Functional analysis of genes involved in the regulation of development of reproductive organs in rice (Oryza sativa)

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Summary

Rice (*Oryza sativa*) is the third main staple food after maize and wheat and is daily consumed by billions of people mainly living in Asia where also most of the rice is grown. Yield is the most important trait in rice which is continuously being improved by breeders and appreciated by farmers. Rice productivity has been enhanced significantly during the so-called Green Revolution during the sixties of the 20th century which was not only about breeding but also about improvement of agricultural management practices including the application of pesticides and fertilizer. Impressive results and progress have been made resulting in yields up to ten tons per hectare under optimal conditions but the global average was in 2009 4.2 tons per hectare (FAO STAT 2009). However, with the rapid economic development in 20th century our lifestyle and consequent food patterns have changed considerably. People now demand for rice with good taste and cooking qualities but also with higher nutritional value. Quality of the rice grain is determined mainly by size, colour, shape, starch and protein content of the endosperm. Although water, fertilizer and temperature have strong effects on development of flowers and final grain quality, it is of course also the genomic content that determines quality. In the last three decades, many efforts have been spend on understanding grain quality using biochemical, physiological and molecular approaches. In this thesis we aimed to gain novel knowledge on grain development using two different approaches. One approach was based on the use of yeast one-hybrid screens and led to the identification of two new transcription factors regulating *GluB-1* which is a major seed storage protein in rice and important for quality. In the second approach which was based on reverse genetics, we gathered a collection of T-DNA and transposon insertion mutants in genes predominantly expressed during flower and grain development that we phenotyped. As a result, a mutant was identified that showed open-staying flowers in combination with elongated grains and of which the corresponding gene was identified and characterized into more detail.

Chapter 1 summarizes the knowledge so far on characteristics of grain quality in rice as well as the genetic and environmental factors that significantly affect grain quality. Previous studies have shown that the quality and quantity of starch and protein in rice endosperm are the main factors affecting grain quality. Therefore, genes encoding starch synthetases and storage proteins are of very importance and are widely studied. The functions of their upstream regulatory factors like transcription factors and the networks in which they operate are also indispensible. Yet the knowledge on these regulatory genes is still relatively limited. In addition, the regulatory networks by which environmental factors determine grain quality are not very well understood either.

Since glutelins are the most abundant seed storage proteins in rice and the fact that the promoter region of one particular gene (*GluB-1)* has been well described, this gene is an obvious candidate for further studies. In **Chapter 2**, we employed yeast one-hybrid assays to search for novel transcription factors regulating *GluB-1*. As a result, two novel CCCH zinc finger proteins, named OsGZF1 and OsGZF2, were identified by screenings of cDNA expression libraries newly derived from developing seed and panicles using the core promoter region of *GluB-1* as bait. The interactions of OsGZF proteins with the bait sequence in yeast were confirmed *in vitro* by Electro Mobility Shift Assays (EMSA). To further elucidate the binding sites in the *GluB-1* promoter, EMSAs were used with oligos with mutations in the so-called GCN4, PROL, AACA and ACGT boxes. Current models for the regulation of *GluB-1* expression imply the importance of these motifs in activation of *GluB-1*. However, no reduction in binding of OsGZF1 and OsGZF2 was found in the EMSAs, suggesting that the sequence motifs involved in activation of *GluB-1* are not the direct

target sites for OsGZF1 and OsGZF2. Detailed expression studies using transgenic rice plants with promoter-GUS constructs and *in situ* hybridization, revealed that both OsGZF1 and OsGZF2 were predominantly expressed in a thin layer of tissue surrounding the scutellum of the developing embryos, instead of the aleurone and subaleurone layers where *GluB-1* is highly expressed. Any regulatory effects of OsGZF1 and OsGZF2 on *GluB-1* promoter were further studied using transient expression assays in rice protoplasts. Experiments in which *GluB-1* promoter-driven *GUS* reporter constructs were co-transformed with *OsGZF1* or *OsGZF2* overexpression constructs into rice protoplasts demonstrated that both proteins can down-regulate reporter gene expression. *OsGZF1* and *OsGZF2* were also able to reduce the activation of the *GluB-1* promoter of a well-known *GluB-1* regulator, RISBZ1, in transient transformation assays. Furthermore, an SDS-PAGE analysis of *OsGZF1* RNAi grains showed increased intensity of glutelin precursor protein as well as two other subunit bands. These results strongly suggest that OsGZF1 and OsGZF2 have functions as repressors of the *GluB-1* promoter.

Certain plant hormones including jasmonate (JA), abscisic acid (ABA) and ethylene (ET) show effects on the development of seeds and flowers. In **Chapter 3,** a *GH3* family protein named OsJAR1 that has a function in the JA pathway was studied in order to further elucidate the molecular mechanism behind the effects of JA on flower development and grain shape. Two *Tos17* transposon mutant lines, *osjar1-2* and *osjar1-3,* showed open-staying florets after flowering and the grains were brownish and more slender like with indica type. The *osjar1* phenotype could be rescued by overexpression of *OsJAR1*. *OsJAR1* was found to be expressed both in reproductive and vegetative organs using promoter-GUS assays. The expression pattern in anthers was further confirmed with RNA *in situ* hybridization. The *osjar1* mutant is relatively insensitive to treatment with JA or MeJA. Unlike in the wild type plants, floret opening in *osjar1-2* cannot be induced by exogenous application of JA and MeJA and inhibition of root growth by MeJA is neither taking place. We also discovered that *osjar1-3* was more vulnerable to infection with rice blast fungus and that overexpression of *OsJAR1* in wild type rice increased the resistance. *In vitro* enzymatic assays were set up to investigate the direct biological function of OsJAR1 and as a result it was found that OsJAR1 can specifically conjugate eight different amino acids to JA. Further *in vivo* analysis confirmed that JA-Ile levels were reduced in *osjar1-2* plants and application of JA induced a significantly higher level of JA-Ile in the wile type than in *osjar1-2*. This indicates OsJAR1 is a JA-Ile synthase with essential functions for the development of flowers and seeds, as well as defence to rice blast fungus and possibly other diseases.

We have demonstrated that OsJAR1 is able to conjugate JA to Ile, but the regulatory connection between JA-Ile and the phenotype of open-staying flowers in *osjar1* is yet unclear. Experiments in **Chapter 4** revealed that during flowering, the K^+ content in *osjar1* lodicules is two times higher than in control lodicules. Swelling and withering of lodicules are the key processes regulating the opening and closure of rice flowers respectively. Therefore we consider accumulation of K^+ in *osjar1* lodicules is one of the major reasons why *osjar1* flowers fail to close after flowering. Based on their high expression levels in flowers, a selection of twelve K^+ transporter genes was selected as candidates for RT-PCR experiments in order to check for expression polymorphisms between control and *osjar1* flowers. Only one gene, named *OsCHX14* from the monovalent cation-proton antiporter 2 family, showed an expression polymorphism. In addition, promoter GUS experiments showed that *OsCHX14* is preferentially expressed in lodicules and styles throughout

floret opening and closure, suggesting a function in flowering. OsCHX14 has 12 potential transmembrane domains at the N-terminus and a GFP-tagged protein was localized on the cytoplasm membrane in rice protoplasts. Functional analysis in yeast confirmed that OsCHX14 is capable of effluxing K^+ outside of cells. Based on these results we conclude that OsCHX14 is one of the key factors responsible for the outward transport of K^+ from lodicules during flowering leading to withering of the lodicules.

In conclusion, the work in this thesis has led to the identification of four genes that we show have functions in development of flowers and seeds. Our results contribute to a better understanding of the effects of regulatory proteins and plant hormones on expression of seed-storage protein in rice and flower opening and closure in rice, respectively.