

Grant Code: AP2725

Title: Wheat alpha-amylase genes and their effect on falling numbers

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Justification/Rationale: Wheat is the second most important cereal crop in the United States. The annual wheat production in the US Pacific Northwest (PNW) is ca. 6.5 million metric tons, and the PNW wheat accounts for a large portion of the US wheat exported abroad.

The falling numbers (FN) method is a viscometric assay involving gelatinization of flour or meal in boiling water and subsequent liquifaction of starch by α -amylases. The FN test is an international measure for grain receipt and trade. Preharvest sprouting (PHS) and late maturity α -amylase (LMA) causes unacceptably high levels of α -amylase or low falling numbers (LFN) in ripe wheat grains (Mares & Mrva, 2014). High levels of α -amylase activity in wheat grains compromise the end-use quality and causes substantially lower prices for wheat growers (Mares & Mrva, 2014).

In 2016, the PNW wheat was seriously affected by LFN, which is basically caused by LMA (Steber, 2017b, Steber, 2017a). Under the LFN challenge, the Idaho Wheat Commission played a pivotal role in organizing two national meetings, the wheat falling number summit and the late maturity alpha-amylase wheat forum, in 2017. We now understand that the LMA-based LFN remains to be a potential threat to the PNW wheat. It is just a matter of when and where it is going to happen again. It is urgent to understand what causes LMA and how to prevent LMA.

α -Amylases (EC 3.2.1.1) hydrolyze α -1,4 internal glucoside linkages in starch and related carbohydrates. Cereal crops have multiple α -amylase isoform genes (Sugimura *et al.*, 2015, Bak-Jensen *et al.*, 2007, Zhang & Li, 2017). Some α -amylase genes may also generate different isoforms by alternate splicing or variable transcription start sites. The alpha-amylase genes are divided into four families (*Amy1* - *Amy4*) each specialized in cellular localization and gene structure (Stanley *et al.*, 2002, Barrero *et al.*, 2013, Zhang & Li, 2017). They are also different in the isoelectric points (pI), classified as the low pI α -amylases (*Amy2* and *Amy4*) and high pI α -amylases (*Amy1* and *Amy3*) (Daussant & Renard, 1987, Barrero *et al.*, 2013, Cheng *et al.*, 2014). In wheat, LMA is caused by a transitory peak expression of high pI α -amylases during grain development (Barrero *et al.*, 2013). All α -amylases have a conserved active site at the C terminus of the protein, but they might have different substrate specificity and they may target different compartments within the cell. Different α -amylase genes have specific expression in germinating seeds, developing caryopses, and/or leaf sheaths (Sugimura *et al.*, 2015, Karrer *et al.*, 1991).

Over the past 30 years, wheat α -amylases have been intensively studied. Due to the lack of the whole genome sequence of wheat, the majority of previous studies inspected only a small subset of the entire α -amylase family in wheat. Recently, several wheat whole genome

databases are publically accessible, which allows us to study the α -amylase genes in a broad sense. However, whether and how the high pI α -amylases (Amy1 and Amy3) affect the wheat falling numbers needs to be confirmed.

Hypothesis & Objectives: LMA is caused by an unusual increase of high pI α -amylases during the grain development (Barrero et al., 2013). An effective approach is to measure the high pI α -amylase protein content in developing grains using the sandwich ELISA (Verity *et al.*, 1999). However, the current ELISA approach only discriminate low or high pI α -amylases, not between different types of high pI α -amylases. How different types of high pI α -amylases affect wheat FN needs to be answered.

We hypothesize: 1) the high pI α -amylases, Amy1 and Amy3, both affect wheat falling numbers, and 2) mutation of the grain-specific Amy3 may affect the wheat falling numbers. Our objectives are to: 1) confirm selected *Amy3* mutant lines with expected mutations, 2) increase seeds of identified *Amy3* mutant lines, 3) perform LMA induction and wheat falling number test to understand how *Amy3* genes affect wheat falling numbers.

Procedures/Plan of Work:

1. Confirm selected *Amy3* mutations

In durum wheat, mutation of three *Amy3* genes, *Amy-A3.1*, *Amy-A3.2*, and *Amy-B3.1*, will be selected for further study. For each gene, at least two independent mutations will be selected to test their effect. Before pursuing any further study, homozygous mutation will be confirmed by TILLING or sequencing. Those with expected homozygous mutations will be propagated in field to increase seeds. In common wheat, mutation of six *Amy3* genes, *Amy-A3.1*, *Amy-A3.2*, *Amy-B3.1*, *Amy-B3.2*, *Amy-D3.1*, and *Amy-D3.2*, will be selected. Again, two or more independent mutations in each gene will be selected to test their effect. By self- or cross-pollination, we can generate homozygous mutants for each target gene on A, B, and/or D genomes. Only homozygous mutation lines will be propagated in greenhouse to increase seeds.

2. Increase seeds of selected *Amy3* mutants

To perform wheat falling number test, adequate flour samples are needed. For that purpose, it is necessary to produce abundant mutant seeds or flours. For durum wheat, we will increase seeds in field. To avoid pollen grains from unwanted genotypes, we will separate each mutant plot with a 2-m bare margin. For common wheat, we will increase them in greenhouse, each spike will be bagged to avoid cross-pollination from unwanted genotypes. Same mutation lines will be mixed to generate at least 500 g kernel or flour for replicated falling number tests.

3. Evaluate the role of *Amy3* on wheat falling numbers

Two types of experiments will be conducted to test *Amy3* effect on falling-number traits. First, using the LMA induction procedures established in Dr. Jianli Chen's lab, we will test both wild-type genotypes (Kronos and CB037) and multiple sets of homozygous mutant lines for their response to LMA induction, which will be done under controlled conditions. Mature grains of wild-type (Kronos and CB037) and multiple sets of homozygous mutant lines will be processed for falling number test. By comparing specific genotypes and the corresponding FN values, we will conclude whether the *Amy3* genes contribute the LMA and LFN traits. Useful data gained in this study will be applied to direct wheat breeding programs.

Duration: One year (FY 2020), Objectives 1-3

Cooperation/Collaboration: Fu will lead the project and work with visiting scholars and/or graduate students on proposed research. Fu will present research findings at professional conferences, and communicate research progress and results to the Idaho Wheat Commission. Visiting scholars, graduate students and other personnel working on this project will report progress to Fu. The research team will regularly communicate via e-mail, phone, and face-to-face meetings. The LMA and FN tests will be done in collaboration with Dr. Jianli Chen located in the Aberdeen Research and Extension Center, the University of Idaho.

Anticipated Benefits, Expected Outcomes and Impacts and Transfer of Information:

- 1) Provide wheat research community with information on the relationship between specific α -amylase genes and wheat falling numbers.
- 2) Provide wheat growers with information on planting wheat cultivars that have less α -amylase activity during late maturity and help growers earn more profit on high FN grains.
- 3) Provide wheat breeders with information about which α -amylase may affect FN and help breeders develop cultivars with good end-use quality.
- 4) Provide the wheat industry with strategic information on how to solve low FN in wheat production.

Literature Review: Preharvest sprouting causes the production and activation of α -amylase inside the wheat kernel, which causes the low falling numbers (LFN) problem in common wheat. However, late maturity α -amylase (LMA) in wheat can cause LFN instead of preharvest sprouting (Mares & Mrva, 2008). Wheat LMA involves the untimely synthesis of high pI α -amylase during the middle to later stages of grain development and ripening (Mares & Mrva, 2008). LMA-prone wheat tends to produce high levels of α -amylase when exposed to cool temperatures during grain development (Mares & Mrva, 2008). It has been suggested that LMA is caused by the activation of the *α -Amy1* genes on wheat chromosomes 6A, 6B, and 6D (Barrero et al., 2013), but overexpression of *Amy3* resulted in LFN similar to the those seen in LMA- or PHS-affected grains (Ral et al., 2016). It will be interesting to look at mutants of the *Amy3* genes in the homeologous group 5 in wheat.

Common wheat is complex because of its hexaploid nature. Most genes have three homeologous copies distributed in the A, B, and D genomes. To simplify wheat research, people develop valuable resources on the diploid and tetraploid wheat. Kronos is a tetraploid wheat with A and B genomes. Recently, Dubcovsky et al. generated a Kronos collection with 4.15 million unique mutations (Krasileva et al., 2017). The Kronos collection and its sequence data enable us to rapidly identify a target gene mutation in the A or B genome. For genes absent in the current Kronos sequence database, Targeting Induced Local Lesions in Genomes (TILLING) will be used to recover mutations of a hiding target gene.

Genome editing allows specific modification of target genes in mammalian and other eukaryotic organisms (Cheng & Alper, 2014). The transcription activator-like effector nuclease (TALEN) and the clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas9 are proved to be functional in wheat (Wang et al., 2014). Recently, a number of feasible and environmentally friendly CRISPR/Cas9 approaches have been used to edit wheat genes (Zhang

et al., 2016, Liang *et al.*, 2017). The target-specific genome editing is practical to generate valued-added agricultural products.

FY2020

IDAHO WHEAT COMMISSION - BUDGET FORM

Principal Investigator: Daolin Fu

Allocated by	Idaho Wheat Commission	during FY 2018	\$	50,000
Allocated by	Idaho Wheat Commission	during FY 2019	\$	50,000

REQUESTED FY2020 SUPPORT:

Budget Categories	(10) Salaries (staff, post-docs, etc.)	(12) Temp Help	(11) Fringe	(20) Travel	(30) OE	(70) Graduate Tuition/ Fees	TOTALS
Idaho Wheat Commission	\$ -	\$ 32,000	\$ 2,784	\$ 3,000	\$ 12,000	\$ -	\$ 49,784

TOTAL BUDGET REQUEST FOR FY 2020: \$ 49,784

BREAKDOWN FOR MULTIPLE SUB-BUDGETS:

Budget Categories	(Insert PI Name)	(Insert CO-PI Name)	(Insert CO-PI Name)	(Insert CO-PI Name)
(10) Salaries	\$ -	\$ -	\$ -	\$ -
(12) Temp Help	\$ -	\$ -	\$ -	\$ -
(11) Fringe Benefits	\$ -	\$ -	\$ -	\$ -
(20) Travel	\$ -	\$ -	\$ -	\$ -
(30) Other Expenses	\$ -	\$ -	\$ -	\$ -
(70) Graduate Student Tuition/F	\$ -	\$ -	\$ -	\$ -
TOTALS	\$ -	\$ -	\$ -	\$ -

Total Sub-budgets \$ -

Explanatory Comments:

Fall 2018 Version

Annual Report

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Title: Wheat alpha-amylases and their effect on falling numbers

Personnel: Mengmeng Lin, Bo Lyu, Daolin Fu, Jianli Chen

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Accomplishments:

***Amy3* mutants of durum wheat:** Durum wheat 'Kronos' is tetraploid, representing a work model for hexaploid wheat. We initially searched *Amy3* mutations in the Kronos database (http://dubcovskylab.ucdavis.edu/wheat_blast). For *Amy-B3.1*, we identified 42 mutations, including 19 missense or truncation mutations. Seventeen mutant lines were planted in greenhouse and further validated using Targeting Induced Local Lesions in Genomes (TILLING). Four mutant lines with expected mutations were identified (Table1).

In durum wheat, *Amy-A3.1*, *Amy-A3.2*, and *Amy-B3.2* were not documented in the Kronos database. We then customized a TILLING protocol to identify their mutations in the Kronos mutant population. Leaf tissues of four plants in a row were mixed to make a DNA row pool, and leaf tissues of four plants in a column were mixed to make a DNA column pool. Eight DNA pools represented 16 mutant lines in a 4×4 design. In total, we prepared 360 DNA row pools and 360 DNA column pools. By screening all DNA pools, we identified 15 mutant lines for *Amy-A3.1* and four mutant lines for *Amy-A3.2* (Table1). Because PCR primers didn't work on *Amy-B3.2*, no mutants were identified for this gene.

Mutant lines will be used for future research. We will use them to generate homozygous single or double mutation lines. Their effect on falling numbers will be studied.

Table 1. Kronos mutants of the *Amy3* gene

Gene	Mutant Lines	Base change	Codon change	Residue change	Heterozygous or Homozygous	Consequence
<i>TaAmy-A3.1</i>	Kronos331	G/A	Gac/Aac	D113N	Hom	missense_variant
	Kronos1090	G/A	Gtg/Atg	V255M	Hom	missense_variant
	Kronos3771	C/T	cCc/cTc	P224L	Hom	missense_variant
	Kronos4387	G/A	cGt/cAt	R163H	Het	missense_variant
	Kronos4387	G/A	Gtg/Atg	V364M	Hom	missense_variant
	Kronos4429	C/T	Ccg/Tcg	P180S	Hom	missense_variant
	Kronos3964	A/T	Aag/Tag	K194*	Hom	stop_gained
	Kronos1159	T/C	ttT/ttC	-	Hom	synonymous_variant
	Kronos3863	C/T	caC/caT	-	Hom	synonymous_variant
	Kronos3236	C/T	gaC/gaT	-	Hom	synonymous_variant
	Kronos244	C/T	caC/caT	-	Hom	synonymous_variant
	Kronos2824	G/A	ggG/ggA	-	Hom	synonymous_variant

	Kronos4273	G/A	aaG/aaA	-	Hom	synonymous_variant
	Kronos4387	G/C	ggG/ggC	-	Hom	synonymous_variant
	Kronos4434	G/A	ggG/ggA	-	Het	synonymous_variant
<i>TaAmy-B3.1</i>	Kronos1323	C/T	Gag/Aag	E185K	Het	missense_variant
	Kronos2314	G/A	Ccg/Tcg	P146S	Het	missense_variant
	Kronos2364	G/A	Ctc/Ttc	L141F	Het	missense_variant
	Kronos2894	G/A	Cgc/Tgc	R181C	Het	missense_variant
<i>TaAmy-A3.2</i>	Kronos2452	C/T	Ctc/Ttc	L259F	Hom	missense_variant
	Kronos2299	G/A	agG/agA	R161S	Hom	missense_variant
	Kronos2184	C/T	taC/taT	-	Hom	synonymous_variant
	Kronos2242	C/T	ggC/ggT	-	Het	synonymous_variant

Gene editing of wheat *Amy3* genes: Clustered Regularly-interspaced Short Palindromic Repeats (CRISPR) is a powerful tool in gene editing. We pursued CRISPR-based gene editing to mutate *Amy3* genes. To knock out all orthologues in A, B, and D genomes, we targeted common regions in three orthologous genes to design specific primers (Table 2).

Table 2. CRISPR targets of *Amy3* genes

Target Genes	Target sites	Target sequence	Primer ID	Oligonucleotide sequence (5'→3')
<i>TaAmy3.1</i>	C1	GCTACTCGGCCGACGTCGCC AGG	U6a-C1-F	gccGCTACTCGGCCGACGTCGCC
			U6a -C1-R	aaacGGCGACGTCGGCCGAGTAG
	C2	CAAGGGCATCCTGCAGTCGG GGG	U3-C2-F	ggcaCAAGGGCATCCTGCAGTCGG
			U3-C2-R	aaacCCGACTGCAGGATGCCCTTG
	C3	ATCCTGCAGTCGGCGGTGCA GGG	U3-C3-F	ggcATCCTGCAGTCGGCGGTGCA
			U3-C3-R	aaacTGCACCGCCGACTGCAGGA
<i>TaAmy3.2</i>	C4	TCTCGCCTCCAGCTTAGCAC AGG	U3-C4-F	ggcaTCTCGCCTCCAGCTTAGCAC
			U3-C4-R	aaacGTGCTAAGCTGGAGGCGAGA
	C5	ACCGACAGCTGCTCGGAAC TGG	U6a-C5-F	gccgACCGACAGCTGCTCGGAAC
			U6a -C5-R	aaacGTTCGCGAGCAGCTGTCCGT
	C6	GGG GCCACGGCGTTTGACTCCC	U6a-C6-F	gccGGGAAGTCAAACGCCGTGGC
			U6a -C6-R	aaacGCCACGGCGTTTGACTTCC

We prepared five CRISPR/Cas9 constructs (each with two target sites) and transformed them into wheat. In total, 657 transgenic wheat plants were obtained with resistance to glufosinate ammonium, which was conferred by *Bar* in the CRISPR/Cas9 construct (Table 3). We are performing high-throughput sequencing to identify DNA variations in transgenic plants. In one pilot experiment, 36% of transgenic plants had mutations in *Amy3* genes. Again, *Amy3* edited mutants will be used to test their effect on wheat falling numbers.

Table 3. Wheat transformation with CRISPR/Cas9 constructs

Target Genes	Constructs	Target sites	Receipt genotypes	No. of calli treated	Herbicide-resistant transgenic plants
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<i>Amy3.1</i>	A1	C1+C3	Kronos	1,260	129
	A2	C1+C2	Kronos	1,080	30
			CB037	2,100	68
<i>Amy3.2</i>	A3	C4+C6	Kronos	1,050	1
			CB037	1,560	41
	A4	C4+C5	Kronos	1,350	117
<i>Amy3.1</i>	A5	C2+C6	Kronos	1,470	167
<i>Amy3.2</i>			CB037	1,470	104
Sum				10,290	657

Projections:

In the past two years, we identified many wheat *Amy3* mutants by TILLING and genome editing in durum and/or hexaploid wheat. These lines will be used to evaluate whether and how the *Amy3* genes affect wheat falling numbers. Mutants and data generated will be used in our next year project, for which we will submit a renewal proposal to the Idaho Wheat Commission.

Publications: No