博　士　論　文（要約）

Study on mediating factors of early wound responses via jasmonic acid signalling in rice

（イネにおいてジャスモン酸を介した傷害ストレス初期応答を担う因子に関する研究）

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Contents

“If your emotional abilities aren`t in hand, if you don`t have self-awareness, if you are not able to manage your distressing emotions, if you can`t have empathy and have effective relationships, then no matter how smart you are, you are not going to get very far.”

Daniel Goleman

Acknowledgement

I have spent five years of my life under the guidance of Associate Professor Kazunori Okada, in the Laboratory of Environmental Biochemistry led by Professor Hideaki Nojiri. This work was performed with a four years financial support from the Japanese Ministry for Education (MEXT) provided as the “Monbukagakusho” scholarship to myself.

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I have received support from so many collaborators in the Japanese research community, from previous lab member, to current ones, as well as from scientists not affiliated to this laboratory. I want to thank each and every one of them. The laboratory life was ever so changing and allowed many interesting social studies in parallel to our scientific endeavours.

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Abstract

論 文 の 内 容 の 要 旨

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論文題目

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Introduction

In the course of life, the rice plant has to adapt and become able to resist numerous biotic and abiotic threatening factors, for example surviving a pest period or adapting to incurring mechanical damages due to either insect damage or strong environmental conditions. Plants in general have shown advanced adaptability and evolved defence mechanisms to a myriad of threatening factors. Rice, as one of the main food sources in Asia and a main crop worldwide, is our plant system of choice to investigate the adaptability and response mechanisms to environmental changes or herbivory resulting in wounding of the rice plant.

One of the defence mechanisms known to be activated is the jasmonic acid pathway. After exposure to biotic or abiotic stresses jasmonic acid (JA) synthesis occurs in rice cells leading to the accumulation and perception of jasmonyl-isoleucine (JA-Ile). This active signalling molecule after recognition by its receptor, the F-box protein OsCOI1, triggers the degradation of the pathway inhibitory factor OsJAZ and with it the release of activity of OsMYC2, the main factor known to activate this system. OsMYC2 is always in position waiting for the repression to be lifted in order to being able to carry on with its activity of

initiating the JA response as a defence form against the initial stress factor. When this defence become unnecessary, OsMYC2 activity is again inhibited by direct binding of OsJAZ. These three factors, a bHLH transcription factor, OsMYC2, F-box protein, OsCOI1, and TIFY motif containing proteins, OsJAZ, are to date the key players of the JA response. In rice there are 15 different OsJAZs known at the moment, characterized by the presence of a TIFY motif for their dimerization as well as a Jas motif responsible for the interaction with COI1 and MYC.

In this study we focused on the activity of two key players, OsMYC2 and OsJAZ, in the context of early wounding response, which in nature could be a daily occurrence in the life of rice plants. At the same time, we brought another factor in this system named RERJ1 (rice early responsive to jasmonates 1) connected to the jasmonic acid pathway. This bHLH transcription factor has been previously identified in microarray studies as a JA early responsive factor. Within 30 minutes after JA treatment and wounding RERJ1 transcript level reaches its peak showing an 800-fold induction compared to the non-treated control leaves.

Of my focus is exposing the early occurrences after wounding in the rice plant with special focus on the JA signalling factors OsMYC2 and OsJAZ, as well as RERJ1, which is a bHLH factor with previously demonstrated transactivation activity, must be involved in the signalling. For achieving my goals, I examined these three factors on the transcript and protein level, exploring their early wound and JA treatment response in transcript levels, as well as their potential to protein-protein interactions, and the RERJ1 regulon harnessing JA-mediated stress responses in rice.

RERJ1 and OsMYC2/OsJAZ directly interact on protein level

Considering possibilities for information input by RERJ1 into the JA signalling, a look at direct protein interactions provided more insight. The results of an yeast two-hybrid assay identified 10 out of 15 OsJAZs as possible interactors of RERJ1 *in vivo*. OsMYC2 has been shown as a potential RERJ1 interactor *in vivo* in Bimolecular Fluorescence Complementation (BiFC) after transient expression in onion epidermis cells.

Considering the weak fluorescence detection for interactions between RERJ1 and OsMYC2, it possibly means that the affinity between these two proteins is not very high and they are occasionally connected by the ability of bHLH proteins to form hetero-dimers. At the same time these findings might signify that both are part of a bigger signalling complex, probably with OsJAZ acting as a possible bridge. All these interactions show the involvement of RERJ1 in the well described feedback loop between JAZ and MYC2.

Early transcripts analysis shows differently regulated OsJAZ dependent on RERJ1

To further our knowledge of the relationship between the JA signalling components, a loss-of-function approach was taken to investigate the involvement of RERJ1 in this context. For this purpose, two different plant materials, the *rerj1-Tos17* mutant and the *osmyc2-RNAi* knock-down lines, were used for the characterization of common signalling factors involved both in wounding and in the JA response, in which the transcript levels of our genes of interests, RERJ1, OsMYC2, and OsJAZ, were analysed.

Firstly, *osmyc2-RNAi* lines have shown that RERJ1 seems to be at least partly dependent on the activity of OsMYC2, mostly after JA treatment and less after wounding, suggesting OsMYC2 is indispensable for the RERJ1 dependent JA response, an already well-established fact. However, it seems to be only partly involved in RERJ1-dependent wounding response, despite showing a certain sensitivity of RERJ1 expression to this kind of stress as well. We hypothesise that there are two different response ways to wounding, one RERJ1 dependent without OsMYC2 being involved, and another OsMYC2 dependent with RERJ1 involvement.

On the contrary, a closer look at the OsMYC2 expression level in the *rerj1-Tos17* knock-down lines showed a RERJ1-dependent slight decrease of the OsMYC2 expression, and this constant small change might seem to signify that RERJ1 is responsible for providing input for OsMYC2 expression at some point along the signalling pathway.

The most probable way for RERJ1 to provide input for the JA signalling and link this system to other pathways involved in defence might be over OsJAZ. Although the temporal transcript pattern of every OsJAZ in response to wounding and JA treatment is similar, it can clearly be distinguished between OsJAZ affected by RERJ1 dependent wounding (for example OsJAZ9 and 11), and those unaffected by it (for example OsJAZ3 and 13). Each OsJAZ has its own function, not yet elucidated for every single one. In this study we could link OsJAZ5, 8, 11 and 12 to RERJ1-dependent early wound responses. Connected to both early responses after wounding and JA treatment, expression of OsJAZ9 and OsJAZ11 seem to be highly dependent on RERJ1. By analysing the transcriptional context, *OsJAZ* genes involved in wounding in a RERJ1-dependent manner were able to be categorised, and thus the transcriptional connection between RERJ1 and OsMYC2 was also partly elucidated.

Search for genes under direct RERJ1 regulation

In previous studies, the involvement of RERJ1 in wounding had been established, however no direct evidence of the mechanism has been provided. Here, by using RERJ1 overexpressing lines, we screened for *in vivo* targets of RERJ1.

One of the interesting aspects of this chapter is the usage of a novel human dopamine receptor derived 10 amino-acids long tag shortly called AGIA. Transgenic lines

overexpressing of N-terminal AGIA-fused RERJ1 were generated and confirmed the overexpression of AGIA-RERJ1 transgene on transcript and protein level. These lines were used for subsequent characterizations through Chromatin Immunoprecipitation (ChIP) analyses with both RERJ1, AGIA and OsMYC2 polyclonal antibodies, as well as for microarray analysis to survey potential candidates of the RERJ1 regulon in this transcriptome. ChIP-qPCR analysis was not able to provide conclusive evidence of the binding of RERJ1 to the promoter region up-stream of the *OsLIS* and *OsJAZ5* gene, encoding a linalool synthase involved in the production of linalool and OsJAZ5 respectively. OsLIS is a monoterpene previously shown to be directly involved in defence against herbivory whereas also being under RERJ1 regulation, thus providing evidence of the involvement of RERJ1 in the defence mechanism to herbivory in rice.

Two colour microarray analysis with AGIA-RERJ1 overexpressing plants provided insight into the transcriptome changes caused by constitutive expression of RERJ1, showing slightly increased RERJ1 and OsMYC2 transcript levels compared to the vector control in two of four lines Used in this study. A wide range of changes in the transcript levels of distinctive OsJAZ family genes was also observed.

The significance of this finding might possibly be linked to the overexpression level of RERJ1 and the activation of its down-stream response genes, such as *OsLIS* and other JA-related stress response genes including the OsJAZ repressor, a core component of the JA signalling.

Conclusion and future prospects

All these findings brought together show that different OsJAZ are responsible for different response connections. A most probable mechanism is RERJ1 providing input in the early stages of the wounding response over OsJAZ 9, 11 and possibly OsMYC2 direct interaction, leading to fast decisions of the rice plant as to signal perception and response pathway activation, eventually leading to the actual defence response involving genes such as *OsLIS* or other factors found responsive in the microarray analysis of RERJ1 overexpressing plants. For further describing the global RERJ1 regulon, other promising E-box *cis*-elements occupied by RERJ1 in the promoters of RERJ1 dependent OsJAZ genes and other candidates of RERJ1-regulon will be revealed by means of ChIP-qPCR to extend our understanding of RERJ1 involvement in the JA signalling system.

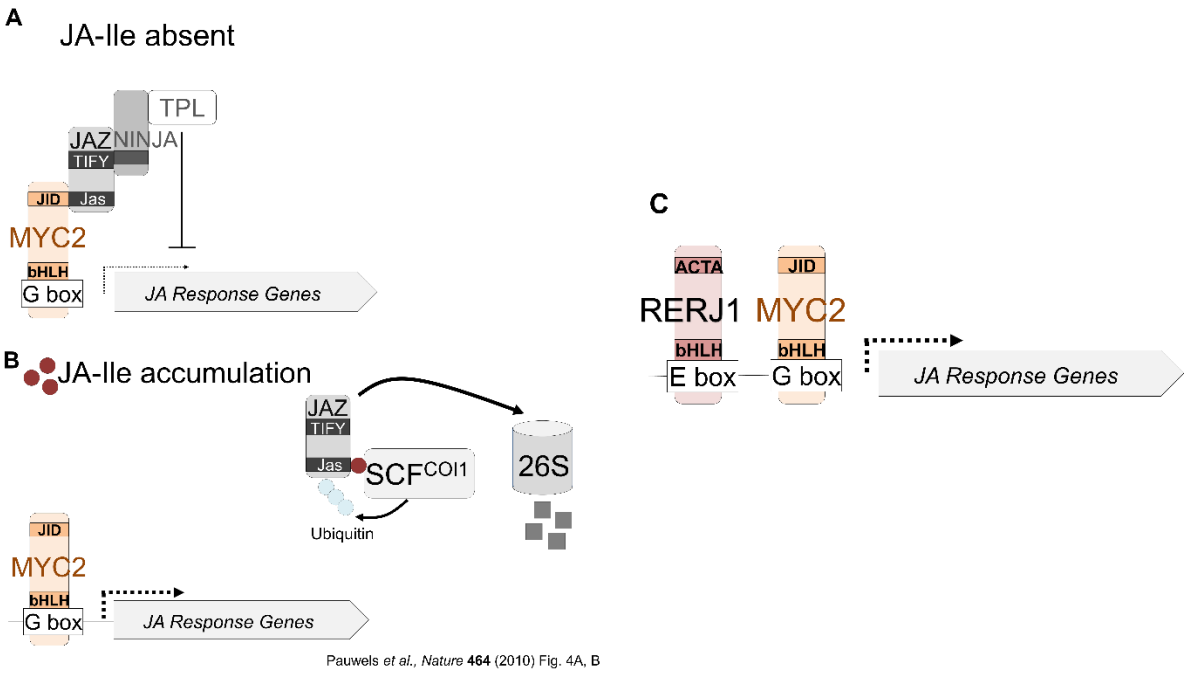


Figure ABC. Model of JA signalling based on Pauwels et al.,2010.

A. Should the active biomolecule JA-Ile be absent, the JAZ-NINJA-TPL complex inhibit the activity of MYC2.

B. Upon accumulation of JA-Ile, the SCF-COI1 complex ubiquitinate JAZ, qualifying it for degradation through the 26s proteasome, allowing MYC2 to read the JA response genes.

C. The bHLH transcription factor RERJ1 described in this study, binds to an E-box *in vivo* and is involved in the transcriptional activation of JA response genes.

List of Abbreviations

Alpha	Amplified Luminescent Proximity Homogeneous Assay
Amp	Ampicillin
BiFC	*Bimolecular Fluorescence Complementation*
Bls	Biotinylation site
bp	Base pair
Cb	Carbenicillin
CDS	Coding sequence
Cm	Chloramphenicol
COI	Coronatine insensitive
dH_2O	Deionized, distilled water
DMSO	Dimethyl sulfoxide
GO	Gene Ontology
h	Hour(s)
His	Histidine
HPT	Hygromycin phosphotransferase
HRP	Horseradish peroxidase
JA-Ile	Jasmonyl-L-isoleucine
JAR	Jasmonic acid resistant
JAs	Jasmonates
JAZ	Jasmonate ZIM-domain
kbp	Kilo base pair
kDa	Kilo-dalton
Km	Kanamycin
Leu	Leucine
LUC	Luciferase
NC	Negative control
NOMT	Naringenin 7-*O*-methyltransferase
OD	Optical density

OX	Overexpression
PANTHER	Protein analysis through evolutionary relationships
PC	Positive control
PEG	Polyethylene glycol
PR	Pathogenesis-related
psi	Pound-force per square inch
qRT-PCR	Quantitative real-time polymerase chain reaction
RAP-DB	The Rice Annotation Project Database
RNAi	RNA-interference
RT	Room temperature
s/sec	Second(s)
SD	Synthetic defined
TPS	Terpene synthase
Tris	Tris (hydroxymethyl) aminomethane
UBQ	Ubiquitin
Ura	Uracil
UTR	Untranslated region
VC	Vector control
WT	Wild-type

Chapter 0 Background

0-1 Plants coping with environmental stresses like wounding

Plants have sessile life styles, spending their whole life cycle in the same location while having to withstand and adapt to every single environmental challenge. Should they fail to do so means they found the one impenetrable barrier that might lead to their death. To survive environmental conditions, harsh or not, plants have to be able to perceive environmental signals, classify them as threatening or not and respond accordingly. A plant in a natural environment can perceive a myriad of signals as stress, from physical constraints such as soil, over periodically changing weather conditions to insect herbivory. They must be able to differentiate between a strong wind with the ability to tear them away from a summer breeze that cannot harm them, just as they must withstand and react accordingly to a tropical rainstorm, a response that has to differ in the case of a light summer shower. Time is of major importance for perceiving such signals. Should a plant not be able to react precisely with the corresponding pathway within the first few minutes of exposure to a certain environmental stress, it might lose in the evolutionary process. Living organisms including plants are prone to react to stress with general or specific life preserving mechanisms. Generally, should a plant be wounded, their sessile living environment restrict their ways of response and they must find the answer to survival within themselves developing internal various mechanisms to defend themselves and thrive despite the conditions available in their environment.

Damage caused by wounding to the plant might have catastrophic effects. Abiotic factors such as wind, rain, snow, water or biotic ones like insects and pathogens can lead to disruptions of the cell wall and allow penetration of the plant internal system. A first general fast response is suberization of cells around the wounded area and increase in cell wall stability (Howe *et al.*, 2004). Wounding responses are characterized as local and systemic, whereas the molecular mechanism of perceiving and transducing the wounding signals are still poorly understood. Early events after wounding observed include ion imbalance and variations in membrane potentials, induction of the Ca^{2+} signalling, production of reactive

oxygen species (ROS), activation of different phytohormonal pathways as well as kinase activities (Maffei *et al.*, 2007).

0-2 Hormonal crosstalk during wounding defence

Jasmonic acid (JA), ethylene (ET), salicylic acid (SA) and gibberellin (GA) are four different phytohormones with evidence of involvement in the wounding response available. A complex network forming between them including activation, inhibition, feedback and loop activity of components are the regulators of the actual wounding response in plants. The centre of attention lies on the jasmonic acid pathway being able to interact with others in order to modulate the plant response. SA pathway is the main antagonist to the JA pathway induced through the production of ROS (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). SA and ROS mediate the hypersensitive response of the plants leading to local cell death around the wounded/infected area.

ET and GA pathways on the other hand enhance the JA response and provide access to regulation of plant features not directly influenced by JA. ET plays a major role in fine tuning the process, by either having a positive or negative effect on the JA response, as needed (Kahl *et al.*, 2000; Onkokesung *et al.*, 2010). (Pieterse *et al.*, 2012) showed that ET is even able to activate MYC and ERF factors of the JA pathway depending on the hormone blend produced. ET shows the same fine-tuning effects on the SA pathway as well.

Gibberellin pathway seems to be involved in the decision of the plant to balance growth and defence since they are both equally resource depleting and prioritization needs to be undertaken (Huot *et al.*, 2014). During intense wounding responses the plant has to divert resources from primary metabolites towards the amplification of the defence pathway by means of defence metabolites production (Cipollini *et al.*, 2014).

In conclusion we can state that plant responses to wounding are a complex undertaking with ramifications in even the most basic plant cell function. The types of these interactions are plant species (tomato or rice), threat type (mechanical wounding or herbivory) and environmental conditions specific, despite the major stress being categorized as wounding.

Even so, plants show the ability to coordinate all available resources for a minimal cost specific defence response with the complex hormonal network at its core.

0-3 Jasmonic Acid – from biosynthesis to signalling – OsMYC2 and OsJAZ introduction

The Jasmonates are a group of non-conventional phytohormones known to influence a wide range of the plant life. From growth and development over defence and adaptation to photomorphogenesis and reproduction, jasmonates are known to be involved in the regulation of each stage in the plant life (Okada *et al.*, 2015). At the basis of this wide interlocution lies the production of active jasmonyl-isoleucine (JA-Ile) activation of the core signalling system in the plant cell nucleus with many players already meticulously described in *Arabidopsis thaliana*, however yet to be characterized in detail in rice.

The biosynthesis of JA has been extensively described with the process, the involved enzymes and catabolites made known. Figure 1-1 illustrates the biosynthesis pathway beginning from phospholipids processed in chloroplasts by the successive activity of key players like phospholipase (PL), lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) for the synthesis of (+)-cis-OPDA (oxo-phytodienoic acid), which is further processed in the peroxisome by the activity of 12-oxo-PDA reductase (OPR) and beta-oxidases to be released as jasmonic acid in the cytosol.

JA however is only able to induce its characteristic signalling upon activation, meaning after addition of characteristic side-groups, like an amino-acid isoleucine (Ile) or a methyl group, respectively. Jasmonyl-isoleucine (JA-Ile) is the first shown active JA molecule able to induce the signalling pathway.

A cyclic induction/inhibition counter play between MYC2, the global regulator, and JAZ (Jasmonate ZIM factor), the main inhibitor, seems to lie at the centre of the JA signalling in plants. While in *A. thaliana* many other participants are also known, in rice the lack of information makes it difficult to draw a conclusive picture depicting this system. OsMYC2 has been identified as the missing master regulator in the JA signalling in rice, as well as 15

different OsJAZ, proteins bearing a Jas domain and a TIFY motif. They are well-known inhibitors able to interact with bHLH factors. All OsJAZ except OsJAZ14 have been shown to directly interact with OsMYC2 in order to modulate the JA dependent defence response in rice (Uji *et al.*, 2016).

Figure 1-2 illustrates the generally assumed JA signalling pathway in plants, as modified from the illustration in Pauwels *et al.,* 2010. In the absence of active JA-Ile, JAZ inhibits the master regulator MYC2 from performing its transcriptional activity over a complex to which Novel Interactor of JAZ (NINJA) and topless (TPL) belong. Upon induction through specific stress factor like wounding, biosynthesis of JA and accumulation of the active JA-Ile leads to its perception by the receptor COI1, which then in a complex with SCF targets JAZ by ubiquitination for degradation through the 26s proteasome, releasing the repression from MYC2 and allowing the JA response gene to be transcribed.

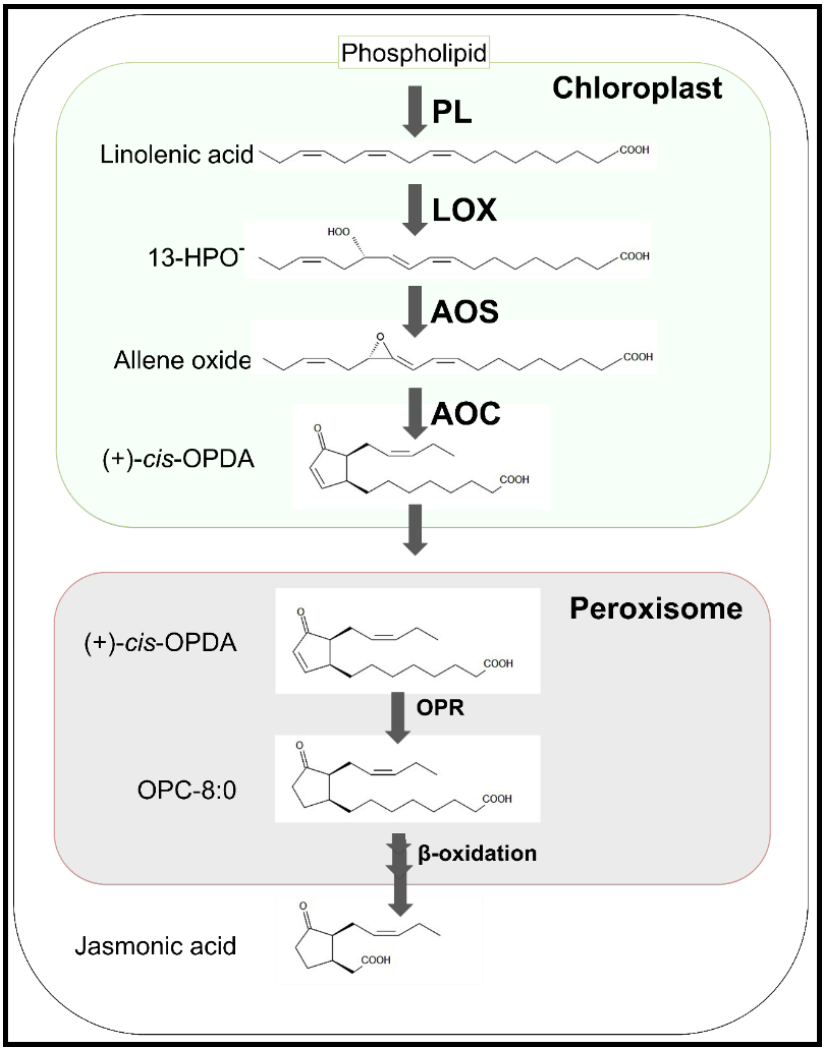
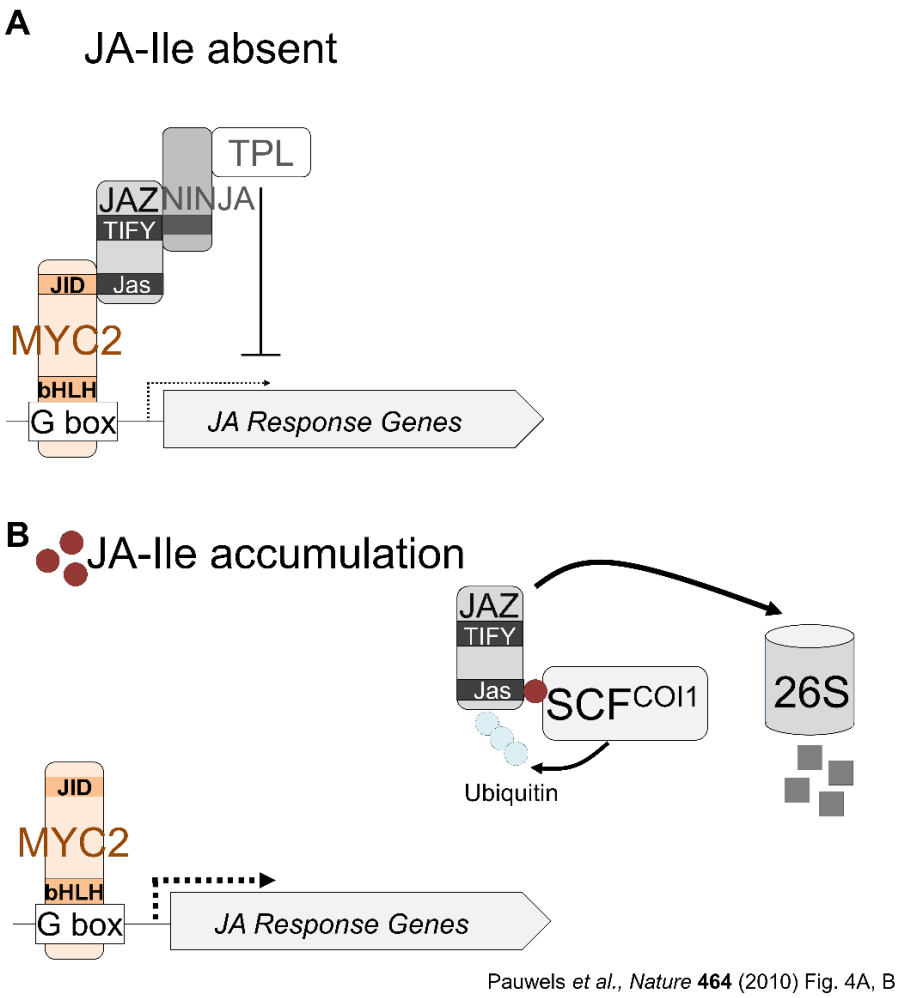


Figure 0-1 Jasmonic acid biosynthesis. Cycle beginning from phospholipids in the chloroplasts, over linolenic acid and OPDA, to the oxidation of OPC in the peroxisome until the release of jasmonic acid in the cytoplasm and begin of the JA response in the rice cell.



Pauwels *et al.*, *Nature* **464** (2010) Fig. 4A, B

Figure 0-2 Model proposed by Pauwels *et al.* for the JA signalling revolving around the interaction between MYC2 and JAZ and their feedback loop in *Arabidopsis thaliana*.

A. In case of JA-Ile absence, MYC2 and JAZ directly interact over the JID and Jas domains, leading to the inhibition of JA response genes transcription with the help of the NINJA-TPL complex.
B. JA-Ile is accumulated in response to stress, being detected by its receptor COI1 and resulting in the degradation of JAZ after ubiquitinoylation by the SCF-COI complex, and setting MYC2 released from inhibition, allowing the transcription of the JA response genes.

0-4 The TIFY protein family

One special family of proteins available in plants and dependent on JA is the TIFY family. This family is divided in two subgroups, (I) with or (II) without a Jas domain, having a total of 20 members, with 15 belonging to the first group. These proteins, JAZ (Thines *et al.*, 2007) are characterized by having a special motif, called TIFY; as well as a Jas domain, responsible for the interaction with MYC2 and the resulting transcriptional inhibition through conformational changes (Zhang *et al.*, 2015). The Jas domain is also known to be responsible for dimerization processes, including interactions with COI1 and NINJA in *Arabidopsis* (Chini *et al.*, 2009).

The following table (1-1) lists all OsJAZ, as well as their interaction partners within the bHLH group of proteins described in the following section. Though this list does not contain all known information, some of the OsJAZ have been previously described in different studies and for some of them the information can be taken from here.

Table 0-1 List of some known OsJAZ interacting bHLH factors and their confirmed functions in rice

JAZ No	Gene ID	Accession	Known Interacting bHLH Partners	Known JAZ Involvement in
OsJAZ1	Os04g0653000	AK069326	OsMYC2, OsbHLH148	Defense
OsJAZ2	Os07g0153000	Os07g05830	OsMYC2	
OsJAZ3	Os08g0428400	AK103459	OsMYC2, OsbHLH148	
OsJAZ4	Os09g0401300	AK065170	OsMYC2, OsbHLH148	
OsJAZ5	Os04g0395800	AK107750	OsMYC2, OsbHLH148	
OsJAZ6	Os03g0402800	AK106184	OsMYC2, OsbHLH148	
OsJAZ7	Os07g0615200	AK068566	OsMYC2, OsbHLH148	
OsJAZ8	Os09g0439200	AK108738	OsMYC2, OsbHLH148	Defense
OsJAZ9	Os03g0180800	AK070649	OsMYC2, OsbHLH148	Defense, Nutrition (root)
OsJAZ10	Os03g0181100	AK120087	OsMYC2, OsbHLH148	Defense,
OsJAZ11	Os03g0180900	AK073589	OsMYC2, OsbHLH148	
OsJAZ12	Os10g0392400	AK061602	OsMYC2, OsbHLH148	
OsJAZ13	Os10g0391400	AK107854	OsMYC2, OsbHLH148	
OsJAZ14	Os10g0391801	Os10g25250		Spikelet
OsJAZ15	Os03g0396500	Os03g27900	OsMYC2	Spikelet

0-5 The bHLH protein family in plants

This protein domain, basic helix-loop helix (bHLH) contains a highly conserved amino acid motif that defines a group of transcription factors initially discovered in animals, however soon after also in all the major eukaryotic organisms. Proteins containing the bHLH domain are involved in a very wide range of regulatory processes, for example modulating secondary metabolism or responses to environmental factors in plants (Buck MJ and Atchley WR, 2003). To the current level of knowledge, the genomes of *Arabidopsis thaliana* (thale cress) and *Oryza sativa* (rice) encode more bHLH motifs than animals with widely unknown functions (Heim *et al.*, 2003).

OsMYC2, the main regulator of the JA signalling in rice, belongs to the bHLH TF family and it is known to be able to bind to DNA through its basic region of the bHLH domain with G-boxes (*CACGTT*) as its *cis*-element; whereas, in the case of bHLH proteins, dimers are a necessity for the interaction process. OsMYC2 also has a JID domain, allowing interaction with OsJAZ (Uji *et al.*, 2016).

There is a wide variety of yet to be characterized bHLH TFs (Chinusammy *et al.*, 2003), however here I will introduce one with special meaning for this study, namely OsbHLH148 (table 0-1). Due to its involvement in stress responses, such as drought, and its previously shown ability to interact with OsJAZ, OsbHLH148 underlines the fact that OsJAZ are able to interact with bHLH proteins (Seo *et al.*, 2011), setting the grounds for the beginnings of our work.

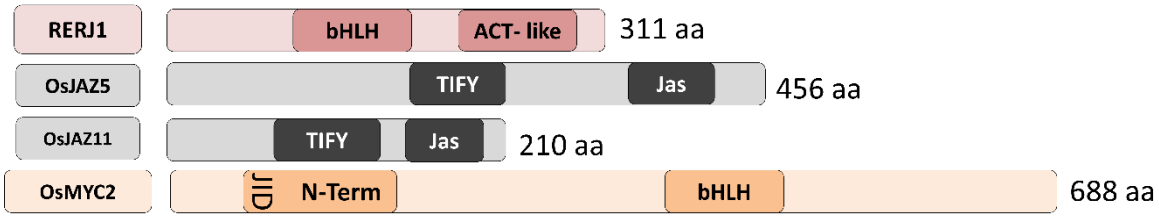


Figure 0-3 Illustration of the protein domains available in RERJ1, OsMYC2 and OsJAZ.

bHLH: basic Helix-Loop-Helix domain

ACT-like: aspartate kinase – chorismite mutase – TyrA (prephenate dehydrogenase)

TIFY: conserved TIFY motif, part of the ZIM domain (GATA-type zinc finger domain)

Jas: MYC2/COI1 interacting destabilizing element (degron)

JAZ: Jasmonate ZIM-domain containing protein

MYC: myelocytomatosis oncogene (human)

JID: JAZ-interacting domain

0-6 RERJ1 (rice early responsive to jasmonates 1)

In this study we further our insight into the functions of the bHLH transcription factor called "**R**ice **E**arly **R**esponsive to **J**asmonates 1" (RERJ1), previously identified in our laboratory. Its transactivation activity has been shown in a reporter gene assay, while as a bHLH transcription factor it is expected to have the ability to bind to E-boxes (figure 0-3).

cDNA of this JA responsive transcription factor, RERJ1, has been isolated during studies on the biological functions of JA in our laboratory (Kiribuchi *et al.*, 2004, 2005). RERJ1 has been shown to be expressed in a JA dependent manner within 15 minutes after elicitor or wound treatment while reaching its peak within 30 minutes after treatment qualifying for an early response gene. Reporter-GUS assays monitoring the activity of the RERJ1 promoter show that in these plant lines RERJ1 is expressed around the wounded locus in a timely manner. (*Miyamoto et al.*, 2013)

In onion cells its nuclear localization has been visualized by a fusion to GFP, results expected considering the bHLH nature of RERJ1. Except for this domain, at the C-terminus of RERJ1 there is also an ACT-like domain, regarding which to this time no possible function has been investigated. Several studies have intended to characterize ACT-like domains in *Arabidopsis* bHLH proteins and propose their necessity for the dimerization process, however these were performed superficially offering no deeper insight in the functionality of ACT-like domains. To bind the DNA, bHLH family proteins were shown to form homo- and/or heterodimers, necessary also for performing other functions. (Cui *et al.*, 2016; Feller *et al.*, 2017)

One function of RERJ1 has been extensively investigated in our laboratory, namely it`s involvement in herbivory by regulating several terpene synthases and protease inhibitor genes. One strongly reacting terpene synthase gene to RERJ1 is the linalool synthase, *OsLIS*, encoding a biosynthetic gene necessary to produce the volatile linalool. Linalool has been previously shown to play a function in herbivory. (Taniguchi et al., 2014a)

0-7 Hypothesis

As a JA dependent transcription factor, the possibility of RERJ1 being involved in JA signalling is very high. The main purpose of this study is firstly to show the regulatory molecular mechanism revolving around RERJ1 as to widen our understanding of the JA signalling system in rice. For this purpose, the connections between RERJ1 and the JA signalling factors OsMYC2 and OsJAZ are relevant and to be revealed on transcriptional and protein interaction level.

Not only JA responsive, but also strongly activated by wounding, another main goal of this study it to reveal in more detail the wounding mechanisms revolving around RERJ1. The same applies to OsJAZ and OsMYC2, especially considering the wide range of the former, with 15 different OsJAZ being characterized up-to date in rice, and the global regulator character of the latter. The expression analyses performed on two different knock-down mutants *rerj1-Tos17* and *osmyc2-RNAi* will reveal a more detailed transcriptional correlation between these two bHLH transcription factors. Combining the physical protein interaction data will reveal a whole new perspective on the working wise and influence range of the JA signalling in rice.

This study has been divided in three different parts, each focusing on a different aspect of the relationship between my JA signalling factors of interest, RERJ1, OsMYC2 and OsJAZ.

In chapter 2 direct protein interactions between our factors show how they directly relate to each other. bHLH proteins are known to form hetero- and/or homodimers. Both RERJ1 and OsMYC2 belonging to this category, make this a very probable interaction on protein level. Also, bHLH proteins have been shown to have potential of interaction with OsJAZ factors, OsMYC2 and other bHLH, like OsbHLH148, belonging to this category. Therefore, it is highly likely that RERJ1 is also able to form the needed platform for direct protein interaction with OsJAZ. Both cases will be elucidated in this chapter by using two distinct methods: bimolecular fluorescence complementation (BiFC) and yeast two-hybrid assay (Y2H).

In chapter 3 the transcript level network between our three factors of interest are to be exposed. Main focus is their expression levels in the *rerj1-Tos17* knock-down mutant. Moreover, transcript levels of RERJ1 and OsMYC2 in *osmyc2-RNAi* knock-down lines, are to

complement our data and complete the missing knowledge regarding a functional correlation between these two bHLH factors.

Chapter 3 will complement our knowledge on the RERJ1 dependent transcriptional network. Having established the transcriptional context on the background of knock-down mutants, one further step to be taken is to characterize the transcriptome of RERJ1 overexpressing plants. Correlations between the transcriptional landscape after repressed and enhanced expression of RERJ1 will complement each other and provide evidence for our hypothesis regarding the involvement of RERJ1 in the JA signalling, as well as a more global characterization of its influence range. The same overexpressing plants are also to be used for revealing protein-DNA interactions within the range of promoters of my factors of interest, OsMYC2 and OsJAZ, another target being the *OsLIS* gene promoter, whose expression dependency on RERJ1 has been characterized in previous studies.

By adding up all these pieces of evidence in chapter 4, I am constructing a new working model of the JA signalling in rice after JA treatment and wounding revolving on the input provided by RERJ1 to the already well characterized system made of OsMYC2 and OsJAZ. At the same time a more in-depth characterization of the early wounding occurrences linked to RERJ1 is attempted.

Chapter 1 Direct protein interactions between the JA signalling factors and RERJ1

1-0 Introduction

The system for JA-Ile perception in Arabidopsis having become accepted includes the SCF-COI1 complex directly binding JAZ, as evidence showing direct COI1-JAZ shows, catalysing the attachment of ubiquitin to the latter, leading to its degradation through the 26s proteasome (Thines *et al.*, 2007). The degradation of ubiquitinated JAZ proteins leads to de-repression of transcription factors, which then can start the transcription of early response genes, including JA response genes and JAZ genes themselves. A negative feedback loop is created through the accumulation of the repressors which then can attenuate the JA signal. The first main regulator known to interact with JAZ in Arabidopsis is MYC2, followed by a range of other bHLH transcription factors. The direct protein interaction between MYC3 and JAZ9 has been characterized *in vitro* through elucidation of the crystal structure of the interaction domains JID in MYC3 and Jas in JAZ9. It was revealed that the Jas domain is responsible through conformational changes for the repression of the transcription initiation. (Zhang *et al.*, 2015)

Although information in rice is not as abundant, OsMYC2 has been identified as the only known AtMYC2 homolog in rice and its possible interaction with 14 out of the 15 know OsJAZ was sown in vivo in a n yeast two-hybrid screen. This interaction is necessary for the feedback loop to be made possible. (Uji *et al.*, 2016)

The main focus of this chapter is to include RERJ1 to the protein interactions and gather information of possible interaction partners for RERJ1 among OsMYC2 and OsJAZ by using *in vivo* methods, bimolecular fluorescence complementation (BiFC) and yeast two-hybrid (Y2H), as well as *in vitro* AlphaScreen with wheat germ produced proteins.

1-1 Material and Methods

In the following the methods used for protein interaction detection are described.

1-1.1 Yeast two-hybrid (Y2H)

The yeast strain used for this experiment was *pJ69-4A* received from Dr. Takafumi Shimizu, having the following genome engineered for the yeast two-hybrid assay:

MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2:: GAL7 - lacZ

Media and solutions used:

YPAD medium（/L）		**SD medium（/L）**	
Bacto Peptone Bacto Yeast Extract	20 g 10 g	Yeast Nitrogen Base w/o Amino Acids (DIFCO)	6.7 g
Adenine	100mg	10x Dropout solution	100 mL
Glucose	20 g	Glucose	20
Agar	20 g	Agar	20 g

10x Dropout solution (/L)			
		L-Isoleucine	300 mg
L-Valine	1500 mg	Adenine Sulfate	200 mg
L-Arginine HCl	200 mg	L-Histidine-HCl	200 mg
L-Leucine	1000 mg	L-Lysine HCl	300 mg
L-Methionine	200 mg	L-Phenylalanine	500 mg
L-Threonine	2000 mg	L-Tryptophan	200 mg
L-Tyrosine	300 mg	Uracil	200 mg

For yeast auxotrophic selection purposes, adenine, histidine, tryptophan and leucine were used and therefore not added to the amino acid mixture; however, they were added to the SD or YPAD medium directly before autoclaving on a need to add basis.
The solutions were autoclaved and stored at room temperature.
Yeast transfection was performed according to the Matchmaker yeast two-hybrid protocol from Clontech Laboratories, with carrier DNA extracted from salmon sperm nuclei.

10xTE		**1xTE buffer (/L)**	
EDTA	10 mM	10xTE	100 mL
Tris base	100 mM	dH_2O	900 mL
Adjust pH to 7.5 and autoclave before storage.			

10xLiAc (/L)	**TE-LiAc buffer (/L)**	
1 M, adjust pH to 7.5 and autoclave.	10xTE	100 mL
	10xLiAc	100 mL
	dH_2O	800 mL

50% PEG (polyethylene glycol)	**PEG-LiAc solution (/L)**	
Dissolve 100mg polyethylene glycol, MW 3375 in 100ml water and autoclave.	10xTE	100 mL
	10xLiAc	100 mL
	50% PEG	800 mL

Yeast transformation was conducted according to the co-transformation protocol from the Matchmaker Kit, Clontech following the PEG/LiAC method.

The transformed yeast was recovered for 4 days on SD medium lacking leucine and tryptophan for plasmid selection, followed by spotting of grown colonies of medium lacking leucine and tryptophan, as well as adenine and histidine for protein interaction selection.

The pictures of the plates were taken 4 days after incubation at 30℃ in darkness.

1-1.2 Bimolecular fluorescence complementation (BiFC)

For this experiment, onion epidermis cells were ballistically transfected by a PDS-1000 He Biolistic Particle Delivery System (Bio-Rad, CA, USA) with constructs of interest fused either N- or C- terminally to either the N- or C-terminus of split EYFP. As a background control of the success of transfection, DsRed1 was used. The constructs of interest were RERJ1 and OsMYC2 CDS.

For the microscopy a BX53 microscope system (Olympus, Japan) was used. The filters used were the following: U-FBNA filter cube (excitation filter, BP470-495 nm; dichromatic mirror, DM505 nm; and suppression filter, BA510-550 nm) and U-FGW cube (excitation filter, BP530-550 nm; dichromatic mirror, DM570 nm; and suppression filter, BA575IF nm) for YFP and dsRED detection respectively.

1-2 Results and Discussion

1-2.1 OsMYC2 and RERJ1 directly interact on protein level *in vivo*

1-2.2 Direct protein interaction between RERJ1 and OsJAZ in yeast two-hybrid

1-2.3 Significance of direct protein interactions

「本章の内容は，学術雑誌論文として出版する計画があるため，公表できない．5年以内に出版予定．」

Chapter 2 RERJ1 dependent transcripts of selected JA responsive genes

2-0 Introduction

Previous microarray data (Kiribuchi *et al.*, 2004; Motegi *et al.*, in preparation) performed with wounded wild-type Nipponbare young rice plants helped discover and gave a first insight into transcriptome changes mediated by RERJ1. Genes affected in this *rerj1*-Tos17 knock-down mutant compared to the wild-type were numerously detected and gave ignition for this study. The genes mostly affected here were belonging to the groups of pathogenesis-related proteins (PR), terpene synthases (TPS) and JAZ genes, a plant specific group of proteins involved in the JA signalling. These findings motivated us to look further into the transcriptome changes believed to be influenced by the presence or absence of the bHLH transcription factor RERJ1 with focus on these groups of genes.

For a very long time RERJ1 was believed to be involved in the JA mediated wounding and stress response in rice (Kiribuchi *et al.*, 2005). Our motivation for this part of the study was to elaborate these hypotheses on the transcriptome level and deliver a more detailed insight of the RERJ1 mediated immediate response to JA treatment and wounding.

In the first chapter of this study my focus is locally oriented with my centre of attention around the JA signalling components OsMYC2 and OsJAZ. On the premises of previous microarray data, I decided to look at early transcriptome occurrences in more detail while utilizing the rerj1-Tos17 mutant compared to the wild-type Nipponbare after two different treatment methods, JA and wounding. An in-depth time course expression analysis is to reveal the dependency of these factors upon RERJ1 as well as establish the possible involvement of RERJ1 in the JA signalling system.

Since the main regulator of this pathway is the bHLH transcription factor OsMYC2, a look at the transcript levels in the *rerj1*-Tos17 mutant and in *osmyc2-RNAi* lines, both of which show knock-down of the respective factor, is to reveal the interdependency mechanism and the common regulatory in/consistencies, within the RERJ1 generated response.

As mentioned in the main introduction chapter, there are 15 different *OsJAZ* known and categorized, each showing a characteristic induction and expression pattern. While disregarding the expected redundancy of these factors, another focus of this chapter is to establish their RERJ1 dependency after JA treatment and wounding in the same rerj1-Tos17 knock-down mutant as used for *OsMYC2* expression analysis.

The clarification of the detailed immediate responses in the transcriptome of rice plants revolving around RERJ1 will hopefully lead to a deeper understanding of early occurrences in defence to wounding happening shortly after JA pathway induction.

2-1 Material and Methods

2-1.1 Plant material

rerj1-Tos17 mutant on the Nipponbare wild-type background

Tos17 seeds possibly down-regulating/knocking-out RERJ1 were received from the National Institute of Agricultural Studies (NIAS), Tsukuba, Japan, and screened by Koji Miyamoto, PhD. He was able to find seeds of one mutant plant showing down-regulated *RERJ1* expression as confirmed by Northern Blotting and propagated them for further use. (Motegi *et al.*, in preparation)

In this *rerj1-Tos17* plants, a 3 kbp Tos17 is inserted in the genome of wild-type *Nipponbare* rice after the first exon from the 5`UTR end of the *RERJ1* gene, disrupting both homologs of the RERJ1 gene, rending the succeeding plants knock-down mutants.

osmyc2-RNAi knock-down mutant lines on the *Nihonmasari* wild-type background

The *Nihonmasari* wild-type background was transformed by Agrobacterium with a construct including the *Hygromycin* gene and a 700 bp long sequence at the 3`UTR of the *OsMYC2* gene under the maize Ubiquitin promoter. This transformation made by Koji Miyamoto, PhD, resulted in the *Nihonmasari* background overexpressing this sequence able to activate the RNAi mechanism in rice. Through this activation, the *OsMYC2* mRNA is being targeted for degradation, leading to a knock-down *osmyc2* mutant.

2-1.2 Wounding and JA treatment

For the wounding and JA treatment two different methods were used, both involving the leaves of 14-d-old plants grown on 0.5% Agar-water, either attached or detached from the plantlet.

For the **JA treatment of intact plants**, 13-d-old plantlets were transferred to water for 24h for acclimation, followed by addition of 500 µM JA to the water, as well as spraying of 100 ml of 500 µM JA on the leaves in a closed environment and incubation in the following time course: 0h (mock, no treatment, sample collection shortly before start of treatment), 30 min, 1, 2, 4 h. Collected as sample were the second and third leaves of one plant per each time-point.

For the **JA treatment in leaves** only, second and third leaves of 13-d-old plants were detached and cut in small pieces of ~0.5 mm. They were water acclimated for 24 h before medium exchange to water containing 500 µM JA. The samples were collected after 30 min, 1, 2, 4, and 12 h after induction. At time point 0, before the start of treatment, leaf pieces were collected as a mock control. Used leaf pieces were a mixture of cut leaves from 7 different individual plantlets.

For the **wounding of intact plants**, 13-d-old plantlets were transferred to water for 24h for acclimation, followed by wounding by pin holder of the second and third leaves. One sample was collected before treatment as time-point 0. The wounded plantlets were incubated on water for another 30 min, 1, 2, 4 h before sampling of the wounded leaves followed by immediate freeze in liquid nitrogen.

For the **wounding of leaves**, 14-d-old second and third leaves of plantlets were cut into small pieces of ~0.5cm by scissors and incubated on water in a Biotron at 28°C for 0 (control), 0.5, 1, 2, 4, 12, 24 h.

After sampling all samples were immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction.

2-1.3 RNA extraction and cDNA synthesis

RNA extraction was performed by using the Promega Maxwell Kit according to the manufacturer`s instructions.

The plant material used for the RNA extraction is leaves of 14-day old plantlets sampled after JA or wound treatment.

The cDNA synthesis was done using the Takara PrimeScript Reverse Transcription kit according to the manufacturer`s instruction from 1ng RNA.

2-1.4 qRT-PCR

The quantitative real time PCR was performed with the above described cDNA as a template. The sequences of the primers used can be found in table 2-1. The reactions, 20ul each, were mixed according to the protocol below and ran according to the PCR conditions described below on an ABI 7300 qRT-PCR cycler.

Table 2-1 Primer List used for RT-qPCR

Primer	Sequence	Mentions
OsUBQ RT Fwd	5'-TCCGAGAGATGGGTTTCATC -3'	*Housekeeping Gene*
OsUBQ RT Rev	5'-GCCAAGATTGCCAAGAAGAC-3'	
RERJ1 qRT Fwd	5'- ATTTTTGCGACACCCCACTA -3'	100 bp region in the
RERJ1 qRT Rev	5'- GCTGGACCACCTTATTATTCATC -3'	3`UTR of RERJ1
OsMYC2 qRT Fwd	5'-TGGACGTGTACCATGCCAGC-3'	
OsMYC2 qRT Rev	5'-TTGAGCTGGTCCTGCGAGTAGAC-3'	
OsMYC2 RNAi Fwd	5'-AGCTCAACCAGCGCTTCTAC-3'	for *osmyc2-RNAi*
OsMYC2 RNAi Rev	5'-GTCTCCTTGTCCGTCTCCAG-3'	

Primer	Sequence	Mentions
OsJAZ1 qRT Fwd	5'- CGTCGCGTTCATTGTTTAGA -3'	*OsJAZ1*
OsJAZ1 qRT Rev	5'- CATAGCAGTTTGCTGTTCGAG -3'	
OsJAZ3 qRT Fwd	5'- AGAGTCGGCTGACCTTGATG -3'	*OsJAZ3*
OsJAZ3 qRT Rev	5'- GCAGCCAGGATAGAAACACG -3'	
OsJAZ4 qRT Fwd	5'- GGCTTGGGAAGAGGCATAAT -3'	*OsJAZ4*
OsJAZ4 qRT Rev	5'- GCACCAGCTAGACCAGCATT -3'	
OsJAZ5 qRT Fwd	5'- CTGTTGATTTGGTCCCCTTG -3'	*OsJAZ5*
OsJAZ5 qRT Rev	5'- CGATCGATCAACACTAAACAGG -3'	
OsJAZ6 qRT Fwd	5'- TGAGATGCCCCTCTCTCATCA -3'	*OsJAZ6*
OsJAZ6 qRT Rev	5'- AGAACATCGCCTTGTCATCG -3'	
OsJAZ7 qRT Fwd	5'- ATGCGCTTGCCGTTAGAGTA -3'	*OsJAZ7*
OsJAZ7 qRT Rev	5'- GCACTGGTCGTCGTCAATGCTAC -3'	
OsJAZ8 qRT Fwd	5'- ACGGGTTGTTTGTTCTCCAC -3'	*OsJAZ8*
OsJAZ8 qRT Rev	5'- ACCGAACGAGAAATGAGGAA -3'	
OsJAZ9 qRT Fwd	5'- GTCACGTCTGCGATTTGAGA -3'	*OsJAZ9*
OsJAZ9 qRT Rev	5'- ATGCGACGAGAACCATCTTC -3'	

Primer	Sequence	Mentions
OsJAZ10 qRT Fwd	5'- GCGCAAGAGGATTGATTCAC -3'	*OsJAZ10*
OsJAZ10 qRT Rev	5'- AAGGGGTTTCCCATCAATTC -3'	
OsJAZ11 qRT Fwd	5'- CGTGTCTGTGGAAAGTGTGG -3'	*OsJAZ11*
OsJAZ11 qRT Rev	5'- GCTACTAATTCCCCCGGAAG -3'	
OsJAZ12 qRT Fwd	5'- TATCTGCCCGGTTTAGAGGA -3'	*OsJAZ12*
OsJAZ12 qRT Rev	5'- TTGGGCCAAAGAAATCTCAA -3'	
OsJAZ13 qRT Fwd	5'- TAATTAACGAGGCCGTGAGG -3'	*OsJAZ13*
OsJAZ13 qRT Rev	5'- ACATTTCAACTCTTTCCAATGAA -3'	
OsMYC2 qRT Fwd	5'- TGGACCTGGACGTGTACCAT -3'	*OsMYC2*
OsMYC2 qRT Rev	5'- AGCTGGTCCTGCAGATAGAC -3'	
OsNOMT Fwd	5'- CTAGCCGGARGCARGAAAGT -3'	*JA treatment marker*
OsNOMT Rev	5'- TGCACGTATAGGCACACACA -3'	*OsNOMT*

PCR protocol

Template DNA	2 µL
Primier mix (10 µM)	0.6 µL
H_2O	7.4 µL
2×SYBR Green PCR Master Mix	10 µL
Total	20 µL

PCR conditions

50℃, 2 min → 94℃, 10 min → ［94℃, 30sec → 58℃, 30sec → 72℃, 30sec］×40 cycles→ 95℃, 15sec → 60℃, 1 min→ 95℃, 15sec → 60℃, 15sec

For all primers a standard dilution curve with 10-fold dilutions of pooled sample cDNA was run (Hellemans *et al.,* 2007). Primer efficiency determined by calculating a linear regression of the cycle threshold (Ct)-values of these serial dilutions, leading to primer selection with an efficiency between 90 and 120% (D'Haene *et al.,* 2010).

qRT-PCR Data Analysis

The quantitative real time PCR was performed by using the ABI 7300 software. The obtained average data of each sample in triplicate was normalized according to the housekeeping gene Ubiquitin (UBQ). The standard error for each sample was calculated and represented by an error bar in each graph.

Statistical Analysis

Selected samples were subjected to the Student`s t-test. The results as a comparison between WT and mutant at each time point can be seen in the according graphs.

2-2 Results and Discussion

In the following the results obtained from the described methods are presented, while also delivering our understanding and interpretation.

2-2.1 OsMYC2 shows subtle changes in expression levels in the *rerj1*-Tos17 knock-down mutant after JA treatment or wounding

2-2.2 RERJ1 shows OsMYC2 dependent expression in *osmyc2-RNAi* lines after JA treatment, however not after wounding

2-2.3 OsJAZ show differential transcript levels in the *rerj1-Tos17* mutant after JA treatment and wounding

2-2.4 Summary and Conclusion

「本章の内容は，学術雑誌論文として出版する計画があるため，公表できない．5年以内に出版予定．」

Chapter 3 Genes under direct RERJ1 regulation

3-0 Project introduction

In the previous chapter a reverse genetic approach was undertaken to reveal the dependency of the JA signalling factors *OsMYC2* and *OsJAZ* on *RERJ1*. In this chapter the main goal is to understand the gene expression in a much more precise way by unravelling protein-DNA interactions.

A specific interaction between RERJ1 and the JA signalling components OsMYC2 and OsJAZ might be between RERJ1 and the promoter regions of the genes under its direct regulation. Therefore, another approach I took to deepening our understanding on the role of RERJ1 in the JA signalling system is to unravel its DNA binding sites with special attention to the promoters of the JA signalling factors as my end goal.

The method of choice here is Chromatin Immunoprecipitation followed by quantitative PCR (ChIP-qPCR) analysis while focusing on E-box containing promoter regions of our genes of interest. Going a step further on this approach means elaborating the global binding sites of RERJ1 by Chromatin Immunoprecipitation followed by Next Generation Sequencing (ChIP-Seq). A methodical challenge posed is the fact that in WT plants, even after stress induction, the RERJ1 protein level is too low for the detection of DNA binding sites. Therefore, a common way in plant biology around this methodical short-coming is to use overexpressing individuals for this analysis. A first step in advancing towards elucidating the *cis*-elements of RERJ1 *in vivo* was the production of RERJ1 overexpressing plants to assure the immunoprecipitation success.

Although in our laboratory we have the possibility of using specific polyclonal antibodies against RERJ1, as a measure of cautiousness combined with a factor of novelty we decided to fuse RERJ1 to a tag, named AGIA, against which there are specific monoclonal antibodies available. This tag, although already successfully used in wheat and other plant systems, has not been tested in rice to the moment of speaking. The AGIA tag is a 10 amino-acids long sequence from the human dopamine receptor against which specific monoclonal antibodies

have been purified in Ehime University, in our collaboration laboratory, the Proteo Science Center (Takeda *et al.*, 2015; Yano *et al.*, 2016). Our minor goal is to use the high specificity to establish a new tag to be used for further rice studies and of course to be able to achieve our main goal of immunoprecipitating RERJ1 for the ChIP assay.

The motivation for this chapter also leads to a deeper understanding of the RERJ1 dependent transcriptome under overexpression conditions. Microarray analysis is bound to reveal how JA signalling factors are differently transcribed in response to the overexpression of RERJ1, as well as deliver an overview of the Nipponbare transcriptional landscape changes under the same overexpressing conditions.

All in all, this chapter harbours three different novelty points, from the possibility of a novel tag with respective monoclonal antibodies use in rice, over the description of the RERJ1 overexpression transcriptome and the search for the *cis*-elements over which this bHLH transcription factor can directly regulate its dependent genes.

3-1 Material and Methods

3-1.1 Transgenic overexpressing plants

Oryza sativa Nipponbare wild-type seeds were undergone Agrobacterium transformation with a construct containing the Hygromycin gene, as well as the *RERJ1* CDS sequence fused to a 10 amino acids short tag linked by 3 amino acids at the N-terminus after the maize Ubiquitin promoter, called p2KG-AGIA-RERJ1. This transformation performed by our collaborators at the Teikyo University in Utsunomiya led to obtaining AGIA-RERJ1 overexpression plants, which I further characterized and used for subsequent microarray and ChIP analysis in this study.

The protocol for plant transfection was based on the one described in Toki *et al.*, 2006 by early transfecting rice scutellum with the p2KG vector as a vector control (VC) or the p2KG-AGIA-RERJ1 construct for the overexpression lines.

All seeds used in this study belong to the T1 generation collected at Teikyo University. T2 generation seeds of each plant introduced in this study (fig. 3-1) were propagated and stored in our laboratory.

The seeds were sterilized for 40 min using 20% Natriumhypochlorite solution and grown on 0.5% wateragar, made with agar melted in tub water from Waterworks TOKYO, in a growth chamber at 27°C under long day conditions (10h dark, 14h light).

3-1.2 Genomic DNA extraction

For the genotyping, genomic DNA was extracted by grinding plant material in TPS extraction buffer (100 mM KCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA) followed by DNA precipitation with 100% ethanol on ice, a washing step with 70% ethanol to eliminate some of the precipitated salts and finally followed by elution in distilled water. The extracted DNA was used as template for the following PCR reaction with Hygromycin specific primers (Primer tables 3-1) to detect transfected plants.

Primer Name	Sequence	Mentions
HPT Fwd	5'- ATGAAAAAGCCTGAACTCACCGCGACGTCTGTC -3'	*Hygromycin*
HPT Rev	5'- CTATTCCTTTGCCCTCGGACGAGTGCTG -3'	*Gene*

PCR protocol

2xKOD Fx Neo buffer	10 µL
dH_2O	6.7 µL
dNTPs (2.0 mM)	1.6 µL
Primer mix (10 µM)	0.6 µL
Template	1 µL
KOD Fx Neo	0.1 µL
Total	20 µL

PCR conditions

94°C, 2 min → ［98°C, 10sec → 58°C, 30sec → 68°C, 30sec］ ×30 cycles→ 4°C, ∞

The results are shown in the Annex

3-1.3 RNA extraction and cDNA synthesis from callus and plant material

RNA extraction was performed by using the same protocol for all plant materials, as described in Chapter2 – Material and Methods. We used the Promega Maxwell Kit, which is an automatized RNA extraction method.

The plant material was frozen in liquid nitrogen and each sample homogenized by hand as to avoid any degradation of the RNA by timely de-freezing.

All extracted RNA was subjected to FA-GE (Formaldehyde Gel-electrophoresis), the concentration was measured by Beckmann and the same concentration of RNA for each sample was used for cDNA synthesis using the Takara PrimeScript Reverse Transcription cDNA Synthesis kit. In the case of callus 1 µg RNA was used for the following reverse transcription step, while for the plant material 400ng RNA were initially used. The cDNA was

used as template for RT-PCR performed with the genes specific primers (Primer table 3-2) and specific PCR conditions listed below.

Primer Name	Sequence	Mentions
		Housekeeping ubiquitin gene
UBQ qRT Fwd	5'-TCCGAGAGATGGGTTTCATC -3'	*Os10g0542200*
UBQ qRT Rev	5'- GCCAAGATTGCCAAGAAGAC -3'	*Ubiquitin-associated/translation elongation factor EF1B, N-terminal*
AGIA Fwd	5'- GAAGAAGCAGCTGGTATTGCT -3'	*AGIA-RERJ1 full length*
RERJ1 CDS Rev	5'- CTAATAGCTCATGGAGCTCAACG -3'	
RERJ1 3`UTR Fwd	5'- ATTTTTGCGACACCCCACTA -3'	*RERJ1 3`UTR*
RERJ1 3`UTR Rev	5'- GCTGGACCACCTTATTATTCATC -3'	

PCR protocol

2xKOD Fx Neo buffer	10 µL
dH_2O	6.7 µL
dNTPs (2.0 mM)	1.6 µL
Primer mix (10 µM each)	0.6 µL
Template	1 µL
KOD Fx Neo	0.1 µL
Total	20 µL

PCR conditions

94°C, 2 min → ［98°C, 10sec → 58°C, 30sec → 68°C, 30sec］ ×28 cycles→ 4°C, ∞

The PCR products were running for 30 min at 180 V on a 2% Agarosegel, stained for 10 min in $EtBr_2$ solution and an UV exposure image was taken after a 10 min de-staining in tub water. The results are shown in figure 3-1.

3-1.4 Nuclear protein extraction from rice calli and leaves

Nuclear protein was extracted from callus and leaves as previously described in Miyamoto *et al.*, 2014. The method is based on a one step nuclei separation in a 70% high-glycerol buffer (20 mM HEPES/NaOH, pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, 50 mM saccharose, 70%[v/v] glycerol, protease inhibitor cocktail (Roche)). After grinding of the cells in this buffer and incubation on ice for 20 min, the plant mixture is filtrated on a quadri-layer of Miracloth (Merck, UK) and centrifuged for 1h at 6000xg at 4°C, followed by washing the pellet with 10% low-glycerol buffer (20 mM HEPES/NaOH, pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, 50 mM saccharose, 10% [v/v] glycerol, protease inhibitor cocktail (Roche)) under mild shaking. After another centrifugation step for 20 min at 6000xg at 4°C, the pellet was resuspended in TE buffer and subjected to sonication for 1 min, with output 1, pulse 1 sec., duty cycle 50%. The sheared nuclei were again centrifuged for 15 min at 20000xg at 4°C, the nuclear debris was removed, and the remaining nuclear protein fraction was used for overnight acetone precipitation (95% [v/v] acetone, -20°C) for concentration of the obtained nuclear protein fraction, after having measured the initial protein concentration by a Bradford test. The pellet was resuspended in Laemmli SDS sample buffer, boiled and subjected to SDS-PAGE on a 12% Tris gel with subsequent transfer to a PVDF membrane for Western Blotting detection of RERJ1 and AGIA by using the respective antibodies.

Each three identical SDS-PAGE gels were run for 20 min at 30 mA until the samples were aligned at the limit to the running gel, followed by a 60 minutes run at 60 mA, under constant mA (Bio-Rad). One gel was washed in dH2O followed by Coomasie Brilliant Blue (CBB) staining for 30 min. The de-staining was performed in dH2O overnight and the resulting protein bands were scanned and saved as a picture file. The second and third identical SDS-PAGE 12% gels were transferred on a PVDF membrane, after 1 min activation in Methanol, under Glycine-Tris buffer (192 mM mM Glycine, 25 mM mM Tris-HCl pH 8.0) for 12h, 4°C at 40 V, under constant voltage.

The PVDF membranes were washed with PBS-T buffer (TBS, 1% Tween-20) and blocked for 1 h at room temperature (RT) with 5% skim-milk dissolved in TBS-T. The first antibody was added in a dilution of 1:2000 in 3% skim-milk TBS-T and lightly shaken for 1 h, at RT. After five washing steps of the membrane in TBS-T, each for 5 min, the second antibody anti-rabbit bound to HRP was added in a dilution of 1:20000 in 3% skim-milk TBS-T, followed by

an incubation of 1 h at RT. Five washing steps with TBS-T followed, before detection using the Immobilon (Bio-Rad) HRP substrate. After a 5 min incubation at RT, the signals on the membrane were detected and the result saved in form of an image file.

The western blotting results can be seen in figure 3-2.

3-1.5 Microarray Analysis

The microarray analysis was performed in the National Institute for Agricultural Studies (NIAS), Tsukuba by myself under the supervision of Prof. Nagamura and his staff in the microarray open laboratory.

For this experiment I prepared RNA extracted from plant leaves as previously described by using the Promega Maxwell kit which allows an efficient extraction of many samples in the same day with less human interference in the main extraction process. These RNA samples were sent to NIAS, where they were analysed by a Bioanalyzer and not degraded samples upon arrival were used for the following microarray analysis.

Two different lines of AGIA-RERJ1ox #3 and #6 represented by RNA extracted from leaves of 4 independent individuals, previously characterized for overexpression of AGIA-RERJ1 on the transcriptome level through qRT-PCR, were used in this two colour 44k Agilent microarray. The RNA of these samples was labelled with Cy5 (red), while also mixed with Cy3 (green) labelled vector control mixed RNA of 4 different control plants. The labelled cRNA mixture of Cy3-VC and Cy5-sample was then hybridized, each sample on a different array of the four available 44k arrays on the slide. The hybridization took place for 17 h at 65°C, followed by washing of the slide and scanning process by the Agilent slide scanner.

The information available in the scanned pictures were extracted by the Agilent Feature Extraction Software and the first step before further processing was the analysis of the processed spots. Should more than 100 non-uniform spots be available, then the scanning procedure should be repeated, whereas not more than one repeat is possible due to the dye signal decreasing with time and light exposure.

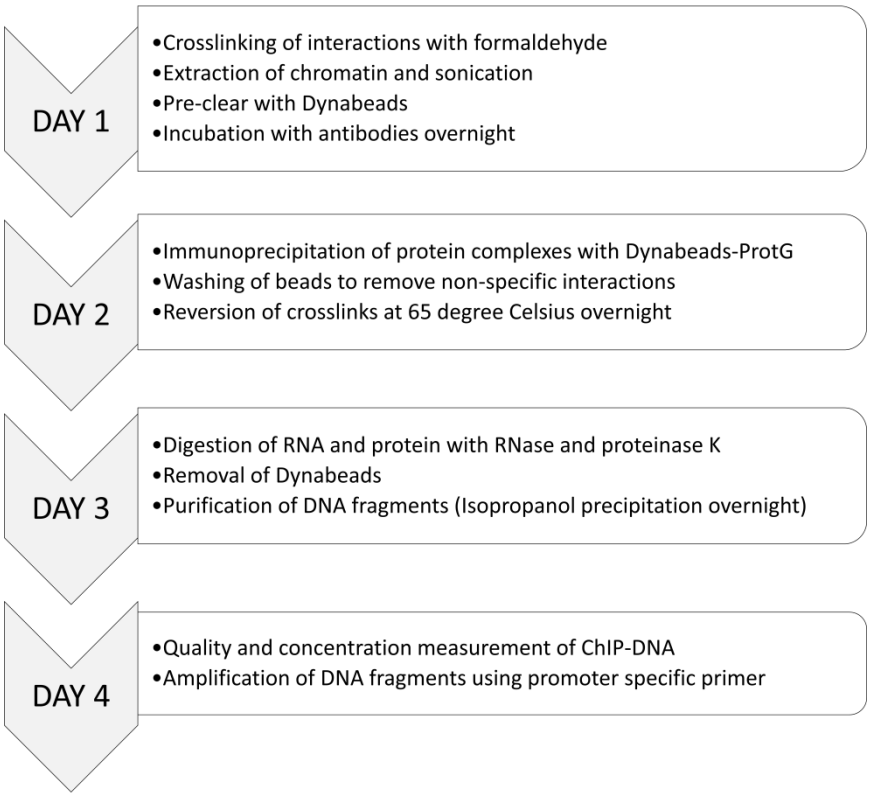
As for the automatic signal processing, the extraction software has a preinstalled limit of 75% signal limit for the rice specific array arrangement. Signals that are over 75% stronger in the sample (green signal) than in the vector control (red signal) are ones that are used for further analysis.

Microarray data analysis

The extracted features are all included in an Excel file, which also facilitates every further processing of the data. By using the advanced filters available in Excel, the unspecific background signals are removed, the fold change can be calculated as the power 10 logarithm of the log ratio and the p-value can be stringently set to lower than 0.1. Following these settings, the genes of interest can be screened. Genes with a positive log ratio are considered up-regulated, while ones with a negative log ratio down-regulated. Generally, genes with a fold change higher than 2 are considered significantly responsive. In this study however, I also consider genes like *OsMYC2*, that have a fold change higher than 1.5.

All genes remaining after the filtering process were used for subsequent analysis. The involved up- and down- regulated genes in all four used overexpressing lines were compared and the results summarized in Venn-Diagrams. Also, the gene landscape was tested by performing a GO analysis and the resulting significantly regulated groups of genes further compared.

3-1.6 ChIP-qPCR



Scheme 3-1 Representation of the work-flow followed in a general ChIP assay with special considerations for this study. Here, we used the Dynabeads-Protein G (Invitrogen) approach for immunoprecipitation.

One of the main experiments of this chapter is the chromatin immunoprecipitation (ChIP, scheme 3-1).

Plant material used here were leaves of over 4-6 weeks old plants, AGIA-RERJ1 overexpression mutant lines #3, #4, #6 and #7. Previously characterized plants on the transcript level numbered 1-7 (figure 3-1), also used for the microarray, were mixed and used for this approach to gather enough starting material and reproducible results. The plant material was sampled and frozen at -80°C until the experimental procedure.

IgG (rabbit) was used for this approach in order to provide the background level for in rabbit produced antibodies. The protein of interest is RERJ1 N-terminal tagged with the AGIA tag,

therefore polyclonal rabbit produced RERJ1 antibodies as well as monoclonal rabbit produced ones against the AGIA tag were used for the immunoprecipitation.

For this experiment preliminary experiments in form of western blotting detection of AGIA-RERJ1 directly after nuclei preparation and after immunoprecipitation of nucleic proteins were performed. The nuclei preparation was previously described following the one step nuclei extraction in high glycerol (70% [v/v]) buffer. After having confirmed the usability of the antibodies, they were used for ChIP analysis.

Frozen plant material was grounded by using the Multi Beads Shocker and resuspended in PBS buffer containing protease inhibitor cocktail and 1% formaldehyde for fixation. The procedure was performed on powdered plant material for 20 min at room temperature under vacuum. The reaction was stopped with 1 M glycine for 10 min at room temperature under vacuum. The PBS buffer was removed, and the crosslinked plant material was washed once with sterile dH_2O to remove all traces of formaldehyde. Resuspension in high glycerol [80% v/v] buffer followed for nuclei preparation as previously described (chapter 3-1.4). After the nuclei were resuspended in TE buffer, the sonication was performed for 5 cycles [15 sec power 2, 30 sec on ice, 10 cycles] on ice, with 2 min on ice incubation between the cycles.

The sonicated material was centrifuged for 20 min at 20.000xg at 4°C and to the supernatant, containing the sonicated nuclear protein in TE buffer, NaCl and NP-40 were added to final concentrations of 100 mM and 0.4% [v/v], respectively, before adjusting the total sample volume to 1600 µl with IP buffer (TE-buffer, pH 8.0, 100 mM NaCl, 0.4% [v/v] NP-40). At this point a 4 h incubation at 4°C under slow rotation followed as an incubation step with 40 µl IP-buffer washed Dynabeads-Protein G to reduce background by removing any unspecific binding between nuclear proteins and Dynabeads bound Protein G. The pre-cleared nuclear protein sample was divided into 500 µl fractions and used for subsequent incubation with each 10 µg antibodies (RERJ1 or AGIA) or 2 µg Rabbit normal IgG, with one 100 µl fraction representing 6.25% of the initial sample volume remaining as Input control. After 18h incubation at 4°C with rotation, 25 µl Dynabeads-Protein G washed with IP buffer was added and another 4h incubation at 4°C with rotation followed. The Dynabeads were then washed 7 times with 500 µl IP buffer and resuspended in 500 µl TE-buffer, pH 8.0. 20 µl of 5 M NaCl were added and the Dynabeads-Protein G complex was incubated at 65°C for

over 18 h. The Input control was also volume adjusted to 500 µl TE-buffer, 20 µl of 5 M NaCl added and incubated together with the immunoprecipitated samples for de-crosslinking at 65°C. The de-crosslinked samples were treated with RNase A for 1h at 65°C, after which the Dynabeads-Protein G protein complex was removed on a magnetic stand. After Proteinase K treatment for 1 h at 46°C the de-crosslinked supernatant was subjected to phenol-chloroform extraction and overnight isopropanol precipitation at -20°C aided with 2 µl Ethachinmate pro reaction (following the Ethachinmate protocol). After washing with 70% Ethanol, the precipitated ChIP-DNA was dissolved in 50 µl DNase free dH_2O. The purity and concentration were measured on a Beckmann luminometer and each 1 µl sample was used as template for subsequent qRT-PCR analysis with focus on promoter regions of our genes of interest, namely *OsLIS, OsJAZ, OsMYC2* and *RERJ1* promoters.

Specifics of the qRT-PCR analysis are listed in the tables below. An *OsLIS* promoter 299 bp specific region was amplified by using the below primers and PCR conditions.

Primer	Sequence	Mentions
OsLISp qRT Fwd	5'- AAGCTTACGCCGCACAACGT -3'	*-500-200 bp region*
OsLISp qRT Rev	5'- AAGCTTATGCTGAGTTGCTG -3'	
OsLISp -300 Fwd	5'- TGGAATGATATGACACAGGAGAG -3'	
OsLISp -300 Rev	5'- ATGGGAAGTGTGTGTGGGAT -3'	
OsLISp -500 Fwd	5'- GCCAAACAACCCAACCCAAT -3'	
OsLISp -500 Rev	5'- TCCTGTGTCATATCATTCCAAAA -3'	
OsLISp -1000 Fwd	5'- ATCAACCTGTCCTCCCATCC -3'	
OsLISp -1000 Rev	5'- TGTCTTCTTCTACCTTACCTCGA -3'	
OsLISp -1500 Fwd	5'- CATTGGACAGTGGGTGATGG-3'	
OsLISp -1500 Rev	5'- GTTTCGACAGTAGGATAAACGA -3'	
OsLISp -2400 Fwd	5'- GGTGGCGTATTTGGATTTCC-3'	
OsLISp -2400 Rev	5'- ACATTGGACTAATTCGTGAG-3'	
OsLISp -2600 Fwd	5'- TTCCAAAGTCCAAACGCAGC-3'	
OsLISp -2600 Rev	5'- GAAATCCAAATACGCCACCT -3'	
OsLISp -4800 Fwd	5'- TGTTCCTTTCTCCGATGATCT-3'	
OsLISp -4800 Rev	5'- TGTCATACTTCGGTCCATTGC -3'	

PCR protocol

Template DNA	2 µL
Primer fwd/rev (1 µM each)	0.6 µL
H_2O	7.4 µL
2×GO Taq PCR Master Mix	10 µL
Total	20 µL

PCR conditions

95°C, 10 min → [95°C, 30 sec → 51.6°C, 30 sec → 68°C, 30 sec] ×45 cycles→ dissociation

For each primer on each plate, two no template controls (NTC) were ran in parallel, in which the sample cDNA was substituted with dH2O to detect potential formation of any PCR by-products. Positive signals in the NTC were determined as insignificant should the dissociation curve peak be at a different temperature than the sample product peak, or if the Ct value of the NTC-signal was at least 8 cycles later than the sample peak (e.g. sample peak at 25, NTC Peak at 33) (D'Haene *et al.*, 2010).

All sample ChIP-DNAs were running in technical triplicates, for each sample Input, IgG and anti-RERJ1 was tested. The *ct* values of the technical triplicates were averaged and used for the subsequent calculation of [Percent Input]. The Δ*ct* value of the Input was adjusted to the total percentage of the sonicated chromatin DNA used for the immunoprecipitation. For 1% Input, 3,3 cycles were subtracted from the Δ*ct* value. The following formula was used for the calculation of the percent input/ sample: 100*2^[adjusted Input (Δ*ct*) – sample (Δ*ct*)]

For comparability the results are shown in relation to the IgG amount. (IgG = 1) in figures 3-4 and 3-5.

3-2 Results and Discussion

Having described the material and methods used in this chapter, in the following I will present the results of experiments.

3-2.1 Characterization of RERJ1 overexpressing plants

3-2.2 Rice transcriptome changes after RERJ1 overexpression

3-2.3 Search for RERJ1 direct target genes

3-2.4 Discussion

3-2.5 Summary and future challenges

「本章の内容は，学術雑誌論文として出版する計画があるため，公表できない．5年以内に出版予定．」

Chapter 4 Conclusions and future of this study

「本章の内容は，学術雑誌論文として出版する計画があるため，公表できない．5年以内に出版予定．」

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Annex