse is more evenly distributed between the embryo distal and proximal ends (Mrva et al., 2006). LMA was found to be associated with randomly distributed patches of cell death in aleurone layer (Bethke et al., 1999; Mrva et al., 2006).

While preharvest sprouting 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 (Mares and Mrva, 2008) and in Washington state (Sjoberg et al., 2019). 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. In order to screen breeding material for LMA susceptibility and develop mapping strategies, this study characterized conditions needed to induce LMA in semi-dwarf U.S. wheat varieties from the Pacific Northwest region including Washington, Idaho, and Oregon.

In order to screen LMA in the breeding programs in the US, a robust screening method is needed. Some preliminary work indicated the lack of correlations between identical experiments and of consistency among samples within experiments. Two replicates of thirty-seven lines from Washington State University spring variety trial were tested side-by-side with LMA induction in the greenhouse environment were not correlated. Such correlation indicates the low repeatability of LMA induction experiments using Mares' (2001a) induction methods with northwest wheat and our greenhouses. Due to the differences in natural climate and wheat germplasm between the Australia and the PNW, it takes different amounts of days for PNW wheat to reach certain developmental stages than it does for Australian wheat (Reviewed by Porter and Gawith, 1999; bom.gov.au/climate/current; weather.wsu.edu). Instead of directly referring Mares' approach to induce LMA, the LMA sensitive window should be determined for the northwest wheat. Meanwhile, when LMA testing results of the untreated control samples were expected to be low, in terms of alpha-amylase activity, since the plants were grown in a standard greenhouse environment without the temperature shock, some of which showed higher activity then treated samples. Cold shock did not elevate alpha-amylase activity in every sample as expected. When the evening temperature is cold enough, condensation was observed in certain growth chamber/room and can introduce inconsistency during induction. Therefore, studies in temperatures in the induction growth chamber/room as well as relative humidity (RH) would

help researchers understanding what type of environment can lead to LMA in the northwest and making suggestions to wheat growers accordingly.

MATERIALS AND METHODS

Germplasm

Grain from the 2011 Washington State University (WSU) soft white spring (SWS) variety trials was obtained from the WSU Cereal Variety Testing, and tested for LMA susceptibility under controlled conditions in the greenhouse. Spring club cultivar 'JD' (PI 656790) and soft white spring breeding line WA8124 (IDO599/S2K00095) were obtained from M. Pumphrey.

Greenhouse LMA induction

Whole plants were tested for LMA induction essentially using the procedure of Mrva and Mares, (2001) except that actual basal temperatures and LMA induction temperatures varied. For LMA induction of the variety trial genotypes, LMA was induced at 18℃ day/ 7.5℃ night in a Conviron™ growth chamber. Three grains were planted into each one gallon pot, and grown in the glasshouse (GH) under a 16 h day/8 h night, with natural sunlight supplemented with sodium lamp light to 300-400 μmol quanta/m2/s, at 21-24℃ day/15-18℃ night temperature until LMA treatment (Figure 2.1 and Expt 1, Table 2.1) or until transfer to a basal chamber at anthesis maintaining a 25℃ day/18℃ night where indicated (Expt 2, Table 2.1). For the first three tillers of each plant, the anthesis date was determined for each spike based on the presence of yellow anthers within florets in the bottom third of the spike. Plants were moved to the cold treatment chamber with a 16 hr day/8 hr night and indicated humidity and temperature conditions at the indicated number of days past anthesis (Miller et al., 2001). Each pot contained three spikes with anthesis dates no more than 2 d apart, providing 3 replicates per pot. Depending on the

experiment, 3 to 6 pots containing 3 tagged spikes per genotype or treatment condition were treated. For spring wheat variety trials, there were 4 plants/genotype*treatment in Experiment 1 and 2 (E1 and E2). After 7 days of high or low temperature treatment, pots were returned to the glasshouse where they were allowed to reach maturity. Untreated controls (no LMA induction) were grown side-by-side with treated plants, but were left in the basal chamber or greenhouse during the entire experiment. Comparisons were made using untreated control spikes that reached anthesis at the same time as the treated spikes. Once material reached harvest maturity, spikes were hand threshed. For variety trial samples, all of the grains from one spike were ground in a coffee grinder as a single sample. For the experiments comparing treatment conditions, 20 random grains were ground from each spike.

Time Course Experiment

A time course experiment was conducted using WA8124 to determine the developmental window for the induction of LMA in terms of GDD. The time course experiment was repeated 3 times, twice beginning with greenhouse-grown plants with 21-24℃ day/15-18℃ night temperature and once beginning with plants grown in a chamber with 25℃ day/18℃ night temperatures. Plants were moved to a reach-in growth chamber 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, (in press) showed that 7 days was the optimal incubation time for LMA induction. Alpha-amylase enzyme assays at each time point were determined as above. For each time point, growing degree days (GDD) were calculated based on Miller et al. (2001). Normal calendar days can be misleading in the field due to variable temperatures each day. GDD are used to improve predictions of crop developmental stages by taking into account daily maximum and minimum temperatures. Daily

maximum and minimum temperatures were recorded beginning at anthesis. Daily growing degree days (DD) were calculated according to the equation:

$$DD\ °C = (Max\ Temp + Min\ Temp)/2$$

The number of daily growing degrees until the indicated number of days past anthesis were summed in order to obtain the number of growing degree days since anthesis. Timepoints giving a significant induction of alpha-amylase in the treatment versus untreated controls samples were determined by Student's t-test.

Temperature Experiments

WA8124 seeds were grown in a glasshouse (21-24℃ day/15-18℃ night), and spikes were labeled with anthesis date. In the first experiment (Expt 1) plants remained in the glasshouse until transfer to the cold chamber. In the second experiment (Expt 2) plants were moved to a basal chamber (Conviron™ GR96) with the indicated controlled temperatures and relative humidity of 55-65%. Humidity was maintained using an EDV-4000 Rotary Desiccant Dehumidifier. When plants reached 21, 23, or 25 dpa (451, 494, or 537 GDDpa), half of the plants were moved to a growth chamber maintaining the indicated LMA treatment temperatures with 45 to 60% humidity (Conviron™ PGR15s). After seven days of treatment, plants were allowed to mature at the basal temperature for 3 to 4 weeks. Due to the practical constraint of using a growth chamber, wheat was planted weekly to allow a single cohort of plants to be incubated at a different LMA treatment temperature each week.

When the basal temperature of interest was different from the default (25℃ day/18℃ night), plants were moved to the treatment chamber at 451, 494, or 537 GDDpa. Approximately 10 spikes were treated for each of the three dpa times points, and a total of 12 to 35 spikes were examined for each treated and untreated condition. Individual spikes were hand-threshed, and 20 grains from each spike were ground for the alpha-amylase enzyme assay.

Humidity Experiment

WA8124 seeds were planted in a glasshouse with three seeds in each pot. First and second tillers were tagged at anthesis, and, meanwhile, the whole plant was moved from the glasshouse into two separate basal (25°C day / 18°C night, 16 hours photoperiod) growth rooms with (~60%) and without (85%) humidity control. After 21 – 25 dpa, 451.5 – 537.5 GDDs, half plants from each basal growth room were moved into cold treatment (18C day / 7.5C night, 16

hours photoperiod) growth chambers with (55%) and without (85%) humidity control, and the rest plants stayed in their basal growth room. When the 7 days treatment was finished, plants were returned to their original basal growth rooms to reach physiological maturity. Each head was hand harvested and threshed. Subsampled 20 grains out of each head and grinded for the Phadebas enzyme assay.

Alpha-amylase enzyme assays

Alpha-amylase enzyme activity was measured using the Phadebas® Amylase Test (Magle Life Sciences™) essentially using the method of Barnes and Blakeney (1974). Total protein extraction was based on the method of Mares et al., 1994; 1 mL of extraction buffer (100 mM Na maleate pH 6.0 and 5 mM $CaCl_2$) was added to 0.2 g of whole meal (for variety trials 2 mL extraction buffer and 0.5 g meal), vortexed, and then incubated for 10 minutes in a 50C water bath. The sample was centrifuged for 10 min at 3000 rpm, and the supernatant used for the enzyme assay. Variety trials samples were assayed according to the manufacturer's instructions while all other experiments were performed in microtiter plates as follows. Phadebas tablets were ground and 2 g suspended in 40 mL of extraction buffer. 200 µl of Phadebas® suspension was aliquoted into each well of a 96-deep well plate. 40 µl of protein extraction was added to each well. Every microtiter plate contained three standards of known falling number and a blank consisting of the extraction buffer. Plates were incubated for 45 min at 50°C. The reaction was stopped by addition of 0.2 mL of 0.5 M NaOH. Plates were centrifuged for 5 min at 4000 rpm, and 0.2 mL of supernatant transferred to another 96-well plate and absorbance at 620 nm determined in a Biotek™ Synergy™ 2 Microplate reader. The A620 value was used as a measure of enzyme activity. ANOVA showed that plate was a significant factor (*p*-value=0.0021) affecting enzyme activity. Three controls of known FN and enzyme activity were

included in each enzyme assay plate and subjected to a linear regression. The linear regression equation was used to calculate the corrected A620 for each plate. Following correction plate was no longer a significant factor affecting Au.

RESULTS

LMA induction in U.S. northwest spring wheat

Examination of falling numbers data from the WSU Cereal Variety Trials revealed the presence of low falling numbers in locations that had not received rain at a time that would induce preharvest sprouting (Sjoberg et al., 2019). This result raised the possibility that U.S. wheat varieties may have LMA susceptibility. To examine whether soft white spring wheat from the northwestern U.S. could induce LMA under conditions previously published for Australian wheat, greenhouse LMA induction experiments were performed using lines from the 2013 WSU Cereal Variety Trials (Mrva and Mares, 2001a). Two independent greenhouse experiments were performed (environment 1 and 2, called E1 and E2). Plants were moved from the glasshouse (22-24°C day/15-17°C night) to a cold chamber (18°C/7.5°C day/night) for 7 days beginning at 26 dpa. When the results of the two independent experiments were compared, they were found to be uncorrelated ($r^2 = 0.11$, *p*-value = 0.14). The lines that induced LMA in both experiments were 'WA8124' and 'Alturas' (Figure 2.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 discovered in U.S. wheat, the results were variable indicating that further experiments were needed to optimize LMA induction conditions.

The effect of the basal temperature and relative humidity on untreated controls

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 alpha-amylase enzyme

levels. Thus, the effect of temperature and relative humidity on alpha-amylase levels without an LMA-inducing cold treatment were examined. Plants were grown continuously under four different temperature regimens, and then alpha-amylase activity was determined for individually harvested spikes. Incubation at the lower temperatures, 20℃ day/10℃ night and 23℃ day/16℃ night, resulted in higher and more variable alpha-amylase activity than incubation at the higher temperatures 25℃ day/18℃ night and 30℃ day/10℃ night (Figure 2.2). This difference in alpha-amylase activity was significant based on a pairwise t-test (Table 2.S1). Thus, future experiments were conducted with a controlled basal temperature of 25℃ day/18℃ night. Next, the effect of differences in humidity on alpha-amylase activity were examined when plants were grown at 25℃ day/18℃ night. Reduced relative humidity, 40-45% day/60-70% night resulted in significantly lower alpha-amylase levels than uncontrolled relative humidity (45-65% day/85-95% night) (ANOVA: *p*-value = 0.0003; Levene's test: *p*-value = 0.0043) (Figure 2.3). Thus, future experiments used a basal chamber with controlled relative humidity. Variability in untreated controls could result from variation in basal temperature or humidity.

Determining the developmental window for LMA induction

Previous work showed that LMA could be induced in Australian cultivar 'Kennedy' between 25 and 30 dpa (Mares and Mrva, 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 were grown under controlled conditions with a 25°C day and 18°C night, until they were transferred to a cold chamber at 18°C day and 7.5°C night. WA8124 showed alpha-amylase induction with a 7-day cold shock treatment initiated between 20 and 24

dpa (Figure 2.4). When Kennedy was subjected to the same LMA induction conditions, alpha-amylase was induced between 25 to 29 dpa, consistent with previously published results (Figure 2.S2A). Thus, WA8124 appears 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 (22-24°C day/15-17°C night) prior to cold treatment (Figure 2.S2B). This suggests that the LMA induction window varies by cultivar and with growth conditions. To estimate the LMA susceptible stage of grain development, we examined the appearance of WA8124 at 20, 24, and 28 dpa during growth at the basal temperature of 25°C day/18°C night (Figure 2.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. 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 was changing 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. Next we examined whether calculating GDD past anthesis (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 when the basal temperature was 22-24°C day/15-17°C night, and between 430 and 516 GDDpa (20 to 24 dpa) when the basal temperature was 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.

LMA induction under varying temperature conditions

Previous work has shown LMA induction by cold and high temperature shock (Mrva and Mares, 2001; Farrell and Kettlewell, 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. Two address this, two experiments (Expt 1 and Expt 2) were conducted to look at the effects of different LMA induction temperatures. Expt 1 was conducted using a basal temperature of 23°C day/16°C night. Expt 2 was conducted using a basal temperature of 25°C day/18°C night after experiments suggested that a higher basal temperature reduces variation in untreated controls (Figure 2.2). Previous work suggested that a cold induction temperature of either 18°C day/12°C or of 10°C day/10°C night should induce LMA, 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 et al., 2001; Mrva et al., 2009; Embiri 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 cooler night temperatures typical of the inland northwestern U.S.. Student's t-test was used to evaluate whether alpha-amylase values were significantly higher in treated spikes than in untreated spikes from plant that remained in the basal chamber during the entire experiment. LMA induction was compared in WA8124 treated for one week before returning the plants to the basal chamber. The 18°C day/7.5°C night treatment resulted in a 4.5-5.1-fold increase, the 10°C day/10°C night treatment a 8.7 to 10.7-fold increase , and 15°C day/4°C night treatment a 1.5 to 3.0-fold increase (Table 2.1). Induction at 15°C day/4°C night had a significant effect only 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 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 2.2). 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 highly 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 but using the mean of untreated values ($\bar{U}$) over the entire run (Expt 1 of 0.26, and Expt 2 of 0.37). Based on this, the 15°C day/4°C night treatment significantly induced a low level LMA in both experiments.

Since WA8124 is known to be LMA susceptible, the frequency of false negatives was examined comparing different basal and treatment temperatures (Table 2.3). The average A620 of the FN 300 sec control sample was 0.24 (n = 11). Based on a definition that a false negative is any treated A620 value below 0.24, we found that the false negative rate was generally lower with the higher 25/18°C than the 23/16°C basal temperature. 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 the higher basal temperature.

While previous work indicated that a high temperature treatment could induce LMA in U.K. wheat (Farrell and Kettlewell, 2008), heat failed to induce LMA in WA8124 (Table 2.1). 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. Since the basal temperatures in the current study were considerably higher, the experiment was repeated using the same temperatures used in Farrell and Kettlewell, 2008. Again, WA8124 failed to induce LMA when temperatures were increased from 20°C day/10°C night to 30°C day/20°C night. Thus, not all LMA susceptible cultivars can respond to high temperature treatment.

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 in soft white spring WA8124. 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 (Mares and Mrva, 2008). This is partly explained by the fact that LMA susceptibility occurs within a relatively short developmental window, previously estimated to occur between 26 and 30 dpa in Australian wheat. Moreover, not all of the grains on a spike induce LMA, likely because they reach anthesis and, therefore, the LMA window asynchronously. By trying to define the conditions that induced LMA in a single line of wheat, the strongest conclusion of this study was that it is 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 actually results in variable

alpha-amylase accumulation in mature grains, and that temperature fluctuations merely exacerbate this condition.

Results in this study suggest that different varieties may become susceptible to cold-induced LMA at different times. The Australian cultivar Kennedy appears to develop more slowly than WA8124, and therefore reached the "window" of LMA susceptibility later than WA8124 (Figure 2.4; Figure 2.S2A). WA8124 itself reached the LMA window later when grown a cooler controlled environment (Figure 2.SB). This suggests that variation in 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. A good approach may be to make use of GDD past anthesis to estimates the correct time for LMA induction, and then visually confirm wheat a grain from the spike is at the late soft dough stage of grain development. Future work will need to test the efficacy of this approach in a larger population.

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. This was not the case (Table 2.1). In fact, it appeared that LMA was not consistently induced at 15°C day/4°C night compared to a 10°C day/10°C night and 18°C day/7.5°C night. These cool induction temperatures were similar to those previously used to induce LMA in Australian and U.K. germplasm (Mrva and Mares, 2001, 2002; Farrell and Kettlewell, 2008). Given that high temperature treatment induced LMA in a U.K. variety, it was also expected that a high temperature treatment would induce LMA in WA8124 (Farrell and Kettlewell, 2008). It did not (Table 2.1). This suggests that there may be varietal differences in whether cold versus high temperature shock can induce LMA. This, in turn, suggests that there may be more than one genetic mechanism leading to this problem. For example, previous work

has shown that the 1R translocation from rye to wheat chromosome 1B increase LMA susceptibility, whereas the GA-insensitive Rht-B1b and Rht-D1b dwarfing alleles decrease LMA susceptibility (Mrva et al., 2009; Farrell et al., 2013).

It appeared that a higher basal temperature reduced the accumulation of alpha-amylase activity in the grain. Initial experiments were performed using a glasshouse with a controlled climate of 22-24°C day/15-17°C night temperatures. This basal temperature was associated with elevated alpha-amylase in untreated controls. When plants were grown continuously in a controlled environment, it was observed that lower temperatures are associated with higher and more variable grain alpha-amylase levels than higher temperatures (Figure 2.2). Interestingly, Derkx and Mares (in press) have suggested using continuous growth in a cool season glasshouse to test LMA instead of a cool temperature treatment. Based on examination of the single LMA-susceptible line WA8124, there was also considerable phenotypic variability after cold temperature induction of LMA for one week (Figure 2.5). It may be the case that LMA susceptibility is a tendency towards variable alpha-amylase expression, and that low temperatures merely increase the range of alpha-amylase activity to a level leading to unacceptable falling number. Future work will need to examine if more consistent results are obtained with constant growth at low temperature or with the one-week cool temperature treatment.

LMA susceptibility is a variable phenotype. But breeding programs need to screen for this trait because farmers receive serious discounts for wheat with falling numbers below 300 sec. This research provided here can help in the development of wheat LMA testing programs for U.S. wheat. While evidence suggests that LMA may not impact end-use quality as strongly as preharvest sprouting, stronger evidence is needed to convince end-users that low falling

number due to LMA does not pose a risk to baking quality (Ral et al., 2016; Kiszonas et al., 2018; Newberry et al., 2018).

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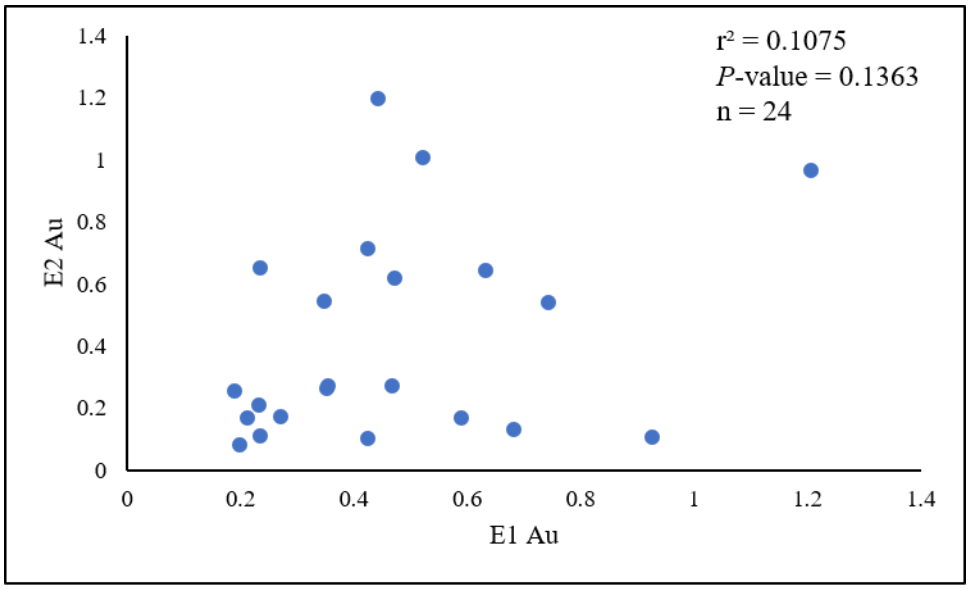

Figure 2.1. The low correlation between E1 and E2, two independent greenhouse LMA induction assays on 24 lines of 2013 SWS Variety Trials, exhibits the high variability in LMA testing.

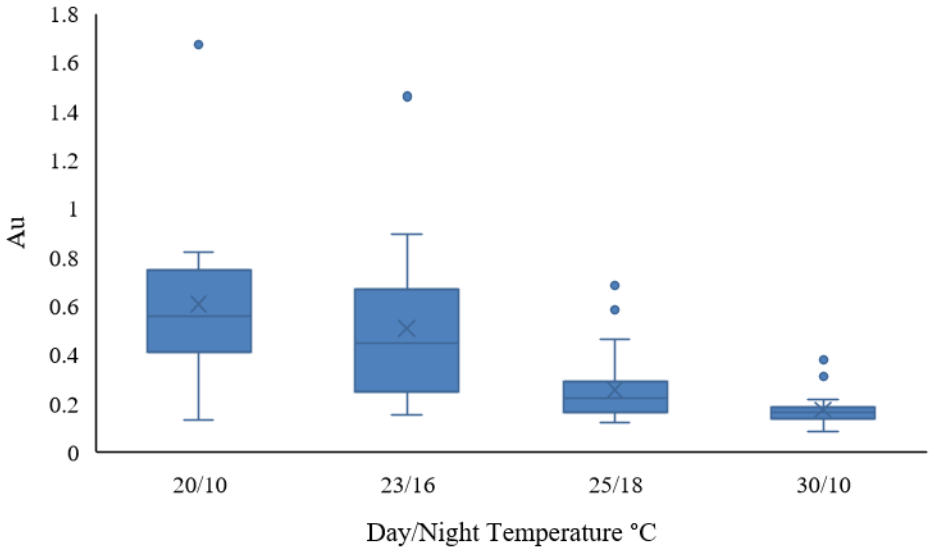

Figure 2.2. The alpha-Amylase activity of the untreated spikes grown at the indicated basal temperatures. Higher alpha-amylase activity and variation were observed at cooler day/night temperatures, 20/10°C (n=18) and 23/16 °C (n=24), than in warmer temperatures, 25/18°C (n=24) and 30/10 °C (n=21).

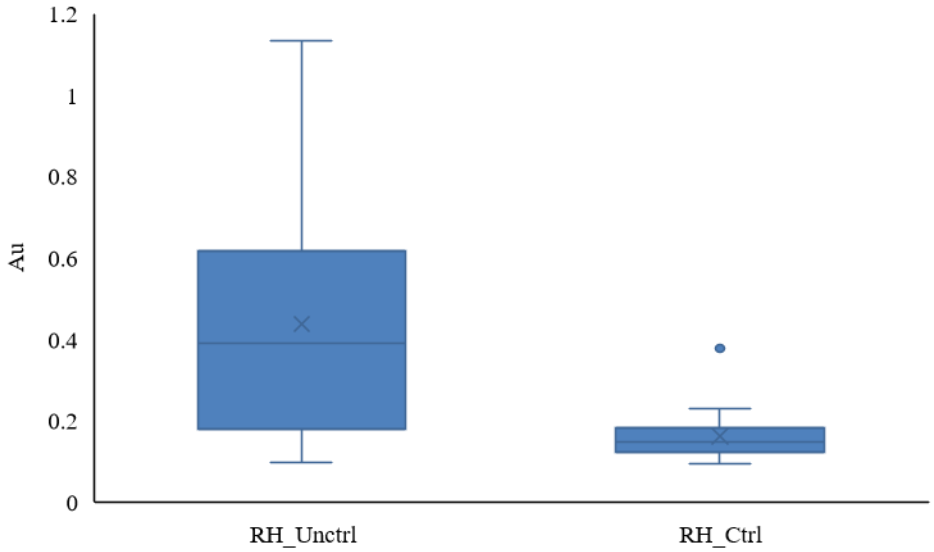

Figure 2.3. By keeping the starting chamber temperature at 25/18°C and changing the presence of relative humidity (RH) control, a dehumidifier, alpha-amylase activity of the untreated spikes was significantly higher (p-value = 0.0003) and more variable (p-value = 0.0043) with the absence of the dehumidifier (n=21) than it was with the RH control (n=21).

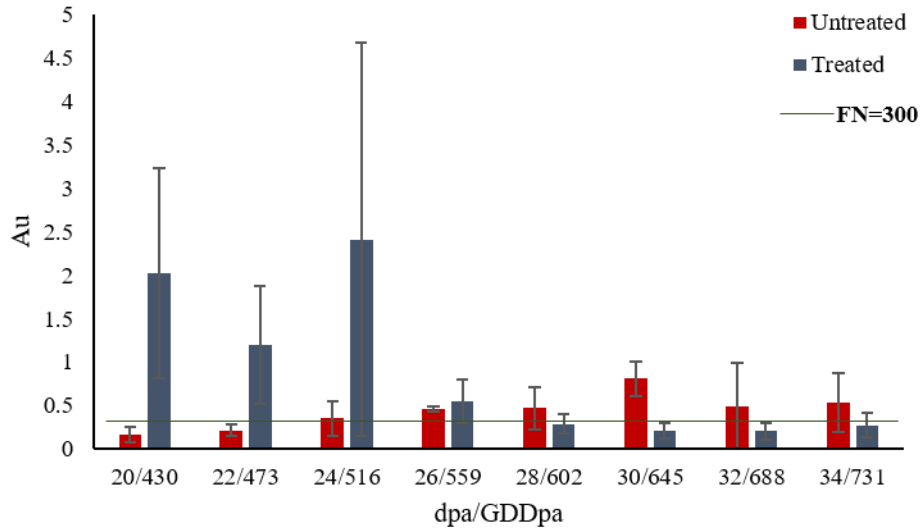

Figure 2.4. LMA time course experiment on WA8124: alpha-amylase was greatly induced in between 20 – 24 dpa, compared to other time points, in WA8124. Each bar represents 3 spikes. The term 'treatment : dpa' was significant (p-value = 0.00259).

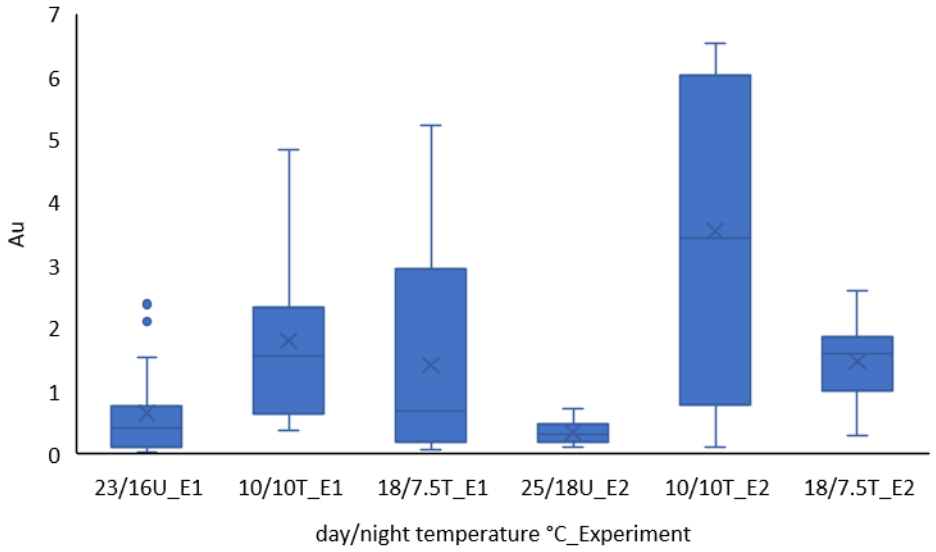

Figure 2.5. Distribution of alpha-amylase activity under two treated (T) temperatures (18°C day/7.5°C night and 10°C day/10°C night) and under two untreated (U) basal temperatures (23°C day/16°C night and 25°C day/18°C night) in Experiment 1 and 2 (E1 and E2).

Table 2.1. The mean of all the alpha-amylase Au values for treated and untreated samples under each condition, the fold-induction (T/U, T/Ū) is treated over untreated values.

Day/Night Temperature °C			A620* Phadebas α-Amylase						
Basal	Induction	Change	Untreated	Treated	T/U[1]	*p*-value[2]	T/Ū[3]	*p*-value[4]	N_T, N_U[5]
23/16	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

[1]The ratio between the average treated samples and their untreated samples
[2]The significance of the treatment for the ratio to its left
[3]The ratio between the average treated samples and the average untreated sample across the whole experiment ($U_1 = 0.26$, $U_2 = 0.37$)
[4]The significance of the treatment for the ratio to its left
[5]The sample sizes of treated sample and untreated samples

Table 2.2. The ANOVA table displays that the cold temperature treatment (treat) significantly induced LMA. The starting temperatures (expt) also significantly affected LMA induction. The various cold temperatures (Temp[1]) are not significantly different from one another.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	4	113.11	28.28	29.31	<0.001
treat	1	77.22	77.22	80.05	<0.001
expt	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[1]	4	113.11	28.28	5.34	0.100

[1]Tests of Hypotheses Using the Type III MS for temp*expt as an Error Term

Table 2.3. The false negative rates for the cold treatment conditions under different starting temperatures: they represent the percentage of the cold treatment of interest failed to induce LMA

	Basal Temperature, day/night °C	
Treatment, °C	23/16	25/18
18 day/7.5 night	33	0
10 day/10 night	0	13
15 day/4 night	35	0

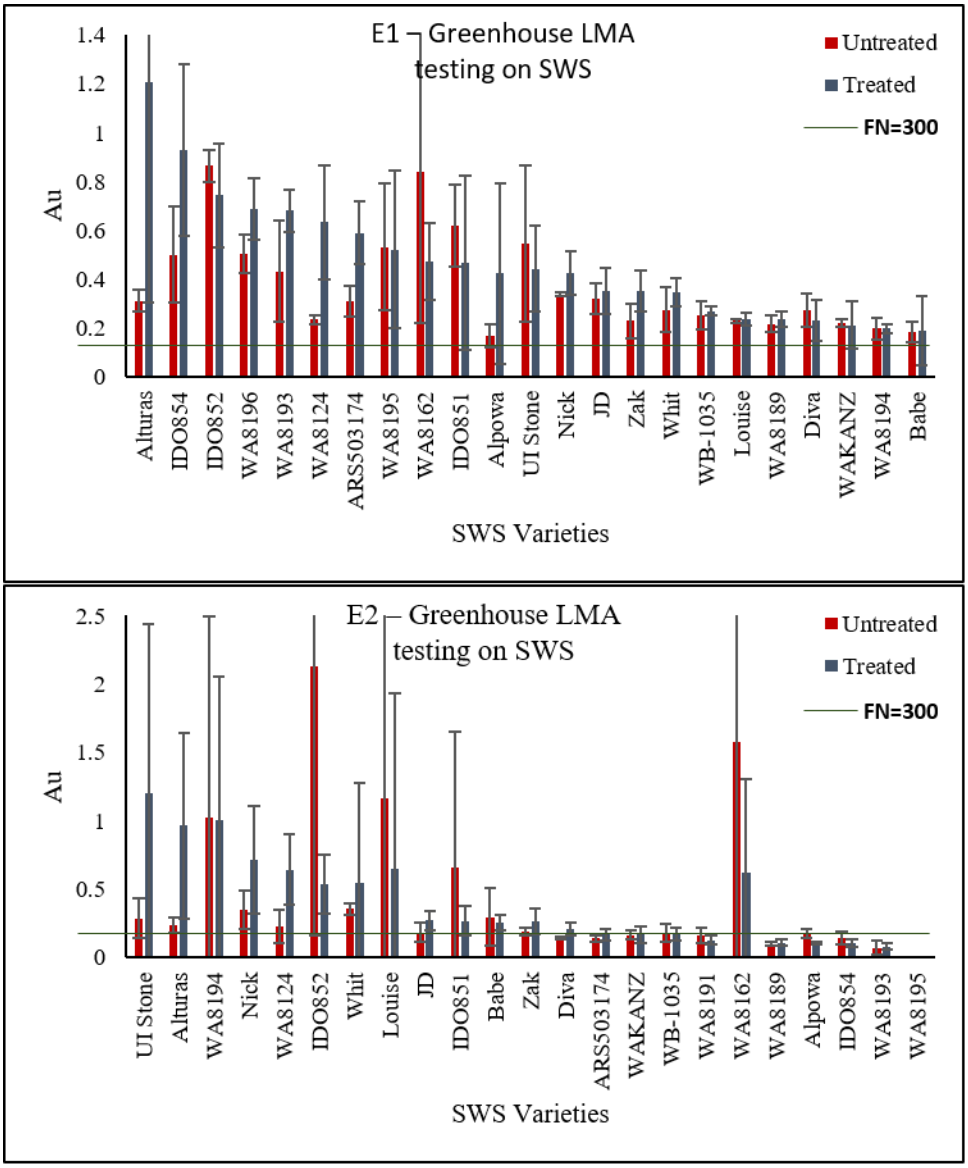

Figure 2.S1. E1 and E2- Greenhouse LMA testing on soft white spring wheat. Alpha-amylase activity is shown in A620 absorbance units (Au). 'Alturas' and 'WA8124' were successfully induced in both independent experiments. The green line represents the alpha-amylase value when FN = 300. Each bar represents 3 - 15 biological replicates ± standard deviation.

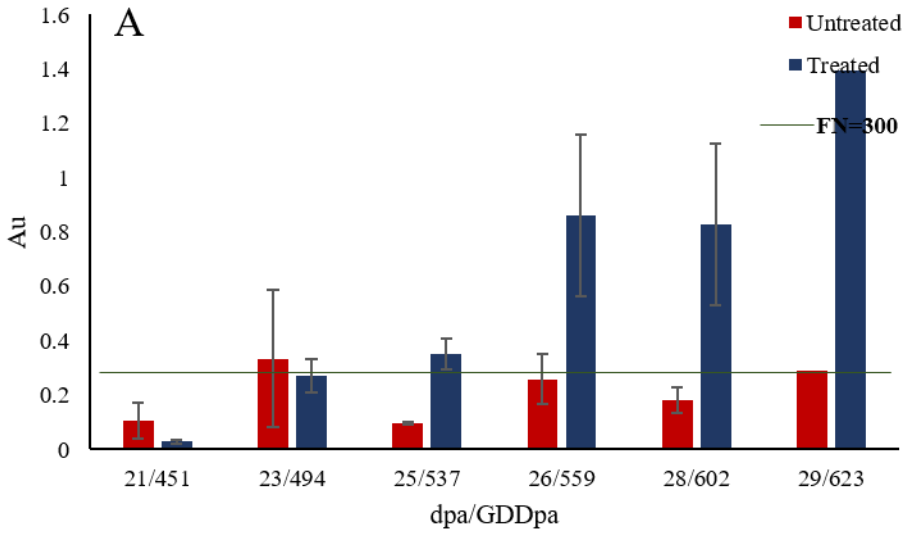

Figure 2.S2A. LMA time course experiment on an Australian line, Kennedy: LMA was successfully induced in between 25 – 29 dpa. Each bar represents n = 1 – 5 spikes.

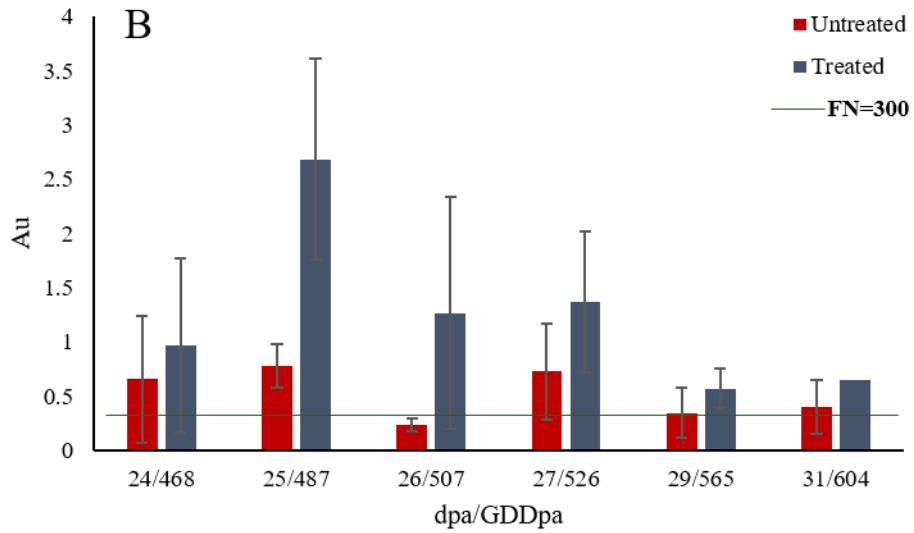

Figure 2.S2B. LMA time course experiment on WA8124 grown under temperature of 22-24°C day/15-17°C night: LMA was successfully induced in between 25-27 dpa.

Figure 2.S3A-H. 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 long LMA susceptible, the vascular bundle has lost its green coloration.

Table 2.S1. A t-test pairwise comparison of alpha-amylase activity after growth at each basal temperature without cold shock (18≤n≤24).

		Day/Night Temperature °C			
	Pairwise t-test *p*-value	20/10	23/16	25/18	30/10
Day/Night Temperature °C	20/10	-	0.1670	<0.0001	<0.0001
	23/16	0.1670	-	0.0003	<0.0001
	25/18	<0.0001	0.0003	-	0.2515
	30/10	<0.0001	<0.0001	0.2515	-

CHAPTER THREE: CHARACTERIZATION OF LMA SUSCEPTIBILITY IN A PANEL OF NORTH AMERICAN SPRING WHEAT GERMPLASM

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*The last two authors contributed equally to this work.

CONTRIBUTIONS: CMS, CCL, MOP, and RSP designed the experiments. RSP and CCL performed GH2018 and GH2019 experiments. CCL performed the Field2018 experiment. KGC, SRR, and MOP provided advice on statistical analyses. Statistical analyses were performed by CCL. The manuscript was written by CCL and CMS.

ABSTRACT

Genetic susceptibility to late maturity alpha-amylase (LMA) in wheat (*Triticum aestivum L.*) results in the induction of alpha-amylase when a cold temperature shock occurs during late grain maturation. The resulting elevated alpha-amylase levels are detected in the wheat industry using the Hagberg-Perten Falling Number (FN) method. Farmers receive a discount for low FN/high alpha-amylase grain due to a belief that this results in higher risk of poor end-product quality, such as cakes that fall and sticky bread or noodles. It is important to assess whether LMA is causing problems with low FN in North American wheat, and to develop methods to select against this problem. To address this problem, we characterized LMA susceptibility in a panel of 256 North American hard red spring wheat lines, representing ten wheat breeding programs. Approximately 79% of the lines showed moderate to severe LMA susceptibility following cold-induction experiments. There was, however, a fairly high degree of variability between independent experiments, with r^2 values ranging from 0.04 to 0.13 ($1.4\times10^{-7} < p < 0.0025$). A preliminary genome-wide association study detected six significant marker-trait

associations on chromosomes 2B, 3A, 3B, 5A, 7B, and 7D. The QLMA.wsu.7B locus detected in this study co-localized a QTL detected in four previous mapping studies using Australian and CIMMYT germplasm.

INTRODUCTION

The Falling Number (FN) method is used by the wheat industry to measure damage due to the presence of the enzyme alpha-amylase in wheat grain (Perten, 1964; Delwiche et al., 2015). Alpha-amylase is activated once it's in contact with water and starts digests starch chains. The water and wheat flour mixture is heated in a near-boiling water bath. There is a negative correlation between the alpha-amylase activity and the flour gelling capacity. While the stirrer falling through the test tube that contains the heated water-flour mixture, Hagberg-Perten falling number machine records how long the stirrer falls from the top to the bottom of the tube. Therefore, high alpha-amylase activity leads to low falling numbers. Uniformly high-quality wheat with undamaged endosperm is more favorable in the Asian wheat market, which is dependent on FN as a quality indicator. Both Late Maturity alpha-amylase (LMA), increased alpha-amylase activity induced by temperature shock during late grain maturation phase, and Pre-harvest Sprouting (PHS), mature grains germinate on mother plants after sprout-induce rain, can elevate alpha-amylase in wheat grain and lower the FN.

Wheat growers in the US, especially in the Pacific Northwest area, are highly dependent on the wheat market in east Asia, which has high standards on the end-use quality and have been severely discounted due to low FN caused by LMA (Washington Grain Commission). Millions U.S. dollars were lost in 2016. Especially when falling number is a significant indicator of grain quality in the wheat market, the excessive amount of alpha-amylase, caused by either LMA or PHS, in wheat grains reduces the gelling capacity by cleaving starch chains into smaller sugar in

the grain endosperm during the FN testing process. Studies have been done conveying the direct negative correlation between FN and low baking quality, such as mushy noodles and unsturdy cakes. Although some research proved that the low FN caused by LMA may not necessarily lead to poor end-use quality, the process of identifying whether the high alpha-amylase activity in the mature grain was caused by LMA or PHS is currently not field friendly outside of Australia (Newberry et al., 2018; Mrva et al., 2006). To reduce the occurrence of low FN in the PNW wheat, resistance to LMA has become an important trait in the breeding programs in North America. It is also important to know the susceptibility of LMA in the breeding programs across North America. One of the fastest ways to decrease the susceptibility for LMA and to protect US wheat growers is breeding against this trait by identifying highly associated QTN's and their corresponding markers and using marker assisted selection in the N. American wheat germplasm.

Several studies have mapped loci associated with elevated risk of LMA in Australian and CIMMYT germplasm (reviewed in Mares and Mrva, 2014). Two major loci were mapped to the centromeric region of chromosome 3B and to the distal end of the long arm of chromosome 7B in multiple studies (Mrva and Mares, 2001a; Emebiri et al., 2010; McNeil et al., 2009). Other loci have been mapped to chromosomes 2DL, 3A, 3B, 3D, 4B, 4D, 5DS and 5BL (Tan et al., 2010). While one study demonstrated that elevated risk of LMA is associated with the Rht loci and the 1B/1R translocation in U.K. germplasm, few studies have examined the genetic mechanisms underlying LMA in non-Australian germplasm.

Genome-wide association studies has been a useful tool to carry out the marker-trait associations in many research areas (Hayes, 2013; Blasco and Toro, 2014; Sukumaran et al., 2015; Wu et al., 2016). The elite lines from different genetic background in the AM panel tend to

have higher allelic recombination rate than the offsprings from certain bi-parental population do. Therefore, the advantage of using an AM panel is allowing way more markers being involved in the gene background and letting more possible loci being detected during the calculation. The hard red spring panel has its 90K high-throughput marker list provides good coverage over the entire wheat genome available (Wang et al., 2014). Even though the genetics similarities among the varieties that came from the same geological region may cause false positives, the usage of principal component analysis (PCA) as well as the kinship matrix in the association calculating software programs can take the population structure into account and solve the issue (Marchini et al., 2004; Liu et al., 2016).

The objectives are assessing the application of LMA testing methods that were developed for spring wheat in N. America on an AM panel, examining the correlation and the repeatability of phenotyping the trait, LMA, among biological replicates and among independent experiments, evaluating the LMA susceptibility in the US, Canadian, and CIMMYT spring wheat varieties, and tentatively mapping the LMA associated loci in the AM panel.

MATERIALS AND METHODS

Germplasm

The association panel was composed of 256 hard red spring varieties from North American wheat breeding programs assembled as part of the Triticeae Coordinated Agricultural Project (www.triticeaecap.org). This panel was previously used to characterize root morphology traits and to map stem rust and stripe rust resistance and essential agronomic traits related loci (Narayanan and Prasad, 2014; Bajgain et al., 2015; Godoy et al., 2018). The panel included elite lines representing North American wheat breeding programs in Mexico: the International Maize and Wheat Improvement Center (CIMMYT), in Canada: Agriculture and Agri-Food Canada

(Ag-Canada) Manitoba, Ag-Canada Saskatchewan, and Ag-Canada Alberta, and in the United States: Montana State University (MSU), South Dakota State University (SDSU), University of California-Davis (UCD), University of Idaho (UI), University of Minnesota (UMN), and Washington State University (WSU).

Greenhouse LMA susceptibility evaluation

Two greenhouse LMA induction experiments, GH2018 and GH2019, were conducted using the method developed in Chapter 2, in the Plant Growth Facility, Pullman, WA. Due to the limited availability of controlled environment chambers, each of the two experiments (expt) were divided into three (GH2018) or two (GH2019) runs. For each genotype, two pots containing two plants each were grown in a glasshouse (22-24°C day / 15-17°C night) with natural light supplemented with sodium lamps as needed to reach a 16h hr photoperiod with 300-400 μmol quanta/m2/s light. The first three tillers of each pot were tagged at anthesis, and then the pots were moved to a warm growth chamber with a 25°C day / 18°C night, 55-65% humidity, and a 16 hr photoperiod (Conviron™ GR96). Based on previous work, the LMA susceptible stage was expected to occur between 430 and 516 Growing Degree Days past anthesis (GDDpa) (20 to 24 dpa with a 25°C day / 18°C night, Chapter 2). At this point in grain development, grains are at the soft dough stage just as the grain transitions to beige from green, and the vascular bundle is yet green, not yet yellow or purple (Chapter 2; Barrero et al., 2013). Because individual lines in the population showed a good deal of variability for the length of time required to transition from anthesis to the LMA susceptible stage, the LMA window was identified based on physical appearance. As the wheat approached 430-516 dpa, a single grain was removed from the center of tagged mother-spikes that had turned lost green coloration but were not yet dry. If the grain appeared to be at the correct stage, then the plant was subjected to cold temperature treated,

ranging from 16 to 30 dpa (Figure 3.S3A-B). For each genotype, one pot was left in the warm chamber (25°C day / 18°C night) as an untreated control and one pot treated for 7 days in a cool chamber (Conviron™ GR48 with high pressure sodium lamps) for LMA induction at 18°C day / 7.5°C night and 16 hr photoperiod. After the 7-day treatment, plants returned to the warm chamber and allowed to mature for 3-4 weeks at 25°C day / 18°C night. Three treated and three untreated spikes that reached anthesis within a 3-day range, were hand harvested and threshed. Note that the 3 spikes from a single pot are considered to be pseudoreplicates.

Greenhouse LMA induction experiment GH2017 was conducted in a single run using a detached tiller method based on Mrva et al., 2001b except that the cold treatment was conducted at 18°C day/7.5°C night instead of 18°C day/12°C. Briefly, two pots containing two plants each were grown in a warm glasshouse (22-24°C day / 15-17°C night). Three spikes per pot were tagged at anthesis. At 26 dpa (~507 GDDpa), tillers were cut at the first internode and placed in a bucket of water. Untreated controls remained in the glasshouse, while cold-treated spikes were incubated for 7 days in a cool chamber (Conviron™ GR48) for LMA induction at 18°C day / 7.5°C night with a 16 hr photoperiod. After the cold treatment, plants were returned to glasshouse until fully mature within 3-4 weeks.

All of the grains from each spike were ground separately with a coffee grinder and alpha-amylase enzyme activity determined with the Phadebas® amylase assay (as in Chapter 2; Kiszonas et al., 2018). The Phadebas® assay uses a water-insoluble starch substrate that is hydrolyzed by alpha-amylase to form water-soluble blue fragments. The absorbance at 620 nm (A620) of the blue solution is given since it is a direct function of alpha-amylase activity. The A620 is referred to as Absorbance units (Au). The results were adjusted for plate-to-plate variation based on a linear regression of three standards of known FN.

Field LMA susceptibility evaluation

A detached-tiller LMA induction protocol based on the procedure of Mrva and Mares (2001a) was used to test field-grown material. Two complete blocks of 213 TCAP lines were planted as single head rows in the last week of April, 2018, on the Spillman Agronomy Farm, Pullman, WA. Occasional irrigation was applied before anthesis. The timing of the LMA susceptible stage of development was determined in a manner similar to the greenhouse experiment except that the anthesis date for each headrow was determined based on the date when at least 50% of the spikes were at antheis. When the lines reached between 430 to 520 GDDpa, headrows were examined every other day to identify plots where most of the tillers were at the LMA susceptible stage, based on the predominance of kernels in the late soft dough stage with a beige seed coat and green vascular bundle in the crease. At this stage, 40 tillers were collected from each head row by cutting near the soil line with scissors. Wheat bouquets were put into buckets of well water immediately after they were cut. 20 tillers were left out-of-doors as an untreated control and 20 tillers were cold treated at 18°C day / 7.5°C night, ~65% relative humidity, and 16 hr photoperiod for 7 days (Reliance™ incubator with EvaDry dehumidifier). Water was changed as needed. After the treatment, the wheat bouquets in water were allowed to mature until dry out-of-doors. Wheat were machine threshed by bundles. and were ground with a Udy Cyclone grinder with a 0.5mm screen. Phadebas alpha-amylase assays were performed on 0.2 g of meal. The results were adjusted for plate-to-plate variation by linear regression based on three standards of known FN (as in Chapter 2).

Genotyping and marker data

Single-nucleotide polymorphisms were genotyped in 242 lines of the panel as described in (Godoy et al., 2018). The panel was genotyped using Illumina iSelect 90K SNP chip array at

the USDA-ARS genotyping laboratory in Fargo, SD (Wang et al., 2014). Allele calls were made using GenomeStudio v2011.1 (Illumina, 2011). Complete genotype information for 34,137 SNPs in the TCAP90K_SpringAM_panel was obtained from the Triticeae Toolbox (https://triticeaetoolbox.org/wheat/). SNPs with a minor allele frequency (MAF) below 0.05 were eliminated from the dataset to reduce the potential for mapping false positives (Tabangin et al., 2009). As a result, 19,192 markers were used for genome-wide association mapping. The marker positions were obtained from the wheat genome consensus map (Wang et al., 2014). Principal components (PC) were calculated using the prcomp function in R (v3.6) on the marker dataset (19192 markers and 242 lines). To visualize the population structure, genetic variation explained by PC1 was plotted against PC2 using plot function in R. Each dot represents a spring wheat variety on the AM panel, and each color represents one out of ten breeding programs in North America. In order to examine the effect of population structure, GWAS was performed without and with the first and first two PCs fitted into the model as fixed effects.

The population was genotyped for specific alleles of the plant height (*Rht-B1* and *Rht-D1*), photoperiod (*Ppd-A1*, *Ppd-B1*, and *Ppd-D1*), and vernalization (*Vrn-A1*, *Vrn-B1*, and *Vrn-D1*) genes by Godoy et al., (2018) (Ellis et al., 2005; Grogan et al., 2016). The Kompetitive Allele Specific Polymerase Chain Reaction (KASP) markers, KASP-wMAS000001 and KASP-wMAS000002, were used to distinguish the tall (*rht-B1a* and *rht-D1a*) and semi-dwarf (*Rht-B1b* and *Rht-D1b*) alleles for *Rht-1*. Marker KASP-Ppd-A1prodel was used for *Ppd-A1*, KASP-wMAS000027 and KASP-TaPpdBJ003 for *Ppd-B1*, and KASP-wMAS000024 for *Ppd-D1*. Vernalization alleles were differentiated using markers KASP-wMAS000033 and KASP-wMAS000035 for *Vrn-A1*, KASP-VrnB1_I_D, KASP-wMAS2000037 and KASP-VrnB1_C for *Vrn-B1*, KASP-wMAS000039 for *Vrn-D1*.

Statistical analysis

Multi-step data normalization as well as comparisons among replications and experiments were performed in R (v3.6). ANOVA were performed using the lme4 package (Bates et al., 2015). Based on lambda from a Box-Cox analysis in the R MASS package in R3.6, a log10 transformation was used to normalize Au data (Butler et.al., 2009, Sakia., 1992). A Mixed Linear Model (MLM) was performed for the Field 2018 data in the lme4 package with genotype as fixed effect and run by genotype interaction as random effect, and used to generate Best Linear Unbiased Estimators (BLUEs) for GWAS (Henderson, 1975; lme4 v1.1-21). BLUEs of both greenhouse and field experiments were used to examine correlations between independent experiments. REML was used as a method to fit an MLM for the combined GH2018 and GH2019 datasets such that genotype, experiment, and run were examined as random effects on alpha-amylase activity in cold-treated samples (lme4 v1.1-21). The MLM for GH2018 and GH2019 was used to calculate Best Linear Unbiased Predictors (BLUPs) using the lmer command in lme4, and the resulting variances were used to calculate repeatability as an estimate of broad-sense heritability using the equation below.

$$h^2 = \frac{V_{genotype}}{V_{genotype} + \frac{V_{residual}}{\#\text{expt}}}$$

BLUPs of the GH2018 and GH2019 experiments and BLUEs of the field experiment were used to perform a genome-wide association study.

Genome-wide association study

FarmCPU (Fixed and random model Circulating Probability Unification, v1.02) was used to identify significant marker-trait associations using default parameters except that the p.threshold was set to 0.05 instead of the default of 0.01 (Liu et al., 2016). The p.threshold

divided by the number of markers, in this case 19,192, determines the number of SNPs considered significant in the first iteration of marker-SNP associations. It is considered appropriate to increase the p.threshold to 0.05 or 0.1 to increase the likelihood of detecting QTL in smaller populations. LMA was mapped using BLUPs calculated from $\log_{10}$ transformed cold-treated plant Phadebas Au values. Untreated plant alpha-amylase enzyme activity was not used for mapping. The resulting BLUPs were used as dependent variables in the GWAS. Markers were identified as significantly associated with the trait after a 1% Bonferroni multiple test correction (p-value < 5.21×10^{-07}; $-\log_{10}(p) > 6.28$). The inputs for Farm CPU include a phenotypic file, marker file, marker position file, and principal component file. Up to 2 PC's were used in the analysis. A Manhattan plot, a QQ-plot, an association table, and an effect of covariate table, if any PC's were included in the analysis, were the outputs. More information, such as the chromosome number, genome position, p-value, and minor allele frequency (MAF) were obtained from the association table. Only QTL with an MAF above 0.05 were reported.

RESULTS

Population Structure

LMA susceptibility was examined in an association mapping (AM) panel of 242 lines selected to represent a wide range of North American hard red spring wheat breeding programs (Bajgain et al., 2015). The panel was previously genotyped for 90,000 gene-based SNPs. A total of 256 were characterized for LMA phenotype. We will briefly describe the population genotype structure previously analyzed by Bajgain et al., 2015 using principal components and hierarchical clustering (Figure 3.S1). The first PC explained 8.9% and the second 4.6% of the genotypic variation. There were two major clusters. The first included Canadian and upper-midwestern U.S. varieties: Saskatewan, Alberta, Manitoba, Minnesota, Michigan, and South

Dakota. The second cluster included Mexico represented by CIMMYT and the western U.S., Idaho, Washington, and California.

LMA phenotyping of a North American spring wheat panel

LMA-induction experiments were conducted three times in the greenhouse and once in the field. A single LMA field induction was performed where it was not possible to control temperature conditions prior to the LMA-inducing cold treatment to examine the feasibility of screening field-grown plants for LMA susceptibility in future breeding efforts. Both GH2017 and F2018 experiments were performed using detached tillers, whereas GH2018 and GH2019 experiments were performed using intact plants. The cold-treatment in GH2017 was performed at 26 days past anthesis (dpa) for all varieties. Because GH2017 did not show good LMA induction based on comparison of untreated and cold-treated spikes, the timing of cold-treatment in the GH2018, GH2019, and F2018 experiments was adjusted based on visual inspection of the plants to identify the late soft dough stage (Zadoks growth stage 85) (Chapter 2; Figure 3.S3). The untreated samples displayed lower alpha-amylase activity (in Au) and lower variation than the treated samples. All of the experiments except for GH2017 showed good induction of alpha-amylase level by cold treatment, and showed sufficient variation to compare LMA induction in different varieties (Figure 3.1). The field experiment, F2018, showed successful LMA induction compared to the untreated control indicating that LMA induction was possible under field-screening conditions.

Both the greenhouse and the field LMA experiments showed a Poisson distribution for alpha-amylase activity after cold treatment, where values were highly skewed towards low Au values (Figure 3.S2). Based on a Box-Cox normality plot, it appeared that a $\log_{10}$ transformation would result in a more normal distribution, and was used for all subsequent statistical

analyses. In each experiment, three treated and three untreated spikes were collected for each genotype. These were treated statistically as pseudoreplicates because they came from two plants in the same pot.

LMA is a highly variable trait such that not all tillers on a plant and not all grains in a spike may induce the phenotype in a given experiment (reviewed in Mares and Mrva, 2008). To assess the reproducibility of LMA induction in each experiment, we examined correlations between the three pseudoreplicates (Table 3.1). There was a weak but significant correlation between pseudoreplicates in GH2017 (r^2 value of 0.24, *p*-value = 1.2×10^{-11}). The correlations between pseudoreplicates in GH2018 and GH2019 were improved, ranging from an r^2 value of 0.26 to 0.43. Thus, pseudoreplicates are moderately correlated. Similar correlations were observed between untreated samples (Table 3.S1). The F2018 experiment was performed using two bulked samples of 20 spikes each from two independent headrows. They showed a weak but significant correlation for treated biological replicates ($r^2 = 0.048$, *p*-value = 0.001) and for untreated biological replicates ($r^2 = 0.130$, *p*-value = 5.103×10^{-8}).

There was limited correlation between independent greenhouse and field LMA induction experiments based on a comparison of BLUEs (Figure 3.2; $0.04 < r^2 < 0.13$). Stronger correlations were obtained when the data were restricted to the highest and lowest tails of alpha-amylase values in GH2018 and GH2019, suggesting that the more extreme LMA resistant and susceptible phenotypes were more reproducible (Table 3.2).

LMA screening of the AM panel allowed us to evaluate its susceptibility in ten North American breeding programs. Varieties were placed in categories based on the number of times that LMA was cold-induced out of eight biological replicates among the two greenhouse (3 replicates each) experiments and one field experiment (2 replicates). A line was considered to

have LMA induction when a cold-treated sample had more than 0.2 Au in the alpha-amylase enzyme assay because control samples with an FN of 300 sec had an average of 0.15 Au (StDev ±0.02) in these experiments. Based on this, there were 55 LMA 'Resistant' lines that induced LMA 0-2 times, 74 LMA 'Susceptible' lines that induced 6-8 times, and 127 inconsistent 'Moderate' lines that induced 3-5 times out of 8 (Figure 3.3A). When these data were examined by breeding program, Montana State University (MSU) and CIMMYT in Mexico had the smallest fraction of LMA resistant varieties; whereas the University of Minnesota (UMN) and Agriculture and Agri-Food Canada (Ag-Canada) Manitoba had the largest LMA resistant lines (Figure 3.3B). The complete testing results are available in Table 3.S2.

Sources of variation in LMA phenotype

Sources of variation in LMA phenotype

Factors contributing to variation between experiments GH2018 and GH2019 were examined using REML with dpa included as a fixed effect (Table 3.S3; Gilmour et al., 1995). The days past anthesis (dpa) at which the LMA induction was performed did not significantly contribute to the variance in alpha-amylase activity (p-value = 0.48). Genotype contributed the most to the variation (Table 3.S3). Since that Run was nested within Expt and that dpa did not significantly contribute to the variation, Genotype and Expt were included to calculate BLUPs for GWAS and for the repeatability (Table 3.S4).

The main reason that independent experiments were poorly correlated was that some varieties had significant LMA induction in one experiment but not in another. It is possible that the timing of LMA induction was correct in one experiment but not in the other. Indeed, the timing of LMA induction in the GH2018 and GH2019 experiments were not significantly correlated (p-value = 0.42; Figure 3.S3A). The GH2019 experiment had a narrower range of

LMA treatment dates than GH2018, likely because GH2019 was conducted in two runs that were only one month apart whereas GH2018 was conducted in three runs spanning 11 months (Figure 3.S3B). One possibility is that seasonal variation in the glasshouse prior to transfer to 25°C day / 18°C night at anthesis impacted subsequent grain development leading to differences between runs performed at different times. Alternatively, the wide range of variation for flowering time in this population may have led to a wide range of variation in the timing of LMA susceptibility (Godoy et al., 2018). A repeatability of 0.40 was calculated based on the MLM of GH2018 and 2019 as an estimate of broad sense heritability (h^2) with genotype included in the model as a random effect and experiment as a fixed effect. This repeatability suggests that it should be possible to detect associations in a genome-wide association mapping study.

Preliminary GWAS of LMA in North American wheat

A preliminary genome-wide association study was performed to identify potential QTLs associated with LMA resistance and susceptibility in the panel of North American hard red spring wheat. Log_{10} transformed alpha-amylase activity (Absorbance 620 units, Au) met the assumptions needed to perform a preliminary study in FarmCPU. FarmCPU was chosen because it was designed to work with large datasets, and to reduce false positive and false negative results by adapting kinship and population structure as covariates in a Mixed Linear Model (MLM) (Liu et al., 2016). The number of principal components used was based on the fit of the Q-Q plots (Figure 3.S4; Appendix 1). Three LMA-associated loci were detected on chromosome 3A, 7B, and 7D in the F2018 environment using the BLUEs of the entire population (n = 206). One locus was detected on chromosome 3B using BLUPs derived from GH2018 and GH2019 for the entire treated population (n = 242) (Table 3.3; Appendix 1). No loci were found in both environments. Since there was greater correlation between the tails of GH2018 and GH2019, a

GWAS was performed using BLUPs for the top and the bottom 25% of the treated population (n = 120). Given the smaller population, the p.threshold was increased from 0.05/number of markers (19,192) to 0.1/number of markers, allowing detection of an additional two significant loci on chromosomes 2A and 5B. The Manhattan plots from the analyses show significant marker-trait as dots above the line representing the negative $\log_{10}$ of the Bonferroni adjusted *p*-value of 0.05 divided by the number of markers used for association mapping (Appendix 2).

Associations to known cloned genes *Rht, Vrn,* and *Ppd*

Previous work has shown that higher LMA susceptibility is associated with having the tall alleles at the *Rht-1* loci, *rht-D1a rht-B1a* (Mrva et al., 2009; Farrell et al., 2013; Derkx and Mares, in press). The association panel was genotyped for the *Rht-1* loci to examine whether the tall genotype is associated with higher LMA susceptibility whereas the semi-dwarf alleles, *Rht-B1b* and/or *Rht-D1b*, are associated with lower LMA susceptibility. Based on a one-way ANOVA of BLUPs from treated greenhouse experiments, there was a weak association between LMA susceptibility and the taller *rht-D1a rht-B1a* genotype (*p*-value = 0.053; Table 3.S5). The association between LMA susceptibility and the taller *rht-D1a rht-B1a* genotype was much stronger using BLUPs from untreated samples (*p*-value < 0.0001). This may be because the *rht-D1a rht-B1a* genotype is associated with an LMA constitutive phenotype, where cold treatment is not needed to express the LMA phenotype (reviewed in Mares and Mrva, 2014). Next, we examined individual genotypes to determine whether the LMA constitutive genotype was found strictly in *rht-D1a rht-B1a* lines. While there were seven *rht-D1a rht-B1a* with a constitutive LMA phenotype, there was one *Rht-D1b* (UC1110) and one *Rht-B1b* (UC1599) line with a constitutive LMA phenotype. Moreover, some *rht-D1a rht-B1a* genotypes expressed LMA only with cold induction, such as Hollis, Briggs, SD3997, Rescue, and Thatcher. Thus, not all *rht-*

D1a rht-B1a genotypes express LMA constitutively and it is possible to observe constitutive LMA expression in a semi-dwarf background.

Since the LMA window appeared to vary between genotypes and between experiments, we hypothesized that genes governing the response of flower development to the environment might influence LMA susceptibility. Thus, we examined the *Vrn-1* vernalization alleles and *Ppd-1* photoperiod sensitivity genes. The population showed variation for the *Ppd-D1* gene, but not for *Ppd-A1* or *Ppd-B1*. Variation at *Ppd-D1* did not significantly affect alpha-amylase expression in treated (*p*-value = 0.47) or untreated (*p*-value = 0.17) samples. Moreover, variation at *Vrn-A1, Vrn-B1,* and *Vrn-D1* also did not have a significant effect (Table 3.S5).

DISCUSSION

While PHS can be seen as a more critical breeding objective due to evidence suggesting a stronger impact on end-use quality, wheat breeding programs still need to select against LMA because farmers can receive substantial discounts for the resulting low falling number grain (Ral et al., 2016; Newberry et al., 2018; Kiszonas et al., 2018; Derkx and Mares, in press). In fact, farmers in Washington state received an estimated $140 million in discounts in 2016, partly due to problems with LMA-induced low FN, and have continued to receive discounts for LMA-affected grain (Steber et al., 2018; D. Herron, personal comm.). This study revealed a substantial degree of LMA susceptibility in a panel of 256 elite spring hard red varieties representing ten major N. American wheat breeding programs (Figure 3.3). In fact, only 21% of varieties ranked as resistant, whereas 50% showed moderate and 29% strong susceptibility. This distribution suggests that there has been little selection for LMA resistance in N. America. This study has identified resistant and susceptible lines that could be used as parents in future biparental

mapping populations, and identified SNPs associated with the LMA trait in a preliminary GWAS.

Several previous studies mapped loci controlling the LMA phenotype (Mrva and Mares, 2001b; Tan et al., 2004; McNeil et al., 2009; Emebiri et al., 2010; Tan et al., 2010). Significant loci were detected on chromosomes 6B and 7B in an association study of 91 CIMMYT lines (Emebiri et al., 2010), and significant loci were mapped on chromosome 3B and 7B using the Cranbrook/Halberd doubled haploid (DH) population of 158 lines (Mrva and Mares 2001ab; 2002; McNeil et al., 2009). The positions of QTL identified in the current study were compared to the genetic map positions of QTL from previous studies (Maccaferri et al., 2015; Martinez et al., 2018). *QLMA-wsu.2B*, *QLMA-wsu.5A*, and *QLMA-wsu.7D* appeared to be unique since no previous studies detected LMA QTL on these chromosomes (Mrva and Mares, 2001b; Tan et al., 2004; McNeil et al., 2009; Emebiri et al., 2010; Tan et al., 2010). The 3B QTL is quite large, associating with different markers in different studies sometimes on the long and sometimes on the short arm of chromosome 3B (Mrva and Mares, 2001; McNeil et al., 2009; Tan et al., 2010). The *QLMA.wsu.3B* locus was 47.72 cM away from the closest LMA QTL, gwm644, suggesting that it may be a unique QTL. *QLMA.wsu.7B*, on the other hand, is located between the flanking markers wPt-3723 and wPt-0884 of the LMA QTL detected by Emebiri et al., (2010) on chromosome 7BL.

The LMA QTL on chromosome 7BL has been mapped in five studies including this current study, using four independent mapping populations (Table 3.3; Mrva and Mares, 2001; Tan et al., 2004; McNeil et al., 2009; Emebiri et al., 2010). It is interesting that *QLMA.wsu.7B* was detected in the field 2018 environment but not in GH2018 and GH2019. F2018 made use of the detached tiller LMA testing method used in the previous studies that mapped the LMA QTL

on 7B, whereas GH2018 and GH2019 made use of intact plants. Future work will need to examine whether LMA testing with intact plants consistently detects different QTL than the detached tiller method.

Given the low correlation between independent LMA experiments in this study, the genome-wide association mapping must be considered a preliminary, exploratory approach (Figure 3.2). The most likely explanation for this was that cultivars in the AM panel showed considerable variation in flowering time and in the timing of the LMA-induction window during grain maturation (Figure 3.S3). Future efforts to map LMA should make use of either biparental populations or association mapping panels containing varieties with very similar phenology including similar vernalization and photoperiod loci. While GWAS did detect significant QTL, future work will need to validate the effectiveness of the markers in selecting for LMA resistance either in biparental mapping populations within breeding programs that have been characterized for LMA phenotype. For example, KASP markers designed to differentiate between these SNPs could be used to genotype breeding lines that have already been subjected to LMA testing. A one-way ANOVA would enable us to determine if the favorable allele is significantly associated with LMA resistance.

Previous studies have tried to develop approaches to overcome the inconsistency of the LMA phenotype. An ELISA assay specific for *TaAmy1* was developed in order to differentiate between the *TaAmy1* induced during LMA and preharvest sprouting, and alpha-amylases like *TaAmy3* that are expressed during development (Verity et al., 1999; Barrero et al., 2013; Mieog et al., 2017). It may be the case that this greatly improves LMA phenotyping given that Emebiri et al., (2010) calculated a heritability of 86.6% in their association mapping of 91 lines compared to the heritability of 40% in the current study. Unfortunately, this ELISA assay is not available

for use by researchers outside of Australia. Researchers have used categorical systems to rank varieties for LMA phenotype, such as determining the number of grains out of seven, that show increased alpha-amylase levels (Mrva and Mares, 2001a). Improved statistical methods for dealing with LMA phenotype data have also been developed (Butler et al., 2009; Tan et al., 2010).

Previous work showed that LMA susceptibility was associated with having the tall *rht-B1a rht-D1a* genotype, with wild-type copies of the wheat DELLA gene on both chromosomes 4B and 4D (Mrva et al., 2009; Farrell et al., 2013). Moreover, individuals with the tall *rht-B1a rht-D1a* genotype sometimes exhibit elevated alpha-amylase without cold temperature treatment (Barrero et al., 2013). Consistent with this, the *rht-B1a rht-D1a* genotype was significantly associated with elevated alpha-amylase in both treated and untreated samples. This was not entirely expected since breeding programs might have selected against the LMA-constitutive phenotype given that falling numbers above 300 sec are an important receival standard. Given that cold treatment is not needed to detect constitutive LMA, breeding programs could easily select against this problem by performing alpha-amylase enzyme assays on mature grain. One way to overcome the high degree of variability in cold-induction of LMA is to map in tall by tall crosses segregating for constitutive LMA, such as the Spica x Maringa RIL population (Tan et al., 2004; Barrero et al., 2013).

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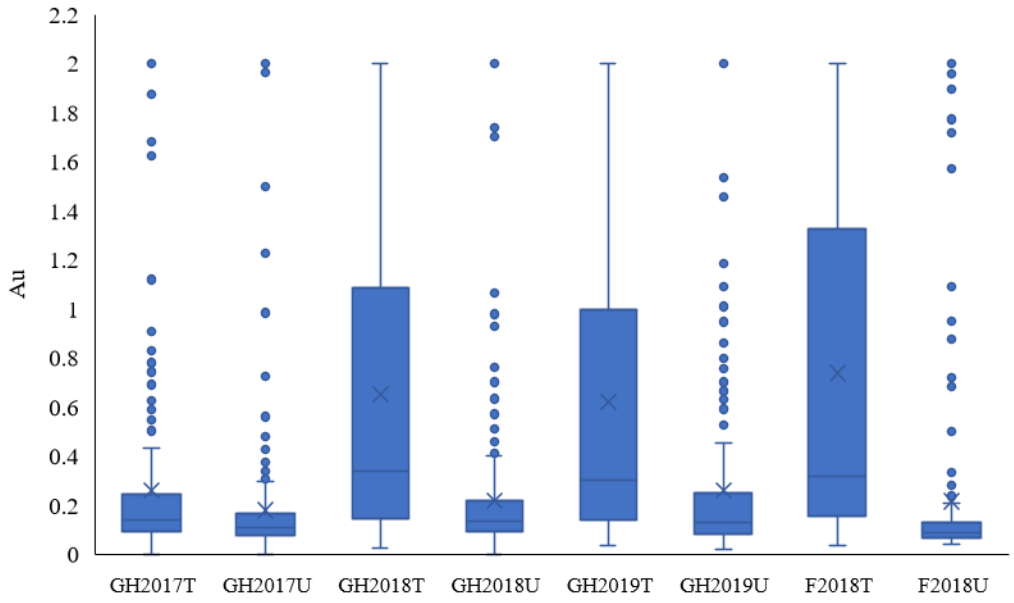


Figure 3.1. The distribution of alpha-amylase absorbance A620 units (Au) in cold-treated (T) and untreated (U) experiments. All three greenhouse (GH) and one field (F) LMA experiments performed on the AM panel are shown.

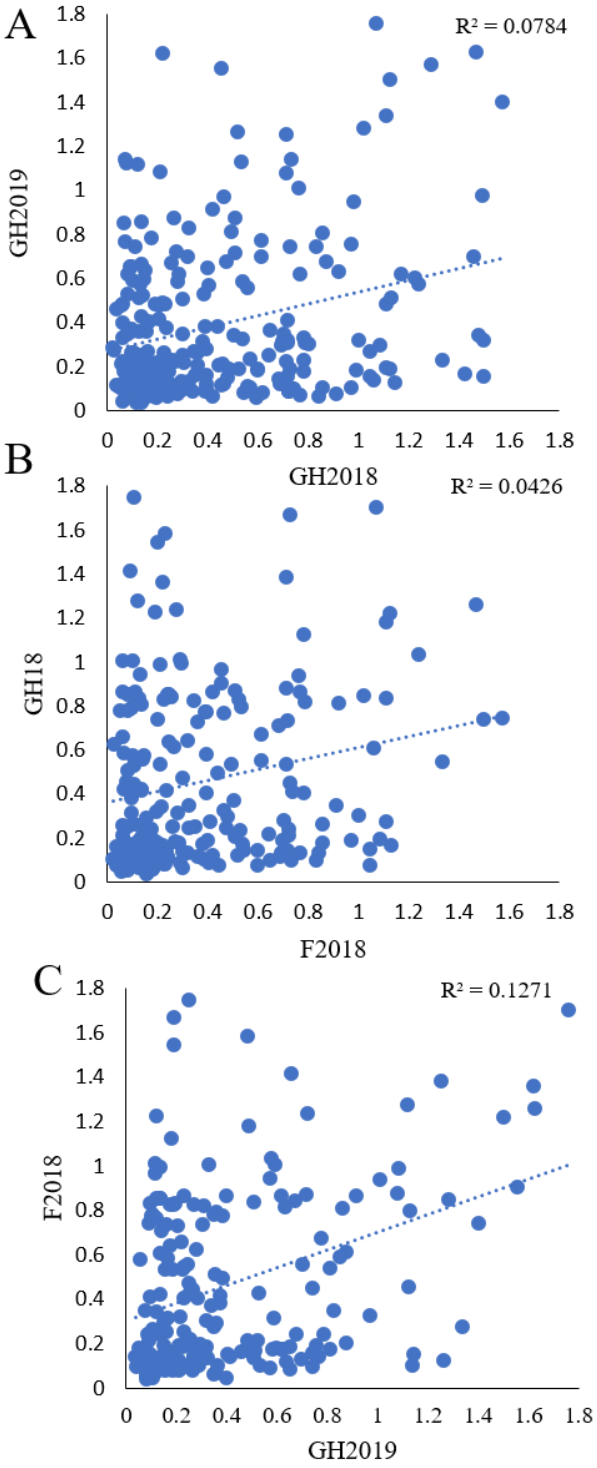


Figure 3.2A-C. Comparison of independent LMA induction experiments. Scatter plots and Pearson correlations among three experiments A) GH2018 vs. GH2019 ($p<7.2\times10^{-6}$) B) GH2018 vs. F2018 ($p=0.0025$) C) GH2019 vs. F2018 ($p<1.4\times10^{-7}$)

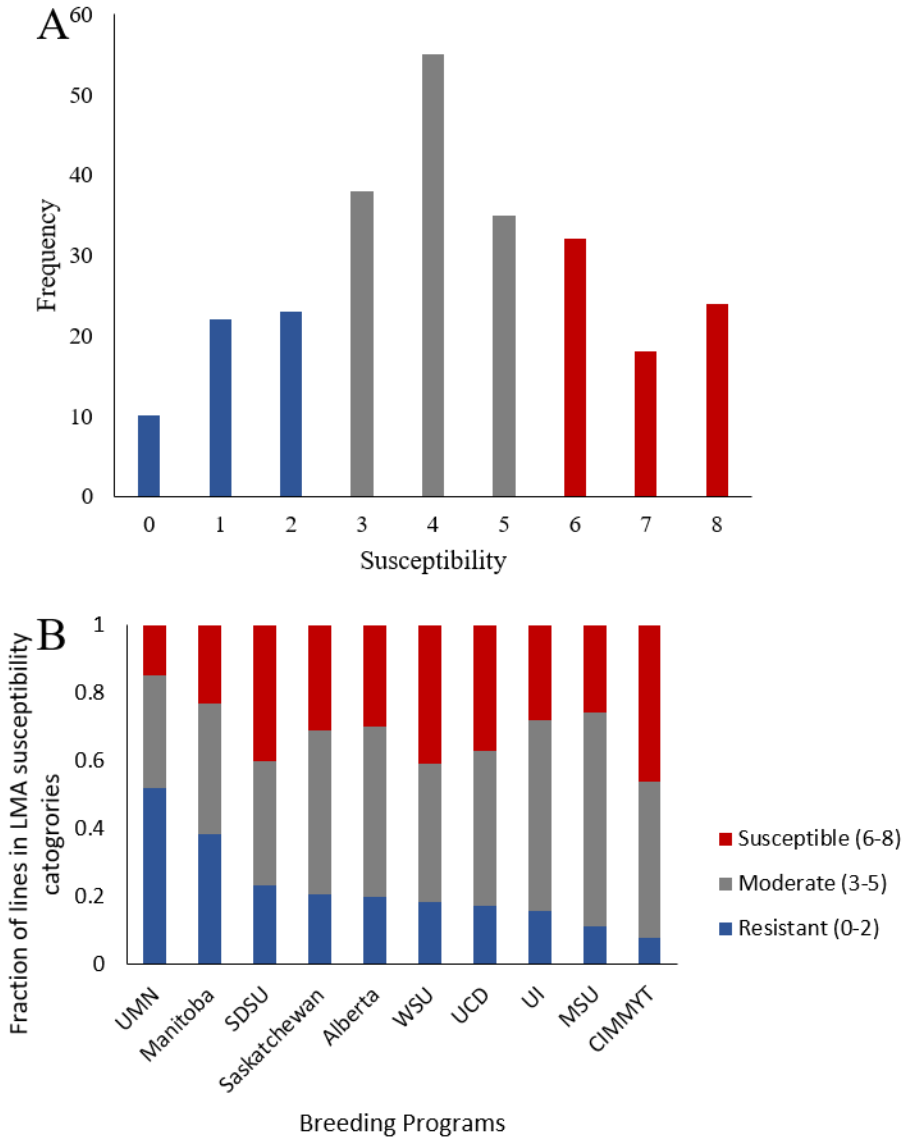


Figure 3.3A-B. The distribution of LMA phenotypes in the AM panel of 256 N. American breeding lines. A) The frequency of each phenotypic category in the AM panel. B) Distribution of LMA phenotype by breeding program. Categories of LMA susceptibility were defined based on the number of biological replicates out of 8 showing LMA susceptibility (A620 > 0.2) in the two greenhouse and one field LMA screening experiments, where, susceptible (red, 0-2 out of 8), moderate (grey, 3-5 of 8), and resistant (blue, 6-8 of 8) . Bars show the fraction susceptible (74), moderate (127), and resistant (55) lines.

Table 3.1A-B. Correlations between treated replications within each greenhouse experiment (n = 250).

A	GH2018.1	GH2018.2	GH2018.3
GH2018.1		$r^2 = 0.412$ $p<2.2\times10^{-16}$	$r^2 = 0.260$ $p<2.2\times10^{-16}$
GH2018.2	$r^2 = 0.412$ $p<2.2\times10^{-16}$		$r^2 = 0.295$ $p<2.2\times10^{-16}$
GH2018.3	$r^2 = 0.260$ $p<2.2\times10^{-16}$	$r^2= 0.295$ $p<2.2\times10^{-16}$	

B	GH2019.1	GH2019.2	GH2019.3
GH2019.1		$r^2 = 0.332$ $p<2.2\times10^{-16}$	$r^2 = 0.425$ $p<2.2\times10^{-16}$
GH2019.2	$r^2 = 0.332$ $p<2.2\times10^{-16}$		$r^2 = 0.314$ $p<2.2\times10^{-16}$
GH2019.3	$r^2 = 0.425$ $p<2.2\times10^{-16}$	$r^2 = 0.314$ $p<2.2\times10^{-16}$	

Table 3.2. Correlations of determination between the BLUEs of GH2018 and GH2019 when only 15%, 25%, and 35% tails were considered.

T_blue_log1	15%GH2018	25%GH2018	35%GH2018	GH2018
15%GH2019	$r^2 = 0.34$ $p=5.54\times10^{-8}$			
25%GH2019		$r^2 = 0.29$ $p=4.20\times10^{-11}$		
35%GH2019			$r^2 = 0.18$ $p= 7.48\times10^{-9}$	
GH2019				$r^2 = 0.08$ $p= 7.23\times10^{-6}$

Table 3.3 Putative significant QTL from GWAS for LMA.

Greenhouse								
QTL	Marker	Chr	Position	-log$_{10}$(p)	[1]Effect	MAF	[2]**Fav** allele	n
QLMA.wsu.3B	IWB63008	3B	801300	8.6	0.08	0.10	**A**/G	242
QLMA.wsu.2B	IWB25007	2B	1071500	9.3	-0.12	0.15	A/**G**	121
QLMA.wsu.5A	IWB25222	5A	517500	7.0	-0.08	0.36	T/**C**	121

Field								
QTL	Marker	Chr	Position	-log$_{10}$(p)	[1]Effect	MAF	[2]**Fav** allele	n
QLMA.wsu.3A	IWB11852	3A	207400	6.9	0.16	0.26	**A**/C	206
QLMA.wsu.7B	IWB25774	7B	1335900	9.8	0.16	0.25	**A**/G	206
QLMA.wsu.7D	IWB48862	7D	861900	9.9	0.17	0.24	**T**/C	206

[1.]The positive Effect values indicate elevated risk of LMA phenotype in N. America hard red spring wheat TCAP association panel

[2.]The major allele that reduce the susceptibility of LMA in N. America hard red spring wheat TCAP association panel

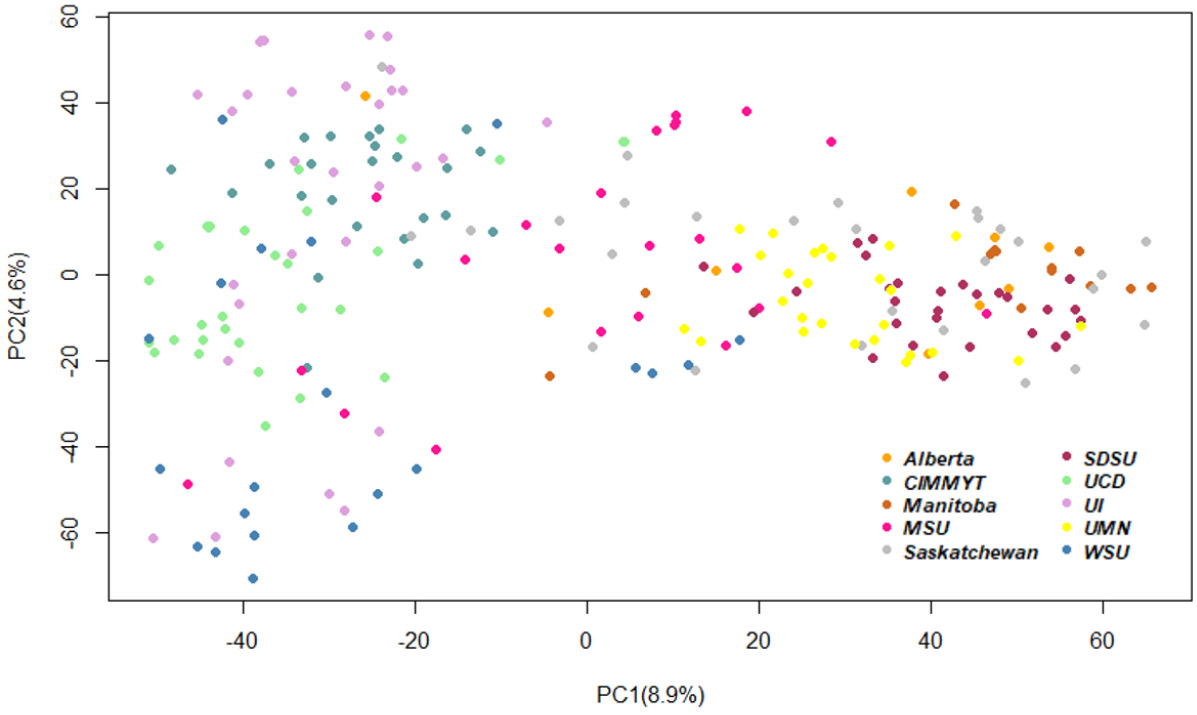


Figure 3.S1. The TCAP population structure. PC1 explains 8.9% genetic variation, and PC2 explains 4.6% genetic variation.

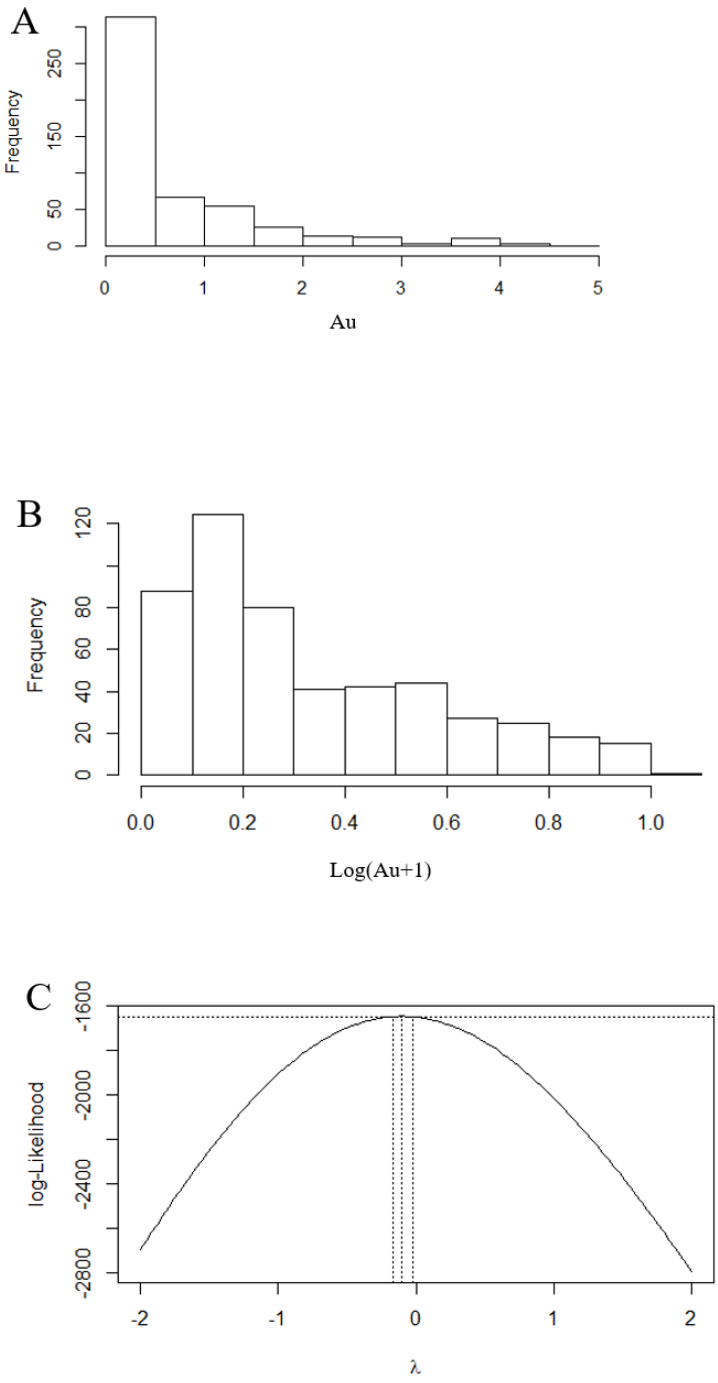
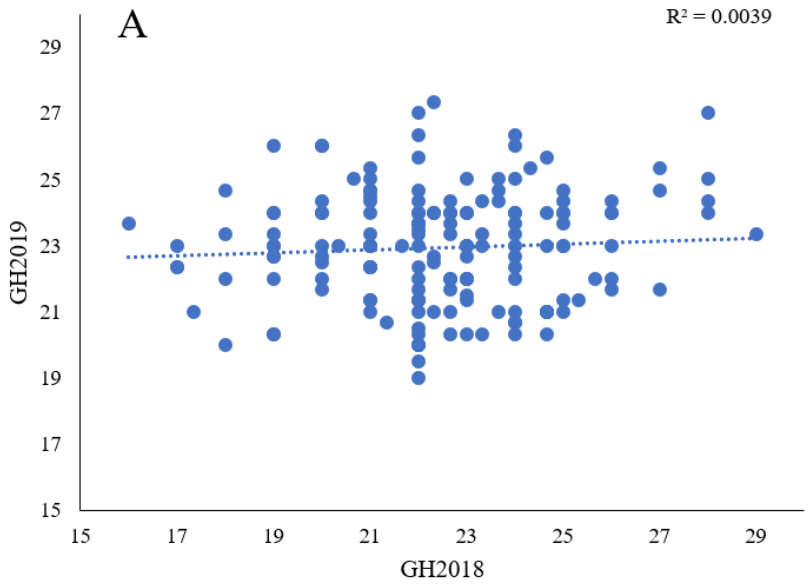


Figure 3.S2A-C. Obtaining a normal distribution. A. The untransformed dataset displays a non-normal, Poisson, distribution. B. After a $\log_{10}$ transformation, the distribution was corrected to a more normal distribution C. A lambda value near 0 in the Box-Cox power transformation estimation, indicated the most appropriate transformation was $\log_{10}$



B

Expt	Run	Lines	dpa range	Anthesis range
GH2018	1	1-125	19-28	Nov 16– Dec 4, 2017
GH2018	2	126-190	16-29	Aug 6-16, 2018
GH2018	3	191-256	NA	Oct, 2018
GH2019	4	1-125	18-27	Mar 9-25, 2019
GH2019	5	126-256	20-27	Apr 26 – May 9, 2019

Figure 3.S3A-B. Comparison of timing of anthesis and LMA treatment (dpa) time in GH2018 and GH2019. A) There is no correlation between LMA treatment time in days past anthesis in GH2018 and GH2019 experiments. B) Comparison of anthesis and LMA treatment (dpa range) times by experiment and run showed that the three runs used to test all lines in GH2018 covered a wider range of dates than the two runs in GH2019.

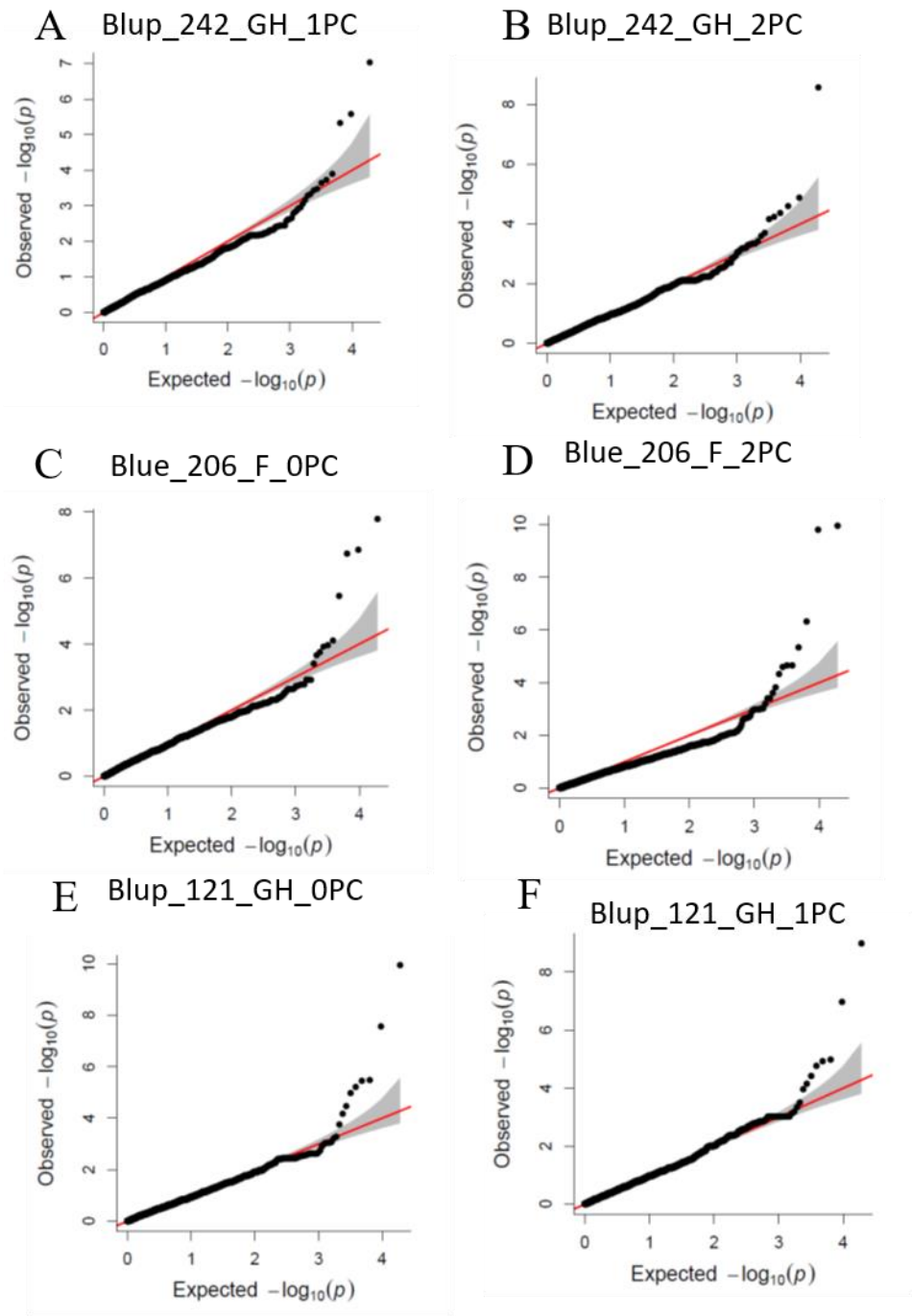


Figure 3.S4A-F. QQ-plots of each GWAS using the greenhouse (GH) and the field (F) data. A-B) The fitting of using two principal components (PC) on the BLUPs of 242 lines of the GH experiments was better than using one PC. C-D) The fitting of using zero PC on the BLUEs of 206 lines of the F experiment was better than using one PC. E-F) The fitting of using one PC on the BLUPs of 121 lines of the GH experiments was better than using zero PC.

Table 3.S1A-B. Correlations between untreated replications within each greenhouse experiment (n = 250)

A	GH2018.1	GH2018.2	GH2018.3
GH2018.1		$r^2 = 0.469$ $p<2.2\times10^{-16}$	$r^2 = 0.176$ $p<1.9\times10^{-11}$
GH2018.2	$r^2 = 0.469$ $p<2.2\times10^{-16}$		$r^2 = 0.358$ $p<2.2\times10^{-16}$
GH2018.3	$r^2 = 0.176$ $p<1.9\times10^{-11}$	$r^2 = 0.358$ $p<2.2\times10^{-16}$	

B	GH2019.1	GH2019.2	GH2019.3
GH2019.1		$r^2 = 0.634$ $p<2.2\times10^{-16}$	$r^2 = 0.504$ $p<2.2\times10^{-16}$
GH2019.2	$r^2 = 0.634$ $p<2.2\times10^{-16}$		$r^2 = 0.518$ $p<2.2\times10^{-16}$
GH2019.3	$r^2 = 0.504$ $p<2.2\times10^{-16}$	$r^2 = 0.518$ $p<2.2\times10^{-16}$	

Table 3.S2. LMA testing results of alpha-amylase activity A620 units (Au) in treated samples

	Genotype	Avg_GH20	Avg_GH20	Avg_F201	Log_GH20	Log_GH20	Log_F2018	Blup_GH	Blue_F201
1	H0800080	2.161308	1.509825	0.209581	0.499867	0.399643	0.082635	0.324796	0.190273
2	H0800103	0.428474	1.749775	0.140533	0.154872	0.439297	0.057108	0.126052	0.131188
3	H0800310	0.365342	1.130842	0.194976	0.135241	0.328551	0.077359	0.041191	0.176206
4	WA8148	0.772756	2.532476	1.293271	0.248649	0.548079	0.360455	0.257896	0.796978
5	H0900009	0.300462	0.257244	0.294094	0.114098	0.09942	0.111966	-0.12171	0.252231
6	H0900081	1.182872	0.417605	0.323471	0.339028	0.151555	0.121714	0.058629	0.277966
7	HOLLIS	2.636439	3.715125	0.315863	0.560676	0.673493	0.119211	0.542624	0.273079
8	HR07005-3	0.549279	0.470225	0.756676	0.19013	0.167384	0.244692	-0.02799	0.495133
9	HR07024-5	0.100741	0.148835	0.150607	0.041685	0.060258	0.060927	-0.19434	0.140189
10	HW080169	0.052419	0.248467	1.045062	0.022189	0.096377	0.310706	-0.18352	0.658091
11	HW09000	0.109699	NA	0.922792	0.045205	NA	0.283932	-0.09714	0.576673
12	HWO9007	0.148605	0.142555	0.534991	0.060171	0.057877	0.186106	-0.18385	0.421993
13	KELSE	0.291339	0.142223	1.984411	0.11104	0.057751	0.474859	-0.15082	0.853469
14	MACON	0.17974	0.309924	0.153734	0.071786	0.117246	0.062106	-0.13765	0.142198
15	OTIS	0.526567	2.078534	1.862112	0.183716	0.488344	0.456687	0.17675	0.865947
16	SCARLET	0.155217	0.921614	0.197668	0.062664	0.283666	0.078336	-0.03527	0.180325
17	SD4265	0.131528	0.459203	0.068026	0.053665	0.164116	0.028582	-0.11894	0.065806
18	TARA2002	0.073424	1.284754	0.152863	0.030771	0.358839	0.061778	-0.00709	0.142222
19	WA8016	0.113938	NA	0.308744	0.046861	NA	0.116855	-0.09564	0.26401
20	WA8034	1.19609	0.376286	0.210322	0.34165	0.138709	0.082901	0.051973	0.188406
21	WA8074	0.322676	1.850685	0.912177	0.121453	0.454949	0.281528	0.114488	0.612722
22	WA8099	0.270977	0.2037	0.179594	0.104138	0.080518	0.071733	-0.1405	0.164906
23	WA8100	0.102805	0.460971	0.529035	0.042499	0.164642	0.184417	-0.12586	0.381065
24	WA8123	0.750472	NA	0.269635	0.243155	NA	0.103679	0.082475	0.237661
25	WA8133	0.14607	0.308977	0.567604	0.059211	0.116932	0.195236	-0.14604	0.419117
26	9263	0.137635	0.150824	0.310209	0.056003	0.061009	0.117341	-0.18453	0.259032
27	9223	0.707567	1.118209	1.960257	0.232378	0.325969	0.471329	0.102735	0.871553
28	9225	0.469467	0.228762	1.220193	0.16716	0.089468	0.346391	-0.09365	0.728337
29	9228	1.98756	0.346677	0.223898	0.475317	0.129263	0.087745	0.132828	0.19779
30	9229	0.474827	0.381888	0.189915	0.168741	0.140473	0.075516	-0.05942	0.172242
31	9232	0.294416	1.253032	1.694426	0.112074	0.352767	0.430466	0.041873	0.842611
32	9233	1.877594	2.794179	1.673257	0.45903	0.579118	0.427041	0.415034	0.845602
33	9245	1.35079	0.362514	1.64184	0.371214	0.134341	0.421907	0.068373	0.817872

Table 3.S3. Mixed Linear Model examining sources of variation, including dpa as a fixed effect and genotype, run, and expt as random effects

Random Effects		
Groups	Variance	Std. Dev
Genotype	5.263×10^{-2}	0.229404
Run	2.008×10^{-2}	0.141710
Expt	2.268×10^{-5}	0.004762
Residual	8.133×10^{-2}	0.285178

Fixed Effects					
	Estimates	Std. Error	df	t	Pr (>|t|)
mean	0.580098	0.213471	136.273426	2.717	0.00743
dpa	-0.006257	0.008881	381.471685	-0.705	0.48152

Table 3.S4. REML output when including genotype as random effect and expt as fixed effect .

Random Effects		
Groups	Variance	Std. Dev
Genotype	0.04049	0.2012
Residual	0.11970	0.3460

Fixed Effects					
	Estimates	Std. Error	df	t	Pr (>|t|)
mean	0.46079	0.02501	473	18.42	2×10^{-16}
expt	-0.04195	0.03085	253	-1.36	0.175

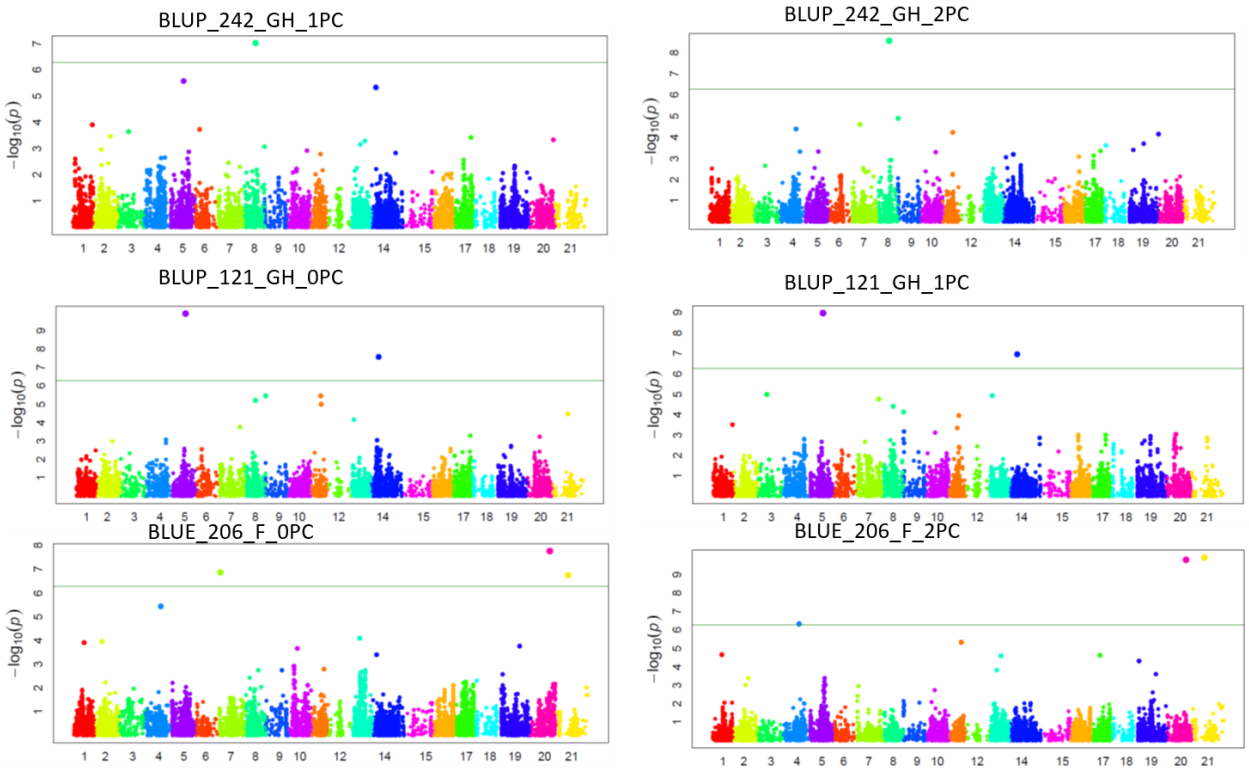
Table 3.S5. One-way ANOVA results (*p*-value) examining the association between the BLUPs of GH2018 and GH2019 and the known genes *Ppd-D1, rht-D1a rht-B1a, and Vrn-A1.*

	Treated	Untreated
rht-D1a rht-B1a	0.053	<0.0001
Ppd-D1	0.47	0.17
Vrn-A1	0.92	0.65

APPENDIX

Appendix 1. Significant QTL that are associated with elevated risk of alpha-amylase activity.

QTL	Marker	Method	PC	p.threshold	Chr	Position	*p*.value	-log(*p*)	Effect	Maf	Envir	**Fav** Allele
QLMA.wsu.2A	IWB55262	blue_206	2	0.05	2A	1151400	4.72E-07	6.3	-0.10122	0.408213	F	T/**C**
QLMA.wsu.3A	IWB11852	blue_206	0	0.05	3A	207400	1.40E-07	6.9	0.156778	0.258454	F	**A**/C
QLMA.wsu.7B	IWB25774	blue_206	0	0.05	7B	1335900	1.71E-08	7.8	0.141969	0.251208	F	**A**/G
QLMA.wsu.7B	IWB25774	blue_206	2	0.05	7B	1335900	1.56E-10	9.8	0.157672	0.251208	F	**A**/G
QLMA.wsu.7D	IWB48862	blue_206	0	0.05	7D	861900	1.85E-07	6.7	0.144909	0.23913	F	**T**/C
QLMA.wsu.7D	IWB48862	blue_206	2	0.05	7D	861900	1.16E-10	9.9	0.174259	0.23913	F	**T**/C
QLMA.wsu.2B	IWB25007	blups_120	0	0.1	2B	1071500	1.57E-08	7.8	-0.10138	0.14876	GH	A/**G**
QLMA.wsu.2B	IWB25007	blups_120	1	0.1	2B	1071500	5.43E-10	9.3	-0.11795	0.14876	GH	A/**G**
QLMA.wsu.3B	IWB63008	blups_242	1	0.05	3B	801300	9.58E-08	7.0	0.075184	0.103306	GH	**A**/G
QLMA.wsu.3B	IWB63008	blups_242	2	0.05	3B	801300	2.75E-09	8.6	0.081083	0.103306	GH	**A**/G
QLMA.wsu.5A	IWB25222	blups_120	0	0.1	5A	517500	2.34E-08	7.6	-0.07952	0.359504	GH	T/**C**
QLMA.wsu.5A	IWB25222	blups_120	1	0.1	5A	517500	1.04E-07	7.0	-0.07561	0.359504	GH	T/**C**



Appendix 2A-F. Manhattan plots in FarmCPU outputs. A-B) Significant locus was detected on chromosome 3B using BLUPs of 242 lines with one or two Principal Components (PC) in the greenhouse (GH) experiments. C-D) Significant loci were detected on chromosomes 2B and 5A using the BLUPs of 121 lines with zero or one PC in the GH experiments. E-F) Significant loci were detected using 206 lines on chromosomes 2A, 3A, 7B, and 7D using zero or two PC in the Field (F) experiment.