# OsRap2.6 transcription factor contributes to rice innate immunity through its interaction with Receptor for Activated Kinase-C 1 (RACK1)

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**OsRap2.6 transcription factor contributes to rice innate immunity through its interaction with Receptor for Activated Kinase-C 1 (RACK1)**

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**Abstract**

The rice small GTPase, *Oryza sativa* Rac1 (OsRac1) is a molecular switch in blast fungus, bacterial blight infection responses, and in elicitor signaling pathways. The Receptor for Activated Kinase-C1 (RACK1), a 36-kDa-scaffold protein interacts with OsRac1 to suppress the growth of the rice blast fungus, *Magnaporthe Oryzae*. RACK1 has two homologs in rice, RACK1A and RACK1B. Overexpressing RACK1A enhances resistance to the rice blast fungus. RACK1A is also involved in various pathways including hormone signaling and defense responses in Arabidopsis and in mammals. RACK1 interacts with NADPH oxidase to produce reactive oxygen species (ROS), and co-chaperones, SGT1 and RAR1, important components of the rice innate immunity complex; however, to date, RACK1A downstream signals are largely unknown. Moreover, identifying genes that confer resistance to the blast fungus is still a challenge. To understand the function of RACK1 during immune responses in rice, we screened for proteins that interact with RACK1A using yeast two-hybrid assays. We sequenced the protein (Os04g0398000 or AK101501) and named it *Oryza sativa* Rap2.6 (OsRap2.6) among others. To identify OsRap2.6 related genes, OsRap2.6 sequence was used as a query for BLAST searches in the rice and Arabidopsis genome database. Surprisingly, we found a 94% similarity between OsRap2.6 AP2 domain and Arabidopsis Rap2.6 (AtRap2.6). Further, I analyzed the interaction between OsRap2.6 and tryptophan-aspartate (WD) repeats, a strong interaction was found between RACK1A and WD repeats 1 and 2. Based on these interesting findings, I selected OsRap2.6 for further analysis.

I fused the entry clones, RACK1A, OsRac1 wild type (WT) and constitutively active (CA) and dominant negative (DN) with the bait vector pBTM116ss and ligated OsRap2.6 coding regions into pVP16 prey vector including pBTM116ss and pVP16-Empty as negative controls in yeast *Saccharomyces cerevisiae* (L40). The positive transformants were selected on media supplemented with 3-amino triazole (-H + 3-AT) at 3mM after 3-5 days at

30 $^0$ C. A strong interaction was found between RACK1A and OsRap2.6; however, interaction was not observed between OsRac1 (WT) and (CA and DN) mutants. The results demonstrated that OsRap2.6 interacts specifically with RACK1A. I therefore hypothesised that OsRap2.6 may have a functional similarity with AtRap2.6 or most members in the AP2/ERF family.

Next, I checked for the subcellular localization of OsRap2.6 and RACK1A in a transient assay in rice protoplasts. I tagged OsRap2.6 to Yellow fluorescent protein (YFP) at N-terminus (Venus-OsRap2.6) and expressed the fusion protein in rice protoplasts with internal positive controls mCherry (YFP), Nuclear localisation signal (NLS)-mCerulean and OsGenL-CFP (NLS). OsRap2.6 localized with mCherry in nucleus and the cytoplasm (94%) and the rest in nuclei (6%). A further comparison using NLS-mCerulean marker showed (90%) of the cells localized within the nucleus and the cytoplasm and (10%) in the nucleus. Altogether, OsRap2.6 localized in the nucleus and cytoplasm in more than (90%) of the rice protoplasts.

I further expressed YFP tagged with RACK1A (mVenus) at the C-terminus and examined its intracellular localization with internal positive controls mCherry (YFP), a rice PAMP receptor, OsCERK1-GFP and OsGenL-CFP with a nuclear localization signal (NLS). RACK1A-mVenus localized mainly in the nucleus and the cytoplasm (CN) (90%) with mCherry. Moreover, in an independent experiment, RACK1A-mVenus co-localized with OsGenL-CFP (NLS) in the cytoplasm and the nucleus (88%) with the remainder of CFP localized in the nucleus (12%). However, a small proportion (6%) was associated with OsCERK1-GFP at the plasma membrane (PM) and endoplasmic reticulum (ER) (3%). Our findings further confirmed the ability of RACK1A to localize as a scaffold protein to different parts of the cell.

Both OsRap2.6 and RACK1A localized in the nucleus and the cytoplasm, but the next question was whether the two proteins interact at the same subcellular region or not. I confirmed their *in vivo* interaction using Bimolecular Fluorescence Complementation (BiFC) methods that detect interactions between two proteins in living cells. I split Venus fluorescent

protein into two halves (Vn/Vc) and tagged (Vn) to OsRap2.6 and (Vc) to RACK1A at N and C-terminus. GUS was used as a negative internal control. The paired constructs (Vn-OsRap2.6 + RACK1A-Vc) and (Vn-OsRap2.6 + GUS-Vc) and their controls mCherry and OsGenL-CFP (NLS) were transfected in rice protoplasts. I found a strong interaction between OsRap2.6 and RACK1A in the cytoplasm and nucleus (92%) and the rest (8%) in the nucleus with mCherry. Moreover, a strong interaction between OsRap2.6 and OsMAPK6 was detected in nuclei and the cytoplasm (76%) and the rest (24%) in the cytoplasm with mCherry, whereas OsRap2.6 and OsMAPK3 showed the strongest BiFC signal (72%) in nuclei and the cytoplasm (with 28%) of the signal localized in the cytoplasm with mCherry control.

I further generated *OsRap2.6* RNAi and *OsRap2.6*-Ox plants by *Agrobacterium* mediated transformation of rice calli in cultivar *Kinmaze.* The transgenic plants were challenged with compatible (007) and incompatible races (031) of the rice blast fungus, *Magnaporthe oryzae*. Disease severity was scored by measuring the relative lesion length on leaves and the amount of the fungus in the rice leaves by performing real time PCR (qPCR) with specific primers for blast fungus DNA. RNAi hence, *OsRap2.6* RNAi resulted in high susceptibility while OsRap2.6 Ox increased resistance to the compatible blast fungus while Overexpressing (Ox) were more resistant and showed significant differences when compared with wild type (p ≤ 0.01) after infection with the rice blast fungus compatible race, 007. However no significant differences were noted in incompatible race, 031 (p ≥ 0.05). These results suggest that OsRap2.6 contributes to rice innate immunity through its interaction with RACK1A in compatible interactions.

From these findings, I concluded that OsRap2.6 contributes to disease resistance in rice by interacting with RACK1A in response to rice blast fungus compatible interactions.

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# Abbreviations and acronyms

| | |
|---|---|
| ABA | abscisic acid |
| AP2/ERF | APETALA2/ethylene-responsive element-binding proteins |
| bHLH | basic Helix Loop Helix |
| BiFC | Bimolecular fluorescence complementation |
| CA-OsRac1 | constitutively active-OsRac1 |
| CCR1 | cinnamoyl CoA reductase 1 |
| CC-NB-LRR | coiled-coil NB-LRR |
| CERK1 | a LysM receptor kinase essential for chitin elicitor signaling |
| CFP | cyan fluorescent protein |
| DN-OsRac1 | dominant negative-OsRac1 |
| DREB | dehydration-responsive element-binding protein |
| DRMs | detergent resistant membranes |
| ER | endoplasmic reticulum |
| ERF | ethylene-responsive factor |
| ETI | effector triggered immunity |
| FLS2 | flagellin sensing 2 |
| GAPs | GTPase-activating proteins |
| GTP | guanosine triphosphate |
| GDP | guanosine diphosphate |
| GEF | guanine exchange factors |
| GFP | green fluorescent protein |
| HR | hypersensitive responses |

| | |
|---|---|
| HsP70 | Heat shock protein 70 |
| HsP90 | Heat shock protein 90 |
| LCM | Leica confocal software |
| MAPK 3/6 | mitogen-activated protein kinases 3 and 6 |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NB-LRR | (NB)-leucine rich repeat (LRR) |
| NB-ARC | ARC: APAF-1, certain R gene products and CED-4 |
| NLS | nuclear localization signal |
| NO | nitric oxide |
| OsRac1 | rice small GTPase, Rac1 |
| OsRACK1 | a rice Receptor for Activated Kinase C1 (RACK1) |
| OsRap2.6 | a rice transcription factor, Rap2.6 |
| Ox | Overexpressing |
| PAL1 | phenylalanine ammonia-lyase 1 |
| PBZ1 | probenazole-induced protein 1 |
| PAMP | pathogen associated molecular pattern. |
| PKC | protein kinase C |
| PM | plasma membrane |
| PR genes | pathogen related genes |
| PTI | pathogen-associated molecular pattern (PAMP) triggered immunity |
| qPCR | real rime polymerase chain reaction |
| R | resistance proteins |
| RAR1 | required for Mla12 resistance |
| RAI1 | Rac immunity 1 |

RACK1  Receptor for activated Kinase C-1 (RACK1A, RACK1B)

RDV  rice dwarf virus

RLK  receptor-like kinases

ROS  reactive oxygen species

RSV  rice stripe virus

RTSV  rice tungro spherical virus

SA  salicyclic acid

TIR  toll/ interleukin-1 receptor (TIR) or

Vn/Vc  Venus N terminus and C terminus

YFP  yellow fluorescent Protein

Y2H  Yeast two hybrid

# Chapter 1: Introduction

## 1.1    Plant defense response system

Rice production is constrained by various pests and diseases, the rice blast fungus, *Magnaporthe oryzae* being among the most prominent (Ribot et al. 2008; Couch et al. 2005; Valent and Chumley, 1991). This fungus accounts for major losses in crops and grain yields (Wilson and Talbot, 2009). *M. Oryzae* produces asexual spores that are dispersed rapidly by wind or by other means. Breeding for resistance is one of the safest ways to counteract *M. oryzae* and other pests; however, understanding the resistance mechanisms for most pests including the blast fungus is still a challenge (Ribot et al. 2008; Valent and Chumley, 1991).

Plants respond to attack by pathogens in two ways; through active defense mechanism referred to as pathogen-associated molecular patterns (PAMPs) triggered by invariant microbial epitopes or the first defense mechanism (Kawano et al. 2010; Zipfel, 2008; Dangl and Jones, 2001) or by the second defense mechanism through microbial effectors (Dangl and McDowell, 2006, Boller and He, 2009). Therefore, PAMP-triggered immunity (PTI) is considered as the primary immune response, and requires membrane receptor proteins known as pattern recognition receptors (PRRs) stimulated by chitin, flagellin or elicitors (He et al. 2007; Bent and Mackey, 2007; Chisholm et al.

2006; Jones and Dangl, 2006).  However, smart pathogens have co-evolved and acquired the ability to overcome PTI by secreting effectors that suppress PTI responses, to the plant cell. Such effectors block the immunity pathway and result to effector-triggered susceptibility. In response, plants evolved specific resistance (R) protein alleles for surveillance of the presence of the pathogen effectors known as effector-triggered immunity (ETI), which constitutes the second line of defense (Dangl and McDowell, 2006; Boller and He, 2009).

The programmed cell death often defined as hypersensitive response (HR) frequently associates R-protein mediated defense responses (Nimchuk et al. 2003). HR mainly restricts the growth and spread of pathogens to other parts of the plant at the infection sites, and the cells undergoing HR are mainly accompanied by reactive oxygen species (ROS) production including superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide (Heath, 2000; Mittler et al. 1999; Jabs et al. 1996). ROS therefore acts as a signaling molecule in plant responses having direct antimicrobial properties.

A large number of R genes, against various pathogens, from various plants have been identified so far (Dodds and Rathjen, 2010; McDowell and Woffenden, 2003), which based on their conserved functional protein domains are classified into several superfamilies, majority being the nucleotide binding sites (NBS) and leucine rich repeats (LRR) families represented by the Toll/ Interleukin-1 receptor (TIR) or Coiled-Coil (CC)-NB-LRR R proteins (Dangyl and Jones, 2001). R genes encode putative receptors that respond to the products of 'Avr genes' (Avr, avirulence) expressed by the pathogen during infection.

Interaction of R protein and Avr protein, directly or indirectly, triggers the defense-signaling pathway (McDowell and Woffenden, 2003). Tremendous research in the last decade has revealed about 37 and 29 R genes against the blast fungus pathogen *Magnaporthe oryzae* and bacterial pathogen *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) (Dai et al. 2007).

But, despite dozens of "major" disease-resistance (R) loci, which are known as Pi, being available (Zeigler et al. 1994), only six, *Pib, Pi-ta, Pi36, Pi9, Pi2,* and *Piz-t*, have been cloned and characterized so far (Liu et al. 2007). It has been shown that the resistance of rice to blast pathogen *M. grisea* is triggered by a physical interaction between the protein products of the host R gene, *Pi-ta*, and the pathogen Avr gene, *AVR-pita* (Jia et al. 2000). Some PAMP receptors, including the LysM domain-containing receptor-like kinases CERK1 (Miya et al. 2007) and LysM RLK1 (Wang et al. 2008) for chitin signaling and resistance to fungal pathogens, the FLS2 (flagellin-sensitive 2) and EFR (elongation factor Tu receptor) for perception of the bacterial flagellin and elongation factor, respectively, that confer resistance to bacterial pathogens have been characterized (Chinchilla et al. 2007). The identification of R proteins and PAMP receptors has revealed the complicated plant defense-signaling pathway, however, downstream these receptors and early events, the relay components and how they activate the signaling cascades that trigger the immune response, are largely unknown.

## 1.2  Small G proteins

Small GTP-binding proteins act as molecular switches to regulate a wide variety of important cell physiological functions, which include cell proliferation, cytoskeleton organization, intracellular trafficking, and immunity response. A typical small G protein is active when bound to GTP and inactive when bound to GDP, that is replaced by free GTP. GTPase-activating proteins (GAPs) accelerates the hydrolysis of GTP, while GTP exchange is catalyzed by Guanine nucleotide exchange factors (GEFs). Activation of a GEF typically activates its cognate G-protein (Paduch et al. 2001). On the other hand, guanosine nucleotide dissociation inhibitors (GDI) are negative regulators whose role is to maintain small GTPases in the inactive state.

Small G-proteins are a superfamily of at least five families (Ras, Rho, Rab, Sar1/Arf and Ran) in animals (Takai et al. 2001). They interact with their regulators and effectors through guanine nucleotide binding motifs as conserved protein domains. When a small G-proteins is stimulated by an upstream signal, it changes into its active form and leads to the conformational change of the downstream effector-binding region so that this region interacts with the downstream effector(s) (Zhu et al. 2011). In this way, this interaction transduces an upstream signal to a downstream effector(s) (Matozaki et al. 2000).

Among the small G proteins, plants lack the Ras family but instead, a Rho-family GTPases, called ROPs (Rho-related GTPase from plants) exist. ROPs share a common ancestor with Rho, cdc42 and Rac in animal and yeast, also

referred to as RACs. ROPs act as predominant GTPase switch that controls the transmission of extracellular signals in plants, and regulate various plant cellular responses including cytoskeletal organization and dynamics, pollen tube growth and development, vesicle traffic and regulates disease resistance (Ying et al. 2004, Agrawal et al. 2003; Berken, 2006).

Chitin oligosaccharides are elicitors that induce defense responses in plants through binding with their cognate receptors. A chitin elicitor receptor, CEBiP, identified from rice, plays a key role in the perception and transduction in the rice cells (Kaku et al. 2006). Receptors generally are thought to transduce an external signal to initiate an immune response. However, the molecular machinery involved in the signal transduction pathway downstream the receptor-ligand recognition, has not yet been well defined. To address this challenging question, an attempt to find clues by researchers who focused on the small G protein was carried out. Rice has 7 members in ROPs family (Christensen et al. 2003). Our laboratory found one of them, OsRac1, a key player in regulation of rice defense response against rice blast fungus.

### 1.3 OsRac1, a Rac/Rop family small GTPase

OsRac1 encodes a GTPase similar to its homologue in mammals. As a molecular switch, OsRac1 hydrolyzes active guanosine triphosphate (GTP) to inactive guanosine diphosphate (GDP) catalyzed by guanine nucleotide exchange factors (GEFs). Recently, plant unique GEFs for small GTPase ROPs family was identified as a group of PRONE (plant-specific Rop nucleotide exchanger) domain containing proteins (Berken et al. 2005, Gu et al. 2006). In rice eleven PRONE- type Rac GEFs candidates have been reported (Wang et al. 2009). But, the big question is among these GEFs, which one is exclusively active towards OsRac1, and how does it finally regulate its GTPase activity and thus control the rice immune response? This is one of the keys to understanding the OsRac1 mediated rice defense mechanisms. Thus, the identification of OsRac1 specific GEF and the study of its regulation against rice immune system is a topic of interest undergoing research in our laboratory.

To further understand the role of OsRac1, transgenic rice expressing constitutively active (CA) OsRac1 (OsRac1-G19V) (GTP-bound), either in leaves or cultured suspension cells, induced ROS production and apoptosis like cell death. In contrast, transgenic rice expressing the dominant negative (DN) OsRac1 (OsRac1-T24N) blocked ROS production and cell death when infected with rice blast fungus (Chen et al. 2010b; Ono et al. 2001), indicating that OsRac1 is required for the activation of ROS production and is a regulator of cell death in rice (Kawasaki et al. 1999). Further experiments demonstrate that

transgenic rice lines expressing CA-OsRac1, but not DN-OsRac1, caused HR-like responses, enhanced resistance against blast fungus and bacterial blight, caused increased production of a phytoalexin and altered expression of defense-related genes (Ono et al. 2001; Kawasaki et al. 1999).

Using proteomics, 100 proteins whose expression levels were altered by OsRac1 and/or SE treatment were systematically up regulated, and 87 were induced by CA-OsRac1 (Fujiwara et al. 2006), suggesting that OsRac1 plays a pivotal role in defense responses induced by elicitor in cultured rice cells. SE elicitor acts as a typical PAMP, thus OsRac1 is an important regulator of rice basal immunity. OsRac1 controls ROS production through regulation of the NADPH oxidase (Wong et al. 2007). Taken together, these findings strongly suggested that OsRac1 has a general role in disease resistance of rice.

It is now clear that, in response to pathogen infection, plant cells produce ROS, which may be involved in direct antimicrobial mechanisms or signaling pathway of defense responses and plays a role in basal resistance through formation of barriers. In the hypersensitive response, ROS may be linked to programmed cell death, and in SAR, ROS interacts with salicylate in signaling. Cell plasma membrane NADPH oxidases and cell wall peroxidases are considered as the two most likely enzymes involved in the generation of ROS (Nanda et al. 2010; Torres, 2010).

OsRac1 also regulates the NADPH oxidases, Rboh (for respiratory burst oxidase homolog), and thus leads to the production of ROS. Overexpression of the CA-OsRac1 increased ROS production and enhanced resistance to virulent

rice fungus and bacterial blight, but the DN-Rac1 caused a reduction of ROS levels. ROS generation depends on NADPH-oxidase (Suharsono et al. 2002; Ono et al. 2001; Kawasaki et al. 1999). CA-OsRac1, but not DN-Rac1, interacted with the N-terminus of rice RBOH (OsRbohB) hence OsRac1 likely potentiates ROS generation through inhibition of the ROS scavenger and down regulates the expression of OsMT2b, a ROS scavenger gene, and rice blast-derived elicitors (Wong et al. 2004). These results suggest that OsRac1 plays a dual role as an inducer of ROS production and a suppressor of ROS scavenging. However, there is still a need to fully understand the control mechanisms of ROS production mediated by the small GTPase.

Heterotrimeric G proteins, made up of alpha ($\alpha$), beta ($\beta$) and gamma ($\gamma$) subunits, have also been demonstrated to be involved in the rice defense response upstream of OsRac1. The rice G$\alpha$ mRNA can be induced by fungal elicitor treatment and by infection with an avirulent race of fungal blast pathogen (Suharsono et al. 2002).

## 1.4  OsRac1 Effectors

The effectors of OsRac1 include enzymes involved in the synthesis of defense related substances and MAPK cascades. Upon pathogen attack, lignin strengthens cell walls and interferes with the enzymatic hydrolysis and mechanical penetration of plant tissue by the pathogen (Lewis et al. 1999). A cinnamoyl-CoA reductase 1, OsCCR1, involved in the first committed step of the lignin biosynthesis, is an effector of OsRac1. The expression of *OsCCR1* is induced by a sphingolipid elicitor and CA-OsRac1 cells increased accumulation of lignin. Furthermore, OsCCR1 interacted with OsRac1 in yeast two-hybrids in a GTP-dependent manner that led to the enzymatic activation of OsCCR1 *in vitro* (Kawasaki et al. 2006). Moreover, the production of other antimicrobial substances such as phytoalexins is related to lignin and lignin-related compounds (Niemann et al. 1990). Therefore, the enzymes involved in the synthesis of defense related substances could be downstream effectors of OsRac1, and through their activation, the defense responses would be initiated in rice.

In addition, the mitogen-activated protein kinase (MAPK) cascade is activated during responses to pathogens or elicitors in rice and is regulated by OsRac1 significantly. A rice MAPK gene, *OsMAPK6*, was posttranslationally activated in cell culture by a sphingolipid elicitor. Silencing of *OsRac1* by RNA interference resulted in a strong reduction of protein levels and kinase activation of OsMAPK6. Furthermore, coimmunoprecipitation experiments showed that OsMAPK6 is closely associated with the active form of OsRac1 (Lieberherr et al.

2005) suggesting that OsRac1 possibly activates the MAPK signaling cascade, which in turn triggers the downstream effectors and leads to the immune response.

The second line of evidence in our laboratory suggests that RAI1, a bHLH transcription factor, is the first transcription factor acting downstream of OsRac1 that interacts with OsMAPK3 and OsMAPK6. RAI1 is up-regulated in suspension cells expressing CA-OsRac1 whereas in *RAI1* T-DNA activation-tagged and *RAI1* RNA interference lines, RAI1 regulated the expression of *PAL* and *WRKY* genes after induction, and showed increased resistance to the rice blast fungus. sphingolipid and chitin elicitors also up regulated the *PAL1* and *OsWRKY19* genes. These results indicated that *RAI1* is involved in defense responses in rice. Furthermore, *RAI1* interacted with OsMAPK3 and OsMAPK6 proteins *in vivo* and *in vitro*. The over-expression of *OsMAPK6* and/or *OsMAPK3* together with *OsMKK4-dd* increased *PAL1* and *OsWRKY19* expression in rice protoplasts. Therefore, the regulation of *PAL1* and *OsWRKY19* expression by *RAI1* could be controlled via OsMKK4-OsMAPK3/6 cascade. A further coimmunoprecipitaton assay indicated that OsMAPK3 and OsRac1 occur in the same complex as OsMAPK6. Taken together, our results indicate that OsRac1 could regulate *RAI1* through an OsMAPK3/6 cascade (Kim et al. 2012).

OsRac1 also regulates the downstream signaling events by interacting with molecular chaperones as well as scaffolding proteins. Among them, are Hsp90 (Sangster and Queitsch, 2005), RAR1 (required for Mla12 resistance) and its interacting partner SGT1 (for suppressor of the G2 allele of skp1), a

homologue of the yeast ubiquitin ligase-associated protein (Austin, et al. 2002, Muskett et al, 2002). Overexpression of OsRAR1 and OsSGT1 in rice significantly increased basal resistance to bacterial blight and fungal blast (Wang et al. 2008). *OsRAR1*-RNAi rice showed impaired basal resistance to a compatible race of the blast fungus *M. grisea* but transgenic rice plants carrying both the CA-OsRac1 and *OsRAR1*-RNAi had the same level of resistance as untransformed control plants, indicating that RAR1 is required for OsRac1-mediated disease resistance. Furthermore, addition of Hsp90-specific inhibitor to the elicitor treated CA-OsRac1 cell cultures resulted in a decrease in mRNA levels of *PBZ1* and *Chitinase1*, two defense response marker genes, suggesting that Hsp90 function is essential for OsRac1-mediated enhancement of PAMP-triggered immune responses. Further experiments showed that OsRac1 forms a complex with RAR1, Hsp90, and Hsp70 *in vivo*. The main function of Hsp90 is maybe to help the complex formation of OsRAR1 and OsRac1 (Thao et al. 2007). Very recently, our laboratory found that the Hsp90 co-chaperone Hop/Sti1 was required for chitin-triggered immune responses and the Hop/Sti1 interacts with the OsRac1 (Chen et al. 2010a).

## 1.5    Receptor for activated Kinase C-1 (RACK1)

The *Oryza sativa* Receptor for activated Kinase C-1 (OsRACK1), a 36-kDa protein (SwissProt accession no. P25388) interacts with OsRac1 and co-chaperones (Nakashima et al. 2008; Thao et al. 2007). OsRACK1 resembles the mammalian RACK1, is homologous to the G-protein *ß*-subunit and is highly

conserved in diverse species including plants (Kwak et al. 1997). RACK1 functions across cells as an adaptor protein as well as binds to phosphatases and transcription factors (Chen et al. 2006). RACK1 serves as a multifunctional scaffolding protein known to be involved in the regulation of various signaling cascades including hormone signaling and development in plants (Chen et al. 2006). RACK1A is characterized by seven WD repeats (Thornton et al. 2004). A previous study in our laboratory showed that RACK1A transcription was induced by a fungal elicitor and by abscisic acid, jasmonate and auxin. Transgenic rice overexpressing RACK1A enhanced ROS production and increased resistance against rice blast infection. Interestingly, RACK1A interacted with the N terminus of NADPH oxidase, RAR1, and SGT1 (Nakashima et al. 2008). As it is known, the scaffolding protein generally serves as molecular glue for kinase anchoring and as an integrative point for diverse signal transduction pathways, in such a way the specificity and efficiency of signal transduction is ensured (Faux and Scott, 1996 Chang et al. 1998).

RACK1A likely functions as a scaffold protein for the formation of an interactive complex including OsRac1, RAR1 and SGT1 and to maintain an effective conformation which is able to activate the downstream effectors and lead to an immune response (Takahashi et al. 2003, Takahashi et al. 2007; Thao et al. 2007). Rice has two RACK1 homologs, RACK1A and RACK1B, but a third homolog, RACK1C, also exists in Arabidopsis. The active form of OsRac1 (CA form) interacts strongly with RACK1A. Plants in which RACK1A is overexpressed have enhanced resistance to a compatible race of the rice blast fungus (007),

and the protein localizes mainly in the cytoplasm (RACK1A-GFP) (Nakashima et al. 2008). The OsRac1-mediated immune response in rice is, therefore, a very complex system, comprised of numerous key players that form a protein complex, the defensome complex (Chen et al. 2010b; Kawano, 2010; Nakashima et al. 2008; Thao et al. 2007; Wong et al. 2007; Ono et al. 2001; Kawasaki et al. 1999).

## 1.6    Rationale of the study

To date, the proteins that interact with RACK1A are largely unknown. Moreover, identifying genes that confer resistance to the blast fungus is still a challenge. To understand the function of RACK1A during immune responses in rice, we screened for proteins that interact with RACK1A using yeast two-hybrid assays. We further analysed for the localisation of RACK1A in rice protoplasts by confocal microscopy. We identified an AP2/ERF transcription factor known as OsRap2.6 and followed its dynamics in terms of localization, interaction with RACK1A, rice Mitogen activated kinase 3 and 6 (OsMAPK3/6) and its potential to contribute to disease resistance like its interacting partner RACK1A. Overexpressing RACK1A plants increased resistance towards the rice blast fungus, compatible race, 007 (Nakashima et al., 2008).

## 1.7    AP2/ERF transcription factors

Rap2.6 and Rap2.6L transcription factors belong to AP2/ERF transcription factor family in Arabidopsis. These APETALA2/ethylene-responsive element-binding proteins or AP2/EREBPs (AP2/ERFs) genes members are 163 in rice and 145 members in Arabidopsis (Sharoni et al. 2011; Sakuma et al. 2002, Riechmann et al. 2000). The *O. sativa* (OsAP2/EREBP) gene family is classified into four subfamilies: AP2, RAV (related to ABI3/VP1), DREB (dehydration-responsive element-binding protein) and ERF (ethylene-responsive factor) (Sharoni et al. 2011). In Arabidopsis, Rap2.6 is a coronatine (COR)-dependent JA-inducible transcription factor (Wang et al. 2008; He et al. 2004) whose expression is strongly induced by a virulent pathogenic bacterial strain, *Pseudomonas syringae* DC3000 (He et al. 2004). AP2/ERFs bind DNA sequences with *cis* elements such as the GCC box (AGCCGCC) and CE1 that regulates plant-pathogen interactions (Ohme-Takagi and Shinshi et al. 1995). Rap2.6 binds to the GCC box (AGCCGCC) and CE1 *cis*-elements, and its expression is responsive to abscisic acid (ABA), high salt, osmotic stress, and cold (Banno et al. 2001).

Both Rap2.6 and Rap2.6L function in plant responses in biotic and abiotic stresses, developmental processes, environmental responses and shoot regeneration (Zhu et al. 2010). Thus, the GCC box is the unique *cis* element regulating plant-pathogen interactions and is sufficient for ethylene responsiveness (Ohme-Takagi and Shinshi et al. 1995). The GCC box is present in promoters of defense-related genes such as $\beta$-1, 3-glucanase, chitinase, and

Pathogen related (PR1) genes but is not present in the promoters of ethylene-responsive genes involved in other functions. AP2/EREBPs are also involved in virus infections in rice for instance in rice stripe virus (RSV), rice tungro spherical virus (RTSV) and rice dwarf virus (RDV) (Sharoni et al. 2011).

Rap2.6 and Rap2.6L act as *trans*-activators as shown in yeast one-hybrid assays and localise within nuclei of onion epidermal cells (Zhu et al. 2010). AvrB induces the expression of Rap2.6, and bacterial growth is enhanced only when AvrB is expressed in plants. Both the *P. syringae* (avrB)-induced Rap2.6 gene expression and the AvrB-mediated bacterial growth require Coronatine. Similarly, RAR1 is required for both *P. syringae* (avrB)-induced Rap2.6 gene expression and the AvrB transgene-dependent susceptibility and defense suppression, meaning that AvrB transgene-dependent susceptibility is intrinsically linked to the virulence function of the *P. syringae*-delivered AvrB. It is likely that RAR1 is targeted by AvrB to suppress PTI; however, AvrB enhances Rap2.6 expression in the absence of coronatine (He et al. 2004).

In this study, we demonstrated for the first time a transcription factor OsRap2.6 that is involved in disease resistance in compatible interactions of rice. The defense genes *PAL1*, *PBZ1* as well as *OsRap2.6* were up regulated in suspension cells induced with chitin. *OsRap2.6* RNAi plants were susceptible while over expressing plants were more resistant to rice blast fungus, compatible race 007. However, no significant differences were found in incompatible race, 031. OsRap2.6 further interacted with RACK1A, the scaffolding protein discussed earlier. Furthermore, OsRap2.6 and RACK1A

localised at the nucleus and the cytoplasm. Moreover, we found a strong interaction between OsRap2.6 and two MAP kinases, OsMAPK6 and OsMAPK3 that had been reported to have a role in defense responses in rice. The results demonstrate the potential of OsRap2.6 in resistance towards rice blast fungus in compatible interactions.

# Chapter 2   Materials and methods

## 2.1   Comparison of predicted amino acid sequences of OsRap2.6

To identify OsRap2.6 related genes, its sequence was used as a query for BLAST searches in the rice and Arabidopsis genome database (http://www.ncbi.nlm.nih.gov/nuccore). Highly similar amino acid sequences were aligned with OsRap2.6 sequence using Genetyx software for Mac-Pro Version 10 (Genetyx, USA).

## 2.2   Yeast two-hybrid assays

The bait constructs, RACK1A, OsRac1 (WT) and (CA and DN) coding regions were ligated into their vector; pBTM116 while OsRap2.6 was ligated into the prey vector, pVP16 as described previously (Nakashima et al. 2008; Kawasaki et al. 1999). The negative controls were pBTM116ss and pVP16. The vectors concentrations ranged between 150-200 ng/µl hosted by yeast *Saccharomyces cerevisiae* L40 (25 µl). The cells were cultured on 2 X YT synthetic complete medium lacking Uracil and tryptophan, either with histidine (SC-UW) or without histidine (SC-UWLH). The inhibitor 3-amino-1, 2, 4-triazole (3-AT) (3 mM) was included in the SC-UWLH media. The bait-prey interaction was analysed based on the histidine requirement for growth as previously described (Kawasaki et al. 2006).

## 2.3    OsRap2.6, RACK1A and OsMAPK3/6 constructs

An entry clone, pENTR-OsRap2.6 was amplified from pVP16-OsRap2.6 (0.5 µl) with forward (5'-CACCATGGTCACCGCGCTAGCCACGT-3`) and reverse (5'-TCACGACGACGAATCCTTCTTCTTG-3`) primers. The blunt-end PCR product was cloned into pENTR-D/TOPO as per the manufacturer's instructions (Invitrogen, USA). Colonies were selected with M13 forward (5`-TGTAAAACGACGGCCAGT-3`) and reverse (5`-CAGGAAACAGCTATGAC-3`) primers. The pENTR-OsRap2.6 was ligated into Gateway destination vectors (GW) with LR clonase 11 enzyme (0.5 µl) (Takara) whose expression was driven by 35S-Cauliflower mosaic virus promoter (35S-Vn-OsRap2.6).

For the subcellular localization pENTR-OsRap2.6 was fused to Venus at the N terminus (Venus-OsRap2.6). In BiFC, Venus was split into two halves (Vn/Vc). The N terminus Venus half (Vn) was tagged to OsRap2.6 (Vn-OsRap2.6) and sequenced with pB12221-35S forward and NOST terminator reverse primers as listed in Table 2. RACK1A-mVenus constructs were provided from our laboratory stocks (Nakashima et al. 2008). The Venus constructs (Vc-OsMAPK3) and (Vc-OsMAPK6) were described previously (Kim et al. 2012). The DNA sequence of all plasmids was confirmed in an ABI-Prism big dye terminator cycle sequencing machine (Applied Biosystems, USA). Data was analysed using Genetyx software for Mac-Pro Version 10 (Genetyx, USA).

**2.4    Isolation of rice protoplasts, transfection and BiFC**

Suspension cells were crushed from primary calluses into small pieces prior to enzyme treatment for effective protoplast isolation. The protoplasts were adjusted to (1.5-2 x $10^7$) cells/ml as stock cells (Kyozuka et al. 1987). For intracellular localization, 100 µl of protoplasts were transfected with 9-10 ng effector (Venus-OsRap2.6) or (RACK1A-mVenus) and/or control plasmids mCherry (YFP) which localize at the nucleus and cytoplasm, a rice chitin receptor OsCERK1-GFP, and two nuclear localization signals (NLS), NLS-mCerulean and OsGenL-CFP (NLS) independently in 1.5 ml tubes. The BiFC system used in this study was as described previously with slight modifications (Chen et al. 2010a; Kawano et al. 2010).

For the interaction studies, protoplasts (100 µl) (1.5-2 × $10^6$ cells) were transformed with 2.5-5 µg of each paired constructs (Vn-OsRap2.6 + RACK1A-Vc), (Vn-OsRap2.6 + Vc-OsMAPK3/6) and a negative control (Vn-OsRap2.6 + GUS-Vc) by Polyethylene glycol (PEG) method with minor modifications (Yoo et al. 2007). The protoplasts were incubated at 30 °C for 15 hours. The localization or co-localization of YFP or CFP proteins and their markers was assessed with a confocal microscope (Leica TCS SP5) in sequential scanning mode. Quantitative assays were accomplished using a method described previously where 50-100 cells of each construct was randomly scanned and categorized according to the plasma membrane (PM), cytoplasm (C), nucleus (N), or cytoplasm and nucleus (CN) localization patterns.

## 2.5    Leica TCS SP5 Confocal scanning microscopy

The Leica TCS SP5 confocal scanning microscope was used to image the rice protoplasts expressing fluorescence proteins. The microscope is equipped with the Leica confocal software (LCS), a 100mW multi-line Argon laser (458nm, 476nm, 488nm, 496nm and 514nm), Diode pumped solid state laser (DPSS) (442nm), a 10mW DPSS (561nm), a 10mW He-Ne Laser (633nm) and a 50mW UV laser (351nm-364nm) at excitation sources. The SP scanner collected the FP signal at various wavelengths and the auto fluorescence of the protoplasts was measured between 440nm and 650nm. The transmitted light image was collected in a separated detector. The rice protoplasts were mounted on a glass slide (Micro slide glass 1.0 mm, TF0410 (S117074) Matsumi, Japan) and covered gently with a thin cover slip. The slide was observed under UV light. Once the desired focus and object lens were chosen, the UV shutter was closed and Leica TCS SP5 scanned the sample. The appropriate Laser intensity was selected in the Leica Control Software (LCS) accordingly and the live images were acquired instantly through sequential scan mode.

The scanner and detector were set to xyz scanning mode, 514 x 514 image size, 8-bit image and 400 Hz scan rate unless otherwise mentioned. The pinhole was set to AE unit 1 as default. The voltage applied to the photomultiplier tube (PMT, AKA `detector gain value)` was adjusted experimentally to obtain the best signal to noise ratio. The z-position and electric zoom were chosen accordingly and the series z-position scans. LCS carried out the image maximal projection. Images were acquired using the 10x/0.4 HC

PLAPO CS object lens and the 40x/0.85 HCX PLAPO CS object lens. The 63x/1.2 HCX PLAPO CS and 40x 1.25-0.70 HCX PL APO CS object lenses were used to obtain images where fluorescent proteins were targeted to the nucleus, cytoplasm or any other locations.

## 2.6    RNAi, Ox constructs and rice transformation

To generate RNAi construct for gene suppression, a 300 base pair fragment was amplified by PCR from pVP16-OsRap2.6 with *OsRap2.6* RNAi primers listed in Table 2. The *OsRap2.6* Ox construct was amplified from Open reading frame (ORF) using *OsRap2.6* Ox primers listed in Table 2. The PCR fragments were cloned into Gateway pENTR/D-TOPO cloning vector with two recombination sites (*attL1 and attL2*) for LR clonase reaction. Subsequently, the derived fragments were transferred into pANDA destination vector by recombinase (LR) reactions. The pANDA vector has kanamycin and hygromycin resistance markers suitable for transformation (Miki and Shimamoto, 2004). The insert and vector sequences were confirmed using *Ubiquitin* 1$^{st}$ Intron 5` and 3`, NOST terminator 5` and 3`, GUS linker 5` and 3`and *attribute* B1 and B2 primers listed in Table 2.

## 2.7    Suspension cells, RNAi and over-expressing plants

*OsRap2.6* RNAi and Ox callus were derived from rice Japonica cv. Kimnaze. The seeds were surface sterilised with 1.2% sodium hypochlorite for 45 min, washed in distilled water and placed on Murashige and Skoog (MS) medium

supplemented with 2 mg/1, 2, 4-dichloroacetic acid (2,4-D) (Murashige and Skoog, 1962). Plants were generated by *Agrobacterium tumifaciens* mediated transformation of rice callus as described previously (Miki and Shimamoto, 2004; Hiei et al. 1994). The transformed callus was selected with Rap2.6 forward (5′-TGGCGGCTACTACCCCTCGTCGT-3′) and reverses primers (5′-GAACGATCGGGGAAATTCGAGCTC-3′) as listed in Table 2. The suspension culture derived from callus was maintained in $R_2S$ medium for various analyses (Ohira et al. 1973). The transformed plants were transferred into smaller pots (50 ml) in a growth chamber for a week at 28 $^0$C, and then transplanted into vermiculite and peat moss in larger pots (500 ml) in the greenhouse (25-30 $^0$C, 12 h: 12 h) for two months. OsRap2.6 RNAi plants were screened with Rap2.6 primers listed in Table 2.

## 2.8    RNA extraction and reverse transcription PCR

For the analysis of gene expression, rice calli from the WT suspension cells was treated with chitin 2-ug/ml (Hepta-N-acetylchitoheptaose, Sigma) and harvested at different time intervals, initial sampling being done at an hour followed by consequent sampling every 3 hours up to 24 hrs (Lieberherr et al. 2005). The samples were frozen in liquid Nitrogen and stored at –80$^o$C.  Briefly, RNA was extracted by TRIzol method (Nacalai tesque, Japan). The samples were digested with DNaseI (Takara, Shiga, Japan). Electrophoresis was done in 1.5 % agarose gels in 1 X TBE buffer, at 100V for 30 min. The gels were stained with ethidium bromide for 15 min. Bands were visualized under UV light.

## 2.9 Infection of rice plants with *M. Oryzae*

*OsRap2.6* RNAi and Ox plants were infected with Rice blast fungus (*M. oryzae*) compatible race 007 and incompatible race (031). The fungal growth conditions and the punch infection method were done following a protocol described previously with minor modifications (Kim et al. 2012; Chen et al. 2010a; Kawano et al. 2010). The spores were estimated to contain ~ $1 \times 10^5$ spores per ml. These were inoculated on leaf blades and kept at 23~30°C in the greenhouse. Disease lesions sizes were measured 7 days after inoculation.

Briefly, the two youngest leaf blades were selected for the infection. Six (6) holes were punched per blade in 4 plants giving a total of 48 infected sampling points (n=48). Measuring lesion progression from the sampling points with a digital calliper scored a lesion length quantitatively. The resistance and susceptibility of each plant was compared with the wild type using cv. Kinmaze in four plants. The experiment was repeated three times. The data was analysed for statistical significance in an Excel program. The means and standard errors were separated accordingly. The p-value was determined by a standard t-test (p<0.05). The asterisk and double asterisk indicate significant differences with $P \leq 0.05$ and $P \leq 0.01$, respectively, compared with wild type (WT) data. Photographs were taken accordingly.

## 2.10 DNA extraction

DNA (100mg) from the infected leaf was extracted with 500 $\mu$L of DNA extraction buffer and 500 $\mu$L of phenol/chloroform solution in liquid nitrogen.

The homogenate was spun at 13,000$g$ for 1 min. The supernatant was precipitated with 400 $\mu$L of isopropanol and purified with 500 $\mu$L of 70% ethanol. The mixture was dissolved in 50 $\mu$L of Tris-EDTA (TE buffer) and DNA was stored at -20$^0$C.

## 2.11    Quantitative Real time PCR (qPCR)

The samples for disease severity were analysed using standard curve quantification method whose absolute values were derived from known quantities. DNA template (5 µl) was added to (14.5 µl) of master mix containing SYBR Green Super mix (Bio-Rad) as the reactive and 0.5 µl (5 pmol) of each primer. The qPCR mixture (20 µl) was loaded into Ultra AMP PCR plates and analysed in an ABM Prism, 7000-sequencer detection system (ABM) for 2.5 hours. The cycle at which the fluorescent reached a significant value above background threshold cycle ($C_T$) was in a linear relationship with the logarithm of the target DNA concentration and was determined in four replicates. From the melting curve, it was possible to measure the melting temperature (Tm) of the amplified product.

Fluorescence data was statistically analyzed using Excel spreadsheet for Mac Pro computers. Means were compared using the standard t-tests. To detect *M. grisea* and rice DNAs, two sets of primers against *M. grisea Pot2* and rice *Ubiquitin* were used in real time PCR (Beruyer et al. 2006)*.* The DNA representing the relative number of fungus cells was quantified per plant cell from the infected rice tissues by calculating an infection ratio with the formula

(N: Mgpot2/ N: *Osubiquitin* x 100) (n=48) as described previously (Kawano et al. 2010). DNA was extracted from the infected lesions and analysed qualitatively in real time PCR (ABM sequencer, 7000 plus) using real time *M. grisea Pot2*, *PAL1*, *PBZ1* and *Ubiquitin* primers listed in Table 2.

## 2.12    Data analysis

The data were analyzed using the Excel program (version 10) for Mac book Pro computer (Apple, USA). Differences in the averages, standard errors and means estimated between two samples were determined using Student's *t*-test.

## 2.13    Gene accession numbers

The sequence data used in this work was retrieved from GenBank entries OsRac1 (AB029508), OsRACK1A (D38231), *PBZ1* (D38170), OsMAPK6, (ABI183398) and OsMAPK3 (AF479883) (Kim et al. 2012; Nakashima et al. 2008; Kawasaki et al. 1999).

# Chapter 3: Results

## 3.1    OsRap2.6 AP2/ERF domain resembles Arabidopsis Rap2.6

Proteins that interacted with RACK1A in the rice cDNA library were screened in yeast two hybrid (Y2H) assays. The primary candidate gene (Os04g0398000 or AK101501) had an AP2/ERF domain whose sequence shared 94% amino acid identity with Arabidopsis Rap2.6 (At1g43160) (shadowed region of Figure 1). We therefore named it as OsRap2.6 and selected it for further analysis. The rest candidate genes included hypothetical proteins with a MATH's domain (Os01g0775300), a CaMK11 association domain, (Os01g0753200), or a ToIA/TF11B domain,(Os12g0112600); Universal stress protein (USP) (Os5g0453700) containing a USP domain; and a V1P1 like protein whose domain was unknown (Os01g0698000) (Table 1).

AP2/ERF genes are involved in various crucial roles in biotic and abiotic stresses as well as defense responses (Agarwal et al. 2006). For example, Rap2.6 confers resistance to *Pseudomonas syringae* DC3000 (He et al. 2004). Overexpression of *AtERF1*, tomato *Pti4/5*, or tobacco *OPBP1* enhances disease resistance (Gu et al. 2002; Guo et al. 2004). *OsBIERF3*, a member of the rice ERFs increases disease resistance against infection by tomato mosaic virus and the bacterial wild fire pathogen, *P. syringae pv tabaci*, as well as tolerance to salt stress in tobacco (Cao et al. 2006).

AP2/EREBPs are also involved in virus infections in rice, for instance in rice stripe virus (RSV), rice tungro spherical virus (RTSV) and rice dwarf virus (RDV) (Sharoni et al. 2011). Such evidence of AP2/ERF being part of defense response pathways formed a basis for my initial hypothesis on the possibility of OsRap2.6 being involved in disease resistance pathways in rice.

## 3.2 OsRap2.6 interacts with RACK1A at WD repeats 1 and 2 in yeast two-hybrid assays

The bait constructs, RACK1A, OsRac1 (WT) and (CA and DN) were fused with the bait vector pBTM116ss. The OsRap2.6 coding regions was ligated into pVP16 prey vector. The negative controls were pBTM116ss and pVP16-Empty. The paired plasmids were transformed into yeast *Saccharomyces cerevisiae* (L40), and positive transformants were selected by plating on media lacking uracil and tryptophan. The interaction of bait and prey was analyzed based on the ability to activate transcription of the histidine 3 (HIS3) reporter gene. The yeast colonies that grew on plates lacking histidine and supplemented with 3-amino triazole (-H + 3-AT) at 3mM after 3-5 days at $30\,^{0}$ C were scored as positive. I found a strong interaction between RACK1A and OsRap2.6. However, this interaction was not observed between OsRac1 (WT) and its (CA and DN) mutants. Moreover, no growth of colonies was recorded in the negative controls, pBTM116ss and pVP16 (Figure 2). These results demonstrated that OsRap2.6 interacts specifically with RACK1A in Y2H. I therefore hypothesised that

OsRap2.6 may have a functional similarity with AtRap2.6 or most members in the AP2/ERF family.

According to a previous report, RACK1 binds proteins and interacts with co-chaperones, phosphatases and transcription factors through the seven WD (1-7) repeat (Adams et al. 2011). It was interesting to further analyze for the interaction between OsRap2.6 and tryptophan-aspartate (WD) repeats in Y2H. The full length RWD repeats (WD 1-7) and (WD 3-7) and (WD 1-2) were fused to the bait vector pBTM116ss, while OsRap2.6 was fused to its prey vector pVP16 in Y2H. A strong interaction between OsRap2.6 and WD repeats 1 and 2 was detected (Figure 3). Thus, WD 1 and 2 repeats may be a common binding site for RACK1A, OsRac1 and OsRap2.6 and may possibly act as a potential interaction site or bridge for the three proteins.

## 3.3    OsRap2.6 localizes in the nucleus and the cytoplasm in rice protoplasts

To determine the intracellular localization of OsRap2.6 protein, I tagged OsRap2.6 with Yellow fluorescent protein (YFP) at the N terminus (Venus-OsRap2.6) and expressed the fusion protein in rice protoplasts with internal positive controls mCherry (YFP), and nuclear localization signals NLS-mCerulean and OsGenL-CFP (NLS) controls. I took a total of 50 to 100 live images with a CCD camera connected to the fluorescent confocal microscope (Leica TCS SP5, Solms, Germany). OsRap2.6 localized with mCherry mainly in the nucleus and the cytoplasm (94%) and nucleus alone (6%) (Figure 4A). A further comparison

using NLS-mCerulean showed (90%) of the marker localized within nucleus and in the cytoplasm and (10%) was distributed mainly in the nucleus (Figure 4B). The localization frequency of Venus-OsRap2.6 with mCherry is as shown (Figure 4C). Altogether, OsRap2.6 localized in the nucleus and the cytoplasm in more than (90%) of rice protoplasts.

In Arabidopsis, Rap2.6-YFP and Rap2.6L-YFP (C-terminus) localize to the nucleus. In our study, we found a discrepancy between the protein localizations in rice and in Arabidopsis, although both proteins shared a similar AP2 domain. The likely reason for the difference was that Rap2.6 was tagged to the C-terminus, whereas OsRap2.6 was tagged to the N-terminus. GFP tagging at N-terminus can to some extent affect the localization of a protein whereas tagging GFP at C-terminus is generally better in preserving the localization of the native protein (Palmer et al. 2004). GFP tag can in principle affect protein function, but fortunately, incase of Ras and Rho family proteins, A GFP tag at the N-terminus seems to have little effect on protein targeting and function, however its important to confirm GFP fusion protein data with own endogenous protein (Michaelson, 2006). In another study, GFP fusion did not affect the function of proteins for filament formation, Ura7p/Ura8p, Psa1p, Git1p, and eIF2/2B (Noree et al. 2010).

## 3.4 RACK1A localizes in the nucleus and the cytoplasm in rice protoplasts

I further expressed YFP tagged with RACK1A (mVenus) at the C-terminus in rice protoplasts for 15 hours and examined its intracellular localization with internal positive controls mCherry (YFP), a rice PAMP receptor, OsCERK1-GFP and OsGenL-CFP (NLS). The localization frequencies were analyzed in 50-100 cells expressing YFP or CFP and compared with the controls. RACK1A-mVenus localized mainly in the nucleus and the cytoplasm (CN) (90%) with mCherry (Figure 5A). Moreover, in an independent experiment, RACK1A-mVenus co-localized with OsGenL-CFP (NLS) in the nucleus and the cytoplasm (88%) with the remainder of CFP localized in the nucleus (12%) (Figure 5B). However, a small proportion (6%) was associated with OsCERK1-GFP at the PM (upper panel) and nucleus (Z-stacks) and ER (3%) (lower panel) (Figure 5C). The localization frequency of RACK1A-mVenus is as shown (Figure 5D). These findings further confirmed the ability of RACK1A to localize as a scaffold protein to different parts of the cell.

According to an earlier report, RACK1A localizes in the cytoplasm in rice protoplasts and modulates its defense responses at posttranscriptional levels through its interaction with OsRac1 at the cytoplasm (Nakashima et al. 2008). In another report, RACK1A and OsRac1 shifted to detergent-resistant membranes (DRM), regions near the plasma membrane (PM) after elicitation with chitin (Fujiwara et al. 2009). Moreover, RACK1A associated with heterotrimeric G proteins gamma ($\gamma$)-subunit 2 (RACK1A-AGG2) and localized

at the plasma membrane (PM), the same cellular component where AGG2 apparently localized; however, RACK1A associated with the gamma ($\gamma$)-subunit 1 (RACK1A-AGG1) throughout the cell (Kamil et al. 2011; Adjobo-Hermans et al. 2006). RACK1A modulates its defense responses at posttranscriptional levels through its interaction with OsRac1 at the cytoplasm (Nakashima et al. 2008).

## 3.5 OsRap2.6 and RACK1A interact in the nucleus and the cytoplasm in rice protoplasts

OsRap2.6 and RACK1A localized in the nucleus and the cytoplasm, but the next question was whether the two proteins interact at the same subcellular region or not. We confirmed the *in vivo* interaction using Bimolecular Fluorescence Complementation (BiFC) methods that detect interactions between two proteins in living cells. The absence of an interaction prevents reassembly of the fluorescent protein and results in background fluorescence (Kerpolla, 2009). I split Venus fluorescent protein into two halves (Vn/Vc) and tagged a half (Vn) to OsRap2.6 and the other half (Vc) to RACK1A at N and C-terminus. GUS was used as a negative internal control. The paired constructs (Vn-OsRap2.6 + RACK1A-Vc) and (Vn-OsRap2.6 + GUS-Vc) and their controls mCherry (YFP) and OsGenL-CFP (NLS) were transfected in rice protoplasts independently. Live images were taken after 15 hours. I found a strong interaction between OsRap2.6 and RACK1A in the cytoplasm and nucleus (92%) and the rest (8%) in the nucleus with mCherry. The negative controls, GUS recorded less than 10% fluorescence in all cells. (Figure 6A). The

quantitative analysis of BiFC positive cells is as shown (Figure 6B). A further comparison with OsGenL-CFP (NLS) gave a similar finding (Figure 6C). Our results further confirmed the potential of RACK1A to interact with OsRap2.6 *in vivo*.

As a scaffold protein, RACK1A translocates to different parts of the cell and interacts with different phosphatases and transcription factors (Adams et al. 2011). According to an earlier report, RACK1A localizes in the cytoplasm in rice protoplasts (Nakashima et al. 2008). In another report, RACK1A and OsRac1 shifts to detergent-resistant membranes (DRM), regions near the plasma membrane (PM) after elicitation with chitin (Fujiwara et al. 2009). Furthermore, RACK1A associated with heterotrimeric G proteins gamma ( $\gamma$ )-subunit 2 (RACK1A-AGG2) and localized at the plasma membrane (PM), the same cellular component where AGG2 apparently localized; however, RACK1A associated with the gamma ( $\gamma$ )-subunit 1 (RACK1A-AGG1) throughout the cell (Kamil et al. 2011; Adjobo-Hermans et al. 2006). According to a recent report, RACK1A interacts with Arabidopsis Nudix hydrolase (AtNUD7) in nuclei and the cytoplasm. AtNUD7 expression is induced rapidly in response to an avirulent bacteria and abiotic stresses (Olejnik et al 2011; Kamil et al. 2011). RACK1A forms an interactive complex including OsRac1, RAR1 and SGT1 and maintains an effective conformation, which is able to activate the downstream effectors and lead to an immune response (Nakashima et al. 2008; Thao et al. 2007).

### 3.6 OsRap2.6 interacts with OsMAPK3 and OsMAPK6 in the nucleus and the cytoplasm in rice protoplasts

Mitogen-activated kinase (MAPK) cascades respond to pathogens or pathogen-derived elicitors, for example OsMAPK6 is activated in response to sphingolipid elicitors in rice cell culture (Kim et al. 2012; Lieberherr et al. 2005). Furthermore, OsMAPK3 and OsMAPK6 are involved in defense responses in rice (Kim et al. 2012; Kishi-Kaboshi et al. 2010, Lieberherr et al. 2005). I therefore investigated whether OsRap2.6 interacts with OsMAPK3 and OsMAPK6. The paired constructs (Vn-OsRap2.6 + Vc-OsMAPK6), (Vn-OsRap2.6 + Vc-OsMAPK3) and their negative control (Vn-OsRap2.6 + GUS-Vc) were transfected in rice protoplasts with mCherry for 15 hours. The interaction between OsRap2.6 and OsMAPK6 was in the nuclei and in the cytoplasm (76%) and (24%) in the cytoplasm. The negative controls had less than 10% fluorescence signal (Figure 7A). Quantitative analysis of BiFC positive cells from (OsRap2.6 + OsMAPK6) is shown (Figure 7B). Frequency of interactions between OsRap2.6 and OsMAPKs in cells is shown (Figure 7C). In addition, OsRap2.6 and OsMAPK3 interacted in the cytoplasm and nuclei (72%) and the remainder (28%) of the signal was in the cytoplasm (Figure 8A). Quantitative analysis of BiFC positive cells from (OsRap2.6 + OsMAPK3) is shown (Figure 8B). Together, these results indicate that OsRap2.6 interacts with OsMAPK6 and OsMAPK3 mainly in the nucleus and the cytoplasm with more than (70%) of the cells fluorescencing at the same location.

OsMAPK6 indirectly interacts with CA-OsRac1 in a complex but not with DN-OsRac1 (Lieberherr et al. 2005). A complete MAPK cascade (comprised of MEKK1, MKK4/MKK5 and MPK3/MPK6) was proposed to be downstream of the flagellin receptor kinase, FLS2, in Arabidopsis. This signaling cascade activates WRKY22 and WRKY29 transcription factors (Asai et al. 2002). Suppression of OsMAPK6 expression by RNAi decreased *PAL1* mRNA levels (Lieberherr et al. 2005). RAI1 transcription factor interacts with OsMAPK3 and OsMAPK6 proteins *in vivo* and *in vitro*. Moreover, OsMAPK3/6 and OsMKK4-dd phosphorylate RAI1 *in vitro*. OsBWMK1 was activated in rice leaves after infection with rice blast fungus, elicitor treatment, and wounding (Cheong et al. 2003; He et al. 1999). OsBWMK1 localises in the nucleus and phosphorylates OsEREBP1, an ERF transcription factor (Cheong et al. 2003). From our findings, we hypothesised that OsRap2.6 may be phosphorylated by OsMAPK3/6 to carry out its transcriptional regulation.

## 3.7 Chitin elicitor in rice suspension cells induces OsRap2.6 expression

Suspension cells derived from wild-type japonica cv. Kinmaze rice were treated with chitin (2 μg/ml), and the expression of potential downstream genes, *PAL1, PBZ1* as well as *OsRap2.6* was examined by reverse transcription qPCR. *Ubiquitin* was used as an internal control. *OsRap2.6* transcripts were rapidly increased after chitin treatment and peaked after 1h but by 3hrs there was no further increase. In contrast to

*OsRap2.6*, *PAL1* transcripts peaked after 1 hr and drastically reduced, while *PBZ1* transcripts peaked at 3hrs after chitin treatment (Figure 9A, B and C). In rice, defense genes *PAL1* and *PBZ1* are rapidly induced by rice blast fungus as previously reported (Chen et al. 2010a; Kawano et al. 2010; Nakashima et al. 2008; Kawasaki et al. 1999). This data agreed with recent findings on RAI1, where a gradual increase in *PAL1* and *OsWRKY19* was noted after OsMAPK6 and OsMAPK3 were overexpressed in rice protoplasts (Kim et al. 2012). In another study, transcription factors including Mybs, WRYKs, NACs and AP2s, were induced in leaves infected with blast fungus, indicating the occurrence of transcriptional reprogramming in rice plants after infection (Ribot et al. 2008). The most highly induced genes in a compatible interaction are PR-1 and PR-5 (thaumatin-like proteins), *PBZ1* (probenazole-inducible gene 1; PR-10), and class 11 chitinase (PR-1a) and *PAL1* (Kim et al. 2012; Chen et al. 2010b; Kawano et al. 2010; Kim et al. 2001). *PAL1* is among the 10 most induced genes in response to *M. oryzae* susceptible interactions (Jantasuriyarat et al. 2005). Defense genes *PAL1* and *PBZ1* are rapidly induced by infection with rice blast fungus as previously reported (Chen et al. 2010a; Kawano et al. 2010; Nakashima et al. 2008; Kawasaki et al. 1999).

## 3.8 *OsRap2.6* RNAi are susceptible to *M. oryzae* compatible race 007

I tested if OsRap2.6 contributes to defense responses in rice by *OsRap2.6* RNAi and over-expression (Ox). *OsRap2.6* mRNA transcripts from three independent RNAi were confirmed by real time PCR (R1, R5 and R10) (Figure 10A). The RNAi and Ox were grown in the greenhouse for two months and inoculated with *M. oryzae* compatible (virulent) Ina 86-137 (race 007) and incompatible (a virulent) TH67-22 (race 031) fungus. From the findings, *OsRap2.6* RNAi showed high susceptibility characterised by larger disease lesions in compatible race (007) as compared to non-transformed plants (WT) as shown in the photograph (Figure 10B), real time PCR analysis of fungal growth ($p \leq 0.01$, n=48) (Figure 10C) and lesion lengths ($p \leq 0.01$, n=48) (Figure 10D). The *PAL1* transcripts were down regulated ($p \leq 0.01$) in selected *OsRap2.6* RNAi plants (Figure 10E). Together, these results demonstrated that fungal growth was enhanced in *OsRap2.6* RNAi as compared to the non-transformed plants. These results suggested that OsRap2.6 contributes to defense responses towards compatible rice blast fungus.

I also investigated if *OsRap2.6* RNAi contributes to increased susceptibility to an incompatible blast fungus race (031) in a similar approach as described for the compatible race. From the findings, the susceptibility to the incompatible blast fungus in RNAi was not significant as shown in the photograph (Figure 12A), real time PCR ($p \geq 0.05$, n= 48) (Figure 12B) and relative lesion length ($p \geq 0.05$, n= 48) (Figure 12C) except in (R1). Expression

of the *PAL1* gene was not significantly reduced in *OsRap2.6* RNAi (p ≥ 0.05) (Figure 12D) except in R10. Therefore, in general not many RNAi showed significant differences. Therefore, our results could suggest that *OsRap2.6* RNAi does not contribute to defense responses in ncompatible interactions.

### 3.9 *OsRap2.6* Ox increases resistance to a compatible race of *M. oryzae*

I further tested if *OsRap2.6* contributes to defense responses in overexpressing plants. I first confirmed the level of OsRap2.6 in at least three independent overexpressing plants by real time PCR (qPCR) (P4, P6 and P14) (Figure 11A). The plants were infected with the rice blast fungus compatible race, 007. From the findings, smaller disease lesions were observed in *OsRap2.6* Ox as compared to the WT as shown in the photograph (Figure 11B), real time PCR ($p \leq 0.01$, n= 48) (Figure 11C) and relative lesion length ($p \leq 0.01$, n= 48) (Figure 11D). The *PAL1* gene was up regulated ($p \leq 0.01$) (Figure 11E). Therefore, *OsRap2.6* Ox showed increased resistance to rice blast fungus compatible interactions.

I also investigated if *OsRap2.6* Ox is resistant to incompatible rice blast fungus race, 031. The only significant resistance noted was only in a single Ox line and the rest were insignificant as shown in the photograph (Figure 13A), real time PCR ($p \geq 0.05$, n= 48) (Figure 13B), and relative lesion length measurements ($p \geq 0.05$, n= 48) (Figure 13C). Expression of the *PAL1* gene did not significantly increase after infection ($p \geq 0.05$) except in (P6) (Figure 13D). Therefore, in general *OsRap2.6* Ox does not contribute to disease resistance in incompatible interactions.

**Chapter 4**


**4.0    Discussion**

This study aimed at understanding the dynamics of OsRap2.6 transcription factor in rice protoplasts and in plants. The most significant finding of this study was for the first time, a transcription factor, OsRap2.6 that interacts with RACK1A and contributes to defense responses in rice was identified. OsRap2.6 AP2/ERF domain resembled Arabidopsis AtRap2.6 (Figure 1). Such evidence of AP2/ERF being part of defense response pathways formed a basis for my initial hypothesis on the possibility of OsRap2.6 being involved in disease resistance pathways in rice. To confirm this hypothesis, I used *OsRap2.6* knockdown (RNAi) and Overexpressing (Ox) transgenic plants. Although various transgenic plants are available to date, complete resistance towards the blast fungus is yet to be achieved successfully. Partial resistance which involves a multiple interaction of several genes has however been reported in rice as well as other plants. In our study, *OsRap2.6* RNAi plants showed increased susceptibility, but when overexpressed, *OsRap2.6*-Ox plants increased resistance towards the blast fungus. Like its counterparts in the AP2/ERF family, the results demonstrated that OsRap2.6 could be a positive regulator of defense related transcription. Furthermore, *OsRap2.6* transcripts and other known defense genes, *PAL1* and *PBZ1* were induced rapidly in the wild type suspension cells in response to chitin treatment (Figure 9). However, the *PAL1* gene was up

regulated faster than *PBZ1*, which started to increase at 3 hrs upon chitin treatment. This trend was also confirmed in the plants during blast fungus infection in *OsRap2.6* RNAi (Figure 10E) and Ox (Figure 11E) in compatible race, 007. Plant defense genes are to a large extent induced in compatible and incompatible interactions, although the kinetics and amplitude of response is attenuated during compatible interactions (Tao et al. 2003; Vergne et al. 2007). In this study, *PAL1* and *PBZ1* were less attenuated in incompatible interactions in RNAi (Figure 12D) and Ox plants (Figure 13D). Moreover, overexpressing RACK1A increased resistance towards *M. oryzae* compatible interactions and *PAL1* and *PBZ1* genes were up regulated (Nakashima et al. 2008). Taken together, OsRap2.6 is involved in rice defense-signalling pathway.

The next question was, how could a transcription factor such as OsRap2.6 be involved in rice defense pathway and what was the mechanism involved? In an attempt to answer this question, I used yeast two-hybrids assays to confirm the possibility of OsRap2.6 interacting with RACK1A and OsRac1, the two main components in the rice defensome complex as outlined in a previous report (Chen et al. 2010b, Nakashima et al. 2008). A strong interaction between OsRap2.6 and RACK1A was noted, however, this interaction did not occur with OsRac1 WT or its mutants (CA and DN) (Figure 2). I further confirmed the interaction between OsRap2.6 and RACK1A using another approach BiFC, which detects interactions between two proteins, interacts in a living cell. Apparently, OsRap2.6 interacted with RACK1A in the nucleus and the cytoplasm (Figure 6), the same place where RACK1A localised (Figure 7). As a

scaffold protein, RACK1A translocates to different parts of the cell and interacts with different phosphatases and transcription factors (Adams et al. 2011). RACK1A modulates its defense responses at posttranscriptional levels through its interaction with OsRac1 at the cytoplasm (Nakashima et al. 2008). It was of prime importance to note that OsRap.6 and RACK1A interact and localize in the nucleus and the cytoplasm.

To examine the possibility of nuclear localization of OsRap2.6, I fused OsRap2.6 with YFP at the N terminus and transfected the construct (Venus-OsRap2.6) transiently in rice protoplasts. Surprisingly, OsRap2.6 also localized to the nucleus and the cytoplasm (Figure 4). Overall, the cell nucleus remains a major target, where target signals lead to the transcriptional activation of a large array of defense genes (Maleck et al. 2000). As expected, transcription factors have a nuclear localisation signal (NLS), which is sufficient to target them to the nucleus. In Arabidopsis, Rap2.6-YFP and Rap2.6L-YFP (C-terminus) had been shown to localize to the nucleus. Rap2.6 acts as a transactivator in yeast and localizes within nuclei in onion epidermal cells. Putative nuclei localization signal sequence (RPPKKYRGY), which indicates a possible nuclear localization, is found near the AP2 domain (Zhu et al. 2010).

The next question was, how is OsRap2.6 able to translocate to the nucleus for transcriptional regulation? One possibility is a MAPK is involved in the phosphorylation process. Earlier on, it was reported that OsMAPK6 indirectly interacts with CA-OsRac1 in a complex but not with DN-OsRac1 and its suppression of OsMAPK6 expression by RNAi decreased *PAL1* mRNA (Lieberherr

et al. 2005). Again, RAI1, a bHLH transcription factor downstream of OsRac1 interacted with OsMAPK3 and OsMAPK6 proteins *in vivo* and *in vitro*. Moreover, OsMAPK3/6 together with OsMKK4-dd phosphorylated RAI1 *in vitro*. A complete MAPK cascade (comprising of MEKK1, MKK4/MKK5 and MPK3/MPK6) was proposed downstream of the flagellin receptor kinase, FLS2, in Arabidopsis. This signaling cascade activates WRKY22 and WRKY29 transcription factors (Asai et al. 2002). OsBWMK1 was activated in rice leaves after infection with rice blast fungus, elicitor treatment, and wounding (Cheong et al. 2003; He et al. 1999). OsBWMK1 localizes in nuclei and phosphorylates OsEREBP1, an ERF transcription factor (Cheong et al. 2003). Transcription factors get phosphorylated to move to the nucleus, but until now, it is not known as yet which kinase is involved to phosphorylate OsRap2.6 to the nucleus. But as discussed earlier, RAI1, a bHLH transcription factor is phosphorylated by OsMAPK3/6 to enter in the nucleus (Kim et al. 2012). I therefore further hypothesised that OsRap2.6 might be phosphorylated by a similar OsMAPK3/6 to carry out its transcriptional regulation. However, due to some logistics, I could only confirm the interaction in BiFC. I used OsRap2.6 and OsMAPK3 and OsMAPK6 fused to half Venus at N and C termini and confirmed the interaction in BIFC as described in methods. A strong interaction was detected between OsRap2.6 and MAPK3 and OsRap2.6 and MAK6 in nucleus and in the cytoplasm (Figure 8 and 9). ). This interaction could suggest a big possibility of OsRap2.6 being phosphorylated by these OsMAPKs however; an invitro kinase phoshorylation assay may be required in future.

**Chapter 5**

## 5.0    Conclusions

Our study confirmed the role of OsRap2.6 in disease resistance in rice blast fungus, and its localization and interaction with RACK1A and MAPK3/6 in rice protoplasts. OsRap2.6 possibly localizes to the nucleus when the cell is active in defense reactions, (during transcriptional regulation), and in the cytoplasm (during normal conditions) or (after a stimulus) like chitin or a fungus is sensed. It is also likely that OsRap2.6 undergoes post-translational modifications in the nucleus that allows it to interact with RACK1A in the cytoplasm. OsRap2.6 localization in the nuclei is significant to its role as a transcription factor; furthermore, its interaction with RACK1A is likely to enable it be involved in disease resistance in rice. The interaction with MAPK3/6 could potentially phosphorylate OsRap2.6 for transcriptional regulation, a step that is yet to be confirmed. We found OsRap2.6 to be a potential positive regulator in *M. oryzae* compatible interactions possibly as a downstream signal of RACK1A. This study has opened up other areas for further research such as analysis of OsRap2.6 target genes in the defense response pathway.

This study has therefore opened up other avenues for further research, which can be summarised in the model shown (Figure 14). The defense model in rice could be described as follows: upon the infection by pathogens, rice cells can recognize the components of pathogens via specific receptors at the plasma membrane or the cytoplasm. Following such recognition, intracellular regulators

are activated and which in turn transduce the signals to the small GTPase OsRac1. The active OsRac1 acts as a molecular switch to turn on the downstream effectors such as the enzymes involved in synthesis of defense related substances, and possibly transcription factors (Kim et al. 2012). This process is likely regulated by the formation of a transient protein complex composed of various effector proteins of osRac1 and the molecular chaperones recruited by OsRac1. In this way, the OsRac1 pathway finally leads to the onset of defense response against pathogens in rice. Identification of these components involved in the OsRac1 signaling pathway sheds a light on the molecular mechanisms for understanding of small G protein mediated defense response against pathogens in rice. However, there are still many questions as to the identity of the defense components, which will likely refine the current model (Nakashima et al. 2008) in the future.

Taken together, the main progress on the small GTPase mediated rice disease resistance is reflected by the findings of critical components involved in the OsRac1 mediated immune responses. Although the OsRac1 signaling pathway is not completely understood now, the accumulated data could allow us to propose tentatively a working model for OsRac1 mediated signal transduction pathway in rice immune responses. In our model, OsRac1 interacts with RACK1A which inturn interacts with OsRap2.6. OsRap2.6 further interacts with osMAPK3 and OsMAPK6. These two OsMAPKs also interact with OsRac1 (Liebherr et al. 2005).

# Chapter 6

## 6.0    Future perspectives

In the current study, *OsRap2.6* RNAi showed an increased susceptibility while Ox plants had an enhanced resistance in the tested lines in compatible interactions. This was, however, not observed in incompatible interactions, suggesting that *OsRap2.6* mutants follow different mechanisms to suppress rice blast infections in compatible and incompatible interactions. Further study may be required to elucidate the pathways in incompatible interactions in rice and compatible interactions in other crops.

The dynamics of how OsRac1 defense response cascades occur is not fully understood to date. For instance, OsRac1 is localized at the plasma membrane (Ono et al. 2001; Kawano et al. 2010), while RACK1A is mainly in the cytoplasm (Nakashima et al. 2008). We found OsRap2.6 to interact with RACK1A in the nucleus and cytoplasm, the same places they localized. Based on an earlier report, RACK1A can translocate to the plasma membrane upon elicitation (Fujiwara et al. 2009). It maybe important in future to use other methods such as coimmunoprecipitation assays to further elucidate the components involved in OsRac1 cascades downstream of RACK1A.

We observed that OsMAPK3 and OsMAPK6 localise at the cytoplasm and the nucleus, which agreed with recent findings (Kim et al. 2012). In Arabidopsis MAPK6 localizes to the plasma membrane, the pre-prophase band, the phragmoplast, and the trans-Golgi network (Müller et al. 2010). Together,

our results suggest that OsMAPK3 and OsMAPK6 might act as signaling intermediates between OsRac1 and OsRap2.6. Whether this process occurs naturally or after activation of OsRac1 by PAMP elicitors or fungus is still in question. Therefore, further studies on the translocation of OsMAPK3/6 in rice cells in response to elicitor treatment will be helpful to understanding the signalling mechanism by OsMAPK3/6 that occurs between OsRac1 and OsRap2.6. Also, OsMAPK3 forms a complex with both OsRac1 WT and CA-OsRac1 in protoplasts (Kim et al. 2012). However, in our yeast two-hybrid assays we did not detect such an interaction, possibly this complex is formed indirectly via RACK1A. This tricomplex formation is yet to be confirmed, although we found a strong interaction between bicomplexes RACK1A and OsRap2.6. Recently it was suggested that OsMAPK3 might interact with the OsRac1 complex under inactivated or less activated conditions, while OsMAPK6 might function only when OsRac1 is activated. Further studies are needed to clearly elucidate the exact functions of OsMAPK3 and OsMAPK6 in the OsRac1-RACK1A-OsRap2.6 signaling pathway. Whether OsMAPK3 and OsMAPK6 phosphorylate OsRap2.6 to the nucleus still remains a possibility.

Table 1: Proteins that interacted with RACK1A in Yeast two-hybrids assays.

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| Protein name | Clone identity | Number of clones | Domain |
|---|---|---|---|
| OsRap2.6 | Os04g0398000 | 1 | AP2 domain |
| Hypothetical protein | Os01g0775300 | 1 | MATH domain |
| Hypothetical protein | Os01g0753200 | 4 | CaMK11 association domain |
| Hypothetical protein | Os12g0112600 | 1 | ToIA/TF11B domain |
| Universal stress protein | Os5g0453700 | 17 | USP domain |

Table 2: Primers used in the study

| Primer | Sequence |
| --- | --- |
| Rap2.6 forward | (5'-TGGCGGCTACTACCCCTCGTCGT-3') |
| Rap2.6 reverse | (5'-GAACGATCGGGGAAATTCGAGCTC-3'). |
| Ubq 1st intron forward | 5`-GCTCTAACCTTGAGTACCTATCTA |
| Ubq 1st intron forward | 3`-TAGCCCTGCCTTCATACGCTATT |
| Nos terminator reverse | 5`-CCATCTCATAAATAACGTCATGCAT |
| Nos terminator reverse | 3`-TACATGCTTAACGTAATTCAACAGA |
| GUS linker forward | 5`-CGTCGGTGAACAGGTATGGAATT |
| GUS linker forward | 3`-TTGCGACCTCGCAAGGCATATT |
| GUS linker reverse | 5`-CACGTAAGTCCGCATCTTCATGA |
| GUS linker reverse | 3`-GTGGTTAATCAGGAACTGTTCGC |
| *attB1* forward | 5`-AGTTTGTACAAAAAAGCAGGCTCC |
| *attB2* reverse | 3`-ACTTTGTACAAGAAAGCTGGGTCG |
| Real time *PAL* forward | 5`-TGAATAACAGTGGAGTGTGGAG-3` |
| Real time *PAL* reverse | 5`-AACCTGCCACTCGTACCAAG-3` |
| Real time *PBZ1* forward | 5`-ATGAAGCTTAACCCTGCCGC-3` |
| Real time *PBZ1* reverse | 5`-GTCTCCGTCGAGTGTGACTTG-3` |
| Real time *ubiquitin* forward | 5`-AACCAGCTGAGGCCCAAGA-3` |

| Real time *ubiquitin* reverse | 5`-ACGATTGATTTAACCAGTCCATGA-3` |
|---|---|
| *M. grisea Pot2* forward | 5`-ACGACCCGTCTTTACTTATTTGG-3` |
| *M. grisea Pot2* reverse | 5`-AAGTAGCGTTGGTTTTGTTGGAT-3` |
| *OsRap2.6* forward | 5`-CACCCGGCACCTGGACAGAACAGATCA-3' |
| *OsRap2.6* reverse | 5 -'AGAATCCTCTCTCTTGCTTTACTTGGAC-3' |
| *Rap2.6* forward | 5`-GAGCCTGACCTATTGCATCTCC-3' |
| *Rap2.6* reverse | 5'-GGCCTCCAGAAGAAGATGTTGG-3' |
| pB12221-35S forward | 5`-ACTGACGTAAGGATGACGC-3` |
| NOST terminator | 5`-GATAATCATCGCAAGACCG-3` |
| *OsRap2.6* Ox forward | 5'-CACCATGGTCACCGCGCTAGCCCACGTCA-3' |
| *OsRap2.6* Ox reverse | 5'-GAACGATCGGGGAAATTCGAGCTC-3' |

```
AtRap2.6 .seq      1 MVSMLTNVVSGETE-----PSASATWTMGHKREREEF-SLPPQPLITGSAVTKECESSMS      54
OsRap2.6 .seq      1 MVTALAHVIRAAPDLHLPHHPSSSASAAAHPQQASSFVPTAAAAASSPSDQLAAAAAAEE      60

AtRap2.6 .seq     55 LERPKKVRGVRQRPWGKWAAEIRDPHKATRVWLGTFETAERAARAYDRAALRFRGSKAKL     114
OsRap2.6 .seq     61 QGRRHYRGVRQRPWGKWAAEIRDPKKARRVWLGTFDTAEDAAIRYDERALRFKGTKAKL     120

AtRap2.6 .seq    115 NFPENVGTQTIQRNSHFLQNSMQPSLTYIDQCPTLLSYSRCMEQQDPLVGMLQPTEEENH     174
OsRap2.6 .seq    121 NFPERVQGRTDLGFLVTRGIPPAATHGGGYVPSSSPAAGACPPPRQQQTVVPYPDLMRYA     180

AtRap2.6 .seq    175 FFEKPWTEYDQYNYSSFG------------------------------------------     192
OsRap2.6 .seq    181 QLLQGGVGGSYMPFGGAATMSSSTVSSSSAPQILDFSTQQLIRAGPPSPMPSSGSGSATA     240

AtRap2.6 .seq    192 -----------------------------                                192
OsRap2.6 .seq    241 AASSTTSASSPGAWPYGGSERKKKDSSS                                 268
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**Figure 1: Comparison of amino acids sequences of rice and Arabidopsis Rap2.6.**

 A BLAST search done in rice and Arabidopsis genome database revealed that OsRap2.6 (AK101501) AP2/ERF domain has 94% amino acid identity with Arabidopsis Rap2.6 (At1g43160) as depicted by an amino acid alignment of the sequences (shadowed region marked by a red arrow).

58
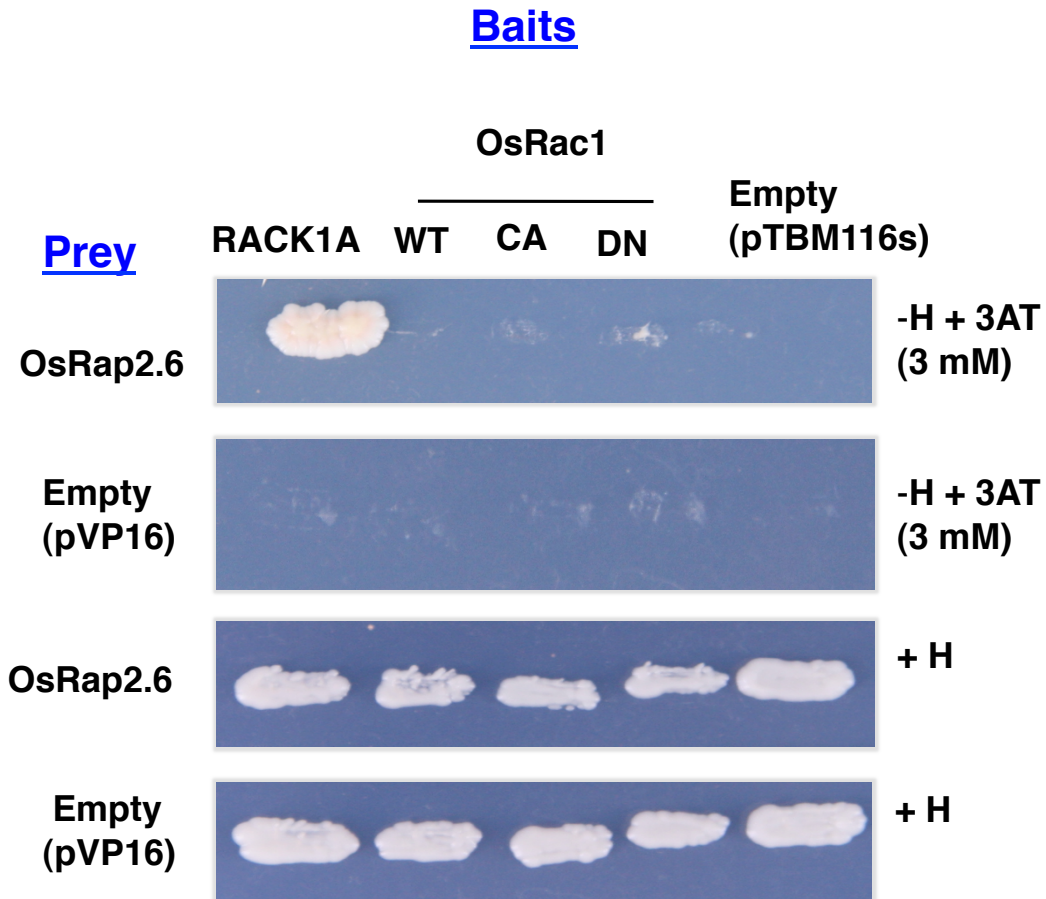
**Figure 2**: Interaction of OsRap2.6 with RACK1A in yeast two-hybrid assays. OsRac1 (WT), constitutively active (CA) and dominant negative (DN)-OsRac1 mutants were examined.

The entry clones, pENTR-RACK1A, OsRac1 (WT) and (CA and DN) were fused with pBTM116ss bait vector while OsRap2.6 coding regions were ligated into pVP16 prey vector. The paired plasmids were transformed into yeast *Saccharomyces cerevisiae* (L40). The positive transformants were selected by plating on media supplemented with 3-amino triazole (-H + 3-AT) at 3 mM. pBTM116ss and pVP16 plasmids fused to empty were used as negative controls.

**bait RACK1A**



**Prey**

**OsRap2.6**

WD 1-7

WD 3-7

**WD 1-2**

**Empty vector**

**-H + 3 AT (3 mM)**
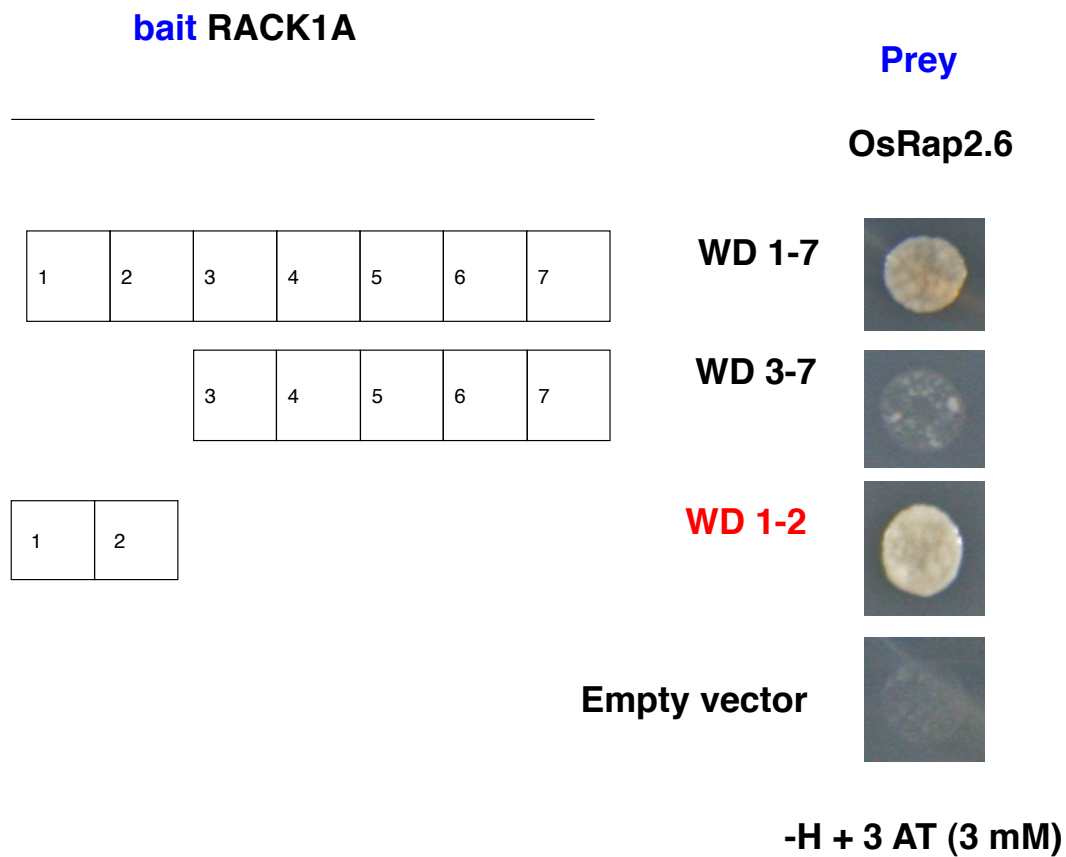
**Figure 3: Interaction of RACK1A with OsRap2.6 at WD repeats 1-2 in yeast two-hybrid assays.**

The entry clones, pENTR-RACK1A (WD 1-7), (WD 3-7), (WD 1-2), were fused with the bait vector pBTM116ss while OsRap2.6 coding regions were ligated into pVP16 prey vector. The paired plasmids were transformed into yeast *Saccharomyces cerevisiae* (L40) and selected on 3-AT media at 3 mM.
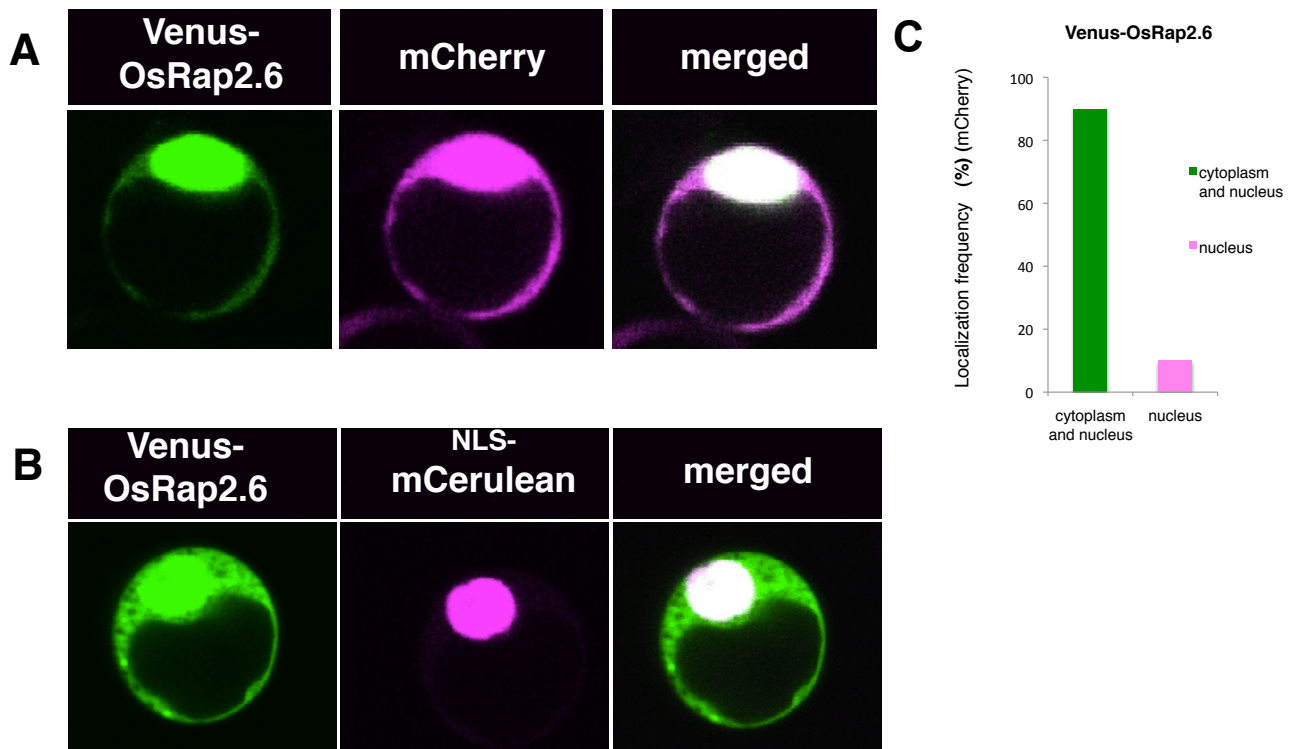
**Figure 4: Subcellular localization of OsRap2.6 in rice protoplasts**

Rice protoplasts were transformed with known fluorescent proteins mCherry (YFP) and NLS-mCerulean constructs. Fluorescence was detected using a CCD camera connected to a confocal microscope. The localization frequency of the cells was analyzed in 50–100 cells expressing YFP/CFP as compared to the positive controls using Excel. Means and standard deviations were separated using Student's $t$-test ($p<0.05$).
(A) Subcellular localization of Venus-OsRap2.6 with the mCherry (YFP).
(B) Subcellular localization of Venus-OsRap2.6 with the NLS-mCerulean.
(C) Localization frequency of Venus-OsRap2.6.
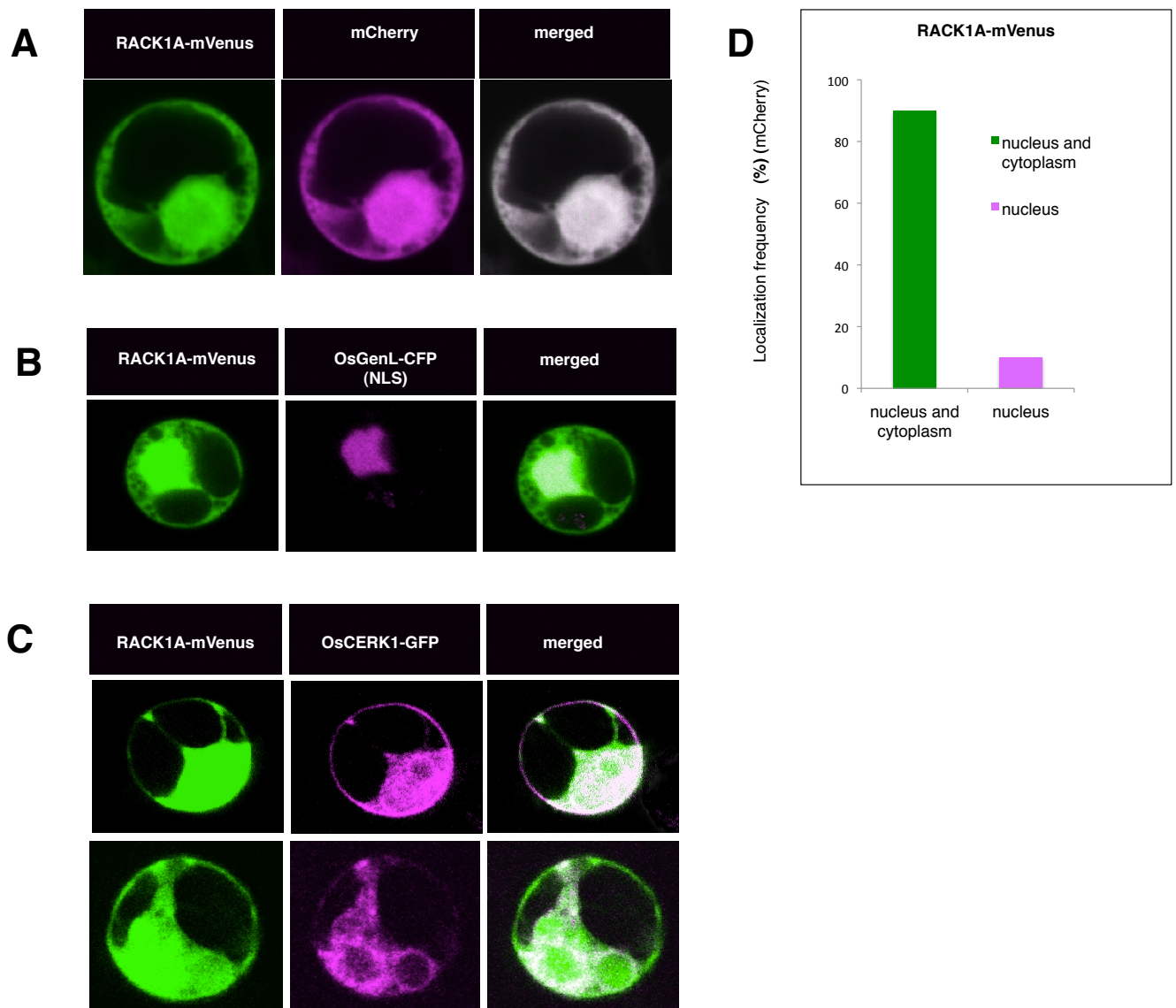
**Figure 5: Subcellular localization of RACK1A in rice protoplasts**

Rice protoplasts were transformed with a plasmid harboring the RACK1A-mVenus construct. Protoplasts transformed with known fluorescent proteins mCherry, OsCERK1-GFP and OsGenL-CFP (NLS) were used as positive controls. Conditions for microscopy and data analysis were identical to those outlined in the legend to Figure 4.
(A) Subcellular localization of RACK1A-mVenus with mCherry (YFP).
(B) Subcellular localization of RACK1A-mVenus with OsGenL-CFP (NLS).
(C) Subcellular localization of RACK1A-mVenus with OsCERK1-GFP.
(D) Localization frequency of RACK1A-mVenus.
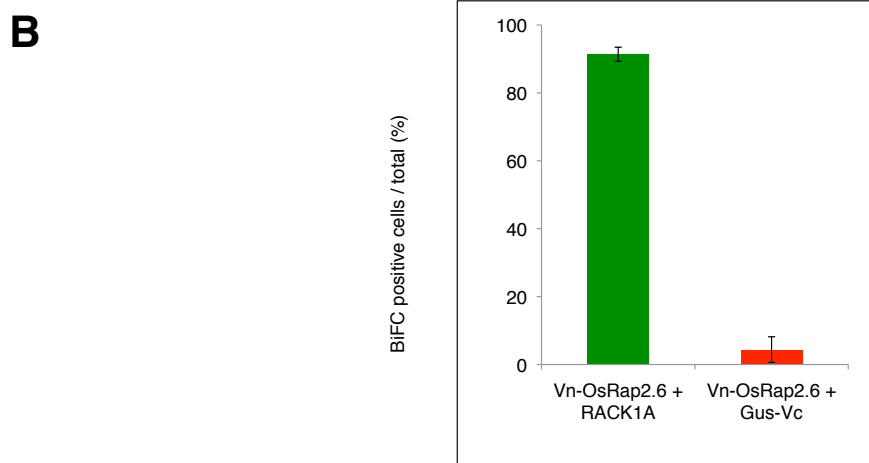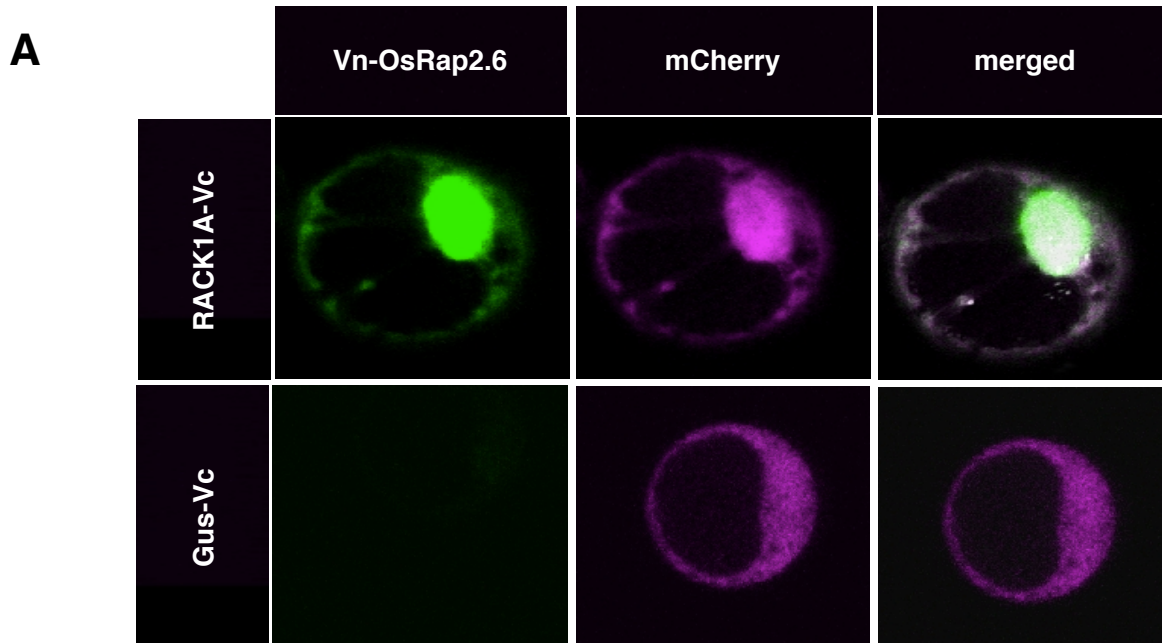
**A**

| | Vn-OsRap2.6 | mCherry | merged |

RACK1A-Vc

Gus-Vc

**B**

BiFC positive cells / total (%)

Vn-OsRap2.6 + RACK1A    Vn-OsRap2.6 + Gus-Vc

**Figure 6: Interaction of OsRap2.6 with RACK1A in rice protoplasts**

Rice protoplasts were co-transfected with the fluorescent construct (Vn- OsRap2.6 + RACK1A-Vc/GUS-Vc). Conditions for microscopy and data analysis were identical to those outlined in the legend to Figure 4
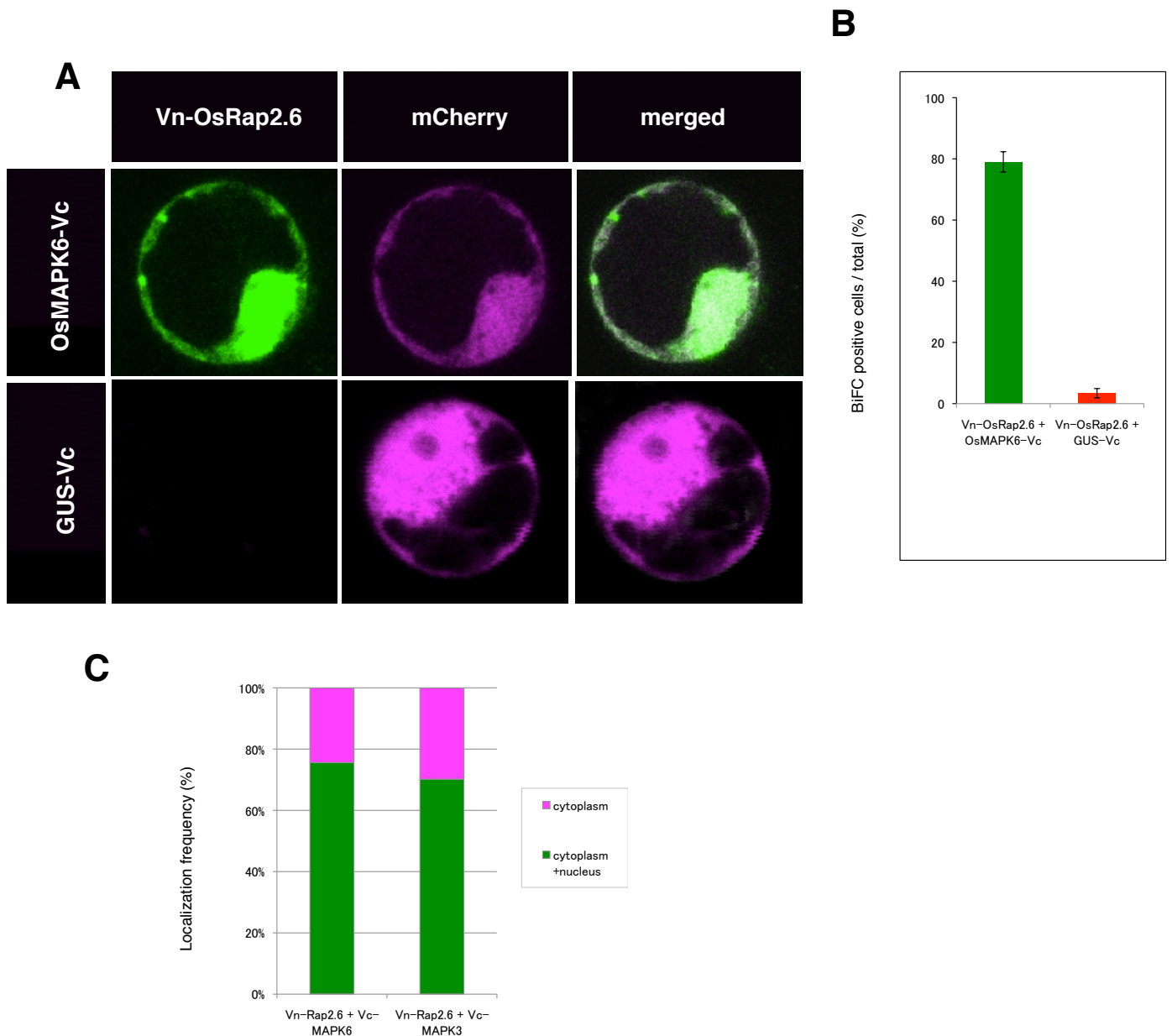
(A) Interaction of OsRap2.6 with RACK1A compared with the mCherry (YFP) construct.
(B) Quantitative analysis of BiFC positive cellS.
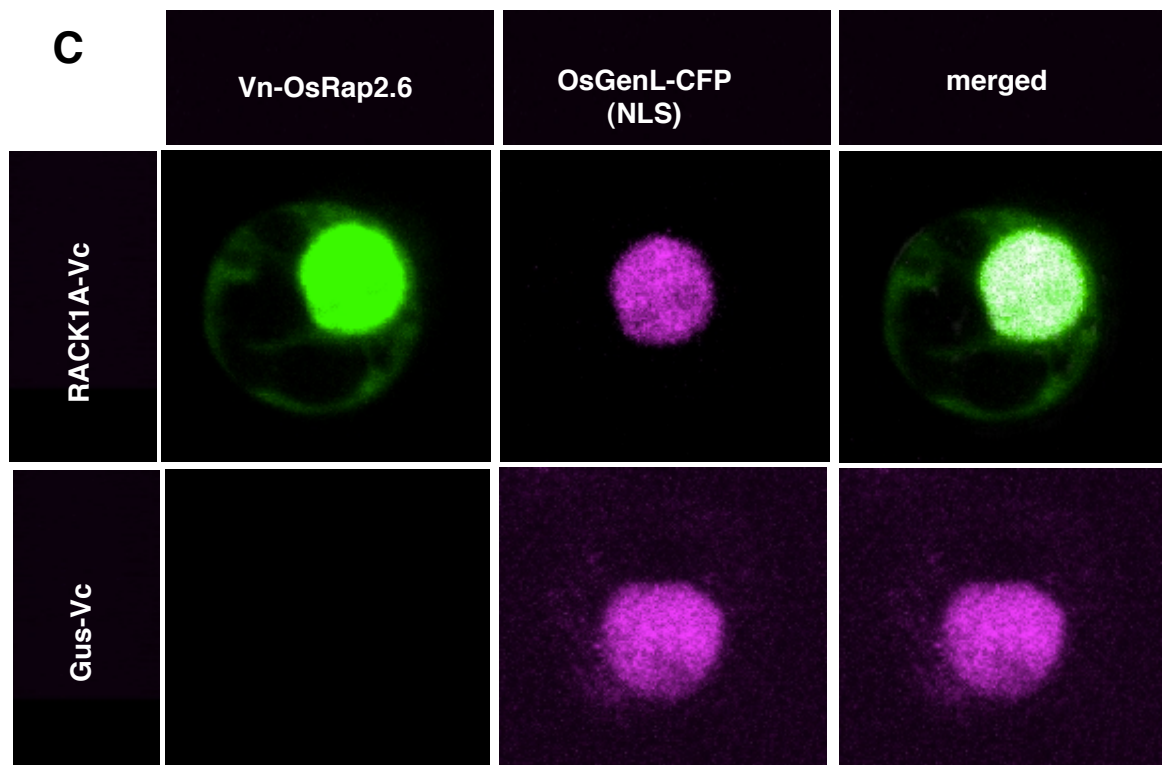
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**Figure 6**: Interaction of OsRap2.6 with RACK1A in rice protoplasts

Rice protoplasts were co-transfected with the fluorescent construct (Vn- OsRap2.6 + RACK1A-Vc/GUS-Vc). Conditions for microscopy and data analysis were identical to those outlined in the legend to Figure 4.
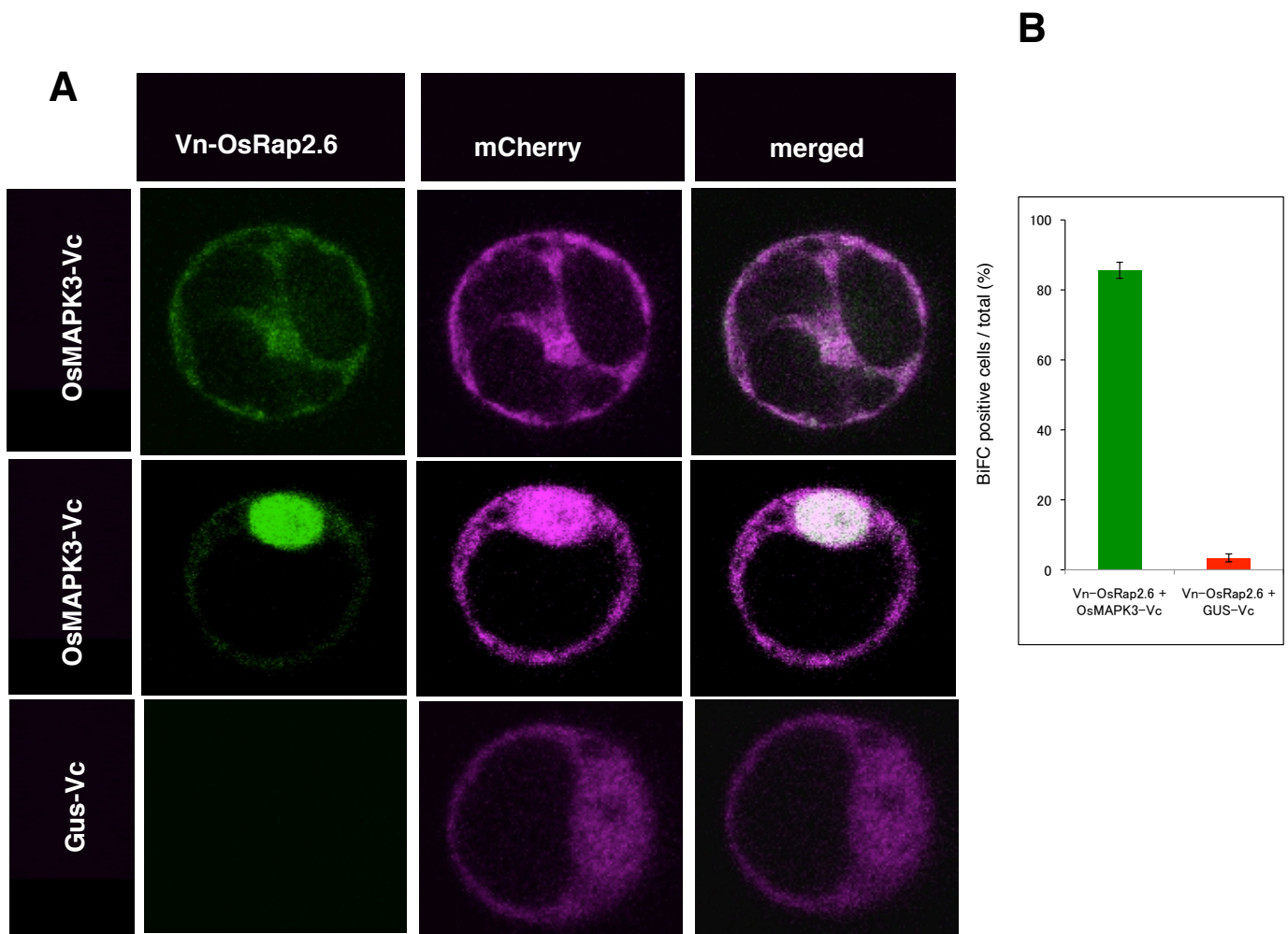
(C) Interaction of OsRap2.6 with RACK1A compared with the OsGenL-CFP construct.

**Figure 7: Interaction of OsRap2.6 with OsMAPK6 in rice protoplasts**

Rice protoplasts were co-transfected with the fluorescent constructs (Vn-osRap2.6 + Vc-OsMAPK6)) and examined under fluorescence, bright field and overlay. Protoplasts transformed with known fluorescent proteins mCherry, OsCERK1-GFP and OsGenL-CFP (NLS) were used as positive controls. Conditions for microscopy and data analysis were identical to those outlined in the legend to Figure 4.

(A) Interaction between OsRap2.6 + OsMAPK6 compared with the mCherry.

(B) Quantitative analysis of BiFC positive cells.

(C) Frequency of interactions between OsRap2.6 and OsMAPKs in cells.

**Figure 8: Interaction of OsRap2.6 with OsMAPK3 in rice protoplasts**

Rice protoplasts were co-transfected with the fluorescent constructs (Vn-OsRap2.6 + Vc-OsMAPK3) and examined under fluorescence, bright field and overlay. Protoplasts transformed with known fluorescent proteins mCherry, OsCERK1-GFP and OsGenL-CFP (NLS) were used as positive controls. Conditions for microscopy and data analysis were identical to those outlined in the legend to Figure 4.
(A)  Interaction between OsRap2.6 and OsMAPK3 compared with the mCherry.
(B)  Quantitative analysis of BiFC positive cells from (OsRap2.6 + OsMAPK3).

66
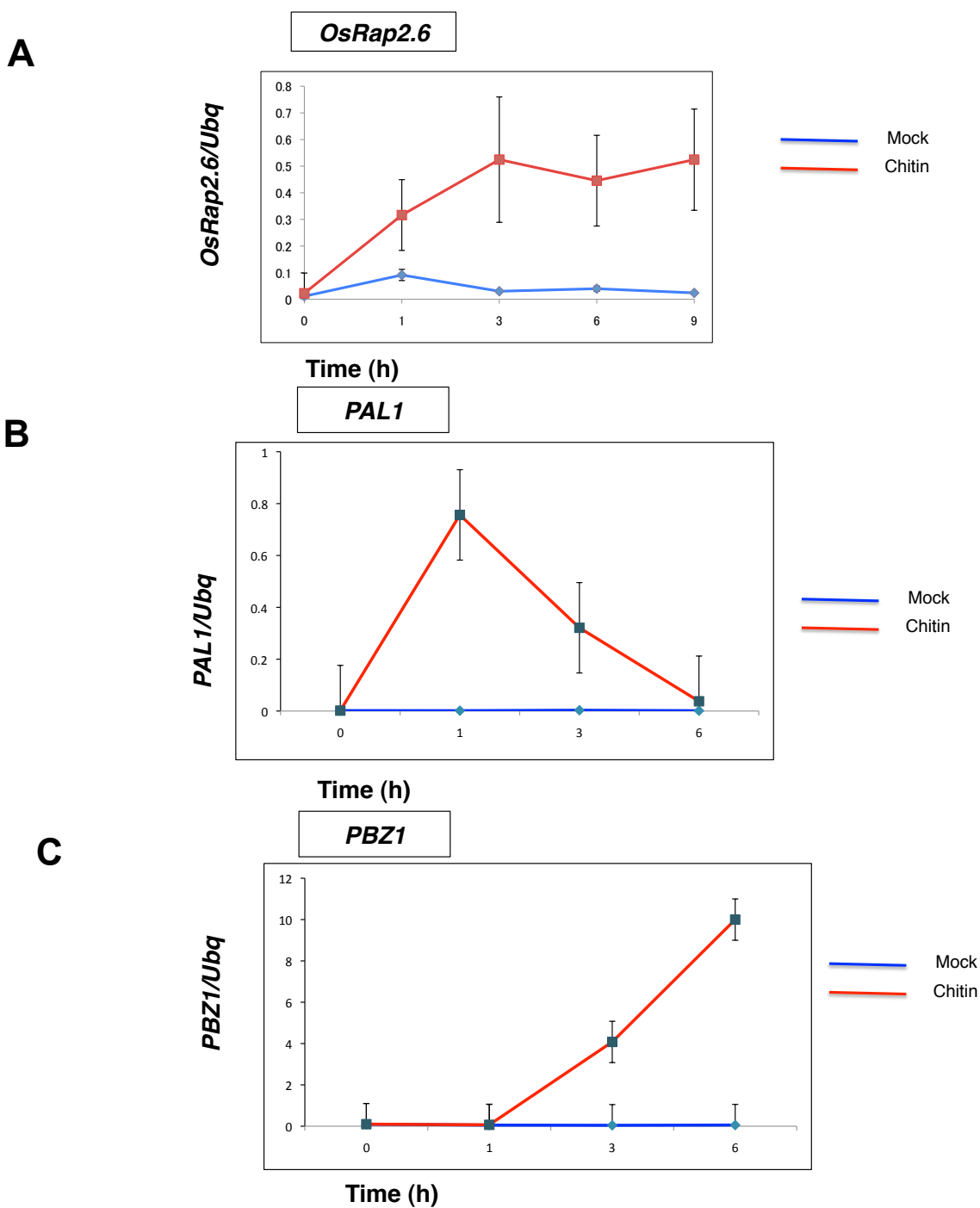
**Figure 9:** Induction of *OsRap2.6*, *PAL1*, and *PBZ1* expression induced by chitin in rice suspension cells.

(A)  *OsRap2.6* expression in WT suspension cells after chitin treatment measured by reverse transcription qPCR. *Ubiquitin* was used as an internal control.

(B)  *PAL1* expression in WT suspension cells after chitin treatment measured by reverse transcription qPCR.

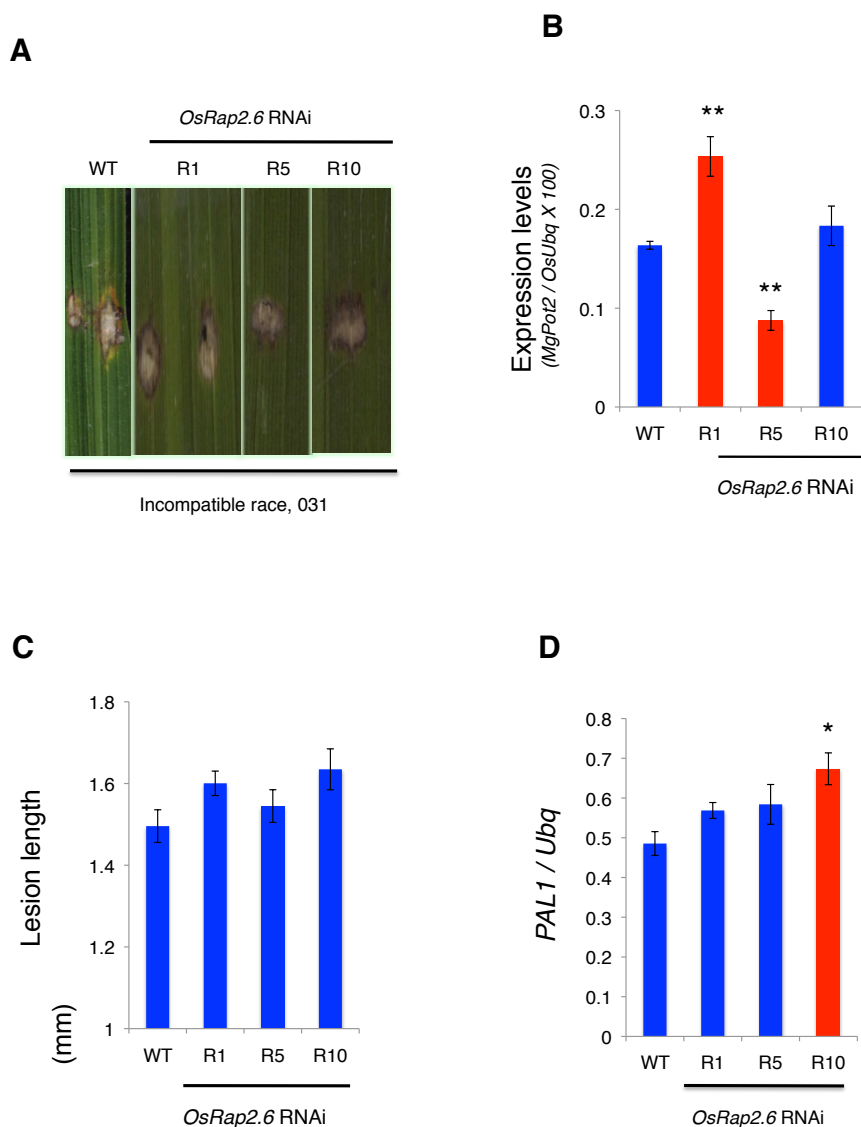(C)  *PBZ1* expression in WT suspension cells after chitin treatment measured by reverse transcription qPCR

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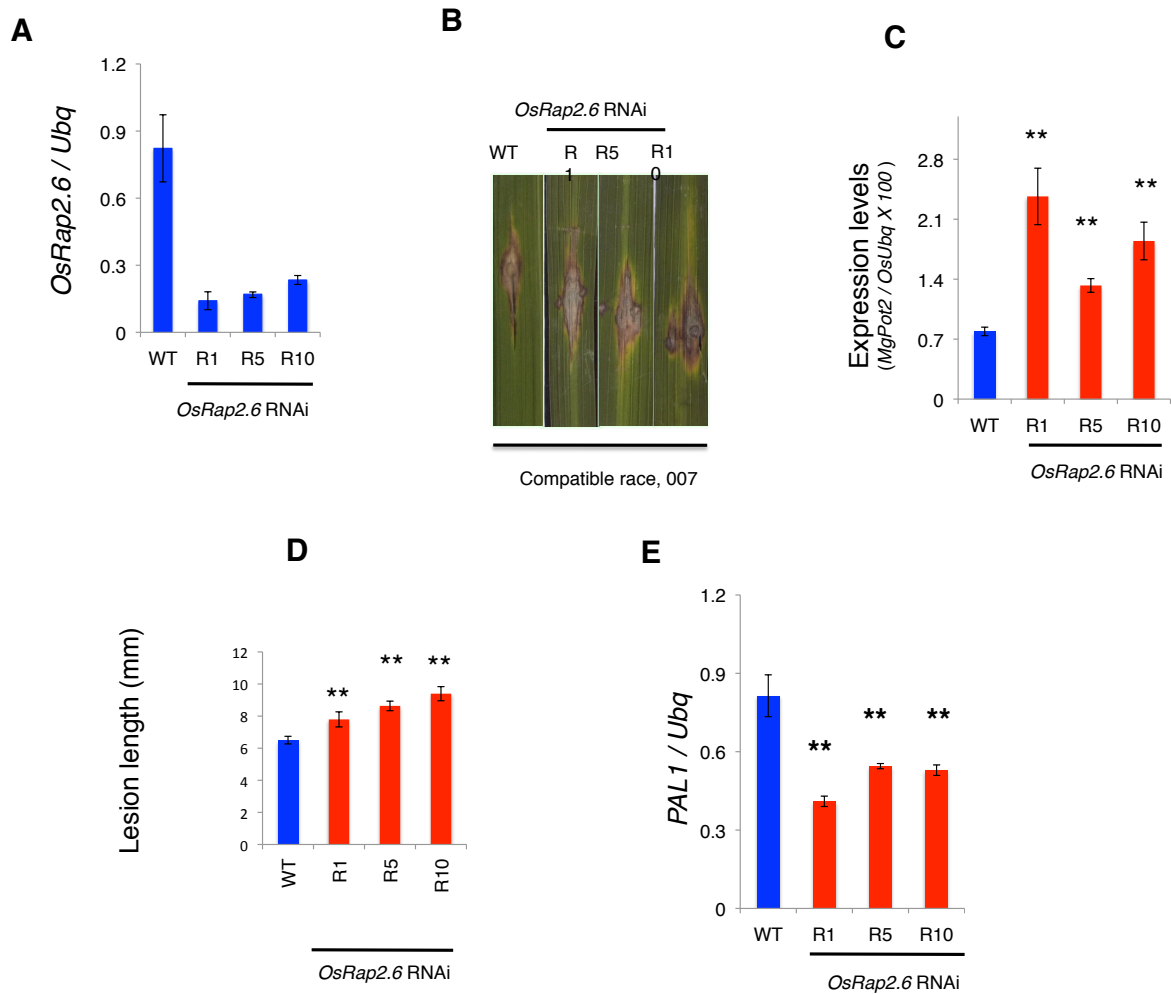
**Figure 10**: *OsRap2.6* RNAi plants are susceptible to a compatible race of *M. Oryzae*

*OsRap2.6* transcript levels in RNAi plants were measured by reverse transcription qPCR for three independently transformed lines, R1, R5 and R10. The RNAi plants were grown in the greenhouse for two months and inoculated with *M. oryzae* compatible (virulent) Ina 86–137 (race 007) fungal spore suspensions. (A) Expression levels of OsRap2.6 transcripts in T1 *OsRap2.6* RNAi plants before infection with rice blast fungus. (B) Photographs showing lesions in leaf blades in WT and *OsRap2.6* RNAi plants. (C) Quantitative analysis of fungal growth showing increased susceptibility in *OsRap2.6* RNAi plants 7 days after infection with a compatible race (007) of rice blast fungus. Rice *Ubiquitin* was used as an internal control. Bars represent the means ± SD calculated using four biological replicates where each consists of three independent technical replicates (p ≤ 0.01, n=48). (D) Lesion length of *OsRap2.6* RNAi plants compared to WT (p ≤ 0.01, n=48). (E) Expression of *PAL1* mRNA in *OsRap2.6* RNAi plants after rice blast infection. Levels of *PAL1* mRNA were down regulated as measured by reverse transcription qPCR (p ≤ 0.01)

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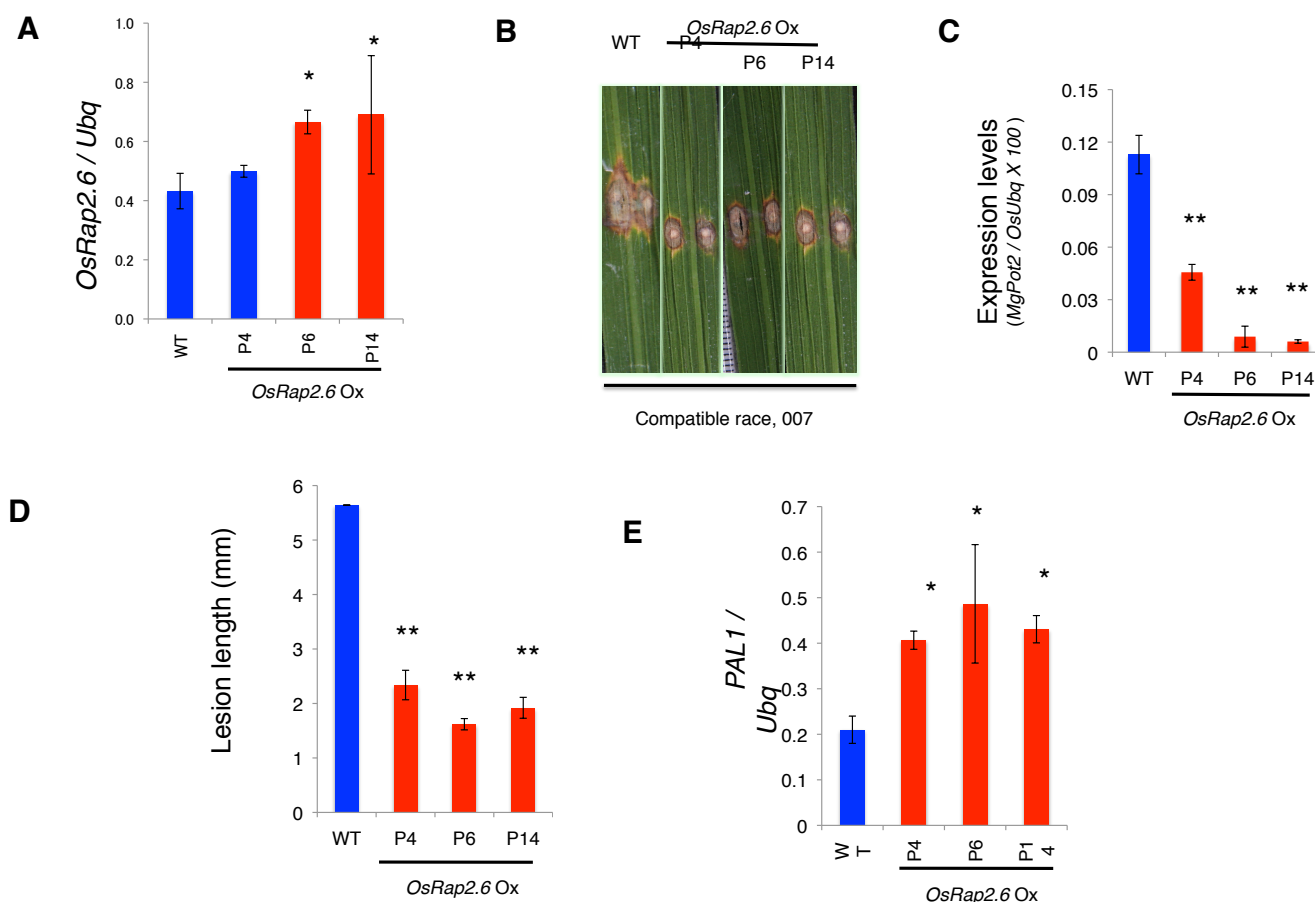
**Figure 11: *OsRap2.6* Ox plants are resistant to a compatible race, 007 of *M. oryzae***

*OsRap2.6* mRNA transcript levels from three independent Ox plants (P1, P6 and P14) were measured by qPCR. The Ox plants were grown in the greenhouse for two months and inoculated with *M. oryzae* compatible (virulent) Ina 86–137 (Race 007) fungal spore suspension. (A) Expression levels of OsRap2.6 transcripts in T1 *OsRap2.6* Ox plants before infection with rice blast fungus. (B) Photographs showing lesions in leaf blades in WT and *OsRap2.6* Ox plants after infection. **(**C) Quantitative analysis of fungal growth showing increased resistance in *OsRap2.6* Ox plants 7 days after infection with rice blast fungus compatible race (007). *Ubiquitin* was used as an internal control. Bars represent the means ± SD calculated using four biological replicates where each replicate consists of three independent technical replicates (p ≤ 0.01, n=48). (D) Lesion length of *OsRap2.6* Ox plants showing increased resistance to blast fungus as compared to WT as shown by increased lesion length after infection (p ≤ 0.01, n=48). (E) Expression of *PAL1* mRNA in *OsRap2.6* Ox plants after infection with the compatible race 007. Levels of *PAL1* mRNA were up regulated as measured by reverse transcription qPCR (p ≤ 0.01, n=48).

**A**

*OsRap2.6* RNAi

WT | R1 | R5 | R10

Incompatible race, 031

**B**

Expression levels
(*MgPot2 / OsUbq* X 100)

WT R1 R5 R10

*OsRap2.6* RNAi

**C**

Lesion length

(mm)

WT R1 R5 R10

*OsRap2.6* RNAi

**D**

*PAL1 / Ubq*

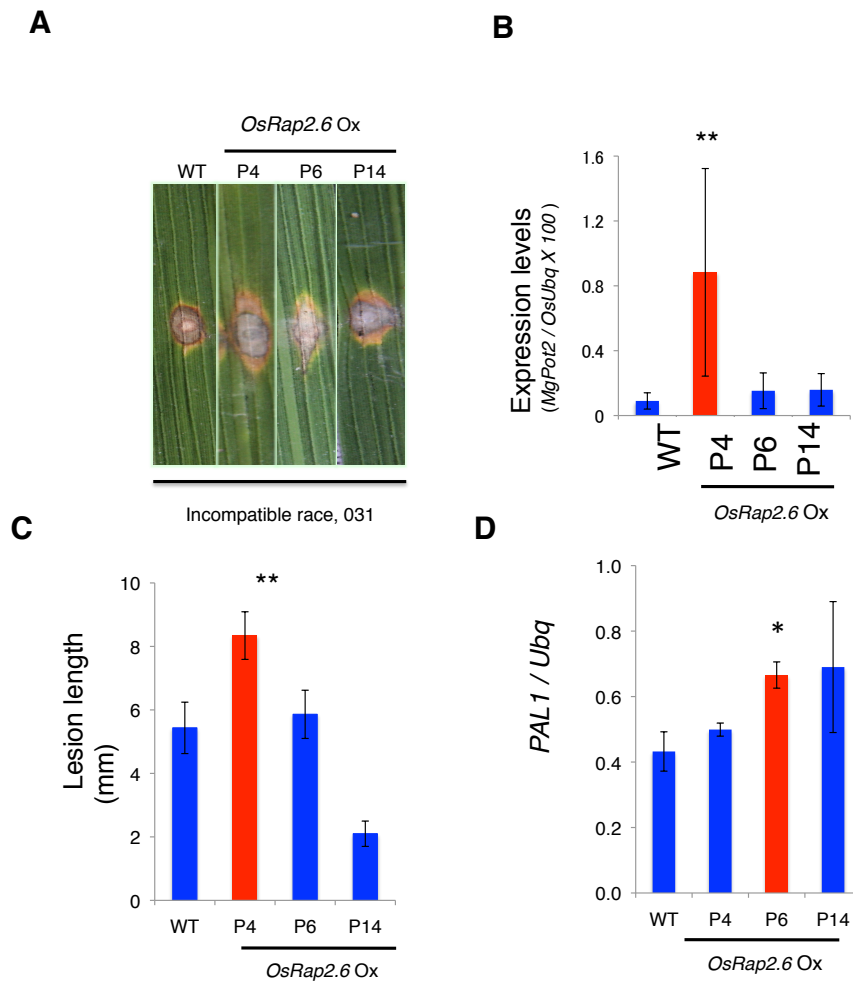WT R1 R5 R10

*OsRap2.6* RNAi

**Figure 12**: *OsRap2.6* **RNAi plants are not susceptible to an incompatible race, 031 of *M. Oryzae***

*OsRap2.6* mRNA transcripts from three independent RNAi were confirmed by real time PCR as shown in R1, R5 and R10. The RNAi plants were inoculated with *M. oryzae* incompatible race (031) fungus.
(A) Fungal infections on leaf blade in WT and *OsRap2.6* RNAi plants in incompatible race 031.
(B) Quantitative analysis showing increased susceptibility in *OsRap2.6* RNAi, 7 days after infection with compatible race (031). *Ubiquitin* is used as an internal control. Bars represent the means ± SD calculated using four biological replicates where each consists of three independent technical replicates (n=48) (p ≤ 0.05).
(C) Expression of *PAL1* mRNA in *OsRap2.6* RNAi after infection with incompatible race 031.

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**A**

*OsRap2.6* Ox

WT  P4  P6  P14

Incompatible race, 031

**B**

Expression levels
(*MgPot2 / OsUbq X 100*)

**

WT  P4  P6  P14

*OsRap2.6* Ox

**C**

Lesion length
(mm)

**

WT  P4  P6  P14

*OsRap2.6* Ox

**D**

*PAL1 / Ubq*

*

WT  P4  P6  P14

*OsRap2.6* Ox

**Figure 13**: *OsRap2.6* **Ox plants are not susceptible to an incompatible race, 031 of *M. Oryzae***

*OsRap2.6* mRNA transcripts from three independent Ox were confirmed by real time PCR as shown in P4, P6 and P14. The Ox plants were inoculated with *M. oryzae* incompatible race (031) fungus.
(A) Overexpressing levels of OsRap2.6 Ox plants after infection with incompatible race, 031.
(B) Fungal infections on leaf blade in WT and *OsRap2.6* Ox plants in incompatible race 031.
(C) Quantitative analysis showing increased susceptibility in *OsRap2.6* Ox, 7 days after infection with incompatible race (031). *Ubiquitin* is used as an internal control. Bars represent the means ± SD calculated using four biological replicates where each consists of three independent technical replicates (n= 48) (p ≤ 0.05).
(D) Expression of *PAL1* mRNA in *OsRap2.6* Ox after infection with incompatible race 031.

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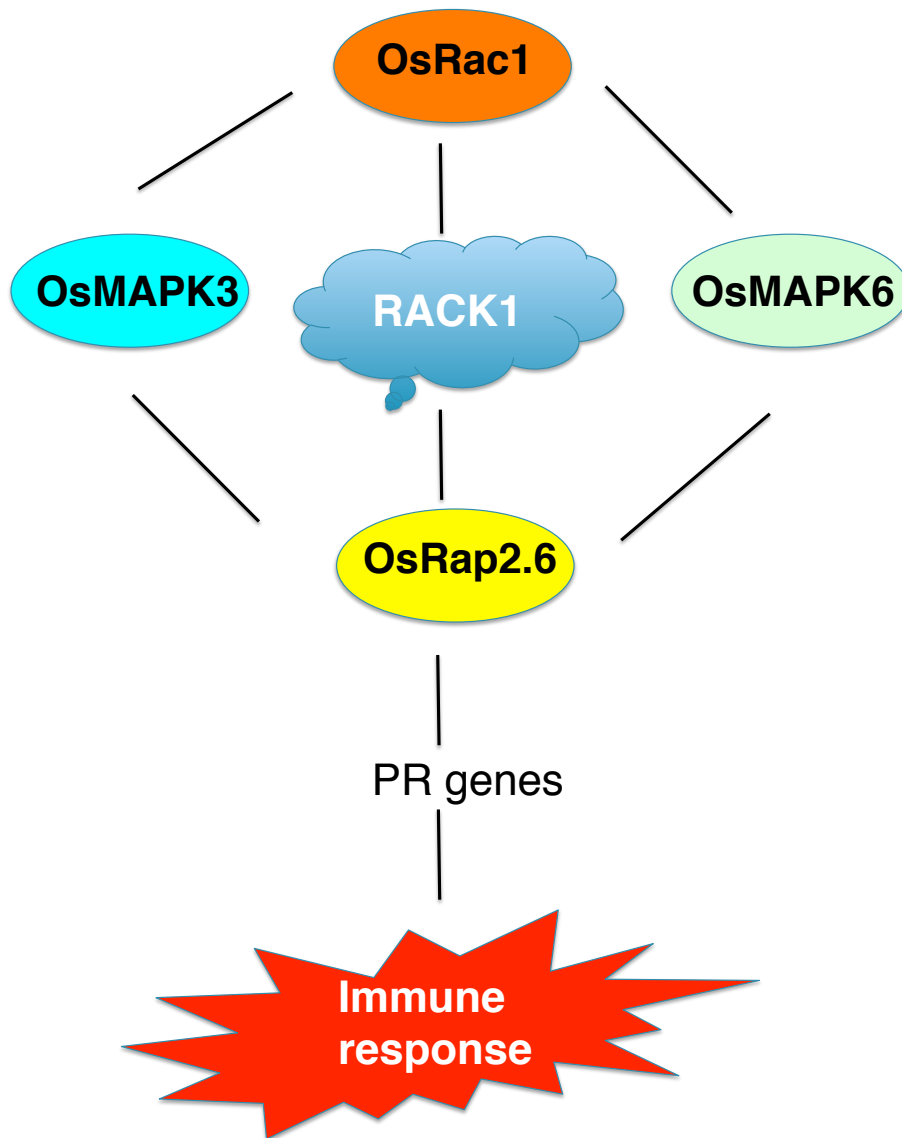
**Figure 14**: Proposed model for OsRap2.6

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**Dedication**

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