IMPROVING MALT BARLEY AGRONOMICS VIA ALLELIC SELECTION OF SENESCENCE AND FLOWERING TIME CONTROLLING GENES

by

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DEDICATION

To my grandparents Dondu Alptekin, Cemal Alptekin, and Hurmuz Esgidir; who inspired me to thrive even under the toughest conditions. Their strength became my inspiration to believe in myself, and their spiritual guidance helped me to follow my dreams of becoming a scientist.

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ABSTRACT

Malt barley (*Hordeum vulgare* L.) is a high-profit crop for farmers; yet, its production raises challenges that need to be addressed. The standards for grain quality in malt barley are stringent, and the rejection of non-qualified grains by maltsters is the leading cause of revenue loss for malt barley producers. Rejection is mainly due to high grain protein content and low kernel plumpness which both cause significant problems in the malting process. While proper growing practices can improve malt quality, the industry requires genotypes that have more stable malt quality. Therefore, understanding the molecular mechanisms associated with grain quality can be applied to improve selection of superior malt varieties. A considerable volume of literature has suggested that regulation of whole-plant senescence and flowering processes in cereals have direct influences on grain yield and quality parameters. The central question in this dissertation examined whether malt barley agronomic and end use quality can be improved by the selection of varying alleles for genes associated with plant development. With this purpose, two whole-plant senescence-regulating NAM, ATAF and CUC (NAC) transcription factors, *HvNAM1* and *HvNAM2*, and a flowering-time controlling Glycine-Rich RNA Binding Protein (*HvGR-RBP1*) were studied. Molecular markers for selection of alleles from varieties 'Karl' (with consistently low grain protein) and 'Lewis' were developed, and an advanced-generation malt barley breeding population was genotyped. Statistical analysis of growth parameters from this population showed that selection of *HvNAM1* 'Karl', *HvNAM2* 'Karl' and *HvGR-RBP1* 'Lewis' alleles ensures a longer grain filling period in malt barley. Plants with prolonged grain filling also exhibited increased kernel plumpness and test weight. Additionally, selection of 'Karl' alleles for both *NAC* genes decreased grain protein content ensuring grain quality for malting. The improvement of grain characteristics correlated with improved malt phenotypes, for example a ~2% increase in malt extract, and improvement in other malt characteristics such as β-glucan content and α-amylase activity was observed. Overall, these data show that molecular genetics and allelic selection for genes controlling plant development is promising for advancing malt quality. Research performed here has a direct potential for improving the profits for malt barley producers.

CHAPTER ONE

INTRODUCTION TO DISSERTATION

Barley has been used to produce malt and alcoholic beverages since the very early ages of civilization. It is accepted that beer brewing using barley began in 3500 - 3100 BCE in ancient Mesopotamia by Sumerians (Homan 2004). Surely, malt production and beer brewing hold their importance in our modern world, and around 20% of grown barley is used by the malt industry on a yearly basis (Nice et al., 2019). Malt barley generally sells at a higher price than barley for other end-uses. However, the acceptance rate of produced barley for malting is only ~25% due to the required high standards of grain quality (Stevens et al., 2015). Barley grain qualified for beer production should possess >90% plump kernels and contain <12% of grain protein based on the guidelines of the American Malting Barley Association (AMBA) (American Malting Barley Association, 2019). Due to the increased effect of climate change, it becomes harder for farmers to meet these stringent parameters causing a big loss-of-revenue (Windes et al., 2019).

Cereal grain quality is under genetic and environmental control (Eagles et al., 1995). Every year, AMBA releases a list of recommended varieties for malting which meet the required high standards (American Malting Barley Association, 2020). This is enabled by malt barley breeding programs which develop new varieties with improved quality. ‘Karl’ was released in 1976, (Wesenberg et al., 1976) due to its consistently low grain protein content under different environments (Burger et al., 1979; Weston et al., 1993). ‘Karl’ was not commercially successful because of its weak straw and low yield

potential (Blake et al., 2011). Instead, it has been used in crosses aimed at lowering grain protein in malt barley breeding programs (See et al., 2002), and experiments have been performed to analyze the genetics of 'Karl's' low-grain protein phenotype

In 2002, See et al. discovered three important Quantitative Trait Loci (QTL) located on chromosomes 2H, 3H and 6H explaining much of the variation in grain protein content, using a population of Recombinant Inbred Lines (RILs) derived from a cross of 'Karl' and a common two-row malting variety, 'Lewis'. This study reported that the 6H-QTL is the major determinant of variation in grain protein in the RIL population. Subsequently, a wheat *NAC* transcription factor from durum wheat was identified as a major regulator of whole-plant senescence and grain protein content (Uauy et al., 2006). Interestingly, this gene (named *GPC-B1*) was located on chromosome 6B. Later, the barley ortholog of this gene, *HvNAM1* was found in a region which is co-linear with *GPC-B1* (Distelfeld et al., 2008, suggesting that *HvNAM1* is responsible for the chromosome 6H QTL identified by See et al., (2002). Distelfeld et al. (2008) also showed the presence of several important differences between the 'Karl' and 'Lewis' *HvNAM1* sequences. Combined with the previous reports, these findings suggest that mutations in *HvNAM1* may cause inefficient nutrient re-cycling in variety 'Karl' during whole-plant senescence, which leads to the accumulation of less protein in barley grains (Distelfeld et al., 2008).

To analyze the function of the barley chromosome 6H grain protein QTL, Jukanti et al., 2008 developed Near-Isogenic Lines (NILs) in both the 'Karl' and 'Lewis' backgrounds. This study identified a set of genes differentially regulated in senescing flag

leaves of ‘Karl’ and its NIL ‘10_11’, carrying the ‘Lewis’ allele at the 6H-QTL. Several of the detected genes were strongly differentially regulated between NIL pairs with >10-fold change in expression; one of them encoded a Glycine-Rich RNA Binding Protein, *HvGR-RBP1* (Jukanti et al., 2008). An *Arabidopsis* ortholog of this gene, *AtGRP7*, showing 60% sequence identity to *HvGR-RBP1* at the protein level, is associated with flowering control in Arabidopsis (Streitner et al., 2008; Lacerenza et al. 2010). A later study also observed differences in flowering initiation between ‘Karl’ and ‘10_11’ (Lacerenza et al., 2010) suggesting a shared role of *HvGR-RBP1* and *AtGRP7* in the regulation of the flowering process.

This dissertation investigated the genetic basis of agronomic and physiological differences observed between lines carrying ‘Karl’ *vs.* ‘Lewis’ alleles for three important genes. With this aim, two whole-plant senescence regulating *NAC* transcription factors (*HvNAM1* and *HvNAM2*) and the flowering-time controlling *HvGR-RBP1* gene were taken into consideration. Molecular markers for *HvGR-RBP1* and *HvNAM2* were designed to differentiate between ’Karl’ and ‘Lewis’ alleles. Combined with the previously designed *HvNAM1* marker (Distelfeld et al., 2008), an advanced-generation malting population was genotyped for the three genes, and their association with agronomic and malt phenotyping traits was investigated.

Chapter Two of this dissertation examines the genetic basis of observed differences in the gene expression of *HvGR-RBP1* in ‘Karl’ and ‘Lewis’ and its effect on plant development. This part also inspects the effects of different *HvNAM1* / *HvGR-RBP1* allele combinations on malt barley agronomics. Chapter Three focuses on a close

ortholog of *HvNAM1* located on chromosome 2H, named *HvNAM2*. The main objective of this chapter is to understand the effect of the 'Karl' *HvNAM2* allele on malt barley agronomics. Lastly, Chapter Four explores the effects of different combinations of alleles of all three genes on malt barley quality. The findings of this dissertation offer a direct benefit to both malt barley breeders and producers by facilitating the selection process for certain traits and improving the grain characteristics of malting genotypes. Furthermore, it establishes a basis for elaborating the role of genes involved in plant development in the control of malt barley grain quality.

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CHAPTER TWO

COMBINED EFFECTS OF GLYCINE-RICH RNA BINDING PROTEIN AND A NAC TRANSCRIPTION FACTOR EXTEND GRAIN FILL DURATION AND IMPROVE MALT BARLEY AGRONOMIC PERFORMANCE

Contribution of Authors and Co-Authors

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Contributions: Performed field studies for Experiment 2 and collected agronomic data

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Contributions: Contributed agronomic data for Experiment 1

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COMBINED EFFECTS OF GLYCINE-RICH RNA BIDING PROTEIN AND A NAC TRANSCRIPTION FACTOR EXTEND GRAIN FILL DURATION AND IMPROVE MALT BARLEY AGRONOMIC PERFORMANCE

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Abstract

Key message **Two key barley genes independently control anthesis and senescence timing, enabling the manipulation of grain fill duration, grain size/plumpness, and grain protein concentration.**

Abstract Plant developmental processes such as flowering and senescence have direct effects on cereal yield and quality. Previous work highlighted the importance of two tightly linked genes encoding a glycine-rich RNA-binding protein (*Hv*GR-RBP1) and a NAC transcription factor (*HvNAM1*), controlling barley anthesis timing, senescence, and percent grain protein. Varieties that differ in *HvGR-RBP1* expression, 'Karl'(low) and 'Lewis'(high), also differ in sequence 1KB upstream of translation start site, including an ~400 bp G rich insertion in the 5'-flanking region of the 'Karl' allele, which is likely disrupting gene expression. To improve malt quality the (low-grain protein, delayed-senescence) 'Karl' *HvNAM1* allele was introgressed into Montana germplasm. After several seasons of selection the resulting germplasm was screened for the allelic combinations of *HvGR-RBP1* and *HvNAM1*, finding lines combining 'Karl' alleles for both genes (-/-), lines combining 'Lewis' (functional, expressed) *HvGR-RBP1* with 'Karl' *HvNAM1* alleles (+/-), and lines combining 'Lewis' alleles for both genes (+/+). Field experiments indicate that the functional ('Lewis', +) *HvGR-RBP1* allele is associated with earlier anthesis and with slightly shorter plants, while the 'Karl' (-) *HvNAM1* allele delays maturation. Genotypes carrying the +/- allele combination therefore had a significantly (3 days) extended grain fill duration, leading to a higher percentage of plump kernels, slightly enhanced test weight, and lower grain protein concentration when compared to the other allele combinations. Overall, our data demonstrate an important function for *HvGR-RBP1* in the control of barley reproductive development and set the stage for a more detailed functional analysis of this gene.

Keywords Barley · Anthesis · Senescence · Grain fill duration · Grain protein concentration · Kernel plumpness

Introduction

Plant developmental processes such as flowering transition and whole plant senescence impact crop yield and quality. Fine-tuning flowering control can affect biomass production during vegetative growth, thereby substantially improving grain yield and its components (Alqudah&Schnurbusch, 2017; Blümel et al., 2015; Mathan et al., 2016). A better understanding of senescence regulation has the potential for advancing the nutritional quality of cereal grains (Distelfeld et al., 2014; Gregersen, 2011) and reducing fertilizer use (Yang&Udvardi, 2018), thus reducing environmental impacts (Omara et al., 2019). Considering the food production needs of the 21st century, research has focused on determining the genetic control of flowering transition and whole-plant senescence. Integrated studies combining classical breeding, association mapping, and -omics techniques have improved our understanding of mechanisms controlling flowering and senescence in both model species and crops (Borràs-Gelonch et al., 2012; Greenup et al., 2009; Ibrahim et al., 2018; Kim et al., 2018; Rangan et al., 2017; Woo et al., 2013).

NAC genes, a large, plant-specific family of transcription factors, are among the genes identified that regulate senescence in several species. In leaves, several *NAC* genes induce leaf senescence so that nutrients are remobilized to seeds or grains. In *Arabidopsis thaliana*, T-DNA insertions in the *AtNAP* gene lead to significantly delayed leaf senescence, while overexpression of this gene causes precocious senescence (Guo&Gan, 2006). In wheat, presence of an allele coding for a functional NAC protein (*TtNAM-B1*)

on chromosome arm 6BS leads to faster leaf and whole-plant senescence and more efficient nutrient remobilization to developing kernels, increasing grain protein, iron and zinc contents (Uauy et al,. 2006; Waters et al., 2009). Reducing the transcript levels of all *NAM* copies in hexaploid wheat using RNA interference leads to a strong stay-green phenotype (delayed senescence) accompanied by a 30 % reduction in percent grain protein (Uauy et al., 2006). A barley gene orthologous to *TtNAM-B1*, *HvNAM1*, is located in a co-linear (with wheat chromosome arm 6BS) region of chromosome six (Distelfeld et al., 2008). Alleles of this gene (GenBank accessions EU368851 and EU368852) found in two varieties ('Lewis' and 'Karl', respectively) (Burger et al., 1979; Hockett et al., 1985; Wesenberg et al., 1976) code for proteins that differ in three positions (W78C, A102P, A357T). Previous work from our lab, comparing near-isogenic germplasm carrying either the 'Lewis' or 'Karl' *HvNAM1* alleles has demonstrated that the 'Karl' allele is associated with delayed senescence and lower percent grain protein, suggesting that its function is impaired (Heidlebaugh et al., 2008; Jukanti et al., 2008).

Our laboratory has previously derived near-isogenic lines varying in the allelic state of *HvNAM1* from a 'Lewis' x 'Karl' mapping population (See et al., 2002; Mickelson et al., 2003). Transcriptomic comparison of flag leaves from variety 'Karl' and near-isogenic line '10_11' (containing the 'Lewis' *HvNAM1* allele) identified an *AtGRP7* ortholog (*HvGR-RBP1*) as one of the most strongly upregulated (in line '10_11' *vs.* 'Karl') genes. For all time points from 7 to 28 days past anthesis, more than 100-fold differences in *HvGR-RBP1* expression also were observed (Jukanti et al., 2008). Similar differences in expression were found in leaves of younger (pre-anthesis) plants

(Lacerenza et al., 2010) with *HvGR-RBP1* either not expressed, or expressed at very low levels, in variety 'Karl'. Intriguingly, comparison of plant development in 'Karl' and '10_11' indicated that, in addition to differences in leaf and whole-plant senescence (faster in line '10_11', attributed to the 'Lewis' *HvNAM1* allele), pre-anthesis plant development was different as well. Starting with leaf eight, and throughout the rest of plant development, leaf development was significantly delayed in 'Karl'. A majority of 'Karl' and '10_11' shoots had 13 leaves (i.e., leaf 13 was the flag leaf), but production of a 14th leaf was about twice as frequent in 'Karl' as in '10_11' and only (very few) 'Karl' plants developed 15 leaves on their main shoots, emphasizing their slower development. This behavior was explained by slower development of the shoot apex and led to delayed anthesis (~5 days) in 'Karl' (Lacerenza et al., 2010). The relationship between *HvNAM1* and *HvGR-RBP1* is unclear; however, recent barley genome information (Mascher et al., 2017) indicates that both are located on chromosome six, with a genetic distance of ~5 cM.

Glycine-rich RNA-binding proteins (GR-RBPs) are small (molecular weight <20 kD) proteins with an N-terminal RNA-binding domain (~ 90 amino acids; also known as an RNA Recognition Motif or RRM) and a C-terminal glycine-rich domain (~70 amino acids) which is intrinsically disordered in the free protein (Ciuzan et al., 2015; Tripet et al., 2014). They comprise a subgroup of the large family of glycine-rich proteins, designated as subfamily IVa (Czolpinska&Rurek 2018; Mangeon et al., 2010). The best-understood plant GR-RBP is *Arabidopsis thaliana* glycine-rich RNA-binding protein 7 (*AtGRP7*). This protein binds both RNA and DNA, with a preference for single-stranded

nucleic acids (Schüttpelz et al., 2008). *AtGRP7* affects plant stress tolerance under high salt and dehydration conditions; the protein is involved in the regulation of stomata and also confers tolerance to low temperatures, most likely through its RNA chaperone activity (Cao et al., 2006; Hecht et al. 2005; Kim et al., 2008; Yang et al., 2014). *AtGRP7* is a component of the flowering autonomous (or earliness *per se*) pathway which promotes floral transition, as demonstrated by the late-flowering phenotype of knockout mutants (Steffen et al., 2019; Streitner et al., 2008). Individual nucleotide resolution crosslinking and immunoprecipitation (iCLIP) has identified several hundred potential *AtGRP7* RNA targets, besides its own pre-mRNA, suggesting its involvement in numerous functions besides flowering time control (Meyer et al., 2017).

Information outlined above suggests that developmental differences observed between variety ‘Karl’ and its near-isogenic line ‘10_11’ are due to both *HvNAM1* and *HvGR-RBP1* function, with *HvNAM1* regulating senescence and *HvGR-RBP1* influencing pre-anthesis development and anthesis date. Using a wild barley nested association mapping population, Maurer et al. (2016) identified a quantitative trait locus (QTL) influencing flowering in the same region of chromosome six, closely linked to *HvNAM1*, and have suggested *HvGR-RBP1* as a candidate gene. In this context, the purpose of research presented here was to 1) characterize differences in *HvGR-RBP1* function between ‘Karl’ and ‘Lewis’; 2) use this information for the development of a molecular marker; and 3) apply this marker and previously developed markers for *HvNAM1* (Distelfeld et al. 2008) to malt barley breeding germplasm, in order to dissect the effects of these genes on plant development and agronomic parameters.

Materials and Methods

Sequence Analysis, Molecular Marker Design and Genotyping

DNA Isolation: Tissue from young barley leaves (~0.5 to 1 g) was finely ground in liquid nitrogen, using mortar and pestle, and mixed with 750 µl of extraction buffer containing 0.1 M Tris/HCl (pH 7.5), 50 mM EDTA, and 1.25% SDS (preheated to 65 °C). After incubation at 65 °C for 30 minutes, samples were cooled on ice and 300 µl of cold (4 °C) 6 mM ammonium acetate was added. Samples were incubated for 15 minutes at 4 °C, followed by centrifugation at 13,300 x g for 15 minutes. The supernatant was transferred to a new tube and mixed gently with 2 volumes of cold isopropanol. After incubation at 4 °C for 5 minutes, samples were centrifuged at 17,500 x g for 15 minutes to pellet the DNA. The pellet was washed twice with 75% ethanol, dried and suspended in 20 µl of H_2O. Subsequently, DNA quantification was performed using a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantified DNA was diluted to 100 ng / µl for genotyping via PCR and restriction digest assays.

Primer Design: To dissect the allelic state of *HvGR-RBP1* in different barley genotypes, we designed a versatile molecular marker. The *HvGR-RBP1* sequence was obtained from NCBI (https://www.ncbi.nlm.nih.gov/) (ID: JX126694.1) and BLASTed against the reference barley genome (variety 'Morex') (Mascher et al., 2017), identifying HORVU6Hr1G055440. Subsequently, primers (see Appendix A: Table S1) were designed, allowing amplification of an approximately 5 kb region encompassing the

HvGR-RBP1 coding, flanking 5'- and flanking 3'-regions. PCR amplification of *HvGR-RBP1* was performed using genomic DNA from barley varieties 'Karl' (Burger et al., 1979; Wesenberg et al., 1976) and 'Lewis' (Hockett et al., 1985). PCR reactions were performed using GoTaq DNA polymerase (Promega, Madison, WI, USA) following the manufacturer's guidelines. An Eppendorf Mastercycler 5333 PCR Thermal Cycler (Eppendorf, Hauppauge, NY, United States) was used for PCR with the following cycling profile: 94 °C for initial denaturation, 30 cycles of 94 °C for 1 minute, T_m for 1 minute (Appendix A: Table S1), 72 °C for 1 minute per kb, and 7 minutes of final elongation at 72 °C. PCR products were then visualized on 1% agarose gels, and Sanger sequencing was performed by Genewiz (South Plainfield, NJ, USA) with PCR samples cleaned using a ZR-96 DNA Clean-Up Kit (Zymo Research, Irvine, CA, USA).

Sequence Analysis: Differences in the sequence of *HvGR-RBP1* between barley varieties 'Karl', 'Lewis', and 'Morex' (reference genome) (Mascher et al., 2017) were analyzed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and pairwise and multiple sequence alignment tools from EBI (https://www.ebi.ac.uk/Tools/psa/, https://www.ebi.ac.uk/Tools/msa/), and Ensembl Plant (http://plants.ensembl.org/index.html/) with default settings. Single nucleotide polymorphisms (SNPs) between varieties were detected manually. PCR amplification and sequencing results identified differences between 'Karl' and 'Lewis' in the promoter region of *HvGR-RBP1*, hence; this region was inspected in more detail. Two kb upstream of the *HvGR-RBP1* start codon were scanned for potential transcription factor binding motifs via an online tool, PlantPAN 3.0 (Chow et al., 2019)

(http://plantpan.itps.ncku.edu.tw/index.html) using the reference sequence from variety 'Morex'. The analysis was performed with default settings chosen for *Brachypodium distachyon,* and transcription factor motifs with a similarity score of 1 were selected as potential candidates.

Analysis of the allelic state of *HvNAM1* was performed using the molecular markers described by Distelfeld et al. (2008). Two marker sequences (UHB6 and UHB7) were amplified with previously described PCR reactions. PCR products were digested with MwoI (for UHB6; New England Biolabs, Ipswich, MA, USA) and HpyCH4III (for UHB7; New England Biolabs) (Distelfeld et al., 2008). For a few barley lines, restriction enzyme cleavage analysis of amplified sequences was complemented with Sanger sequencing to confirm *HvNAM1* allelic state.

Plant Material, Field Studies and Data Collection

To understand the impact of different *HvGR-RBP1* and *HvNAM1* alleles, we genotyped a subset (95 lines) of the material described as the "Malt Panel" by Pauli et al. (2015), resulting from a set of crosses aimed at introgressing the 'Karl' *HvNAM1* allele into malt barley breeding germplasm (Table 2.1; see Appendix A: Table S2 for pedigrees). Subsequent to genotyping, we re-analyzed agronomic traits that had been collected as described in Pauli et al. (2015) and results from the re-assessment are listed as Experiment 1 throughout this manuscript. In order to test the interaction of *HvGR-RBP1* and *HvNAM1* allelic states with nitrogen fertilization and irrigation, we also performed a smaller experiment (13 varieties and lines; Tables 2.1 and Appendix A: Table S2) which is referred to as Experiment 2 throughout this manuscript. Experiment 2

was grown for three different location-years, namely Bozeman 2016, Bozeman 2017 (Arthur Post Research Farm, Bozeman, MT, USA; 45º40’40.78 N, 111º09’07.14 W) and Conrad 2017 (Western Triangle Agricultural Research Station, Conrad, MT, USA; 48º18’26.05 N, 111º55’29.24 W), with three replicates for each location-year and treatment. For each location-year, plant material was grown under two water treatments in separate but adjacent blocks. Post Farm plots received 119 mm of rainfall from May to July during the 2016 season and 124.5 mm during 2017, while rainfall (2017) amounted to 89.9 mm for the Western Triangle location. All plots had adequate pre-season rainfall to reach soil field capacity. Approximately 150 mm water was added to the irrigated plots over three events throughout the season to ensure that plots did not dry below the barley wilting point; irrigation was stopped in the first week of July. Each location-year and water treatment also consisted of two nitrogen treatments in a split-plot randomized complete block design where the main plots were nitrogen treatment and the subplots were the genotypes. Prior to treatment, field soil samples were analyzed for total nitrogen content by AGVISE Laboratories (Benson, MN, USA) on a field level in 2016 and a replication level in 2017. This information was used to adjust the field nitrogen to the desired level in each individual split-plot, which was calculated by using the Montana Barley Production Guide (McVay et al. 2017) for malting barley nitrogen application (61.77 g nitrogen * expected yield in kg ha^{-1}). The exact amount of nitrogen (applied as urea) used in this experiment for each location and treatment is listed in Appendix A: Table S3.

Taking advantage of the smaller experiment size, we collected more data from Experiment 2, particularly for developmental parameters. Plants from this experiment were analyzed for developmental traits including plant height, anthesis date, maturity date, grain fill duration, and tiller numbers. Agronomic traits included percentage of plump kernels, test weight, grain protein concentration, yield, and harvest index. Grain protein concentration and kernel plumpness were measured and reported in the same way as Experiment 1 (Pauli et al., 2014). Plant height was measured at maturity and was determined by extending the tip of the barley ear upward in order to record the maximum height including awns. Two measurements per plot were performed and the averages were recorded. Tiller count was measured at maturity by counting the number of productive tillers per 30.5 cm or row length. Again, two measurements were performed per plot (replicate), and the average was recorded. Anthesis date was recorded when approximately 50% of plants in each plot had reached Zadoks growth stage 49 (‘awn tipping’) (Alqudah and Schnurbusch 2017; Zadoks et al., 1974). Maturity dates were recorded only at the Post Farm (2016, 2017) location-years as the date at which approximately 50% of barley ears had reached Zadoks stage 92. The grain filling period was estimated by subtracting anthesis from maturity dates for each plot/replicate. Harvest index was calculated as grain weight divided by total row biomass weight (only for Post Farm location-years). Test weights were determined using a GAC2500- UGMA (Dickey-john Corporation, Auburn, IL, USA) and reported as kg hL^{-1}.

Screening of *HvGR-RBP1* Allelic State in the USDA Barley Core Collection

The USDA Barley Core Collection is a genetically diverse group of 2465 6-row and 2-row lines consisting of advanced breeding lines, cultivars, and landraces collected from different geographical locations around the world (Muñoz-Amatriaín et al., 2014). PCA analysis of 2-row genotypes consisting of 6906 polymorphic markers from a 9K Infinium SNP chip (https://triticeaetoolbox.org/) was used to select a subset of 138 lines representing the genetic diversity of the population. To determine the frequency of the nonfunctional ('Karl') allele in a more genetically diverse population than the breeding population, the allelic state of *HvGR-RPB1* was analyzed in 138 accessions using the newly designed molecular marker for the gene.

Data Analysis

In this study, R software (v.3.5.3) was used to investigate the statistical significance of collected agronomic and developmental data (R Core Team, 2018). Experiment 1 was treated as a randomized incomplete block design with a 2-level irrigation treatment (Pauli et al., 2015). Experiment 2 was treated as a randomized complete block design with 2-level irrigation and 2-level fertilizer treatments. In both experiments, due to the non-independence of the barley lines with shared pedigrees, the genetic relatedness of lines used in the study was taken into consideration during statistical analysis. For this purpose, we performed Analysis of Variance (ANOVA) with the R package lme4qtl. This package considers the genetic relatedness of individual lines as a random factor (Ziyatdinov et al., 2018).

For Experiment 1, a relationship matrix was constructed from the SNP genotypes consisting of 333 markers obtained by Pauli et al., (2015). Method-of-moments estimators were used to construct a kinship matrix, $Gn{\times}n$ where n is the number of genotypes and the kinship estimate between the most distant subpopulations is zero on average using the R package popkin (Ochoa and Storey 2019). The following linear mixed model was fit to these data

$$Y_{ijk} = \mu + alleleCombination_i + irrigation_j + (alleleCombination \times irrigation)_{ij} + genotype_k + \varepsilon_{ijk},$$

where Y_{ijk} is a single phenotypic observation, μ is the grand mean, $alleleCombination_i$ is a dummy variable capturing the effect of the allele combinations at the *HvNAM1* and *HvGR-RBP1* loci, $irrigation_j$ is the effect of the jth irrigation treatment, $(alleleCombination \times irrigation)_{ij}$ is the effect of the interaction between the ith alleleCombination and the jth irrigation treatment, $genotype_k$ is the effect of the kth genotype following $\mathcal{N}(0,Gn{\times}n)$, and ε_{ijk} is the random error following $\mathcal{N}(0,\sigma^2)$. The model terms allelic combination and irrigation were modeled as fixed effects and the other terms were modeled as random effects.

In Experiment 2, the pedigree of the 13 utilized varieties and lines was obtained from T3/Barley (https://triticeaetoolbox.org/barley/) with manual extraction until there was an ‘unknown’ ancestor for the variety. The genetic relatedness matrix $Gn{\times}n$ where n is the number of genotypes was then built using this information with the R package

synbreed with the kin function (Wimmer et al., 2012). The following linear mixed model was fit to these data

$$Y_{ijkl} = \mu + alleleCombination_i + irrigation_j + nitrogen_k + (alleleCombination \times irrigation)_{ij} + (alleleCombination \times nitrogen)_{ik} + (irrigation \times nitrogen)_{kj} + (alleleCombination \times irrigation \times nitrogen)_{ijk} + genotype_l + \varepsilon_{ijkl},$$

where Y_{ijk} is a single phenotypic observation, μ is the grand mean, $alleleCombination_i$ is a dummy variable capturing the effect of the allele combinations at the *HvNAM1* and *HvGR-RBP1* loci, $irrigation_j$ is the effect of the jth irrigation treatment, $(alleleCombination \times irrigation)_{ij}$ is the effect of the interaction between the ith alleleCombination and the jth irrigation treatment, $genotype_k$ is the effect of the kth genotype following $\mathcal{N}(0, Gn \times n)$, and ε_{ijk} is the random error following $\mathcal{N}(0, \sigma^2)$.

The model terms allelic combination and irrigation were modeled as fixed effects and the other terms were modeled as random effects. The normality of model residuals was tested with the R package ‘fitdistrplus’ (Delignette-Muller&Dutang, 2015), and datasets that did not fit a normal distribution were normalized via the R package ‘bestNormalize’ (Peterson&Cavanaugh, 2019). Comparisons between different allelic groups and treatments were made with the Wilcoxon test, with the significance cut-off value $p < 0.05$; since no multiple testing corrections were made, p values are recorded in Supplemental Appendix A: Table S4.

Results

A GC-rich Insertion in the Promoter Region Disrupts *HvGR-RBP1* Function in Barley Variety 'Karl'

Previous studies from our laboratory have indicated that *HvGR-RBP1* is not expressed, or expressed at a very low level, in barley variety 'Karl' (see introduction). In order to discover the reason for this finding, a ~5 kb region, including ~ 2 kb of 5' UTR, the 500 bp coding region and ~ 3 kb of 3' UTR, was amplified from genomic DNA of varieties 'Karl' and 'Lewis' (with high HvGR-RBP1 expression) and sequenced (Figure 2.1a). The primer combinations PM, P1 and P2 amplified the 5' UTR while P5 and P6 amplified the 3' UTR (Figure 2.1a, Appendix A: Table S1). The coding region is captured by P3 and P4, with P4 including both part of the coding region and 3' UTR. A gene model, which is based on previous information about this gene (Tripet et al. 2014), the reference barley (var. 'Morex') genome (Mascher et al., 2017), and novel sequence information from this study is presented in Figure 2.1b. Gel electrophoretic analysis of PCR products showed a prominent difference in the DNA sequence amplified with the PM primer combination, with the fragment from 'Karl' ~ 400 bp longer than the 'Lewis' fragment (Figure 2.2a). Sequencing of this amplicon identified a GC-rich insertion in 'Karl' which is absent in the reference genome, and in variety 'Lewis' (Appendix B, Supplementary File 1). This insertion may be responsible for the lack of gene transcription (Jukanti et al., 2008; Lacerenza et al., 2010) that was reported in var. 'Karl.' In addition to the insertion, sequencing identified three single nucleotide differences that resulted in three amino acid substitutions, asparagine to serine at the 59th position, alanine

to glycine at the 114th position, and tyrosine to histidine at the 115th position, between 'Lewis' and 'Karl'. However, these differences are unlikely to be of functional importance since *HvGR-RBP1* is not expressed in var. 'Karl'.

The promoter region of *HvGR-RBP1* was scanned for potential transcription factor binding motifs. Our analysis indicated that this region is almost identical in varieties 'Lewis' and 'Morex'; hence, the 'Morex' (reference) sequence was used for this part of the study. Several transcription factor binding motifs were identified using the PlantPAN (v. 3.0) tool (http://plantpan.itps.ncku.edu.tw/) (Chow et al., 2019). Motifs included those which may be recognized by members of the MADF, WRKY, Myb/SANT, and NAC families, suggesting roles for HvGR-RBP1 in plant developmental and stress response processes (Figure 2.1b).

Screening for HvGR-RBP1 Allelic State in Breeding Germplasm Suggests a Phenotypic Selection for the Functional Allele

To understand the allelic effect of *HvGR-RBP1* on agronomic traits in malt barley, we screened germplasm from the Montana State University malt barley breeding program included in Experiments 1 and 2. The molecular marker exploited the amplicon length difference in the 5' UTR of *HvGR-RBP1*, enabling easy differentiation of homozygous and heterozygous genotypes (Figure 2.2a). The germplasm screened represents efforts to control grain protein concentration by introgressing the 'Karl' (low grain protein; delayed senescence) *HvNAM1* allele; lines were advanced and phenotypically selected for yield, low grain protein, and a high percentage of plump kernels. As expected, the 'Karl' *HvNAM1* allele was present in a majority of tested lines.

Interestingly, the functional (high gene expression; 'Lewis') *HvGR-RBP1* allele was present in a considerably larger percentage of lines than expected based on genetic distance between the two genes (~5 cM; see introduction) (Table 2.1, Figure 2.2b, Appendix A: Table S2). Phenotypic selection may have favored lines carrying the functional *HvGR-RBP1* allele.

Barley Plants Carrying a Functional *HvGR-RBP1* Allele Head Earlier and are Slightly Reduced in Height

To understand whether the combination of the functional 'Lewis' allele of *HvGR-RBP1* and a 'Karl' *HvNAM1* was due to phenotypic selection during breeding we compared agronomic traits in lines with different allele combinations. Lines carrying a 'Karl' allele of *HvGR-RBP1* and a 'Lewis' allele of *HvNAM1* were rare and have therefore not been included in our analyses. Data from Experiment 1 (re-analysis of agronomic data from Pauli et al., (2015) based on new molecular marker analyses) indicate that germplasm homozygous for the functional ('Lewis') *HvGR-RBP1* allele reaches the heading stage 0.5 days earlier (Table 2.2). The effect was stronger in Experiment 2, with 1.4 days of difference in anthesis date (Table 2.3), with measured differences significant in both experiments. Lines combining functional ('Lewis') *HvGR-RBP1* with 'Karl' *HvNAM1* alleles had the earliest heading or anthesis dates (Tables 2.2 and 2.3). Lines with this allele combination were also reduced in height; separate analysis of *HvGR-RBP1* and *HvNAM1* effects suggests a small but consistent effect of *HvGR-RBP1* on height, while the influence of *HvNAM1* was not consistent between the two experiments (Tables 2.2 and 2.3).

Experiment 2 also analyzed maturity dates. Based on these, plants carrying the 'Karl' *HvNAM1* allele matured ~2 days later (Table 2.3). This finding agrees with previous research from our laboratory comparing barley lines with 'Lewis' *vs.* 'Karl' *HvNAM1* alleles (Heidlebaugh et al., 2008; Jukanti and Fischer 2008; Jukanti et al., 2008; Lacerenza et al., 2010), and with the recent literature regarding the function of *NAC* genes in senescence regulation (Distelfeld et al., 2014; Podzimska-Sroka et al., 2015). Interestingly, as the functional ('Lewis') *HvGR-RBP1* allele accelerates flowering and heading while the 'Karl' *HvNAM1* allele delays plant maturity, the combination of these two alleles (+/- group in Table 2.3) resulted in a significantly increased (~3 days longer) grain fill duration.

Experiments also analyzed the effect of irrigation and N fertilizer treatments on flowering/heading dates, maturity dates, and plant height (Tables 2.2, 2.3 and Appendix A: Table S5). The statistical model used indicates that both genes contribute significantly to the control of grain fill duration. The model also suggests that the effects of *HvGR-RBP1* alleles are independent of the environmental conditions tested, while *HvNAM1* allelic effects are slightly affected by irrigation treatments. As expected, both irrigation and N treatments had significant effects on plant height, with irrigated plants ~20 cm taller in Experiment 1 and ~13 cm taller in Experiment 2 (Appendix A: Table S5). Significant interactions between allele combinations and irrigation treatments occurred in both experiments (Tables 2.2c and 2.3c). In contrast to heading, maturity, and grain fill duration, tillering (i.e., numbers of tillers per meter) was not influenced by the genes

studied here (Table 2.3a), while both nitrogen fertilization and irrigation significantly influenced this parameter (Appendix A: Table S5).

Germplasm Combining Functional (‘Lewis’) *HvGR-RBP1* with ‘Karl’ *HvNAM1* Alleles Has a Lower Grain Protein Concentration and a Higher Percentage of Plump Kernels

The agronomic traits most important to malt quality, grain protein and percentage of plump kernels, were also examined to determine their regulation by HvGR-RBP1 and HvNAM1 allelic states. In both experiments, germplasm homozygous for ‘Lewis’ *HvGR-RBP1* and ‘Karl’ *HvNAM1* alleles presented a strongly enhanced percentage of plump kernels when compared to the other allele combinations (Tables 2.2b and 2.3b). The statistical model indicates that, in both experiments, the influence of alleles and allele combination on this trait are highly significant (Tables 2.2c and 2.3c). Lines with this allele combination also had the lowest grain protein concentrations (Tables 2.2b and 2.3b), with the influence of *HvNAM1* consistent between the two experiments. The ‘Karl’ *HvNAM1* allele lowered grain protein from 13.4 to 12.4% in Experiment 1 and from 13.1 to 11.8% in experiment 2, confirming previous studies in barley and wheat (Heidlebaugh et al., 2008; Jukanti et al., 2008; Uauy et al., 2006).

Irrigation treatments applied in this study significantly enhanced the percentage of plump kernels and lowered percent grain protein in Experiment 1, while applied N fertilization (Experiment 2) slightly enhanced grain protein concentration (Figure 2.3, Appendix A: Table S5). Germplasm homozygous for ‘Lewis’ *HvGR-RBP1* and ‘Karl’ *HvNAM1* alleles had the highest percentage of plump kernels and the lowest grain protein concentration, irrespective of irrigation treatment, in both Experiments 1 (Figure 2.3) and

2 (see Appendix A: Tables S6, S7 and S8). This allele combination also maintained its advantage for malt barley production under the two N levels tested in Experiment 2 (Appendix A: Table S8), suggesting that observed effects are robust under a range of growing conditions.

Germplasm Combining Functional (‘Lewis’) *HvGR-RBP1* with ‘Karl’ *HvNAM1* Alleles Has Slightly Enhanced Test Weight, But Yield is not Increased

Based on data presented in Tables 2.2 and 2.3, lines homozygous for the functional (‘Lewis’) *HvGR-RBP1* and ‘Karl’ *HvNAM1* alleles have slightly enhanced test weight; this effect can be mostly attributed to the functional *HvGR-RBP1* allele. In Experiment 1, this allele combination appears associated with a slight yield penalty, while essentially no yield effects associated with alleles and allele combinations were observed in Experiment 2.

As expected, tested environmental parameters had a significant influence on test weight and yield in both experiments. Irrigation increased test weight by 1-3 kg hL^{-1} and yield by 2,000 to 3,000 kg ha^{-1} (Figure 2.3, Appendix A: Table S7), while higher N levels were associated with slightly (but not significantly) enhanced yield in Experiment 2 (Appendix A: Table S8). The statistical model confirms that effects of irrigation on test weight and yield were significant in both experiments (Tables 2.2c and 2.3c), while no significant effect of applied N treatments was observed in Experiment 2 (Table 2.3c). Some combinations between studied genes/alleles and irrigation affected test weight, but not yield (Table 2.3c).

The *HvGR-RBP1* Promoter Insertion is not Unique to Variety 'Karl'

In our analysis, we studied the influence of the functional ('Lewis') vs. nonfunctional ('Karl') *HvGR-RBP1* alleles on plant developmental and agronomic parameters. To determine if the nonfunctional *HvGR-RBP1* allele is unique to 'Karl', we used the newly developed marker (Figure 2.2a) to screen a subset (138 accessions) from the USDA Barley Core Collection (Muñoz-Amatriaín et al. 2014). Based on this analysis, 22 accessions (15.9 %) possessed an upstream insertion identical or similar to the one found in var. 'Karl' (Appendix A: Table S9). Thirteen (13) of the accessions carrying the insertion were landraces, three were breeding lines, and two were cultivars, while the status of the remaining four accessions is unclear (Appendix A: Table S9). Presence of the upstream insertion in landraces with a geographically diverse origin (including Asia, Africa and South America) indicates that this variation is not particularly rare. However, it stands as an intruging question whether the functional vs. nonfunctional allele provides a different selective advantage depending on the environment.

Discussion

Flowering and senescence are important phases of a plant's life cycle, particularly in annual species with monocarpic senescence. They define adaptation to a particular environment; in annual crops including cereals, timing of flowering and senescence control yield and quality (Distelfeld et al., 2014; Hill&Li 2016; Woo et al., 2018). Interactions between flowering and senescence control appear particularly relevant, and both flowering time-dependent and -independent inputs into the control of senescence have been identified

(Bogard et al., 2011; Hensel et al., 1993; Kim et al., 2004; Miryeganeh et al., 2018; Parrott et al., 2012; Wingler et al., 2010; Wu et al., 2008).

The observation that *HvGR-RBP1* is essentially non-functional in barley variety 'Karl'(Jukanti et al., 2008; Lacerenza et al., 2010) appears to be due to changes in the 5' flanking sequence about 1KB upstream of the translation start site, which includes an ~400 bp G-rich insertion (Figure 2.2a). The amplicon size difference is directly applicable as a molecular marker, which has been utilized here to screen malt barley breeding germplasm (Appendix A: Table S2) and a geographically diverse subset of the USDA Barley Core Collection, finding that the non-functional insertion is not rare (Appendix A: Table S9) (Muñoz-Amatriaín et al. 2014). The insertion could disrupt transcription for a variety of reasons, including disruption of enhancers, or attraction of silencers. The fact that the insertion includes repeated G sequence could be important as such sequences are known to interact with interfering RNA (Pernitzsch et al. 2014) or could form secondary structures that impedes expression (Yang et al. 2018). Further analysis of the region will allow discrimination between these possibilities.

Data shown in Tables 2.1 and 2.2 indicate that germplasm with a functional *HvGR-RBP1* allele reaches anthesis earlier and is slightly reduced in height when compared with germplasm carrying the 'Karl' allele which is not expressed. These findings fit with previous analyses comparing variety 'Karl' with a near-isogenic line ('10_11') carrying the functional allele from variety 'Lewis' (Lacerenza et al., 2010; Parrott et al. 2012). Furthermore, a homologous gene in *A. thaliana* (*AtGRP7*) is important for the autonomous (or earliness *per se*) flowering pathway; knockout mutants

exhibit substantially delayed flowering (Steffen et al., 2019; Streitner et al., 2008). Together, these data indicate that *At*GRP7 and *Hv*GR-RBP1 have similar or identical functions in *A. thaliana* and barley flowering time control. Future research will focus on dissecting the molecular interactions through which *Hv*GR-RBP1 modulates barley flowering, considering known differences between flowering time control in *A. thaliana* and cereals (Blümel et al., 2015; Greenup et al., 2009). The fact that vernalization treatments eliminate developmental differences between germplasm differing in GR-RBP function (Parrott et al., 2012; Streitner et al., 2008) may be helpful in this context.

The 'Lewis' allele of *HvNAM1* leads to plants reaching maturity 2 days earlier than the 'Karl' allele in experiment 2 (Table 2.3a). This finding from field experiments agrees with our previous (greenhouse) based comparison of flag leaf senescence in near-isogenic germplasm varying in *HvNAM1* allelic state (Heidlebaugh et al., 2008; Jukanti et al., 2008; Mason et al., 2016). Importantly, based on experiment 2, effects of *HvGR-RBP1* and *HvNAM1* are additive; genotypes combining functional ('Lewis') *HvGR-RBP1* with 'Karl' *HvNAM1* alleles have a grain fill duration which is ~3 days longer. While this difference may not appear large, it corresponds to an ~9% extension of this developmental phase. This extension in grain fill duration may explain the substantial and significant increase in the percentage of plump kernels, and the slight (but significant) increase in test weight and decrease in grain protein concentration seen in germplasm with this allele combination (Table 2.3b). While maturity was not measured in experiment 1, the same effects on kernel plumpness, test weight and grain protein as in experiment 2 were observed when comparing lines combining functional ('Lewis')

HvGR-RBP1 with 'Karl' *HvNAM1* alleles with the other allele combinations (Table 2.2b), confirming conclusions drawn from experiment 2. Analysis of seed samples from the Bozeman 2017 (non-irrigated) location indicates that enhanced kernel plumpness is associated with a slight (~3%) increase in kernel diameter, an ~10% increase in single kernel weight, but no change in seed length (data not shown).

Maltsters can reject barley for malt if grain protein is too high and/or the percentage of plump kernels too low, reducing the farmer's profit by half. A variety of conditions can increase grain protein and reduce plump seed, including too much soil nitrogen, lack of rainfall, and heat during grain fill. In the current study, the positive effects on kernel plumpness and protein content contributed by the combination of the 'Lewis' *HvGR-RBP1* and the 'Karl' *HvNAM1* alleles were stable under the different environments (irrigation, nitrogen) tested (Figure 2.3). Weston et al. (1993) found that low grain protein lines from 'Karl' had lower protein across nitrogen treatments when compared with types not carrying low protein gene. However, the low protein types also had lower plumps and kernel weight across treatments, making them undesirable. Our data suggest that this is due to linkage between the 'Karl' *HvGR-RBP1* and *HvNAM1* alleles. Recombinants between the genes studied here satisfy a breeding goal that benefits growers and end-users - varieties with stable low protein and high plumps. It might be expected that a genotype with increased grain fill duration leads to a yield increase.

Extended grain fill duration (known as a 'stay-green phenotype' if maturation is delayed) has been extensively discussed in the literature and is often suggested to increase yield (Distelfeld et al., 2014; Thomas and Howarth 2000; Thomas and Ougham

2014), yet no significant yield increase was seen in our experiments when considering lines carrying the 'Karl' *HvNAM1* allele (delayed maturity) combined with either the 'Lewis' or 'Karl' *HvGR-RBP1* alleles (Tables 2.2b and 2.3b). Significantly increased kernel plumpness and test weight, combined with no or marginal yield effects suggest that lines combining functional ('Lewis') *HvGR-RBP1* with 'Karl' *HvNAM1* alleles produce fewer, but larger kernels. While this is still desirable in malting barley, the system may be sink-limited. As tillering is not affected (Table 2.3), one possible explanation is that the early-flowering ('Lewis' allele for *HvGR-RBP1*) genotype is associated with the production of fewer fertile spikelets, i.e., faster exhaustion of the determinate inflorescence meristem, and/or enhanced seed abortion. This is clearly a problem which warrants further analysis.

Overall, data presented here combined with past work indicate an important function for *Hv*GR-RBP1 in the control of barley reproductive development, similar or identical to *At*GRP7 function in *Arabidopsis* development. This research sets the stage for a detailed functional analysis of *Hv*GR-RBP1; furthermore, our data indicate that screening for the allelic state of *HvGR-RBP1*, particularly in combination with *HvNAM1*, is of high practical value in malt barley breeding.

Declarations

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Ethics Approval: Not applicable

Consent to Participate: Not applicable

Consent for Publication: All authors have read the manuscript and approve of its publication.

Availability of Data and Material: All data utilized are contained within the manuscript, or within Supplementary Material. Raw data are shown in Table

Code Availability: Not applicable

Table 2.1. Description of Experiments

	Experiment 1: Re-analysis of Pauli et al. 2014 data with new markers	**Experiment 2: Detailed agronomic analysis**
Description	Subset of an association mapping population described in Pauli et al., 2014	Five varieties and eight lines; partial overlap with experiment 1 (Table S2 for details)
Number of screened lines and varieties	95 lines	13 varieties and lines
Experimental design	Randomized incomplete block design	Randomized complete block design
Replication	Two location-years	Three location-years
Treatments	Irrigation	Irrigation and nitrogen

Table 2.2. Influence of *HvNAM1* and *HvGR-RBP1* Allelic States on Agronomic and Physiological Traits in Experiment 1 (with 95 lines). Data represent mean values and standard deviations averaged across both location-years and treatments. Two-sided differences between the means of different alleles/allele combinations were calculated using the Wilcoxon test (*P* value < 0.05) and are represented with superscript letters. For allele combinations, the plus (+) symbol represents the wild-type (functional, 'Lewis') allele, while the minus (–) symbol denotes the 'Karl' allele of both *HvGR-RBP1* and *HvNAM1*. For ANOVA analyses, *P* values are represented by a dot (.) for *P* < 0.1, one star (*) for *P* < 0.05), two stars (**) for *P* <0.01, or three stars (***) for *P* < 0.001.

Table 2.2a. Separate analysis of *HvGR-RBP1* and *HvNAM1* effects

Single gene effect	Number of lines	Heading [Julian days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
HvGR-RBP1^{+}	53	187.9 ± 2.1 ^{b}	66.6 ± 10.8 ^{a}	69.8 ± 15.1 ^{a}	67.4 ± 1.8 ^{a}	12.6 ± 0.8 ^{a}	5834 ± 1829 ^{a}
HvGR-RBP1^{-}	42	188.4 ± 1.9 ^{a}	68.4 ± 12.0 ^{a}	61.7 ± 14.2 ^{b}	66.5 ± 1.7 ^{b}	12.6 ± 0.6 ^{a}	5775 ± 1706 ^{a}
HvNAM1^{+}	18	188.3 ± 1.9 ^{a}	69.1 ± 11.7 ^{a}	66.9 ± 15.0 ^{a}	67.2 ± 1.9 ^{a}	13.4 ± 0.7 ^{a}	6107 ± 1786 ^{a}
HvNAM1^{-}	77	188.1 ± 2.1 ^{a}	67.0 ± 11.3 ^{a}	66.0 ± 15.3 ^{a}	66.9 ± 1.8 ^{a}	12.4 ± 0.6 ^{b}	5738 ± 1765 ^{b}

Table 2.2b. Analysis of allele combinations

Allele combinations (*HvGR-RBP1*/*HvNAM1*)	Number of lines	Heading [Julian days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
+/+	18	188.3 ± 1.9 $^{a, b}$	69.1 ± 11.7 ^{a}	66.9 ± 15.0 ^{b}	67.2 ± 1.9 ^{a}	13.4 ± 0.7 ^{a}	6107 ± 1786 ^{a}
+/-	35	187.7 ± 2.2 ^{b}	65.3 ± 10.1 ^{b}	71.3 ± 15.0 ^{a}	67.4 ± 1.7 ^{a}	12.2 ± 0.6 ^{c}	5693 ± 1841 ^{b}
-/-	42	188.4 ± 1.9 ^{a}	68.4 ± 12.0 ^{a}	61.8 ± 14.2 ^{c}	66.5 ± 1.7 ^{b}	12.6 ± 0.6 ^{b}	5775 ± 1706 $^{a, b}$

Table 2.2c. Analysis of variance for fixed effects

Fixed effects	Heading [Julian days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
HvNAM1 allele	*	***	**		***	***
HvGR-RBP1 allele	**	***	***	***	***	*
Irrigation	***	***	**	***	***	***
HvNAM1- *HvGR-RBP1* alleles	**	***	***	***	***	***

HvNAM1 allele*irrigation		*			*	
HvGR-RPB1 allele*irrigation		*				.
HvNAM1- HvGR-RBP1 alleles *irrigation		*			.	
Transformation	None	None	None	None	None	None

Table 2.3. Influence of *HvNAM1* and *HvGR-RBP1* Allelic States on Agronomic and Physiological Traits in Experiment 2 (with 13 lines and varieties). Data represent mean values and standard deviations averaged across all location-years and treatments. Two-sided differences between the untransformed means of different alleles/allele combination were calculated using the Wilcoxon test (*P* value < 0.05), and are represented with superscript letters. For allele combinations, the plus (+) symbol represents the wild-type (functional, 'Lewis') allele, while the minus (–) symbol denotes the 'Karl' allele of both *HvGR-RBP1* and *HvNAM1*. For ANOVA analyses, *P* values are represented by a dot (.) for *P* < 0.1, one star (*) for *P* < 0.05), two stars (**) for *P* < 0.01, or three stars (***) for *P* <0.001. dap, days after planting.

Table 2.3a. Separate analysis of *HvGR-RBP1* and *HvNAM1* effects

Single gene effect	Number of lines	Anthesis [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Tillers [tillers m^{-1}]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]	Harvest index
HvGR-RBP1^{+}	10	58.0 ± 3.3 ^{b}	93.3 ± 3.5 ^{a}	35.2 ± 3.7 ^{a}	70.5 ± 9.7 ^{b}	160.4 ± 33.8 ^{a}	79.5 ± 16.3 ^{a}	65.0 ± 2.3 ^{a}	12.5 ± 2.0 ^{a}	6461 ± 1501 ^{a}	0.442 ± 0.042 ^{a}
HvGR-RBP1^{-}	3	59.2 ± 3.1 ^{a}	94.0 ± 3.0 ^{a}	34.1 ± 2.5 ^{b}	73.5 ± 8.7 ^{a}	157.7 ± 35.4 ^{a}	79.3 ± 13.9 ^{a}	64.0 ± 2.8 ^{b}	12.0 ± 1.7 ^{b}	6328 ± 1722 ^{a}	0.442 ± 0.043 ^{a}
HvNAM1^{+}	6	58.3 ± 2.6 ^{a}	92.4 ± 3.3 ^{b}	33.9 ± 2.8 ^{b}	70.3 ± 9.7 ^{a}	160.4 ± 33.1 ^{a}	74.8 ± 17.3 ^{b}	64.5 ± 2.4 ^{b}	13.1 ± 2.0 ^{a}	6420 ± 1478 ^{a}	0.443 ± 0.044 ^{a}
HvNAM1^{-}	7	58.2 ± 3.8 ^{a}	94.4 ± 3.3 ^{a}	35.9 ± 3.8 ^{a}	71.8 ± 9.3 ^{a}	159.3 ± 35.1 ^{a}	83.4 ± 13.0 ^{a}	65.0 ± 2.5 ^{a}	11.8 ± 1.6 ^{b}	6440 ± 1619 ^{a}	0.441 ± 0.040 ^{a}

Table 2.3b. Analysis of allele combinations

Allele combination (*HvGR-RBP1*/*HvNAM1*)	Number of lines	Anthesis [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Tillers [tillers m^{-1}]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]	Harvest index
+/+	6	58.3 ± 2.6 ^{b}	92.4 ± 3.3 ^{b}	33.9 ± 2.8 ^{b}	70.3 ± 9.7 ^{b}	160.4 ± 33.1 ^{a}	74.8 ± 17.3 ^{a}	64.5 ± 2.4 ^{b}	13.1 ± 2.0 ^{a}	6420 ± 1478 ^{a}	0.443 ± 0.044 ^{a}
+/-	4	57.5 ± 4.2 ^{b}	94.7 ± 3.4 ^{a}	37.2 ± 4.0 ^{a}	70.6 ± 9.5 ^{b}	160.5 ± 35.0 ^{a}	86.5 ± 11.5 ^{b}	65.7 ± 2.0 ^{a}	11.6 ± 1.5 ^{c}	6523 ± 1538 ^{a}	0.440 ± 0.038 ^{a}
-/-	3	59.2 ± 3.1 ^{a}	94.0 ± 3.0 ^{a}	34.1 ± 2.5 ^{b}	73.5 ± 8.7 ^{a}	157.7 ± 35.4 ^{a}	79.3 ± 13.8 ^{a}	64.0 ± 2.8 ^{b}	12.0 ± 1.7 ^{b}	6328 ± 1722 ^{a}	0.442 ± 0.043 ^{a}

Table 2.3c. Analysis of variance for fixed effects

Fixed effects	Anthesis [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Tillers [tillers m^{-1}]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]	Harvest index
HvNAM1 allele		***	***	*		***	***	***		
HvGR-RBP1 allele	***	.	***	*		***	***	**	*	
HvNAM1- *HvGR-RBP1* alleles	***	***	***	**		***	***	***	.	
Irrigation		*	**	**	*		***		**	
Nitrogen				.	*					

HvGR-RPB1 allele*irrigation							**			
HvGR-RPB1 allele*nitrogen							.			
HvNAM1 allele*irrigation		.	*	.						
HvNAM1 allele*nitrogen							.	.		
HvNAM1- HvGR-RBP1 alleles *irrigation		*	*	*			*			
HvNAM1- HvGR-RBP1 alleles *nitrogen		.	.							
Transformation	None	None	None	None	None	Ordnorm	None	None	None	LambertFX

Figure 2.1. Amplification of *HvGR-RBP1* and Potential Gene Model. 1a. The amplified PCR products of *HvGR-RPB1* from variety 'Lewis' is shown on %1 agarose gel. PM is the amplicon from 'Lewis' that varied for the insertion with 'Karl'. 1b. A potential gene model for HvGR-RBP1 is represented. The promoter region of the gene possesses several potential transcription factor binding motifs. The G-rich insertion in 'Karl' may disrupts gene expression. *HvGR-RPB1* consist of two exons and an intron, which are highly similar between 'Karl' and 'Lewis' with only three single nucleotide polymorphisms (A261G, C426G, T428C) that are located in Exon-2 and result in missense mutations Asparagine to Serine at 59th, Alanine to Glycine at 114th and Tyrosine to Histidine at 115th positions in 'Karl'. The locations for forward and reverse primers used for amplification of PCR products from Figure 1a are also shown as *PM, P1-P6.*

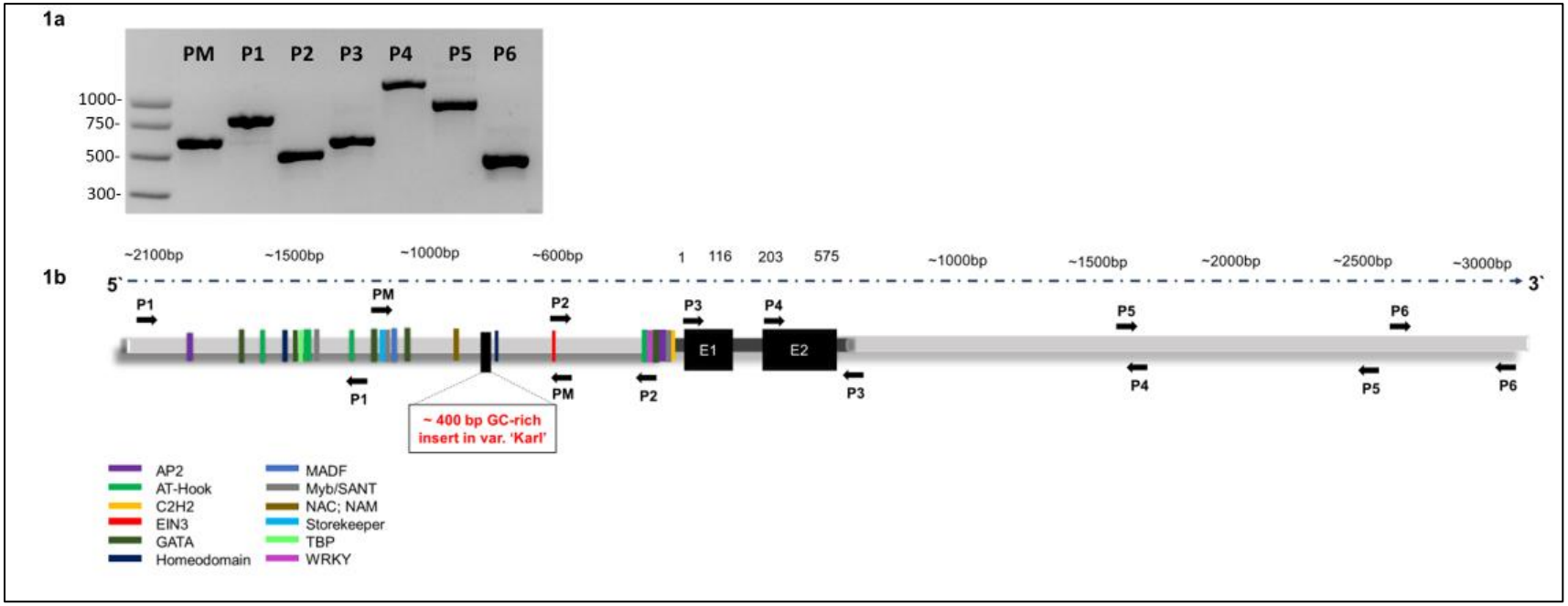


Figure 2.2. *HvGR-RBP1* Marker Development and Genotyping. 2a. The G-rich insertion in variety 'Karl' is used as a marker for genotyping of *HvGR-RBP1* gene. Here, four different varieties are represented: Karl, Lewis, MT124017 and 10_11. The difference in amplification length in the marker region is ~300 to 400 bp differentiates 'Karl' and 'Lewis' genotypes, as well as the heterozygote 'MT124017'. 2b. A pie chart representing the distribution of each allelic group in the screened MSU breeding population. +/+ indicating functioning alleles for both *HvGR-RPB1* and *HvNAM1*. -/- indicating non-functioning alleles for both genes. +/- indicating functional 'Lewis' allele for *HvGR-RBP1* and non-functioning 'Karl' allele for *HvNAM1*.

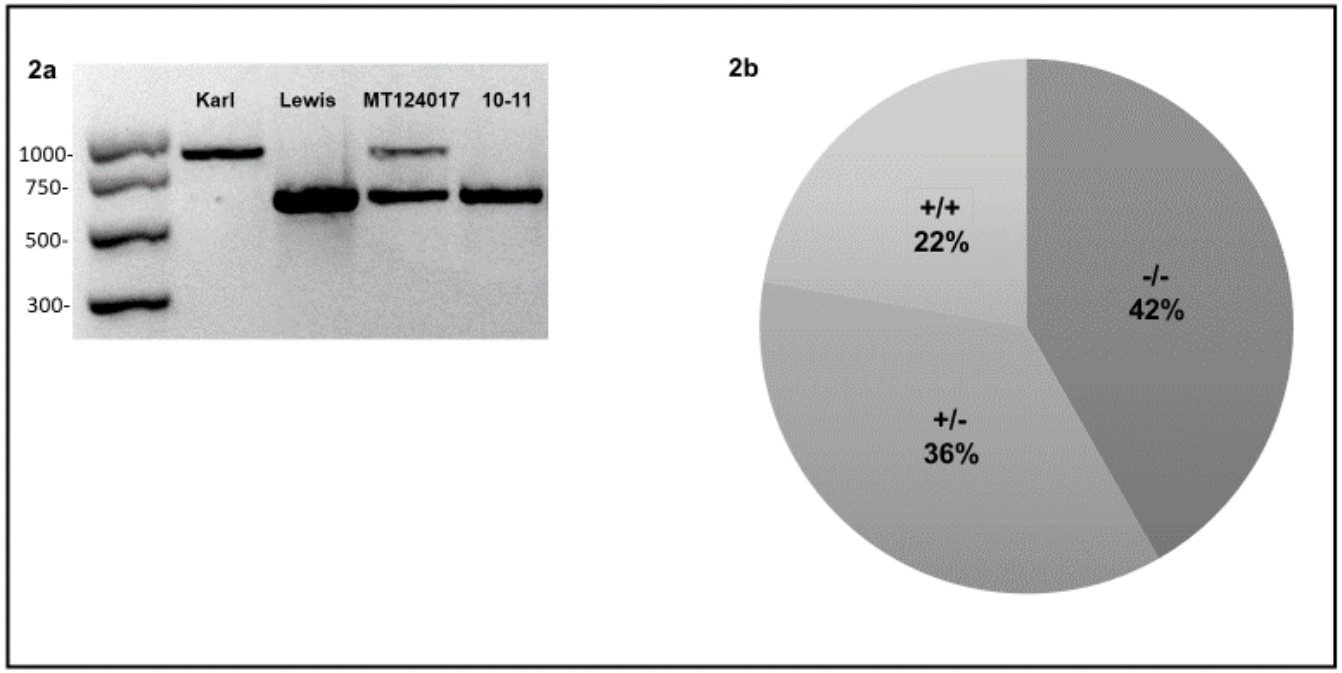
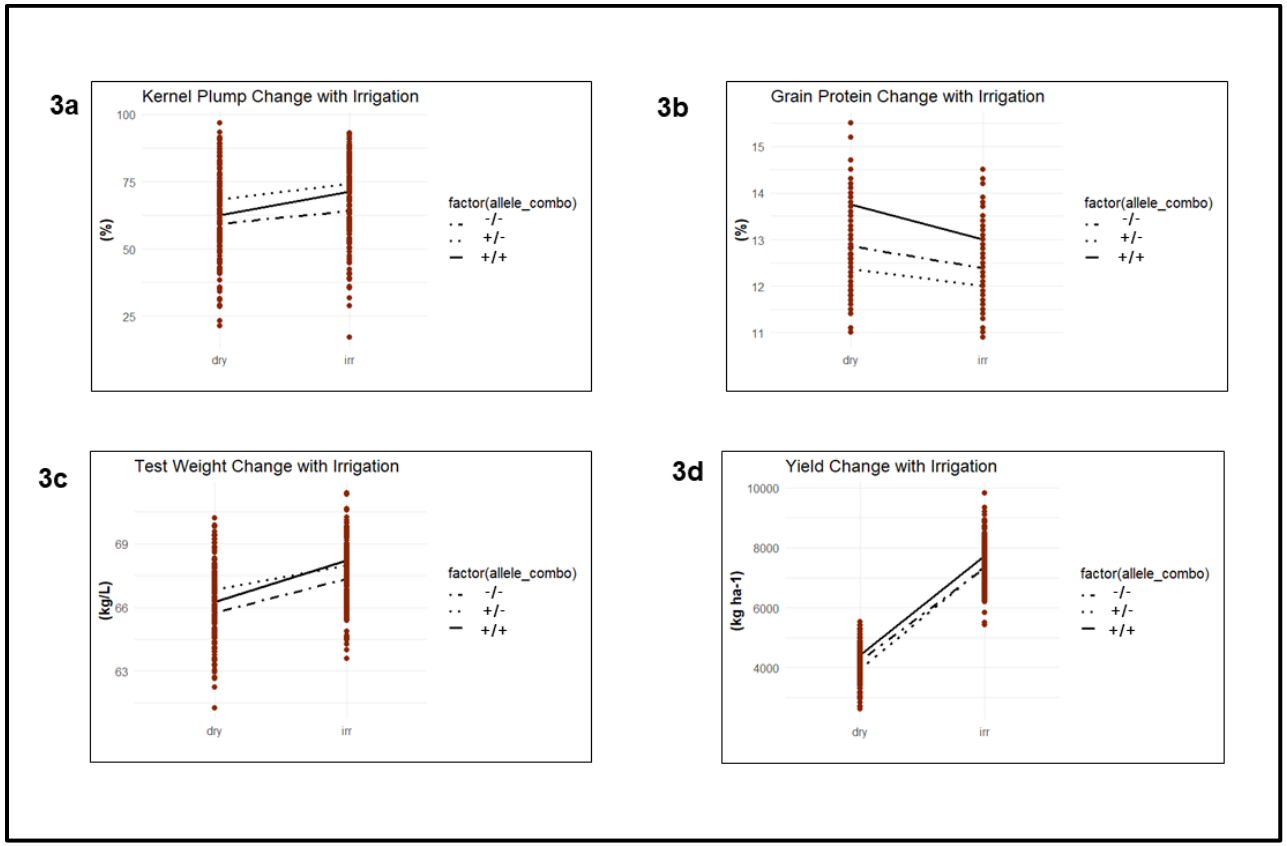


Figure 2.3. Changes in Agronomic Traits Under Irrigation Treatment in Re-analysis of Pauli *et.* al 2015. Data collected on lines segregating for *HvGR-RBP1* and *HvNAM1* and for traits from dry and irrigated conditions. Lines intersect data from the two treatments at the mean value for each trait for each allelic combination. Agronomic traits are represented as (3a) kernel plumpness (%), (3b) grain protein (%) (3c) test weight (kg/L), (3d) yield (kg/ha). Note that change in mean between dry and irrigated is less for allelic combo +/- except for in the case of yield



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CHAPTER THREE

DISSECTING THE EFFECTS OF SENESCENCE-REGULATING *HvNAM1* AND *HvNAM2* TRANSCRIPTION FACTORS ON MALT BARLEY AGRONOMICS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Performed lab experiments, analyzed data and wrote the manuscript

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Contributions: Performed field studies for Experiment 2 and collected agronomic data

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Contributions: Contributed agronomic data for Experiment 1

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Molecular Breeding

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Molecular Breeding

DISSECTING THE EFFECTS OF SENESCENCE-REGULATING *HvNAM1* AND *HvNAM2* TRANSCRIPTION FACTORS ON MALT BARLEY AGRONOMICS

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Abstract

Whole-plant senescence is a critical stage of plant development for determining cereal grain yield and quality. NAC transcription factors have been identified as senescence regulators in several plant species, and molecular manipulation of several *NAC* genes has indicated their essential contribution to grain quality including grain protein and micronutrient content. Allelic variation in the barley *NAC* transcription

factors *HvNAM1* and *HvNAM2* has been associated with variation in grain protein content in previous studies. However, the separate and combined contributions of these transcription factors to malt barley agronomics and quality has not previously been addressed. Here, we designed a molecular marker for the *HvNAM2* transcription factor to differentiate between alleles of the 6-row low-grain protein variety ‘Karl’ and a 2-row higher-protein line, ‘Lewis’. Analysis of the different *HvNAM1* and *HvNAM2* alleles suggests that *HvNAM1* controls grain protein content while *HvNAM2* is strongly associated with kernel plumpness and test weight. Results from this study indicate that plants carrying ‘Karl’ alleles for both genes have higher kernel plumpness and lower grain protein content. The newly designed *HvNAM2* molecular marker is of considerable value for malt barley breeding.

Introduction

Senescence is the last developmental stage of plant cells, plant organs, and even entire plants in species with monocarpic senescence (Fischer, 2012). During senescence, nutrients are remobilized and transported to the developing seeds of annual crops such as barley (Distelfeld, Avni, & Fischer, 2014). Efficiency of nutrient recycling during senescence is associated with variation in grain protein and micronutrient content in *Arabidopsis* (Pottier, Dumont, Masclaux-Daubresse, & Thomine, 2019), wheat (Shi et al., 2012; Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006), and barley (Cai et al., 2013). In durum wheat, stay-green mutants with delayed leaf senescence exhibited higher grain yield and kernel weight (Spano et al., 2003). These examples show that the timing of senescence substantially influence cereal agronomic traits and grain quality (Gregersen, Holm, & Krupinska, 2008). An improved functional understanding of whole-plant senescence is crucial for the enhancement of grain nutritional quality and yield in cereal crops, which are needed to meet the food demand of the 21st century.

Several approaches have been utilized to dissect the molecular mechanisms controlling plant (particularly leaf) senescence in crops and model species (Distelfeld, Avni, & Fischer, 2014; Wingler, Masclaux-Daubresse, & Fischer, 2009). Transcriptomic studies have identified a diverse set of senescence-associated genes (SAGs) (Guo, Cai, & Gan, 2004; Woo et al., 2016; Gregersen & Holm, 2007; Lin et al., 2015). These studies indicate that the senescence process is controlled by both internal and external factors including plant hormones (Kim, Chang, & Tucker, 2015), light intensity (Brouwer, Ziolkowska, Bagard, & Keech, 2012; Liebsch & Keech, 2016) and biotic/abiotic stress

(Sade, Del Mar Rubio-Wilhelmi, Umnajkitikorn, & Blumwald, 2018; Wingler & Roitsch, 2008). They also underline the importance of senescence regulation by transcription factors; in this context, NAC and WRKY genes/proteins have received particular attention (Fischer, 2008; Fischer, 2012) (Hickman et al., 2013; Kim, Nam, & Lim, 2016).

NAC genes (‘No Apical Meristem,’ NAM; *‘Arabidopsis* Transcription Activating Factor,’ ATAF; ‘Cup-Shaped Cotyledons,’ CUC) code for a large family of plant-specific transcription factors with functions in developmental regulation, abiotic stress control, and defense (Jensen & Skriver, 2014). The importance of *NAC* genes in senescence regulation has emerged from several studies (Borrill, Harrington, Simmonds, & Uauy, 2019; Buchanan-Wollaston et al., 2005; Gregersen & Holm, 2007; Guo, Cai, & Gan, 2004). Overexpression of *AtNAP* (Guo & Gan, 2006), *ORE1* (Balazadeh et al., 2010), and *ANAC016* (Kim, Sakuraba, Han, Yoo, & Paek, 2013) has resulted in early onset of senescence. In wheat, the *GPC-B1*/*NAM-B1* gene accelerates senescence and increases nutrient remobilization to the grains. RNAi-mediated knockdown of four related (to *NAM-B1*) *NAC* genes results in a strong stay-green phenotype, delaying senescence of main spike peduncles more than 30 days, and substantially lowering grain protein, zinc, and iron concentrations (Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006).

Grain protein content (GPC) is an important characteristic of malt barley due to its impact on malt quality. High GPC is associated with lower starch content and a decrease in malt extract; therefore, it is not desirable (Muñoz-Amatriaín, Cuesta-Marcos, Hayes, & Muehlbauer, 2014; Paynter, 1996). A barley variety, ‘Karl,’ has been identified as a source for low GPC in the 1970s (Wesenberg et al., 1976), and used in malt barley

breeding programs. Variety ‘Karl’ is characterized by low GPC under varying nitrogen and irrigation regimes (Burger, Wesenberg, Carden, & Pawlisch, 1979; See, Kanazin, Kephart, & Blake, 2002), thus serving as a reliable source for lowering GPC. In 2002, See et al. discovered two Quantitative Trait Loci (QTL) on chromosomes 6H and 2H associated with grain protein based on their crosses of ‘Karl’ with a standard 2-row malt barley variety, ‘Lewis’ (See, Kanazin, Kephart, & Blake, 2002). Co-linearity between the barley chromosome 6H GPC QTL and the wheat *GPC-B1* region (Distelfeld et al., 2008) has allowed the identification of the barley *HvNAM1* gene which, based on the analysis of near-isogenic germplasm varying in the chromosome six QTL/*HvNAM1* allelic state, is functionally identical to wheat *GPC-B1*/*NAM-B1* (Jukanti et al., 2008; Distelfeld, Avni, & Fischer, 2014). Data obtained in these studies suggest that mutations in the var. ‘Karl’ *HvNAM1* coding region disrupt protein function, leading to delayed senescence, less-efficient nutrient remobilization and lower GPC. Later studies have confirmed the association between *HvNAM1* allelic variation and GPC (Cai et al., 2013; Jamar, Loffet, Frettinger, Ramsay, & Fauconnier, 2010). Cai et al. (2013) also described variation in GPC associated with the allelic state of a closely related (to *HvNAM1*) *NAC* gene, *HvNAM2*, but did not characterize the impact of this gene on malt barley agronomic and quality parameters.

Both the GPC QTL mapping study resulting from the original ‘Lewis’ x ‘Karl’ cross (See, Kanazin, Kephart, & Blake, 2002) and comparison of the parents with near-isogenic germplasm varying in the chromosome 6H QTL/*HvNAM1* allelic state suggested that ‘Karl’ contains low-GPC alleles of additional genes, with *HvNAM2* as a good

candidate based on the data of Cai et al. (2013) and high sequence similarity between *HvNAM1* and *-2*. In the present study, we therefore designed a molecular marker differentiating var. 'Karl' and 'Lewis' *HvNAM2* alleles. The new marker, together with previously developed *HvNAM1* markers (Distelfeld et al. 2008), was applied to a malt barley breeding population to dissect separate and combined effects of both genes on plant developmental and grain quality parameters. We further explored the performance of alleles of the two genes under varying (irrigation, nitrogen) environments. Our results suggest that the new *HvNAM2* marker allows the selection of malt barley varieties with higher kernel plumpness, higher test weight, and lower GPC.

Materials and Methods

Designing a Molecular Marker for *HvNAM2*

DNA Isolation: DNA was isolated from barley varieties 'Karl' (Wesenberg et al., 1976) and 'Lewis' (Hockett et al., 1985) with the method described in Alptekin et al., 2020 using young barley leaves. The isolated DNA was quantified with a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and diluted to 100 ng/µl for PCR amplification of the *HvNAM2* gene.

Primer Design and *HvNAM2* Sequencing: Primers for the *HvNAM2* gene were designed with the following method: The sequence of *HvNAM2* was obtained from NCBI (https://www.ncbi.nlm.nih.gov/) (ID: DQ869679.1) and BLASTed against the reference barley genome (variety 'Morex') (Mascher et al., 2017), identifying HORVU2Hr1G039640 as a corresponding *HvNAM2* sequence in the reference genome.

Next, the primers listed in Appendix C: Table S1 were designed and used for amplification of an approximately 5 kb region covering the *HvNAM2* coding and flanking 5'- and 3'-regions. PCR amplification of *HvNAM2* was performed using GoTaq DNA polymerase (Promega, Madison, WI, USA) following the manufacturer's guidelines with the isolated genomic DNA from 'Karl' and 'Lewis'. PCR was performed with an Eppendorf Mastercycler 5333 PCR Thermal Cycler (Eppendorf, Hauppauge, NY, USA) with the following cycling profile: 95 °C for initial denaturation, 30 cycles of 95 °C for 1 minute, T_m for 1 minute (Appendix C: Table S1), 72 °C for 1 minute per kb, and 5 minutes of final elongation at 72 °C. PCR amplicons were then visualized on 1% agarose gels and sequenced via Sanger sequencing by Genewiz (South Plainfield, NJ, USA).

Sequence Analysis and Detection of Restriction Sites: Sanger sequencing data for *HvNAM2* were analyzed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and pairwise and multiple sequence alignment tools from EBI (https://www.ebi.ac.uk/Tools/psa/, https://www.ebi.ac.uk/Tools/msa/), and Ensembl Plants (http://plants.ensembl.org/index.html/) with default settings aiming to detect differences between 'Karl', 'Lewis', and the reference genome sequence from variety 'Morex' (Mascher et al., 2017). Single nucleotide polymorphisms (SNPs) between varieties were detected manually and compared with previously identified SNPs (Cai et al., 2013). Subsequently, sequence differences were investigated with respect to restriction digest sites using an online tool (http://insilico.ehu.es/restriction/two_seq/) (Bikandi, Millán, Rementeria, & Garaizar, 2004). After careful examination, the SNP located in the first intron of *HvNAM2* (at the 307th nucleotide, G in 'Lewis' and C in

‘Karl’) was identified as a possible site to design a restriction digest-based molecular marker. Several restriction enzymes were identified for the analysis of this SNP, namely BseGI, BstF5I, BtsCI, and FokI. These enzymes were predicted to cut ‘Karl’, but not ‘Lewis’ DNA at the analyzed SNP. Primers were then designed to amplify a 404-bp long region covering the intronic SNP (Appendix C: Table S1). Restriction enzyme BtsCI was chosen for the digestion, as it requires a single restriction recognition site and cuts the amplicon only once. Potential restriction products for ‘Karl’ and ‘Lewis’ varieties were predicted using another online tool (http://www.molbiotools.com/restrictioncomparator.html).

Restriction Digest: PCR reactions for restriction digests were completed using GoTaq DNA polymerase (Promega, Madison, WI, USA) following the manufacturer’s guidelines. Five X (5X) Colorless GoTaq® Flexi Buffer reaction buffer was used for PCR to avoid interference with subsequent restriction digests. An Eppendorf Mastercycler 5333 PCR Thermal Cycler was then used for PCR reactions with the following cycling profile: 95 °C for initial denaturation, 30 cycles of 95 °C for 1 minute, 63 °C for 1 minute, 72 °C for 1 minute per kb, and 5 minutes of final elongation at 72 °C. Digestion reactions were performed using BtsCI (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s guidelines. Digested amplicons were visualized on 2% agarose gels to identify digested and undigested bands.

Plant Material and Genotyping

To understand the impact of different *HvNAM2* alleles on malt barley agronomics, we genotyped the plant material described in Alptekin et al., 2020 using the newly

designed marker. Experiment 1 from that study contained 95 advanced malt barley breeding lines which were first described in (Pauli, Brown-guedira, & Blake, 2015). These lines were grown under two different irrigation regimes, designated as 'irrigated' and 'dry.' Experiment 2 (Alptekin et al., 2020) contained 13 varieties grown under two different irrigation (irrigated and dryland) and fertilizer regimes (normal and high nitrogen). This population was first described in Alptekin et al., 2020; it is a sub-population of plant material used in experiment 1. Heading (Julian days), height (cm), percentage of plump kernels (%), test weight (kg hL^{-1}), grain protein content (GPC; %) and yield (kg ha^{-1}) measurements were obtained in experiment 1. In experiment 2, maturity (days after planting, dap), grain filling duration (days), tiller count (tillers m^{-1}) and harvest index were measured in addition to traits in experiment 1.To compare the effects of *HvNAM1* and *HvNAM2* on malt barley agronomics, we used the previously performed screening results for *HvNAM1*, described in Alptekin et al., 2020. The genotyping information of *HvNAM1* and *HvNAM2* for all varieties and lines used in this study is provided in Appendix C: Table S2.

Statistical Analysis

In this study, statistical analysis of agronomic data was performed with R software (v.3.5.3) (R Development Core Team, 2017) with the method described in Alptekin et al., 2020. Experiment 1 from Alptekin et al., 2020 was treated as a randomized incomplete block design with 2-level irrigation while experiment 2 was considered as randomized complete block design with 2-level irrigation and fertilizer treatments. In both experiments, the genetic relationship of the different lines was taken

into consideration with the method described in Alptekin et al., 2020. An Analysis of Variance (ANOVA) was performed for each trait separately using the R package lme4qtl (Ziyatdinov et al., 2018). A linear mixed model was used to identify the contribution of allelic combinations × irrigation (Experiment 1) and allelic combinations × irrigation × fertilizer (Experiment 2) as fixed factors where allelic combinations were defined with alleles of *HvNAM1* and *HvNAM2*. Different allelic groups and treatments were compared using the Wilcoxon test, with a significance cut-off value of $p < 0.05$. Multiple testing corrections were not performed.

Results

Characterization of the *HvNAM2* Gene and Marker Design

It was previously reported that the *HvNAM2* gene consists of three exons and two introns (Cai et al., 2013). Those authors identified several SNPs located in both introns, exon 2, and exon 3 between domesticated and wild barley varieties, but varieties 'Karl' and 'Lewis' were not included in their analyses. Aiming to design a molecular marker for *HvNAM2* which allows the differentiation of alleles inherited from these genotypes, we first sequenced *HvNAM2* in both varieties. Our analysis identified 3 SNPs, namely C307G, A798C, and T979G, using the start codon as a reference point. SNPs located in the 307th and 798th positions are intronic, while the difference in position 979 (ccT in 'Karl'; ccG in 'Lewis') is silent, coding for the amino acid proline in both instances. We also sequenced the 5'- and 3'-UTR regions of *HvNAM2* and found them to be highly similar.

The SNP in position 307 was used for the development of a molecular marker (Figure 3.1). PCR primers covering this region were designed (Appendix C: Table S1); digestion of amplification products with BtsCI yielded two bands (~100 and ~300 bp) in var. 'Karl', while the amplification product of var. 'Lewis' was not digested (Figure 3.1b). PCR also yielded an additional product; nevertheless, observed size differences clearly allowed the differentiation of 'Karl' and 'Lewis' *HvNAM2* alleles (Figure 3.1c). The new marker, together with previously described markers allowing the differentiation of 'Karl' and 'Lewis' *HvNAM1* alleles (Distelfeld et al., 2008) was used for genotyping the plant material described in Alptekin et al. (2020).

The Allelic State of *HvNAM2* Influences Plant Development

It is known that the 'Karl' *HvNAM1* allele causes a later maturation from previous studies of our group (Distelfeld, Avni, & Fischer, 2008, Jukanti & Fischer, 2008; Lacerenza, Parrott, & Fischer, 2010). Alptekin et al., 2020 also showed that plants carrying 'Karl' allele of *HvNAM1* mature ~2 days later (Adapted data from Alptekin et al., 2020 is shown in Appendix C: Table S3a). Here, data from experiment 2 suggest that 'Karl' allele of *HvNAM2* was also associated with ~1-day delay in maturation (Appendix C: Table S3a). This delay in maturation is more obvious when both genes have 'Karl' allele; however, observed differences between allelic groups were not statistically significant based on Wilcoxon pair-wise comparisons (Appendix C: Table S3b). The statistical modeling of maturation data also suggested *HvNAM1* as the only important gene for this trait. Thus, it is not clear from our observations whether *HvNAM2* delays the plant senescence.

Interestingly, ‘Karl’ allele of the *HvNAM2* gene was associated with earlier heading in experiment 1 and earlier anthesis in experiment 2. Plants carrying ‘Lewis’ allele for *HvNAM2* had an average heading date of 188.5 ± 1.9 while those with ‘Karl’ allele headed ~1 day earlier (187.3 ± 2.2, Figure 3.2a). Anthesis date data from experiment 2 also agreed with these results (Appendix C: Table S3a). Plants combining ‘Karl’ alleles for both *HvNAC*s showed earlier heading compared to other groups (Table 3.1). Statistical modeling of this trait in experiment 1 suggested that *HvNAM2* alone contributes to the variation in heading date (Table 3.2). However, the model from experiment 2 suggested that both genes contribute to the variation in anthesis date (Appendix C: Table S3c). Our earlier study (Alptekin et al., 2020) showed that *HvGR-RBP1* which is linked to *HvNAM1* on 6H-GPC QTL is associated with flowering-time control. Based on Alptekin et al. (2020), plants carrying *HvGR-RBP1* ‘Lewis’ allele headed 1.5 days earlier compared to those with ‘Karl’ allele. Therefore, the model suggestion from experiment 2 might be confounded. In order to confirm the effect of *HvNAM2* gene on heading date, we compared the plants carrying LKL vs LKK genotypes where alleles show *HvGR-RBP1*, *HvNAM1* and *HvNAM2* respectively (Table 3.3). Our previous study suggested the ‘Lewis’ allele for *HvGR-RBP1* was promoting earlier heading (Alptekin et al., 2020). Here, data from Table 3.3 shows that plants carrying ‘Karl’ allele for *HvNAM2* (LKK) head ~1.5 days earlier than LLK background. Thus, this confirms that *HvNAM2* ‘Karl’ allele has a significant effect on heading date.

The grain filling duration data from experiment 2 suggested that both *NAC* genes affect this trait (Appendix C: Table S3). When both alleles were 'Karl', plants have ~3 days longer grain filling compared to other groups.

Our analysis also suggests that plants carrying the 'Karl' allele for both *NAC* genes tend to be shorter (Table 3.1 and Table 3.2). In experiment 1, plants carrying *HvNAM1* 'Karl' allele were 67.0 ± 11.3 cm in height on average while those with the 'Lewis' allele were 69.1 ± 11.7 cm; however, the difference was not statistically significant based on Wilcoxon test ($p<0.05$). Plants carrying *HvNAM2* 'Karl' allele showed 64.9 ± 9.9 cm average height while those with the 'Lewis' allele were 68.4 ± 11.8 in Experiment 1. The variation arising from the different alleles of *HvNAM2* were statistically significant based on Wilcoxon test ($p< 0.05$). Statistical modelling of height trait showed that both NAC genes are important for height (Table 3.2), and KK genotypes (both *HvNAM1* and *HvNAM2* is 'Karl') are shorter than others (Table 3.1). Interestingly, a strong positive correlation between yield and height was observed ($R= 0.83$) (Table 3.4). This trait was also slightly correlated with heading (Table 3.4), thus; it might suggest that the locus where *HvNAM2* is located may affect heading, height and yield. Table S3b (Appendix C) shows that plants in experiment 2 with KK genotypes tillered slightly more; however, the difference was not statistically significant. No changes were observed in harvest index (Appendix C: Table S3).

The 'Karl' Allele of *HvNAM2* Causes a Decrease in Grain Protein Content

Variety 'Karl' has been used as a source of the low-GPC trait in malt barley breeding programs over the last decades (Burger et al., 1979). We have previously

demonstrated that the ‘Karl’ *HvNAM1* allele lowers GPC (Jukanti & Fischer 2008; Jukanti et al., 2008); however, the effect of the ‘Karl’ *HvNAM2* allele remained unknown. Here, our analysis from experiment 1 suggests that the ‘Karl’ *HvNAM2* allele further decreases GPC; plant lines with the ‘Karl’ allele had a GPC of 12.2 ± 0.6%, while those with the ‘Lewis’ allele averaged 12.8 ± 0.7% ($p = 0.05$ based on Wilcoxon test). Results from experiment 2 confirmed these data (Appendix C: Table S3a). Table 3.1 indicates that lines combining ‘Karl’ alleles of both NAC genes had the lowest GPC compared to alternate alleles, averaging 12.0 ± 0.5% in experiment 1. Statistical modelling of grain protein trait suggests that both genes are important for GPC (Table 3.2).

Plants with ‘Karl’ Allele of *HvNAM2* are Increased in Kernel Plumpness and Test Weight, But Slightly Decreased in Yield

Kernel plumpness is one of the most important traits for malt barley breeders due to its impact on malt extract. Here, we were able to show that selection for the ‘Karl’ *HvNAM2* allele increases kernel plumpness ~12% (Figure 3.2c). Kernel plumpness for genotypes carrying the ‘Karl’ allele was 74.5 ± 15 %, as compared to 62.8 ± 14% for the ‘Lewis’ allele. Table 3.1 also shows that plants with the ‘Karl’ *HvNAM2* allele produce a higher percentage of plump kernels, independently from the allelic state of *HvNAM1*. Statistical modeling of kernel plumpness from both experiments suggests that only *HvNAM2* contributes to the variation in kernel plumpness (Table 3.2, Appendix C: Table S3c), while pairwise comparison of lines carrying ‘Karl’ vs. ‘Lewis’ alleles suggest a contribution of the ‘Karl’ *HvNAM1* allele to this trait (Appendix C: Table S3a).

In addition to increased kernel plumpness, plants carrying the var. ‘Karl’ *HvNAM2* allele also exhibited higher test weight (Figure 3.2d). Plant lines with the ‘Karl’ allele showed a mean test weight of 67.8 ± 1.7 kg hL^{-1}, while this value was ~1 kg hL^{-1} lower in plants carrying the ‘Lewis’ allele. Statistical modeling indicates that the influence of *HvNAM2* on test weight is highly significant in both experiments (Tables 3.2 and S3c in Appendix C), but the model only suggests a significant influence of *HvNAM1* in experiment 2. Correlation analysis indicates a strong positive correlation between the percentage of plump kernels and test weight (R = 0.73) (Table 3.4).

We also observed minor effects of the studied genes on yield. In experiment 1, plants carrying the *HvNAM2* ‘Karl’ allele yielded 5622 ± 1735 kg ha^{-1} on average, while this number was 5883 ± 1785 kg ha^{-1} for genotypes with the ‘Lewis’ allele. The ~200 kg ha^{-1} was not significant. One the other hand, there was a statistically significant difference of ~400 kg ha^{-1} between *HvNAM1* ‘Karl’ and ‘Lewis’ alleles in experiment 1. Plants carrying the *HvNAM1* ‘Karl’ allele yielded 5738 ± 1765 kg ha^{-1} on average, while the ones with the ‘Lewis’ allele yielded 6107 ± 1786 kg ha^{-1}. Table 3.1 also shows that plants with ‘Karl’ alleles for both *NAC* genes yielded less compared to the other groups, and statistical modeling of yield suggested both alleles as important (Table 3.2). These results indicate that the ‘Karl’ alleles of *HvNAM1* and *HvNAM2* are associated with slightly decreased yield. These results suggest that the increases in test weight and kernel plumpness associated with the genes studied here are also accompanied by a slight decrease in yield across the tested environments.

Plants Carrying ‘Karl’ Alleles for both *HvNAM1* and *HvNAM2* Show Stable High Kernel Plumpness and Low Grain Protein Under Dryland Conditions

For this study, we collected agronomic data under varying irrigation and nitrogen fertilizer regimes; this enabled us to dissect the behavior of *HvNAM1* and *HvNAM2* under different environments. The ‘Karl’ *HvNAM2* allele stably increased kernel plumpness under both irrigated and dryland conditions (Figure 3.3a). The mean value for the ‘Karl’ allele under dryland conditions was 71.8%, as compared to 59.7% for the ‘Lewis’ allele. In contrast, we did not observe significant differences in kernel plumpness for *HvNAM1* ‘Karl’ vs. ‘Lewis’ alleles, neither under dryland nor under irrigated conditions (Figure 3.3a). Table 3.5 also shows that plants carrying the ‘Karl’ *HvNAM2* allele have the highest kernel plumpness regardless of the environmental conditions. Plants with the ‘Karl’ *HvNAM2* allele produced >70% plump kernels under both irrigated and dryland conditions, while the other allele combinations were much less favorable (Table 3.5). These data suggest that selection for the ‘Karl’ HvNAM2 allele is highly valuable in malt barley breeding.

Figure 3.3b shows that the ‘Karl’ *HvNAM2* allele increases test weight regardless of environment in experiment 1; however, the increase is higher under irrigation conforming the *HvNAM2* irrigation* interaction shown in Table 3.2. Similar to kernel plumpness, our data do not support an influence of HvNAM1 on test weight (Figure 3.3b).

‘Lewis’ alleles for both *HvNAM1* and *HvNAM2* significantly increase GPC regardless of the environment (Figure 3.3c). The effect of the ‘Karl’ *HvNAM1* allele is

larger than that of the ’Karl’ *HvNAM2* allele (Figure 3.3c). Plants carrying the KK allele combination have the lowest GPC in both irrigated and dryland environments (Table 3.5). Our results suggest that the KK genotype has a stable phenotype in terms of GPC, kernel plumpness, and test weight under both irrigated and dryland conditions (Table 3.5). The results from experiment 2 support these findings (data not shown). Since nitrogen did not significantly affect agronomic traits in this study, it was not further analyzed (Appendix C: Table S3c).

Discussion

Whole-plant senescence is an important part of cereal development due to its effect on grain quality and yield (Distelfeld, Avni, & Fischer, 2014). The efficiency of whole-plant senescence is associated with GPC (Cai et al., 2013), grain micronutrient content (Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006), and nitrogen-use efficiency (Ishida & Makino, 2018). Each crop requires different grain characteristics based on end use. Crops used as human food or animal feed require high nutritional value with certain minimum standards of GPC and micronutrient content (Baik & Ullrich, 2008). On the other hand, grains like barley that are generally used for malting require high kernel plumpness with more extractable sugars, which are negatively correlated with GPC (Fang, Zhang, & Xue, 2019). Understanding the regulation of whole-plant senescence will therefore allow the development of genotypes with desired grain characteristics.

Work performed here has dissected the effects of different alleles of two NAC transcription factors, HvNAM1 and HvNAM2, on malt barley quality and agronomics.

Variety ‘Karl’ has been used in malt barley breeding programs since the 1980s to decrease GPC, and to improve the performance of malting genotypes (Burger et al., 1979). Molecular markers for the differentiation of *HvNAM1* ‘Karl’ and ‘Lewis’ alleles have been designed previously, and have enabled selection for the low-GPC trait associated with the ‘Karl’ allele (Distelfeld et al., 2008). Here, the new molecular marker designed to distinguish ‘Karl’ and ‘Lewis’ *HvNAM2* alleles indicates that the two *NAC* genes are associated with different agronomic and quality traits in malt barley. This finding corroborates the observations of Pearce et al. (2014) on *GPC1* and *GPC2*, wheat orthologs of *HvNAM1* and *HvNAM2*, which suggest that the two transcription factors regulate the expression of substantially different groups of genes. While our study does not focus on the molecular biology of *HvNAM1* and *HvNAM2* function, we have observed differences in plant developmental traits associated with *HvNAM1 vs. HvNAM2*, providing support for the hypothesis forwarded by Pearce et al. 2014 suggesting that *NAC* genes possess some functional divergence despite their high similarity at the DNA level.

Single-nucleotide polymorphisms (SNPs) detected between ‘Karl’ and ‘Lewis’ *HvNAM2* sequences can be compared with the previously identified SNPs in Cai et al., (2013). All three SNPs detected in our study were also identified by those authors in their population of cultivated and wild Tibetan barley genotypes (158 genotypes, 59 cultivated and 99 wild barley varieties). Cai et al. (2013) grouped their genotypes into 6 haplotypes based on variation in *HvNAM2* sequences. Interestingly, neither ‘Karl’ nor ‘Lewis’ *HvNAM2* matched any of the haplotypes reported in the previous analysis. For the SNP located in the first intron (C307G, Figure 3.1a), all genotypes reported in Cai et al. (2013)

showed ‘G’, as in ‘Lewis’. Haplotypes 2,3 and 4 possessed the ‘Lewis’ sequence (C) for the SNP located in the second intron (position 798), while haplotypes 1, 5 and 6 carried an ‘A’ in this position, identical to ‘Karl’. For the silent mutation in the third exon, haplotypes 1 and 2 carried the ‘Karl’ sequence, while haplotypes 3,4,5 and 6 were identical to ‘Lewis’. ‘Karl’ and ‘Lewis’ were also compared with the sequence variants reported in Lilja (2015) for Nordic barley varieties. All those sequences were identical to ‘Lewis’, while the reference barley sequence (var. ‘Morex’) (Mascher et al. 2017) was identical to ‘Karl’. Clearly, substantial variation exists in the *HvNAM2* gene sequence, but functional implications of variants detected both previously (Cai et al. 2013) and here will require careful molecular and biochemical characterization.

In contrast to *HvNAM1*, no study has so far addressed the effect of *HvNAM2* on plant senescence and maturation. Efforts aimed at understanding the effect of its wheat ortholog (*GPC2*) on plant senescence did not provide a clear answer (Distelfeld et al., 2012; Pearce et al., 2014). Data presented in Table S3 (Appendix C) indicate that the ‘Karl’ *HvNAM2* allele is associated with ~1-day delay in maturation and an increase in the duration of grain filling. Surprisingly, plants carrying the *HvNAM2* ‘Karl’ allele also head ~1 day earlier compared to other genotypes. A NAC transcription factor from *Arabidopsis*, *AtNAP*, showed regulatory roles in both senescence and fertility (Guo & Gan, 2006). A previous study knocking out the rice ortholog of wheat *GPC2* showed functions of that gene in pollen fertility (Distelfeld et al., 2012). Taken together, findings of our study match those mentioned in the earlier studies, and provide additional evidence for multifunctionality of NAC transcription factors in plant development. Nonetheless,

we cannot rule out the possibility that observed effects of the ‘Karl’ *HvNAM2* allele on anthesis are due to genes linked to *HvNAM2* on barley chromosome 2.

Results presented in Figure 3.2 and Tables 3.1-3.2 suggest that germplasm carrying the ‘Karl’ *HvNAM2* allele produces a higher percentage of plump kernels and higher test weight. Data shown in Table S3 (Appendix C) suggest that *HvNAM1* also influences these traits, but, ANOVA analysis of both experiments does not support an influence of *HvNAM1* on kernel plumpness (Tables 3.2 and Appendix C: Table S3). Analyzed alleles of neither *HvNAM1* nor *HvNAM2* were associated with clear effects on yield; therefore, the enhanced percentage of plump kernels is most likely due to the production of larger, but fewer kernels. Importantly, the ‘Karl’ *HvNAM2* allele provides higher kernel plumpness under dryland as well as under irrigated conditions (Figure 3). A high percentage of plump kernels and low GPC are important quality parameters which, if not met, cause rejection of malt barley with significant revenue losses for growers (American Malting Barley Association, 2019). The *HvNAM2* marker developed here ensures the selection for high kernel plumpness under varying environments, while both *HvNAM1* and *-2* contribute to the control of GPC, providing important tools for malt barley breeding and selection.

There is an extensive amount of literature showing the association of *HvNAM1* and its orthologs with GPC (Avni et al., 2013; Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006; Wang, Ren, Sun, & Sun, 2015), and a few showing an association of *HvNAM2* with this trait (Cai et al., 2013; Lilja, 2015; Östensson, 2016). Our observations regarding *HvNAM1* and *HvNAM2* effects on GPC match previous studies. The ‘Karl’

alleles for both *NAC* genes significantly decrease GPC; however, the effect of *HvNAM1* is larger (Tables 3.1 and 3.2, Figure 3.3). For *HvNAM2*, the observed effect may also be due to the increase in kernel plumpness associated with the same ('Karl') allele. Increased kernel plumpness may result in dilution of proteins thereby lowering GPC. Further studies addressing this problem need to be undertaken to fully understand the *HvNAM2* effect on this trait.

Conclusion

This study aimed to dissect the effects of the *HvNAM1* and *HvNAM2* genes on malt barley grain agronomics and quality. Our data show that the alleles present in low-GPC variety 'Karl' decrease GPC. The 'Karl' *HvNAM2* allele also significantly increases kernel plumpness and test weight, but yield is not enhanced. Overall, data presented here show that the 'Karl' alleles of both genes are highly useful for the selection of superior malting genotypes with high kernel plumpness and low grain protein.

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Declaration of Interest

The authors declare that they have no conflict of interest.

Table 3.1. Influence of *HvNAM1* and *HvNAM2* allelic combinations on agronomic and physiological traits in Experiment 1 (with 95 lines). Data represent mean values and standard deviations averaged across both location-years and treatments. Two-sided differences between the means of allelic combinations were calculated using the Wilcoxon test (P value < 0.05), and are represented with superscript letters. For allele combinations, K symbol represents 'Karl' allele, L symbol denotes the 'Lewis' allele of both *HvNAM1* and *HvNAM2*. Comparisons were only made between KK-KL and LK-LL aiming to analyze the effect of 'Karl' and Lewis' alleles in a single gene at a time.

Allelic combinations [*HvNAM1*/*HvNAM2*]	**Number of lines**	**Heading [Julian days]**	**Height [cm]**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**
KK	24	187.3 ± 2.3 ^{b}	64.8 ± 9.9 ^{b}	73.8 ± 15.7 ^{a}	67.7 ± 1.8 ^{a}	12.0 ± 0.5 ^{b}	5542 ± 1712 ^{a}
KL	53	188.4 ± 1.9 ^{a}	68.0 ± 11.7 ^{a}	62.6 ± 13.8 ^{b}	66.6 ± 1.6 ^{b}	12.6 ± 0.6 ^{a}	5824 ± 1785 ^{a}
LK	4	187.4 ± 1.7 ^{a}	65.9 ± 10.4 ^{a}	78.3 ± 9.7 ^{a}	68.4 ± 1.2 ^{a}	13.1 ± 0.5 ^{b}	6087 ± 1851 ^{a}
LL	14	188.5 ± 2.0 ^{a}	70.1 ± 12.0 ^{a}	63.6 ± 14.7 ^{b}	66.9 ± 1.9 ^{b}	13.5 ± 0.7 ^{a}	6112 ± 1784 ^{a}

Table 3.2. Analysis of Variance for fixed effects in Experiment 1 (with 95 lines). Significance of fixed effects were analyzed based on linear-mixed model. *P* values are represented by a dot (.) for $P < 0.1$, one star (*) for $P < 0.05$), two stars (**) for $P < 0.01$, or three stars (***) for $P < 0.001$.

Fixed Effect	**Heading [Julian days]**	**Height [cm]**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**
HvNAM1 allele		*			***	***
HvNAM2 allele	***	***	***	***	***	***
Irrigation	***	***	**	***	***	***
HvNAM1-HvNAM2 alleles	***	***	***	***	***	***
HvNAM1 allele * irrigation					*	
HvNAM2 allele * irrigation		**		.		
HvNAM1-HvNAM2 alleles * Irrigation		.				
Transformation	None	None	None	None	None	None

Table 3.3. Influence of *HvNAM2* allelic status in combination with *HvGR-RBP1* 'Lewis' and *HvNAM1* 'Karl' alleles on agronomic and physiological traits in Experiment 1. Data represent mean values and standard deviations averaged across both location-years and treatments. Two-sided differences between the means of allelic combinations were calculated using the Wilcoxon test (*P* value < 0.05), and are represented with superscript letters. For allele combinations, K symbol represents 'Karl' allele, L symbol denotes the 'Lewis' allele for *HvGR-RBP1*, *HvNAM1* and *HvNAM2*. Comparisons were only made between LKL and LKK aiming to analyze the effect of 'Karl' and Lewis' alleles of *HvNAM2* in *LK* background for *HvGR-RBP1* and *HvNAM1*.

Allelic combinations [HvGR-RBP1/*HvNAM1*/*HvNAM2*]	**Number of Individuals**	**Heading [Julian days]**	**Height [cm]**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**
LKL	12	188.7 ± 1.9 ^{a}	67.2 ± 11.1 ^{a}	66.2 ± 12.1 ^{b}	66.9 ± 1.4 ^{b}	12.6 ± 0.6 ^{a}	6007 ± 2044 ^{a}
LKK	23	187.2 ± 2.2 ^{b}	64.3 ± 9.4 ^{a}	74.1 ± 15.8 ^{a}	67.7 ± 1.8 ^{a}	12.0 ± 0.4 ^{b}	5524 ± 1710 ^{b}

Table 3.4. Correlation of different agronomic and physiological traits with each other in Experiment-1 (95 lines). Data represent the 'Spearman' correlation coefficients for each trait which was calculated by combining data across both location-years and treatments. Only, statistically significant correlation coefficients are represented. Correlations which did not show statistical significance are represented with 'NS' which stands for 'Not Significant'. Coefficients higher than 0.5 are written as bold to emphasize its importance.

Trait	Heading	Height	Yield	Plump	Test Weight	Protein
Heading	**…**	**0.58**	**0.51**	-0.26	-0.14	NS
Height		**…**	**0.83**	0.14	0.32	-0.22
Yield			**…**	0.28	0.46	-0.31
Plump				**…**	**0.73**	-0.36
Test Weight					**…**	-0.26
Protein						**…**

Table 3.5. Influence of *HvNAM1* and *HvNAM2* allelic combinations on agronomic and physiological traits in Experiment 1 (with 95 lines) under varying irrigation regimes. Data represent mean values and standard deviations averaged across both location-years and treatments. Two-sided differences between the means of allelic combinations were calculated using the Wilcoxon test (*P* value < 0.05) and are represented with superscript letters. For allele combinations, K symbol represents 'Karl' allele, L symbol denotes the 'Lewis' allele of both *HvNAM1* and *HvNAM2*. Comparisons were only made between different irrigation regimes of the same allelic combination aiming to understand the effect of irrigation on the trait.

	Allelic combinations	**Heading**	**Height**	**Plump kernels**	**Test weight**	**Grain protein**	**Yield**
	[*HvNAM1*/*HvNAM2*]	**[Julian days]**	**[cm]**	**[%]**	**[kg hL^{-1}]**	**[%]**	**[kg ha^{-1}]**
Irrigated	KK	188.4 ± 2.1 ^{a}	73 ± 6.1 ^{a}	76.2 ± 14.5 ^{a}	68.2 ± 1.5 ^{a}	11.9 ± 0.4 ^{b}	7090 ± 614 ^{a}
Dry	KK	186.0 ± 1.8 ^{b}	56 ± 3.3 ^{b}	71.3 ± 16.7 ^{a}	67.1 ± 1.9 ^{b}	12.2 ± 0.5 ^{a}	3891 ± 568 ^{b}
Irrigated	KL	189.5 ± 1.6 ^{a}	78.1 ± 7.5 ^{a}	65.2 ± 13.9 ^{a}	67.4 ± 1.4 ^{a}	12.4 ± 0.5 ^{b}	7469 ± 787 ^{a}
Dry	KL	187.4 ± 1.4 ^{b}	58 ± 3.8 ^{b}	60.0 ± 13.3 ^{b}	65.8 ± 1.5 ^{b}	12.9 ± 0.6 ^{a}	4178 ± 563 ^{b}
Irrigated	LK	188.9 ± 0.8 ^{a}	74.4 ± 7.3 ^{a}	81.7 ± 6.2 ^{a}	69.1 ± 0.9 ^{a}	12.7 ± 0.3 ^{b}	7797 ± 566 ^{a}
Dry	LK	186.0 ± 0.8 ^{b}	57.4 ± 3.5 ^{b}	74.9 ± 11.7 ^{a}	67.7 ± 1.2 ^{b}	13.4 ± 0.3 ^{a}	4376 ± 579 ^{b}
Irrigated	LL	189.4 ± 1.7 ^{a}	80.1 ± 7.8 ^{a}	68.3 ± 12.6 ^{a}	67.9 ± 1.6 ^{a}	13.1 ± 0.6 ^{b}	7721 ± 736 ^{a}
Dry	LL	187.7 ± 1.8 ^{b}	59.6 ± 3.5 ^{b}	58.7 ± 15.3 ^{b}	65.8 ± 1.6 ^{b}	13.9 ± 0.7 ^{a}	4443 ± 611 ^{b}

Figure 3.1. Molecular marker design for *HvNAM2* and genotyping. A) The SNP located at the 307th position in the first intron was used to design a molecular marker for differentiation of 'Karl' and 'Lewis' alleles. A restriction digest site recognized by BtsCI (TC/AG) was present in variety 'Karl' but not in 'Lewis'. B) Amplification of an ~400 bp long region covering the SNP in position 307 is shown. Digestion of the amplicon from variety 'Karl' yields two bands of ~100 and 300 bp. The 'Lewis' amplicon is not digested. C) The designed molecular marker is used for genotyping of plant material described in Alptekin et. al. 2020. A non-specific band was also amplified; however, the patterns produced from 'Karl' vs. 'Lewis' alleles are clearly distinct. Samples 1,2,3,5,6, and 7 are detected as carrying the 'Karl' allele, while 4,8 and 9 carry the 'Lewis' allele.

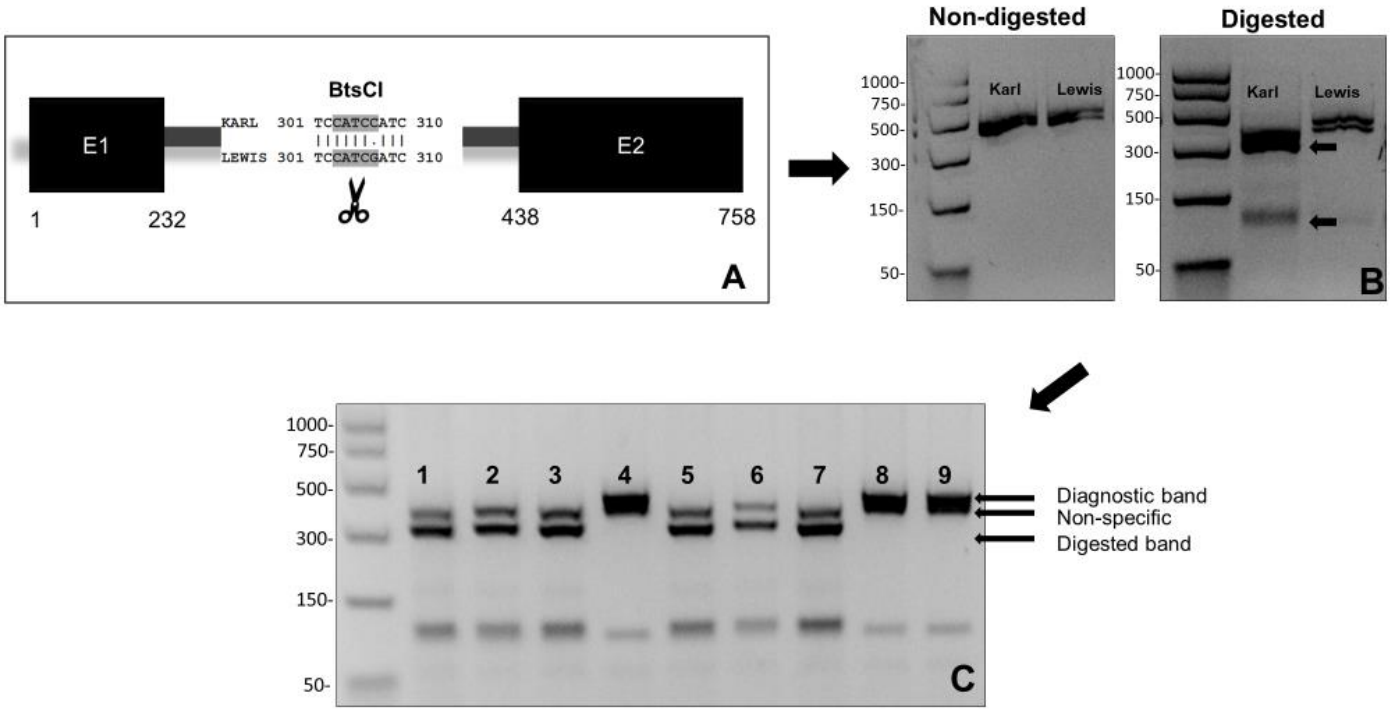


Figure 3.2. Influence of *HvNAM2* 'Karl' and 'Lewis' alleles on A) Heading Date [Julian Days] B) Grain Protein [%] C) Plump Kernels [%], and D) Test Weight [kg hL^{-1}]. K symbol represents 'Karl' allele, L symbol denotes the 'Lewis' allele of *HvNAM2* gene.

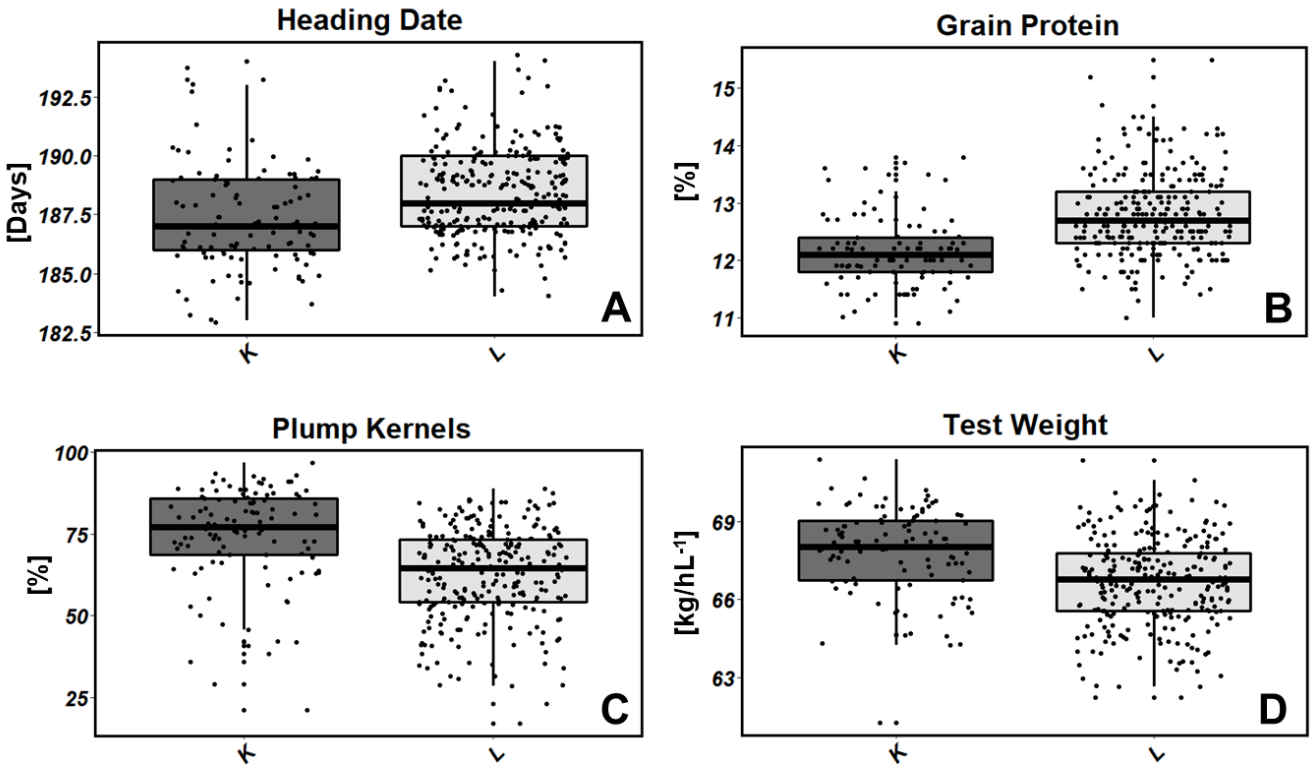
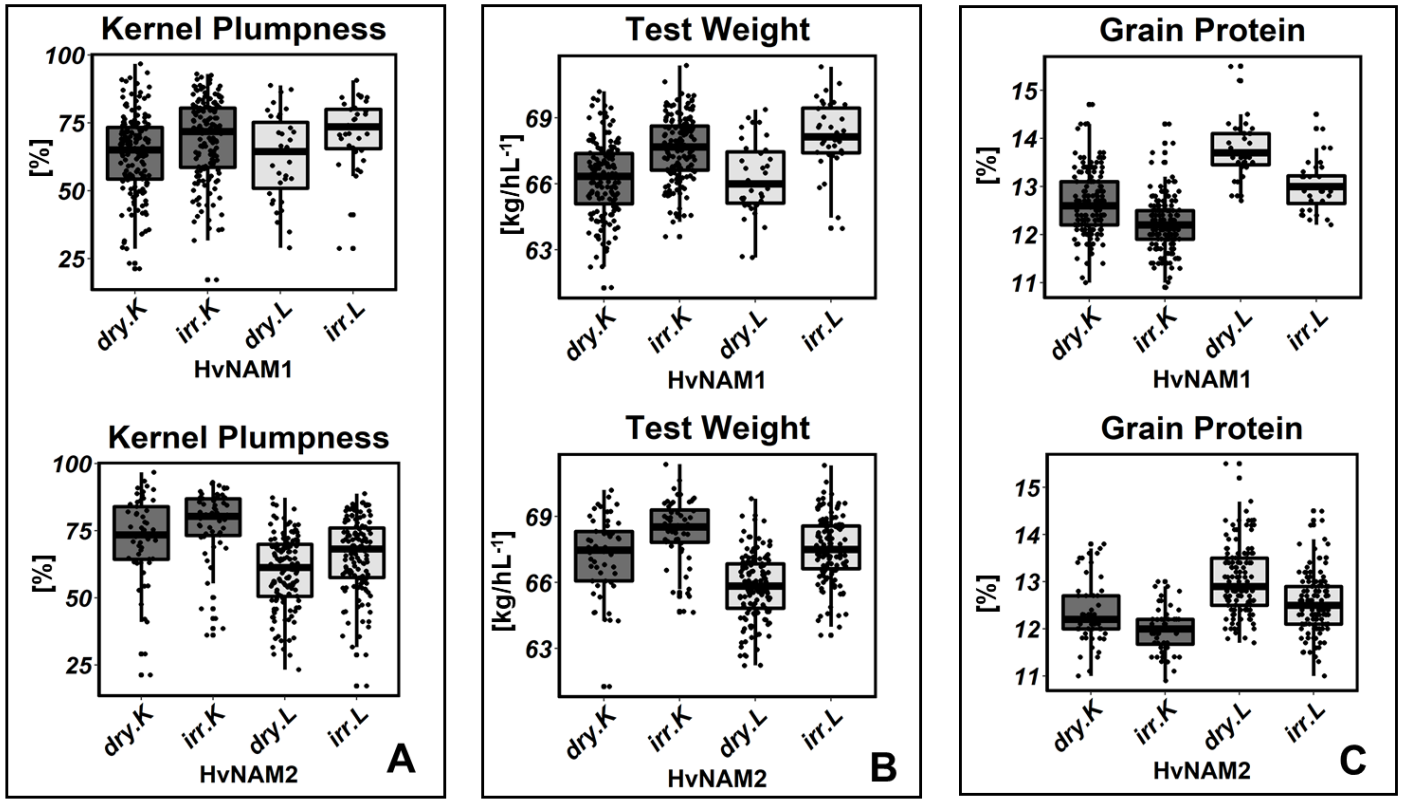


Figure 3.3. Influence of *HvNAM1* and *HvNAM2* genes on A) Grain Protein [%], B) Percentage of Plump Kernels [%] and, C) Test Weight [kg hL^{-1}] under different irrigation regimes. Symbol are used as follows: *dry.K* = 'Karl' allele under dryland, *irr.K* = 'Karl' allele under irrigation, *dry.L* = 'Lewis' allele under dryland, *irr.L* = 'Lewis' allele under irrigation. The upper half of the figure shows the effects of *HvNAM1* alleles, and the lower half shows the effect of *HvNAM2*.



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CHAPTER FOUR

ALLELIC SELECTION OF SENESCENCE AND FLOWERING TIME CONTROLLING GENES CONFERS MALT EXTRACT STABILITY

Contribution of Authors and Co-Authors

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Contributions: Performed field studies and collected malt data for Experiment 2

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Contributions: Contributed malt data for Experiment 1

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Co-Author: Jamie Sherman

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ALLELIC SELECTION OF SENESCENCE AND FLOWERING TIME CONTROLLING GENES CONFERS MALT EXTRACT STABILITY

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Abstract

Malt barley (*Hordeum vulgare L.*) is an important cash crop for farmers with stringent grain quality standards. Grain quality is influenced by management practices such as irrigation regime; however, genetic control also has strong effects. Malt barley breeders have long focused on the improvement of barley grain quality by selecting varieties possessing low grain protein and high kernel plumpness. Despite the success of classical breeding for selection of better malt varieties, unperceived molecular

mechanisms behind the desired traits stand as an obstacle for a more controlled, precise selection. Different stages of plant development such as flowering, grain filling, and senescence comprise major processes affecting the cereal grain quality and yield. Therefore, understanding the control and manifestation of these processes can streamline genotypic selection. Here, we showed in barley the effect on malt quality traits of three genes controlling whole-plant senescence and flowering time. We demonstrated that the selection of alleles for each gene can cause drastic changes in malt phenotype. The allele from 'Karl' genotype for *HvNAM1* and *HvNAM2* genes combined with 'Lewis' allele of *HvGR-RBP1* presented a ~2% increase in malt extract while improving soluble to total protein ratios and α-amylase activity. 'Karl' alleles for two *NAC* transcription factors controlling senescence also decreased malt protein content and consequently, improved wort protein and FAN values significantly. This study shows that the markers we designed for *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* can be used directly for the selection of varieties with higher malt extract and overall better malt performance. The observed genotype effects are also stable under dryland conditions suggesting that these genes might be promising for adaptation of malt barley into dryland.

Introduction

Barley, *Hordeum vulgare L.*, is an important crop grown worldwide with more than 40 million hectares of annual production (FAOSTAT, accessed at http://faostat.fao.org/site/291/default.aspx). Barley has been used as the main ingredient of alcoholic beverages such as beer and whiskey for many centuries (Homan, 2004). Today, around 20% of grown barley is used by the malt industry on a yearly basis (Baik & Ullrich, 2008; Nice et al., 2019). Even though malt barley pays high premiums for farmers and stands as a high-profit crop, its production is demanding (Chappell et al., 2017). In the U.S., the region suitable for malt barley production is mainly limited to states with dry and cool climates such as Montana and North Dakota (USDA, 2019). Even in these regions, malt barley production requires careful management practices to meet with the high standards for grain quality. Despite management efforts, only ¼ of barley grown for malt is accepted by maltsters, resulting in a substantial revenue loss for farmers (O'Donovan et al., 2008; Stevens, Sainju, Caesar-TonThat, & Iversen, 2015).

The endosperm of barley grains contains proteins (~17%), starch (~65%), and β-glucans (~4 to 9%) which are degraded during germination (Fang et al., 2019; Gupta, Abu-Ghannam, & Gallaghar, 2010). The conversion of these compounds to amino acids and extractable sugars in the course of malting is the key parameter for malt quality. This rate is directly related to the quantity of starting material in barley grains such as the amount of grain protein and available starch (Fang et al., 2019). The physical characteristics of grain size, grain width, and grain hardness also have an effect on malt quality (Gupta et al., 2010). There characteristics and barley grain composition is

determined through the complex interaction of genotype and the environment (Brouwer, Schwarz, Barr, Hayes, Murphy, & Jones, 2016; Eagles, Bedggood, Panozzo, & Martin, 1995). Differences between genotypes have significant effects on both agronomic and malt traits (Burger, Wesenberg, Carden, & Pawlisch, 1979; Elía et al., 2010). Therefore, malt barley breeders have been selecting for certain genotypes with desired grain characteristics over many years which mainly include low grain protein content and high kernel plumpness (Brouwer, Schwarz, Barr, Hayes, Murphy, Jones, et al., 2016).

Over the last decade, there has been an effort for easing the process of breeding selection. With this aim, many important Quantitative Trait Loci (QTL) controlling traits associated with grain phenotype, e.g. grain protein content (GPC), kernel size and, kernel width have been identified (Ayoub, Symons, Edney, & Mather, 2002; Fan et al., 2017; Pauli, Brown-guedira, & Blake, 2015; Stevens et al., 2015). In addition, QTL controlling malt attributes such as malt extract, wort protein, and diastatic power have been detected on multiple barley chromosomes (Elía et al., 2010; Fang, Zhang, & Xue, 2019; Pauli et al., 2015). Biological mechanisms underlying the phenotypic effect of QTL mainly remain to be understood. However, the observed grain phenotypes are associated with changes in plant development (Walker, Ford, Muñoz-Amatriaín, & Panozzo, 2013). Thus far, numerous studies suggested that genes controlling flowering time (Bingham, Blake, Foulkes, & Spink, 2007; Coventry, Barr, Eglinton, & McDonald, 2003), onset of whole-plant senescence (Distelfeld, Avni, & Fischer, 2014) and grain filling duration (Coventry et al., 2003) have an important influence on grain quality. These processes are suggested to be influencing source strength, carbohydrate deposition, starch and protein structure of

the endosperm, therefore having an impact on the malting performance of the grain (Fang et al., 2019; Walker et al., 2013). An important example of this is represented by a gene controlling whole-plant senescence, *HvNAM1*, in barley (Distelfeld et al., 2008). The discovery of this gene was initiated by the findings of See et. al. (2002) regarding a QTL for grain protein located on chromosome 6H. In the low grain protein-containing barley genotype 'Karl', the *HvNAM1* gene possess several missense mutations which can potentially affect the function of the encoded protein. It is thought that these mutations result in lower GPC because of 1) inefficient nutrient recycling in the course of senescence and/or 2) dilution of grain protein with the effect of delayed senescence (Distelfeld, Avni, & Fischer, 2014). Even though the mechanism underlying the control of *HvNAM1* on senescence process is still not completely understood, incorporation of the 'Karl' *HvNAM1* allele has conferred lower grain protein for malting varieties over the last decade (See, Kanazin, Kephart, & Blake, 2002).

QTL regions controlling grain quality and malt phenotype generally demonstrate linkage and/or pleiotropy due to the multicomponent nature of these traits (Kuczyńska, Mikołajczak, & Ćwiek, 2014; Wang et al., 2019). Therefore, dissection of QTL regions and identifying candidate genes can improve the understanding of QTL effects and facilitate/stimulate breeding selection. In the context of leaf senescence and its impact on grain quality, we have generated near-isogenic lines from a 'Karl' x 'Lewis' mapping population (See et al. 2002) to further investigate the 6H-GPC QTL (Jukanti et al., 2008). In 2010, we showed that this region controls both whole-plant senescence and flowering time (Lacerenza, Parrott, & Fischer, 2010). We later demonstrated that the flowering-

time effect of this QTL was due to the linkage of a flowering time controlling gene, *HvGR-RBP1*, while whole-plant senescence is controlled by *HvNAM1* (Alptekin et al., 2020a). In our recent study we verified that breakage of this linkage (combining *HvNAM1* 'Karl' allele with *HvGR-RBP1* 'Lewis' allele) results in an extended period of grain filling duration and elevated kernel plumpness (Alptekin et al., 2020a). Following this study, we designed molecular markers for the *HvNAM2* gene, a gene highly similar to *HvNAM1* controlling whole-plant senescence located on chromosome 2H, to dissect its allelic effect on grain protein (Alptekin et al., 2020b). Here, we investigate the effect of allelic selection for *HvGR-RBP1*, *HvNAM1* and *HvNAM2* genes on malt phenotypes to analyze 1) the influence of observed increase in kernel plumpness and decrease in grain protein content on malt phenotype; and 2) the applicability of these markers for direct selection of improved malt barley varieties. Our results show that marker assisted selection for these three genes controlling plant development can significantly improve malt phenotype by providing more control over the breeding selection process.

Materials and Methods

Plant Material and Malt Phenotyping

In this study, we re-analyzed a sub-population of plant material described in Pauli et al. (2015) to understand the impact of allelic differences in three genes on malt phenotype: two senescence-controlling NAC transcription factors (*HvNAM1* and *HvNAM2*) and a flowering-time controlling RNA-binding gene (*HvGR-RBP1*). The sub-population contained 95 elite malting lines which were a part of the population described as "malt panel" in the original study (Appendix D: Table S1) (Pauli et al., 2015). The

families used in the original study were chosen based on their superior malt performance, and some varieties were released as “advanced malt germplasm” by the Montana State University Malt Barley Breeding Program (e.g., Buzz, a variety named MT124112 in this study (Sherman, J. 2019) . The plant material was grown in 2012 at the Arthur Post Research Farm, Bozeman, MT, USA (45º40’40.78 N, 111º09’07.14 W) under irrigated conditions. Only one replicate of grown material was submitted for malt phenotyping. The full malt phenotyping was performed at the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Cereal Crops Research Unit, Madison, WI. All traits were collected based on the American Society of Brewing Chemists protocols by Pauli et. al. 2015. Traits measured in this study were the following: kernel weight (mg), kernel plumpness (% of kernels retained by a 6/64th inch / 2.38 mm sieve), grain protein (%), malt extract (%), wort protein (%), soluble to total protein ratio (%), diastatic power (°ASBC), α-amylase activity (20 dextrinizing units [°DU]), β-glucan (ppm), and free amino nitrogen (FAN) (ppm) (Pauli et al., 2015).

To investigate the effect of *gene x environment* interactions on malt phenotype, a smaller population of plant material was grown under 4 different environments: Irrigated-Normal Nitrogen, Irrigated-High Nitrogen, Dry-Normal Nitrogen, and Dry-High Nitrogen. This material is a sub-population of the above-described malt panel. It consists of 13 varieties including some parental lines (Appendix D: Table S1), and it is subsequently referred to as ‘sub-malt panel’. Plant material from the sub-malt panel was grown in two location-years, namely Bozeman-2017 (Arthur Post Research Farm, Bozeman, MT, USA; 45º40’40.78 N, 111º09’07.14 W) and Conrad 2017 (Western

Triangle Agricultural Research Station, Conrad, MT, USA; 48º18’26.05 N, 111º55’29.24 W). The plant material was grown with three replicates for each location-year and treatment, and all the treatments were subjected to full malt phenotyping. The details regarding the treatments are described in full in Alptekin et. al., 2020a.

All malt phenotyping for the sub-malt panel was conducted at the Montana State University Malt Quality Lab (http://www.montana.edu/barleybreeding/malt-quality-lab/) with the following method: The barley grains were passed over an initial screening of plumpness on a 2.18 mm (5.5/64th inch) screen. Next, all three replications from each treatment were pooled for malt phenotyping (which was 120 grams of grains) and steeped until the grain reached a 45% moisture on average. The steeping step was performed with the following regime: 10-hour steep, 18-hour rest, 6-hour steep, 10-hour rest, and 4-hour steep over 2 days at 15 ºC. Barley grains were then germinated over 96 hours at 15 ºC in containers with a 5-minute rotation every 30 min and aeration for 1 min out of every 10 min. Kilning was performed for 24 hours with a gradual increase in temperature from 60 ºC to 85 ºC, with 12 hours at 60 ºC, 6 hours at 65 ºC, 2 hours at 75 ºC, and 4 hours at 85 ºC to kill and dry the grain down to 3-5% moisture. All the steps of steeping, germinating and kilning were performed in a CLP micro-malter (SGK ‘Combi’ and SG Steep Germinator, CLP, Milton Keynes, UK). After kilning, chit was separated from malt, and the malt was ground on a mill to 0.2 mm for fine grind or 1 mm for coarse grind (DFLU Laboratory Disc Mill, Buhler Scientific, Lake Bluff, Illinois).

Subsequent to malting, the malt quality parameters were determined based on the guidelines of the American Society of Brewing Chemists protocols: Malt 4, Malt 6, Wort

17, and Wort 18 (*ASBC Methods of Analysis, Online*, 2003; *ASBC Methods of Analysis, Online*, 2010a; *ASBC Methods of Analysis, Online*, 2010b; *ASBC Methods of Analysis, Online*, 2011). Traits measured in this study were the following: malt extract (%), malt protein (%), wort protein (%), soluble to total protein ratio (%), diastatic power (°ASBC), α-amylase activity (20 dextrinizing units [°DU]), β-glucan content (ppm), and free amino nitrogen (FAN) (ppm). Malt protein (%) was measured with the grain analyzer (Foss Infratec 1241 Grain Analyzer, Foss North America, Eden Prairie, MN, USA). Soluble protein, diastatic power, α-amylase, free amino nitrogen, and β-glucan levels were measured using the Gallery Basic analyzer (Thermo Fisher Scientific, Waltham, MA) based on Fisher Scientific established protocols ("Diastatic Power," 2016; "α-amylase in malt," 2016; " β-glucan," 2016; for soluble protein "Total Protein (Biuret)," 2011; for free amino acid nitrogen "TON (Total Oxidized Nitrogen) as N and Nitrate by calculation (TON-Nitrite)," 2014). Malt extract was measured for 40 grams of grain on a fine grind dry basis for all samples using the ASBC Malt procedure**.** The density of the filtrates was measured in Degrees Plato with an Anton-Paar density meter (DMA 5000 M, Ashland, VA 23005) and Degrees Plato together with the grain moisture, was calculated with the Malt-4 Extract procedure, were then used to calculate percent malt extract. Additionally, two agronomic traits which were reported by Alptekin et al., (2020a), kernel plumpness (%) and grain protein content (%), were re-analyzed for the sub-malt panel to reports its association with malt attributes.

Genotyping for *HvGR-RBP1*, *HvNAM1* and *HvNAM2*

The plant material from both the malt and sub-malt panels was genotyped to identify the allelic states of *HvGR-RBP1* (Gene ID: HORVU6Hr1G055440), *HvNAM1* (Gene ID: HORVU6Hr1G019380), and *HvNAM2* (Gene ID: HORVU2Hr1G039640). Prior to genotyping, the DNA was isolated from all plants as previously described (Alptekin et al., 2020a) using young barley leaves. The isolated DNA was quantified with a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and diluted to 100 ng/µl for genotyping. The allelic state of *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* was determined using the methods described in Alptekin et al., 2020a (for *HvGR-RBP1* and *HvNAM1*) and Alptekin et al., 2020b (for *HvNAM2*). The 5` UTR region of *HvGR-RBP1* which reveals a ~400bp difference in length between 'Karl' and 'Lewis' genotype was amplified with PCR using GoTaq DNA polymerase (Promega, Madison, WI, USA) following the manufacturer's guidelines and analyzed using agarose gel electrophoresis assay. The allelic state of *HvNAM1* and *HvNAM2* was determined using restriction digest assays after the amplification of marker regions using the primers listed in Appendix D: Table S2. PCR products from UHB6 and UHB7 were digested with MwoI (for UHB6; New England Biolabs, Ipswich, MA, USA) and HpyCH4III (for UHB7; New England Biolabs) (Distelfeld et al. 2008) to analyze the allelic state of *HvNAM1*. The BtcSI enzyme (New England Biolabs, Ipswich, MA, USA) was used for the digestion of PCR products to determine the allelic state of the *HvNAM2* gene. The genotypes of all varieties used in this study are given in Appendix D: Table S1.

Statistical Analysis

In this study, R software (v 3.5.3) was used to perform statistical analyses of collected malt attributes (R Development Core Team, 2017). For the malt panel, one-way Analysis of Variance (ANOVA) with multi-allelic groups as a factor was used to compare malt quality and agronomic traits. The different allelic groups were statistically compared with Tukey`s HSD post-hoc test subsequent to one-way ANOVA. Assumptions of ANOVA was controlled with using R package car. The data homogeneity was tested with Levene`s Test ($p < 0.05$) while the normality of residuals was checked with Shapiro-Wilk Test ($p<0.05$). In case the assumptions of one-way ANOVA)were not met, the Kruskal-Wallis test was conducted instead. If the Kruskal-Wallis Test was used for calculating the statistical differences, the Dunn`s test was performed for post-hoc comparisons of multi-allelic groups using the R package FSA. Wilcoxon-tests were performed for pairwise comparisons with the significance cut-off value $p < 0.05$.

The statistical analysis of sub-malt panel was conducted treating it as a complete randomized design. This design included two levels of irrigation and fertilizer treatments since different blocks were pooled for malt phenotyping. A linear-mixed model was fit to the data using allele combination*irrigation*fertilizer as fixed factors. Because of the known non-independence of the varieties examined, their genetic relatedness was taken into consideration in the course of statistical analysis. With this aim, R package lme4qtl was used for conducting ANOVA which considers the genetic relatedness of used varieties as a random factor (Ziyatdinov et al., 2018). A relationship matrix built from the pedigrees of the varieties using R package synbreed with the kin function (Wimmer et al.

2012) and included in the in the model as a random factor (details described in Alptekin et al., 2020a). The R package 'fitdistrplus' was used to test the normality of model residuals (Delignette-Muller & Dutang, 2015). Data that did not fit a normal distribution were normalized using the R package 'bestNormalize' (Peterson & Cavanaugh, 2019). Pair-wise comparisons between different allelic groups were also performed with the Wilcoxon-test with the significance cut-off value $p < 0.05$.

Results

Allele Frequencies at *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* Indicate Linkage or Selection at These Loci

Aiming to understand the effect of allelic combinations in *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* on malt quality, we genotyped the plant material described in the 'Experimental' section as the malt and sub-malt panels with the markers previously designed in Alptekin et al., 2020a and 2020b. Genotyping this germplasm showed that the 'Karl' allele (represented by the letter 'K' from here on) for *HvNAM1* was present in a large fraction of the population (80 lines out of 103) (Appendix D: Table S1), which demonstrates its frequent incorporation into the breeding program. For *HvNAM2,* the 'Lewis' allele (represented by the letter 'L' from here on) was more frequent (72 lines) while the differences in the frequencies of the *HvGR-RBP1* alleles ('K' for 43 lines, 'L' for 60 lines) was not as drastic. Comparison of allelic combinations for the three genes showed that some genotypes are missing in the malt and sub-malt panels, such as KLL and KLK, where the allelic states are shown for *HvGR-RBP1*, *HvNAM1*, and HvNAM2 genes respectively. The best-represented allelic combinations were KKL and LKK with

42 and 25 lines possessing these genotypes, respectively (Appendix D: Table S1). The KKK allelic combination was only present in one line and was removed from further analyses because of low representation. This genotyping data shows that there is an imbalance in the representation of different allelic groups which may have arisen through breeding selection or linkage.

Combining the 'Lewis' allele of *HvGR-RBP1*
with Karl alleles of *HvNAM1* and *HvNAM2*
Maximizes Kernel Size, Weight and Malt
Extract

Using these genotypes, we re-analyzed the malt phenotyping data from Pauli et al., (2015). In addition to malt attributes, three agronomic traits (kernel weight, kernel plumpness and grain protein) with close relation to malt quality (Eagles, Bedggood, Panozzo, & Martin, 1995; Daba et al., 2018) were also examined. Our previous analysis (Alptekin et al., 2020a and 2020b) showed that kernel plumpness increases with the presence of the L allele for *HvGR-RBP1* and K allele for *HvNAM2* in both irrigated and dryland conditions. Here, we observe the same results in the re-analysis of the malt panel which was grown under irrigation (Table 4.1): the L allele for *HvGR-RBP1* and K allele for *HvNAM2* each individually increase kernel plumpness ~3% compared to the alternate alleles ($p = 0.05$, Wilcoxon test, two-sided). Next, we examined the changes in collected agronomic traits and malt attributes arising from different allelic combinations at *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* (Table 4.2). Plants carrying LKK and LLK allelic combinations (*HvGR-RBP1*, *HvNAM1* and *HvNAM2*, respectively) exhibited the highest kernel plumpness (Table 4.2). KKL plants overall had lower kernel plumpness compared to alternate allelic groups, and the most drastic change in kernel plumpness was observed

between KKL and LLK genotypes, with a change of 4.5% in percent plump (Table 4.2). Additionally, a statistically significant difference of 3.5 % between the mean kernel plumpness of KKL and LKK genotypes was recorded ($p < 0.01$, Dunn's test, Table 4.3).

Dissection of the allelic effects showed that increase in kernel plumpness arising from *HvGR-RBP1*^{L} and *HvNAM2*^{K} affect kernel weight. Both alleles increase the kernel weight ~2 mg (*HvGR-RBP1*: $p = 0.05$, Wilcoxon test; *HvNAM2*, $p = 0.05$, Wilcoxon test), while the *HvNAM1* gene did not have a detectable influence on this trait (Table 1). Similar to plumpness, kernels with LKK and LLK genotypes showed higher kernel weight compared to other allelic combinations (Table 2). Grains with LKK genotypes were ~2 mg heavier than KKL ($p < 0.05$, Tukey's test, Table 3).

We also observed that increases in kernel plumpness and kernel weight are associated with malt extract. The results, shown in Table 4.1, indicated that *HvNAM2*^{K} and *HvGR-RBP1*^{L} alleles individually increase malt extract ~1.2% ($p = 0.05$, Wilcoxon test) and ~0.8% ($p = 0.05$, Wilcoxon test) respectively. Data for malt extract on Table 4.2 can also be compared to kernel plumpness and weight, and plants carrying LKK genotype present the highest percentage of extract with an average of 79.2%. Comparison of LKL and LKK combinations also highlighted a ~2% difference in the extract ($p < 0.001$, Tukey's test, Table 4.3). Similar to the trend in kernel plumpness and weight, *HvNAM1* did not show any effect on malt extract (Table 4.1).

To understand the stability of genotype effect on malt extract, we analyzed the malt attributes from the sub-malt panel under different environments. Modelling of malt extract in sub-malt panel supported that the only fixed effect causing significant variation

on malt extract is the allele combination (Appendix D: Table S3c), with no detectable contribution by the irrigation and nitrogen treatments. Additionally, ANOVA analysis did not suggest any significant *gene x environment* interaction (Appendix D: Table S3c). We then analyzed individual contributions of genes on malt extract under different irrigation regimes (Figure 4.1). Under irrigation, different alleles for all of the genes behaved similarly; however, differences were observed under dryland conditions. Figure 4.1a shows that the L allele of *HvGR-RBP1* gene increases the malt extract under dryland conditions. The K alleles for *HvNAM1* and *HvNAM2* shows similar effects, but with more stability compared to *HvGR-RBP1*^{L} (Figure 4.1b, c). Despite the observed effects arising from allelic differences were not significant based on Wilcoxon test (p = 0.05), data structure presented in Figure 4.1 suggests that the presence of three alleles may improve the malt extract under dryland conditions.

HvNAM1 and *HvNAM2* 'Karl' Alleles Decrease Grain Protein Content, and Improve S/T Values

In our previous studies, we showed that the K allele for *HvNAM1* and *HvNAM2* decreases grain protein content (Alptekin et al., 2020a, 2020b), and here we observed the same effect under irrigated conditions in the malt panel. The K allele at both genes individually decrease the grain protein content 0.6% and 0.5% respectively while *HvGR-RBP1* does not show any effect on this trait (Table 4.1). Examination of the multiallelic combinations showed that grains with LKK genotype exhibit lower grain protein content compared to alternate alleles (Table 4.2) with a mean value of 12.7%. These differences between multi-allelic groups were statistically significant based on post-hoc test (Table 4.3). Assessment of post-hoc results suggested that allelic variation in both NAC genes

individually affect the grain protein. In order to lower the grain protein, Karl allele for both *HvNAM1* and *HvNAM2* seems as necessary based on comparisons of allelic groups LLL vs LKL, LLK vs LKK and LLL vs LKK (Table 4.3). Results from sub-malt panel also supported these observations and LKK genotype showed lower grain protein content relative to allelic groups KKL, LLK and LLL. Statistical modelling from sub-malt panel suggested that genotype is the only fixed effect causing significant variation on grain protein. Irrigation and nitrogen did not contribute to the variation for grain protein in the sub-malt panel based on statistical modelling of this trait.

In the sub-malt panel, malt protein data was also collected in addition to grain protein (Appendix D: Table S3). The malt protein values from this panel show that *HvNAM1* influences this trait and K allele of the gene significantly decreases malt protein ~0.6% ($p = 0.05$, Wilcoxon test) (Appendix D: Table S3a). Similar to observations from grain protein, LKK genotype exhibits a lower malt protein compared to other allelic groups (Appendix D: Table S3b). The statistical modelling of malt protein data also suggested the allelic combination as the only fixed effect contributing the variation in malt protein trait, agreeing with the observations from mean comparisons (Appendix D: Table S3a). Although the ANOVA results suggests that *HvGR-RBP1* and *HvNAM2* have a significant effect on this trait, this was not observable in the mean comparisons performed with Wilcoxon test (p value = 0.05) (Appendix D: Table S3c).

In this study, we observed that grains with the LKK genotype exhibit improvement in the in S/T values from the malt panel. Table 4.1 shows that the K allele of *HvNAM2* elevates S/T values approximately 5% compared to the L allele of this gene.

This increase is vital because S/T values must be in the range of 38% to 45% based on AMBA standards (AMBA 2019). Lines carrying the K allele for *HvNAM2* reached this 38% threshold while other lines cannot meet with the criteria. The K allele for *HvNAM2* alone also promoted a significant 5 % increase in wort protein (Wilcoxon test, p value = 0.05) that is related to changes in S/T (Table 1). In the multi-allelic comparisons, lines with the LKK allele combination exhibited the highest ratio of soluble to total protein, with an difference of 39.3 to 33.8 between LKK and KLL (Dunn's test, $p < 0.01$), suggesting that these genotypes are going through malt modification faster compared to other allele combinations (Table 4.2 and Table 4.3). Our data also show that the effect of genotypes on S/T was observable under different irrigation regimes. Figure 4.2a demonstrates that grains with the LKK genotype have a tendency for higher S/T values under dryland compared to LLL and LLK genotypes. Overall, observations from soluble to total protein ratios indicated that grains carrying the LKK genotype have a faster modification during malting.

LKK Genotypes Exhibit Characteristics of Faster and Better Malt Modification

Malt attribute values for free amino nitrogen (FAN), β-glucans, α-amylase and diastatic power (DP) suggested that different alleles of *HvGR-RBP1*, *HvNAM1* and *HvNAM2* are associated with useful changes in these traits. $HvNAM2^{K}$ and $HvGR\text{-}RBP1^{L}$ alleles significantly increase the amount of free amino acid nitrogen (FAN) in the process of malting while *HvNAM1* is does not influence this trait. ($p = 0.05$, Wilcoxon test, Table 4.1). This increase in FAN is promising because the FAN values remain in the AMBA threshold of 140 to 190 ppm (AMBA 2019). An opposite effect of alleles was observed in

the malt panel for DP where *HvNAM2*^{K} and *HvGR-RBP1*^{L} alleles significantly decrease the malt diastatic power (p = 0.05, Wilcoxon test, Table 4.1). Similar to FAN values, the decrease in DP arising from *HvNAM2*^{K} and *HvGR-RBP1*^{L} alleles was in the expected range of AMBA standards (AMBA 2019). Like for DP, β-glucan content in malt was affected by genotype, wherein *HvNAM2*^{K} and *HvGR-RBP1*^{L} alleles caused a significant decrease relative to the alternative allele (Table 4.1). α-amylase activity showed a different association with the genotypes than the other traits, and only the *HvNAM1* gene gave rise to a significant change in this trait (Table 4.1). The *HvNAM1*^{K} allele significantly increased the enzyme activity while alleles from the other genes did not result in any detectable modification.

The trend of faster and better malt modification in LKK genotypes compared to alternate allelic groups we observed in S/T values for LKK grains was also present for FAN, β-glucans, α-amylase and DP values. The LKK group exhibited the highest α-amylase activity compared to other groups. Also, these grains showed high FAN and relatively low β-glucan amounts in the malt (Table 4.2). Statistical modelling of these traits from the sub-malt panel suggested that irrigation has a significant effect on DP, FAN and α-amylase values. Different allelic groups behave differently under varying irrigation conditions for these traits (Figure 4.2). For example, all allelic groups have high DP values under irrigation (Figure 4.2b). The LKK genotype also exhibits the highest α-amylase activity under dryland. (Figure 4.2c). For FAN values, LKK allelic combination showed high FAN values both irrigated and dryland conditions. All these results suggest that genotype effect significantly improves malt barley performance under dryland

conditions. The enhanced performance of lines carrying the LKK genotype is promising for adaptation of malt barley to dryland cultivation.

Discussion

Grain quality in cereals is affected by certain agronomic parameters such as grain yield and availability of nutrients from the sink to source (Walker et al., 2013). Thus, developmental processes such as heading/flowering and whole-plant senescence are vital for determining the physical and nutritional quality of grain. In the course of whole-plant senescence, the nutrients in different plant parts such as leaves are recycled via autophagy-like mechanisms, and transferred to the kernels for deposition (Distelfeld et al., 2014). The efficiency and duration of this process are associated with parameters like grain protein content, micronutrient content, and yield in both wheat and barley (Cai et al., 2013; Distelfeld et al., 2008; Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006). Accordingly, it is not a coincidence that the major gene responsible for grain protein content in wheat and barley (*GPC-B1* / *HvNAM1*) is a transcription factor controlling the whole-plant senescence process (Distelfeld et al., 2008; Wu et al., 2013). 'Karl', a 6-row barley variety, carrying missense mutations in this gene has been used in malt barley breeding programs starting several decades ago when the its low-grain protein trait was discovered by Burger et al. in 1979 (Burger et al., 1979). In this study, we focused on 'Karl' alleles for two genes coding for senescence-regulating NAC transcription factors: *HvNAM1* and *HvNAM2*. Previous work showed that the 'Karl' alleles for *HvNAM1* and *HvNAM2* decrease the grain protein content (Alptekin et al., 2020a, 2020b), and this was the main reason for their incorporation into the Montana State University Malt Barley

Breeding Program. Here, we showed that the decrease in grain protein content is directly effective on malt protein. The decreased malt protein values are also associated with an increase in wort protein, improvement in S/T ratio, increase in FAN and DP values. There might be other additional genes linked to *HvNAM1* and *HvNAM2* which influence the nutritional composition of grain, i.e., diastatic enzyme content, and improve the malt phenotype. However, overall, we can conclude that the markers we developed in Alptekin et al., 2020a, 2020b are beneficial for the selection of varieties with less grain protein and better phenotypes for other associated traits.

Even though variety 'Karl' was useful for decreasing the grain protein content, reports were showing that this variety does not have all required qualities for malt barley. Barley grains from 'Karl' have a dark color, low diastatic power, and low-yield tendencies, hence; it was not suitable for malting by itself (Goblirsch, Horsley, & Schwarz, 1996; Weston, Horsley, Schwarz, & Goos, 1993). Despite its usefulness for decreasing the grain protein content, it is possible that variety 'Karl' transfers some alleles in the breeding process which are not beneficial for maltsters. Aiming to understand the genetic background of low grain protein trait associated with 'Karl', our group created Near-Isogenic Lines (NILs) from 'Karl' and a common 2-row malt variety 'Lewis' (Jukanti et al., 2008). The chosen NIL pair of 'Karl' namely '10_11' was backcrossed to in 'Karl' for four times (BC4Fx) making this genotype mostly 'Karl', and it carried 'Lewis' segment for 6H-QTL controlling the grain protein content. The comparative analysis of transcriptome profiles between 'Karl' and '10_11' identified several genes in the region as potential candidates for genotypic selection. In 2020, we

showed that variety ‘Karl’ is defective in an important gene controlling flowering time, *HvGR-RBP1* (Alptekin et al., 2020a). The selection of right alleles for this gene extends grain filling duration and significantly increases grain plumpness (Alptekin et al., 2020a). In this study, our results show that the ‘Lewis’ allele of the *HvGR-RBP1* gene increases kernel weight. This increase in percentage of plump kernels and kernel weight results in more malt extract in LKK genotypes. This genotype also showed substantially lower DP and β-glucan content (Table 4.2). Together, these findings indicate that increased plumpness of LKK kernels combined with extended grain filling period allow them to accumulate more starch which is directly convertible to extractable sugar yield in the malting process.

One intriguing result of this study was observations regarding the important effect of the *HvNAM2* gene on malt parameters such as malt extract, S/T, DP, α-amylase, β-glucan, and FAN. It is known that the *HvNAM2* gene influences the protein content of barley grains (Cai et al., 2013; Distelfeld et al., 2008), consequently; its impact on malt protein and S/T values is expected. In our recent study, we also showed that *HvNAM2* influences kernel plumpness in addition to its effect on grain protein content (Alptekin et al., 2020b). The *HvNAM2* gene is located in a region on chromosome 2H where many different QTLs controlling flowering time, grain size, grain weight and grain length were detected (Pauli et al., 2015; Walker et al., 2013; Wang et al., 2019). There are also several other QTLs associated with malt traits such as malt extract, β-glucan, S/T and α-amylase located in this region (Fang et al., 2019), suggesting that our HvNAM-2 marker may select favorable alleles of additional linked genes.

Interestingly, the allele providing the improvement on malt phenotypes for the *HvNAM2* gene was the ‘Karl’ allele (Table 4.1). One parental 2-row line named ‘Hockett’ also carries the K allele for *HvNAM2* (Appendix D: Table S1). This line is a popular malting barley variety with high kernel plumpness which is consistent under dryland conditions (Sherman, 2019; Varella et al., 2018). While variety ‘Karl’ was used to lower grain protein in malt barley breeding, ‘Hockett’ (genotype LLK; ’Lewis’ alleles for *HvGR-RBP1* and *HvNAM1*, ‘Karl’ allele for *HvNAM2*) was used to increase the plumpness (Varella et al., 2018). Crosses of these two varieties show ideal quality for malting, e. g., the newly released malt barley variety Buzz (Sherman, 2019) which has an LKK genotype (genotype name in this study MT124112, Appendix D: Table S1). Another popular barley variety developed by the University of Minnesota in the 1970s (‘Morex’) also carries the LLK genotype, like Hockett (Sherman, 2019). The name for the variety was derived from ‘more extract’ since it always produced ~2% more extract compared to other varieties (Rasmusson & Wilcoxson, 1979). Mapping populations of ‘Morex’ (6-row) with ‘Triumph’ (2-row) (Elía et al., 2010) and with ‘Harrington’ (2-row) (Marquez-Cedillo et al., 2000) both marked a QTL region near the *vrs1* locus on chromosome 2H associated with malt extract where the high extract allele was provided by ‘Morex.’ In the ‘Morex’ by ‘Triumph’ population, the same region on chromosome 2H was also associated with DP, grain protein content, FAN, fermentability and S/T ratios (Elía et al., 2010). Analysis from Alptekin et al., 2020b which consisted of 2-row advanced malting barley genotypes showed that the *HvNAM2* (Gene ID: HORVU2Hr1G039640) is also located near the *vrs1* locus on chromosome 2H. Data

from this study supports the previous findings regarding the association of the region on chromosome 2H with malt phenotype traits. It remains unclear whether *HvNAM2* is the main gene controlling grain characteristics or the malt attributes in this region. Nevertheless, two varieties with high kernel plumpness/kernel weight characteristics carrying the 'Karl' allele for *HvNAM2* suggest that a region around this gene may have unusual genes/alleles controlling grain size and composition in these varieties. In this study, we did not focus on the other genes in the region; however, our marker for *HvNAM2* enables for selection of high malt extract/fermentability alleles in malt barley genotypes. Further investigation of *HvNAM2* and other genes located in this region may provide more information regarding genes controlling these traits.

Conclusions

In conclusion, we were able to show that the markers we designed in our previous studies do not only improve malt barley agronomics but also enable the selection of varieties with the best malt phenotype. Many previous studies aiming to improve malt phenotype attributes focused on identification of QTLs; however, genes located in the QTL regions were not studies in detail.

Studies focusing on the investigation of candidate genes in QTLs are rare (Daba et al., 2018). This is mainly due to the complex nature of cereal genomes, and the labor-intensive nature of fine-mapping studies. Despite the challenges, the new advances in barley genomics and sequencing technologies instill hope for the future of malt barley breeding. The barley reference genome (Mascher et al., 2017) can help us to reinterpret the genome-wide association studies for improvement of both barley grain and malting

characteristics. Here, we demonstrate that a careful examination of known QTL regions such as 6H-GPC can advance the selection of better malt genotypes. The chromosome region on 2H marked with *vrs1* in previous studies (Elía et al., 2010; Marquez-Cedillo et al., 2000) also seems promising for advancing the selection process. The *HvNAM2* marker designed by our group represents a first and important step for a more detailed analysis of this region.

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Declaration of Interest

The authors declare that they have no conflict of interest.

Table 4.1. Individual effects of *HvGR-RBP1*, *HvNAM1* and *HvNAM2* genes on malt attributes. This table describes the effect of 'Karl' and 'Lewis' alleles for each gene on malt attributes in malt panel which was grown under irrigated conditions. The 'Karl' allele is represented with letter K as a superscripted, i.e. *HvGR-RBP1*^{K}, while the 'Lewis' allele is represented with letter L in the table. The mean values and standard deviations for each trait are provided here. Two-sided comparisons between the means of different alleles for each gene were performed with Wilcoxon-test with the significance cut-off value $p < 0.05$ and represented here via superscript letter if the difference was statistically significant.

	HvGR-RBP1		***HvNAM1***		***HvNAM2***	
Allele	*HvGR-RBP1*^{K}	*HvGR-RBP1*^{L}	*HvNAM1*^{K}	*HvNAM1*^{L}	*HvNAM2*^{K}	*HvNAM2*^{L}
Number of Individuals	41	53	76	18	27	67
Kernel Weight [mg]	40.7 ± 2.8 ^{b}	42.1 ± 2.8 ^{a}	41.4 ± 3.0 ^{a}	41.9 ± 2.4 ^{a}	42.9 ± 2.8 ^{a}	40.9 ± 2.7 ^{b}
Plump Percent [%]	90.7 ± 5.3 ^{a}	93.8 ± 4.1 ^{b}	92.3 ± 5.0 ^{a}	93.3 ± 4.1 ^{a}	94.6 ± 4.3 ^{a}	91.6 ± 4.9 ^{b}
Malt Extract [%]	77.4 ± 1.2 ^{b}	78.2 ± 1.9 ^{a}	77.8 ± 1.6 ^{a}	77.7 ± 1.8 ^{a}	79.0 ± 1.6 ^{a}	77.3 ± 1.4 ^{b}
Grain Protein [%]	13.2 ± 0.4 ^{a}	13.1 ± 0.6 ^{a}	13.0 ± 0.5 ^{b}	13.6 ± 0.5 ^{a}	12.8 ± 0.5 ^{a}	13.3 ± 0.5 ^{b}
Wort Protein [%]	4.3 ± 0.6 ^{a}	4.6 ± 0.7 ^{a}	4.4 ± 0.7 ^{a}	4.6 ± 0.6 ^{a}	4.8 ± 0.7 ^{a}	4.3 ± 0.6 ^{b}
S/T [%]	33.8 ± 4.9 ^{a}	36.2 ± 6.1 ^{a}	35.2 ± 5.9 ^{a}	34.8 ± 4.9 ^{a}	38.7 ± 6.3 ^{a}	33.7 ± 4.8 ^{b}
DP [°ASBC]	151.9 ± 34.2 ^{a}	135.0 ± 39.9 ^{b}	138.5 ± 38.3 ^{a}	158.7 ± 34.4 ^{a}	119.0 ± 27.6 ^{b}	151.8 ± 38.1 ^{a}
α-Amylase Activity [°DU]	72.5 ± 18.9 ^{a}	78.4 ± 18.9 ^{a}	76.4 ± 19.8 ^{a}	73.6 ± 15.5 ^{a}	86.3 ± 19.4 ^{a}	71.6 ± 17.3 ^{b}
β-Glucan [ppm]	302.0 ± 140.0 ^{a}	233.4 ± 135.9 ^{b}	263.3 ± 134.6 ^{a}	263.5 ± 170.5 ^{a}	178.5 ± 91.6 ^{b}	297.5 ± 143.7 ^{a}
FAN [ppm]	150.6 ± 41.0 ^{b}	172.8 ± 44.9 ^{a}	161.8 ± 45.8 ^{a}	168.8 ± 38.8 ^{a}	189.6 ± 45.7 ^{a}	152.4 ± 39.4 ^{b}

Table 4.2. Combined effects of *HvGR-RBP1*, *HvNAM1* and *HvNAM2* genes on malt attributes. This table describes the effect of allelic combinations for three genes on malt attributes and their statistical significance. The "Karl" allele is represented with letter K while "Lewis" allele is represented with letter L in the table. The alleles show the genotype for *HvGR-RBP1*, *HvNAM1* and *HvNAM2*, respectively. Some combinations are missing since they were not detected in population used in the malt panel. Mean values, number of represented varieties (N), standard deviations, and 95% confidence boundaries are shown for a total of 10 traits.

Allelic Group, Trait	**N**	**Mean**	**Standard Deviation**	**95% Confidence Interval for Mean**	
				Lower Bound	**Upper Bound**
Kernel Weight [mg]					
KKL	41	40.7	2.8	39.9	41.6
LKK	23	42.9	3.1	41.7	44.2
LKL	12	41.0	2.3	39.7	42.2
LLK	4	42.7	0.4	42.3	43.0
LLL	14	41.6	2.7	40.3	43.0
Plump Percent [%]					
KKL	41	90.7	5.3	89.1	92.3
LKK	23	94.5	4.6	92.5	96.1
LKL	12	93.5	2.7	92.0	94.9
LLK	4	95.3	1.6	93.9	96.7
LLL	14	92.7	4.5	90.2	94.7
Malt Extract [%]					
KKL	41	77.4	1.3	77.0	77.8
LKK	23	79.2	1.6	78.6	79.8
LKL	12	76.9	1.5	76.1	77.8
LLK	4	78.0	1.7	76.6	79.4
LLL	14	77.6	1.8	76.7	78.5
Grain Protein [%]					
KKL	41	13.2	0.4	13.1	13.3
LKK	23	12.7	0.5	12.5	12.9
LKL	12	13.1	0.5	12.8	13.4
LLK	4	13.4	0.3	13.2	13.7
LLL	14	13.7	0.6	13.4	14.0
Wort Protein [%]					
KKL	41	4.3	0.6	4.1	4.5
LKK	23	4.8	0.7	4.6	5.1
LKL	12	4.1	0.5	3.8	4.4
LLK	4	4.5	0.7	3.9	5.1
LLL	14	4.6	0.6	4.2	4.9
S/T [%]					
KKL	41	33.8	4.9	32.4	35.4
LKK	23	39.3	6.3	36.9	41.8

LKL	12	32.1	4.0	30.1	34.5
LLK	4	35.2	5.7	30.5	39.9
LLL	14	34.6	4.9	32.1	37.1
DP [°ASBC]					
KKL	41	151.9	34.3	141.0	162.0
LKK	23	117.2	27.2	106.0	128.0
LKL	12	133.7	51.3	105.0	162.0
LLK	4	129.9	31.1	99.9	149.0
LLL	14	166.9	31.6	151.0	183.0
α-Amylase Activity [°DU]					
KKL	41	72.6	18.9	67.0	78.6
LKK	23	88.4	19.2	80.4	95.9
LKL	12	66.6	13.8	59.6	74.3
LLK	4	74.4	18.7	58.3	90.4
LLL	14	73.3	15.2	65.9	81.3
β-Glucan [ppm]					
KKL	41	302.0	140.1	261.0	346.0
LKK	23	180.1	95.5	144.0	220.0
LKL	12	290.5	114.7	230.0	355.0
LLK	4	169.2	76.4	105.0	233.0
LLL	14	290.4	182.1	202.0	385.0
FAN [ppm]					
KKL	41	150.6	41.0	139.0	163.0
LKK	23	192.8	46.5	174.0	211.0
LKL	12	140.4	29.5	125.0	158.0
LLK	4	171.5	41.9	137.0	206.0
LLL	14	168.1	39.5	148.0	189.0

Table 4.3. Significantly Different Allelic Groups Based on Post-Hoc Test. Table describes the assessment of statistical significances for the differences between multi-allelic groups. Multi-group comparisons between the means of allelic combinations were performed with post-hoc tests followed by one-way ANOVA and Kruskal-Wallis test with the significance cut-off value $p < 0.05$. P-values are represented with the significance codes: $p < 0.1$ '.', $p<0.05$'*', $p<0.01$ '**', $p<0.001$'***'.

Trait	Significantly Different Allelic Groups				Statistical Test	Significance
	Allelic Group 1	**Mean of Allelic Group 1**	**Allelic Group 2**	**Mean of Allelic Group 2**		
Kernel Weight [mg]	*LKK*	42.9	*KKL*	40.7	Tukey`s HSD	*
Plump Percent [%]	*LKK*	94.5	*KKL*	90.7	Dunn`s Test	**
Malt Extract [%]	*LKK*	79.2	*KKL*	77.4	Tukey`s HSD	***
	LKL	76.9	*LKK*	79.2		***
	LLL	77.6	*LKK*	79.2		*
Grain Protein [%]	*LKK*	12.7	*KKL*	13.2	Tukey`s HSD	**
	LLL	13.7	*KKL*	13.2		**
	LLK	12.7	*LKK*	12.7		*
	LLL	13.7	*LKK*	12.7		***
	LLL	13.7	*LKL*	13.1		*
Wort Protein [%]	*LKK*	4.8	*KKL*	4.3	Dunn`s Test	*
	LKK	4.8	*LKL*	4.1		*
S/T [%]	*LKK*	39.3	*LKL*	32.1	Dunn`s Test	**
	LKK	39.3	*KKL*	33.8		**
DP	*LKK*	117.2	*LLL*	166.9	Dunn`s Test	***
	LKK	117.2	*KKL*	151.9		***
α-Amylase Activity [°ASBC]	*LKK*	88.4	*KKL*	72.6	Dunn`s Test	*
	LKK	88.4	*LKL*	66.6		*
β-Glucan [ppm]	*LKK*	180.1	*KKL*	302.0	Dunn`s Test	**
	LKK	180.1	*LKL*	290.5		.
FAN [ppm]	*LKK*	192.8	*KKL*	150.6	Dunn`s Test	**
	LKK	192.8	*LKL*	140.4		**

Figure 4.1. The effect of different alleles of *HvGR-RBP1* (1a), *HvNAM1* (1b) and *HvNAM2* (1c) genes on malt extract under varying irrigation treatments in sub-malt panel. The notations on x-axis represent the allele and irrigation respectively, i. e., *K.Dry* represents K alleles of the respective gene under dry land environment.

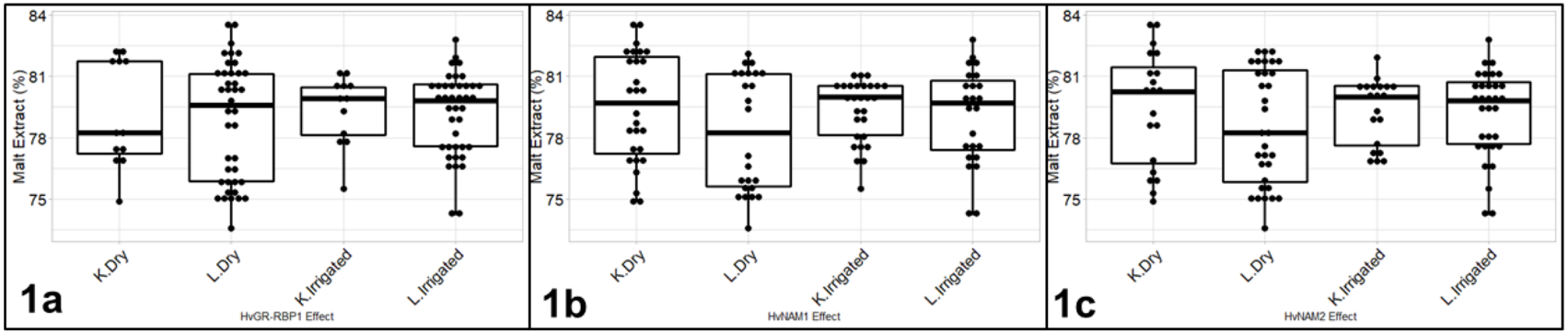
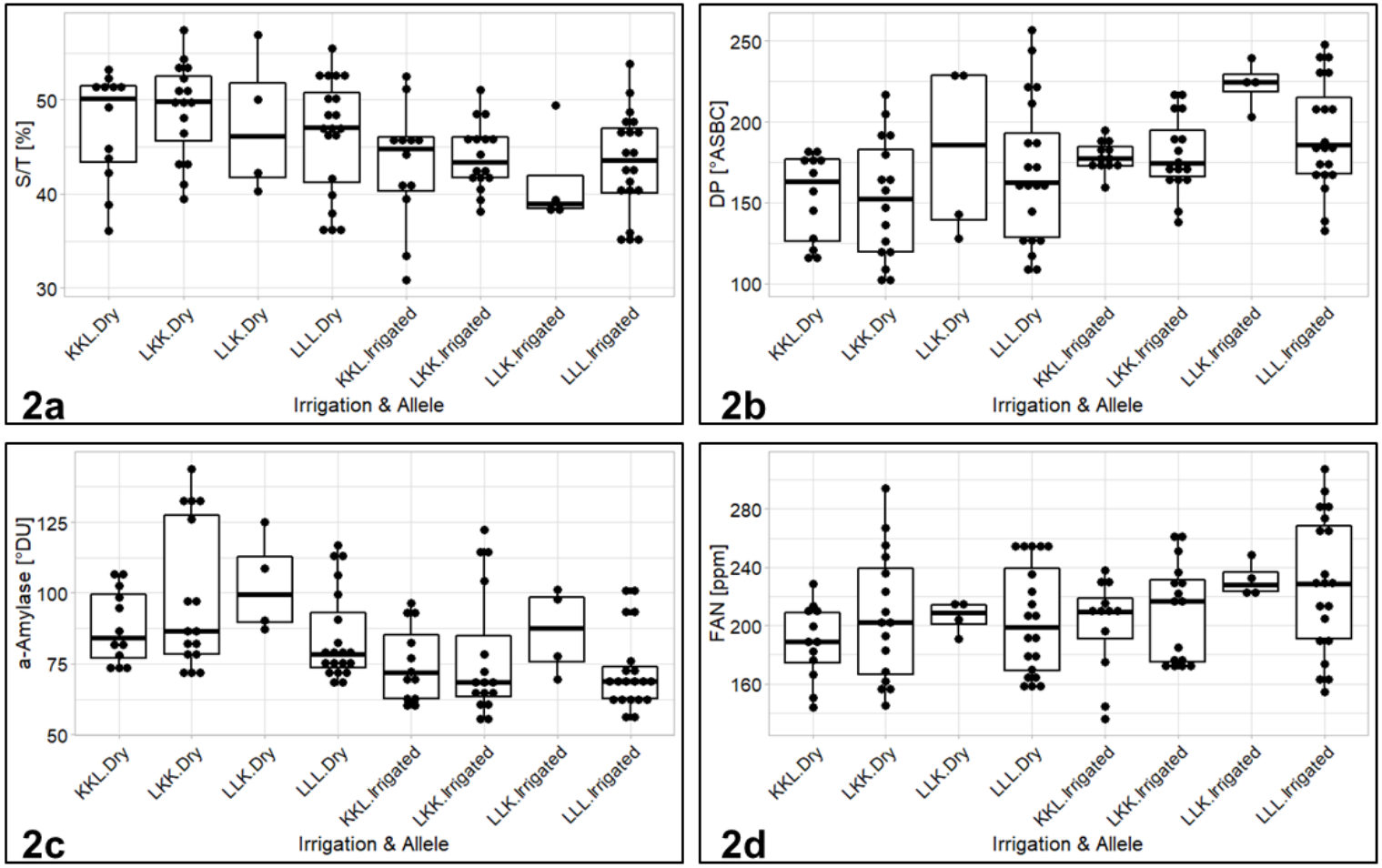


Figure 4.2. The behavior of different allelic group under irrigated and dry conditions for S/T (3a), DP (3b), α-Amylase (3c), and FAN (3d) in sub-malt panel. The alleles represent status of the *HvGR-RBP1*, *HvNAM1* and *HvNAM2* genes respectively. The notations on x-axis represent the allelic combination and irrigation respectively, i. e., *KKL.Dry* represents KKL alleles under dry land environment.



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CHAPTER FIVE

CONCLUSION and FUTURE DIRECTIONS

Conclusions

The last few decades have been an era of change and advancement for plant breeding. Studies of Quantitative Trait Loci (QTL) were one of the first steps toward revolutionizing the plant breeding process, and they substantially facilitated the selection of desired agronomic traits (Kumar et al., 2017). In the 2000s, QTL studies started to be accompanied by microarrays which provided extensive information regarding the gene expression patterns of QTL candidate genes (Jukanti et al., 2008; Golkari, Gilbert, Ban, & Procunier, 2009, p. 414). Gene expression studies brought the perspective of molecular biology to plant breeding by hinting at major mechanisms controlling agronomic traits; they also paved the way for a more targeted selection. In the last few years, massively improved DNA sequencing technologies pushed plant breeding to the era of genomics. Cheaper and higher-throughput sequencing technologies made genome sequencing possible for economically important crops with large genomes such as wheat (Appels et al., 2018) and barley (Mascher et al., 2017); resulting data are publicly available. Use of these data in combination with the knowledge from previous efforts of classical breeding offers potential for advancing plant breeding in novel ways. Understanding the molecular biology behind the desired traits will help breeders to achieve a more focused selection, allowing the generation of varieties which are more resilient to challenges arising from climate change.

Work presented here has focused on a low-protein barley variety (‘Karl’) (Wesenberg et al., 1976) which, due to its consistently low grain protein content, has been extensively used in malt barley breeding programs over the last four decades. Combining information from previous breeding studies with the current reference genome of barley, we were able to generate molecular markers that allow the selection of desired traits for malt barley. These molecular markers offer a high practical value for barley breeders who are seeking improved kernel plumpness and low grain protein content in malt barley.

This thesis has also analyzed the effect of genetic selection on attributes of the malt phenotype. We were able to show that the selection of certain alleles of genes controlling flowering and senescence timing improves the malt phenotype with increasing malt extract, improving diastatic power, α-amylase, and FAN values. Our findings show that the improvement in grain characteristics with genetic selection is directly convertible to a better malt performance. A significant increase of 1% in malt extract can lead to substantial changes in revenue for malting and beer production. Therefore, the findings of this study ensure economical gain both for barley producers and maltsters.

Besides the agronomic and economic importance, findings of this study also offer a base for molecular research analyzing the regulation of whole-plant senescence and flowering in cereals. Here, we were able to show that the flowering time controlling *earliness per se* (*EPS*) gene *HvGR-RBP1* (Streitner et al., 2008; Tripet et al., 2014) is silenced in several barley accessions collected in different parts of the world (Muñoz-

Amatriaín et al., 2014). Whether this silencing provides a regional adaptation for flowering control remains unanswered. *EPS* genes hold importance for controlling flowering time due to their independence from day length or temperature-dependent signaling, and they can offer alternative paths for improvement of grain filling duration in cereals. Further studies characterizing the details of flowering regulation by *HvGR-RBP1* and other *EPS* genes in barley should be undertaken.

One notable finding of this study was the association between different alleles of *HvNAM2*, plant developmental parameters, and grain quality. Although the molecular mechanisms governing this association remain elusive, we were able to show that the *HvNAM2* 'Karl' allele substantially improves malt barley agronomic performance. Follow-up studies will be necessary to distinguish between *HvNAM2* effects and those of linked genes. Despite a considerable number of studies carried out on *NAC* genes in cereals, particularly wheat *GPC-B1* and barley *HvNAM1* (Distelfeld et al., 2008, Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006, Pearce et al., 2014), the molecular targets of these transcription factors remain largely unknown. Identification of these targets may lead to an improved understanding of gene regulatory networks controlling cereal grain characteristics. Investigation of allelic differences in such genes will advance our knowledge regarding the source of phenotypic variation and ease the way for targeted selection.

To conclude, work presented in this thesis demonstrates how combining knowledge from classical breeding with information arising from sequencing data can improve the process of selection and facilitate the breeding process. Molecular markers

developed in this dissertation will serve as practical tools for barley breeders, saving significant amounts of time during the selection of desired traits in malt barley. Breeders will be able to use the presented methods for the generation of advanced malting genotypes that meet the demands of malt barley producers and maltsters.

Future Directions

Besides the work presented here, several lines of research were initiated but not completed due to time constraints. This work is summarized below with some suggested future directions:

Studies Regarding to *HvNAM1* Transcription Factor

In this dissertation, it was shown that variety 'Karl' *vs.* 'Lewis' alleles of the *HvNAM1* transcription factor gene are associated with substantial developmental and agronomic differences. Research published prior to this dissertation has also associated different alleles of *HvNAM1* and *GPC-B1*, the wheat ortholog of *HvNAM1*, with variation in plant maturation and consequently in grain protein and micronutrient content (Distelfeld et al., 2008, Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006, Jukanti et al., 2008). Several hypotheses have been forwarded to explain these observations (Distelfeld et al., 2014). One idea is that the missense mutations present in the 'Karl' allele of *HvNAM1* affect the protein structure and result in impairment of protein function (Distelfeld et al., 2008 and 2014). To explore this idea further, we conducted a series of computational analyses investigating the potential effect of missense mutations present in variety 'Karl'. Observations from these analyses showed that there are three important

mutations between variety ‘Karl’ and ‘Lewis’: W78C, A102P, A357T. Among these, W78C is located in the NAC domain of the protein.

Up to date, several NAC proteins have been structurally characterized (Olsen et al. 2005; Welner et al. 2012). Based on these studies, NAC proteins contain a conserved N-terminal domain with DNA-binding ability and a diverse C-terminal domain that functions as a transcription regulatory domain (Welner et al. 2012). NAC domains bind to DNA as dimers where the core recognition sequence was identified as CTG[GA] (Olsen et al. 2005). Also, the crystal structure of a NAC protein namely ANAC19 has been determined in association with DNA (Welner et al. 2012). Using the available structure from Welner et al., 2012, we computationally modeled the *HvNAM1* ‘Karl’ protein and analyzed the effect of the W78C mutation located in the NAC domain of *HvNAM1*. Modeling suggested that this mutation may substantially alter protein function. Tryptophan is an important hydrophobic amino acid which is often located in the core of the protein molecules. However, it was located on the protein surface in our analysis suggesting that it may be involved in protein-protein interactions. Considering that NAC transcription factors work as dimers to regulate gene expression, missense mutation W78C may interfere with the dimerization process of *HvNAM1* with other partners and may cause changes in target gene expression in the course of senescence. Further investigation of this mutation may provide insights regarding the molecular background for mode-of-action of the *HvNAM1* ‘Karl’ allele. This mutation might be analyzed further with the following experiments:

- The *HvNAM1* gene can be sequenced from different varieties containing differential grain protein, and mutations/SNPs present in these varieties can be characterized for their effect on protein structure.
- The DNA binding ability of the *Hv*NAM1 protein can be analyzed using two variants from 'Karl' and 'Lewis' varieties.
- The targets of the *HvNAM1* gene can be investigated in both varieties.

Studies Regarding Other Genes Located in 6H

Grain Protein QTL

QTL regions are generally large and encompass several genes. Each of these genes can be a candidate for the associated phenotype, thus; QTL regions require a careful examination in order to find which gene is responsible for the observed phenotype. The 6H-QTL associated with grain protein (See et al., 2002) also possesses several genes which are differentially expressed in the course of senescence (Jukanti et al., 2008). A few of these genes which are differentially expressed between 'Karl' and its NIL '10_11' have been described in earlier studies from our group (Jukanti et al., 2008; Tripet et al., 2014). In this dissertation, we showed that a better understanding of the *HvGR-RBP1* gene located in this region and selection of the 'Lewis' allele of this gene can substantiall improve the malt barley phenotype. Based on a preliminary analysis performed here, we detected some changes in the DNA sequence of contig 6206_s_at (coding for a leucine-rich repeat transmembrane protein kinase) (Jukanti et al., 2008) between varieties 'Karl' and 'Lewis', suggesting that this locus may contain additional agronomically interesting genes.

Studies Regarding Other Genes Located in 2H

Grain Protein QTL

See et al. (2002) described an additional grain protein QTL located on chromosome 2H; however, this QTL was not explored much compared to the 6HQTL. In this dissertation, we focused on the *HvNAM2* gene (Chapter 3), a close paralog of *HvNAM1*, located on chromosome 2H. We were able to show that the 'Karl' *HvNAM2* allele decreases grain protein 0.5-1%, in addition to the decrease arising from the 'Karl' *HvNAM1* allele. We also observed slight changes in plant maturation arising from the 'Karl' allele of *HvNAM2*. It is known that several NAC transcription factors are involved in the regulation of plant senescence (Fischer et al., 2012), thus; have a potential for improvement of malt barley characteristics. Using RNA-Seq data from 'Karl' and its NIL '10_11', we were able to detect seven other NAC transcription factors located on chromosome 2H and differentially expressed in the course of senescence. We sequenced these genes in varieties 'Karl' and 'Lewis' and detected several SNPs. Further investigation of these genes may improve our understanding of their contribution to malt barley agronomics. The detected SNPs between varieties 'Karl' and 'Lewis' can be used for the generation of molecular markers, and the effects of different alleles on malt barley agronomics can be analyzed in a similar way to Chapter 2 of this dissertation.

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APPENDICES

APPENDIX A

ADDITIONAL TABLES FOR CHAPTER TWO

Table S1. List of all primers used in this study. Primers for *HvGR-RBP1* is newly designed in this study. Primers for dissecting the allelic state of *HvNAM1* are taken from Distelfeld et al., 2008.

Primer Combination	**Primer Name**	**Primer Sequence (5`to 3`)**	**Tm (°C)**	**Expected Amplicon Length (bp)**	**Binding Site**
HvGR-RBP-1 Primer Combination-M (PM)	PM-Forward	CGATCACCTTCCTAGCGGAC	59	595 bp for Lewis, ~1000 bp for Karl	5`UTR, Marker Region
	PM-Reverse	GCTTTCCATGGGTTTCATGGG	58		
HvGR-RBP-1 Primer Combination-1 (P1)	P1-Forward	CAACATCCACACCACACCATG	59	780 bp	5` UTR
	P1-Reverse	AGGTCGGGACTTACTTGCTAT	57		
HvGR-RBP-1 Primer Combination-3 (P2)	P2-Forward	CAGAGGGCGTTATTTCTCAATCT	57	485 bp	5`UTR
	P2-Reverse	AGGTGGATATGTCTCGGTTTCG	60		
HvGR-RBP-1 Primer Combination-C (P3)	P3-Forward	ATGGCAGAGTCGGACGGCG	65	577 bp	Coding Region
	P3-Reverse	GGCAACTCCGACGGATACTGAAG	61		
HvGR-RBP-1 Primer Combination-4 (P4)	P4-Forward	GGCGCGTGCAGATCATCAACG	64	1234 bp	Coding Region + 3` UTR
	P4-Reverse	GGTGGGTGTGGGATCGAGAA	58		
HvGR-RBP-1 Primer Combination-5 (P5)	P5-Forward	TTGGAGCATCATGGCTTGGC	58	922 bp	3` UTR
	P5-Reverse	CCTTGGTGAGACCGATCAGTG	59		
HvGR-RBP-1 Primer Combination-6 (P6)	P6-Forward	GCGATTAGCGATGGCTTAAGA	56	436 bp	3` UTR
	P6-Reverse	GAGGTCCGCAGTCGCATATC	60		
HvNAM1 Primer Combination for *UHB6`*	UHB6`Reverse	CGATGAGACGGCGTACAATA	58	469 bp	Coding Region
	UHB6`Forward	GGGATCATCATCCATCAGAGA	58		
HvNAM1 Primer Combination for UHB7`	UHB7`Reverse	TTCACGCCGGATATTTGGAC	58	301 bp	Coding Region
	UHB7`Forward	CAACCCCGTTCAACTGGCT	58		

Table S2: The list of all varieties used in this research and their genotypes for *HvGR-RBP1* and *HvNAM1*.

Variety	**Pedigree**	**Allele for *HvGR-RBP*1**	**Allele for *HvNAM1***	**Allele Combination (H*vGR-RBP*, *HvNAM1*)**	**Represented Experiment**
21-7	Parent	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 2
Amsterdam	Parent	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 2
Craft	Parent	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 2
Eslick	Parent	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 2
Hockett	Parent	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 2
Lewis	Parent	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 2
MT090182	Parent	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 2
MT090190	Parent	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 2
MT124071	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1 and Experiment 2
MT124128	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1 and Experiment 2
MT124148	Craft/MT07 0174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1 and Experiment 2
MT124601	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1 and Experiment 2
MT124673	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1 and Experiment 2
MT124084	Craft/MT07 0175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124099	Craft/MT07 0175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1

MT124127	Hockett/MT 070174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124136	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124168	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124180	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124322	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124665	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124690	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124764	MT020204/ MT070174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124879	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124075	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124140	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124238	MT040073/ MT070175	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124388	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124499	Craft/MT07 0175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124555	MT040073/ MT040075	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124570	MT040073/ MT040075	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124602	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1

MT124645	MT010158/ MT070176	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124688	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124868	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124061	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124164	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124242	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124340	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124381	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124502	Craft/MT07 0175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124512	Craft/MT07 0175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124663	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124695	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124026	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124118	Hockett/MT 070174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124163	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124315	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124377	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1

MT124580	MT020204/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124649	MT010158/ MT070176	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124661	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124862	MT040073/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124901	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124001	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124009	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124073	MT010158/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124093	Craft/MT07 0175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124162	Craft/MT07 0174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124172	Craft/MT07 0174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124223	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124243	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124652	MT010158/ MT070176	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124007	MT010158/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124064	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124124	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1

MT124138	Craft/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124177	Craft/MT070174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124411	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124571	MT040073/ MT040075	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124582	MT020204/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124659	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124664	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124792	MT020204/ MT070174	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124167	Craft/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124300	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124303	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124336	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124370	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124380	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124457	MT010158/ MT070176	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124650	MT010158/ MT070176	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124677	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1

MT124025	MT010158/ MT070175	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124056	MT010158/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124080	Craft/MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124113	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124134	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124232	MT040073/ MT070175	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124289	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124331	MT020204/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124406	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124691	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124705	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124728	MT010158/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124929	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124945	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124069	MT010158/ MT070175	Lewis Type, Functional (+)	Lewis Type, Functional (+)	+/+	Experiment 1
MT124112	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124282	Hockett/MT070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1

MT124669	Hockett/MT 070174	Lewis Type, Functional (+)	Karl Type, Impaired Functioning (-)	+/-	Experiment 1
MT124696	MT040073/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1
MT124936	MT020204/ MT070175	Karl Type, Non-Functional (-)	Karl Type, Impaired Functioning (-)	-/-	Experiment 1

Table S3. The amount of fertilizer used in Experiment 2.

Year	Location	Irrigation Regime	Normal Treatment	High Nitrogen Treatment
2016	Bozeman Post Farm	Irrigated	112.0 kg/ha	168 kg/ha
		Dry	112.0 kg/ha	168 kg/ha
2017	Bozeman Post Farm	Irrigated	97.5 kg/ha	168 kg/ha
		Dry	97.5 kg/ha	168 kg/ha
2017	Conrad Western Triangle	Irrigated	112.0 kg/ha	168 kg/ha
		Dry	56.0 kg/ha	112 kg/ha

Table S4. The actual P-values for main Tables 2.2 and2.3 are provided

P-values for Table 2.2a						
Single Gene Effect	**Heading**	**Height**	**Plump kernels**	**Test weight**	**Grain protein**	**Yield**
HvGR-RBP1^{+}*vs HvGR-RBP1*$^{-}$	2.32E-02	1.00E-01	3.48E-08	3.63E-06	2.66E-01	8.10E-01
HvNAM1^{+}vs *HvNAM1*$^{-}$	4.33E-01	1.59E-01	5.81E-01	1.96E-01	< 2.20E-16	3.86E-02

P-values for Table 2.2b						
Allele Combinations (HvGR-RBP1/HvNAM1)	**Heading**	**Height**	**Plump kernels**	**Test weight**	**Grain protein**	**Yield**
+/+ and +/-	5.68E-02	1.90E-02	3.42E-02	5.03E-01	< 2.20E-16	3.56E-02
+/+ and -/-	7.77E-01	6.87E-01	6.59E-03	6.02E-03	2.95E-13	8.53E-02
+/- and -/-	3.45E-03	1.23E-02	6.23E-09	4.58E-06	6.55E-10	4.43E-01

P-values for Table 2.3a										
Single Gene Effect	**Anthesis**	**Maturity**	**Grain fill**	**Height**	**Tillers**	**Plump kernels**	**Test weight**	**Grain protein**	**Yield**	**Harvest index**
HvGR-RBP1^{+}*vs HvGR-RBP1*$^{-}$	1.72E-03	1.32E-01	4.13E-02	3.58E-03	7.09E-01	4.95E-01	4.19E-03	2.04E-02	5.32E-01	8.24E-01
HvNAM1^{+}vs *HvNAM1*$^{-}$	9.06E-01	1.82E-07	2.86E-06	1.25E-01	8.98E-01	1.47E-07	4.78E-03	< 2.20E-16	8.26E-01	4.20E-01

P-values for Table 2.3b										
Allele Combination (HvGR-RBP1/HvNAM1)	**Anthesis**	**Maturity**	**Grain fill**	**Height**	**Tillers**	**Plump kernels**	**Test weight**	**Grain protein**	**Yield**	**Harvest index**
+/+ and +/-	6.00E-02	5.11E-07	6.53E-11	9.19E-01	9.58E-01	2.50E-10	4.38E-07	2.93E-16	5.20E-01	2.84E-01
+/+ and -/-	1.19E-02	5.46E-04	5.24E-01	7.16E-03	7.65E-01	5.19E-02	3.32E-01	2.48E-08	7.05E-01	8.60E-01
+/- and -/-	1.12E-03	1.21E-01	1.35E-07	1.12E-02	6.99E-01	3.09E-05	1.45E-06	6.01E-03	4.12E-01	4.69E-01

Table S5. Analysis of agronomic and physiological traits under different treatments. Irrigation was used as the only treatment in Experiment 1 while Experiment 2 included both irrigation and fertilizer treatments. Two-sided differences between the untransformed means of different alleles/allele combination were calculated using the Wilcoxon test (P value < 0.05) and are represented with superscript letters.

Analysis of Traits Based on Their Environment							
Experiment 1		**Heading [Julian days]**	**Height [cm]**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**
Irrigation	**Irrigated**	189.2 ± 1.8 ^{a}	76.9 ± 7.6 ^{a}	69.2 ± 14.6 ^{a}	67.7 ± 1.5 ^{a}	12.4 ± 0.6 ^{a}	7424 ± 759 ^{a}
	Dry	187.0 ± 1.6 ^{b}	57.7 ± 3.8 ^{b}	63.2 ± 15.3 ^{b}	66.2 ± 1.7 ^{b}	12.9 ± 0.8 ^{b}	4156 ± 594 ^{b}

Analysis of Traits Based on Their Environment						
Experiment 2		**Heading [dap]**	**Maturity [dap]**	**Grain fill [days]**	**Height [cm]**	**Tillers [tillers m^{-1}]**
Nitrogen	**High N**	58.2 ± 3.3 ^{a}	93.7 ± 3.4 ^{a}	35.1 ± 3.4 ^{a}	73.1 ± 10.2 ^{a}	167.5 ± 35.1 ^{a}
	Low N	58.3 ± 3.3 ^{a}	93.2 ± 3.4 ^{a}	34.8 ± 3.6 ^{a}	69.2 ± 8.3 ^{b}	152.1 ± 31.4 ^{b}
Irrigation	**Irrigated**	57.9 ± 3.6 ^{b}	95.0 ± 3.5 ^{a}	36.0 ± 3.7 ^{a}	77.5 ± 7.6 ^{a}	170.9 ± 31.3 ^{a}
	Dry	58.6 ± 2.9 ^{a}	92.0 ± 2.7 ^{b}	34.0 ± 3.0 ^{b}	64.8 ± 6.5 ^{b}	148.7 ± 33.3 ^{b}

Analysis of Traits Based on Their Environment						
Experiment 2 Continued		**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**	**Harv. index**
Nitrogen	**High N**	78.7 ± 16.2 ^{a}	64.8 ± 2.3 ^{a}	12.6 ± 1.9 ^{a}	6628 ± 1511 ^{a}	0.442 ± 0.043 ^{a}
	Low N	80.2 ± 15.3 ^{a}	64.7 ± 2.7 ^{a}	12.2 ± 1.9 ^{b}	6232 ± 1574 ^{b}	0.442 ± 0.040 ^{a}
Irrigation	**Irrigated**	81.2 ± 17.0 ^{a}	66.1 ± 2.0 ^{a}	12.6 ± 1.3 ^{a}	7349 ± 1305 ^{a}	0.431 ± 0.048 ^{b}
	Dry	77.7 ± 14.2 ^{b}	63.4 ± 2.1 ^{b}	12.2 ± 2.4 ^{a}	5511 ± 1199 ^{b}	0.453 ± 0.032 ^{a}

Table S6. Analysis of the effect of allelic combinations on agronomic and physiological traits under different irrigation treatments in Experiment 1. The two-sided comparisons are provided between the different treatment of same allele (i.e. irrigated +/- vs dry +/-). Two-sided differences between the untransformed means of different alleles/allele combination were calculated using the Wilcoxon test (P value < 0.05) and are represented with superscript letters.

Experiment 1		**Allele Type**	**Heading [Julian days]**	**Height [cm]**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**
Irrigation	**Irrigated**	+/+	189.3 ± 1.6 ^{a}	78.8 ± 8.0 ^{a}	71.3 ± 12.7 ^{a}	68.2 ± 1.6 ^{a}	13.0 ± 0.5 ^{a}	7738 ± 696 ^{a}
	Dry	+/+	187.3 ± 1.8 ^{b}	59.1 ± 3.6 ^{b}	62.4 ± 15.9 ^{b}	66.2 ± 1.7 ^{b}	13.8 ± 0.6 ^{b}	4429 ± 596 ^{b}
	Irrigated	+/-	188.9 ± 2.0 ^{a}	73.8 ± 6.4 ^{a}	74.2 ± 13.7 ^{a}	68.0 ± 1.5 ^{a}	12.0 ± 0.5 ^{a}	7373 ± 740^{a}
	Dry	+/-	186.5 ± 1.7 ^{b}	56.4 ± 3.3 ^{b}	68.3 ± 15.8 ^{b}	66.8 ± 1.7 ^{b}	12.4 ± 0.6 ^{b}	3939 ± 546 ^{b}
	Irrigated	-/-	189.4 ± 1.7 ^{a}	78.8 ± 7.6 ^{a}	64.1 ± 14.5 ^{a}	67.3 ± 1.4 ^{a}	12.4 ± 0.5 ^{a}	7334 ± 775 ^{a}
	Dry	-/-	187.4 ± 1.4 ^{b}	58.1 ± 4.0 ^{b}	59.4 ± 13.6 ^{b}	65.7 ± 1.6 ^{b}	12.9 ± 0.6 ^{b}	4216 ± 577 ^{b}

Table S7. Analysis of the effect of allelic combinations on agronomic and physiological traits under different irrigation treatments in Experiment 2. The two-sided comparisons are provided between the different treatment of same allele (i.e. irrigated +/- vs dry +/-). Two-sided differences between the untransformed means of different alleles/allele combination were calculated using the Wilcoxon test (P value < 0.05) and are represented with superscript letters.

Analysis of Traits Based on Their Environment							
Experiment 2		**Allele Type**	**Heading [dap]**	**Maturity [dap]**	**Grain fill [days]**	**Height [cm]**	**Tillers [tillers m^{-1}]**
Irrigation	**Irrigated**	+/+	57.8 ± 2.8 [a]	93.8 ± 3.5 [a]	34.8 ± 2.8 [a]	77.1 ± 7.2 [a]	171.0 ± 31.1 [a]
	Dry	+/+	58.7 ± 2.2 [b]	91.0 ± 2.5 [b]	33.0 ± 2.4 [b]	63.5 ± 6.7 [b]	149.7 ± 31.7 [b]
	Irrigated	+/-	57.1 ± 4.4 [a]	96.5 ± 3.1 [a]	38.6 ± 4.3 [a]	76.1 ± 8.6 [a]	171.8 ± 32.8 [a]
	Dry	+/-	57.9 ± 3.9 [a]	92.9 ± 2.7 [b]	35.9 ± 3.1 [b]	65.1 ± 6.7 [b]	149.3 ± 33.8 [b]
	Irrigated	-/-	59.0 ± 3.6 [a]	95.4 ± 3.0 [a]	34.7 ± 2.2 [a]	80.0 ± 6.1 [a]	169.3 ± 30.6 [a]
	Dry	-/-	59.4 ± 2.6 [a]	92.6 ± 2.3 [b]	33.4 ± 2.6 [b]	67.0 ± 5.4 [b]	146.0 ± 36.4 [b]

Analysis of Traits Based on Their Environment							
Experiment 2 Continued		**Allele Type**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**	**Harv. index**
Irrigation	**Irrigated**	+/+	76.9 ± 19.3 [a]	65.8 ± 2.1 [a]	13.3 ± 1.2 [a]	7356 ± 1244 [a]	0.431 ± 0.048 [a]
	Dry	+/+	72.8 ± 14.9 [b]	63.2 ± 1.9 [b]	12.9 ± 2.5 [a]	5482 ± 1034 [b]	0.455 ± 0.037 [b]
	Irrigated	+/-	87.4 ± 11.7 [a]	66.9 ± 1.6 [a]	11.8 ± 0.9 [a]	7418 ± 1246 [a]	0.432 ± 0.045 [a]
	Dry	+/-	85.6 ± 11.2 [a]	64.6 ± 1.7 [b]	11.4 ± 2.0 [a]	5628 ± 1257 [b]	0.448 ± 0.027 [a]
	Irrigated	-/-	81.4 ± 15.5 [a]	65.8 ± 2.2 [a]	12.2 ± 1.0 [a]	7241 ± 1506 [a]	0.431 ± 0.052 [a]
	Dry	-/-	77.2 ± 11.7 [b]	62.3 ± 2.2 [b]	11.8 ± 2.2 [a]	5414 ± 1419 [b]	0.454 ± 0.026 [a]

Table S8. Analysis of the effect of allelic combinations on agronomic and physiological traits under different fertilizer treatments in Experiment 2. The two-sided comparisons are provided between the different treatment of same allele (i.e. High N +/- vs Low N +/-). Two-sided differences between the untransformed means of different alleles/allele combination were calculated using the Wilcoxon test (P value < 0.05) and are represented with superscript letters.

Analysis of Traits Based on Their Environment							
Experiment 2		**Allele Type**	**Heading [dap]**	**Maturity [dap]**	**Grain fill [days]**	**Height [cm]**	**Tillers [tillers m^{-1}]**
Nitrogen	**High N**	+/+	58.5 ± 2.7 [a]	92.6 ± 3.4 [a]	33.7 ± 2.5 [a]	72.3 ± 10.3 [a]	166.9 ± 34.8 [a]
	Low N	+/+	58.0 ± 2.4 [a]	92.1 ± 3.3 [a]	34.0 ± 3.0 [a]	68.4 ± 8.8 [b]	153.8 ± 30.1 [b]
	High N	+/-	57.3 ± 4.1 [a]	95.2 ± 3.3 [a]	37.8 ± 3.7 [a]	72.6 ± 10.4 [a]	170.2 ± 35.7 [a]
	Low N	+/-	57.7 ± 4.3 [a]	94.2 ± 3.4 [a]	36.7 ± 4.1 [a]	68.6 ± 8.1 [b]	150.8 ± 31.9 [b]
	High N	-/-	59.1 ± 3.0 [a]	94.1 ± 3.0 [a]	34.3 ± 2.1 [a]	75.1 ± 9.6 [a]	165.0 ± 35.9 [a]
	Low N	-/-	59.3 ± 3.2 [a]	94.0 ± 3.1 [a]	33.8 ± 2.9 [a]	71.8 ± 7.5 [a]	150.3 ± 33.9 [a]

Analysis of Traits Based on Their Environment							
Experiment 2 Continued		**Allele Type**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**	**Harv. index**
Nitrogen	**High N**	+/+	73.5 ± 18.0 [a]	64.4 ± 2.1 [a]	13.4 ± 2.0 [a]	6583 ± 1405 [a]	0.442 ± 0.043 [a]
	Low N	+/+	76.2 ± 16.6 [a]	64.5 ± 2.6 [a]	12.8 ± 2.0 [b]	6256 ± 1537 [a]	0.445 ± 0.046 [a]
	High N	+/-	85.9 ± 11.8 [a]	65.8 ± 1.8 [a]	11.8 ± 1.5 [a]	6710 ± 1561 [a]	0.439 ± 0.042 [a]
	Low N	+/-	87.1 ± 11.2 [a]	65.7 ± 2.2 [a]	11.4 ± 1.5 [a]	6337 ± 1502 [a]	0.441 ± 0.035 [a]
	High N	-/-	79.4 ± 13.5 [a]	64.3 ± 2.6 [a]	12.2 ± 1.6 [a]	6610 ± 1666 [a]	0.447 ± 0.048 [a]
	Low N	-/-	79.1 ± 14.3 [a]	63.8 ± 2.9 [a]	11.9 ± 1.7 [a]	6046 ± 1746 [a]	0.438 ± 0.037 [a]

Table S9. Genotyping results of *HvGR-RBP1* allelic state in USDA Barley Core Collection. The variety type and geographical location is also provided. In case this information was not available, it is represented with NA.

Variety Name	**Allele for *HvGR-RBP1***	**Variety Type**	**Geographic Location**
PI57095	Karl Type, Non-Functional (-)	Landrace	Georgia
PI447051	Karl Type, Non-Functional (-)	Breeding	Spain
PI525199	Karl Type, Non-Functional (-)	Cultivar	France
Stepford	Karl Type, Non-Functional (-)	NA	NA
PI286389	Karl Type, Non-Functional (-)	Landrace	Eritrea
CIho11789	Karl Type, Non-Functional (-)	Uncertain	Saudi Arabia
PI573682	Karl Type, Non-Functional (-)	Landrace	Georgia
PI573663	Karl Type, Non-Functional (-)	Landrace	Georgia
PI227449	Karl Type, Non-Functional (-)	Landrace	Iran
PI573668	Karl Type, Non-Functional (-)	Landrace	Georgia
PI467519	Karl Type, Non-Functional (-)	Cultivar	Germany
PI449279	Karl Type, Non-Functional (-)	Breeding	Spain
PI436154	Karl Type, Non-Functional (-)	NA	NA
PI296472	Karl Type, Non-Functional (-)	Landrace	Ethiopia
PI436136	Karl Type, Non-Functional (-)	Landrace	Chile
PI270692	Karl Type, Non-Functional (-)	Landrace	Peru
CIho14978	Karl Type, Non-Functional (-)	Landrace	Ethiopia
PI94834	Karl Type, Non-Functional (-)	Landrace	Ukraine
PI392481	Karl Type, Non-Functional (-)	Breeding	South Africa
PI264251	Karl Type, Non-Functional (-)	NA	NA
PI268255	Karl Type, Non-Functional (-)	Landrace	Iran
PI573675	Karl Type, Non-Functional (-)	Landrace	Georgia
PI506293	Lewis Type, Functional (+)	Cultivar	United Kingdom
Hays	Lewis Type, Functional (+)	NA	NA
PI327716	Lewis Type, Functional (+)	NA	NA
PI467786	Lewis Type, Functional (+)	Cultivar	Netherlands
PI447054	Lewis Type, Functional (+)	Breeding	Spain
PI494099	Lewis Type, Functional (+)	Cultivar	United States
PI436149	Lewis Type, Functional (+)	Landrace	Chile
PI436146	Lewis Type, Functional (+)	Landrace	Chile
PI498438	Lewis Type, Functional (+)	Cultivar	New Zealand
PI498437	Lewis Type, Functional (+)	Cultivar	New Zealand
PI467847	Lewis Type, Functional (+)	Cultivar	Brazil
PI95175	Lewis Type, Functional (+)	Landrace	Unknown
PI349896	Lewis Type, Functional (+)	Landrace	Serbia
PI362203	Lewis Type, Functional (+)	Cultivar	Belgium

PI467831	Lewis Type, Functional (+)	Cultivar	Poland
CIho13135	Lewis Type, Functional (+)	Cultivar	Germany
Lavina	Lewis Type, Functional (+)	NA	NA
PI371377	Lewis Type, Functional (+)	Landrace	Switzerland
CIho11588	Lewis Type, Functional (+)	Cultivar	Israel
PI321823	Lewis Type, Functional (+)	Cultivar	Czechoslovakia
PI467834	Lewis Type, Functional (+)	Cultivar	Poland
Conlon	Lewis Type, Functional (+)	NA	NA
PI328742	Lewis Type, Functional (+)	NA	NA
PI95192	Lewis Type, Functional (+)	Landrace	Unknown
PI338354	Lewis Type, Functional (+)	Cultivar	Belgium
PI422233	Lewis Type, Functional (+)	Landrace	Yemen
CIho14345	Lewis Type, Functional (+)	Landrace	Azerbaijan
PI268158	Lewis Type, Functional (+)	NA	NA
PI599621	Lewis Type, Functional (+)	Cultivar	Czech Republic
PI321854	Lewis Type, Functional (+)	Cultivar	Croatia
PI573703	Lewis Type, Functional (+)	Landrace	Georgia
CIho5440	Lewis Type, Functional (+)	Breeding	United States
PI342331	Lewis Type, Functional (+)	Landrace	Turkey
PI564502	Lewis Type, Functional (+)	Cultivar	Bolivia
PI323352	Lewis Type, Functional (+)	Cultivar	Canada
PI285636	Lewis Type, Functional (+)	Cultivar	Poland
PI414409	Lewis Type, Functional (+)	NA	NA
PI73292	Lewis Type, Functional (+)	Landrace	Armenia
PI328015	Lewis Type, Functional (+)	NA	NA
PI506299	Lewis Type, Functional (+)	Breeding	United Kingdom
PI498440	Lewis Type, Functional (+)	Cultivar	New Zealand
PI467424	Lewis Type, Functional (+)	NA	NA
PI371327	Lewis Type, Functional (+)	Landrace	Switzerland
PI268176	Lewis Type, Functional (+)	NA	NA
PI280420	Lewis Type, Functional (+)	Cultivar	United Kingdom
PI564461	Lewis Type, Functional (+)	Cultivar	Bulgaria
PI467416	Lewis Type, Functional (+)	NA	NA
PI573617	Lewis Type, Functional (+)	Cultivar	Latvia
CIho14394	Lewis Type, Functional (+)	Landrace	Armenia
PI285118	Lewis Type, Functional (+)	Landrace	Eritrea
PI95167	Lewis Type, Functional (+)	Landrace	Unknown
PI566574	Lewis Type, Functional (+)	Uncertain	China
PI388645	Lewis Type, Functional (+)	Cultivar	Ukraine
PI573659	Lewis Type, Functional (+)	Landrace	Georgia
PI371017	Lewis Type, Functional (+)	Landrace	Switzerland

PI95181	Lewis Type, Functional (+)	Landrace	Unknown
PI371248	Lewis Type, Functional (+)	Landrace	Switzerland
PI327969	Lewis Type, Functional (+)	NA	NA
PI467422	Lewis Type, Functional (+)	NA	NA
PI361038	Lewis Type, Functional (+)	NA	NA
PI190203	Lewis Type, Functional (+)	Cultivar	Germany
PI467808	Lewis Type, Functional (+)	Cultivar	Austria
PI573608	Lewis Type, Functional (+)	Cultivar	Lithuania
PI73291	Lewis Type, Functional (+)	Landrace	Armenia
PI296188	Lewis Type, Functional (+)	Cultivar	Russian Federation
PI467454	Lewis Type, Functional (+)	Uncertain	Hungary
PI268169	Lewis Type, Functional (+)	NA	NA
PI467657	Lewis Type, Functional (+)	Landrace	United Kingdom
PI290165	Lewis Type, Functional (+)	NA	NA
PI184880	Lewis Type, Functional (+)	Breeding	Sweden
PI573594	Lewis Type, Functional (+)	Cultivar	Lithuania
PI61510	Lewis Type, Functional (+)	Landrace	Armenia
PI290743	Lewis Type, Functional (+)	Cultivar	Kenya
PI315932	Lewis Type, Functional (+)	Cultivar	France
PI372081	Lewis Type, Functional (+)	NA	NA
PI60701	Lewis Type, Functional (+)	Landrace	Egypt
PI467443	Lewis Type, Functional (+)	Cultivar	Poland
PI41250	Lewis Type, Functional (+)	Uncertain	Russian Federation
PI304912	Lewis Type, Functional (+)	Cultivar	Morocco
PI350713	Lewis Type, Functional (+)	Landrace	Austria
PI467844	Lewis Type, Functional (+)	Cultivar	Brazil
PI467551	Lewis Type, Functional (+)	NA	NA
CIho14333	Lewis Type, Functional (+)	Landrace	Azerbaijan
PI330397	Lewis Type, Functional (+)	Cultivar	Czechoslovakia
PI412946	Lewis Type, Functional (+)	Uncertain	South Africa
PI564487	Lewis Type, Functional (+)	Cultivar	Germany
PI599625	Lewis Type, Functional (+)	Cultivar	Czech Republic
PI573611	Lewis Type, Functional (+)	Uncertain	Cameroon
PI321845	Lewis Type, Functional (+)	Uncertain	Slovenia
PI57015	Lewis Type, Functional (+)	Landrace	Iraq
PI473574	Lewis Type, Functional (+)	Cultivar	Canada
PI346387	Lewis Type, Functional (+)	Cultivar	Argentina
PI467839	Lewis Type, Functional (+)	Cultivar	Poland
CIho3694	Lewis Type, Functional (+)	Landrace	Egypt
PI372091	Lewis Type, Functional (+)	Cultivar	Armenia
PI283464	Lewis Type, Functional (+)	NA	NA

PI48641	Lewis Type, Functional (+)	Landrace	Iran
PI283428	Lewis Type, Functional (+)	NA	NA
PI290193	Lewis Type, Functional (+)	Cultivar	Hungary
PI498439	Lewis Type, Functional (+)	Cultivar	New Zealand
PI189764	Lewis Type, Functional (+)	Cultivar	Sweden
PI467840	Lewis Type, Functional (+)	Cultivar	Poland
PI328336	Lewis Type, Functional (+)	Landrace	Turkey
PI190200	Lewis Type, Functional (+)	NA	NA
PI467749	Lewis Type, Functional (+)	Cultivar	Netherlands
PI573660	Lewis Type, Functional (+)	Landrace	Georgia
PI392484	Lewis Type, Functional (+)	Breeding	South Africa
PI296185	Lewis Type, Functional (+)	Landrace	Yemen
PI372102	Lewis Type, Functional (+)	Cultivar	Belarus
PI268180	Lewis Type, Functional (+)	Cultivar	Czech Republic
PI485524	Lewis Type, Functional (+)	Cultivar	United Kingdom
PI73301	Lewis Type, Functional (+)	Landrace	Armenia
PI234846	Lewis Type, Functional (+)	Cultivar	Kenya
PI485536	Lewis Type, Functional (+)	Breeding	United Kingdom
PI467789	Lewis Type, Functional (+)	Cultivar	Netherlands
PI294743	Lewis Type, Functional (+)	Uncertain	Romania

APPENDIX B

SUPPLEMENTARY FILE FOR CHAPTER TWO

Supplementary File 1: The sequencing alignment of the *HvGR-RBP1* marker region is shown below. Amplicons from varieties 'Karl', 'Lewis' and '10_11' were sequenced *via* Sanger sequencing and aligned with the reference genome sequence from variety 'Morex' (Gene ID: HORVU6Hr1G055440) using the multiple sequence alignment tool 'ClustalW' (https://www.ebi.ac.uk/Tools/msa/clustalo/). The start codon was used as a reference point; thus, -1203 represents 1203 bp upstream of the start codon in the 5' UTR. The insert site is shown with both forward and reverse sequencing.

FORWARD SEQUENCING

```
Karl                                      GGTGGTGGTGTGTGATAGAAGTTGGCGTG
10_11                                     GGTGGTGGTGTGTGATAGAAGTTGGCGTG
Lewis                                     GGTGGTGGTGTGTGATAGAAGTTGGCGTG
Morex                                     GGTGGTGGTGTGTGATAGAAGTTGGCGTG   -1203
                                          ****************************

Karl       TTCAATGTGATCCTGTTGTCGCTCTCCATGACACTATTTCTAGCTATCATCAACTATGAT
10_11      TTCAATGTGATCCTGTTGTCGCTCTCCATGACACTATTTCTAGCTATCATCAACTATGAT
Lewis      TTCAATGTGATCCTGTTGTCGCTCTCCATGACACTATTTCTAGCTATCATCAACTATGAT
Morex      TTCAATGTGATCCTGTTGTCGCTCTCCATGACACTATTTCTAGCTATCATCAACTATGAT   -1143
           ************************************************************

Karl       GGTACCCTCCCCCACTACTCTACCTTCAACATCAAAGTGAAGTCCCCTCCAACATATCAA
10_11      GGTACCCTCCCCCACTACTCTACCTTCAACATCAAAGTGAAGTCCCCTCCAACATATCAA
Lewis      GGTACCCTCCCCCACTACTCTACCTTCAACATCAAAGTGAAGTCCCCTCCAACATATCAA
Morex      GGTACCCTCCCCCACTACTCTACCTTCAACATCAAAGTGAAGTCCCCTCCAACATATCAA   -1083
           ************************************************************

Karl       TCTTGCTTCCTTCTAGGAATTAGTAAAGTAAAAAATGTCTTTACATCCCTTTATGTATTT
10_11      TCTTGCTTCCTTCTAGGAATTAGTAAAGTAAAAAATGTCTTTACATCCCTTTATGTATTT
Lewis      TCTTGCTTCCTTCTAGGAATTAGTAAAGTAAAAAATGTCTTTACATCCCTTTATGTATTT
Morex      TCTTGCTTCCTTCTAGGAATTAGTAAAGTAAAAAATGTCTTTACATCCCTTTATGTATTT   -1023
           ************************************************************
```

Difference between 'Karl' and 'Lewis' starts here.

```
Karl       AAGAGGCTCCATGTTTCCCTTCTCTCCAATCCCCAAGTTGGCGATTCACAAGAGAAATAG
10_11      AAGAGGCTCCATGTTTCCCTTCTCTCCAATCCCCAAGTTGGCTCTTCAGAATATCGAGCA
Lewis      AAGAGGCTCCATGTTTCCCTTCTCTCCAATCCCCAAGTTGGCTCTTCAGAATATCGAGCA
Morex      AAGAGGCTCCATGTTTCCCTTCTCTCCAATCCCCAAGTTGGCTCTTCAGAATATCGAGCA   -963
           ****************************************  **** ** *   *

Karl       CAATGTCGGTAGTTCTGTATCTTTATTGGTAATGTTTTATAT--ATATCTCTATTCATTA
10_11      CATCAAAATTCATGCTATCTTCCATGG-CGATTATTATCTCGGTGTCGCTGAAGGCAATA
Lewis      CATCAAAATTCATGCTATCTTCCATGG-CGATTATTATCTCGGTGTCGCTGAAGGCAATA
Morex      CATCAAAATTCATGCTATCTTCCATGG-CGATTATTATCTCGGTGTCGCTGAAGGCAATA   -903
           **       *  * ** * *          * * ** * *     *  **  *  ** **

Karl       TTATTTTGATGTTTATATTAAAGCCCACCCTCAGTTTTTGTTTT-----------TCCCT
10_11      TCATCAACT---ACATTACAACTTTTTGTGTCAATTTACATGCATCAACTTGGGAACAAC
Lewis      TCATCAACT---ACATTACAACTTTTTGTGTCAATTTACATGCATCAACTTGGGAACAAC
Morex      TCATCAACT---ACATTACAACTTTTTGTGTCAATTTACATGCATCAACTTGGGAACAAC   -843
           * **          **   **         *** ***   *               *
```

```
Karl       AGCCCTTGCAAACTC-------------------------AGGACCAAAGTTAACTACT
10_11      AGACATGAGAAACTCTTTGAGCCCATTGACATTTTGCTTTCCGTGATAGCGAACCCTCCT
Lewis      AGACATGAGAAACTCTTTGAGCCCATTGACATTTTGCTTTCCGTGATAGCGAACCCTCCT
Morex      AGACATGAGAAACTCTTTGAGCCCATTGACATTTTGCTTTCCGTGATAGCGAACCCTCCT   -783
           ** * *   ******                           *    *  *    ** **

Karl       TGTGCATCTCCTGGTGTTGGAGGGGGTTGGTGTGCAAGTATTTATGGTTGAATTTTTTTT
10_11      TGAGCTTTCCATGGGTTTCATG-GGTAGTTTGAATGTGGCTTCACAATATTATCTGCTTT
Lewis      TGAGCTTTCCATGGGTTTCATG-GGTAGTTTGAATGTGGCTTCACAATATTATCTGCTTT
Morex      TGAGCTTTCCATGGGTTTCATG-GGTAGTTTGAATGTGGCTTCACAATATTATCTGCTTT   -723
           ** ** *  * ***  **   * **     **     *  ** *   *   ** *  ***
```

The sequencing signal is completely lost at this position for var. 'Karl'.

```
Karl       GGGGGGGGGGGGGGGGG-----------------------------------------
10_11      CAGAGGGCGTTATTTCTCAATCTACACTCATAGTCATGCAGTGAAGGTCTATGCATCCCC
Lewis      CAGAGGGCGTTATTTCTCAATCTACACTCATAGTCATGCAGTGAAGGTCTATGCATCCCC
Morex      CAGAGGGCGTTATTTCTCAATCTACACTCATAGTCATGCAGTGAAGGTCTATGCATCCCC   -663
             * *** *

Karl       ------------------------------------------------------------
10_11      ATGGCATGCTTGCACCAGATGGTTCTTAGCATTGATTTAAAAATAGCAGCACAATAGCCA
Lewis      ATGGCATGCTTGCACCAGATGGTTCTTAGCATTGATTTAAAAATANCAGCACAATAGCCA
Morex      ATGGCATGCTTGCACCAGATGGTTCTTAGCATTGATTTAAAAATAGCAGCACAATAGCCA   -603

Karl       ------------------------------------------------------------
10_11      GCGAACTTTCCCTGGATGACTTGGTATTTGTGAATGTTTTTATGGCAAATTCATCGAAAA
Lewis      GCGAACTTTCCCTGGATGACTTGGTATTTGTGAATGTTTTTATGGCAAATTCATCGAAAA
Morex      GCGAACTTTCCCTGGATGACTTGGTATTTGTGAATGTTTTTATGGCAAATTCATCGAAAA   -543

Karl       ------------------------------------------------------------
10_11      CATATGACGATTTTTTCGAAGACGGGTGCCAATTACTTCCGGCAGACAAGGGATTTACCG
Lewis      CATATGACGATTTTTTCNAAGACGGGTGCCAATTACTTCCGGCAGACAAGGGATTTACCG
Morex      CATATGACGATTTTTTCGAAGACGGGTGCCAATTACTTCCGGCAGACAAGGGATTTACCG   -483

Karl       ------------------------------------------------------------
10_11      TAAAAATTGCAATGTTCGCTTGAA-AGGATTGTTACCCGAACATAAATTGCCGCGAAAAA
Lewis      TAAAAATTGCAATGTTCGCTTGGAAAGGATTGTTACCCGAACATAAATTGCCGCGAAAAA
Morex      TAAAAATTGCAATGTTCGCTTGAA-AGGATTGTTACCCGAACATAAATTGCCGCGAAAAA   -423
```

REVERSE SEQUENCING

```
Karl       GATTTTTAAACGGATAAACGGTGTCACAAATTTACAAACATACNGTAAAATTGGAGTTCC
Morex      GATTTTTAAACGGATAAACGGTGTCACAAATTTACAAACATACAGTAAAATTGGAGTTCC   -360
Lewis      GATTTTTAAACGGATAAACGGTGTCACAAATTTACAAACATACAGTAAAATTGGAGTTCC
10_11      GATTTTTAAACGGATAAACGGTGTCACAAATTTACAAACATACAGTAAAATTGGAGTTCC
           ******************************************* ****************

Karl       GCTCTTTTCCTTACAGTAATGTCATTCAGAGAACGTTCCCTGGGGGACCATGGCAAACGA
Morex      GCTCTTTTCCTTACAGTAATGTCATTCAGAGAACGTTCCCTGGGGGACCAGGGCAAACGA   -420
Lewis      GCTCTTTTCCTTACAGTAATGTCATTCAGAGAACGTTCCCTGGGGGACCAGGGCAAACGA
10_11      GCTCTTTTCCTTACAGTAATGTCATTCAGAGAACGTTCCCTGGGGGACCAGGGCAAACGA
           ************************************************** *********
```

```
Karl       ACATTTTTCGCGGCAATTTATGTTCGGGTAACAATCCTTTCAAGCGAACATTGCAATTTT
Morex      ACATTTTTCGCGGCAATTTATGTTCGGGTAACAATCCTTTCAAGCGAACATTGCAATTTT   -480
Lewis      ACATTTTTCGCGGCAATTTATGTTCGGGTAACAATCCTTTCAAGCGAACATTGCAATTTT
10_11      ACATTTTTCGCGGCAATTTATGTTCGGGTAACAATCCTTTCAAGCGAACATTGCAATTTT
           ************************************************************

Karl       TACGGTAAATCCCTTGTCTGCCAGAAGTAATTGGCACCCGTCTTCGAAAAAATCGTCATA
Morex      TACGGTAAATCCCTTGTCTGCCGGAAGTAATTGGCACCCGTCTTCGAAAAAATCGTCATA   -540
Lewis      TACGGTAAATCCCTTGTCTGCCGGAAGTAATTGGCACCCGTCTTCGAAAAAATCGTCATA
10_11      TACGGTAAATCCCTTGTCTGCCGGAAGTAATTGGCACCCGTCTTCGAAAAAATCGTCATA
           ********************** *************************************

Karl       TGTTTTCGATGAATTTGCCATAAAAACATTCACAAATACCAAGTCATCCAGGGAAAGTTC
Morex      TGTTTTCGATGAATTTGCCATAAAAACATTCACAAATACCAAGTCATCCAGGGAAAGTTC   -600
Lewis      TGTTTTCGATGAATTTGCCATAAAAACATTCACAAATACCAAGTCATCCAGGGAAAGTTC
10_11      TGTTTTCGATGAATTTGCCATAAAAACATTCACAAATACCAAGTCATCCAGGGAAAGTTC
           ************************************************************

Karl       GCTGGCTATTGTGCTGCTATTTTTAAATCAATGCTAAGAACCATCTGGTGCAAGCATGCC
Morex      GCTGGCTATTGTGCTGCTATTTTTAAATCAATGCTAAGAACCATCTGGTGCAAGCATGCC   -660
Lewis      GCTGGCTATTGTGCTGCTATTTTTAAATCAATGCTAAGAACCATCTGGTGCAAGCATGCC
10_11      GCTGGCTATTGTGCTGCTATTTTTAAATCAATGCTAAGAACCATCTGGTGCAAGCATGCC
           ************************************************************

Karl       ATGGGGATGCATAGACCTTCACTGCATGACTATGAGTGTAGATTGAGAAATAACGCCCTC
Morex      ATGGGGATGCATAGACCTTCACTGCATGACTATGAGTGTAGATTGAGAAATAACGCCCTC   -720
Lewis      ATGGGGATGCATAGACCTTCACTGCATGACTATGAGTGTAGATTGAGAAATAACGCCCTC
10_11      ATGGGGATGCATAGACCTTCACTGCATGACTATGAGTGTAGATTGAGAAATAACGCCCTC
           ************************************************************

Karl       TGAAAGCAGATAATATTGTGAAGCCACATTCAAACTACCCATGAAACCCATGGAAAGCTC
Morex      TGAAAGCAGATAATATTGTGAAGCCACATTCAAACTACCCATGAAACCCATGGAAAGCTC   -780
Lewis      TGAAAGCAGATAATATTGTGAAGCCACATTCAAACTACCCATGAAACCCATGGAAAGCTC
10_11      TGAAAGCAGATAATATTGTGAAGCCACATTCAAACTACCCATGAAACCCATGGAAAGCTC
           ************************************************************

Karl       AAGGAGGGTTCGCTATCACGGAAAGCAAAATGTCAATGGGCTCAAAGAGTTTCTCATGTC
Morex      AAGGAGGGTTCGCTATCACGGAAAGCAAAATGTCAATGGGCTCAAAGAGTTTCTCATGTC   -840
Lewis      AAGGAGGGTTCGCTATCACGGAAAGCAAAATGTCAATGGGCTCAAAGAGTTTCTCATGTC
10_11      AAGGAGGGTTCGCTATCACGGAAAGCAAAATGTCAATGGGCTCAAAGAGTTTCTCATGTC
           ************************************************************

Karl       TGTTGTTCCCAAGTTGATGCATGTAAATTGACACAAAAAGTTGTAATGTAGTTGATGATA
Morex      TGTTGTTCCCAAGTTGATGCATGTAAATTGACACAAAAAGTTGTAATGTAGTTGATGATA   -900
Lewis      TGTTGTTCCCAAGTTGATGCATGTAAATTGACACAAAAAGTTGTAATGTAGTTGATGATA
10_11      TGTTGTTCCCAAGTTGATGCATGTAAATTGACACAAAAAGTTGTAATGTAGTTGATGATA
           ************************************************************

Karl       TTGCCTTCAGCGACACCGAGATAATAATCGCCATGGAAGATAGCATGAATTTTGATGTGC
Morex      TTGCCTTCAGCGACACCGAGATAATAATCGCCATGGAAGATAGCATGAATTTTGATGTGC   -960
Lewis      TTGCCTTCAGCGACACCGAGATAATAATCGCCATGGAAGATAGCATGAATTTTGATGTGC
10_11      TTGCCTTCAGCGACACCGAGATAATAATCGCCATGGAAGATAGCATGAATTTTGATGTGC
           ************************************************************
```

The sequencing signal is disrupted and lost for var. 'Karl'

```
Karl       TCGATATTCTGAAGAGNTTTCCCACTCCCCCCCCCCCCCCCAAAAAAANNTTNNCCCNNA
Morex      TCGATATTCTGAAGAGCCAACTTGGGGATT-GGAGAGAAGGGAAACATGGAGCCTCTTAA   -1019
Lewis      TCGATATTCTGAAGAGCCAACTTGGGGANTGGGAGAGAAGGGAAACATGGAGCCTCTTAA
10_11      TCGATATTCTGAAGAGCCAACTTGGGGATT-GGAGAGAAGGGAAACATGGAGCCTCTTAA
           ****************    *                    *** *        *   *

Karl       ANNNNTTGGNNNNNNNNNNNNNNNN---
Morex      ATACATAAAGGGATGTAAAGACATTTTTTACTTTACTAATTCCTAGAAGGAAGC-AAGAT   -1078
Lewis      ATACATAAAGGGATGTAAAGACATTTTTTACTTTACTAATTCCTAGAANGAAGC-AAGAT
10_11      ATACATAAAGGGATGTAAAGACATTTTTTACTTTACTAATTCCTAGAAAGGAAG-CAAGA
           *    *

Karl
Morex      TG-ATATGTTGGAGGGGACTTCACTTT
Lewis      TGATATGTTGGGAGGGGACTTCACTTT
10_11      TTGATATGTTGGAGGGGACTTCACTTT
```

APPENDIX C

SUPPLEMENTARY TABLES FOR CHAPTER THREE

Table S1. List of all primers used in this study. Primers for *HvNAM2* is newly designed in this study. Primers for dissecting the allelic state of *HvNAM1* are taken from Distelfeld et. al. 2008.

Purpose	**Primer Name**	**Primer Sequence (5`to 3`)**	**Tm (°C)**	**Expected Amplicon Length (bp)**	**Binding Site**
***HvNAM2* Sequencing**	NAM2_OF1	ACACGAGTTCATGGGCCAGCC	64	1396 bp	5` UTR
	NAM2_O1R	CAACAACCGATCGATCCCCTTCC	60		
	NAM2_C1F	GTCCGGTGGCGATCTGACACG	63	892 bp	5` UTR + Coding Region
	NAM2_C1R	CGCGCTGGCGTTCATAGGGT	64		
	NAM2_C2F	GTTCATGCGCTCGCTCGGGAT	65	1305 bp	Coding Region + 3` UTR
	NAM2_C2R	CGGAGTACCCGTGCATGAACACG	64		
	NAM2_O2F	TATCCGGCGTGAACTGGAATCCC	61	1220 bp	3` UTR
	NAM2_O2R	GGAAGGTGAAAAGGGATGCAGC	59		
Molecular Marker for *HvNAM2*	NAM2-SNP1-F	GGGCAGCTCGGACTCATCTTCCG	65	404 bp	Intronic Region
	NAM2-SNP1-R	CACTAGGACAGCACGAACAAGTA	60		
Molecular Marker for *HvNAM1*	UHB6`Reverse	CGATGAGACGGCGTACAATA	58	469 bp	Coding Region
	UHB6`Forward	GGGATCATCATCCATCAGAGA	58		
	UHB7`Reverse	TTCACGCCGGATATTTGGAC	58	301 bp	Coding Region
	UHB7`Forward	CAACCCCGTTCAACTGGCT	58		

Table S2. The list of all varieties used in this research and their genotypes for *HvNAM1* and *HvNAM2*.

Variety	**Pedigree**	**Allele for *HvNAM1***	**Allele for *HvNAM2***	**Represented Experiment**
21-7	Parent	Karl Type	Lewis Type	Experiment 2
Amsterdam	Parent	Lewis Type	Lewis Type	Experiment 2
Craft	Parent	Lewis Type	Lewis Type	Experiment 2
Eslick	Parent	Lewis Type	Lewis Type	Experiment 2
Hockett	Parent	Lewis Type	Karl Type	Experiment 2
Lewis	Parent	Lewis Type	Lewis Type	Experiment 2
MT090182	Parent	Karl Type	Karl Type	Experiment 2
MT090190	Parent	Karl Type	Karl Type	Experiment 2
MT124071	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1 and Experiment 2
MT124128	Hockett/MT070174	Karl Type	Karl Type	Experiment 1 and Experiment 2
MT124148	Craft/MT070174	Lewis Type	Lewis Type	Experiment 1 and Experiment 2
MT124601	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1 and Experiment 2
MT124673	Hockett/MT070174	Karl Type	Karl Type	Experiment 1 and Experiment 2
MT124084	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124099	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124127	Hockett/MT070174	Lewis Type	Karl Type	Experiment 1
MT124136	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124168	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124180	Craft/MT070174	Karl Type	Lewis Type	Experiment 1
MT124322	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124665	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124690	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124764	MT020204/MT070174	Lewis Type	Lewis Type	Experiment 1
MT124879	Craft/MT070174	Karl Type	Lewis Type	Experiment 1
MT124075	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124140	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124238	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124388	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124499	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124555	MT040073/MT040075	Karl Type	Lewis Type	Experiment 1
MT124570	MT040073/MT040075	Karl Type	Lewis Type	Experiment 1
MT124602	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124645	MT010158/MT070176	Lewis Type	Lewis Type	Experiment 1
MT124688	MT040073/MT070175	Karl Type	Karl Type	Experiment 1
MT124868	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1

MT124061	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124164	Craft/MT070174	Karl Type	Lewis Type	Experiment 1
MT124242	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124340	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124381	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124502	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124512	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124663	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124695	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124026	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124118	Hockett/MT070174	Lewis Type	Karl Type	Experiment 1
MT124163	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124315	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124377	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124580	MT020204/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124649	MT010158/MT070176	Karl Type	Lewis Type	Experiment 1
MT124661	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124862	MT040073/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124901	Craft/MT070174	Karl Type	Lewis Type	Experiment 1
MT124001	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124009	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124073	MT010158/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124093	Craft/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124162	Craft/MT070174	Lewis Type	Karl Type	Experiment 1
MT124172	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124223	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124243	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124652	MT010158/MT070176	Karl Type	Lewis Type	Experiment 1
MT124007	MT010158/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124064	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124124	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124138	Craft/MT070174	Karl Type	Karl Type	Experiment 1
MT124177	Craft/MT070174	Lewis Type	Karl Type	Experiment 1
MT124411	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124571	MT040073/MT040075	Karl Type	Lewis Type	Experiment 1
MT124582	MT020204/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124659	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124664	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124792	MT020204/MT070174	Lewis Type	Lewis Type	Experiment 1
MT124167	Craft/MT070174	Karl Type	Lewis Type	Experiment 1
MT124300	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124303	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124336	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1

MT124370	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124380	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124457	MT010158/MT070176	Lewis Type	Lewis Type	Experiment 1
MT124650	MT010158/MT070176	Karl Type	Lewis Type	Experiment 1
MT124677	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124025	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124056	MT010158/MT070175	Karl Type	Lewis Type	Experiment 1
MT124080	Craft/MT070175	Karl Type	Lewis Type	Experiment 1
MT124113	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124134	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124232	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124289	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124331	MT020204/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124406	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124691	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124705	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124728	MT010158/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124929	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124945	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1
MT124069	MT010158/MT070175	Lewis Type	Lewis Type	Experiment 1
MT124112	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124282	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124669	Hockett/MT070174	Karl Type	Karl Type	Experiment 1
MT124696	MT040073/MT070175	Karl Type	Lewis Type	Experiment 1
MT124936	MT020204/MT070175	Karl Type	Lewis Type	Experiment 1

Table S3. Influence of *HvNAM1* and *HvNAM2* allelic states on agronomic and physiological traits in Experiment 2 (with 13 lines and varieties). Data represent mean values and standard deviations averaged across all location-years and treatments. Two-sided differences between the means of different alleles/allele combination were calculated using the Wilcoxon test (P value < 0.05) and are represented with superscript letters. For allele combinations, K symbol represents 'Karl' allele, L symbol denotes the 'Lewis' allele of both *HvNAM1* and *HvNAM2*. For ANOVA analyses, P values are represented by a dot (.) for $P < 0.1$, one star (*) for $P < 0.05$), two stars (**) for $P < 0.01$, or three stars (***) for $P < 0.001$. dap, days after planting.

Table S3a. Separate analysis of *HvNAM1* and *HvNAM2* effects

Single gene effect	Number of lines	Heading [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Tillers [tillers m^{-1}]
$HvNAM1^K$	7	58.3 ± 3.8 ^{a}	94.3 ± 3.2 ^{a}	35.9 ± 3.8 ^{a}	71.8 ± 9.3 ^{a}	159.3 ± 35.1 ^{a}
$HvNAM1^L$	6	58.2 ± 2.5 ^{a}	92.3 ± 3.3 ^{b}	33.9 ± 2.8 ^{b}	70.3 ± 9.7 ^{a}	160.4 ± 33.1 ^{a}
$HvNAM2^K$	5	57.5 ± 3.9 ^{b}	94.1 ± 3.6 ^{a}	36.5 ± 4.1 ^{a}	70.6 ± 9.3 ^{a}	160.3 ± 33 ^{a}
$HvNAM2^L$	8	58.7 ± 2.8 ^{a}	93 ± 3.3 ^{b}	34.0 ± 2.6 ^{b}	71.5 ± 9.7 ^{a}	159.5 ± 34.9 ^{a}

Table S3a Continued. Separate analysis of *HvNAM1* and *HvNAM2* effects

Single gene effect	Number of lines	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]	Harv. index
$HvNAM1^K$	7	83.4 ± 13.01 ^{a}	65 ± 2.5 ^{a}	11.8 ± 1.6 ^{b}	6440 ± 1619 ^{a}	0.441 ± 0.040 ^{a}
$HvNAM1^L$	6	74.8 ± 17.3 ^{b}	64.5 ± 2.4 ^{b}	13.1 ± 2 ^{a}	6420 ± 1478 ^{a}	0.443 ± 0.044 ^{a}
$HvNAM2^K$	5	84.2 ± 12.7 ^{a}	65.2 ± 2.3 ^{a}	11.9 ± 1.7 ^{b}	6397 ± 1477 ^{a}	0.437 ± 0.042 ^{a}
$HvNAM2^L$	8	76.5 ± 16.7 ^{b}	64.5 ± 2.5 ^{b}	12.7 ± 2 ^{a}	6450 ± 1601 ^{a}	0.445 ± 0.042 ^{a}

Table S3b. Analysis of allele combinations						
Allelic combinations [HvNAM1/HvNAM2]	**Number of lines**	**Heading [dap]**	**Maturity [dap]**	**Grain fill [days]**	**Height [cm]**	**Tillers [tillers m^{-1}]**
KK	4	57.5 ± 4.2	94.7 ± 3.4	37.2 ± 4.0	70.6 ± 9.5	160.5 ± 35.0
KL	3	59.3 ± 3.1	93.9 ± 3.0	34.1 ± 2.5	73.5 ± 8.7	157.7 ± 35.4
LK	1	57.7 ± 2.4	91.5 ± 3.3	33.3 ± 2.9	70.7 ± 8.4	159.1 ± 23.9
LL	5	58.3 ± 2.5	92.4 ± 3.3	34.0 ± 2.7	70.3 ± 10.0	160.6 ± 34.7

Table S3b Continued. Analysis of allele combinations						
Allelic combinations [HvNAM1/HvNAM2]	**Number of lines**	**Plump kernels [%]**	**Test weight [kg hL^{-1}]**	**Grain protein [%]**	**Yield [kg ha^{-1}]**	**Harv. index**
KK	4	86.5 ± 11.5	65.7 ± 2.0	11.6 ± 1.5	6523 ± 1537	0.440 ± 0.038
KL	3	79.3 ± 13.8	64.0 ± 2.8	12.0 ± 1.7	6327 ± 1721	0.442 ± 0.043
LK	1	75.2 ± 13.7	63.1 ± 2.3	12.9 ± 1.9	5894 ± 1085	0.424 ± 0.053
LL	5	74.8 ± 18.0	64.7 ± 2.3	13.1 ± 2.0	6524 ± 1525	0.447 ± 0.042

Table S3c. Analysis of variance for fixed effects

Fixed Effects	Heading [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Tillers [tillers m^{-1}]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]	Harv. index
HvNAM1 allele	**	***					***	***	**	
HvNAM2 allele	***		***			**	***	***	***	
HvNAM1-HvNAM2 alleles	**	***	***	*		***	***	***	**	
Irrigation		*	*	**	*		**		**	
Nitrogen				.	*				*	
HvNAM2 allele * irrigation		*	**	**			.		*	
HvNAM2 allele * nitrogen		*	*							
HvNAM1 allele * irrigation		.		.						
HvNAM1 allele * nitrogen							.			
HvNAM1-HvNAM2 alleles * irrigation		.	.	*			*		*	
HvNAM1-HvNAM2 alleles * nitrogen		.								
Transformation	None	None	None	None	None	Ordnorm	None	None	None	LambertFX

APPENDIX D

SUPPLEMENTARY TABLES FOR CHAPTER FOUR

Table S1. List of all genotypes used in this study together with their allelic state for each gene

Variety	**Pedigree**	**Allele for *HvGR-RBP1***	**Allele for *HvNAM1***	**Allele for *HvNAM2***	**Allele Combinations (*HvGR-RBP1, HvNAM1, HvNAM2*)**	**Represented Experiment**
21-7	Parent	K	K	L	KKL	sub-malt panel
Amsterdam	Parent	L	L	L	LLL	sub-malt panel
Craft	Parent	L	L	L	LLL	sub-malt panel
Eslick	Parent	L	L	L	LLL	sub-malt panel
Hockett	Parent	L	L	K	LLK	sub-malt panel
Lewis	Parent	L	L	L	LLL	sub-malt panel
MT090182	Parent	L	K	K	LKK	sub-malt panel
MT090190	Parent	L	K	K	LKK	sub-malt panel
MT124071	MT010158/ MT070175	K	K	L	KKL	malt panel and sub-malt panel
MT124128	Hockett/M T070174	L	K	K	LKK	malt panel and sub-malt panel
MT124148	Craft/MT07 0174	L	L	L	LLL	malt panel and sub-malt panel
MT124601	MT020204/ MT070175	K	K	L	KKL	malt panel and sub-malt panel
MT124673	Hockett/M T070174	L	K	K	LKK	malt panel and sub-malt panel
MT124084	Craft/MT07 0175	K	K	L	KKL	malt panel
MT124099	Craft/MT07 0175	K	K	L	KKL	malt panel
MT124127	Hockett/M T070174	L	L	K	LLK	malt panel
MT124136	Craft/MT07 0174	L	K	K	LKK	malt panel
MT124168	Craft/MT07 0174	L	K	K	LKK	malt panel
MT124180	Craft/MT07 0174	L	K	L	LKL	malt panel
MT124322	MT020204/ MT070175	K	K	L	KKL	malt panel

MT124665	Hockett/MT070174	L	K	K	LKK	malt panel
MT124690	MT040073/MT070175	K	K	L	KKL	malt panel
MT124764	MT020204/MT070174	L	L	L	LLL	malt panel
MT124879	Craft/MT070174	L	K	L	LKL	malt panel
MT124075	MT010158/MT070175	K	K	L	KKL	malt panel
MT124140	Craft/MT070174	L	K	K	LKK	malt panel
MT124238	MT040073/MT070175	L	K	L	LKL	malt panel
MT124388	MT020204/MT070175	K	K	L	KKL	malt panel
MT124499	Craft/MT070175	K	K	L	KKL	malt panel
MT124555	MT040073/MT040075	L	K	L	LKL	malt panel
MT124570	MT040073/MT040075	K	K	L	KKL	malt panel
MT124602	MT020204/MT070175	K	K	L	KKL	malt panel
MT124645	MT010158/MT070176	L	L	L	LLL	malt panel
MT124688	MT040073/MT070175	K	K	K	KKK	malt panel
MT124868	MT040073/MT070175	K	K	L	KKL	malt panel
MT124061	MT010158/MT070175	K	K	L	KKL	malt panel
MT124164	Craft/MT070174	L	K	L	LKL	malt panel
MT124242	MT040073/MT070175	K	K	L	KKL	malt panel
MT124340	MT020204/MT070175	K	K	L	KKL	malt panel
MT124381	MT020204/MT070175	K	K	L	KKL	malt panel
MT124502	Craft/MT070175	K	K	L	KKL	malt panel
MT124512	Craft/MT070175	K	K	L	KKL	malt panel
MT124663	Hockett/MT070174	L	K	K	LKK	malt panel
MT124695	MT040073/MT070175	K	K	L	KKL	malt panel
MT124026	MT010158/MT070175	K	K	L	KKL	malt panel
MT124118	Hockett/MT070174	L	L	K	LLK	malt panel
MT124163	Craft/MT070174	L	K	K	LKK	malt panel

MT124315	MT020204/ MT070175	K	K	L	KKL	malt panel
MT124377	MT020204/ MT070175	K	K	L	KKL	malt panel
MT124580	MT020204/ MT070175	L	L	L	LLL	malt panel
MT124649	MT010158/ MT070176	L	K	L	LKL	malt panel
MT124661	Hockett/M T070174	L	K	K	LKK	malt panel
MT124862	MT040073/ MT070175	L	L	L	LLL	malt panel
MT124901	Craft/MT07 0174	L	K	L	LKL	malt panel
MT124001	MT010158/ MT070175	K	K	L	KKL	malt panel
MT124009	MT010158/ MT070175	K	K	L	KKL	malt panel
MT124073	MT010158/ MT070175	L	L	L	LLL	malt panel
MT124093	Craft/MT07 0175	L	L	L	LLL	malt panel
MT124162	Craft/MT07 0174	L	L	K	LLK	malt panel
MT124172	Craft/MT07 0174	L	K	K	LKK	malt panel
MT124223	MT040073/ MT070175	K	K	L	KKL	malt panel
MT124243	MT040073/ MT070175	K	K	L	KKL	malt panel
MT124652	MT010158/ MT070176	L	K	L	LKL	malt panel
MT124007	MT010158/ MT070175	L	L	L	LLL	malt panel
MT124064	MT010158/ MT070175	K	K	L	KKL	malt panel
MT124124	Hockett/M T070174	L	K	K	LKK	malt panel
MT124138	Craft/MT07 0174	L	K	K	LKK	malt panel
MT124177	Craft/MT07 0174	L	L	K	LLK	malt panel
MT124411	MT020204/ MT070175	K	K	L	KKL	malt panel
MT124571	MT040073/ MT040075	K	K	L	KKL	malt panel
MT124582	MT020204/ MT070175	L	L	L	LLL	malt panel
MT124659	Hockett/M T070174	L	K	K	LKK	malt panel
MT124664	Hockett/M T070174	L	K	K	LKK	malt panel
MT124792	MT020204/ MT070174	L	L	L	LLL	malt panel

MT124167	Craft/MT070174	L	K	L	LKL	malt panel
MT124300	Hockett/MT070174	L	K	K	LKK	malt panel
MT124303	Hockett/MT070174	L	K	K	LKK	malt panel
MT124336	MT020204/MT070175	K	K	L	KKL	malt panel
MT124370	MT020204/MT070175	K	K	L	KKL	malt panel
MT124380	MT020204/MT070175	K	K	L	KKL	malt panel
MT124457	MT010158/MT070176	L	L	L	LLL	malt panel
MT124650	MT010158/MT070176	L	K	L	LKL	malt panel
MT124677	Hockett/MT070174	L	K	K	LKK	malt panel
MT124025	MT010158/MT070175	L	K	L	LKL	malt panel
MT124056	MT010158/MT070175	K	K	L	KKL	malt panel
MT124080	Craft/MT070175	K	K	L	KKL	malt panel
MT124113	Hockett/MT070174	L	K	K	LKK	malt panel
MT124134	Hockett/MT070174	L	K	K	LKK	malt panel
MT124232	MT040073/MT070175	L	K	L	LKL	malt panel
MT124289	Hockett/MT070174	L	K	K	LKK	malt panel
MT124331	MT020204/MT070175	L	L	L	LLL	malt panel
MT124406	MT020204/MT070175	K	K	L	KKL	malt panel
MT124691	MT040073/MT070175	K	K	L	KKL	malt panel
MT124705	MT040073/MT070175	K	K	L	KKL	malt panel
MT124728	MT010158/MT070175	L	L	L	LLL	malt panel
MT124929	MT020204/MT070175	K	K	L	KKL	malt panel
MT124945	MT020204/MT070175	K	K	L	KKL	malt panel
MT124069	MT010158/MT070175	L	L	L	LLL	malt panel
MT124112	Hockett/MT070174	L	K	K	LKK	malt panel
MT124282	Hockett/MT070174	L	K	K	LKK	malt panel
MT124669	Hockett/MT070174	L	K	K	LKK	malt panel

MT124696	MT040073/ MT070175	K	K	L	KKL	malt panel
MT124936	MT020204/ MT070175	K	K	L	KKL	malt panel

		HvGR-RBP1	*HvNAM1*	*HvNAM2*
Total Count (Alleles)	K	43	80	31
	L	60	23	72

Total Count (Allele Combination)	KKL	42
	LLL	18
	LLK	5
	LKK	25
	LKL	12
	KKK	1

Table S2. List of all markers used in this study. The primers and restriction enzymes used in the assays are also given.

Marker Name	**Primer Name**	**Primer Sequence (5`to 3`)**	**Tm (°C)**	**Expected Amplicon Length (bp)**	**Digestion Assay**	**Restriction Digest**
HvGR-RBP1 Marker	PM-Forward	CGATCACCTTCCTAGCGGAC	59	~600 bp for Lewis, ~1000 bp for Karl	No	NA
	PM-Reverse	GCTTTCCATGGGTTTCATGGG	58			
HvNAM1 Marker (UHB6')	UHB6`Forward	GGGATCATCATCCATCAGAGA	58	~470 bp	Yes	Digestion with Mwo1
	UHB6`Reverse	CGATGAGACGGCGTACAATA	58			
HvNAM1 Marker (UHB7')	UHB6`Forward	GGGATCATCATCCATCAGAGA	58	~300 bp	Yes	Digestion with HpyCH4III
	UHB6`Reverse	CGATGAGACGGCGTACAATA	58			
HvNAM2 Marker	P2-Forward	CAGAGGGCGTTATTTCTCAATCT	57	~500 bp	Yes	Digestion with BtsCI
	P2-Reverse	AGGTGGATATGTCTCGGTTTCG	60			

Table S3. Analysis of malt attributes from sub-malt panel. A total of 10 traits were analyzed in this sub-population of malt panel. The mean values with standard deviations for each trait are given in each column. Table S3a shows the effect of different alleles of each gene. Two-sided comparisons between different alleles of the same gene were performed with Wilcoxon-test with the significance cut-off value $p < 0.05$ and represented here via superscript letter if the different was statistically significant. Table S3b describes the effect of allelic combinations for three genes on malt attributes. The "Karl" allele is represented with letter K while "Lewis" allele is represented with letter L in the table. The alleles show the genotype for HvGR-*RBP1*, *HvNAM1* and *HvNAM2*, respectively. Some combinations are missing since they were not detected in population used in sub-malt panel. Table S3c describes the effect of each fixed effect on malt phenotyping traits in the linear-mixed model. P-values are represented with the significance codes: $p< 0.1$ '**.**', $p<0.05$'*', $p<0.01$ '**', $p<0.001$'***'.

Table S3a. Analysis of Traits Based on HvGR-RBP1, HvNAM1 and HvNAM2 individually

Single Gene Effect	Number of Individuals	Kernel Plump [%]	Malt Extract [%]	Grain Protein [%]	Malt Protein [%]	Wort Protein [%]
HvGR-RBP1^{K}	3	84.6 ± 10.6 ^{a}	79.2 ± 2.2 ^{a}	11.6 ± 1.6 ^{a}	10.9 ± 1.2 ^{a}	4.9 ± 0.5 ^{a}
HvGR-RBP1^{L}	10	83.3 ± 14.2 ^{a}	79.0 ± 2.5 ^{a}	12.0 ± 1.9 ^{a}	11.1 ± 1.3 ^{a}	5.0 ± 0.5 ^{a}
HvNAM1^{K}	7	87.3 ± 9.9 ^{a}	79.4 ± 2.2 ^{a}	11.2 ± 1.5 ^{b}	10.8 ± 1.1 ^{b}	4.9 ± 0.5 ^{a}
HvNAM1^{L}	6	79.4 ± 15.7 ^{b}	78.6 ± 2.6 ^{a}	12.6 ± 1.9 ^{a}	11.4 ± 1.4 ^{a}	5.0 ± 0.5 ^{a}
HvNAM2^{K}	5	87.0 ± 11.3 ^{a}	79.4 ± 2.2 ^{a}	11.3 ± 1.7 ^{b}	10.9 ± 1.2 ^{a}	5.0 ± 0.5 ^{a}
HvNAM2^{L}	8	81.5 ± 14.2 ^{b}	78.8 ± 2.5 ^{a}	12.2 ± 1.8 ^{a}	11.2 ± 1.3 ^{a}	5.0 ± 0.5 ^{a}

Table S3a Continued. Analysis of Traits Based on HvGR-RBP1, HvNAM1 and HvNAM2 individually

Single Gene Effect	Number of Individuals	S/T [%]	DP [°ASBC]	α-Amylase Activity [°DU]	β-Glucan [ppm]	FAN [ppm]
HvGR-RBP1^{K}	3	45.1± 6.3 ^{a}	166.1 ± 23.5 ^{a}	81.5 ± 14.6 ^{a}	209.6 ± 161.3 ^{a}	194.4 ± 29.5 ^{a}
HvGR-RBP1^{L}	10	45.4 ± 5.7 ^{a}	176.7 ± 39.4 ^{a}	83.9 ± 21.4 ^{a}	185.7 ± 163.2 ^{a}	212.9 ± 39.7 ^{a}
HvNAM1^{K}	7	45.9 ± 5.6 ^{a}	166.0 ± 29.7 ^{b}	85.2 ± 22.0 ^{a}	213.6 ± 179.2 ^{a}	202.1 ± 35.5 ^{a}
HvNAM1^{L}	6	44.7 ± 6.0 ^{a}	183.9 ± 41.4 ^{a}	81.2 ± 17.2 ^{a}	165.1 ± 137.3 ^{a}	216.2 ± 40.3 ^{a}
HvNAM2^{K}	5	46.1 ± 5.4 ^{a}	173.2 ± 38.3 ^{a}	89.2 ± 24.7 ^{a}	206.7 ± 188.9 ^{a}	210.1 ± 35.7 ^{a}
HvNAM2^{L}	8	44.9 ± 6 ^{a}	174.9 ± 35.6 ^{a}	79.6 ± 15.4 ^{a}	181.5 ± 144 ^{a}	207.7 ± 40.0 ^{a}

Table S3b. Analysis of Traits Based on Combination of all Three Genes						
Allele Combination	**Number of Individuals**	**Kernel Plump [%]**	**Malt Extract [%]**	**Grain Protein [%]**	**Malt Protein [%]**	**Wort Protein [%]**
KKL	*3*	84.6 ± 10.6	79.2 ± 2.2	11.6 ± 1.6	10.9 ± 1.2	4.9 ± 0.5
LKK	*4*	89.3 ± 9.0	79.5 ± 2.2	11.0 ± 1.4	10.7 ± 1.0	4.9 ± 0.5
LLK	*1*	77.9 ± 15.5	78.8 ± 2.6	12.6 ± 2.2	11.8 ± 1.7	5.2 ± 0.3
LLL	*5*	79.7 ± 15.9	78.6 ± 2.7	12.6 ± 1.9	11.3 ± 1.4	5.0 ± 0.5

Table S3b Continued. Analysis of Traits Based on Combination of all Three Genes						
	Number of Individuals	**S/T [%]**	**DP [°ASBC]**	**α-Amylase Activity [°DU]**	**β-Glucan [ppm]**	**FAN [ppm]**
KKL	*3*	45.1 ± 6.3	166.1 ± 23.5	81.5 ± 14.5	209.6 ± 161.3	194.4 ± 29.5
LKK	*4*	46.5 ± 5.0	165.8 ± 34.0	87.9 ± 26.2	216.6 ± 194.0	207.9 ± 38.9
LLK	*1*	44.4 ± 6.9	202.4 ± 42.9	94.7 ± 17.5	167.2 ± 172.7	218.8 ± 17.4
LLL	*5*	44.8 ± 5.9	180.2 ± 40.6	78.5 ± 16.0	164.6 ± 131.7	215.7 ± 43.6

Table S3c. Analysis of Variance for Fixed Effects										
Fixed Effects	**Kernel Plump [%]**	**Malt Extract [%]**	**Grain Protein [%]**	**Wort Protein [%]**	**Malt Protein [%]**	**ST [%]**	**DP [°ASBC]**	**α-Amylase Activity [°DU]**	**β-Glucan [ppm]**	**FAN [ppm]**
Allele Combination (HvGR-RBP1, HvNAM1 and HvNAM2)	***	*	***		***		***	.		
HvGR-RBP1			.		**		*			
HvNAM1	**		***		***		***			
HvNAM2					*		*	*		
Irrigation						.		*	*	*

Nitrogen										
Allele										
Comb*Irrigation										
Allele										
Comb*Nitrogen										
Transformation	None	None	None	None	None	None	None	None	OrderNorm	None