Closing report

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Investigation of heat stress associated RNA interference and crop quality determinant genes with genome editing techniques in barley.

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Analyses of barley RNA interference factors

To identify barley's RNA silencing associated genes, the A. thaliana DCL, AGO and RDR protein's amino acid sequences were used as queries to build a Hidden Markov-model (HMM). According to HMM profile analysis, a total number of five HvDCLs, eleven HvAGOs and seven HvRDR genes were predicted in the barley genome (Figure 1.). H. vulgare silencing components were named based on the closest relative in A. thaliana, or monocot species O. sativa and Z. mays. The barley RNA interference components were analyzed in depth, the protein structures and chromatin localization of the candidate genes were demonstrated.

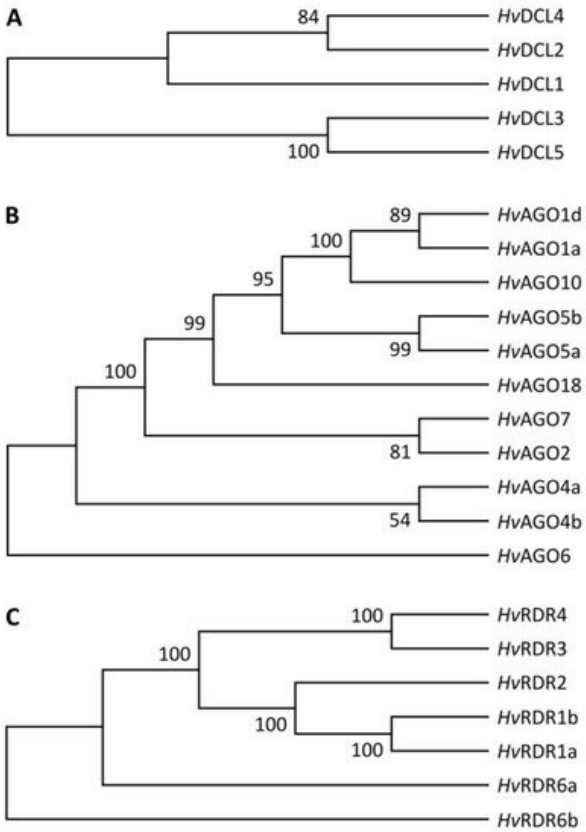

Figure 1.

Investigation of barley heat-stress responsive RNA silencing factors

To unravel heat-induced transcriptional regulation of silencing trans factors in barley, we studied RNA-seq data available. Based on these, we selected silencing 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. The highly conserved miR390/TAS3/ARF pathway exerts its function via the siRNA-based subgroup of silencing factors. We searched for TAS3-derived tasiRNA targets in barley and identified three potential ARF target transcripts. Two of these targets were significantly down-regulated upon heat stress suggesting that the activity of siRNA-based silencing is potentially elevated at higher temperature. Contrarily, the principal trans factors of miRNA pathway, HvDCL1 and HvAGO1a seem to be much stable under the investigated circumstances. In summary, data from RNA-seq, semi-quantitative and RT-qPCR

measurements all converge and points towards the transcriptional accumulation of factors enrolled primarily in siRNA-based silencing, including hc-siRNA, pha-siRNA and RDR6-dependent sRNA pathways. Importantly, our gradient heat treatment mimics natural situations, e.g., temperature changes during a summer day, therefore may be relevant in field conditions.

Altogether, we have identified members of gene families having key roles in RNA silencing of barley and provided basic data on their genomic location, clade, phylogenetic relations, domain and motif organisation, and catalytic core build-up. Our data firmly suggests that these players are potentially functional and likely required at some point during barley's lifecycle. Transcriptional accumulation of siRNA pathway factors hints to a probable role in environmental adaptation. This work will be a stepping-stone to ask further fundamental and exciting questions that remained pending in barley and monocot RNA silencing field.

These data were published in 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 4,634)

CRISPR/Cas9 mediated knock off of barley genes

Based on the results of heat shock studies we selected DCL3 and AGO6 for further experiments to investigate the role of RNA silencing in heat stress responses.

In both cases specific single guide RNAs were created targeting important domains of the genes. In general, transgenic plants were generated expressing the specific CRSPR/Cas9 – guide RNA system and target mutations in the T0 plants were identified.

DCL3 mutant barley

In the case of DCL3 barley mutants we were not able to prove the action of of sgRNA 1 but we were able to identify mutants having immature stop codon at the position of sgRNA2 (Figure 2). Tihis mutations results in truncated RNase III domain strongly hindering the function of DCL3. We had difficulties with germination of the mutated lines indicating that DCL3 may

have important developmental related functions. We managed to propagate one T0 line and now we are waiting for the production of T1 seeds for further investigations.

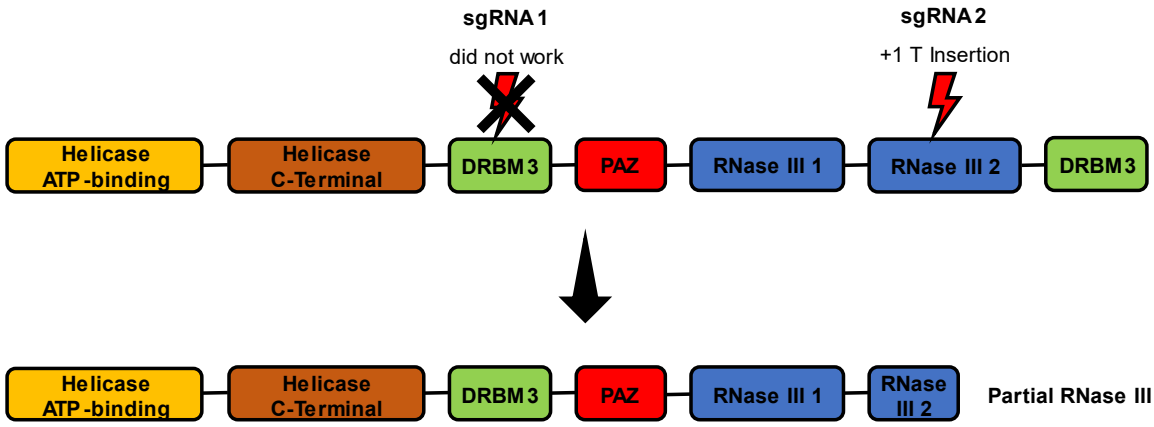

Figure 2.

However, we experienced the increased sensitivity of drought and heat of mutant plants (Figure 3.). This observation is very promising, since it is indicated that the 24 nt siRNA pathway indeed has an pivotal role in in perception and/or response to environmental stresses. This line will be further investigated to understand biological processes compensating environmental stresses in barley.

AGO6 mutant barley

We also selected HvAGO6 as potentially important component of RNAi responsible for heat shock sensing. To generate HvAGO6 mutant barley lines 3 possibly efficient sgRNA sequences were selected bioinformatically. To test their biological activity in N. benthamiana transient leaf infiltration system, dsRED expressing sensor constructions were created. According to transient tests the best performing guide RNAs were selected to make a 2 sgRNA coding CRISPR/Cas9 construction to transform immature barley embryos. Four plants were successfully regenerated after transformation experiments. According to T7 endonuclease tests

all T0 regenerants carried mutations on both sg5 and sg21 target sites. However, the plants were infertile, no T1 generation have been generated. We produced more T0 plants and eventually we managed to produce T1 plants caryy homozygous biallelic mutations.

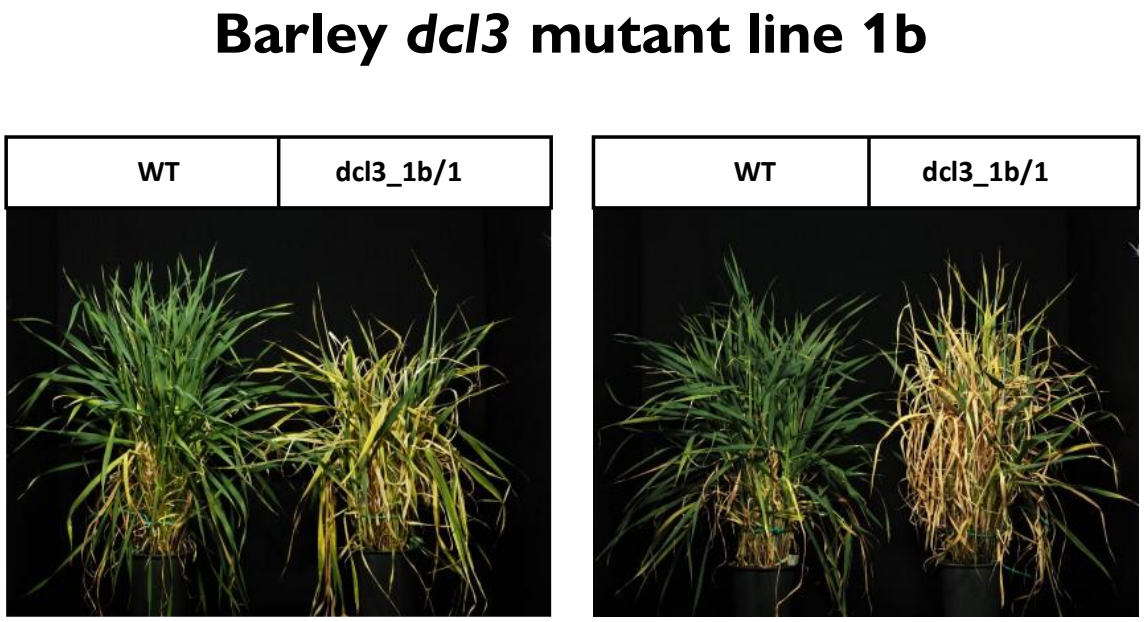

Figure 3. Response of representatives of dcl3 barley line to environmental stresses.

The ago6 transgenic plant will be further propagated and the fine molecular roles of AGO6 in development and stress responses will be investigated.

pHUE Hv AGO6 2sg 1/a.3

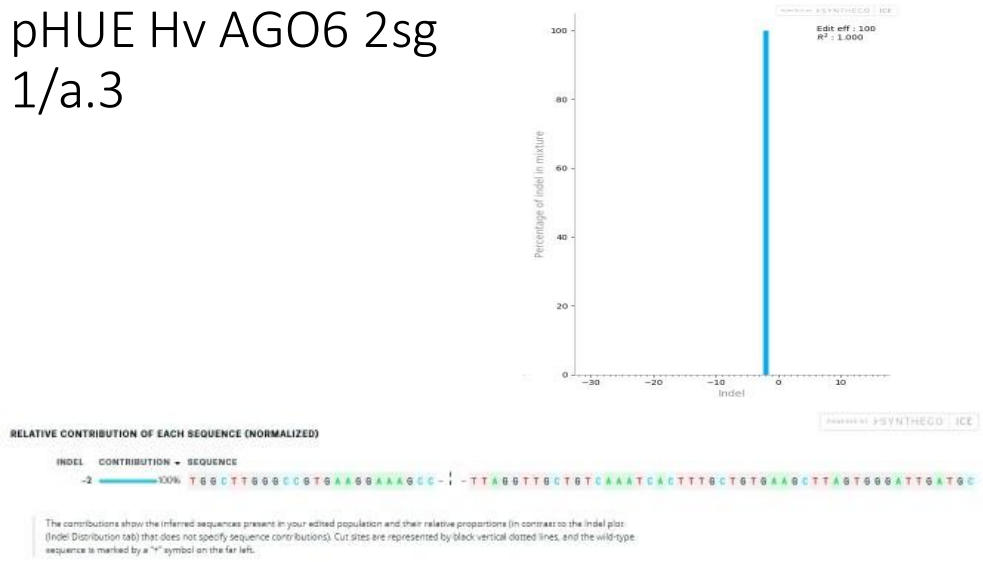

Figure 4. An example for ago6 biallelic mutant.

GW2 mutant barley

A GW2 orthologue (B0FLE0) was previously identified in a Tibetan barley and named Yield-related gene (HvYrg1, GenBank No. EU333863). Later two *GW2* orthologs have been identified in barley, named HvYrg1and HvYrg2. Two genes show 86.62% identity. We also identify the barleys GW2/HvYrg1 orthologue, using the rice GW2 protein (ABO31101) for blast search in the Uniprot database. The target genomic sequence was identified in the Ensemble database (HORVU6Hr1G044080.2) identifying the barley HvYrg1 gene.

Next we identified CRISPR/Cas9 gRNA target sites on HvYrg1 gene using CRISPOR software. The generated gRNA sequences were investigated for off-target activity and gRNAs exhibiting no potential *in silico* predicted off-targets were selected for further experiments. Based on the gained data two gRNAs have been chosen for targeting 5’ region of the HvYrg1 gene in first exon. The selected target sequences contain restriction cleavage sites overlapping with RISPR/Cas9 target sites facilitating the detection of mutations. Two gRNA expression cassettes were built expressing the gRNA1and 2 independently. The pHUER vector backbone contains expression cassettes for Cas9, DsRed marker protein and sgRNA driven by rice U3 promoter. The presence and accuracy of the introduced sgRNA sequences were confirmed in the vectors by sequencing.

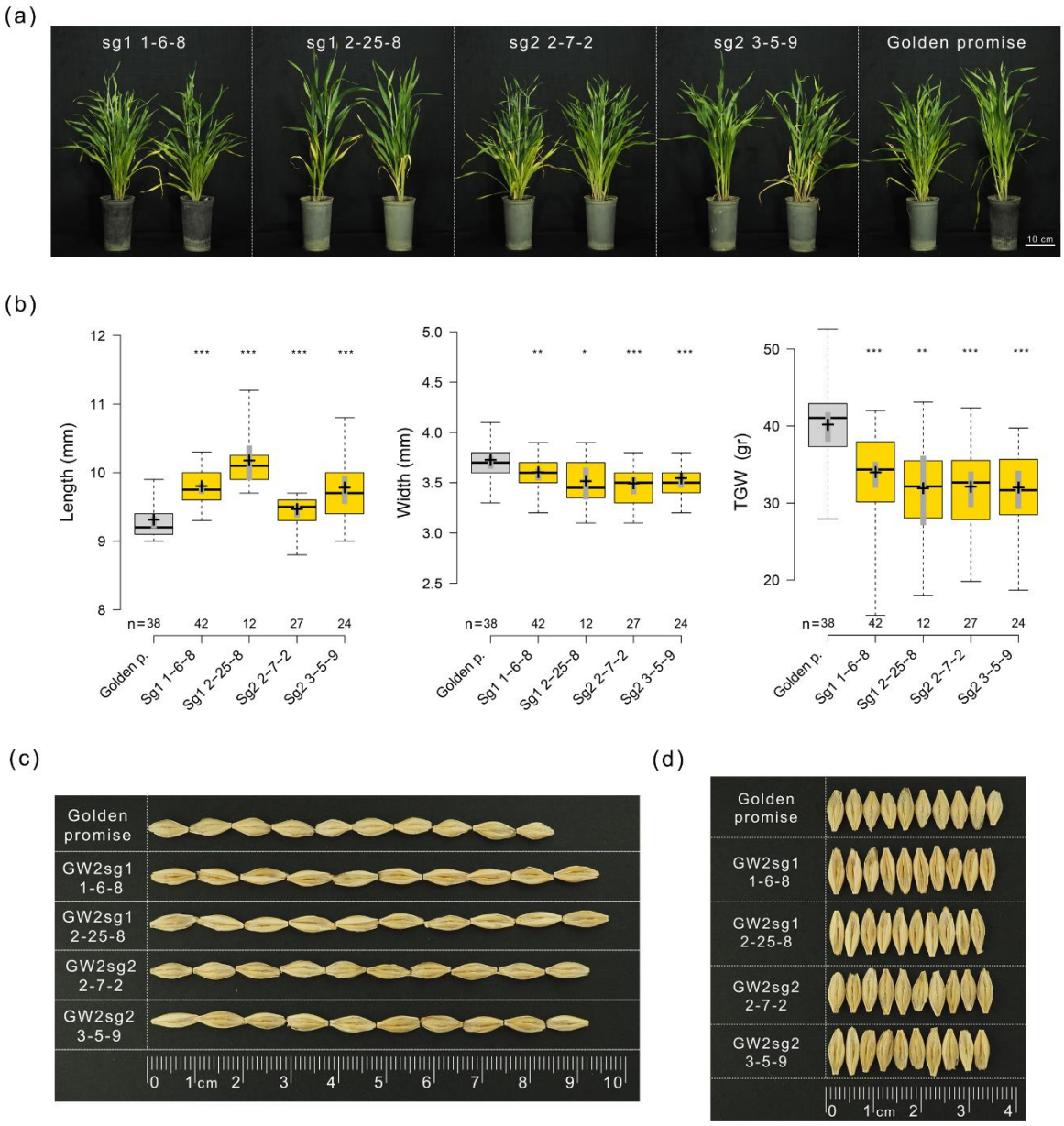

Figure 5.

Embryos of barley plants (*cv*. Golden Promise) were used for *Agrobacterium*-mediated transformation. DsRed marker gene activity was used to identify primarily transgenic plantlets. The selected lines were checked for the presence of the transgene cassette by PCR analyses. CRISPR-targeted mutations in PCR positive lines were identified by PCR/RE treatment in T0 plants. In the case of gRNA1 we identified 12 plants bearing mutations out 16 plants. No biallelic mutants have been identified in T0 only heterozygous and/or mosaic transgenic lines were identified. Transgenic lines expressing gRNA2 exhibited mutations in 9 out of 10 lines

including two biallelic mutants. We selected two independent mutated lines per specific gRNA which were further propagated for T1 generation. In The T1 generation mutant biallelic lines were identified PCR/RE in both cases and the selected biallelic lines were sequenced to identify homozygous mutations. To prove the heritability of the identified mutations they were further propagated for T2 generation and the introduced mutations were re-sequenced. The sequence analyses revealed that the mutations were stably heritable. Next, we selected two-two independent lines representing mutants generated by each gRNAs. Form the instigated mutants three contained one base insertion generating premature stop codon. In one of the gRNA2 lines the insertion of 1 base and the deletion of 4 bases resulted in the production of mutated protein, lacking one amino acid, exhibiting severe conformational changes.

The majority of the transgenic plants were mostly indistinguishable from the wild-type barley plants although 2-25-8 plants exhibited slightly elongated pathotype (Figure 5). This finding indicates that the presence of the transgene cassette does not interfere markedly with the normal developmental processes. Next, we analysed the harvested kernel for seed length, width and thousand weights. We observed the significant elongation and thinner morphology of the grains (Figure 5). The analyses of the dry grain thousand weight (TGW) showed significant decrease in all mutants.

VRS1 mutant barley

Increased seed production has been aa important goal during the domestication of cereal crops. The wild-type *Vrs1* allele encodes a transcription factor. Expression of *Vrs1* was strictly localized in the lateral-spikelet primordia of immature spikes. This suggests that the VRS1 protein suppresses development of the lateral rows. Loss of function of *Vrs1* resulted in complete conversion of the lateral spikelets in two-rowed barley into fully developed fertile spikelets in the six-rowed phenotype.

So far no six-rowed barley generated by genome editing has been described. We developed a homozigous vrs1 barley mutant plant by CRISPR/Cas9 in a two-rowed barley cultivar ‚Golden promise'. We were able to create the six-rowed phenotype, but in some cases we experienced aberration in inflorescence development (Figure 6). In the future we will further investigate the generated lines to understand biological processes lying behind the observed phenomenon.

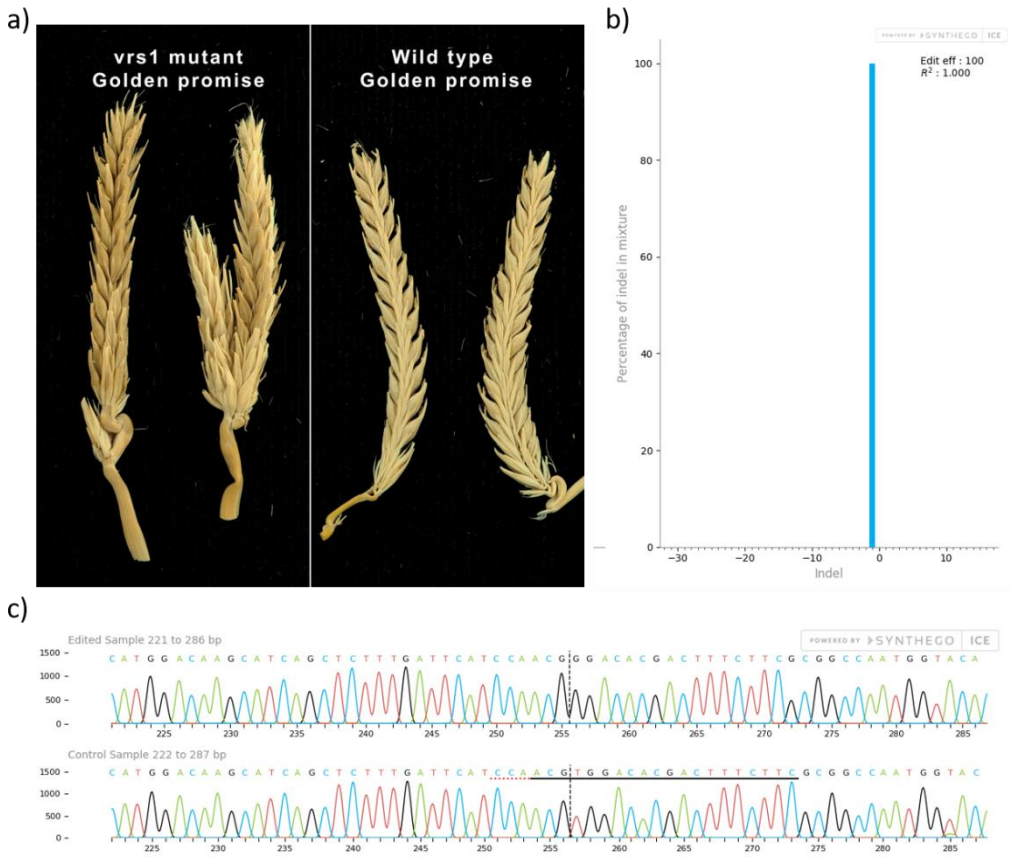

Figure 6. *a)* vrs1 knock-out mutant's phenotype. *b)* The extent of indel mutation in the knock-out line. *c)* Mutated sequences of vrs1 gene compare to the wild type.