Closing Report

Establishment of genome editing in barley and other crop species for research and crop improvement.

NKFI K125300, 2017-12-01 - 2023-11-30, PI Dr Zoltan Havelda

The main planned goals of the closing research project were the following:

1. Generation of gene knock-out lines for investigating RNA interference regulators in barley.

2. Targeted genome editing by CRISPR/Cas9 system in barley to establish virus resistance and modify economically important traits.

1.

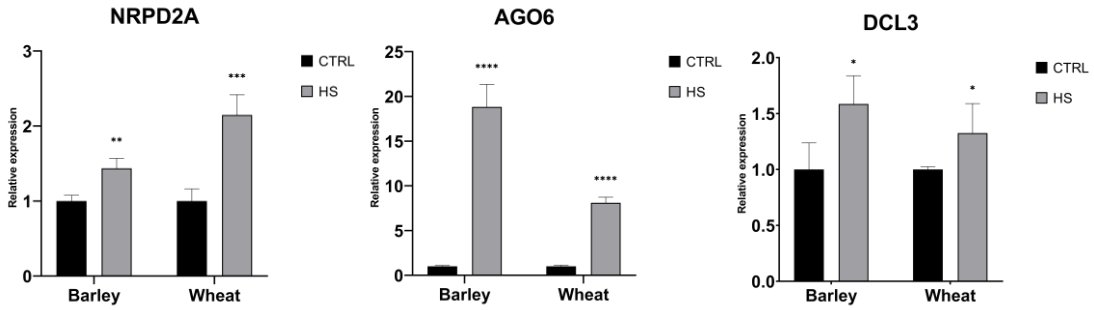
RNA interference (RNAi) is associated with small regulatory RNAs and are involved in many aspects of plant development, including leaf, shoot, flower, and fruit and root development, phase-change, auxin signaling and response to environmental/biotic stresses. One of the important RNAi pathways is associated with the activity of small interfering RNAs (siRNAs), typically24-nt in length, generated by mainly by DICER 3 (DCL3) via processing of Pol IV/RNA DEPENDENT RNA POLYMERASE 2 (RDR2)-dependent double-stranded RNA (dsRNA) species originating mainly from intergenic or repetitive regions of the genome. These 24-nt-long siRNAs are loaded into the RNA-induced transcriptional silencing (RITS) complexes, containing ARGONAUTE4 and 6 (AGO4 and AGO6) executor proteins. This pathway plays fundamental roles in maintaining genome integrity, by initiating de novo DNA methylation on transposable and repetitive elements mainly during reproductive transitions (meiosis, gametogenesis and embryogenesis), especially in plants with larger, repetitive genomes. The aim of these experiments to understand the function and biological importance of this pathway in developmental processes and stress perception in monocots especially in barley, possessing a large genome (5GB) compared to *Arabidopsis thaliana* (235 Mb).

Identification barley RNAi factors

To investigate the barley RNAi pathway first we identified and analyzed the RNAi components of barley. In that work, we identified the members of barley DCL (HvDCL), AGO (HvAGO), and RDR (HvRDR) gene families, important members of the RNA Directed DNA Methylation (RdDM) pathway, analyzed their phylogenetic relationship to model and crop plants, investigated their domain architecture and core catalytic motifs/regions. Transcriptional

changes of RNA silencing trans factors suggests that siRNA-based silencing may play a role in adaptation to heat stress conditions. We selected various RNAi factors and assessed their change during three different heat stress regimes, including moderate HS (35.5 °C/48 h), prolonged heat (40 °C/24 h) and gradient elevation of heat (21–37 °C/4 h). Significant accumulation of HvDCL3, HvAGO2, HvAGO6, HvRDR2 and HvRDR6a mRNA was confirmed. Contrarily, the principal trans factors of the miRNA pathway, HvDCL1 and HvAGO1a seem to be much stable under the investigated circumstances.

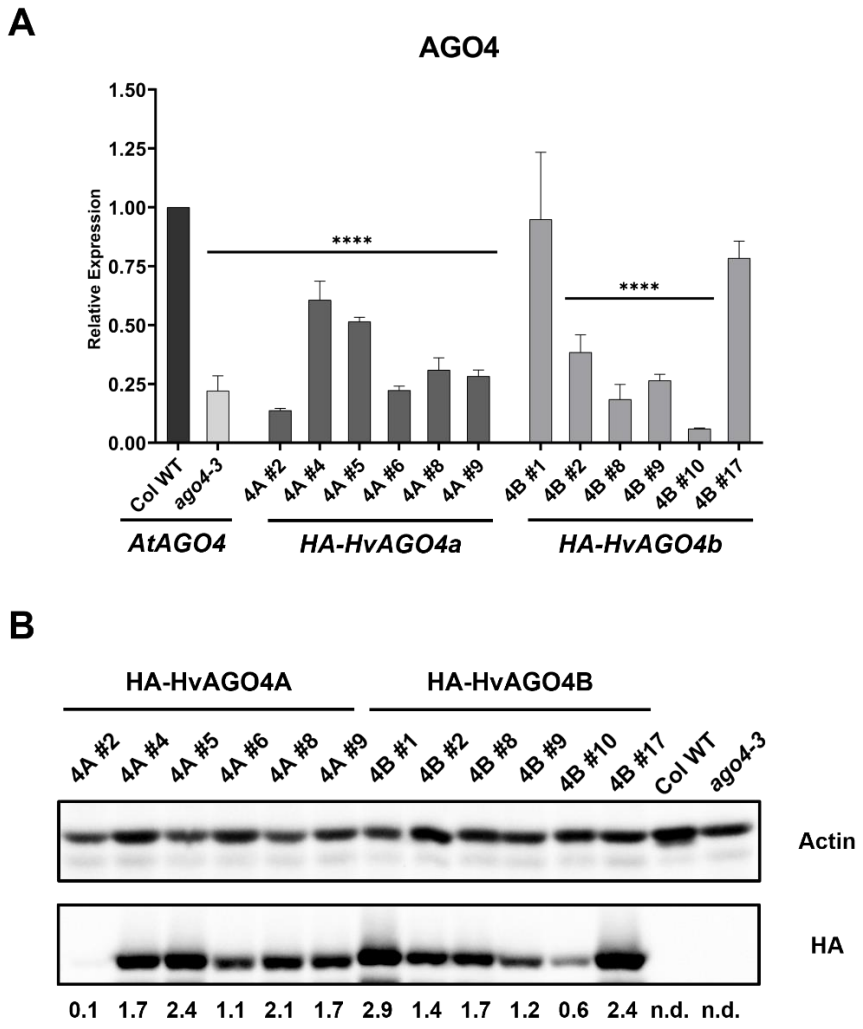
This data was published: Hamar E., Szaker H.M., Kis A., Dalmadi A., Miloro F., Szittya G., Taller J., Gyula P.*, Csorba T.*, Havelda Z.*. (2020). **Genome-wide identification of RNA silencing-related genes and their expressional analysis in response to heat stress in barley** (Hordeum vulgare L.). Biomolecules 10, 929; doi:10.3390/biom10060929. (IF 4,082)



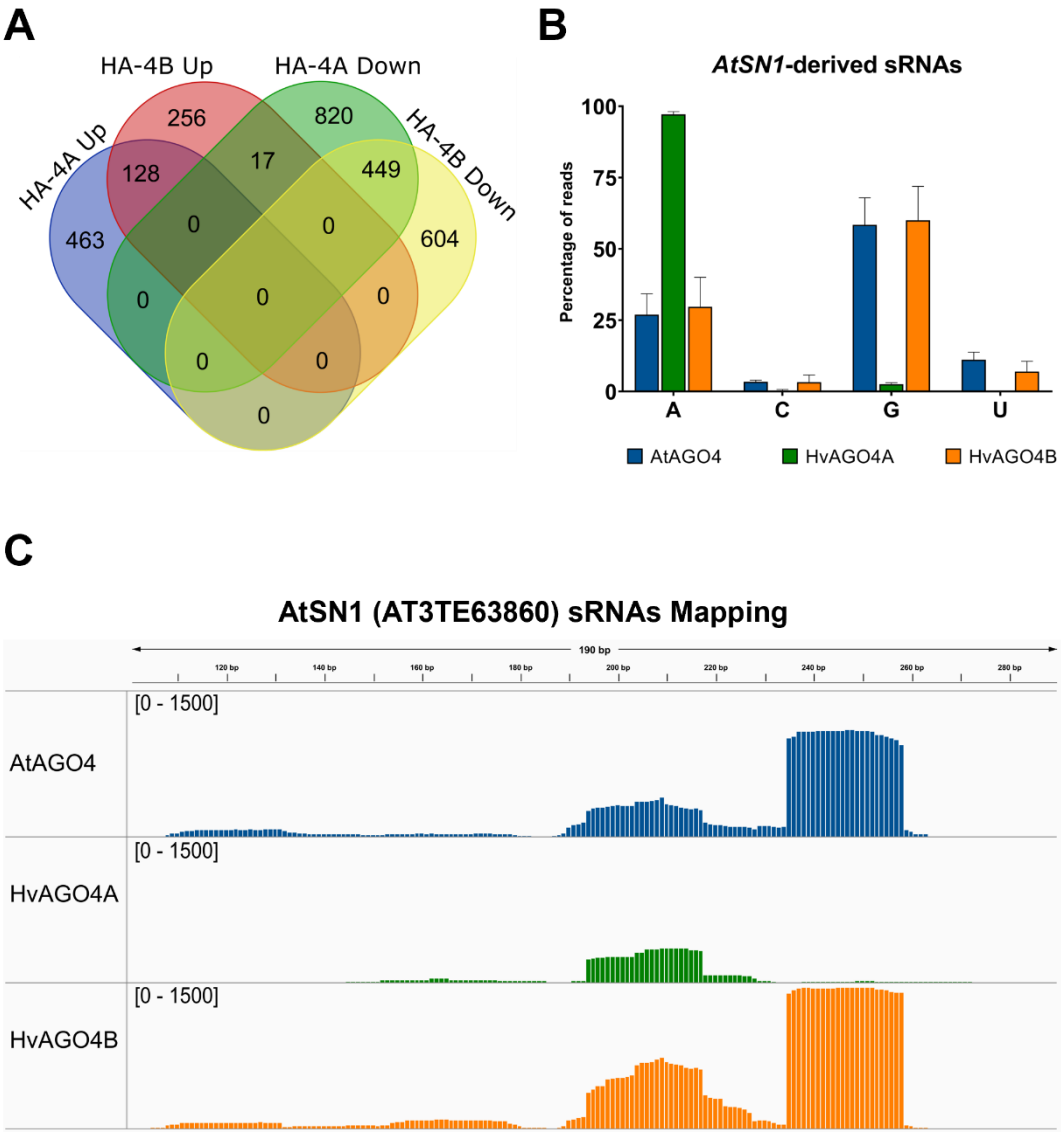
Later we showed that members of the RdDM pathway induced not only in barley but also in wheat due to heat stress indicating that this pathway can a major component of heat perception.

Characterization of barley AGO4 proteins

First, we have identified two active paralogous barley genes and one presumed pseudogene within the AGO4 clade by *in silico* analysis. Next, we functionally investigated the two active AGO4 proteins by transforming their HA tagged version in Arabidopsis ago4 knock-out background and investigated their complementation ability. For AGO4-associated sRNAs, mixed-stage inflorescences of 7-week-old plants from 3 independent T3 complementation lines of HvAGO4a and HvAGO4b were collected and processed using Dynabeads Protein G Immunoprecipitation Kit with anti-HA-peroxidase antibody according to the manufacturer's instructions. The immunoprecipitated fractions were eluted and used for RNA purification and protein samples. RNA was extracted using standard phenol-chloroform method and used to generate cDNA libraries for sRNA-IP sequencing using Truseq Small RNA Library Preparation Kit (Illumina).



AtAGO4 and HA-tagged barley AGO4 gene expression levels determined by RT-qPCR of T1 mixed-stage inflorescences. Data were normalized using AtUBC9 and AtACT2. The average of 3 independent biological replicates was calculated and statistically significant differences from Columbia wild type (Col WT) are indicated with asterisks.



Venn diagram showing the number of TEs where the amount of sRNAs show at least 2-fold statistically significant (p-value<0.05) change compared to AtAGO4. B. Percentage of the AtSN1-derived sRNAs in the categories based on 5' end nucleotide. Error bars represent the SD of the three sequenced lines. C. SRNAs mapped on AtSN1 genomic locus. The reads are merged from the three independent biological replicate IP datasets. The quantity of mapped reads (counts) is written on the left side of the image inside the square brackets. Different colors indicate individual IP datasets: AtAGO4 (blue), HA-HvAGO4A (green) and HA-HvAGO4B (orange).

We demonstrated different properties in sRNA binding and functionality of barley AGO4 proteins via heterologous complementation in Arabidopsis ago4 mutant background. This approach facilitated the investigation of the distinct ways of action of the two HvAGO4 proteins in the downregulation of RdDM targets, and their specific preferences for sRNAs located in different regions of repeats and TEs. Bioinformatic analysis of RNA sequencing data identified two active AGO4 genes in barley, HvAGO4a and HvAGO4b. Arabidopsis

heterologous complementation assay verified their function reminiscent to AtAGO4 as both of them reversed the molecular consequences of ago4-3 mutation in terms of the investigated loci. As expected, they incorporate preferably 24 nucleotides long small RNAs (sRNAs) having adenine at their 5’ end, but in a various extent. While HvAGO4A exclusively binds sRNAs with 5' A, HvAGO4B, like AtAGO4, accepts also 5' G, U and C. This diverse binding capacity is reflected by the wide range of TE-derived sRNAs and, in some cases, in their varying loading. Both barley AGO4 proteins effectively restore the extrachromosomal DNA and transcript levels of heat-activated ONSEN retrotransposon to the wild type level.

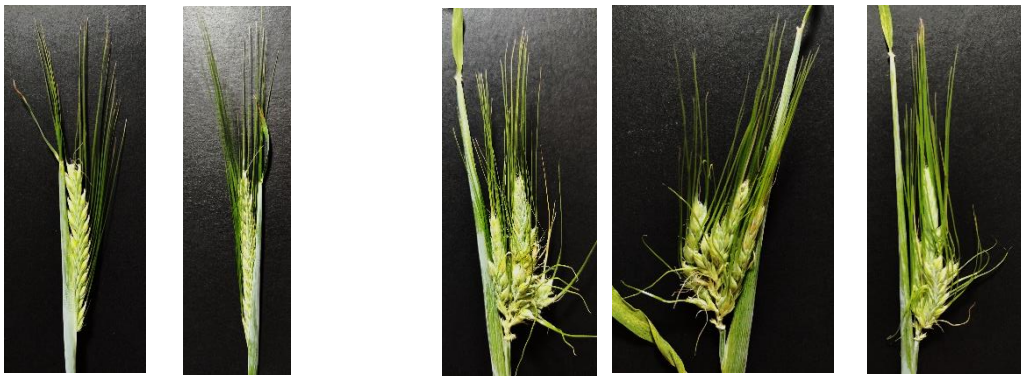
Manuscript in preparation: Fabio Miloro, András Kis, Zoltán Havelda, Ágnes Dalmadi (2023) **Functional Characterization of Barley AGO4 paralogs in Arabidopsis heterologous Complementation**.

Analyses ago4 knock-out mutants

Following this in silico investigation, to study the function of the two active putative genes, mutations were performed on each and both genes using a multiplexed CRISPR/Cas9 system. This system uses a series of gRNAs and tRNAs that are cleaved by the plant's endogenous endonucleases to yield sgRNAs, but with a single promoter and increased expression. This technique allows for higher efficiency precisely because of the increased expression. 2 sgRNAs were designed to induce a mutation in each AGO4 gene, and all 4 were used to generate a double mutant. The sgRNAs were also designed to target regions of the gene important to produce protein domains essential for protein function, and were also designed to be spaced about 1000-1500 bp apart to induce a deletion of a larger region. In this way, both the effects of the single gene and the understanding of its actual function in the plant can be studied, as well as how the two genes manage to restore the phenotype in the case of a single mutated gene and the phenotype in the case of a double mutant. In the case of HvAGO4a, both sgRNAs induced different types of mutations, including single base indels, but a line with a 1324 bp deletion was also observed. In contrast, in the case of HvAGO4b, only one sgRNA effectively induced mutations, specifically indels of 1-2 bp, deletions of 36 bp and 875 bp. Finally, in the case of double mutants, mainly 1 sgRNA for HvAGO4a and 1 sgRNA for HvAGO4b functioned effectively, again resulting in small indels but also large deletions.

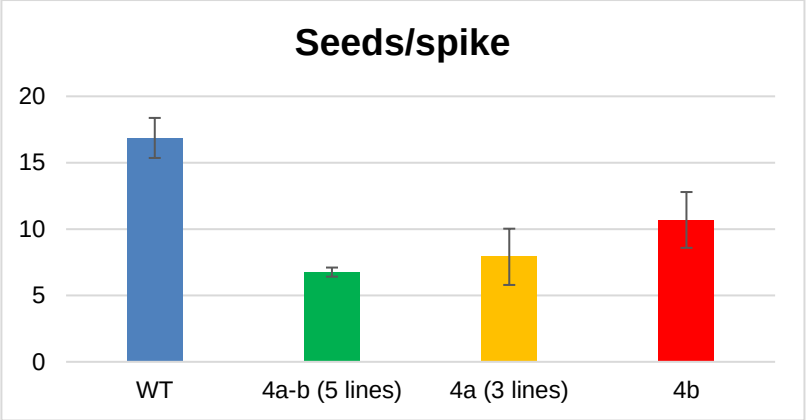
Several T0 lines were selected that were positive for Cas9 and dsRED (fluorescent marker) and showed mutations after in vitro regeneration. From these T0 lines, T1 lines were selected that were mutated in at least one region of the affected gene, resulting in 3 lines for ago4a, 3 lines for ago4b, and 5 lines for ago4a-b. Plants of the mutants were grown together with wild type Golden Promise plants as controls in a controlled environment with a day temperature of 20°C and a night temperature of 18°C. The mutant plants grew under these conditions with a wild type phenotype, although a slight reduction in height and a bushier appearance was observed in the double mutant. Subsequently, the plants were visually analysed during spike emergence and it was observed that in the case of the single mutant plants there were spikes with fewer seeds or completely without seeds, while the double

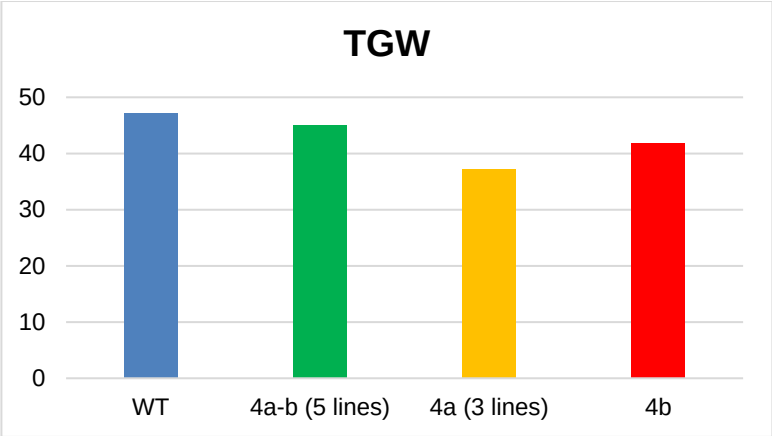
mutant plants showed abnormal spikes with multiple branching or sterile central floret and the presence of some fertile lateral florets that produced seeds, an abnormal phenomenon in Golden Promise, which is a 2-row barley and where only the central floret is capable of producing fertile seeds.



wt **ago4a-b double mutants**

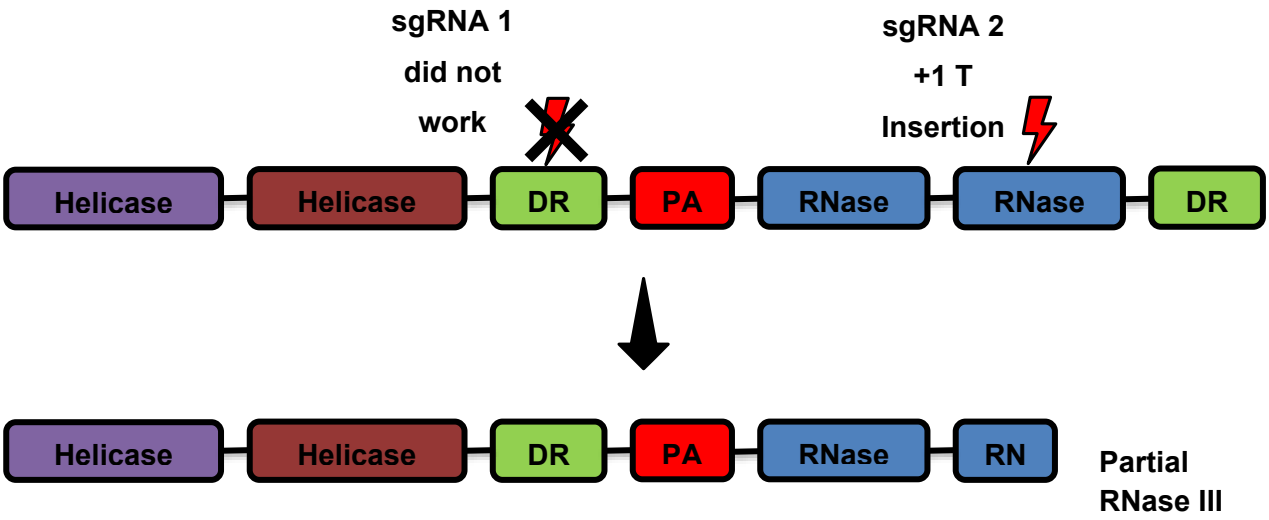
These differences, found only in some double mutant plants, showed that mutating only one of the two genes is not enough to see their function. For each plant, we took 15 spikes and collected the seeds from these spikes to calculate the average seeds per spike and the Thousand Grain Weight (TGW).



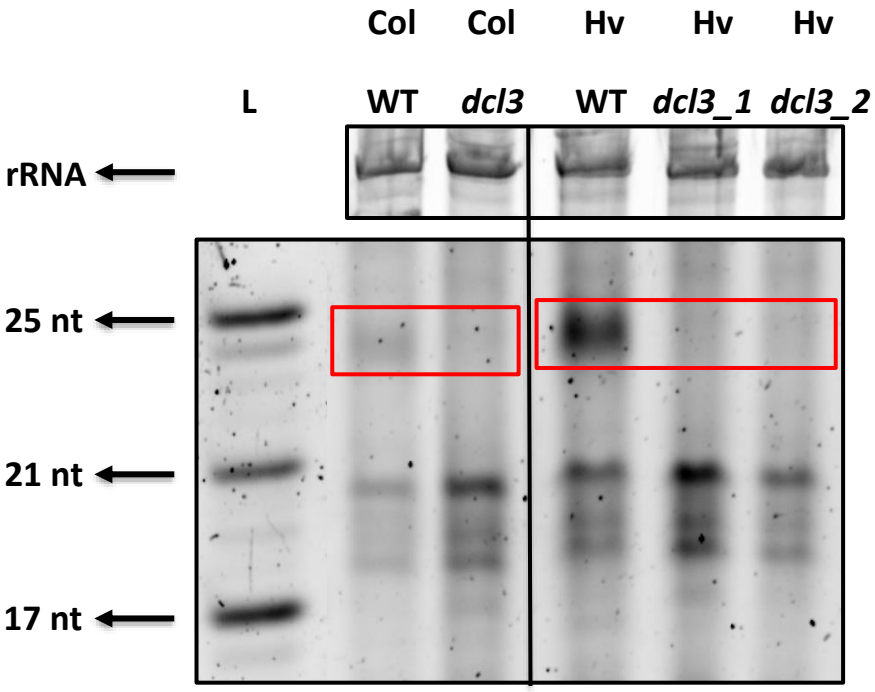


These data indicate that **AGO4 proteins determine pivotal functions in barley during development of generative organs**.

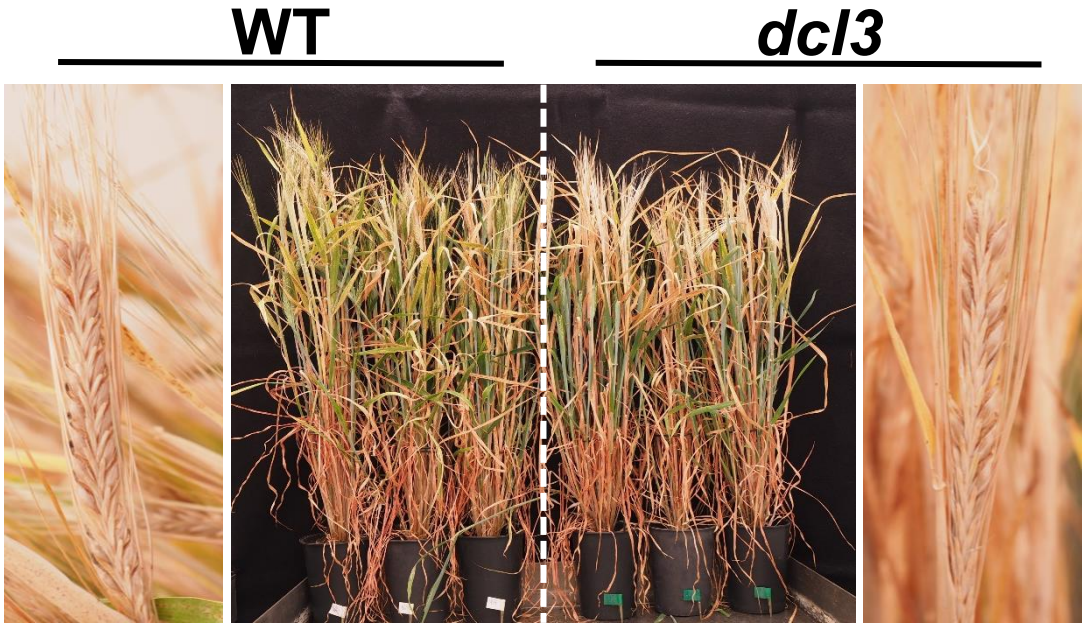
Analyses dcl3 knock-out mutants



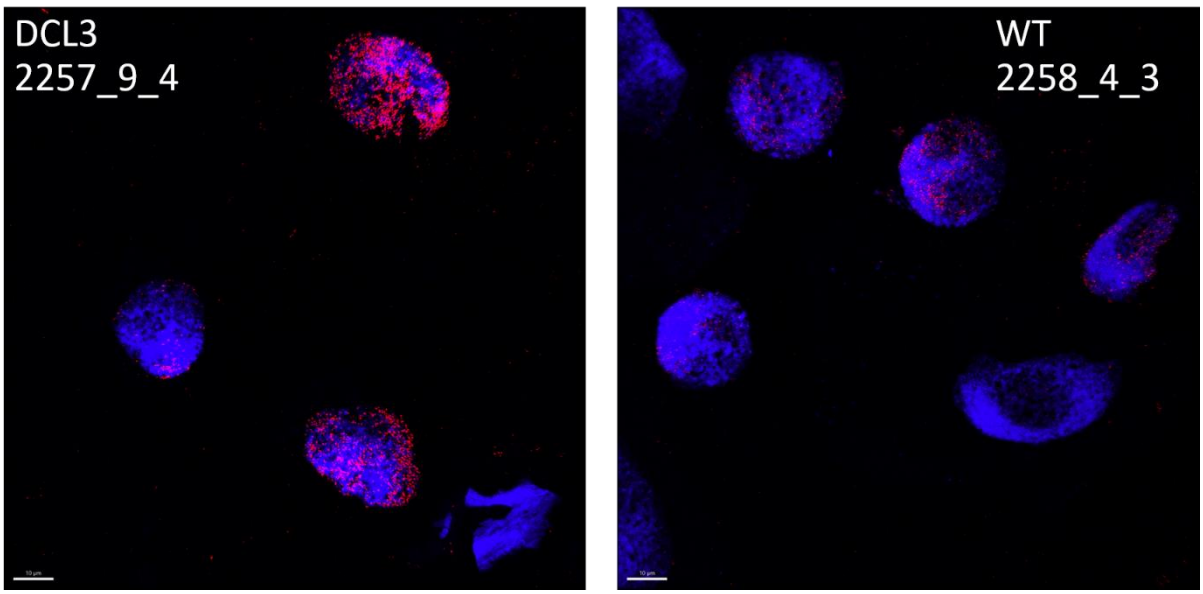
We have generated CRISPR/Cas9 mediated dcl3 mutant lines. Unfortunately, one of the designed guide RNAs doesn't work, so we get mutants only at the RNAase domain. We identified three independent mutants, interestingly the three mutants carried the same insertion (+1T) generating a premature stop codon rendering the RNAase domain to be truncated. We further propagated the mutant lines to generate homozygous T3 knock-out lines.



It is expected that dcl3 mutant lines, since inhibited in the production of 24 nt siRNA, the accumulation of these siRNA species is inhibited. Indeed, Sybergold staining of total RNA samples derived form young endosperm reveled that the siRNA accumulation in barley mutants severely inhibited (red rectangular), demonstrating the presence and effect of the introduced mutation.



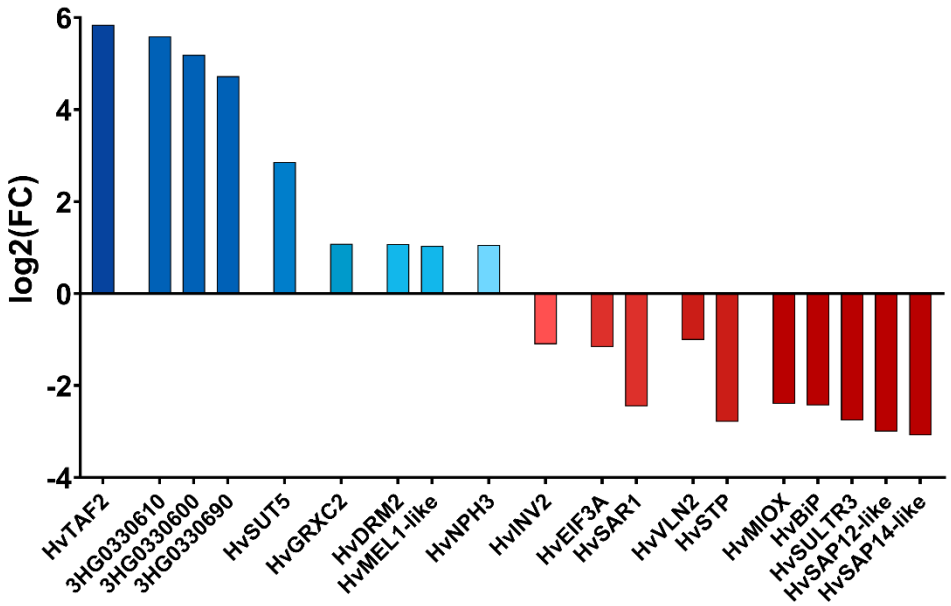
The mutant plants, showed normal vegetive growth at normal conditions (middle panels) but showed some infertility and somewhat reduced seed set in the spikes.



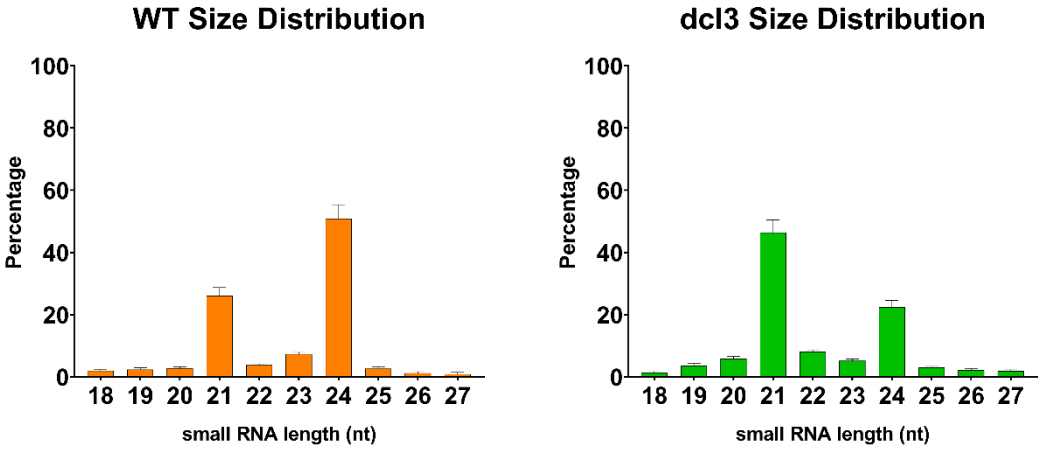
Since DCL3 is producing the 24 nt siRNA species necessary to the establishment of RdDM, we have checked the methylation status of dcl3 mutant relative to wild type plants, by

immunohistochemical staining (provided by Adél Sepsi) of methylated cytosines in the nucleus. Intriguingly we found that the methylation level increased in mutants, suggesting that different methylation systems cross-regulate each other. Further experiments are necessary to understand this very interesting phenomenon.

Next, we collected flower samples of the mutant and wild type plants for RNAseq and small RNA seq analyses on Illumina platform. RNA-Seq inflorescences (1.5-2.5 cm) dcl3 mutants (3 lines) vs WT. We identified 87 up- and 62 down-regulated genes.

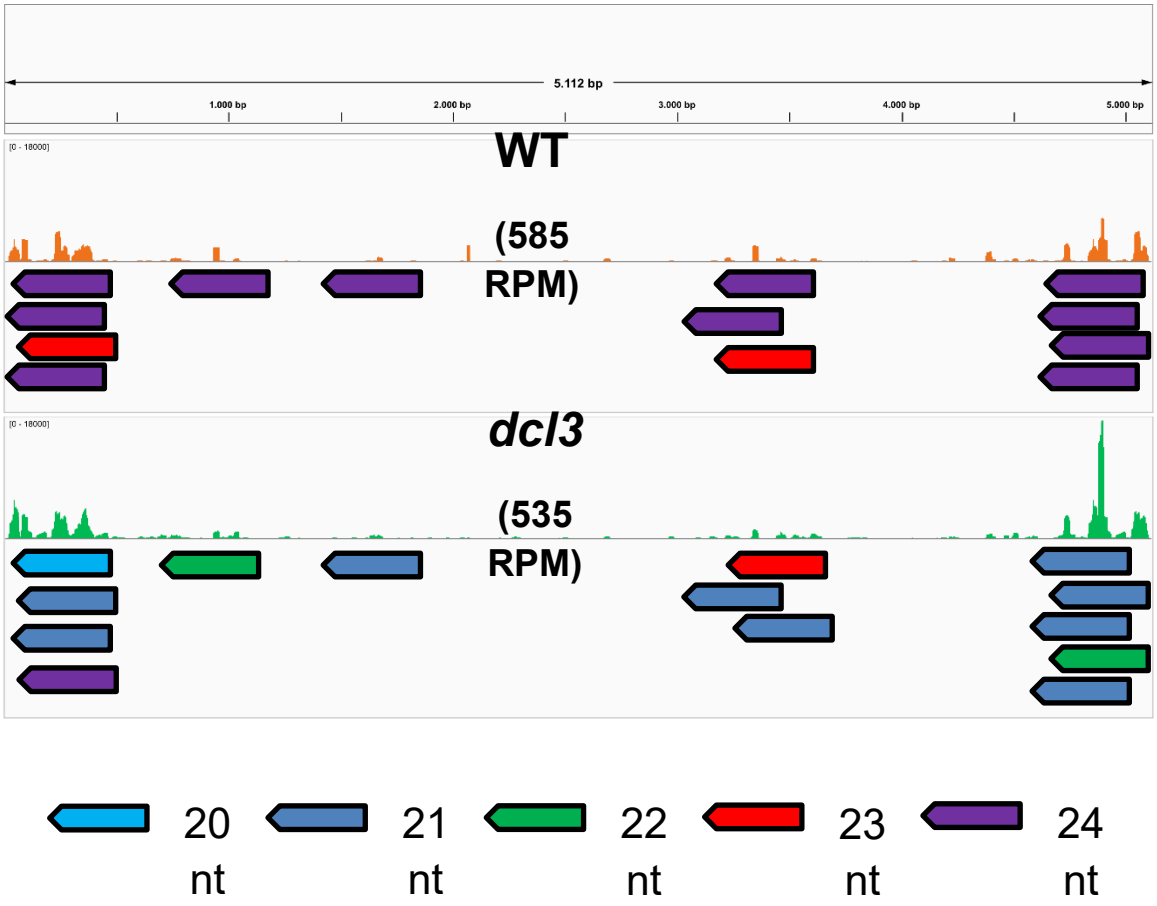


Representatives of the affected genes focusing inflorescence-specific, RdDM pathway and TE-related genes.



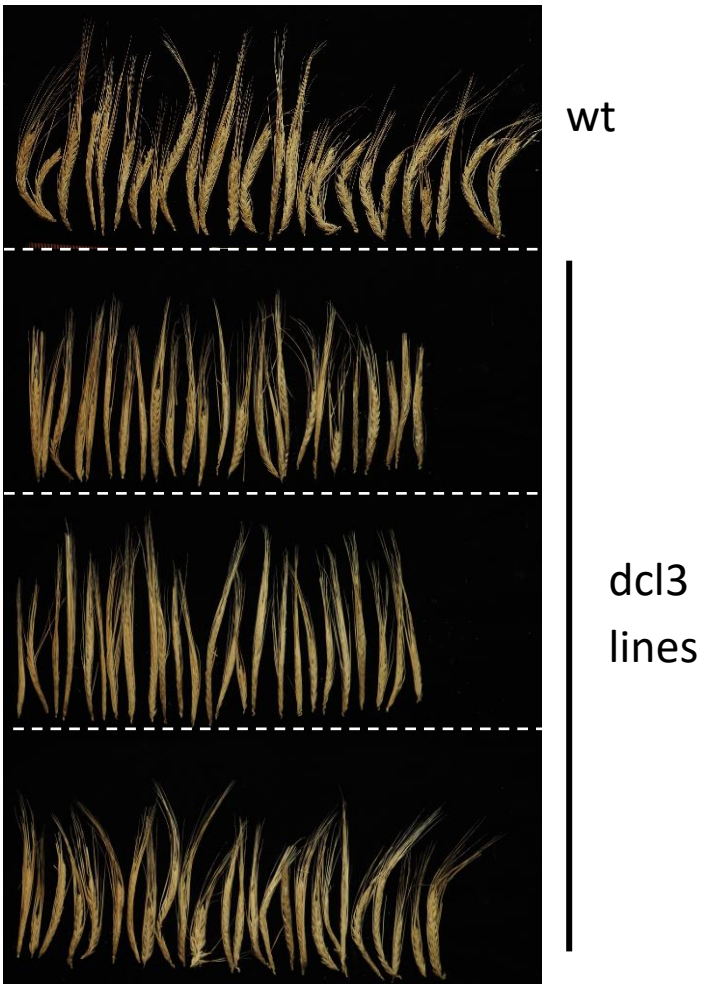
We also carried out small RNA HTS experiments on Illumina platform to reveal changes in the small RNA population of dcl3 mutant plants. Samples were taken from young inflorescence tissues (1.5-2.5 cm). We found that the drastic reduction, as expected, but not the complete elimination of 24 siRNA species. This finding indicates that a redundant function of another DCL, perhaps DCL5, can compensate the impact of the introduced mutations.

HORPIA2 (LTR Retrotransposon)



However, we identified change in sRNAs size distribution on the TE-derived sRNAs species originating from the same regions, different size composition of small RNA population is generated exhibiting the dominance of 21 nt small RNAs. These data show that mutation in DCL3 gene drastically modulate the small landscape in the mutant plants.

Since we identified the RdDM pathway as the main determinant of heat stress perception we applied heat stress treatment to dcl3 mutants, under greenhouse conditions, to see the response of mutant plants compared to the wild type plants. The plants were grown under continuously stressed environment during the cultivation period till maturity. Then we investigated spike development. 25 representatives of spikes of the mutant lines showed spike development abnormalities compared to the control plants indicating **the DCL3 controlled pathway is one of the main determinants of heat stress responses.**

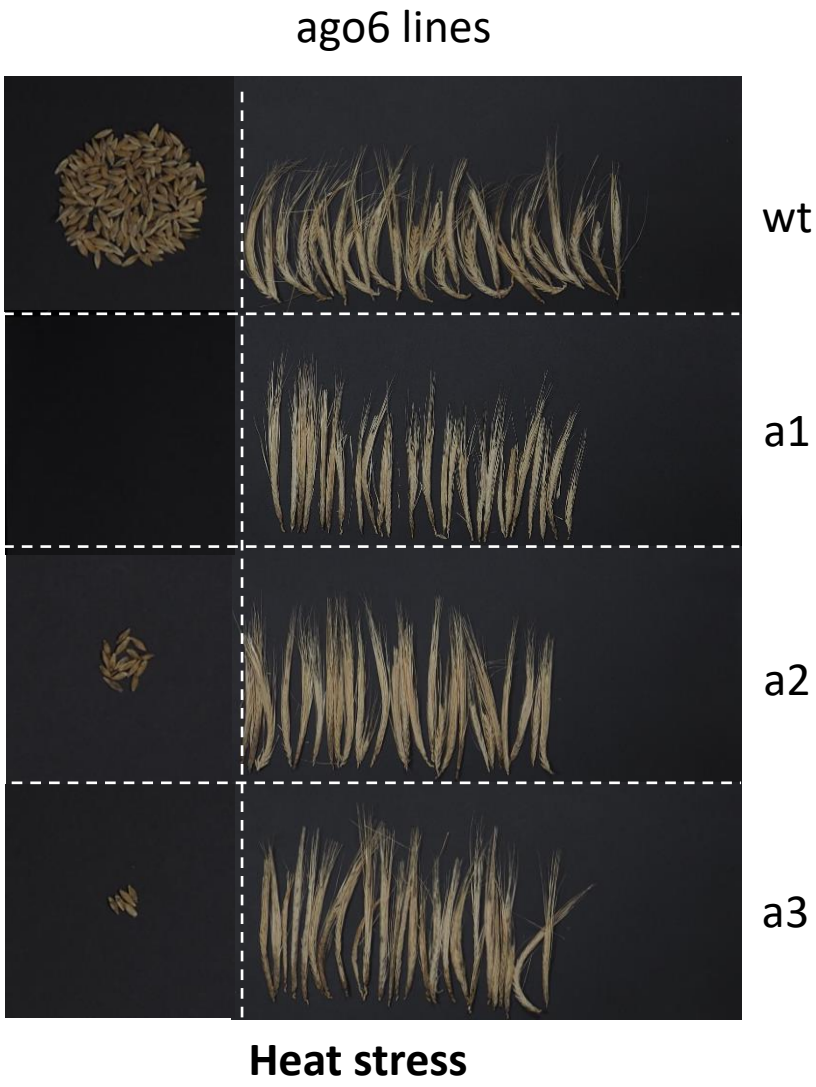


Heat stress

Analyses ago6 knock-out mutants

Our previous experiments demonstrated the drastic upregulation of AGO6 mRNA level due to heat stress. The level of upregulation of AGO6 mRNA level was for more the highest among the components of RdDM pathway in barley and also in wheat. To investigate the role of this gene in heat shock response we performed knock-out of AGO6 using CRISPR/Cas9 in barley. Several T0 lines were selected in which a partial or complete mutation of the AGO6 gene was observed and from which fully mutated and homozygous T1 lines were subsequently selected. Some of these selected T1 lines were also negative for Cas9 and

hygromycin resistance. We propagated three selected lines to T3 and used them to apply heat stress treatment. Similarly to dcl3 mutants, the plants were grown under continuously stressed environment during the cultivation period till maturity. Then we investigated spike development. 25 representatives of spikes of the ago6 mutant lines showed very strong development abnormalities (at normal condition 18-9 ℃ the mutant plants develop close normal spikes) and their seed production was strongly affected.



These observations identify AGO6 as one of the main plant components alleviating the effect of heat stress.

The profound effect of AGO6 in heat shocked plants also raising the possibility of future applications in breeding programs to produce mor heat resistant parley cultivars. To understand the biological mechanism lying behind this phenomenon controlled AGO6

transgenic expression systems have been building using two different promoters, one inducing an over-expression of AGO6 in the mutant (ubiquitin promoter) and the other directly cloned from the barley AGO6 gene to maintain an expression pattern as similar as possible to the wild type gene. In order to control protein accumulation, we tagged the AGO6 with HA tag, so that an anti-HA antibody could be used to quantify and localize the protein. These two different complementation groups will allow us to observe the phenotype of the plant during heat stress with an expression similar to the wild type or with an over-expression of AGO6 and to compare them with plants mutated for this gene. In this way, we could better understand the function of this protein during heat stress and the molecular responses induced in the plant.

These lines are under propagation and construction.

1. Summary

During our work, we have generated T2-T4 homozygous knock-out mutant barley lines for AGO4, AGO5 and DCL3 genes. We have started the molecular characterization and functional analyses of the mutants. Our preliminary data reveled important observation of these genes in development processes and especially in heat perception. However, these experiments are incomplete and further intensive work necessary to complete the experiments**. We think that these projects have great potentials and will provide important data about the role of RdDM in basic development processes and heat stress responses in monocots having large genomes in the future.** Additional RdDM mutants, such as mutations RdDR2 and NRPD2 genes, have been also generating and experiments are in progress to produce the knock-out barley lines.

Finally, I would like indicate that the delays, which our projects suffered, not only because of the scientific difficulties associated with the projects. We faced severe problems during Covid periods affecting plant growing and working conditions. Moreover, the infrastructural conditions of the University also posed difficulties, growing of barley plants requires space and well controlled conditions. Thirdly, the problems of the newly intruded ordering system at the MATE significantly set back our scientific advance and consumed large amount of human power of scientists. Every effort will be made to complete the experiments.

2.

Targeted genome editing by CRISPR/Cas9 system in barley to establish virus resistance and modify economically important traits.

Direct utilization of CRISPR/Cas9 system against plant DNA virus

Wheat dwarf virus (WDV) is an economically important, insect transmitted, virus belonging to *Geminiviridae* family. WDV strains infect both wheat and barley causing severe yield losses and the natural resistance resources are limited. Direct utilization of CRISPR/Cas9 system to inhibit geminivirus replication has been described in model plants. Here we show the direct anti-viral utilization of CRISPR/Ca9 system in an important crop plant, barley, to establish effective WDV resistance. Artificial transient expression system has been used to test the biological activity of selected WDV specific single guide RNAs (sgRNAs). Stable introduction of polycistronic construct expressing four WDV specific sgRNAs and Cas9 in barley lines resulted in extreme resistance in the majority of challenge infection experiments mediated by virus carrying leafhoppers. The introduced resistance was stably heritable providing resistance to the progeny lines. However, we also found that in few cases WDV escaped under the control of CRISPR/Cas9 system. Molecular analyses of the infected barley lines revealed that only one sgRNA was active probably imposing high but in some cases surmountable selection pressure on the replicating virus. Our results show that CRISPR/Cas9 mediated direct virus targeting can be a powerful tool to engineer full resistance against economically important geminiviruses *in* monocotyledonary crop plants but the design of multiple, biologically highly active sgRNAs is the prerequisite of its durable utilization.

These data have been published in: Kis A, Hamar É, Tholt G, Bán R, Havelda Z. (2019**) Creating highly efficient resistance against wheat dwarf virus in barley by employing CRISPR/Cas9 system.** Plant Biotechnol J. 2019 Jan 11. doi: 10.1111/pbi.13077. (IF 8.14)

Producing virus resistant plant via knocking-out eIF4E by CRISPR/Cas9 system

Utilizing CRISPR/Cas9 technology we successfully disabled eIF4E encoding gene in barley. We produced T2 and T3 homozygous generation. However, unfortunately at this stage of the

experiments publications were presented about the investigations of the biological role eIF4E in virus resistance in barley. That is why **we abandoned this line of research** and did not continue with provoked challenges of BaYMV infection.

Producing gw2 knock-out barley plants

Grain Width and Weight 2 (GW2) is an E3-ubiquitin ligase-encoding gene that negatively regulates the size and weight of the grain in cereal species. Therefore, disabling GW2 gene activity was suggested for enhancing crop productivity. We show here that CRISPR/Cas-mediated mutagenesis of the barley GW2.1 homologue results in the development of elongated grains and increased protein content. At the same time, GW2.1 loss of function induces a significant grain yield deficit caused by reduced spike numbers and low grain setting. We also show that the converse effect caused by GW2.1 absence on crop yield and protein content is largely independent of cultivation conditions. These findings indicate that the barley GW2.1 gene is necessary for the optimization between yield and grain traits. Altogether, our data show that the loss of GW2.1 gene activity in barley is associated with pleiotropic effects negatively affecting the development of generative organs and consequently the grain production. Our findings contribute to the better understanding of grain development and the utilisation of GW2.1 control in quantitative and qualitative genetic improvement of barley.

András Kis, Dávid Polgária, Ágnes Dalmadi, Imtiaz Ahmad, Marianna Rakszegi, László Sági Tibor Csorba and Zoltán Havelda (2023) **Targeted mutations in the GW2.1 gene modulate grain traits and induce yield loss in barley**

Submitted, under revision.

Manuscript attached to the report.

Elaboration traceless CRISPR/Cas9 mutagenesis in bread wheat

CRISPR/Cas9 system is widely used to generate mutations and study their impact on crop improvement. The basic CRISPR/Cas system requires two components: a Cas nuclease, such as Cas9, Cpf1, and a programmable guide RNA (gRNA). The efficiency of genome editing is greatly affected by the mode of execution. It is most effective to incorporate the genes encoding the two major elements into the plant genome and then remove them after mutations are created. However, short foreign DNA fragments may remain in the plant genome. No matter how transgene is integrated into the plant genomes raises important legislative concerns regarding genetically modified organisms. Another concern is the

delivery of genome editing machinery because established methods show strong genotype-dependency. We devised hybridization-based editing technology (Hi-Breeder) to overcome these problems, which enables direct editing of wheat lines via a single cross with transgenic barley (*Hordeum vulgare* L.) line. In the first step, wheat is crossed with a 'Golden Promise' barley carrying a reporter gene (*DsRed*) and a wheat-specific (*TaMlo gene*) guide RNA-Cas9 CRISPR construct. In the next step, F1 hybrid plants carrying the CRISPR construct are selected from the hybrid population based on the expression of the reporter gene and using barley chromosome-specific PCR markers. These hybrid plants are backcrossed with the original wheat parent, resulting in wheat with diploid genome and monosomic chromosome additions of barley. In the F1BC1 generation plants that still contain the chromosome(s) carrying the CRISPR construct are simply selected based on the phenotype of the reporter gene to allow another round of genome editing in the recurrent (paternal) wheat genome. Following self-fertilization, the genome-edited diploid wheat plants without barley chromosomes can be identified in the progeny.

This approach will also be presented as patent. A method for altering genome structure and gene expression in wheat

Inventors: András Kis, Dávid Polgári, Zoltán Havelda, László Sági

SZTNH registration number: 2312463

A manuscript submitted, addressing the easy and fast genotyping of barley x wheat hybrids.

Mohammad Ali, Dávid Polgári, Adél Sepsi, Levente Kontra, Ágnes Dalmadi, Zoltán Havelda, László Sági and András Kis (2023) **Rapid and cost-effective molecular karyotyping in wheat, barley, and their cross-progeny by chromosome-specific multiplex PCR**

Interspecific hybridisation is a powerful tool for increasing genetic diversity in plant breeding programmes. Hexaploid wheat (Triticum aestivum, 2n=42) × barley (Hordeum vulgare, 2n=14) intergeneric hybrids can contribute to the transfer of agronomically useful traits by creating chromosome addition or translocation lines as well as full hybrids. Information on the karyotype of hybrid progenies possessing various combinations of wheat and barley chromosomes is thus essential for the subsequent breeding steps. Since the standard technique of chromosome in situ hybridisation is labour-intensive and requires specific skills. a routine, cost-efficient, and technically less demanding approach is beneficial both for research and breeding.

We developed a Multiplex Polymerase Chain Reaction (MPCR) method to identify individual wheat and barley chromosomes. Chromosome-specific primer pairs were designed based on the whole genome sequences of 'Chinese Spring' wheat and 'Golden Promise' barley as reference cultivars. A pool of potential primers was generated by applying a 20-nucleotide sliding window with consecutive one-nucleotide shifts on the reference genomes. After filtering for optimal primer properties and defined amplicon sizes to produce an ordered

ladder-like pattern, the primer pool was manually curated and sorted into four MPCR primer sets for the wheat A, B, and D sub-genomes, and for the barley genome. The designed MPCR primer sets showed high chromosome specificity in silico for the genome sequences of all 18 wheat and barley cultivars tested. The MPCR primers proved experimentally also chromosome-specific for the reference cultivars as well as for 13 additional wheat and four barley genotypes. Analyses of 16 wheat × barley F1 hybrid plants demonstrated that the MPCR primer sets enable the fast and one-step detection of all wheat and barley chromosomes. Finally, the established genotyping system was fully corroborated with the standard genomic in situ hybridisation (GISH) technique.
Wheat and barley chromosome-specific MPCR offers a fast, labour-friendly, and versatile alternative to molecular cytogenetic detection of individual chromosomes. This method is also suitable for the high-throughput analysis of distinct (sub)genomes, and, in contrast to GISH, can be performed with any tissue type. The designed primer sets proved to be highly chromosome-specific over a wide range of wheat and barley genotypes as well as in wheat × barley hybrids. The described primer design strategy can be extended to many species with precise genome sequence information.

Attachment

Targeted mutations in the *GW2.1* gene modulate grain traits and induce yield loss in barley

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Highlights

Mutations in the barley *GW2.1* gene result in elongated grains possessing increased protein content.

gw2.1 mutants exhibit reduced seed setting and yield.

Grain production capacity of *gw2.1* mutants are unaffected by temperature fluctuation.

Abstract

Grain Width and Weight 2 (*GW2*) is an E3-ubiquitin ligase-encoding gene that negatively regulates the size and weight of the grain in cereal species. Therefore, disabling *GW2* gene activity was suggested for enhancing crop productivity. We show here that CRISPR/Cas-mediated mutagenesis of the barley *GW2.1* homologue results in the development of elongated grains and increased protein content. At the same time, GW2.1 loss of function induces a significant grain yield deficit caused by reduced spike numbers and low grain setting. We also show that the converse effect caused by GW2.1 absence on crop yield and protein content is largely independent of cultivation conditions. These findings indicate that the barley *GW2.1* gene is necessary for the optimization between yield and grain traits. Altogether, our data show that the loss of *GW2.1* gene activity in barley is associated with pleiotropic effects negatively affecting the development of generative organs and consequently the grain production. Our findings contribute to the better understanding of grain development and the utilisation of GW2.1 control in quantitative and qualitative genetic improvement of barley.

Keywords

crop yield, genome editing, *Grain Width and Weight 2* (*GW2*), *Hordeum vulgare*, protein content

1. Introduction

The reliable and efficient food production relies on stable crop yields; these are affected by climatic and soil conditions, abiotic and biotic stresses, and by crop production technologies (Miladinovic et al., 2021). Due to the fast increase of the world's population intensive breeding efforts must be taken to improve crop production rates. An additional urgent task of the agricultural developments is to adapt important crops to the effects of climate changes, securing their productivity (Raza et al., 2019). Cereals, members of the Poaceae family encompassing wheat, rice, maize, and barley are major sources of food production and provide more than 50% of the food energy (Yu and Tian, 2018).

The seed quantity and quality collectively determine the reproductive success and agronomic performance of crop plants. In cereals, the endosperm is the most valuable part of the grain due to its high starch and protein content (Wu et al., 2022). Grain quantity is defined by the grain weight and grain number (grain number per panicle/spike and the panicle/spike number per plant) that determine the final yield (Li and Lubberstedt, 2018; Nadolska-Orczyk et al., 2017; Xue et al., 2008). Grain number and weight are polygenic traits. There is a negative correlation between these traits, caused by the competition between grains for the limited available resources during the grain filling period. Plant adaptation to environmental cues, such as the available nutrients and/or diverse stress conditions, aims to maximise their productivity.

In recent years, an increasing number of molecular regulators or quantitative trait loci (QTLs) influencing grain yield have been identified and studied (Li et al., 2010; Song et al., 2007; Yan et al., 2011). The *Grain Width and Weight 2* (*GW2*) gene was discovered in rice as a QTL (*OsGW2*) that negatively regulates grain size and potentially the yield (Song et al., 2007). *OsGW2* encodes a RING-type E3 ubiquitin ligase constitutively expressing in the leaves, roots, flower organs and grains (Choi et al., 2018; Lee et al., 2018; Song et al., 2007; Yamaguchi et al., 2020). The ubiquitin-26S proteasome system (UPS) is an essential protein-degradation pathway in plant growth and development (Linden and Callis, 2020). UPS is actively involved in regulation of grain development (Li and Li, 2014; Li et al., 2019; Linden and Callis, 2020). Like in rice, wheat TaGW2 homoeologues also possess E3 ubiquitin ligase activity, suggesting that targeting proteins into the ubiquitin-proteasome system (UPS) may be a conserved mode of action for these factors (Bednarek et al., 2012). Further evidences showed that *GW2*-like gene homologues are functionally conserved in maize, wheat, sorghum, barley, mosquito-grass (*Dasypyrum villosum*), and dicot *Gossypium* species (Feng et al., 2021; Huang et al., 2022b; Li et al., 2010; Sestili et al., 2019; Su et al., 2011; Tao et al., 2017; Zombori et al., 2020). The two *GW2* homologues in maize, *ZmGW2-CHR4* and *ZmGW2-CHR5*, were associated with kernel size and weight (Li et al., 2010). In tetraploid and hexaploid wheat plants a mis-spliced variant of the A-genome *GW2* homoeologue *TaGW2-A1* was associated with significant increase in grain width, length, and weight.

Differences in carpel size and weight between wild type (WT) and *tagw2-a1* mutant plants were identified, suggesting that *TaGW2-A1* restricts grain size within the maternal tissue before anthesis (Simmonds et al., 2016). Targeted mutagenesis or RNA interference-mediated knock-down of wheat *TaGW2* homoeologues positively influenced grain development in tetraploid and hexaploid wheats (Hong et al., 2014; Sestili et al., 2019; Wang et al., 2018; Zhang et al., 2018). The functional conservation of GW2-like factors is further supported by the observation that overexpression of *Gossypium hirsutum GW2-2D* decreased grain size in *A. thaliana* (Huang et al., 2022b).

GW2 protein may be involved in multiple molecular mechanisms: (i) *gw2* mutations increased cell number through cell cycle regulation (Song et al., 2007) and a molecular network was revealed controlling cell division during grain development via GW2-WG1-OsbZIP47 regulatory module (Hao et al., 2021); (ii) OsGW2 interacts with, ubiquitinates and destabilises the Expansin-like 1 (EXPLA1) protein, responsible for loosening cell walls and expansion of cells (Choi et al., 2018): rice and wheat *GW2* homoeologous both control grain size through increasing cell number and length under various environmental conditions (Sestili et al., 2019; Verma et al., 2021; Zhang et al., 2018); (iii) OsGW2 indirectly controls phosphoglycerate kinase (PGK) levels, therefore may regulate carbohydrate metabolism during grain development: in support, the natural *gw2* mutant rice 'Oochikara' has a floury endosperm phenotype consisting of small, loosely packed starch granules (Choi et al., 2018; Lee et al., 2018); (iv) OsGW2 may regulate protein folding and/or stability during grain development because a disulfide isomerase-like 1-1 protein that is needed for disulfide bond formation within proteins accumulates to high levels in the *gw2* mutant (Lee et al., 2018); (v) OsGW2 suppresses chitinase 14 (CHT14) protein levels therefore may control immunity (pathogen defence) during grain development (Lee et al., 2018); (vi) GW2 regulates diverse vegetative phases of plants: GW2 knock-out rice grain germinated faster, had an improved biomass, and displayed vigorous growth, improved root and shoot architecture when compared to control plants (Achary and Reddy, 2021).Absence of OsGW2 increased chlorophyll content and accelerated leaf senescence in rice (Shim et al., 2020). The effects of GW2 on plants' vegetative growth were suggested to derive from its role on proteostasis (ubiquitination and decay through proteasome) and/or potentially hormonal signalling (Achary and Reddy, 2021; Geng et al., 2017; Verma et al., 2021). GW2 also affects grain protein content in wheat (Zhang et al., 2018). Rice CRISPR-mediated *gw2* mutated lines produced grains with enhanced protein and free amino-acid contents (Achary and Reddy, 2021);

The function of *GW2* gene was also examined in barley, where two *GW2* orthologues were described: the *HvYrg1* (*HvGW2.1*) and *HvYrg2* (*HvGW2.2*) (Zombori et al., 2020). RNAi-mediated silencing of either *HvGW2.1* (30–50% down-regulation) or *HvGW2.2* (20–27% down-regulation) activities in 'Golden Promise' barley, resulted in longer and wider grains. The RNAi lines also showed increased thousand grain weight and alterations in vegetative growth.

In summary, numerous independent evidence in various cereal species suggest a positive effect of *GW2* mutation on grain size (length, width, and weight) and nutritional values (protein and

polysaccharide/starch content), potentially as the result of the combined effect on vegetative and reproductive molecular events. Noticeably however, there are some contradictory findings regarding the impact/roles of GW2. The knock-down of *HvGW2.1* and *HvGW2.2* homologues in barley resulted in opposite effects on vegetative traits: *HvGW2.1-RNAi* plants exhibited earlier heading, prolonged grain-filling period, and enhanced root growth, while *HvGW2.2-RNAi* plants showed delayed flowering and inhibited root development (Zombori et al., 2020). Furthermore, the simultaneous RNAi-mediated knock-down of all *GW2* homoeologous in hexaploid wheat brought about significant reduction in endosperm cell number causing reduced grain size (Bednarek et al., 2012). The reasons for the discrepancy between these findings are not known so far. Nutrient/resource availability or environmental stress exposure may be some factors that regulate the penetrance of *GW2* mutations. Finally, although grain size, and consequently thousand grain weight, are increased in the absence of GW2, the way how the crop yield is altered remains less studied and poorly understood.

In the present work, we aimed to elucidate the roles of *GW2.1* gene in barley grain development. To this end, we generated independent CRISPR/Cas9 mutant lines and evaluated their development and reproductive fitness in independent, greenhouse cultivation regimes. Our data suggests that GW2.1 activity fine-tunes the balance between different grain traits and yield. However, the adverse effects associated with the lack of GW2.1 activity questions the direct use of this gene in barley breeding programs.

2. Materials and methods

2.1. Plant materials and growth conditions

Barley (*Hordeum vulgare*) 'Golden Promise' plants were grown in growth cabinet (Versatile Environmental Test Chamber MLR-350; Sanyo, Tokyo, Japan) under 15 °C daytime and 12 °C night temperatures with 16-h light (50 μE $m^{-2}s^{-1}$) and 8-h dark periods to obtain explants for *Agrobacterium*-mediated genetic transformation. Transformant plants were grown in the greenhouses and subsequently propagated to produce homozygous mutant lines. Selected members of T3 generation were first pre-cultivated in Jiffy-7 pellets, then planted into pots, containing 1:1:1 ratio of peat moss (DSM 3 W; Kekkilä, Vantaa, Finland), fine quartz sand (0.1-0.4 mm), and fertile soil from fallow, at 2-3 leaves stage and moved to the greenhouse. The plants were placed in the greenhouse on 15th of December, 2019 (2020 cultivation period) and on 07th of February, 2022 (2022 cultivation period). Under the greenhouse conditions the water supply, light supplementation and basal heating were provided, however the external weather conditions strongly affected the light and temperature values during the cultivation periods. Comparative cultivation studies were carried out with T3 homozygous mutant *gw2.1* lines randomly arranged in the greenhouse. As a CRISPR/Cas9-expressing transgenic control plant, a transgenic barley plant expressing Cas9 and four virus specific single guide (sg)RNAs was used (Kis et al., 2019).

2.2. Generation of CRISPR/Cas9 constructs

To identify the *GW2* ortholog in the 'Golden Promise' barley, we used the rice GW2 protein sequence (GenBank ID ABO31101) (Song et al., 2007) to run BLAST search in the UniProt database. A *GW2* ortholog (B0FLE0) was previously identified in a Tibetan barley and named *Yield-related gene* (*HvYrg1*, EU333863). The two genes show 86.62% identity. The genomic sequence of *GW2.1* gene was acquired using the Ensembl Plants database (HORVU6Hr1G044080.2). We used the CRISPOR software (Concordet and Haeussler, 2018) to select CRISPR/Cas9 target sites in the first exon of the *GW2.1* gene, overlapping with suitable restriction cleavage sites. SgRNAs exhibiting minimal off-target activities were selected for further experiments. The *HvGW2.1-sg1* targets the locus at positions +126-145 downstream to translational start site, while the *HvGW2.1-sg2* at +60-79 nucleotides of the *GW2.1* gene. The selected target sequences contained restriction cleavage sites overlapping with the CRISPR/Cas9 target sites to facilitate the detection of the obtained mutations (Fig. 1B). Two sgRNA expression cassettes were built expressing *HvGW2.1-sg1* and *HvGW2.1-sg2* sgRNAs separately (Figure 1C). To facilitate the detection of the integrated T-DNA, a *35S*::*DsRed* construct was inserted into the *Pme*I site of the pHUE411 vector (Xing et al., 2014), which was amplified from the pC61KdsRED vector (Kis et al., 2019) generating the pHUER plasmid. The CRISPR/Cas9 vector, containing the two unique sgRNAs, was prepared as described previously (Xing et al., 2014). The presence and accuracy of the introduced sgRNA sequences in the generated vectors were confirmed by sequencing. Primers used are listed in Table S1.

2.3. Transformation and detection of targeted mutations

Immature barley embryos were transformed by *A. tumefaciens* (AGL1 strain) as described previously (Kis et al., 2016), harbouring the pHUER vectors containing the *Cas9* gene under the control of the maize ubiquitin (*Ubi*) promoter and two different sgRNAs specific for the first exon of *GW2.1* gene. Transgenic plants that originated from the same callus were considered as sibling lines. The presence of the transgene was detected by DsRed marker protein fluorescence followed by PCR reaction using primer pairs specific for the *Ubi* promoter (Table S1). For direct PCR, samples were collected from the leaves of transgenic plants (~0.25 cm^2) using 100 µL of Extraction solution (Sigma-Aldrich, E7526) and 100 µL of Dilution solution (Sigma-Aldrich, D5688) following the manufacturer's instructions. The target sites were amplified with specific primers (Table S1) by PCR using Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F537S) with 1 µL of DNA extract in 50 µL of final volume. PCR composition and cycling conditions were set based on the manufacturer's instructions. The samples were further analysed with restriction enzyme analysis, using 3 µL of unpurified PCR products in 10 µL of final volume. The non-digested amplicons were directly analysed by Sanger sequencing (Eurofins Genomics) to identify homozygous mutations.

2.4. Analysis of grain morphology, composition and yield

The Marvin System (MarviTech GmbH, Wittenburg, Germany) was used for determination of the thousand grain weight (TGW), grain width (GW) and grain length (GL) per individual plants according to the relevant industrial standard (MSZ 6367/4-86, 1986). The collected data were presented and statistically explored with boxplots (box-and-whisker plots) made by BoxPlotR (http://shiny.chemgrid.org/boxplotr/, (Spitzer et al., 2014)). On the boxplots, whiskers extend to data points that are less than 1.5x of the interquartile range (boxes) away from the 1st and 3rd quartiles (bottom and top box lines, called hinges) as originally defined by Tukey (1977). Data points located further away from the whiskers can be viewed as outliers. Median (bold line) and mean values (+) are indicated within (or near) the box. The grey stripe within the box corresponds to the 95% confidence interval (CI95%) of the mean. As a conservative measure, non-overlapping (or just touching) CI95% ranges are considered statistically different at a high alpha value (down to $p<0.01$) when independent treatments have a relatively high (n>10) and similar numbers of replicates (Cumming and Finch, 2005; Payton et al., 2003). With the same conditions for treatments, CI95% ranges overlapping by about 25% or less still represent a significant difference at $p<0.05$ or lower (Cumming, 2009; Julious, 2004; Van Belle, 2011).

For measuring the grain's composition, 6-to-14 grams of grain pools were milled using a ball mill (Retsch Mixer Mill MM 200), to produce wholemeal samples of control and mutant lines respectively. The total protein content of wholemeal samples was measured by the Dumas method with an Elementar Rapid N III Analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) according to the ICC 167 standard method (1995). Duplicate analyses were carried out on each sample and when the difference between them was higher than 10%, two more replications were measured.

The total amount of mixed-linkage β-glucan was determined in wholemeal samples using a Megazyme kit (Megazyme, Bray, Ireland) according to the ICC 166 standard method (1998). The amylose content of the starch was measured by Megazyme method (Yun and Matheson, 1990). Duplicate analyses were carried out on each sample, and if the difference between them was higher than 10%, then two more replicas were measured. Statistical analysis of the compositional traits on Box Plot data was carried out by Statistica 14.0 (2020) (TIBCO). Two-factor analysis of variance (two-way ANOVA test) was carried out by Microsoft Excel.

2.5. Heat stress treatment

Heat stress treatments were done as described before (Hamar et al., 2020; Szadeczky-Kardoss et al., 2022). Briefly, for thermotolerance to moderately high temperatures, 7-day-old seedlings grown on Jiffy were exposed in a preheated plant growth cabinet (Versatile Environmental Test Chamber MLR-350; Sanyo, Tokyo, Japan) to 37 °C for 2 days starting at ZT8, long day conditions (16 h light/8 h dark). For mild heat stress treatment 30 and 75 (at the stage of early spike development) days old barley plants were kept at 30 °C for 1 day (16 h light/8 h dark). For molecular analysis, leaf and young green spike section samples were taken immediately following heat treatments alongside non-treated controls,

frozen in liquid nitrogen, and stored at -80 °C. RNA and protein samples were extracted as described previously (Szaker et al., 2019).

2.6. Protein extraction and Western blotting

For protein extraction, non-treated and heat-treated samples were ground in 2x Laemmli buffer (150 mM Tris–HCl, pH 7.5, 6 M urea, 2% SDS and 5% beta-mercaptoethanol), boiled for 5 min and cell debris removed by centrifugation at 18 000 × g at 4 °C for 10 min. The resulting supernatants were separated using 10% SDS-PAGE and transferred to Hybond PVDF membranes (GE Healthcare) and subjected to western blot analysis. The antibodies used were anti-sHSP-CI (Agrisera, AS15 3029), anti-HSP70 (Agrisera, AS08 371), and anti-HSP90-1 (Agrisera, AS08 346). For detection, we used the monoclonal HRP-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, A6154). The signal was visualised and quantified by Image Lab 5.1 software (Bio-Rad). Protein signals were normalised to the Rubisco large subunit (RbcL).

3. Results

3.1. Generation of CRISPR/Cas9-mediated gw2.1 knock-out barley lines

To address the role of *GW2* gene product in the development of barley plants we identified the sequence of the *GW2.1* orthologue (HORVU6Hr1G044080.2) in 'Golden Promise'. Recent research revealed two *GW2* orthologs in barley, *HvGW2.1* (*HvGWYrg1*) and *HvGW2.2* (*HvYrg2*) (Zombori et al., 2020). Since the partial inhibition of *HvGW2.2* activity brings about adverse effects on vegetative traits (Zombori et al., 2020), the *HvGW2.1* gene was considered as the primary *GW2* homologue suitable for enhancing crop productivity (Fig. 1A). To obtain *gw2.1* mutant barley lines, the designed sgRNA sequences exhibiting limited *in silico* predicted off-target activities for *GW2.2* and other potential non-specific targets were selected. Two sgRNAs were chosen for targeting the 5' region of the *GW2.1* gene in the first exon (Fig. 1B, C). *Agrobacterium*-mediated transformations of 100-100 immature barley embryos with pHUER vector constructs, containing *HvGW2.1-sg1* or *HvGW2.1-sg2,* were carried out. PCR/RE analysis of the T0 transgenic plants expressing *HvGW2.1-sg1* identified 12 mutant plants from four independent calli while transgenic plants expressing *HvGW2.1-sg2* exhibited mutations in 9 plants, including two entirely digestion-resistant plants, from three independent calli (Fig. 2A). For further experiments, two T0 plants from different calli for each sgRNA with the highest mutation rates were selected (Fig. 1A) and selfed for producing T1 generation. In T1 generations of the four selected T0 plants, 30-30 individuals were selected and analysed with PCR/RE to identify digestion-resistant mutant plants, which were sequenced to identify mutations at nucleotide level. Based on PCR fragment sequencing data we selected homozygous mutant plants, if possible without DsRed expression. To prove the heritability of the mutations, the selected T1 mutant plants were further propagated for the next (T2) generation, and the *GW2.1* specific PCR amplicons of 30-30 mutants, using pooled DNA samples of 5-

5 plants, were re-sequenced. Sequence analyses of the selected representatives of mutant plants revealed that the *gw2.1* mutations were stably heritable (Fig. 2B). These selected T2 mutant plants were further propagated to T3. To analyse the potential off-target effects of the *HvGW2.1-sg1* and *-sg2* on *HvGW2.2* orthologue we PCR amplified the section of *HvGW2.2* gene overlapping with the two sgRNA target sites, using pooled leaf samples of 5-5 members of the various T3 lines. PCR fragment sequencing analysis revealed no mutations in the analysed *HvGW2.2* gene sequences (Fig S1). At T3 generation the selected mutant plants were also investigated for the presence of transgene (*Cas9* and *DsRed*). Based on the gained data all of the sg1_1-6-8 and sg1_2-25-8 progenies were positive, while sg2_2-7-2 and sg2_3-5-9 progenies were negative for the presence of *Cas9* and *DsRed*.

3.2. Genomic characterization of the selected gw2.1 mutant lines

We selected two independent mutant plants corresponding to each sgRNAs (the sg1_1-6-8, sg1_2-25-8 and sg2_2-7-2, sg2_3-5-9, respectively) for further work. Plant sg1_1-6-8 has a single T nucleotide insertion, while plant sg1_2-25-8 exhibits a single A nucleotide insertion compared to the wild type *GW2* nucleotide sequence (Fig. 2B). The mutant plant sg2_2-7-2 contains a G to C substitution and three nucleotide deletion (Fig. 2B), while plant sg2_3-5-9 possesses a T insertion (Fig. 2B). The one nucleotide insertion in plants sg1_1-6-8, sg1_2-25-8 and sg2_3-5-9 induces frameshift mutations resulting in the presence of pre-mature stop codon and long 3'UTR region (Fig. 2C) therefore becoming a presumed target of the Non-sense Mediated Decay (NMD) pathway (Kertesz et al., 2006). In theory, mutant *gw2* mRNA transcripts still could be translated leading to production of truncated proteins. Notably, these truncated proteins would lack the RING domain motif located within the C61-C103 residues (RING-C2 type, ^{61}C-(X)$_2$-C-(X)$_{11}$-CC-(X)$_4$-C-(X)$_2$-C- (X)$_{14}$-C-(X)$_2$-C^{103}, since STOP codons are created upstream to this domain (Fig. 2C). Based on these observations the introduced genetic alterations in *GW2.1* gene can be considered as null mutations (Fig. 2B, C). Bioinformatic analyses of sg2_2-7-2 plant revealed that the genetic alteration resulted in the lack of one glutamic acid (E25) in the mutated protein which neither affects its putative bipartite nuclear localization signal (^{8}RRK(X)$_{21}$KKLRK^{36}), nor the RING domain (Fig. 2C). According to these data, the sg2_2-7-2 mutant plant may act either as a hypomorph or a silent mutant.

3.3. GW2 is a negative regulator of grain length in barley

The *gw2.1* homozygous barley transgenic plants (T3) were tested in greenhouse: the water intake was controlled, but light and temperature changes were affected by natural weather conditions, including diurnal and seasonal changes. Analyses of the growth in the early cultivation stage revealed that most of the transgenic plants were indistinguishable from the wild type (WT) barley plants in respect of vegetative development (Fig. 2D). Plants belonging to sg1_2-25-8 line exhibited slightly elongated

phenotype. These findings indicate that the mutation of *gw2* gene does not, or only marginally alters the vegetative developmental processes of barley under our growing conditions.

Based on the literature data obtained in other monocot crops, we decided to study the impact of *gw2.1* mutation on reproductive development of barley during two independent cultivation periods. We harvested fully grown dry grains of WT and transgenic plants in 2020 and 2022 cultivation years. First, we analysed the grain length (GL), grain width (GW) and the thousand grain weight (TGW) as major indicators of grain production (Fig. 3).

In 2020 all mutant plants produced elongated grains in comparison to the WT plants (Fig. 3A, C). However, the width of the elongated grains was significantly decreased in the mutants compared to the WT control (Fig. 3A, D), which also resulted in decrease in TGW (Fig. 3A, E). In the sg2_2-7-2 hypomorph mutant line the length of the grains was similar to the WT grains, but the GW of the grains and TGW were also reduced (Fig. 3A, C-E).

We repeated the grain analysis (GL, GW and TGW measurements) in the 2022 cultivation year. At this point, we introduced an additional control line, which expressed the Cas9 protein alongside non-related virus specific sgRNAs (Kis et al., 2019) to monitor the impact of the presence of CRISPR/Cas9 system elements. The experiments revealed that similarly to the previous year, the GL was increased in the mutant plants compared to the WT but not in the hypomorph and Cas9-control plants (Fig. 3B, C).

Surprisingly, the investigation of other grain parameters showed opposite changes in 2022 (vs 2020) since the mutant plants typically possessed increased GW (Fig. 3B, D) and TGW (Fig. 3B, E) in comparison to the control plants. The hypomorph sg2_2-7-2 and the Cas9-control transgenic plants produced grains with GL, GW and TGW values like the WT plants in the 2022 cultivation period. The close to normal phenotype of the hypomorph and control plants reinforced the notion that the presence of the CRISPR/Cas9 components is not likely to be the cause of the observed grain size alterations. Our data also show that WT plants were more sensitive to the effects of cultivation periods than the *gw2.1* mutants since the observed relative differences in GW and TGW mainly originated from the strongly altered production rates of WT plants in 2020 and 2022 compared to the more consistent productivities of mutant plants (Fig. 3.).

These observations indicate that GW2.1 acts as a negative regulator of grain elongation in barley and this effect is largely penetrant independently on cultivation conditions.

3.4. Loss of GW2 alters conversely the agro-economic traits

The apparently contradictory data obtained in 2020 and 2022 cultivation years regarding GW and TGW (Fig. 3D, E) data urged us for further investigation. To understand the GW2.1 gene's impact on the overall reproductive fitness of plants, we compared the total seed number per plant and the final crop yield of *gw2.1* mutants and control plants. Seed number per plant in mutant plants was consistently decreased in both years compared to their respective controls (WT and CRISPR/Cas9-control plants)

(Fig. 4A). Moreover, the yield of the *gw2.1* mutant plants were also significantly diminished compared to the control plants in both years (Fig. 4B). The yield decline observed in the mutant lines could be the direct consequence of the reduced seed numbers per plant. Additionally, the number of spikes and the seed set per spikes were also significantly reduced in the mutant plants (Fig. S2). The hypomorph sg2_2-7-2 mutant plant exhibited a mild phenotype, displaying moderate reduction in grain number and yield (Fig. 4A, B) in both years.

The absence of GW2.1 reduced the seed set and the yield of plants in both cultivation periods (Fig. 4A, B), in spite of the fact that in 2022 the GW and the calculated TGW values were significantly higher in *gw2.1* mutant plants than in the control plants (Fig. 3B, D, E). In conclusion, *gw2.1* mutants produced fewer but bigger grains compared to WT plants in 2022 but not in 2020, likely due to different cultivation conditions.

Grains compete for resources during the grain filling period; fewer produced grains generally grow bigger and may have higher nutritional content, due to the accumulated sugars, starch, fibre, and protein contents. *gw2.1* mutant lines produced low yields in both cultivation periods (vs WT plants), and fewer but larger grains in 2022 (Fig. 3 and 4). To further address the trade-off between grain quality and quantity, and how this depends on the cultivation conditions, we quantified total starch (amylose, amylopectin and β-glucan), and protein content of grains harvested from *gw2.1* and WT plants in both cultivation periods. Typically no genotype-dependent differences were found in grain parameters (Fig. S3) except the protein content of the grains (Fig. 4C). The absence of GW2.1 induced a massive and consistent increase of protein content in the whole grain: cca. 1,4-fold (40%) increase in 2020 and 1,5-fold (50%) increase in 2022. In 2022 CRISPR/Cas9 control plants also exhibited a negligible (although significant) increase in protein content. These data also show that the absence of GW2.1 specifically affects protein homeostasis of grains, but does not influence sugar metabolism. The elevated protein content of *gw2.1* grain is in correlation with earlier observations made in wheat and rice (Achary and Reddy, 2021; Zhang et al., 2018) and is fully consistent with the molecular function of GW2.1 as E3-ligase.

In summary, our observations indicate that the *gw2.1* mutations in barley severely compromise the yield (Fig. 4B) and are associated with enhanced protein content of grains (Fig. 4C).

3.5. Cultivation conditions dependent and independent effects of gw2.1 mutations

To better understand the impact of the environmental changes on reproductive performance of both WT and *gw2.1* mutant plants, we analysed temperature data available throughout the two cultivation periods (Fig. 4D). In 2020 the average temperature was balanced, with mild transitions typically between 19–25 ºC (Δt=6 ºC). In contrast, in 2022 the average temperature showed drastic fluctuations between 16–28 ºC (Δt=12 ºC) along the cultivation period. In 2022 the plants were exposed to more drastic maximum

and minimum temperature extremes during sensitive developmental stages, such as meiosis and heading periods, compared to the 2020 cultivation conditions (Fig. 4D).

By directly comparing the performance of WT plants between the two cultivation periods, a consistent reduction was observed in 2022 regarding the grain quantitative attributes, such as GW (1.2-fold reduction in 2022 vs 2020), TGW (1,9-fold reduction), and consequently yield per plants (1,3-fold reduction) (Fig. 3D, E and Fig. 4B). GL was mostly unaffected with a non-significant decreased value in 2022 (Fig. 3C). Furthermore, besides quantitative differences, the grain protein content was also decreased; the cca. 18% protein content in 2020 was reduced to cca. 12% in 2022 (Fig. 4C).

Data from both of the investigated years (2020 vs 2022) were compared also in the cases of mutant plants. Generally, the mutant plants performed more consistently regarding the quantitative attributes GL, GW and TGW (Fig. 3, 4). However, the grain protein content was significantly diminished in 2022 vs 2020 also in the *gw2.1* plants, from cca. 22-24% to cca. 17-19% (Fig. 4C). The optimal temperature for barley growth and development is in the range of 15-20 °C. The higher average daily temperatures measured in 2022 may have contributed to the low performance of WT plants in 2022 in comparison to 2020, having a dramatic impact on quantitative and/or qualitative grain traits. Notably, the environmental conditions did not alter the impact of GW2.1 mutation on GL and protein content increase.

3.6. Heat stress response regulation of GW2.1 gene expression

The average daily temperatures up to 26-28 ºC during 2022 (Fig. 4D) may have contained heat stress temperature spikes. Indeed, when analysed in detail, we observed that daily temperatures could sometimes rise even above 50 ºC, suggesting that plants are affected by severe temperature stresses (Fig. 4D). Therefore, we exposed WT and selected *gw2.1* mutant lines to moderate HS at 37 ºC and tested the early (following 1 h) and the late (1 day) HS-induced changes. We found that *GW2.1* mRNA level was significantly decreased after 1 h HS treatment and was drastically down-regulated following 1 day HS treatment (Fig. S4A). The mutant lines exhibited a reduced basal mRNA expression (cca. 0.5-fold lower compared to WT levels), as expected, likely due to the activity of NMD-mediated decay induced by their long 3' UTR (Kertesz et al., 2006). Heat-induced down-regulation of *gw2.1* mRNA also indicates the transcriptional regulation of the *GW2.1* locus in response to heat (Fig. S4A). To directly test the requirement of *GW2.1* gene activity/product during HS response, we analysed accumulation of heat stress proteins HSP70 and HSP90 in response to heat in WT and *gw2.1* mutants. HSP70 and HSP90 proteins accumulated to a similar level in response to 1 hour and 1 day HS treatments in WT and mutant plants (Fig. S4B). Heat tolerance of *gw2.1* mutant seedlings was also tested. No significant differences were found in survival rates of *gw2.1* plants compared to control plants (*data not shown*). To reveal that mild heat stress treatment, which spring barley likely encounters on field, can also alter the expression level of *GW2.1* gene, we applied 30 ºC for 1 day long treatment to young (30 days old) and green spike

developing (75 days old) plants which were grown at 18 ºC. We found that mild heat stress treated leaf samples showed a tendency of moderate (albeit not significant) reduction of *GW2.1* mRNA level (Fig. S4C). In contrast, at same time, samples originated from young green spikes exhibited the significant down-regultion of *GW2.1* mRNA content relative to the control plants grown at 18 ºC (Fig. S4C).

These observations indicate that *GW2.1* gene expression sensitively responds to heat stress treatment, especially in developing spikes, but does not directly affect the production of HSP chaperones and heat stress tolerance.

4. Discussion

The role of UPS in controlling grain size through the action of a RING-type E3 ubiquitin ligase GW2 was first described in rice (Song et al., 2007). Since then, a number of studies have been conducted in several economically important crops including wheat, rice, maize and analysed the roles of GW2 on vegetative and reproductive development. These studies suggested that manipulation of *GW2* gene may be directly and successfully used in breeding programs for increasing yield productivity (Achary and Reddy, 2021; Huang et al., 2022a; Yamaguchi et al., 2020; Zhang et al., 2018). Notably, most of these works mainly analysed GL, GW and TGW values, but the grain yields and grain quality/quantity measurements in near natural cultivation experiments were less investigated (Huang et al., 2022a; Sestili et al., 2019; Song et al., 2007). In addition, contradictory findings in some reports raised uncertainty regarding the exact roles of GW2 in crop productivity (Bednarek et al., 2012; Zombori et al., 2020).

The knowledge on roles of GW2 in barley is scarce. In a recent publication two *GW2* gene variants were described (Zombori et al., 2020). Partial RNAi mediated down-regulation of mRNA expression of both genes (*GW2.1* and *GW2.2*) induced the production of enhanced TGW. However, detailed phenotyping revealed that the suppressed activity of *GW2.1* and *GW2.2* associated with various phenotypic alterations. Down-regulation of the activity of both genes increased the number of side shoots. While *GW2.1* specific RNAi lines showed earlier heading time, prolonged grain-filling period, and stimulated root growth, the *GW2.2* specific RNAi lines exhibited delayed flowering and reduced root system.

To clarify the roles of *HvGW2.1* gene, a canonical E3-ubiquitin ligase *GW2.1* homologue in barley, we created independent knock-out mutant lines using CRISPR/Cas9 mutagenesis system (Fig.2A-C). Importantly, our mutagenesis did not impact *HvGW2.2* locus allowing the investigation of the specific biological role of the *HvGW2.1* gene. Employing these, alongside multiple controls (WT, CRISPR/ Cas9-control and *gw2.1* hypomorph plants), we characterised the effects of *gw2.1* mutation on barley's vegetative growth and reproductive development. Greenhouse observations revealed only marginal involvement for GW2.1 during early vegetative growth regulation. From the mutant plants sg1_2-25-8 has slightly elongated plant body structure (Fig. 2D). Since this phenotype was observed only in sg1_2-25-8 plants, we cannot exclude a CRISPR/Cas9 off-target or a transgene insertional effect. However,

more detailed phenotyping measurements are necessary to precisely reveal the effect of *gw2.1* mutation on barley vegetative and reproductive development.

We performed analysis on reproductive fitness by measuring grain size, quality, and yield (Fig. 3, 4). We have shown that absence of GW2.1 provokes grain elongation, as vastly documented in the literature. However, grain shape changes were found to be coupled to a low grain set and yield decrease. In both cultivation periods the mutant lines performed equally and their seemingly altered performances in 2020 and 2022 originated from the under performance of WT plants in 2022 (Fig. 3). This observation indicates that control WT plants exhibited high sensitivity to temperature fluctuations in 2022 while the mutant lines were tolerant and performed similarly to 2020. This feature of *gw2.1* plants perhaps gives an explanation to the discrepant findings observed by other investigators (Bednarek et al., 2012; Zombori et al., 2020).

The lack of GW2 in rice enhances grain weight but also induces phenotypic alterations increasing the panicle number per plant, days to heading and decreasing main panicle length, grain numbers per main panicle (Song et al., 2007). Genetic knock-out of *GW2* gene via genome editing in Indica (var. MTU1010) resulted in enhanced grain protein content and increased grain width compared to the control plant (Achary and Reddy, 2021). As a pleiotropic effect these mutant plants showed improved root-shoot length and biomass production. A natural single nucleotide substitution in the coding sequence of *GW2* gene in rice, line jf42, induced enlarged grain size which was associated with no significant differences in the number of tillers and filled grain per panicle (Huang et al., 2022a). Enhanced grain size was also associated with natural and genome editing generated rice *gw2* mutants which showed thicker internodes as pleiotropic effect (Yamaguchi et al., 2020). In hexaploid wheat there are three *GW2* homeologues (*TaGW2A*, *TaGW2B*, and *TaGW2D*). RNAi mediated inhibition of the activities of wheat homeologues generated controversial data. *GW2* gene specific RNAi construct induced the down-regulation of transcript levels of homeologue mRNAs inducing decrease in grain size while no changes were observed in the number of grains per spike and the number of spikes per plant (Bednarek et al., 2012). In contrast, the simultaneous RNAi mediated down-regulation of *GW2* homeologue transcripts in a Chinese bread wheat variety brought about an increase in grain width and weight but similarly to the previous work was not associated with changes in spike and grain number (Hong et al., 2014). In addition, the paralleled RNAi mediated down-regulation *GW2* homeologues in tetraploid durum wheat elicited similar or higher grain yield per plant and somewhat increased spike numbers (Sestili et al., 2019). These observations indicate that loss of *GW2* activity, in addition to altered grain size, exerts pleiotropic effects influencing the development of other tissue types and/or reproductive organs. These various changes are divergent in the different *gw2* mutants indicating that the induced pleiotropic effects are species/cultivar specific and/or potentially determined by the nature of the particular mutation.

In separate cultivation experiments we also revealed strong pleiotropic effects of barley *gw2.1* mutations influencing economically important traits. The yield production of the mutant plants was significantly less in both of the investigated years, even in that year where the mutant plants exhibited

higher TGW value. To investigate the biological background of the general reduction of grain productivity of the *gw2.1* mutant lines we also measured the spike and the grain numbers (per spike) in the 2022 cultivation experiment. It was found that mutant lines typically produced less spikes and the number of grains per spike was also reduced. These data show that pleiotropic effects of *gw2.1* barley mutants strongly affect the basic developmental processes indicating the limitation of the practical use of *gw2.1* mutants in breeding programs.

The tendency of changes in grain traits of the knock-out mutant lines (sg1_1-6-8, sg1_2-25-8 and sg2_3-5-9) relative to the control (WT or unspecific CRISPR/Cas9 cassette containing) plants were consistent in the experiments, providing a strong basis for our conclusions. The three *gw2.1* knock-out lines show consistent and similar changes in cases of GW and TGW, but display significant variances in GL, grain number and yield when they are compared to each other in different cultivation experiments. The observed differences between knock-out lines could be due to diverse reasons. The observed differences between knock-out lines might be attributed to off-target effects associated with the presence of CRISPR/Cas9 transgene cassette. Two knock-out lines (sg1_1-6-8, sg1_2-25-8) do have, while one line (sg2_3-5-9) does not have the CRISPR/Cas9 transgene. Since all the three lines showed the similar tendency of changes and variances we hypothesise that CRISPR/Cas9 transgene off-target activities might not be the main cause of the observed variances. Even though the *in silico* off-targets were thoroughly analysed, we can not exclude that unintended integration of fragments of the transgene cassette into other unknown genomic off-target positions might contribute to the observed differences. However, the variances between lines are likely not line specific since the performances of individual lines show variances depending on the particular cultivation periods (Fig. 3C and Fig. 4A, B). As an alternative explanation it is possible that temperature dependent regulation of *GW2.1* gene might be a pivotal regulatory factor during spike development. The lack of GW2.1 could render the transgenic lines sensitive to variation in their micro-environment conditions (micronutrients, light availability or local temperature spikes) during greenhouse cultivation periods mounting more variable phenotypic responses.

The RING-type E3 ligases are the most expanded components of the UPS system (Jiménez-López et al., 2018). As based on our knowledge the number of RING-type E3 ligase homologues in barley is not known so far. However, in the closely related rice and maize genomes 399 and 478 putative homologues were identified, respectively (Jiménez-López et al., 2018). This suggests a divided workshare and perhaps lots of redundancies in terms of targets between these components. The impressive 40-to-50% change in the total protein content of the *gw2.1* mutants (Fig. 4C), lacking a single RING-type E3 ligase suggests a dominant and specific role for it during barley grain filling and maturation. Whether the protein surplus is a specific type of protein (e.g. gluten, hordein) or a mixture of different proteins, remains to be determined. Notably, the protein content of *gw2.1* mutants was significantly elevated, regardless of the cultivation period, but the starch content was largely unaffected both qualitatively and quantitatively, as based on amylose, amylopectin and amylose/amylopectin ratio measurements (Fig 4C,

S3). These results are fully consistent with findings in wheat (Zhang et al., 2018). In summary, the gathering data indicate that GW2.1 specifically governs protein, but not polysaccharide metabolism of barley grains. Since barley grain protein content is increased in the absence of GW2.1 independently from environmental conditions the modulation of *GW2* gene expression can be a foundation of genetic improvement of grains with high protein content.

GW2.1 level seems to be dynamically regulated by environmental changes. The different grain quality and quantity data originating from the investigation *gw2.1* mutant lines and *GW2.1* mRNA down-regulation during heat stress (37 ºC and 30 ºC) treatments support this assumption. The hypothesis that *GW2.1* gene can be part of a stress response network is also backed by previous findings showing that the expression level of rice *GW2* mRNA can be altered in response to fluoride toxicity in a cultivar-dependent manner (Banerjee et al., 2021). Heat stress mediated down-regulation of *GW2.1* mRNA level (Fig. S4A, C) suggests that its absence may be favourable under adverse conditions. For this we can envision multiple scenarios. In the absence of GW2.1 seed set and spike development are decreased which can lead to fewer but greater grains. This phenomenon may be a strategy to maximise viability of the progenies in the next vegetation period. Indeed, in 2022 cultivation period elevated average daily temperature conditions lead to increased GW, TGW but decreased spike and grain number in *gw2.1* mutants compared to control plants. The other possibility is that perhaps higher protein content has a positive effect on germination and/or seedling growth in the next generation under an unfriendly environment. Finally, we cannot exclude that *GW2.1* dramatic down-regulation at 37 ºC is simply a secondary effect of heat stress. It was shown earlier that the housekeeping transcriptome program is shut-off and sharply shifted to a HS transcriptome (Szadeczky-Kardoss et al., 2022). Altogether, our data indicate that *GW2.1* gene may be an element of heat perception and consequently tightly regulated by temperature conditions.

With the baseline set in our study, now several further questions can be asked: (i) what the target specificity and what sort of proteins are specifically ubiquitinated and targeted for decay by GW2; (ii) how GW2 activity is modulated by ambient temperature fluctuations; (iii) through which molecular routes regulates GW2 processes upstream to grain development, such spike and grain number determination; (iv) how much conserved are the effects of GW2 actions on protein content and grain traits in the different monocot crops and beyond; (v) what is the best strategy to modulate GW2 expression and/or activity for its agronomic use?

5. Conclusions

The basal function of the plant seeds is to supply energy to the developing seedlings. Crop grains also provide nutrition and energy for animal and human organisms. Using *gw2.1* CRISPR/Cas9 barley mutants and diverse cultivation conditions we have shown that GW2.1 regulates the balance between yield and grain traits, likely to optimise evolutionary fitness on a long term. However, loss of GW2.1

function is also associated with severe pleiotropic effects reducing the grain set and yield of the mutant plants. Further studies are needed to reveal the complex molecular network of grain development in barley to exploit the potential economic benefits linked to the disabled function of GW2.1.

Author contribution

AK and ZH conceived and designed the study. AK, ÁD, IA, DP and MR performed the experiments; AK, TC, LS, MR and ZH analysed data; ZH wrote the manuscript with the help of TC. All authors approved the final version.

Declaration of Competing Interest

All the authors declare that they have no conflict of interest.

Acknowledgement

This work was supported by the Hungarian Research, Development and Innovation Office (NKFIH), grant numbers FK134264, K125300, K137722 and the Hungarian Academy of Sciences. Hungarian National Laboratory Program, grant number RRF-2.3.1-21-2022-00007. AK was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. IA is a Stipendium Hungaricum Scholar (scholarship no 2022-516933).

Appendix A. Supplementary material

Supplementary material, Fig.S1-S4, Table S1.

Data Availability

Data will be made available on request.

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Figures

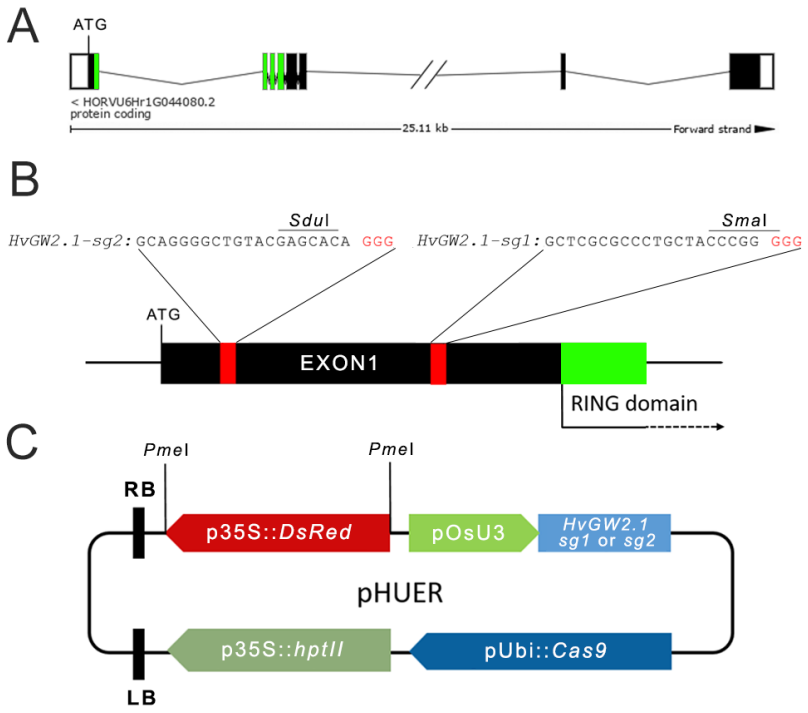

Fig. 1. Structural organization of barley *GW2.1* gene and Cas9 expression cassette. (A) Barley *GW2.1* gene consists of 8 exons; black boxes indicate exons while thin black lines represent intron sequences. Green colour indicates the region encoding the RING domain; (not to scale). (B) 20-nt sgRNA sequences along with *Sdu*I and *Sma*I restriction sites target two separate positions in the first exon of *GW2.1* disrupting the RING-U box domain of the gene; (sg1 and sg2, respectively, PAM sequences are shown in red). (C) Constitution of CRISPR/Cas9 vector, showing the rice U3 promoter driven sgRNA expressing cassettes, 35S promoter driven *DsRed* reporter gene, selection marker *hptII* constructs and Ubiquitin promoter driven *Cas9* cassette.

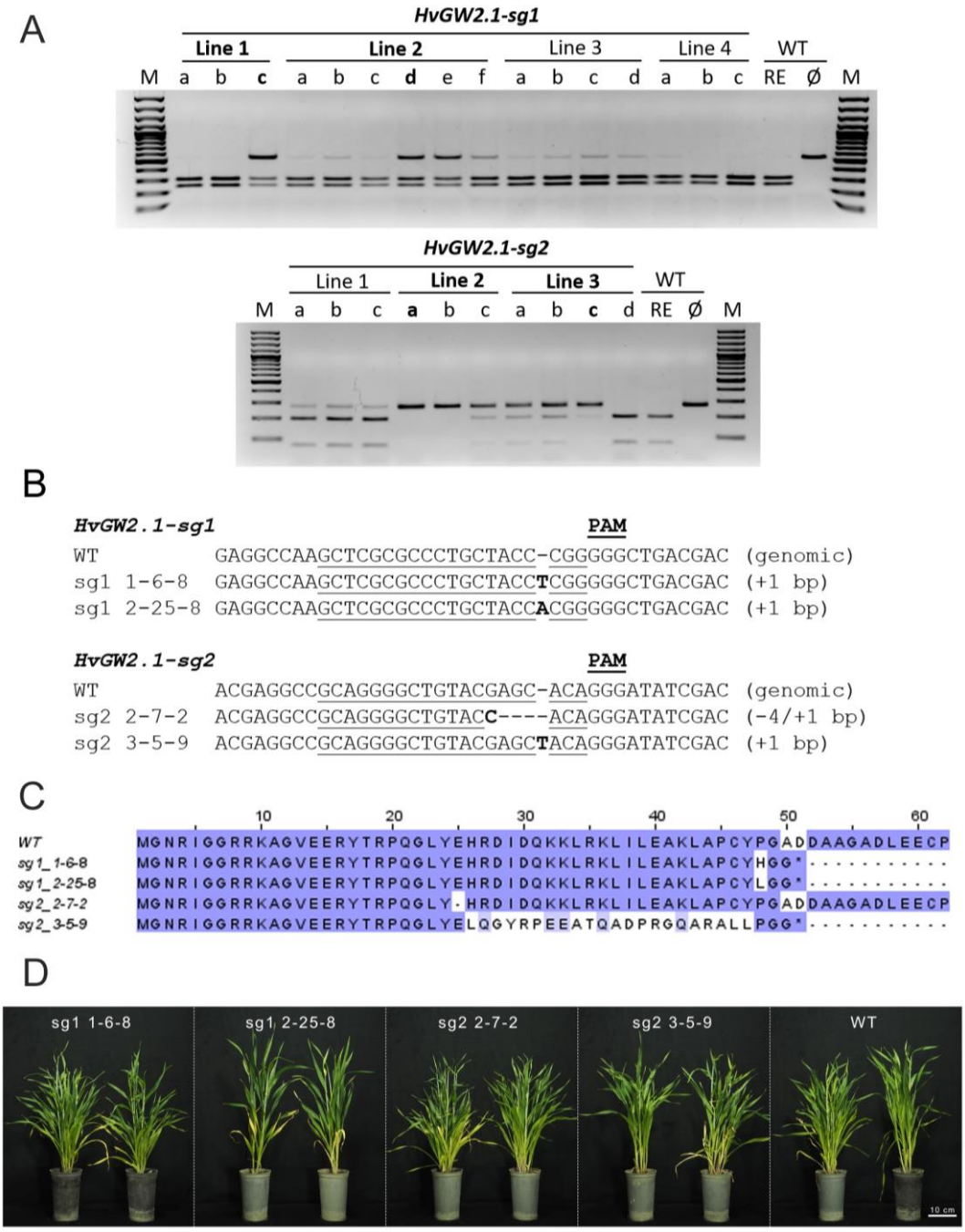

Fig. 2. Genome editing mediated targeted mutagenesis of *GW2.1* gene and verification of *GW2.1* disruption in selected transformant plants. (A) Restriction enzyme digestion analyses of PCR products encompassing sgRNA target sites amplified from genomic DNA samples of T0 transgenic barley plants expressing sgRNA1 or sgRNA2 and wild type control plant. Lines 1-4 represent independent calli. Small letters a-f represent sibling plants originated from the same callus. Bold letters indicate the selected T0 lines. RE, digested; Ø, non-digested wild type DNA specific PCR products. M, molecular weight marker. (B) Genotyping of *gw2.1* mutations in independent transgenic progeny plants at T2 generation expressing sgRNA1 or sgRNA2. The underlined sections represent the target sites of the sgRNAs. Bold letters show the nucleotide insertions. (C) *In silico* translation analysis of the generated *gw2.1* variants. N terminal fragment (62 amino acids) of GW2.1 is shown. (D) Phenotypic analyses of young developing mutant barley plants compared to the wild type control (*cv.* 'Golden Promise').

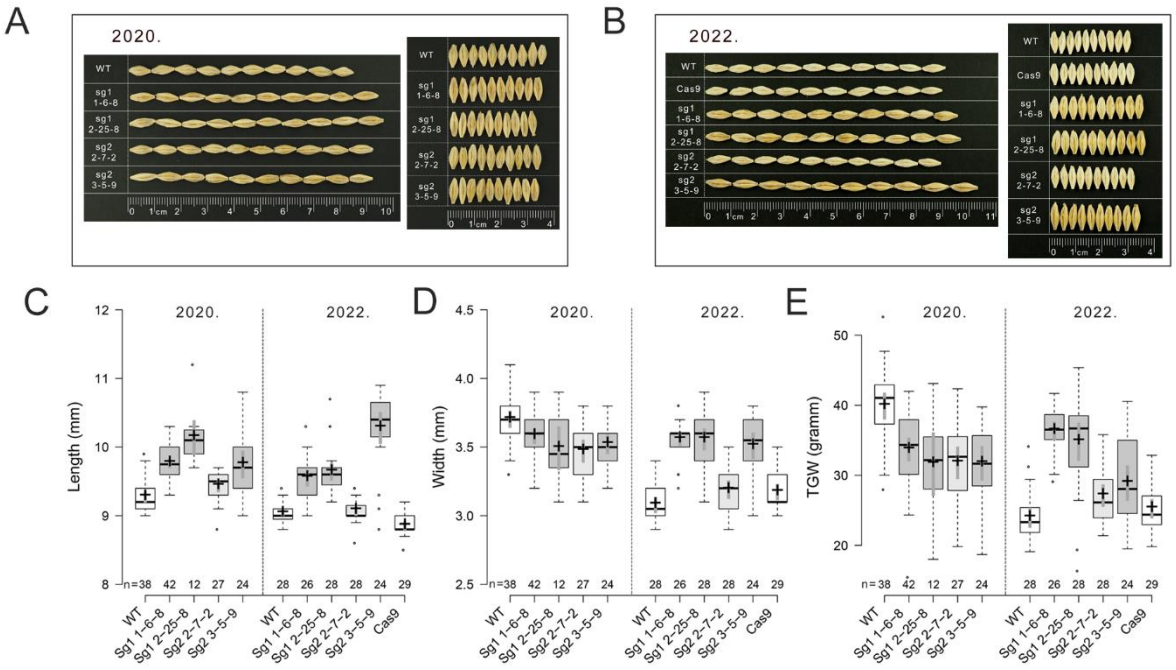

Fig.3. Analyses of grain and production parameters of *gw2.1* mutant lines compared to control plants in 2020 and 2022 cultivation periods. (A) Grain length and width of mutant and control plants' seeds (ten each) in 2020 cultivation periods. (B) Grain length (GL) and width (GW) of mutant and control plants' seeds (ten each) in 2022 cultivation period. Comparison of grain length (C), grain width (D) and calculated thousand grain weight (TGW) (E) of mutant and control plants in 2020 and 2022 cultivation periods, as indicated. Numbers at the bottom (n) indicate the number of the individual plants investigated in the particular study. Light grey boxes indicate the hypomorph line. The collected data were presented and statistically explored with boxplots (box-and-whisker plots) made by BoxPlotR (*see Materials and methods*).

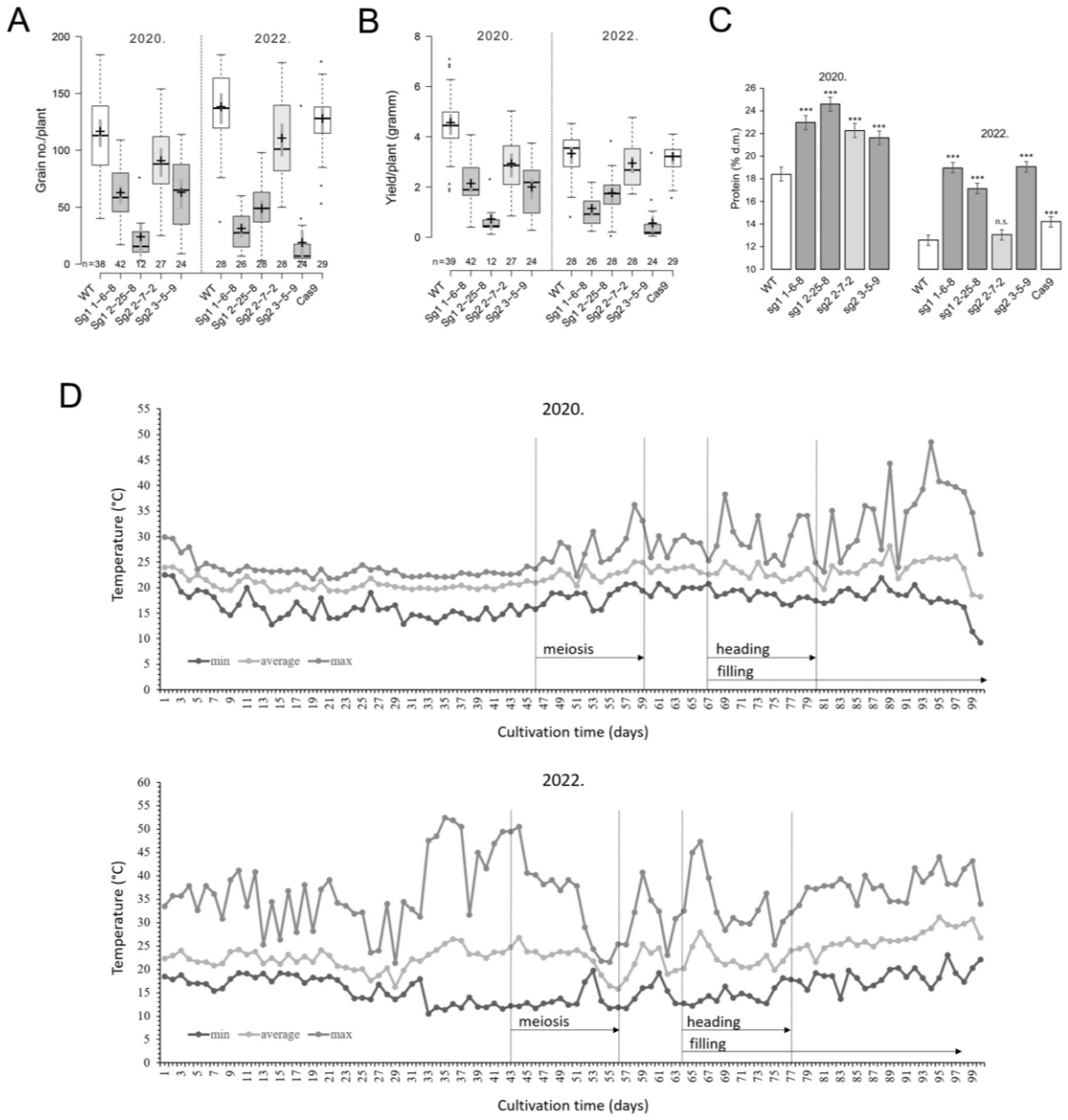

Fig.4. Analyses of grain number, total yield and grain protein content of *gw2.1* mutants compared to control plants in 2020 and 2022 cultivation periods. (A) Grain numbers per plant of mutant and control plants. Numbers at the bottom (n) indicate the number of the individual plants investigated in the particular experiment. Light grey box indicates the hypomorph line. The collected data were presented and statistically explored with boxplots (box-and-whisker plots) made by BoxPlotR (*see Materials and methods*) (B) Total yield per plant of *gw2.1* mutants and control plants. (C) Protein content of wholemeal samples of *gw2.1* mutants compared to control plants. Two-factor analysis of variance (two-way ANOVA) test was used to determine difference between samples (non-significant, n.s.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significance against wild-type control). (D) Temperature profiles of the 2020 (top) and 2022 (bottom) cultivation periods. Light grey lines indicate the daily average, grey lines the daily maximum, dark lines the daily minimum temperature values.

Supplementary material

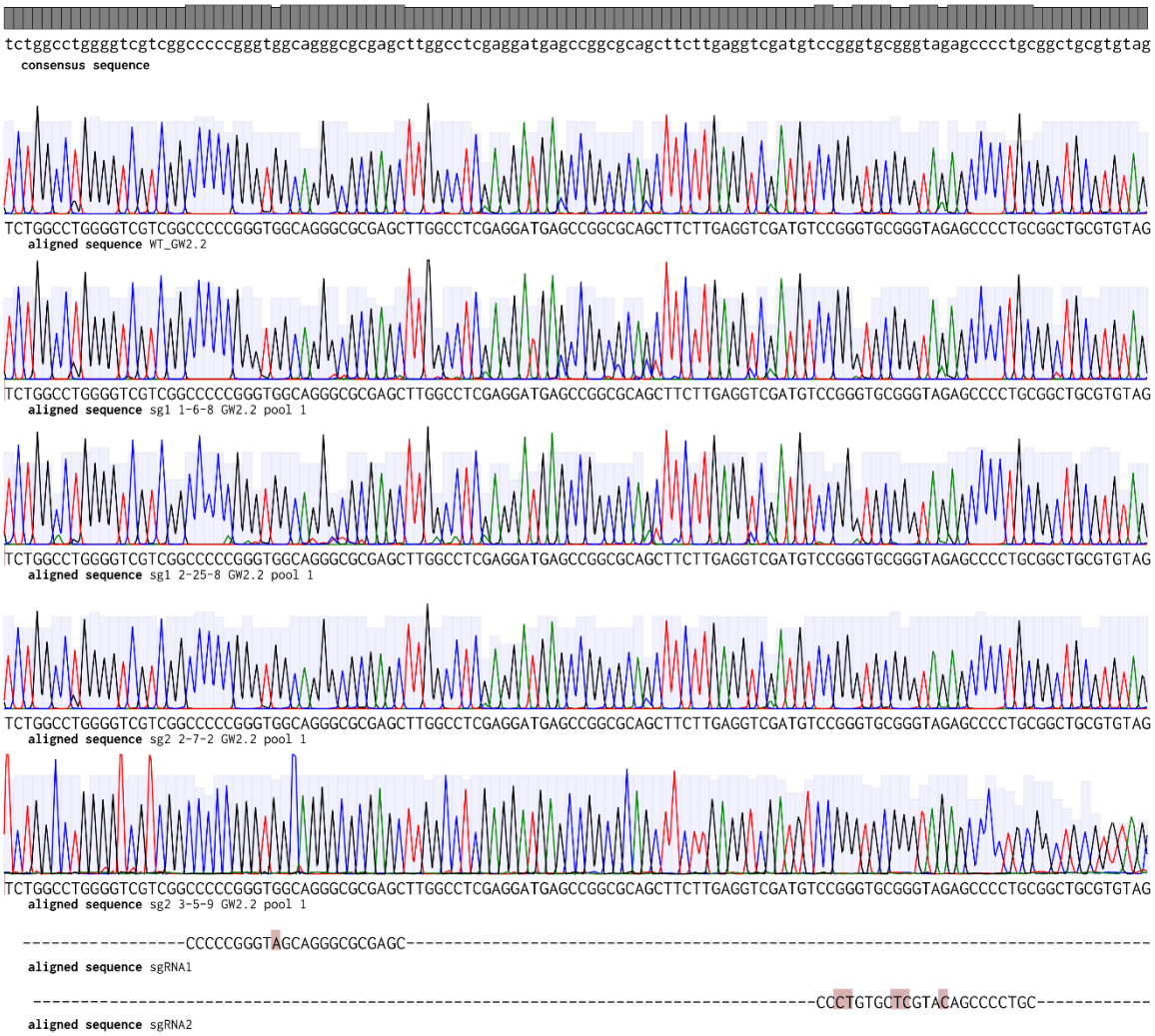

Fig. S1. *GW2.2* gene is not affected in *gw2.1* mutants. Sequence analyses of *GW2.2* potential target site specific PCR products of pooled T3 representatives (5-5 plants) of mutated *gw2.1* lines compared to WT plants.

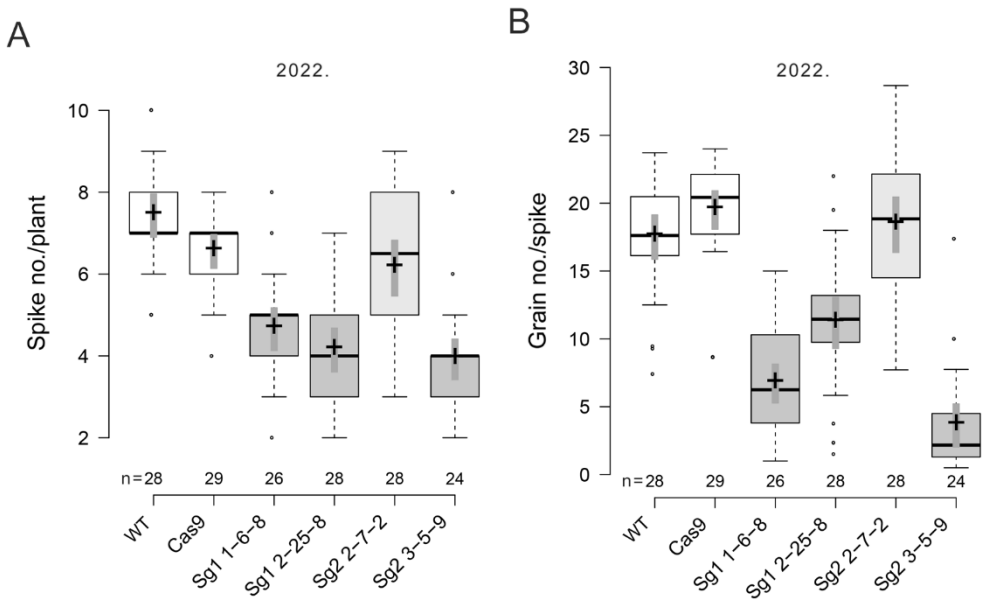

Fig. S2. Analyses of spike number and grain number per spike of control and *gw2.1* mutant plants in the 2022 cultivation period. (A) Spike number per plant. (B) Grain number per spike. Numbers at the bottom (n) indicate the number of the individual plants investigated in the particular experiment. Light grey box indicates the hypomorph line. The collected data were presented and statistically explored with boxplots (box-and-whisker plots) made by BoxPlotR (*see Materials and methods*).

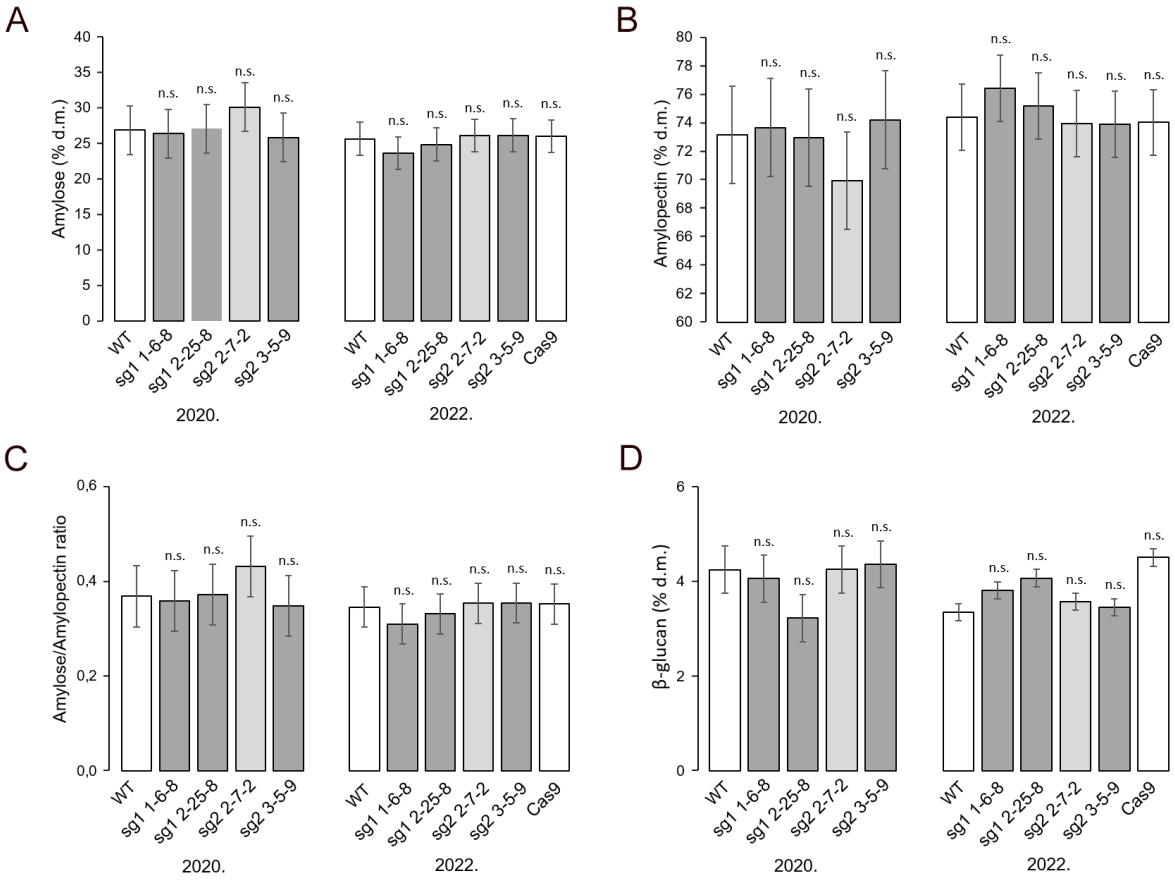

Fig. S3. Compositional traits of the wholemeal samples of the *gw2.1* mutants compared to the control plants in 2020 and 2022 cultivation periods. (A) Amylose, (B) Amylopectin, (C) Amylose/Amylopectin ratio, (D) β-glucan contents of grain samples. Light grey bars indicate the hypomorph line. Two-factor analysis of variance (two-way ANOVA) test was used to determine difference between samples (non-significant, n.s.; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 significance against wild-type control).

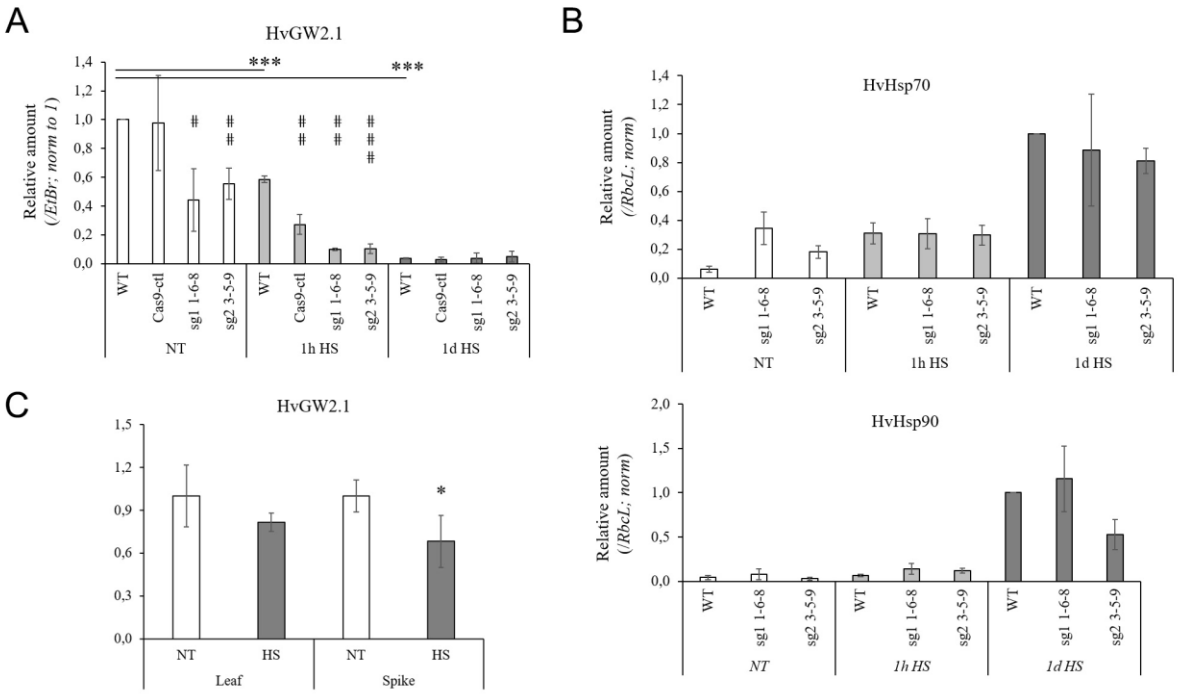

Fig. S4. GW2 regulation and HSP protein accumulation changes in control and representative *gw2.1* lines during HS. (A) *GW2.1* mRNA expression changes in response to heat stress treatment (non-treated, NT; 1 hour, 1h HS; or 1 day, 1d HS at 37 ºC) as determined by semi-quantitative PCR measurements; values were normalised to *Actin* mRNA expression. (B) *GW2.1* mRNA expression changes in response to moderately high temperature (1h 30 ºC) in barley leaves and spikes, as measured by qrtPCR; values were normalised to *Actin* mRNA expression. (C) HSP70 and (D) HSP90 protein accumulation changes in response to HS treatments (non-treated, NT; 1 hour, 1h HS; or 1 day, 1d HS at 37 ºC); values were normalised to Rubisco large subunit (RbcL) stain free signals. Samples are shown below (wild type, WT, *Cas9-control, gw2.1* mutants sg1_1-6-8 and sg2_3-5-9); P-values based on two-tailed Student's t-test ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ significance against WT control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significance against NT control).

Table S1: DNA oligonucleotides used in the study.

Name	Sequence (5' - 3')	Utilization
gRNA1_insert_F	GGCGCTCGCGCCCTGCTACCCGG	to create guide RNA spacer for pHUER vector
gRNA1_insert_R	AAACCCGGGTAGCAGGGCGCGAG	
gRNA2_insert_F	GGCGCAGGGGCTGTACGAGCACA	
gRNA2_insert_R	AAACTGTGCTCGTACAGCCCCTG	
gRNA1_det_F	CAGGGTAATCCCACCTCGCCTCGG	to detect genomic mutation on *HvGW2.1*
gRNA1_det_R	CTCACGGCAGCGGCACTCTACG	
gRNA2_det_F	ATGGGGAACAGAATAGGAGGGAGG	
gRNA2_det_R	GGCACTCAAACAACCACCAACATC	
35Sp_PmeI_F	TACGTTTAAACGCCAACATGGTGGAGCACGAC	to create 35SdsRED cassette
35Sp_PmeI_R	GCCGTTTAAACGGGGGATCTGGATTTTAGTACTG	
Ubi_det_F	AACCAGATCTCCCCCAAATC	Zea Mays Ubi1 promoter detection primer
Ubi_det_R	AAACCAAACCCTATGCAACG	
HvGW2.1_qPCR_F	CAGGCTAACATGCGGTCTTTC	oligoes for qPCR
HvGW2.1_qPCR_R	ACGTGTTGGTTGCTCAAAAGG	
HvActin_qPCR_F	ATGTTCCCAGGTATCGCTGAC	
HvActin_qPCR_R	ACTCGTCGTACTCATCCTTGG	
HvGW2.2_det_F	GGG GGG ATG GGG AAC CGG GTG	to detect genomic mutation on *HvGW2.2*
HvGW2.2_det_R	CCT TGG CGC AGC ACT TTG ACC GG	