Final report of the project

Identification and characterization of loci conferring tolerance to iron toxicity in rice

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Abiotic Stress Tolerance in Crops

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Summary

Iron toxicity is one of the major nutrient disorders affecting rice production and causes substantial yield losses, especially in Africa and parts of Southeast Asia. In a previous project, we had conducted a genome-wide association study (GWAS) by screening 329 diverse rice accessions representing the entire genetic diversity of Asian rice (*Oryza sativa* L.) under iron toxic conditions in nutrient solutions, and determined seven different phenotypic traits related to biomass and foliar symptom formation. The aims of this project were (i) bioinformatic analyses of previously generated data to identify candidate loci and genes associated with tolerance to iron toxicity; (ii) determining physiological factors associated with tolerance loci through experiments with selected lines, and (iii) preparation of mutant lines for selected candidate genes for further experiments. Bioinformatic analysis using mixed model analysis identified 18 distinct loci associated with seven different phenotypic traits. Local linkage disequilibrium analysis was used to identify candidate genes within these loci. In the physiological analyses we focused on one locus located on chromosome 1, which was associated with foliar symptom formation. Contrasting haplotypes for this locus showed significant differences in antioxidant status, and more specifically in dehydroascorbate reductase activity. One candidate gene underlying this locus (putative glutathione-S-transferases) showed sequence polymorphisms in the protein coding region, which caused substantial amino acid substitutions. Mutant lines for several candidate genes were identified in public gene banks and are currently being genotyped. In summary, this project was successful in identifying candidate loci and genes associated with tolerance to iron toxicity, determining physiological factors underlying tolerance, and paved the way for further characterization of candidate genes using mutant experiments. These investigations

advance our understanding of iron stress biology in rice and will be useful in the breeding of adapted rice varieties, thereby benefitting resource poor farmers in developing countries.

Research Report

Details of the results obtained in this project, including detailed methods, graphical representation of results and data tables, are summarized in the enclosed manuscript, which has been submitted for publication. Therefore, only a brief summary is given in this report.

Bioinformatic analyses of previously generated data

For the association mapping of phenotypic data generated in previous experiments funded by the *fiat panis* foundation, we used 44 100 publicly available single nucleotide polymorphism (SNP) markers (Zhao et al., 2011) and the software TASSEL (Bradbury et al., 2007). Mixed model analysis identified 20 SNP markers exceeding the significance threshold of $P<0.0001$ for the associations with the seven different phenotypic traits (shoot and root biomass, shoot and root length, foliar symptom formation after three and five days, and shoot iron concentrations). Linkage disequilibrium (LD) analysis using the software Haploview (Barrett et al., 2005) suggested that these markers represented 18 different loci. A list of genes contained within these loci was curated, and screened for possible candidate genes based on their physical position and functional annotation based on the MSU database (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). We opted to focus our further analysis on one locus associated with foliar symptom formation located on chromosome 1 at around 28 Mb for the following reasons: (i) the trait 'foliar symptom formation' had the highest heritability; (iii) the locus had the most highly significant P-value among all significant loci; (iii) the identified locus co-localized with several previously reported quantitative trait loci (QTL) from experiments with bi-parental populations (Dufey et al., 2014); (iv) the locus contained plausible candidate genes. Two out of these possible candidate genes, *i.e.* the putative glutathione-S-transferases LOC_Os01g49710 and LOC_Os01g49720, were chosen for re-sequencing and local disequilibrium analysis. These investigations revealed that one of these genes (LOC_Os01g49710) had nucleotide insertions/deletions in the protein coding region of the gene, which were in complete LD with the most significant SNP marker. We therefore concluded that this gene could be associated with tolerance to iron toxicity. Glutathione-S-transferases (GSTs) are a diverse class of enzymes with multiple functions in plants including detoxification, stress responses and antioxidant activity (Marrs, 1996). Therefore, we decided to focus on antioxidant responses of contrasting haplotypes for this locus in the subsequent experiments.

Experiments with selected lines from the population

In these experiments we decided to investigate contrasting haplotypes for the above mentioned locus on chromosome 1. Haplotypes were defined as lines carrying different alleles at three significant SNP markers located within the candidate locus. Five lines representing the tolerant and intolerant haplotype, respectively, were tested in an additional iron stress experiment. The contrasting lines did not differ significantly in their shoot iron concentrations, indicating that tolerance was associated with a shoot-based tolerance mechanism rather than iron exclusion at the root surface. This was in line with the hypothesis that the previously identified GST gene was associated with a shoot-based detoxification mechanism. Antioxidant activity was tested in all lines, including the foliar concentrations and activity of substrates and enzymes involved in the ascorbate-glutathione cycle (Noctor and Foyer, 1998). These analyses revealed that tolerant lines had significantly lower dehydroascorbate reductase (DHAR) activity, which represented one of the possible functions of GSTs (Edwards and Dixon, 2005). Gene expression was also investigated for the two GST genes identified previously, indicating that the genes were highly responsive to iron but did not show genotypic differences in mRNA level. Thus, differences in DHAR activity may be caused by functional alterations due to amino acid substitutions, rather than differences in expression level. We concluded that the continuous restoring of the reduced form of ascorbate through the DHAR activity may stimulate the generation of harmful reactive oxygen species (ROS) via the Fenton reaction (Becana et al., 1998).

In addition, shoot iron concentrations were investigated in all lines included in the association mapping panel employing atomic absorption spectrometry. This allowed us to classify tolerant lines as 'includers' and 'excluders', and also led to the identification of loci associated with iron exclusion.

Preparation of mutant lines for further experiments

To verify the involvement of some of the candidate genes identified through GWAS in tolerance to iron toxicity, public gene banks were screened for available gene knock-out or activation tagged mutants (Hirochika, 2001; Jeong et al., 2002). Database research led to the identification of four available mutant lines as shown in Table 1. Most of these mutants were T-DNA insertion lines, which were transformed with a vector facilitating either gene-knock-out or activation tagging (=over-expression), depending on the position of the insertion (Jeong et al., 2002). In addition, one TOS17 line was identified, which was generated through the activation of an endogenous retrotransposon, thereby potentially causing gene knockouts (Hirochika, 2001). The advantage of these plants is that they are considered as non-transgenic and therefore can be grown without restrictions. Seeds of all mutant lines listed in Table 1 were ordered from the respective gene banks and are currently being

genotyped. Experiments with this material will be conducted during the second half of 2015, when seeds will be available.

Table 1: List of available mutant lines for candidate genes for iron toxicity tolerance determined through a genome-wide association study

Candidate gene MSU-ID	Annotated gene function	Associated trait in GWAS study	Mutant line IDs	Type of mutant
LOC_Os01g49710	Glutathione-S-transferase	Foliar symptom formation	2D-41470 NC0465	T-DNA insertion (activation tagged); TOS17 (knock-out)
LOC_Os01g49350	Arabino lactone oxygenase	Foliar symptom formation	K-05774	T-DNA insertion (knock-out)
LOC_Os02g56560	Casein kinase	Shoot iron concentration	1B-20104	T-DNA insertion (activation tagged)

Conclusion, Outlook, and Relevance for Global Food Security

In conclusion this project was successful in identifying candidate genes and loci associated with tolerance to iron toxicity in rice. We also found possible important sequence polymorphisms in a candidate gene (glutathione-S-transferase, LOC_Os01g49710), which were consistent with differential antioxidant activity in contrasting rice lines. Seeds of mutant lines for several candidate genes are currently being produced. Experiments with this material will possibly verify the involvement of these genes in tolerance to iron toxicity and shed further light on the underlying physiological mechanisms.

The work will be carried on by a PhD student currently employed in a different project (funded by Deutsche Forschungsgemeinschaft) and master students. Elsa Matthus, who was in charge of conducting the experiments described in this report, was offered a full PhD scholarship at the University of Cambridge. She received this offer before the 'Studienstiftung des deutschen Volkes' began to even review her proposal for a PhD scholarship to carry on the work described above, and therefore she accepted this offer. This is understandable, because obtaining a PhD from one of the most prestigious universities in the world is a unique opportunity for a talented young scientist. Currently, she is working within a Food Security Research Scheme, focusing on the fundamental

mechanisms of root-to-root interaction and root system architectural changes under phosphate deprivation, both topics of agricultural relevance.

Nevertheless, it can be concluded that the project laid the foundation for the coming years of iron toxicity research in my work group. We are planning to expand our research into three directions: (i) Understanding the molecular and physiological basis of tolerance to iron toxicity, in which the genes and mutant lines identified in this project will play an important role. (ii) Exploiting possible synergies between tolerance to iron toxicity and 'biofortification' of rice. In other words, we are aiming to identify genes and mechanisms enabling plants to tolerate high levels of iron in the foliar tissue and eventually translocate excess iron to the grain to enhance the nutritional value. The extensive genotype screening and classification into iron 'includers' and 'excluders' plays an important role in these efforts; (iii) Field-based verification of research results may be organized with two potential partners: the International Rice Research Institute (IRRI), which already acted as a partner in the field experiments conducted by Elsa Matthus during her master thesis, and *AfricaRice*, who can offer access to field sites in Madagascar. I have scheduled a trip to Madagascar from March 15th to 23rd to meet potential collaboration partners and explore the possibilities for field-based research.

With this approach, we are hoping to contribute to global food security by simultaneously tackling an important abiotic stress affecting rice production, and the problem of 'hidden hunger' through enrichment of rice grains with iron.

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