Number:

Rac/Rop Small GTPases in Rice Innate Immunity

（イネ自然免疫における Rac/Rop 低分子量 GTPase の役割）

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ABSTRACT

Plant-specific Rac/Rop small GTPases function as molecular switches for numerous signal transduction events, including defense responses. To understand the function of each of the seven Rac/Rop family members in rice, we studied the subcellular localization of Rac/Rop using GFP fusion proteins in transient expression systems. We also investigated the roles of these genes in disease resistance by testing single *Rac/Rop*-RNAi plants against the rice blast pathogen *Magnaporthe grisea*. Our studies revealed a strong correlation between the number of lysine/arginine (KR) residues in the polybasic region of Rac/Rop GTPases and their subcellular distribution *in vivo*. Infection assays showed that *OsRac1* is a positive regulator of blast resistance, confirming previous observations, whereas *OsRac4* and *OsRac5* are negative regulators of blast resistance. *OsRac6* may make minor contributions to disease resistance, while *OsRac3* and *OsRac7* are probably not involved in defense. Therefore, our study suggests that the rice Rac/Rop family plays multiple roles in diverse cellular activities and has both positive and negative functions in disease resistance.

Recognition of pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRRs) represents a critical first step of innate defense in plants and animals. However, maturation and transport of PRRs is not well understood. To extend our previous study, we studied the function of a novel OsRac1 interactor Hop/Sti1 and found that co-chaperone Hop/Sti1 was required for chitin-triggered immunity and resistance to rice blast fungus. Further, Hop/Sti1 interacts with Hsp90 and both bind the rice chitin receptor OsCERK1 in the endoplasmic reticulum (ER). Hop/Sti1 and Hsp90 are required for efficient transport of OsCERK1 from the ER to the plasma membrane (PM) via a pathway dependent on Sar1, a small GTPase which

regulates ER-to-Golgi trafficking. Finally, Hop/Sti1 and Hsp90 are present at the PM in a complex (designated the ‘defensome’) with OsRac1, a plant specific Rho-type GTPase. Our results suggest that the Hop/Sti1-Hsp90 chaperone complex plays an important and likely conserved role in the maturation and transport of PRRs and may function to link PRRs and Rac/Rop GTPases.

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CHAPTER ONE

Analysis of the Rac/Rop Small GTPase Family in Rice

1-1. INTRODUCTION

Rac/Rop small GTPases are plant-specific Rho subfamily members and participate in diverse signal transduction events including defense responses, pollen tube growth, root hair development, reactive oxygen species (ROS) generation, and hormone responses in plants (Nibau et al. 2006, Yang and Fu 2007). Rice OsRac1 was shown to be involved in ROS production and cell death (Kawasaki et al. 1999), and regulates ROS production by interacting directly with the N-terminal region of NADPH oxidase (Wong et al. 2007) and lignin production by interacting with cinnamoyl CoA reductase (Kawasaki et al. 2006). OsRac1 was also shown to be a positive regulator of resistance to fungal and bacterial pathogens in rice (Ono et al. 2001). OsRac1 forms a complex with MAPK6, RAR1, SGT1, Hsp90 and RACK1, thereby transmitting signals to downstream components of innate immunity (Lieberherr et al. 2005, Thao et al. 2007, Nakashima et al. 2008, Fujiwara et al. 2009).

Dominant negative OsRac1 impairs *N* gene-mediated resistance in tobacco (Moeder et al. 2005). Maize Rac/Rops induce ROS production when expressed in mammalian cells (Hassanain et al. 2000). In addition, Rac/Rop small GTPases are up-regulated in pathogen-inoculated chickpea leaf and elicitor-treated cell cultures (Ichinose et al. 1999). Transformation with constitutively active cotton Rac13 also increases ROS production in soybean and *Arabidopsis* (Potikha et al. 1999). Moreover, human Rac promotes an oxidative burst in soybean (Park et al. 2000). Silencing of *Rac/Rop* in tobacco by

heterologous expression of *Medicago Ms-Rac1* antisense cDNA causes defects in defense responses (Schiene et al. 2000). *Arabidopsis* Rop2 promotes ROS production and is involved in phosphatidic acid-induced cell death (Park et al. 2004).

In barley, *HvRacB* is a negative regulator of disease resistance to a powdery mildew pathogen (Schultheiss et al. 2002, 2003). The closest ortholog of *HvRacB* in rice, *OsRacB,* also known as *OsRac6* (Miki et al. 2005), was proposed as a negative regulator in disease resistance (Jung et al. 2006). Similarly, tobacco NtRac5 is considered as a negative regulator of NADPH oxidase involved in ROS production in tobacco (Morel et al. 2004). There are seven *Rac/Rop* members in rice (Miki et al. 2005), among which only *OsRac1* (Ono et al. 2001) and *OsRacB* (Jung et al. 2006) had been previously associated with defense. The contributions of other *OsRac*s to defense responses in rice remain unknown.

Most Rac/Rop GTPases carry a polybasic region (PBR) characterized by a high density of lysine and/or arginine (KR) residues at their C termini. The PBR is important for membrane association and signaling function of small GTPases (Williams 2003). Rac/Rops can be divided into two types based on their C-terminal motifs. Type I Rac/Rops possess a conserved CaaL (a, aliphatic amino acid) motif, whereas Type II proteins lack this motif but retain a cysteine-containing element for membrane anchoring (Winge et al. 2000). The subcellular localization of Type II Rac/Rops has been characterized in *Arabidopsis* (Lavy et al. 2002); however, no systematic analysis of the subcellular localization of rice Rac/Rop proteins has been reported.

In this study, I analyzed the polybasic region of Rac/Rop family in rice and defined the subcellular distribution *in vivo* and defense contribution *in planta* of different Rac/Rops. These experiments revealed a strong correlation between the number of KR

residues in the PBR of each rice Rac/Rop protein and its subcellular distribution *in vivo*, and also showed that *OsRac4* and *OsRac5* are two negative regulators of blast disease resistance. *OsRac6* may make minor contributions to blast resistance, whereas *OsRac3* and *OsRac7* probably play no role in defense.

1-2. MATERIALS AND METHODS

1-2-1 Transgenic Plants

The constructs for single gene knock-downs of each *OsRac/Rop* gene were described previously (Miki and Shimamoto 2004, Miki et al. 2005). *Agrobacterium*-mediated transformation of rice calli was performed according to the procedure of Hiei et al. (1994). T_0 plants were regenerated from transformed calli selected for hygromycin resistance. Seeds from T_0 plants were then germinated and grown as T_1 plants for infection assays.

1-2-2 PEG Transformation of Rice Protoplasts and Subcellullar Localization

CA-OsRac1, *DN-OsRac1* and *CA-OsRac2-6* have been described previously (Ono et al. 2001, Nakashima et al. 2008). DN mutants of *OsRac2-7* were produced by a standard site-directed mutagenesis protocol, and designated *OsRac2-T21N*, *OsRac3-T22N*, *OsRac4-T22N*, *OsRac5-T20N*, *OsRac6-T20N*, and *OsRac7-T20N*. All these mutants were subcloned into a GFP Gateway destination vector through LR reactions, resulting in GFP fusion at the N terminus.

Methods for protoplast isolation from rice cultured cells were essentially as previously described (Kyozuka and Shimamoto 1991). Plasmid transformation was

performed using the PEG method (Sheen 2002, Chen et al. 2006). Protoplasts isolated from rice Oc suspension culture cells were adjusted to 2.5×10^6 cells/ml. Plasmid DNAs (5 μg DNA of each construct) were mixed with 100-μl aliquots of suspended protoplasts in each transformation experiment. Confocal microscopy (LSM510-META, Carl Zeiss, Germany) was used to examine the protoplasts between 12 and 16 h after transformation at 30 °C. For quantitative assays of the frequency of cells with PM-exclusive localization, a number of cells ($33 \leqq n \leqq 45$) for each construct were randomly scanned and categorized as displaying a PM-exclusive (PM) or PM-nucleus-cytoplasm (PNC) localization pattern. Colocalization of GFP-Rac/Rop proteins and a nuclear marker CFP-GEN-L (Moritoh et al. 2005) was assessed with a confocal microscope (Leica TCS SP5, Germany) in sequential scanning mode.

1-2-3 Particle Bombardment of Onion Epidermal Cells

For each GFP fusion Rac/Rop construct, 5 μg DNA was coated with 1.0-μm diameter gold particle Au Powder (Bio-Rad, USA). Resuspended coated particles were spread and dried on Microcarrier (Millipore, USA) and delivered into onion (*Allium cepa*) epidermal cells using a PDS-1000/He Particle Delivery System (Bio-Rad, USA). After overnight incubation (12-16 h) at 30 °C, expression and localization of the GFP fusion protein were observed with a confocal microscope (LSM510-META, Carl Zeiss, Germany) using an excitation filter of 488 nm. For plasmolysis, the samples were treated with 0.5 M NaCl and further incubated for 15 min at room temperature before observation. Quantification of the fluorescence intensity of GFP-Rac/Rops in the nucleus and the PM was performed based on a previous report (Jeon et al. 2008). The average fluorescence intensity of two points in the PM and the nucleus was used to

calculate the ratio of GFP signal intensity in the nucleus and the PM (Nu/PM).

1-2-4 Semi-quantitative RT- PCR Analysis

Total RNA was extracted from rice blades using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen). cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). Semi-quantitative reverse transcript PCR was performed with samples of cDNA and gene-specific primers.

1-2-5 Infection of Rice Plants with Rice Blast Fungus

Growth conditions for the blast fungus and methods for punch inoculation of leaf blades have been described previously (Thao et al., 2007). Prior to flag leaf emergence, new rice leaves emerge every 12-15 d. For each plant/tiller, only the youngest two leaves (aged about one month) were used for infection. Lengths of lesions were measured 12 d after inoculation. To facilitate the comparison and integration of data sets produced at different stages of the growing season, and to minimize the variation caused by environmental conditions, a “relative lesion length” was defined as the measured lesion length normalized against the mean lesion length observed in wild-type leaves.

1-3. RESULTS

1-3-1 Analysis of the Polybasic Region of Rice Rac/Rop Small GTPases

Amino acid analysis of the polybasic region in seven Rac/Rop members in rice revealed that three members (OsRac5-7) belong to type I Rac/Rop proteins that possess a conserved CaaL motif at the C-terminus. The remaining four proteins (OsRac1-4)

belong to the type II Rac/Rop proteins that contain a truncated but functional post-translational modification motif. Moreover, OsRac2 harbors a GC-CG box, in which cysteines (C) are flanked by glycines (G) and separated by five to six non-polar residues (Lavy and Yalovsky 2006), whereas OsRac1, 3, and 4 contain only half of the GC or CG pair of the GC-CG box in the PBR. In contrast, type I Rac/Rop proteins lack the GC-CG box (Fig. 1-1). Sequence analysis further showed that OsRac3 and OsRac4 carried 12 and 14 lysine/arginine (KR) residues in the polybasic region, respectively, whereas the other members harbor about 8 KR residues (Fig. 1-1). In addition, OsRac3 terminates with CFKS which is closer to the CaaL motif than other type II Racs. Interestingly, most Rac/Rops (OsRac2-6), contain canonical nuclear targeting signal sequences (K-K/R-x-K/R) that also consists of the basic amino acids, lysine (K) and arginine (R).

1-3-2 Subcellular Localization of Rice Rac/Rop GTPases *in vivo*

To understand the subcellular localization of rice Rac/Rop GTPases *in vivo*, We isolated seven *Rac /Rop* full length cDNAs (*OsRac1-7*) from the rice genome (Miki et al. 2005) and generated both constitutively active (CA) and dominant negative (DN) mutants of all seven *Rac/Rop* members based on the method previously used to generate CA-*OsRac1* and DN-*OsRac1* (Kawasaki et al. 1999). All these mutants were fused to the intact *GFP* coding sequence at their N-terminus. The localization pattern of each GFP-tagged Rac/Rop mutant was first analyzed in transient rice protoplast experiments. Generally, Rac/Rop proteins were predominantly localized at the PM, but some signals were observed in the nucleus and the cytoplasm (Fig. 1-2). In order to evaluate *in vivo* Rac/Rops distribution quantitatively, two types of analyses were further performed in

transient assays. The first was to analyze the frequency in which the Rac/Rop protein was exclusively distributed in the PM in transformed cells. I noted that for type I Rac/Rops (OsRac5-7), more cells showed GFP signal in the nucleus and the cytoplasm than type II (OsRac1-4; Fig. 1-3A). Most CA forms of Rac/Rop members showed a higher frequency of PM-exclusive localization pattern than their DN forms (Fig. 1-3A). OsRac3 and OsRac4 showed the highest percentage of PM-exclusive localization signals among rice Rac/Rop GTPases (Fig. 1-3A). Similar results were obtained when the same set of constructs were transiently expressed in onion epidermal cells (Fig. 1-3B). In order to further verify the plasma membrane localization of GTPases, I induced plasmolysis of onion epidermal cells expressing GFP-CA-OsRac3 and GFP-CA-OsRac6, two extreme cases of PM-exclusive (PM) localization and PM-nucleus-cytoplasm (PNC) localization, by treatment with a high-concentration of NaCl solution. The control GFP protein was found in both the cytoplasm and the nucleus (Fig. 1-3C, left). Similarly, GFP-OsRac6 was observed in the cytoplasm and the nucleus as well as in the PM after plasmolysis (Fig. 1-3C, middle). In contrast, GFP-OsRac3 was exclusively detected in the PM and shrinking with the PM after plasmolysis (Fig. 1-3C, right).

The second analysis was to quantify the GFP fusion protein intensity ratio in the nucleus (Nu) and the PM as shown in Fig. 1-3D, lower. The average of the GFP fluorescence intensity of two points in the PM (P_{1-2}) and the nucleus (N_{1-2}) were used to calculate the ratio of Nu/PM fluorescence intensity (Fig. 1-3D, upper). Consistent with the first analysis, the GFP intensity ratio of Nu/PM in CA-Rac/Rop was always lower than for DN-Rac/Rop. Among seven rice Rac/Rop proteins, GFP-OsRac3 and GFP-OsRac4 showed the lowest fluorescence intensity ratio of Nu/PM (Fig. 1-3E),

further supporting the notion that OsRac3 and 4 are PM-exclusive Rac/Rop proteins in rice.

1-3-3 Roles of Rac/Rop GTPases in Blast Resistance in Rice

To study the roles of different *Rac/Rop* GTPases in rice defense, we generated transgenic plants by silencing individual *Rac/Rop* genes by RNAi (Miki and Shimamoto 2004, Miki et al. 2005) and investigated basal defense responses to virulent rice blast pathogen in T_1 generation plants. Plants #61-1, #18-1, #45-1, #61-2 and #47-1 were *OsRac7*-silenced plants and #61-3 and #45-2 were two T_1 segregants without an RNAi effect. Phenotypes of all these transgenic plants were similar to those of wild type (Fig. 1-4A) indicating that loss of *OsRac7* did not cause a defect in disease resistance. Although *OsRac6* was strongly silenced by RNAi, the resistance of *OsRac6*-RNAi plants was slightly enhanced. The *P* values for plants #125-1 and #150-2 from Student's tests indicated the difference between *OsRac6*-RNAi lines and wild type plants was not significant (Fig. 1-4B); plants #3-1, #3-2 and #3-3 were the progeny of a single T_0 plant of *OsRac5*-RNAi, plant #1-1 was another independent *OsRac5*-RNAi plant. In contrast to the positive role of *OsRac1* in defense shown in previous studies (Ono et al. 2001), silencing of *OsRac5* significantly enhanced disease resistance to the virulent blast fungus in *OsRac5*-RNAi plants #3-2 and #1-1, except #3-1 which showed a higher expression level of *OsRac5* (Fig. 1-4C), indicating that *OsRac5* plays a negative role in basal resistance to blast infection. Results from three type I *Rac/Rop*-RNAi plants indicated that *OsRac5* is a negative regulator of blast resistance. *OsRac6* may be a minor component in defense, whereas *OsRac7* may not be involved in blast resistance.

The basal resistance to blast infection in type II *OsRac/Rop*-RNAi T_1 plants was also

examined by infection with virulent rice blast pathogen. Four independent *OsRac4*-RNAi plants #2-2, #19-1, #7-1 and #16-4 showed an enhanced disease resistance phenotype like *OsRac5*-RNAi plants in infection assays, indicating that *OsRac4* is another a negative regulator in the rice defense pathway (Fig. 1-4D). Since *OsRac4* is a type II *Rac/Rop* that functions as a negative regulator, I further reasoned that *OsRac3*, the most similar *Rac* homolog of *OsRac4* in sequence structure, tissue expression and subcellular localization pattern, might be also a negative regulator in defense. However, this supposition was not the case for one *OsRac3*-RNAi plant. Impairment of *OsRac3* expression (plant #59-5) did not cause a clear reduction in defense resistance (Fig. 1-4E).

1-3-4 OsRac1 Is a Positive Regulator in *Pi-a*-mediated Blast Resistance

The role of *OsRac1* in basal resistance has been intensively studied. *CA-OsRac1* plants show enhanced basal resistance to blast infection, whereas *DN-OsRac1* impairs resistance (Ono et al. 2001). *CA-OsRac1* also offsets the loss function of *RAR1* in basal defense in rice (Thao et al. 2007). In the present study, *OsRac1*-RNAi plants were inoculated with an avirulent race 041 corresponding to *Pi-a*. The results indicated that *OsRac1* knock-down significantly affected *Pi-a*-mediated defense responses to the avirulent blast fungus in *OsRac1*-RNAi T_1 plants (Fig. 1-5Aand B). These results support the hypothesis that *OsRac1* has a positive role in *Pi-a*-mediated blast resistance.

1-4. DISCUSSION

1-4-1 Subcellular Localization of Rac/Rop Proteins

The polybasic region (PBR), CaaL motif and GC-CG box are known to be important for Rac/Rop membrane association in *Arabidopsis* (Lavy et al. 2002, Lavy and Yalovsky 2006). In this study, the subcellular localization of individual rice Rac/Rop members was investigated with GFP fusion proteins. Quantitative results showed that most CA-Rac/Rop-transformed cells showed a higher frequency of PM-exclusive localization (Fig. 1-2, 1-3A and B), and a lower ratio of nucleus versus plasma membrane localization than DN forms (Fig. 1-3D). These findings suggest that active Rac/Rops are more efficiently targeted to the PM. Alternatively, active Rac/Rops might be more stable than corresponding inactive form in the PM. Detailed amino acid sequence analysis revealed that in contrast to *Arabidopsis*, in rice only OsRac2 possesses an intact GC-CG box in the PBR of type II Rac/Rops. OsRac1 contains only a downstream CG pair, whereas OsRac3 and 4 carry only an upstream GC pair. Instead, OsRac3 and OsRac4 possess the highest lysine/arginine (KR) content in their polybasic region, a finding that is strongly correlated with their PM localization frequency and efficiency. This observation is consistent with results from the mutational study on the PBR of K-ras4B in mammalian cells (Roy et al. 2000). Based on our studies, I propose that the membrane association of Rac/Rops is determined by two properties. The KR content of the PBR is a determinant of the PM targeting, and the cysteine in the CaaL motif or the GC-CG box undergoes diverse post-translational modification to anchor Rac/Rop proteins to the PM. The completeness of the CaaL motif and the GC-CG box may contribute little to plasma membrane targeting, but the association of Rac/Rops to the PM, as long as the C (cysteine) residue remains at the C terminal end. In addition, our data also support the idea that activation of Rac/Rop protein promotes the PM localization of Rac/Rop small GTPases (Fig. 1-3A, B and D).

Comparative sequence analysis also showed that potential nuclear localization signal (NLS) sequences (K-K/R-x-K/R) are present in OsRac2-6. The mammalian Rac family consists of three members, Rac1-3. Only Rac1 has a functional NLS in its PBR and directs SmgGDS, the ARM family protein which has guanine nucleotide exchange factor (GEF) activity, into the nucleus (Lanning et al. 2003, 2004). Recent study further shows that mammalian Rac1 interacts with the STAT transcription factor and GTPase-activating protein (GAP) and facilitates the nuclear translocation of the complex containing these proteins (Kawashima et al. 2006, 2009). However, in rice, the presence of NLS was not obligatory for nuclear localization as exemplified by OsRac3 and OsRac4 that are the most membrane-exclusive Rac/Rop proteins in rice. On the other hand, the NLS sequence was dispensable for nuclear targeting of OsRac1 and OsRac7, since both OsRacs were present in the nucleus without a NLS in the PBR, indicating that they may enter the nucleus with the help of other components under certain conditions. Interestingly, recent findings indicate that certain disease resistance proteins are translocated into the nucleus, followed by transcriptional activation of downstream signaling in the nucleus (Shen et al. 2007, Burch-Smith et al. 2007). Our recent study indicates that OsRac1 interacts with multiple NBS-LRR proteins and is required for R protein-mediated defense responses (Kawano et al. unpublished data). Our results showing that rice Rac/Rops are predominantly localized in the PM and are able to enter the nucleus suggest that OsRac proteins may have multiple functions in the PM, the cytoplasm and the nucleus in various cellular signaling processes.

1-4-2 Dual Roles of Rac/Rop GTPases in Disease Resistance

That different Rac/Rop GTPases have opposite functions in the same signaling

pathway seems to be common in plants. In Arabidopsis, the same Rops are involved in two antagonistic pathways when they interact with different effectors. For example, Rop2 and Rop4 are redundantly required for pavement cell morphogenesis (Fu et al. 2005). In one instance, Rop2/4 interacts with and activates RIC4 to promote localized outgrowth of pavement cells. In other instances, Rop2 inactivates another interactor RIC1, a suppressor of outgrowth in the indentation zones. A recent study shows that both Rop2 and Rop6 interact with RIC1, but play opposing roles in pavement cell morphogenesis (Fu et al. 2009). Rop2 inactivates RIC1 and activates RIC4 to accumulate cortical actin microfilaments for lobe formation in the outgrowing region, whereas Rop6 might activate RIC1 to promote well-ordered cortical microtubules restricting expansion in the indenting region.

In rice defense response, constitutively active OsRac1 (CA-OsRac1) promotes HR-like cell death, whereas dominant negative OsRac1 (DN-OsRac1) suppresses cell death in rice and tobacco (Kawasaki et al. 1999, Moeder et al. 2005). Cell death is thought to function as a physical block to further pathogen ingress (Greenberg and Yao. 2004). Consistent with these results, CA-OsRac1 transgenic plants display enhanced resistance to a compatible race of M. oryzae, whereas DN-OsRac1 impairs race-specific resistance to blast infection (Ono et al. 2001). RAR1 is an important protein for defense responses in plants (Shirasu. 2009). *RAR1*-silenced rice plants are compromised for resistance to virulent fungal infection, whereas overexpression of CA-OsRac1 compensates for the loss of RAR1 function, showing a genetic link between RAR1 function and the OsRac1-mediated defense pathway (Thao et al. 2007). Most recently, OsRac1 was shown to play a role in Pit-mediated blast resistance (Kawano et al. unpublished data). OsRac1 interacts with NADPH oxidase (Rboh) to

promote ROS production. On the other hand, OsRac1 suppresses the function of the ROS scavenger, OsMT2b, to maintain ROS accumulation (Wong et al. 2004, 2007). OsRac1 is known to physically interact with cinnamoyl CoA reductase (CCR), a key enzyme for lignin biosynthesis to produce monolignols (Kawasaki et al. 2006). Monolignols require ROS/peroxidase for lignin polymerization (Boerjan et al. 2003). The burst of ROS production has an antimicrobial function and is also considered a signal activating plant defense responses (Kotchoni and Gachomo. 2006). A high ROS level is also important for lignification during defense responses. All these studies suggest that OsRac1 has a positive role in plant disease resistance.

In contrast, the infection study with *OsRac*-RNAi plants indicates that *OsRac4* and *OsRac5* negatively regulate blast resistance (Fig.1-4C and D). Consistent with this conclusion, a microarray assay showed a defense-related marker gene, PBZ1, was highly induced in elicitor-treated *OsRac4*-RNAi cultured cells compared with wild type cells (data not shown). In barley, overexpression of *HvRacB* enhances accessibility of powdery mildew fungus to barley epidermal cells, whereas *HvRacB*-RNAi and DN-*HvRacB* prevent the fungal pathogen from penetrating into plant cells (Schultheiss et al. 2002, 2003). Furthermore, CA-*HvRacB* inhibits actin filament targeting to the attempted penetration site, whereas *HvRacB*-RNAi supports fungal invasion (Opalski et al. 2005). These results indicate that *HvRacB* is a negative regulator of basal defense. In tobacco, overexpression of *NtRac5* decreases cryptogein-induced ROS production, implying that *NtRac5* plays a negative role in ROS production and perhaps defense (Morel et al. 2004). The closest homolog of *OsRac1* in barley, CA-*HvRac1*, promotes ROS production and cell death in response to fungal pathogens, having a distinct function in contrast to other *HvRac* member,

such as CA-*HvRacB* and CA-*HvRac3* (Pathuri et al. 2008).

Our present data support our previous results indicating *OsRac1* positively regulates disease resistance (Fig. 1-5), whereas *OsRac4* and *OsRac5* are negative regulators to blast resistance. *OsRac7*, *OsRac6* and *OsRac3* do not seem to be involved in disease resistance. The positive and negative regulators may function antagonistically in the disease resistance pathway to fine tune defense responses. Since OsRac3 and OsRac4 are the most membrane-exclusive OsRacs in rice, there is no obvious correlation between defense function and subcellular localization pattern. *OsRac6* plays a modest role in defense based on the present infection studies with *OsRac6*-RNAi plants; however, based on overexpression, *OsRac6*, also known as *OsRacB* in another study, was proposed as a negative regulator in defense (Jung et al. 2006). In our study, I observed that some *OsRac6*-RNAi plants (plants #125-1 and #150-2, Fig. 1-4B) became slightly more resistant to the compatible pathogen; however, the level was not significant compared to wild type. This finding could be due to a minor contribution of OsRac6 to defense that was not significantly reflected in RNAi knock-down plants. In our screening of T_1 plants, I failed to obtain *OsRac2*-RNAi plants, thus I were not able to investigate the contribution of *OsRac2* to disease resistance. The role of *OsRac2* in defense remains to be studied in the future. The existence of positive and negative roles for Rac/Rop GTPases in rice innate immunity suggests the complexity of Rac/Rop function in disease resistance.

1-5. FIGURES

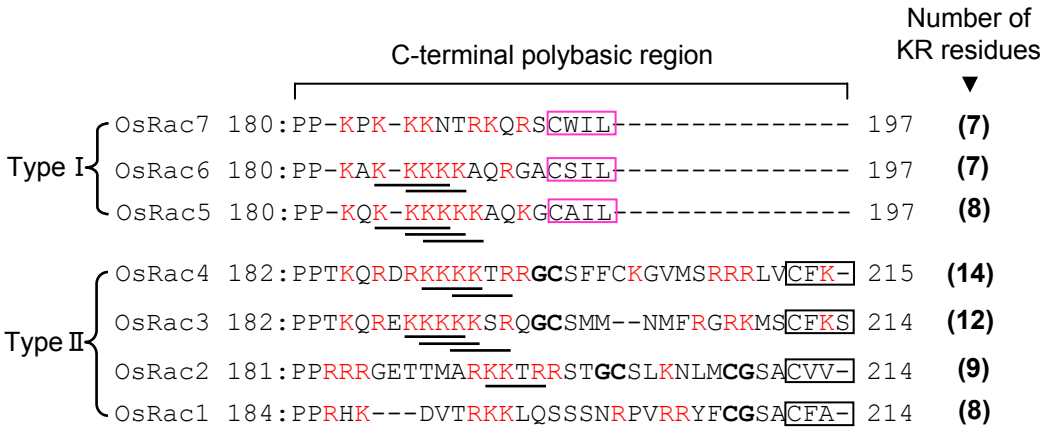


Figure 1-1. Amino acid alignment of the polybasic regions of rice Rac/Rop family members. Rac/Rop proteins are divided into two types according to the conserved CaaL motif at the C-terminus. Type I Rac/Rops possess a conserved CaaL (a, aliphatic amino acid) motif, whereas Type II proteins lack an intact CaaL motif and instead contain a GC-CG box, in which cysteines are flanked by glycines and separated by five to six non-polar residues (Lavy and Yalovsky 2006). The amino acid glycine-cysteine pair (GC or CG) shown in boldface and basic amino acid lysine/arginine (KR) residues in red. Conserved NLSs (K-K/R-x-K/R) are underlined. Numbers indicate positions in the polypetide sequence. The numbers in parentheses indicate the total number of KR residues in each polybasic region. Sequence were aliged with Gentyx7, and dashes indicate gaps introduced to optimize the alignments.

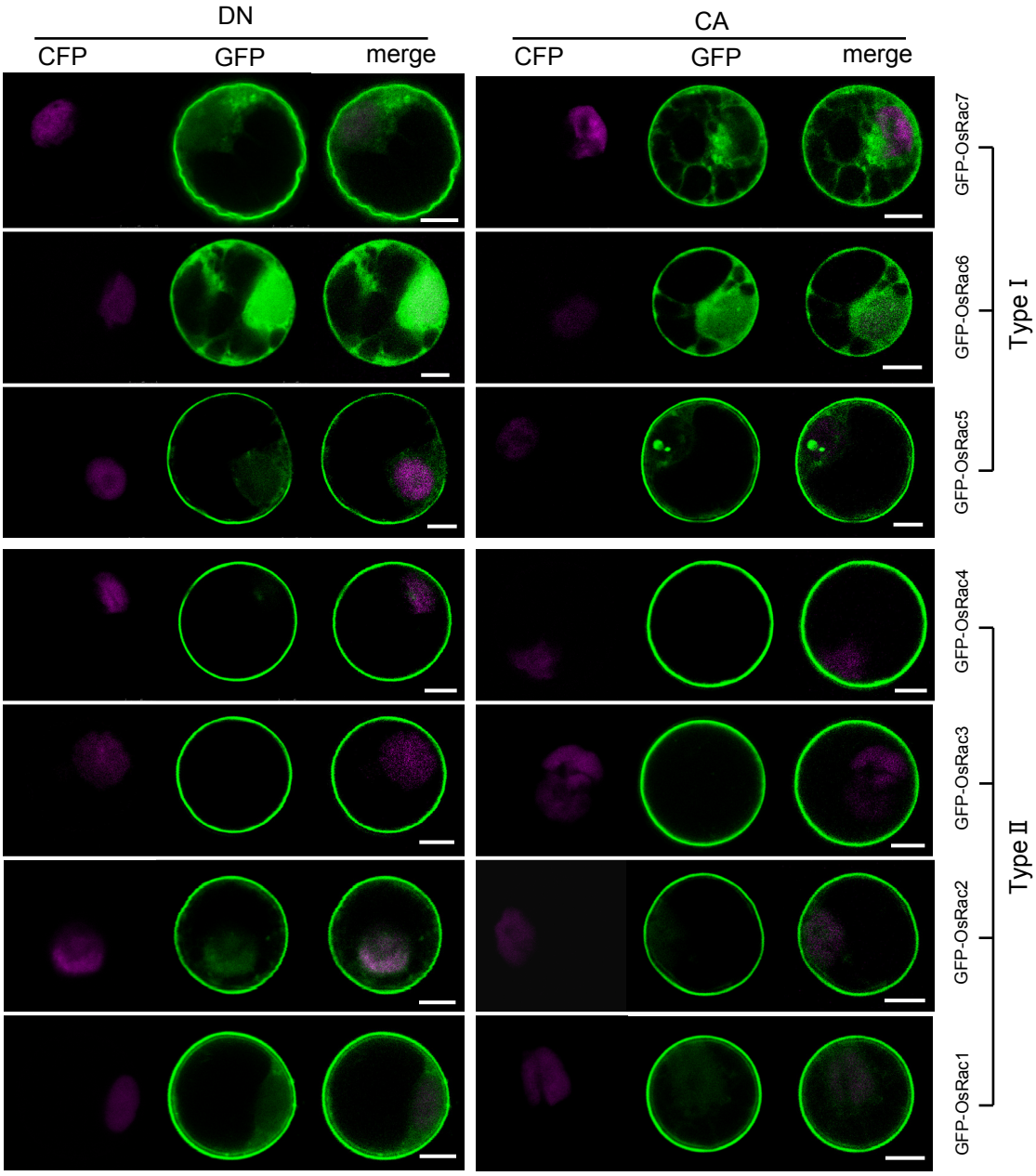


Figure 1-2. Subcellular localization of rice Rac/Rop proteins in protoplasts. Typical subcellular localization patterns of dominant negative (DN) and constitutively active (CA) Rac/Rop fusion GFP signal in rice protoplasts. CFP fused GEN-L serves as nuclear marker. Bar = 5 μm.

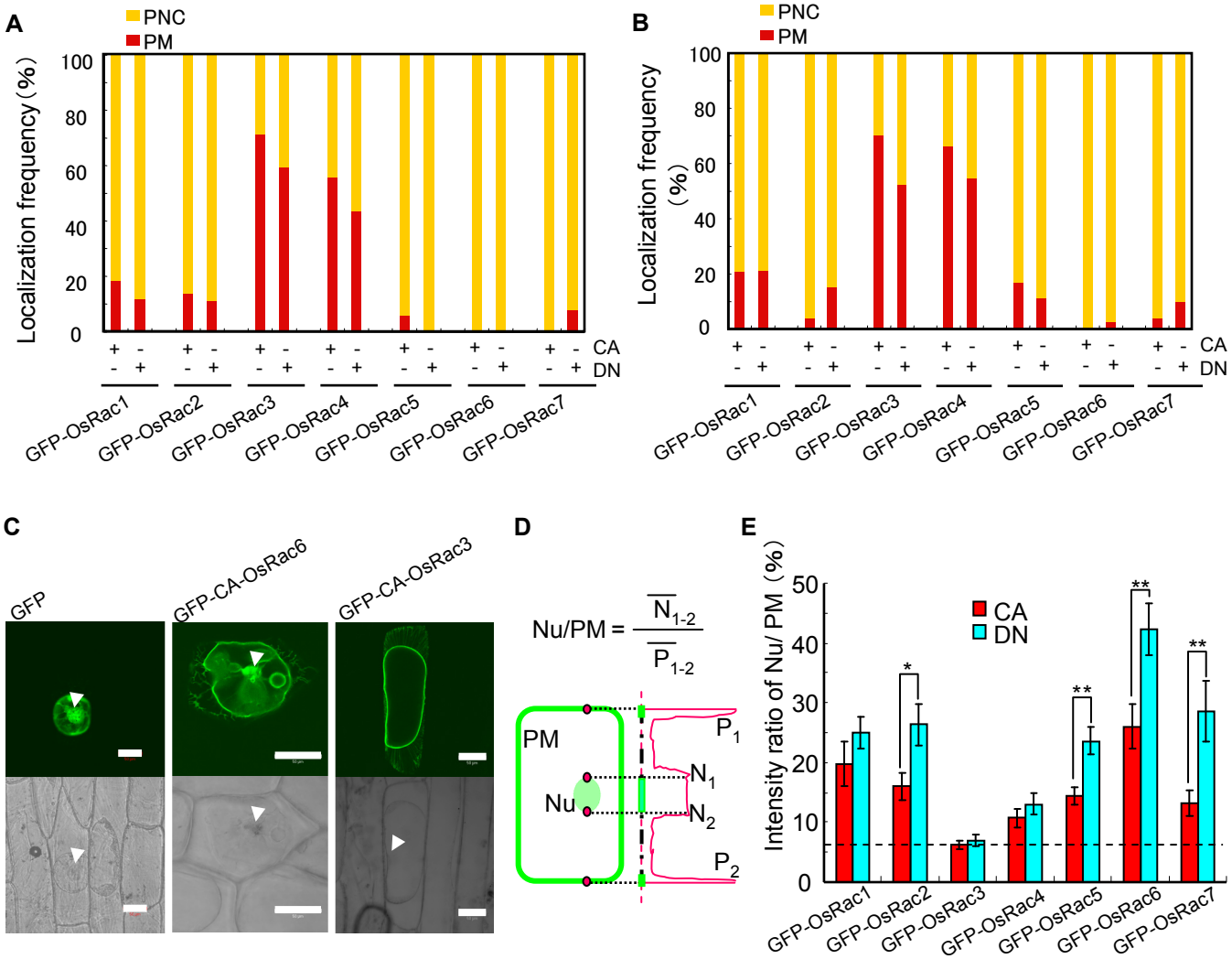


Figure 1-3. Localization frequency and distribution of GFP-DN- or GFP-CA-Rac/Rop *in vivo*. (A) Localization frequency of GFP-DN- or GFP-CA-Rac/Rop signal in transformed rice protoplasts. Red bars indicate the percentage of cells with the PM-exclusive (PM) localization. Yellow bars indicate the percentage of cells with PM-Nucleus-Cytoplasm (PNC) localization. 74 ≦ n ≦ 100 (B) Localization frequency of GFP-DN- or GFP-CA-Rac/Rop signal in transformed onion epidermal cells. 33 ≦ n ≦ 45. (C) Plasmolysis assays of GFP-OsRac3 (right) and GFP-OsRac6 (middle) induced by 0.5M NaCl solution. GFP (upper panel) and bright field images (lower panel) are shown. The CaMV promoter 35S-GFP (left) serves as a control. Arrowheads indicate nuclei; Bars = 50 μm. (D) the measurement of GFP intensity in in the plasma membrane (PM) and the nucleus (Nu). Average of two data points in the PM ($P_{1\text{-}2}$) and the nucleus ($N_{1\text{-}2}$) were used to calculate the ratio of GFP in the nucleus and the PM (Nu/PM). (E) Quantification of GFP-CA- or GFP-DN-Rac/Rop fluorescence intensity in the plasma membrane (PM) and the nucleus (Nu) *in vivo*. GFP-CA- or GFP-DN-Rac/Rop intensity ratio in the PM and the nucleus, 34 ≦ n ≦ 40. Dot line indicates the basal ratio of Nu/PM; * indicates 0.01 < *P* < 0.05, ** indicates 0.001 < *P* < 0.01. Data shown are means ± SE.

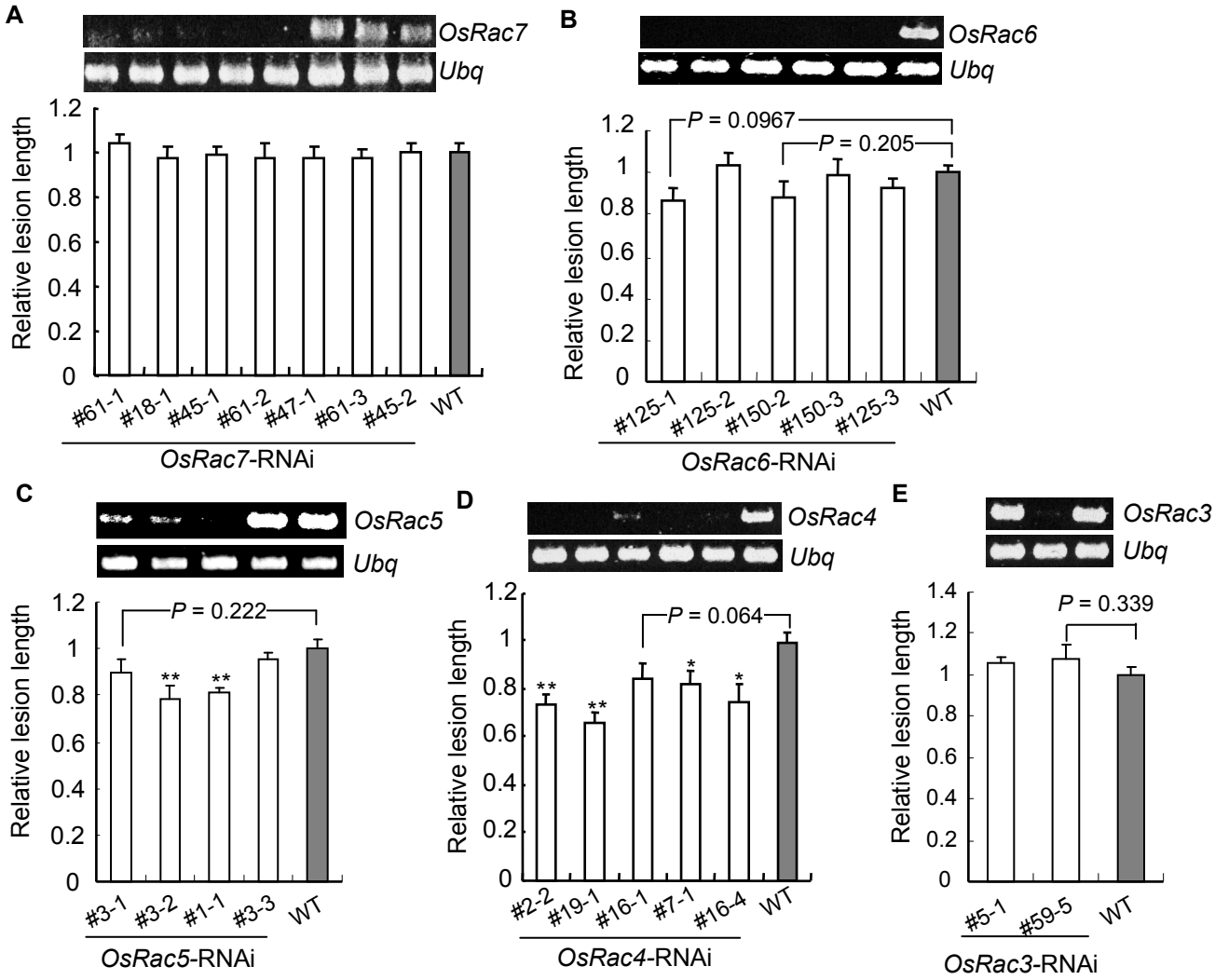
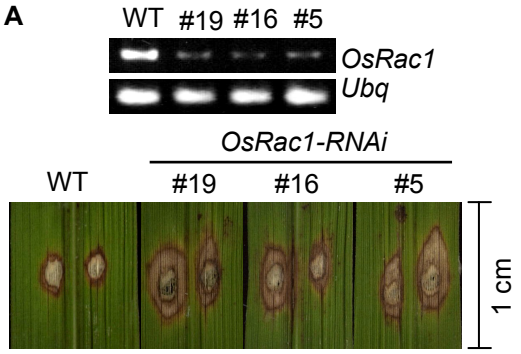


Figure 1-4. Roles of Rac/Rop GTPases in basal resistance against blast infection in rice. The order of the RT-PCR lanes corresponds to the order in the graph showing relative lesion lengths. Lines with different initial arabic numbers are independent T_1 plants; lines with the same initial arabic number indicate the T_1 lines derived from the same T_0 plants. Data shown are means ± SE. (A) Expression of *OsRac7* in RNAi transgenic T_1 plants (upper panel) and infection assays of *OsRac7*-RNAi plants with a virulent rice blast pathogen race 007 (lower graph). Plants # 61-3 and # 45-2 are two segregants without an RNAi effect in the *OsRac7*-RNAi T_1 generation. $12 \leqq n \leqq 26$. (B) Expression of *OsRac6* in RNAi transgenic T_1 plants (upper panel) and infection assays of *OsRac6*-RNAi with a virulent rice blast pathogen race 007 (lower graph), $7 \leqq n \leqq 28$, *P* values were calculated by the Student's t-test against wild type. (C) Expression of *OsRac5* in RNAi transgenic T_1 plants (upper panel) and their resistance to a virulent rice blast pathogen race 007 (lower graph). Plant # 3-3 is a segregant without an RNAi effect in the T_1 generation. $16 \leqq n \leqq 45$; * indicates $P < 0.01$, ** indicates $P < 0.005$. (D) Expression of *OsRac4* in RNAi transgenic T_1 plants (upper panel) and their resistance to a virulent rice blast pathogen race 007 (lower graph), $9 \leqq n \leqq 25$; * indicates $P < 0.01$, ** indicates $P < 0.005$. (E) Expression of *OsRac3* in RNAi transgenic T_1 plants (upper panel) and their resistance to a virulent rice blast pathogen race 007 (lower graph). # 5-1 is a segregant without an RNAi effect in the T_1 generation. $18 < n < 54$.

A



B

Table 1 Lesion length of *OsRac1-RNAi* plants

Plants	Mean (cm)	SE	n
WT	0.28	± 0.008	26
#19 **	0.45	± 0.028	21
#16 *	0.39	± 0.021	24
#5 **	0.42	± 0.044	12

* $P < 0.01$ ** $P < 0.005$

Figure 1-5. *OsRac1* is a positive regulator of *Pia*-mediated blast resistance in rice. (A) (upper panel) Expression of *OsRac1* in *OsRac1*-RNAi transgenic T1 plants. (Lower panel) Representative pictures showing *OsRac1*-RNAi plants infected with avirulent rice blast pathogen 041. Bar = 1 cm. (B) Silencing of *OsRac1* significantly affects *Pi-a*-mediated blast resistance in rice. * indicates $P < 0.01$, ** indicates $P < 0.005$.

CHAPTER TWO

The Hop/Sti1-Hsp90 Chaperone Complex Facilitates the Maturation and Transport of a PAMP Receptor in Rice Innate Immunity

2-1. INTRODUCTION

Pattern recognition receptors (PRRs) are the first layer of defense against pathogen infection at the cell surface (Jones and Dangl, 2006). Pathogen-specific molecules recognized by PRRs are called pathogen-associated molecular patterns (PAMPs) (Chisholm et al., 2006; Zipfel, 2008). In plants, host perception of PAMPs activates rapid defense responses such as calcium influx, production of reactive oxygen species (ROS), induction of defense-related genes, and accumulation of antimicrobial compounds (Jones and Dangl, 2006). Most plant PAMP receptors characterized to date are receptor-like kinases (RLKs) or receptor-like proteins (RLPs). RLKs possess an extracellular domain, a transmembrane domain, and a kinase domain, whereas RLPs lack the intracellular kinase domain. Protein structural analysises indicate that RLKs perceive signals through their extracellular domain and transmit signals via their intracellular kinase domain. *Arabidopsis* and rice encode more than 600 and 1100 RLK/Ps, respectively (Shiu et al., 2004), which are involved in numerous cellular signaling and developmental events.

RLKs function in plant-microbe interactions and defense responses. FLS2 and EFR , for example, are receptors for bacterial flagellin and elongation factor Tu (EF-Tu), respectively (Gomez-Gomez et al., 2000; Zipfel et al., 2006), while CEBiP and LysM-type CERK1 are receptors for fungal chitin (Kaku et al., 2006; Miya et al., 2007;

Wan et al., 2008). Recently, BRI-associated kinase (BAK1) was found to be important for innate immunity as well as cell death (Chinchilla et al., 2007; He et al., 2007; Kemmerling et al., 2007), suggesting that different RLK/P-mediated signaling pathways share common components. RLK/Ps are located in the plasma membrane (PM) and are assumed to move to endosomes through endocytosis (Russinova et al., 2004; Geldner and Robatzek, 2008). However, the modes of maturation, trafficking and PM localization of RLK/Ps are largely unknown.

OsRac1 plays a role in basal resistance and is involved in the immune response induced by two PAMPs derived from fungal pathogens, N-acetylchitooligosaccharide (chitin) and sphingolipids (Ono et al., 2001; Suharsono et al., 2002; Fujiwara et al., 2006). OsRac1 controls cell death, the activation of *PR* gene expression, and phytoalexin production (Kawasaki et al., 1999; Ono et al., 2001; Suharsono et al., 2002; Wong et al., 2004). It also regulates ROS production by interacting directly with the N-terminal region of NADPH oxidase (Wong et al., 2007), and lignin production by interacting with cinnamoyl CoA reductase (Kawasaki et al., 2006). Furthermore, PAMP-induced activation of rice MAPK6 requires OsRac1, which forms a complex with MAPK6 in rice cell extracts (Lieberherr et al., 2005).

Chaperones and co-chaperones also play critical roles in plant innate immunity. Cytoplasmic Hsp90 functions in effector-triggered immunity together with the co-chaperone-like proteins RAR1 and SGT1 (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004; Hubert et al., 2009; Shirasu, 2009). We recently demonstrated that RAR1, Hsp90, and Hsp70 are present in the OsRac1 complex, but none of them appear to interact directly with OsRac1 (Thao et al., 2007). Scaffold protein RACK1A directly interacts with OsRac1 (Nakashima et al., 2008). In

mammalian cells, Hsp90 and Hsp70 form a complex through the co-chaperone Hop; this complex is important for steroid hormone receptor maturation (Pratt and Toft, 2003). Structure of Hop protein in eukaryotes is conserved, which include three TPR domains and two DP domains. Plant-encoded Hop, which is also known as stress-induced protein 1 (Sti1), interacts with Hsp90 *in vitro* (Torres et al., 1995; Zhang et al., 2003), although its biological function has not yet been elucidated. We have identified Hop/Sti1 as an OsRac1interactor (Nakashima et al., 2008).

In this study, I further studied a novel OsRac1 interacting cochaperone Hop/Sti1 and demonstrated that Hop/Sti1 and a cytoplasmic Hsp90 interact with the rice chitin receptor OsCERK1. I show, moreover, that Hop/Sti1a and Hsp90 are required for efficient transport of OsCERK1 from the endoplasmic reticulum (ER) to the PM via the Sar1-dependent trafficking system. I further provide evidence that Hop/Sti1a and Hsp90 are present in an OsRac1 immune complex, designated the "defensome" at the PM.

2-2. MATERIALS AND METHODS

2-2-1 Transgenic Plants and Cultured Cells

Rice *Hop/Sti1a* and *Hop/Sti1b* cDNAs were provided by the Rice Genome Resource Center, Tsukuba, Japan. For overexpression of *Hop/Sti1a*, the coding region was amplified by PCR, cloned into the pENTR/D-TOPO vector (Invitrogen), and subcloned into the p2K1 vector by an LR reaction. To make RNAi constructs for *Hop/Sti1* suppression, a 350-bp *Hop/Sti1a* 3'-UTR fragment was amplified by PCR and subcloned in inverse orientation into the pANDA vector (Miki and Shimamoto 2004) through an intermediate pENTR/D-TOPO vector. A 665-bp chimeric fragment

containing 350 bp of the *Hop/Sti1a* 3'-UTR region and 315 bp of the *Hop/Sti1b* 3'-UTR region was amplified by two PCR series by the overlap extension method, and cloned into the pANDA vector.

2-2-2 Chitin Treatment and Quantitative Real-Time PCR Analysis

Cultured rice cells were treated with 20 μg/ml chitin, hexa-N-acetylchitohexaose (Seikagaku, Japan), and harvested at the indicated times after chitin treatment. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen). cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with samples of cDNA and standard plasmids using SYBR Green PCR master mix (Applied Biosystems) and the gene-specific primers listed in Supplemental Table 1. Data were collected using the ABI PRISM 7000 sequence detection system according to the manufacturer's instructions.

2-2-3 Yeast Two-hybrid Assays

For bait constructs, the *Hop/Sti1a* and *Hop/Sti1b* coding regions were ligated into the pBTM116 vector at the *Spe*I and *Pst*I sites. For prey constructs, *Hop/Sti1a* was subcloned into the pVP16 prey vector at the *Sal*I and *Kpn*I sites, and *Hop/Sti1b* at the *Kpn*I site. The prey constructs of *SGT1*, *RAR1*, and *Hsp90* for Y2H assays have been described previously (Nakashima et al., 2008).

2-2-4 Split-ubiquitin Two/Three Hybrid Assay

OsHop/Sti1a and *OsCERK1* full-length coding regions and their deletion fragments

were amplified by PCR, using specific primers shown in Table 1, and subcloned into pENTR-TOPO (Invitrogen). *OsFLS2, OsBAK1* and *OsCERK1*, as well as *OsCERK1* deletion mutants, were subcloned into the bait vector pMet-GWY-Cub-RUr3A, and full-length CA-*OsRac1*, *HvHsp90* and *Hop/Sti1a*, as well as *Hop/Sti1a* deletion mutants, were subcloned into the prey vector pCup-Nul-GWY-Cyc1 through the Gateway system (Kim et al., 2002). *OsFLS2* (AK120799) and *OsBAK1* (AK103038) were provided by the Rice Genome Resource Center, Tsukuba, Japan. Yeast cells expressing bait and prey constructs were grown to 5×10^6 cells/μl and spotted in serial dilutions on selective agarose plates with or without 5-fluroorotic acid (FOA). At least three independent colonies were repeated for each two-hybrid pair.

2-2-5 BiFC Assays in Rice Protoplasts

The BiFC system used for this study was described previously (Kakita et al., 2007). Gus, OsCERK1, OsCERK1-KD, OsCERK1-NT, OsCERK1-TM, OsCERK1-ED, OsRac1, and Hop/Sti1a were cloned into the pVN/gw vector for N-terminal fusion using LR reactions. Gus, Hsp90, Hop/Sti1a, OsRac1 were cloned into the pVC/gw vector for C-terminal fusion in the same way. Protoplasts isolated from rice Oc cells were adjusted to 2.5×10^6 cells/ml. Plasmid DNAs (about 5 μg DNA of each construct) were mixed with 100-μl aliquots of suspended protoplasts in each transformation experiment. BFA (50 μg/ml) or Geldanamycin (10 μM) was added immediately to protoplasts. Confocal microscopy (LSM510-META, Carl Zeiss) was carried out after the protoplasts had been incubated for 24 h at 30 °C. For quantitative assays, cells with visible fluorescence were considered positive and the number of positive cells was counted using a fluorescent microscope. The data were normalized with the mean of the negative control.

2-2-6 Immunoblot Analysis

Sample proteins were separated by 10.5% SDS-PAGE and electrotransferred onto an Immobilon-P membrane (Millipore). The membrane was blocked for 30 min in Blocking One (Nacalai Tesque) and incubated for 2 h with specific antibodies followed by incubation with anti-rabbit/rat/mouse/IgG conjugated to horseradish peroxidase (GE Healthcare). To produce antibodies against Hop/Sti1a, an N-terminal fragment of *Hop/Sti1* (encoding amino acids 1-198) was cloned into the pDEST17 vector (Invitrogen) through an LR reaction. The recombinant protein was produced in *E. coli* strain BL21 and purified with a HiTrap chelating HP column (GE Healthcare) to be used as an antigen to raise antibodies in rabbits. Chemical enhancement was performed using ECL Plus western blot detection reagents (GE Healthcare). The enhanced signals were detected by an LAS-4000 system (Fujifilm).

2-3. RESULTS

2-3-1 The Hsp90 Co-chaperone Hop/Sti1 Interacts with OsRac1 and Is Localized to the ER

In order to understand the mechanism of OsRac1-mediated defense response in rice, we investigated OsRac1 interacting proteins. Based on affinity chromatography and database analysis, we identified two highly similar Hop/Sti1s in rice (Nakashima et al., 2008), hereafter designated Hop/Sti1a and Hop/Sti1b, which share 80.4% amino acid identity (Fig. 2-1A and 2-1B). Three Hop/Sti1 homologs are found in *Arabidopsis* (Fig. 2-1A). Sequence analysis suggested that the rice Hop/Sti1 proteins share a conserved structure with yeast and mammalian Hops (Fig. 2-1B; Nicolet and Craig, 1989).

Since Hop/Sti1 was identified through affinity chromatography with OsRac1, I first used *in vitro* binding assays to confirm the interaction of Hop/Sti1 with OsRac1. Hop/Sti1 interacted with wild-type (WT)-, constitutively active (CA)-, and dominant negative (DN)-OsRac1 (Fig. 2-2A). These results were further substantiated by co-immunoprecipetation assays with crude lysates of cultured suspension cells (Nakashima et al., 2008) (Fig. 2-2B). Moreover, the split-ubiquitin two-hybrid system (SUS) showed that Hop/Sti1a, one of the two rice homologs, interacted with CA-OsRac1 but not with DN-OsRac1 (Fig. 2-2C). Although the reasons for the differences between the results of the two-hybrid and *in vitro* binding assays remain unclear, these results indicate that Hop/Sti1 interacts with OsRac1 both *in vitro* and *in vivo*.

I next analyzed the interactions of Hop/Sti1 with known defense-related (co-)chaperones in the yeast two-hybrid (Y2H) system. Hop/Sti1a did not interact with RAR1 or SGT1 (Fig. 2-2D). A strong interaction between Hop/Sti1a and cytoplasmic Hsp90 was observed, while the interaction of Hop/Sti1b, the other rice homolog, with Hsp90 was weak (Fig. 2-2D, 2-3B and 2-3C). As previously reported for *Arabidopsis*, RAR1 interacted with SGT1, and both interacted with Hsp90 (Fig. 2-2D). Hop/Sti1 did not interact with MAPK6, RbohB, or RACK1, components of the OsRac1 immune complex (Nakashima et al., 2008). OsRac1 did not directly interact with RAR1, SGT1, or Hsp90 (Fig. 2-3B and 2-3D). Since Hop/Sti1a strongly interacted with OsRac1 and Hsp90 (Fig. 2-3B and 2-3C), I chose Hop/Sti1a for further studies.

I examined the intracellular localization of Hop/Sti1 by expressing the C-terminal YFP-tagged Hop/Sti1a protein in rice protoplasts isolated from suspension cell cultures. Hop/Sti1a-YFP co-localized with a CFP-fused ER marker, showing a typical ER pattern

suggesting that Hop/Sti1 targets to the ER *in vivo* (Fig. 2-3E), which is consistent with the presence of ER targeting signals in Hop/Sti1 sequence (Fig. 2-1B).

2-3-2 Hop/Sti1a Is Involved in Chitin-triggered Immune Responses and Rice Blast Resistance

To examine the function of Hop/Sti1 in rice innate immunity, I produced transgenic rice cell cultures and plants in which *Hop/Sti1* expression was silenced by RNAi, as well as cells and plants that overexpressed Hop/Sti1a (Fig. 2-4A). In *Hop/Sti1a*-RNAi cell lines, *Hop/Sti1a* expression was strongly reduced (Fig. 2-4B and 2-5A). In *Hop/Sti1ab*-RNAi plants, levels of both *Hop/Sti1a* and *Hop/Sti1b* mRNAs were strongly reduced (Fig. 2-4C). In contrast, *Hop/Sti1a* expression was significantly increased in *Hop/Sti1a*-OX plants (Fig. 2-4D).

Hop/Sti1a-RNAi and wild-type cell cultures were treated with chitin, and chitin-induced defense-related gene expression was monitored by quantitative PCR (qPCR). Chitin-induced defense-related gene expression of *PAL1* and *PBZ1* was strongly reduced and was delayed for 1-2 h in *Hop/Sti1a*-RNAi cells compared to the wild-type cells (Fig. 2-5B), indicating that Hop/Sti1a plays a role in chitin-triggered immunity. Consistent with this phenotype, rice cultivar Kinmaze possesses the chitin receptor OsCERK1 (Fig. 2-S1), a rice ortholog of the *Arabidopsis* chitin receptor AtCERK1 that is involved in chitin-triggered immune responses (Shimizu et al., submitted). However, since cv. Kinmaze lacks the flg22 receptor OsFLS2, flg22-triggered immune responses are absent (Takai et al., 2008; Fig. 2-S1).

In infection assays with a virulent strain of the rice blast fungus (*Magnaporthe grisea*; strain 2403-1, race 007), *Hop/Sti1ab*-RNAi plants were more susceptible than

wild-type plants to rice blast infection (Fig. 2-5C), whereas *Hop/Sti1a*-OX plants were more resistant (Fig. 2-5D). Taken together, these results demonstrate that Hop/Sti1a is involved in chitin-triggered immune responses and resistance to blast infection in rice.

2-3-3 Hop/Sti1a and Hsp90 Interact with the Rice Chitin Receptor OsCERK1 via Its Transmembrane Domain

During steroid receptor maturation in mammalian cells, Hop passes client proteins from Hsp70 to Hsp90 in a chaperone complex (Pratt and Toft, 2003). Since the Hop/Sti1-Hsp90 (co-)chaperone complex is involved in PAMP-triggered immune responses in rice, I wondered whether rice RLKs could also interact with the Hop/Sti1-Hsp90 complex. I chose the rice chitin receptor OsCERK1 to test this idea. Interestingly, Hop/Sti1 and Hsp90, but not CA-OsRac1, interacted with OsCERK1 in SUS assays (Fig. 2-6A). Similarly, rice homologs of two other RLKs, OsFLS2 and OsBAK1, also interacted with Hop/Sti1a (Fig. 2-6A). I further studied the interaction between Hop/Sti1a and OsCERK1 using truncated fragments of OsCERK1 and found that the transmembrane (TM) domain of OsCERK1, but not the extracellular LysM domain (ED) or the kinase domain (KD), interacted with Hop/Sti1a (Fig. 2-6B and C). Similarly, Hsp90 interacted with the TM domain of OsCERK1 (Fig. 2-6B and C). A Hop/Sti1a deletion assay showed that either the TPR1 or the TPR2A domain of Hop/Sti1a alone was sufficient for its interaction with all three interacting proteins (Fig. 2-6D). OsRac1 also interacted weakly with the DP1 domain of Hop/Sti1a. These results showed that Hop/Sti1a and Hsp90 interact with the TM region of OsCERK1, thus connecting an RLK with OsRac1 and its signaling partners in rice.

2-3-4 OsCERK1 Matures in the ER and Is Transported to the PM through a Vesicle Trafficking Pathway

It has been suggested that RLK receptors are PM proteins whose localization is regulated by endocytosis (Russinova et al., 2004; Geldner and Robatzek, 2008). However, the processes of maturation and transport of plant RLKs and PAMP receptors are largely unknown. The finding that Hop/Sti1a localizes to the ER and physically interacts with a PAMP receptor (Fig. 2-3E and 2-6) raises the question of how Hop/Sti1a, an ER protein, interacts with the PM-localized RLK. To address this question, I used GFP-fused intact OsCERK1 (OsCERK1-GFP) to investigate the subcellular localization of OsCERK1 in rice protoplasts.

OsCERK1-GFP was found predominantly in the PM. However, some signals were also detected in vesicle-like organelles and typical ER structures. To analyze GFP signals more precisely in transformed protoplasts, the cells were categorized into four types (P1-P4) according to the distribution of the OsCERK1-GFP signal in different organelles (Fig. 2-7A), and the frequency of each type was determined (Fig. 2-7B). Under normal conditions, P1, in which the GFP signal was observed only in the PM, contributed 4% of the total transformed cells; P2, in which signals were detected in the PM and in dot-like vesicles, comprised 50%; and P3, in which signals were detected in the PM, vesicles and the ER network, accounted for 46%. No P4 cells, in which the signal was observed only in the ER, were found (Fig. 2-7B, upper panel).

These results suggested that OsCERK1 may be transported from the ER to the PM through a vesicular trafficking pathway. Consistent with this, brefeldin A (BFA), a well known inhibitor of vesicle trafficking (Satiat-Jeunemaître et al., 1996), dramatically changed the OsCERK1-GFP localization pattern. In 87% of the protoplasts examined,

OsCERK1-GFP was confined to the ER (P4 type), while the remaining 13% of the protoplasts were type P3, with signals detected in the ER and the PM (Fig. 2-7B, lower panel). No P1 or P2 cells were detected in the presence of BFA.

I observed dynamic changes in the subcellular localization of OsCERK1-GFP, which depended on the time after transformation and the presence of BFA. OsCERK1-GFP became visible at 3.5-4 h after transformation. The GFP signal appeared mainly in the ER around the nucleus, while only a faint signal was detected in the PM at 4 h after transformation (Fig. 2-7C, upper left). The GFP signal in the PM became distinct by 7 h after transformation (Fig. 2-7C, upper right). These results showed that OsCERK1-GFP occurred first in the ER, where it was probably synthesized, before it reached the PM.

When BFA was added to protoplasts at 3 h after transformation and the protoplasts were further incubated for 1 h, the pattern was similar to the control (Fig. 2-7C, lower left). However, after 4 h of BFA treatment, the GFP fluorescence increased in the ER, whereas no such increase was detectable in the PM (Fig. 2-7C, lower right). These results suggest that OsCERK1-GFP was synthesized normally in the presence of BFA but then accumulated in the ER due to the inhibition of vesicle trafficking by BFA.

I next examined whether the exit of OsCERK1 from the ER depends on COPII-mediated ER-to-Golgi traffic. AtSar1 is a small GTPase which regulates the ER-to-Golgi trafficking in *Arabidopsis* (Takeuchi et al., 2000). A constitutively active Sar1 (CA-AtSar1) mutant inhibits the transport of the Golgi membrane protein AtRer1B from the ER to the Golgi (Takeuchi et al., 2000). I co-transformed *OsCERK1-CFP*, *GFP-AtRer1B*, and either WT-*AtSar1* or CA-*AtSar1* into rice protoplasts and examined their fluorescence signals. OsCERK1-CFP predominantly localized to the PM, while

AtRer1B-GFP was found in the Golgi-like organelles when WT-*AtSar1* was co-transformed (Fig. 2-7D, upper panel). In contrast, when CA-*AtSar1* was co-transformed, the AtRer1B-GFP signal was restricted to the ER, as was the co-expressed OsCERK1-CFP signal (Fig. 2-7D, lower panel). It was noted that when WT-*AtSar1* was co-transformed, GFP-AtRer1B signals overlapped punctate organelles, most probably the endosomes, en route to the PM (Fig. 2-7D, upper panel). Together, these results indicate that OsCERK1 matures in the ER and is subsequently transported from the ER to the PM through a Sar1-dependent vesicle trafficking pathway.

2-3-5 OsCERK1 Receptor Interacts with Hop/Sti1a *in vivo* and the Complex Is Transported via Trafficking System

To characterize proteins that may be involved in maturation and transport of OsCERK1, I studied protein interactions by bimolecular fluorescence complementation (BiFC) assays (Hu et al., 2002). When BiFC constructs were transformed into rice protoplasts, they sometimes generated background fluorescence, as has been reported previously (Walter et al., 2004). To quantify protein interactions in BiFC assays, I measured the frequency of reconstituted YFP-positive protoplasts in each combination of constructs and found that OsCERK1/Hsp90, Sti1a/Hsp90, OsCERK1/Sti1a and CA-OsRac1/Sti1a all provided unambiguous evidence for protein interactions (Fig. 2-8A, C lower right and 2-9B lower right).

Hop/Sti1a-OsCERK1 complexes were primarily detected in the PM, but also in the ER and dot-like organelles (Fig. 2-8C-1), which resembled the pattern of OsCERK1-GFP localization (Fig. 2-7B, upper panel). Addition of BFA to the BiFC assays prevented the transport of Hop/Sti1a-OsCERK1 complexes to the PM (Fig.

2-8D). However, BFA had no effect on the localization of the nuclear protein GenL-GFP or CFP-GenL (Moritoh et al, 2005; Fig. 2-8C-1 and 2-8D). Thus, Hop/Sti1a appears to form a complex with OsCERK1 which is transported from the ER to the PM through punctate intermediate organelles. Furthermore, the NT fragment of OsCERK1, which contains both the ED and TM domains (Fig. 2-8B), formed a complex with Hop/Sti1a in the ER as well as the PM (Fig. 2-8C-2). In contrast, when the TM domain alone was used in BiFC assays with Hop/Sti1a, the Hop/Sti1a-OsCERK1 complex was mainly restricted to the ER, with no clear signal from the PM (Fig. 2-8C-3), indicating that the complex was retained in the ER and failed to translocate to the PM (Fig. 2-8C-3). These results imply that the ED is mainly responsible for the proper ER exit of OsCERK1; consistent with this, deletion of the ED caused retention of the truncated OsCERK1 in the ER (Fig. 2-8E).

I next analyzed subcellular localization of Hsp90 and its interaction with OsCERK1 by BiFC assays. Hsp90 mainly localized to the cytoplasm, as expected but could also be detected in the ER (Fig. 2-9A). However, the BiFC signal for the Hsp90-OsCERK1 complex was predominantly localized to the PM (Fig. 2-9B-1). The interactions of the NT and TM fragments of OsCERK1 with Hsp90 were also examined. The intracellular localization of the BiFC signals (Fig. 2-9B-2, B-3) resembled that obtained with Hop/Sti1a (Fig. 2-9B-2, B-3), indicating that Hsp90 also interacts with the TM domain of OsCERK1. When CA-*AtSar1* was co-transformed in the BiFC assay for Hsp90 and OsCERK1, the fluorescence was mainly detected in the ER (Fig. 2-9C), suggesting that the OsCERK1-Hsp90 complex is transported from the ER to the PM through the Sar1-dependent trafficking system. I also analyzed Hop/Sti1a-CA-OsRac1 interactions by BiFC and found low levels of fluorescence

signals in the ER, besides the cytoplasm and the PM (Fig. 2-9D). A similar subcellular localization pattern was observed for Hop/Sti1a-Hsp90 interactions (Fig. 2-9E).

For Hop/Sti1a and Hsp90 interacted with OsCERK1 in the ER and at the PM (Fig. 2-6, 2-8 and 2-9), and for Hop/Sti1a-RNAi impaired chitin-triggered defense gene induction (Fig. 2-5B), I investigated whether an impairment of Hop/Sti1a or Hsp90 function affected the transport of OsCERK1 from the ER to the PM by determining the percentage of OsCERK1-GFP-expressing protoplasts in which ER-localized GFP signals were detected. In Hop/Sti1a-RNAi protoplasts, the frequency of protoplasts with ER-localized OsCERK1-GFP signals was higher than that in wild-type protoplasts (Fig. 2-10), suggesting that Hop/Sti1a is required for the correct targeting of OsCERK1 to the PM. Next, I examined Hsp90 function in the transport of OsCERK1 using geldanamycin (GDA), an inhibitor of Hsp90 (Richter and Buchner, 2001). Levels of ER signals were similar in GDA-treated wild-type and *Hop/Sti1a*-RNAi protoplasts (Fig. 2-10). However, when *Hop/Sti1a*-RNAi protoplasts were treated with GDA, no additive effect on the percentage of protoplasts showing ER signals was found (Fig. 2-10), suggesting that Hop/Sti1a and Hsp90 function in the same pathway to facilitate ER exit of OsCERK1. Together, these results indicate that both Hop/Sti1a and Hsp90 interact with OsCERK1 and regulate its transport from the ER to the PM.

2-3-6 OsCERK1, Hop/Sti1a, Hsp90 and OsRac1 May Form a Complex in the ER

Since our BiFC assays revealed that OsCERK1 can form a complex with Hop/Sti1a and Hsp90 in the ER as well as at the PM (Fig. 2-8 and 2-9), I further analyzed the co-localization of these three proteins by a BiFC-based method (Fig. 2-11A and C).

First, a pair of OsCERK1 and Hop/Sti1a BiFC constructs was transformed into rice protoplasts together with CFP-OsRac1 (WT), and the YFP and CFP signals arising respectively from the OsCERK1-Hop/Sti1 interaction and from OsRac1 were monitored. A BiFC-generated YFP signal was detected in the ER and at the PM, and co-localized with CFP, indicating that the OsCERK1-Hop/Sti1a complex and OsRac1 were present in the same subcellular compartments (Fig. 2-11B). Similarly, OsCERK1-Hsp90 and Hop/Sti1a were present in the same regions (Fig. 2-11D), which was consistent with the other interaction experiments. Taken together, these findings suggest that OsCERK1 interacts with Hop/Sti1a and Hsp90, and possibly with OsRac1 through Hop/Sti1a, in the ER, and that these interactions may be important for the efficient targeting of OsCERK1 to the PM where it functions as a PAMP receptor.

2-3-7 Hop/Sti1 Is Associated with the OsRac1 Immune Complex at the PM

I have proposed that a protein complex at the PM, containing OsRac1, Hsp90, Hsp70, RAR1, SGT1, RACK1A, RbohB and MAPK6, plays a role in PAMP-triggered immunity in rice, based on a number of protein-protein interaction and functional studies (Lieberherr et al., 2005; Thao et al., 2007; Wong et al., 2007; Nakashima et al., 2008). In the current study, I showed that Hop/Sti1a, Hsp90 and OsRac1 combine with the PAMP receptor OsCERK1 in the ER and are subsequently transported to the PM. At the PM, these proteins are likely to form a complex (Nakashima et al., 2008; and Fig. 2-11). Therefore, I name the OsRac1 complex present at the plasma membrane the "defensome". The proposed protein network of the defensome is shown in Fig. 2-12A. Although direct interactions of MAPK6 with other members of the defensome have not been demonstrated, MAPK6 appears to participate in the same complex as OsRac1

based on co-IP experiments (Lieberherr et al., 2005). Detailed biochemical and cell biological studies of the defensome complex will be required to understand the molecular mechanisms of PAMP-triggered innate immunity in rice.

2-4. DISCUSSION

2-4-1 Importance of the Hop/Sti1-Hsp90 Chaperone Complex in PAMP-triggered Immunity in Plants

In animals, Hop/Sti1 is best known as one of the co-chaperones for the cytoplasmic Hsp90 chaperone, which participates in a complex that regulates steroid hormone receptor biogenesis and maturation. The complex contains Hsp90, Hsp70, Hop, p23 and other co-chaperones (Pratt and Toft, 2003). In yeast, Hop/Sti1 has been shown to stabilize the Hsp90 complex with Ste11, a MAPKKK; a defect in Hop/Sti1 inhibits MAPK signaling (Lee et al., 2004). In plants, *in vitro* binding assays indicated that soybean Hop/Sti1 interacts with Hsp90 (Zhang et al., 2003).

Cytoplasmic Hsp90 and two co-chaperone-like molecules, RAR1 and SGT1, form a ternary complex and play a critical role in innate immune responses triggered by resistance (R) proteins in *Arabidopsis* and tobacco (Shirasu, 2009). In contrast, the interaction of these three (co)-chaperones seems to contribute mainly to basal resistance in rice (Thao et al., 2007; Wang et al., 2008). Although the involvement of the Hsp90 chaperone complex and other co-chaperone-like proteins in plant innate immunity has been well established, the molecular mechanisms of their functions are not yet understood (Hubert et al., 2009; Shirasu, 2009).

We have shown previously that GDA treatment suppresses PAMP-triggered immune

responses in rice cells and disrupts OsRac1-Hsp90 complex formation (Thao et al., 2007). It is possible that the OsRac1-Hsp90 complex analyzed in our previous study is a component of a larger PM defensome complex, identified in the present study, which contains RLK, Hop/Sti1 and the PM-anchored OsRac1. Since I showed that *Hop/Sti1a*-RNAi and GDA decreased the efficiency of the PM targeting of OsCERK1, and thereby impaired chitin-triggered defense gene expression (Fig. 2-5B and 2-10), it seems possible that the Hsp90 chaperone complex, including Hop/Sti1a, has a dual function in rice innate immunity: one function is related to efficient export from the ER and PM localization of PAMP receptors, and the other to signaling in the defensome at the PM.

2-4-2 Roles of the ER Maturation and Trafficking of PAMP Receptors in Plant Innate Immunity

Our results indicate that maturation of the rice chitin receptor OsCERK1 occurs in the ER, and that the receptor is subsequently transported to the PM through the endomembrane network including the ER and the Golgi (Fig. 2-7).

Most newly synthesized polypeptides can fold without the aid of chaperones, but some hydrophobic proteins strictly require chaperones for proper folding and assembly. Plant PAMP receptors often possess an extracellular hydrophobic LRR or LysM region and putative N-glycosylation sites. The ER is a critical site for protein N-glycosylation and folding. Glycosylated proteins are folded and assembled in the ER and exported from the ER by the coatomer complex II (COPII) machinery (Gurkan et al., 2006). Defective glycosylation leads to misfolding and aggregate formation; misfolded proteins are retained in the ER or degraded through the ER-associated degradation

(ERAD) pathway (Helenius and Aebi, 2001).

Endosome localization of plant RLKs has been reported recently (Russinova et al., 2004; Geldner and Robatzek, 2008). Analysis of the OsCERK1-GFP localization patterns in a large protoplast population showed that although OsCERK1-GFP was mostly present in the PM, very few cells (4%) showed OsCERK1-GFP fluorescence exclusively in the PM. In the majority of protoplasts (96%), GFP fluorescence was also observed in punctate organelles or the ER (Fig. 2-7B, upper panel). These results, together with the findings in the presence of BFA and CA-Sar1, strongly suggest that the ER is the site for OsCERK1 biogenesis and maturation (Fig. 2-12B). Our results from Y2H and BiFC assays indicate that Hop/Sti1a also interacts with OsFLS2 and OsBAK1 (Fig. 2-6A and 2-S2). OsFLS2 is a functional ortholog of AtFLS2, which perceives flg22 PAMP signals in some rice cultivars (Takai et al., 2008). Therefore, it is possible that Hop/Sti1a functions as a general facilitator of the ER exit and transport of PAMP receptors to the PM.

Two recent studies of the mutant BRI1 steroid receptors bri1-9 and bri1-5 in *Arabidopsis* demonstrated that the ER lumen protein UGGT, which is a folding sensor, and calnexin /calreticulin (CNX/CRT) and BiP, which are ER-resident chaperones, function in the quality control of BRI1 receptors (Jin et al., 2007; Hong et al., 2008). These proteins retain the mutant receptors in the ER so that they are not transported to the PM, thereby abolishing their receptor function. The trafficking system is known to be important for plant innate immunity (Collins et al., 2003; Wang et al., 2005; Nomura et al., 2006; Kwon et al., 2008; Speth et al., 2009), but how trafficking affects innate immunity is largely unknown. Recent studies have indicated that key components of the ER quality control (ERQC) system are involved in EFR- and N protein-mediated

immune responses in plants (Caplan et al., 2009; Li et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). Our results suggest that Hop/Sti1 and Hsp90 regulate OsCERK1 maturation by assembling a complex (es) with OsRac1 in the ER and subsequently transporting OsCERK1 from the ER to the PM (Fig. 2-12B). The precise mechanisms by which these proteins regulate maturation, ER export and trafficking of OsCERK1 remain to be elucidated, and how the ERQC system cooperates with the Hsp90-Hop/Sti1 co-chaperone machinery will be an interesting topic for the future.

2-4-3 Conservation of the Hsp90 Chaperone System for Maturation and Trafficking of Cell-Surface Proteins in Animals and Plants

The role of the Hsp90 chaperone system in the maturation of nuclear steroid receptors is well established in animals (reviewed by Wegele et al., 2004), and more general functions of the system in the maturation, trafficking and expression of animal cell surface proteins have recently begun to emerge. Wang et al. (2006) demonstrated that the Hsp90 chaperone system helps to stabilize the cystic fibrosis transmembrane conductance regulator (CFTR) and promotes its efficient transport to the cell surface. CFTR is a chloride channel expressed at the cell surface of epithelial cells, and defects in its folding and ER export cause CF disease (Riordan, 2005). Down-regulation of the Hsp90 co-chaperone Aha1 rescues a transport-defective CFTR mutation, and it has been proposed that modulations of ER exit by the Hop-containing Hsp90 chaperone complex regulate the transport of CFTR to the cell surface (Wang et al., 2006). Maturation of the human cardiac potassium channel ether-a-gogo-related protein (hERG) in the ER is also facilitated by cytoplasmic Hsp90 and Hsp70, and GDA treatment suppresses hERG cell-surface targeting (Ficker et al., 2003). Furthermore, other Hsp90 co-chaperones

such as FKBP38 are involved in the maturation and cell-surface targeting of hERG (Walker et al., 2007); Hop was identified as an interactor of hERG in that study, although its role in hERG transport and function was not analyzed. All available studies highlight the importance of the cytoplasmic Hsp90 chaperone system in protein folding and maturation in the ER, and in the efficient cell-surface localization of physiologically important animal proteins, but the precise molecular mechanisms underlying these processes remain obscure.

I have shown here that Hop/Sti1 and Hsp90 are likewise involved in the maturation, trafficking and PM targeting of the rice PAMP receptor OsCERK1 (Fig. 2-8 to 2-11, and 2-12B). I further showed that Hop/Sti1 and Hsp90 interact with the TM domain of OsCERK1 (Fig. 2-6C, 2-8C and 2-9B). These results imply a function for the Hop/Sti1-Hsp90 chaperone complex in the ER exit of OsCERK1. Since the specific interacting regions of CFTR and hERG with Hsp90 and its co-chaperones have not yet been identified, our results shed light on the questions of which regions of animal cell-surface proteins are likely to interact with the cytoplasmic Hsp90 chaperone complex, and of the exact function of these interactions in protein maturation in the ER and cell-surface targeting. Furthermore, our observation that OsRac1, a Rac GTPase, is colocalized with the OsCERK1 complex in the ER, suggests that Rac or other small GTPases may likewise participate in the regulation and trafficking of CFTR, hERG and other physiologically important mammalian cell-surface proteins. I also have demonstrated the involvement of Sar1 GTPase in the trafficking of OsCERK1 (Fig. 2-7D and 2-12B); a similar observation regarding the trafficking of the hERG channel in mammalian cells was reported recently (Delisle et al., 2009), suggesting that trafficking systems for cell-surface proteins are highly conserved between animals and

plants. Our results on the regulation of the rice OsCERK1 receptor by the Hop/Sti1-Hsp90 chaperone complex may therefore contribute to a deeper understanding of conserved regulatory processes that control cell-surface targeting of proteins in animals and plants.

2-4-4 A Defensome Model for PAMP-triggered Innate Immunity in Rice

Extending our previous studies on the interactions of a number of proteins during rice immune responses, using various experimental approaches including Y2H, co-IP, BiFC and gel filtration experiments together with functional studies, I propose the existence of a protein complex, the defensome, which regulates rice innate immunity (Fig. 2-12). Two major components of the defensome are PAMP receptors, represented by OsCERK1, and OsRac1, which is a molecular switch activating most of the downstream signaling components in PAMP-triggered immunity. These two key components are linked by Hop/Sti1. Thus, Hop/Sti1 has a critical role in connecting the RLK receptor with both the OsRac1 molecular switch and the Hsp90 chaperone complex, the latter including SGT1 and RAR1 which are required for innate immune responses. I propose that the components of the defensome have two functions: some, mainly the co-chaperone proteins, contribute to receptor maturation/transport and the formation of the signaling complex, while others are recruited later to act as signaling components at the PM. It is also possible that OsCERK1 and OsRac1 interact independently with the Hop/Sti1-Hsp90 complex.

We have recently found, using a fluorescence resonance energy transfer (FRET)-based biosensor, that OsRac1 is activated within a few minutes of exposure to chitin, and also identified a novel guanine nucleotide exchange factor (GEF) that is

specific for OsRac1 (A. Akamatsu et al., unpublished results). OsCERK1 may activate this GEF upon perception of chitin, thereby leading to OsRac1 activation at the PM; these events appear to occur in the defensome, whose components may thus undergo rapid modifications after pathogen infection. Future analysis of defensome dynamics is likely to illuminate the molecular mechanisms that underlie signaling in rice innate immunity.

2-5. FIGURES

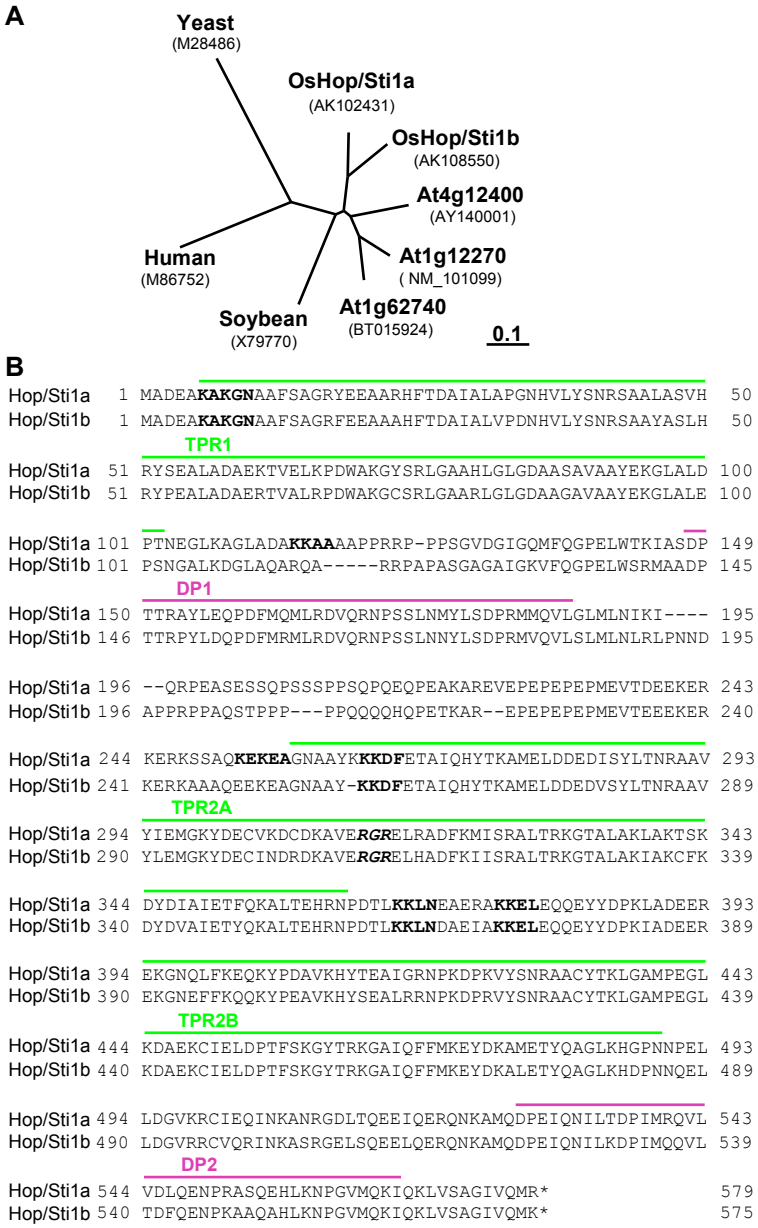


Figure 2-1. Conserved Domains of Hop/Sti1 Proteins. (A) Phylogenetic analysis of Hop/Sti1 from various organisms. Accession numbers are given in brackets. *Hop/Sti1* is a single-copy gene in yeast and human. There are two *Hop/Sti1* genes in rice (Os) and three in *Arabidopsis* (At). The unrooted phylogenetic tree was drawn with ClustalW. The scale bar represents evolutionary distance (amino acid replacements). (B) Comparison of the rice Hop/Sti1 homologs Hop/Sti1a and Hop/Sti1b. Hop/Sti1 contains three conserved tetratricopeptide repeat (TPR) domains (green lines) and two aspartate-proline (DP) repeat domains (pink lines). In addition, Hop/Sti1a carries six dilysine motifs (KKXX or KXKXX) shown in bold print and one RXR motif shown in bold and italic print, both of which are known ER retention signals (Shikano et al., 2003). Hop/Sti1b contains four dilysine motifs and one RXR motif.

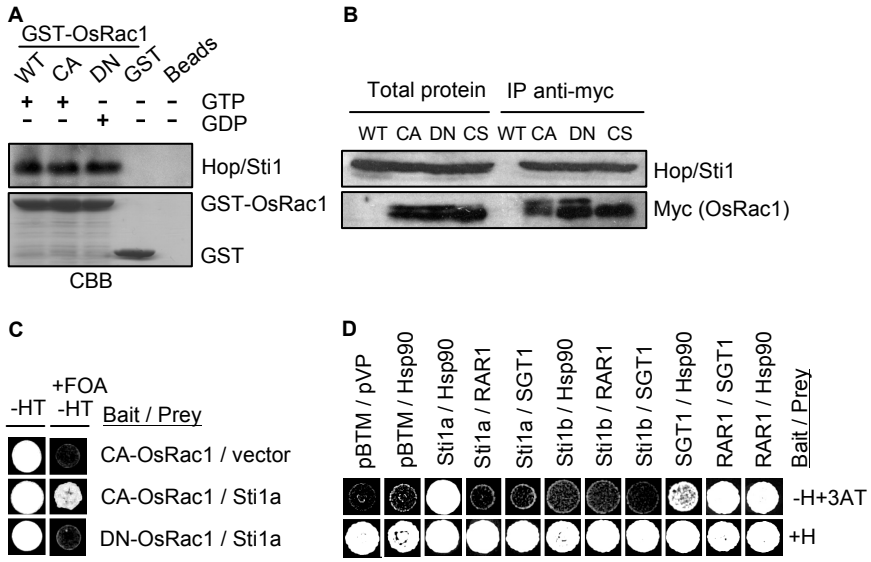


Figure 2-2. The Hsp90 Co-chaperone Hop/Sti1 Interacts with OsRac1. (A) Interaction of Hop/Sti1a and OsRac1 in *in vitro* binding assays. Upper image, purified GST-tagged OsRac1 and His-tagged Hop/Sti1a were subjected to pull-down assays with anti-GST beads and detected with anti-Hop/Sti1a antibody. Lower image, Coomassie Brilliant Blue (CBB)-stained gel after blotting. (B) Co-immunoprecipitation (Co-IP) assays of OsRac1 with Hop/Sti1 *in vivo*. Protein extracts from wild type (WT), Myc-tagged constitutive active (CA-), dominant negative (DN-) and C terminal point mutant (CS-) OsRac1 cultured cells were subjected to Co-IP with anti-Myc antibody. Western blotting was performed with anti-Hop/Sti1a and anti-Myc antibodies. (C) Two-hybrid assays of Hop/Sti1a and OsRac1 in SUS. (D) A survey of Hop/Sti1-interacting proteins among known (co-)chaperones in the Y2H system.

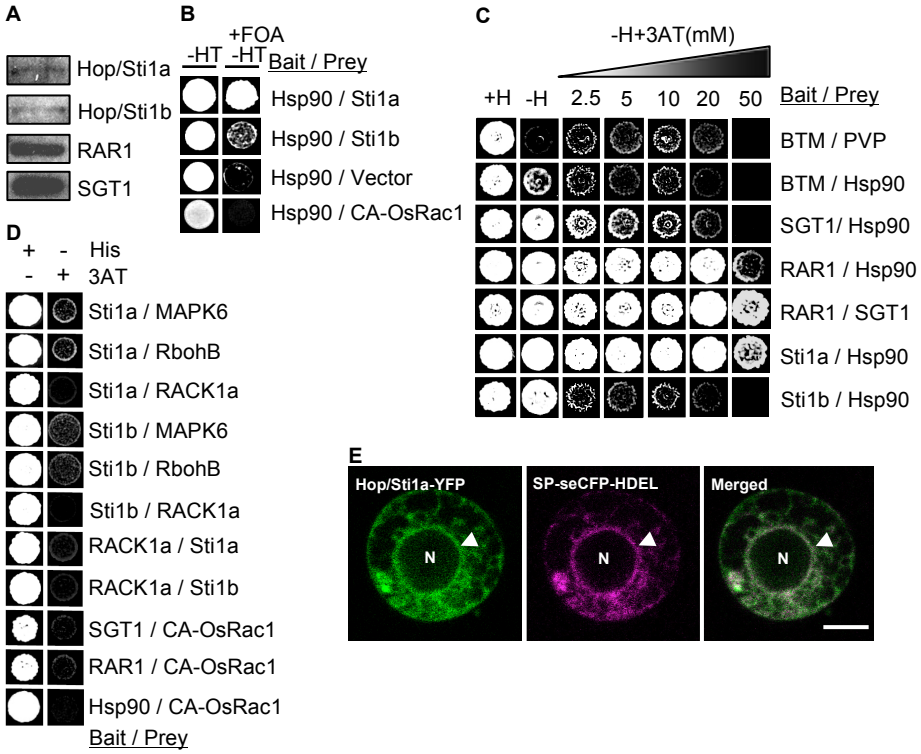


Figure 2-3. Hop/Sti1a has a stronger interaction with Hsp90 than Hop/Sti1b and Is Localized to the ER. (A) Protein expression in yeast. Total proteins were extracted from yeast cultures, followed by western blotting with antibodies recognizing the indicated proteins. (B) Interaction between Hop/Sti1a or Hop/Sti1b and Hsp90 in SUS assays. (C) Semi-quantitative assay for the interaction strength of different two-hybrid pairs. The strength of interactions was estimated from the concentration of 3AT in the plates. (D) Negative combinations in a survey of Hop/Sti1 and OsRac1 interacting proteins in Y2H assays. (E) Subcellular localization of Hop/Sti1a protein. Hop/Sti1a-YFP was colocalized predominantly with the ER marker SP-seCFP-HDEL (middle). The arrowheads indicate the typical ER ring structure surrounding the perinuclear membrane. N, nucleus; bars = 5 μm.

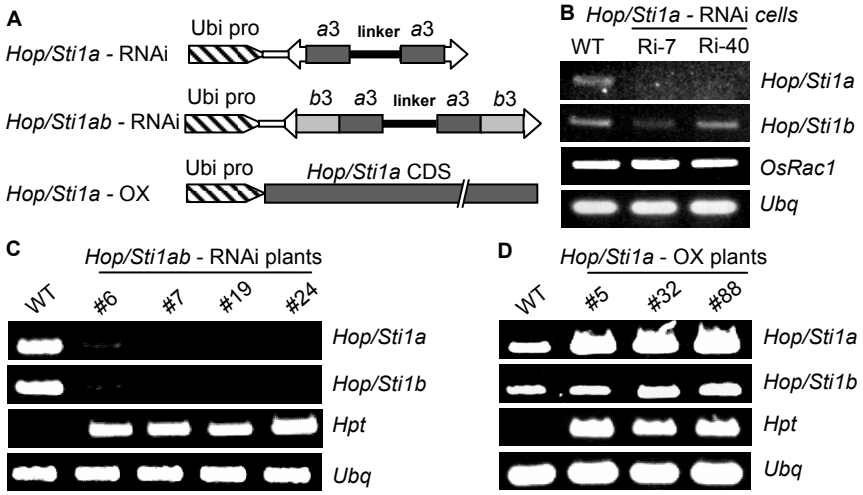


Figure 2-4. *Hop/Sti1* Expression in Transgenic Rice Plants. (A) Constructs for *Hop*/*Sti1* RNAi and *Hop*/*Sti1* overexpression (OX) in transgenic cells and plants. a3, 3' UTRs of *Hop/Sti1a* and b3, 3' UTRs of *Hop/Sti1b*, were used as trigger regions for RNAi silencing. The construct for *Hop/Sti1a* overexpression carries the coding sequence (CDS) of *Hop/Sti1a*. (B) RT-PCR of *Hop/Sti1a*, *Hop/Sti1b* and *OsRac1* in *Hop/Sti1a*-RNAi cultured cell lines Ri-7 and Ri-40. *Ubq* was used as an internal control. (C) RT-PCR of *Hop/Sti1a* and *Hop/Sti1b* in *Hop/Sti1ab*-RNAi plants. The *Hpt* gene for hygromycin resistance was used as a marker of transgenic plants. #6, #7, #19, #24 are independent *Hop*/*Sti1ab*-RNAi lines. (D) RT-PCR of *Hop/Sti1a* and *Hop/Sti1b* in *Hop/Sti1a*-OX plants. #5, #32, #88 are independent *Hop/Sti1a*-OX lines.

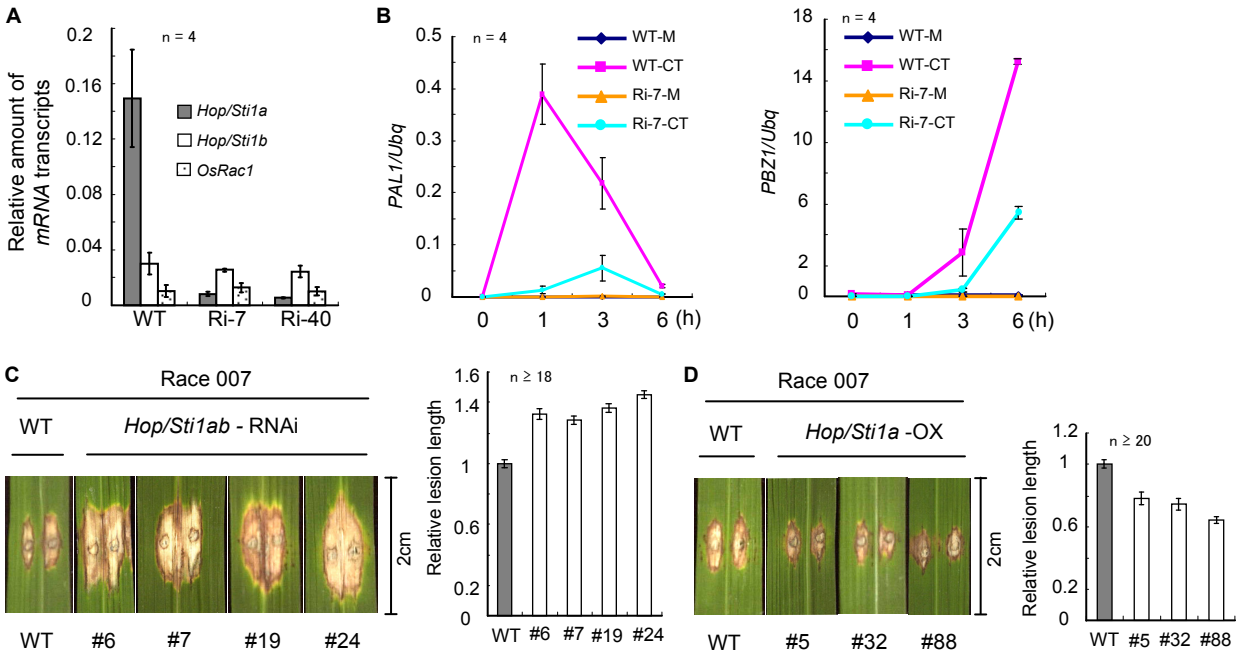


Figure 2-5. Hop/Sti1a Is Involved in the Chitin-triggered Immune Response and Rice Blast Resistance. **(**A) Expression of *Hop*/*Sti1* and *OsRac1* in *Hop*/*Sti1a*-RNAi cultured cells Ri-7 and Ri-40 was analyzed by qPCR and normalized with endogenous *Ubq*. Data shown are means ± SE; n = 4. (B) Induction of defense-related genes, *PAL1* (left) and *PBZ1* (right), in *Hop*/*Sti1a*-RNAi (Ri-7) and wild-type (WT) cells, following chitin treatment (CT), was monitored at the indicated time points using qPCR. M, mock. Data are means ± SE; n = 4. (C) and (D) Infection assays of *Hop/Sti1ab*-RNAi (C) and *Hop/Sti1ab*-OX (D) plants with the virulent rice blast fungus (race 007). Pictures (left) show typical phenotypes of WT and representative transgenic plants. Relative lesion lengths (right; WT = 1) are shown with means ± SE; n ≥ 18.

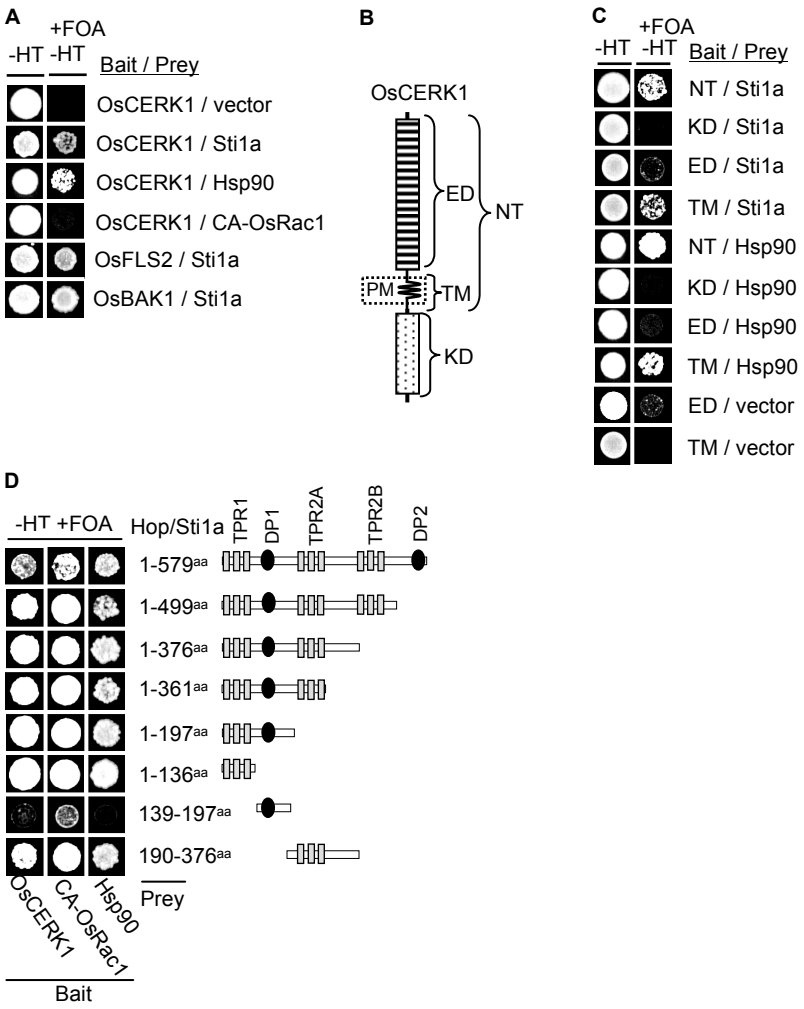


Figure 2-6. Hop/Sti1a Interacts with Rice Chitin Receptor via Its Transmembrane Domain. (A) Interactions of Hop/Sti1a, Hsp90 and CA-OsRac1 with the rice RLK PAMP receptors in SUS. (B) Schematic diagram of OsCERK1 and its four deletion mutants used for SUS assays. (C) Interactions of OsCERK1 deletion mutants with Hop/Sti1a and Hsp90 in SUS assays. (D) Interactions of a series of Hop/Sti1a deletion mutants with OsCERK1, CA-OsRac1, and Hsp90 in SUS assays. A schematic diagram of the Hop/Sti1a deletion mutants is shown on the right. Numbers indicate the first and last amino acids of Hop/Sti1a (intact, 579 amino acids; top line) that are retained in the mutants.

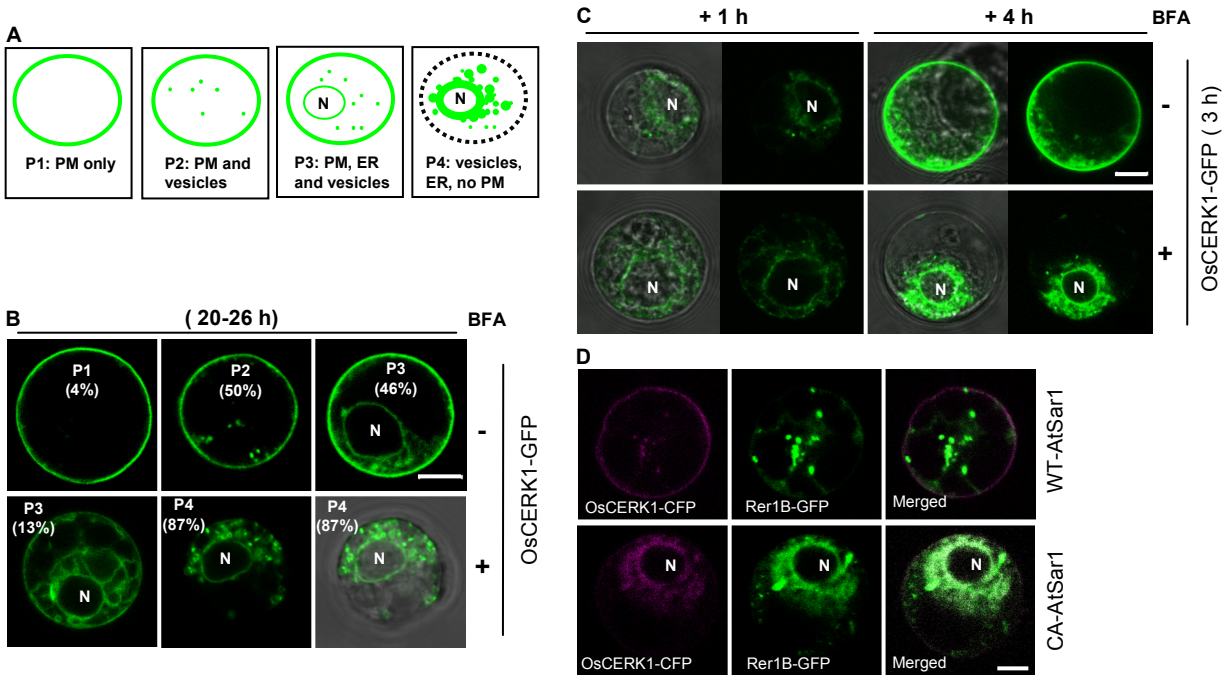


Figure 2-7. OsCERK1 Matures in the ER and Is Subsequently Transported to the PM by a Vesicle Trafficking Pathway.

(A) Four types of OsCERK1-GFP localization. Green color indicates GFP signal. N, nucleus. (B) Subcellular localization of OsCERK1-GFP in the absence (upper panel) or presence (lower panel) of BFA. The percentage of each pattern is based on 100-120 random scanned cells. (C) OsCERK1-GFP localization pattern at the early stage of its biogenesis. Protoplasts transformed with OsCERK1-GFP were pre-incubated for 3 h before BFA was added to the medium. Microscopic observation was performed at 1 h and 4 h after BFA treatment. (D) OsCERK1-GFP localization patterns in the presence of WT- or CA-AtSar1. WT-AtSar1-Rer1B-GFP or CA-AtSar1-Rer1B-GFP was co-transformed with OsCERK1-CFP into protoplasts. N, nucleus; bars in B-D = 5 μm.

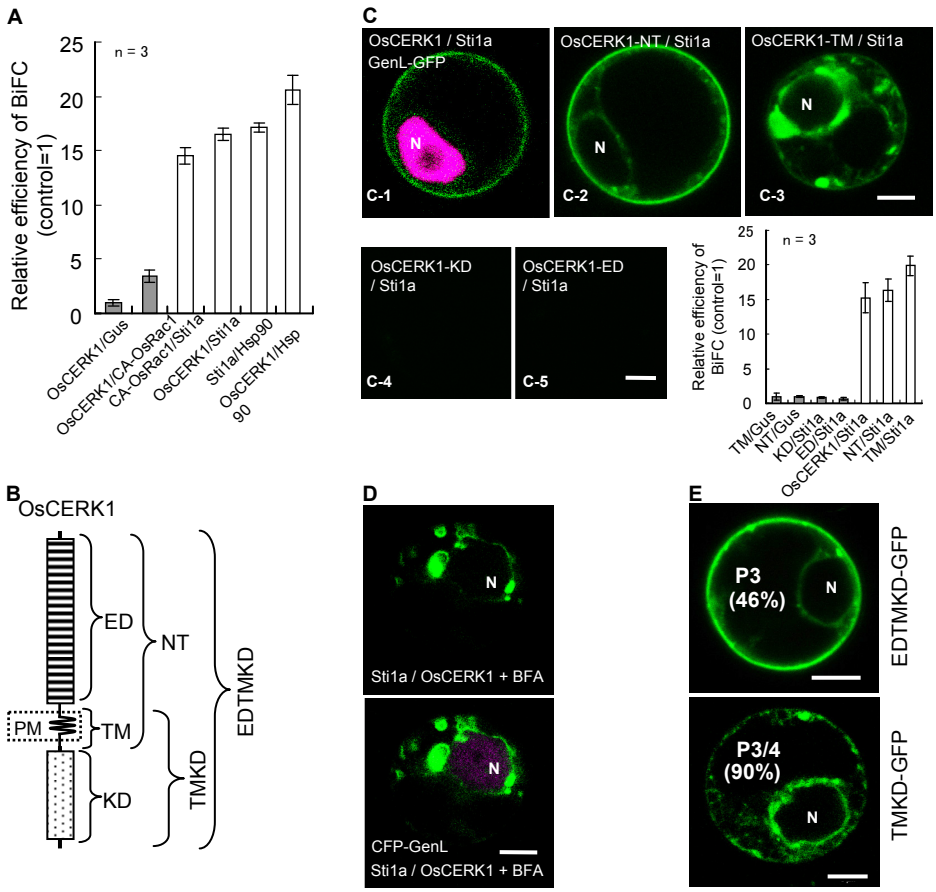


Figure 2-8. OsCERK1 Receptor Interacts with Hop/Sti1a *in vivo* and the Hop/Sti1a - OsCERK1 Complex Is Transported via Trafficking System. (A) Quantitative evaluation of the five BiFC pairs. Data presented are means ± SD. OsCERK1/Gus serves as a negative control. (B) Schematic diagram of OsCERK1 and its deletion mutnats for BiFC and subcellular localization assays. (C) BiFC assays of Hop/Sti1a and OsCERK1. NT and TM deletion mutants of OsCERK1 are shown in Figure 2-8B. C1-5 represent typical patterns of BiFC-reconstituted YFP fluorescence. GenL-GFP, nuclear marker. The graph (lower right) is a quantitative evaluation of each BiFC pair analyzed. TM/Gus and NT/Gus serve as negative controls. Bars in C-G = 5 μm; N, nucleus. (D) Hop/Sti1a-OsCERK1 BiFC signals in the presence of BFA. CFP-GenL, nuclear marker. (E) Typical subcellular localization patterns of intact OsCERK1 and ED-deleted OsCERK1 in protoplasts.

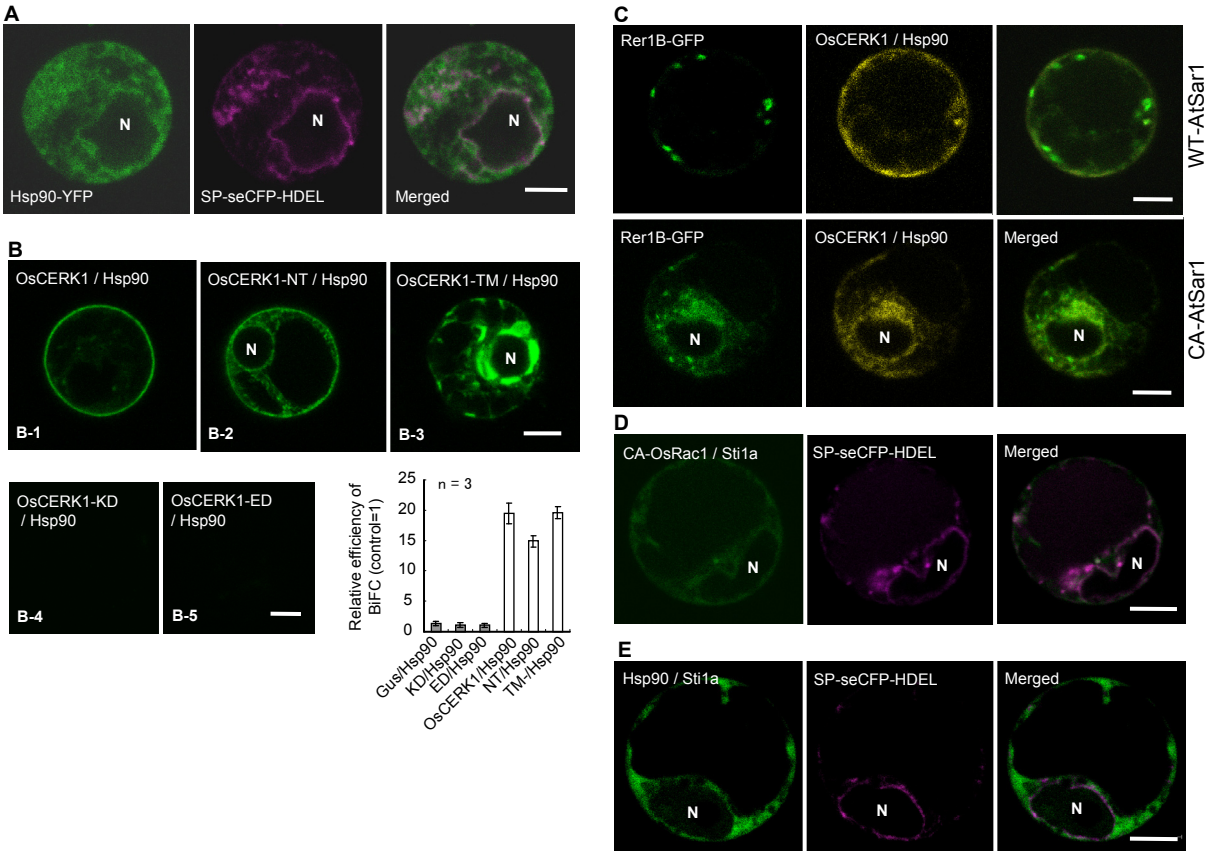


Figure 2-9. OsCERK1 Receptor Interacts with Hsp90 Chaperone *in vivo* and the Transport of OsCERK1-Hsp90 Complex Is Regulated by Sar1. (A) Subcellular localization of Hsp90-YFP in protoplasts. SP-seCFP-HDEL, ER marker. (B) BiFC assays of Hsp90 and OsCERK1. NT and TM fragments of OsCERK1 are shown in Figure 2-8B. B1-5 represent typical patterns of BiFC-reconstituted YFP fluorescence. The graph (lower right) is a quantitative evaluation of each BiFC pair analyzed. Gus/Hsp90 serves as a negative control. Data shown are means ± SD. (C) Effect of CA-Sar1 on OsCERK1-Hsp90 complex localization in BiFC assays. WT-Sar1-Rer1B-GFP or CA-Sar1-Rer1B-GFP was co-transformed with OsCERK1-Hsp90 BiFC constructs into protoplasts. (D) BiFC pattern of CA-Rac1 and Hop/Sti1a. (E) BiFC pattern of Hsp90 and Hop/Sti1a. SP-seCFP-HDEL, ER marker

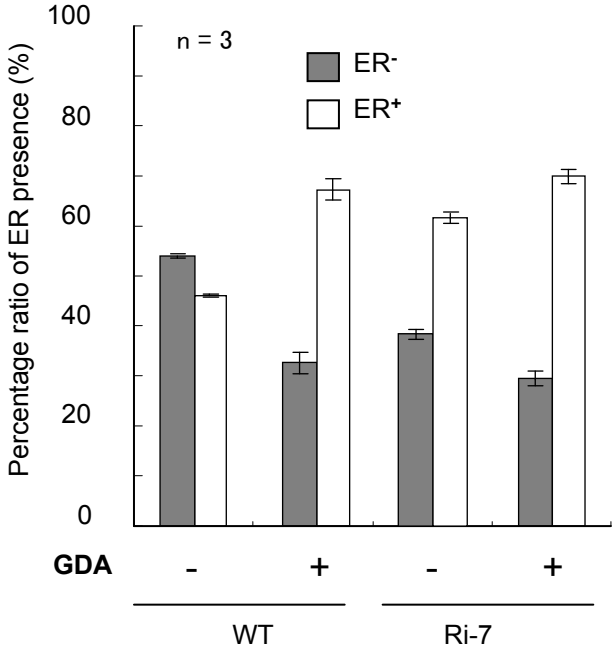


Figure 2-10. OsCERK1-GFP Subcellular Localization Patterns in Wild-type (WT) and *Hop/Sti1a*-RNAi Line Ri-7 in the Presence or Absence of GDA. Gray and white bars indicate the percentages of cells without (ER^-) and with (ER^+) GFP signal in the ER, respectively. Geldanamycin (GDA), the Hsp90 Inhibitor. Data shown are means ± SD.

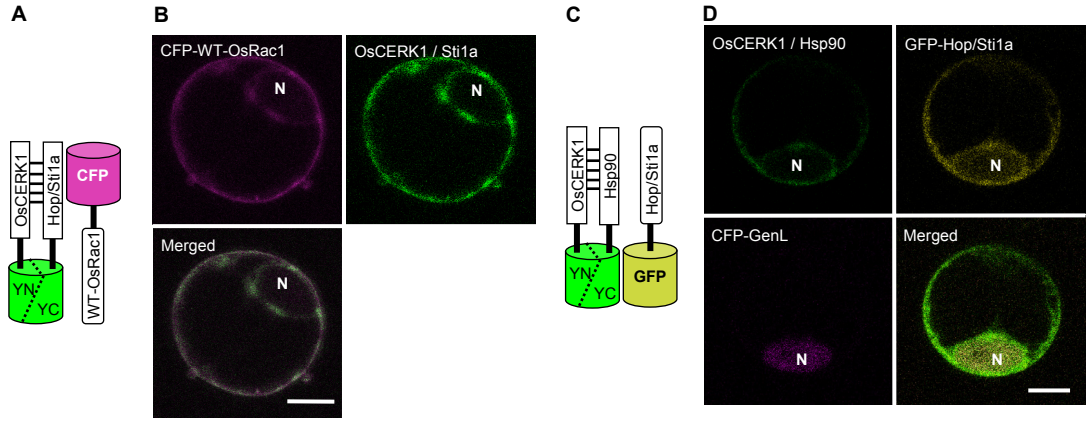


Figure 2-11. OsCERK1, Hop/Sti1a, Hsp90, and OsRac1 May Form a Complex in the ER and PM. (A) OsCERK1-YN, Hop/Sti1a-YC and CFP-WT-OsRac1 constructs used for BiFC-based colocalization assay. (B) Colocalization of OsCERK1, Hop/Sti1a, and OsRac1 at the PM and the ER. Bars in B and D = 5 μm; N, nucleus. (C) OsCERK1-YN, Hsp90-YC and Hop/Sti1a-GFP constructs used for BiFC-based colocalization assays. (D) Colocalization of OsCERK1, Hsp90, and Hop/Sti1 at the PM and the ER. CFP-GenL, nuclear marker.

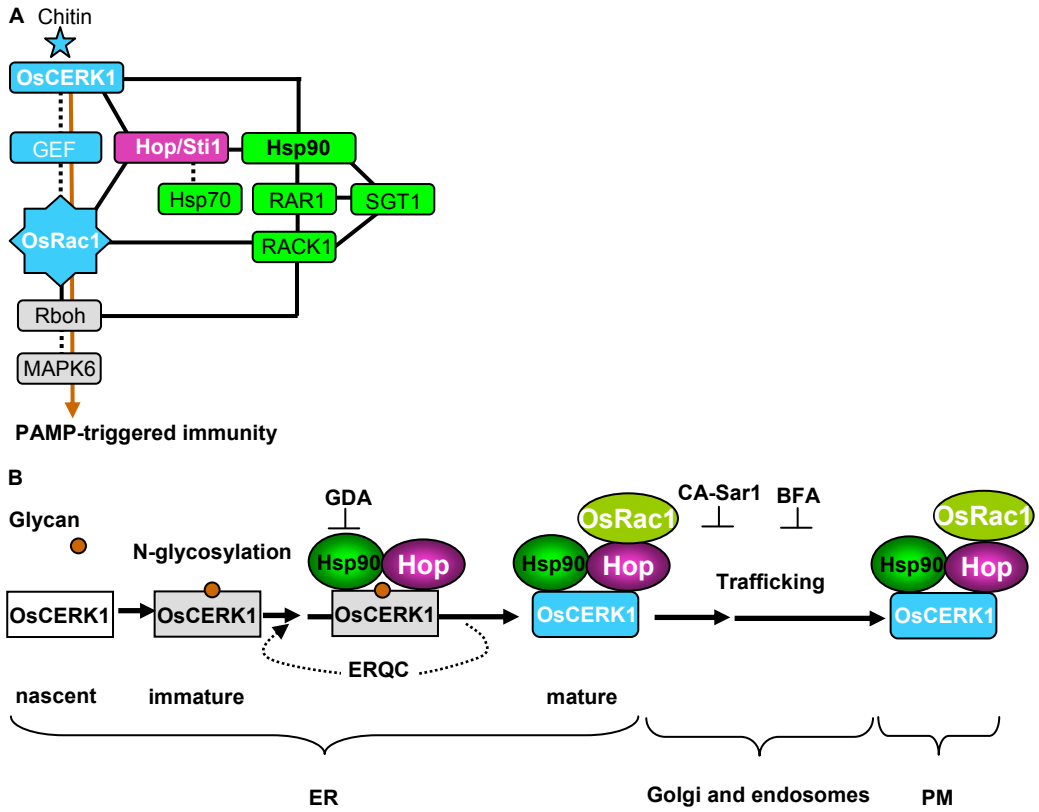


Figure 2-12. Protein Network in the OsRac1 Immune Complex (Defensome) and a Model of Hop/Sti1-Hsp90 Chaperone Function in Maturation and Trafficking of OsCERK1
(A) "Defensome" protein network involved in PAMP-triggered innate immunity in rice. The defensome is present at the PM. A chitin signal (blue star) is received by OsCERK1 and transmitted to a putative GEF for OsRac1 activation. Activated OsRac1 induces activation of Rboh for reactive oxygen species production, MAPK cascade triggering and activation of other downstream components. Hsp90 and the co-chaperones Hop/Sti1, RAR1, SGT1 and Hsp70 interact with OsRac1 and OsCERK1 through Hop/Sti1 and the scaffolding protein RACK1. Solid lines linking two proteins indicate established direct interactions, while dotted lines indicate possible interactions that remain to be demonstrated. The brown arrow indicates signal transduction. (B) A model for the maturation, trafficking, and PM localization of OsCERK1. Nascent OsCERK1 is glycosylated and folded in the ER. The Hop/Sti1-Hsp90 chaperone complex binds OsCERK1 in the ER and assists its ER exit. OsRac1 and other proteins may form a complex with mature OsCERK1 (blue), which then exits the ER and is transported to the PM via the Sar1-dependent vesicle trafficking system, while misfolded protein may be recycled by the ERQC system. At the PM, OsCERK1-Hop/Sti1-Hsp90 forms the defensome together with several (co-)chaperones and other signaling proteins for PAMP reception and signal transduction.

2-6. SUPPLEMENTAL FIGURES

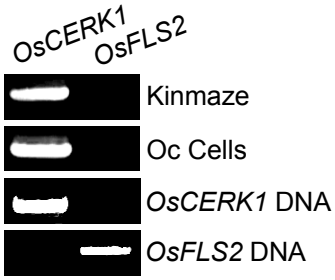


Figure 2-S1. OsFLS2 is Absent in Rice Cultivar Kinmaze and Oc Cells. Rice cultivar Kinmaze and Oc cells possess the chitin receptor OsCERK1, but lack the flg22 receptor OsFLS2. Template cDNA was extracted from Kinmaze and Oc suspension cells. Plasmid DNAs of *OsCERK1* and *OsFLS2* served as positive controls. Gene-specific primers of *OsCERK1* and *OsFLS2* were used to amplify the kinase domain (KD) of each RLK receptor.

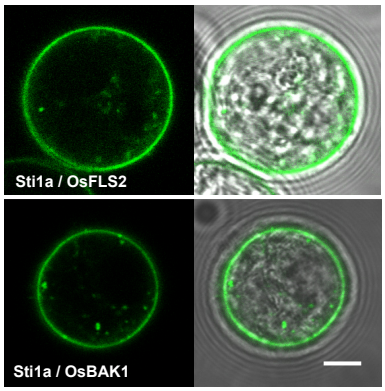


Figure 2-S2. Interaction of Hop/Sti1a with Rice RLKs *in vivo*. Interaction of Hop/Sti1 with OsFLS2 (upper) and OsBAK1 (lower) in the BiFC system in rice protoplasts. Reconstituted YFP BiFC signal shown on the left; YFP and bright field images merged on the right. Bars = 5 μm.

2-7. Table

Table 1. Primers used in this study

Primer name	Sequence (5'-3')
Primers for conventional yeast two-hybrid constructs	
Hop/Sti1 Bait-F	ACTAGTCATGGCCGACGAGGCGAAGG (For both Hop/Sti1a and b Bait)
Hop/Sti1a Bait-R	CTGCAGCTATCTCATTTGAACTATT
Hop/Sti1b Bait-R	CTGCAGCTACTTCATTTGGACTATC
Hop/Sti1a Prey-F	CGTCGACCG ATGGCCGACGAGGCGAAGG
Hop/Sti1a-Prey-R	CGGTACCCCTATCTCATTTGAACTATT
Hop/Sti1b Prey-F	GGTACCGAATGGCCGACGAGGCGAAGG
Hop/Sti1b Prey-R	CGGTACCCCTACTTCATTTGGACTATC
Primers for overexpression	
Hop/Sti1a OX-F	CACCATGGCCGACGAGGCGAAGG
Hop/Sti1a OX-R	CTATCTCATTTGAACTATT
Primers for RNAi	
Hop/Sti1a RNAi-F	CACCTCGACCAAAGCAAGAAGCGCTGTTC
Hop/Sti1a RNAi-R	CTTTCAGCATGTGGCCAGACCCATGACCAAAA CCGA
Hop/Sti1b RNAi-F	ATGGGTCTGGCCACATGCTGAAAGGGCCAGG GATAG
Hop/Sti1b RNAi-R	TAGGTCGTGGTGACTGATTAGGCA
Primers for RT-PCR	
Hop/Sti1 RT-F	ATGGCCGACGAGGCGAAGG (For both a and b)
Hop/Sti1a RT-R	TATGCCGTCAACGCCGCTG (a-specific)
Hop/Sti1b RT-R	TTCGTCTCCGGCTGGTGCT (b-specific)
Primers for quantitative real time PCR	
UBQ qRT -F	AACCAGCTGAGGCCCAAGA
UBQ qRT -R	ACGATTGATTTAACCAGTCCATGA
PBZ1 qRT -F	ATGAAGCTTAACCCTGCCGC
PBZ1 qRT -R	GTCTCCGTCGAGTGTGACTTG
PAL1 qRT -F	TGAATAACAGTGGAGTGTGGAG
PAL1 qRT -R	AACCTGCCACTCGTACCAAG

Table 1. Primers used in this study

Primer name	Sequence (5'-3')
Hop/Sti1a qRT-F	GAGGAAATCGTCTGCTCAGA
Hop/Sti1a qRT-R	GCGGTCTCGAAGTCCTTT
Hop/Sti1b qRT-F	GTTGCTTGATGGTGTTAGG
Hop/Sti1b qRT-R	CTGTCTTTCTTGCAATTCC
Primers for Hop/Sti1a deletion assays	
Hop/Sti1a 1^{aa}-F = Hop/Sti1a OX-F	
Hop/Sti1a579^{aa}-R = Hop/Sti1a OX-R	
Hop/Sti1a 136^{aa}-R	CTAGAACATCTGGCCTATGCCGTC
Hop/Sti1a139^{aa}-F	CACCATGGAGCTCTGGACCAAGATCGCC
Hop/Sti1a190^{aa}-F	CACCATGCTTAACATCAAGATCCAGAGA
Hop/Sti1a 197^{aa}-R	CTATCTCTGGATCTTGATGTTAAG
Hop/Sti1a 361^{aa}-R	CTAATTCCGATGCTCAGTTAGAGC
Hop/Sti1a 376^{aa}-R	CTATTTCTTTGCCCTCTCAGCCTC
Hop/Sti1a 499^{aa}-R	CTACCTTTTCACACCGTCCAGCAG
Primers for OsCERK1 deletion assays	
OsCERK1 FL-F	CACCATGGAAGCTTCCACCTCCCTCCT
OsCERK1 FL-R	TCTCCCGGACATTAGGTTGAC
OsCERK1 ED-F = OsCERK1 FL-F	
OsCERK1 ED-R	TCCTGCAGAAGCTCCCTTTCC
OsCERK1 NT-F = OsCERK1 FL-F	
OsCERK1 NT-R	CCCCTGTGTAGCATTAGAAAGTT
OsCERK1 TM-F	CACCATGGCTATAGCAGGAGGTGTTGTG
OsCERK1 TM-R = OsCERK1 NT-R	
OsCERK1 KD-F	CACCATGTTTAGTATTGGCAATAAAATAGG
OsCERK1 KD-R	AAGCGTCATCAGCGCGACGACCA

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I would like to dedicate this thesis to my ex-mentor Prof. Yao-Gung Liu, who leads me to science; my wife, who always supports me behind; and my son, whom I should have more time to play with in his childhood.

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