Universidade de Lisboa

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Departamento de Biologia Vegetal

The role of *OsRMC* gene in the JA signaling pathway: a transgenic approach in Arabidopsis and by an Y1H assay

João Guilherme Costa de Alvarez Cortes

Dissertação

Mestrado em Biologia Celular e Biotecnologia

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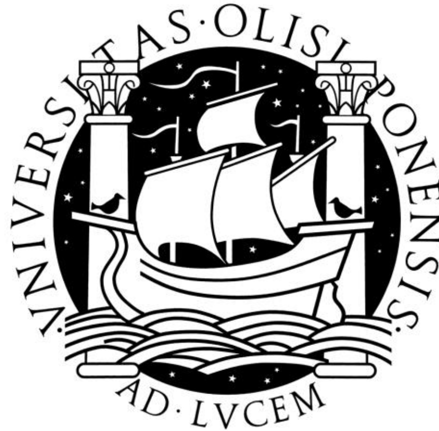

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Orientador interno: Prof. Doutora Helena Trindade (Centro de Biotecnologia Vegetal – IBB, Faculdade de Ciências da Universidade de Lisboa)

Orientador externo: Doutor Tiago Lourenço (Laboratório de Genómica de Plantas em Stress – GplantS, Instituto de Tecnologia Química e Biológica – ITQB)

2012

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List of Abbreviations

3-AT	3-amino-1,2,4-triazole
bp	Base pairs
cDNA	Codifying DNA
CEF	Cefotaxime
Col-0	*Arabidopsis thaliana* Columbia ecotype
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EREBP	Ethylene-responsive element binding protein
g, mg, µg, ng	Gram, miligram, microgram, nanogram
JAs	Jasmonates
JA	Jasmonic acid
JAZ	Jasmonate ZIM domain
L, mL, µL	Liter, mililiter, microlitre
M, mM, µM	Molar, milimolar, micromolar
NaCl	Sodium Chloride
min	Minutes
ºC	Degree Celsius
PR	Pathogen related
PEB	Protein Extraction Buffer
PCR	Polymerase chain reaction
RT	Room temperature
RT-PCR	Semi-quantitative reverse transcriptase PCR
RMC	Root meander curling
RFP	Ref fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
TF	Transcription factor
Vol	Volume
Y1H	Yeast one hybrid
WT	Wild-type

Abstract

Jasmonic acid is a plant hormone that has been involved in several biological processes such as development, biotic and abiotic stress. Previously, it was found in the host lab that a rice RNA interference (RNAi) – OsHOS1 line had up-regulation of *OsRMC* gene expression. In rice, this gene seems to be involved in the modulation of abiotic stress and root curling, acting as a negative regulator of the jasmonic acid signaling. *OsRMC* does not have a known homologue in *Arabidopsis* which makes this model species an important system to study the function of this gene. To achieve this goal, we overexpressed *OsRMC* in Arabidopsis in two different systems either in a cell suspension cultures (to study gene expression changes of JA-responsive genes) or whole plants (phenotypic changes induced by JA). We were able to obtain stable transgenic cell suspension culture overexpressing *OsRMC* as well for the empty vector (negative control). However, we were not able to see gene expression changes induced by JA even in untransformed cell suspension cultures for the analyzed genes (*PDF1.2*, *VSP1* and *MYC2*). Regarding the transformation of whole plants, we generated three independent lines overexpressing *OsRMC*. These lines were characterized for transgene expression level and corresponding protein (OsRMC-RFP).

We also performed a direct yeast one hybrid (Y1H) assay to complement the work done with *Arabidopsis* plants and cell suspension cultures regarding JA-signaling. In this assay, the promoter sequences of three genes involved in JA-signaling from rice (*JAZ*-like 1, *JAZ*-like 2 and *JAZ*-like 3) were analyzed. Promoter fragments corresponding to 1000bp upstream of the ATG codon of each gene were used as bait sequences. As prey, we used two transcription factors from the EREBP subfamily, previously identified as binding to the *OsRMC* gene promoter. However, we couldn't detect interaction between the two TFs analyzed and the bait sequences from the *JAZ* genes. As future work, these baits strains will be screened by Y1H with cDNA libraries induced by salt or JA.

As main achievements of this work, we were able to produce transgenic Arabidopsis plants overexpressing *OsRMC* which were fully characterized at transgene expression and protein level. These lines will be used in further studies such as to

monitor expression changes in JA-responsive genes, determination of the sub-cellular localization of the OsRMC-RFP, and JA-induced phenotypic characterization.

Resumo

O ácido jasmónico é uma hormona vegetal que está envolvida em vários processos biológicos tais como vias de desenvolvimento, stress biótico e abiótico. Foi descoberto anteriormente no laboratório anfitrião que uma linha de arroz de RNA de interferência (RNAi) – OsHOS1 apresentava uma expressão génica do gene *OsRMC* aumentada. Em arroz, este gene parece estar envolvido na modulação do stress abiótico e do encaracolamento das raizes, agindo como um regulador negativo da via de sinalização do ácido jasmónico. O gene *OsRMC* não tem nenhum homólogo conhecido em *Arabidopsis*, tornando esta espécie modelo num sistema importante para estudar a função deste gene. De modo a alcançar o objectivo principal, nós sobrexpressamos *OsRMC* em Arabidopsis em dois sistemas diferentes, quer em cultures de células em suspensão (para o estudo das alterações da expressão génica de genes que respondem ao ácido jasmónico) quer em plantas (alterações fenotípicas induzidas por ácido jasmónico) Fomos capazes de obter culturas estáveis transformadas de células em suspensão sobrexpressando *OsRMC* e também culturas transformadas com o vector vazio (controlo negativo). Contudo, não fomos capazes de vizualisar quaisquer alterações de expressão génica (para os genes analisados *PDF1.2*, *VSP* e *MYC2*) induzidas por ácido jasmónico mesmo em culturas de células em suspensão não transformadas. Em relação à transformação de plantas, foram geradas três linhas independentes sobreexpressando *OsRMC*. Estas linhas foram caracterizadas em termos de nível de expressão do transgene e da proteína correspondente (OsRMC-RFP)

Neste trabalho também se inclui um ensaio directo de yeast one hybrid (Y1H) de modo a complementar o trabalho realizado com as plantas de *Arabidopsis* e as culturas de células em suspensão, no que diz respeito à sinalização pelo ácido jasmónico. Neste ensaio, as sequências dos promotores de três genes de arroz envolvidos na sinalização pelo ácido jasmónico (*JAZ*-like1, *JAZ*-like2 e *JAZ*-like3) from analizadas. Fragmentos dos promotores, correspondendo a 1000bp a montante do codão ATG de cada gene, foram usados como sequências "isca". Como "presas", usamos dois factores de transcrição da subfamilia EREBP, previamente reportados com capacidade de se ligarem ao promotor do gene *OsRMC*. Contudo, não conseguimos detectar qualquer interacção entre os dois TFs analisados e as sequências "isca" dos genes *JAZ*. Como trabalho futuro, estas estirpes

criadas irão ser examinadas por Y1H com bibliotecas de cDNA induzidas por sal ou ácido jasmónico.

Este trabalho tem como principais realizações a produção de plantas transgénicas de Arabidopsis sobrexpressando o gene *OsRMC*, as quais foram caracterizadas nos níveis de expressão do transgene e da proteína. Estas linhas serão usadas em estudos futuros de modo a monitorizar alterações de expressão de genes que respondem ao ácido jasmónico, na determinação da localização sub-celular da proteína OsRMC-RFP e na caracterização fenotípica induzida por ácido jasmónico.

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General Introduction

General Introduction

Plant abiotic and biotic stress responses

Terrestrial plants, as sessile organisms, are constantly exposed to a variety of environmental challenges. These varying conditions can cause stress in plants reducing its fitness. In fact, in most crops, actual yields are only 20% of attainable yields and a dominant factor limiting yield is abiotic stress such as excess or deficient water, high or low temperature, and high salinity (Boyer, 1982). Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels (Cramer *et al.*, 2011). Plant responses to abiotic stress are dynamic and complex; they are both elastic (reversible) and plastic (irreversible) (Cramer *et al.*, 2011). To survive these challenges, plants have developed elaborate mechanisms to perceive external signals and to manifest adaptive responses with proper physiological changes, at cellular and molecular levels. At the molecular level, the perception of environmental stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades (Hashimoto *et al.*, 2004). Understanding the mechanisms by which plants perceive and transduce these stress signals to initiate adaptive responses is essential for engineering stress-tolerant crop plants (Xiong *et al.*, 2001). A large number of abiotic stress responsive genes have been reported in a variety of plants including rice and Arabidopsis. These genes induced during stress conditions may function not only in the protection of cells from stress by production of important metabolic proteins, but also in the regulation of genes, including transcription factors (TFs), for signal transduction in the stress responses. These TFs regulate expression of multiple downstream target genes under stress conditions (Todaka *et al.*, 2012). These regulatory systems are achieved through specific cis-elements in the promoter regions of target genes, which are termed 'regulons'. Several regulons involved in abiotic stress responses have been identified in Arabidopsis (Todaka *et al.*, 2012).

Plant hormones constitute a battery of regulators involved in control of developmental, physiological and metabolic processes and are also likely to mediate the responses of plants to environmental stresses (Rup Kumar Kar, 2011). Beyond their role as developmental keys in a plant's life, the action of several plant hormones is also crucial

in responses to biotic and abiotic challenges. The five classical plant hormones are auxins, cytokinins, gibberellins, abscisic acid and ethylene, but other important group of plant hormones are also involved in responses to abiotic and biotic stress and these include the well characterized group designated as jasmonates.

Hormones are important regulators of plant responses to abiotic stress, with the two most important being abscisic acid (ABA) and ethylene. The essential components of ABA signaling have been identified, and their mode of action was clarified (Cramer *et al.*, 2011). Plants consistently face challenges from a wide array of biotic stresses, including insect herbivory, and fungal, bacterial and viral attacks (Dreher *et al.*, 2007). One area in which the details of the defense responses are becoming more evident is in the plant's production of pathogen-related (PR) proteins. PR proteins have been defined as plant proteins that are induced during pathogen infection or wounding. This term describes the collective induction of numerous plant genes that are induced in a variety of species in response to pathological attacks (Hashimoto et al., 2004). The major families of PR proteins have been grouped at least in 14 different classes, primarily on the basis of their amino acid sequence identity (Van Loon *et al.*, 1999). The PR10 class of proteins, first identified as a major pollen allergen (Bet v1) from white birch (Breiteneder *et al.*, 1989), are induced by pathogen attack in a wide variety of plant species, including parsley (Somssich *et al.*, 1986), potato (Matton and Brisson, 1989), pea (Barratt and Clark, 1991), soybean (Crowell *et al.*, 1992), asparagus (Warner *et al.*, 1993), sorghum (Lo *et al.*, 1999) and rice (Midoh and Iwata, 1996, McGee *et al.*, 2001). The biotic and abiotic stress-inducible nature of the PR10 genes has been investigated including pathogen infection (Midoh and Iwata 1996, McGee *et al*,. 2001, Tanaka *et al.*, 2003), salt tolerance (Moons *et al.*, 1997), UV irradiation (Rakwal *et al.*, 1999), and ozone stress (Agrawal *et al.*, 2002).

Jasmonates

Among plant hormones, jasmonates (JA) are one of the best characterized and studied. The signalling pathway of JA is involved in the modulation of either biotic (Kloek *et al.*, 2001) or abiotic (Sugano *et al.*, 2003) stress although most of the reports implicate this hormone in the organization of the plant defense mechanism. Nevertheless, the signalling pathway of JA has been well characterized in the recent years

especially in dicots like *Arabidopsis* and tomato (Chini *et al.*, 2007 and 2009, Staswick, 2008, Thines *et al.*, 2007) but not in monocots and in particular rice.

Jasmonates (JAs) are fatty acid derivates that act as signaling molecules. These signaling molecules share notable structural and functional properties with prostaglandins found in animals. They are mainly derived from linolenic acid and function in normal developmental pathways but also play a crucial role in allowing plants to mount a defense to biotic challenges. JAs affect different processes such as pollen development and fruit ripening, and as such, they modulate vital physiological processes such as flower development, and fertility. Jasmonates are essential to complete the last steps of stamen development, specifically in three processes: (a) pollen maturation after the trinucleated stage to guarantee viability and fertility; (b) elongation of stamen filaments so that the anthers can reach the stigma in preparation for pollination; and (c) dehiscence of anther locules for pollen release (Acosta and Farmer, 2010). Impairments of these functions in jasmonate synthesis or perception mutants results in male sterility. Jasmonates also control seed size although the specific tissue types affected in seeds are not yet identified (Farmer and Dubugnon, 2009). Jasmonates also modulate petal size and affects vein structure. They also promote resistance to insects and pathogens, and in fact, in Arabidopsis it is estimated that 67 to 85% of genes activated by wound or insects are regulated, in terms of expression, by jasmonates (Acosta and Farmer, 2010). The genes activated in a wound response, for example, in leaf damage, fall into a series of categories including jasmonate synthesis, jasmonate signaling components, defense genes, defensins, resource allocation genes, general stress response genes, etc. The cyclopentanones JA and methyl-jasmonate (MeJA) are produced via the octadecanoid pathway, JA being the key terminal product of this pathway. The octadecanoid pathway has been extensively studied in *Arabidopsis thaliana* and tomato, but not as well in rice.

The JAs for which there is most evidence for biological activity are: (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.*, 2009), which is the ligand that seems to play the major role in jasmonate signaling in Arabidopsis leaves and possibly flowers; Jasmonoyl-L-tryptophan, as an auxin signaling inhibitor active in Arabidopsis roots (Staswick, 2009); and OPDA and dinor-OPDA, which have signaling properties either independent of canonical jasmonate signaling (Stintzi *et al.*, 2001; Taki *et al.*, 2005) or using part of the jasmonate signaling machinery (Ribot *et al.*, 2008).

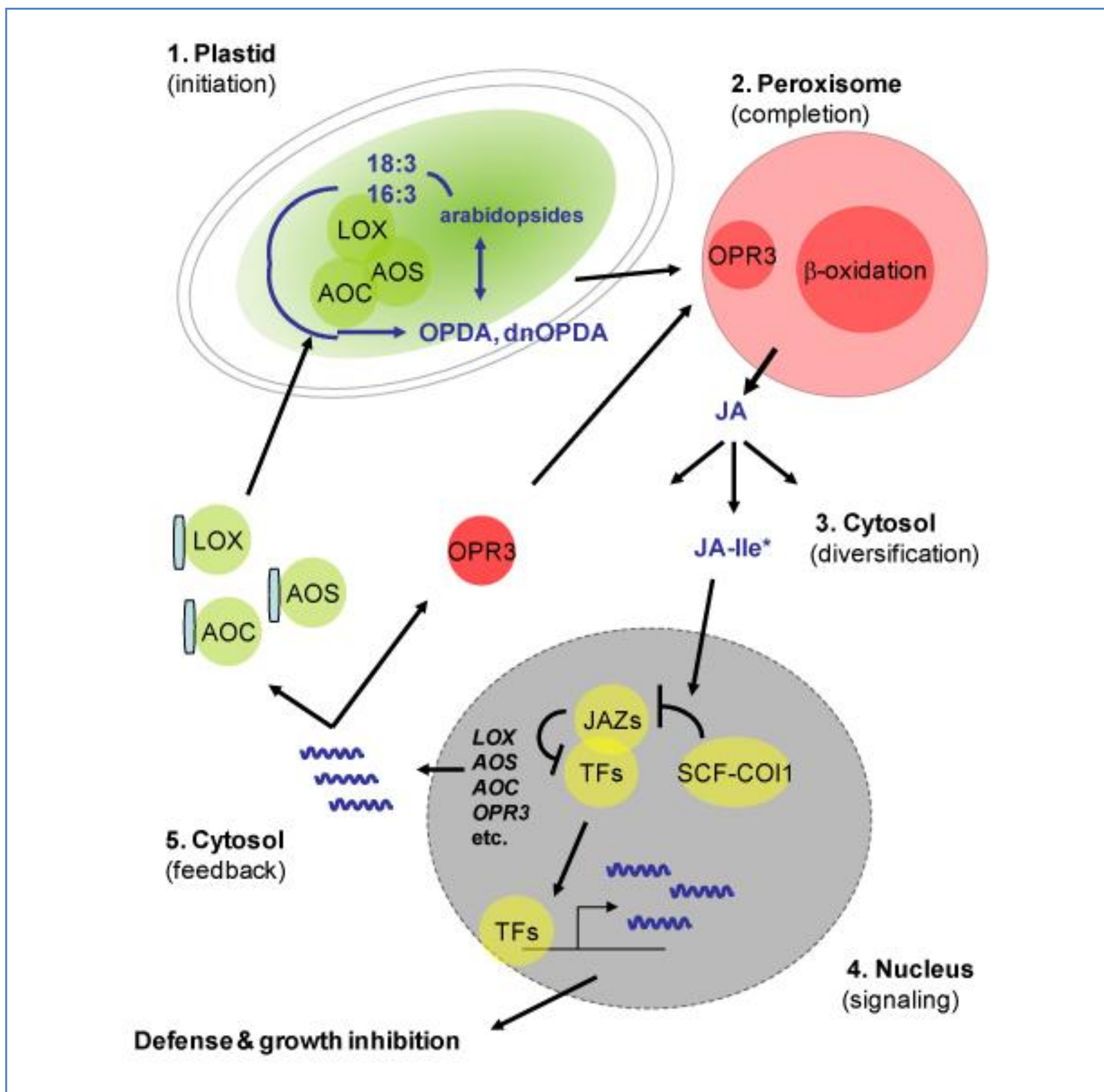
The analysis of mutants, mainly from *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) that are deficient in jasmonate biosynthesis or jasmonate signaling has been extremely useful for dissecting jasmonate function (Wasternack, 2010). Among these mutants, the Arabidopsis mutant *coi1-1*, which was already isolated in 1994, has been exceptional, because it is deficient in nearly all jasmonate responses. Jasmonate research has come full circle by the recent discovery that COI1 protein is a jasmonate receptor. Following the perception of JA a number of cellular signaling processes occur that result in the posttranslational modification of upstream regulatory proteins, transcriptional activation of JA-responsive transcription factors and downstream genes.

The first step in the JA signaling takes place in the nucleus, where JA-Ile binds to its receptor, the CORONATINE INSENSITIVE 1 (COI1) protein which forms part of an SCF ubiquitin E3 ligase (see Scheme 1). SCF complexes are a type of E3 ubiquitin ligases, multisubunit machines that specify and mediate protein ubiquitination for targeted degradation by the 26S proteosome. SCF ubiquitin E3-ligases contain a variable F-box protein that directly binds the targets of the ubiquitination complex thereby conferring its specificity. The F-box protein COI1 defines an SCF complex that is specifically involved in jasmonate signaling (Santner *et al.*, 2007). The other components of the SCF-COI1 complex are: either ASK1 or ASK2, which serve as adaptors for COI1 to form the substrate-recognition subunit; RBX1, a RING finger protein that recruits an E2 ubiquitin conjugating enzyme to bring it close to the substrate; and the scaffolding CULLIN1 protein that holds the substrate-recognition portion of the complex at its N-terminus and RBX1 at its C-terminus. RBX1 and CULLIN1 are necessary for normal jasmonate signaling and so are other compounds known to interact with or regulate SCF complexes such as AXR1, the COP9 signalosome and SGT1b. The main targets of this SCF-COI1 complex are the Jasmonate ZIM-domain (JAZ) repressors proteins which are ubiquitinated. The destruction of JAZ proteins by the 26S proteosome releases transcription factors, and allows gene expression JA-dependent (Browse, 2009; Chung *et al.*, 2009; Fonseca *et al.*, 2009[a]). JAZ proteins lack recognizable DNA-binding domains, however, their interaction with the transcription factor MYC2 suggests that JAZ proteins control jasmonate related gene expression by preventing the function of transcriptional activators, but the exact mechanism is still not known. MYC2 is a basic helix-loop-helix (bHLH) transcription factor that activates a first wave of gene transcription upon jasmonate perception, including the JAZ genes and the putative jasmonate biosynthesis gene LOX3 (Chini *et al.*, 2007; Pauwels *et al.*, 2008). In Arabidopsis, JAZ proteins are

encoded by 12 different genes (JAZ1 through JAZ12), which are predicted to produce over 20 protein variants, including two characterized alternative splice products, JAZ10.3 and JAZ10.4 (Staswick, 2008). Most *JAZ* genes are rapidly induced by MYC2 upon activation of jasmonate signaling. In general, the promoters of *JAZ* genes contain putative MYC2 binding motifs that have been proven functional for JAZ3.

Among JAZ proteins, there are two conserved features which are the Jas motif and the ZIM domain. The Jas motif is towards the C-terminus and constitutes the binding site of COI1 in the presence of JA-Ile; therefore, the Jas motif is required for timely JAZ protein degradation upon jasmonate perception. This motif is also necessary and sufficient for the interactions of JAZ proteins with MYC2 (Chini *et al.*, 2009). This motif might also be important for nuclear localization of JAZ proteins, even if its absence does not necessarily prevents their entry into the nucleus. The ZIM domain is located in the center of the JAZ proteins and contains a conserved TIFY motif (TIFYXG). This domain mediates homo- and heteromeric interactions between many JAZ proteins, a capability that is critical for their function as negative regulators of jasmonate signaling.

As for any other hormone, jasmonate produces strong effects in cells. It is important then to control hormone signaling after the initial response. In the case of jasmonates, one way to accomplish this is to metabolize the bioactive forms of the hormone to make them inactive. The fact that *JAZ* gene transcription is rapidly induced upon jasmonate signaling suggests a negative feedback loop control mechanism, whereby newly made JAZ proteins would repress again their corresponding transcription factors. Cells may rely on alternative splice variants of JAZ proteins, such as JAZ10.3 and JAZ10.4, which lack the Jas motif (Yan *et al.*, 2007; Chung and Howe, 2009). In consequence, they are not recognized efficiently by COI1 and are not subject to effective jasmonate-induced proteosome degradation. Rapid accumulation of these variants is predicted to desensitize cells to the presence of the hormone (Acosta and Farmer, 2010).



Scheme 1 - An overview of cellular compartments in JA synthesis and signaling (from Acosta and Farmer, 2010)

Thesis objectives

The main goal of this work is to understand how a rice gene, *OsRMC* (*ROOT MEANDER CURLING*), may influence the JA-signaling pathway in *Arabidopsis* by overexpressing it and monitoring gene expression changes of JA-responsive genes either in wild-type (WT) and in transgenic cell-suspension cultures and plants.

This rice gene was shown to be involved in the modulation of abiotic stress (salinity stress; Zhang *et al.*, 2009) and root curling (Jiang *et al.*, 2007) mediated by JA in rice. According to homology analysis, OsRMC belongs to the cysteine-rich-repeat receptor-

like protein kinase subfamily (CRK, also known as DUF26 - domain unknown function 26 - subfamily receptor-like kinase) but it has no transmembrane domain or kinase domain.

Previously studies developed in the thesis host-lab, have putatively linked the function of this gene in root mechanosensing in rice through a negative regulation of the JA-signalling pathway (Lourenço, T. *et al.*, unpublished results). The *OsRMC* gene expression is also up-regulated in a rice RNA interference (RNAi) line that silences the expression of an ubiquitin E3-ligase, OsHOS1 (Lourenço, T. et al. unpublished results), which suggest that its expression can be modulated through the action of the proteasome (Lourenço, T. et al. unpublished results).

The *OsRMC* gene does not have any known homologue in *Arabidopsis*. Therefore, the use of a model-plant such as *Arabidopsis*, with a well-characterized JA-signaling pathway, seemed appropriate to study the function of *OsRMC* to complement other studies being developed in the host-lab.

To further complement the work developed, we also decided to perform a Yeast-One hybrid assay using the promoter region of three rice *JAZ* genes. These genes have been shown to be up-regulated in the RNAi::OsHOS1 line. Therefore, and since *JAZ* genes are well known negative regulators of the JA-signaling pathway, we thought it could be interesting to test the binding of two EREBP TFs, previously identified as binding to the *OsRMC* promoter region (Serra, T. et al., unpublished results).

As main outputs of this work, we were able to generate three independent *Arabidopsis* transgenic lines overexpressing the *OsRMC* gene. These three lines were characterized in transgene expression (RT-PCR) and protein expression (Western blot). Due to the fusion construct between OsRMC and a fluorescent tag (Red Fluorescent Protein; RFP), we were also able to visualize the sub-cellular localization of OsRMC in *Arabidopsis* roots. We were also able to generate stable transgenic *Arabidopsis* cell-culture lines overexpressing *OsRMC*. However, this approach failed to deliver consistent results regarding JA-responsive genes expression changes. As for the Yeast-One hybrid approach, we were able to produce yeast bait-strains for the three rice *JAZ* genes selected and test the binding of the two EREBP TFs.

The results of this work and future perspectives will be discussed in the next chapters.

CHAPTER ONE: Preparation and analysis of *Arabidopsis thaliana* cell suspension cultures and whole plants overexpressing *OsRMC*

Introduction

The main goal of this work was to overexpress the rice *OsRMC* gene both in *Arabidopsis* cell suspension cultures and in whole plants. *OsRMC* gene has been described in rice as being negatively involved in root curling (Jiang *et al.*, 2007) and salinity tolerance (Zhang *et al.*, 2008). This gene has also been showed to be involved in JA-mediated root curling in rice (Jiang *et al.*, 2007)) but the mechanism is still unclear. Since *Arabidopsis* does not have a known homologue for *OsRMC*, we considered this model suitable for this work. Ultimately, our goal was to study the JA responses using two different approaches overexpressing *OsRMC*: 1) using *Arabidopsis* cell suspension cultures to rapidly observe JA-dependent gene expression changes in JA-marker genes such as *VEGETATIVE STORAGE PROTEIN* (*VSP*, Benedetti *et al.*, 1995) and *PLANT DEFENSIN1.2* (*PDF1.2*, Penninckx *et al.*, 1996), and a gene involved in JA-signaling, *MYC2*, overcoming the tissue culture step and 2) using whole *Arabidopsis* plants to observe JA-dependent phenotypic changes. In addition, we also wanted to clarify the subcellular localization of *OsRMC* since the two reports describing work with this gene had contradictory results, placing this protein either in the plasma membrane or the apoplast (Jiang *et al.*, 2007; Zhang *et al.*, 2008).

In this chapter, all the work done with *Arabidopsis thaliana* cell suspension cultures and whole plants is presented. We were able to establish transformed cell suspension cultures and attempted to use them to analyze gene expression of JA-responsive genes. The *Arabidopsis* cell suspension cultures were also used to produce protoplasts and these were also subject to an assay involving JA. We were also able to produce three independent transgenic *Arabidopsis* plants which were characterized for their transgene expression and corresponding protein level. The identification of the sub-cellular localization of OsRMC-RFP protein was also attempted in the transgenic plants.

JA-marker genes for expression analysis

Vegetative storage proteins (VSP) serve as proteinaceous storage reserves. These protein have been identified in numerous plants, such as soybean (*Glycine max*; Wittenbach, 1983), potato (*Solanum tuberosum*; Mignery et al., 1984, 1988), sweet potato (*Ipomoea batatas*; Maeshima et al., 1985), white clover (*Trifolium repens*; Goulas *et al.*, 2003), alfalfa (*Medicago sativa*; Meuriot *et al.*, 2004b), and in the bark of deciduous trees such as poplar (*Populus deltoides*; Coleman *et al.*, 1991) and elderberry (*Sambucus nigra*; Van Damme *et al.*, 1997). Arabidopsis VSP transcripts are induced by mechanical wounding, jasmonic acid (JA), insect herbivory, and osmotic and nutritional stresses (Liu *et al.*, 2005).

Plant defensins may be highly expressed or upregulated in plants challenged by various abiotic and biotic stresses. For example PDF1.2 is highly and constitutively expressed in the halophyte salt cress *Thellungiella halophila* (Taji *et al.*, 2004). A defensin-like protein from wheat was also found to be induced during cold acclimatization (Koike *et al.*, 2002). Induction of gene expression of plant defensins by drought or salt stresses has also been reported (Yamada *et al.*, 1997; Maitra and Cushman, 1998) but their potential roles in abiotic stress tolerance are not known.

MYC2, a basic helix-loop-helix (bHLH) domain-containing transcription factor, participates in the jasmonate (JA) signaling pathway and is involved in the modulation of diverse JA functions. However, a comprehensive list of MYC2-dependent JA-responsive proteins has yet to be defined (Guo *et al.*, 2012). Nonetheless, results have been published that support a positive role for MYC2 in regulating JA-mediated carbohydrate metabolism and oxidative stress tolerance.

Biological material

Arabidopsis thaliana

The virtues of *Arabidopsis thaliana* as an experimental model plant for genetic, biochemical, and molecular biological studies have been extensively reviewed *Arabidopsis thaliana* is a small dicotyledonous species, member of the mustard (Brassicaceae) family, which includes economically important plants such as cabbage,

turnip, broccoli, radish and canola. Although this species is not of major agronomic importance, the important advantages it presents for basic research in genetics and molecular biology makes it an important model plant worldwide, and as a fact, it was the first one to be established as such. The advantages this species brings to basic research are: (1) it has a small genome, which has already been sequenced and annotated; (2) there are extensive genetic and physical maps of all its 5 chromosomes; (3) it has a short life cycle, of approximately about 6 weeks from germination to seed maturation; (4) the plant is of small size and easily cultivated; (5) it produces numerous self progeny; (6) its genome is efficiently manipulated by genetic engineering; (7) there are already a large number of mutant lines and genomic resources available; and additionally (8) it has been elected as a flowering plant model for a long time now, leading to an endless collection of studies and information gathered for more than 40 years.

Cell suspension cultures

Suspension culture of isolated plant cells is a valuable tool for providing the material for high-throughput studies such as metabolic analyses, production of secondary plant products, and herbicide discovery (Boisson *et al.*, 2012). Also, plant cell suspension cultures are useful tools for investigations of physiological phenomena such as cell proliferation and differentiation. In such experimental systems, the environment should be completely controlled and the population of target cells should, if possible, be homogenous (Fukuda *et al.*, 1994). Although *Arabidopsis thaliana* represents a model plant, it remains somewhat recalcitrant in tissue culture (Gleddie, 1989). In addition to providing cells in quantity for biochemical and molecular analysis, it may be possible to use Arabidopsis cell suspensions for protoplast isolation, regeneration and fusion experiments since plant cell cultures are excellent sources of protoplasts (Xuan and Menczel, 1980). Techniques have been developed for establishing and maintaining cell suspensions of Arabidopsis cells in order to have efficient regeneration and transformation protocols available (Gleddie, 1989).

Transformation techniques

The Floral Dip Method

Whole plants were transformed with the floral dip method (Clough and Bent, 1998). The Arabidopsis floral dip transformation is notable for a number of reasons. First, it is strikingly easy to perform, no plant tissue culture is required and the procedure can be performed by nonspecialists. Generally, success rates are high: it is common that 1% of the progeny seedlings are transgenic. In Arabidopsis floral dip transformation, essentially all T1 transformants are the product of germline transformation events, meaning that the plants are uniformly transgenic in all cells. It is also intriguing that bacteria other than *Agrobacterium* have recently been used for floral dip transformation. Many different *Agrobacterium tumefaciens* strain backgrounds have been used successfully in the floral dip method. GV3101 is the most commonly used and the most suited for *Arabidopsis* transformation. LBA4404 is also commonly used but generally works less well than GV3101, and this aspect of LBA4404 can be exploited to reduce the proportion of transformants that carry more than one T-DNA insertion.

Transformation of cell suspension cultures

Reports of transformation of *Arabidopsis* cell suspension cultures date back to 1997, where a stably transformed cell suspension culture was obtained overexpressing the firefly luciferase gene and that could be readily used for protoplasting, PEG-mediated transformation and transient expression studies (Forreiter *et al.*, 1997)

Material and Methods

Genetic Construct

A genetic construct was made in order to overexpress *OsRMC* in plants and cell suspension cultures. This genetic construct was made using a GATEWAY (Life Technologies, USA)-based vector (pB7RWG2.0; VIB, University of Gent, Belgium) (Fig. 1) that allows our protein of interest to be fused in frame with the Red Fluorescence Protein (RFP) tag in the C-terminal and it has the Glufosinate-ammonium gene that confers tolerance to BASTA (Sigma-Aldrich, Germany) as the plant selective marker. The generated genetic construct was designated as pB7::OsRMC-RFP (courtesy of Tânia Serra, GPlantS). Also, the promoter present in this vector is the p35S promoter from the Cauliflower mosaic virus, which permits constitutive expression of the OsRMC-RFP transcriptional fusion.

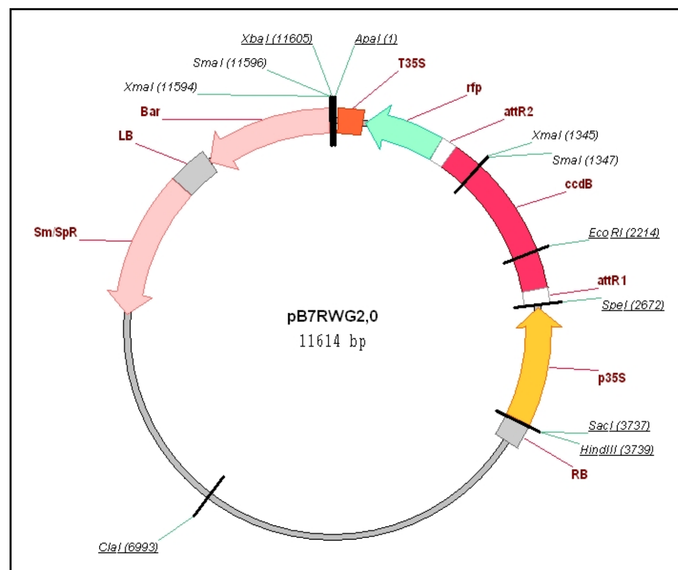


Figure 1 – GATEWAY based vector used in the preparation of the genetic contruct

Arabidopsis cell suspension cultures

Arabidopsis thaliana cell suspension cultures (kindly provided by Prof. Laszlo Bogre, Royal Holloway, University of London, England), were used in all experiments as

genetic background. These cell suspension cultures were diluted on a weekly basis with fresh Arabidopsis Medium (see appendix for composition), in a laminar air flow chamber (Braun Horizontal BBH6). In brief, 10mL of a one week-old culture was diluted in 40mL of fresh Arabidopsis medium in a sterilized 250mL flask.

Arabidopsis plant material

Three week old *Arabidopsis thaliana* plants grown in soil (Shamrock Professional Range Specialist Pot Plant Medium, Ireland), ecotype Columbia-0 (Col-0), were used in all transformations with the floral dip method (modified from Clough and Bent, 1998). A total of two sets of transformations were performed with the floral dip method.

Agrobacterium tumefaciens transformation

Agrobacterium, strain LBA4404, was transformed with the vector pB7::OsRMC-RFP using the following protocol: 0.1-1μg of plasmid was added to frozen competent cells and the mixture was incubated for 5 minutes at 37ºC. The cells were then incubated on ice for 30 minutes and plated on LB solid medium (see appendix for composition) with the antibiotics Rifampicin and Spectinomicin at concentrations 25mg/L and 100mg/L respectively. The plates were grown on a chamber for 2-3 days at 28ºC. The colonies grown in this plate were re-streaked in a fresh LB solid medium with the same selection pressure as above. To further confirm the *Agrobacterium* transformation, we performed a colony-PCR to the re-streaked colonies with the appropriate primers to amplify *OsRMC*.

Agrobacterium tumefaciens growth

First an *Agrobacterium* 5mL pre-culture was made, inoculating a single colony into 5mL of LB medium supplemented with 25mg/mL of Rifampicin and 100mg/mL of Spectinomicin. The culture was grown at 28ºC with 220 rpm. From the pre-culture, a larger culture was established transferring 1-5mL of the pre-culture to 200 mL of LB

medium supplemented with the antibiotics Rifampicin and Spectinomicin at the same concentrations as above. The larger culture wan grown overnight at 28ºC with 220 rpm.

Arabidopsis thaliana transformation – Floral Dip Method

The *Agrobacterium* cultures were grown until an OD_{600nm} of ~1 was achieved. At this point, the bacteria were spinned down gently by centrifuging at 4.000xg for 10 minutes at room temperature. The pellet was ressuspended in a same volume of a freshly made solution composed of 5% sucrose, 10nM $MgCl_2$ and 0.05% Silwet L-77. The ressuspended solution was transferred to a 5L plastic beaker for the dipping phase of the transformation method. Above-ground parts of *Arabidopsis thaliana* plants were dipped in the *Agrobacterium* solutions with gentle agitation. The pots were labeled and the plants were wrapped in Saran Wrap, and laid on their sides overnight. The next day, the Saran Wrap was removed and plants were placed straight up and under standard growth conditions. In one of the transformations the whole dipping procedure was repeated 5 days after the first dipping. The plants received no more water when the siliques became mature, and the seeds were harvested.

Seed sterilization

Seeds were surface sterilized with a solution containing equal parts of double-distilled water and commercial bleach along with Tween-20 0.1% for 10 minutes at room temperature in an eppendorf tubes rotator. The next steps were performed in a horizontal laminar air flow chamber (Braun Horizontal BBH6). The seeds were washed with sterile double-distilled water for at least 7 times at room temperature. The seeds were then left in the last wash water and were stored in the dark at 4ºC for 2-3 days for stratification. After stratification, the selection of transformed seeds was made by plating them on MS Medium (see appendix for composition) supplemented with BASTA at a concentration of 20ug/L. BASTA-resistant seedlings would became visible after a week of growth at 22ºC with a 16h photoperiod.

Plant genotyping

Quick Plant Extract buffer was used to extract DNA from leaf of transformed *Arabidopsis* T0 or T1 plants. The following protocol was used: first 20μL of Quick Extract Plant Buffer (Epicenter Biotechnologies, USA) was added to more or less 1 mm^2 of leaf tissue and the mixture was left to incubate for 6 minutes at 65ºC; next, the mixture was transferred to a new bath at 98ºC and let to incubate for 2 more minutes. The DNA extracted was used to perform PCR for the confirmation of insertion of the transgene. A total of 2μL of DNA extracted with the protocol mentioned above was used to perform the PCR.

Arabidopsis cell culture transformation

The cell suspension cultures were transformed using the protocol as follow: 100 μL of *Agrobacterium tumefaciens* LBA4404 grown to an optical density (OD_{600}) of 0.8, and carrying either the empty vector pB7RWG2.0, or the pB7::RMC-RFP, were added to 3 to 4 days old cell suspension cultures. After adding the *Agrobacterium* the cell suspension cultures were co-cultured for 3 days at 25ºC with agitation at 120 rpm. After the incubation with the *Agrobacterium* and on a weekly basis, the cultures were diluted and treated with Cefotaxime and BASTA at 500mg/L and 20μg/mL respectively. The concentration of Cefotaxime was gradually decreased in steps of 100mg/L each week, until 100mg/L was achieved. This antibiotic was suspended and no longer added to the cultures in the later stages after confirmation of Agrobacterium-free cultures.

Jasmonic acid assays

The wild type and transformed cell suspension cultures were used to perform assays with jasmonic-acid (JA). In these assays, 495μL of the 3-4 days old cultures were treated with 5μL of JA at concentrations ranging from 50μM to 500μM. The culture was left to incubate with the JA in dark conditions. The total volume of the treated culture were collected at different time points of 1h, 2.5h, 5h and 7h and centrifuged. The

supernatant was discarded and the cellular pellet was snap-frozen in liquid nitrogen and stored at -80ºC until use.

RNA extraction

For all total RNA extraction procedures the following protocol was used. This TRIzol RNA extraction Protocol uses TRIzol (Life Technologies, USA) as the main reagent.

The following protocol for total RNA extraction was used:

1 mL of TRIzol was added to 100 mg of grinded frozen samples immediately vortexed to allow the tissue to thaw in the TRIzol reagent. Then, the homogenate was passed over a qiashredder (Qiagen, USA) column by centrifuging at max speed for 30 seconds. The cleared homogenate was then centrifuged at 10.000xg for 5 minutes at room temperature. The liquid was quickly transferred to a new 2mL tube and incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complexes. After this incubation step, 200µL of chloroform was added to the solution and this was vigorously shaken for 15 seconds, and incubated at room temperature for 3 minutes. The solution was then centrifuged for 10 minutes at full speed, at room temperature. After centrifugation, different phases could be identified. The RNA, present exclusively in the clear upper-phase of the solution, was transferred to a new 2 mL tube. Isopropanol (500µL) was added to the RNA solution and incubated for 10 minutes at room temperature. Then, the solution was centrifuged for 10 minutes at 10.000xg at room temperature for precipitation of the RNA. The supernatant was discarded and the RNA-pellet was washed with 1mL of a 75% EtOH solution. Another centrifugation step of 5 minutes at 7.500xg at room temperature was performed. After discarding the supernatant the RNA pellet was dried for 10 minutes in a laminar air flow chamber The RNA was ressuspended in RNase-free water (treated with DEPC).

DNase treatment procedures (TURBO DNA-free Life Technologies, USA)

First the DNase digestion reagents were added to the RNA samples: typically 0,1X volume of TURBO DNase Buffer and 0.75 µL of TURBO DNase, and the mixture was gently mixed. This mixture was incubated for 30 minutes at 37ºC. After the 30 minute incubation, 0.75 µL of TURBO DNase was again added to the RNA samples, and incubated for another 30 more minutes at 37ºC. After the second incubation: DNase Inactivation Reagent (typically 0.1 volumes) was added to the samples and mixed well. This mixture was incubated for 5 minutes at room temperature, mixing occasionally. After this incubation, the samples were centrifuged at 10.000xg for 1.5 minutes and after the RNA samples were transferred to a fresh tube. After this treatment to remove any contaminating DNA, all RNA samples were checked for their quality through gel electrophoresis and followed by approximate quantification using NanoDrop (Nanodrop 3330, ThermoScientific, USA)

cDNA synthesis

All cDNA was synthesized using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Switzerland). The procedure to obtain cDNA from the RNA samples was carried exactly in the same way for all samples, and as described below.

Five hundred nanograms of total were used to produce cDNA. The first-strand using oligo-dT as primer. This mixture was incubated for 10 minutes at 65ºC in the thermocycler. After the ten minutes incubation a mixture composed of 4µL of buffer, 0.5µL of RNase inhibitor, 2µL of dNTPs, 1µL of DTT and 1.1µL of retrotranscriptase was added to each RNA sample. This mixture was then put in the thermocycler for the actual synthesis of the cDNA. The program steps for the cDNA synthesis can be viewed in the appendix:

Protein extraction

We tested two protocols [Laccus Buffer and Protein Extraction Buffer (PEB) protocol] for protein extraction of the cell suspension cultures and the PEB protocol for the whole plants

The first protein extraction protocol was done with the Laccus Buffer (see appendix for composition). Briefly, 50µL of Laccus Buffer was added to each sample, and then centrifuged for 20 minutes at 10.000xg. The supernatant was passed to a new tube and stored at -20ºC until use.

The PEB protocol (see appendix for composition) allowed for a Bradford protein quantification assay and was used to extract total protein from transformed *Arabidopsis* seedlings. The following protocol for the total protein extraction with PEB was used: 10 days after germination seedlings were grinded in liquid nitrogen in a mortar until a fine powder was obtained. For each 100 mg of grinded material 200µL of PEB with 2x complete protease inhibitor was added and mixed well. The mixture was centrifuged at maximum speed at 4ºC for 15 minutes. The supernatant was transferred to a new 1.5mL tube and stored at -20ºC until use.

Protein quantification

Protein quantification was done using the Bradford reagent (Bio-rad, Germany). A calibration curve was made with BSA incubating the Bradford reagent for 30 minutes in BSA concentrations of 2.5, 1, 0.5, 0.25 and 0.1mg/mL. All samples were read on a plate reader with the Gen5 software, measuring absorbance at 595nm.

Western blot

Twenty micrograms of each sample total protein, mixed with Loading Buffer, were loaded per lane on a 12% SDS-PAGE and gels were blotted into PVDF membranes. OsRMC-RFP detection was carried out using a 1:1000 dilution (0.2µg/mL) of a polyclonal α-GFP antibody (200µg/mL Santa Cruz Biotechnology; USA) raised against rabbit (1:20000 dilution (0.52µg/mL), 1mg/mL from Abm Inc; Canada). Chemioluminescent

detection was performed with Western Lightning Plus-ECL (Perkin-Helmer, USA), according to manufacturer's instructions. Coomassie Brilliant Blue staining was as total protein loading control.

Protoplast preparation

Protoplasts were prepared from wild type *Arabidopsis* cell suspension cultures, and from the transformed *Arabidopsis* cell suspension culture with the empty vector pB7RWG2.0 and the vector carrying the genetic construct pB7RWG::OsRMC-RFP. The following protocol was used: 3-4 days old *Arabidopsis* cell suspension cultures were collected by centrifuging (in a swing out rotor) in a 50 mL Falcon tube for 5 minutes at 1500rpm. After, the supernatant was discarded by decanting it. The cells were then mixed with 25mL of enzyme solution (see appendix for composition) and the falcon was filled up with B5-GM medium (see appendix for composition). The 50mL content was then split into two large Petri plates and to each of them another 25mL of B5-GM medium was added. The plates were then shaken carefully and slowly at 30-40 rpm/min for 3 hours in the dark. The protoplasts were checked in an inverted microscope to decide the time of harvest. When about 80% of the cells started to look spherical another 20-30 minutes were allowed for further shaking. After, the protoplasts were transferred to two 50mL falcon tubes. The protoplasts were then centrifuged for 5 minutes at 1500 rpm at room temperature. The supernatant was discarded by decanting it and the cellular pellet was ressuspended in 25mL of B5-GM medium. The cells were then centrifuged for 5 minutes at 1000rpm at room temperature. After, the supernatant was discarded and the cellular pellet was ressuspended in 5mL of B5 0.28M sucrose medium (see appendix for composition). The cells were transferred to 15mL falcon tubes and centrifuged for 7 minutes at 800rpm at room temperature. The floating cells were carefully transferred to a new 15mL falcon tube using a sterile plastic wide-mouth Pasteur pipette. The protoplasts were counted in an inverted microscope using a Fuchs-Rosenthal counting chamber and the concentration of protoplasts was calculated. A total of 3 different protoplasts preparation were prepared in this way, one for the wild-type culture, and one for each transformed culture. These protoplasts preparation were used to view in an inverted microscope to check for RFP localization. Also, the protoplasts were also used in a jasmonic acid assay.

Results

In this section of the chapter all results will be shown concerning the work done with *Arabidopsis* cell suspension cultures and whole plants.

Cell suspension cultures

Figure 2 - Transformed Arabidopsis cell suspension cultures; the two flasks on the left correspond to the pB7RWG:: OsRMC-RFP cultures and the two on the right to the pB7RWG2.0 cultures

The transformed (confirmed by PCR analysis in to the culture overexpressing *OsRMC* using specific primers; data not shown) *Arabidopsis thaliana* cell suspension cultures were used in a JA assay and were also used to produce protoplasts. For accurate measure, each transformed culture was kept in duplicate. These transformed cultures were used in only one JA assay, the first ones being performed with wild type cultures. The reason to start the JA assays only with the wild type cultures comes from the fact that the transformed cultures were still in the process of homogenization through rounds of dilution and treatment with BASTA, and because the JA assay still needed optimization in terms of concentration of JA to be used and times of exposure. These transformed cultures were very prone to contamination, especially the pB7RWG:: OsRMC-RFP cultures. Figure 2 shows one week old transformed cell suspension cultures,

where it can be seen that the growth of the cultures was not exactly the same for all cultures.

Gene expression analysis under JA

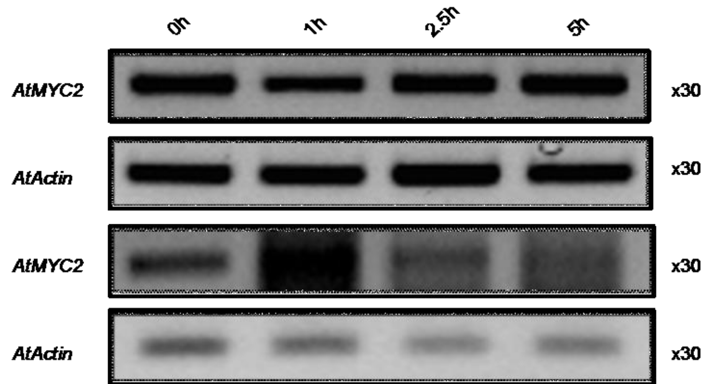


Figure 3- Transcript accumulation from 0 hours to 5 hours of AtMYC2 and AtACTIN as control; the top two panels refer to the first Jasmonic acid assay conducted with wild type Arabidopsis cell suspension cultures; the bottom two panels refer to a repetition of the first assay; treatment - 50µM JA

Figure 3 shows results for the RT-PCRs obtained with cDNA from wild type *Arabidopsis* cell suspension cultures. Results for the gene *MYC2* are shown at time points of 0, 1, 2.5 and 5 hours of treatment with 100µM of JA. In this assay RT-PCRs for the genes *PDF1.2* and VSP were also performed. In the RT-PCRs mentioned above no bands were seen in the gels and so no results are shown for the RT-PCRs for these genes. The RT-PCRs for the first assay showed that the gene *MYC2* seems to be consistently transcribed along the 5 hours of treatment, with the same happening for the control gene *Actin*. This assay was repeated because no results were obtained for the genes *PDF1.2* and *VSP*. The bottom two panels refer to the assay repetition. In this assay still no bands were visible for the genes *PDF1.2* and *VSP* and also the results for the gene *MYC2* seem consistent with the first assay, although in this case the cDNA appeared contaminated with genomic DNA.

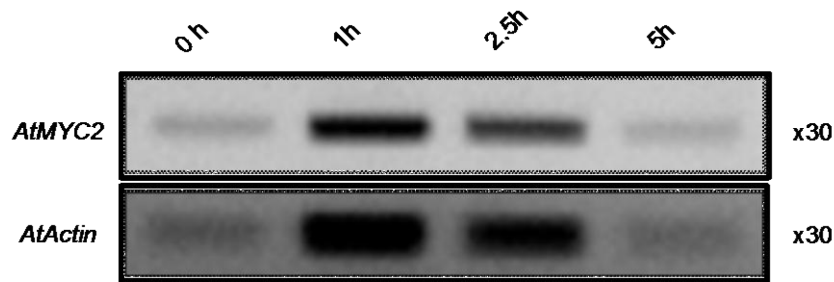


Figure 4 - Transcript accumulation from 0 hours to 5 hours of AtMYC2 and AtACTIN as control; the top two squares refer to the first repetition of the Jasmonic acid assay conducted with wild type Arabidopsis cell suspension cultures; treatment - 50µM JA

A third JA assay with wild type cultures was conducted, but in this case the treatment consisted of 100µM of JA. Still the RT-PCRs for the genes *PDF1.2* and *VSP* resulted in no bands and the results for the *MYC2* gene seem to be inconsistent with the first two assays, although in this case the gene *Actin*, which served as a endogenous control, did not show consistent accumulation of transcript at the different time points. This can be due to the fact that the concentration of cDNA for all time points was not homogenous.

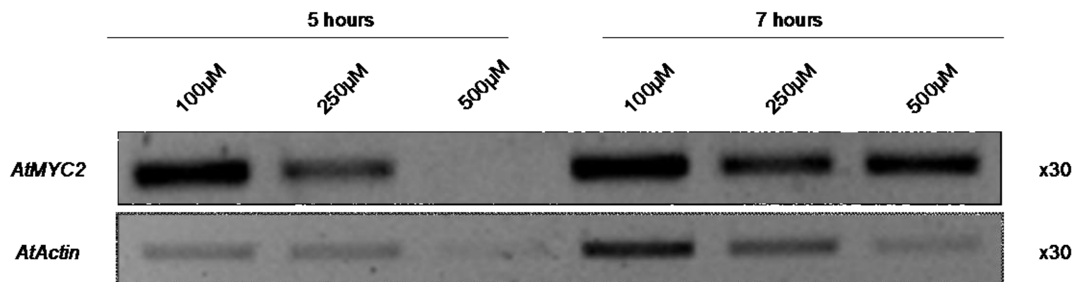


Figure 5 - semi-quantitative RT-PCR results for the gene *AtMYC2*; the concentrations refer to the JA used; the gene *AtActin* was used as a control

A fourth JA assay with wild type cell suspension cultures was performed. In this assay only time points of 5 and 7 hours were gathered but a range of concentrations of JA was used in this assay (see figure 5). Still no results for the genes *PDF1.2* and *VSP* are shown because no bands were visible in the gels of all the RT-PCRs performed with primers for the genes mentioned above. As for the gene *MYC2,* there seems to be no consistent accumulation with respect to the treatment involved, either for the hours or

the concentration of JA used. The gene *Actin* still showed no consistent accumulation in the different treatments, and so this gene was discarded as a potential housekeeping gene to serve as control and another gene was selected, namely *Tubulin*.

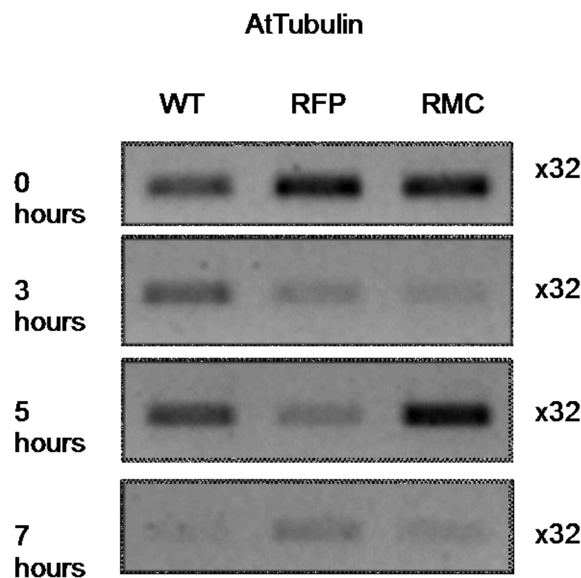


Figure 6 – semi-quantitative RT-PCR results for the gene AtTubulin

In the final JA assay, RNA was extracted from the protoplasts produced from all the transformed and wild type cell suspension cultures and cDNA was produced. This gene was also not a good candidate because it showed different accumulation in the different treatments. RT-PCRs for all the JA-responsive genes analyzed in this work were also performed but showed no convincing results, and so no results are shown for this final assay.

Protoplasts

Protoplasts were produced from the transformed *Arabidopsis thaliana* cell suspension cultures. These protoplasts were used in a JA assay but were also used to visualize the RFP protein in an inverted fluorescent microscope (Leica, Germany). The protoplasts of the pB7RWG2.0 culture confirmed that the RFP protein was being expressed (Fig. 7). No images for the protoplasts of the pB7RWG::OsRMC-RFP culture is

shown because there was no signal detected in the protoplasts of the mentioned culture. This negative result could be an indication of the sub-cellular localization of OsRMC.

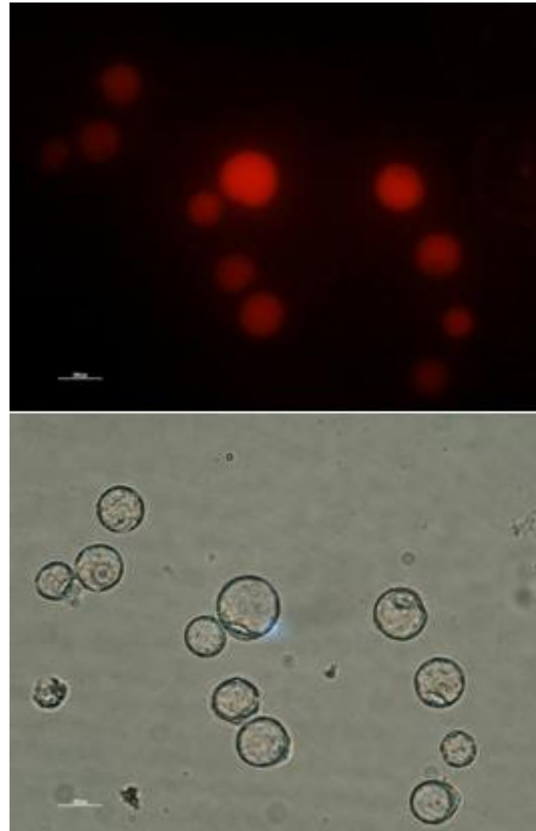


Figure 7 - Protoplasts of pB7RWG2.0 culture. In these photos it is clear that the RFP gene is being transcribed and translated into the RFP protein. The image below is the bright field of the upper image; the upper image was gathered with a fluorescence inverted microscope and with the filter TRITC

Arabidopsis plants

Arabidopsis thaliana Col-0 plants were used for the transformation with the floral dip method. In figure 8 (right panel) the plants are in their right stage to perform the floral dip.

Figure 8 - Pots where (left) the Arabidopsis Col-0 plants were grown and (right) at the stage of development to perform the floral dip method

In the first transformation with the floral dip method only one transformant was obtained and was designed line RMC#1. The seedling resistant to BASTA was transferred to soil (see figure 9) to produce more seeds.

Figure 9 - Transgenic line RMC#1 plant overexpressing the OsRMC gene

In order to access if line RMC#1 was truly transformed with the *OsRMC* gene DNA was extracted from the plant and a simple PCR was performed with primers for the *OsRMC* gene. In figure 10 it is shown that the DNA extracted from line RMC#1 showed some amplification for the gene.

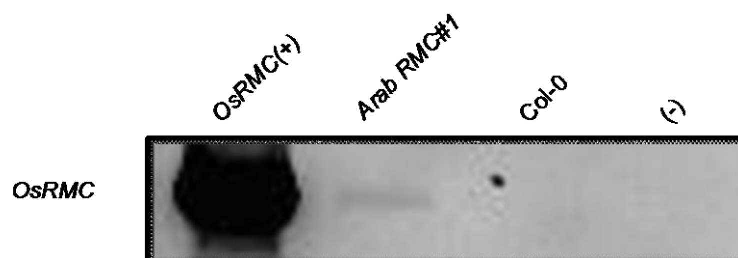


Figure 10 - Genotyping of line RMC#1. Negative controls correspond to amplification from *Arabidopsis* col-0 genomic DNA; positive control (pB7RWG::OsRMC-RFP) demonstrated over amplification

With the second floral dip method transformation two more lines overexpressing the *OsRMC* gene were obtained and these lines were designated line RMC#2 and RMC#3. Figure 6 shows the results for the segregation analysis for the mentioned lines. The main objective of this segregation analysis was to access if any of the sublines was homozygous for the gene. It is shown that all seeds plated in MS medium with BASTA for the lines RMC#2.5 and RMC#3.1 germinated and grow, demonstrating that these sublines are homozygous for the gene. In this segregation analysis the seeds were also plated in non-selective medium (MS medium without BASTA) to access possible problems with germination of the seeds.

In the second floral dip method transformation also two positive seedlings (resistant to BASTA) were gathered for the empty vector (pB7RWG2.0). These seedlings were also transferred to soil to produce more seed. The plants obtained from these seedlings produced very few seeds and only one of these seeds germinated again under selective medium (see figure 11 bottom). This seedling was not transferred to soil because it showed abnormal phenotype in the plate. This abnormal phenotype was mainly characterized by very short roots and may be due to the local of insertion of the construction in the plant genome.

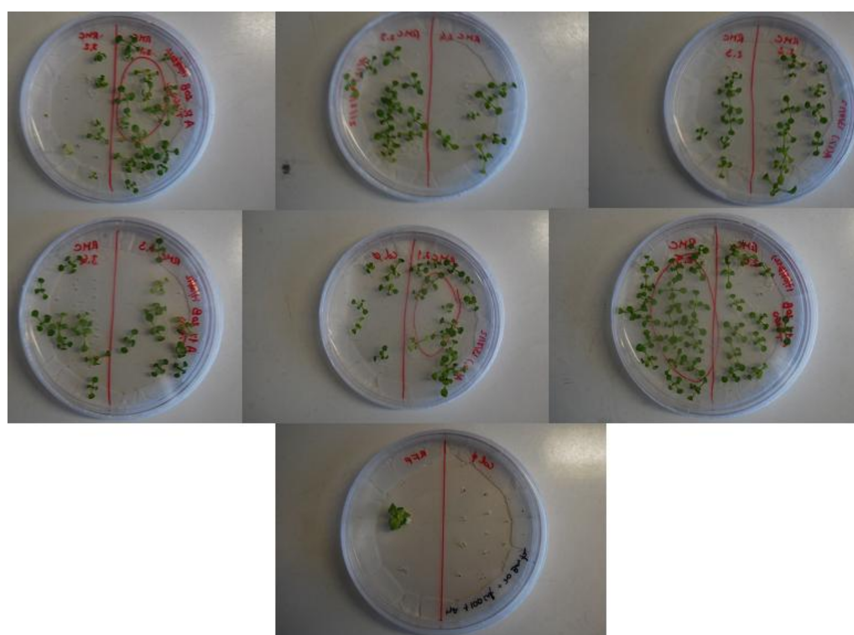


Figure 11 - Segregation analysis of lines RMC#2 and RMC#3

RNA was extracted from all the three obtained lines, RMC#1 and homozygous lines RMC#2.5 and RMC#3.1 and cDNA was produced from the RNA of these lines. The figure 12 shows results for the RT-PCR from the three lines, with Actin as an endogenous control and cDNA from Col-O plants as a negative control. The figure 12 shows that the three lines transcribe the gene *OsRMC* although the accumulation of the transcript is clearly different in the three lines, with line RMC#2.5 being the one that shows higher accumulation, and line RMC#1 showing very little accumulation. The fact that line RMC#1 shows very little accumulation of the transcript may be due to the fact that this line is an heterozygous line.

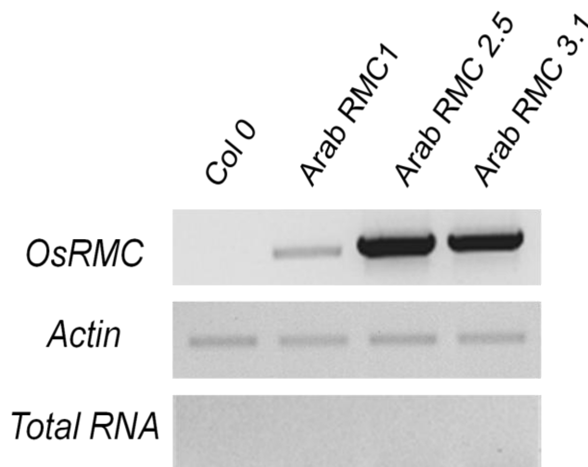


Figure 12 - Analysis of transcription of *OsRMC* in Col-0, RMC#1 (heterozygous) and lines RMC#2.5 and RMC#3.1 (homozygous)

Western Blots

A western blot was also conducted with protein extracts from the transgenic lines and Col-0 in order to access if the gene was being transduced. The *OsRMC* gene was transcriptionally fused with the tag RFP in this construction, and so, the detection of the *OsRMC* gene was done with an antibody to detect the tag. Although no antibody was available in the lab specifically for the RFP gene, there was one available for the GFP gene, and since these two proteins (RFP and GFP) share considerable similarity, and

since the antibody used in this assay was a polyclonal one, some results could still be obtained.

Figure 13 shows bands below the 63kDa band in the protein extracts from lines RMC#2.5 and RMC#3.1 that were absent for the protein extracted from Col-O plants, showing that the fused protein RMC-RFP could still be detected in this western blot. Although we could not detect any band for the RMC#1 line this could be due to low *OsRMC* gene expression observed in the RT-PCR analysis (Fig. 12)

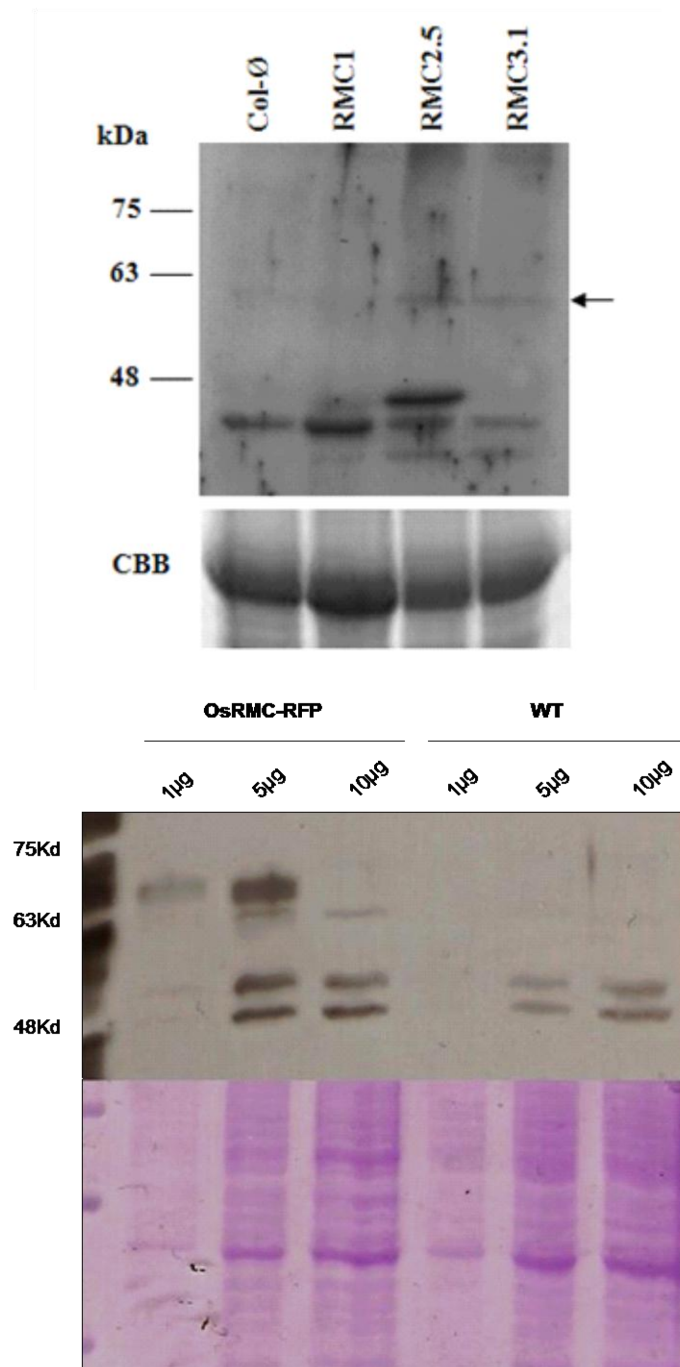


Figure 13 - Western Blot with anti-GFP polyclonal antibody to detect RFP; protein was extracted from transformed *Arabidopsis* line RMC#1, RMC#2.5, RMC#3.1 and Col-0. . The arrow on the right of the panel shows the bands corresponding to the OsRMC-RFP fusion protein (~57-60kDa). Coomassie Brilliant Blue (CBB) staining was used as loading control.

Discussion

Arabidopsis cell suspension cultures

The Jasmonic acid assays were repeated a total of three times due to lack of consistent results in the analysis of gene expression of Jasmonic acid responsive genes. These assays still needed to be optimized in order to obtain good quality cDNA for the RT-PCRs. Although much optimization will be needed in these assays in order to do a gene expression analysis much of the optimization does not concern, in my opinion, the way the assay was performed, but rather the quality of the material used in the assay.

The lack of consistent results in this part of the work may be explained by several reasons. First the transformed cell suspension cultures were not homogenously transformed, despite several weeks growing in selective medium with BASTA. The *Arabidopsis* cell suspension cultures were co-cultured with the *Agrobacterium* for 3 days, and despite this method proved successful in obtaining good transformed cultures with *Arabidopsis* cell suspension cultures with other work done previously in the laboratory by other colleagues, it seemed that these cultures were not efficiently transformed/homogenous to proceed with the Jasmonic-acid assays and all the work done after obtaining the samples. We cannot also discard the hypothesis of the influence of the *OsRMC* gene in the transformed cell cultures, as these cultures would grow poorer and were prone to contaminate more easily. It is thus tempting to speculate that this gene may have an influence in the cellular response of *Arabidopsis* cell suspension cultures affecting the responses to biotic stress (which may be related to JA sensitivity)

Another reason for the lack of consistent results in this part of the work was the RNA extracted from the samples. The RNA extracted from all the time points, and even in the 0h time point, was of low quality and in low concentrations. The cDNA synthesized from this RNA suffered from the same problems and so the RT-PCRs

The RT-PCRs for the genes *VSP* and *PDF1.2* did not show any transcript accumulation in the gel. Only the gene *AtMYC2* showed bands, which showed show very little differences in terms of accumulation. There seems to be a decrease in accumulation

at one hour in this gene. As for the gene *AtActin*, it showed in these RT-PCRs to be stable even at 5 hours of treatment. So, since the genes *VSP* and *PDF1.2* were not up-regulated under JA, the jasmonic acid assay was repeated with some modifications.

Nevertheless, and despite several attempts, we were not able to have a suitable system to analyze the gene expression changes under JA in cell suspension cultures. This may be due to the lack of homogeneous cultures when we overexpress the *OsRMC* gene in this biological system.

Sub-cellular localization of OsRMC

One of the objectives of this work was to access the localization of the OsRMC protein in the cell. Reports that this protein is localized in the plasma membrane are not in accordance with other reports that localized this protein in the apoplast. In one of them, the authors claim that OsRMC is localized in plasma membrane (Jiang *et al.*, 2007)), however in a second one the authors refer to OsRMC as an apoplastic protein (Zhang *et al.*, 2009). In this work the transformed *Arabidopsis thaliana* cell suspension cultures were visualized several times in the microscope. The localization of the RMC protein was tracked by the signal of the Red Fluorescent Protein (RFP) protein. In the first attempts to visualize the transformed cultures some difficulties were encountered in getting a clear picture of the cells. Normally the cell suspension in the slide tended to form clumps of cells, making the process of getting clear pictures of individualized cells somewhat difficult. In one attempt to visualize the cell suspension cultures the cells were incubated with pectin previously to the visualization, but this measure proved to be insufficient to provide clear pictures. Another difficulty in the process of localization of the OsRMC protein was due to the lack of signal provided by the tag (the RFP protein). The use of protoplasts from the transgenic cell suspension cultures proved useful to give a good indication regarding the sub-cellular localization of OsRMC protein. We were not able to detect any signal in the membrane of protoplasts isolated from the transgenic cell culture overexpressing the *OsRMC-RFP*. However, we could detect a strong signal inside the protoplasts (Fig. 7) isolated from the culture transformed with the empty vector (pB7RWG2.0). Altogether, our results regarding the localization of OsRMC may support the idea that OsRMC is in fact an apoplastic protein.

Arabidopsis plants

Two rounds of transformation with the floral dip method were made on two different sets of plants. In the first round of transformation two pots with more or less 5 *Arabidopsis* plants were transformed with the vector carrying the transgene and two pots were transformed with the empty vector. From this round of transformation only one transformant for the vector carrying the transgene OsRMC was obtained. One reason that might explain such a low efficiency of the method is that the plants used in this first transformation were not in their perfect conditions. The leaves of most of the plants were turning purple, denoting that the plants might have been suffering some kind of stress, and this is one of the factors that most influences the efficiency of the floral dip method.

The To transformant was grown in soil to produce seed, and from the To seeds, 12 positive lines, growing in selective medium were obtained. Seeds were obtained from these 12 lines (1.1 through 1.12), after the plants were grown in soil. The segregation analysis of the transgene was made with these 12 lines, all derived from line 1, in order to look for possible homozygous lines. Unfortunately, not a single line proved to be homozygous, according to the results of the segregation analysis. In this analysis, a lot of seeds didn't even germinate, and in all the lines at least two or three seeds failed to germinate and grow in selective medium. These results might be explained by the fact that the original insertion site of the transgene in line 1 was not suitable to obtain homozygous lines.

The second round of transformation using the floral dip method was done on much more healthier plants, in comparison with the plants used in the first transformation. In this second round the floral dip method was repeated twice on the same plants in order to improve the efficiency of the method and to obtain more transformants. Two more transformants for the vector carrying the transgene were obtained with this set of transformations, and two transformants for the empty vector were also obtained. These two lines were also grown on soil to produce seeds for the segregation analysis of the transgene. These last two lines produced very few seeds, and so a few were selected to grow in soil to produce more seeds. The segregation analysis proved that at least one homozygous T1 plant was obtained for the lines 2 and 3.

We could also make the molecular characterization of the 3 transgenic lines as compared to Col-0. All the transgenic plants showed *OsRMC* transgene expression with different levels. This may be due of the localization of the insertion of the T-DNA in the plant genome. We could also detect a correlation between the transgene expression and the level of protein (Fig. 13) in the different transgenic lines.

Altogether, our results show that the cell suspension culture is not a good system to test JA-induced gene expression, at least when overexpressing *OsRMC*, and future work has to be carried out in the transgenic *Arabidopsis* plants developed and characterized.

CHAPTER TWO: Yeast-One-Hybrid direct screening of JAZ-like promoter fragments with two EREBP transcription factors

Introduction

The One-hybrid (Y1H) screening in yeast is a powerful method to rapidly identify heterologous transcription factors that can interact with a specific regulatory DNA sequence of interest (the bait sequence). This method is essentially derived from the two-hybrid concept. In the two-hybrid concept the interaction between two proteins (bait and prey) is detected via *in vivo* reconstitution of a transcriptional activator that turns on expression of a reporter gene. In the one-hybrid system, detection is based on the interaction of a transcription factor (prey) with a bait DNA sequence upstream of a reporter gene (Ouwerkerk and Meijer, 2001). The bait sequence can be a well defined *cis*-acting element, but larger regulatory sequences can also be used.

In order to complement the work presented in this thesis related to the influence of *OsRMC* in the modulation of JA signaling we developed a Y1H direct screening taking into consideration some important factors. It was known from previous work in the host laboratory that an RNA interference (RNAi) rice line for the gene *OsHOS1* had increased transcript levels of *OsRMC* as compared to wild-type plants (Lourenço *et al.*, unpublished results). Moreover, three selected JAZ-like rice genes (known repressors of the JA signaling) also showed up-regulation in the RNAi-OsHOS1 line (Lourenço *et al.*, unpublished results). These three genes were denoted as JAZ1-like (LOC_Os03g08320), JAZ2-like (LOC_Os03g28940) and JAZ3-like (LOC_Os10g25290) and were previously reported as highly responsive to JA (Yoshii *et al.*, 2010).

Having this background knowledge in mind, it was tempting to speculate that two transcription factors from the EREBP subfamily (AP2 family), isolated from a cDNA salt induced library in a Y1H screening using the promoter of *OsRMC* as bait (Serra et al. unpublished results) could also bind to the promoter of the JAZ-like genes. The TFs from this subfamily are known to be involved in response to several kind of stresses, including abiotic and biotic (Santos *et al.*, 2011).

To further complement our hypothesis, the analysis of these JAZ-like genes promoters showed that they possess GCC-like motifs in their sequences that might act as *cis*-elements for recruiting transcription factors from the EREBP subfamily. Therefore, and to test out hypothesis, we decided to use the promoter region of the three JAZ-like genes (retrieved from the Rice Genome Annotation Project;

http://rice.plantbiology.msu.edu) as bait for a direct-Y1H screening using the two EREBP TFs mentioned above, as prey.

JAZ genes / JAZ proteins

JAZ proteins belong to a larger group of so-called ZIM-domain proteins, named after the putative transcription factor Zinc-finger inflorescence meristem (ZIM). ZIM family members were assigned to this group based on a conserved 28 amino acid ZIM motif, even though most do not have the GATA Zn-finger DNA binding region found in the original ZIM protein (Staswick, 2008). This inconsistency recently led to the renaming of the family to TIFY, after a conserved sequence in many ZIM motifs. In addition to ZIM, other JAZ family members also have the Jas motif of 26 amino acids. In *Arabidopsis thaliana* twelve proteins were tentatively assigned to the JAZ family based on features of the Jas motif, although other TIFY members also contain a closely related region (Staswick, 2008). Apart from these two conserved regions JAZ proteins diverge considerably, ranging from 131 to 352 amino acids in length, and the relative position of ZIM and Jas varies. Other plants besides *Arabidopsis* also contain JAZ-like proteins, but they are apparently not found in other organisms.

An attempt to find homologs of JAZ proteins in *Oryza sativa* has been made and led to the finding of twelve members based on BLAST search in the rice genome RAP database, specifically searching the consensus sequence motif of the ZIM domain, including TIF[Y/F]XG (Seo *et al.*, 2011).

Material and Methods

Preparation of bait sequences

Primer design

First, primers were designed in order to amplify the bait promoter sequences. Each primer was designed with left and right adaptors corresponding to restriction sites for restriction enzymes to be used for cloning the promoter fragments in the pINTHIS3NB vector, the vector which will be used to transform yeast in order to obtain the bait strains. The designing of the primers with the left and right adaptors took into account the possibility of the promoter fragments having restriction sites for the restriction enzymes to be used for cloning the promoter fragment in the pINTHIS3NB vector. For this a number of useful websites such as http://tools.neb.com/NEBcutter2/ were used.

The promoter sequence to be analyzed corresponds to 1000bp upstream of the ATG codon of each of the three *JAZ*-like genes. This search for the upstream sequence of the ATG codon was done with a BLAST search in www.gramene.org. The promoters sequence did not serve as a full bait sequence; instead it was cut into two fragments with overlapping regions of 50bp (see Fig 1). The promoter fragments were designated as JZx.y with x corresponding to the number of the gene and y corresponding to the first or second 500bp upstream of the ATG codon. So in total six promoter fragments were amplified for this assay, designated JZ1.1, JZ1.2, JZ2.1, JZ2.2, JZ3.1 and JZ3.2. The right adaptors for all six primers to amplify the six fragments were all for the restriction enzyme *SpeI*. As for the left adaptors, the primers to amplify JZ1.1 and JZ1.2 had the restriction site for *PspOMI*, and the primers to amplify the fragments for JAZ-like 2 and JAZ-like 3 had the restriction site for the restriction enzyme *NotI*. All primers were designed with Primer3 (version 0.4.0) (Table 1).

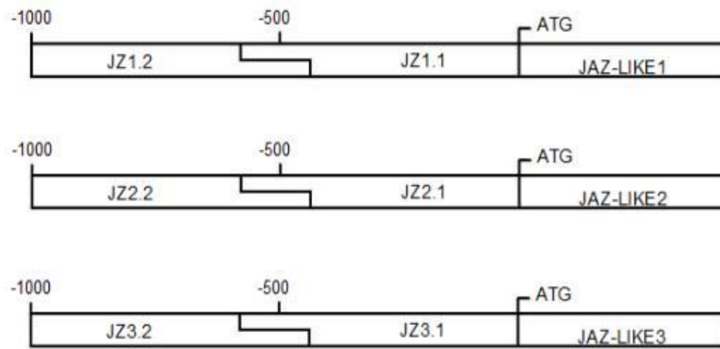


Figure 1 - Picture showing the overlapping fragments to serve as bait sequences in the yeast one hybrid assay

Table 1 - Specific primer sequences for all three JAZ-like genes along with the sequences for the adaptors, preferential annealing temperature and expected amplicon size.

Primer name	Primer sequence (5'-3')	Annealing temperature (ºC)	Amplicon size
JZ1.1FW	GGGCCCATTTAGCAGGCTTACTATTATATTTGC	57.21	493
JZ1.1RV	ACTAGTGTTGTAGCTCTAGCAAAATCAGC	57.58	
JZ1.2FW	GGGCCCTTCCGCTTGTAAAGTGCTAGA	57.88	489
JZ1.2RV	ACTAGTGAATGGGGCAAATGACAAC	58.76	
JZ2.1FW	GCGGCCGCCCAGATTGGTAGGTAGGATTGG	59.72	478
JZ2.1RV	ACTAGTCTTCTTCTTCTTCTGCCCACA	59.60	
JZ2.2FW	GCGGCCGCCAAACTTCCATATTGTGTAAGGAG	57.01	446
JZ2.2RV	ACTAGTGGGCCCTTCGTCCTAAAAT	60.27	
JZ3.1FW	GCGGCCGCACAGATGGCGTATACACATAAAGTA	57.03	505
JZ3.1RV	ACTAGTGTCCACCTCCACCACCTC	58.81	
JZ3.2FW	GCGGCCGCTTGCATTCCGAATTCTAACC	57.70	465
JZ3.2RV	ACTAGTATAGGATTGCTAGGGCACGA	59.69	

Amplification and sequencing

The promoter fragments were amplified with the PhusionTaq (Finnzymes, Finland) from genomic DNA of *Oryza sativa* from Nipponbare cv. This enzyme is was chosen to perform the PCR reactions instead of a regular Taq polymerase because it offers an extreme performance with accuracy and speed compared to a regular Taq, thus reducing the risks of errors in the amplification of the promoter fragments. The PCR reactions were performed using the following reagents in the reaction mixture (Table 2). The PCR reactions were performed on a thermocycler (T300 Thermocycler, Biometra) with a pre-heated lid (at 99ºC) to reduce evaporation of the PCR solution. The initial denaturation step was performed at 98ºC for 30 seconds and each following cycle comprised the following conditions: a first denaturation step at 98ºC for 10 seconds, followed by an annealing step of 20 seconds at 58ºC and an extension step of 15 seconds at 72ºC.A final extension step of 5 minutes at 72ºC was performed and the reaction had a final cooling step until it reached 10ºC.

The PCR amplification products originated by PhusionTaq are blunted which is useful for the cloning step shown next.

Table 2 - Reaction mixture for the PCR reactions performed to amplify the promoter fragments

PCR MIX		
Component	20µL reaction	Final conc.
H_2O	add to 20µL	
5x Phusion HF Buffer	4µL	1x
10mM dNTPs	0.4µL	200µM each
Forward Primer	0.8µL	400µM
Reverse Primer	0.8µL	400µM
Template DNA	2µL	
Phusion DNA Polymerase	0.2µL	0.02U/µL

All PCR products were isolated with a High Pure PCR Product Purification kit from Roche. The purified PCR products of each of the PCR reactions were cloned in the

pJET1.2/blunt Cloning Vector (Fermentas, Thermo Fischer Scientific, USA) according to the protocol below (see Table 3).

Blunt-End Cloning Protocol

Incubate the ligation mixture at room temperature (22ºC) for 5 minutes

Use the ligation mixture directly for transformation

Table 3 - Ligation mixture for pJET blunt end cloning of the promoter fragments amplified with PhusionTaq

Ligation mixture		
Component	10µL reaction	Final conc.
H_2O (nuclease free)	to 10µL	
2x Reaction Buffer	5µL	1x
Purified PCR product	0,5µL	
pJET1.2/blunt Cloning Vector	0,5µL	
T4 DNA Ligase	0,5µL	

Transformation of TOP10 *E.coli* with pJET1.2/blunt Cloning vector

A total of six transformations were carried, one for each promoter fragment, using the following protocol. In brief, 2µL of plasmid was added to 50µL of TOP10 *E.coli* competent cells. This mixture was left in ice for 30 minutes and then quickly put on a thermo stated bath at 42ºC for 45 seconds after which the mixture was again quickly put on ice again for 2 minutes. Sterile LB medium was then added to the mixture in a volume 5x that of the cells. After, the cells were incubated shaking for 75 minutes at 37ºC and at 225rpm. Finally, the cells were plated on LB solid medium supplemented with 100mg/L of Ampicilin and left to incubate at 37ºC overnight.

Individual colonies were then sub-cultured in liquid LB medium- supplemented with the same selection pressure as above, for a further amplification of the plasmid. These subcultures were then used to extract the plasmid to send for sequencing. All plasmids were extracted with a MiniPreps kit, SP-PMN-250 EasySpin® (Citomed, Portugal) (for protocol see Appendix).). The purified plasmids were then sent for sequencing (Beckman-Coulter Genomics, UK). The plasmids with the confirmed sequence for the JAZ promoter fragments were used in the following cloning procedures.

Cloning of the bait sequence in the pINTHIS3NB vector

The sequenced promoter fragments in the pJET1.2/blunt plasmids were then cut with the restriction enzymes *NotI* and *SpeI* (for the fragments JZ2.1, JZ2.2, JZ3.1 and JZ3.2) or *PspOMI* and *SpeI* (for the fragments JZ1.1 and JZ1.2) present in the adaptors of the primers and ligated to the digested pINTHIS3NB plasmid with the same restriction enzymes. In this way, the bait sequence was cloned in the polylinker of pINTHIS3NB in order to make a transcriptional fusion with the auxotrophic marker present in this plasmid, *HIS3* (histidine). The plasmid resulting from this cloning was transformed in *E.coli* (DH5α) for amplification. The plasmid was extracted as before and digested with *SacI* and *NcoI* to isolate a fragment containing our promoter sequence and auxotrophic selective marker. This fragment was purified and used in the preparation of the yeast bait strain.

Preparation of bait strains

Growth and preparation of yeast cells for transformation (competent cells)

Yeast Y187 strain (see Appendix for genome) was transformed with a lithium acetate transformation protocol. First, 50mL cultures of Y187 yeast was grown overnight in YAPD medium (YPD medium supplemented with 20 mg/L adenine hemisulfate). The cultures were diluted in the following day to an OD_{600} of ~0.25 in liquid YAPD medium and grown for an additional 3 hours with shaking at 30ºC. The cells were then harvested in 50-mL centrifuge tubes by centrifugation in a swing-out tabletop centrifuge. The supernatant was discarded and the cells were ressuspended in 50mL sterile water by vigorous shaking. The centrifugation step was then repeated, as well as the ressuspension step with sterile water. Next, after one final centrifugation, cells were ressuspended in 1mL of 1xTE/1xlithium acetate. The yeast suspension was then transferred to a 1.5-mL microcentrifuge tube and centrifuged for 30 seconds at maximum

speed. The supernatant was discarded and the cells ressuspended in 250μL of 1xTE/1xlithium acetate.

Prior to every transformation, the required amount of YEASTMAKER carrier DNA (Clontech, USA) was denatured in a boiling bath for 10 minutes and then snap cooled on ice. For each transformation a mix composed of 2.5μL (25μg *per* reaction) of carrier DNA, 50μL of yeast suspension, 300μL of freshly prepared 40%PEG/1xTE/1xlithium acetate and ~500ng of the bait sequence fragment. The mixture was incubated at 30ºC for 30 minutes shaking at 180rpm and then transferred to a water bath at 42ºC for 15 minutes. Afterwards, the cells were harvested by centrifuging at max speed for 30 seconds and ressuspended in 1 mL of YAPD medium.

Then, the cells were allowed to recover, by incubating for 4 and half hours at 30ºC with shaking (180rpm) in 15 mL tubes. After the recuperation step the cells were transferred to 1.5 mL tubes and harvested by centrifuging at maximum speed for 30 seconds. The supernatant was discarded and the cells were ressuspended in 100μL of 1xTE buffer and then plated on YAPD-G418 (150mg/L) plates. The plates were incubated at 30ºC for 2-3 days until the first colonies appear. The colonies were picked to a fresh YAPD-G148 plate.

Titulation of bait strains

3-amino-1,2,4-triazole (3-AT) was used for titulation of bait strains concerning possible leaky expression of the *HIS3* gene. This leaky expression is due to unspecific binding of endogenous transcription factors that activate *HIS3* gene. The positive colonies were picked for each bait strain from YAPD-G418 plates and plated on CM medium (see Appendix for composition) supplemented with Methionine (M), Adenine (A), Tryptophan (T), Histidine (H) and Leucine (L) (20mg/L Methionine, 20mg/L Adenine, 20mg/L Tryptophan, 40mg/L Histidine, 30mg/L Leucine) MATHL, CM MATH and CM MAT plates with various concentrations of 3-AT. Concentrations of 5, 10, 15 and 25mM of 3-AT were used, and all transformed bait strains were plated on such plates. This way the concentration of 3-AT required to reduce leaky growth was determined for all bait strains.

Direct Screening

All bait strains were then transformed with the pACTII vector carrying the EREBP TFs either the F3-1 or the F3-61 (or the empty vector for internal control) to screen for possibly interaction with the JAZ promoter fragments. All preparations of yeast bait strains for transformation was as already described and transformations were done using the same lithium acetate method. Transformed bait strains were plated on CM MAT plates with the accordingly concentration of 3-AT for each strain.

Results

Primer testing

After the designing of the primers, they needed to be tested to check for the right amplification of the promoter fragments (Fig 2).

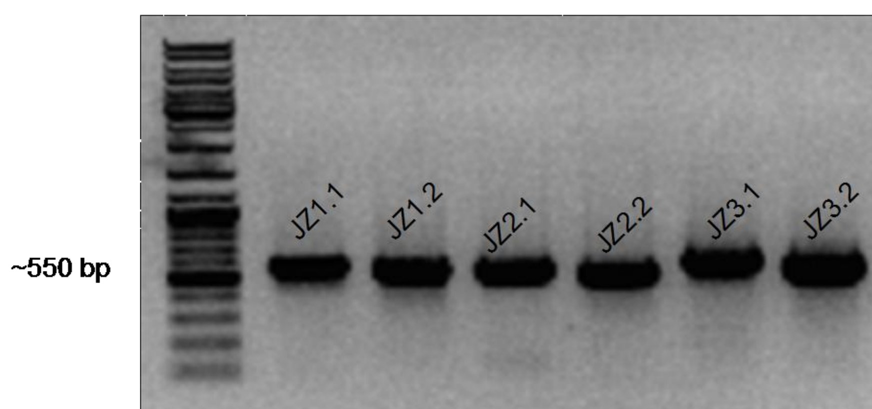


Figure 2 Results of a PCR to test all pairs or primers; amplicon size for all amplifications is in accordance with the sizes predicted for each amplification; annealing temperature of 54ºC

When the primers were tested with the PhusionTaq the 54ºC annealing temperature was no longer suitable, and so the primers were tested with different programs, namely, with different annealing temperatures (56º and 58ºC), and with different reaction mixtures, namely with and without DMSO. In final, all the primers proved successful at amplifying all the fragments at 58ºC without DMSO in the reaction mixture.

Titulation of bait strains

After the titulation process it was determined that the 5mM of 3-AT was sufficient to reduce growth for the bait strains JZ1.1, JZ1.2, JZ2.2 and JZ3.2 (Fig 3 for example of JZ1.1). For the bait strains JZ2.1 and JZ3.1 the titulation process was more

problematic. Bait strains JZ2.1 and JZ3.1 demonstrated growth even at high concentrations of 3-AT. Bait strain JZ3.1 demonstrated high growth in the absence of histidine demonstrating leaky expression of the *HIS3* gene (see figure 4.B). Bait strain JZ2.1 demonstrated complete growth in MAT plates with concentrations of 15 and 25mM of 3-AT.

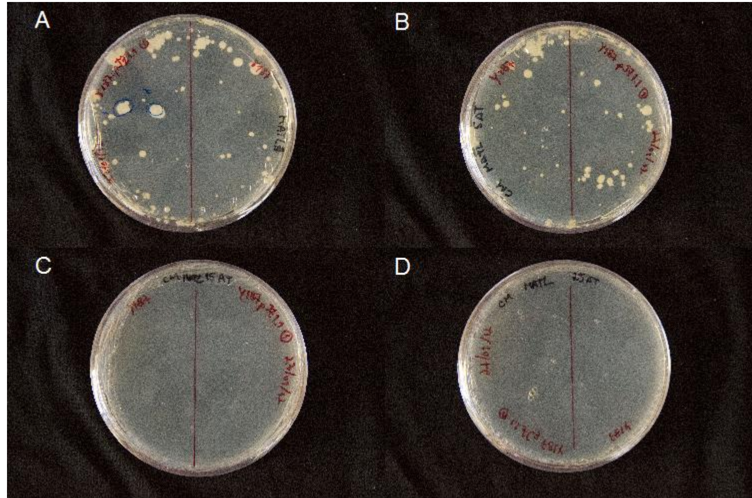


Figure 3 - Titulation for bait strain JZ1.1: (A) MATL plate; (B) 5mM of 3-AT; (C) 15mM of 3-AT; (D) 25mM of 3-AT

The titulation process for the bait strain JZ1.1 showed that concentrations of 15mM or higher were sufficient to repress all growth on plates in the absence of histidine (see Fig 3)

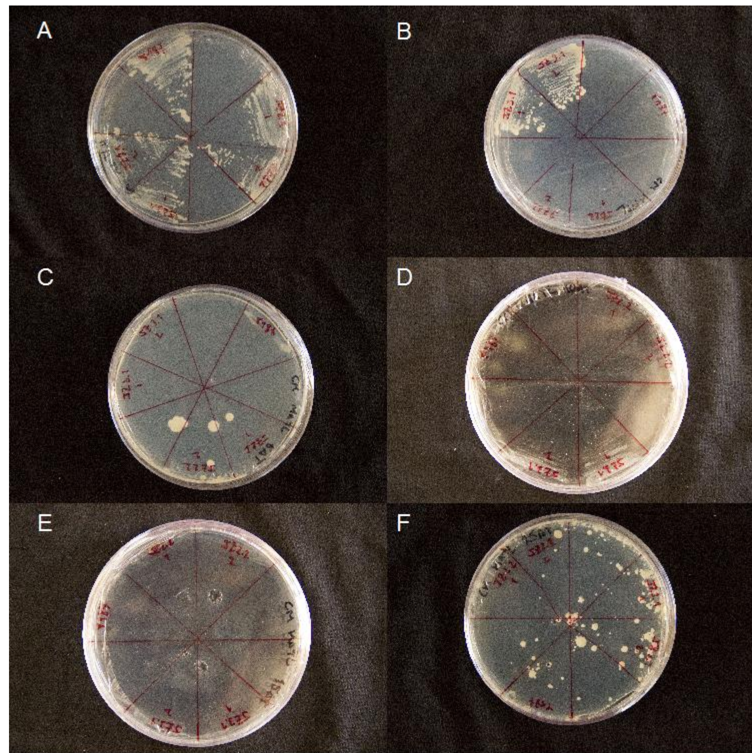


Figure 4 - Titulation for bait strains JZ2.2 and JZ3.1: (A) MATLH plate; (B) MATL plate (growth of bait strain JZ3.1); (C) 5mM of 3-AT; (D) 10mM of 3-AT; (E) 15mM of 3-AT; (F) 25mM of 3-AT

For bait strains JZ2.2 and JZ3.1 the titulation process revealed that bait strain JZ32.2 with 3-AT concentrations of 10mM or higher couldn't grow in the absence of histidine and that bait strain JZ3.1 could grow on the absence of histidine, even with 25mM of 3-AT in the medium (Fig 4).

Direct screening analysis

There was no interaction between TF F3-1 with each of the bait strains. The growth demonstrated by bait strains JZ2.1 and JZ3.1 on figure 4 are due to expression of the *HIS3* gene by other endogenous transcription factors present in the yeast. To corroborate this statement we can see that in figure 5C the empty vector also demonstrated the same growth as the transformed bait JZ3.1. For the bait strain JZ2.1 the same was verified, with the bait strain transformed with the empty vector pACTII also growing in MAT plates with high concentrations of 3-AT.

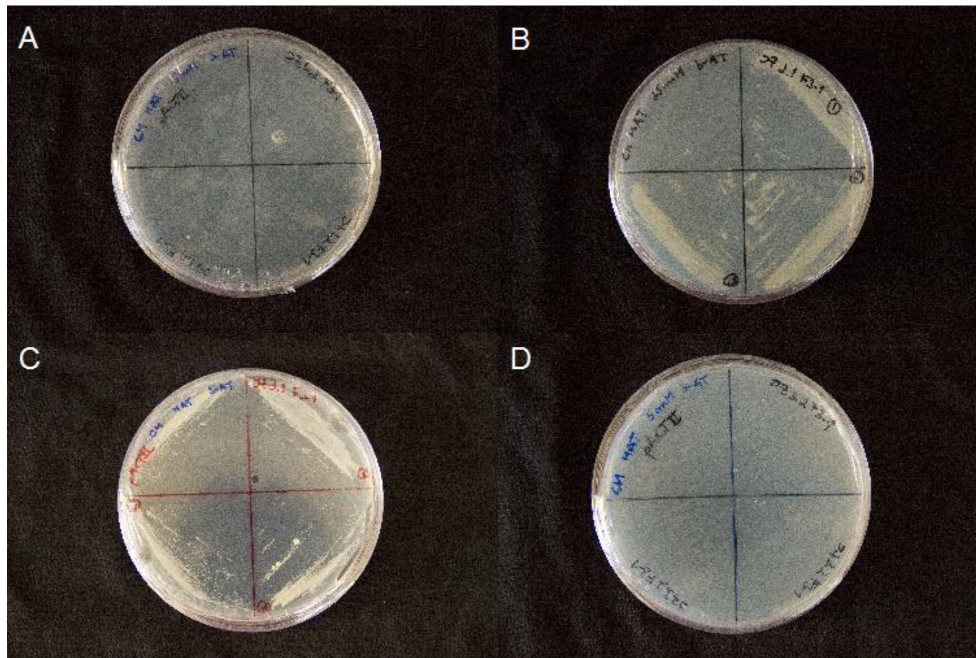


Figure 5 - Direct screening analysis with the transcription factor F3-1: (A) JZ1.2 with 5mM of 3-AT; (B) JZ2.1 with 25mM of 3-AT; (C) JZ3.1 with 5mM of 3-AT; (D) JZ3.2 with 5mM of 3-AT

The results for the transcription factor F3-61 (figure 6) are the same for the transcription factor F3-1. The bait strains JZ2.1 and JZ3.1 also showed growth in MAT plates with high concentration of 3-AT reinforcing the fact that this growth is caused by unspecific activation of the *HIS3* gene by endogenous factors. Also the nature of the sequences for these two bait strains mentioned above plays a role in the behavior of the bait strains, these two probably being more prone to interact with yeast endogenous factors.

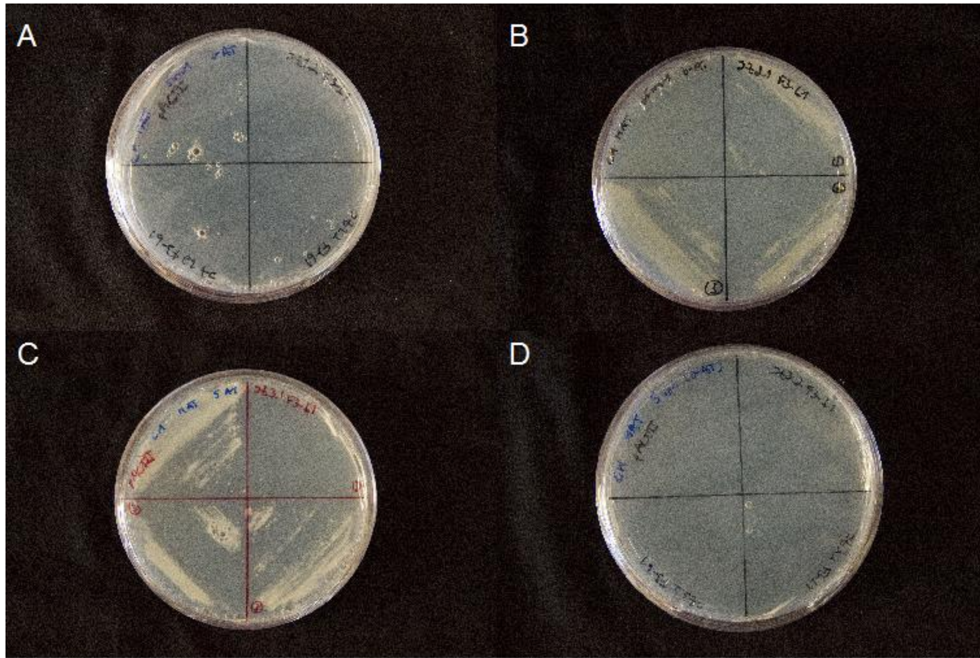


Figure 6 - Direct screen analysis with the transcription factor F3-61: (A) JZ1.2 with 5mM of 3-AT; (B) JZ2.1 with 25mM of 3-AT; (C) JZ3.1 with 5mM of 3-AT; (D) JZ3.2 with 5mM of 3-AT

Discussion

The primers designed to perform this yeast one hybrid assay resulted well in amplifying 550bp fragments from genomic DNA, with an annealing temperature of 54ºC. However, the primers did not perform homogenously at 54ºC when the read-proof Taq Polymerase PhusionTaq was used in the PCR reactions. The PCR program had to be calibrated, namely in the annealing temperature. DMSO was also applied in the reaction mixture to check for possible improvement of the reaction. The annealing temperatures of 56ºC and 58ºC were tried, with 58ºC yielding better results to all pairs of primers.

The results from the yeast one hybrid assay were all negative, meaning, there was no bait strain that showed differential growth in the absence of histidine in the medium. Although there was no ligation detected between the bait and prey sequences, the nature of the fragments was different since some did display a lot more leaky expression of the HIS gene. The fragments 2.1 even displayed growth at 25 nm of 3-AT

The bait strains with the JAZ promoter fragments demonstrated differential growth in MATH plates, and in the titulation process. Four of the six bait strains, namely for fragments JZ1.1, JZ1.2, JZ2.2 and JZ3.1, showed reduced growth when 5 and 10mM of 3-AT was applied to the medium, whereas for bait strains JZ2.1 and JZ3.1, as high as 25mM was applied to the medium and still these bait strains achieved complete growth in the plate after a week of incubation in MATH. Curiously, the bait strain for JZ2.1 was the most difficult to transform, with the plates showing very few colonies, sometimes just one or two colonies would appear after 3-4 days of incubation. The bait strain for JZ2.1 showed no colonies when was first transformed with the empty vector pACTII. The reason for this may be due to the presence in this promoter fragment (of JZ2.1 and JZ3.1) of deleterious sequences that alter the behavior of the bait strain. One possible solution to overcome this problem would be to sub-divide the ~550bp of each of the promoter fragments into smaller fragments. This way we could, eventually, reduce the deleterious effect of the initial promoter fragment to be used in the Y1H screening.

The results from the Y1H assay were all negative, meaning, there was no interaction between the 2 EREBP TFs and the bait strains carrying the JAZ-like promoter fragments. This means that the *JAZ*-like genes selected for this screening have a different mechanism of regulation from the one observed for the *OsRMC* gene which was our

initial hypothesis. Nevertheless, and regarding what was described in the Introduction of this chapter about the up-regulation of *JAZ*-like genes in a RNAi-OsHOS1 rice line, it would be interesting to follow the analysis of these promoter fragments in Y1H screenings. For future screenings some alterations could be made in order to access better the possibility of the promoter of the JAZ-like genes being target of *cis*-acting elements. Only 1000bp upstream of the ATG codon were analyzed. In a possible future assay with these promoters, 2000bp upstream of the ATG codon could be analyzed. Also, and since it is available at the host lab, a cDNA salt induced library could be used to identify transcription factors binding to the JAZ-like promoters.

Although the JAZ genes are extensively well studied in Arabidopsis and this was, to the best of our knowledge, the first attempt to study the transcriptional regulation of JAZ-like genes from *Oryza sativa*.

Final Conclusions / Future Work

The main goal of this work was to establish a link between the *OsRMC* gene and the jasmonate signaling pathway, given the hypothesis that it might be involved in that role as a negative regulator in rice. The *OsRMC* gene does not have a known homologue in *Arabidopsis*, which made this system a proper model to test our hypothesis. To test our hypothesis, we had to develop the biological material (*Arabidopsis* overexpressing *OsRMC*) in order to perform the envisaged assays.

Aiming to observe phenotypic changes in response to JA in *Arabidopsis* we have developed three independent *Arabidopsis* lines overexpressing the gene *OsRMC*; using as a method of transformation the floral dip method. These lines had different levels of transgene expression which we could also correlate with the protein level (OsRMC-RFP). With segregation analysis we obtained two homozygous lines (RMC#2 and RMC#3). For the RMC#1 line, even after two rounds of segregation and seed amplification we were not able to find any homozygous line (on-going work).

Due to time constraints, we were not able to further characterize these lines in response to JA. Nonetheless, these lines will be important in future assays to test our initial hypothesis regarding the function of OsRMC. One of our interests will be the root growth rate in the presence of JA. A classical effect of JA is the reduction of the root growth rate. As a future assay, we will determine the root growth rate of Col-0 (wild-type) and transgenic lines in the presence or absence of JA (vertical growth). This assay will give us information regarding the JA sensing ability of the different lines. Other JA-insensitive phenotypes are related to development (apical meristem transition to floral) and seed development. JA has been shown to be a critical signal for anther dehiscence, and hence, pollen release, although other hormones, particularly auxin, are also involved (Wilson *et al.*, 2011). The overexpression of *OsRMC* in the homozygous lines might be hampering the release of pollen and hence reducing the number of seeds formed. As future assays, we will determine the time of transition from the apical meristem to floral, and also observe the siliques to determine the number of seeds formed in each line. These results may also give us insight of the ability of the transgenic lines to sense JA.

We would also like to visualize the roots phenotype when facing a mechanical barrier (root mechanosensing). The absence of root curling in rice RNAi-OsHOS1 plants

when challenged by a mechanical barrier was correlated with the up-regulation of *OsRMC* (Lourenço, T., unpublished results). So as future work, these transformed Arabidopsis plant lines will be used to evaluate the contribution of the *OsRMC* gene in the development of the roots with this part of the work is already under way. The mechanosensing assays envisaged takes into account the effect of the *OsRMC* gene in the modulation of root development in rice, and so it will be interesting to observe the effects in the roots of the transformed Arabidopsis plants when challenged with a barrier. The roots of the transformed homozygous lines will also be used to access the subcellular localization of the OsRMC protein which was one of the main objectives of this work. This part of the work is already being performed and the roots will be visualized in a confocal microscope to try to get a clear picture of the RFP signal in the homozygous lines.

Regarding the gene expression analysis of JA responsive genes using the cell suspension cultures, clearly some optimization has to be performed, particularly in the RNA extraction process in order to obtain results for all the three genes studied (for results for the RNA extraction see Appendix). The transformation process of the cultures was successful because the cultures have grown in selective medium, and the protoplasts prepared from such cultures proved that the genetic construct was being expressed (at least for the empty vector of RFP). Although the transformation of the cell suspension cultures proved successful with the LBA4404 Agrobacterium strain, other strains could be used that are more virulent. This could lead to a more homogenous transformed cell suspension culture. The JA assays might have been done with larger volumes of cells to ensure that more transformed cells were being sampled and that a higher concentration of RNA could be extracted. The analysis done for the *MYC2* gene demonstrates that this gene is consistently expressed during the times of treatment with JA. The other two genes, *PDF1.2* and *VSP* are known JA-responsive genes but were consistently lacking in the RT-PCRs with the cDNA prepared from RNA from any culture studied, including the wild-type cultures. In a future repetition of these assays the transformed *Arabidopsis* plants will be used as biological material instead of the cell suspension cultures since with this work we could establish that the cell suspension cultures were not a good system to study the genes mentioned above.

Yeast One Hybrid

No interaction was observed between the bait sequences (the JAZ promoter fragments) and the preys (the two EREBP transcription factors F3-1 and F3-61). This assay could be repeated in the future using extended promoter sequences (the majority of *cis*-elements are located in the first 1000bp upstream of the ATG codon (Figueiredo *et al.*, 2012) but larger regulatory sequences of up to 2000bp might be used in these kinds of assays to access possible interactions. Nonetheless, the primers designed in this work proved successful and the bait strains obtained could be used in other Y1H screening assays with cDNA libraries (salt induced, JA induced) in order to identify TFs that act in the regulation of these JAZ-like genes.

Personal remarks

I can say that, even though no positive results were obtained with this assay, I gained a lot of experience in the bench with a lot of techniques. First, I learned to design and test primers and how to get the sequences to analyze. Also I learned to work with yeast, which is quite different from working with bacteria, both in terms of safety measures to avoid contaminations and in terms of growth mediums and conditions of growth. Also, this assay allowed me to continue working in the lab in periods of time where the major part of the work was in standby (waiting for plants to grow and produce seed). This part of the work was very rewarding for me for the reasons mentioned above but also because this work started from the very beginning (except for the fact that the two TFs had already been isolated) which was a very important factor for me. Also, the fact that this was the first attempt, to best of our knowledge, to study JAZ-like genes, is something that I feel proud of.

Regarding the major part of the work, concerning Arabidopsis plants and cell suspension cultures, I feel that the work was delayed by the fact the RMC#1 didn't produce, until now, any homozygous lines, and so, some assays could not be done in time.

References

Acosta, ÍF. & Farmer, EE. (2010). Jasmonates. *Arabidopsis book* 8:e0129.

Agrawal, GK., Rakwal, R., Yonekura, M., Kubo, A. & Saji, H. (2002). Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (*Oryza sativa* L.) seedlings. *Proteomics* 2:947–959.

Barratt, DHP. & Clark, JA. (1991). Proteins arising during the late stages of embryogenesis in *Pisum sativum* L. *Planta* 184:14–23.

Benedetti CE., Xie D., Turner JG. (1995). *COI1*-dependent expression of an *Arabidopsis* vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. *Plant Physiology* 12:567–572.

Boisson, AM., Gout, E., Bligny, R., & Rivasseau, C. (2012). A simple and efficient method for the long-term preservation of plant cell suspension cultures. *Plant methods 8*(1):4.

Boyer, JS. (1982). Plant productivity and environment. *Science* 218:443–448.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. & Breitenbach, M. (1989). The gene coding for the major birch pollen allergen *Bet*v1, is highly homologous to a pea disease resistance response gene. *EMBO J* 8:1935–1938.

Browse, J. (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Reviews in Plant Biology* 60: 183-205.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, JM., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F. M., Ponce, M. R., Micol, JL. & Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signaling. *Nature 448*(7154):666–671.

Chini, A., Fonseca, S., Chico, JM., Fernández-Calvo, P., & Solano, R. (2009). The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. *The Plant journal : for cell and molecular biology 59*(1):77–87.

Chung, HS. & Howe, GA. (2009). A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. *Plant Cell* 21: 131-145.

Clough, SJ. & Bent, AF. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana *The Plant Journal* 16(6):735-743

Coleman, GD., Chen, THH., Ernst, SG. & Fuchigami, L. (1991). Photoperiod control of poplar bark storage protein accumulation. *Plant Physiology* 96:686–692.

Cramer, GR., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: a systems biology perspective. *BMC plant biology* 11(1):163.

Crowell, DN., John, ME., Russell, D. & Amasino, RM. (1992). Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Molecular Biology* 18:459–466.

Dreher, K. & Callis, J. (2007). Ubiquitin, hormones and biotic stress in plants. *Annals of botany 99*(5):787–822.

Farmer, EE. & Dubugnon, L. (2009). Detritivorous crustaceans become herbivores on jasmonate-deficient plants. *Proceedings of the National Academy of Sciences USA* 106:935-940.

Figueiredo, DD., Barros, PM., Cordeiro, AM., Serra, TS., Lourenço, T., Chander, S., Oliveira, MM. & Saibo, NJ. (2012) Seven zinc-finger transcription factors are novel regulators of the stress responsive gene OsDREB1B. *Journal of Experimental Botany* 63(10):3643-3656

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C. & Solano, R. (2009). (+)-7-iso- Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature Chemical Biology* 5:344-350.

Fonseca, S., Chico, J. M., & Solano, R. (2009ª). The jasmonate pathway: the ligand, the receptor and the core signaling module. *Current Opinion in Plant Biology* 12: 539-547.

Forreiter, C., Kirschner, M. & Nover, L. (1997). Stable transformation of an Arabidopsis cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in vivo. *The Plant cell 9*(12):2171–2181.

Fukuda H, Ito, M., Sugiyama, M. & Komamine, A. (1994). Mechanisms of the proliferation and differentiation of plant cells in cell culture systems. *International Journal of Developmental Biology* 38:287–299.

Gleddie, S. (1989). Plant regeneration from cell suspension cultures of *Arabidopsis thaliana* heynh. *Plant Cell Reports* 8: 1-5

Goulas, E., Le Dily, F., Ozouf, J. & Ourry, A. (2003). Effects of a cold treatment of the root system on white clover (*Trifolium repens* L.) morphogenesis and nitrogen reserve accumulation. *Journal of Plant Physiology* 160:893–902.

Guo, J., Pang, Q., Wang, L., Yu, P., Li, N. & Yan, X. (2012). Proteomic identification of MYC2-dependent jasmonate-regulated proteins in Arabidopsis thaliana. *Proteome science 10*(1):57.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. & Koshiba, T. (2004). A novel rice PR10 protein, RSOsPR10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. *Plant Cell Physiology* 45(5):550–559.

Jiang, J., Li, J., Xu, Y., Han, Y., Bai, Y., Zhou, G., Lou, Y., Xu, Z. & Chong, K. (2007). RNAi knockdown of Oryza sativa root meander curling gene led to altered root development and coiling which were mediated by jasmonic acid signalling in rice. *Plant Cell and Environment* 30(6):690-699.

Kar, RK. (2011). Plant responses to water stress: role of reactive oxygen species. *Plant signaling & behavior* 6(11):1741–1745.

Kloek, AP., Verbsky, ML., Sharma, SB., Schoelz, JE., Vogel, J., Klessig, DF. & Kunkel, BN. (2001). Resistance to Pseudomonas syringae conferred by an Arabidopsis thaliana coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. *Plant Journal* 26(5):509-522.

Koike, M., Okamoto, T., Tsuda, S. & Imai, R. (2002). A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. *Biochemical and Biophysical Research Communications* 298:46–53.

Liu, YL., Ahn, JE., Datta, S., Salzman, RA., Moon, J., Huyghues-Despointes, B., Pittendrigh, B., Murdock, LL., Koiwa, H. & Zhu-Salzman, K. (2005). Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. *Plant Physiology* 139: 1545–1556.

Lo, SCC., Hipskind, JD. & Nicholson, RL. (1999). cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. *Molecular Plant-Microbe Interactions* 12:479–489.

Maeshima, M., Sasaki, T. & Asahi, T. (1985). Characterization of major proteins in sweet-potato tuberous roots. *Phytochemistry* 24:1899–1902.

Maitra, N. & Cushman, J. (1998). Characterization of a drought-induced soybean cDNA encoding a plant defensin. *Plant Physiology* 118:1536.

Matton, DP. & Brisson, N. (1989). Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* 2:325–331.

McGee, JD., Hamer, JE. & Hodges, TK. (2001). Characterization of a *PR-10* pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 14:877–886.

Meuriot, F., Noquet, C., Avice, JC., Volenec, JJ., Cunningham, SM., Sors, TG., Caillot, S. & Ourry, A. (2004[b]). Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. *Plant Physiology* 120:113–123.

Midoh, N. & Iwata, M. (1996). Cloning and characterization of a probenazole inducible gene for an intracellular pathogenesis-related protein in rice. *Plant Cell Physiology* 37:9–18.

Mignery, GA., Pikaard, CS., Hannapel, DJ. & Park, WD. (1984). Isolation and sequence analysis of cDNAs for the major potato tuber protein, patatin. *Nucleic Acids Research* 12:7987–8000.

Mignery, GA., Pikaard, CS. & Park, WD. (1988). Molecular characterization of the patatin multigene family of potato. *Gene* 62:27–44.

Moons, A., Prinsen, E., Bauw, G. & Van Montagu, M. (1997). Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *Plant Cell* 9:2243–2259.

Ouwerkerk, PBF. & Meijer, AH. (2001). Yeast One-Hybrid Screening for DNA-Protein Interactions. *Current Protocols in Molecular Biology* 55:12.12.1–12.12.12.

Pauwels, L., Morreel, K., De Witte, E., Lammertyn, F., Van Montagu, M., Boerjan, W., Inze, D. & Goossens, A. (2008). Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. *Proceedings of the National Academy of Sciences USA* 105:1380-1385.

Penninckx, IAMA., Eggermont, K., Terras, FRG., Thomma, BPHJ., DeSamblanx, GW., Buchala, A., Metraux, JP., Manners, JM. & Broekaert, WF. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid–independent pathway. *Plant Cell* 8:2309–2323

Rakwal, R., Agrawal, GK. & Yonekura, M. (1999). Separation of proteins from stressed rice (*Oryza sativa* L.) leaf tissues by two-dimensional polyacrylamide gel electrophoresis: induction of

pathogenesis-related and cellular protectant proteins by jasmonic acid, UV irradiation and copper chloride. *Electrophoresis* 20:3472–3478.

Ribot, C., Zimmerli, C., Farmer, EE., Reymond, P. & Poirier, Y. (2008). Induction of the Arabidopsis PHO1;H10 gene by 12-oxo-phytodienoic acid but not jasmonic acid via a CORONATINE INSENSITIVE1- dependent pathway. *Plant Physiology* 147:696-706.

Santner, A., & Estelle, M. (2007). The JAZ proteins link jasmonate perception with transcriptional changes. *Plant Cell 19*(12):3839–3842.

Seo, JS., Joo, J., Kim, MJ., Kim, YK., Nahm, BH., Song, SI., Cheong, JJ., Lee, JS., Kim, JK. & Choi, YD. (2011). OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. *Plant Journal* 65:907–921.

Somssich, IE., Schmelzer, E., Bollmann, J. & Hahlbrock, K. (1986). Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. *Proceedings of the National Academy of Sciences USA* 83:2427–2430.

Staswick, PE. (2008). JAZing up jasmonate signaling. *Trends in Plant Science* 13(2):66-71.

Staswick, P.E. (2009). The tryptophan conjugates of jasmonic and indole-3-acetic acids are endogenous auxin inhibitors. *Plant Physiology* 150:1310-1321.

Stintzi, A., Weber, H., Reymond, P., Browse, J. & Farmer, EE. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proceedings of the National Academy of Sciences USA* 98: 12837-12842.

Sugano, S., Kaminaka, H., Rybka, Z., Catala, R., Salinas, J., Matsui, K., Ohme-Takagi, M. & Takatsuji, H. (2003). Stress-responsive zinc finger gene ZPT2-3 plays a role in drought tolerance in petunia. *Plant Journal* 36(6):830-841.

Taji, T., Seki, M., Satou, M., Sakurai, T., Kobayashi, M., Ishiyama, K., Narusaka, Y., Narusaka, M., Zhu, JK. & Shinozaki, K. (2004). Comparative genomics in salt tolerance between Arabidopsis and Arabidopsis-related halophyte salt cress using Arabidopsis microarray. *Plant Physiology* 135:1697–1709

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., Yagi, K., Sakurai, N., Suzuki, H., Masuda, T., Takamiya, K., Shibata, D., Kobayashi, Y., & Ohta, H. (2005). 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. *Plant Physiology* 139:1268-1283

Tanaka, N., Che, FS., Watanabe, N., Fujiwara, S., Takayama, S. & Isogai, A. (2003). Flagellin from an incompatible strain of *Acidovorax avenae* mediates H_2O_2 generation accompanying hypersensitive cell death and expression of *PAL*, *Cht-1*, and *PBZ*1, but not of *LOX* in rice. *Molecular Plant-Microbe Interactions* 16:422–428.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, SY., Howe, GA. & Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448(7154):661-665.

Todaka, D., Nakashima, K., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. *Rice* 5(1):6.

Van Damme, EJM., Roy, S., Barre, A., Citores, L., Mostafapous, K., Rouge, P., VanLeuven, F., Girbes, T., Goldstein, IJ. & Peumans, WJ. (1997). Elderberry (*Sambucus nigra*) bark contains two structurally different Neu5Ac(alpha 2,6)Gal/GalNAc-binding type 2 ribosome-inactivating proteins. *European Journal of Biochemistry* 245:648–656

Van Loon, LC. & Van Strien, EA. (1999). The families of pathogenesis related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology* 55:85–97.

Warner, SAJ., Scott, R. and Draper, J. (1993). Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. *Plant Journal* 3:191–201.

Wasternack, C., & Kombrink, E. (2010). Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. *ACS Chemical Biology* 5(1):63–77.

Wittenbach, VA. (1983). Purification and characterization of a soybean leaf storage glycoprotein. *Plant Physiology* 73:125–129.

Xiong, L., & Zhu, JK. (2001). Abiotic stress signal transduction in plants: Molecular and genetic perspectives. *Physiologia Plantarum* 112:152–166.

Yamada, S., Komori, T. & Imaseki, H. (1997). Expression of plasma membrane water channel genes under water stress in Nicotiana excelsior. *Plant Cell Physiology* 38(11):1226-1231

Xuan, LT. & Menczel, L. (1980). Improved protoplast culture and plant regeneration from protoplast derived callus in *Arabidopsis thaliana*. *Pflanzenphysiol* 96:77-80.

Yan, Y., Stolz, S., Chetelat, A., Reymond, P., Pagni, M., Dubugnon, L. & Farmer, EE. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. *Plant Cell* 19:2470-2483.

Zhang, L., Tian, L., Zhao, J., Song, Y., Zhang, C. & Guo, Y. (2009). Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. *Plant Physiology* 149(2):916-928.

Appendix

Growth Media

Arabidopsis Medium

- 4,414 g/L MS + B5 vitamins (Duchefa:M0231)
- 30 g/L Sucrose
- 1 mg/L 2,4-D
- Adjust pH to 5.7 with 0,1M KOH, autoclave

B5 0.34 M glucose-mannitol Medium (B5-GM)

- 3163.98 mg/L B5 powder (Duchefa MO2310050)
- 0, 34 M glucose
- 0, 34 M mannitol
- Adjust pH to 5,5 with 0,1M KOH, autoclave

B5 0,28M sucrose Medium

- 3163, 98 mg/L B5 powder (Duchefa MO2310050)
- 0, 28 M sucrose
- Adjust pH to 5.5 with 0,1M KOH autoclave

Enzyme solution

- Prepare freshly before use.
- 1% Cellulase (Yakult or Serva)
- 0, 2% Macerozyme (Yakult or Serva) in B5-GM medium
- Stir for 30 minutes and filter through Whatmann paper or centrifuge 4000 rpm for 10 minutes. Sterilize by filter.

CM Medium

- 0.17% YNB (yeast nitrogen base without amino-acid or ammonium sulfate)
- 2% Glucose
- 5g/L Ammonium sulfate ($(NH_4)_2SO_4$)
- 2% Microagar

LB Medium

- 5g/L Yeast Extract
- 10g/L NaCl
- 10g/L Triptona
- 15g/L Agar (for solid LB medium)

Protein extraction buffers

Laccus buffer

Tris-HCl	25mM
$MgCl_2$	10mM
DTT	1mM
NaF	1mM
$NaVO_3$	0.5mM
B-glycerolphosphate	15mM
p-nitrophenylphosphate	15mM
Tween 20	0.1%
Phenylmethylsulfonylfluoride (PMSF)	0.5mM
Leupeptine	5µg/mL
Aprotinin	5µg/mL

Store at -20ºC

PEB (Protein Extraction Buffer)

Tris HCl pH 8.0	50mM
NaCl	150mM
EDTA pH 8.0	2mM
Triton X-100	0.4%
Protease inhibitor	2x complete
Water	

PEB should be stored at -20ºC without 2x complete

Semi-Quantitative PCR reactions

cDNA synthesis

Template-primer mix	
RNA solution	500-1000ng
Oligo-dT primers	1μL
RNA-se free water	Until 11.4μL

65ºC – 10 min

Immediately place on ice

Master RT Mix	
Buffer 5x	4μL
RNAse inhibitor	0.5μL
Deoxynucleotide mix 10mM	2μL
DTT	1μL
RT (Reverse Transcriptase)	1.1μL

RT-PCR Program	
25ºC	10 min
55ºC	30 min
85ºC	5 min

Western blots

Gel preparation and solutions

Resolving Gel 10%

Component	10mL gel	Final conc,
TrisHCl pH 8.8 1,5mM	2,5mL	375mM
SDS 10%	100µL	0,1%
H_2Odd	4,796mL	
Acrylamide mix 40%	2,5mL	10%
APS 10%	100µL	0,1%
TEMED	4µL	0,04%(w/w)

Stacking Gel 10%

Component	5mL gel	Final conc,
TrisHCl pH 8.8 0,5mM	1,25mL	125mM
SDS 10%	50µL	0,1%
H_2Odd	3,02mL	
Acrylamide mix 40%	2,5mL	10%
APS 10%	50µL	0,1%
TEMED	5µL	0,1%(w/w)

DNA sequences

OsRMC coding sequence

***ATGGCGCGGTGCACTTTGCTCGTTCTCCTCGTGGCGGCGGCGGTGGCGGTCGTC
CCGCTCGCCGCCGGCCAGCCGTGGGCTACCTGCGGCGACGGCACGTACGAGCAGGGGAG
CGCCTACGAGAACAACCTCCTGAACCTGGCCCTCACCCTCCGCGACGGCGCCTCCTCCC
AGGAGATCCTCTTCTCCACGGGCTCCAACGGCGCCGCCCCGAACACCGTCTACGGCCTC
CTCCTCTGCCGCGGCGACATCTCCCGCGCCGCCTGCTACGACTGCGGCACCAGCGTGTG
GAGGGACGCCGGGAGCGCGTGCCGCCGCGCCAAGGACGTCGCCCTCGTCTACAACGAGT
GCTACGCCCGCCTCTCCGACAAGGACGACTTCCTCGCCGACAAGGTGGGGCCGGGGCAG
CTGACGACCCTCATGAGCAGCACCAACATCAGCAGCGGCGCCGACGTCGCCGCCTACGA
CCGCGCGGTGACGCGGCTGCTGGCGGCCACCGCGGAGTACGCGGCGGGGGACATCGCGA
GGAAGCTGTTCGCGACGGGGCAGCGGGTGGGCGCCGACCCGGGGTTCCCCAACCTGTAC
GCGACGGCGCAGTGCGCGTTCGACATCACGCTGGAGGCGTGCCGCGGCTGCCTCGAGGG
CCTCGTCGCCAGGTGGTGGGACACGTTCCCGGCGAACGTCGACGGCGCCAGGATCGCCG
GGCCGAGGTGCCTCCTCAGGTCGGAGGTGTACCCGTTCTACACCGGCGCCCCGATGGTG
GTGCTGCGTGAGTAG***

OsRMC protein sequence

***MARCTLLVLLVAAAVAVVPLAAGQPWATCGDGTYEQGSAYENNLLNLALTLRDG
ASSQEILFSTGSNGAAPNTVYGLLLCRGDISRAACYDCGTSVWRDAGSACRRAKDVALV
YNECYARLSDKDDFLADKVGPGQLTTLMSSTNISSGADVAAYDRAVTRLLAATAEYAAG
DIARKLFATGQRVGADPGFPNLYATAQCAFDITLEACRGCLEGLVARWWDTFPANVDGA
RIAGPRCLLRSEVYPFYTGAPMVVLRE***

Yeast One Hybrid Promoter sequences

Locus: LOC_Os03g08320 (JAZ-like 1)

Promoter sequence

ATTACATATTCCGCTTGTAAAGTGCTAGACAAATTTATTAAGTATAATTAATCCGTCATT
AATAAATATTTACTGTAGTACAATATTATCAAATCATATAGCAATTATGCTTAAAAGATT
TGTCTCGTAATTTACACGTAATCTGTGTAATTTTTTTTATTTAATACTCTATACATGTGA
CTAAACATTTAACGTGACAAGGTGAAAAGTTTTTACGTGGGAACTAGATAGGGCCTTTAA
CCGATAAGAGCAAGATTAATAATACAGCCAACTTACTGGCTATAAGGTTCTTTATAGTAT
TCTCTCAGCGTACCTTTATAATATAGTTAGCTTTTTATCATTAATATTGGGTACACTTGC
CTCTCTCACAGAGTTTCTTGGTTCTTATGTCCAAACCGGCTGTTAAGTTTACAGCTCGCT
TCTCCTCTCTCTCCTCTCTTCTCTCCTCCACCTTATCATTTAGCAGGCTTACTATTATAT
TTGCTCTAACCCAAAACCAACCAGACCATTTTCCCAGCGTTGTCATTTGCCCCATTCGCA
CGCCACCACCACCGGTGGATTTACACCGCATCGCTGGCGCGGCCGCACGACGCACGTGAT
CCCCCCCACGCAGCCACGCTGCGGAGGCAGAGGTAGCGCGCCGCCGCCGAAACAACCC
AACCCAACCCAACGTCCCGACCCGATCGAACAACCACCGCGAAAACAGCGAGAGACGTGC
GGCGCGTGCCCGCAATTTCTCGTCTTTTTTTCCGCGCGGGCGCCGCGCTTCCCTCCGCTC
GCGTTTCACGTGCCGCTTTCCTACCCGAGAAACAAAGATTATACTAGGCGTTTAGGCCTC
GTCTTCCTTCCTTCCACATGTACACCTAGTATATAACAAGAGGGAGACAGGCTACAGATT
CACGGGAAGGTTTCGTCCTCGTTTCGTTTCTTGCAGCAGGAAGCCAAAGTGTATTTCCCG
AGCTTTGATGTGAGCTAGCTGATTTTGCTAGAGCTACAAC

Yeast One Hybrid Promoter sequences

Locus: LOC_Os03g28940 (JAZ-like 2)

Promoter sequence

AAATATTTTTTCTAAAATCTAAATATACCATAAAAAAATTTCAAACTTGTATACAAACTT
CCATATTGTGTAAGGAGGCCACTACAAAGTTTTCATATTTTTTTAAAACTTTAGATGAGA
TAAATCAATTGTTAATCTTGTATGTTCGTAAGGGTATTTTGGATATATTTGAAAAGAAGG
CCCAACCCAATGACATATTGTAGAAGAATTGATAAAGTTACTGGTATATTATAAAAAGAG
ATAATGTCGATGGCAAATCGTTGAACTCGAACAAACTTAATGGTATAGAATATATTCTCT
TTTAATTCTATTGGTGCATACATTATAGTTATTTGGATGATCTAAACTATGAGATGATAA
TAGAGTCTTATATTCTTATATTTTAGAATGGAGACAGTATCTTGAGATGAGGAAGGAAGT
ACTACTCCATCCAAAAGAACGAATTCATAGCTACGAACCTGAACACATATATATATCCAG
ATTGGTAGGTAGGATTGGTTTTTTCTGGTACGGAAGGTTATGCATTTTAGGACGAAGGGC
CCCACTCCTTTGTCGGCCACGTTGCATCCAATCAAACCTAACGTCACGGCATACCACACC
GGCACACAGTAAACACAAACCAGGAAAAAGATAACGAGAGAATAGGAATCTGCGCACCTT
GCCAATCTCCACAACAATCACGTGCTCAAACAAAGGCACCAACACCCAAACTAACTTTTT
TTTTTCCCTCGCAACTTGCGATTACAAAAATCGAAACTTTTTCGTCTTAAAGATAATCCC
CACCATTATTGTTATTGTTAGTCTCGTGCGTCCCTTATATAGAACCTGAACCGCCGGACG
GAGGAGGAAGACAACTCAGAGACAGACAAGGACGAGCAACCCGCAACCGCGACCAGAGGA
GAAGACTCGATCGAATCGAATCGAAGCGGGAGGATTTCGGGGGTCCGTGACTCCGTTCGG
AGAAAAGGATTCAGCTTGTGGGCAGAAGAAGAAGAAGAAG

Yeast One Hybrid Promoter sequences

Locus: LOC_Os10g25290 (JAZ-like 3)

Promoter sequence

CAGTTAATTATTGCATTCCGAATTCTAACCCTTTCTTTGAATTCAGAACAAGGTACGAAT
TGTGCTAGATTTATATCAGTTCGTAGATATTAAAATTAGGAAGACAATAAAAGTGTTAAA
AATAATATACGTCAAGAAAAAATGTGTGCAATACCAATTTGAGCTTGTAAAAATAGTAAC
TGACTAACTTTTATTATATTTATAGGTATTTTGGTCGTTTGGATAAAAATGTTAAGTACT
AGGCGGAGGTTAACAGGACGGTGGTGGTAAATGTAGAAGTTAAGCAAAAATCGATGACAT
ATTATAAAAAGTGACAAAGTCAGTGACAATCATAGACCTGTAACAAACTCAACTCAACGG
TCAGCTGGTGGTTCTACTCAAGGTGCGGGACACGAGAGCACAGTGTGAGGCATGACGGAG
GCACGGACTCATGAAGATGAAGTGGAGGCACAGATGGCGTATACACATAAAGTATACCAT
ACCTGGGACATAATATCGTGCCCTAGCAATCCTATGCATAGTATCCCACAACCAAAAAAA
ATCAATAAAGAAAAGATATTTTTTTCTCTTGCAAATTAAGCAAAAATAATAAAATAGAAA
CAAAGGATCCACAGAGAGAGAGAGAGGCCTAGACGAATTTTAGAAACCACAGGAAGATAA
ACCAAAGGAGCTAATGGCGTGTTTTAGTCCCTCTAAAAATAGATAGATAGATAAACCAGT
CGACGAGGTTCCAAGGAGAGAAGCGACAATTGTACTACTCCTCCCTCGCCCTCGCCGCCG
CGCGTCGCTCTCCCTTCCCGCGCTCGCATTTCACGTGCTCCTTTTCCAACCCAAAGCCAA
AGGAAGCCCATCAGCTTAGCTATATAGCGCGCACCACCACTCGGCGTGTACACACACACA
CATCCCACCTCCCCACACGCAAGCTTCGCAGCGCAGCTTCGCGGAGACAACGTGAGAAAG
AAAGAAACGAGACGAACGCGAGGTGGTGGAGGTGGACGCC

Yeast One Hybrid

Yeast Y187 strain genotype

Strain	**Genotype**	**References**
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met⁻, gal80Δ,URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ*	Harper *et al.*, 1993