**Genome-wide Association Studies (GWAS) and Genetic Diversity of Barley** (*Hordeum vulgare* L.) **genotypes** **Towards to Cold Stress Tolerance in Central Highlands of Ethiopia**

**Chapter 1. General Introduction**

Barley (*Hardeam valgare* L.) is consumed all around the world as the main cereal crop. According to the FAO data, nowadays, it is one of the ancient grain crops cultivated and used worldwide grown over a broader environmental range than any of the cereal, and the average harvested area for barley is estimated at 504,000 ha worldwide (FAO, 2020). Abiotic stress tolerant crops will probably be keys to food security by helping agriculture to cope with climatic changes. Barley has gained particular interest owing to providing a wide range of allelic variants, which could explain the degree of adaptive competence and plasticity of Hordeum and help plant breeding efforts for stress tolerance. It is an excellent model system to study plant response to adverse environmental conditions (Francia et al. 2004). Cold stress in barley is a complex quantitative trait significantly influenced by the environment. Due to this low heritability and dependency on environmental conditions, the direct selection of barley for cold tolerance is time-consuming and less effective (Adhikari *et al.,* 2022). Cold stress can cause foret and spike abortion as well as damage to the developing grain, which could have a significant impact on barley yield and yield components (Tyrka *et al.,* 2015). To reduce the negative effects of cold stress on barley production, it is necessary to identify genomic regions associated with cold tolerance (Fiust *et al.,* 2020).

Plant breeders require access to novel genetic variants to develop barley varieties that can adapt to changing abiotic stressors, such as cold stress. In addition, research on the genetic mechanisms and discovery of genes affecting cold tolerance would be helpful for developing cultivars with strong cold tolerance (Pan et al. 2015). Therefore, efforts to improve cold tolerance need to be continued and strengthened since this improvement has been a major target of global plant breeding programs (Bengtsson *et al.,* 2017). Even though crop-breeding programs have not overtly bred for improving low-stress tolerance traditionally, they have focused on maximizing yield instead when selection pressure for these factors is relatively low (Gilliham *et al.,* 2017). Hence, breeders are extremely interested in new technology offers, such as the possibility to improve the selection strategies in barley breeding by adopting a wide range of novel approaches. Nowadays, the molecular markers system is an effective tool and an important strategy for improving the cold tolerance between various varieties of barley. Information value of the SNPs technique, which is a powerful tool for genetic diversity and association analysis in barley breeding, has been frequently confirmed in several investigations (Lai *et al.,* 2017; Wabila *et al.*, 2019; Jabbari et al. 2021). Moreover, this technique can be valuable for marker-assisted selection to improve cold tolerance in barley breeding (Adhikari *et al.,* 2022). The genetic diversity within and between crop plant species permits the breeders to develop new cold-tolerant varieties with desirable characteristics (Pan *et al.,* 2015). In addition, evaluating the genetic diversity of barley lines using SSR markers is important in barley breeding for successful exploration, genetic stability, and effective conservation, because morphological characters are limited in number and unstable (Teklemariam *et al.,* 2022). QTL mapping is well suited to the detection of genes associated with particular traits, yet is costly and time-consuming for developing the mapping population (just a few cycles of recombination). To overcome these limitations, the identification of molecular markers associated with traits of interest through multiple regression analysis has been adopted in barley so far (Wang *et al.,* 2012; Cheghamirza *et al.,* 2017; Jamali *et al.,* 2017; Beheshtizade *et al.,* 2018). The identification of molecular markers associated with a trait of interest would be an efficient approach to enhancing barley breeding programs (the collections of germplasms, the detection and analysis of potential in specific genotypes, the identification of desirable alleles, the validation of candidate markers linked to quantitative traits, and indirect selection using marker-assisted selection method) (Singh *et al.,* 2020). The objectives of the current study were as follows:

* 1. **Objectives**
* To assess the severity and incidence of cold stress on Barley in most hotspot areas in Central Highlands of Ethiopia
* To see the field response and genetic variability of Barley genotypes for Cold stress in Central Highlands of Ethiopia
* To understand the genome-wide association Analysis of Cold Stress for adult plant resistance of Barley genotypes in Central Highlands of Ethiopia
* To investigate the genome-wide association mapping of Cold Stress Tolerance of Barley genotypes in Central Highlands of Ethiopia

**2. General Materials and Methods**

**Activity 1: Assessment of the Severity and Incidence of Cold Stress on Barley (***Hordeum vulgare*L.**) in most hotspot areas in Central Highlands of Ethiopia**

* 1. **Introduction**
  2. **Materials and Methods**

**Activity 2: Field response and genetic variability of Barley (**Hordeum vulgareL**.) genotypes for Cold stress in Central Highlands of Ethiopia**

**2.1. Introduction**

**2.2. MATERIALS AND METHODS**

**2.2.1. Description of study area**

The experiment will conduct at Mekdela Amba University College of agricultural and natural resource plant science demonstration site in 2017 E.C under off-season irrigation system. The area is geographically located 481km from Addis Abeba to southwest direction. The latitude is about 10.988962 and 39.255822 longitude its altitude is 3206. 34100ma.s.l. the annual rainfall of the area is 150-750 mm and the annual average temperature is 15-200C0 (south wollo zone Agricultural office, 2019).

**2.2.2. Planting Material**

The cold stress-screening panel will contain 240 barley genotypes from Ethiopian biodiversity institute (EBI) and 10 Ethiopian released cultivars making 250 wheat lines.

**2.2.3. Filed experiments**

The field experiment will conduct under natural infestation conditions during the 2026 and 2027 growing seasons at Mekdela Amba University College of agricultural and natural resource plant science demonstration site under off-season irrigation system in Ethiopia. The trial will conduct in a partially balanced lattice design with two replications, where each replication will further divided into 25 rows and 10 columns. Each genotype will planted in a plot of 1 m long and 0.4 m wide, consisting of two rows per plot. The distance between adjacent plots was 0.4 m. Seed rate will be150 kg/ha, with seeds drilled evenly in rows, and fertilizer will apply as per the recommendation of each specific area.

**2.2.4. Data collection**

The set of genotypes will evaluate for cold tolerance screening in the field under natural conditions on a cold tolerance rating (CTR) scale of 1-9 (where, 1 = no visible damage of seedlings, 2 = highly tolerant, 3 = tolerant, 4 = moderately tolerant, 5 = intermediate, 6 = moderately susceptible, 7 = susceptible, 8 = and highly susceptible, and 9 = 100% total seedling damage or dead), as described by Singh et al (1989). Data will recorded for cold stress tolerance in the month of the first sowing after the plants exposure to cold temperature in the fall of winter. Seedlings will evaluate for cold tolerance by the following formula:

Incidence of cold stress (%) = Dead seedlings X 100

Total seedlings (Dead +Normal seedlings)

|  |  |
| --- | --- |
| Score | Description of Cold stress Severity |
| 1 | No cold stress/ Highly resistant/ |
| 2 | 1-5 % affected/ |
| 3 | 6-10 % affected/Resistant/ |
| 4 | 11-20 % leaves affected |
| 5 | 21-30% affected/ Intermediate/ |
| 6 | 31-40% leaves affected |
| 7 | 41-60% affected/ Susceptible/ |
| 8 | 61-80% damage |
| 9 | 81-100% affected, almost all seedlings withered and bare stem seen /Highly susceptible/ |

**2.2.5. Other agronomic data recording**

The phenotypic data will record days to heading (when the spikes of 50% of the plants are fully visible), plant height (average height of five plants will measure from the ground to the tip of the spike excluding the own [cm]), spike length (average length of five spikes containing grains [cm]), and grain yield (gm/plot). These yield data will take from the whole plot and will convert to kilograms per hectare (kg ha-1) at 12.5% moisture content using plot size as a factor.

**2.2.6. Data analysis of phenotypic traits**

The phenotypic data of each trial and the combined environment will analyze using the Multi-environment Trial Analysis with R ‘metan’ software package (Olivoto and Lúcio, 2020). The analysis of variance (ANOVA) of the trait will calculate, including genotype, year, location, block, and replication (R Software Core Team, 2020)

**Activity 3: Genome-Wide Association Analysis of Cold Stress for adult plant resistance of Barley (***Hordeum vulgare* L.**) genotypes in Central Highlands of Ethiopia**

**3.1. Introduction**

Cold stress is one of the climate change consequences affecting stable food production. In the last decade, temperature increases and extremely decreases expected for many agricultural land so of the world (jabbari *et al.,* 2018). Thus, agricultural lands are the most severely affected by climate change with extremely low temperature. Several morphological and physiological traits in barley contribute to cold stress tolerance (Chen et al., 2010; Del Pozo et al., 2012) which indicates the interactions of the environment and the genotype. Understanding the genetic basis of important traits under stress conditions can improve breeding approach (jabbari *et al.,* 2018).

Cold acclimation initiates when plants integrate and respond to environmental signals received from gradual reductions in photoperiod, daily temperature, light intensity, and red/far-red ratio in incoming light10, 11. An early start and long acclimation period result in higher winter hardiness for winter cereals, and thus, the timing of floral transition impacts the total amount of accumulated freezing tolerance12. Cold acclimated cereals further increase their freezing tolerance when exposed to slightly below 0 °C temperatures, which stimulate a second hardening process13. Transcriptional analyses show different sets of genes are induced during the above-zero and sub-zero cold hardening processes14. Winter-hardy plants undergo a multitude of developmental adjustments during cold acclimation to build up frost resistance8. Te morphological changes observed in cereals generally include a switch to prostrate growth habit (PGH), compact growth, strengthening of cell walls, changes in membrane structures, and increased number of leaf initials formed at SAM12,15-18. The biochemical changes involve production of various cry protectants, antioxidants, and antifreeze proteins to aid protection against future frost damage19,20. An enhanced photosynthetic performance is observed for the most winter-hardy cereals and results in higher biomass production and reduced susceptibility to photo inhibition as compared to tender genotypes21. Through the modification of photosynthesis, winter-hardy cereals can effectively increase their carbon pools in crown tissues to be retrieved during deaclamation incidents throughout winter and regrowth in the spring. Cold acclimation also causes epigenetic changes at the DNA and histone levels with effects on gene activities22, 23. Overall, cold responses are coordinated by an extensive cross-talk between cold-induced Ca2+, reactive oxygen species, phytohormone, and light signaling pathways10, 24-26, of which retrograde/anterograde stress signaling between plastids and nucleus are suggested to play a central role Monica (Baga et al., 2022).

**3.2. Materials and Methods**

**3.2.1. DNA Extraction**

**3.2.1. DNA Sequencing**

**3.2.1. Multi-environment Trails**

The study materials will evaluate under natural infestation conditions during the 2026 and 2027 growing seasons at two seasons and two locations in Ethiopia. The trials will plant. The experimental design was a partially balanced lattice with two replications, each of which was further divided into 25 rows and ten columns. Each genotype will plant in two rows, with a length of 1 meter and a row-to-row distance of 20 cm, and a plot-to-plot distance of 0.4 meters. The distance between adjacent plots was 0.4 m. The seed rate will be 150 kg/ha, with seeds drilled evenly in rows, and fertilizer will apply according to the recommendations for each specific area.

**3.2.2. Cold stress Tolerance evaluation**

The set of genotypes will evaluate for cold tolerance screening in the field under natural conditions on a cold tolerance rating (CTR) scale of 1-9 (where, 1 = no visible damage of seedlings, 2 = highly tolerant, 3 = tolerant, 4 = moderately tolerant, 5 = intermediate, 6 = moderately susceptible, 7 = susceptible, 8 = and highly susceptible, and 9 = 100% total seedling damage or dead), as described by Singh et al (1989)(See above section, 2.2.4).

**3.2.3. Data analysis**

**3.2.3.1. Phenotypic data analysis**

The combined analysis of variance (ANOVA) will conduct for four environment, and three variance components, the environmental variance, genotypic variance and interaction of the genotype and environmental variance will calculate for cold stress tolerance using restricted maximum likelihood estimation procedure using MATA-R version 6.0 software.

**3.2.3.1. Population structure analysis**

To know the population structure of barley cold stress panel, a model-based Bayesian cluster analysis will perform using STRUCTURE software (v.2.3.4). The program will run for three replicates for every suppose subpopulation ranking from k=1 to k=10 under the admixture model of population structure. Burn-in iteration will 20,000 followed by 20,000 Markov chain Monte Carlo (MCMC) replications after burn-in for each run. To identify the optimum number of sub-populations/clusters the best K value will used. The best K value will obtain as Delta K (ρK) from structure harvester using the log probability of the successive structure repetitions (Evanno et al., 2005).

**3.2.3.2. Genotyping and genome-wide association analysis**

The barley cold stress tolerance panel will genotype using 20K SNP array from Trait Genetics containing 23,197 SNP markers. After quality control, such aount of SNP markers will search to be informative and applicable for GWAS analysis. Association mapping of cold stress tolerance trait with genome-wide SNPs will determine by performing a single-trait GWAS following a single locusmixed linear model (MLM) analysis using the statgen GWAS R software package (van Rossum and Kruijer, 2022) in the R software (R Core Team, 2024). The vanRaden kinship matrix will include to account for hidden relatedness (VanRaden, 2008). Quantile-quantile (QQ-plot) generated using–log10 p-value will visually assesse to determine how well the model accounte for population structure and family relatedness between study samples. An arbitrary threshold to declare significant marker-trait associations (MTA) P < 0.001(-log10 [P] > 3.0) will used as describ by (Alemu et al., 2021a, 2021b). StatgenGWAS R will used to visualize the Manhattan and Q Q plots. High-confidence candidate genes from the identified resistance-associated regions will retrieve using the latest IWGSC RefSeq Annotation v2.1, available on (https://wheat-urgi.versailles.inrae.fr/Seq-Repository/Annotations).

**Activity 4: Genome-Wide Association Mapping of Cold Stress Tolerance of Barley (***Hordeum vulgare*L.**) genotypes in Central Highlands of Ethiopia**

**4.1. Introduction**

The genetic components underlying the response of plants to the photo-thermal environmental cues affecting seasonal and local adaptation are key factors that limit crops’ geographical distribution and yield potential (Andrea *et al.,* 2013). Therefore, as they have critical implications for agricultural productivity, such components have become an important focus of applied research. The winter hardiness of temperate cereals refers to the ability of plants to withstand the chilling and freezing temperatures that occur during the winter season (cold tolerance), and is also associated with vernalization requirement and photoperiod sensitivity, to maximize yield potential whilst minimizing the risk of damage due to abiotic stresses. In economically important temperate cereals, like wheat and barley, the range of cultivated germplasm can be divided into winter, facultative and spring types, depending upon their requirement for vernalization to flower and set seed. In particular, facultative genotypes although cold tolerant and able to survive winters can be sown in spring as well and set seed without vernalizing (Hayes *et al.,* 1993). The genetic control of frost tolerance is complex and is the final manifestation of several component traits. Plants become tolerant to the effects of freezing through the acclimation process or hardening, i.e. a relatively slow, adaptive response during late autumn, signaled by a gradual decrease in temperature, day length and light intensity leading to a series of biochemical changes enhancing the cold resistance of sensitive tissues. Frost tolerance also depends on the intrinsic capacity of the vegetative tissues to survive freeze-induced desiccation, and their efficient recovery from the stress (Pecchioni et al 2012).

Frost tolerance is a key trait with economic and agronomic importance in barley because it is a major component of winter hardiness, and therefore limits the geographical distribution of the crop and the effective transfer of quality traits between spring and winter crop types. Three main frost tolerance QTL (Fr-H1, Fr-H2 and Fr-H3) have been identified from bi-parental genetic mapping but it can be argued that those mapping populations only capture a portion of the genetic diversity of the species (Andrea *et al.,*2013).

**4.2. Materials and methods**

**4.2.1. Genetic materials and experimental design**

About 250 barley genotypes with their passport data will source from the Ethiopian Biodiversity Institute (EBI), Addis Ababa. Additionally, 9 improve varieties and 5 released landraces will used as check cultivars will acquired from the National barley Improvement program at Holeta Agricultural Research Center, part of the Ethiopian Institute of Agricultural Research (EIAR), Addis Ababa. The SNP markers dataset will extract from the resequencing of barley accessions. The field experiment will conduct under natural infestation conditions during the 2026 and 2027 growing seasons at Mekdela Amba University College of agricultural and natural resource plant science demonstration site under off-season irrigation system in Ethiopia (See detail above section, 2.2.3).

**4.2.2. Phenotyping**

Accurate and well-characterized data for the traits of interest, specifically agronomic and yield-related traits will collect. Five plants from each row will randomly select according to the type of traits being measure, including days to flowering, days to maturity, plant height, seed number per plant, grain yield, and thousand seed weight. The missing and unrepresentative phenotypic data was imputed by SAS JMP V.5 [72]. Data will normalized and standardized by the Shapiro-Wilk statistics test at P>0.05 (Soto-Cerda, 2020).

**4.2.3. Genotyping**

GWAS was conducted using genotyping by sequencing (GBS) [73]. The GBS procedure [74], utilized the ApeKI restriction/incision enzyme (recognition site of G|CWCG) to generate the GBS library, which was then sequenced on Illumina HiSeq2500 lanes [75]. SNP markers were extracted from the resequencing data of 1,628 sorghum accessions [76]. The SNP dataset was filtered to exclude SNPs with an MAF of less than 0.05 missing values. The remaining missing values were imputed using the Beagle 5.0 software package [77], resulting in 50,165 SNPs. To ensure data quality, the SNP dataset was again filtered to exclude any SNPs with a MAF of 0.00002, calculated from the expression 4×4.614×4.61, which corresponds to a likelihood ratio test derived from an LOD score of 4. Under the null hypothesis, this likelihood ratio follows a chi-square (χ²) distribution with one degree of freedom [78]. Furthermore, only SNP markers identified in at least three different models were considered reliable for agronomic and yield-related QTNs. Similarly, QTNs that were detected in three or more models and demonstrated a phenotypic variation of R² > 10% were classified as major QTNs.

**4.2.4. Data analysis**

The phenotypic data were analyzed using a mixed linear model (MLM) approach implemented in the “asreml-R” R package [79].

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