Mol Breeding (2022) 42:59
https://doi.org/10.1007/s11032-022-01331-7

Selection of favorable alleles of genes controlling flowering and senescence improves malt barley quality

Burcu Alptekin · Mohammad Erfatpour · Dylan Mangel · Duke Pauli · Tom Blake · Hannah Turner · Jennifer Lachowiec · Jamie Sherman · Andreas Fischer

Received: 7 April 2022 / Accepted: 14 September 2022

Abstract Malt barley (*Hordeum vulgare* L.) is an important cash crop with stringent grain quality standards. Timing of the switch from vegetative to reproductive growth and timing of whole-plant senescence and nutrient remobilization are critical for cereal grain yield and quality. Understanding the genetic variation in genes associated with these developmental traits can streamline genotypic selection of superior malt barley germplasm. Here, we determined the effects of allelic variation in three genes encoding a glycine-rich RNA-binding protein (*Hv*GR-RBP1) and two NAC transcription factors (*Hv*NAM1 and *Hv*NAM2) on malt barley agronomics and quality using previously developed markers for *HvGR-RBP1* and *HvNAM1* and a novel marker for *HvNAM2*. Based on a single-nucleotide polymorphism (SNP) in the first intron, the utilized marker differentiates *NAM2* alleles of low-grain protein variety 'Karl' and of higher protein variety 'Lewis'. We demonstrate that the selection of favorable alleles for each gene impacts heading date, senescence timing, grain size, grain protein concentration, and malt quality. Specifically, combining 'Karl' alleles for the two *NAC* genes with the 'Lewis' *HvGR-RBP1* allele extends grain fill duration, increases the percentage of plump kernels, decreases grain protein, and provides malt quality stability. Molecular markers for these genes are therefore highly useful tools in malt barley breeding.

Keywords Barley (*Hordeum vulgare* L.) · Flowering · Senescence · Grain quality · Malt quality · Molecular marker

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11032-022-01331-7.

B. Alptekin · M. Erfatpour · D. Mangel · D. Pauli · T. Blake · H. Turner · J. Lachowiec · J. Sherman · A. Fischer (✉)
Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA
e-mail: fischer@montana.edu

Present Address:
B. Alptekin
Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA

Present Address:
M. Erfatpour
Department of Plant Sciences, North Dakota State University, Fargo, ND 58108, USA

Present Address:
D. Mangel
Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, USA

Present Address:
D. Pauli
School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

Published online: 22 September 2022

Introduction

Barley (*Hordeum vulgare* L.) is an important crop grown worldwide with more than 40 million hectares of annual production (FAOSTAT, accessed at https://www.fao.org/faostat/en/#home). Barley has been used as the main ingredient of alcoholic beverages such as beer and whiskey for many centuries (Homan 2004). Today, about 20% of barley produced is used by the malt industry (Baik and Ullrich 2008; Nice et al. 2019). The value of malt barley can reach twice that of feed barley, but malt barley production is demanding (Chappell et al. 2017). In the USA, the region suitable for malt barley production is mainly limited to states with dry and cool climates such as Idaho, Montana, and North Dakota (USDA Small Grains Annual Summary, accessed at https://usda.library.cornell.edu/concern/publications/5t34sj573). Even in that region, malt barley production requires careful management to meet the high standards for grain quality. Despite such management efforts, only ~25% of barley grown for malt is accepted by maltsters, resulting in a substantial revenue loss to farmers (O'Donovan et al. 2008; Stevens et al. 2015).

Stringent barley quality requirements are due to variable grain quality impacting the malting process. Malting consists of several steps, starting with the steeping of barley seeds in water to initiate germination. Barley grains store proteins (10–17%) and starch (65–68%) inside cell walls that are in part composed of β-glucans (~4 to 9%); all these components are degraded during germination (Fang et al. 2019; Gupta et al. 2010). The conversion rate of storage compounds to amino acids and sugars during malting is key to malt quality. This rate is directly related to grain characteristics including grain protein and starch contents (Fang et al. 2019). Physical properties (grain size, grain width, and grain hardness) also impact malt quality (Gupta et al. 2010), justifying the analysis of kernel plumpness as a basis for malt acceptance. The physical and compositional characteristics of grains are quantitative traits; they are determined by the complex interaction of genotype and environment (Brouwer et al. 2016; Burger et al. 1979; Eagles et al. 1995; Elía et al. 2010).

Numerous studies have genetically dissected grain traits (e.g., grain protein concentration (GPC), kernel size, and kernel width) (Ayoub et al. 2002; Fan et al. 2017; Pauli et al. 2015; Stevens et al. 2015). Several authors have concluded that genes controlling plant development also impact grain characteristics and thereby malt quality (Walker et al. 2013). For example, genes controlling flowering time (Bingham et al. 2007; Coventry et al. 2003), onset or rate of whole-plant senescence (Distelfeld et al. 2014), and grain fill duration (Coventry et al. 2003) have a vital impact on grain quality. An important quantitative trait locus (QTL) on chromosome 6H which influences GPC (See et al. 2002; Jukanti et al. 2008) controls both flowering time and whole-plant senescence (Laccerenza et al. 2010). Using barley reference genome information (Mascher et al. 2017), we demonstrated that the impact of this QTL on plant development is due to linkage between a flowering time controlling gene, *HvGR-RBP1*, and a senescence controlling gene, *HvNAM1* (Alptekin et al. 2021).

Glycine-rich RNA-binding proteins (GR-RBPs) including *Hv*GR-RBP1 are small (< 20 kD) proteins with an N-terminal RNA-binding domain and a C-terminal glycine-rich domain (Ciuzan et al. 2015; Tripet et al. 2014). The best-understood plant GR-RBP is *Arabidopsis thaliana* glycine-rich RNA-binding protein 7 (*At*GRP7). This protein binds both RNA and DNA, with a preference for single-stranded nucleic acids (Schüttpelz et al. 2008), and has several known functions (Cao et al. 2006; Kim et al. 2008; Yang et al. 2014). *At*GRP7 is a component of the flowering autonomous (or earliness per se) pathway which promotes floral transition, as demonstrated by late-flowering knockout mutants (Steffen et al. 2019; Streitner et al. 2008). Previous biochemical and genetic analyses by our lab suggest that *Hv*GR-RBP1, similarly to *At*GRP7, preferentially binds single-stranded nucleic acids and that it is involved in flowering time control (Tripet et al. 2014; Parrott et al. 2012).

HvNAM1 is a member of the *NAC* gene family, a large family of plant-specific transcription factors with functions in developmental regulation, abiotic stress control, and defense (Jensen and Skriver 2014). The importance of *NAC* genes for senescence regulation has emerged from several studies. Mutations in the wheat *TtNAM-B1* gene, or loss of this gene, are associated with delayed senescence and lower grain protein and micronutrient (Fe, Zn) contents (Uauy et al. 2006; Waters et al. 2009). The sequences of *HvNAM1*, and of a second barley *NAC* gene, *HvNAM2*, are highly similar to wheat *TtNAM-B1* (Uauy et al. 2006). *HvNAM1* is located within

the chromosome 6H grain protein QTL mentioned above, in a region that is co-linear with the wheat *TtNAM-B1* region (Distelfeld et al. 2008), and allelic variation in this barley gene controls senescence timing and GPC (Jukanti and Fischer 2008; Jukanti et al. 2008; Alptekin et al. 2021). Interestingly, a QTL for malt quality has been identified on chromosome 2H near *HvNAM2* (Pauli et al. 2015), and Cai et al. (2013) identified an association between *HvNAM2* allelic state and GPC.

In this study, we designed a molecular marker which allows the distinction between *HvNAM2* alleles of the low-GPC variety 'Karl' (Burger et al. 1979; Wesenberg et al. 1976) and a higher-GPC variety, 'Lewis' (Hockett et al. 1985), parents of a cross previously used for GPC QTL mapping (See et al. 2002; Mickelson et al. 2003). We utilized this marker by itself and in combination with previously established molecular markers for *HvNAM1* (Distelfeld et al. 2008) and *HvGR-RBP1* (Alptekin et al. 2021) to dissect effects of allelic differences in these developmental genes on barley grain and malt quality.

Materials and methods

Plant material and malt phenotyping

In this study, we re-analyzed a subset (95 lines) of the plant material described as the "Malt Panel" by Pauli et al. (2015). That panel represents elite malting germplasm of the Montana State University (Bozeman, MT, USA) breeding program. It is derived from crosses between advanced-generation malting lines (Online Resource 1, pedigree), with the parents having desirable malting quality. The panel was genotyped in the F_6 generation, using 384 markers showing, as expected, ~3% remaining heterozygosity (Pauli et al. 2015). Analysis of 95 lines from the Malt Panel was performed here to understand the impact of allelic differences in three genes on agronomics and malt phenotype: two genes coding for senescence-controlling NAC transcription factors (*HvNAM1* and *HvNAM2*) and a gene coding for a flowering-time controlling RNA-binding protein (*HvGR-RBP1*). The set of 95 lines is designated as experiment 1 throughout the rest of this manuscript (Online Resource 1). The plant material was grown in 2012 at the Arthur Post Research Farm, Bozeman, MT, USA (45°40′40.78 N, 111°09′07.14 W) under both dryland and irrigated conditions, in two replicates, but only one replicate of material grown under irrigated conditions was submitted for malt phenotyping 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 (https://www.asbcnet.org/methods/pages/default.aspx). Traits measured in this study were the following: kernel weight (mg), kernel plumpness (percent of kernels retained by a 6/64th inch/2.38 mm sieve), GPC (%), malt extract (%), wort protein (%), soluble to total protein ratio (S/T) (%), diastatic power (DP) (°ASBC), α-amylase activity (20 dextrinizing units [°DU]), β-glucans (ppm), and free amino nitrogen (FAN) (ppm) (Pauli et al. 2015).

To investigate the effect of *gene* × *environment* interactions on physiological/agronomic characteristics, a smaller set of barley lines and varieties was grown under irrigated and dryland conditions, and under two nitrogen fertilization levels, in the same field (experiment 2). This set consists of 13 varieties and lines including some parental lines (Online Resource 1). Experiment 2 was grown for three location-years, namely Bozeman-2016, Bozeman-2017 (Arthur Post Research Farm, Bozeman, MT, USA; 45°40′40.78 N, 111°09′07.14 W), and Conrad-2017 (Western Triangle Agricultural Research Station, Conrad, MT, USA; 48°18′26.05 N, 111°55′29.24 W). The plant material was grown with three replicates for each location-year and treatment as described by Alptekin et al. (2021). Raw agronomic data for experiments 1 and 2 are shown in Online Resources 2 and 3, respectively, while malt quality data for experiment 1 are provided in Online Resource 4.

HvNAM2 genomic DNA sequencing and analysis

Prior to sequencing and genotyping, DNA was isolated as previously described (Alptekin et al. 2021) 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^{-1}.

For sequencing, DNA was isolated from barley varieties 'Karl' (Wesenberg et al. 1976; Burger et al. 1979) and 'Lewis' (Hockett et al. 1985). The *HvNAM2* sequence (DQ869679) was obtained

from NCBI and used to identify the gene in the reference barley genome (HORVU2Hr1G039640) (Mascher et al. 2017). Primers amplifying a 3 kb region encompassing the *HvNAM2* coding region (exons and introns), 700 bp of upstream, and 800 bp of downstream sequences were designed using the Integrated DNA Technologies (Coralville, IA, USA) PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index) (Online Resource 5). Amplicons of 667 to 794 bp were generated for each genotype using Taq 2×Master Mix (New England Biolabs, Ipswich, MA, USA) following the manufacturer's guidelines with a BIO-RAD (Hercules, CA, USA) CFX96 real-time PCR system. The PCR cycling program included an initial denaturation step for 5 min at 95 °C, followed by 35 cycles each of 30 s at 94 °C (denaturation), 1 min at annealing temperature (3 °C above the melting temperature of primer), 30 s at 72 °C (extension), and a final extension of 5 min at 72 °C. PCR amplicons were then loaded on 1% agarose gels to visualize DNA bands with expected sizes, quantified by fluorometry (Qubit, ThermoFisher Scientific, Waltham, MA, USA), and normalized to 4 ng μl^{-1} for Sanger sequencing by the Genomics Core Laboratory at the University of Montana (Missoula, MT, USA) (https://hs.umt.edu/dbs/labs/genomics/laboratory-services/default.php).

HvNAM2 sequences were analyzed using the "Clustal Omega" multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al. 2019) with default settings to identify differences between 'Karl', 'Lewis', and the reference genome sequence from variety 'Morex' (HORVU2Hr1G039640) (Mascher et al. 2017). Sequences were also compared with those in Cai et al. (2013); the resulting alignment is shown as Online Resource 6.

Genotyping for HvGR-RBP1, HvNAM1, and HvNAM2

Based on sequence analysis, two molecular markers differentiating 'Karl' and 'Lewis' *HvNAM2* alleles were developed. For the first marker (primers shown as *HvNAM2* marker 1 in Online Resource 5), a 404 bp region was amplified using GoTaq DNA polymerase (Promega, Madison, WI, USA) and the following PCR protocol: 95 °C for initial denaturation, 30 cycles of 95 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min per kb, and 5 min of final elongation at 72 °C. Amplicons were then digested using *Bts*CI (New England Biolabs, Ipswich, MA, USA), and digested amplicons were visualized on 2% agarose gels to differentiate digested and undigested bands (Fig. 1). For the second marker, primers amplifying a 1171 bp region in lines carrying the 'Karl' allele were designed (Online Resources 5 and 6), with no amplification in lines carrying the 'Lewis' allele (Online Resource 7), using the same PCR reagents and cycling conditions as for sequencing. All lines in experiments 1 and 2 were screened with both markers; for the three lines for which the two markers apparently disagreed (MT124113, MT124659, MT124118), allele assignments were based on marker 1, which allows the detection of heterozygous lines.

Germplasm was also genotyped to identify the allelic states of *HvGR-RBP1* (HORVU6Hr1G055440) and *HvNAM1* (HORVU6Hr1G019380) as previously reported by Alptekin et al. (2021), using the primers and enzymes listed in Online Resource 5. Detection of *HvNAM1* 'Lewis' and 'Karl' alleles using markers *uhb6* and *uhb7* is based on SNPs which can be detected by restriction digests using *Mwo*I and *HpyCH4*III, respectively (Online Resource 5; Distelfeld et al. 2008). For *HvGR-RBP1*, PCR amplification of a region ~ 1 kb upstream of the translation start site results in DNA fragments with ~ 400 bp size difference, due to the presence of an insert in the 'Karl' sequence which is absent in 'Lewis' (Alptekin et al. 2021); the marker therefore readily distinguishes homo- and heterozygous lines.

The genotypes for *HvNAM2*, *HvGR-RBP1*, and *HvNAM1* of all lines and varieties used in this study are provided in Online Resource 1.

Statistical analysis

In this study, the statistical significance of collected agronomic, developmental, and malt quality data was investigated with R software (https://r-project.org) using the methods described in Alptekin et al. (2021). 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 nitrogen fertilizer treatments.

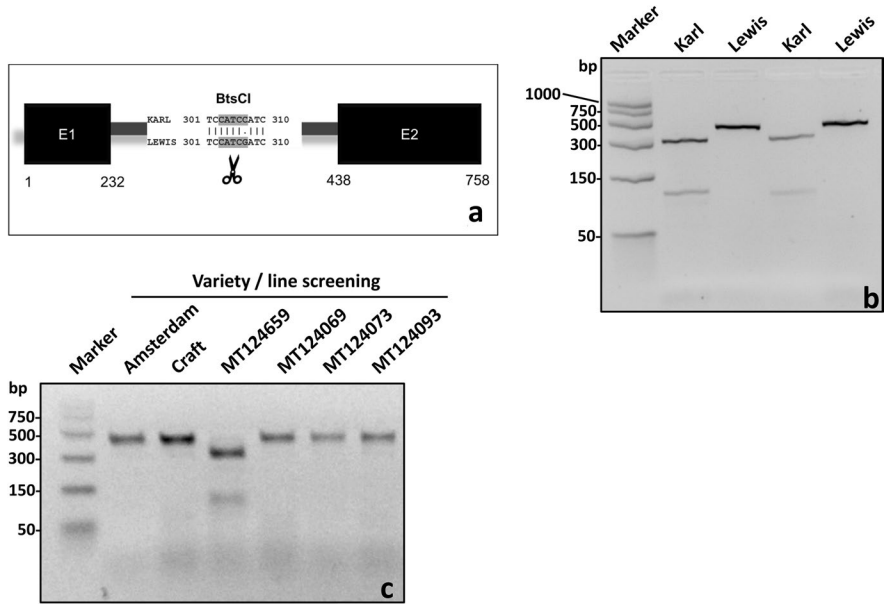

Fig. 1 *HvNAM2* molecular marker design. **a** A single-nucleotide polymorphism (SNP) located at the 307th position from the start codon (within the first intron) was used to design a molecular marker allowing the differentiation of 'Karl' and 'Lewis' alleles. A *Bts*CI restriction digest site is present in variety 'Karl' but absent in 'Lewis'. **b** Amplification of a 400 bp long region covering the SNP in position 307 is shown. Digestion of the amplicon in variety 'Karl' resulted in two bands of 300 and 100 bp. The 'Lewis' amplicon is not digested. **c** The molecular marker was used for genotyping of barley lines and varieties used in this study. Varieties 'Amsterdam' and 'Craft' and lines M124069, MT124073, and MT124093 carry the 'Lewis' allele, while line MT124659 carries the 'Karl' allele. Sequencing information including location of PCR primers and SNPs is shown in Online Resource 6, and complete genotyping data are given in Online Resource 1

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. 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, $G_{n\times n}$ 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 2021). The following linear mixed model was fitted 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 loci *HvGR-RBP1, HvNAM1,* and *HvNAM2*; $irrigation_j$ 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 $\mathcal{N}(0,G_{n\times n})$; 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.

For analyzing the agronomic and developmental data from 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 as previously described in Alptekin et al. (2021). The genetic relatedness matrix $G_{n\times n}$ 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:

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

where Y_{ijkl} is a single phenotypic observation; μ is the grand mean; $alleleCombination_i$ is a dummy variable capturing the effect of the allele combinations at loci *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*; $irrigation_j$ is the effect of the jth irrigation treatment; $nitrogen_k$ is the effect of the kth nitrogen treatment; $(alleleCombination \times irrigation)_{ij}$ is the effect of the interaction between the ith alleleCombination and the jth irrigation treatment; $(alleleCombination \times nitrogen)_{ik}$ is the effect of the interaction between the ith alleleCombination and the kth nitrogen treatment; $(irrigation \times nitrogen)_{jk}$ is the effect of the interaction between the jth irrigation treatment and the kth nitrogen treatment; and $(alleleCombination \times irrigation \times nitrogen)_{ijk}$ captures the three-way interaction among these factors. $genotype_l$ is the effect of the lth genotype following $\mathcal{N}(0,G_{n\times n})$, and ε_{ijkl} is the random error following $\mathcal{N}(0,\sigma^2)$. The factor allelic combination, irrigation, and nitrogen were modeled as fixed effects, and the other terms were modeled as random effects.

During analysis of agronomic and developmental data, the normality of model residuals was tested with the R package "fitdistrplus" (Delignette-Muller and Dutang 2015), and datasets that did not fit a normal distribution were normalized via the R package "bestNormalize" (Peterson and Cavanaugh 2020).

Statistical analyses agnostic to the pedigree were also performed. Pairwise comparisons between different allelic groups and treatments were made with the Wilcoxon test, with $\alpha = 0.05$; no multiple testing corrections were performed. Multiple comparisons were made after one-way ANOVA for allelic groups using Tukey's test except where assumption violations occurred. In those cases, Kruskal–Wallis one-way ANOVA tests were performed, followed by Dunn's test for multiple comparisons with $\alpha = 0.05$.

Results

Characterization of HvNAM2 alleles

It has previously been reported by Cai et al. (2013) that the *HvNAM2* gene consists of three exons and two introns. Those authors identified several single-nucleotide polymorphisms (SNPs) located in both introns, in exon 2, and in exon 3 between domesticated and wild barley types, but varieties 'Karl' and 'Lewis' were not included in their analysis. Recently, Hagenblad et al. (2022) sequenced exons and introns (start to stop codon) of *HvNAM2* and (and also *HvNAM1*) in 80 accessions of Nordic barley using 'Karl' and 'Lewis' for comparison, reporting that *HvNAM2* sequences of all these accessions were identical to variety 'Lewis'. For this study, we sequenced exons and introns of *HvNAM2*, as well as ~ 700 bp of upstream (from the start codon) and ~ 800 bp of downstream (from the stop codon) sequence in varieties 'Karl' and 'Lewis'. Sequence comparison identified two SNPs, one in each intron, in positions for which variability has previously been identified in the germplasm analyzed by Cai et al. (2013) (Online Resources 6 and 8). The SNP in position 307 (from the start codon) was utilized to develop a molecular marker (Fig. 1). Additionally, sequencing revealed that variety 'Karl' possesses a variable number tandem repeat (VNTR) composed of two repeats of a 20-bp sequence located 587 bp upstream from the *HvNAM2* start codon (Online Resources 6 and 7). Variety 'Lewis' and the reference genome sequence (variety 'Morex') contain only a single copy of this sequence in the corresponding region. This difference was exploited for the development of a second molecular marker, with amplification of an 1171-bp DNA fragment observed in varieties and lines carrying the 'Karl' *HvNAM2* allele, but not in those carrying the 'Lewis' allele (Online Resources 6 and 7). As mentioned under the "Materials and methods" section, all lines in experiments 1 and 2 were screened with both markers. For the three lines for which the two markers disagreed (MT124113, MT124659, MT124118), allele assignments shown in Online Resource 1 are based on marker 1, which allows distinction of homo- and heterozygous lines. For future studies, particularly

those analyzing panels in which substantial heterozygosity is expected, the 20 bp VNTR could be exploited for development of an InDel marker to increase throughput.

Allele frequencies for HvGR-RBP1, HvNAM1, and HvNAM2 indicate linkage or selection at these loci

We genotyped all varieties and lines used in experiments 1 and 2 for *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, differentiating between the 'Karl' allele (K) and the 'Lewis' allele (L) for each gene (Online Resource 1). Analyses of *HvGR-RBP1* and *HvNAM1* allelic state have previously been reported by Alptekin et al. (2021). Since the original panel was part of a breeding program, we focused on lines that had been advanced. Genotyping this germplasm showed that the K allele for *HvNAM1* was present in a large fraction of the lines (80 lines out of 103) (Online Resource 1), which demonstrates its value in controlling GPC. For *HvNAM2*, perhaps surprisingly, the L allele was more frequent (72 lines); the small negative (albeit not significant in our dataset; Table 1) yield effect of the K allele may have led to elimination of lines carrying it in the breeding process. The fact that the (desirable) L allele for *HvGR-RBP1* occurs more frequently than expected from its linkage to *HvNAM1* (in 43 out of 103 lines) has previously been discussed by Alptekin et al. (2021).

Comparison of allelic combinations for the three genes showed that some genotypes are missing, such as KLL and KLK, where the allelic states are shown for *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, respectively. The best-represented allelic combinations were KKL and LKK with 42 and 25 lines possessing these genotypes, respectively (Online Resource 1, bottom). The KKK allelic combination was present only in one line (MT124688). With only a single representative line for this allele combination in experiment 1 (and none in experiment 2), comparison with the other allele combinations is not appropriate. We therefore removed KKK from further analyses.

The allelic state of HvNAM2 influences plant development and agronomics

Plants carrying the L allele for *HvNAM2* had an average heading date of 188.5 ± 1.9 days, while those with the K allele headed one day earlier (187.3 ± 2.2 days) in experiment 1 (Table 1). Similarly, the K allele for *HvNAM2* was associated with earlier anthesis (1.2 days) and delayed

Table 1 Influence of *HvNAM2* alleles on agronomic and physiological traits in experiments 1 (95 lines) and 2 (13 varieties and lines)

Experiment 1									
Gene/ allele	Number of lines	Heading [Julian days]	Maturity [Julian days]	Grain fill [days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
$HvNAM2^K$	28	187.3 ± 2.2^b	n.d	n.d	64.9 ± 9.9^b	74.5 ± 15.0^a	67.8 ± 1.7^a	12.2 ± 0.6^b	5622 ± 1735^a
$HvNAM2^L$	67	188.5 ± 1.9^a	n.d	n.d	68.4 ± 11.8^a	62.8 ± 14.0^b	66.7 ± 1.7^b	12.8 ± 0.7^a	5884 ± 1786^a
Experiment 2									
Gene/ allele	Number of lines	Anthesis [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
$HvNAM2^K$	5	57.5 ± 3.9^b	94.1 ± 3.6^a	36.5 ± 4.1^a	70.6 ± 9.3^a	84.2 ± 12.7^a	65.2 ± 2.3^a	11.9 ± 1.7^b	6398 ± 1478^a
$HvNAM2^L$	8	58.7 ± 2.8^a	93.1 ± 3.3^b	34.0 ± 2.6^b	71.5 ± 9.7^a	76.5 ± 16.7^b	64.5 ± 2.5^b	12.7 ± 2.0^a	6451 ± 1602^a

Data represent mean values and standard deviations averaged across treatments (experiment 1) and across location-years and treatments (experiment 2). Two-sided differences between the means were calculated using the Wilcoxon test (p-value < 0.05) and are represented with superscript letters. The "K" symbol indicates the 'Karl' allele and the "L" symbol denotes the 'Lewis' allele of *HvNAM2*

n.d., not determined. *dap*, days after planting

maturity (1 day), together resulting in an increase in grain fill duration in experiment 2 (2.5 days; Table 1). The *HvNAM2* K allele was also associated with reduced plant height (in experiment 1), a substantially higher percentage of plump kernels, reduced GPC, and increased test weight in both experiments (Table 1).

Combined effects of HvGR-RBP1, HvNAM1, and HvNAM2 on plant development and agronomics

HvGR-RBP1 and HvNAM1 separately and together impact plant development and agronomics (Alptekin et al. 2021). Therefore, we aimed to determine their interaction with *HvNAM2*. Lines with the L allele for *HvGR-RBP1* and the K allele for *HvNAM2* (LKK and LLK) had the earliest heading dates, shortest plants, highest percentage of plump kernels, and highest test weight in experiment 1 (Table 2). Although these two genotypes vary for *HvNAM1*, the lack of a significant difference for heading date, height, percentage of plump kernels, and test weight between LKK and LLK suggests that the allelic difference for *HvNAM1* does not significantly impact these traits. Lines with the LKK genotype had the lowest grain protein, confirming the impact of *HvNAM1* on that trait. In experiment 2, plants with the LKK genotype had the longest grain fill duration, highest percentage of plump kernels, highest test weight, and lowest grain protein, confirming that this allele combination is favorable for malt barley (Table 2).

Grain fill duration is typically shorter under dryland than under irrigated conditions, and the percentage of plump kernels, test weight, and yield are lower, while GPC is higher (Online Resource 9). Irrigation shows significant effects on all analyzed traits in experiment 1 and on maturity dates, grain fill duration, plant height, test weight, and yield in experiment 2 (Online Resource 10). Importantly, plants with the LKK and LLK allele combinations performed well both in dryland and irrigated plots, with the highest percentages of plump kernels and highest test weight in experiment 1. For experiment 2, the LKK combination (represented by 4 varieties/lines) had the longest grain fill duration, the highest percentage of plump kernels, and the highest test weight in both dryland and irrigated plots (Online Resource 9). These results suggest that selection for the alleles/molecular markers analyzed in our study allows identification of germplasm with stable quality under varying environments.

Statistical analysis (Online Resource 10) further dissects the influence of alleles, allele combinations, and combinations of alleles and environmental conditions on measured traits. The *HvNAM2* allelic state influences all measured traits in experiment 1 and impacts maturity, percentage of plump kernels, test weight, grain protein, and yield in experiment 2. Importantly, analysis of both experiments suggests a significant influence of the *HvGR-RBP1*HvNAM1*HvNAM2* allele combination on all analyzed developmental and agronomic parameters.

Effects of HvGR-RBP, HvNAM1, and HvNAM2 on malt quality

The individual effects of *HvGR-RBP1* and *HvNAM1* on agronomic traits were previously reported by Alptekin et al. (2021), where the L allele of *HvGR-RBP1* significantly increased seed size and the K allele of *HvNAM1* decreased GPC. The L allele of *HvGR-RBP1* is also associated with higher malt extract, lower diastatic power (DP), lower β-glucan levels, and higher free amino nitrogen (FAN) levels, while *HvNAM1* is not significantly associated with any malt quality trait in experiment 1 (Table 3). In contrast to *HvNAM1*, *HvNAM2* allelic identity significantly impacts all the malt quality traits, with the K allele increasing kernel weight, malt extract, wort protein, the ratio of soluble to total protein (S/T), α-amylase activity, and FAN, while decreasing GPC, diastatic power, and β-glucan levels (Tables 1 and 3).

Table 4 reports the combined effects of *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* on malt quality. Malt extract was highest in lines with the L allele for *HvGR-RBP* and K alleles for *HvNAM1* and *HvNAM2* (LKK). These lines also had the highest wort protein, ratio of soluble to total protein (S/T), and free amino nitrogen (FAN), with low β-glucan levels, indicating that the malt was better modified than in grains sourced from the other allelic combinations. LKK lines also had the highest α-amylase activity but the lowest diastatic power.

Table 2 Influence of *HvGR-RBP1*/*HvNAM1*/*HvNAM2* allele combinations on agronomic and physiological traits in experiments 1 (94 lines) and 2 (13 varieties and lines)

Experiment 1									
Allele combination	Number of lines	Heading [Julian days]	Maturity [Julian days]	Grain fill [days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
LKK	23	187.2 ± 2.2^{a}	n.d	n.d	64.3 ± 9.4^{ab}	74.1 ± 15.8^{a}	67.7 ± 1.8^{a}	12.0 ± 0.4^{a}	5524 ± 1710^{a}
KKL	41	188.4 ± 1.8^{b}	n.d	n.d	68.3 ± 11.9^{c}	61.6 ± 14.2^{b}	66.5 ± 1.7^{b}	12.6 ± 0.6^{b}	5771 ± 1705^{a}
LKL	12	188.7 ± 1.9^{b}	n.d	n.d	67.2 ± 11.1^{bc}	66.2 ± 12.1^{b}	66.9 ± 1.4^{b}	12.6 ± 0.6^{b}	6007 ± 2045^{a}
LLK	4	187.4 ± 1.7^{a}	n.d	n.d	65.9 ± 10.4^{bc}	78.3 ± 9.7^{a}	68.4 ± 1.2^{a}	13.1 ± 0.5^{c}	6087 ± 1851^{a}
LLL	14	188.5 ± 2.0^{b}	n.d	n.d	70.1 ± 12.0^{c}	63.6 ± 14.7^{b}	66.9 ± 1.9^{b}	13.5 ± 0.7^{d}	6112 ± 1785^{a}
Experiment 2									
Allele combination	Number of varieties/lines	Anthesis [dap]	Maturity [dap]	Grain fill [days]	Height [cm]	Plump kernels [%]	Test weight [kg hL^{-1}]	Grain protein [%]	Yield [kg ha^{-1}]
LKK	4	57.5 ± 4.2^{b}	94.7 ± 3.4^{a}	37.2 ± 4.0^{a}	70.6 ± 9.5^{ab}	86.5 ± 11.5^{a}	65.7 ± 2.0^{a}	11.6 ± 1.5^{c}	6523 ± 1538^{a}
KKL	3	59.2 ± 3.1^{a}	94.1 ± 3.0^{a}	34.1 ± 2.5^{b}	73.5 ± 8.7^{a}	79.3 ± 13.8^{b}	64.0 ± 2.8^{bc}	12.0 ± 1.7^{bc}	6328 ± 1722^{a}
LLK	1	57.7 ± 2.4^{ab}	91.5 ± 3.3^{b}	33.3 ± 2.9^{b}	70.7 ± 8.4^{ab}	75.2 ± 13.7^{b}	63.1 ± 2.3^{c}	12.9 ± 1.9^{ab}	5894 ± 1086^{a}
LLL	5	58.4 ± 2.6^{ab}	92.5 ± 3.3^{b}	34.0 ± 2.7^{b}	70.3 ± 10.0^{b}	74.8 ± 18.0^{b}	64.7 ± 2.3^{b}	13.1 ± 2.0^{a}	6525 ± 1526^{a}

Data represent mean values and standard deviations averaged across treatments (experiment 1) and across location-years and treatments (experiment 2). Multiple comparisons between the means of allele combinations were performed using a Kruskal–Wallis one-way ANOVA followed by Dunn's post-hoc test. Superscript letters indicate significant differences (p-value < 0.05). For allele combinations, the "K" symbol represents the 'Karl' allele, and the "L" symbol denotes the 'Lewis' allele of *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, respectively

n.d., not determined. *dap*, days after planting

Table 3 Individual effects of *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* alleles on malt attributes in experiment 1 (94 lines) under irrigated conditions

Gene	*HvGR-RBP1*		*HvNAM1*		*HvNAM2*	
Allele	Karl	Lewis	Karl	Lewis	Karl	Lewis
Number of lines	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}
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}
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]	152 ± 34.2^{a}	135 ± 39.9^{b}	139 ± 38.3^{a}	159 ± 34.4^{a}	119 ± 27.6^{b}	152 ± 38.1^{a}
α-amylase[°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-glucans [ppm]	302 ± 140^{a}	233 ± 136^{b}	263 ± 135^{a}	264 ± 171^{a}	179 ± 91.6^{b}	298 ± 144^{a}
FAN [ppm]	151 ± 41.0^{b}	173 ± 44.9^{a}	162 ± 45.8^{a}	169 ± 38.8^{a}	190 ± 45.7^{a}	152 ± 39.4^{b}

Data represent mean values and standard deviations. Differences were analyzed by two-sided comparisons between the means of different alleles for each gene using the Wilcoxon-test (p-value < 0.05) and are represented with superscript letters

Table 4 Influence of *HvGR-RBP1*/*HvNAM1*/*HvNAM2* allele combinations on malt quality traits in experiment 1 (94 lines) under irrigated conditions

Allele combination	Number of lines	Kernel weight [mg][1]	Malt extract [%][1]	Wort protein [%][2]	S/T [%][2]	DP [°ASBC][2]	α-amylase [°DU][2]	β-glucans [ppm][2]	FAN [ppm][2]
LKK	23	42.9 ± 3.1^{a}	79.2 ± 1.6^{a}	4.8 ± 0.7^{ab}	39.3 ± 6.3^{ab}	117.2 ± 27.2^{ab}	88.4 ± 19.2^{ab}	180.1 ± 95.5^{a}	192.8 ± 46.5^{b}
KKL	41	40.7 ± 2.8^{b}	77.4 ± 1.3^{b}	4.3 ± 0.6^{c}	33.8 ± 4.9^{c}	151.9 ± 34.3^{c}	72.6 ± 18.9^{c}	302.0 ± 140.1^{b}	150.6 ± 41.0^{c}
LKL	12	41.0 ± 2.3^{ab}	76.9 ± 1.5^{b}	4.1 ± 0.5^{c}	32.1 ± 4.0^{c}	133.7 ± 51.3^{bc}	66.6 ± 13.8^{c}	290.5 ± 114.7^{b}	140.4 ± 29.5^{c}
LLK	4	42.7 ± 0.4^{ab}	78.0 ± 1.7^{ab}	4.5 ± 0.7^{bc}	35.2 ± 5.7^{bc}	129.9 ± 31.1^{bc}	74.4 ± 18.7^{bc}	169.2 ± 76.4^{a}	171.5 ± 41.9^{bc}
LLL	14	41.6 ± 2.7^{ab}	77.6 ± 1.8^{b}	4.6 ± 0.6^{bc}	34.6 ± 4.9^{bc}	166.9 ± 31.6^{c}	73.3 ± 15.2^{bc}	290.4 ± 182.1^{b}	168.1 ± 39.5^{bc}
		*	***	*	***	***	**	**	**

Statistical assessments of the effects of allelic combinations on different malt traits were calculated using one-way ANOVA. If assumptions of one-way ANOVA were not met, the Kruskal–Wallis test was used. Allele combinations which show significant differences were further analyzed with an appropriate post hoc test. p values are represented by one star (*) for $p < 0.05$, two stars (**) for $p < 0.01$, or three stars (***) for $p < 0.001$. Differences between the means of allele combinations were analyzed using [1]Tukey's HSD or [2]Dunn's test, with superscript letters indicating significant differences (p-value < 0.05). For allele combinations, the "K" symbol represents the 'Karl' allele, and the "L" symbol denotes the 'Lewis' allele of *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, respectively

Discussion

HvNAM2 sequencing and marker development

The influence of *NAC* genes on senescence, nitrogen remobilization, and grain protein concentration (GPC) has emerged from several studies, starting in 2006 (e.g., Distelfeld et al. 2014; Guo and Gan 2006; Jukanti et al. 2008; Mao et al. 2017; Podzimska-Sroka et al. 2015; Uauy et al. 2006). NAC gene/protein function is often associated with accelerated senescence, although examples of senescence-delaying NAC transcription factors also exist (Sakuraba et al. 2015; Wu et al. 2012). Numerous studies have dissected the molecular underpinnings of flowering time control, with both environmental and endogenous (or autonomous) inputs controlling this developmental transition (e.g., Freytes et al. 2021; Hill and Li, 2016; Woods and Amasino, 2015). Previous work in *Arabidopsis* and in barley indicates that *At*GRP7 and *Hv*GR-RBP1 are involved in autonomous flowering control, with loss-of-function resulting in delayed flowering (see Introduction; Alptekin et al. 2021; Steffen et al. 2019; Streitner et al. 2008). The timing of both flowering and senescence determines grain yield and quality; local adaption of these

traits is therefore of primary importance in crop breeding (Bingham et al. 2007; Coventry et al. 2003; Distelfeld et al. 2014).

Barley variety 'Karl' has been used in malt barley breeding programs since the 1980s to decrease GPC and to improve the performance of malting genotypes (Wesenberg et al. 1976; Burger et al. 1979). Molecular markers for the differentiation of *HvNAM1* 'Karl' and 'Lewis' alleles on chromosome 6H were designed previously and have enabled selection for the delayed-senescence/low-GPC 'Karl' allele (Distelfeld et al. 2008). The recent creation of a marker for *HvGR-RBP1* has allowed the analysis of a second gene on chromosome 6H, which is linked to *HvNAM1* (Alptekin et al. 2021). Here, we have developed a marker for *HvNAM2*, which is associated with an important chromosome 2H QTL influencing grain size, grain weight, and grain length (Pauli et al. 2015; Walker et al. 2013), and malt traits such as malt extract, β-glucans, S/T, and α-amylase activity (Pauli et al. 2015). The two SNPs identified by comparing *HvNAM2* in 'Karl' and 'Lewis' were also identified by Cai et al. (2013) in a population of cultivated and wild Tibetan barley genotypes (158 genotypes, 59 cultivated, and 99 wild barley varieties; Online Resource 8). Hagenblad et al. (2022) recently analyzed a group of Nordic barley varieties and reported that their *HvNAM2* sequences are monomorphic, matching the 'Lewis' sequence. However, 5′ and 3′ flanking sequences were not analyzed by those previous studies. In the present study, a VNTR was identified in the 5′ flanking region, with two repeats of a 20 bp sequence in variety 'Karl', whereas the sequence is present only once in variety 'Lewis' and in the reference genome sequence (Online Resources 6 and 7). While the emphasis of work presented here was on marker development and genotyping, bioinformatic analysis indicates that both the single and the repeated sequences may serve as targets for different transcription factors (Online Resource 11). Variation in repeat number may also enable binding of transcription factor dimers, with head to tail protein dimers recognizing direct sequence repeats (Strader et al. 2022). Implications of the identified VNTR for *HvNAM2* function, if any, remain to be identified in future studies.

The new finding on *HvNAM2*, together with our recent identification of an ~ 400 bp insertion in the 5′-flanking region of the 'Karl' *HvGR-RBP1* gene (Alptekin et al. 2021), emphasizes the importance of analyzing gene flanking regions. For future studies, particularly those analyzing panels in which substantial heterozygosity is expected, the 20 bp VNTR will allow development of an InDel marker with increased throughput.

HvNAM2 allelic state influences plant development, kernel plumpness, grain protein, and malt quality

Data presented here indicate that the 'Karl' *HvNAM2* allele is associated with earlier heading and delayed senescence, thereby increasing the duration of grain filling (Table 1). Delayed senescence is also associated with the 'Karl' *HvNAM1* allele (Alptekin et al. 2021; Jukanti et al. 2008). Although we cannot exclude the possibility that genes linked to *HvNAM2* are responsible for some of the effects observed here, the newly developed *HvNAM2* marker allows selection of germplasm with a higher percentage of plump kernels, higher test weight, and lower GPC (Table 1). Plant development could impact these traits, with early transition to reproduction and delayed senescence extending grain fill duration, allowing the development of larger seeds with lower GPC. This study validates the impact of *HvNAM2* on GPC (Cai et al. 2013) and adds its impact on kernel plumpness. As seed size and GPC are known to affect malt quality (Eagles et al. 1995; Daba et al. 2019), it is not surprising that *HvNAM2* influences every malt quality trait tested (Table 3). The impact of the K allele was mostly positive; it is associated with more extract, higher modification, higher α-amylase activity, and lower β-glucan levels. One problem related to low GPC is reduced diastatic power, which may be sufficient for all-malt but not for adjunct-malt brewing.

Grain and malt quality is improved by selecting favorable allele combinations of HvGR-RBP1, HvNAM1, and HvNAM2

The three genes we interrogated, *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, both separately and together impact barley flowering, senescence, agronomics, and malt quality. Our data confirm previous reports indicating that the *HvNAM1* 'Karl' allele delays senescence and lowers GPC (Jukanti et al. 2008; Lacerenza et al. 2010; Distelfeld et al. 2014). Alptekin et al. (2021) have recently reported the effect of

HvGR-RBP1 on heading date and seed size and the combined effects of *HvGR-RBP1* and *HvNAM1* on grain fill duration. The L allele of *HvGR-RBP1* and the K allele of *HvNAM2* are associated with earlier heading and larger seeds, which improve malt quality. K alleles for *HvNAM1* and *HvNAM2* are associated with lower GPC (Tables 1 and 2) (Alptekin et al. 2021). The observation that *HvNAM1* primarily impacts GPC and *HvNAM2* affects both GPC and seed size may indicate that the two *NAC* genes regulate overlapping but not identical gene sets or that observed effects of *HvNAM2* allelic state are partially due to the activity of linked genes.

One confounding factor could be the linkage between K alleles for *HvNAM1* (low protein) and *HvGR-RBP1* (smaller seeds). However, the creation of the marker for *HvGR-RBP1* (Alptekin et al. 2021) allows breaking this linkage; here, we identify lines with the favorable alleles for both *HvGR-RBP1* (L) and *HvNAM1* (K). In fact, 36% of the lines tested were recombinants between *HvGR-RBP1* and *HvNAM1* (Online Resource 1), indicating selection for the LK combination on chromosome 6H. The observation that previously developed low-GPC lines also had a low percentage of plump seeds compared to high-protein lines (Weston et al. 1993) may be explained by the negative effects of linkage between *HvGR-RBP1* (K) and *HvNAM1* (K) alleles.

In experiment 1, *HvNAM1* had little effect on malt quality (only impacting GPC), while *HvGR-RBP1* impacted kernel weight and plumpness, extract, β-glucan levels, and FAN, and *HvNAM2* impacted seed size, GPC, and all malt quality traits (Tables 1 and 3) (Alptekin et al. 2021). More importantly, the combination of favorable alleles for all three genes improved malt quality significantly. The LKK genotype had the lowest GPC, highest extract, and best malt modification, although diastatic power (DP) was too low for adjunct brewing (Tables 2 and 4). However, observing the effects of *HvNAM1* and *HvNAM2* on DP individually and in combination suggests that DP can be modulated by selection for these two genes. Choosing the K allele of either *HvNAM1* or *HvNAM2* should result in higher DP than including both, and from their individual effects, it appears that the *HvNAM1* K allele has a less negative effect on DP than the *HvNAM2* K allele (Table 3). An additional advantage of the LKK genotype may be its performance under dryland conditions, where grain fill duration was extended, seeds were larger, and GPC lower than most genotypes under irrigation, suggesting more stable malt quality from dryland agriculture (Online Resource 9). Analyses performed for this study indicate that the recently released variety 'Buzz' (https://www.montana.edu/barleybreeding/learning-center/barley-variety-dictionary/two-row/buzz-barley.html) possesses the LKK allele combination, providing improved malt quality.

Concluding remarks

Taken together, data from this study support previous findings regarding the association of the studied chromosome 2H region with malt phenotype traits and provide a new marker allowing selection for the 'Karl' *HvNAM2* allele in breeding programs. The study also indicates that selection for favorable allele combinations of three genes influencing barley flowering and senescence, namely *HvGR-RBP1*, *HvNAM1*, and *HvNAM2*, improves both agronomic and malt quality parameters.

Acknowledgements The authors would like to thank Christopher Martens for malt phenotyping.

Author contribution BA contributed to *HvNAM2* sequencing and marker development, performed *HvNAM1* and *HvGR-RBP1* genotyping, performed all statistical analyses (guided and supported by JL), and wrote a draft manuscript. ME contributed to *HvNAM2* sequencing, developed the VNTR marker, and performed *HvNAM2* genotyping. DM performed experiment 2 field studies, supported by JS's breeding program. DP and TB shared field data for experiment 1. HT helped with malt phenotyping. AMF and JS obtained funding and directed the study. All authors have reviewed and approved the manuscript, as submitted to *Molecular Breeding*.

Funding This project was supported by funding from the Montana Agricultural Experiment Station (MAES), the Montana Wheat and Barley Committee, the Brewers Association, the American Malting Barley Association, and the United States of Agriculture—National Institute of Food and Agriculture (USDA-NIFA) (2017–68008-26209).

Data availability *HvNAM2* sequences from varieties 'Karl' and 'Lewis' are shown in Online Resource 6. Genotyping data for *HvGR-RBP1*, *HvNAM1*, and *HvNAM2* are shown in Online Resource 1; agronomic and malt quality data for experiment 1 (Pauli et al. 2015) are shown in Online Resources 2 and 4, and agronomic data for experiment 2 (Alptekin et al. 2021) are shown in Online Resource 3.

Code availability No new code was generated as a part of this study.

Declarations

Conflict of interest The authors declare no competing interests.

References

Alptekin B, Mangel D, Pauli D, Blake T, Lachowiec J, Hoogland T, Fischer A, Sherman J (2021) Combined effects of a glycine-rich RNA-binding protein and a NAC transcription factor extend grain fill duration and improve malt barley agronomic performance. Theor Appl Genet 134:351–366. https://doi.org/10.1007/s00122-020-03701-1

Ayoub M, Symons J, Edney J, Mather E (2002) QTLs affecting kernel size and shape in a two-rowed by six-rowed barley cross. Theor Appl Genet 105:237–247. https://doi.org/10.1007/s00122-002-0941-1

Baik B-K, Ullrich SE (2008) Barley for food: characteristics, improvement, and renewed interest. J Cereal Sci 48:233–242. https://doi.org/10.1016/j.jcs.2008.02.002

Bingham IJ, Blake J, Foulkes MJ, Spink J (2007) Is barley yield in the UK sink limited? II. Factors affecting potential grain size. Field Crops Res 101:212–220. https://doi.org/10.1016/j.fcr.2006.11.004

Brouwer BO, Schwarz PB, Barr JM, Hayes PM, Murphy KM, Jones SS (2016) Evaluating barley for the emerging craft malting industry in western Washington. Agron J 108:939–949. https://doi.org/10.2134/agronj2015.0385

Burger WC, Wesenberg DM, Carden JE, Pawlisch PE (1979) Protein content and composition of Karl and related barleys. Crop Sci 19:235–238. https://doi.org/10.2135/cropsci1979.0011183X001900020016x

Cai S, Yu G, Chen X, Huang Y, Jiang X, Zhang G, Jin X (2013) Grain protein content variation and its association analysis in barley. BMC Plant Biol 13:35. https://doi.org/10.1186/1471-2229-13-35

Cao S, Jiang L, Song S, Jing R, Xu G (2006) *AtGRP7* is involved in the regulation of abscisic acid and stress responses in *Arabidopsis* Cell Mol Biol Lett 11:526–535. https://doi.org/10.2478/s11658-006-0042-2

Chappell A, Scott KP, Griffiths IA, Cowan AA, Hawes C, Wishart J, Martin P (2017) The agronomic performance and nutritional content of oat and barley varieties grown in a northern maritime environment depends on variety and growing conditions. J Cereal Sci 74:1–10. https://doi.org/10.1016/j.jcs.2017.01.005

Ciuzan O, Hancock J, Pamfil D, Wilson I, Ladomery M (2015) The evolutionarily conserved multifunctional glycine-rich RNA-binding proteins play key roles in development and stress adaptation. Physiol Plant 153:1–11. https://doi.org/10.1111/ppl.12286

Coventry SJ, Barr AR, Eglinton JK, McDonald GK (2003) The determinants and genome locations influencing grain weight and size in barley (*Hordeum vulgare* L.). Aust J Agric Res 54:1103–1115. https://doi.org/10.1071/ar02194

Daba S, Horsley R, Schwarz P, Chao S, Capettini F, Mohammadi M (2019) Association and genome analyses to propose putative candidate genes for malt quality traits. J Sci Food Agric 99:2775–2785. https://doi.org/10.1002/jsfa.9485

Delignette-Muller ML, Dutang C (2015) fitdistrplus: an R package for fitting distributions. J Stat Softw 64:1–34. https://doi.org/10.18637/jss.v064.i04

Distelfeld A, Avni R, Fischer AM (2014) Senescence, nutrient remobilization, and yield in wheat and barley. J Exp Bot 65:3783–3798. https://doi.org/10.1093/jxb/ert477

Distelfeld A, Korol A, Dubcovsky J, Uauy C, Blake T, Fahima T (2008) Colinearity between the barley grain protein content (GPC) QTL on chromosome arm 6HS and the wheat *Gpc-B1* region. Mol Breed 22:25–38. https://doi.org/10.1007/s11032-007-9153-3

Eagles HA, Bedggood AG, Panozzo JF, Martin PJ (1995) Cultivar and environmental effects on malting quality in barley. Aust J Agric Res 46:831–844. https://doi.org/10.1071/ar9950831

Elía M, Swanston JS, Moralejo M, Casas A, Pérez-Vendrell AM, Ciudad FJ, Thomas WTB, Smith PL, Ullrich SE, Molina-Cano JL (2010) A model of the genetic differences in malting quality between European and North American barley cultivars based on a QTL study of the cross Triumph × Morex. Plant Breed 129:280–290. https://doi.org/10.1111/j.1439-0523.2009.01694.x

Fan C, Zhai H, Wang H, Yue Y, Zhang M, Li J, Wen S, Guo G, Zeng Y, Ni Z, You M (2017) Identification of QTLs controlling grain protein concentration using a high-density SNP and SSR linkage map in barley (*Hordeum vulgare* L.). BMC Plant Biol 17:122. https://doi.org/10.1186/s12870-017-1067-6

Fang Y, Zhang X, Xue D (2019) Genetic analysis and molecular breeding applications of malting quality QTLs in barley. Front Genet 10:352. https://doi.org/10.3389/fgene.2019.00352

Freytes SN, Canelo M, Cerdán PD (2021) Regulation of flowering time: when and where? Curr Opin Plant Biol 63:102049. https://doi.org/10.1016/j.pbi.2021.102049

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601–612. https://doi.org/10.1111/j.1365-313X.2006.02723.x

Gupta M, Abu-Ghannam N, Gallaghar E (2010) Barley for brewing: characteristic changes during malting, brewing and applications of its by-products. Compr Rev Food Sci Food Saf 9:318–328. https://doi.org/10.1111/j.1541-4337.2010.00112.x

Hagenblad J, Vanhala T, Madhavan S, Leino MW (2022) Protein content and *HvNAM* alleles in Nordic barley (*Hordeum vulgare*) during a century of breeding. Hereditas 159:12. https://doi.org/10.1186/s41065-022-00227-y

Hill CB, Li C (2016) Genetic architecture of flowering phenology in cereals and opportunities for crop improvement. Front Plant Sci 7:1906. https://doi.org/10.3389/fpls.2016.01906

Hockett EA, Gilbertson KM, McGuire CF, Bergman LE, Wiesner LE, Robbins GS (1985) Registration of 'Lewis' barley. Crop Sci 25:570–571. https://doi.org/10.2135/cropsci1985.0011183X002500030036x

Homan MM (2004) Beer and its drinkers: an ancient Near Eastern love story. Near East Archaeol 67:84–95. https://doi.org/10.2307/4132364

Jensen MK, Skriver K (2014) NAC transcription factor gene regulatory and protein-protein interaction networks in plant stress responses and senescence. IUBMB Life 66:156–166. https://doi.org/10.1002/iub.1256

Jukanti AK, Fischer AM (2008) A high-grain protein content locus on barley (*Hordeum vulgare*) chromosome 6 is associated with increased flag leaf proteolysis and nitrogen remobilization. Physiol Plant 132:426–439. https://doi.org/10.1111/j.1399-3054.2007.01044.x

Jukanti AK, Heidlebaugh NM, Parrott DL, Fischer IA, McInnerney K, Fischer AM (2008) Comparative transcriptome profiling of near-isogenic barley (*Hordeum vulgare*) lines differing in the allelic state of a major grain protein content locus identifies genes with possible roles in leaf senescence and nitrogen reallocation. New Phytol 177:333–349. https://doi.org/10.1111/j.1469-8137.2007.02270.x

Kim JS, Jung HJ, Lee HJ, Kim KA, Goh C-H, Woo Y, Oh SH, Han YS, Kang H (2008) Glycine-rich RNA-binding protein 7 affects abiotic stress responses by regulating stomata opening and closing in *Arabidopsis thaliana* Plant J 55:455–466. https://doi.org/10.1111/j.1365-313X.2008.03518.x

Lacerenza JA, Parrott DL, Fischer AM (2010) A major grain protein content locus on barley (*Hordeum vulgare* L.) chromosome 6 influences flowering time and sequential leaf senescence. J Exp Bot 61:3137–3149. https://doi.org/10.1093/jxb/erq139

Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, Basutkar P, Tivey ARN, Potter SC, Finn RD, Lopez R (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res 47:W636–W641. https://doi.org/10.1093/nar/gkz268

Mao C, Lu S, Lv B, Zhang B, Shen J, He J, Luo L, Xi D, Chen X, Ming F (2017) A rice NAC transcription factor promotes leaf senescence via ABA biosynthesis. Plant Physiol 174:1747–1763. https://doi.org/10.1104/pp.17.00542

Mascher M, Gundlach H, Himmelbach A et al (2017) A chromosome conformation capture ordered sequence of the barley genome. Nature 544:427–433. https://doi.org/10.1038/nature22043

Mickelson S, See D, Meyer FD, Garner JP, Foster CR, Blake TK, Fischer AM (2003) Mapping of QTL associated with nitrogen storage and remobilization in barley (*Hordeum vulgare* L.) leaves. J Exp Bot 54:801–812. https://doi.org/10.1093/jxb/erg084

Nice L, Huang Y, Steffenson BJ, Gyenis L, Schwarz P, Smith KP, Muehlbauer GJ (2019) Mapping malting quality and yield characteristics in a north American two-rowed malting barley × wild barley advanced backcross population. Mol Breed 39:121. https://doi.org/10.1007/s11032-019-1030-3

O'Donovan JT, Clayton GW, Grant CA, Harker KN, Turkington TK, Lupwayi NZ (2008) Effect of nitrogen rate and placement and seeding rate on barley productivity and wild oat fecundity in a zero tillage system. Crop Sci 48:1569–1574. https://doi.org/10.2135/cropsci2007.10.0587

Ochoa A, Storey JD (2021) Estimating F_{ST} and kinship for arbitrary population structures. PLoS Genet 17:e1009241. https://doi.org/10.1371/journal.pgen.1009241

Parrott DL, Downs EP, Fischer AM (2012) Control of barley (*Hordeum vulgare* L.) development and senescence by the interaction between a chromosome six grain protein content locus, day length, and vernalization. J Exp Bot 63:1329–1339. https://doi.org/10.1093/jxb/err360

Pauli D, Brown-Guedira G, Blake TK (2015) Identification of malting quality QTLs in advanced generation breeding germplasm. J Am Soc Brew Chem 73:29–40. https://doi.org/10.1094/asbcj-2015-0129-01

Peterson RA, Cavanaugh JE (2020) Ordered quantile normalization: a semiparametric transformation built for the cross-validation era. J Appl Stat 47:2312–2327. https://doi.org/10.1080/02664763.2019.1630372

Podzimska-Sroka D, O'Shea C, Gregersen P, Skriver K (2015) NAC transcription factors in senescence: from molecular structure to function in crops. Plants 4:412–448. https://doi.org/10.3390/plants4030412

Sakuraba Y, Piao W, Lim J-H, Han S-H, Kim Y-S, An G, Paek N-C (2015) Rice ONAC106 inhibits leaf senescence and increases salt tolerance and tiller angle. Plant Cell Physiol 56:2325–2339. https://doi.org/10.1093/pcp/pcv144

Schüttpelz M, Schöning JC, Doose S, Neuweiler H, Peters E, Staiger D, Sauer M (2008) Changes in conformational dynamics of mRNA upon *At*GRP7 binding studied by fluorescence correlation spectroscopy. J Am Chem Soc 130:9507–9513. https://doi.org/10.1021/ja801994z

See D, Kanazin V, Kephart K, Blake T (2002) Mapping genes controlling variation in barley grain protein concentration. Crop Sci 42:680–685. https://doi.org/10.2135/cropsci2002.6800

Steffen A, Elgner M, Staiger D (2019) Regulation of flowering time by the RNA-binding proteins *At*GRP7 and *At*GRP8. Plant Cell Physiol 60:2040–2050. https://doi.org/10.1093/pcp/pcz124

Stevens WB, Sainju UM, Caesar-TonThat T, Iversen WM (2015) Malt barley yield and quality affected by irrigation, tillage, crop rotation, and nitrogen fertilization. Agron J 107:2107–2119. https://doi.org/10.2134/agronj15.0027

Strader L, Weijers D, Wagner D (2022) Plant transcription factors - being in the right place with the right company. Curr Opin Plant Biol 65:102136. https://doi.org/10.1016/j.pbi.2021.102136

Streitner C, Danisman S, Wehrle F, Schöning JC, Alfano JR, Staiger D (2008) The small glycine-rich RNA binding protein *At*GRP7 promotes floral transition in *Arabidopsis thaliana* Plant J 56:239–250. https://doi.org/10.1111/j.1365-313X.2008.03591.x

Tripet BP, Mason KE, Eilers BJ, Burns J, Powell P, Fischer AM, Copié V (2014) Structural and biochemical analysis of the *Hordeum vulgare* L. *Hv*GR-RBP1 protein, a glycine-rich RNA-binding protein involved in the regulation of barley plant development and stress response. Biochemistry 53:7945–7960. https://doi.org/10.1021/bi5007223

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A *NAC* gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301. https://doi.org/10.1126/science.1133649

Walker CK, Ford R, Muñoz-Amatriain M, Panozzo JF (2013) The detection of QTLs in barley associated with endosperm hardness, grain density, grain size and malting quality using rapid phenotyping tools. Theor Appl Genet 126:2533–2551. https://doi.org/10.1007/s00122-013-2153-2

Waters BM, Uauy C, Dubcovsky J, Grusak MA (2009) Wheat (*Triticum aestivum*) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. J Exp Bot 60:4263–4274. https://doi.org/10.1093/jxb/erp257

Wesenberg DM, Hayes RM, Standridge NN, Burger WC, Goplin ED, Petr FC (1976) Registration of Karl barley (Reg. No. 147). Crop Sci 16:737. https://doi.org/10.2135/cropsci1976.0011183X001600050039x

Weston DT, Horsley RD, Schwarz PB, Goos RJ (1993) Nitrogen and planting date effects on low-protein spring barley. Agron J 85:1170–1174. https://doi.org/10.2134/agronj1993.00021962008500060015x

Wimmer V, Albrecht T, Auinger H-J, Schön C-C (2012) synbreed: a framework for the analysis of genomic prediction data using R. Bioinformatics 28:2086–2087. https://doi.org/10.1093/bioinformatics/bts335

Woods DP, Amasino R (2015) Dissecting the control of flowering time in grasses using *Brachypodium distachyon*. In: Vogel J (ed) Genetics and genomics of *Brachypodium*. Springer, Cham, pp 259–273. https://doi.org/10.1007/7397_2015_10

Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munné-Bosch S, Antonio C, Tohge T, Fernie AR, Kaufmann K, Xue GP, Mueller-Roeber B, Balazadeh S (2012) JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in *Arabidopsis* Plant Cell 24:482–506. https://doi.org/10.1105/tpc.111.090894

Yang DH, Kwak KJ, Kim MK, Park SJ, Yang KY, Kang H (2014) Expression of *Arabidopsis* glycine-rich RNA-binding protein *At*GRP2 or *At*GRP7 improves grain yield of rice (*Oryza sativa*) under drought stress conditions. Plant Sci 214:106–112. https://doi.org/10.1016/j.plantsci.2013.10.006

Ziyatdinov A, Vázquez-Santiago M, Brunel H, Martinez-Perez A, Aschard H, Soria JM (2018) lme4qtl: linear mixed models with flexible covariance structure for genetic studies of related individuals. BMC Bioinform 19:68. https://doi.org/10.1186/s12859-018-2057-x