

Introduction

The applied goal of this project is to increase the profitability and sustainability of US agriculture by developing winter malting barley varieties that will benefit farmers, processors, and consumers. These varieties will be more cold-tolerant and have malting quality that meets or exceeds current benchmarks. Greater cold tolerance will expand the potential winter malting barley acreage and superior quality will ensure value for producers and end users. **The basic research goal is to advance our fundamental understanding of the genetics of low temperature tolerance and vernalization sensitivity in the Triticeae, using barley as a model.** We will specifically address the question, “Is vernalization sensitivity required for maximum cold tolerance?” In doing so, we will assess the biological and commercial potential of facultative germplasm. Our specific objectives will be to generate comprehensive phenotype and genotype data on doubled haploid (DH) and backcross marker assisted selection (BC-MAS) breeding lines developed from crosses between Nebraska (superior cold tolerance) and Oregon (superior malting quality) germplasm. One of the DH populations is between two facultative parents and therefore does not segregate for vernalization sensitivity; the other DH population does segregate for vernalization sensitivity. In the BC-MAS populations we will use “perfect markers” to select for vernalization-sensitive and -insensitive lines in the same genetic background. Together, the DH and BC-MAS germplasm will allow us to determine the main effects and interactions of alleles at the two principal low temperature tolerance QTL (*Fr-H1* and *FrH-2*) and to potentially discover other genes determining this key trait.

The experiments and analyses proposed for this AFRI project are an integrated extension of an ongoing winter malting barley breeding program and a Barley Coordinated Agricultural Research (CAP) project. The breeding program is comprised of the following components; crossing, generation advance (field, single seed descent, and doubled haploid), selection (phenotypic and marker-assisted), multi-environment phenotyping, and variety release. In the Barley CAP, 96 lines from the breeding program are genotyped annually for 3,072 SNP loci, phenotyped in multi-environment trials, and the resulting data are used for association analyses. **The germplasm that is the foundation for the proposed AFRI project was developed for the breeding program and the Barley CAP: the AFRI program would provide an opportunity to leverage and mine this germplasm by profiling at both the genotypic and phenotypic levels to a degree that is not employed in the breeding program or available via the Barley CAP.** The experiments proposed for AFRI-support are research investments with a high probability of accelerating variety release and the development of a deep germplasm pool that can be exploited for years to come.

In the interest of brevity, the proposed AFRI experiments are described as discrete entities. In reality, the AFRI germplasm and data will be fully integrated with the breeding program and the Barley CAP. For example, marker and phenotype data generated via the AFRI program will be used to identify lines for use as parents in the breeding program; promising AFRI germplasm will be put into additional trials to determine its potential for variety release; and new genes/QTL discovered via association mapping in the Barley CAP will be applied to

the AFRI germplasm. Finally, the AFRI-supported marker assisted selection (MAS) component of this project will focus on a subset of germplasm selected via foreground and genomic selection. **The un-selected germplasm will not be discarded - it will be advanced to field assessment for phenotypic selection and potentially future use in future MAS-based studies.**

Genetics of winterhardiness: Winterhardiness in barley (and other members of the Triticeae) has three principal components – low temperature (LT) tolerance, vernalization (VRN) response, and photoperiod (PPD) sensitivity. Vegetative stage LT is an induced response: plants show maximum cold tolerance only after a period of acclimation during which hundreds of Cold-Regulated (COR) genes are up- and down-regulated (Fowler and Thomashow, 2002). Reproductive stage LT is a distinct phenomenon (Reinheimer et al., 2004) and is not a primary focus of this research, although there may be spin-offs (see *Approach – possible additional phenotyping*). Under field conditions, hardening typically occurs during the same inductive conditions that determine VRN response and PPD sensitivity (e.g. low temperatures and short days). Once the plant transitions from a vegetative to a reproductive state, the level of cold tolerance is reduced (Galiba et al., 2009). VRN and PPD are straightforward phenotypes to measure whereas phenotyping LT is more complicated due to challenges of obtaining uniform and differential field assays and the high cost of controlled environment assays. In this proposal, the genetics of the three traits will be briefly reviewed in the order LT, VRN, and PPD and in the context of candidate genes for each of the three phenotypes.

LT genetics: In the Triticeae genomes, the group 5 chromosomes are the site of the two principal LT QTL, which are termed *Fr1* and *Fr2* (Francia et al. 2004; Skinner et al., 2005; Galiba et al., 2009). In barley, these two QTL are approximately 30 cM apart. The candidate gene for *Fr-H1* is *Vrn-H1* (synonymous with *HvBM5A*) (Fu et al., 2005; Von Zitzewitz et al., 2005). The candidate gene (or genes) for *Fr-H2* is (or are) one (or more) members of two physically linked clusters of at least 11 C-repeat Binding Factor (CBF) genes (Skinner et al., 2005; Francia et al., 2007; Galiba et al., 2009). There is evidence that the presence or absence of specific gene family members (Francia et al., 2007), or the copy number of specific gene family members (Stockinger et al., 2007), may be responsible for the phenotypic variation in LT associated with this complex locus. A deeper analysis of the copy number issues is the focus of a proposal submitted to this same AFRI panel by Dr. E. Stockinger and our projects are very complementary.

A probable role for the 5H CBF transcription factors in LT is apparent and therefore *Fr-H2* is one of the targets for QTL mapping and MAS in this proposal (see *Approach*). The *Fr-H1* locus is also a target for QTL mapping and MAS in this proposal. However, a casual effect of the candidate gene is not as apparent as in the case of *Fr-H2* since it is not yet clear whether *Fr-H1* is a pleiotropic effect of *Vrn-H1* or the effect of a physically linked gene (or genes). Support for the former comes from the observation that timing of maximum cold tolerance is usually coincident with the timing of vernalization saturation (Limin et al., 2007) and an unsatisfied vernalization requirement maintains the vegetative state. On the other hand, there is evidence that LT is not necessarily a function of VRN. The variety “Dicktoo”, a check in winterhardiness nurseries and a standard for

barley LT research, is not vernalization-sensitive: it is what is termed a “facultative” (see below - *Facultative growth habit and the genetics of VRN*). LT QTL map to *Fr-H1* in the Dicktoo x Morex population, which does not segregate for VRN sensitivity (Pan et al., 1994) and Dicktoo achieves a high degree of LT without any VRN sensitivity (Limin et al., 2007). Resolving the question of whether the *Fr-H1* is due to allelic variation at *Vrn-H1* or to a tightly linked gene (or genes) will require multiple lines of evidence. The proposed AFRI research will assess the contributions and interactions of *Fr-H1* as a QTL. We are also collaborating on a project with Dr. Kazuhiro Sato (Resource Institute for Bioresources), involving comparative sequencing of BAC clones that contain *Vrn-H1* from different *Hordeum* accessions. Since the two new BAC libraries are derived from accessions with “winter” alleles at *Vrn-H1* (see below - *Facultative growth habit and the genetics of VRN*), whereas the reference BAC sequence is from Morex (a spring variety), this endeavor will provide new insights into sequence variation in *Vrn-H1* and in adjacent genes. It will also supply a resource for identifying polymorphisms that will be necessary for genotyping *Vrn-H1* alleles in this proposed research (see *Approach – Genotyping*).

Facultative growth habit and the genetics of VRN: The role of VRN sensitivity in LT is relevant to this proposal in view of the three growth habit types in barley: spring, winter, and facultative. Winter varieties are LT tolerant, highly responsive to VRN, and vary in PPD sensitivity. Spring varieties have minimal LT tolerance, do not respond to VRN, and sensitivity/insensitivity to short day PPD is not relevant if they are grown under long-day (spring planted) conditions. The term “facultative” is generally used to describe genotypes that are LT tolerant, are not VRN sensitive, and may be PPD sensitive. Facultative varieties have “winter” allele haplotypes at the *VRN-H1* locus, and complete deletions of the *VRN-H2* locus on 4H (Karsai et al. 2005; von Zitzewitz et al. 2005; Szucs et al., 2007). The “winter allele” haplotype refers to the sequence of the first intron of *Vrn-H1*: “winter” haplotypes have a full-length intron that includes a highly conserved 0.44 kb “vernalization critical” region (Fu et al. 2005; von Zitzewitz et al. 2005). This critical region is the putative binding site, under long day conditions, for the repressor encoded by *Vrn-H2*. Deletions of various lengths are associated with variation in flower time (Szucs et al., 2007), with large deletions (~2.8 kb) characteristic of spring growth habit types.

The preceding review of the genetics of growth habit and VRN is in accordance with the two models proposed for the interactions of the three genes that determine VRN sensitivity, with the caveat “under long-day conditions”. Using current nomenclature and chromosome designations, the loci are *VRN-H1* (5H), *VRN-H2* (4H) and *VRN-H3* (7H). Winter genotypes are *Vrn-H2*/*vrn-H1vrn-H1*/*vrn-H3vrn-H3* and all other allelic configurations lead to a lack of significant vernalization-response (i.e. spring or facultative growth habits). *HvBM5A*, a MADS-box floral meristem identity gene and member of the AP1 transcription factor family, is considered the determinant of *VRN-H1* (von Zitzewitz et al. 2005; Trevaskis et al. 2007). A zinc finger–CCT (*CONSTANS*, *CONSTANS*-like, and *TOC*) domain transcription factor (*ZCCT*), encoding a flowering repressor down-regulated by vernalization, is considered the determinant of *VRN-H2* (Yan et al. 2004). Allelic variation at this complex locus (there are actually three tightly linked *ZCCT* genes in barley; *ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc*) is ascribed to loss-of-function mutations or

complete deletion (Dubcovsky et al. 2005; Karsai et al. 2005). *Vrn-H3* is attributed to the effects of *HvFt1* (Yan et al., 2006; Faure et al., 2007). *HvFT1* expression is induced by long days and may mediate the long-day flowering response (Turner et al., 2005). Allelic variation at *HvFT1* is attributed to mutations in the first intron, with the relatively rare dominant alleles conferring very early flowering (Yan et al., 2006).

According to the model of Yan et al. (2004) *Vrn-2* encodes a dominant repressor – down-regulated by vernalization – that inhibits the expression of *vrn-1* alleles and therefore no vernalization-sensitivity is predicted in genotypes with recessive *vrn-2* alleles (loss-of-function mutations or complete deletions of *ZCCT*), regardless of allelic state at *VRN-1*. Likewise, genotypes with both dominant *Vrn-2* and *Vrn-1* alleles (lacking a repressor binding site in the promoter and/or in the intron 1) are also predicted to show minimal vernalization-sensitivity. *Vrn-H3* did not figure in this model, as first proposed, since most cultivated barley accessions are monomorphic for the recessive allele at this locus. The alternative model proposed by Trevaskis (2007) is based on the argument that since *Vrn-H2* expression is low under short-day conditions, which are precisely those that occur under field conditions, it is unlikely that *Vrn-H2* plays a major role in regulating *Vrn-H1*. Per this model, the dominant *Vrn-H2* allele represses the activity of *Vrn-H3* under long day conditions prior to the onset of winter and as winter progresses, after sufficient exposure to low temperature, the recessive *Vrn-H1* is up-regulated and represses *Vrn-H2*, allowing the long-day induction of *Vrn-H3*, which in turn further enhances the activity of *Vrn-H1*. A similar model of a feedback regulatory loop among the three vernalization genes has been proposed for wheat (Distelfeld et al., 2009).

Practically speaking, the two VRN models are not mutually exclusive. The Trevaskis et al. (2007) model is applicable to conditions encountered under normal autumn-sowing conditions. The Yan et al. (2004) model fits conditions in which vernalization-sensitive germplasm is spring-sown. Spring-sowing of “winter cereals” is not unreasonable. There are agronomic and breeding advantages to facultative growth habit. In terms of agronomics, a facultative variety could be planted under either fall- or spring-sown conditions. This would simplify variety distribution and it would also provide farmers with the option of re-planting with the same variety in the spring if the variety suffered extensive winter injury after fall-planting. For breeding purposes, facultative growth habit would be desirable from the standpoint of allowing for accelerated cycles of generation advance in controlled environments (e.g. single seed descent) or off-season increases. With vernalization sensitive germplasm, there is an obligatory delay of at least six weeks of low temperature treatment between each generation under controlled environment conditions. Seed production under field conditions where vernalization requirements are not met (e.g. counter-season nurseries) is at best sluggish and at worst impossible. **Given the potential advantages of facultative growth habit, one of the objectives of this project is to empirically assess the cold tolerance of facultative vs. vernalization-sensitive germplasm in QTL mapping and MAS populations (see Introduction).**

Genetics of PPD: Szucs et al. (2006) identified *HvPhyC*, 1 cM from *Vrn-H1*, as a candidate for a short-day sensitivity QTL. The tight linkage of this candidate gene with *Vrn-H1* may be of adaptive significance, as short-day sensitivity is an alternative mechanism for delaying the vegetative to reproductive transition. **In fact, short-day sensitivity may be a more effective mechanism than vernalization sensitivity because variation in daylength is not subject to perturbation whereas temperature regimes may become more volatile as a consequence of global climate change.** Two cloned genes have been shown to determine photoperiod sensitivity. *HvFT3* is the candidate for *Ppd-H2* (chromosome 1H). Allelic variation at this locus is due to complete deletion of the gene in genotypes that are sensitive (e.g. remain vegetative) under short-day conditions (Faure et al., 2007). In earlier QTL studies, *Ppd-H2* was shown to have significant effects on flowering under short photoperiods and in autumn-sown field experiments (Pan et al. 1994). Allelic variation at *Ppd-H1* (on chromosome 2H) is particularly important in spring barley as the recessive allele confers insensitivity to long-day conditions, allowing for a prolonged growing period and consequently higher yield (Turner et al., 2005; Jones et al., 2008). Allelic variation at this locus is attributed to amino acid changes in the CCT domain leading to the recessive allele.

Genetics of malting quality: The unique properties of germinating barley account for the worldwide importance of this crop for malting and brewing. The conversion of starch and protein reserves in the grain to simple sugars, amino acids, and other components in malt allows yeast growth and flavor formation during subsequent fermentation (Hertrich, 2005). Historically, the malting and brewing industries have used general guidelines for chemical composition and enzymatic capacity to ascertain whether a given malt is adaptable to their commercial processes. The guidelines generally refer to broad categorical composition rather than specific metabolite content, addressing the endpoints of multiple, complex and poorly understood metabolic pathways. Beyond the general malting quality guidelines, each maltster and brewer must determine specific process alterations to effectively utilize good quality malt in production, since target beer profiles and brewing processes vary around the world and change with time.

Malting quality, therefore, in the most general sense, has a complex genetic basis, although there is an extensive catalog of genes with known, or putative, effects on malting quality (reviewed by Hayes et al., 2003). Some examples include the alpha-amylases, beta-amylases, alpha-glucosidases, and various proteases (Jones 2005, 2008) - page limitations preclude an extensive review of these genes in this proposal. In order to meet the expectations of farmers and of the industry, barley breeders must at least maintain malting quality while improving productivity (low temperature tolerance in the case of this proposed research). In most cases, both quality and productivity must be improved simultaneously to meet the needs of both end users and producers. An integrated approach to molecular breeding for malting quality will involve targeting quantitative trait loci (QTL) for quality traits using linked markers and allele-specific markers for genes with known roles in malting quality.

The process of making malting quality QTL tractable targets for MAS has been accelerated by the resources provided by the Barley CAP. We recently reported

the development of a 2,383 locus linkage map that allows for objective positioning of malting quality QTL reported in the literature on the current generation of SNP-based maps (Szucs et al., 2009). This paper (*see Appendix*) is supported by extensive online resources at <http://wheat.pw.usda.gov/ggpages/maps/OWB/>. As described therein, genes with known (or predicted) roles in malting quality were mapped directly via their inclusion in one of the Illumina Oligonucleotide Polymorphism Arrays (OPAs), and these genes can therefore be objectively mapped along with 154 malting quality QTL for 18 traits. Obviously, MAS for 154 QTL determining 18 traits contributing to malting quality is not feasible. A considerable amount of complexity reduction can be accomplished by aligning coincident QTL on a single foundation genotype relevant to this particular research objective (*see Unpublished data-Characterization of 88Ab536-B*).

QTL analysis: There is a long history of QTL analysis in completely inbred barley mapping populations derived from bi-parental crosses, leading to hundreds of reports in the past 20 years. Statistical methods for QTL detection in such germplasm have been continuously improved. Details on the many different methods available are described in Wang et al., 2005. The two principal features of this approach are (i) members of the populations trace to a single cross, usually between two lines contrasting for the target trait(s), and (ii) there is extensive linkage disequilibrium (LD) between QTL and markers. This allows for the estimation of QTL positions (within a confidence interval), effects, and interactions between QTL without the necessity of high density of markers (Piepho et al., 2000). There are, however, limitations to QTL detection in bi-parental populations - including bias in the estimation of QTL effects due to reduced sample size (Vales et al., 2005), narrow genetic bases and consequent limited scope of inference (Crepieux et al., 2005), and broad confidence intervals for QTL positions and effects (Darvasi et al. 1993; Hyne et al. 1995) - which can compromise the success of MAS. Association mapping (also called LD mapping or genome-wide association analysis) provides a complementary approach to bi-parental mapping. A wider genetic base, including actual breeding lines, can be sampled (Flint-Garcia et al., 2003; Varshney et al., 2005) and high density genotyping can provide better estimates of the actual polymorphisms in linkage disequilibrium with the QTL (Zhao et al., 2007). Association mapping is one of the objectives of the Barley CAP and its linked program AGOUEB in Europe (Hayes and Szucs, 2007), based on the relatively long-range LD in barley (Rostoks et al., 2006; Cockram et al., 2007). Genome-wide association mapping has its limitations: population structure and relatedness of individuals usually lead to false positives and false negatives in the association (Pritchard et al., 2000), and a high density of markers is needed since in cases of short-range LD (Rafalski, et al., 2002).

We propose, with AFRI support, to complement our ongoing and parallel association mapping research for winter hardiness and malting quality traits in our breeding germplasm with targeted bi-parental mapping in two carefully planned DH populations (one derived from the cross of Oregon and Nebraska facultative parents and the other from the cross of an Oregon facultative with a Nebraska vernalization-sensitive type). The two DH populations are of sufficient size, and we will thoroughly genotype and phenotype

them in order to obtain the best possible estimates of QTL for target winterhardiness and malting quality traits. We propose to use a subset of the same SNPs used for profiling Barley CAP germplasm for genotyping the DH mapping populations (see *Approach-Genotyping*), which will establish connectivity between the AFRI bi-parental and CAP association mapping efforts.

Marker assisted selection: The use of molecular markers has enabled the dissection of complex traits into quantitative trait loci (QTL) (Dudley, 1993, Tanksley, 1993; Lee 1995; Zeng et al., 1999). Marker assisted selection (MAS) is a useful tool for plant breeding that involves using markers closely linked to these QTL or in candidate genes (perfect markers). MAS improves the efficiency of selection in segregating populations by predicting which individuals carry target alleles (Lande and Thompson, 1990; Knapp, 1998; Ribaut and Betran, 1999). The main advantages of MAS, as summarized by Collard et al. (2005) include: time and cost savings accrued by the substitution of complex field or laboratory assays with molecular tests; efficiencies achieved by elimination of unreliable phenotypic assays; accelerated cycle time and space savings by selection of genotypes at seedling stages; gene ‘pyramiding’; reduction of linkage drag; and selection for traits with low heritability. **All of these advantages of MAS apply to our proposed AFRI project.**

First generation MAS methods are best suited for manipulating a few genes with large effects, rather than many genes with small effects (Tanksley, 1993; Ribaut and Betran, 1999; Dekkers and Hospital, 2002). Marker-assisted backcrossing has been especially successful for good varieties that need to be improved for a few key traits (reviewed by Xu and Crouch, 2008) and as applied to barley for specific components of malting quality (Han et al., 1997; Schmierer et al., 2005.) and disease resistance (Toojinda et al., 1998; Jefferies et al., 2002) yet population sizes become impractical for MAS when multiple loci are involved and many of the traits that breeders would target for MAS involve multiple loci (Crosbie et al., 2003). Under these situations, MAS efforts may be better targeted at determining optimum combinations of QTL alleles rather than pyramiding specific alleles (Zhu et al., 1999). An example of such a strategy is genomic selection, which is a form of MAS that simultaneously estimates all locus, haplotype, or marker effects across the entire genome to calculate genomic estimated breeding values (GEBVs) (Meuwissen et al., 2001). The process involves using a population of individuals with both phenotypic and genotypic data, known as the “training population”, to estimate model parameters that will subsequently be used to calculate GEBVs for selection candidates (e.g., breeding lines) having only genotypic data. These GEBVs are then used to select the individuals for advancement to the next breeding cycle (Meuwissen et al., 2001; reviewed by Heffner et al, 2009).

Since we are proposing to integrate MAS for low temperature tolerance and malting quality, we have designed a scheme that focuses on a limited number of target genes for the former and genomic selection for the latter.

The genomic selection will be facilitated by the allele enrichment that ensues from backcrossing to a malting quality parent (OR813). The term backcrossing is used a bit loosely here in that OR813 is a sib line of OR76. We have abundant malting quality data sets data on germplasm characterized with 3,072 Barley CAP SNPs

(including SNPs in nine genes with known functions in malting quality). The resources include the 88Ab536-B project (*see next section*), the comparison of OR813 vs. OR76, the “Beer Genes set” (36 lines evaluated in 21 environments, the OSU CAP I breeding lines (96 lines, 3 environments) and the OSU CAPII breeding lines (71 lines, 3 environments). In addition, there are data from genotyped and phenotyped CAP breeding lines from nine other breeding programs. The SNP haplotype/QTL alignments assist in identifying higher resolution QTL targets and the populations will serve as the training populations to predict best allelic combinations for malting quality in our BC-MAS populations.

Unpublished data:

Characterization of 88Ab536-B: In order to integrate SNP haplotypes, QTL, and functional genomics we are, in collaboration with Dr. Gary Muehlbauer’s group at the University of Minnesota, characterizing a facultative six-row accession called 88Ab536-B using structural (CAP SNPs) and functional (Barley Affymetrix I GeneChip) genomics resources. This work is relevant to this AFRI proposal because 88Ab536-B is a grandparent of OR71, OR72, OR76 and OR813 (*see Experimental plan - Germplasm*) and the presumed donor of most (if not all) malting quality alleles in the four “OR lines”. 88Ab536-B was selected in the F6 generation after an exceptionally cold winter at Aberdeen, Idaho and went on to become the first winter six-row approved by the American Malting Barley Association (AMBA). The pedigree of 88Ab536-B is Ne76129/Morex, F1//Morex. Ne76129 was an experimental winter feed barley germplasm line from the University of Nebraska breeding program. Morex was the US spring malting barley standard for years and it appears in the pedigree of nearly all, US six-row malting barley germplasm. As shown in the Figure titled “88Ab536 graphical haplotype” posted at <http://barleyworld.org/OrNe.html> the 154 malting quality QTL reported by Szucs et al. (2009) can be reduced to 55 discrete genomic regions by grouping QTL into ~ 5 cM bins and assigning them map coordinates based on 4,608 Barley CAP SNPs. Because Ne76129 is a feed barley with no reported favorable malting quality attributes, most favorable alleles for malting quality can be reasonably assumed to trace to Morex. Conversely, because Morex is very susceptible to winter injury and most favorable alleles for low temperature tolerance can be reasonably assumed to trace to Ne76129. Overall, the graphical haplotype and QTL summary support these assumptions: ~70% of the malting quality QTL are in regions where 88Ab536 alleles trace to Morex. A subset of these regions, which we have confirmed as important determinants of malting quality via association analyses, will be targeted for genomic selection (*see Approach – Genotyping*). Caution must be exercised in making assumptions is that “most” favorable alleles trace to the parent with the target phenotype: there are many reports in the literature of favorable alleles tracing to parents with overall unfavorable phenotypes and in this particular case, we have confirmed through allele re-sequencing that the 88Ab536-B *Bmy1* allele on the long arm of chromosome 4 traces to Ne76129. Clark et al. (2003) reported that this particular allele confers higher beta amylase enzyme activity and thermostability than the Morex allele. The *Fr-H1* and *Fr-H2* regions on 5H in 88Ab536-B trace to Ne 76129, providing additional evidence for the importance of this genomic region in LT. The chromosome 4HL introgression accounts for the

facultative growth habit of 88Ab536 – it includes a complete deletion of *Vrn-H2*. The fact the Ne76129 was facultative due to a deletion of *Vrn-H2* is not entirely unexpected: as noted previously, Dicktoo is facultative and as described below (*see below NB03437-facultative*) we have also found *Vrn-H2* deletion lines in populations of accessions where most individuals are *Vrn-H2* dominant (i.e. the gene is present).

NB03437-facultative: Validation for the *Vrn-H2*-represses-*Vrn-H1* model (under long day conditions) and additional evidence that maximum cold tolerance should be achievable without vernalization sensitivity is provided by the identification, genotyping, and phenotyping of a facultative version of NB03437. We determined that ~ 1% of plants grown from seed lots of this Nebraska accession flowered, without vernalization, under long-day conditions. This is shown in Figure 1, where the phenotype and genotype of one of the early-flowering plants is shown. We genotyped these early-flowering plants and determined that all are facultative (e.g. *Vrn-H2* deletions and *Vrn-H1* full-length intron 1). They are visually indistinguishable from vernalized NB03437 plants in terms of morphological characteristics (e.g. height and spike type. In order to determine the cold tolerance of the NB03437-facultative (hereafter referred to as NB03437-f) type, we sent seed (tracing to the individual head selection shown in Figure 1) to the Martonvasar Research Institute (Hungary) for controlled freeze testing. **Per these data, NB03437-f is as cold tolerant as the vernalization-sensitive NB018199 and the Dicktoo winter hardiness check (Table 1).** In terms of additional genotyping, samples from a single NB03437-f accessions and NB03437 (“wild type”) accessions is scheduled (June, 2009) for genotyping with Barley (B) OPA 1 to determine their degree of genetic similarity. NB03437-f was the parent of all germplasm proposed for use in this AFRI project.

Rationale and Significance

AFRI program priorities: **The proposed research directly addresses Research Project Priority 1 of the Plant Genome, Genetics, Breeding program as it involves “cultivar development, selection theory, applied quantitative genetics, and breeding for improved local adaptation to abiotic stress”.**

Winter malting barley: a new crop with an established market: Winter malting barley offers a new opportunity for farmers, processors, and consumers. The crop will bring needed genetic diversity and production options to areas where wheat monocultures predominate. **Winter malting barley is a “new crop” in that the first commercial-scale production of a winter malting variety in the U.S. will be harvested in 2009. However, winter malting barley is a new crop with established markets. The infrastructure for producing, receiving, storing, transporting, and processing winter malting barley is already in place due to the historical prevalence of spring malting barley.** Spring malting barley production is at its lowest level in years due to a combination of factors, including Fusarium Head Blight (FHB) infection in the Midwest, drought under dryland production in the western states, and the high cost of irrigation. As a consequence the development of winter malting barley varieties is a high priority for the U.S. industry (*see letters of support from Drs. Mike Davis and Gary Hanning*).

Realizing the potential of winter malting barley: **Winter malting barley offers solutions to many of the problems facing spring barley because it can be produced in areas where FHB is not such a serious risk (e.g. Western states) and its early maturity may allow for production in FHB-prone regions such as the upper Midwest. In regions that receive winter precipitation (e.g. the Columbia Basin and Palouse of the Pacific Northwest), dryland winter barley yields are typically 20% higher than those of spring barley. In production areas where winter precipitation is limiting but irrigation is available (e.g. the Snake River Plain of Idaho), winter and spring barley yields are similar but a 10 MT/ha winter barley crop can be produced with one to two fewer irrigation applications than a spring crop.**

The potential of winter malting barley remains underexploited because although the ancestral state of barley is winter habit, spring growth habit forms were selected ~ 10,000 years ago and established the foundation for barley production in most of Europe. Early agriculturists, and subsequently plant breeders, selected for malting quality in this spring germplasm base. When barley was introduced into North America, malting types were accordingly of spring habit. Subsequently, sophisticated cropping, contracting, and insurance systems developed for spring barley. As a consequence, needed improvements in winter barley were not made.

Approach

Germplasm: Two types of germplasm resources will be used: (1) doubled haploid (DH) populations and (2) backcross (BC) MAS-derived populations. Space limitations preclude detailed descriptions of the germplasm resources: these are available online at <http://barleyworld.org/OrNe.html>; pedigrees and germplasm flow timelines are shown in Tables 3 and 4. OR71, OR76, and OR813 are sib lines derived from same initial cross. 88Ab536-B is a “grandparent” on both sides of the pedigree. For morphological traits, the three sib lines are quite similar. However, they differ for key malting quality traits and show genetic diversity in SNP haplotype. The cold tolerance levels of OR71 and OR76 are not significantly different (Table 1). OR813 was not included in the Martonvasar freeze test due to seed limitations at the time of the test – subsequent data from controlled environment and field tests suggest it is similar to its sib lines. OR71 was considered for release as a malting variety but was rejected after two years of testing in the AMBA Pilot program. OR76 is in its second year of AMBA testing and is scheduled for seed increase with the prospect of release as a commercial variety. OR813 was re-selected during head row purification of OR76: it has an outstanding quality profile superior to OR76 (Table 2). Both OR71 and OR76 are facultative (*Vrn-H2* deletions); OR813 is a winter (it has *Vrn-H2*); the dominant allele at this locus traces to either the Kold or Strider winter feed barley parents. The Nebraska germplasm accessions trace to very different pedigrees than the Oregon germplasm. All have been advanced through the Nebraska breeding program and shown excellent levels of survival in national and international trials and in our comparative assessment (Table 1). Malting quality profiles of the NB lines are unacceptable (Table 2).

NB03437 (not NB03437-f) was grown in the trials that produced seed for these malting analyses, due to limited seed supply of the latter at the time of these trials.

The BOPA1 and 2 graphical haplotypes of the OR and NB germplasm reveal diversity within and between the two germplasm groups. The full data are too extensive for inclusion in the proposal; for illustrative purposes only the *Fr-H1* and *Fr-H2* regions are shown in Figure 2. Full genome graphical haplotypes are available online at <http://barleyworld.org/OrNe.html>. Interestingly, for these SNPs, there is abundant allelic diversity at *Fr-H2* but monomorphism at *Fr-H1*; this is addressed below under “*Genotyping*”.

Experiments - overview: The DH and MAS germplasm will follow similar paths for genotyping and phenotyping (Tables 3 and 4). Details on specific procedures follows, where appropriate. **In overview, all germplasm resources will be genotyped at Oregon State University (OSU) for specific candidate and target genes and at the Scottish Crops Research Institute (SCRI) for 384-OPA SNPs. All the DH and selected BC-MAS germplasm will be phenotyped for low temperature tolerance in three field environments (Fort Collins, Colorado; Lincoln, Nebraska; and Pendleton, Oregon) over a two year period and in a controlled environment test at the Martonvasar Research Institute (MRI), Hungary. All the DH and selected BC-MAS germplasm will be phenotyped for agronomic traits at two locations in Oregon, over a two year period and samples from these trials will be characterized for malting quality traits at the USDA-ARS Cereal Crops Research Unit (CCRU). In order to fully assess the germplasm produced by this project and to make the germplasm widely available, we propose an International Winter Malting Barley Performance Nursery (IWMBPN) that will test the most winter hardy, high quality lines against the best available checks.** AFRI funds will not support the IWMBPN, as this will be grown as a voluntary effort by interested cooperators after the project is completed.

Genotyping

Target genes: We will genotype target LT, VRN, PPD loci in each of the four germplasm arrays (two DH, two BC-MAS (at BC1F1)). DNA extractions from all germplasm are completed and the screening of target genes is underway. For simplicity, in the remainder of this proposal these target genes will be referred to as *Fr-H1* (where the candidate gene is *Vrn-H1* = HvBm5A); *Fr-H2* (where the candidate gene is one or more of the CBF gene family members); *Vrn-H2* (where the candidate genes are one or more ZCCT gene family members); *Ppd-H1*; and *Ppd-H2*. We have developed and routinely applied primer sets for all loci in various germplasm arrays and have completed screening (and in some cases allele re-sequencing) of the six parents in the panel: the results of the polymorphism screen are online at <http://barleyworld.org/OrNe.html>. As shown in Figure 2, there is SNP monomorphism at *Fr-H1* in the parental germplasm. This monomorphism suggests a lack of functional variation at the determinant(s) of LT in this region. However, it is entirely possible that there are undetected functional variants. In order to test this empirically, we need to test for QTL at this position (in the DH populations) and determine the selection response (in the BC-MAS populations). We are confident of finding polymorphisms for *Fr-H1* based on abundant

polymorphisms in *Vrn-H1* and adjoining sequence based on alignments of BAC clone sequence (unpublished data). If no polymorphisms are found in *Vrn-H1*, we will extend our search for mappable polymorphisms by re-sequencing nearby genes of interest (e.g. *HvPhyC*) and the genomic equivalents of the monomorphic EST-based SNP loci.

Design and application of a new Illumina 384 OPA: A custom synthesized BeadXpress 384-plex Oligo Pool Assay (OPA) will be developed by choosing SNP assays that have high performance quality, high polymorphism information content (PIC) values in the target germplasm, and show even genetic distribution across the barley genetic map. SNPs in genes targeted in the AFRI project (e.g. LT, VRN, PPD, and malting quality-related genes) will be preferentially included if they meet the aforementioned criteria. SNPs will be selected based on data from the two 1,536-Plex OPAs (BOPA1 and BOPA2) which have been used to profile the target Oregon and Nebraska parental germplasm as well as over 5,000 other barley genotypes, including the base germplasm used in this study, as part of the USDA CSREES Barley CAP and UK AGOUEB projects (Rostoks et al., 2006; Hayes and Szucs, 2006). The entire genotyping procedure will be performed as recommended in the Goldengate Genotyping Assay for VeraCode Manual (Illumina VC-901-1001) and will be conducted in the barley genotyping facility at the Scottish Crop Research Institute (SCRI) using DNA provided by Oregon State University (OSU). After conducting the assays, the Veracode bead plates will be scanned using default settings in the VeraScan software on the BeadXpress Reader System and the output analysis files incorporated for analysis into BeadStudio Software. Both raw data and QC-checked and analysed data will be sent to OSU for analysis.

Phenotyping

Controlled environments: By the grant start-date, vernalization sensitivity will have been determined for both DH populations at OSU greenhouse facilities under long-day conditions, following the procedures described by Szucs et al. (2007). The phenotypes measured are final leaf number (FLN) and heading date. The final selections from BC-MAS will be tested for vernalization sensitivity in Year 3 of the grant. The low temperature tolerance of the two DH populations and the final selections from the BC-MAS (F4 generation) will be measured at the MRI phytotron following the procedures first described by (Tischner et al. 1997) and subsequently used for all of our collaborative barley LT projects (Skinner et al. 2006 and citations therein). Briefly, 10 plants of each genotype are grown in each of four replicates, along with standard cold tolerant and cold-susceptible checks. Plants are hardened following a progressive protocol of lowering temperature and shorter photoperiod and finally frozen at the test temperature (typically -13°C for barley) for 24 hours. Percent survival is recorded at the end of a recovery period of 21 days under optimal growth conditions.

Field environments: Three types of data will be recorded from field trials: agronomic, malting quality, and low temperature stress resistance. There are standard protocols for measuring these phenotypes that are used routinely in the participating breeding programs. In the interest of page limitations, these traits are simply listed without describing protocols. Plot sizes, seeding rates, and agronomic

practices (e.g. fertility and weed control) will be in accordance with experience and standard practice at each location. The Corvallis, Oregon test site usually provides intense disease pressure from the fungal pathogens that incite barley stripe rust and scald. The Nebraska parental germplasm is quite susceptible to these pathogens whereas the Oregon germplasm has quantitative resistance. Accordingly, all of the germplasm grown at this location will be fungicide-protected. Based on past experience, even with fungicide protection, disease data (percent severity for stripe rust and 1-9 scale ratings for scald) can be obtained later in the season. These disease data will allow us to leverage mapping of disease resistance genes, although this is not a primary objective of the AFRI project. If necessary and resources permit, trials without fungicide will also be conducted. In all tests of the DH lines, an augmented design 8 times and 35 lines per block will be used. If necessary and resources permit, trials without fungicide will also be conducted. In all tests of the DH lines, an augmented design with 6 checks repeated 8 times will be used (280 DH lines and 48 checks). In all tests of the BC-MAS lines, an augmented design with 6 checks repeated 3 times will be used (96 BC-MAS lines and 18 checks).

Agronomic traits: The following traits will be measured at Corvallis (a high (~1000 mm/year) rainfall site) and Pendleton, Oregon (a moderate (~300 mm/year) rainfall site) on all germplasm for each of two years: heading date, plant height, test weight, lodging, stripe rust and scald resistance, and other traits as opportunity presents (e.g. resistance to Barley Yellow Dwarf Virus, net blotch, leaf rust, mildew, and/or common root rot).

Malting quality traits: The following standard set of malting quality traits will be measured (or calculated) on/for samples of each entry, and checks, from the Corvallis and Pendleton experiments for both the DH and BC-MAS germplasm: kernel weight, 6/64 sizing, barley color, malt extract, wort color, wort clarity, barley protein, wort protein, the soluble/total protein ratio, diastatic power, alpha amylase activity, and wort beta glucan. These traits will be measured following standard American Society of Brewing Chemist (ASBC) protocols, as described in detail by Duke and Henson (2008). We have budgeted for malt analyses on a subset of samples from the Nebraska winter hardiness screening trials (see next section). There is no way to know in advance what percentage of lines will survive and produce sufficient seed (~250g) for malt analysis. Busch Agricultural Resources, LLC has an in-house malt analysis lab. If capacity and resources allow, they may be able to conduct malt analyses on a subset of samples from the Fort Collins winter hardiness screening trials.

Winter hardiness : The same DH and BC-MAS germplasm phenotyped at Corvallis and Pendleton will be grown at Fort Collins, Colorado and Lincoln (or Mead) Nebraska for field assessment of cold tolerance. Because the objective of these screening trials is to assess cold tolerance (measured as a visual estimate of percent survival) and not agronomic or malting quality traits, the germplasm will be grown in smaller plots with more replications. In all tests at these locations, an alpha lattice design with three replicates will be used. This design allows for optimum estimates of line performance as well as spatial analysis to correct for non-uniformity of the field. There will be a common set of checks in all field trials consisting of the parental lines of the DH populations and the BC-MAS derived lines.

Possible additional phenotyping: Cooperators in the US and abroad have expressed interest in assessing the DH and/or BC-MAS germplasm for agronomics, malting quality, and/or field survival. Facultative lines could be of interest for reproductive LT research and as potential varieties under spring-sown conditions.

Analyses

Linkage and QTL mapping in DH populations: Linkage maps for each DH population will be created using Joinmap 4.0. The population sizes (130 and 150) will complement estimates of OPA SNP map positions based on consensus maps (HarvEST; <http://www.harvest-web.org/>) or 96 individuals (Szucs et al., 2009). A skeleton map for QTL analysis will be created by removal of co-segregating markers. Positions and additive affects of the QTL will be estimated using composite interval mapping (CIM) (Zeng 1994) as implemented in Windows QTL Cartographer 2.5 (Wang et al. 2005). Epistatic interactions between QTL will be tested using Multiple Interval Mapping (MIM, Zeng et al. 1999) using QTL Cartographer. We will purchase MapQTL 6.0 (<http://www.kyazma.nl>) with other funds prior to the start of the AFRI project. This software will be useful for integrating results from the DH and BC-MAS as it allows for QTL analysis for any advanced generation derived from a backcross.

The following data sets (and number of environments per set) will be available for QTL analysis: (1) vernalization response (2 traits); (2) agronomics (~ 8 traits x 2 years x 2 locations); (3) malting quality (~ 12 traits x 2 years x 2 locations); (4) low temperature tolerance (1 controlled freeze test; minimum of 4 field tests (2 per year for 2 years in Nebraska and Colorado) and possibly at 2 locations in Oregon for 2 years (if low temperature stresses occur). The DH populations are larger than those previously used for mapping the target traits, and this should provide better estimates of QTL parameters. The use of a common parent for both populations offers additional prospects for analysis. The large number of environments sampled will create a robust dataset, allowing for optimum estimates of main effects and interactions. The QTL and candidate gene information from these DH populations will be essential for designing future MAS efforts.

Marker assisted selection: The MAS scheme for only one population is shown in Figure 3 due to space limitations: the full scheme is online at <http://barleyworld.org/OrNe.html>). For simplicity, alleles at *Fr-H1* and *Fr-H2* are referred to as “Or” or “Ne” if they trace to Oregon (OR76 and/or OR813) vs. Nebraska (NB03437-f or NB18199). This scheme assumes that we will identify polymorphisms in *Vrn-H1*, or linked markers, that will allow us to distinguish between the two Or lines (since the MAS populations have three-parent pedigrees, each with two OR parents) and each of the Ne parents. If we are not able to identify polymorphisms at *Fr-H1*, we will increase in the number of plants in the other two allele combination classes. With or without *Vrn-H1*, there will still be 96 BC1F3 and 96 BC1F4 plants. We will start MAS with 120 and 150 BC1F1 plants in the OR76/NB03437-f //OR813 (hereafter referred to as MAS-1) and OR76/NB18199 //OR813 (hereafter referred to as MAS-2) populations, respectively. In order to empirically assess the impact of vernalization sensitivity on low temperature tolerance, we will target *Vrn-H2* heterozygotes in the first round of MAS in both

populations. In MAS-1, we will start with $n = 120$ *Vrn-H2* heterozygotes. In MAS-2 we will pre-screen for the ~ 129 *Vrn-H2* heterozygotes. Subsequently, population sizes and procedures will be comparable for the two populations. All MAS-1 BC1s and the ~ 129 MAS2-BC1s will be genotyped with the 384 OPA (SCRI). For *Fr-H1* and *Fr-H2* we will target the following allele configurations at *Fr-H1/ Fr-H2*: OrOrOrNe; OrNeOrOr; and OrNeOrNe. After selection based on the target *Vrn* and *Fr* loci, we will use the 384-OPA data for genomic selection for malting quality based on breeding value prediction from markers in malting quality QTL regions (*see Introduction – Marker assisted selection*). In the BC1F2 we will start with ~ 960 plants and select ~ 68 , representing homozygotes for the following combinations at *Fr-H1* and *Fr-H2*: OrOrNeNe; NeNeOrOr; and NeNeNeNe. These homozygotes will occur in various combinations with alleles at *Vrn-H2* and *Ppd-H2* (also in the homozygous condition). In the BC1F3 we will start with ~ 68 homozygotes for target loci and reduce this to ~ 48 for phenotyping and advance. These ~ 48 BC1-F4 MAS-derived lines will be genotyped, at a minimum, with the 384-OPA. If resources permit, full BOPAs 1 and 2 will be used, or the most comprehensive genotyping platform available in 2012. The phenotyping procedures for agronomic traits, malting quality, and low temperature tolerance are described in the preceding *Phenotyping* section.

International Winter Malting Barley Performance Trial (IWMBPT)

An international collaborative trial is proposed after the AFRI project end date (2012-2013). Participation will require commitments by participants (*see Letters of support*). The IWMBPT will serve as a mechanism for generating international visibility for AFRI-supported research, a validation of AFRI-supported research findings, and a mechanism for germplasm distribution. Optimistically, it will also be useful for generating data for variety release. The replicated trial will consist of a limited number of entries (~ 50) that can be accommodated by the ongoing testing programs of all participants. These entries will consist of the most cold tolerant lines, with superior quality profiles, developed during the AFRI project and the best varieties/promising lines submitted by cooperators. Agronomic, malting quality, growth habit, and low temperature tolerance data will be obtained by each participant following standard protocols. The results will be made available in an international peer-reviewed publication and the data deposited with GrainGenes. OSU will coordinate the trial, analysis, and reporting.

Storage and distribution of tools and resources

Samples of the BD and BC-MMAS germplasm will be deposited with the National Small Grains Collection (*see Letter of Support from Dr. Harold Bockleman, Curator*). All genotype and phenotype data will be curated and deposited with GrainGenes (*see Letter of Support from Dr. Victoria Blake, Curator*), as we have with prior map data sets. All data, analyses and interpretation will be subject to peer review for publication in international journals. A standard Research Materials Transfer Agreement accompanies all germplasm distributed under the auspices of Oregon State University.

Figure 1. Genotype (left panels) and phenotype (right panel) images for one of two NB03437 facultative (f) plants in a population of ~ 200 winter (w) plants. Results were consistent for both 'f' vs. all 'w' plants. All 'f' and 'w' plants had a full-length first intron in *HvBM5 (VRN-H1)* (upper left). *ZCCT-H (VRN-H2)* was present in all 'w' plants and deleted in the two 'f' plants (lower left).

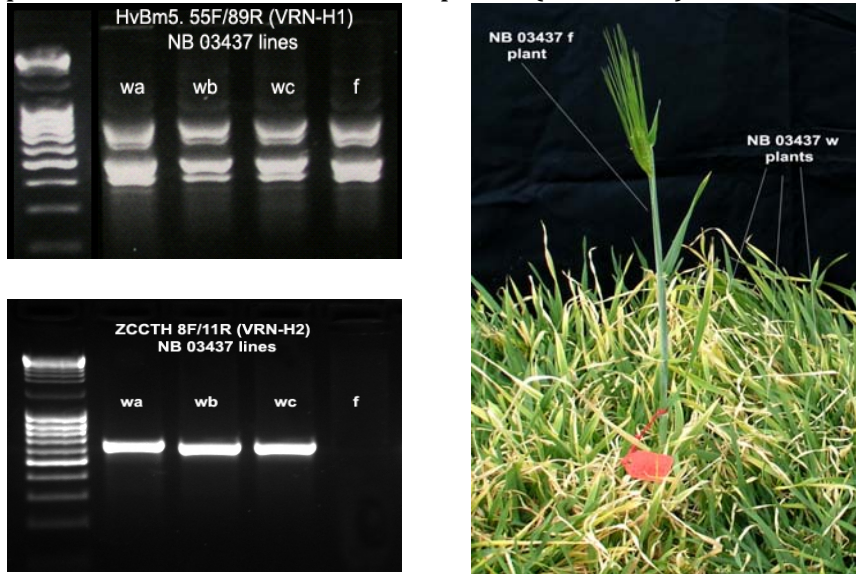


Table 1. Percent survival of AFR germplasm parents after freezing at -13.5° C in the Martonvasar Research Institute (Hungary) phytotron. NB03437f is the facultative re-selection from NB03437.

Accession	Survival %	Growth habit
NB018199	90.9	Winter
NB03437-f	87.8	Facultative
DICKTOO	84.2	Facultative
NB713	77.7	Winter
OR76	75.8	Facultative
OR71	72.9	Facultative
MOREX	7.3	Spring
	LSD(.05) = 8.9%	

Table 2. Malting quality of Oregon (OR) and Nebraska (NB) germplasm from three environments; 2008 harvest.

Accession	On 6/64" (%)	Barley Protein (%)	Malt Extract (%)	S/T (%)	DP (°ASBC)	Alpha-amylase (20° DU)	Beta-glucan (ppm)
OR71	94	10.8	79.6	34.8	89	44.0	301
OR76	97	12.4	79.6	38.8	91	64.3	179
OR813	98	11.4	81.7	42.8	110	76.8	124
NB018199	48	11.9	72.4	36.2	99	36.2	331
NB03437*	54	11.9	73.2	34.9	90	38.9	260
NB713	76	11.6	74.7	33.3	87	33.1	602

*NB03437-w. Data courtesy of Cereal Crops Research Unit (CCRU; Madison, WI).

Table 3. Flow of doubled haploid (DH) populations for the AFRI proposal.

Season	OR71/ NB03437-f (n = 130)	OR71/ NB713 (n = 150)	Action
Spring 09	+	+	DNA extraction (OSU)
	+		FLN, HD (OSU)
Summer 09		+	FLN, HD (OSU)
Fall 09	+		Freeze test (MRI)
Winter 09/ 10	+	+	Plant Agronomics (AG) (OSU); Plant winterhardiness (WH) trials (BARI,UNL)
Spring/ Summer 10	+	+	Rate WH trials (BARI,UNL) Harvest AG trials (OSU)
	+	+	OSU Samples to CCRU for malt quality
Fall 10	+	+	QTL analysis (OSU)
		+	Freeze test (MRI)
Winter 10/11	+	+	Plant AG (OSU); Plant WH (BARI,UNL)
Spring / Summer 11	+	+	Rate WH trials (BARI,UNL) Harvest AG trials (OSU)
	+	+	OSU Samples to CCRU
Fall 11	+	+	QTL analysis (OSU)
Winter 11/12	+	+	Final genotyping/ phenotyping (OSU)
Spring 12	+	+	Publish
<i>Winter 12/13</i>	<i>subset</i>	<i>subset</i>	<i>International performance nursery</i>

Table 4. Flow for BC-MAS populations - OR76/NB03437-f//OR813 (n = 120) and OR76/NB18199//OR813 (n = 257) - for the AFRI proposal.

Season	Generation	Action
Spring 09		DNA extraction (OSU)
Summer 09	BC1F1	Foreground genotyping (OSU)
Fall 09	BC1F1	Background genotyping (SCRI, OPA)
	BC1F1	MAS (OSU)
Winter 09/10	BC1F2	Foreground genotyping (OSU)
Spring 10	BC1F2	MAS (OSU)
Winter 10/11	BC1F3	Plant AG (OSU); Plant WH(BARI,UNL)
Spring/Summer 11	BC1F3	Rate WH (BARI,UNL); Harvest AG (OSU)
	BC1F3	OSU Samples to CCRU
Winter 11/12	BC1F4	Plant AG (OSU); Plant WH (BARI,UNL) Freeze test (MRI); Final genotyping (SCRI)
Spring/Summer 12	BC1F4	Rate WH (BARI,UNL); Harvest AG trials (OSU)
Summer 12	BC1F4	OSU Samples to CCRU
Fall 12		Publish
<i>Fall 12/Winter 13</i>	<i>subset</i>	<i>International performance nursery</i>