

## **Establishment of a genome editing platform: reducing cadmium accumulation in barley and durum wheat by de- and re-functionalization of transport protein-encoding genes as an example to study the societal acceptance of new genetic engineering methods.**

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- Project duration: June 2020 – December 2021
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- Post-doc: Dr. Santiago Alejandro Martinez
- Other research collaborations: Dr. Martin Mascher (IPK); Dr. Thorsten Schnurbusch (IPK)

### **I. General consideration**

- a) The project is highly interdisciplinary since it links the molecular plant sciences and biotechnology with the social sciences. The ScienceCampus Halle pillars are inherently interconnected in this project: It is concerned with the development and production of cereal grains with improved health benefits and the evaluation of the societal implications and acceptance of this employed technology.
- b) The research is highly innovative as it employs and evaluates a novel technology to tackle an important problem in agronomy and human nutrition.
- c) The project is integrated within the ScienceCampus Halle by sharing expertise with other projects. As a case study for cereal crop improvement, it is highly relevant for the plant-based bioeconomy and the Saxony-Anhalt region.
- d) Results of the project are a basis for subsequent research projects that study and exploit mechanisms of Cd reduction (BMBF, DFG).

### **II. Topic, open questions and purpose**

Due to its carcinogenic potential, long-term intake of cadmium (Cd) presents a critical risk for humans. A reduction of Cd levels in cereal grains by biotechnological means will therefore have direct health benefits and may serve as a showcase to improve public acceptance of genome editing technologies. This project aimed (1) to identify gene variants in Cd transporters in the model cereal crop barley that may be targeted by genome editing; (2) to decrease Cd levels in grains of the cereal crop durum wheat by genome editing-mediated re-functionalization of a vacuolar Cd transport protein; and (3) to evaluate the public acceptance of genome editing.

### III. Theory and methods

Plants take up Cd from the soil by transporters of the ZIP and NRAMP families, the physiological function of which is to acquire micronutrients, such as Fe, Mn, and Zn (Kailasam and Peiter, 2021). Following loading of the xylem, Cd is translocated to the shoot and further on to the grains. Studies in rice suggest that transporters of the LCT family are likely to play a role in this transfer process. Deposition of Cd in grains can be reduced by minimizing the Cd influx into root cells, which, however, would also compromise micronutrient uptake. This problem may be solved by improving transporter selectivity by directed evolution. Although feasible in principle, such a strategy is technically highly demanding (Kailasam and Peiter, 2021). Alternatively, Cd may be sequestered in root cell vacuoles by Heavy Metal ATPases, such as HMA3. The presence and activity of this pump has been shown to be strongly associated with low Cd levels in grains. However, HMA3 also transports zinc and hence reduces the transport of Zn to the shoot. Natural variation may exist in the efficiency and selectivity of Cd transport by HMA3.

The problem of grain Cd was chosen as case study to demonstrate the usefulness of genome editing approaches. In durum wheat, a duplication of 17 nucleotides that occurred during domestication has caused the loss of HMA3 gene function. Targeted excision of the duplicated section using RNA-guided Cas9 endonuclease was employed to restore the functional gene sequence. Since such precise deletions are hardly possible to achieve by standard Cas endonuclease-mediated site-directed mutagenesis, we took a novel editing concept employing homology-mediated end-joining instead of the commonly used non-homologous end-joining DNA repair pathway. In barley, the function of HMA3 variants was analysed in a panel of exome-capture-sequenced accessions to identify proteoforms with improved ability to translocate Cd into the vacuole. Furthermore, Cd levels in grains of 250 re-sequenced barley accessions were determined by inductively coupled plasma - mass spectrometry (ICP-MS), and a genome-wide association study (GWAS) was conducted to identify new targets for genome editing-mediated Cd reduction. The originally envisaged deletion of the *HMA3* gene in barley was not pursued, as this was published by another group in the course of the project.

The development of the MoralAtlas is based on our previous research on genome editing attitude and related moral beliefs. Its structure is rooted in Moral Foundations Theory.

### IV. Results and perspectives

An analysis of 98 barley accessions differing in HMA3 alleles identified a substantial variation of the root-to-shoot translocation of Cd, which was not correlated with Zn translocation (Figure 1). Most interestingly, the variation in Cd translocation is associated with HMA3 haplotypes, despite this association being affected by the *Succula* transposable element recently described to increase HMA3 expression. This result indicates that it is likely feasible to identify HMA3 proteoforms with increased

efficiency and selectivity for Cd sequestration. This approach is currently further pursued in collaboration with Martin Mascher (IPK). Superior proteoforms may eventually be introduced into elite varieties by gene editing approaches (Kailsam and Peiter, 2021).

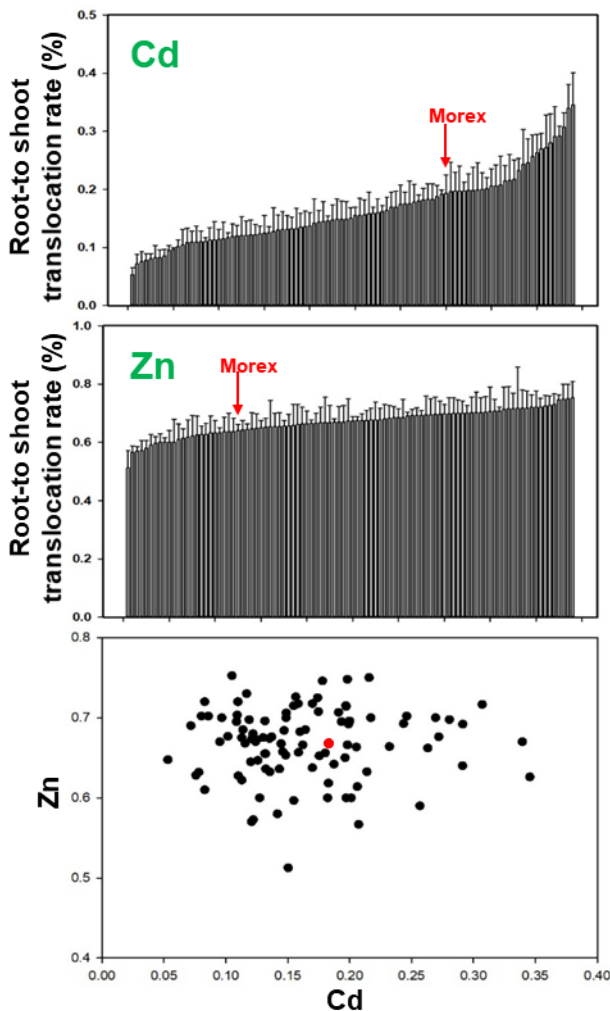


Figure 1: Cd and Zn translocation by barley genotypes differing in HMA3 alleles

An analysis of 250 barley accessions revealed a tremendous variation in Cd levels of grains grown on the same uncontaminated field, ranging from 5 to 170  $\mu\text{g kg}^{-1}$  DW (Figure 2). The currently running GWAS analysis, performed in collaboration with Thorsten Schnurbusch (IPK), will identify candidate genes for targeted genome editing approaches to reduce Cd accumulation. Intriguingly, preliminary data indicate that HMA3 is amongst the candidate genes associated with low Cd concentration.

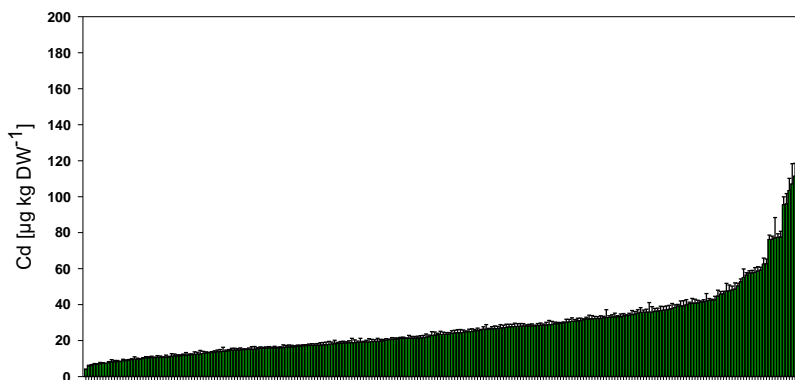


Figure 2. Cd accumulation in grains of barley genotypes grown in non-polluted soil.

Based on previous work in rice, it was originally planned to analyse a barley homolog of the LCT family, HvLCT2, for its relevance in Cd transfer to the grain. However, sequence analyses revealed that the HvLCT2 gene is not present in the sequenced Morex cultivar, rendering the design of guide RNAs for Cas9-mediated modification difficult and questioning the relevance of this gene. This part of the project was therefore postponed in favour of the more promising approaches based on HMA3.

Based upon homology-mediated end-joining of the DNA ends that result from cleavage of an appropriate Cas9 target motif in durum wheat, the defective HMA3-B1b allele was converted into a functional gene variant as is present in the ancestor and some cultivars of this crop (Figure 3). Currently, progenies produced from primary HMA3 restoration mutants carrying the functional allele are being screened for homozygous mutant segregants that are free from Cas9 and gRNA transgenes as well. Selected lines will be tested for their Cd concentrations in roots, leaves, and grains in comparison with their HMA3-dysfunctional donor lines.

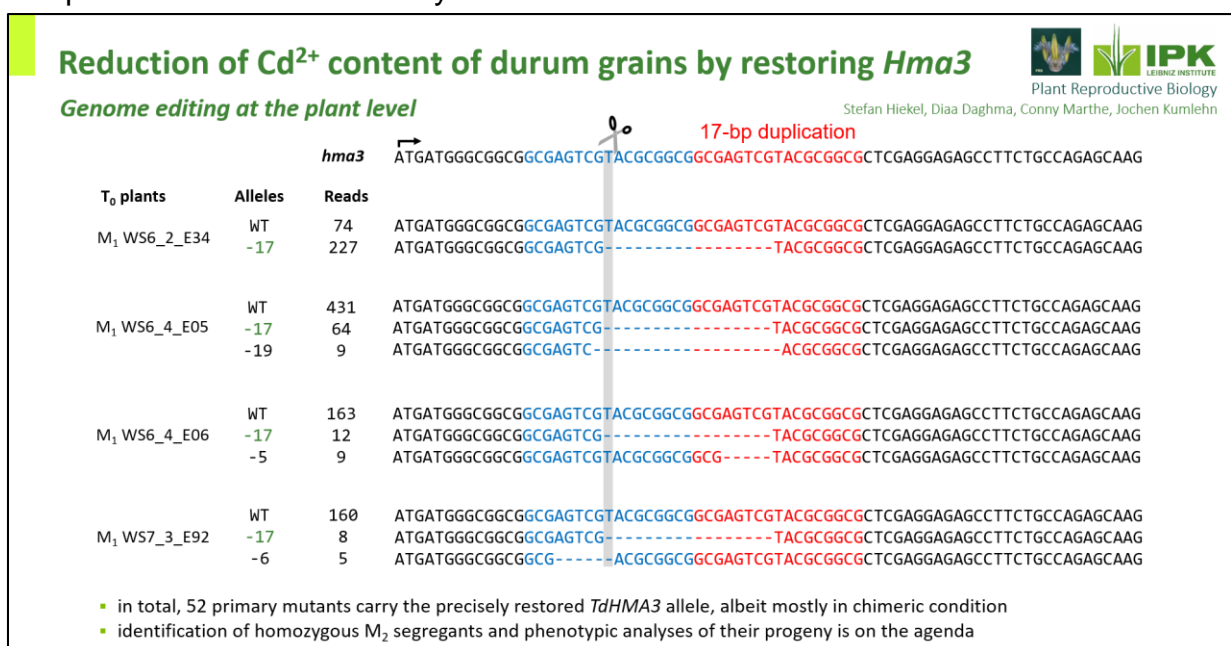


Figure 3. Restoration of functional HMA3 allele in genome-edited durum lines

As an interactive tool, the Moralatlas contributes to constructive science communication about genetic engineering.

## V. Index (publications, talks, poster, patents)

### Publications:

Budhagatapalli N, Halbach T, Hiekel S, Büchner H, Müller A, Kumlehn J (2020) Site-directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA-transgenic maize used as haploidy inducer. *Plant Biotechnology Journal* 18: 2376-2378

Kailasam S, Peiter E (2021) A path toward concurrent biofortification and cadmium mitigation in plant-based foods. *New Phytologist* 232: 17-24

Koeppel I, Hertig C, Hoffie R, Kumlehn J (2019) Cas endonuclease technology - a quantum leap in the advancement of barley and wheat genetic engineering. *International Journal of Molecular Sciences* 20: 2647

### Talks:

Kumlehn J (2020) Cas endonuclease technology in cereals. CRISPR Symposium, 13 October 2020, Lund, Sweden

Kumlehn J (2021) Site-directed genetic engineering in cereals - Principles and applications. 6th Conference on Cereal Biotechnology and Breeding, 3-5 November 2021, Budapest, Hungary (video conference)

Kumlehn J (2022) Site-directed genetic engineering in cereals - Principles and applications. Conference on CRISPR & Genome Editing, 8-9 July 2022, Vienna, Austria

Kumlehn J (2022) Cas endonuclease technology in cereals - Ways to increased precision. International CEPLAS/IPK Summer School, Translational Plant Biodiversity Research, 9-13 May 2022, Kloster Steinfeld, Kall-Steinfeld, Germany

Peiter E (2021) Genome Editing - Die Lösung für die Landwirtschaft? Studentische Förderinitiative der Naturwissenschaften e. V., 16 December 2021, Halle, Germany (online).