

IMPROVING BARLEY RESILIENCE TO HEAT AND DROUGHT: GENETIC AND  
PHYSIOLOGICAL INSIGHTS INTO STAY-GREEN, ROOT-MEDIATED GENE  
EXPRESSION, AND DUAL-PURPOSE QUALITY TRAITS

by

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## ABSTRACT

Barley (*Hordeum vulgare* L.), a major cereal crop grown globally for feed, forage, and malting, is increasingly threatened by abiotic stresses such as heat and drought. These conditions reduce biomass, digestibility, starch accumulation, and grain filling, while increasing grain protein concentration by altering the timing of senescence. Such losses undermine profitability for growers, reducing forage value and compromising malting quality. This dissertation investigates the genetic and physiological basis of barley's response to heat and drought through three integrated studies. First, genome-wide association study (GWAS) was conducted in two diverse iCore populations across irrigated and dryland environments to dissect the genetic architecture of forage traits. Biomass and development traits showed high heritability, while digestibility traits were more environmentally responsive. Several QTL were identified that maintained forage quality without compromising yield. The second study assessed the performance of near-isogenic lines (NILs) harboring stay-green alleles at four QTL (*QGFhd-2H*, *QLN-2H*, *QGFmt-6H*, and *QGF-7H*) under heat stress and control conditions. Under heat stress, stay-green NILs demonstrated improved tillering and reduced grain protein compared to non-stay-green lines. Trait correlations revealed key trade-offs, particularly between vegetative growth and grain protein under stress, emphasizing the importance of optimized assimilate allocation. The third project evaluated the role of *HvNAM-1*, a NAC transcription factor regulating senescence and protein accumulation, in modulating root responses under both control and combined heat-drought conditions. Root transcriptomic analysis of NILs differing in alleles for *HvNAM-1* revealed an allele that promoted root longevity and activated stress-protective pathways under combined heat and drought. These responses included enhanced expression of genes related to oxidative defense, and membrane stabilization in ND lines, suggesting root-based resilience complements the stay-green phenotype. The identified QTL and gene expression profiles offer promising targets for breeding cultivars with improved yield, quality, and resilience across diverse environments.

## CHAPTER ONE

## INTRODUCTION

Global agriculture today faces rising challenges from a rapidly changing climate, threatening both crop productivity and quality. Among the most pressing concerns are abiotic stresses such as extreme temperatures, drought, and salinity, which are increasingly frequent and intense across major agricultural regions. Plants, being sessile organisms are affected by adverse environmental conditions such as non-optimal temperatures alone or in combination with non-optimal water, metal and nutrient availability. These stresses, especially when occurring during critical growth stages, impose substantial limitations on yield and quality. The variability in crop yields is largely driven by such abiotic stresses, underscoring the urgent need for breeding stress-resilient cultivars to ensure global food security. Among the various stresses, high temperature and drought are especially detrimental, and their frequency is rising with each passing decade. These stresses can occur individually or in combination, with synergistic effects that amplify the damage to plant systems. For example, the duration, frequency and intensity of heat waves in US have increased notably (NOAA, 2024), while elevated temperatures contributed to 61% of the 2020-2022 drought severity through increased evaporation (NOAA.Climate.gov). Furthermore, heat events without accompanying precipitation lead to significant water imbalances (Kopecká et al., 2023).

The combined impact of heat and drought stress can be particularly severe, often exceeding the effects of either stress alone. The extent of damage depends greatly on the growth stage at which the stress occurs. During the vegetative stage, high temperatures and water deficit reduce photosynthetic rates, ultimately leading to lower biomass and forage yield (Buxton & Fales, 1994).

When stress occurs at the reproductive stage, it impairs critical processes such as pollen germination, viability, tube growth, and ovule fertility (Callens et al., 2023), ultimately resulting in advanced senescence, reduced grain number, lower weight and starch content (Hör et al., 2025; Shirdelmoghanloo et al., 2022).

Barley (*Hordeum vulgare* L.), the fourth most important cereal crop globally, serves diverse end uses including animal feed (80–90%), malt production (10%), and human consumption. Morphologically, barley is categorized into two-row and six-row types based on kernel arrangement on the spike. Two-row barley is typically preferred for malt production due to its higher starch content, whereas six-row types are favored for feed due to their greater protein content (Miralles et al., 2021). Barley seeds generally contain 8-30% protein and approximately 60% starch, the most abundant component (Jaeger et al., 2021), which includes 3-11% of the polysaccharide  $\beta$ -glucan (Skendi et al., 2003).

As a forage crop, barley is valued for its balanced composition of digestible fiber, starch content, and protein levels (Nikkhah, 2013). Forage/biomass yield and forage quality are the key traits of focus to benefit forage growers. Forage quality is often evaluated through measures of intake potential and digestibility, typically assessed using neutral detergent fiber (NDF) and acid detergent fiber (ADF). NDF estimates levels of cellulose, hemicellulose, and lignin, while ADF captures cellulose and lignin components. Lower NDF and ADF levels correspond to improved digestibility. However, it is well documented that forage yield and quality are negatively correlated because higher biomass often results in increased stem-to-leaf ratio and greater lignification, which diminishes digestibility (Lin et al., 2021). As lignin accumulation intensifies with plant maturation, forage quality is also affected by the plant's developmental timing, which is something that abiotic

stress factors such as drought and high temperature can alter. The degree and timing of these stresses ultimately determine their impact on forage attributes. Given the sensitivity of these traits to both developmental stage and environmental cues, exploring the genetic basis of their variation becomes essential for improving stress-resilient forage varieties. Prior research has shown substantial genetic variation in forage quality traits, and mapping this variation to specific genomic regions can help guide breeding decisions

Although QTL for ADF and NDF have been reported in a biparental population of cross between Steptoe and Morex under irrigated conditions (Siahsar et al., 2009; Surber et al., 2011) these studies largely focused on performance under optimal environments. For instance, (Surber et al., 2011) identified QTL for forage traits measured at anthesis and peak biomass stages and reported greater heritability of traits at anthesis, suggesting better selection efficiency at that stage. However, the genetic basis of forage yield and quality under abiotic stress, particularly high temperature and water deficit, remains underexplored in barley. As the frequency of climate induced stresses increases, identifying genotypes with resilience to heat and drought without compromising forage value becomes crucial. To address this gap, we utilized a genome-wide association study (GWAS) approach in a diverse barley panel to uncover QTL linked to forage yield and quality under variable moisture and temperature conditions.

Malt barley quality is highly sensitive to environmental conditions, particularly temperature and water availability. For optimal malting performance, grain protein content should typically fall between 10-12%, while higher levels are preferable for animal feed. Beta-glucan and protein content, both critical to end-use quality, are known to fluctuate under abiotic stress (Eagles et al., 1995; Zhang et al., 2001). High temperatures and water deficit accelerate senescence and

reduce the grain filling period. Combined heat and drought stress has been reported to cause 3-17% yield losses in barley (Xie et al., 2018). Studies have highlighted the importance of timing and rate of accumulation of assimilates in the grain (Eagles et al., 1995; Hong & Zhang, 2020; Wu et al., 2017). Protein content is largely determined at early grain fill, whereas carbohydrates and beta-glucans accumulate at later stages. Therefore, advance senescence triggered by stress can lead to high grain protein but reduced grain starch and plumpness compromising both yield and end-use quality (Hong & Zhang, 2020).

To develop stress-tolerant varieties, it is essential to investigate how plants respond at morphological, physiological, and molecular levels. Morphologically, heat and drought stress alter plant architecture, including senescence timing, root system depth, and leaf area (Gous et al., 2016; Shirdelmoghanloo et al., 2022; Williams et al., 2024; Williams et al., 2022). Physiological changes include shifts in stomatal conductance and water/nutrient uptake (Guo et al., 2019). At the cellular level, these stresses can modulate gene expression, initiate reactive oxygen species responses, and trigger stress memory mechanisms (Elkelish et al., 2025; Janiak et al., 2018). One promising approach involves deploying the stay-green trait to mitigate yield and quality losses. Stay-green genotypes often show extended grain fill periods through delayed senescence or early heading and are associated with increased grain plumpness and improved yields (Shirdelmoghanloo et al., 2022). However, the underlying mechanisms, especially those related to root dynamics, remain poorly understood. Stay-green sorghum lines, for instance, exhibit deeper and more extensive root systems under drought and conserve moisture through reduced transpiration rates (Djanaguiraman et al., 2024). Similarly, traits like narrower nodal angle have been linked to stay-green adaptation under drought conditions (Mace et al., 2012). Root systems are central to plant survival under

abiotic stress, acting as the primary interface for water and nutrient acquisition (Wong et al., 2023). Stay-green traits are often associated with continued water uptake and nitrogen assimilation after anthesis, attributed to enhanced root growth (Borrell & Hammer, 2000). Nitrogen efficient wheat cultivars have been reported to show increased root growth and distribution before anthesis facilitated by upregulation of nitrate transporter genes which enhance N<sub>2</sub> uptake (Guo et al., 2019). (Borrell et al., 2022) reported that sorghum stay green QTL increases drought tolerance of stay green sorghum genotypes via modification of plant canopy and root architecture. The close relationship between senescence and root function suggests that genetic regulators of stay-green traits may also influence root development under stress.

Many NAC transcription factors have been reported to regulate senescence in barley (Christiansen & Gregersen, 2014). One of the most studied among them is *NAM-B1* (also called as *Gpc-B1*) gene that regulates grain protein content in wheat. It was identified via map-based cloning and the allele for high grain protein content (GPC) and accelerated senescence was initially reported in a wild emmer wheat accession (Avivi, 1979; Distelfeld et al., 2014; Uauy, Distelfeld, et al., 2006), which was later crossed to durum wheat (Joppa & Cantrell, 1990). From the resulting population, a QTL on 6BS was mapped (Joppa et al., 1997) that not only regulates GPC but also senescence timing (Uauy, Brevis, et al., 2006) and Fe and Zn concentrations in the grain (Çakmak et al., 2004; Distelfeld et al., 2007). The modern wheat varieties carry a nonfunctional *NAM-B1* allele which results in delayed senescence and reduced GPC, Zn and Fe in grains (Uauy, Distelfeld, et al., 2006). Using the *Gpc-B1* wheat sequence, two barley genes *HvNAM-1* and *HvNAM-2* were identified, showing 98% similarity. These genes are located on 6H and 2H respectively (Uauy, Distelfeld, et al., 2006), with *HvNAM-1* on 6H showing high collinearity with the wheat 6BS QTL,

thereby being recognized as its ortholog (Distelfeld et al., 2008). Similar to wheat, expression of *HvNAM-1* in flag leaves increases from anthesis through maturity (Uauy, Distelfeld, et al., 2006) and is associated with increased proteolysis and nitrogen remobilization in flag leaves, resulting in higher GPC (Jukanti & Fischer, 2008). Although *HvNAM-2* has also been suggested to contribute to GPC (Cai et al., 2013; Distelfeld et al., 2014; Jamar et al., 2010; See et al., 2002), its role appears more limited. Additionally, *HvNAM-1* is linked on 6H to a gene, *HvGR-RBPI*, encoding a Glycine-rich RNA binding protein 1. The function allele of *HvGR-RBPI* promotes early anthesis while the non-functional *HvNAM-1* allele delays senescence (Alptekin et al., 2021).

While many studies have established the association between *HvNAM-1* expression in flag leaves and delayed senescence or reduced GPC, little is known about its expression in roots. Christiansen et al., (2011) reported no detectable *HvNAM-1* expression in the roots at the three-leaf stage. However, more recent findings by (Liu et al., 2019) described the plant age-mediated root senescence process in barley and observed *HvNAM-1* expression in roots at later growth stages. This highlights the need to explore *HvNAM-1* expression in roots at these growth stages, particularly under abiotic stress conditions.

Recent studies using a recombinant inbred line (RIL) population segregating for the stay-green trait in semi-arid environment identified co-locating QTL for seminal root traits, grain fill duration and grain quality (Williams et al., 2024). This population had parents differing at the *HvNAM-1* locus and one of the identified QTL, QGFmt-6H, also associated with seminal root-to-shoot length ratio and co-located with *HvNAM-1*. Additional QTL, *QGFhd-2H*, *QLN-2H*, *GGFhd-7H*, and *QGF-7H*, were associated with seedling root architecture and grain fill-related traits in semi-arid environments, highlighting the importance of root traits in stress adaptation (Williams

et al., 2024). Given the increasing frequency of combined heat and drought stress discussed earlier, the potential role of these root-associated QTL in conferring stress resilience warrants further exploration. For example, (Gous et al., 2016) mapped QTL for stay-green under heat stress in barley adjacent to QTL for root length and root-to-shoot ratio. Interestingly, stay-green expression under heat was not associated with spike number, unlike under drought, leading the authors to suggest greater selection value of the trait under drought conditions. In wheat, (Kumar et al., 2022) showed that stay-green genotypes maintained stable yields under combined heat and drought via enhanced photosynthesis and carbon fixation during anthesis. Similar results were observed in other wheat studies (Latif et al., 2020; Pinto et al., 2016). In barley, stay green, under heat stress, was associated with increased grain filling rate and grain plumpness (Shirdelmoghanloo et al., 2022). Despite these insights, research on the stay-green trait in barley remains limited (Kamal et al., 2019). Greater emphasis is needed on understanding root contributions to stress adaptation. Currently, the expression of *HvNAM-1* in roots during late grain fill and its potential role in delaying senescence under combined heat and drought stress remains unexplored in barley. A deeper understanding of this via gene expression studies could uncover how stay-green genotypes maintain water uptake during stress. Transcriptomic studies, such as the one by (Klaus et al., 2024), have begun to explore drought-responsive gene expression across different root zones in barley seedlings. Additionally, methods like weighted gene co expression network analysis (WGCNA) are increasingly employed to identify stress-responsive gene modules.

This dissertation is comprised of three research projects addressing abiotic stress (heat and drought) tolerance in forage and malt barley. The goal of the first project involves identifying QTL associated with climate resilient traits that could be used for forage barley improvement using

genome wide association studies (GWAS) and genetically dissect important forage traits to empower forage breeding programs, with emphasis on understanding the genetic relationship between environment, grain yield, forage yield and quality. The second project evaluates stay green and non-stay green barley near isogenic lines (NILs) for previously identified QTL to confirm their effects. We also evaluated these NILs under controlled heat stress conditions to examine how allelic variation at stay-green QTL influences barley performance under heat. The third project applies transcriptomic approaches to assess whether the ND allele for *HvNAM-1* alters the expression of senescence-related genes in roots, thereby promoting continued root growth and water uptake during late grain fill under stress conditions.

Together, these projects aim to advance our understanding of the genetic and physiological bases of heat and drought resilience in barley and to support the development of more climate-resilient cultivars.

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CHAPTER TWO

DISSECTING GENETIC RESILIENCE TO HEAT AND WATER  
DEFICIT IN FORAGE BARLEY VIA GENOME WIDE  
ASSOCIATION STUDY

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### Abstract

Barley is a vital forage crop valued for its favorable balance of digestible fiber, protein, and starch, making it an important component of livestock systems in both irrigated and dryland environments. Forage yield and quality are two important traits for forage breeders. While higher biomass supports overall productivity, it often comes at the cost of digestibility. Forage quality, commonly measured using acid detergent fiber (ADF) and neutral detergent fiber (NDF), declines with plant maturity and is further modulated by abiotic stresses such as heat and drought. As climate variability intensifies, understanding how these environmental stresses affect the genetic control of forage traits is essential for developing resilient cultivars. This study used genome-wide association studies (GWAS) to dissect the genetic architecture of forage yield and quality traits under contrasting environments in barley. Two iCore barley populations (WC1 and WC2), each comprising 260 diverse lines, were evaluated under dryland and irrigated conditions during multiple field seasons. We observed that while biomass and developmental traits exhibited high heritability and stability across environments, quality traits such as ADF and NDF were more sensitive to environmental variation. Moderate drought, in some cases, improved forage digestibility, while heat stress and irrigated conditions reduced the digestibility. GWAS identified several significant quantitative trait loci (QTL) linked to biomass, grain yield, and forage quality, with distinct sets of loci responding to heat, drought, or irrigated conditions. Of particular interest were *HvGYIHL* (irrigated), *HvADF7HL* (2016 dryland), *HvNDF1HL* (avg, 2016 dryland), *HvNDF6HL* (irrigated), that maintained or enhanced digestibility without compromising yield, representing a genetic route to overcome the traditional yield and quality trade-off in forage breeding. These QTL could be beneficial to improve biomass, grain yield and quality in both dryland and irrigated environments. These findings offer new insight into the genomic basis of forage trait resilience under climate stress. They support the application of marker-assisted selection for barley cultivars that combine high biomass with improved forage quality, adapted to both dryland and irrigated systems.

**Abbreviations:** **GWAS** Genome wide association studies, **BY** Biomass yield, **GY** Grain yield, **ADF** Acid detergent fiber, **NDF** Neutral detergent fiber, **WC** World core, **QTL** Quantitative Trait Loci

## Introduction

Cow-calf operations contribute significantly to Montana's agricultural GDP, and winter-feeding costs are the largest expense for most operations (USDA National Agricultural Statistics Service, 2020). While alfalfa hay remains a popular option for forage production in Montana, annual cereal forages such as barley provide a valuable alternative and are an important substitute during rotation out of alfalfa. Where alfalfa requires a year to establish, annual cereals grow rapidly and produce forage in a single season, providing flexibility and affordability for growers. Additionally, barley is more drought and saline tolerant than alfalfa (Maas, 1993; Steppuhn & Raney, 2005), making it well adapted to areas without access to irrigation.

Forage yield and quality are of the utmost importance to livestock producers and forage growers, and thus they are major traits of interest to forage breeders. Increasing biomass yield is of obvious benefit as more forage can be produced on fewer acres. Improved forage nutritional quality means less acreage is required to have the same nutritional gain. Two important quality metrics are animal intake—how much the animal can consume—and forage digestibility, once consumed how efficiently the forage is utilized. Both quality parameters are negatively impacted by high fiber content (Mertens, 1987; Reid et al., 1988) and are commonly approximated by measuring neutral detergent fiber which is primarily affected by the amount of cellulose, hemicellulose and lignin (NDF, animal intake), and acid detergent fiber primarily impacted by the amount of lignin and cellulose (ADF, digestibility). A conservative estimate is that a one percent increase in forage digestibility can lead to a three percent increase in the average daily weight gains of steers (Casler & Vogel, 1999; Mohammed et al., 1967).

Forage yield and quality are complex quantitative traits. They are the phenotypic result of the interaction of many different genes, most of which have yet to be identified. Barley biomass yields tend to increase with later heading, greater height, and increased tillers (Neumann et al., 2017). In fact, positive correlations between days to heading, number of tillers and plant height have been reported (Haaning et al., 2020). The main plant biochemical components responsible for variation in nutritional quality of forages are plant structural components such as hemicellulose, cellulose, and lignin. These complex structural components make up much of the plant cell wall and are the primary constituents of what is referred to as fiber in forage analysis, typically measured as neutral detergent fiber (NDF) and acid detergent fiber (ADF). NDF includes cellulose, hemicellulose, and lignin, serving as a measure of animal intake potential. ADF, includes cellulose and lignin and is more directly related to digestibility. Because most animals lack the enzymes to digest these components, any gene related to their deposition can impact nutritional quality. Other genes controlling biomass including those that affect plant architecture, flowering, and senescence which in turn can impact the deposition of these fibers. Plants resistant to lodging can have more of these structural fibers. Also, as plants mature, increased amounts of structural fiber and lignin are deposited, so as biomass increases available nutrients decrease (Cherney & Marten, 1982a, 1982b). The digestibility of cell wall components decreases with lignification. However, in cereals the decrease in digestibility generally levels off during grain fill as the highly digestible grain head becomes a greater proportion of the total biomass yield (Cherney & Marten, 1982a, 1982b; Garnsworthy & Stokes, 1993). When grain proportion becomes substantial, i.e. at dough stage (Zadok stage 80), digestibility could even increase (Ben-Ghedalia et al., 1995; Crovetto et al., 1998; Hesel & Thomas, 1987).

Genome wide association studies (GWAS) have been utilized successfully to map QTL for forage quality and yield in alfalfa (Biazzi et al., 2017; Jia et al., 2022; Lin et al., 2020; Liu & Yu, 2017; Sakiroglu & Brummer, 2017; Yu, 2017), maize (López-Malvar et al., 2019; Wang et al., 2016), sorghum (Li et al., 2018; Zhao et al., 2016), and perennial ryegrass (Arojju et al., 2016). A number of Quantitative Trait Loci (QTL) mapping studies in barley have been performed using biparental mapping population from a cross between variety ‘Steptoe’, a feed barley, and variety ‘Morex’, a malt barley, identifying QTL for grain yield and malt quality (Hayes et al., 1993; Kleinhofs et al., 1993; Romagosa et al., 1996), as well as forage traits, (Siahsar et al., 2009; Surber et al., 2011). However, both forage studies were conducted under irrigation. Many forage growers in Montana do not have access to irrigation, with 80% of production on dry land. The Northern Great Plains region of the United States often has short growing seasons for spring crops, due to late snowfall and terminal heat and drought, and thus, may require forages with specific phenologies. Also, as heat and drought events increase, resilient forage varieties need to be identified.

Buxton & Fales. (1994) stated that “no single factor impacts forage traits more than plant maturity, but plant environment modifies the impact of plant maturity” (Buxton & Fales, 1994; Fahey Jr. & Hussein, 1999). Although most studies have been performed to determine the environmental impact on forage production and quality in non-cereals, forage traits are impacted by various environmental factors such as high temperature, drought, soil nutrient availability, and solar radiation (Buxton, 1996). Heat and drought during development impact both yield and nutritional quality (Buxton & Fales, 1994; Fahey Jr. & Hussein, 1999). Heat and drought during the vegetative stage can reduce tillering and height of cereals, constraining biomass production

(Mmbando, 2025). According to studies conducted by Thorvaldsson. (1987), in cool season forages, an increase in 1°C temperature reduces digestibility by 3-7g/kg. This reduction in digestibility is due to increased lignification (Buxton & Fales, 1994) and indigestible cell wall content as reported in tall fescue (Fales, 1986). The increase in length of photoperiod and light intensity improves digestibility through increasing amino acid and non-structural carbohydrates and decreasing cell wall content due to enhancing the rate of photosynthesis (Mueller & Orloff, 1994). Water deficit, when not severe, slows plant maturation, increasing leaf/stem ratio and thus increasing forage yield and digestibility (Buxton, 1996; Coleman et al., 2004). However, under severe drought stress, leaf growth is inhibited, and existing leaves may senesce prematurely resulting in reduced digestibility (Buxton, 1996). Several studies have reported the impact of environment on forage quality in other crops such as alfalfa (Sanz-Sáez et al., 2012), *Festuca arundinacea* and *Dactylis glomerata* (Sanaullah et al., 2014), *Panicum maximum* (Habermann et al., 2019) and various legumes (Kuchenmeister et al., 2013), but there still exists a gap in understanding how different gradients of these stresses impact the productivity and digestibility of forage barley .

The goal of this study was to identify gene regions that promote dry land forage production, quality and stability. To perform a genome wide association study (GWAS), we evaluated two different populations of 260 lines each from the barley USDA Barley World Core Collection Informative Core (iCore) for forage traits under varying environmental conditions two of which involved lower precipitation and higher temperatures than the 10-year average. iCore includes 1,860 lines to represent the total global barley diversity. Our objectives for this study were to (i) identify QTL associated with climate resilience traits that could be used for forage barley

improvement, (ii) and genetically dissect important forage traits to empower forage breeding programs, with emphasis on understanding the genetic relationship between environment, grain yield, forage yield and quality.

## Materials and Methods

### Plant Materials

The iCore 1,860 lines were genotyped with a barley SNP iSelect platform with 7,842 markers (Muñoz-Amatriaín et al., 2014). Only 621 lines in the iCore are spring, two-row barley, which is the type most commonly grown in the western United States and known for higher productive tiller number than six-row barley (Haaning et al., 2020). From the spring, two-row subset of the iCore, 260 barley lines were randomly selected for testing in 2016 and 2017 (WC1). The WC1 lines originated from 69 different countries, and the breakdown of their genetic improvement classification was 52% cultivar, 38% landrace, and 10% genetic and breeding accessions. A second set of 260 lines was randomly selected from the remaining untested spring, two-row iCore and designated WC2, including lines from 57 different countries; 51% of the lines were classified as cultivars, 29% as landrace, and 20% as genetic and breeding accessions.

### Field Trials

The 2016-17 WC1 trials were planted between May 4-6, and the 2018, 2019 WC2 trials were planted on April 28. Field plots in 2016 were planted as two row plots, 2.44 meters in length and planted on 2.74 meter centers. Plots in 2017 through 2019 were planted as three row plots, 4.57 meters in length and planted on 5.49 meter centers. All plots were seeded at a rate of 12 g/m<sup>2</sup> with a row spacing of 0.3 meters. The WC1 was grown under both irrigated and rainfed (dryland)

treatment conditions in field trials at the Arthur H. Post Research Farm in Bozeman, MT (Latitude: 45.6729, Longitude: -111.1547, Elevation: 1455 m) on Amsterdam silt loam soil in both the 2016 and 2017 field seasons. The irrigated treatment received 127 mm of irrigation applied to the field in two, 63.5 mm increments immediately prior to the heading stage of development. The WC2 population was also grown at the Arthur H. Post Research Farm but only under rainfed (dryland) conditions during the 2018 and 2019 field seasons.

In all location-years, an augmented randomized complete block design (Federer, 1956, 1961) with 10 blocks and 4 check plots per block was deployed. The check varieties, Lavina, Hays, Conlon, and Stepford, were randomly assigned plots within each block, and the 260 experimental lines were randomly assigned to the remaining plots. This design resulted in 30 plots per block and 300 plots per treatment: 260 plots for the genetically diverse lines and 40 plots for the checks.

#### Agronomic Data Collection

Plots were monitored daily throughout the growing season and data were collected on plot heading date (Zadoks stage 51), soft-dough date (Zadoks stage 85), maturity date (Zadoks stage 92), and mature plant height. Zadok stages were defined as when 50% of the main tillers in a plot reached the designated stage. The Zadok stage 92 definition was modified to be assessed visually with maturity defined as when no green color remained in 50% of the main tiller heads, including the awns and glumes. From heading, soft-dough, and maturity dates, two additional parameters were calculated: the number of days between heading and soft-dough, and the number of days between soft-dough and maturity. Plant height was measured on mature plants from ground level to the top of the plant excluding awns.

To ensure that observed variation between lines was not due to stage differences at tissue collection, when a plot reached the soft-dough stage (Zadoks stage 85), six 6-inch forage samples were collected from representative areas of the plot and bulked. Soft-dough is often recommended as the optimal stage for forage harvest due to the balance of peak biomass yield and desirable forage quality. Samples were cut one inch from the soil surface, and wet weights determined. Collected forage samples were dried in a forced-air oven at 40°C for 96 hours, weighed, and then ground using a Wiley cutting mill to pass through a 2-mm screen. From dried sample weights, an estimated dry ton/acre biomass yield was calculated. Two hundred fifty forage barley samples from the 2016 field season (WC1) were analyzed in technical triplicate for acid detergent fiber (ADF) and neutral detergent fiber (NDF) using an ANKOM 2000 Fiber Analyzer as per the manufacturer's instructions. The ADF on these samples was determined via Method 973.18 (Grant & Mertens, 1992). The NDF was determined using heat-stable alpha-amylase and sodium sulfite via the methods described by Van Soest et al. (1991).

From these samples, a custom NIR calibration curve was developed using a Foss NIRSystems 6500 with proprietary WinISI software. Calibration equations were validated on 20 additional 2016 WC1 samples not included in the sample subset used in calibration creation. All subsequent forage samples were analyzed for ADF and NDF using this custom NIR calibration via Method 4.2 of the National Forage Testing Association ("Fiber (Acid Detergent) and Protein (Crude) in Animal Feed and Forages: Near-infrared Reflectance Spectroscopic Method (989.03)," 1990; (Martin et al., 1989). This calibration was updated as needed with additional samples to ensure that it remained robust from year-to-year.

It is important to note that although digestibility is the forage quality variable of interest, the laboratory proxies ADF and NDF measure % non-digestibility – thus, as ADF or NDF values decrease, digestibility and intake increase. In addition to forage trait data, grain yield was also collected on each plot. If they are to be successfully produced by seed growers for the forage market, forage barley varieties must have sufficient grain yield and selection for grain yield is requisite for cultivar success. Best linear unbiased prediction (BLUP) values of each trait of each line were obtained and used as phenotypic data for mapping analysis (see Statistical Analysis below). Experimental design, data collection, and sampling for WC2 were the same as described for WC1.

#### Marker and Map Data

The SNP data and genetic map used for this study were downloaded from the Triticeae Toolbox public repository (<https://triticeaetoolbox.org>). The lines were genotyped with the barley iSelect SNP chip as previously described (Muñoz-Amatriáin et al., 2014). The Morex 2016, IBSC physical map was utilized (Beier et al., 2017). Markers with a minor allele frequency of less than 5% or which were missing more than 50% of allele data were removed, resulting in 6,585 remaining markers. Of the remaining markers, 6,383 had known marker positions while 202 markers were unaligned.

#### Statistical Analysis

All data processing and statistical modeling were performed in the open-source statistical platform R (R Core Team, 2020) through the graphical user interface RStudio (RStudio Team, 2020). To adjust WC1 agronomic data for across field variation within an environment, a mixed

model with factors for block, check, and accession was used to estimate best linear unbiased predictors (BLUPS) using the restricted maximum likelihood (REML) method in the ‘lme4’ package (Bates et al., 2015). BLUPs were adjusted with environment trait grand means to restore traits to original scales. Agronomic trait BLUPs for each accession were then averaged within and across treatments to generate accession level BLUP data for the Dryland, Irrigation, and Average treatments. All statistical analyses were then repeated for the WC2 population. Pearson’s correlation coefficient was calculated to estimate the correlation between agronomic trait BLUPs using averages of all populations and treatments.

#### Population structure

The population structure of both WC1 and WC2 was inferred by conducting a principal component (PC) analysis. Missing marker data was first imputed separately for each population using the expectation maximization (EM) algorithm in the “A mat” function of the rrBLUP package. The EM algorithm replaces missing marker data based on the multivariate normal distribution of the population (Endelman, 2011). The resulting genotypic data including imputed marker values were then scaled and centered using the “scale()” function and the “svd()” function was applied to the scaled data. Multiplying a matrix of the scaled data by the “v” vector, which was created by the svd function, produced the principal component values and a screeplot that was utilized to assess the variation accounted for by the first 10 PCs. By the elbow criterion, the screeplots for both WC populations indicated that the first 2 PCs should be utilized for subsequent statistical procedures

### Genome-Wide Association Mapping

Genome-Wide Association Mapping (AM) was conducted using the marker dataset and BLUP adjusted phenotypic datasets from WC1 field trials as follows: line trait means across dryland environments (Dryland), line trait means across irrigated environments (Irrigated), and line trait means for each environment. The WC2 field trial was analyzed as individual environments (2018, 2019) and the mean of 2018 and 2019 environments. The AM analysis was conducted in the R package Genome Association and Prediction Integrated Tool (GAPIT) using FarmCPU method (Lipka et al., 2012). Significant QTL were identified using a Bonferroni-corrected threshold, calculated as  $\log_{10}(0.05/N)$ , where N is the total number of markers tested. This correction for multiple testing was applied automatically by GAPIT and is commonly used in GWAS to minimize false positives while preserving statistical power in moderately sized panels. Candidate gene identification was guided by the observed linkage disequilibrium (LD) decay within the population, which occurred at approximately 9 kb. Therefore, genes located within  $\pm 9$  kb (a total 18 kb window) of each significant SNP were considered potential candidates underlying the trait-associated loci.

### Results

The weather was variable over the course of the study (Table 1.1). Since forage sampling was completed for all trials by the end of July, August weather had no impact on forage measurements and were only relevant for grain yield. Over the growing seasons, precipitation was about 30 percent lower than the long-term average (LTA) in 2016 and 2017, while 2018 and 2019 were similar to the LTA. However, precipitation was not similarly distributed across the months in

any year. For example, in June 2016, precipitation was about 30 percent lower than the LTA, while in 2017 and 2019 both May and June were near the LTA. In 2018, June was above the LTA such that better-than-average precipitation in June compensated for lower-than-average precipitation during July. 2017 had the least precipitation, especially in July. Temperatures also varied across years, with average high temperatures higher than the LTA and more days above 32°C in 2016 and 2017, while 2018 and 2019 were more similar to the LTA. The timing of the heat varied in 2016 and 2017 with the average high temperature 14°C above the LTA and two more days above 32°C in June 2016, while in July 2017 the average high and low temperatures were both about 15°C above the LTA and about double the days above 32°C. To discriminate between the effects of heat and drought together and heat alone, five additional mm of irrigation were applied to the irrigated environments prior to heading for a total of ~266.7 mm of in-season water during 2016 and 2017. Irrigation was not applied in 2018 and 2019, since precipitation was closer to LTA.

Month	Temperature (°C); Precipitation (mm)	L.T.A.	2016	2017	2018	2019
May	Avg High (°C)	18.9	17.8	19.4	20.0	15.6
	Avg Low (°C)	3.3	3.9	3.9	6.7	3.9
	Precip (mm)	73.7	68.6	66.0	73.7	43.2
	Days >32°C	0	0	0	0	0
June	Avg High (°C)	23.3	26.7	24.4	22.2	22.8
	Avg Low (°C)	7.2	8.9	7.8	8.3	7.2
	Precip (mm)	71.1	<b>20.3</b>	55.9	91.4	53.3
	Days >32°C	1	3	2	0	0
July	Avg High (°C)	28.9	28.3	31.1	27.8	26.7
	Avg Low (°C)	10.6	10.6	12.8	10.6	10.0
	Precip (mm)	35.6	30.5	<b>2.5</b>	5.1	68.6
	Days >32°C	8	6	<b>15</b>	3	1
August	Avg High (°C)	28.9	28.9	28.3	26.7	27.8
	Avg Low (°C)	9.4	9.4	10.6	9.4	10.0
	Precip (mm)	33.0	22.9	15.2	33.0	17.8
	Days >32°C	7	7	1	5	0
Total Season	Precip (mm)	213.4	142.2	139.7	203.2	182.9

Table 1.1. Summary of Field Season Precipitation and Temperatures (in metrics). Arthur H. Post Farm, Bozeman, MT (2016–2019). L.T.A = Long Term Average.

Significant variation was observed for all traits between populations and treatments and confirms impact of both water deficit and high temperature on traits (Table 1.2). Environmental variation impacted the progression of development. In most environments, heading occurred at about 59 days after planting. The fastest progression through grain fill was observed in 2017 (25 days) followed by 2016 (29 days) on dryland. In 2016, irrigation delayed the progression to soft dough by 2 days. However, irrigation did not delay soft dough in 2017. Progression through grain fill was longer in 2018 (34 days) and 2019 (37 days) as compared to 2016 and 2017. 2016 dryland had the lowest mean biomass, coinciding with low precipitation in June. Irrigation in 2016 increased biomass yield to be similar to 2018 and 2019. Grain yield was also positively impacted by irrigation in 2016. In 2017, the grain and biomass yields were not different between dryland and irrigated, indicating that the negative impacts of extreme heat in July were not remedied by irrigation. Grain yield in 2017 was lower than 2016 in both dryland and especially irrigated, emphasizing the importance of heat events that impacted progression through grain fill. Both low precipitation and heat impacted forage quality. Livestock intake is higher when NDF is lower and nutritional quality is improved with lower ADF. Better quality was observed in 2016 and 2017 dryland when compared to irrigated or 2018 and 2019. However, the heat during July of 2017 reduced quality when compared to 2016.

Trait	Heritability, Mean, standard deviation	WC1				WC2	
		Dryland		Irrigated		Dryland	
		2016	2017	2016	2017	2018	2019
Days to heading	Heritability	0.95	0.96	0.97	0.96	0.83	0.98
	Mean and standard deviation	58.82 ± 4.28 <sup>a</sup>	59.05 ± 3.73 <sup>a</sup>	58.12 ± 4.17 <sup>a</sup>	58.19 ± 3.99 <sup>a</sup>	65.58 ± 5.53 <sup>b</sup>	58.33 ± 4.04 <sup>a</sup>
Days to soft-dough	Heritability	0.94	0.88	0.93	0.91	0.8	0.86
	Mean and standard deviation	79.28 ± 5.75 <sup>a</sup>	73.75 ± 2.87 <sup>c</sup>	80.49 ± 4.92 <sup>b</sup>	73.10 ± 3.48 <sup>c</sup>	81.13 ± 4.05 <sup>b</sup>	77.53 ± 3.02 <sup>d</sup>
Days to maturity	Heritability	0.85	0.79	0.93	0.87	0.54	0.87
	Mean and standard deviation	88.34 ± 3.19 <sup>a</sup>	84.42 ± 3.19 <sup>b</sup>	89.1 ± 3.11 <sup>c</sup>	86.53 ± 2.67 <sup>d</sup>	99.8 ± 3.59 <sup>e</sup>	95.54 ± 3.53 <sup>f</sup>
Forage yield (ton/ac)	Heritability	0.8	0.29	0.71	0.64	0.67	0.39
	Mean and standard deviation	3.84 ± 0.9 <sup>a</sup>	4.2 ± 0.63 <sup>b</sup>	5.5 ± 1.15 <sup>c</sup>	4.4 ± 0.76 <sup>b</sup>	5.04 ± 1.03 <sup>d</sup>	5.72 ± 0.71 <sup>c</sup>
Grain yield (kg/ha)	Heritability	0.54	0.41	0.77	0.46	0.67	0.72
	Mean and standard deviation	3771.27 ± 784.26 <sup>a</sup>	3384.96 ± 589.87 <sup>b</sup>	4917.30 ± 1092.25 <sup>c</sup>	3458.14 ± 728.61 <sup>b</sup>	4219.97 ± 878.32 <sup>d</sup>	4839.46 ± 1208.79 <sup>c</sup>
Acid detergent fiber (%)	Heritability	0.59	0.24	0	0.36	0.55	0.24
	Mean and standard deviation	27.85 ± 3.28 <sup>a</sup>	31.27 ± 2.98 <sup>b</sup>	30.3 ± 3.14 <sup>c</sup>	33.79 ± 3.14 <sup>d</sup>	34.5 ± 2.91 <sup>d</sup>	37.33 ± 3.18 <sup>c</sup>
Neutral detergent fiber (%)	Heritability	0.59	0.24	0	0.15	0.58	0
	Mean and standard deviation	52.9 ± 3.11 <sup>a</sup>	56.58 ± 3.42 <sup>b</sup>	54.21 ± 2.79 <sup>d</sup>	59.07 ± 3.63 <sup>c</sup>	54.8 ± 3.3 <sup>d</sup>	59.21 ± 3.54 <sup>c</sup>
Plant height (cm)	Heritability	0.84	0.8	0.88	0.77	0.89	0.92
	Mean and standard deviation	59.03 ± 8.56 <sup>a</sup>	66.87 ± 8.35 <sup>b</sup>	79.36 ± 13.41 <sup>c</sup>	69.65 ± 9.68 <sup>d</sup>	83.48 ± 12.89 <sup>e</sup>	85.47 ± 9.55 <sup>c</sup>

Table 1.2. Trait estimates by experimental population and treatment with significance groupings based on Tukey's Honest Significant Difference test ( $p < 0.05$ ). Means within each trait sharing the same letter are not significantly different. Letters are assigned separately for each trait and have no meaning across different traits.

### Heritability of forage traits:

Developmental traits such as days to heading, days to soft-dough, and plant height exhibited high heritability across environments, indicating that most of variation observed was due to genetic variation even in varying environments (Table 1.2). In contrast, grain yield showed low heritability in both dryland environments of WC1, with a notable increase under irrigated conditions in 2016. However, this increase was not observed in 2017, likely due to elevated temperatures that reduced the genetic expression of yield potential under irrigation. Heritability of forage yield dropped substantially in 2017 dryland compared to 2016 dryland. This sharp decline suggests that the extremely hot and dry 2017 season increased environmental variation and reduced the ability to detect genetic differences for forage yield and may have amplified genotype-by-environment ( $G \times E$ ) interactions. Under irrigated conditions, heritability was moderate in 2016 but decreased in 2017, again reflecting the suppressive effect of heat stress even with irrigation. The years 2018 and 2019 showed moderate to low heritability for forage yield, indicating some year-to-year environmental variability but not as extreme as in 2017 dryland. Both NDF and ADF had lower heritability in 2017. While NDF and ADF had no heritability under irrigation in 2016, NDF had no heritability in 2019 as well.

### Correlations among traits

Agronomic trait correlations were assessed using accession BLUPs. On average, biomass yield was positively correlated with all the traits (Figure 1.1). Delayed development was related to increased height, increased grain yield and more biomass in all environments. In most environments, higher biomass correlated with poorer quality, higher NDF and ADF, but in some environments the relationship was not significant. Grain yield also positively correlated with later

development in most environments. Grain yield correlation with plant height varied across environment. The correlation was not significant in 2016 and 2017 dryland as well as in 2018, and 2019. However, it was positive in 2017 irrigated and was negative in 2016 irrigated conditions. Grain yield correlated with ADF and NDF varied across environments. While the correlations of grain yield with both ADF and NDF were positive in 2017 irrigated, they were not significant in 2017 dryland. Grain yield was positively correlated to NDF in 2016 dryland and 2018 and it was negatively correlated to ADF in 2016 irrigated and 2019. Quality, measured as lower NDF and ADF, correlated with earlier development and less height, less grain yield and less biomass in most environments. However, the relationship between development and ADF was the opposite in 2016, with improved digestibility (lower ADF) correlating with later development, and higher grain yield. Although similar correlations were observed across most environments, there was a breakdown in correlations across many traits in 2017 dryland, although the biomass correlations to development, height and grain yield held.

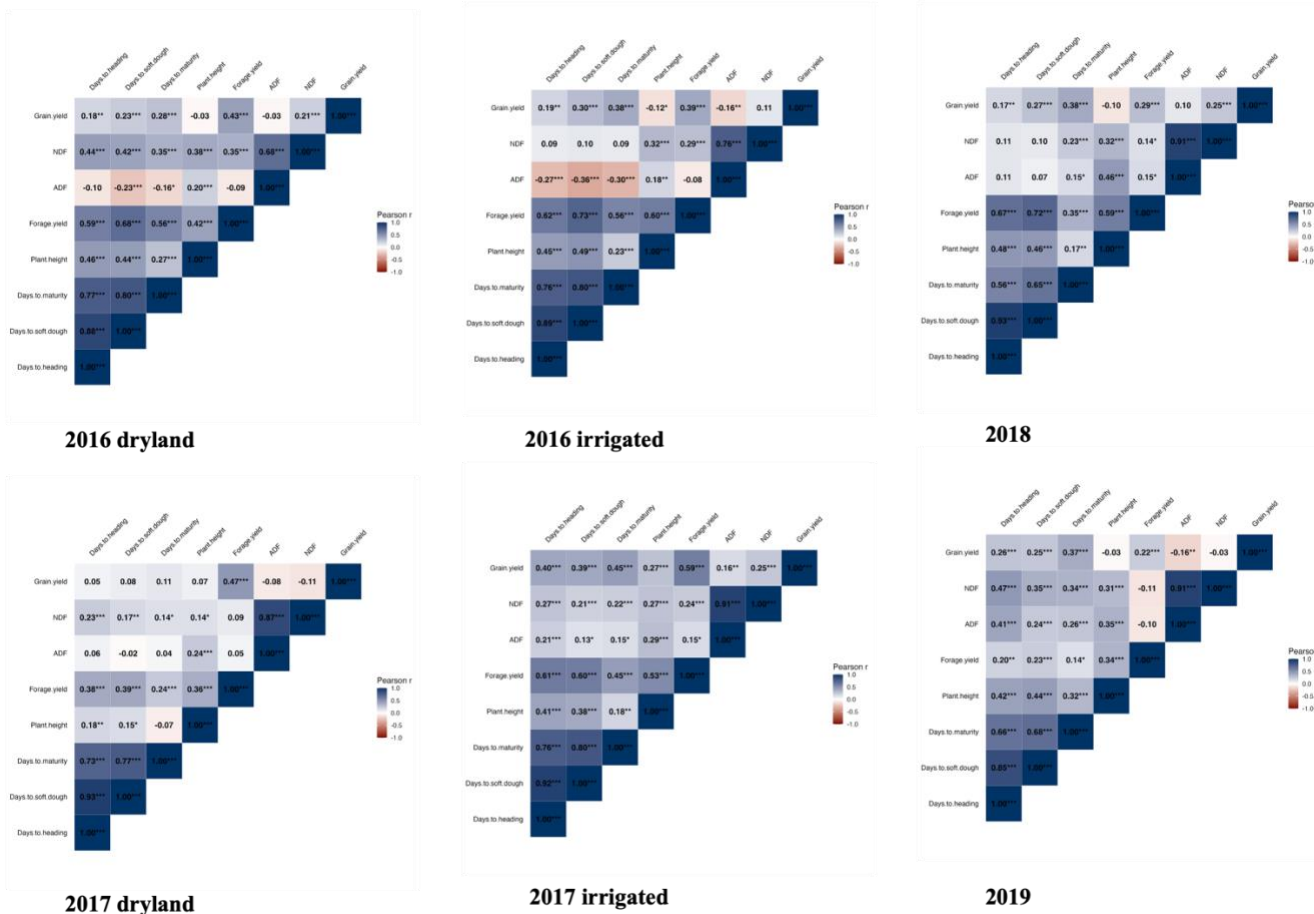


Figure 1.1. Pearson correlation matrices among agronomic and forage traits across dry and irrigated environments in WC1 and WC2 barley populations. Each subplot displays pairwise Pearson correlation coefficients between traits for WC1: 2016 dryland, 2017 dryland, 2016 irrigated, 2017 irrigated, and WC2: 2018, and 2019. Values in the matrix represent Pearson's correlation coefficient. \*, \*\*, \*\*\* indicate the significance of the correlation coefficient at  $p < 0.05$ ,  $0.01$ , and  $0.001$ , respectively.

### Genetic dissection of forage traits

Principal component analysis was conducted on genotypic data of WC1 and WC2 with the goals of determining the genetic structure for both, as well as, evaluating the similarity in genetic variation between the two populations (Figure 1.2). In the WC1 population, the first 2 PCs accounted for 12.8 and 5.2% of the genetic variation while in the WC2 population PC1 and PC2 accounted for 11.5 and 4.1%. We note that when PCA was completed on the two populations together there is overlap. The populations WC1 and WC2 share some genetic similarities that can be seen in the cluster formation. The genetic overlap in the populations suggests WC2 could be used to confirm associations found in WC1.

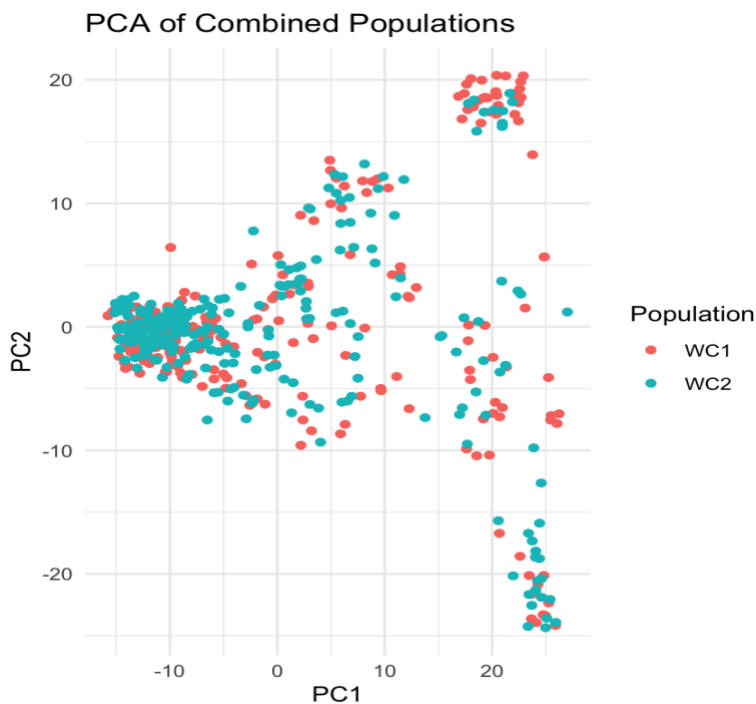


Figure 1.2. Principal component analysis (PCA) of the WC1 and WC2 populations. Each point represents a genotype, colored by population (WC1 in red, WC2 in blue). The plot shows partial

genetic overlap, suggesting shared structure and potential for cross-population validation of GWAS results.

#### Forage trait quantitative trait loci (QTL)- WC1

Across environment A total of 21 QTL were identified for important agronomic and quality traits across locations and environments (Table 1.3). QTL identified when averaged across 2016 and 2017 dry and irrigated environments include: biomass yield QTL on the short arm of chromosome 2H (*HvBY2HS*), long arm of 4H (*HvBY4HL*), and long arm of 5H (*HvBY5HL*); NDF QTL on the long arm of 1H (*HvNDF1HL*), short arm of 2H (*HvNDF2HS*), long arm of 2H (*HvNDF2HL*) and two on the long arm of 5H (*HvNDF5HL.1* and *HvNDF5HL.2*). No significant grain yield and ADF QTL were observed by averaging across 2016 and 2017 environments.

Dry vs irrigated To identify QTL important to dryland productions, GWAS was performed on the mean BLUPs of the dry land and irrigated environments separately from 2016 and 2017. Not surprisingly some of the QTL observed across the treatments were observed in the separate treatments. For example, *HvBY2HS* and *HvNDF5HL.2* were observed in both dryland and irrigated treatments. However, QTL unique to dryland were also observed, for example the most significant QTL for biomass yield was unique to dryland conditions and located on the short arm of chromosome 4H (*HvBYD4HS*). *HvBYD7HL* was also unique to dryland conditions. Two QTL for grain yield were unique to dryland conditions (*HvGYD3HL* and *HvGYD6HL*) and two were unique to irrigated conditions (*HvGYI1HL* and *HvGYI7HL*). QTL *HvNDF2HS* was specific to dryland for NDF. Irrigated specific QTL for NDF were *HvNDF2HL* and *HvNDF6HL*. Dryland specific QTL for ADF were on the short arm of chromosome 2H (*HvADFD2HS*) while the irrigation specific QTL for ADF was on the long arm of 6H (*HvADFI6HL*).

Heat during June vs July We also observed differences in QTL between 2016 and 2017, which are likely due to differences in weather patterns between the two years. When comparing dryland biomass yield QTL in individual environments, *HvBYD4HS* was unique to 2016 and the most significant QTL for biomass. *HvBYD7HL* was also only observed in 2016. Three QTL unique to dryland 2017 were *HvBYD1HL* and *HvBYD6HL* and *HvBYD7HS*. Two ADF QTL unique to 2016 dryland were *HvADFD2HS* and *HvADF7HL*. No 2017 dryland specific NDF QTL were detected. NDF QTL specific to 2016 dryland were *HvNDF1HL*, *HvNDF2HS*, *HvNDF5HL.2*.

QTL Designation	SNP	Environments QTL observed	Chromosome	Position	P.value	maf	effect	Co-Segregating traits with effects
HvBY2HS	BK_12	avg, dry, irr, 2018	2H	29.125791	3.74660223362379e-07	0.1646	-0.151	days to heading (-2.24), days to soft dough (-1.86), height (-0.840), NDF (-0.243)
HvBY2HS	BK_16	dry	2H	29.124351	3.77016412045768e-06	0.1796	-0.122	days to heading (-2.25), days to soft dough (-2.22), height (-0.72), ADF, NDF (-0.463)
HvBY4HL	SCRI_RS_198888	avg	4H	464.141603,	5.47823632192873e-07	0.28	-0.105	thousand weight (0.474), grain yield (-1.51)
HvBY5HL	SCRI_RS_128407	avg	5H	605.366696,	5.82489567665179e-06	0.05	-0.185	days to soft dough (-1.124), height (-0.78), NDF (-0.54), grain yield (-2.54)
HvBY5HL	11_20736	2016 dry	5H	514.827552	1.59526645529218e-06	0.064	-0.31	days to heading (1.42), height (-0.72), NDF (0.620)
HvBY4HS	12_11175	dry	4H	16.2	3.17370084938609e-08	0.003	0.90	
HvBY7HL	11_21201	dry	7H	580.5	4.11803507482469e-07	0.277	0.106	days to soft dough (1.145), thousand weight (2.189)

Table 1.3 Continued

HvBY1HL	11_11481	2017 dry	1H	540.8	4.49897921 669388e-07	0.28	0.0424	
HvBY6HL	11_20784	2017 dry	6H	507.5	2.19987793 990303e-07	0.490	0.042	
HvBY7HS	12_30344	2017 dry	7H	180.4	6.70139352 10604e-07	0.23	0.056	
HvGYD3HL	SCRI_RS_ 109716	dry	3H	482.1	2.09703470 212738e-06	0.261	1.44	
HvGYD6HL	12_30956	dry	6H	583.3	9.77498021 898235e-07	0.5	-1.11	
HvGY1HL	SCRI_RS_ 155439	irr	1H	345.3	1.10575525 147287e-06	0.46	2.55	
HVG7HL	12_21016	irr	7H	644.4	5.24140252 36116e-08	0.0117	-10.78	height (1.67), thousand weight (2.217)
HvADFD2H	11_10943	dry	2H	21.5- 70.5	6.05254278 344014e-07	0.410	-0.243	days to heading (- 2.255), days to soft dough (-2.22), height (-0.72), biomass yield (- 0.122), NDF
HvADFD2H	scri_rs_702 6	2016 dry	2H	34.8790 52	8.41311840 530248e-07	0.3826	0.419	days to heading (- 2.376), days to soft dough (-3.44), NDF (-1.004)
HvADF7H	12_31395	2016 dry	7H	578.3	2.41504595 130334e-07	0.3153	-0.614	ADF, days to soft dough (1.64)
HvADF6HL	scri_rs_201 988	irr	6H	554.8	5.20321739 59007e-06	0.474	-0.22	thousand weight (- 0.364)
HvNDF1HL	SCRI_RS_ 213455	avg	1H	415.225 482	5.47299750 992226e-06	0.48	0.138	
HvNDF2HS	12_30872	avg	2H	29.1245 97	8.75939283 625259e-08	0.162	-0.243	days to heading (- 2.24), days to soft dough (-1.8), height (- 0.84),biomass yield (-0.151)
HvNDF2HL	12_10151	avg	2H	615-728	2.05939870 530004e-06	0.0158	0.765	height (0.667), grain yield (1.28), thousand weight (- 3.63)
HvNDF2HL	scri_rs_159 212	irr	2H	726.022 609	3.32657030 287198e-06	0.270	0.156	height (-1.174)
HvNDF5HL. 1	11_20239	avg	5H	373.454 403	1.22533621 077145e-06	0.03	0.3598	
HvNDF5HL. 2	SCRI_RS_ 135254	avg	5H	582.644 477, 613.480 320	6.71024216 815946e-06	0.345	-0.126	days to soft dough (-1.124), height (- 0.78), biomass (- 0.185), grain yield (-2.542)
HvNDF5HL. 2	12_31182	avg	5H	613.480 32	5.04342355 099457e-07	0.0337	-0.549	days to soft dough (-1.124), height (- 0.78), biomass (- 0.185), grain yield (-2.54)
HvNDF5HL. 2	11_10521	dry	5H	597.317 333	4.38583769 598122e-09	0.121	0.413	height (-0.62), days to heading (- 1.26), days to soft dough (-0.706)

Table 1.3 Continued

HvNDF5HL.2	scri_rs_38003	irr	5H	582.424	8.59076323941315e-08	0.1450	-0.220	days to maturity (-0.396), height (-0.94), NDF, thousand weight (-2.26)
HvNDF6HL	scri_rs_175593	irr	6H	343.2	4.65586507684719e-06	0.4901	-0.109	

Table 1.3. Environment-Specific QTL associated with forage yield, quality, and agronomic traits in barley under dry, hot and irrigated conditions identified using GWAS in WC1 population.

### Pleiotropy

Several SNPs associated with biomass, grain yield, and quality traits exhibited consistent pleiotropic effects, often matching expectations due to trait correlations (Table 1.4). The major alleles at *HvNDF2HS* and *HvADF2HS* were associated with increased plant height, delayed heading, higher biomass, higher grain yield, and reduced quality (lower NDF and ADF). The minor allele at *HvBY4HS* increased biomass, plant height, NDF, ADF, while SNPs at *HvBY2HS* and *HvBY5HL* revealed that alleles promoting biomass and grain yield were also linked to taller plants, later heading and higher NDF. Notably, under dryland conditions, the minor allele at *HvNDF5HL.2* was associated with reduced plant height, earlier heading, and lower biomass, grain yield, ADF, and NDF values. Conversely, under irrigation, this SNP showed opposite allelic effects, highlighting the influence of environment (Table 1.2). Together, these results support the observed correlation in most environments where higher grain and biomass yields are associated with later development, taller stature and poorer quality (higher NDF and ADF), making it difficult to improve both yield and quality. However, several QTL especially under dryland conditions broke negative association between yield and quality. For example, the minor alleles at *HvADF7HL* and *HvGYI1HL* and major allele of *HvNDF1HL* were all associated with delayed heading, increased biomass yield, grain yield and reduced ADF. The minor allele of *HvADF6HL* associates with

reduced ADF and NDF and later heading. Likewise, the major alleles at *HvGYD3HL* and *HvGYD6HL* and minor allele of *HvNDF6HL*, were associated with later heading, more grain yield and reduced ADF. These QTL match the correlations observed in 2016 where delayed development correlated with improved quality. Other QTL pleiotropic effects indicate that improved quality is often associated with higher grain yield. For example, the major alleles at *HvBY6HL* and *HvNDF5HL.1* were linked with lower ADF, and in the case of *HvBY6HL*, also with higher grain yield, making them particularly valuable for balancing forage quality and productivity.

#### Confirmation in WC2

Since few QTL overlapped between WC1 and WC2, we wanted to determine if lack of overlap was due to the absence of the genetic impacts or if the environments were reducing the significance of the QTL. Therefore, we observed trait means for minor and major alleles in the same QTL regions identified in WC1 in WC2 (Table 1.4). The same trends were observed between WC1 and WC2 for the following QTL: biomass yield (*HvBY2HS*, *HvBY5HL*, *HvBY7HL*), grain yield (*HvGYI7HL*), NDF (*HvNDF1HL*, *HvNDF2HS*, *HvNDF2HL*, *HvNDF5HL.2*). Most of these QTL were detected in the average of dry and irrigated environments in WC1 with exceptions of *HvBY7HL* and *HvGYI7HL* which are detected in dry and irrigated environments respectively.

QTL	SNP			Biomass Yield (ton/acre)		Grain (kg/ha)		NDF		ADF		Heading Date		Height (cm)		WC 2	
	Position range (kb)	Id	Environments QTL observed	Minor allele	Major Allele	Minor allele	Major Allele	Minor allele	Major Allele	Minor allele	Major Allele	Minor allele	Major Allele	Minor allele	Major Allele	Minor allele	Major Allele
HvBY 2HS	29.12	BK_12, BK_16	avg, dry, irr, 2018	3.6939	4.65***	3450.985	3893.89**	53.99*	55.944	30.33	30.77	53.39	59.86*	59.909	70.67***	4.732	5.5***
HvBY 4HL	464.1	SCRI_RS_19888	avg	4.36	4.5481	3858.56	3807.952	55.69	55.619	30.51	30.80	58.99	58.719	67.153	69.53	5.44	5.3814
HvBY 5HL	605.3	SCRI_RS_128407, 11_20736, scri_rs_146093	avg, 2016 dry, 2018	3.26	4.56***	3109.47	3861.63**	53.88*	55.738	30.332	30.74	53.17	59.11*	56.75	69.59***	5.15	5.4249
HvBY 4HS	16.2	12_11175	dry, 2016 dry	6.79**	4.03	2847.33	3511.76	58.78	54.68	32.64	29.44	66.5	59.14	80.37*	62.93	4.6015	5.409260
HvBY 7HL	580.5	11_21201	dry, 2016 dry	4.17*	3.99	3261.905	3604.06**	55.10	54.55	30.235	29.16**	59.40	59.081	67.40	61.32***	5.51	5.36228
HvBY 1HL	540.8	11_11481	2017 dry	4.45**	4.13	3375.97	3330.39	56.42	56.76	31.23	31.3666	59.72	59.11	69.55**	65.880	5.37	5.41842
HvBY 6HL	507.5	11_20784	2017 dry	4.24	4.22	3240.50	3445.37**	56.72	56.57	31.7	30.94*	58.94	59.613	67.22	66.6818	5.3213	5.48
HvBY 7HS	180.4	12_30344	2017 dry	4.2212	4.23	3249.24	3374.385	56.41	56.71	31.69	31.19	57.34	59.88*	66.60	67.05	4.98	5.50**

Table 1.4 Continued

HvGY D3HL	482.1	SCRI_RS_1097 16	dry, 2018	3.96	4.0744	3618.36	3470.459	54.55	54.7586	30.35	29.14***	56.75	60.03*	62.39	63.2191	4081.42	4457.7
HvGY D6HL	583.3	12_30956	dry, 2018	4.01	4.07	3531.580	3486.757	54.768	54.642	29.99	28.92***	58.09375	60.25*	62.81	63.19	4016.26	4665.6
HvGYI IHL	345.3	SCRI_RS_1554 39	irrigate d	5.12*	4.82	4384.78*	3911.03	56.60	56.56	31.51*	32.35	59.38**	57.60	75.82	73.95	4017.077	4715.0
HVGYI 7HL	644.4	12_21016	irrigate d	3.54	4.98**	3088.07	4136.04*	55.67	56.586	32.605	31.96	49.5	58.54*	60.14	75.00*	3894.06	4371.7
HvADF 2HS	21.5- 70.5	11_10943, scri_rs_7026, 12_10219	dry, 2016 dry, 2019	3.94	4.12*	3424.6	3567.97*	54.48	54.86	29.09*	29.71	59.02	59.27	60.550	64.71***	36.169	35.60
HvADF 7HL	578.3	12_31395	2016 dry	4.24** *	3.667	3968.97*	3539.356	52.92	52.78	25.92* **	28.46	61.50***	58	59.17	59.28	35.89	35.80
HvADF 6HL	554.8	scri_rs_201988	irrigate d, 2018	4.97	4.940	4141.54	4107.615	56.084 9**	57.019	31.29* **	32.585	59.2892**	57.656 7	74.4130	75.2144	36.19747	35.53*
HvNDF IHL	415.2	SCRI_RS_2134 55	avg, 2016 dry	4.36	4.62**	3612.61	4019.15** *	55.71	55.54	31.190	30.25***	58.28	59.29*	68.78	69.03	57.10	56.721
HvNDF 2HS	29.12	12_30872	avg, dry, 2016 dry	3.693	4.65***	3450.9	3895.08** *	53.99* **	55.963	30.331	30.79	53.39	59.85* **	59.90	70.68***	55.39***	57.18
HvNDF 2HL	615- 728	12_10151, scri_rs_159212, 11_21251, scri_rs_114164	avg, irr, 2018, 2019	3.69	4.5102*	3131.147 5	3833.98* 5	53.673 125	55.674	30.81	30.72	54.1875	58.87*	66.384	68.96	56.06812 5	56.901

Table 1.4 Continued

HvNDF 5HL.1	373.4	11_20239	<b>avg</b>	4.2349 09040 3	4.508	3493.491 75	3836.44	56.269 5	55.61	32.546 25	30.64**	57.375	58.860	69.778	68.88	54.73*	56.93
HvNDF 5HL.2	582.- 613	SCRI_RS_1352 54, 12_31182, 11_10521, scri_rs_38003	<b>avg.</b>	4.20	4.65***	3732.34	3870.541	54.60* **	56.19	30.3*	30.94	56.655	59.93* **	63.69	71.68***	56.09**	57.148
			<b>irr</b>	5.270	4.9152	3857.50	4160.556	57.338	56.4701	32.863	31.84930	59.725	58.25	84.527	73.49		
HvNDF 6HL	343.2	scri_rs_175593	<b>irrigate d</b>	5.0594	4.861	4247.03*	4005.135	56.366 76	56.776	31.466 ***	32.45	59.3***	57.596 1	74.7018	74.9614	57.0296	56.777 5

Table 1.4. Pleiotropic effects of significant forage SNPs on other agronomic and forage traits in the WC1 population. Trait means are shown for both the major (Maj.) and minor (Min.) alleles. Statistical significance of the minor allele effect was determined using Type III ANOVA: \*\*\* =  $p < 0.0001$ ; \*\* =  $p < 0.001$ ; \* =  $p < 0.05$ . The reported environment is highlighted in bold; similar trends were observed across other environments. Asterisks (\*) in the WC2 column indicate significant effects of the same SNP on the corresponding trait in WC2.

SNP effects and candidate gene identification:

To identify candidate genes underlying beneficial allelic effects, we explored the linkage disequilibrium (LD) blocks surrounding SNPs with significant effects on forage traits. Several SNPs were associated with both biomass yield and forage quality under dry and irrigated environments, and gene annotations within an 18 kb window revealed plausible biological mechanisms for their effects (Table 1.5).

QTL	Physical position (kb)	SNP	Gene Name
<i>HvBY1HL</i>	540.80	11_11481	60S ribosomal protein L18A-1
<i>HvBY6HL</i>	507.50	11_20784	ONAC010 (NAC transcription factor)
<i>HvGYD3HL</i>	482.10	SCRI_RS_109716	Glutamate synthase 2
<i>HvGYD6HL</i>	583.30	12_30956	Multidrug transporter
<i>HvGYI1HL</i>	345.30	SCRI_RS_155439	Trihelix transcription factor GT-2
<i>HvGYI7HL</i>	644.40	12_21016	Chaperone protein HtpG
<i>HvADF7HL</i>	578.30	12_31395	Lysophosphatidylcholine acyltransferase 2
<i>HvADF6HL</i>	554.80	SCRI_RS_201988	Pheophorbide a oxygenase
<i>HvNDF1HL</i>	415.23	SCRI_RS_213455	UDP-glucose 4-epimerase
<i>HvNDF2HL</i>	615	12_10151	Cysteine proteinases superfamily protein
<i>HvNDF5HL.1</i>	373.45	11_20239	Asparagine synthetase, Adhesin FhaB

Table 1.5. List of putative genes within 18kb window of candidate SNPs associated with forage yield and quality under various environments.

## Discussion

To better understand the loci related to forage yield and quality in barley, 260 spring, two-row barley lines were selected, and data were generated on each accession's forage and agronomic traits. The developmental traits and plant height showed relatively high heritability across all environments. This matches the prior report of Alqudah et al. (2016) where heritability for plant height was reported to be high to very high. In contrast, grain yield had much lower heritability, particularly under dry environments and dropped even more in 2017. In that year, genotype differences in yield were close to zero indicating that environmental stress amplifies non-genetic variance. For example, under drought, grain yield heritability is very low compared to non-stress conditions (Blum & Jordan, 1985). Likewise, Marzougui & Chargui. (2018) found lower barley yield heritability than heading date heritability in semi-arid environments. The drop in heritability of forage yield under hot conditions (especially 2017 dryland) suggests biomass production was also environmentally constrained. ADF showed strong environmental sensitivity, with heritability nearly zero under irrigated conditions in 2016 and remaining low in 2017. This implies that digestibility is not stable across environments and is highly influenced by abiotic factors like temperature and water availability. Like ADF, NDF also appears to be poorly heritable under hot and irrigated conditions. Similarly, heritability values of ADF and NDF were reported to be low in lucerne breeding lines under arid conditions indicating a strong influence of the environment on digestibility and intake (Tlahig et al., 2024). The zero heritability values under 2016 irrigation and 2019 suggest that quality traits are buffered by environmental effects. With increased water availability (as in 2016 irrigated and 2019), there is increased biomass and higher ADF, NDF indicating that most of the observed variation is due to the environment rather than the genotype.

Biomass yield and grain yield were strongly and positively correlated in every environment, implying that enhanced biomass accumulation observed in high-yielding cultivars is frequently linked to their capacity for efficient resource utilization (Boukerrou & Rasmusson, 1990; Cui et al., 2025). A negative relationship between biomass yield and forage quality, as observed in most of our environments, has also been reported in triticale (Cui et al., 2025). These findings indicate that biomass and fiber content increase in parallel, eventually reducing digestibility and intake (Jung & Allen, 1995). Biomass yield was also positively associated with increased duration of vegetative development. Hot and dry environments result in an early onset of the reproductive stage (earlier in 2017 than 2016) and therefore reduced biomass yields were observed in both 2016 and 2017 dry environments. Increased precipitation in 2018 and 2019 extended the vegetative stage, boosting biomass yields. While irrigation improved yield in 2016, no such benefit was seen in 2017 due to extreme heat. Delayed heading and maturity generally enhanced grain yield, except in 2017 dryland. Additionally, grain yield and biomass were positively associated with plant height. Marzougui & Chargui. (2018) found that early-heading barley had significantly higher grain yield, and taller stature under semi-arid conditions.

In most environments increased water correlated with lower digestibility likely due to increased biomass and delayed development. However, in both environments in 2016, improved digestibility (lower ADF) was associated with delayed development. Moderate drought conditions reportedly inhibit new tiller growth and increase leaf/stem ratio resulting in increased digestibility (Grant et al., 2014; Katoch, 2022). Under short to medium durations of water stress, increase in water soluble carbohydrates and decrease in cell wall constituents such as cellulose, hemicellulose, and lignin was observed in grasses (Coleman et al., 2004). Reduction in ADF and NDF under water

deficit conditions was reported in forage legumes due to delayed plant development (Fariaszewska et al., 2020; Peterson et al., 1992; Vincent et al., 2005). While NDF showed positive correlations with delayed development in all environments, these were non significant under 2016 irrigated conditions. Conversely, we observed non significant correlations between ADF and development in 2017 dryland, and positive correlations between higher ADF and delayed development were observed in 2017 irrigated. In 2017, early development and higher ADF and NDF values were observed as compared to 2016, which can be explained by higher temperatures in 2017. Increased temperature reduces the leaf to shoot ratio, decreasing biomass and digestibility. With an increase in 1°C, the dry matter digestibility of cool season grasses declined by 6.6g/kg (Minson & Wilson, 1994). This occurs because higher ambient temperatures causes a reduction of water soluble carbohydrates in the stem and an increase of cell wall components (Ford et al., 1979). In alfalfa and triticale, lignin and NDF were observed to increase with rise in temperatures (Cui et al., 2025; Moyo & Nsahlai, 2021; Sanz-Sáez et al., 2012). Temperature is the key factor shaping length of the growing season and plant development, but changes in precipitation and CO<sub>2</sub> levels could result in shifts from these trends (Izaurrealde et al., 2011). All this supporting evidence highlights that both temperature and water deficit variations can impact overall biomass accumulation and digestibility in barley possibly via modifications in cell wall compositions.

We identified many ADF and NDF associated SNPs that coincide with previously reported forage quality QTL. A population of hulled × hulless barley was grown in irrigated and dryland environments and in which QTL were observed for ADF on chromosomes 2H, and 7H that explained 74-80% of variation in both environments (Abdel-Haleem et al., 2010). We identified QTL associated with biomass yield (HvBY2HS), ADF (HvADF2HS) and NDF (HvNDF2HS) in

close proximity to this chromosome 2H locus, but comparing the pleiotropic effects revealed tradeoffs between biomass yield and digestibility. Han et al. (2003) reported QTL for ADF on chromosomes 1H, 2H, 4H that explained 64.5% of total variation. A doubled haploid population of Steptoe × Morex was studied for forage quality and QTL for digestible dry matter and ADF on chromosomes 1H, 2H, 3H, 5H and NDF on chromosomes 1H, 2H, 3H, 5H, 6H were identified (Siahsar et al., 2009). The current study identified QTL associated with NDF (HvNDF5HL.2) in close proximity to their NDF locus on 5H. In addition to this, Naz et al. (2017) reported SNP for thioglycolic acid lignin (TGAL) near HvNDF5HL.2, which underlines the role of lignin in impacting forage quality traits. Using 145 of the lines from the same population, Surber et al. (2011) evaluated forage samples at two different maturity points, anthesis and peak forage yield. At anthesis, QTL for ADF were identified on chromosomes 1H, 2H, and 7H, while QTL for NDF were found on 2H, 4H, and 7H. At the later stage of maturity QTL for both ADF and NDF were only found on chromosome 2H (Surber et al., 2011). All the QTL on 2H reported in their study collocate with HvADF2HS and HvNDF2HS in ours. We also identified biomass yield QTL HvBY4HS in proximity to their NDF locus on 4H. In the current study, this QTL was detected in dry environments, with the minor allele associated with increased plant height and biomass yield but was estimated to have a negative effect on grain yield. Eight of the observed QTL showed pleiotropic effects of lower NDF and ADF, with earlier development, shorter stature, and lower biomass and yield potential. These QTL were detected mostly across dry environments and average of dry and irrigated environments: HvBY2HS (avg, dryland, irrigated, 2018), HvBY5HL (avg, 2016 dryland, 2018), HvBY4HS (dryland, 2016 dryland), HvADF2HS (dryland, 2016 dryland, 2019), HvNDF2HS (avg, dryland, 2016 dryland), HvNDF5HL.2 (avg, 2016 dryland,

dryland, irrigated). Since growers need both improved yield and quality these QTL showing negative correlation to quality and yield are less useful. However, *HvBY6HL* (2017 dry), was associated with increased yield and improve quality and so could be beneficial. QTL *HvGYI7HL* (irrigated) showed association of taller stature, more biomass, delayed heading and higher grain yield. Association of higher biomass with taller plants was also shown by *HvBY1HL* (2017 dryland) and these QTL could be deployed under irrigated and severe heat events respectively. However taller plants were not always associated with high biomass. QTL *HvBY7HL* (dryland, 2016 dryland) associated more biomass and ADF with shorter height and lower grain yield.

Two QTL (*HvGYD3HL*, *HvGYD6HL*) detected in dryland environments showed effects of lower ADF and delayed heading. In 2016, correlations of delayed development with lower ADF values were perhaps in part due to these dryland environment QTL. However, we also observed pleiotropic effects not fitting the correlations observed. For instance, in addition to later heading, and reduced ADF, *HvGYI1HL* (irrigated), *HvADF7HL* (2016 dryland), *HvNDF1HL* (avg, 2016 dryland) also showed increased biomass and grain yield potential. QTL *HvNDF6HL* (irrigated) showed lower ADF, delayed heading and increased grain yield. These QTL could be beneficial to improve biomass, grain yield and quality in both dryland and irrigated environments.

The QTL observed in WC1 that showed similar pleiotropic trends in WC2 were *HvBY2HS*, *HvBY5HL*, *HvBY7HL*, *HvGYI7HL*, *HvNDF2HS*, and *HvNDF5HL.2*. This means that these QTL show stable effects across populations and environments and could be deployed broadly. However, the effects of ADF QTL were not consistent across populations. Some QTL were not confirmed in WC2, perhaps due to differences in temperature and precipitation between the environments, although genetic differences cannot currently be ruled out. Although breeders tend to deploy QTL

that have consistent effects across environments, this choice may eliminate QTL with positive effects in specific environments. It is our goal to identify QTL that provide heat and drought tolerance without displaying negative effects when those stresses are not present.

Since both 2016 and 2017 trials were exposed to heat and drought, some of the QTL with positive effects in those environments could be related to abiotic stress tolerance. For instance, SNP 11\_11481 (*HvBY1HL*), associated with increased biomass yield and plant height in 2017, co-localized with 60S ribosomal protein L18A-1. This gene was also implicated previously, where overexpression of BrPP5.2 in *Brassica rapa* improved heat shock tolerance via inherent chaperone activity and altered expression of RPL18AA (Muthusamy et al., 2021). These findings support the potential role of L18A-1 in promoting translational stability and stress-responsive protein synthesis during abiotic stress. SNP 11\_20784 (*HvBY6HL*), linked to higher grain yield and reduced ADF in 2017 was near the ONAC010, a transcription factor in rice, known to be induced under drought stress (Fang et al., 2008; Sperotto et al., 2009), and to function in nutrient remobilization and senescence regulation. Notably, ONAC010 is also the closest rice ortholog of *HvNAM-1*, a regulator of developmental senescence (Uauy et al., 2006). This co-localization suggests a genetic basis for relating senescence timing with productivity and forage digestibility under terminal drought. SNP SCRI\_RS\_109716 (*HvGYD3HL*), associated with improved biomass and grain yield and reduced ADF under dry conditions, included a gene potentially encoding GLUTAMATE SYNTHASE 2, key to nitrogen assimilation and photorespiration, supporting a mechanism where sustained nitrogen metabolism contributes to better forage quality under stress (Pageau et al., 2006; Zhang et al., 2024). In the dry 2016 season, the minor allele of SNP 12\_31395 (*HvADF7HL*) increased biomass, grain yield, and days to heading while reducing ADF, and co-localized with

lysophosphatidyl choline acyltransferase 2, involved in osmotic stress tolerance via ABA signaling (Shaikh et al., 2022). Notably, the LD block of SCRI\_RS\_213455 (*HvNDFIHL*) (avg, 2016 dryland), whose major allele delays heading, increases biomass yield, grain yield and reduces ADF, includes UDP-glucose 4-epimerase. This gene when overexpressed showed increased sucrose and reduced cellulose levels under sub optimal N levels perhaps via repressing cellulose synthesis related genes (Guevara et al., 2014).

Together, these loci highlight stress adaptive pathways contributing to productivity and forage quality under drought and heat. These SNP candidate gene associations provide insights into the genetic control of forage productivity and quality, offering promising targets for selection in barley breeding programs aimed at resilience under increasingly variable climates. We are currently exploring these candidate genes further to validate their functional roles and breeding utility.

### Conclusions

Barley provides a valuable option for growers looking for a crop that can produce forage in a single growing season. But forage yield and quality are largely dependent on timing and duration of plant development which, in turn is influenced by the environment. This study focused on the impact of high temperature and water deficit on barley forage and identified loci associated with forage and key agronomic traits within and among irrigated and dryland environments. Temperatures above long-term average advanced the plant development and decreased the forage quality whereas moderate water deficit conditions delayed the plant development and resulted in reduced biomass yields and better forage quality. While the genetics of forage traits in barley had

not been well studied, these findings are consistent with research conducted in other grass and legume forages. Estimates of pleiotropic effects showed that the relationship between forage quality, forage yield, and maturity dates varied by locus and additional investigation was warranted. We shortlisted candidate SNPs and putative genes that shows beneficial effects on biomass yield, grain yield and forage quality despite the extreme weather events. For plant breeders developing forage barley cultivars, along with improving forage quality and yield, deploying resilient forage QTL is a goal. Those loci that are detected in the dryland *HvGYD3HL*, *HvGYD6HL*, *HvADF7HL* and *HvNDF1HL* are of the most interest to Montana forage barley breeders since most of the cropland in Montana is under dryland farming practices. The heat specific QTL *HvBY1HL* and *HvBY6HL* observed would be beneficial during the times of extreme heat. *HvADF6HL*, *HvGYI7HL*, *HvGYI1HL*, are the QTL that could be beneficial in irrigated environments. While *HvNDF2HL* may be deployed across environments, *HvNDF5HL.2* may be used to explore in depth the effect of water deficit on yield and quality traits as this QTL showed opposite effects between dry and irrigated conditions. The associations detected across the treatments could be expected to be the most environmentally stable and would be expected to have consistent impacts in future environments regardless of whether the crop was grown under dryland or irrigated conditions. The above-mentioned loci must be further validated, and they have great potential to benefit to forage barley breeders and growers via increased biomass yields and digestibility.

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CHAPTER THREE

EVALUATION OF STAY-GREEN QTL FOR ENHANCING  
HEAT STRESS RESILIENCE IN BARLEY NEAR ISOGENIC  
LINES

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### Abstract

Climate-induced heat stress poses a significant threat to barley (*Hordeum vulgare* L.) productivity and grain quality, especially during reproductive development. Williams et al (2024) identified stay green quantitative trait loci (QTL) associated with seedling root traits, grain quality and yield using a biparental population developed by crossing stay green (MT124118) and non-stay green (ND19119) parents. This study evaluated the performance of near-isogenic lines (NILs) carrying stay-green (ND) and non-stay green (MT) alleles generated for four QTL (*QGFhd-2H*, *QLN-2H*, *QGFmt-6H*, and *QGF-7H*) under controlled and heat-stressed environments. NILs were phenotyped for agronomic traits including plant height, tiller number, grain yield components, and grain protein content. Genotypic identity was confirmed via KASP assays targeting stay-green-associated SNPs.

Under heat stress, stay-green (ND) NILs showed enhanced tillering, and lower grain protein content relative to non-stay-green (MT) lines for most QTL. ND NILs for *QGFmt-6H* showed reduced grain protein both under control and heat stress conditions. The ND allele at *QGFhd-2H* enhanced reproductive traits and grain protein under stress despite reduced plant height, suggesting a beneficial balance of vegetative investment and reproductive output.

Trait correlations revealed stress-induced trade-offs between vegetative growth and grain protein content, likely due to altered assimilate partitioning. The results suggest that these root-associated stay-green QTL modulate heat tolerance via morphological and physiological pathways. These findings provide a foundation for targeted breeding of climate-adapted barley cultivars, particularly for dryland and high-temperature environments.

**Keywords:** Barley, Heat stress, Stay Green, NILs, QTL

## Introduction

Over the course of the 20th century, global temperatures have steadily increased and continue to rise, resulting in heightened temperature anomalies (Walthall et al., 2013) (IPCC). Consequently, the duration, frequency and intensity of heat waves are increasing with each decade (NOAA.gov/ 2024). In the United States, a heat wave is defined as ambient temperatures exceeding the 85<sup>th</sup> percentile of the average distribution (Bouchama et al., 2024). This changing climate directly impacts agriculture, including barley (*Hordeum vulgare* L.), with the United States accounting for approximately 3% of global barley production, yielding around 4.05 million metric tons annually (fas.usda.gov 2023/2024). Studies show that climate change has significantly reduced grain yields in barley worldwide, including in the U.S. (Ray et al., 2019). These reductions in productivity have cascading effects on global supply, trade, and commodity prices. Projections indicate that by 2050, climate-driven losses in barley production will likely lead to higher price volatility and reduced profitability for growers (Baker et al., 2018).

Barley is the fourth most important cereal crop worldwide, serving diverse purposes including animal feed, human food, and malt production. As a cool-season crop, barley is especially sensitive to deviations from optimal temperatures, making it vulnerable to climate-induced stress (Savin et al., 1997). Elevated temperatures negatively affect both yield and grain quality, primarily by decreasing starch accumulation, which not only reduces seed size but also increases grain protein concentration (Ni et al., 2020). These changes are particularly critical for malt barley, which commands higher market prices but requires strict quality standards, especially regarding grain protein and beta-glucan levels both of which are sensitive to heat fluctuations (Correll et al., 1994; Zhang et al., 2001). The extent of damage from heat stress depends on its

timing, duration, and intensity, with all developmental stages susceptible but flowering and early grain filling being especially vulnerable. For instance, high temperatures during tillering can reduce the number of tillers formed (Porter & Gawith, 1999; Wollenweber et al., 2003). Heat stress during early floret development alters anther shape and size, reduces pollen viability, and accelerates reproductive development (Callens et al., 2023). Additionally, kernel abortion can occur within 10 days after pollination associated with increased ethylene production (Hays et al., 2007), and cytokinin levels may also decline, reducing both kernel number and weight (Banowitz et al., 1999). During the early grain filling stage, heat stress has been shown to increase grain length but decrease grain width in barley (Shirdelmoghanloo et al., 2022). These traits are governed by distinct developmental processes; grain length is associated with caryopsis cell division and elongation, while grain width is influenced by starch accumulation in the endosperm (Shirdelmoghanloo et al., 2022). At this stage, heat stress also reduces starch synthesis enzyme activity (Wallwork et al., 1998a) and alters endosperm composition (Wallwork et al., 1998b), ultimately affecting final grain quality.

Plants employ a range of adaptive strategies to cope with heat stress, involving morphological and physiological traits that help maintain grain filling under elevated temperatures (Kumar et al., 2013). These adaptations may include changes in shoot and root biomass, as well as shifts in flowering and senescence timing. One promising trait under investigation for improving stress tolerance is “stay-green”, which has shown potential across several crops including barley (Shirdelmoghanloo et al., 2019; Williams et al., 2024), maize (Cerrudo et al., 2017), rye (Zhang et al., 2022), sorghum (Burke et al., 2010; Johnson et al., 2015), and wheat (Kumar et al., 2021). Stay-green genotypes tend to prolong grain filling either by initiating early heading or by delaying

senescence (Williams et al., 2024). Historically, the first mention of this trait was in broad bean, where stay-green lines exhibited delayed maturity and more uniform seed size (Steinbuch et al., 1962). At the physiological level, stay-green phenotypes are associated with delayed transitions from carbon (C) and nitrogen (N) assimilation to remobilization, thereby slowing the onset of senescence (Thomas & Ougham, 2014).

For deploying this trait to mitigate abiotic stress effects, researchers have focused on understanding its physiological basis and genetic regulation. In barley, stay-green was shown to maintain extended grain fill period despite heat stress (Shirdelmoghanloo et al., 2019). Delayed senescence enhances carbohydrate accumulation and limits nitrogen remobilization, thereby increasing grain starch and reducing grain protein content (Cohen et al., 2022; Gregersen, 2011). Enhanced photosynthetic capacity in these lines results in greater assimilate production, which supports extended grain filling even under stress. Furthermore, at the anthesis stage, stay-green cultivars under combined heat and drought conditions upregulate chlorophyll biosynthesis genes and downregulate chlorophyll degradation genes as compared to non-stay green cultivars (Kumar et al., 2022). In wheat, stay-green also improved heat tolerance by supporting translocation of assimilates to grains during the critical grain-filling window (Yang et al., 2016). However, the performance of stay-green traits can be environmentally dependent. In some cases, no correlations were observed between stay-green and yield under irrigated conditions, whereas positive associations emerged under rainfed environments (Cook et al., 2021). Similarly, stay-green wheat cultivars produced higher yields in regions with deep soil moisture but failed to express the trait and yield advantage in areas where deep soil moisture was lacking (Christopher et al., 2008). These findings suggest that water availability plays a key role in stay-green expression and effectiveness.

Importantly, root systems may mediate these environmental interactions. The ability to retain green leaf area during late growth stages appears linked to enhanced water access, which may depend on root depth and distribution (Borrell et al., 2014; Christopher et al., 2008; Mace et al., 2012). In barley, the stay-green trait was positively correlated with deeper rooting, particularly under dryland conditions (Williams et al., 2022). Comparable results in wheat show that higher root length density at depth improves grain yield under rainfed but not irrigated environments (Postic et al., 2019). One QTL on chromosome 5H, associated with heat stress tolerance in barley, was found to co-locate with root traits (Gous et al., 2016), suggesting a genetic link between root function and stress resilience. Studies in wheat provide further evidence: genotypes with cooler canopy temperatures exhibited contrasting root distributions depending on the environment; more shallow roots under irrigated, heat-stressed conditions, and deeper roots under drought (Pinto & Reynolds, 2015). On the other hand, some research has shown no overlap in QTL for heat and drought tolerance in barley, suggesting these two stress responses might be governed by distinct genetic pathways (Gous et al., 2016).

Despite extensive research on the stay-green phenotype across various crop species, its interaction with root traits in barley under heat stress remains poorly understood. This is a significant gap, given that roots are highly sensitive to elevated temperatures more so than shoots and have a narrower optimal temperature range for growth (Porter & Gawith, 1999; Tiwari et al., 2022). Within a malt barley population segregating for the stay-green phenotype, QTL were discovered that co-located with seminal root traits (assessed under greenhouse conditions) as well as grain fill duration and grain quality traits (measured under semi-arid field environments) (Williams et al., 2024). To validate these findings, near-isogenic lines (NILs) for the identified

QTL were developed and evaluated under both control and heat stress conditions. This work seeks to address the central research question: Do stay-green lines associated with seedling root traits exhibit improved agronomic performance under heat stress compared to non-stay-green lines?

## Materials and methods

### Experimental design and management

A biparental population was generated as described in Williams et al. (2024) by crossing a pollen donor, stay green ND19119 (ND15403.3/ND15368/ND16453) from North Dakota State University and female parent, non-stay green MT124118 (Hockett/MT070174) from Montana State University. The resulting F1 progeny from the parental cross were subsequently advanced by single seed descent for six generations, yielding recombinant inbred lines (RILs). Four QTL were identified for extended grain fill, one through earlier heading, *QGFhd-2H*, one through later maturity, *QGFmt-6H*, and another through both earlier heading and later maturity, *QGF-7H*. In addition, QTL were identified that associated with changes in early root development, *QLN-2H*. For most of the QTL the allele from the ND parent extended grain fill or increased root development.

### Development of NILs

Lines from the developing mapping population at the F<sub>5</sub> generation were screened using Kompetitive Allele Specific PCR (KASP) to identify heterozygotes at each QTL. SNP markers previously associated with stay-green traits in barley (including markers on chromosomes 2H, 6H, and 7H) (Williams et al., 2024) were targeted using allele-specific primers (Table 2.1). The recommended KASP protocol by He et al. (2014) was followed with 25ng DNA, 5 µl Master Mix

and 0.14  $\mu$ l primers. Genotyping was performed using the standard KASP thermal cycling protocol with a touchdown profile (61–55 °C). Each heterozygote was allowed to self-pollinate, and 20 of its progeny were screened for lines homozygous for either the MT or ND allele. For each of these QTL, five pairs of near isogenic lines (NILs), 50% carrying the ND allele and the other 50% with the MT allele were developed. Head rows for each homozygote were planted in the field to increase the seed and seed from each head row was increased in full plots the following year. Lines were re genotyped to ensure allelic assignments.

QTL	SNP	Allele MT	Allele ND	Sequence	CG %_X	CG %_Y	CG%_co mmon
QGFhd-2H	BOPA1_2634-2228	A	C	GATTTTCCAAGRGGTCCGCAA ACTCTTCCAACCGGCAAAGTT CTCCCTGG[A/C]AA TAACAATAGTTATGATAAATG CCGCCGTAGATTTGATCTTGG AGATGC	54.5	65	37.9
QGFhd-2H	JHI-Hv50k-2016-94875	T	C	GATCACAGATAATATACATTA TGTAACCTTTCTTTCAAGCTCAA GTTTGAA[T/C]AGG TCAAAAGCACGATGTGTAGCA AACTAGTCATTAATGGTGTA TTTCT	30	33.3	37.9
QLN-2H	JHI-Hv50k-2016-114164	C	G	AAGGTGATAACTAGGACGGTA GGAGCCATTCGCTCCTGTAAG TTTGTAC[C/G]G TGCCGAAACATAGCSGGCATT GTGGCAGCAGGCAGGCACATC ACACAAT	44	44	59.1
QLN-2H	JHI-Hv50k-2016-115620	A	G	CCATAWCAAATACCTTATGCC GTGGTATATAGTGATGCCAAT TGACAGCA[A/G]A CCTTTCGTAAAGTGAAGGAAA CCCGCCGTGAATTTCTCTTAAT GACTTG	42.3	44	37.9

Table 2.1 Continued

QGFmt-6H	JHI-Hv50k-2016-385462	G	C	TTTGTA AAAATGCAAGATYTGC AAAGGTTTGGACTTCATTCAC GGGCGTGG[C/G]GT AGCACACCTTGACGTGAAACC AGATAACATATATGTCAGGAA TGGTAT	52.2	52.2	41.1
	<i>HvNAM-1</i>	ccgcg cgaccgc aag tacg	ccgcg cgacc gcaag tacc	ACGAGGAGCTGGTCGTGCACT ACCTCAAGAAGAAGGCCGCCA AGGCGCCGCTCCCCGTCACCA TCATCGCCGAGGTGGACCTCT ACAAGTTCGACCCATG[C/G]GA GCTCCCCGGTATGTACTACTA GTTAGTACTATGTCTATCCCTA TCTCGTCGATCGTGCTTGCTTG CTCTATCAAGCGCCGTAATTC CCGGTGAATT			
QGF-7H	JHI-Hv50k-2016-481205	T	G	TCAYGACAATATCGTTGTAA CAGGGGAAACATCCTGATGAT GAGCATCC[T/G]GT ACTTATGACACATTCTAAAGC TGGATTTAGCGTCCGGCACAG GAGAAG	44	45.8	48

Table 2.1. KASP Primer Sequences for Allele-Specific SNP Genotyping

Plants were grown in two environments: greenhouse or a controlled growth room. Three seeds per pot were sown in Sunshine Mix #1 (Sungro Horticulture) and replicated three times per environment. In the greenhouse, conditions were maintained at 22 °C (day) and 18 °C (night) with a 16-hour photoperiod. In the growth room, identical temperature and light intensity were provided from germination through tillering. From tillering to maturity, heat stress was imposed by raising the daytime temperature to 32 °C and nighttime to 17 °C in the growth room. A simulated heatwave was introduced for one week during early grain filling by further elevating temperatures to 35 °C/20 °C (day/night).

All plants were adequately watered and fertilized to avoid drought stress and ensure uniform growth conditions across treatments.

### Agronomic trait evaluation

At maturity, plant height was measured from the base to the tip of the spike, excluding awns, using a meter stick. The number of productive tillers was counted from all three plants in the pot. Seed was harvested, threshed and cleaned manually from all plants within a pot (three per replicate). Grain number and total grain weight were recorded per plant, and average grain number and grain weight per head were calculated. Values from the three plants per pot were averaged to represent one biological replicate.

For nitrogen analysis, grain samples from each replicate were ground to a fine flour. Total nitrogen content was determined using a Costech ECS 4010 Elemental Analyzer, based on dry combustion. Nitrogen values were converted to percent grain protein by multiplying by a factor of 6.25.

### Statistical analysis

#### Pearson correlation analysis

Pearson correlation analysis was conducted to evaluate pairwise associations among plant height, number of productive tillers per plant, grain number per head, grain weight per head and grain protein content within stay green (ND) and non-stay green (MT) NIL groups of four QTL *QGFhd-2H*, *QLN-2H*, *QGFmt-6H*, *QGF-7H* under control and heat stress conditions. Correlation coefficients ( $r$ ) and p-values were calculated using `cor.test()` function in R. Heat maps were generated to show the strength of correlations.

### T test

To understand the effects of allele (stay green/non stay green) on the traits plant height, number of productive tillers per plant, grain number per head, grain weight per head and grain protein content, two sample t-test was conducted by comparing trait means between alleles within control and heat stress treatments. As equal variance could not be assumed in all cases, Welch's t-test (which does not assume equal variances) was applied.

### Results

Near-isogenic lines (NILs) were developed such that each NIL pair contrasted in their alleles across all four QTL: *QGFhd-2H*, *QLN-2H*, *QGFmt-6H*, and *QGF-7H*. In each pair, one line carried the ND alleles and the other carried the MT alleles. As a result, 50% of the NILs carried the ND allele and 50% carried the MT allele at a given QTL. These were evaluated under control conditions and under heat stress to determine if any of the alleles provides better agronomic performances under these conditions. Traits were measured including plant height, number of productive tillers per plant, grain number per head, grain weight per head and percent grain protein content. Allele (ND/MT) effects on these traits were estimated under control and heat stress (Table 2.2).

#### Agronomic Performance of ND and MT NILs Under Control and Heat Stress Conditions

*QGFhd-2H* (2.1H) Under control conditions, the ND allele showed significantly higher productive tiller number than the MT allele, while MT had slightly higher seeds per head and seed

weight per head, but these differences were not statistically significant. Grain protein content was significantly lower in ND compared to MT.

Under heat stress, the NILs with the ND allele were shorter, produced significantly higher seed number and weight per head than MT NILs. MT showed a lower grain protein content than ND, though ND had higher productive tiller number, albeit not significantly.

QLN-2H (2.2H) Under control conditions, MT NILs were significantly taller than ND, while no significant differences were observed for tiller number, seeds per head, or seed weight. ND exhibited significantly lower grain protein content than MT.

Under heat stress, ND and MT were similar across all traits, including height, tiller number, and seed components. Although MT had slightly higher protein content, the difference was not statistically significant.

QGFmt-6H (6H) Under control conditions, ND NILs were significantly lower in percent grain protein than MT NILs, but not significantly different for other traits.

Under heat stress, ND NILs had significantly more productive tillers than MT. Grain protein content was markedly lower in ND NILs compared to MT. For all other traits, including seeds per head, seed weight, and height, the differences were not significant.

QGF-7H (7.2H) Under control conditions, no significant differences were observed between MT and ND NILs for any traits, including productive tiller number, seed yield components, or grain protein content.

Under heat stress, ND NILs were significantly taller than MT NILs and had significantly more productive tillers. The grain protein content was significantly lower in ND NILs than MT, while other traits were not significantly different.

QTL	Treatment	Allele	Height (cm)	Productive tiller number	Seeds/head	Seed weight/ head (g)	Grain protein (%)
<i>QGFhd-2H</i>	Control	MT	55.88 ± 5.23	2 ± 1.31	13.96 ± 10.86	0.67 ± 0.531	15.77 ± 0.53
		ND	54.61 ± 6.37	3.1 ± 2.2**	11.91 ± 4.18	0.58 ± 0.22	15.03 ± 0.68****
	Heat	MT	56.76 ± 6.95*	2.35 ± 0.98	4.64 ± 2.78	0.16 ± 0.12	13.46 ± 1.54**
		ND	51.76 ± 5.61	2.82 ± 0.46	6.98 ± 2.99**	0.23 ± 0.12*	15.21 ± 2.42
<i>QLN-2H</i>	Control	MT	62.59 ± 8.47*	2.37 ± 1.33	15.87 ± 4.47	0.81 ± 0.27	17.93 ± 0.8
		ND	58.71 ± 7.9	2.32 ± 1.41	16.38 ± 2.85	0.82 ± 0.18	16.3 ± 0.84****
	Heat	MT	64 ± 5.33	2.37 ± 0.94	5.74 ± 3.28	0.17 ± 0.12	16 ± 2.1
		ND	63.01 ± 6.74	2.8 ± 1.16	4.58 ± 2.82	0.16 ± 0.11	15.16 ± 1.84
<i>QGFmt-6H</i>	Control	MT	66.7 ± 9.3	3.52 ± 2.34	15.33 ± 7.02	0.9 ± 0.87	16.29 ± 0.5
		ND	64.74 ± 7.62	2.95 ± 1.91	16.59 ± 3.54	0.81 ± 0.23	14.78 ± 0.45****
	Heat	MT	61.11 ± 4.29	1.88 ± 0.69	5.46 ± 2.16	0.19 ± 0.14	18.46 ± 1.37
		ND	62.99 ± 5.18	2.73 ± 1.03*	6.14 ± 3.38	0.22 ± 0.12	14.16 ± 1.57****
<i>QGF-7H</i>	Control	MT	61.54 ± 6.87	4.56 ± 3.25	12.92 ± 2.95	0.56 ± 0.15	15.76 ± 1.18
		ND	62.68 ± 6.21	4.35 ± 3.3	13.24 ± 3.23	0.61 ± 0.16	15.89 ± 1.3
	Heat	MT	57.48 ± 5.52	2.71 ± 0.65	9.24 ± 2.83	0.28 ± 0.09	15.79 ± 0.82
		ND	63.92 ± 7.54****	3.23 ± 0.84**	8.9 ± 2.97	0.32 ± 0.11	13.86 ± 1.62****

Table 2.2: NILs for Four Stay Green QTL Response to Heat Treatment During Grain Fill.

\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$

Agronomic Trait Correlation Patterns in ND and MT NILs  
Under Control and Heat Stress

*QGFhd-2H (2.1H)* Plant height was positively associated with number of productive tillers per plant for both ND and MT alleles under control and heat stress conditions, although non-significant for ND under heat stress (Figure 2.1). Number of productive tillers per plant and percent grain protein negatively correlated with grain number and weight per head for ND under both control and heat stress conditions. Plant height was positively correlated with grain protein for MT under control and heat stress. For both MT and ND alleles, plant height under heat stress was positively correlated to grain number per head, grain weight per head. Plant height and number of productive tillers per plant were negatively correlated to percent grain protein for ND under heat stress.

*QLN-2H (2.2H)* Plant height positively correlated with grain number and grain weight per head for MT allele and with number productive tillers per plant for ND allele under control conditions (Figure 2.1). Number of productive tillers per plant negatively correlated with grain number and weight per head for both ND and MT under control conditions. Plant height positively correlated with number of productive tillers per plant, grain number and weight per head for both ND and MT alleles under heat stress. Percent grain protein negatively correlates with plant height, number of productive tillers per plant, grain number and weight per head both for MT and ND under heat stress.

*QGFmt-6H (6H)* Plant height positively correlates with number of productive tillers per plant both for ND and MT alleles under control and heat stress conditions (Figure 2.1). Plant height also positively correlated with grain number and weight per head for ND under heat stress. Number of productive tillers per plant negatively correlates with percent grain protein for MT, grain number per head and grain weight per head both for MT and ND under control conditions. Both plant

height and number of productive tillers per plant negatively correlate with percent grain protein for ND under heat stress.

*QGF-7H (7.2H)* Plant height positively correlated with number of productive tillers per plant both for ND and MT alleles under control and heat stress (Figure 2.1). Number of productive tillers per plant negatively correlated with grain number, grain weight per head and percent grain protein for MT and ND alleles under control conditions. Plant height positively correlated with grain number, grain weight per head for ND and MT alleles under heat stress and this association was negative for both alleles under control conditions. While number of productive tillers per plant and plant height positively correlated with grain protein for MT, they negatively associated for ND allele under heat stress.

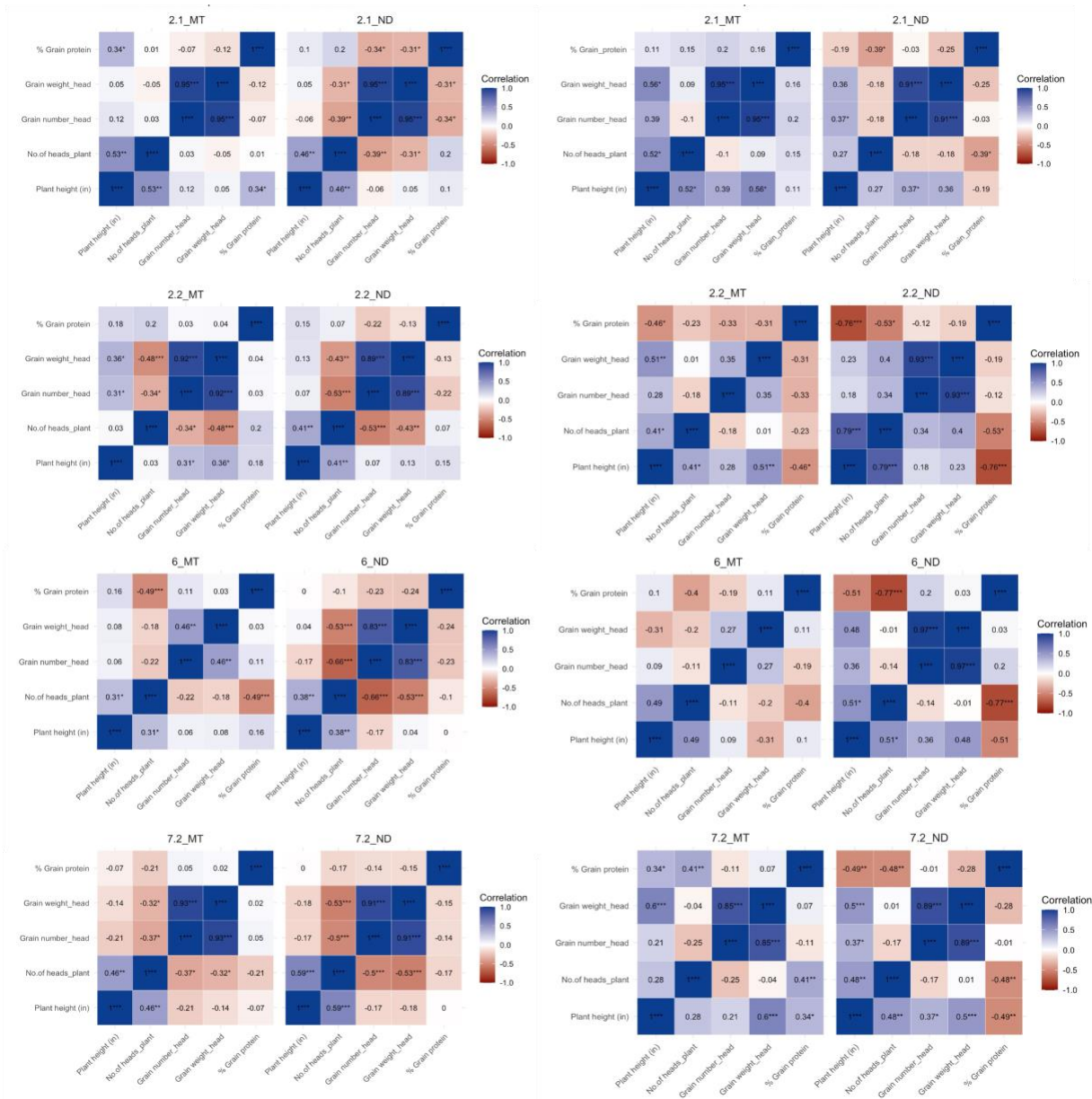


Figure 2.1. Trait-wise Pearson correlation matrices for stay green (ND) and non-stay green (MT) groups of QGFhd-2H, QLN-2H, QGFmt-6H, QGF-7H under control (left), heat stress (right) conditions. Values are colored by correlation strength (red = negative, blue = positive), with significance indicated by stars ( $p < 0.05 = *$ ,  $< 0.01 = **$ ,  $< 0.001 = ***$ ).

## Discussion

In this study, we evaluated the potential of previously identified stay-green QTL from semi-arid conditions for conferring heat tolerance by assessing the agronomic performance of four sets of near-isogenic lines (NILs) under control and heat stressed environments. These NILs differed in heading and maturity timing. One of the most critical stages influencing heat tolerance is the flowering, as it directly impacts reproductive success and yield stability under stress. Hill et al. (2024) reported that barley plants exposed to temperatures above 35 °C during flowering exhibited an average 20% reduction in seed set, with late-maturing genotypes experiencing up to 80% loss. To ensure stress was applied at equivalent developmental stages, heat stress was initiated when each of the NIL sets reached tillering.

The following sections present QTL-specific responses, focusing on how each locus modulated yield components and physiological traits under heat. These insights not only validate the utility of previously identified stay-green QTL but also reveal novel allele-specific trade-offs and advantages under elevated temperatures.

### *QGFhd-2H*

*QGFhd-2H* exhibited clear signs of yield stability under heat. This supports the findings from Williams et al. (2024), where the ND allele at this locus was associated with early heading and enhanced grain yield and protein across environments. Notably in our study, ND lines also exhibited higher grain protein content under heat stress. A similar trend was reported by Gous et al. (2017), where stay-green phenotypes under heat stress were linked with elevated grain protein levels in barley. While the ND allele was also associated to shorter seedling root length and lower

lateral root count, it contributed positively to grain production under both irrigated and dryland conditions (Williams et al., 2024). This QTL may be particularly beneficial for heat-stressed forage or feed barley, where protein content thresholds are more flexible.

### *QLN-2H*

The consistent performance of NILs carrying *QLN-2H* across both control and heat stress environments suggests a stable, though relatively neutral role in heat stress adaptation. Under control conditions, the ND allele exhibited significantly lower grain protein content than the MT allele, despite no major differences in yield components or tiller number. Interestingly, Williams et al. (2024) reported that the ND allele at this locus was associated with increased seedling root length, which may enhance early-stage water and nutrient uptake. While this allele may still be valuable in environments with moderate or prolonged stress events where extended grain fill is possible, our findings suggest that its contribution is limited under acute heat stress during reproductive stages.

### *QGFmt-6H*

The *QGFmt-6H* NILs exhibited a more distinctive response under heat stress, suggesting a potential role in heat adaptation. Under both control and heat stress conditions, the ND allele consistently showed lower grain protein content than MT, which aligns with previous findings (Williams et al., 2024), where this allele was associated with reduced protein concentration across environments. This reduction in protein could be advantageous for malt quality. Under heat stress, ND lines produced significantly more productive tillers than MT, despite no major differences in seed number or weight. This indicates that tiller number, rather than per head yield, may be a key

contributor to total yield under stress in these NILs. Interestingly, Williams et al. (2024) also noted that the ND allele was associated with delayed maturity and reduced seedling root length and provides a positive effect on yield in both irrigated and dryland conditions. The present study supports the positive impact on tillering and yield components, even though the greenhouse heat stress was more intense than field conditions previously tested (Williams et al., 2024), potentially amplifying the negative effects on grain number and weight. This QTL region is reported to co-locate with *HvNAM-1*, a major regulator of leaf senescence and grain protein content in barley. The observed reduction in protein and improvement in tillering may reflect delayed senescence or extended assimilate production under stress. This supports the idea that *QGFmt-6H* offers heat resilience via traits that stabilize vegetative growth and grain composition.

#### *QGF-7H*

The performance of NILs carrying *QGF-7H* under heat stress points to its potential utility in improving shoot-related traits associated with heat resilience. Under stress conditions, the ND allele was associated with a significant increase in plant height and productive tiller number, though seed yield components such as seed number and weight per head were not significantly different between alleles. These observations suggest that the ND allele may enhance vegetative growth under elevated temperatures, contributing to greater biomass and potentially buffering yield losses. Interestingly, grain protein content was significantly lower in ND under heat. These findings correspond with Williams et al. (2024), where the ND allele for this QTL was reported to promote earlier heading, greater plant height, and increased grain yield under dryland and irrigated environments.

Building on these locus specific effects, we investigated how heat stress alters whole plant physiology, beginning with height. Heat stress has been shown to reduce plant height in cereal crops such as wheat (Joshi et al., 2016), and a similar trend was observed in several NILs in our study. Notably, under heat stress, plant height was negatively associated with grain protein percentage in all ND NILs, while this relationship was absent under control conditions. A similar negative correlation was seen for MT NILs in the 2.2H background under heat stress. Likewise, the number of productive tillers per plant was inversely related to grain protein in all the ND NILs and in MT NILs for 2.2H under stress. All ND NILs, except for 2.1H, exhibited reduced grain protein percentage under heat stress, while ND NILs for 2.1H showed reduced plant height. This pattern suggests a potential trade-off, where taller plants may allocate more nitrogen toward sustaining vegetative biomass at the expense of grain protein accumulation. A similar mechanism could explain the observed negative correlation between grain protein content and number of productive tillers per plant in ND NILs under heat stress, as increased sink strength from more productive tillers may dilute available nitrogen per grain (Blum, 1998). The observed negative association between grain protein and other growth traits may also reflect a shift in assimilate partitioning under heat stress. Barley endosperm typically comprises 60% starch and 8-30% protein (Jaeger et al., 2021). Heat stress during early grain filling reduces the activity of enzymes needed for starch biosynthesis (Blum, 1998; Mangelsen et al., 2011), resulting in disproportionately lower starch accumulation. Consequently, grain protein concentration increases, not due to enhanced protein production, but due to the relative decline in carbohydrate deposition, a phenomenon known as the growth dilution effect (Pleijel et al., 1999). This biochemical response, widely observed in cereals including wheat (Bhullar & Jenner, 1985;

Sofield et al., 1977; Viswanathan & Khanna-Chopra, 2001), provides a plausible explanation for the grain protein trends observed in our NILs under terminal heat stress.

Interestingly, plant height in ND NILs was positively correlated with grain number and grain weight per head under heat stress. Taller barley genotypes have been reported to contribute more pre-anthesis stem reserves to grain yield compared to shorter genotypes (Daniels et al., 1982). However, this same study found that absolute yields between tall and short genotypes were similar, suggesting that taller plants might suffer reduced current assimilation, possibly due to self-shading, particularly under stress. Another study showed that plant height was positively correlated with SPAD values of the penultimate leaf, a proxy for chlorophyll content, and explained 10.9% of grain yield in wheat (Roy et al., 2021). This suggests that stem reserves remain a key source for grain filling, but under elevated temperatures, stay-green genotypes may gain an additional advantage by prolonging the grain-filling period. This extended duration allows more effective mobilization of reserves to grains, mitigating sink limitations often induced by heat (Blum, 1998; Blum et al., 1994).

Although root traits were not directly assessed in this study, previous work has shown that these QTL are associated with seedling root traits (Williams et al., 2024). Additional studies support a correlation between plant height and root length (Bai et al., 2013; Wang et al., 2018). Stay-green genotypes with their deeper root systems can access moisture from deeper soil levels and support continued grain filling (Manschadi et al., 2006). However, heat tolerance in stay-green genotypes may also stem from mechanisms beyond root architecture. For example, ND NILs for 2.1H showed reduced plant height and increased grain protein under heat, while simultaneously producing more grains, and higher seed weight per head. Notably, 2.1H is located within the

*qGL2H* region, a QTL associated with grain length in barley (Watt et al., 2020). Candidate genes within this QTL include a MYB transcription factor, hexosyltransferase, and root UV-B sensitive 2 protein, which may contribute to the observed phenotypes (Watt et al., 2020). High temperatures during grain fill are known to increase grain length while reducing width and thickness in barley (Shirdelmoghanloo et al., 2022). Moreover, grain protein content has been reported to correlate positively with grain length and other physical traits across environments (Shirdelmoghanloo et al., 2022), potentially explaining the increased protein observed in 2.1H ND NILs under heat.

Collectively, these observations suggest that multiple physiological and morphological traits such as plant height, tillering, grain number, and protein interact to shape heat stress resilience in barley NILs carrying stay-green QTL. The favorable trait combinations observed in ND lines, particularly those balancing yield and protein content under terminal heat stress, underscore their value in breeding for climate-adaptive barley. These ND allele-specific associations highlight the potential of stay-green genotypes to maintain productivity under adverse heat conditions. Several of these heat-tolerant NILs are already integrated into the Montana barley breeding pipeline, facilitating their deployment in hot and dry environments. While ND NILs for *QGFhd-2H* may be more suitable for forage and feed purposes due to their high grain protein content, ND NILs for *QGFmt-6H*, and *QGF-7H* appear promising for malt barley production under heat stress conditions.

### Conclusions

This study evaluated the role of stay-green QTL in barley under terminal heat stress by analyzing the performance of NILs differing in heading, maturity dates and allelic background.

Significant allele effects were observed, particularly for grain protein and number of productive tillers per plant. The ND allele at *QGFhd-2H* enhanced reproductive traits and grain protein under stress despite reduced plant height, suggesting a beneficial balance of vegetative investment and reproductive output. Correlation analyses also revealed key tradeoffs, including negative associations between plant height and number of tillers/grain protein content, indicative of nitrogen dilution. These findings highlight the complex interplay between stay-green traits, assimilate partitioning, and stress adaptation. Importantly, the QTL on chromosome 6H, which co-locates with *HvNAM-1*, a gene involved in nitrogen remobilization, and overlaps with seedling root trait QTL, represents a promising target for further investigation. A gene expression study evaluating *HvNAM-1* activity in roots under control vs heat stress conditions could help elucidate its functional role in root-mediated stay-green responses. The superior performance of ND NILs under heat stress, particularly for QTL *QGFmt-6H*, and *QGF-7H*, supports their potential deployment in breeding programs targeting hot, dry environments. One next goal is to validate the QTL for agronomic performance in field conditions. Continued efforts to fine-map these QTL will be instrumental in identifying the underlying genes and optimizing stay-green traits for improving heat resilience in barley.

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CHAPTER FOUR

ROOT-DRIVEN RESILIENCE: TRANSCRIPTOMIC INSIGHTS  
INTO HvNAM-1 MEDIATED SENESCENCE AND STRESS  
ADAPTATION IN BARLEY

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Abstract

Heat and drought stresses, particularly in combination, substantially reduce barley productivity and grain quality. The stay-green trait, characterized by delayed senescence and extended grain filling, has emerged as a promising mechanism for stress adaptation. Stay-green has also been associated with differences in roots. This study investigates the potential role of the NAC transcription factor *HvNAM-1*, a known regulator of developmental senescence, in modulating root responses under both control and combined heat-drought conditions. Using near-isogenic lines (NILs) differing at the *HvNAM-1* locus, we conducted RNA sequencing across key developmental stages. The NILs were created from a bi-parental cross between two experimental lines MT124128 and ND19119, where the MT allele at *HvNAM1* was associated with earlier above ground senescence and higher grain protein, while the ND allele was associated with delayed senescence and lower grain protein. Transcriptomic analysis revealed that ND roots, particularly at the booting stage under stress, upregulated genes associated with continued root growth, while downregulating senescence-related transcription factors. In contrast, MT lines activated canonical senescence and nutrient export pathways. Weighted Gene Co-expression Network Analysis (WGCNA) further supported these findings, identifying stress-specific modules in ND enriched for RNA metabolism and cellular protection functions. The transcriptional strategy in ND lines may contribute to prolonged grain filling and improved malt quality by maintaining root activity under stress. While delayed root senescence confers clear advantages under abiotic stress, potential trade-offs related to nutrient remobilization efficiency remain to be explored. Overall, this study highlights the potential of *HvNAM-1* as a target for breeding climate-resilient barley with stable yield and malting quality under heat and drought stress. Future proteomic and metabolomic studies are warranted to clarify the physiological implications of this strategy.

Key words: *HvNAM-1*, senescence, roots, heat stress, drought stress, differentially expressed genes (DEGs)

### Introduction

Heat and drought stresses are among the major abiotic stress factors that cause irreversible damage to crop productivity and quality. Both these stresses in combination cause even more detrimental effects in plants at reproductive stages (Cohen et al., 2021). Barley faces 3-17% average yield losses globally due to severe heat and drought episodes (Xie et al., 2018). In wheat-producing regions, the combination of heat waves and drought resulted in variability around 40% of annual production (Zampieri et al., 2017). Drought and heat stresses result in closure of stomata that restricts evapotranspiration cooling (Rizhsky et al., 2002), enhances carbohydrate demand for cellular respiration (Lambers et al., 2008; Yue et al., 2012), reduces photosynthesis and carbon assimilation, decreases grain filling duration (Awasthi et al., 2014), and prematurely ends the crop life cycle (Cohen et al., 2021). Additionally, this combination of stresses reduces malt quality (Mahalingam, 2017), by decreasing starch accumulation and increasing seed nitrogen (Savin & Nicolas, 1996). This is critical for barley growers as they lose half the crop value when it cannot be sold for malt.

The effects of heat and drought are exacerbated when combined. Under elevated temperatures, plants tend to lose water through evapotranspiration, which can cool the canopy (Lopes & Reynolds, 2010) but this cooling is limited by available soil moisture and root activity under drought conditions (Heckathorn et al., 2013). Moreover, roots are sensitive to stress conditions (Heckathorn et al., 2013) and are directly affected by elevated temperatures and moisture deficit (Calleja-Cabrera et al., 2020). Additionally, heat and drought also reduce root growth, and thereby water and nutrient absorption (Calleja-Cabrera et al., 2020; Heckathorn et al., 2013). Heat stress reduces carbon partitioning to roots resulting in reduced root development (Batts

et al., 1998; Heckathorn et al., 2013). With prolonged drought stress the transportation of carbon from leaves to roots is restricted resulting in reduction in root length (Muller et al., 2011). Reduction of root biomass of up to 40% was observed when combined heat and drought stress was induced at heading stage in barley (Mahalingam et al., 2022; Muller et al., 2011).

Plants adapt several morphological and physiological traits to stabilize yields under such stresses, including divergent senescence strategies. Some accelerate senescence to complete their life cycle quickly, while others maintain green tissues longer, a stay green phenotype, to support grain filling. In parallel, acclimatization to stress conditions involves adjustments in root system function, particularly in hydraulic conductance which can change over the course of stress exposure and with environment cues (Lipiec et al., 2013). In a study conducted in wheat, heat and drought tolerance was associated with limiting root hydraulic conductivity through smaller central meta xylem vessels, stele, and smaller but numerous stomata on flag leaves (Kulkarni et al., 2017; Shahinnia et al., 2016). QTL associated with root traits have been associated with both heat and drought adaptation in wheat (Acuña-Galindo et al., 2015). The stay green phenotype may also be a protection against heat and drought. Stay green cereals have an increase in grain filling duration due to early heading or delayed senescence (Mace et al., 2012). The potential of the stay green trait to improve heat and drought tolerance has been previously reported in barley (Gous et al., 2016), wheat, sorghum (Awika, 2016), and maize (Cerrudo et al., 2017). Williams et al. (2022) found that barley stay-green lines had higher percentage of deep roots and maintained roots through grainfill. In wheat, stay green lines have reportedly shown better yields under combined heat-drought conditions (Kumar et al., 2021). A study revealed that stay green QTL in sorghum modified root

architecture and increased biomass partitioning to roots providing conducive conditions for increased water availability during grain filling under water deficit conditions (Borrell et al., 2014).

We identified QTL for stay green (grainfill duration) that collocated with grain quality and root traits in a RIL population from ND19119-X MT124118 (Williams et al., 2024). Genetic mapping associated the locus including *HvNAM1* with variation in maturity, root development, grain protein content and plump seed. The ND19119 allele was associated with delayed above ground senescence, lower grain protein, and plumper seed as well as the *HvNAM-1* allele that has also been associated with those traits (Alptekin et al., 2021). *HvNAM-1*, in barley, is an orthologue of wheat *NAM-B1* (also known as Grain protein content 1, Gpc-B1). The 6H GPC QTL in barley was originally mapped using a RIL population from a cross between a high GPC variety Lewis and low GPC variety Karl (Mickelson et al., 2003; See et al., 2002). The ‘Karl’ and ‘Lewis’ alleles, associated with low and high grain protein content (GPC) respectively, and differ at two SNP positions that lead to changes in two amino acids (Distelfeld et al., 2008). Comparison of sequences of Gpc-B1 from modern wheat and wild emmer showed that most modern durum and bread wheat lines contain a frameshift mutation in the first intron, along with either a thymine insertion at position 11 or a full gene deletion, resulting in loss of protein function (Distelfeld et al., 2014; Hagenblad et al., 2022; Uauy et al., 2006). The expression of *HvNAM-1* was previously detected in barley flag leaves post spike emergence (Hagenblad et al., 2022) and the major differences in protein accumulation in seeds were observed at 21 days post anthesis (Jukanti et al., 2008).

Since, Williams et al. (2024) identified an association of stay green (delayed senescence) phenotype with root traits at *HvNAM-1* under semi-arid environment, we wanted to further investigate if this QTL region delayed senescence in roots and impacted tolerance to hot and dry

conditions via modulating root developmental mechanisms. Although Christiansen et al. (2011) reported no expression of *HvNAM-1* in barley roots at the three-leaf stage, Liu et al. (2019) described the plant age-mediated root senescence process in barley and observed *HvNAM-1* expression in roots at later growth stages. Therefore, we hypothesize that the genomic region harboring the allelic difference for *HvNAM-1* would change expression of senescence related genes in roots through development as well as under combined heat-drought stress. The overarching question we ask is – Does the ND allele for *HvNAM-1* delay expression of senescence genes in roots allowing more access to water during late grain fill and continued root growth during grain fill even under abiotic stress conditions?

## Materials and methods

### Plant materials

A biparental population was generated as described in Williams et al. (2024) by crossing pollen donor ND19119 (ND15403.3/ND15368/ND16453) from North Dakota State University and female parent MT124118 (Hockett/MT070174) from MSU. This population was used to identify QTL related to extended grainfill, agronomic, quality and root traits. For the identified QTL of interest, five pairs of near isogenic lines (NILs) were developed for *QGFmt-6H*, with 50% carrying the stay green (ND) allele and 50% with the non-stay green (MT) allele to validate the QTL by selecting heterozygous F5s for each QTL, allowing them to self and selecting homozygotes for each allele using KASP genotyping assay.

### Experiment conditions

Plants were grown using the polyvinyl chloride (PVC) pot assay used by Williams et al. (2022). Three seeds of each genotype were planted in each pot and one seedling was retained. The control set was grown under greenhouse conditions of 22°C day and 18°C night, with a 16-hour photoperiod. The treatment set was grown in the controlled growth room where the conditions from germination to the tillering stage were maintained with a day temperature of 22°C, a night temperature of 18°C, a 16-hour photoperiod, and a light intensity of 498  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  photosynthetically active radiation (PAR). From the three-leaf stage until sampling, heat stress was induced in the growth room by increasing the day temperature to 32°C while keeping the night temperature at 17°C. Additionally, heat wave conditions were mimicked by increasing the day and night temperatures to 35°C/20°C for a week. To induce drought conditions, watering was completely cut off starting from three leaf stage until sampling. During sampling, the PVC tubes were placed on a bench horizontally and the whole root system was pulled out carefully and shaken gently to remove any attached surface. The roots were washed using tap water for 30-60 seconds, patted dry using paper towels, and flash frozen in liquid nitrogen and stored at -70° C.

#### RNA extraction and sequencing

RNA was extracted from MT and ND NILs at the three-leaf stage under control conditions, and at the two node and booting stages under control as well as treated (combined heat-drought) conditions in triplicates. Bulk RNA-Sequencing was conducted by grouping NILs based on allele under each treatment (control or heat-drought) and growth stage (three-leaf, two-node, and booting) to determine if expression differences occurred between groups.

RNA-seq was performed on three biological replicates of MT and ND lines at the booting stage under both control and heat-drought conditions, resulting in a total of 12 samples. Differential

gene expression analysis was conducted to examine the effect of genotype on gene expression under each condition, focusing on the main effects. The differentially expressed genes (DEGs) were calculated with allele as main effect for a) ND vs MT under control and b) ND vs MT under combined heat-drought; with Treatment as main effect for c) Combined heat-drought vs control for ND and d) Combined heat-drought vs control for MT. The genes with p-adjusted  $< 0.05$  and  $> 1$  log<sub>2</sub>-fold change were designated as DEGs.

The frozen whole root system collected for the respective genotypes, growth stages and treatments were ground. Total RNA was further isolated from a subsample of bulk ground tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the standard protocol. RNA quality and integrity were evaluated using a Qubit fluorometer for accurate quantification, and a NanoDrop spectrophotometer to assess purity. The samples were sequenced using Illumina NovaSeq platform (Azenta Life Sciences). The quality of the raw reads was assessed using FastQC tool (Brown et al., 2017). To remove adapter sequences and poor-quality reads, Trimmomatic v0.39 (Bolger et al., 2014) was used (Suffix array using ensemble ) STAR 2.7.11b software was used to map the trimmed reads to barley reference genome Morex v3 pseudomolecules assembly.

Differential gene expression was conducted on the read counts using DESeq2 package (Love et al., 2014) on R and p-adj  $< 0.05$  was set to extract significant differences. The differentially expressed genes obtained for each factor were analyzed for Gene ontology enrichment analysis using topGO package on R (Alexa & Rahnenführer, 2009). Hierarchical clustering of samples was performed using the hclust () function in R (Langfelder & Horvath, 2012).

#### Weighted Gene Co-expression Network Construction

Weighted Gene Co-expression Network Analysis (WGCNA) was performed separately for two gene expression datasets using the WGCNA package in R (Langfelder & Horvath, 2008). To construct the co-expression network, we first assessed the relationship between genes by calculating pairwise correlations of gene expression across samples. A soft-thresholding power of 11 was selected for network construction to ensure a balance between network connectivity and sparsity, and the minimum module size was set to 30 genes. A topological overlap matrix (TOM) was calculated based on the unsigned correlation network where negative correlations were treated as having the same significance as positive correlations. The genes were grouped into dynamic modules and Pearson correlations revealed associations of these modules with module trait Treatment. A heatmap of module-trait relationships was generated to visualize the strength of associations between modules and sample traits. The modules were filtered using a threshold of 0.8 for module membership (MM) and gene significance (GS), and top highly connected genes with edge weight  $> 0.5$  were selected for visualization using Cytoscape (Shannon et al., 2003). The high correlation of GS and MM for a gene indicate that it is significantly associated with the trait and is possibly a hub gene within the specific module. To capture genes involved in multiple dense subnetworks, maximal clique centrality (MCC) on cytohubba plugin (Chin et al., 2014) was used to rank the top 30 hub genes.

#### Transcription factor and Transcription factor binding sites

##### prediction

The TF prediction tool in PlantTFDB (Jin et al., 2014) was used to scan for TF in protein sequences of genes in GO clusters of interest. The binding site prediction tool in PlantTFDB was used to scan for TF binding sites in the promoter sequences of the DEGs. This tool adopts FIMO

(Find individual Motif Occurrences), MEME Suite 5.5.7 and a threshold p value of  $1e^{-4}$  was set. Further, regulation prediction was done by assigning an interaction if there are  $\geq 1$  binding sites of a TF in the promoter of gene. The background positions relative to TSS of genes is set to range of -2000bp and threshold p value for binding site prediction was set to  $1e^{-5}$ .

#### Sparse Partial Least Squares Discriminant Analysis (sPLS-DA)

The gene expression matrices were normalized as TPM (Transcripts Per Million), log-transformed, and subsequently analyzed using sPLS-DA implemented in the mixOmics R package (Rohart et al., 2017). Further, Variable Importance in Projection (VIP) scores were computed for stay green and non-stay green samples to highlight how much each gene contributes to the distinction between groups and these scores are plotted against  $\log_2$ -fold changes as volcano plots with significant threshold set to  $VIP > 1.2$ ,  $|\log_2FC| > 2$  i.e., a 4-fold change. Out of these genes, top 30 are ranked by VIP scores are plotted in dot and tile plots. While the VIP scores and genes are plotted on x and y axes in the left panel, regulation patterns are depicted in the right panel categorized as upregulated and downregulated.

#### Validation of RNA seq results using RT-qPCR

To validate the differential expression of genes from RNA sequencing analysis, quantitative real-time PCR (RT-qPCR) was conducted. Total RNA was extracted from root tissue as described above, and RNA quality and concentration were measured using Nanodrop spectrophotometer. cDNA was reverse transcribed using oligo-dT from 1  $\mu$ g of total RNA (Biorad iScript reverse transcription supermix). The thermal cycle conditions used were 25°C for 5 min, 46°C for 1 hour

30 min, 95°C for 1 min, 4°C infinity hold. The cDNA was diluted by 1:10. Barley actin gene was used as the housekeeping gene, and the primers for candidate genes are shown in the Table 3.1. Each 8µl reaction contained 5 µl of SYBR Green mix (Biorad iTaq Universal SYBR Green Supermix), 1 µl of cDNA, and 2 µl of nuclease free water. The thermal cycle conditions were 95°C for 30 sec, 95°C for 5 sec, 55°C for 30 sec, 39 cycles of 95°C, 65°C for 5 sec, 95°C 0.5°C/cycle. To confirm the specificity of primers, melt curve analysis was conducted.

Gene	Primer name	Primers	Product size
<i>HvNAM-1</i>	qHvNAM-1 Fw	GTATGTCGCTGTCATCCACG	98
	qHvNAM-1 Rev	GTCTTGACACGGGGAGATACA	
<i>HvNAM-2</i>	qHvNAM-2 Fw	GTGGAGGCAATGCGATGAAC	91
	qHvNAM-2 Rev	CCGAGGATGACATGCTGTTG	
<i>HvGR-RBP1</i>	qHvGR-RBP1 Fw	GTCAACGAGGCCAGTCT	95
	qHvGR-RBP1 Rev	CCACCACCTCCGTACCTG	
<i>Actin</i>	qACT Fw	GGCATGGAGTCTTCTGGAATCC	115
	qACT Rev	CCACCACTGAGCACTATGTTTC	

Table 3.1. Candidate genes and their primers

Cq values obtained from the instrument were used to calculate fold changes in gene expression using the  $2^{-\Delta\Delta Cq}$  method (Livak & Schmittgen, 2001) or comparative Cq method. For each sample, the Cq value of the target gene was first normalized to that of the reference (housekeeping) gene (ACTIN) to obtain  $\Delta Cq$ :

$$\Delta Cq = Cq_{\text{target}} - Cq_{\text{ACTIN}}$$

The  $\Delta Cq$  of each treatment sample was then compared to the average  $\Delta Cq$  of the control group to obtain  $\Delta\Delta Cq$  values. Fold changes in gene expression were computed as  $2^{-\Delta\Delta Cq}$ . Calculations were made for group-wise average expression, standard deviation (SD), standard error (SE), and statistical significance using a two-tailed t-test.

## Results

### Developmental differential gene expression

To identify the most informative developmental stage, we compared the two node and booting stages to three leaf stage for both MT and ND NILs and used absolute  $\log_2$ -fold change ranking as a proxy to identify candidate genes with strong expression differences across growth stages.

### Stage-Specific Transcriptome Shifts in Barley Roots with under Control Conditions (MT)

To evaluate the dynamics of gene expression differences related to root growth across barley development stages, we examined the MT NILs at three leaf, two node, and booting stages under normal greenhouse conditions. When comparing the two-node stage to the three-leaf stage, upregulated genes were largely associated with putative proteins for stress signaling and nutrient transport (Supplementary Master file, two node vs three leaf (MT)), including *Small Auxin-Up RNA*, *O-methyltransferase COMT-type*, *SWEET repeat sugar transporters*, ABA/WDS-induced proteins, apoptotic protease-activating factors, dehydrins, amino acid transporters, and heat shock proteins. Concurrently, several genes were downregulated, including those encoding putative

ubiquitin-like domains, NRT1/PTR family transporters, dirigent proteins, WAT1-related proteins, terpenoid cyclases, sucrose synthases and heat shock proteins.

When comparing the booting stage to the three leaf (Supplementary Master file, booting vs three leaf (MT)), upregulated genes encoded putative apoptotic protease-activating factors, ubiquitin-like domain-containing genes, various transporters, *HvNAM-1*, and jacalin-like lectins. On the other hand, downregulation was observed for genes encoding putative auxin efflux carriers, WAT1-related proteins, and maintenance-of-meristems-like proteins.

Stage-Specific Transcriptome Shifts in Barley Roots under Control Conditions (ND) To determine patterns of transcription in developing roots of ND NILs for the *HvNAM-1* locus, the ND NILs at three leaf, two node, and booting stages under normal greenhouse conditions were examined. When comparing the three leaf and two-node stages (Supplementary Master file, two node vs three leaf (ND)), ND upregulated genes encoding putative proteins sugar transporters, heat shock proteins, small auxin up RNA, chlorophyll a/b binding proteins, phloem protein 2-like, terpenoid cyclases, and dirigent proteins, and downregulated genes encoding putative proteins O-methyltransferase COMT-type, maintenance-of-meristems-like proteins, amino acid transporters, and WAT1-related proteins.

At the booting stage (Supplementary Master file, booting vs three leaf (ND)), upregulated genes included putative proteins serpin family proteins, leaf development regulators, terpenoid cyclases, and C2-domain ABA-related proteins. Meanwhile, downregulated genes featured putative proteins maintenance-of-meristems-like proteins, ubiquitin-specific protease domain proteins, heat shock factors, wall-associated receptor kinases, and TAR1.

Differences in gene expression between ND and MT alleles during development To identify expression differences related to genomic region varying for the *HvNAM-1* locus during development, bulk RNA Seq analysis was done to compare developmental DEGs between the ND *HvNAM-1* and MT *HvNAM-1* NILs at two node and booting stage.

At the two-node stage (Supplementary Master file, ND vs MT (control, two node)), ND NILs exhibited upregulation of several genes encoding putative proteins TAR1 (*HORVU.MOREX.r3.1HG0028870*), stomatal development and plant interaction regulator, root meristem growth factor, small auxin up RNA, laccase, glabrous1 enhancer binding protein like, NRT1/PTR, heat shock factor, chalcone synthase (*HORVU.MOREX.r3.6HG00592150*), dirigent protein, and SWEET sugar transporter. Downregulated genes included putative proteins potassium transporters, auxin efflux carriers, male flower specific protein 18, RADIALIS like TF, and subtilisin-like proteases.

At the booting stage (Supplementary Master file, ND vs MT (control, booting)), ND NILs showed upregulation of genes encoding putative proteins auxin efflux carrier, protein TAR1, WAT1-related protein, sexual differentiation modulator, flowering promoting factor, and *Pyricularia oryzae* resistance 21. Among the downregulated genes were genes encoding putative proteins nitrate transporter, NAC domains, peptidases and AP2/ERF domains.

In order to explore further the effects of the genomic region varying for *HvNAM-1* on roots during grain fill, we explored expression differences between ND and MT under control conditions by conducting RNA sequencing at booting stage, for 3 lines with the ND allele and 3 lines with the MT allele. The number of upregulated and downregulated genes between ND and MT under control conditions were 125 and 54 respectively (Figure 3.1c). The GO analysis of these

differentially expressed genes resulted in top significantly enriched GO clusters for biological process which include response to water (GO:0009415), response to abscisic acid (GO:0009737), response to stimulus (GO:0050896), transport (GO:0006810), response to temperature stimulus (GO:0009266). The significant enriched GO clusters for molecular function include endo-1,4-beta-xylanase activity (GO:0031176), oxidoreductase activity (GO:0016491), endopeptidase activity (GO:0004866), and hydrolase activity (GO:0004553). Several genes encoding transcription factors were identified among the DEG including the NAC transcription factor, *HvNAM-1*, which is known to regulate senescence in flag leaves as well as roots.

Analysis of promoter regions in candidate target genes (DEGs between ND and MT under control conditions) revealed variable distributions of putative *HvNAM-1* binding sites, based on conserved NAC transcription factor recognition motifs. The binding motif was obtained from PlantTFDB and corresponds to *HvNAM-2*, it was applied here due to high structural similarity (89%) in the N-terminal DNA-binding domains of *HvNAM-1* and *HvNAM-2*, suggesting potential overlap in their target specificity. Notably, most genes contained a single binding site, while a subset exhibited two or more sites, suggesting potential for enhanced or cooperative regulation. Specifically, eight genes were identified with two or more *HvNAM-1* binding sites: seven with two sites and one with three. The gene *HORVU.MOREX.r3.1HG0054020*, encoding a *putative S-adenosyl-L-methionine-dependent methyltransferase*, contained three *HvNAM-1* binding motifs and was upregulated in the ND genotype. Among the genes with two *HvNAM-1* binding sites, *HORVU.MOREX.r3.1HG0019960* (*chloramphenicol acetyltransferase-like domain*), *HORVU.MOREX.r3.3HG0298280* (*F-box associated interaction domain*), and *HORVU.MOREX.r3.7HG0746640* (*isopenicillin N synthase-like, Fe(2+) 2OG dioxygenase*),

*HORVU.MOREX.r3.7HG0721830* (germin), *HORVU.MOREX.r3.2HG0193470* (unannotated), were all upregulated in ND. In contrast, *HORVU.MOREX.r3.2HG0203800*, annotated as *phospholipase C/P1 nuclease domain*, *HORVU.MOREX.r3.2HG0135690* (*putative nuclease HARBII-like*) were downregulated.

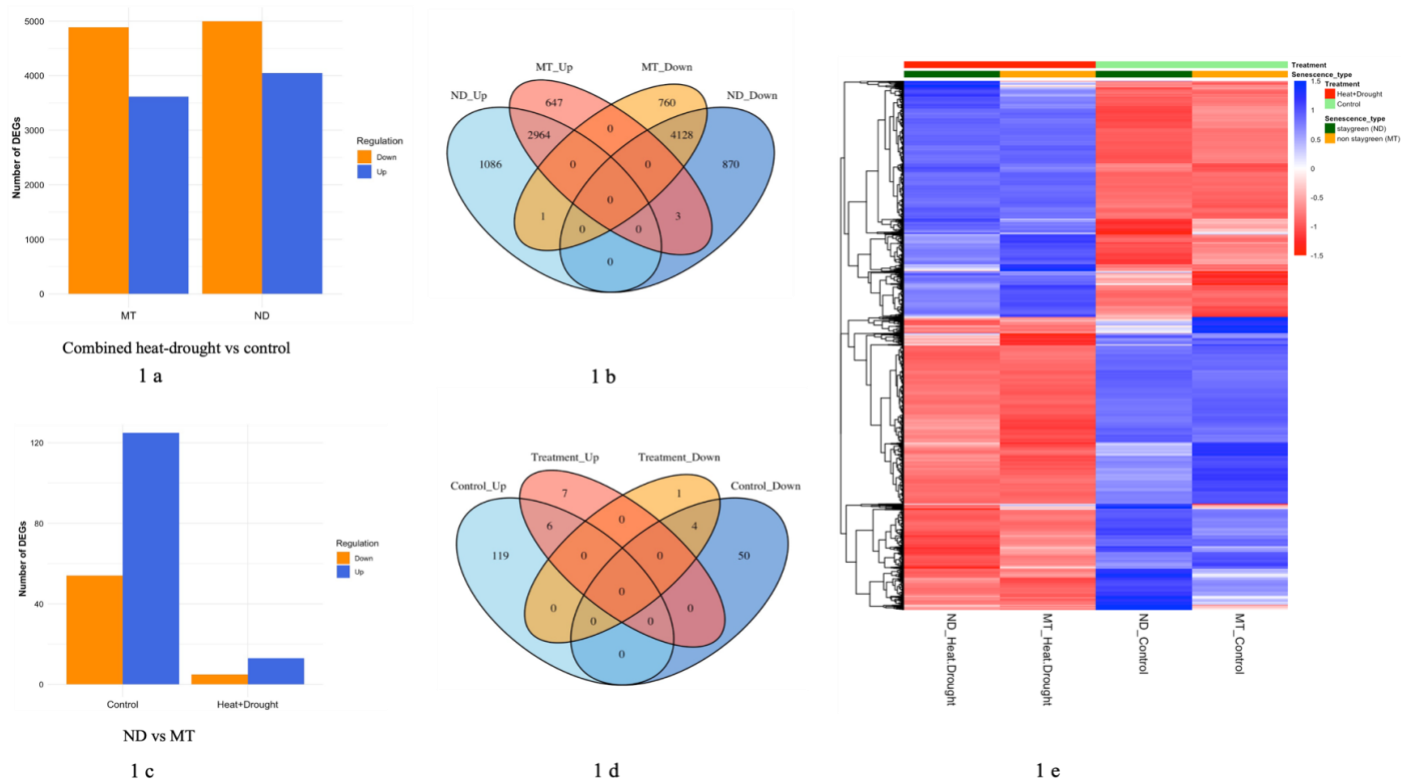


Figure 3.1. Booting stage-specific DEGs. Bar chart showing number of DEGs in MT and ND under combined heat-drought stress, each compared to their respective control conditions (1a), control and heat-drought of ND, each compared to MT (1c); up (blue), down (orange). Venn diagrams showing common and unique DEGs between ND and MT under heat-drought, each compared to their control conditions (1b), between control and heat-drought of ND, each compared to MT (1d). Heat map showing comparing DEGs between ND and MT under control and heat-drought (1e); up (blue), down (red).

### Heat and drought differential gene expression

Differences in gene expression between MT and ND alleles under heat/drought stress To identify expression differences associated with the genomic region containing *HvNAM-1* under combined heat and drought stress, bulk RNA Seq analysis was performed by comparing DEGs between NILs with MT and ND alleles. Bulk RNA Seq comparing MT and ND NILs gene expression at the 2-node stage under treatment (Supplementary Master file, ND vs MT (at two node, treated)) indicated upregulation of genes encoding putative proteins in ND lines such as light-dependent protochlorophyllide reductase, BPS1 (chloroplastic), sugar and nitrate transporters, heat shock factor, NAC domain transcription factors, MIZU-KUSSEI 1-like (*HORVU.MOREX.r3.5HG0487490*) and plant development regulators. Downregulated genes encoding putative proteins in ND included heat shock proteins, sugar transporters, chlorophyll a/b binding proteins, leaf development regulators, O-methyltransferase COMT, stress-related chloroplast proteins, WAT1-related proteins, and osmotin/thaumatin-like superfamily proteins.

At the booting stage (Supplementary Master file, ND vs MT (at booting, treated)), which is a critical phase for reproductive development and yield determination, ND showed broader transcriptomic changes. Upregulated genes encoding putative proteins included C2-domain ABA-related protein, BPS1, osmotin/thaumatin-like proteins, *Small Auxin Up RNA (SAUR)*, sugar transporters, cellulose synthase, and epidermal patterning factor-like proteins. In contrast, genes with putative functions such as root cap formation, mitochondrial/chloroplast MTERF superfamily, chloroplast-localized quinone oxidoreductase, nitrate/sugar transport, and OCTOPUS-like proteins (*HORVU.MOREX.r3.2HG0216220*) were downregulated.

The gene expression trends at booting indicated booting can be the critical stage for capturing *HvNAM1*-related expression differences, particularly those associated to root longevity and function under combined heat-drought stress. Therefore, RNA sequencing was conducted for NILs varying for *HvNAM-1* locus at booting stage under combined heat-drought conditions. At booting stage, the number of upregulated and downregulated DEGs between ND and MT combined heat-drought are very low, 13 and 5 respectively (Figures 3.1 c). The reduced number of DEGs between ND and MT under combined heat-drought stress suggests that stress exposure may partially override the delayed senescence program typically observed in ND genotypes. *HvNAM-1* was shown to be upregulated in both ND and MT roots under combined heat-drought as compared to control conditions; therefore, it did not appear in this set of DEGs (Supplementary file 2. ND vs MT at booting. Treatment). Amino acid transport (GO:0006865), methylation (GO:0032259) were among the significantly enriched GO clusters for biological processes and protein dimerization activity (GO:0046982), structural constituent of chromatin (GO:0030527) among the enriched GO clusters for molecular function. Notably, *HORVU.MOREX.r3.6HG0564030* (AP2/ERF transcription factor) and *HORVU.MOREX.r3.6HG0563070* (C2H2-type zinc finger domain protein) were downregulated in ND. Similarly, *Glabrous1 Enhanced Binding Protein (GeBPI)* also showed lower expression in ND. In contrast, multiple genes were upregulated in ND including gene *HORVU.MOREX.r3.6HG0557230*, encoding terpenoid cyclase/protein prenyltransferase. Two laccase genes, *HORVU.MOREX.r3.1HG0073550* and *HORVU.MOREX.r3.4HG0388460* were upregulated in ND. A strong induction was observed for *HORVU.MOREX.r3.6HG0556250*, encoding GDA1/CD39-type Apyrase, and *HORVU.MOREX.r3.1HG0028870*, encoding TAR1.

Additionally, a gene encoding phenylcoumaran benzylic ether reductase-like and four genes encoding leucine-rich repeat (LRR) domains (*HORVU.MOREX.r3.6HG0560100*, *HORVU.MOREX.r3.6HG0558220*, *HORVU.MOREX.r3.6HG0558500*, *HORVU.MOREX.r3.6HG0557770*) were also upregulated in ND under stress.

With MT allele Transcriptomic analysis comparing combined heat-drought and control in NILs with MT allele at two node and booting stage revealed several differentially expressed genes.

At the two-node stage (Supplementary Master file, Treated vs control (MT, two node)), upregulated genes in MT included sodium potassium root defective 1-3 (*HORVU.MOREX.r3.1HG0081470*), water stress and hypersensitive response domain protein (*HORVU.MOREX.r3.3HG0248140*), gibberellin-regulated protein (*HORVU.MOREX.r3.4HG0342540*), chlorophyll a/b binding protein (*HORVU.MOREX.r3.4HG0395490*), and stomatal development (*HORVU.MOREX.r3.7HG0743130*). Downregulated genes included flowering promoting factor (*HORVU.MOREX.r3.2HG0101800*), chloroplast-localized quinone oxidoreductase (*HORVU.MOREX.r3.2HG0160760*), Nakanori-like (*HORVU.MOREX.r3.2HG0205090*), osmotin/thaumatin-like protein (*HORVU.MOREX.r3.5HG0500130*), and phloem protein 2-like (*HORVU.MOREX.r3.7HG0746860*).

At the booting stage (Supplementary Master file, Treated vs control (MT, booting)), upregulated genes encoding putative proteins included peroxidases, and peptidase inhibitors, while several laccases (e.g., *HORVU.MOREX.r3.1HG0073550*), cold shock domain proteins, sugar

transporters, wave dampened 2-like (*HORVU.MOREX.r3.3HG0219790*), and root cap regulators (*HORVU.MOREX.r3.3HG0292090*) were downregulated under stress.

We examined MT lines at booting stage comparing combined heat-drought with control conditions to capture of key transcriptional shifts associated with genomic region harboring MT allele and root senescence initiation, which may not be evident at two node stage. We identified 647 uniquely upregulated and 760 uniquely downregulated sequences in the MT NILs relative to the ND NILs in response to the treatment (Figure 3.1 b). To highlight genes directly associated with the differences between combined heat-drought and control, we conducted sparse Partial Least Squares Discriminant Analysis (sPLS-DA) for MT background (Supplementary file 3.). The first two components of the sPLS-DA model revealed 84% and 6% (Figure 3.2c) with the component 1 showing distinct clustering between treatment and control samples. Here we report top ranked genes based on VIP scores (Figure 3.2 e). Genes encoding putative proteins laccase, 14-3-3, annexins, plant 2-oxoglutarate-dependent oxidoreductases genes were all downregulated in MT under combined heat-drought. Upregulation of a gene encoding putative protein PPM-type phosphatase like domain was observed in MT, which is known to mediated by ABA (Zhang & Gan, 2012). In addition to that, upregulation of genes encoding putative proteins ubiquitin-like domain (related to macromolecule degradation, regulation) and bZIP, MADS-box (associated with signaling) was observed.

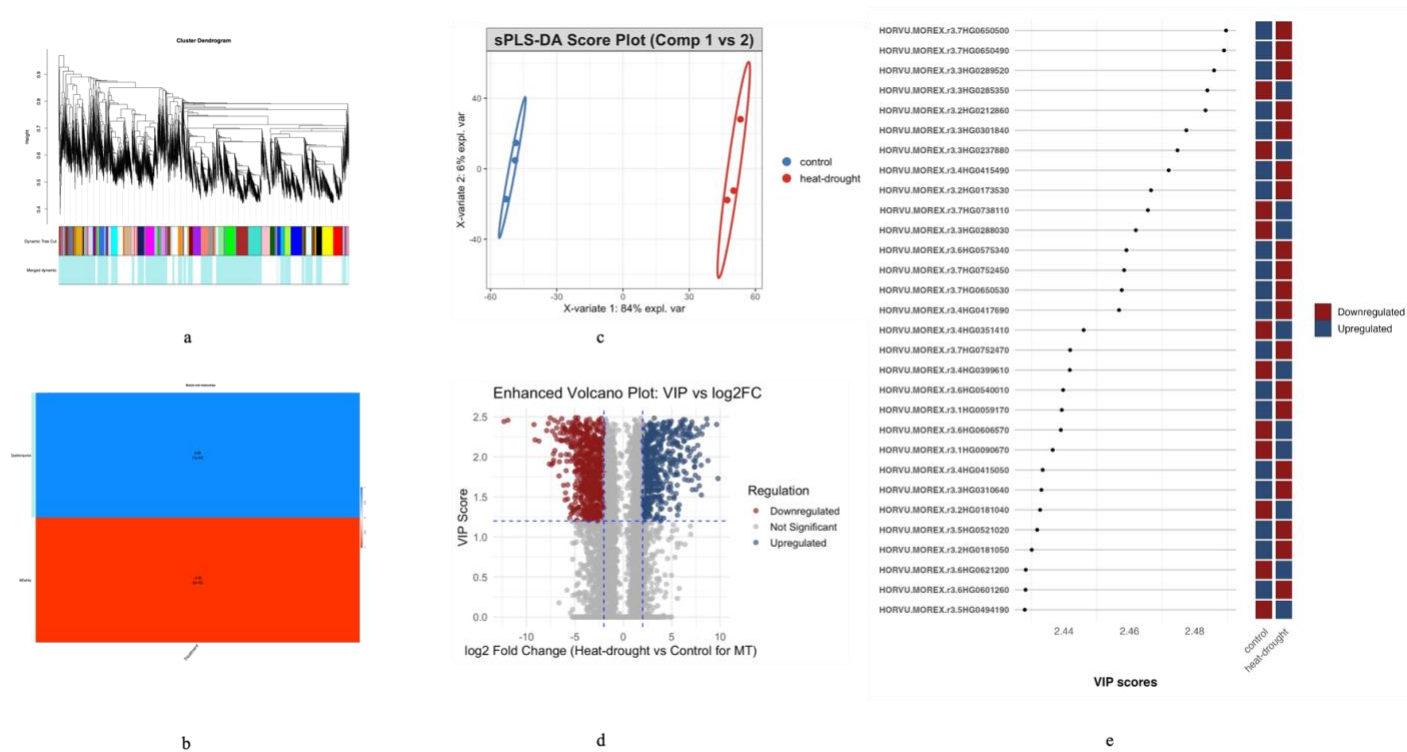


Figure 3.2. Non stay green (MT) specific DEGs. a) Hierarchical clustering dendrogram of genes in WGCNA, with color-coded modules. b) Heatmap showing module-trait correlations between WGCNA modules on y axis (pale turquoise (+ve) and white (-ve)) and treatment on x axis. c) sPLS-DA score plot showing distinction between control and combined heat-drought samples across components 1 and 2. d) Volcano plot displaying VIP scores (y-axis) against log<sub>2</sub> fold changes (x-axis) for all genes. Significantly up and downregulated genes are colored blue and red, respectively e) Dot and tile plot of the top 30 VIP-scoring genes.

With ND allele In the ND background, transcriptomic comparison between control and combined heat-drought conditions revealed distinct sets of differentially expressed genes at both the two-node and booting stages.

At the two-node stage (Supplementary Master file, Treated vs control (ND, two node)), stress conditions upregulated several genes encoding putative proteins including starch synthase (*HORVU.MOREX.r3.1HG0022500*), Protein SOSEKI (*HORVU.MOREX.r3.4HG0400630*), heat shock proteins, osmotin/thaumatin-like protein (*HORVU.MOREX.r3.5HG0423060*), gibberellin-regulated protein (*HORVU.MOREX.r3.5HG0512390*), Bax inhibitor 1-related gene (*HORVU.MOREX.r3.5HG0510030*), chlorophyll a/b binding protein (*HORVU.MOREX.r3.5HG0481920*), and a Late embryogenesis abundant protein, (*HORVU.MOREX.r3.1HG0061780*). Meanwhile, phloem protein 2-like (*HORVU.MOREX.r3.7HG0746860*), rhodanese-like domain protein (*HORVU.MOREX.r3.6HG0540160*), and oxidative stress response regulator (*HORVU.MOREX.r3.2HG0185880*) were significantly downregulated.

At the booting stage (Supplementary Master file, Treated vs control (ND, booting)), upregulated genes included *Flowering-Promoting Factor 1* (*HORVU.MOREX.r3.2HG0113820*), *HvNAM-2* (*HORVU.MOREX.r3.2HG0134090*), *HvNAM-1* (*HORVU.MOREX.r3.6HG0556820*), and putative proteins including a seed development and stress response regulator (*HORVU.MOREX.r3.2HG0199880*), along with laccases and heat shock factors. Downregulated genes under stress at booting included Casparian Strip Membrane Protein (*HORVU.MOREX.r3.1HG0085320*), *Root Cap Associated* gene

(*HORVU.MOREX.r3.2HG0192880*), and protein OCTOPUS-like (*HORVU.MOREX.r3.2HG0216220*).

Examination of ND lines at the booting stage under combined heat-drought and control conditions was used to gain insights into how ND roots initiate or delay senescence. We identified 1086 uniquely upregulated and 870 uniquely downregulated genes in the ND NILs in response to the treatment (Figure 3.1 b). Some GO term enrichments for biological processes unique to ND lines are late endosome to vacuole transport via multivesicular body sorting pathway (GO:0032511), regulation of stomatal movement (GO:0010119), regulation of flower development (GO:0009909), regulation of reproductive process (GO:2000241). To highlight genes directly associated with the differences between combined heat-drought and control, we conducted sparse Partial Least Squares Discriminant Analysis (sPLS-DA) for ND background (Supplementary file 3.). The first two components of the sPLS-DA model revealed 86% and 4% of total variance in ND (Figure 3.3 c) with the component 1 showing distinct clustering between treatment and control samples. Here we report top ranked genes based on VIP scores (Figure 3.3 e).

We observed *HORVU.MOREX.r3.4HG0382640* encoding *TIM23* like gene to be upregulated in ND under heat-drought as compared to control. We also observed down regulation of genes encoding peroxidases and upregulation of LEA (Late Embryogenesis Abundant protein), and AWP 19-like protein in ND. Interestingly, we observed an upregulation of Thiamine DiPhosphate (TDP) binding fold genes in the ND under combined heat-drought stress compared to control. ND roots also showed downregulation of genes encoding jacalin-like lectin and dirigent proteins under combined heat-drought. Gene encoding Pyridoxal phosphate-dependent transferase

was observed to be upregulated in ND under heat-drought. Furthermore, downregulation of senescence associated genes encoding tropinone reductase, transport related genes encoding Major Facilitator Sugar Transporter like, defense related genes encoding peroxidases, regulation and signaling associated genes encoding WRKY transcription factors was observed in ND under combined heat-drought.

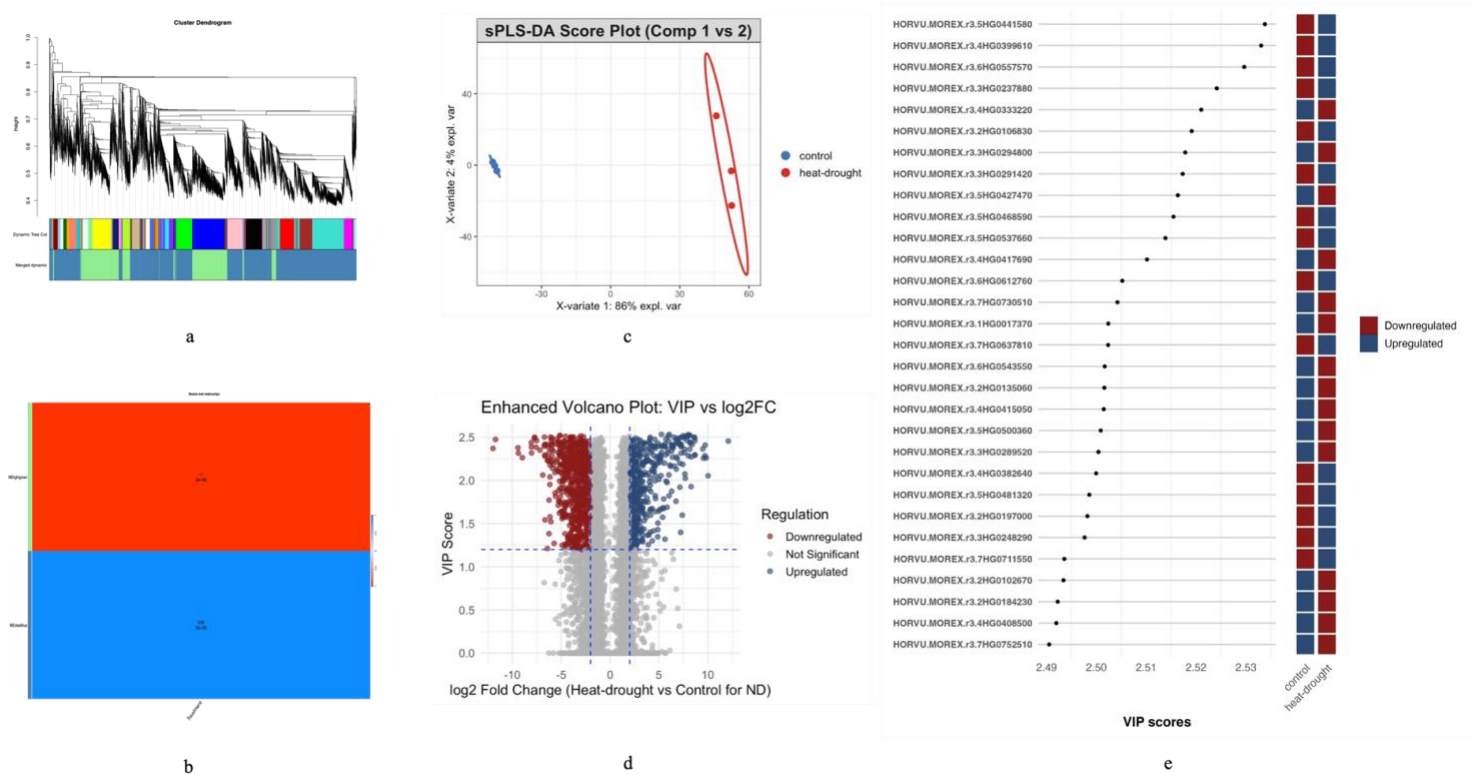


Figure 3.3. Stay green (ND) specific DEGs. a) Hierarchical clustering dendrogram of genes in WGCNA, with color-coded modules. b) Heatmap showing module-trait correlations ( $r$ ,  $p$  value) between WGCNA modules on y axis (lightgreen (-ve) and steelblue (+ve)) and treatment on x axis. c) sPLS-DA score plot showing distinction between control and combined heat-drought samples across components 1 and 2. d) Volcano plot displaying VIP scores (y-axis) against  $\log_2$  fold changes (x-axis) for all genes. Significantly up and downregulated genes are colored blue and red, respectively e) Dot and tile plot of the top 30 VIP-scoring genes.

### Weighted gene coexpression network analysis

To identify gene coexpression network patterns in response to the combined heat-drought stress, WGCNA analysis was conducted separately for ND and MT using DEGs from combined heat-drought vs control conditions. Genes that showed similar expression patterns across treatment were grouped together in the same module (Figures 3.2 a and 3.3 a). These co-expressed genes within a module are considered to share common regulatory mechanisms or biological functions (Langfelder et al 2008). Correlations between the treatment factor and gene expression data were calculated (Figures. 3.2b and 3.3b). In the ND NILs, the light green ( $r = -1$ ) and steel blue ( $r = 0.99$ ) modules correlated with treatment (Figure 3.3b). In the MT NILs, pale turquoise ( $r = 0.99$ ) and white ( $r = -0.99$ ) modules correlated with treatment (Figure 3.2b).

### Hub gene identification

Hub genes play a key role in regulating gene expression by being central to the biological pathways. To explore the functional relevance of these stress-associated modules, hub genes were identified using the Maximal Clique Centrality (MCC) algorithm in Cytoscape. We focused our analysis on the steel blue module from the ND NILs, given its strong positive correlation with treatment ( $r = 0.99$ ) (Figure 3.4). This module was of particular interest as it represents the stress-responsive transcriptional program in NILs carrying the genomic region harboring the ND *HvNAM-1* allele. Most of the hub genes from ND (combined heat-drought vs control) steel blue module overlapped with the significant genes resulted based on VIP scores. In addition to that, the significant genes from ND dot and tile plot (Figure. 3.3e) included three hub genes from the steel blue module; *HORVU.MOREX.r3.2HG0135060* encoding major facilitator, sugar transporter like, *HORVU.MOREX.r3.7HG0637810* encoding LEA (Late Embryogenesis Abundant protein),

*HORVU.MOREX.r3.3HG0294800* (unannotated). We observed *HvNAM-1* to be one of the hub genes in this network, which was most likely interacting with a gene *HORVU.MOREX.r3.7HG0752450*, encoding kelch repeat. Although *HvNAM-1* is a key transcription factor, its limited connectivity in the network may reflect condition-specific regulation or the stringency of correlation thresholds, which can miss biologically relevant but indirect or weakly co-expressed targets. In addition to this, other hub genes observed in ND in response to combined heat-drought encode upregulating HSP90, HS chaperone binding, alpha-amylase, LEA, Pi21 encoding a proline-rich protein, MAIGO2 and downregulating peroxidases, Fe2OG\_OXY, CAT-like aspartic peptidases, COMT, and beta glucanase.

To explore the regulatory potential of *HvNAM-1*, its promoter was analyzed using the PlantCARE tool (Lescot et al., 2002). This revealed multiple *ABA-responsive elements (ABREs)*, including those conserved across barley, rice, maize, and Arabidopsis. Notably, the core ABRE sequence ‘ACGTG’ and its variants were identified at position 658, indicating that *HvNAM-1* could be ABA-regulated. The presence of *ABRE3a*, *ABRE4*, and *AT-ABRE*, along with light and stress-associated motifs like *G-box* and *ACE*, further indicates *HvNAM-1* may integrate multiple environmental signals. These findings align with the observed transcriptional upregulation of *HvNAM-1* under stress, suggesting that ABA signaling may mediate its expression. This supports the hypothesis that *HvNAM-1* contributes to stress-induced senescence regulation through ABA-responsive pathway.

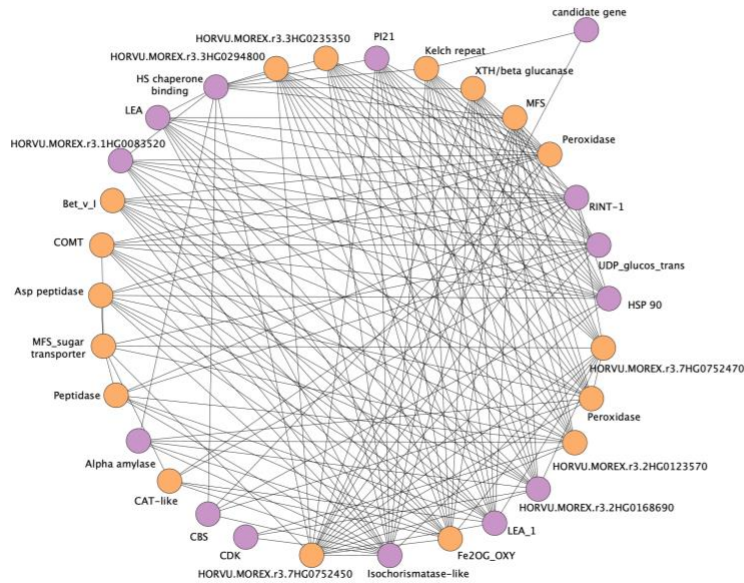


Figure 3.4. Hub gene network of steelblue module identified from ND DEGs under combined heat-drought compared to control conditions. Orange-colored nodes represent significantly downregulated genes, while purple-colored nodes represent upregulated genes. Edges denote predicted gene co-expression relationships based on WGCNA and TOM-based adjacency. (Candidate gene:*HvNAM-1*).

#### Validation of RNA seq using RT-qPCR

To validate the gene expression differences obtained from RNA sequencing results, we performed RT-qPCR on three DEGs, *HvNAM-1*, *HvNAM-2*, *HvGR-RBP1* (Figure 3.5). We observed the qPCR expression pattern to be consistent with RNA seq data, indicating the accuracy of transcriptomics study conducted.

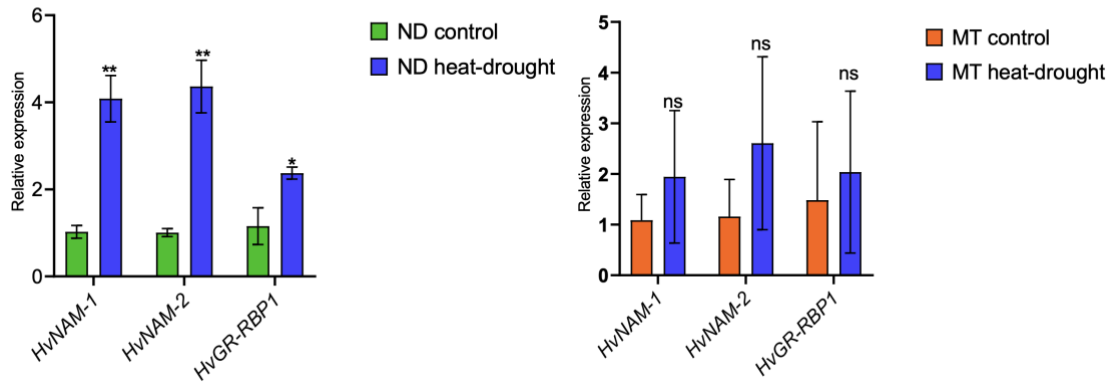


Figure 3.5. RT-qPCR validation of HvNAM-1, HvNAM-2, and HvGR-RBP1 expression in ND (left) and MT (right) roots under control and combined heat-drought conditions at the booting stage. Relative expression levels were measured using  $2^{-\Delta\Delta Cq}$  method. Expression levels between the two panels (ND vs MT) are not directly comparable due to separate normalization. Data represent the mean  $\pm$  SE of two technical replicates for each of three biological replicates. Asterisks denote significant differences between treatments (\* $P < 0.05$ , \*\* $P < 0.01$ ; ns- non significant).

## Discussion

The stay green phenotype has been deployed to increase grain yield in many crops including barley, and its delayed senescence is associated with conferring heat and drought tolerance at grain filling stage. Williams et al. (2022) studied the association of yield stability of stay green genotypes under semi-arid conditions with root traits. Williams et al. (2024) identified stay green QTL associated with seedling root traits and yield stability under semi-arid environments, and one of the QTL, *QGFmt-6H* coincides with genomic location of a NAC transcription factor, *HvNAM-1*. *HvNAM-1* is known to regulate developmental senescence and nutrient remobilization in barley, with lines functional at *Gpc-1* (*NAM-1*) having early senescence and increased grain protein content in wheat and barley (Distelfeld et al., 2014). In this study, we generated NILs, where the MT lines carried the non-stay green allele and ND lines carried the stay green allele, to study gene expression differences between NILs varying for genomic region harboring *HvNAM-1*. Since we observed a divergent transcriptional response of ND and MT associated with root senescence, particularly of *HvNAM-1* at booting stage, we examined this stage under control and combined heat-drought conditions.

### Developmental differential gene expression

ND vs MT root senescence from three leaf to booting stage Root development in barley undergoes a timed transition during vegetative to reproductive stages (Liu et al., 2019). In both NIL types, those carrying the MT and the ND alleles, we observed similar expression trends from the three-leaf to booting stage. Notably, *HvNAM-1* and *HvNAM-2* were not expressed in either genotype at the three-leaf stage under control conditions, aligning with previous findings

(Christiansen et al., 2011). As development progressed, both genotypes exhibited a marked decline in the expression of genes related to putative protein functions such as ubiquitin-mediated protein turnover, terpene biosynthesis, and heat shock responses, particularly at the two-node and booting stages. The ubiquitin-proteasome pathway is a known regulator of senescence and hormone signaling, and its repression has been associated with the maintenance of cellular function and root longevity (Lei et al., 2023).

Despite this shared pattern, key differences emerged between the ND and MT genotypes when their transcriptional responses at the two-node stage were compared to the three-leaf stage. At the two-node stage, ND roots displayed suppressed expression of Protein Phosphatase 2C (*HORVU.MOREX.r3.1HG0090670*), a known ABA-induced senescence promoter (Zhang & Gan, 2012). Simultaneously, ND upregulated genes encoding putative proteins linked to cell survival, such as Bax inhibitor (*HORVU.MOREX.r3.3HG0318760*) and C2H2-type zinc finger proteins (*HORVU.MOREX.r3.3HG0229460*), which are critical in stress defense and longevity pathways (Yue et al., 2012; Zhang et al., 2022).

At booting stage when compared to three leaf, in MT, we observed significant upregulation of genes encoding an F-box protein (*HORVU.MOREX.r3.3HG0220950*) associated with proteasome-mediated degradation and the acceleration of root senescence (Chen et al., 2013). In contrast, ND continued to favor a maintenance mode, with increased expression of Wall-Associated Receptor Kinase (*HORVU.MOREX.r3.3HG0226190*), a gene known to suppress senescence through structural signaling pathways (L. Li et al., 2021). This was further supported by the downregulation of *S40*, a senescence-activating gene in ND, and upregulation in MT of *HvNAM-1*, activating senescence and nutrient remobilization pathways (Jehanzeb et al., 2017).

Together, these findings illustrate a clear dichotomy in root aging programs from the three-leaf to two-node and booting stages: the MT genotype may accelerate root senescence as part of a resource remobilization strategy, while ND maintains metabolic stability and delays senescence, possibly supporting deeper root function and resilience under stress.

Differences in gene expression between ND and MT alleles during development At the two-node stage, ND exhibited a transcriptional profile consistent with delayed senescence and sustained root function. Notably, genes associated with mitochondrial energy production such as TAR1, involved in oxidative phosphorylation, were upregulated in ND, suggesting that these lines maintain more active respiration (Bonawitz et al., 2008). Additionally, ND roots upregulated gene encoding putative chalcone synthase, a key enzyme in flavonoid biosynthesis, potentially enhancing antioxidant defenses and reducing oxidative damage during early reproductive development (Xue et al., 2021). Interestingly, while *HvNAM-1* and *HvNAM-2* were slightly upregulated in ND relative to MT at this stage, their expression did not translate into activation of typical downstream targets associated with senescence and nutrient remobilization. Notably, previous studies have observed increased *HvNAM-1* expression in roots around 32 days after germination, approximately corresponding to the two-node stage (Liu et al., 2019), supporting the developmental relevance of this comparison. This study also reported that at 39 days after germination, barley root senescence is marked by root discoloration, disruption of cortical cells, and arrest of root elongation. While at 46 days after germination, oxidative stress response, reduced root activity, auxin/cytokinin hormones additionally were observed.

As development advanced to the booting stage, *HvNAM-1* expression differences increased between ND and MT. From RNA-Seq results, a downregulation of *HvNAM-1* was

observed (*HORVU.MOREX.R3.6HG0556820*) in ND (Supplementary file 2. ND vs MT at booting. Control), possibly indicating loss of its function in ND. Also, a different interpretation would be that *HvNAM1* expressed at lower levels in ND lines is precisely because they senesce more slowly; yet expression could become measurable at later development dates. The *HvNAM1* protein differs in three amino acids between MT and ND, and these differences are likely involved in reduced function of the ND allele. The *HvNAM-1* MT allele may be involved in a NAC-dependent senescence cascade. NAC proteins, including barley *HvNAC005*, are known to promote nutrient remobilization and stress signaling; their overexpression accelerates senescence by activating proteases, hormone pathways, and transporters (Christiansen et al., 2016; Gregersen, 2011). NAC proteins are well-characterized as dimeric DNA-binding proteins, capable of forming both homodimers and heterodimers with other NAC family members (Olsen et al., 2005). Among the DEG (ND vs MT under control), we identified the following genes with at least two binding sites for *HvNAM-1* in their promoter region. Genes encoding PPM type Phosphatase Like Domain (induce leaf senescence) (Zhang & Gan, 2012) and Phospholipase C/P1 Nuclease like domain (protein and peptide hydrolyzing enzymes) showed upregulation in MT lines. ND lines showed upregulation of a gene encoding Fe(2+) 2OG dioxygenase domain which was also upregulated in wheat roots to maintain root growth under drought (Dalal et al., 2018). Gene encoding Germin protein belongs to cupin superfamily, is upregulated in ND and known to increase lateral root development regulated by ABA and auxin signaling (Nguyen et al., 2023). In *Medicago truncatula*, knockdown of a serpin, part of the cupin superfamily, resulted in premature nodule senescence, confirming their protective role (Cohen et al., 2019; Dhanushkodi et al., 2018). The presence of a single *HvNAM-1* binding site in the promoters of genes encoding other transcription factors such

as WRKY, bZIP, TFIIB, and C2H2 likely indicates a mode of direct but limited regulatory influence. Unlike promoters with multiple binding sites that may support strong activation, a single motif suggests a possibly conditional regulation. Additionally, ND lines also maintained low expression of senescence-related target genes (amino acid transporters and proteases). Arabidopsis *CAT2* and *CAT5* (cationic amino acid transporter) genes were upregulated in senescing leaves (Couturier et al., 2010). These observations reinforce the role of *HvNAM-1* in coordinating transcriptional programs for promoting root senescence. Concurrently, rising ABA levels in MT roots after the two-node stage may work in concert with *HvNAM-1* to amplify the senescence response, as observed in the roots of the barley cultivar ‘Golden Promise’ (Liu et al., 2019). Prior studies show that ABA-induced senescence requires proteasome-mediated removal of ABA signaling repressors, allowing downstream genes to be activated (Wang & Schippers, 2019). Indeed, ABA has been shown to enhance proteolysis during senescence in rice and oilseed rape (Fukayama et al., 2010; Poret et al., 2017). Similarly, we have identified cis acting elements involved in abscisic acid responsiveness in the promoter region of *HvNAM-1*. Although further functional validation is required, these results likely predict regulation of *HvNAM-1* and ABA in modulating root senescence.

#### Heat and drought differential gene expression

As roots respond to combined heat-drought stress, their development and senescence trajectories are strongly influenced by hormonal signaling and transcriptional regulation. In this section, we examine altered responses of MT and ND lines at the two-node and booting stages under stress conditions.

Differences in gene expression between MT and ND alleles under heat-drought stress At the two-node stage, ND roots under heat-drought stress showed upregulation of *MIZU-KUSSEI 1-like (MIZ)*, a gene involved in ABA-regulated hydrotropism (Moriwaki et al., 2012), while it was suppressed under control conditions. This suggests that ND roots activate water-acquisition behavior under stress. In contrast, MT lines upregulated *HvNAM-1* and *HvNAM-2*, reflecting a stress-induced promotion of senescence and nutrient remobilization.

By the booting stage, key transcription factors diverged in expression between genotypes. ND showed downregulation of AP2/ERF and GeBP1, which are senescence-associated regulators (Khare et al., 2017; Mahalingam et al., 2022), suggesting that ND delays senescence under stress. A similar pattern has been observed in tolerant barley genotypes (Liu et al., 2019). Huang et al. (2022) reported that overexpression of the ERF012 factor inhibited root growth and promoted leaf senescence. Additionally, ND upregulated a gene encoding squalene synthase as well as its upstream isogenes *HMGR4* and *HMGR6*, known for enhancing triterpenoid production and root elongation (Wang et al., 2023; Zhang et al., 2023). These isoprenoid biosynthesis genes, when overexpressed, have been shown to increase root biomass and squalene content. Drought-associated QTL, correlating with root pulling force, deeper root length and dry weight, grains per plant, leaf senescence, days to flowering and yield, overlapped with a gene encoding terpenoid cyclase/protein prenyltransferase in rice (Al-Bader, 2019). Further support for reinforcement of root vascular tissues in ND comes from the increased expression of terpenoid cyclase and laccase genes, which promote secondary cell wall thickening and water transport capacity (Ashoub et al., 2018; Niu et al., 2021). In *Arabidopsis*, overexpression of *miR408* negatively regulates drought tolerance via downregulating genes encoding cupredoxin, laccases (Ma et al., 2015). ND also

upregulated GDA1/CD39 apyrase gene, which links to improved ABA signaling and stomatal regulation (Chowdhury et al., 2023; Osakabe et al., 2005; Zhang et al., 2021). Apyrase also participates to maintain auxin and ethylene levels; *apy2* null mutants showed reduced polar auxin transport levels resulting in reduced root lengths in *Arabidopsis* (Liu et al., 2012). Upregulation of *TARI* in ND suggests preserved oxidative phosphorylation capacity (Bonawitz et al., 2008), while expression of gene encoding phenylcoumaran benzylic ether reductase-like supports stress tolerance through lignin biosynthesis and ROS detoxification (Zhang et al., 2016) and over expression of this gene conferred tolerance to salt and oxidative stress in poplar via ROS detoxification and reducing chlorophyll loss (Wei et al., 2022). Together, these findings suggest that ND delays root senescence and enhances root functionality under heat-drought, consistent with investment in deeper roots for water acquisition levels in times of stress.

Stress Responses in MT lines At the two-node and booting stages, MT upregulated PPM-type Phosphatase-Like Domain genes, known mediators of ABA-induced senescence. However, important drought tolerance positive regulators encoding 14-3-3 proteins (Zhang et al., 2023) and annexins (Konopka-Postupolska et al., 2009; Mikołajczak et al., 2023) were downregulated, implying compromised protective signaling. Additional genes downregulated in MT include 2-oxoglutarate-dependent oxidoreductases (2-ODDs). Overexpression of leucoanthocyanidin dioxygenase genes belonging to 2-ODDs family in *Arabidopsis*, increased length of primary roots, chlorophyll content and biomass under drought stress (N. Li et al., 2021), thus their repression may limit drought resilience. These findings suggest that MT *HvNAM-1* lines prioritize resource remobilization over stress adaptation, promoting root senescence even at the cost of survival traits (Espinoza et al., 2007).

Stress Responses in ND lines ND lines displayed transcriptional signatures of delayed root senescence in response to combined heat-drought stress. Notably, ND upregulated TIM23, a protein involved in protein import into mitochondria, known to enhance heat and drought tolerance by stabilizing damaged proteins (Mendoza, 2018). Downregulation of genes encoding peroxidases and upregulation of LEA proteins and AWP19-like proteins further reinforce a stress mitigation strategy; these genes have been linked to ABA-dependent drought responses and membrane stabilization (Akbulak et al., 2018; Kamarudin et al., 2019; Yao et al., 2018). ND also showed induction of thiamine diphosphate (TDP)-binding fold genes, known to support metabolic flexibility during osmotic stress by activating several TDP dependent enzymes (Rapala-Kozik et al., 2012). Genes encoding Jacalin-like lectin and dirigent proteins were downregulated in ND, a trend consistent with wheat where specific TaJRLs are suppressed under hormone and abiotic stress signals (Song et al., 2014). This may reflect a hormonal tuning of ND roots away from senescence-linked signaling. Upregulation of gene encoding pyridoxal phosphate-dependent transferase (Aminotransferase/transaminase) in ND supports enhanced amino acid metabolism and has been associated with greater biomass accumulation under drought in maize (Khan et al., 2022). Finally, ND downregulated several senescence-associated genes, encoding tropinone reductase, major facilitator sugar transporter-like proteins, and WRKY transcription factors, which are typically induced during stress and senescence (Espinoza et al., 2007). In Arabidopsis, SAG13 (Senescence associated gene 13/ At2g29350) encodes a putative tropinone reductase. This possibly underscores a consistent suppression of the senescence program in ND, reinforcing the hypothesis that low *HvNAM-1* delays root aging under stress while enhancing resilience.

### Shared Heat-Drought Stress Responses Between ND and MT

Despite the divergent root aging trajectories of ND and MT, some core genes and pathways were consistently regulated across both genotypes in response to combined heat-drought stress, suggesting fundamental heat-drought response programs. Among the DEGs identified in ND and MT in response to combined heat-drought as compared to control, four genes, *HORVU.MOREX.r3.2HG0097640*, *HORVU.MOREX.r3.2HG0184620*, *HORVU.MOREX.r3.3HG0325670*, and *HORVU.MOREX.r3.2HG0187910*, overlapped with stress memory-associated loci from a barley GWAS study by (Elkelish et al., 2025). These encode a protein kinase, a Hsp70-Hsp90 organizing protein, an F-box domain-containing protein, and a RWP-RK domain-containing protein respectively. Each has been linked to important physiological traits like proline content, chlorophyll retention, spike length, thousand kernel weight, and stress adaptation across generations. Their consistent regulation suggests that these loci may function as part of a core short and long-term stress memory mechanism, regardless of *HvNAM-1* allelic differences. Additionally, VIP score calculations (Figures 3.2e and 3.3e) identified several high-confidence DEGs contributing to treatment differentiation (combined heat-drought vs control). Notably, three top DEG encoding putative cupin (upregulated), S-adenosyl-L-methionine-dependent methyltransferase superfamily (downregulated), and glycosyl hydrolase (upregulated), showed identical regulation in both ND and MT under heat-drought, indicating that certain metabolic and cell wall remodeling processes are universally stress-inducible in barley roots.

### *HvGR-RBP1* and *HvNAM-1* Crosstalk: Implications for Increased Grain Fill Duration and Grain Quality

Both *HvNAM-1* and *HvNAM-2* were downregulated under control conditions relative to their levels in heat-drought treatments at the booting stage, with ND exhibiting lower basal expression than MT. Notably, *HvGR-RBPI* (Glycine-rich RNA Binding Protein 1) showed increased expression in both genotypes under stress. This gene is closely associated with flowering time regulation and is in tight linkage to *HvNAM-1* (Alptekin et al., 2021). Elevated *HvGR-RBPI* expression corresponds to earlier anthesis, which is often accelerated under stress conditions. Alptekin et al. (2022) found *HvGR-RBPI* and *HvNAM-1* had additive effects extending grain filling periods by three days, resulting in plumper grains with lower protein content, a favorable trait combination for malting quality. Our data concur, with the ND at *HvNAM-1* combined with functional *HvGR-RBPI* may support better grain quality outcomes under stress by delaying root senescence and prolonging grain development.

#### Co-Expression Network Differences Highlight Regulatory Divergence

Although many DEGs overlap between ND and MT under stress, their network-level regulation differs dramatically, reflecting genotype-specific transcriptional rewiring. Interestingly, in ND lines, *HvNAM-1* appeared as a top MCC ranked gene in the “steel blue” module of the ND co-expression network under stress. This could reflect *HvNAM-1*'s function in stress response due to its integration within broader regulatory modules. However, the steel blue module, unique to ND under combined heat-drought, was functionally distinct, centered on stress protection and maintenance rather than NAC-driven senescence. ND hub genes encoded LEA proteins, MAIGO2, HSP90, and Pi21, all known for roles in protein stabilization, osmotic protection, and ABA-mediated resilience. For example, *Arabidopsis maigo2* mutants display hypersensitivity to ABA

and osmotic stress, due to impaired Golgi/ER protein trafficking (Zhao et al., 2013; Zhao & Lu, 2014). Additionally, upregulation of ABA-associated stress genes like *LEA*, as also noted by (Collin et al., 2025) in barley roots, confirms ND's orientation toward longer-term stress endurance. ND's downregulation of peptidases, alongside its activation of protective and metabolic maintenance genes, suggests strategic suppression of proteolysis and oxidative damage. This represents a clear shift in regulatory strategy, in contrast to MT lines, where senescence is promoted. These findings confirm that co-expression network of ND supports delayed senescence and prolonged root activity under adverse conditions.

#### Implications and Future Directions: Is There a Cost to Delayed Root Senescence?

While our findings demonstrate that ND allele for *HvNAM-1* confers advantages under both control and stress conditions, such as delayed root senescence, enhanced root maintenance, and potential improvements in grain quality, it remains unclear whether this strategy carries long-term physiological or agronomic trade-offs. One possible limitation is that nutrient remobilization from senescing roots may be incomplete or inefficient in ND lines, potentially affecting nitrogen availability for grain filling under non-stress environments. Alternatively, the process may be merely delayed rather than impaired, suggesting a slower but sustained contribution to reproductive sinks.

#### Conclusions

Barley growers benefit most from cultivars that meet malt-grade quality standards while remaining profitable. To achieve this, grain must conform to stringent parameters, including

optimal levels of protein and beta-glucans, which are often disrupted by environmental stresses such as heat and drought. The stay-green phenotype has emerged as a promising trait that enables plants to maintain yield and quality under such stresses by sustaining photosynthetic capacity and delaying senescence. In this transcriptomics study, we examined whether genotypes with ND allele at genomic region for *HvNAMI* exhibit root-mediated gene expression patterns that delay senescence and enhance grain filling under both normal and heat-drought stress conditions.

Our findings reveal that ND lines display a distinct transcriptional strategy both under control and stress conditions, prioritizing root maintenance, stress tolerance, and delayed remobilization, in contrast to MT lines, which undergo coordinated root and shoot senescence to mobilize resources for reproductive growth. These patterns are consistent with field observations that stay-green cereals tend to invest more in root function during stress. ND roots maintained activity longer into development, thereby supporting water and nutrient uptake, prolonging photosynthesis, and potentially extending grain filling duration.

In summary, ND roots engage a stay-green-associated gene expression program that delays senescence and enhances stress resilience. These below-ground insights complement previous shoot-based studies and underscore the potential of targeting root senescence pathways to improve heat-drought adaptation in barley and related cereals. However, it remains an open question whether such delayed root senescence may compromise complete nutrient remobilization, particularly under nutrient-limited conditions, and whether this strategy affects yield stability across environments. Addressing these questions will require integrative studies involving proteomics and metabolomics, to further elucidate the gene networks and physiological trade-offs

identified here. Ultimately, this work lays the foundation for breeding climate-resilient cereal crops by incorporating root traits into selection frameworks.

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## CHAPTER FIVE

## CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation is the result of an integrated investigation into the genetic, physiological, and molecular responses of barley to heat and drought stress, with direct implications for both forage and malt breeding. Through a combination of genome-wide association studies, controlled environment-based evaluation of stay-green and non-stay-green NILs, and transcriptomic profiling of root development, this work underscores the complexity of stress response and adaptation and highlights promising mechanisms and loci for breeding resilient barley cultivars. Across all chapters, a unifying goal emerges: uncovering the genetic and physiological mechanisms that allow barley to adapt to environmental stress. This research offers a foundation for future studies and will serve as a useful resource for barley breeders and researchers seeking to enhance crop performance under increasingly variable climates.

Chapter 2 of this thesis is focused on dissecting the genetic architecture of forage yield and quality traits under contrasting environments (dryland and irrigated), identifying key SNPs and candidate genes with pleiotropic effects that could support selection for improved biomass and quality traits in adverse environments. To carry out this study two different populations of 260 lines each from the barley iCore were evaluated for forage traits under varying environmental conditions. The WC1 population was grown under both irrigated and rainfed (dryland) treatment conditions, while the WC2 population was grown only under rainfed (dryland) conditions during the 2018 and 2019 field seasons. Forage yield and quality (higher quality means lower ADF and NDF) are the important traits for forage breeders. These are complex quantitative traits easily

influenced by environmental stresses such as high temperature and water deficit (Buxton & Fales, 1994). Genome wide association studies of these populations across environments resulted in identifying significant QTL that promote biomass yield, grain yield and quality during conditions of drought and heat. We observed that biomass yield, developmental traits, and plant height exhibited higher heritability and stability across environments, while forage quality traits such as ADF and NDF were far more environmentally influenced. Temperature and water availability significantly modulated biomass production and digestibility, with moderate drought improving forage quality through altered development and reduced fiber content. Negative correlations between biomass yield and digestibility reflect the inherent trade-offs in breeding forage barley. Several robust QTL related to forage yield, digestibility, and grain yield were identified, which aligned with previously reported loci across different populations (Siahsar et al., 2009; Surber et al., 2011), lending confidence to their stability. Among the loci identified, those consistently detected under dryland conditions, such as *HvGYD3HL*, *HvGY6HL*, *HvADF7HL*, and *HvNDF1HL*, are of particular relevance to forage barley breeding programs in Montana, where most cultivation relies on non-irrigated systems. These QTL represent strong candidates for selection in breeding efforts aimed at improving yield and quality under water-limited environments. In contrast, heat-specific QTL such as *HvBY1HL* and *HvBY6HL* may provide strategic advantages during episodes of high temperature stress, which are becoming increasingly common. Conversely, QTL including *HvADF6HL*, *HvGY7HL*, and *HvGY1HL* appear to confer benefits primarily under irrigated conditions, offering targeted improvement opportunities in more controlled settings. Pleiotropic loci that enhanced biomass or grain yield while maintaining or improving forage quality, such as *HvGYD3HL* and *HvADF7HL*, were of interest. The identification

of consistent QTL across both WC1 and WC2 populations suggests the possibility of marker-assisted selection for reliable improvement of forage traits. Future work will focus on functional validation of key candidate genes, particularly those associated with heat and drought response. Given the increasing variability in climate, developing lines that sustain both high biomass and forage quality under stress will be critical. The QTL and gene targets identified here form a foundational toolkit for breeding forage barley adapted to dryland and irrigated systems.

In a malt barley population segregating for the stay-green trait, (Williams et al., 2024) identified QTL associated with seminal root architecture (under greenhouse conditions), as well as grain fill duration and quality traits (in semi-arid field environments). To validate these findings, near-isogenic lines (NILs) targeting the identified QTL regions with 50% carrying the stay green allele and 50% carrying the non-stay green allele were developed. Chapter 3 explored the contribution of these stay-green QTL to terminal heat stress adaptation in barley by evaluating the generated NILs. By imposing heat stress during tillering the study isolated the genetic and physiological responses of each QTL under stress and control conditions. The temperatures imposed for heat stress were intense and above average summer temperatures typical for Montana drylands. Significant allelic effects were observed for traits central to yield and quality, such as grain number, grain weight, and grain protein concentration. Importantly, correlation analyses revealed that under heat stress, traits like plant height and number of heads per plant were negatively associated with grain protein, suggesting trade-offs driven by nitrogen dilution and assimilate partitioning. In some environments, increased plant height can reduce grain yield due to excessive allocation of resources to biomass, but under stress conditions, greater height may enhance yield. Grain yield itself typically exhibits a negative correlation with grain protein, a

relationship that is well established in cereals. Heat stress during early grain filling reduces starch accumulation in barley due to impaired enzyme activity, leading to a relative increase in grain protein content (Ni et al., 2020). This likely explains the protein trends observed in NILs under terminal heat stress. These findings highlight how physiological traits interact with developmental timing and genetic background to shape adaptation to elevated temperatures.

Among the QTL examined, *QGFhd-2H* carrying the ND allele demonstrated a favorable shift in resource allocation under heat stress, exhibiting improved grain protein and yield traits despite shorter stature. QTL such as *QGFmt-6H* and *QGF-7H* provided more stable performance, suggesting their suitability for selection in adverse conditions. Interestingly, the *QGFmt-6H* region co-locates with *HvNAM-1*, a gene implicated in nitrogen remobilization (Distelfeld et al., 2014), and also with seedling root trait QTL (Williams et al., 2024).

Collectively, the results underscore the value of stay-green traits in enhancing barley resilience under heat, particularly when integrated with favorable phenological and root-related characteristics (Williams et al., 2024). NILs carrying ND alleles for *QGFhd-2H*, *QGFmt-6H* and *QGF-7H* represent promising genetic resources for breeding programs targeting hot, dry environments. To further elucidate the genetic mechanisms underlying *QGFmt-6H*, we have pursued a root-focused gene expression study of *HvNAM-1* under heat-drought stress, which is presented in our subsequent work.

Chapter 4 focused on the QTL *QGFmt-6H*, which co-localizes with the NAC transcription factor *HvNAM-1*, to investigate whether ND allele for this gene contributes to delayed senescence in barley roots under both control and combined heat-drought conditions. By developing near-

isogenic lines (NILs) differing in genomic regions harboring *HvNAM-1*, we examined the mechanisms underlying the stay-green phenotype in barley roots.

According to (Liu et al., 2019), root senescence in barley follows a genetically programmed trajectory involving hormonal signals and transcriptional reprogramming. As barley seminal roots age, a notable peak in abscisic acid (ABA) concentration occurred at the point when root growth ceased and cell degradation was initiated. This is also associated with decreased nitrate uptake activity indicating reduced root activity. The ABA accumulation was tightly associated with the upregulation of senescence-related transcription factors such as NAC, WRKY, and AP2 that play central regulatory roles in orchestrating stress and oxidative response genes. Altogether, this indicates that root senescence follows a genetically programmed, ABA-modulated trajectory involving TF driven transcriptional reprogramming distinct from but partially analogous to leaf senescence (Liu et al., 2019). Although direct interaction between *HvNAM-1* and ABA has not been confirmed, a related NAC-a6 subgroup member, HvNAC005, has been shown to be ABA-responsive (Christiansen et al., 2016) suggesting a conserved transcriptional module regulating root senescence. We have also identified multiple ABA-responsive elements (ABREs), including those conserved across barley, rice, maize, and Arabidopsis in the promoter region of *HvNAM-1* confirming that *HvNAM-1* is likely ABA-regulated, although further experimental validation is required.

Under both control and heat-drought regimes, ND lines exhibited a delayed senescence response, in contrast to MT lines. RNA-Seq analyses revealed that ND lines suppress canonical senescence pathways while maintaining elevated expression of genes involved in stress adaptation,

continued root growth, and cell wall remodeling. In contrast, MT lines showed activation of nutrient remobilization, and senescence-associated transcription factors, particularly under stress conditions. These contrasting gene expression profiles, most evident at the booting stage, underscore the regulatory role of *HvNAM-1* in coordinating both developmental and stress-induced root senescence. Through NAC-mediated cascades, MT lines appear to accelerate resource reallocation, while ND lines delay degradation and sustain root structural integrity. Notably, ND lines activated a distinct co-expression network enriched for protective genes encoding LEA, HSP90, and MAIGO2, indicative of a rewired transcriptional program that supports continued root function during grain filling. These physiological distinctions likely underpin the improved stress tolerance and grain quality consistently observed in ND genotypes under terminal stress. Importantly, shared and unique differentially expressed genes (DEGs) and hub genes were identified between ND and MT genotypes under heat-drought conditions. Among these, the connection between *HvNAM-1*, *HvGR-RBPI*, and prolonged grain filling provides molecular support for delayed senescence as a viable strategy to enhance grain quality (Alptekin et al., 2022).

This work establishes root senescence as a dynamic, genetically regulated process in barley, shaped not only by environmental signals but also by allelic variation within the *HvNAM-1*-containing genomic region. By systematically comparing NILs under both optimal and stress conditions, we observed changes in transcriptional networks that could maintain metabolic function. These findings extend the stay-green paradigm beyond leaves, positioning root longevity as a targetable trait for improving resilience and grain quality under climate-related stress.

The findings presented in this chapter open new avenues for understanding how root senescence is coordinated under stress, particularly via *HvNAM-1* activity. Yet, several questions

remain unanswered and addressing them will be crucial for advancing both fundamental knowledge and applied crop improvement. One immediate priority is to uncover the direct transcriptional targets of *HvNAM-1* in roots. While our expression data suggest clear regulatory roles, it remains unknown whether *HvNAM-1* directly binds the promoters of the senescence and stress-related genes it appears to control. Techniques such as ChIP-seq, paired with RNA-seq, will be essential to chart *HvNAM-1*'s precise downstream network and its interactions with other transcription factors under different environmental regimes. Another frontier lies in exploring the spatial and temporal dynamics of root senescence at high resolution, as (Klaus et al., 2024) have shown that root responses to water deficit are not uniform across tissues or developmental stages. Future studies should employ spatio-temporal transcriptomics across distinct root zones, to uncover zone-specific reprogramming patterns that may explain how some regions persist while others degrade.

These molecular efforts must be mirrored by advanced phenotyping, capable of capturing root growth, decay, and architecture dynamically under stress. Platforms such as automated rhizotrons and X-ray computed tomography offer non-invasive ways to quantify root traits in soil-like conditions (Nagel et al., 2012), enabling the correlation of gene expression profiles with real-time physiological changes. Such integrative datasets will provide critical insights into how transcriptional reprogramming translates into functional resilience. By resolving how *HvNAM-1* controls root senescence and interfaces with hormonal signaling and stress adaptation, we move closer to designing barley cultivars that remain productive under challenging environments, cultivars that delay root degradation just enough to sustain uptake, grain filling, and quality without compromising developmental timing.

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