**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, and malting quality in a winter/facultative double haploid and a spring 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. It also funded phenotyping for dormancy, PHS, and malting quality data collection on the spring malting barley population. For the 2021-2022 cycle, we have continued our work measuring germination and PHS of our winter/facultative and spring barley populations. This award also provided funding for us to travel to the USDA CCRU to micro malt and analyze a subset of our winter and spring malting barley populations. The data collected in this study (susceptibility to PHS, germination rate, germination energy over time, and micro malting quality analysis) has increased understanding of winter and spring malting barley seed dormancy, dormancy persistence, and the relationships between PHS susceptibility and malting quality. It will allow for the confident selection of PHS resistant lines with good malting quality.

**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. 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 headrows 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 (444For sampling in 2022, we 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 sampling of lines that had sufficient amounts of seed for both germination tests and teaball malt samples.

In addition to measuring germination, a 120-line subset previously mentioned were sent to the USDA Cereal Crops 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 and 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 using the methods described by Schmitt & Budde 2011. We are still waiting for the last batch to be analyzed and we will 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, as indicated by 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 winter and facultative experimental lines and parent genotypes were monomorphic for dormant HvMKK3. The check lines Charles and Endeavor were used as PHS susceptibility checks in 2020 and 2021, respectively, and 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 50k 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 stepwise 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 within years and for 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 subsampled the population for phs scoring. While the 2020 data followed the same trend as the 2021 results, the observations were insufficient 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 of evaluating 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 PHS for most of the lines is encouraging, however PHS must be tested in more than one year to account for different environmental effects. This is particularly important given the significantly increased dormancy 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 slightly for GE at later time points due to reduced variation but still retained heritability values a minimum of ~0.75.

Genome wide association results

For single timepoint GWAS, we found a total of 37 significant marker-trait associations for 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 avoid 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 significant markers detected included 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 aand increases in ABA maintain seed dormancy and decreases in ABA reduce seed dormancy (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 categorywas included for comparison to Charles for 2020 and Endeavor for 2021. Parents KWS 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 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 later and did not occur until timepoints 3.5 (68 days post PM) and 4 (96 days post PM). The significant effect of the year 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 maltster would start malting barley approximately 47 days after harvest from the field, we will want to select lines that break dormancy by then. 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 each year, we can select genotypes that increase germination rate with less concern of reducing our PHS resistance for the given AlaAT haplotypes.

Table 1: Summary of number of plots sampled 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 single time point genome wide association of markers with 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 (+/- 1 Mbp) 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/non-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 funded by the Organic Research and Education Initiative (OREI) lead by Mark Sorrells

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**Section 2 Spring Barley: The relationship of malting quality traits to PHS resistance.**

**Objectives: Determination of the genetic relationship between PHS resistance and malting quality traits, determine the relationship between the germination traits at the time of malting to the malting quality.**

**Materials and methods:**

**Spring barley germplasm**

The population used is described in detail in (Sweeney et al., 2020; Sweeney, Rooney, & Sorrells, 2021) and is described briefly here. Parents ‘Conlon’ (PI 597789), ‘Pinnacle’ (PI643354), ‘ND Genesis’ (PI 677345), ‘Craft’ (PI 646158) (from North Dakota State University and University of Montana), ‘KWS Tinka’ (PI 681721) (a European variety from KWS), ‘Newdale’, and ‘Bentley’ (Canadian varieties) (Juskiw et al., 2009; Legge et al., 2008) were all crossed to the Canadian variety ‘AAC Synergy’ (Legge et al., 2014). Selection decisions and crossing schemes are described in detail in (Sweeney, Rooney, & Sorrells, 2021). A stratified random sampling of the seven families generated was used to create a base population (C0). Two individuals from all families except the ‘AAC Synergy’ by ‘Newdale’ and ‘Bentley’ families were selected with genomic selection or phenotypic selection and intermated to create the cycle 1 genotypically (C1G) selected taxa and the cycle 1 phenotypically (C1P) selected taxa. Selection was performed using an index selection scheme heavily weighted for PHS resistance, moderately for spot blotch, and lightly for heading date, leaf rust, grain protein, and height. In the early stages of C1G, F2 taxa within this group were selected on the basis of genomic selection using an undated index with higher weights for disease resistance and intermated to create cycle 2 genotypically (C2G) selected taxa. The top performing 25% of F2 taxa in C1G based on genomic selection using the initial index were used to create the 88 taxa used as C1G representatives. In total the population used was comprised of the eight parents, 105 C0 taxa (~15 from each half-sibling family), 87 C1G taxa, 108 C1P taxa, and 114 C2G taxa.

**Data collection**

All taxa were grown in 2019 and 2020 at two locations and half the population (represented by 66 taxa from C0, 37 taxa from C1G, 64 taxa from C1P, 58 taxa from C2G, and the 8 parents) was grown in one location in 2021. All trails were conducted according to an augmented block design with parents replicated across blocks and all other taxa present only once. Grain samples were harvested, germination traits measured, and PHS assessed on all taxa in these locations according to the methodology presented in the Winter Barley section. For the spring barleys germination traits were measured at 6, 20, 34, 48, 69, 110, and 160 days post PM and are labeled sTP1-sTP7. Previous work has shown that GI measured at sTP1 (6 days post PM) is an excellent proxy for PHS susceptibility. A higher GI value corresponds to PHS susceptibility.

Malting data was collected from one location in 2020 and the one location in 2021 according to (Schmitt & Budde, 2011) on one replicate of 2.25 grams of the sampled barley for each taxa at each malting timepoint. Malting was performed at two timepoints in 2020 and 2021 on the half the population (the same taxa in 2020 and 2021), 48 days post PM (corresponding to sTP4) and 110 days post PM (corresponding to sTP6). In 2020 the rest of the population was malted only at 110 days post PM. This was done using batch freezing at -20 C, and after-ripening at room temperature such that all taxa went into the malting process at exactly the correct number of days post PM. Malting was performed at the USDA-ARS Cereal Crops Research Unit (CCRU) in Madison, Wisconsin. Seven traits were collected per sample according to those scaled ASBC methods, AA (20˚ DU), DP (˚ASBC), FAN (PPM), BG (PPM), ME (% dry basis), SP (%), WP (%) and ST (%). Internal quality checks were included within every analysis such that daily assay runs were controlled to the standard, know values of those quality checks.

Based on the results of variance partitioning (described in detail in an upcoming publication) a group of 368 taxa from this population from the 2019 trails that were malted and analyzed without control over after-ripening times – taxa were malted between 150 and 200 days post PM – were also used. This group was comprised of 99 C0, 74 C1G, 81 C1P, and 106 C2G lines, as well as the 8 parents. Malting quality data was collected in the same manner, except for the controlled harvest and after-ripening before malting, rather samples were pulled from typical harvest bags. This data was included upon analysis of the 2020 and 2021 data which revealed that changes over time (between 47 to 110 days post PM) were small in magnitude, and likely unable to be detected on a per taxa basis under the malting procedure used. Pairwise t-tests between malting timepoints on a per taxa basis revealed only marginal numbers of significant changes over time, which could purely be due to random chance. The rate of change of malting quality declines during after-ripening time until the grain loses viability (Woonton et al., 2005) and often has a significant plateau for a number of months. Based off germination data and modeling (Rooney et al., 2021), these lines had already likely reached their asymptotic germination values by 110 days post PM, so it may be the case that even if the assay was sensitive enough to detect the differences between malting at 110 vs 150-200 days post PM there would be little difference. The 2019 data collected was assigned a value of TP6 for the malting quality timepoint. *At this time all malting quality traits except for wort protein have been entirely collected. Mechanical failures have delay collection of WP several weeks. For this reason, traits WP and ST will not be discussed here. Please see upcoming publications from the Sorrells Lab and the PhD dissertation of Travis Edward Rooney, to be published in the next 3 months for more detailed analysis of all traits including these.*

**Genetic data**

The genetic data used is the same as that used for the CU population in (Rooney et al., 2021). with slight differences due to lack of a significant outgroup. Briefly, all lines were genotyped with the 50k SNP chip (Bayer et al., 2017) at the USDA Small Grains Genotyping Laboratory in Fargo, ND. Marker data with >10% heterozygosity and minor allele frequency < 0.05 was filtered. Redundant markers were eliminated using LD pruning with the SNPrelate R package (Zheng et al., 2012) with a 25,000-bp sliding window and a cutoff of 0.95. This resulted in 11615 markers used for the full population, and a slightly reduced number when the half population was used. Two KASP markers, AlaAT\_L214F and MKK3\_E165Q whose development is detailed in (Sweeney, Rooney, Walling, et al., 2021) were also included in the genetic data. These genotype, respectively, the causative SNP in *HvAlaAT1* identified by (Sato et al., 2016) and the SNP identified by (Vetch et al., 2020) in *HvMKK3* as the putatively causative mutation in North American germplasm in the Seed Dormancy 2 (*SD2*) region. This *HvMKK3* mutation was different than that cloned by (Nakamura et al., 2016). (Sweeney, Rooney, Walling, et al., 2021) used the MKK3\_E165Q KASP marker and marker JHI-Hv50k-2016-367342 from the 50k SNP chip to distinguish three alleles of *HvMKK3* also shown to exist within this germplasm in that study. These alleles were called the “highly non-dormant” (MKK3N\*), the “non-dormant” (MKK3N), and the “dormant” (MKK3D) based on the phenotypes they associated with, and this notation will be followed here.

**Statistical analysis**

All data analysis was performed within the R statistical language version 4.0.3 (R Core Team, 2020) in the RStudio IDE (RStudio Team, 2021).

Genome wide association

Taxa effects (Best linear unbiased estimates, BLUEs) were extracted in a combined analysis for all three years of data (2019, 2020, and 2021) using a linear model of the form

Where value was the malting quality trait value, year, TP, year:TP, and taxa were fixed effects for the year, timepoint, year-by-timepoint interaction, and taxa respectively, error was assumed to be normally distributed with variance σ2error. These effects were then used as the phenotypes in a genome wide association (GWA) analysis. GWA analysis was performed within the GAPIT framework (Lipka et al., 2012) with multiple locus mixed model (MLMM) (Segura et al., 2012). The MLMM model includes highly significant markers as fixed effects within the model in a forward backward stepwise regression, which can allow for the identification of multiple factors in close physical proximity to be distinguished easily. Two principal components from eigen value decomposition of the relationship matrix were used as fixed effects in the model to account for population structure and kinship was used as a random effect to account for relatedness. A significance threshold of p-value < 5E-5 was used.

*Genetic correlations*

Genetic correlations of GI at 5 days post PM (GI TP1) and at the times of malting (TP4 and TP6) to the malting quality traits were calculated using bivariate mixed models. Plot level means (means of all technical replicates for a plot) were always used as the input to these models to allow for paired observations. All models were of the form

Where value1, value2 correspond to the paired plot level observations of traits one (malting quality trait) and two (germination trait). Year was a fixed effect for that factor per trait, and taxa was a random effect with a general heterogeneous correlation or covariance structure, with the variances of each trait along the diagonal of the matrix and the correlations or the covariances between each trait on the off diagonal. The model was compared to an equivalent model with a diagonal variance structure (assuming no correlations between traits) for the taxa effect with a likelihood ratio test to assess improvements in model fit, and hence significance of correlation. *HvMKK3* has shown to be strongly associated with differences in germination phenotype. Large effect loci have been shown to lead to changes in marker effects genome wide therefore all correlations were also calculated on a per *HvMKK3* allele basis. This analysis was performed within ASReml-R (Butler et al., 2018). For BG, any plot level estimates >900 ppm were excluded from the analysis.

**Results**

*GWA*

Table 2 shows the significant marker trait associations (MTA) for the malting quality traits. AA was most strongly associated with MKK3\_E165Q, as was FAN. SP was also strongly associated with a marker in the SD2 region approximately 7 Mbp proximal to the HvMKK3. This SNP was in moderate LD (correlation = -.76) with MKK3\_E165Q and was lowly to moderately correlated to the other alleles of HvMKK3 (MKK3D: -0.07, MKK3N: -0.56). Traits BG, DP, FAN, and SP were associated with a region on Chromosome 6H. All significant markers in the region were in high LD (correlation>0.94). AA was associated with a different region on Chr6H. FAN was associated with one marker near the distal end of Chr7H, and SP was associated with a region near the distal end of Chr1H.

Table 2: Significant marker trait associations of the malting quality traits to genetic loci. P.value, significance of association; Minor Allele Frequency of SNP

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **SNP** | **trait** | **Chromosome** | **Position** | **P.value** | **MAF** |
| JHI-Hv50k-2016-415600 | AA | 6 | 526130710 | 1.32E-08 | 0.15 |
| MKK3\_E165Q | AA | 5 | 596732030 | 1.82E-09 | 0.21 |
| JHI-Hv50k-2016-388539 | BG | 6 | 69601728 | 5.7E-07 | 0.28 |
| JHI-Hv50k-2016-386349 | DP | 6 | 47982141 | 3.35E-07 | 0.30 |
| JHI-Hv50k-2016-385463 | FAN | 6 | 42618343 | 1.3E-06 | 0.29 |
| MKK3\_E165Q | FAN | 5 | 596732030 | 5.87E-22 | 0.21 |
| SCRI\_RS\_4520 | FAN | 7 | 610967042 | 4.14E-08 | 0.07 |
| JHI-Hv50k-2016-365992 | SP | 5 | 594326624 | 8.4E-16 | 0.31 |
| JHI-Hv50k-2016-385463 | SP | 6 | 42618343 | 9.4E-07 | 0.29 |
| JHI-Hv50k-2016-48870 | SP | 1 | 497105966 | 3.42E-05 | 0.15 |
| JHI-Hv50k-2016-49136 | SP | 1 | 499170798 | 2.4E-05 | 0.15 |

*Genetic Correlations*

Table 3 shows the results of examining the genetic correlations of the malting quality traits to GI at TP1, TP4, and TP6. FAN, SP, and AA were strongly genetically correlated to the germination traits over time. BG and ME were moderately correlated to the germination traits and DP was largely unrelated to the germination traits. Table 4 shows the correlations of these traits on a per *HvMKK3* allele basis. AA is moderately correlated within the MKK3D allele to the germination traits over time, but lowly correlated within the MKK3N allele, and uncorrelated to GI at sTP1 within the MKK3N\* allele. BG content is most strongly related to germination traits within the MKK3D allele. DP is largely uncorrelated with germination traits, except for the MKK3D allele at GI sTP6. FAN is moderately to highly correlated over all alleles. ME was uncorrelated to the germination traits. SP followed a similar pattern to FAN.

Table 3: Genetic correlation of the malting quality traits to the germination traits over time. Asterisks indicate significant incrase in model likelihood and hence significance of genetic correlations, p-value< 0.01.

|  |  |  |  |
| --- | --- | --- | --- |
| **trait** | **GI\_sTP1** | **GI\_sTP4** | **GI\_sTP6** |
| AA | 0.643\* | 0.634\* | 0.42\* |
| BG | -0.419\* | -0.482\* | -0.377\* |
| DP | 0.092 | 0.205\* | 0.134 |
| FAN | 0.906\* | 0.763\* | 0.603\* |
| ME | 0.309\* | 0.391\* | 0.225 |
| SP | 0.771\* | 0.673\* | 0.465\* |

Table 4: Genetic correlations of the germination traits to malting quality traits on a per *HvMKK3* allele basis. Asterisks indicate significant incrase in model likelihood and hence significance of genetic correlations, p-value< 0.01.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **trait** | **SD2Code** | **GI\_sTP1** | **GI\_sTP4** | **GI\_sTP6** |
| AA | D | 0.447\* | 0.692\* | 0.518\* |
| AA | N | 0.263\* | 0.268\* | 0.089 |
| AA | N\* | -0.089 | 0.054 | -0.397\* |
| BG | D | -0.343 | -0.574\* | -0.766\* |
| BG | N | 0.008 | -0.109 | 0.092 |
| BG | N\* | 0.114 | -0.153 | 0.071 |
| DP | D | -0.056 | 0.285\* | 0.339\* |
| DP | N | 0.033 | 0.183 | 0.128 |
| DP | N\* | -0.108 | -0.097 | -0.373\* |
| FAN | D | 0.781\* | 0.647\* | 0.527\* |
| FAN | N | 0.5\* | 0.477\* | 0.142 |
| FAN | N\* | 0.865\* | 0.494 | 0.807\* |
| ME | D | -0.105 | 0.166 | -0.006 |
| ME | N | 0.07 | 0.29 | 0.132 |
| ME | N\* | -0.16 | 0.166 | 0.047 |
| SP | D | 0.458\* | 0.516\* | 0.365\* |
| SP | N | 0.412\* | 0.547\* | 0.191 |
| SP | N\* | 0.658\* | 0.204 | 0.119 |

**Discussion**

Genetic correlation and GWA strongly point to a to common genetic basis of PHS resistance and several malting quality traits. Much of the genetic association focused around the SD2 region. In the SD2 region *HvMKK3* has been determined to be the causative gene effecting dormancy and PHS resistance. The common association of the AA and FAN malting quality traits to the causative mutation is strong evidence that the *HvMKK3* may have pleiotropic effects on malting quality. Surprisingly, SP did not also associate with a SNP in higher linkage with the MKK3\_E165Q mutation (or the mutation itself) that AA and FAN associated with, instead it was most strongly associated with a SNP seven mega base pairs (7 million base pairs) away from the mutation. This may indicate more measurement error associated with SP, however, this trait did have the highest heritability on a year basis (genetic variance/phenotypic variance, see dissertation) which would point away from this hypothesis. It may be that there are two genes in the larger *SD2* region that effect malting quality, one being the mentioned *HvMKK3* which has effects on germination trait, AA, and FAN, while another unknown gene effects SP. This is not the most parsimonious hypothesis however, and the data we have collected only allows speculation in this matter. Follow-up experiments should be performed to knock out and restore the *HvMKK3* alleles and examine their effects on malting quality and thus causality of this gene.

From the perspective of breeding, if the *HvMKK3* is not causal, AA and FAN are likely linked so tightly to *HvMKK3* that breaking the linkage would take an extremely large population size, luck, and a concerted effort. From the same perspective, alleles of *HvMKK3* that are shown to be compatible with PHS resistance, MKK3D and MKK3N, do have significant correlations to germination traits, both at TP1 (PHS resistance measure) and somewhere around the time of malting (47 to 110 days post PM) and so GI at various times may be suitable for making selections in some cases. Correlations can be deceptive however, especially since the malting quality trait goals are related to absolute ranges rather than vague goals of increased or decreased values. It is helpful to look at these values in relation to the AMBA malting barley breeding targets (Figure 4). Examination of this is informative. The MKK3N\* appears to be more associated with a trait profile for adjunct brewing, while the other alleles more generally fall into the all-malt categories, especially for those traits that are seen to have a high correlation with the germination traits. However, for both these categories there are taxa that meet the guidelines with any allele. The exception to this is BG, which was inflated for all alleles. Studies recently published from this lab indicate that the MKK3N\* was enriched in Canadian and western US breeding programs targeted for adjunct malting quality. The result corroborates this and give an indication as to why those programs are enriched for this allele: it generally produces a line with a higher chance of meeting the adjunct style malting quality guidelines, especially for AA, FAN, and SP. Whether this is due to causation is, again, more than this study can answer, but results strongly implicate pleiotropy of *HvMKK3* leading to the classic, sought-after, Canadian style adjunct malt.

This work was undertaken in the context of expanding malting barley acreage to novel areas and answering questions on the relationship between PHS resistance malting quality. We have answered the latter question; there are strong relationships between some malting quality traits and PHS resistance, more than what is caused by a the *HvMKK3* allele, as the correlations are still significant upon sub setting to individual alleles and performing correlations. The first question has yet to be answered directly. Areas that are seeing expanding acreage are producing barley for craft malting facilities which themselves feed into craft malting operations, which in general are producing an all-malt style of beer. The areas of expanding acreage are the areas that most urgently need PHS protection within the barley cultivars they produce. These results directly inform towards this goal. It is possible to have substantial PHS protection while still having the potential to produce high quality malt as early as 47 days post PM (see dissertation), which certainly does not introduce any burdensome storage time to break dormancy. Lastly, craft malting operations are, in general, more flexible than the larger scale malting operations that produce adjunct malt for large brewing companies. This could be taken advantage of in the production of craft malts, as these facilities might be more flexible in the production of a malt that can be grown locally, successfully, without large risk of PHS.

Figure 4: Malting quality trait values in relationship to the allele of *HvMKK3* and the germination rate at TP1 (6 days post PM) as a measure of PHS susceptibility. Malting quality traits are listed on the top of each facet, *HvMKK3* allele is shown by color, and GI TP1 is shown on the x-axis. Blue shaded graph area is the acceptable breeding target published by AMBA for all malt brewing, red shaded area is the acceptable breeding target for adjunct brewing.

