THESES OF PHD DISSERTATION

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GENOME EDITING IN BARLEY (*HORDEUM VULGARE* L.) USING CRISPR/CAS9

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1. INTRODUCTION

Barley is among the world's four most cultivated crops with remarkable economic importance. It is a great subject for both applied and basic research, as its diploid genome is fully sequenced and there are several effective genetic transformation methods available. Being closely related with *Triticum aestivum* makes it an excellent model of hexaploid bread wheat.

Like in many plants, RNA interference (RNAi) plays an important role in developmental processes, pathogen resistance and genome stability of barley. Key components of this regulatory system are Argonaute (AGO), Dicer-like (DCL), RNA-dependent-RNA polymerase (RDR) proteins. The genes of these proteins are well studied in molecular biology's model plant *Arabidopsis thaliana* and some crop species, however in barley there were no genome-wide investigation available in this field prior to our study.

Wheat Dwarf Virus (WDV) is among the most hazardous virus pathogens of barley and wheat. As a member of *Geminiviridae*, it has circular single stranded DNS genome which becomes double stranded during replication. Endogenous RNAi pathways are ineffective against WDV and till this day there is no resistance gene identified.

CRISPR/Cas9 genome engineering system can be a great solution for these scientific problems. Its programmable endonuclease can create site specific double stranded DNA breaks, leading to mutations as a result of endogenous DNA repair systems' work. If we use it to target RNAi genes or replicating WDV genome, the resulting mutant barley lines can be a basis of future studies on barley RNAi or a new solution for plant virus resistance.

2. OBJECTIVES:

1. Identification of barley's RNAi components and their phylogenetic relationship *in silico*
2. Expression analyses of predicted barley RNAi genes
3. Test of gRNAs efficiency in *Nicotiana benthamiana* transient expression system
4. Creating RNAi mutant barley lines using CRISPR/Cas9
5. Studying CRISPR/Cas9 system's operation in transgenic WDV resistant barleys

3. MATERIALS AND METHODS

3.1 Bioinformatic methods

We have downloaded the DCL, AGO and RDR sequences of *Arabidopsis thaliana*, rice and maize from UniProt database. Multiple sequence alignments were performed using ClustalW. Neighbour-joining (NJ) phylogenetic trees were constructed in Mega 7 and Mega 10. HMMer online software was employed to create HMM-profiles then profile based search was performed in Ensembl Genomes database. Following manual filtering of resulting sequences Pfam and MEME web server were used to detect functional domains and conservative molecular motifs. We have used IdentAndSim software to calculate our predicted barley sequences identity and similarity to references. Further sequence editing and data visualisation was performed using Jalview and Microsoft PowerPoint.

3.2 Plant material and environmental conditions

Hordeum vulgare cv. Golden promise was chosen as a subject to our investigations. Plants were grown in Sanyo MLR-350 (Sanyo Electric Co, Japan) growth chambers on 18°C, 6 h darkness and 18 h light. Young grains and leaves for expression analyses were originated from 2 months old plants. 16-day-old plants were used for abiotic stress treatments. Drought stress: 1 week without watering. Heat stress: 24h on 40°C. Heat and drought stress: the combination of the previous treatments.

Sampling: the youngest leaves of plants were collected and frozen in liquid nitrogen. Samples were stored on -70°C till processing.

3.3 RNA based methods

RNA was isolated from frozen leaves and young grains using TRIzol reagent (Thermo Fischer Scientific, USA) following the manufacturer's instructions. Nanodrop (Thermo Fischer Scientific, USA) was used to measure RNA concentration. For cDNA synthesis we have used 1µg of RNA with RevertAid kit (Thermo Fischer Scientific, USA) following the manufacturer's instructions.

Norther hybridisation of WDV was performed according to Kis et al. 2016's protocol using 5 µg RNA per sample.

3.4 DNA based methods

PCR primers were designed manually. Reactions were preformed using PhireII hot start DNA polymerase (Thermo Fischer Scientific, USA). Endoganous control for semiquantitative PCR was barley actin (GeneBank ID: AZ145451.1).

5µl of PCR products were separated on 1.2% agarose gel (Lonza, Switzerland.). ChemiDoc XRS+ (BioRad Inc. USA) system was used for imaging.

T7 (NEB, USA) assay was employed to detect mutations according to manufacturer's instructions. CloneJet PRC cloning kit (Thermo Scientific) was used to clone PCR fragments to *E. coli*. Eurofins Genomics was responsible for sequencing of samples.

3.5 Protein based methods

Cas9 (Agrisera, Sweden) antibody was used with goat-anti rabbit (Agrisera, Sweden) secondary antibody to detect Cas9 protein in samples originating from 2 months old barley plants which were separated on 8% SDS-PAGE previously. The detailed description of this method can be found on Agrisera's website.

3.6 CRISPR/Cas9 construction design

In the beginning of our work, gRNAs were designed manually. Later CRISPOR online software was used for this purpose. Xing et al. (2014)'s methods and plasmids were used to create all (sensor)constructions for transient tests and barley transformation.

3.7 WDV inoculation

We have used WDV's natural insect vector, *Psammotettix alienus* grasshopper for virus transmission between plants. The method was described in Kis et al. (2016).

4. RESULTS

Using *Arabidopsis* DCL, AGO and RDR sequences as a reference 5 *Hv*DCLs, 11 *Hv*AGOs and 7 *Hv*RDRs were predicted *in silico*. The resulting barley sequences underwent phylogenetic investigations and

compared to RNAi components of dicot (*Arabidopsis*) and monocot (rice, maize) models. Phylogenetic trees of barley and model sequences were constructed to explore evolutional relationships and to create nomenclature for predicted barley RNAi sequences. From the results of phylogenetic analyses, AGO 18 clade can be highlighted as it is relatively young on evolutional timescale and as a result of it being the only clade without dicot members. Conservative molecular patterns were analysed with MEME web toolkit. The RNAi components of barley and reference species exhibited similar conservative motif patterns within the same clades. Among clades these patterns were a slightly different, but still specific to the respective protein family.

Following bioinformatic investigations, expression analyses were performed to detect patterns of RNAi components' expression in young, immature grain and leaf samples. Almost all genes were exhibited stronger expression in immature grain tissues. We were unable to detect any expression in the case of *HvAGO1d, HvAGO5a HvAGO10, HvRDR4* and *HvRDR6b* in these samples with semiquantitative RT-PCR.

After abiotic stress treatments of 16-day-old barleys *HvAGO2, HvAGO6, HvRDR2* and *HvRDR6a* showed heat induction. Under combined heat and drought stress conditions *HvAGO6* exhibited an alternative splice variant. It may indicate *HvAGO6*'s abiotic stress regulated expression.

After expression analyses, we have chosen 4 barley RNAi genes *(HvAGO4a, HvDCL3, HvAGO6* and *HvDCL5*) which are possibly key components of siRNA pathway and targeted them with CRISPR/Cas9 constructions.

Mutations were detected with T7 assay and sequencing. Most of them resulted frameshift and immature STOP codon which can lead to translational termination. In some cases, like in *HvAGO4a* 3/a, b, c T_0 lines,

one allele of the gene carried 15 bp insertion. As it is divisible by 3, it won’t shift the reading frame of *HvAGO4a*.

On *HvAGO4a* and *HvDCL3* plants, excluding dwarf and sterile *HvDCL3* 12/a, no visible macroscopic alteration from wild type plants’ phenotype were observed. *HAGO4a* lines showed later flowering then control plants. The delay was line specific. For further examinations we have chosen 3 mutant lines *(HvAGO4a* 1a/4, 3c/10, 16/a) and observed late flowering in T_1 and T_2 generations too.

In the case of *HvDCL5* and *Hv*AGO6 we weren’t able to produce T_1 generation till the writing of current thesis.

CRISPR/Cas9 system can be used to target pathogen genomes beside endogenous targets. Taking advantage of this we constructed genome editing cassettes to target WDV genome with 4 gRNAs in multiplex manner and performed examinations on each CRISPR/Cas9 component’s operation both in transient system and in transgenic barley lines. 3 out of 4 gRNAs showed expression *in vivo* and 3 out of 4 lines exhibited complete inhibition of WDV systemisation. The 4th line in which the pathogen was able to spread exhibited high level of virus tolerance , no visible signs of WDV infection could be detected.

5. New Scientific Results

1. With bioinformatic tools, main components of barley's RNAi machinery: 5 *Hv*DCLs, 11 *Hv*AGOs and 7 *Hv*RDR proteins and the genes coding them were identified.

2. According to our experiments, *HvAGO2, HvAGO6, HvRDR2* and *HvRDR6a* showed heat induction. In the case of *HvAGO6* under combined environmental abiotic stress conditions (drought and heat) an alternative splice variant was identified.

3. Using CRISPR/Cas9 genome engineering system, we generated mutations in *HvAGO4a, HvAGO6, HvDCL3*, and *HvDCL5* genes, *HvAGO4a* and *HvDCL3* mutants' T_0 generation and progeny were genotyped.

4. The expression of WDVGuide4Guard construction in transgenic barley lines limited the systemization of wheat dwarf virus. We found that the amount of Cas9 protein in transgenic plants does not correlate with level of WDV resistance.

5. PUBLICATIONS

Scientific articles in reviewed journals with impact factor

Hamar E., Szaker H M, Kis A, Dalmadi A, Miloro F, Szittya Gy, 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.)" MDPI biomolecules 10(6), 929; https://doi.org/10.3390/biom10060929

Kis, A., Hamar, E., Bán, R, Tholt G, Havelda Z. (2019). "Creating Highly Efficient Resistance against Wheat Dwarf Virus in Barley by Employing CRISPR / Cas9 System," Plant Biotechnology Journal 1004–6. https://doi.org/10.1111/pbi.13077.

Hungarian scientific articles in reviewed journals

Hamar, E., Kocsis L. (2018). "A gyökérzet oxigénellátása talajmentes termesztésben és in vitro kultúrában" Kertgazdaság 50(4) 47-52

Kis A., Hamar É., Tholt G., Taller J., Havelda Z. (2018) „WDV toleráns árpavonalak előállítása CRISPR/Cas9 rendszerrel" Georgikon for Agriculture 22. vol. 1. no. / 2018 23-28

Full length conference publications

Hamar É., Kis A., Taller J., Havelda Z. (2018) „Dicer-like, Argonauta és RNS-függő RNS polimeráz géncsaládok azonosítása árpában (*Hordeum vulgare* L.) és lehetséges szerepük a növényi stresszválaszban" Doktoranduszok Országos szövetsége, Tavaszi szél 2018 Tanulmánykötet ISBN 978-615-5586-31-6, DOI: 10.23715/TSZ.2018.1

Summary conference publications

É. Hamar, A. Kis, Á. Dalmadi, J. Taller, Z. Havelda (2019) "Identification of RNA interference-related genes in barley (*Hordeum vulgare* L.)" Hungarian Molecular Life Sciences 2019 March 29-31

Hamar É., Kis A., Taller J., Havelda Z. (2017): „Investigation of AGO4 and DCL3 RNAi components in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 system" Hungarian Molecular Life Sciences 2017 31 March-2 April ISBN: 978-615-5270-34-5, pp 170

Kis A., Hamar É., Tholt G., Havelda Z. (2017)" Establishment of a highly efficient wheat dwarf geminivirus resistance in barley by multiple CRISPR/Cas9 system" Hungarian Molecular Life Sciences 2017 31 March-2 April ISBN: 978-615-5270-34-5, pp 172

Bálint J., Gyula P., Taller D., Dalmadi Á., Hamar É., Kis A., Szittya Gy., Várallyay É., Taller J., Havelda Z. (2017) Investigation of the regulation and activity of RNA interference executor complexes in model and crop plants. Hungarian Molecular Life Sciences 2017 31 March-2 April ISBN: 978-615-5270-34-5, pp 171

Lectures in Hungarian language

Hamar É., Kis A., Taller J., Havelda Z., (2017): Az RNS interferencia fehérjekomponenseinek vizsgálata árpában (*Hordeum vulgare* L.). Professzorok az Európai Magyarországért Egyesület XV. nemzetközi tudományos konferenciája, Budapest 2017 november 8.

Hamar É., Kis A., Taller J., Havelda Z. (2017): Az RNSi fehérjekomponenseinek vizsgálata árpában (*Hordeum vulgare* L.) CRISPR/Cas9 rendszerrel.; RNS kutatók fóruma, Gödöllő 2017. június 23

6. References

Kis, A., Tholt, G., Ivanics, M., Várallyay, É., Jenes, B. 2016. "Polycistronic Artificial MiRNA-Mediated Resistance to Wheat Dwarf Virus in Barley Is Highly Efficient at Low Temperature." https://doi.org/10.1111/mpp.12291.

Xing, H., Dong, L., Wang, Z., Zhang, H., Han, C, Liu, B, Wang, X., and Qi-jun Chen. 2014. "A CRISPR / Cas9 Toolkit for Multiplex Genome Editing in Plants."