IDRIP – Improving drought resistance in barley by transcriptional silencing of genes with suppressor function

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Trockenheit während der letzten Lebensphase von Nutzpflanzen kann schwerwiegende Auswirkungen auf die Pflanzenproduktion haben. Besonders sensibel reagieren die Pflanzen, wenn die Trockenheit in der reproduktiven Phase auftritt. In diesen Fällen kann es zu dramatischen Ertragsverlusten kommen. Während es durch die Trockenheit vor der Blüte zu einer Verringerung der Körnerzahl kommt, führt Wassermangel nach der Blüte zu einer verringerten Kornfüllung. Neben dem Ertrag wird auch die Kornqualität beeinfluss, was Auswirkungen auf die weitere Prozessierung der Körner, wie z.B. dem Vermälzen hat. In vorausgehenden Arbeiten konnten Gene identifiziert werden, welche eine Suppressor-funktion für die Trockenstressresistenz und die Korngröße zeigen. Die Promotorregionen dieser ausgewählten Gene sollen transkriptionell inaktiviert werden. Hierfür wurde der Mechanismus der RNA vermittelten DNA Methylierung angewandt werden, um die ausgewählten Gene in Gerste (*Hordeum vulgare)* transkriptionell stillzulegen.

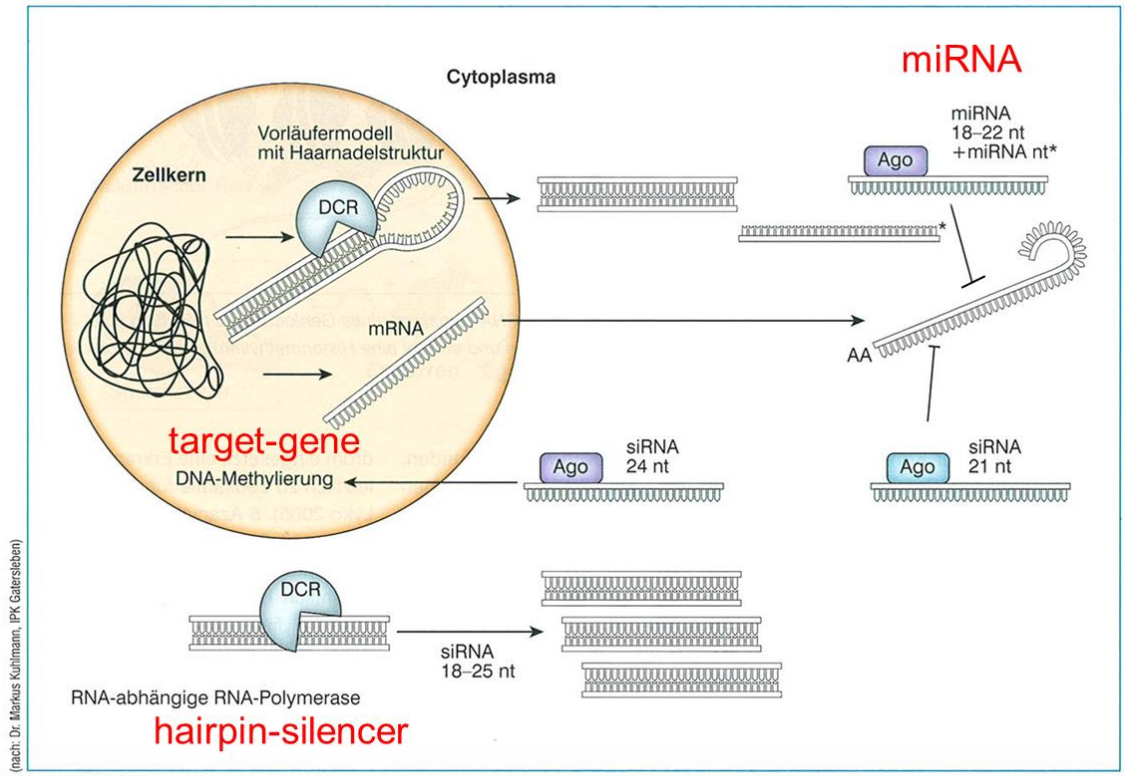
I. Project parameters

1. Topic, purpose and state of scientific knowledge

Drought stress in the terminal phase of barley development leads to severe losses in grain yield.

Figure 1:
Barley plants (*Hordeum vulgare*) during their reproductive phase of development , left: Greenhouse control conditions, normally watered; right: after 4 days without watering (drought stress).

It has been shown that during the reproductive phase of development the plant is most sensitive to water deficit. Drought stress leads to a reduction in grain number and reduced seed filling. In our previous research we identified genes involved in drought stress response with suppressor function. The promotor regions of these target genes will be transcriptionally inactivated to improve drought resistance in barley. The mechanism of RNA-directed-DNA-methylation will be applied to silence genes with suppressor function for drought resistance and grain size in barley (*Hordeum vulgare)*.
Transcriptional gene silencing is a mechanism inactivating the targeted gene via methylation of its promotor region.



mod. from Unterricht Biologie, 2014

Figure 2:
Scheme for inactivation of genes with suppressor function in barley drought stress response.
A silencer transgene is transferred to barley plants. The silencer contains promotor fragments arranged in hairpin-formation. Expression of the silencer is required for the generation of heterochromatic 24 nt sized siRNA, which are generated by processing ofdouble stranded RNA (dsRNA). Presence of heterochromatic small RNAs is the prerequisite for inactivation
of target genes via DNA methylation (transcriptional silencing). MiRNAs lead to post transcriptional gene suppression of their respective mRNA targets. Drought stress specific miRNA genes active in the caryopsis are mutated using CrispCas-technology.

Sequence specific methylation will be achieved by stable and transient expression of a hairpin construct directed against promotors of target genes with suppressor function in barley drought resistance. Applying this method to barley may establish a new epigenetic method of stable gene activity modification other than genetic engineering of plants. Further three microRNAs were identified, being expressed during this phase under drought stress condition. The miRNA genes were targeted by CrispCas mediated mutation to interfere the suppressive function mediated via post transcriptional gene silencing by miRNAs.
This project will help investigating a new technology that can be applied to improve crop plant performance during drought stress. The stability of grain yield under drought stress is an important issue, not only for local breeders, but will also help to secure food and feed worldwide under changing climate conditions.

2. Interdisciplinarity of the project

Stability of barley yield clearly affects all three research pillars. Better yield with less drought stress mediated effects on grain quality will improve the malting quality and the use of barley as food and feed.

3. Innovativity of the research

Utilizing sequence specific DNA methylation a novel technology will be applied to reduce expression of genes with suppressor function during barley drought response.

4. Application-oriented research

Due to climate change an increase of drought stress during the barley grain filling phase can be expected. An applicable method which helps to stabilize yield under unfavorable conditions would be beneficial.

5. Relevance of the project for the bioeconomy, the ScienceCampus and the state of Saxony-Anhalt

Yield stability under drought stress conditions aims a general feature, which will also be an important trait for Saxony-Anhalt. Predictions of the local climate propose an increase of hot and dry summers (example 2018) which will affect the most sensitive grain filling period of barley.

II. Theory and methods

Barley plants (cv. Golden Promise) were modified to achieve improved performance under drought stress conditions. Three major steps will be taken:

1. Transcriptional silencing of target genes in barley via stable transformation and virus induced gene silencing.
2. Performance analysis of silenced plants under drought stress conditions.
3. Test of transcriptional inactivation of target genes in the absence of the silencer transgene and performance analysis of silenced non transgenic plants under drought stress conditions.

III. Results and perspectives

1. Results

Plants with stable expression of silencing effectors in barley were generated.
The silencer constructs, containing 300-400bp regions of the promoters of the target genes *HvRD22, HvUSPL1, HvABA8´OH-1* were chosen. The *Agrobacterium* mediated transformation of the T-DNA into *H. vulgare* cv. Golden Promise and selection of transgenic plants was performed by Edgar Peiter in Halle and in parallel by Jochen Kumlehn at IPK. The transformation procedure resulted in positive transformed plants **(Table 1)**.

	Construct	Plant/lines #
EP-IPK	empty vector control	23
BG834	empty vector control	18
EP101	ProABAOH	7
BG831	ProABAOH	8

EP102	ProRD22	8
BG832	ProRD22	15

EP103	ProUSPL	33
BG833	ProUSPL	16

	Construct	Plant/lines #
BG828	miR-drought	9
BG829	miR5071	11
BG830	miR9662	20

***Table 1**: Number of generated plant lines, selected and positively tested. Empty vector lines are generated as negative reference lines. Pro refers to selected Promotor region used for transcriptional genes silencing.*

In the transferred T-DNA construct the expression of the hairpin construct is controlled by the *UBIQUITIN1*-promotor (*UBIpro*) from maize. The transient expression of the hairpin constructs was achieved using the method of virus-induced-gene-silencing (VIGS). Successful TGS was tested by qPCR and related DNA methylation determined by MSRE-qPCR and bisulfite sequencing. Three experimental series applying VIGS for introduction of site specific DNA methylation was performed. To achieve higher rate of methylation VIGS will be repeated in the next generation of treated plants. The plants inoculated with the ProABA construct showed more tillers at the end of the experiment in agreement with the hypothesis and prior experiments suppressing the ABA-hydoxylases by PTGS.

Derived from a small RNA sequencing approach (Surdonja et al., 2017) miRNAs were identified unique present in caryopses under drought stress conditions. The respective miRNA-genes are targeted by CrispCas directed mutagenesis and plants impaired in this genes with suppressor function were generated **(Table 1)**. The grains derived from the selected lines were analysed and quantified (MARVIN) **(Figure 3)**.

Figure 3:
Spikes of transgenic barley plants with CrispCas-modified miRNA genes.
Left: Mutation of a novel drought stress caryopsis specific miRNA gene lead to increase termination of grain development, reduced seed filling, resulting in reduced seed number and grain weight. Right: T0 - Plants with CrispCas mediated mutation of miRNA9262 gene shown difference in spike development compared to Golden promise wildtype plants.

Analysis of plant performance under control and drought stress conditions:

The plants selected for transcriptional inactivation of the target genes were analysed by methylation sensitive restriction digestion and quantitative real time PCR and sequenced for the CrispCas-mediated mutation of the miRNA genes. To analyse yield parameters plants were grown in pots under greenhouse conditions. Interestingly, CrispCas knock-out of miR-9662, lead to an increase in thousand grain weight (**Figure 4**)

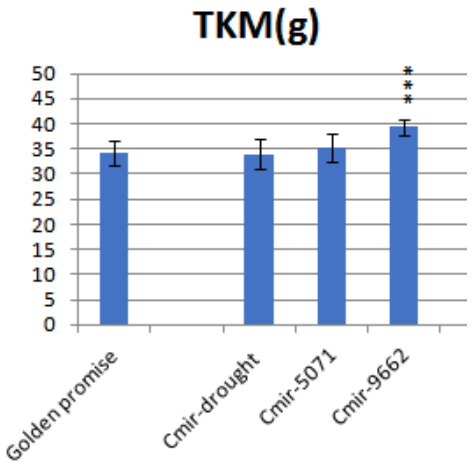


Figure 4:
Thousand grain weight (TKM) of miR-mutant-barley plants (T3). Golden promise was used as reference, as this cultivar is the genetic background for the plant transformation process. Error bars indicate standard deviation, T-test was applied for determination of statistical differences: $p<0.005$ = ***. N=12, Three lines for the respective miRNAs were analysed: miRdrought as drought and development specific miRNA, predicted in silico by Barturen etal., 2014, miR5071 and miR9662 are described miRNAs found to be expressed in caryopses, specifically under drought stress.

The detectable increase was caused by higher seed length and width. Currently plants are growing in S1 field like containments to apply drought stress under field like conditions. Stress will be applied 4 days after fertilisation and parameters “number of tillers”, “spikes”, “seeds”, “TGW” and “yield” will be measured (MARVIN).

Transcriptional inactivation of target genes in the absence of the silencer transgene and performance of silenced post-transgenic plant under drought stress conditions.
To analyse the stability of the inherited DNA, methylation will be analysed one to three generation after elimination of the silencer. Comparison of different target genes will show whether the efficiency of silencing and also the persistence of the methylation is similar between the target genes. As the process for generation of the transgenic lines took more time than expected

2. Prospects for further external funding

Breeders have a high interest in an improvement of elite breeding lines. This new method will help to transfer features into crop plants without genetic manipulation of the genome.

IV. Index

Butardo Jr V. M., Seiler C., Sreeenivasulu N., Kuhlmann M. Obtaining High-Quality Transcriptome Data from Cereal Seeds by a Modified Method for Gene Expression Profiling in press

Kuhlmann M (2019) DNA-Methylierung – das 5. Element. Biol. Unserer Zeit 49 342-349. dx.doi.org/10.1002/biuz.201910686

Lück S, Kreszies T, Strickert M, Schweizer P, Kuhlmann M, Douchkov D siRNA-Finder (si-Fi) software for RNAi-target design and off-target prediction. Front. Plant Sci. 10 (2019) 1023. dx.doi.org/10.3389/fpls.2019.01023

Tedeschi F, Rizzo P, Huong B, Czihal A, Rutten T, Altschmied L, Scharfenberg S, Grosse I, Becker C, Weigel D, Bäumlein H, Kuhlmann M (2019) EFFECTOR OF TRANSCRIPTION factors are novel plant-specific regulators associated with genomic DNA methylation in Arabidopsis. New Phytol. 221 261-278. dx.doi.org/10.1111/nph.15439

Demidov D, Heckmann S, Weiss O, Rutten T, Tomaštíková E D, Kuhlmann M, Scholl P, Municio C M, Lermontova I, Houben A (2019) Deregulated phosphorylation of CENH3 at Ser65 affects the development of floral meristems in Arabidopsis thaliana. Front. Plant Sci. 10 928. dx.doi.org/10.3389/Fpls.2019.00928

Seiler C, Kuhlmann M (2019) Quantification of DNA methylation as biomarker for grain quality. In: Sreenivasulu N (Ed.): Rice grain quality: methods and protocols. (Series: Methods in molecular biology, Vol. 1892) New York, NY: Humana Press 301-310. doi.org/10.1007/978-1-4939-8914-0_17 ISBN 978-1-4939-8912-6

Püffeld M, Seiler C, Kuhlmann M, Sreenivasulu N, Butardo V M (2019) Analysis of developing rice grain transcriptome using the agilent microarray platform. In: Sreenivasulu N (Ed.): Rice grain quality: methods and protocols. (Series: Methods in molecular biology, Vol. 1892) New York, NY: Humana Press 277-300. doi.org/10.1007/978-1-4939-8914-0_16 ISBN 978-1-4939-8912-6

Anacleto R, Badoni S, Parween S, Butardo V M, Jr., Misra G, Cuevas R P, Kuhlmann M, Trinidad T P, Mallillin A C, Acuin C, Bird A R, Morell M K, Sreenivasulu N (2018) Integrating a genome-wide association study with a large-scale transcriptome analysis to predict genetic regions influencing the glycaemic index and texture in rice. Plant Biotechnol. J. Epub ahead of print. dx.doi.org/10.1111/pbi.13051

Grehl C, Kuhlmann M, Becker C, Glaser B, Grosse I (2018) How to design a whole-genome bisulfite sequencing experiment. Epigenomes 2 21. dx.doi.org/10.3390/epigenomes2040021

Hashemipetroudi, S. H., Nematzadeh, G., Ahmadian, G., Yamchi, A., Kuhlmann, M. Assessment of DNA Contamination in RNA Samples Based on Ribosomal DNA. (2018) *J. Vis. Exp.* (131), e55451, doi:10.3791/55451.

Wang H, Chen W, Eggert K, Charnikhova T, Bouwmeester H, Schweizer P, Hajirezaei M R, Seiler C, Sreenivasulu N, von Wirén N, Kuhlmann M (2018) Abscisic acid influences tillering by modulation of strigolactones in barley. J. Exp. Bot. 69 3883-3898. dx.doi.org/10.1093/jxb/ery200

Doll, S.; Kuhlmann, M.; Rutten, T.; Mette, M.F.; Scharfenberg, S.; Petridis, A.; Berreth, D.C.; Mock, H.P. (2018**)** Accumulation of the coumarin scopolin under abiotic stress conditions is mediated by the arabidopsis thaliana tho/trex complex. *Plant J. 93 431-444. dx.doi.org/10.1111/tpj.13797*

Thirulogachandar, V.; Alqudah, A.M.; Koppolu, R.; Rutten, T.; Graner, A.; Hensel, G.; Kumlehn, J.; Brautigam, A.; Sreenivasulu, N.; Schnurbusch, T.*, et al.* (2017**)** Leaf primordium size specifies leaf width and vein number among row-type classes in barley. *Plant J*, *91*, 601-612.

Surdonja, K., Eggert, K., Hajirezaei, M.-R., Harshavardhan, V., Seiler, C., von Wirén, N., Sreenivasulu, N., and Kuhlmann, M. (2017). Increase of DNA Methylation at the HvCKX2.1 Promoter by Terminal Drought Stress in Barley. Epigenomes 1, 9.

Sandmann M, Talbert P, Demidov D, Kuhlmann M, Rutten T, Conrad U, Lermontova I (2017) Targeting of A. thaliana KNL2 to centromeres depends on the conserved CENPC-k motif in its C-terminus. Plant Cell 29 144-155. dx.doi.org/10.1105/tpc.16.00720