**Genetic Characterization of Germination Traits and Their Relationship to Preharvest Sprouting in Winter and Spring Barley**

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**Executive summary**

This grant supported a single-year project to better understand the genetic control of preharvest sprouting (PHS), seed dormancy in a winter/facultative double haploid population and the relationship of malting quality on a subset of the winter malting barley population. In the previous cycle, this project produced genome-wide Illumina 50K marker data on a population of Cornell winter and facultative breeding lines and characterized these same populations for PHS, germination rate, and germination energy from physiological maturity to 6 months post-harvest. For the 2021-2022 cycle, we have continued our work on measuring germination and PHS of our winter/facultative barley population. This award also provided funding for us to travel to the USDA CCRU to micro malt and analyze a subset of our winter malting barley population. The data collected in this study (susceptibility to PHS, germination rate, germination energy, and micro malting quality analysis) has increased understanding of winter malting barley seed dormancy and dormancy persistence and will allow for the selection of PHS resistant lines, with quick dormancy break, rapid germination rate gain and understand the relationship to malting quality characteristics.

**Detailed Report on Objectives, Methodology and Results – AMBA Funded Project**

**Section 1: Winter Barley**

Objective 1:  Continuation of genetic characterization of a winter barley double haploid population for dormancy, PHS, and malting quality using 50K Illumina sequencing marker data and KASP markers

The winter barley germplasm was developed by crosses to the common parent DH130910 (now cv. Lightning) to parents Flavia, KWS Scala, SY Tepee, and Wintmalt. At the time of crossing in fall 2017, parents KWS Scala, SY Tepee and Flavia were prominent winter barley lines that had good yield, moderate disease resistance, and high malting quality in Cornell regional trials. As of 2021, KWS Scala continues to be the most widely grown winter barley in New York state. Wintmalt was chosen as a superior malt quality donor. The common parent DH130910 was developed by Oregon State University (OSU) and was chosen for its high yield, moderate disease resistance, and facultative growth habit. F1 seed from each cross was sent to OSU for double haploidization and DH plants were returned to Ithaca in summer 2018. Two locations of headrows were planted in the 2019-2020 growing season. Checks for the 2020 populations included the parental lines for the population and Charles, a PHS and disease susceptible line in New York. Seed from 2019-2020 was used for yield plots at two locations for the 2020-2021 growing season and additional seed was sent to OSU for further collaborative projects. Two preliminary yield trials were planted for the 2021-2022 growing season. Each location consisted of 435 unique DH lines replicated once at each location. Checks for both locations included the common parent Lightning(DH130910), KWS Scala and Endeavor. Endeavor was selected over Charles due to its favorability as an AMBA recommended variety but also due to its susceptibility to PHS and foliar disease in the New York growing environment.

Materials and methods

PHS was measured by harvesting 5 spikes per headrow at physiological maturity (PM), after-ripening for 3 days, and then misting in a greenhouse for 3 days, after which the spikes were assessed for PHS on a 0 to 9 scale. Due to labor constraints brought on by the pandemic in the 2020 field season, phenotyping capacity was limited and germination was prioritized for that year. PHS was phenotyped on a sub-sample of 100 lines from one location. The sub-sample consisted of all facultative types across all four families, parental checks, and Charles. For 2021, both preliminary yield trials(480 plots at two locations) were measured for PHS.

For germination measurements in 2020, twelve to fifteen additional spikes were sampled from headrows of 444 lines in two locations two days after 50% of the headrow reached PM for use in germination tests. Some lines were omitted due to poor winter survival and low seed quality. In 2021, fifteen to twenty spikes were sampled from one complete location (480 plots) and approximately 25% at the second location (120 plots). Furthermore, a subset of 240 plots were sampled for an additional 35-40 spikes for malt quality. Of the 240 plots, 120 were selected with shared lines between both locations (60 lines at both locations) and the remaining 120 plots sampled were split evenly based on seed availability after we appropriated amounts for our germination tests.

Spikes were dried for 48 hours before being hand threshed and frozen at -20 C. Grain was removed from the freezer 24 hours prior to starting the assays and stored at ambient lab temperature and humidity for the duration of the experiment. Germination energy (GE) and germination index or rate (GI) were measured at five time points for 2020: 5 (TP1), 19 (TP2), 47 (TP3), 96 (TP4), and 152 (TP5) days post PM. Germination Energy(GE) and germination index or rate (GI) were measured at eight time points for 2021: 5(TP1), 12(TP1.5), 19 (TP2), 33(TP2.5), 47(TP3), 68 (TP3.5), 96 (TP4) and 152(TP5) days post PM. Germination tests followed the American Society of Brewing Chemists (ASBC) method for GE with two modifications. The first modification was the use of 30 kernels instead of 100 kernels. The second modification was an extended germination count from 3 days to 5 days in lieu of counting for 3 days and using H2O2 to break dormancy. In brief, GE was calculated as the percentage germinated kernels after three days and for five days. GI was calculated as:

*3 Day GI =10 (N24+N48 +N72)/ (N24+2N48 +3N72)*

*5 Day GI =10 (N24+N48 +N72 +N96+N120)/ (N24+2N48 +3N72+4N96 +5N120)*

where N24, N48, N72, N96 and N120 were the number of germinated kernels at 24, 48, 72, 96 and 120 hours after the start of the assay. For analysis GI was scaled by GE as GIscale = GI\*GE to account for low germination at TP1 and TP2.  For TP1 in 2020, a subset of the lines (223) was phenotyped compared to the remaining timepoints (444). The TP1 subsample included for the remaining lines. For sampling in 2022, we only sampled one complete location (480 plots) and 120 plots at the second location. For the second location, 60 of the 120 lines were sampled based on a range of their respective GE and GI rates measured from the previous field season. The remaining 60 samples consisted of sampling check lines and available seed.

In addition to measuring germination, a 120 line subset previously mentioned were sent to the USDA Cereal Crops and research unit(CCRU) in Madison, WI. The small grain samples were frozen upon arrival. Each line was malted in single replicates at two different time points 67 days post PM and 152 post PM, a modification from our original plan of 47/111 days post PM due to substantial observed dormancy at 47 days post PM. Due to limits on how many tea ball samples could be malted at one time, the samples were malted in batches every week for two months. Malting procedure and analysis were performed in the methods described by Schmitt & Budde 2011. We are still waiting on the last batch to be analyzed and aim to write a supplemental report once the complete malting data for the winter barley becomes available. Preliminary results indicate that there was significant dormancy for some lines when the grain was malted at 68 days post PM, given the high beta glucan levels.

We previously reported development of a high-throughput Kompetitive Allele Specific Primer (KASP) marker AlaAT1\_L214F for the causal mutation in *HvAlaAT1* discovered by Sato et al. (2016). We also developed KASP assays for a SNP in the 5’ UTR of *HvGA20ox1* (GA20ox1\_331\_5UTR), the E165Q mutation in *HvMKK3* identified in Vetch et al. (2020) for both spring barley and winter barley. A summary of the winter barley haplotypes for the parent lines and checks are found in QTL x environment modeling of malting barley preharvest sprouting (Sweeney et al. 2021). All experimental lines and parent genotypes were monomorphic for dormant HvMKK3. The check lines Charles and Endeavor used as a phs susceptibility check in 2020 and 2021 respectively, have the highly non-dormant (N\*) allele for MKK3 and the dormant AlaAT(D) allele, however we cannot make specific conclusions about this haplotype given the low representation in the winter barley population.

All lines were genotyped with the 50Fk Illumina Infinium iSelect SNP array at the USDA Small Grains Genotyping Lab in Fargo, ND. After filtering poor quality markers, minor allele frequency (MAF) below 0.05, and monomorphic sites, 15,467 polymorphic markers remained and were used for genome-wide association (GWA). After conducting linkage disequilibrium (LD) pruning to reduce high LD blocks that exist in double haploid populations, we retained 8,384 markers for analysis. The *GAPIT* R package (Tang et al., 2016) was used for GWA. Multi-locus mixed models (MLMM) were fit for germination traits at each time point and for PHS. MLMM models have fewer false positive associations and use forward and backward step-wise regression compared to the standard mixed linear model.  P-values below the Bonferroni cutoff (p=0.05) of 3.57x10-6 were considered significant. A summary of the GWA results is presented in Table 1. Models were run for all trait/time point combinations, including the 5-day extensions. In addition to single timepoint GWAS, we used time series analysis to develop models that represent dormancy breakage over time. Details of the methods can be found in Rooney. et. al 2022. Briefly, each line was modelled in a logistic curve representing the GE and rate over time, both with years and combined years. We derived components of the logistic curve, such as time to 95% germination or time to 5.0 GI, to determine what novel loci, if any, can be detected if we use the timepoint data as a complete model compared to conducting GWA on each timepoint independently.

Results

Pre-harvest sprouting

For the 2020 year, we only did a subsample of phs scoring. While the 2020 data did follow the trend explained for the 2021 results, we did not have enough observations to determine whether all experimental lines in the 2020 year were resistant or susceptible to phs in the 2020 environment mainly due to low variation and sample size. In our first complete year observation of the 435 unique lines in our trials, we found that 95% of our experimental lines were pre-harvest sprouting resistant (0-2 score) across two locations. Approximately 4.4 % were somewhat resistant (2-4) and only 1.6 % of our lines were PHS susceptible (above 4). The low mean of phs for most of the lines is encouraging, however phs needs to be tested in at least more than one year to account for different environmental effects. This is particularly important given the significant increased dormancy we observed for the 2021 year. Given the low variation of phs scores, correlations were low to most of the GE and GI timepoints. PHS was only moderately correlated with the first timepoint (PM 5) for GE(0.633) and GI(0.683). Even with a week of after ripening, correlation with phs scores dropped significantly at time point 1.5(12 days post PM) for GE(0.367) and GI(0.415). This suggests that for our winter barley population, there is potential to select for increased dormancy break while maintaining PHS resistance. Broad sense heritability for all germination traits, timepoints, years and combinations were very high(0.9). Heritability dropped slight for GE at later time points due to reduced variation but still retained heritability values a minimum~0.75.

Genome wide association results

For single timepoint GWAS, we found a total of 37 significant marker trait associations associated with GE and GI across all timepoints and years. One marker per LD group of the significant single time point GWA was selected in table 1 to prevent redundancy. Significant marker trait associations from the logistic models were also included. The most significant association was the KASP marker for AlaAT\_L214F(Qsd1) and the closely associated 50K JHI-Hv50k-2016-276836(r=0.91) marker. Other potential hits detected in the study include Isoamylase (*HvISA3*,HORVU.MOREX.r2.5HG0404420) which is a starch-debranching enzyme located at 475796690-475807295 bp, 705025 bp distal to marker JHI-Hv50k-2016-311435. Isoamylases hydrolyze α-(1,6) glycosidic linkages and debranch amylopectin during grain filling (Gous and Fox, 2017). Shu and Rasmussen (2014) identified a MTA for amylose content highly associated with the contig MLOC\_10776, which includes *HvISA3* (https://ics.hutton.ac.uk/morexGenes/). Starch with higher amylose content is hydrolyzed more slowly by amylolytic enzymes and higher amylose content has been hypothesized to be a contributing factor to grain dormancy (Chu et al., 2014). Although the role of amylose content in seed dormancy has been hypothesized, prior evidence for the role of *HvISA3* is limited. This locus may be useful for PHS resistance but a consistent decrease in GI is not desirable. Negative impacts on starch related malting quality traits like malt extract may also be present.  Another gene of interest in this region is an abscisic acid responsive protein (Liu et al. 2013). Abscisic acid (ABA) is an important regulator of seed dormancy in barley: increases in ABA maintain seed dormancy and decreases in ABA reduce seed dormancy in barley (Gómez-Cadenas et. al 1999). Other potential novel loci include HvVP1 (Viviparous-1) which may be associated with JHI-Hv50k-2016-165725 on chromosome 3H and segregating in the SY Tepee family. HvVP1 is a master transcription factor regulator that controls switching between seed maturation and germination in barley (Abraham et al. 2016).

Role of alanine amino transferase

Alanine amino transferase played a significant role in dormancy break in our winter malting barley population. Figures 3a-c show germination rate and energy for each family of the population grouped by haplotype for AlaAT over years 2020, 2021, and combined analysis 2020/2021. A check “family” was included for comparison to Charles for 2020 and Endeavor for 2021. Parents Scala, SY Tepee and Wintmalt contained the non-dormant AlaAT allele(N) and the common parent DH130910 and Flavia contained the dormant allele(D). In both years, GE showed clear differentiation over time based on AlaAT haplotype, particularly at time point 2(19 days post PM) for 2020 and timepoints 1.5-3.5(19 days post PM through 68 days post PM). GI showed clear differentiation both across timepoints and within timepoints based on the AlaAT haplotype. The differences in GE and GI for both years based on each haplotype demonstrate that AlaAT significantly affects germination rate, energy and subsequently the rate of dormancy breakage. We also observed significant differences between GE and GI depending on the year. In 2020, dormancy break occurred around time point 3(47 days post PM). In 2021, dormancy break was substantially higher and did not occur until timepoints 3.5(68 days post PM) and 4(96 days post PM). The significant effect of the year environment on dormancy demonstrates the need to test seed germination traits across multiple years.

The observed dormancy for most non dormant haplotypes at earlier post physiological maturity (PM) days is encouraging for balancing the selection of high germination rate and maintaining initial seed dormancy for PHS. If we assume that a malster, at the earliest, would start malting barley approximately 47 days after harvest from the field, we will want to select lines that maintain dormancy until 50-60 days post PM. Most non-dormant AlaAT haplotypes fit this profile for the 2020 and 2021 crop year. However, given the substantial dormancy we observed in 2021, we must be careful in considering how it relates to our PHS values observed for that same year. PHS will need to be tested in multiple years where conditions would develop less dormant barley. If we continue to have low PHS mean and variation across multiple years with potentially differing levels of dormancy per year, we can then focus the selection pressure to increase germination rate with less concern of reducing our PHS resistance for the given AlaAT haplotypes.

Table 1: Summary of number of plots sampled from for each time point. The corresponding days post physiological maturity (PM) are also included for reference.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **TP1** | **TP1.5** | **TP2** | **TP2.5** | **TP3** | **TP3.5** | **TP4** | **TP5** |
| Days post maturity (PM) | **5** | **12** | **19** | **33** | **47** | **68** | **96** | **152** |
| 2020 | 441 | x | 894 | x | 894 | x | 894 | 894 |
| 2021 | 872 | 872 | 872 | 872 | 872 | 872 | 868 | 872 |

Figure 1: Visualization of the per single time point genome wide association of germination energy (GE), germination index (GI) across timepoints (TP) and years.

Chart

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Table 1: Marker-trait association summary for germination trait genome-wide association for both single time point and logistic time analysis. The “Genes of interest” column indicates nearby (+/- 1000000 base pairs) genes that have been implicated in seed germination or dormancy in the literature. Empty cells indicate no previously reported genes in the marker region

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Trait\*** | **Marker** | **Chr** | **Position** | **MAF\*\*** | **p values\*\*\*** | **Genes of interest** |
| 2021\_TP3\_GI, Logistic GE 2021\_Lower | JHI-Hv50k-2016-8002 | 1 | 6931684 | 0.36721 | 1.02E-06 |  |
| Logistic GE, 2020/2021\_Centering | JHI-Hv50k-2016-15198 | 1 | 18049005 | 0.49654 | 0 |  |
| 2021\_TP1\_GE | JHI-Hv50k-2016-59425 | 2 | 1081341 | 0.49885 | 8.16E-09 |  |
| Logistic GI 2021\_Lower | SCRI\_RS\_204158 | 2 | 12096571 | 0.49726 | 3.56E-14 |  |
| Logistic GE 2020\_Rate | JHI-Hv50k-2016-77653 | 2 | 40257373 | 0.23148 | 1.15E-10 |  |
| Logistic GE 2020\_Rate | JHI-Hv50k-2016-77964 | 2 | 41814315 | 0.22917 | 2.24E-09 |  |
| Logistic GE 2021\_Lower | JHI-Hv50k-2016-77945 | 2 | 41820733 | 0.4988 | 2.06E-09 |  |
| 2021\_TP1\_GI, Logistic GI 2021\_Lower | JHI-Hv50k-2016-165725 | 3 | 66720210 | 0.5 | 5.73E-11 | HvVP1 |
| 2021\_TP3\_GI | JHI-Hv50k-2016-228126 | 4 | 5948962 | 0.47344 | 1.31E-06 |  |
| Logistic GI 2020/2021\_TimeTo5.0 | JHI-Hv50k-2016-228195 | 4 | 6032545 | 0.49043 | 2.06E-06 |  |
| 2020\_TP1\_GE, Logistic GI 2020\_Lower | JHI-Hv50k-2016-273301 | 4 | 618718558 | 0.39238 | 1.86E-06 |  |
| 2020/2021\_TP3\_GE | JHI-Hv50k-2016-273434 | 4 | 618750390 | 0.48998 | 9.79E-11 |  |
| 2020/2021\_TP3\_GI, Logistic GI 2020/2021\_TimeTo5.0 | JHI-Hv50k-2016-276836 | 4 | 622826452 | 0.48998 | 3.10E-15 |  |
| 2020\_TP3\_GE | JHI-Hv50k-2016-276707 | 4 | 623071816 | 0.4955 | 2.58E-10 |
| 2020/2021\_TP1.5\_GI | JHI-Hv50k-2016-276688 | 4 | 623232032 | 0.49654 | 9.50E-07 |  |
| 2021\_TP1\_GE, Logistic GE 2021\_Lower | JHI-Hv50k-2016-276604 | 4 | 623528344 | 0.49885 | 2.41E-07 |  |
| All germination traits | Qsd1 | 5 | 442160000 | 0.35189 | 1.04E-59 | *HvAlaAT* (Qsd1) |
| 2020/2021\_TP2\_GI, Logistic GI 2020/2021\_TimeTo5.0 | JHI-Hv50k-2016-308652 | 5 | 446691785 | 0.36526 | 8.79E-14 |  |
| 2020/2021\_TP4\_GE | JHI-Hv50k-2016-308712 | 5 | 446936428 | 0.22717 | 6.86E-07 |  |
| Logistic GI 2021\_Lower | JHI-Hv50k-2016-308862 | 5 | 447841431 | 0.31781 | 2.40E-11 | HORVU.MOREX.r2.5HG0399930.1, S-adenosylmethionine decarboxylase |
| 2020\_TP1\_GE, Logistic GE 2020/2021\_Lower | JHI-Hv50k-2016-308899 | 5 | 448089400 | 0.30717 | 2.17E-11 |  |
| 2021\_TP1\_GE, Logistic GE 2021\_Lower | JHI-Hv50k-2016-309905 | 5 | 458001626 | 0.24249 | 3.05E-12 |  |
| 2020/2021\_TP5\_GE, Logistic GE 2020\_rTimeTo95 | JHI-Hv50k-2016-311435 | 5 | 475091665 | 0.10245 | 2.27E-12 | GRAM-containing/ABA-responsive protein, ISA3 |
| 2021\_TP1\_GE, Logistic GE 2021\_Lower | JHI-Hv50k-2016-311700 | 5 | 475995048 | 0.10393 | 6.85E-16 | GRAM-containing/ABA-responsive protein, ISA3 |
| 2020\_TP5\_GE | JHI-Hv50k-2016-312060 | 5 | 477830016 | 0.1036 | 3.78E-13 |  |
| Logistic GE 2020\_rTimeTo95 | JHI-Hv50k-2016-316773 | 5 | 493655229 | 0.09259 | 1.5E-50 | Altered Abscisic acid signaling and drought |
| Logistic GE 2020\_rTimeTo95 | JHI-Hv50k-2016-316958 | 5 | 497027186 | 0.09028 | 1.5E-50 | Altered Abscisic acid signaling and drought |
| 2020\_TP5\_GE | JHI-Hv50k-2016-336814 | 5 | 538533757 | 0.21396 | 1.39E-09 |  |
| 2020\_TP5\_GE | JHI-Hv50k-2016-337656 | 5 | 540818357 | 0.35586 | 1.37E-14 |  |
| Logistic GE 2020/2021\_Centering | JHI-Hv50k-2016-380542 | 6 | 27552556 | 0.46544 | 1.5E-50 |  |
| Logistic GE 2020/2021\_Centering | JHI-Hv50k-2016-380636 | 6 | 27999396 | 0.45968 | 1.5E-50 |  |
| Logistic GI 2020\_Lower | JHI-Hv50k-2016-435773 | 7 | 1396559 | 0.47947 | 4.66E-10 |  |
| Logistic GI 2020\_Lower | JHI-Hv50k-2016-435980 | 7 | 1493018 | 0.47705 | 5.25E-09 |  |
| 2020\_TP1\_GI | JHI-Hv50k-2016-507370 | 7 | 611934447 | 0.15138 | 3.03E-11 |  |

Trait abbreviations: Preharvest sprouting (PHS), germination energy (GE), germination index (GI), time point 1 (TP1), time point 1.5(TP1.5) time point 2 (TP2), timepoint 2.5(TP2.5), time point 3 (TP3), timepoint 3.5(TP3.5) time point 4 (TP4), time point 5 (TP5)

\*\*MAF: Minor allele frequency

\*\*\*P-values below the Bonferroni cutoff (p=0.05) of 3.57x10-6 were considered significant

Figure 3a-c: Display of germination rates based on lines with the dormant/don-dormant Qsd1 haplotype across the time points measured for each year. Each right facet represents germination index(GI) and germination energy(GE) Charles/Endeavor was included as a check comparison.

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**Other Barley Research**

-Two-row winter malting barley breeding program evaluating winter double haploid population and RIL population in augmented design in yield plots at two locations

-Multi-purpose organic naked barley research NAM F2 selection experiment funded by Organic Research and Education Initiative (OREI)

-Value added grains experiment funded by the Organic Research and Education Initiative (OREI) lead by Mark Sorrells

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