Analysis of the OsRac1 Protein Complex Involved in Defense Signaling of Rice.

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25.12.2007

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Abbreviaties and Acronyms

Avr	avirulence
CBF3	centromere binding factor3
CC	coiled-coil
CRIB	Cdc42/Rac-interactive binding
CS	CHORD-SGT1
DEX	dexamethasone
ETI	effector-triggered immunity
GAP	GTPase-activating protein
GDA	geldanamycin
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GST	glutathione S transferase
GTP	guanosine triphosphate
Hop	hsc70/hsp90-organizing protein
HR	hypersensitive response
HSP70	heat shock cognate protein 70
HSP90	heat shock cognate protein 70
IP	immunoprecipitation
LRR	leucine-rich repeat
NBS	nucleotide-binding site
NLR	NOD-like receptor
NTD	N-terminal domain

PAMP	pathogen-associated molecular patterns
PBZ1	*Probenazole1*
PRR	pattern recognition receptor
PTI	PAMP-triggered innate immunity
RAR1	required for Mla12 resistance
RIC	Rop-interacting CRIB-containing protein
RLK	receptor-like kinases
ROS	reactive oxygen species
R protein	resistance protein
RWD/RACK	WD-repeat-containing receptor of activated C kianase homolog
SCF	SKP1/CULLIN1/F-box protein
SE	sphingolipid elicitor
SGS	SGT1-specific
SGT1	suppressor of the G2 allele of *skp1*
Sti1	stress inducible protein 1
TPR	tetratricopeptide repeat
TTSS	type III secretion system

CHAPTER 1: Introduction

Plant innate immunity

Plants use two innate immune systems to respond to pathogen infection. One is pathogen-associated molecular patterns (PAMPs)-triggered innate immunity (PTI) and the other is effector-triggered immunity (ETI) (Zipfel and Felix, 2005; Chisholm et al., 2006; Jones and Dangl, 2006).

PTI, the first line of defense of plants, is relatively non-specific resistance. PTI is achieved through a set of defined receptors, also referred as pattern recognition receptors (PRRs) that recognize conserved PAMPs (Figure 1A: Zipfel and Felix, 2005). Upon PAMP recognition, primary defense responses are induced such as cell wall alterations, deposition of callose and the accumulation of defense-related proteins including chitinases, glucanases and proteases, which all negatively affect colonization by potential pathogens (Van Loon et al., 2006). Several PAMPs have been identified for plant pathogens include FLS2 and EFR recognizing flagellin and EF-Tu-derived peptides flg22 and elf18, respectively, in *Arabidopsis*. FLS2 and EFR are plasma membrane-resident receptor-like kinases (RLKs) with extracellular leucine-rich repeats (LRRs) and a cytoplasmic serine/threonine kinase domain (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). In addition to RLKs, the plasma membrane-anchored CEBiP

chitin receptor which contains two extracellular LysM domains, a module implicated in

peptidoglycan-binding, but lacks an intracellular kinase domain, was identified (Kaku et

al., 2006). Interestingly, whereas flg22 and elf18 are recognized by different RLKs, the

primary defense responses induced upon their recognition are largely similar (Zipfel et

al., 2006). This seems to be an evolutionary advantage, as in this way recognition of

different PAMPs by different PRRs converges into a limited number of signaling

pathways that all activate primary defense responses. A complete MAPK pathway and

WRKY transcription factors for response to flagellin have been identified downstream

of FLS2 (Asai et al., 2002).

 Some successful pathogens of plants are able to break or suppress basal defense

activated in PTI (Figure 1B). Plant and animal pathogenic bacteria contain four

secretory systems, of which the type III secretion system (TTSS) appears to be the most

important for virulence. By the TTSS, plant pathogenic bacteria inject multiple effectors

into the host plant; without a functional TTSS, they only induce the primary defense

response and are not pathogenic (Jakobek et al., 1993; Chang et al., 2005). A pathogenic

P. syringae strain mutated in the TTSS, and unable to deliver any type III effectors,

triggers a faster and stronger transcriptional re-programming in bean than does the

wild-type strain (Jakobek et al., 1993). It has been shown that many TTSS - injected

effectors of plant pathogenic bacteria suppress PTI of plants allow successful colonization (Nomura et al., 2005). For a subset of effectors, the mechanism of suppression has been elucidated: *P. syringae* effectors AvrPto, AvrRpt2 and AvrRpm1 all compromise responses elicited by PAMPs (Hauck et al., 2003; Kim et al., 2005). The functions and mechanisms of delivery of fungal and oomycete effectors are still poorly understood.

Effectors that enable pathogens to overcome PTI are recognized by specific disease resistance (*R*) genes direct or indirectly. If one effector is recognized by a corresponding R protein, ETI ensues (Figure 1C). The recognized effector is termed an avirulence (Avr) protein. The recognition of pathogen effectors by these R proteins activates a common defense mechanism known as hypersensitive response (HR), which includes the oxidative burst, the induction of defense-related genes, and localized cell death (Shirasu and Schulze-Lefert, 2000). A number of *R* and *Avr* genes have been cloned from a variety of host pathogen systems. Although similarities among *Avr* gene products are limited, in general, plant disease *R* genes share a number of known structural motifs (Martin et al., 2003). The largest class of *R* genes encodes proteins with nucleotide-binding site (NBS) and Leu-rich repeat (LRR) motifs. Members of this group confer resistance to a number of pathogens including bacteria, viruses, fungi, nematodes,

and insects, suggesting the existence of a common signal transduction pathway that results in resistance to these diverse organisms (Martin et al., 2003). The NBS-LRR class of *R* genes could be subdivided into two major groups based on the presence of domains similar to the *Toll* and interleukin-1 receptor or coiled-coil (CC) domain at the amino terminus. Very little is known about the signalling events required to activate NBS-LRR-mediated ETI. NBS-LRR proteins are probably folded in a signal competent state by cytosolic heat shock protein 90 and other receptor co-chaperones (Schulze-Lefert, 2004; Holt et al., 2005). The LRRs seem to act as negative regulators that block inappropriate NBS activation. NBS-LRR activation involves both intra- and intermolecular conformational changes and may resemble the induced proximity mechanism by which the related animal Apaf-1 protein activates programmed cell death. The LRR domain is believed to be involved in recognition of effector-perturbed host targets or in direct recognition of effectors, whereas the NBS domain represents the activation domain that is triggered by the exchange of a nucleotide diphosphate by a nucleotide triphosphate. (Takken et al., 2006)

The role of Rac/Rop GTPase in plant innate immunity

Plants seem to lack Ras family of small GTPases, which has a crucial signaling role in

growth and development of animals and most other eukaryotes. However, plants have a large number of Rho-like proteins termed Rop (Rho related GTPase from plant) or Rac (OsRac for rice Rac) (Yang, 2002; Zeng & Yang, 2000), which are highly conserved in the plant kingdom. Rac/Rop is activated by an upstream signal, and like mammals, is regulated by at least three regulators: the guanine nucleotide exchange factors (GEFs), the GTPase-activating proteins (GAPs), and the guanine nucleotide dissociation inhibitors (GDIs). The guanosine diphosphate (GDP)-bound inactive form of Rac/Rop is converted into its active guanosine triphosphate (GTP)-bound form by GEF that interacts with one or more downstream cellular target proteins and produces a variety of response. The active GTP form can be inactivated by the hydrolysis of GTP to GDP through either the intrinsic ability of the GTP form or through association with GAP. GDI negatively regulates the GDP form (Zheng and Yang, 2000; Yang, 2002; Vernoud et al., 2003).

The involvement of Racs/Rops in the regulation of plant defense response has been studied in several species. There are seven Rac/Rop genes in rice (Miki et al., 2005), and 11 in *Arabidopsis* (Gu et al., 2004). In rice, OsRac1, which is located in the plasma membrane, is involved in reactive oxygen species (ROS) production through an NADPH oxidase, and in the initiation of cell death during defense signaling (Kawasaki

et al., 1999; Ono et al., 2001). OsRac1 functions as a positive regulator of NADPH oxidase activation in PAMP signaling (Ono et al., 2001), and at the same time suppresses expression of a scavenger metallothionein (Wong et al., 2004) in transient accumulation of ROS. Expression of CA-OsRac1 enhances resistance to rice blast and bacterial blight infections (Ono et al., 2001, Suharsono et al., 2002) whereas DN-OsRac1 suppresses HR induced by incompatible races of rice blast fungus, indicating that OsRac1 is one of the key regulators of rice innate immunity (Ono et al., 2001). OsRac1 regulates the stability and activation of OsMAPK6, a rice mitogen-activated protein (MAP) kinase, by sphingolipid elicitors (Lieberherr et al., 2005). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is activated by OsRac1 in rice innate immunity (Kawasaki et al., 2006). There are number of other studies aimed at understanding the molecular mechanism of rice innate immunity (Yang et al., 2004; Gu et al., 2006; Chu at al., 2006; Takahashi et al., 2007), but the details of how these pathways are triggered, controlled, and ended remain unknown. Recently, a direct interaction between CA-OsRac1 and the N terminus of the NADPH oxidases, OsRbohs and StRbohB has been found and this direct OsRac1-Rboh interaction was shown to be important for activating NADPH oxidase activity (Wong et al., 2007).

The role of Rac/Rop proteins in defense response has been also shown in other species. In chickpea, a Rac homologue showed enhanced expression during defense response 3–5 days after inoculation (Ichinose et al., 1999). DN-OsRac1 inhibits N-mediated resistance to TMV infection in tobacco (Moeder et al., 2005), and Rac GTPase has been established to be involved in ROS production in Arabidopsi*s* (Potikha et al., 1999, Pak et al., 2000). Interestingly, in barley and tobacco, it has been shown that some Racs play negative roles in innate immunity. HvRacB is required for invasion by the powdery mildew pathogen *Blumeria gramisis* f. sp. *Hordei* in barley (Schutheiss et al., 2002, 2003, 2005). RNAi of *HvRacB* was shown to enhance resistance, whereas expression of CA-*HvRacB* increased susceptibility to *B. graminis*. In tobacco, *NtRac5* mRNA is repressed when tobacco leaves and cells are treated with the fungal elicitor cryptogein. Moreover, the regulation of NtRbohD is affected at both transcriptional and translational levels in cells overexpressing *NtRac5*. Thus, NtRac5 could be considered as a negative regulator of NtRbohD.

Plants seemingly lack receptor tyrosine kinases that can induce Rho-mediated pathways in animals in response to ligand binding (Kjoller and Hall, 2005), and much attention has been drawn to RKL as the prominent cell surface receptors for extracellular signals in plants. The link between RLKs and Rac/Rop was shown by the

fact that the tomato RopGEF KPP was characterized as binding partner of the cytosolic domains of the pollen-specific tomato RLKs – LePRK1 and LePRK2 (Kaothien et al., 2005). Recently, AtRopGEF12 was shown to interact with an Arabidopsis pollen receptor kinase AtPRK2a in pollen tubes through its C terminus (Zhang and McCormick, 2007). RLKs are not the only upstream players to feed into Rac/Rop pathway. Heterotrimeric G proteins have been linked with OsRac1 in disease resistance mechanisms of rice (Suharsono et al., 2002; Lieberherr et al., 2005). It was shown that the *PR* gene expression and H_2O_2 generation are reduced in *d1* mutant lacking Gα subunit of the heterotrimeric G protein. On the other hand, the restoration of PR gene expression and H_2O_2 production by constitutively active OsRac1 in *d1* mutants indicated a model in which OsRac1 functions downstream of Gα in the early step of signaling (Suharsono et al., 2002). However, the connection between Gα and OsRac1 remains to be elucidated.

In animals and yeast, CRIB (Cdc42/Rac-interactive binding) motif is present in many effectors and allows them to bind to the active Racs (Apenstrom, 1999). In plants, no homolog of animal Rho targets was found. Instead of those targets, new proteins containing CRIB motif, called RICs (Rop-interacting CRIB-containing proteins), are shown to interact with the active form of Arabidopsis Rop1 (Gu et al., 2005). However,

functions of RICs are still unclear and it is possible that there are some other Rac/Rop

targets, which do not use CRIB motif to interact with these GTPases. Therefore, it is

necessary to further study about Rac/Rop effectors and the mechanism of their

interactions with Rac/Rop proteins.

Conserved components of plant innate immunity

Several conserved proteins in vertebrates and plants that interact with R proteins or are

required for *R* function have been recently identified. The co-chaperone-like proteins

RAR1 and SGT1, as well as the cytosolic HSP90 chaperone were originally identified

in plants by mutational screens as essential components of a subset of R

protein-triggered immune responses to diverse plant pathogens (Shirasu and

Schulze-Lefert, 2003).

Screening of mutants that have defect on R protein mediated defense response, *RAR1*

(required for *Mla12* resistance) was originally isolated from barley (*Hordeum vulgare*)

and identified as a requirement for resistance to powdery mildew (*Blumeria graminis* f.

sp. *Hordei*) mediated by *Mla12* (Torp and Jorgensen, 1986). *RAR1* homologs are present

in eukaryotes, except for yeast (*Saccharomyces cerevisiae*), and recently have been

predicted to function as a cochaperone in plants (Figure 2). In addition, *RAR1* is required

for a subset of *R*-gene-mediated resistance responses in monocot and dicot plant species (Shirasu et al*.,* 1999; Tornero et al., 2002; Muskett et al., 2002). In plants, RAR1 interacts directly with SGT1 (<u>s</u>uppressor of the <u>G</u>2 allele of *skp1*), and HSP90 (Azevedo et al., 2002; Liu et al., 2002, Takahashi et al., 2003). SGT1 also contributes to *R*-gene-mediated resistance (Austin et al., 2002; Azevedo et al., 2002, 2006; Liu et al., 2002, 2004). As SGT1 and (metazoan) RAR1 share structural similarities with co-chaperones and bind to each other as well as to HSP90 (Azevedo et al., 2002; Shirasu and Schulze-Lefert, 2003; Takahashi et al., 2003; Bieri et al., 2004; Liu et al., 2004), they are thought to act as co-chaperones, possibly by positively modulating HSP90 activity on its R protein clients. Distinct isoforms of Arabidopsis *HSP90* are required for specific *R*-gene-mediated resistance responses. For example, *AtHSP90.1* is required for the full function of *RPS2* that confers resistance to *Pseudomonas syringae* expressing *AvrRpt2* (Takahashi et al., 2003), while *AtHSP90.2* is required for the function of *RPM1* resistance to *P. syringae* expressing *AvrRPM1* (Hubert et al., 2003). Similarly, the requirement for *Hsp90* in *R*-gene-mediated resistance in solanaceous plants has been shown. These include *Rx*-mediated resistance to *Potato virus X*, *N*-mediated resistance to *Tobacco mosaic virus*, and *Pto*-mediated resistance to *P. syringa*e expressing *AvrPto.* Thus, *Hsp90* plays an important role in disease resistance

signaling (Lu et al., 2003; Liu et al., 2004).

Both HSP90 and SGT1 are required for the stabilization and assembly of the yeast

CBF3 (for centromere binding factor3) and the human kinetochore complexes (Bansal et

al., 2004; Lingelbach and Kaplan, 2004; Steensgaard et al., 2004; Niikura et al., 2006).

As yeast does not contain any RAR1 homolog, CBF3 complex assembly is a

RAR1-independent process. Whether CHP1, a RAR1 homolog in human, is required for

the kinetochore complex assembly is not known, although the *Aspergillus* homolog

CHPA seems not to be involved in this process (Sadanandom et al., 2004). Interestingly,

SGT1 is also involved in degradative processes, as it is required for the function of

several SCF (for SKP1/CULLIN1/F-box protein) complexes in *Saccharomyces*

cerevisiae and plants (Kitagawa et al., 1999; Gray et al., 2003; Bansal et al., 2004). In

these organisms, SGT1 interacts with SCF subunit SKP1, indicating its role in the

degradation of a potentially large number of proteins. Thus, SGT1 may be a key

regulator coupling the stabilization and degradation of proteins. HSP90 and SGT1 also

play a key role in the activation of mammalian immune responses induced by the

NOD-like receptor (NLR) protein family, a group that contains an NBS-LRR domain

(Hahn 2005; da Silva Correia et al., 2007; Mayor et al., 2007). SGT1 and HSP90

interact with NLR proteins to form an 'inflammasome' complex and are required for

activation of the complex. Therefore, regulatory networks for immune responses in plants and mammals share at least some common components, and understanding the molecular mechanisms involved in protein complexes containing R proteins or NLP proteins, SGT1, and HSP90 is becoming crucial in the study of innate immunity in higher eukaryotes.

HSP90 is composed of three domains: an N-terminal domain (NTD) containing an ATP binding module, a middle client binding domain, and a C-terminal dimerization domain (Pearl and Prodromou, 2006). SGT1 is composed of three conserved regions: an N-terminal TPR (for tetratricopeptide repeat) domain, a central CS (for CHORD-SGT1) domain, and a C-terminal SGS (for SGT1-specific) domain (Azevedo et al., 2002). RAR1 is made up of two homologous zinc binding domains, CHORD I and CHORD II (Shirasu et al., 1999; Heise et al., 2007). The N-terminal domain of HSP90 (HSP90-NTD) and the CS domain of SGT1 are known to interact with the CHORD I and CHORD II domains of RAR1, respectively (Azevedo et al., 2002; Takahashi et al., 2003). Moreover, HSP90-NTD and the CS domain of SGT1 interact with each other in plants (Takahashi et al., 2003). Therefore, competition or synergy between RAR1 and SGT1 for binding HSP90 might occur either through the CS domain, interacting with both the HSP90-NTD and the CHORD II domains, or through HSP90-NTD, interacting

with both the CHORD I and CS domains (Figure 3).

Despite these advances in assigning RAR1, SGT1, and HSP90 a function in folding/stability of preactivated immune sensors, it remains possible that they fulfill additional roles in post-activation signaling (Shen, Q.H., and Schulze-Lefert, 2007).

OsRac1 interacting proteins

Recently, in our lab, to understand the molecular mechanisms of disease resistance induced by Rac GTPase, OsRac1-interacting proteins was isolated with GST-OsRac1 affinity column chromatography (A. Nakashima et al., submitted). Among those proteins identified, A. Nakashima found 5 NBS-LRR type proteins, a stress inducible protein 1 (Sti1), and a WD-repeat-containing receptor of activated C kianase homolog (RWD/RACK1).

Sti1 (stress inducible protein 1) is a cochaperone protein that is homologous to the human heat shock cognate protein 70 (hsc70)/heat shock protein 90 (hsp90)-organizing protein (Hop), and has been demonstrated to be a cochaperone for HSP90 and HSP70 (Pratt and Toft, 2003). Sti1 and human Hop share over 50% amino acid sequence similarity and have a common domain structure consisting of three TPR repeat domains (TPR1, TPR2a, and TPR2b) and two small domains (DP1 and DP2) containing a

characteristic DP repeat motif (Praparanich et al., 1998). Several lines of evidence suggest that the highly conserved EEVD sequence that terminates many eukaryotic HSP90 and HSP70 proteins directly interacts with a TPR binding pocket in co-chaperones. In a current model, client proteins are transferred from HSP70 to HSP90 in a process mediated by the co-chaperone Sti1/Hop, which may simultaneously interact with HSP70 and HSP90 via separate TPR domains, but the mechanism and *in vivo* importance of this function is unclear. HSP90 client proteins first interact with the molecular chaperones HSP70 and HSP40 (Hernandez et al., 2002), then the client protein is transferred from HSP70 to HSP90, resulting in HSP70/HSP40 release (Wegele et al., 2004; Hernandez et al., 2002; Wegele et al., 2006). In mammalian cells, it constitutes a component of steroid receptor complex containing HSP90 and HSP70 (Smith et al., 1995). Homologs of Sti1/Hop have been identified in several organisms (Chen and Smith, 1998), however, few information on a plant Hop homolog is available. Sti1 is highly conserved in eukaryotes and plant Sti1 has been shown to interact with HSP90 *in vitro* (Zhang et al., 2003).There are two Sti1 homologues in rice termed Sti1a and Sti1b. The structure of rice Sti1 proteins predicted from the amino acids sequences indicate that they are similar to the structure of yeast and mammalian Sti1/Hop and contain three TPR domains and two DP repeats (Figure 4).

The data from A. Nakashima also identified RWD/RACK1, which has been recently shown to be involved in hormone signaling and development (Chen et al., 2006). RACK1 was originally isolated as an interacting protein for the activated form of protein kinase C and has homology with G protein β subunit (Ron et al., 1994). RACK1 carries seven WD40 repeats and serves as a scaffold protein by binding with kinases, phosphatase, and membrane receptors in various signaling pathways in mammals (Schechtman and Mochly-Rosen, 2001; Patterson et al., 2004; Yaka et al., 2002; Lopez-Bergami et al., 2005). In rice *RACK1* gene was isolated as a homolog of Gβ and termed *RWD* (Iwasaki et al., 1995). RWD specifically binds CA-OsRac1 and interacts directly with OsRAR1 in yeast two-hybrid and *in vivo* immunoprecipitation assays. RWD was shown to be essential for basal resistance to rice blast and PAMP-triggered immunity in rice (A. Nakashima et al., submitted).

CA-OsRac1 activates ROS production and HR in rice, and confers resistance to rice blast and bacterial blight (Kawasaki et al., 1999; Ono et al., 2001; Suharsono et al., 2002). However, the molecular mechanisms of OsRac1 immune response regulation are largely unknown. In this study, I present evidence suggesting that OsRac1 associates with well-studied components of plant innate immunity. I will describe about the identification of protein components in OsRac1 complex which include RAR1, HSP70,

HSP90, SGT1, Sti1 and RWD. Interactions among components of the protein complex were confirmed *in vivo* by co-IP experiments as well as *in vitro* pull down assays. Since RAR1, SGT1 and Sti1 were found in the protein complex containing OsRac1, I am then interested in determining the role of these proteins in PAMP-triggered immune responses, such as the pathogen related (*PR*) gene expression and ROS production, together with their functions in the protein complex containing OsRac1. Finally, I will discuss about the possible mechanism how this network of proteins involved in rice innate immunity.

CHAPTER 2: Results

OsRac1 forms a complex with OsRAR1, HSP90, and HSP70 *in vivo*

   OsRac1 plays a central role in disease resistance in rice, and RAR1 is required for

function of multiple R genes such as *Mla*, *RPM1*, *RPS* and *N* gene (Shirasu et al*.,* 1999;

Tornero et al., 2002; Muskett et al., 2002b; Liu et al., 2002; Takahashi et al., 2003). I

was therefore interested in determining whether rice RAR1 (OsRAR1) physically

associates with OsRac1 *in vivo*. For an analysis of OsRAR1 interaction with OsRac1 *in*

vivo, suspension cell cultures expressing myc-tagged CA-OsRac1, DN-OsRac1, and

CS-OsRac1 driven by the maize (*Zea mays*) Ubiquitin promoter (Lieberherr et al.,

2005) were used for coimmunoprecipitation (co-IP) experiments. Results of co-IP

experiments using anti-myc and anti-RAR1 antibodies indicate that CA-OsRac1 and

DN-OsRac1 associate with endogenous OsRAR1, but there is no association in extracts

from CS-OsRac1, which has impaired membrane localization, suggesting that

membrane localization of OsRac1 is required for its association with OsRAR1 (Figure 5,

top panel). However, in these and other co-IP experiments, we always detected two

protein bands for Rac1 by anti-myc antibody (Figure 5, bottom panel; Lieberherr et al.,

2005). However, the reasons for this observation are not known.

   To confirm the association of OsRac1 and OsRAR1, I immunoprecipitated OsRAR1

in protein extracts from three transgenic OsRac1 cell cultures with anti-RAR1 antibody and then examined the precipitate with anti-myc antibody (Figure 6). OsRac1 was found in the OsRAR1 complex in CA- and DN-OsRac1 extracts but not in the CS-OsRac1 mutant, whereas RAR1 protein was detected in co-IPs from three OsRac1 mutants and the untransformed control (Figure 6, top two panels). These results indicate that OsRAR1 and OsRac1 are part of the same protein complex in rice cell cultures.

Because in plants, RAR1 interacts directly with SGT1 and HSP90 (Azevedo et al., 2002; Liu et al., 2002, Takahashi et al 2003), and the interaction of OsRAR1 and HSP90 was confirmed in yeast two-hybrid assays (L. Chen, unpublished data), I tested whether HSP90 is part of the OsRac1 complex *in vivo*. The co-IP experiments showed that HSP90 coprecipitated with Rac1 in extracts from the three myc-OsRac1 transgenic cell cultures but not from untransformed cultured cells (Figure 5, second panel). The association between OsRac1 and HSP90 was confirmed by co-IP with anti-HSP90 antibody followed by immunoblots with anti-myc antibody (Figure 7). These results demonstrate that OsRac1 and HSP90 associate with each other in vivo.

 HSP70 is known to participate with HSP90 in almost all cochaperone complexes studied in eukaryotes (Pratt and Toft, 2003). Therefore, I examined whether HSP70 also coprecipitated with OsRac1 by anti-myc antibody (Figure 5, third panel). HSP70

precipitated with anti-myc, suggesting that it is part of the OsRac1 complex. These data suggest that Rac1 forms a multiprotein complex containing OsRAR1, HSP90, and HSP70, which is mainly localized at the plasma membrane, although other possibilities remain to be studied.

SGT1 interacts with RAR1 and HSP90 (Figure 4: Shirasu and Schulze-Lefert, 2003), and the SGT1-HSP90 association was recently shown to be crucial in NLR-mediated immune responses in mammals (da Silva Correia et al., 2007; Mayor et al., 2007). However, repeated experiments clearly indicate that SGT1 does not coimmunoprecipitate with OsRac1 (Figure 5), OsRAR1, or HSP90 (unpublished data) under the conditions used. Because two-hybrid assays with OsRAR1, SGT1, and HSP90 showed that SGT1 is able to interact with RAR1 and HSP90 (L. Chen, unpublished data), it is possible that SGT1 interaction with the OsRac1 complex may be unstable and transient in rice cell cultures. Alternatively, SGT1 may complex with OsRAR1 and HSP90 in some other subcellular localizations.

Sti1 interacts with OsRac1 both *in vitro* and *in vivo*

Recently, a research in our lab has identified Sti1 as an OsRac1 interacting protein from an affinity column chromatography (A. Nakashima et al., submitted). Together

with the fact that Sti1/Hop associates with HSP90 and HSP70 in plants (Pratt and Toft, 2003; Zhang et al., 2003), and OsRac1, OsRAR1 and HSP90 proteins do not have direct interactions (L.Chen, unpublished data), this data prompted an investigation whether Sti1 is a protein link between OsRac1 and the other components found in the complex.

To confirm its interaction of Sti1 with OsRac1, I purified CA-, DN-, WT- OsRac1 protein fused to glutathione S transferase (GST) at the N-teminus and performed *in vitro* binding assays (Figure 8). Sti1 tagged with MBP was pulled down together with all types of OsRac1, and signals were detected after Western blotting with anti-MBP antibody, indicating that OsRac1 can bind Sti1 irrespective of its form. GST protein and agarose beads were negative controls for the assays. There was no signal from GST and beads lanes, suggesting that the OsRac1-Sti1 interaction was specific (Figure 8).

To examine whether Sti1 is part of the protein complex containing OsRac1 *in vivo*, I also used transgenic rice cell cultures expressing myc-CA-OsRac1, myc-DN-OsRac1, and myc-CS-OsRac1. Western blot analysis of proteins immunoprecipitated with the anti-myc antibody showed that Sti1 was coimmunoprecipitated with myc-CA-OsRac1, myc-DN-OsRac1, and myc-CS-OsRac1, indicating that Sti1 is in the same protein complex with OsRac1 *in vivo* (Figure 9). These results showed that Sti1 forms a

complex with OsRac1 irrespective of its form, consistent with *in vitro* binding experiments (Figure 8).

Sti1 interacts with HSP90 *in vivo*

Sti1 is cochaperone and interacts with HSP90 in many species. To confirm the *in vivo* interaction between HSP90 and Sti1 in rice, a co-IP experiment was performed. Suspension cell cultures expressing myc-tagged CA-OsRac1, DN-OsRac1, and CS-OsRac1 were used. Results of co-IP experiments using anti-HSP90 antibody following by immunobloting using anti-Sti1 antibody indicated that Sti1 associates with HSP90 in all forms of OsRac1 cultured cells (Figure 10), suggesting a conserved role of Sti1 in rice as cochaperone of HSP90.

Together, these results indicate that Sti1 interacts with OsRac1 as well as HSP90 *in vivo*, suggesting that Sti1 and HSP90 are components of a protein complex containing OsRac1.

RWD interacts with OsRAR1 and SGT1 *in vivo*

By using affinity chromatography, A. Nakashima also identified RWD as another OsRac1 interacting protein, and yeast two-hybrid assays indicated that RWD interacted

with OsRAR1 and SGT1, but not with HSP90 (L. Chen, unpublished data). To determine whether RWD interacts with SGT1 and OsRAR1 *in vivo*, I performed co-IP experiments using wild type and *OsRAR1*-RNAi cell cultures. RWD co-immunoprecipitated with RAR1, but no RWD band was detected in the control experiments using *OsRAR1*-RNAi cell cultures, confirming that OsRAR1 interacts with RWD (Figure 11). Similar results were obtained for SGT1 (Figure 12) though I was not able to use *SGT1*-RNAi cell cultures, since rice has a single SGT1 gene and *SGT1*-RNAi cell cultures were non-viable. These results showed that both SGT1 and OsRAR1 interact with RWD *in vivo*.

OsRAR1 is essential for OsRac1-mediated enhancement of sphingolipid-triggered immune responses in rice cell cultures

To investigate the function of OsRAR1 in rice disease resistance, OsRAR1 expression was knocked down by using RNA interference (RNAi) method (Hara, 2004). Generally, OsRAR1 expression was greatly decreased in *OsRAR1*-RNAi lines (Appendix-Figure 1). Because OsRAR1 complexes with OsRac1, it was tempting to investigate whether PAMP-triggered immune responses require RAR1. For this purpose, I analyzed *PR* gene expression, which was previously shown to be involved in the defense of rice

(Takahashi et al., 1999, Suharsono et al., 2002), in cultured cells in response to sphingolipid elicitors (SEs). SEs were isolated from the membranes of rice blast fungus and were shown to induce the accumulation of phytoalexins, cell death, increased resistance to infection by virulent pathogens, and *PR* gene expression (Koga et al., 1998; Umemura et al., 2000; Suharsono et al., 2002). Expression of the *PR* genes *Probenazole1* (*PBZ1*) and *Chitinase1* was induced in the wild type and was strongly enhanced in CA-OsRac1 cells (Figure 12). *OsRAR1*-RNAi did not affect induction of *PBZ1* or *Chitinase1* expression. Interestingly, however, the increase in PR gene expression (Figure 13) as well as H_2O_2 production was diminished in the double transgenic CA-OsRac1/*OsRAR1*-RNAi cell cultures (Figure 14), indicating that, like PR gene induction, OsRAR1 is involved in OsRac1-mediated H_2O_2 production in rice cell cultures.

OsRAR1 is involved in basal resistance against rice blast and bacterial blight infection in rice

To examine the role of OsRAR1 in rice innate immunity, L. Chen performed infection experiments of rice plants with blast fungus and bacterial blight. Japonica rice var Kinmaze, which was used for the production of transgenic plants, carries the *Pi-a* blast

resistance gene that is incompatible with race 031 but is compatible with race 007 of

Magnaporthe grisea. Two T1 transgenic lines carrying the *OsRAR1*-RNAi construct

(Appendix - Figure 1, lines 18 and 61) showed no difference in response to infection

with race 031 from the wildtype plant or a T1 segregant without a transgene (Appendix

- Figure 1, line 15), making it unlikely that *Pi-a*–mediated blast resistance requires

OsRAR1. To further test for possible OsRAR1 requirements in *R* gene–mediated blast

resistance, the same infection analysis using the *OsRAR1*-RNAi transformants in the

Kanto IL 5 and Kanto IL 14 backgrounds that carry *Pi-z* and *Pi-b* blast resistance genes,

respectively, was performed. These experiments clearly showed that OsRAR1 is not

required by either of these two *R* genes for blast resistance (L. Chen, unpublished data).

That OsRAR1 is not required for the three *R* genes for rice blast resistance is consistent

with previous results indicating that RAR1 is required for many but not all *R* genes

examined (Shirasu and Schulze-Lefert, 2003).

To determine whether OsRAR1 is involved in basal resistance to virulent blast isolates,

OsRAR1-RNAi plants were infected with compatible race 007. Leaves of untransformed

control plants developed typical disease symptoms (Appendix - Figures 2A and 2B, race

007/WT). Of the four segregants in the T1 generation, the two that carried the

OsRAR1-RNAi transgene had larger lesions (Appendix - Figures 2A and 2B, lines 26

and 28), whereas the two segregants that carried no transgenes had lesion lengths similar to the wild-type control (Appendix - Figures 2A and 2B, lines 15 and 27). Furthermore, infection of T1 generation plants with the rice bacterial blight pathogen *Xanthomonas oryzae* pv *oryzae* race 1 (T7174), which is also compatible with var Kinmaze, gave results similar to blast infection (Appendix - Figures 2C and 2D). These results demonstrate that OsRAR1 is involved in basal resistance to both rice blast fungus and bacterial blight. This is consistent with the previous reports that RAR1 functions in the basal resistance of Arabidopsis to *Pseudomonas syringae* pv tomato (Holt et al., 2005) and of mlo barley (*Hordeum vulgare*) to *M. grisea* (Jarosch et al., 2005).

OsRac1 and OsRAR1 are functionally linked in basal resistance to rice blast

To examine whether OsRac1 and OsRAR1 are functionally linked in rice disease resistance, transgenic rice carrying both CA-OsRac1 and *OsRAR1*-RNAi was generated by introducing the *OsRAR1*-RNAi construct into CA-OsRac1 transgenic calli and regenerating plants for infection experiments.

The infection data from L. Chen indicated that, as previously reported, CA-OsRac1 rice plants were more resistant to a compatible race of rice blast (Ono et al., 2001).

Transgenic plants carrying both the CA-OsRac1 and *OsRAR1*-RNAi constructs were more resistant to rice blast than *OsRAR1*-RNAi plants and had the same level of resistance as untransformed control plants (Appendix – Figure 3), indicating that OsRAR1 is required for OsRac1-mediated basal resistance to rice blast. Similar results were obtained for basal resistance to bacterial blight (L. Chen, unpublished data).

HSP90 is essential for OsRac1-mediated enhancement of sphingolipid-triggered immune responses in rice cell cultures

Because HSP90 is essential for disease resistance in plants (Schulze-Lefert, 2004) and for some NLR-mediated immune responses in mammals (da Silva Correia et al., 2007; Mayor et al., 2007), its role in PAMP-triggered immune responses mediated by OsRac1 was determined using geldanamycin (GDA), an HSP90-specific inhibitor. Wild type and CA-OsRac1 cell cultures were pretreated with GDA overnight and then treated with SE for 3, 6, and 9 h. *PBZ1* and *Chitinase1* were induced by SE in wild-type cells, but expression was much greater in CA-OsRac1 cells. A high level of *PR* gene expression was observed even at time point 0 (i.e., without SE treatment) in CA-OsRac1 transgenic cells, peaking by 6 h after SE treatment. Treatment of cell cultures with GDA resulted in a substantial decrease in *PBZ1* and *Chitinase1* mRNA in CA-OsRac1 cell culture

(Figure 15). These results indicate that HSP90 function is essential for OsRac1-mediated enhancement of PAMP signaling. However, there was no reduction in *PR* gene induction in wild-type cells with GDA treatment. Together, these findings show that HSP90 is essential for OsRac1-mediated enhancement of PAMP-triggered immune responses in rice cell cultures.

Expression level of Sti1 protein regulates *PR* gene expression level in rice cultured cells

To examine the role of Sti1 in induction of PR gene expression, I used the transgenic cell cultures silenced for *Sti1a* using RNAi method and overexpressing *Sti1a* which was provided by Dr. L. Chen. The promoter used is a constitutive maize *Ubq* promoter. *Sti1ab*-RNAi construct carries 3' UTRs of *Sti1a* and *Sti1b* to suppress expression of both genes by a single RNAi construct. *Sti1a*-RNAi construct carries 3' UTR of *Sti1a* alone. A construct for overexpresion carries *Sti1a* cDNA.

Sti1 protein levels were highly suppressed in two *Sti1a*-RNAi lines au6 and au7 (Figure 16). *PBZ1* and *Chitinase3* expressions were suppressed in these two lines whereas the overexpression of Sti1 in 2 overexpressing lines (aO93 and aO1, which have the expression level of Sti1 protein clearly increased) strongly enhanced this *PR*

gene expression in the transgenic cultured cells. *PR* gene expression in two other *Sti1a*-overexpressing lines aO88 and aO3, which do not have the Sti1 protein expression level changed comparing with the wild type level, are not significantly different from this in the non-transgenic control (Figure 16). These results indicate that in rice cell cultures the levels of *PR* expression were closely related to the level of Sti1 protein, suggesting that Sti1 plays a key role in *PR* gene expression.

The protein expression of HSP90 does not alter in both *Sti1a*-RNAi and overexpressing lines, suggesting that there is no regulation at the protein level of HSP90 by Sti1a.

Sti1a regulates sphingolipid - triggered ROS production in rice cell cultures

To test whether Sti1 is also required for SE-dependant ROS production, the *Sti1a*-RNAi and overxpressing cultured cells were treated with SE for 0, 3, 6, 9 hr. Aliquots were collected for ROS measurement.

The ROS production induced by SE strongly enhanced in Sti1a overepressing lines and reduced in *Sti1a*-RNAi lines (Figure 17), indicating that, like with PR gene induction, Sti1 is also involved in SE-triggered H_2O_2 production in rice cell cultures.

Sti1 has a role in blast resistance in rice

To examine whether Sti1 plays a role in disease resistance of rice, transgenic rice plants overexpressing or silenced for *Sti* gene expression were analyzed (L. Chen, unpublished data). When these plants were infected with a virulent strain of the rice blast fungus (*Magnaporthe grisea*) strain 2403-1 (race 007), *Sti1a*-overxpressing plants, 4, 5, and 32, showed enhanced resistance to rice blast (Appendix - Figure 4). In contrast, *Sti1*-RNAi plants, a3, ab5, ab6, and ab7, showed decreased resistance to rice blast infection (Appendix - Figure 4). These results clearly indicate a critical role of Sti1 in the immune responses to of rice to blast infection. Together with my data suggesting the role of Sti1 in PAMP- triggered signaling in rice cell cultures, these results indicate that Sti1 is an important regulator of defense responses in rice and suggest that Sti1 can regulate disease resistance by the formation of a protein complex with OsRac1 and HSP90.

HSP90 but not OsRAR1 may be essential for association with the OsRac1 complex

The nucleotide-bound state of HSP90 is known to be essential for function of its client adaptor proteins in the complex (Catlett and Kaplan, 2006). Furthermore, HSP90 function was shown to be crucial for complex formation with HSP90, SGT1, and NLR proteins such as NOD1 and NALP3 and activation of immune responses in

mammals (da Silva Correia et al., 2007; Mayor et al., 2007). To determine if HSP90 function is required for the association of HSP90 and OsRAR1, rice cell cultures were treated with 10 μM GDA, and the interaction of OsRac1 with HSP90 was measured with co-IP. There was no HSP90 signal with GDA treatment, indicating that functional HSP90 is essential for its association with OsRac1 (Figure 18A). GDA treatment also caused dissociation of OsRAR1 from the OsRac1 complex (Figure 18B). Furthermore, anti-RAR1 antibody was used to test whether intact HSP90 is required for HSP90 - OsRAR1 associations. GDA treatment reduced precipitation with anti-RAR1 to nearly undetectable levels, suggesting that HSP90 is essential for association HSP90 and OsRAR1 (Figure 18C). I also examined whether HSP90 function is also important for the association of OsRac1 and Sti1. After GDA treatment, the OsRac1 protein complex was immunoprecipitated with anti-myc antibody from the cultured cells of WT and CA-OsRac1 mutant. Interestingly, although Sti1 directly interacts with OsRac1, the signal of Sti1 protein was not detected from OsRac1 complex after GDA treatment, suggesting that treatment of GDA also causes OsRac1-Sti1 complex disassociation (Figure 19).

Finally, I tested whether OsRAR1 is required for association of HSP90 in the OsRac1 complex using *OsRAR1*-RNAi / myc-CA-OsRac1 double transgenic cell culture and

found that OsRAR1 is not required for the association of HSP90 in the OsRac1 complex (Figure 20). Together, these results indicate that HSP90 function is essential for association of HSP90, OsRAR1 and Sti1 in the OsRac1 complex in rice cell cultures.

OsRAR1 expression is regulated by OsRac1

OsRAR1 protein was apparently present in higher concentrations in CA-OsRac1 cell cultures, suggesting that OsRAR1 expression is affected by OsRac1. To test this hypothesis, *OsRAR1* mRNA accumulation was measured by RT-PCR in wild-type and CA-OsRac1 cell cultures. *OsSGT1* mRNA was also measured because SGT1 transcripts are induced by pathogen infection in rice and Arabidopsis (Cooper et al., 2003; Zimmermann et al., 2004). CA-OsRac1 overexpression cultures had much higher mRNA levels of both *OsRAR1* and *OsSGT1* (Figure 21), suggesting that OsRac1 regulates OsRAR1 and SGT1 expression. To further examine the regulatory relationship between OsRac1, OsRAR1, and SGT1, a cell culture in which CA-OsRac1 can be induced (ICA) by treatment with dexamethasone (DEX) (Wong et al., 2004) and an *OsRac1*-RNAi cell culture (Miki et al., 2005) were examined. Induction of CA-OsRac1 with DEX treatment clearly increased both transcript (Figure 22) and protein (Figure 22) accumulation levels of OsRAR1 compared with the ethanol treated control

(ICA/DEX and ICA/ethanol), and OsRAR1 protein was reduced in the *OsRac1*-RNAi

cell culture. By contrast, SGT1 mRNA accumulation correlated with OsRac1 expression,

but SGT1 protein levels did not change in these cell cultures (Figure 22). These results

indicate that OsRAR1 could be transcriptionally regulated by OsRac1, but any control

of SGT1 by OsRac1 would be at the posttranscriptional level. The presence of some

OsRAR1 transcript, but no protein in the *OsRac1*-RNAi line, could indicate that there is

another regulatory component besides OsRac1 or that OsRac1 also has some

posttranscriptional roles in OsRAR1 expression. Nevertheless, these results suggest a

close regulatory link between OsRac1 and OsRAR1 at the transcriptional and possibly

posttranscriptional levels.

CHAPTER 3: Discussion

OsRAR1 is an important component in innate immunity in rice

In barley, Arabidopsis, and tobacco, RAR1, together with HSP90 and SGT1, plays a key role in R gene–mediated resistance (Shirasu and Schulze-Lefert, 2003). The plant infection results demonstrate that OsRAR1 is required for basal resistance against compatible races of rice blast and bacterial blight (Appendix-Figures 2) and that this resistance is mediated by OsRac1 (Appendix-Figure 3). Interestingly, OsRAR1 is not required by the three examined rice R genes for blast resistance (L. Chen, unpublished data; Appendix-Figured 1). Recently, it was shown that OsRAR1 is not required for *Pish*-mediated blast resistance (Takahashi et al., 2007). Since RAR1 is not required for all R genes (Shirasu and Schulze-Lefert, 2003), these results in rice are not unexpected. In my work, I also demonstrated that OsRAR1 is critical for OsRac1–mediated enhancement of PAMP signaling (Figures 13 and 14). Therefore, OsRAR1 activity in rice innate immunity may be on a broader scale than in those other species.

The results shown here are consistent with the involvement of RAR1 in basal resistance in Arabidopsis and barley (Holt et al., 2005; Jarosch et al., 2005). Basal resistance is considered to be a weak response to virulent pathogens, and its molecular mechanism is not known. Thus, it is possible that basal resistance is induced by weak R

proteins that recognize cognate effectors (Jones and Dangl, 2006). In this model, RAR1 function associated with basal resistance is also mediated by R proteins. More recently, RAR1 was shown to be a target of the *P. syringae* effector AvrB (Shang et al., 2006). Therefore, considering the observation that there are many PAMP receptors in plant cells and that RAR1 could potentially form complexes containing such receptors and other key signaling proteins, the extent of RAR1 involvement in various defense signaling pathways remains to be studied.

Results of RT-PCR and immunoblotting showed that the overexpression of OsRac1 strongly increased OsRAR1 mRNA and protein accumulation as well as transcript levels of Os*SGT1* (Figure 21, Figure 22), suggesting that OsRac1 may function upstream of OsRAR1 in defense signaling and that the presence of a regulatory link between OsRac1 and OsRAR1 in SE-induced signaling, and that OsRac1 is a central mediator of disease resistance in rice and that it coordinates the activity of other important factors such as OsRAR1, HSP90, and the MAPK cascade.

SGT1 plays diverse roles in plants, whereas RAR1 has much more specialized resistance functions (Shirasu and Schulze-Lefert, 2003). SGT1 was not detected in immunoprecipitated OsRAR1 complexes in rice cell cultures under the conditions used, whereas yeast two-hybrid analysis showed that rice SGT1 and OsRAR1 directly interact

(L. Chen, unpublished data). One explanation for this is that the interaction between OsSGT1 and OsRAR1 is transient *in planta*. Attempts to generate *OsSGT1*-RNAi rice cell cultures and whole plants by the same transformation protocol used for generating *OsRAR1*-RNAi cell culture and plants were unsuccessful. This is likely due to the fact that *OsSGT1* is an essential single copy gene in rice. Therefore, the function of SGT1 in rice innate immunity has not as yet been determined by genetic methods.

HSP90 is a critical regulator in OsRac1–mediated PAMP signaling in rice

Heat shock proteins are well known for regulating the maturation of protein complexes, for degrading damaged or misfolded peptides, and for involvement in the activity of many signal transduction proteins (Pratt and Toft, 2003; Rutherford, 2003). Previous studies have demonstrated that HSP90 plays a general role in R protein–mediated immunity in plants and that it acts physically close to R proteins (Hubert et al., 2003; Kanzaki et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004). However, no specific role for HSP90 in protein complex formation or specific immune responses in plants has been identified.

The close association of HSP90 with OsRac1 (Figure 5, Figure 7) prompted an investigation into the possible involvement of HSP90 in OsRac1-mediated enhancement

of PAMP signaling in rice cell cultures. To examine the function of HSP90, I used GDA, which binds the N-terminal structural domain of HSP90 and inhibits ATPase activity by blocking its highly conserved ATP binding pocket, and it has little effect on prokaryotic pathogens (Stebbins et al., 1997; Picard, 2002). GDA experiments on rice cell cultures demonstrated the requirement of HSP90 for OsRac1- mediated enhancement of PAMP signaling to induce *PR* genes (Figure 15). These results could be explained by the dissociation of OsRAR1, HSP90 and Sti1 from the OsRac1 complex caused by GDA (Figure 18, Figure 19), since it has been demonstrated that GDA treatment alters the conformation and dissociation of cochaperones, such as p23, Hop, and HSP70, from the steroid receptor complex in mammalian cells (Whitesell et al., 1994; Bagatell et al., 2001; Waza et al., 2006). These findings are similar to those in recent studies of innate immunity in mammals (da Silva Correia et al., 2007; Mayor et al., 2007), which demonstrate that GDA causes inactivation of NLR-containing protein complexes and that HSP90 is required for stability of the signaling complex.

I demonstrated the critical role of HSP90 in OsRac1– mediated enhancement of PAMP signaling; thus, I tested whether HSP90 is also required for SE-dependant ROS production. The ROS production after GDA treatment followed by SE treatment was examined. However, GDA failed to suppress SE-induced ROS production in

CA-OsRac1 cell cultures (data not shown). One possible interpretation of this result is that GDA itself increases ROS generation (Dikalov et al., 2002).

With regard to the possible function of HSP70 in innate immune responses in rice, I have no information at the moment. The rice genome contains 14 HSP70 genes that can be classified into three groups (unpublished data), much like Arabidopsis, which also contains 14 genes for HSP70 (Sung et al., 2001). I attempted to knock down expression of nine genes in group 1 and three genes in group 2 by RNAi methods employing conserved coding sequences as targets (Miki et al., 2005). However, I was not able to obtain transgenic plants in which expression of HSP70 genes in groups 1 or 2 was clearly decreased (unpublished data). A different approach would therefore be required to determine the function of HSP70, if any, in rice innate immunity.

Sti1 is a novel factor for innate immunity in rice

HSP90 and HSP70 are abundant and highly conserved molecular chaperones that act in concert with other chaperones (e.g., Hsp40) and cochaperones (e.g., Cdc37) to mediate the folding of client proteins into functional conformations. Detailed mechanistic studies of the folding of steroid receptors into functional conformations have yielded a generalized paradigm for protein folding by HSP90 and HSP70 (Pratt

and Toft, 2003). In this model, a so-called "early" protein folding complex is formed by the binding of HSP70 and HSP40 to the client protein (Kosano et al., 1998; Dittmar et al., 1998). The HSP70/HSP90 organizing protein (Hop) then stabilizes the interaction of HSP90 with HSP70 and the client protein, resulting in the formation of an "intermediate complex" (Kosano et al., 1998; Dittmar et al., 1998; Chen et al., 1996). Further maturation of the complex is accompanied by the loss of HSP70 and Hop from the complex and the interaction of other HSP90 cochaperones (e.g., p23) and immunophilins, such as the FK506-binding protein FKBP52, with HSP90 and its client protein (Johnson and Toft, 1995; Hutchison et al., 1995; Owens-Grillo et al., 1995; Ratajczak et al., 1996).

The results of the present study indicated that the general characteristics of plant Hop are similar to animal and yeast Hop (Zhang et al., 2003). Here, the conserved associations of Sti1 with HSP90 were confirmed *in vitro* in rice (Figure 10). Together with the interaction of Sti1 with HSP90 in yeast two-hybrid assay (L. Chen, unpublished data), this data suggests that the plant Hop may be a general cochaperone of HSP90. Moreover, the direct interaction between Sti1 and OsRac1 found by affinity chromatography was also confirmed both *in vivo* and *in vitro* (Figure 8, Figure 9), which partially clarify the mechanism of how OsRac1, HSP90 and OsRAR1 form the

complex together. In yeast Sti1 is shown to stabilize HSP90 in complex with ste11, a MAPKKK, and defect in Sti1 reduces MAPK signaling (Lee et al., 2004). In rice PAMP-induced MAPK6 activation has been shown to require OsRac1 activity (Lieberherr et al., 2005). Therefore, one possible role of Sti1 in the complex might be to stabilize interaction of HSP90 and kinases involved in MAPK cascade. Plant kinases involved in the MAPK cascade may be stabilized by Sti1 and HSP90 during immune response to pathogens.

By using cell cultures of *Sti1a*-RNAi and overexpressing lines, I could show the critical role of Sti1 in the enhancement of *PR* gene expression in PAMP signaling (Figure 16). However, I did not observe this correlation at the mRNA level of Sti1 (unpublished data), indicating a post-transcriptional mechanism of Sti1 to regulate *PR* gene expression in rice cultured cells. I also tested whether Sti1 is required for SE-dependant ROS production. The ROS production in *Sti1*-overexpressing and RNAi cell cultures was examined (Figure 17), clearly suggesting an involvement of Sti1 in rice defense signaling.

Despite interest in determining how Sti1 regulates HSP70 and HSP90 function, no prior *in vivo* mutational analysis targeting specific domains of Sti1 has been completed, in part because disruption of *sti1* in yeast causes only minor growth defects (Nicolet and

Craig, 1989). In contrast to a proposed critical role in transfer of client proteins from HSP70 to HSP90, deletion of *Sti1* does not have dramatic effects on glucocorticoid receptor (GR) complexes isolated out of yeast (Chang et al., 1997). However, consistent with an *in vivo* role in maturation of HSP90 client proteins, deletion of *Sti1* affects the activity of heterologous HSP90 substrates and therefore inhibits steroid receptor function (Chang et al., 1997), as well as the native yeast transcription factor HSF (Liu et al., 1999) and protein kinase Stell (Lee et al., 2004). Moreover, deletion of *Sti1* in combination with deletion in genes encoding HSP90 or HSP70 cochaperones causes a lethal phenotype or enhanced temperature-sensitive growth defects (Chang et al., 1997; Liu et al., 1999; Abbas-Terki et al., 2002). In this work, it has been shown that Sti1 has the direct association with HSP90 and HSP70 in rice cells, as well as with OsRac1 protein. However, which domain of Sti1 is responsible for its association with OsRac1 and the mechanism of their association remains unknown. Another recent work in our lab attempts to find out these answers (L. Chen, unpublished data). *Sti1*-RNAi plants were also found to be compromised to virulent fungus, while *Sti1*-overexpressing plants became more resistant (Appendix-Figure 4). *R* gene *Pi-a* mediated pathway was also affected by the loss of Sti1 (L. Chen, unpublish data), together with my results, indicate Sti1/Hop is a novel player of defense signaling.

A network of proteins involved in innate immunity in rice

 Together with the data from affinity column chromatography and yeast two-hybrid system, my results indicate a network of proteins containing OsRac1 which involved in rice innate immunity (Figure 23A). I show here that OsRac1 associates with well-studied components of plant innate immunity such as RAR1, HSP90, and HSP70. The yeast two-hybrid analysis showed that OsRAR1 and HSP90 do not directly interact with OsRac1, although OsRAR1 interacts directly with HSP90 (L. Chen, unpublished data). Thus, it seems that OsRac1 forms a complex with OsRAR1 and HSP90 by indirect interactions. In another experiment, I show that OsRac1 directly interacts with Sti1/Hop, a cochaperone in the complex with HSP90 and HSP70, *in vivo* and *in vitro* (Figure 8, Figure 9). Thus, OsRAR1 and HSP90 are likely to form a complex with OsRac1 through Sti1/Hop. I have previously found that only CA-OsRac1 associates with OsMAPK6 (Lieberherr et al., 2005). Furthermore, other studies in our lab indicated that NBS-LRR proteins and RACK1/RWD also specifically bind CA-OsRac1 (A. Nakashima et al., submitted, Y. Kawano, unpublished data). RACK1/RWD is likely to be a second link between OsRac1 and OsRAR1, because RWD interacts directly with OsRAR1 in yeast two-hybrid and *in vivo* immunoprecipitation assays (A. Nakashima et al., submitted), thus confirming the observation that OsRAR1 is part of the OsRac1

complex. These results connect Rac/Rop GTPase with known players in plant defense pathways and reveal the general importance of Rac/Rop GTPase in plant innate immunity. Recently, a direct interaction between CA-OsRac1 and the N terminus of the NADPH oxidases, OsRbohs and StRbohB, has been found (Wong et al., 2007). Thus, it is possible that OsRAR1 and HSP90 are required for full activation of the OsRac1 complex, at least partially through regulation of NADPH oxidase. Moreover, RWD interacts with OsRAR1 and SGT1 (Figure 11, Figure 12) and Sti1/Hop interacts with HSP90 (Figure 10). However, in co-IP experiments using anti-myc antibody to detect a complex with OsRac1, I could not detect SGT1, a conserved regulator of immune responses in plants. Therefore, there is no clear evidence for the involvement of SGT1 in the complex containing OsRac1. It may be a dynamic complex and its components could change after pathogens infect rice cell. Some components may be transiently present in the complex. SGT1 may be such a protein and may be in the complex transiently so that we could not detect it in our co-IP experiments. It remains to be shown whether OsRAR1 and/or SGT1 associate with HSP90 and OsRac1 and R proteins in a single complex, or whether they simultaneously co-regulate other complexes required for downstream signaling events. One current model derived from these observations is that a network of proteins including some known components of

plant innate immunity such as OsRac1, OsRAR1, SGT1, HSP90, HSP70, Sti1, RWD,

NBS-LRR, OsMAPK6, and RBOH (NADPH oxidase), can form one or more protein

complexes (Figure 23).

We name that complex defensome and propose a working model in which defensome

complex regulates both PAMP-mediated resistance and R protein-mediated resistance

(Figure 23B). The key component of the defensome is OsRac1 which is a molecular

switch for both PAMP- and R protein-mediated immune response in rice. OsRac1 has

been shown to have a role in PAMP- as well as R protein-mediated resistance to rice

blast fungus and bacterial blight (Ono et al., 2001, Suharsono et al., 2002). In the

defensome model, OsRac1 which first forms a complex with Sti1, HSP90, HSP70, and

OsRAR1 is in an inactive GDP-bound form. The inactive OsRac1 may be converted to

the active GTP-bound form by GEF. PAMP signals derived from pathogens may induce

activation of GEF through the kinase domain of RLK once signals are recognized by

receptors. As discussed before, the link between RLKs and Rac/Rop has been shown

(Kaothien et al., 2005; Zhang and McCormick, 2007). At this stage, the active form of

OsRac1 can now interact with RWD (A. Nakashima et al., submitted) to form an active

defensome. This active defensome containing active OsRac1, Sti1, HSP90, HSP70,

OsRAR1, RWD, and possibly SGT1 play a major role in PAMP-mediated resistance.

The active defensome regulates ROS production through an NADPH oxidase activity which can be activated by a direct interaction of active OsRac1 and Rboh. The active defensome also contains NBS-LRR proteins bound to active OsRac1. When the corresponding Avr protein enters rice cells and bind to LRR motif of the NBS-LRR protein (Jia et al., 2000; Dodds et al., 2006), it induces conformational changes of the NBS-LRR protein. This active R protein then may cause a strongly activation of OsRac1 and possibly other proteins such as MAP kinase, which also found in the active defensome, leads to strong immune responses including HR.

Our defensome model, in which the protein complex containing OsRac1 functions in both PAMP- and R protein-mediated resistances (Figure 23B), is consistent with recent reports that the signaling pathways for PAMP-triggered immunity and R protein–mediated race-specific resistance substantially share common regulatory components. There have been several reports suggesting a link between PAMP-triggered resistance and R protein–mediated resistance at the molecular level. In *Arabidopsis*, RIN4 is a negative regulator of PAMP signaling and is targeted by *Pseudomonas syringae* type-III effector AvrRpt2 for degradation, leading to the activation of an R protein, RPS2 (Kim et al., 2005). AvrB, a *P. syringae* effector protein, suppresses PAMP-triggered immunity through RAR1, which is indispensable for the stabilization of

RPM1, the R protein corresponding to AvrB (Shang et al., 2006). *rar1* mutations in *Arabidopsis* allowed enhanced growth of the virulent bacterial strain *P. syringae* DC3000 (Holt et al., 2005). NbSGT1 is required not only for R protein–mediated HR induction but also for some non-host resistance responses (Peart et al., 2002).

With respect to the intracellular localization of OsRac1 complex, the results obtained in this study and previous studies (Ono et al., 2001) suggest that the primary location of defensome is likely to be at the plasma membrane. However, since recent studies indicate that some R proteins also function in the nucleus (Shen and Schulze-Lefert, 2007), whether the defensome could be also located in the nucleus or the cytoplasm remains to be studied.

Materials and Methods

Plasmid Constructs and Rice Transformation

Oryza sativa L. *japonica* var. Kinmaze was used as wild type. The *OsRac1* mutants *OsRac1*-19V (CA-*OsRac1*), *OsRac1*-24N (DN-*OsRac1*) and *OsRac1*-212S (CS-*OsRac1*) were described previously (Kawasaki et al., 1999; Ono et al., 2001). *Os*Rac1 protein was tagged with the myc epitope at the N terminus and the expression of each construct was under the control of maize *Ubiquitin* promoters. To make OsRAR1-RNAi or Sti-RNAi constructs, cDNA fragments amplified by PCR using two primers, OsRAR1-RNAi-F and OsRAR1-RNAi-R, or Sti1-RNAi-F and Sti1-RNAi-R (Appendix - Table 1), respectively, was inserted into pANDA developed as a vector for RNAi. *Sti1ab* RNAi construct using Sti1a-RNAi-F and Sti1b-RNAi-R primers to suppress expression of both genes by a single RNAi construct.

Agrobacterium-mediated transformation of rice calli was performed according to a published method (Hiei et al., 1994). Plants regenerated from transformed calli were selected by hygromycin resistance. Rice suspension cell cultures expressing *OsRAR1*-RNAi and CA-*OsRac1 / OsRAR1*-RNAi were also produced.

Rice Cell Cultures and Elicitor Treatment

Rice cell cultures expressing CA- and DN-OsRac1 were generated as described previously (Kawasaki et al., 1999; Ono et al., 2001; Suharsono et al., 2002). For analysis of gene expression, rice cell cultures were collected after treatment with 5µg/ml of an SE prepared from rice blast fungus *Magnaporthe grisea* (Koga et al., 1998; Umemura et al., 2002).

Gene Expression

Total RNA was extracted from cultured cells and seedlings using RNeasy plant RNA extraction kit (TAKARA). One µg RNA was digested with DNaseI (TAKARA) and reverse-transcribed with Super Script II (Invitrogen). PCR reactions were performed with specific primer sets as shown in Appendix - Table 1.

Protein Analysis

Rice suspension cell cultures expressing CA-*OsRac1*, DN-*OsRac1* and C212S-*OsRac1* were ground in cold extraction buffer (137 mM NaCl, 8.1 mM Na_2HPO_4 anhydrous, 1.47 mM Na_2HPO_4, pH 7.4, 10% sucrose, and complete protein inhibitor tablets (Roche). Cell debris was removed by centrifugation at 12000 x *g* for 25 min. Protein concentrations were determined using Bradford protein assay with BSA as the standard.

Proteins were separated in 10% or 12.5% SDS polyacrylamide gels and blotted onto nitrocellulose membranes (Millipore) with a semi-dry electroblotting-Trans blot SD cell (Bio-Rad).

For immunodetection, membranes were incubated for 1 h with primary antibodies against the myc epitope mouse monoclonal (Invitrogen) or rabbit polyclonal (Santa Cruz Biotechnology) HvRAR1 (Takahashi et al., 2003), HvHSP90 (Takahashi et al., 2003), SGT1 (Takahashi et al., 2003), HSP70 (Stressgen Biotechnologies), Sti1 (Zhang et al., 2000), and RWD, followed by anti-mouse/rat/rabbit/ IgG were conjugated to horseradish peroxidase (Sigma). Specific protein bands were visualized with the ECL chemiluminescent Western Blotting Detection Reagent (GE Healthcare) and Hyperfilm ECL (GE Healthcare).

For immunoprecipitations, 1g extract (in a 1 ml volume) was incubated with protein A or G Sepharose 4 Fast Flow beads (GE Healthcare) at 4°C for 3 h. Supernatants were collected and combined with 20 µl anti-myc antibody and rotated end-over-end at 4°C for 1 h. 50 µl protein A or G were added and the incubation was continued overnight. Immuno-complexes were washed three times with 1ml ice-cold washing buffer (extraction buffer plus 150 mM NaCl, 0.5% Triton X-100), re-suspended in 20 µl SDS-PAGE sample buffer, heated to 90°C for 3 min and separated by SDS-PAGE as

described.

Quantification of H_2O_2

  Quantification of sphingolipid elicitor-induced H_2O_2 production was performed

according to He et al. (2000), with modifications described by Suharsono et al. (2002).

Briefly, 0.4 g of suspension cultured cells were pre-cultured in a fresh R_2S medium at

30°C for 16 h. Cells were transferred to 2 ml of fresh medium containing SE (10 μg/ml).

Aliquots were collected following incubation and filtered through a 0.22 μm filter. 100

μl of the filtered aliquot was mixed with 1ml xylenol orange buffer (0.25 mM $FeSO_4$,

0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 10 mM sorbitol, 12.5 mM xylenol orange) and

incubated for 2 h at room temperature. Absorbance was measured in a

spectrophotometer (Beckman) at 650 nm, and H_2O_2 levels were determined based on a

standard curve made from known concentrations of H_2O_2 dissolved in the R_2S medium.

  For geldanamycin (GDA) treatment, GDA was diluted from a 10 mM stock in

dimethylsulfoxide (DMSO) into fresh R2S medium in the pre-culture step (described

above) to a concentration of 10 μM. DMSO alone was added into the R_2S medium as a

control.

In vitro pull down assay

The appropriate plasmids of OsRac1 (pGEX-4T-1 CARac1 and pGEX-4T-1 DNRac1) and Sti1a in pMAL vector were use. GST fusion proteins of OsRac1 and MBP tagged Sti1 proteins were expressed in BL21 cells (Stratagene) and induced by the addition of 1mM isopropyl-β-D-thiogalactopyranoside. The harvested bacteria were homogenized in extraction buffer (PBS) containing 1% Triton X-100, and centrifuged to remove the insoluble proteins and cell debris right after the extraction process. The glutathione-Sepharose 4B beads (Amersham) were washed with extraction buffer and used for the GST pull-down assay. GST-OsRac1-GTP and –GDP were separately immobilized onto Sepharose beads. The immobilized beads were incubated with the supernatants containing MBP-Sti1a fusion proteins for 1 h at 4°C. The beads were washed four times with PBS buffer, and the washed beads were suspended with SDS-PAGE sampling buffer. The bound proteins were subjected to an immunoblot analysis with anti-MBP antibody.

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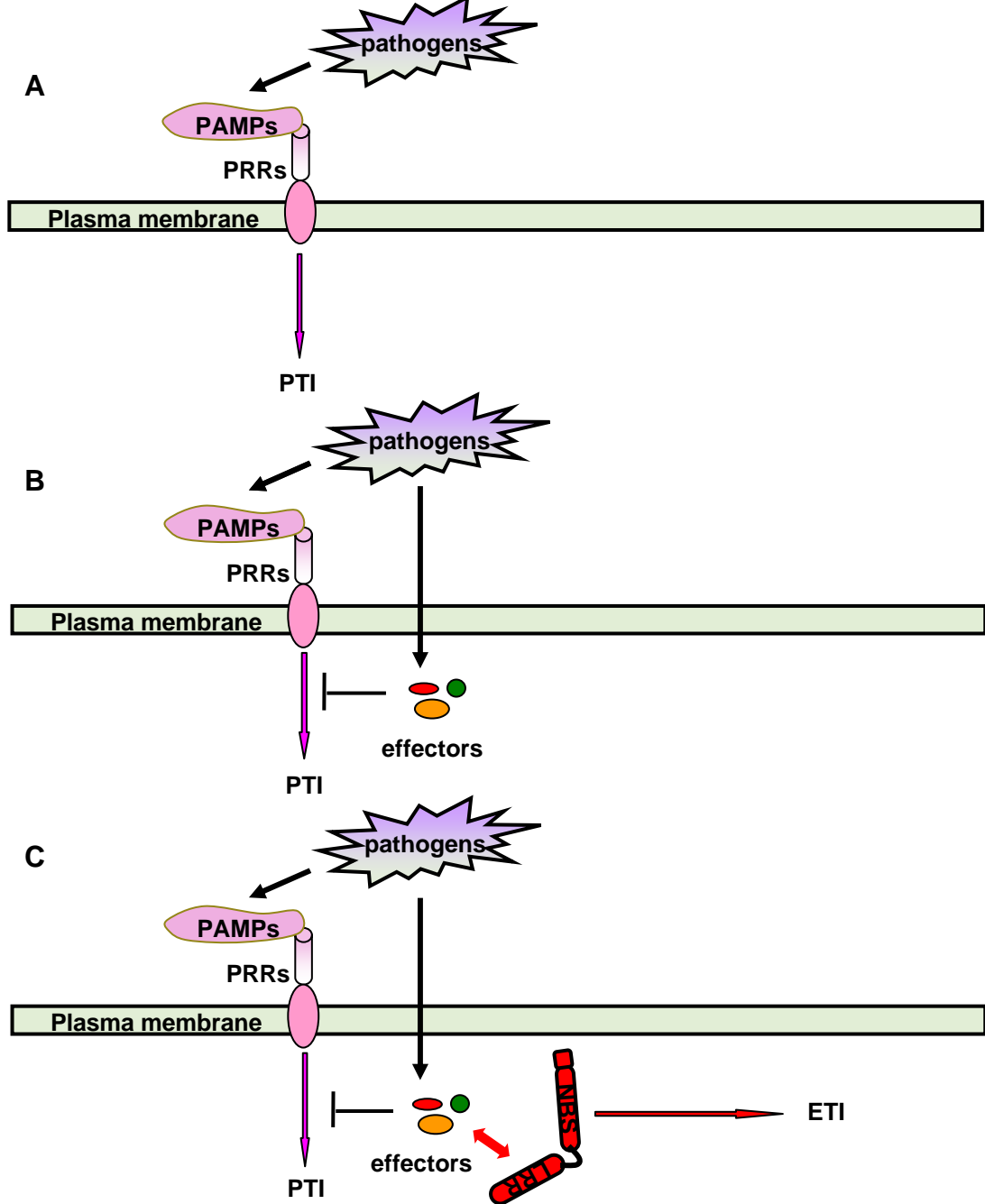
Figure 1. Model of plant innate immunity.

(A) pathogen-associated molecular patterns (PAMPs) of pathogens are recognized by parttern recognition receptors (PRRs), which trigger PAMP-trigged immunity (PTI).

(B) True pathogens secrete effectors that suppress PTI.

(C) Resistant plants carry R (NBS-LRR) proteins that recognize effectors, which trigger efector-triggered immunity (ETI).
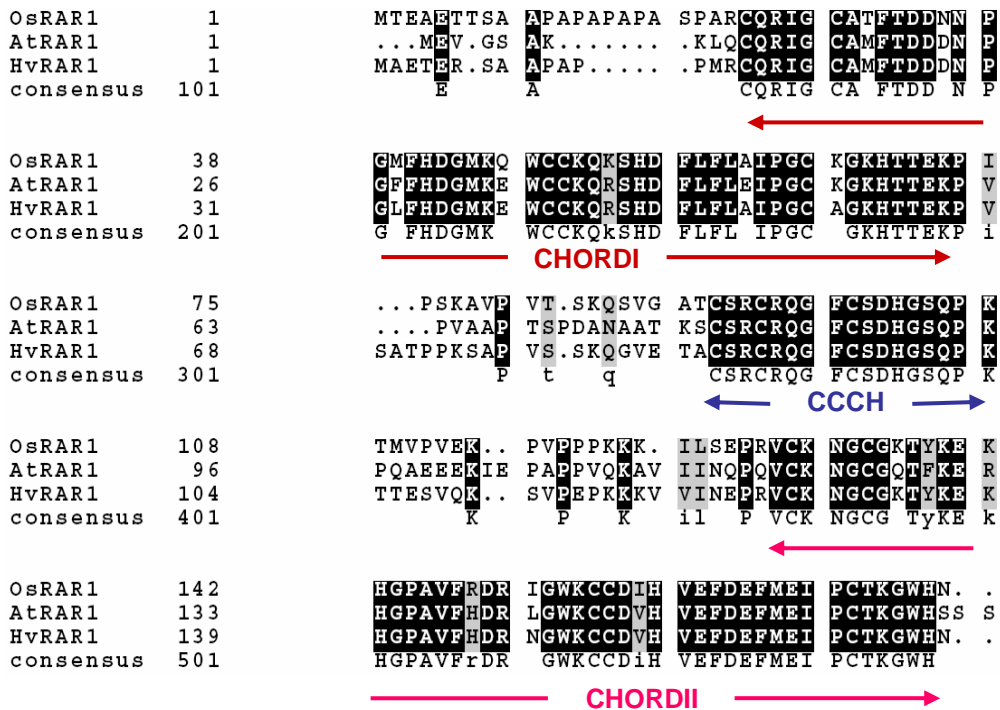
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OsRAR1       1              MTEAETTSA  APAPAPAPA  SPARCQRIG  CATFTDDNN  P
AtRAR1       1              ...MEV.GS  AK.......  .KLQCQRIG  CAMFTDDDN  P
HvRAR1       1              MAETER.SA  APAP.....  .PMRCQRIG  CAMFTDDDN  P
consensus  101                   E         A          CQRIG  CA FTDD N  P

OsRAR1      38              GMFHDGMKQ  WCCKQKSHD  FLFLAIPGC  KGKHTTEKP  I
AtRAR1      26              GFFHDGMKE  WCCKQRSHD  FLFLEIPGC  KGKHTTEKP  V
HvRAR1      31              GLFHDGMKE  WCCKQRSHD  FLFLAIPGC  AGKHTTEKP  V
consensus  201             G FHDGMK   WCCKQkSHD  FLFL IPGC   GKHTTEKP  i
                                                 CHORDI

OsRAR1      75              ...PSKAVP  VT.SKQSVG  ATCSRCRQG  FCSDHGSQP  K
AtRAR1      63              ....PVAAP  TSPDANAAT  KSCSRCRQG  FCSDHGSQP  K
HvRAR1      68              SATPPKSAP  VS.SKQGVE  TACSRCRQG  FCSDHGSQP  K
consensus  301                     P    t    q      CSRCRQG  FCSDHGSQP  K
                                                      CCCH

OsRAR1     108              TMVPVEK..  PVPPPKKK.  ILSEPRVCK  NGCGKTYKE  K
AtRAR1      96              PQAEEEKIE  PAPPVQKAV  IINQPQVCK  NGCGQTFKE  R
HvRAR1     104              TTESVQK..  SVPEPKKKV  VINEPRVCK  NGCGKTYKE  K
consensus  401                     K       P    K   il   P VCK  NGCG TyKE  k

OsRAR1     142              HGPAVFRDR  IGWKCCDIH  VEFDEFMEI  PCTKGWHN.  .
AtRAR1     133              HGPAVFHDR  LGWKCCDVH  VEFDEFMEI  PCTKGWHSS  S
HvRAR1     139              HGPAVFHDR  NGWKCCDVH  VEFDEFMEI  PCTKGWHN.  .
consensus  501             HGPAVFrDR   GWKCCDiH  VEFDEFMEI  PCTKGWH
                                                 CHORDII
```

Figure 2. Amino acid sequences alignment of *Rar1*.

Sequences can be found in the Genbank/EMBL data libraries under the following accession number: AK111881 (OsRAR1), AF192262 (AtRAR1), and AF192261 (HvRAR1).
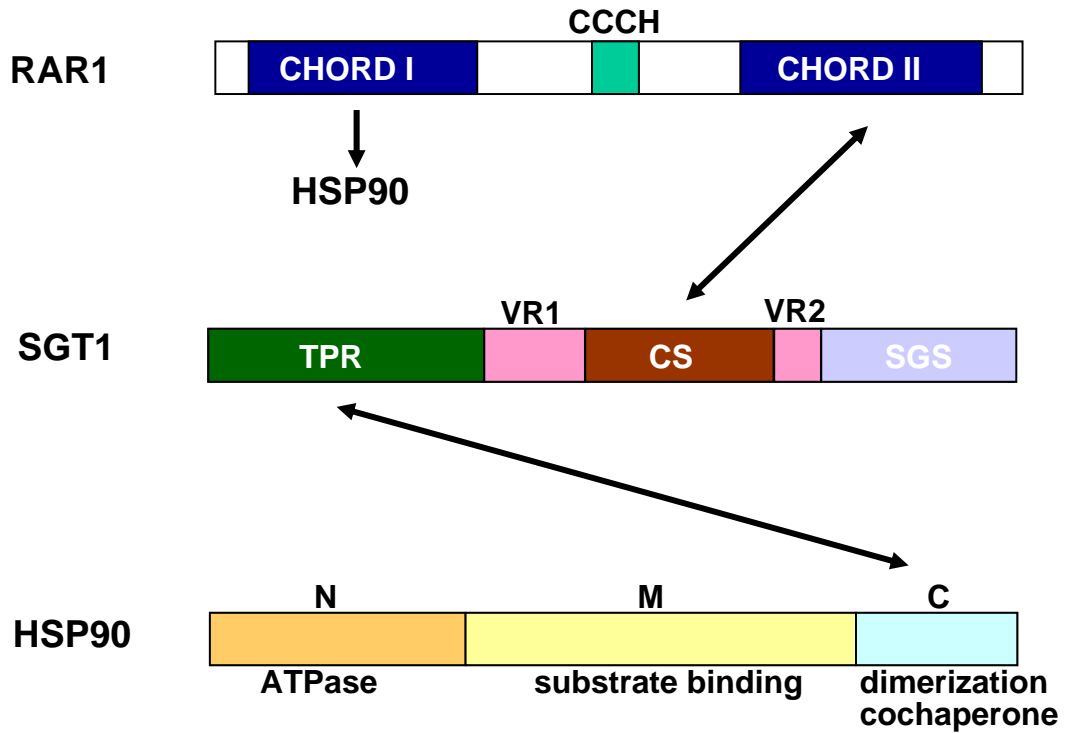
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Figure 3. Regulators for R-protein stability

RAR1 (required for *Mla12* resistance) contains two zinc-binding finger motifs termed CHORD-I and CHORD-II (cysteine- and histidine-rich domains). In plants, RAR1 interacts directly with SGT1 (suppressor of the G2 allele of *skp1*), and HSP90. The CHORD-I domain of RAR1 interacts directly with the N terminus of HSP90, and SGT1 binds the CHORD-II domain of RAR1.
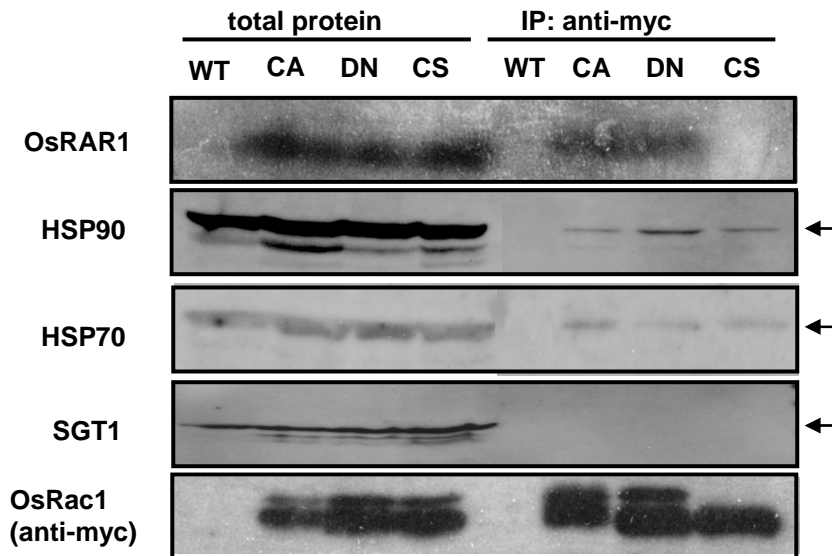
A

```
                                                    TPR1
Sti1a  MADEAKAKGNAAFSAGRYEEAARHFTDAIALAPGNHVLYSNRSAALASVHRYSEALADAE   60
Sti1b  MADEAKAKGNAAFSAGRFEEAAAHFTDAIALVPDNHVLYSNRSAAYASLHRYPEALADAE   60
       ***************** ****  ******** * *********** ** *** *******

Sti1a  KTVELKPDWAKGYSRLGAAHLGLGDAASAVAAYEKGLALDPTNEGLKAGLADAKKAAAAP  120
Sti1b  RTVALRPDWAKGCSRLGAARLGLGDAAGAVAAYEKGLALEPSNGALKDGLAQARQARRPA  116
       :**  *:****** ******:*********.************:*:* ***:*::*   ..
                                          Sti1/DP1
Sti1a  PRRPPPSGVDGIGQMFQGPELWTKIASDPTTRAYLEQPDFMQMLRDVQRNPSSLNMYLSD  180
Sti1b  P----ASGAGAIGKVFQGPELWSRMAADPTTRPYLDQPDFMRMLRDVQRNPSSLNNYLSD  176
       *    .**..**::*******::*:**** **:****** ***************** ****

Sti1a  PRMMQVLGLMLNIKIQRPEASESSQPSSSPPSQPQEQ-PEAKAREVEPEPEPEPMEVTDE  239
Sti1b  PRMVQVLSLMLNLRLPNNDAPPRPPAQSTPPPPPQQQQHQPETKAREPEPEPEPMEVTEE  236
       ***:***.****::: . :*.   ..*:**. **:*  :.:::  ************.*

Sti1a  EKERKERKSSAQKEKEAGNAAYKKKDFETAIQHYTKAMELDDEDISYLTNRAAVYIEMGK  299
Sti1b  EKERKERKAAAQEEKEAGNAAYKK-DFETAIQHYTKAMELDDEDVSYLTNRAAVYLEMGK  295
       ********::**:************ ****************:***********:****
                                    TPR2A
Sti1a  YDECVKDCDKAVERGRELRADFKMISRALTRKGTALAKLAKTSKDYDIAIETFQKALTEH  359
Sti1b  YDECINDRDKAVERGRELHADFKIISRALTRKGTALAKIAKCFKDYDVAIETYQKALTEH  355
       ****::* **********:****:********** **** ****:****:****:******

Sti1a  RNPDTLKKLNEAERAKKELEQQEYYDPKLADEEREKGNQLFKEQKYPDAVKHYTEAIGRN  419
Sti1b  RNPDTLKKLNDAEIAKKELEQQEYYDPKIADEEREKGNEFFKQQKYPEAVKHYSEALRRN  415
       **********:** ************** ********:.**:**** *****:**: **
                                    TPR2B
Sti1a  PKDPKVYSNRAACYTKLGAMPEGLKDAEKCIELDPTFSKGYTRKGAIQFFMKEYDKAMET  479
Sti1b  PKDPRVYSNRAACYTKLGAMPEGLKDAEKCIELDPTFSKGYTRKGAIQFFMKEYDKALET  475
       ****:*******************************************************:**

Sti1a  YQAGLKHGPNNPELLDGVKRCIEQINKANRGDLTQEEIQERQNKAMQDPEIQNILTDPIM  539
Sti1b  YQAGLKHDPNNQELLDGVRRCVQRINKASRGELSQEELQERQNKAMQDPEIQNILKDPIM  535
       *******.*** ****** **:;:**** ** :*:*;*** ****************.****
                     Sti1/DP2
Sti1a  RQVLVDLQENPRASQEHLKNPGVMQKIQKLVSAGIVQMR                       578
Sti1b  QQVLTDFQENPKAAQAHLKNPGVMQKIQKLVSAGIVQMK                       574
       :***.*:****:*:* ********************:
```

B

Figure 4. Amino acid alignment of *Sti1a* and *Sti1b* (A) and Phylogenetic tree (B) of Hop/Sti1.

Figure 5. Co-IP of OsRac1 and OsRAR1, HSP90, and HSP70.

Total protein extracts from Os Rac1 transgenic mutants were incubated with anti-myc antibody and protein A Sepharose beads. Precipitates were washed, collected by centrifugation, and separated by SDS-PAGE. Total extracted and immunoprecipitated samples from wild type cell culture were used as a control. Immunoblot analyses were performed with anti-RAR1 antibody. OsRAR1 was detected in CA and DN-OsRac1 immune complexes but not in CS-OsRac1 (top panel). All three OsRac1 mutants contained HSP90 (second panel). HSP70 was also detected in all three OsRac1 mutants. SGT1 was not detected in OsRac1 protein complexes (bottom panel).
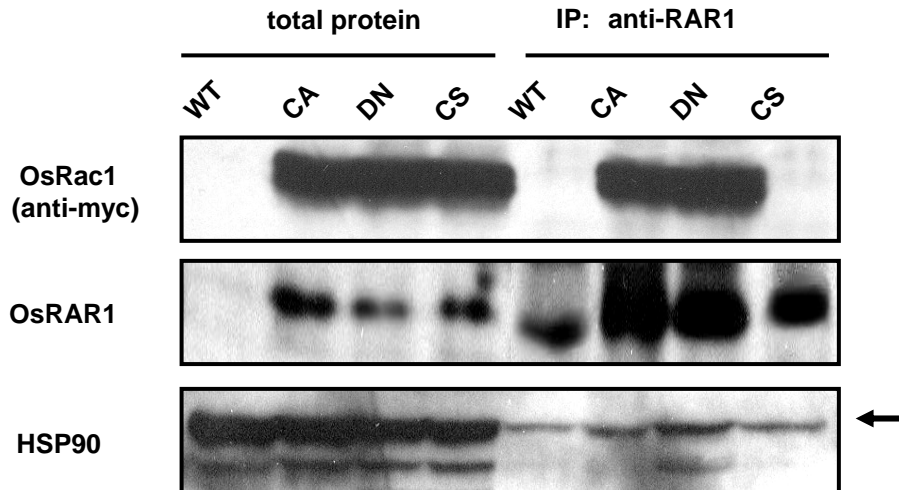
Figure 6. Immunoprecipitation with anti-RAR1 antibody and immunobloting with anti-myc antibody indicates the association of OsRAR1 and OsRac1 in complexes from CA- and DN-OsRac1 cells but not from CS-OsRac1 cells.

Signals were also detected from crude extracts of transgenic but not of the nontransgenic wild-type cell culture (top panel). As a positive control, OsRAR1 was detected in crude extracts as well as in precipitated complexes from all cell cultures (middle panel).
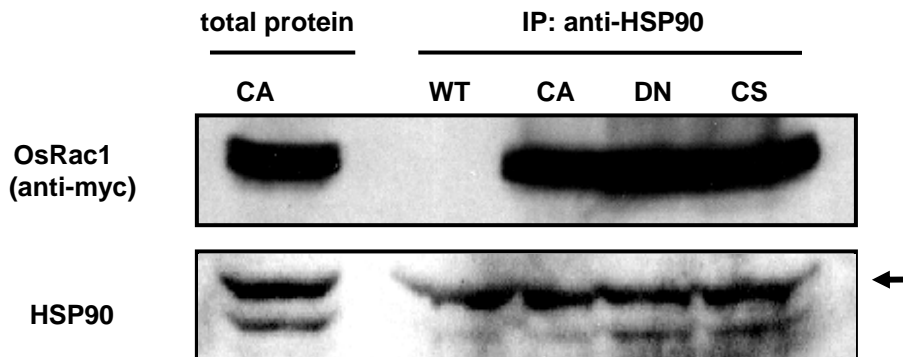
Figure 7. Association of HSP90 with OsRac1 *in vivo*.

Immunoprecipitation was performed with anti-HSP90 antibody and immunoblotted with anti-OsRac1 (top panel) and anti-HSP90 (bottom panel) antibodies.
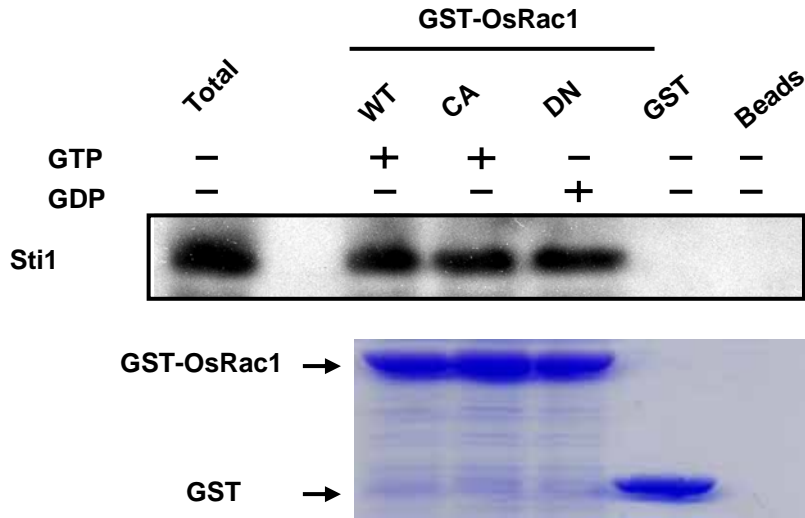
Figure 8. *in vitro* **pull down assays**

CA-, DN-, WT-OsRac1 protein fused to glutathione S transferase (GST) at the N-teminus was purified and used for *in vitro* binding assays. Western blotting with anti-MBP antibody indicates that OsRac1 can bind Sti1 irrespective of its form. GST protein and agarose beads were negative controls for the assays.
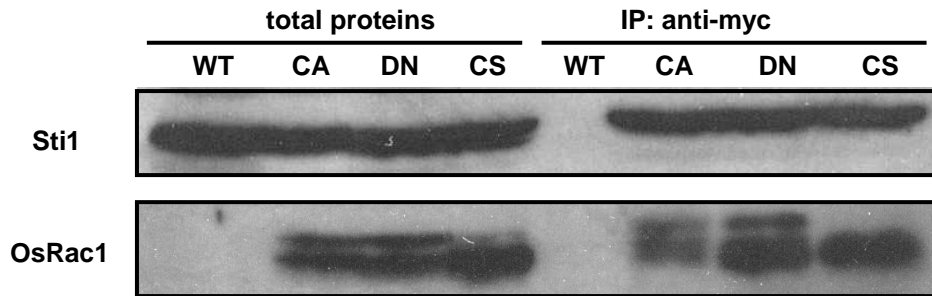
Figure 9. Sti1 interacts with OsRac1 *in vivo*.

Immunoprecipitation was performed with anti-myc antibody and immunoblotted with anti-Sti1 (top panel) and anti-myc (bottom panel) antibodies.

	total proteins				IP: anti-HSP90			
	WT	CA	DN	CS	WT	CA	DN	CS
Sti1								
HSP90								
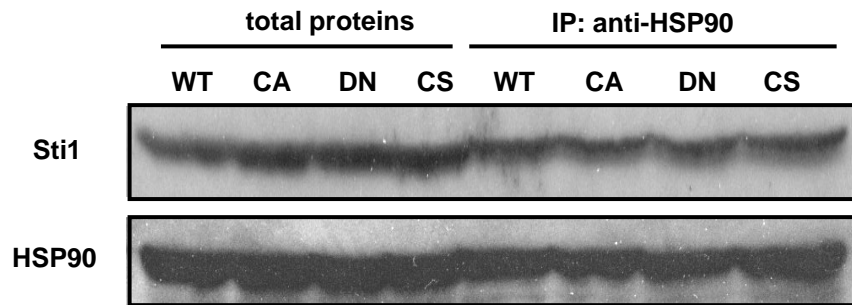
Figure 10. HSP90 interacts with Sti1 *in vivo*.

Immunoprecipitation was performed with anti-HSP90 antibody and immunoblotted with anti-Sti1 (top panel) and anti-HSP90 (bottom panel) antibodies
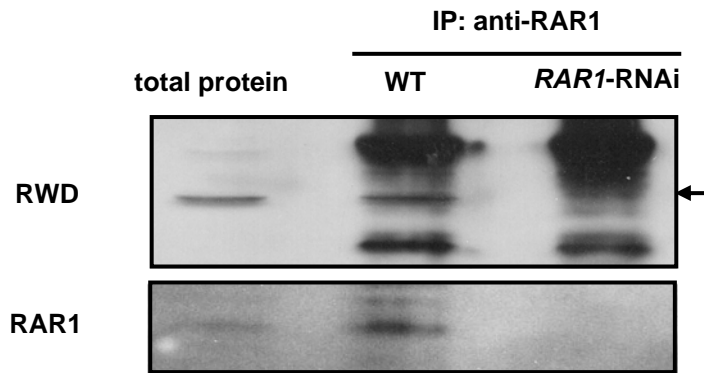
Figure 11. RWD interacts with OsRAR1 *in vivo*.

Co-IP experiments using wild type and *OsRAR1*-RNAi cell cultures were performed. RWD co-immunoprecipitated with OsRAR1, but no RWD band was detected in the control experiments using *OsRAR1*-RNAi cell cultures.
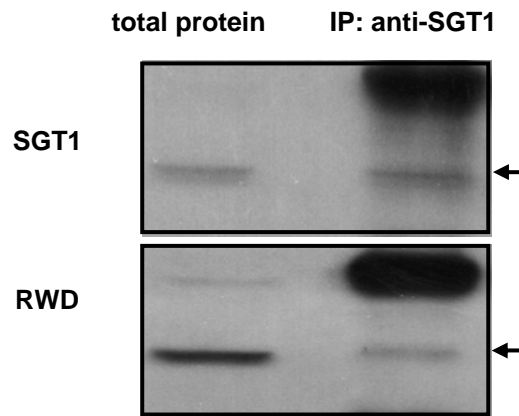
Figure 12. RWD interacts with SGT1 *in vivo*.

Co-IP experiments using anti-SGT1 antibody and immunoblots using SGT1 and
RWD antibodies suggested an *in vivo* interaction between SGT1 and RWD.
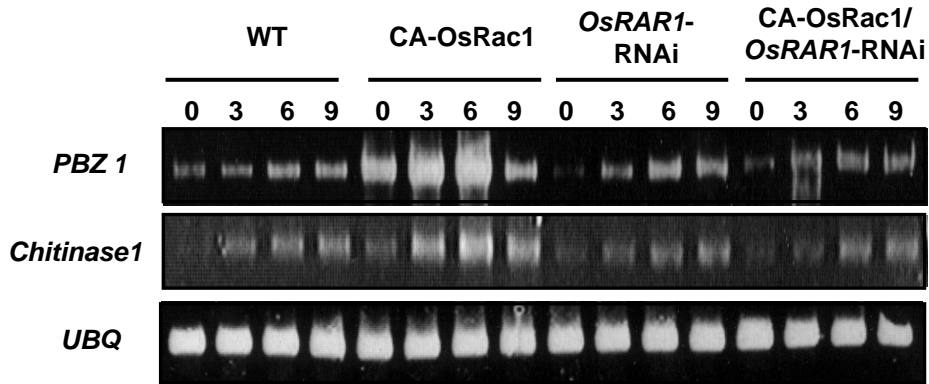
Figure 13. Suppression of SE-induced *PBZ1* and *Chitinase1* by *OsRAR1*-RNAi in CA-OsRac1 cell cultures.

SE induction of *PBZ1* and *Chitinase1* was examined in the wild type, CA-OsRac1, *OsRAR1*-RNAi, and *OsRAR1*-RNAi/CA-OsRac1 double mutants after 3, 6, or 9 h of treatment with SE. Activation of SE-induced *PBZ1* and *Chitinase1* expression in the CA-OsRac1 cell cultures (left) was suppressed by *OsRAR1*-RNAi (right).
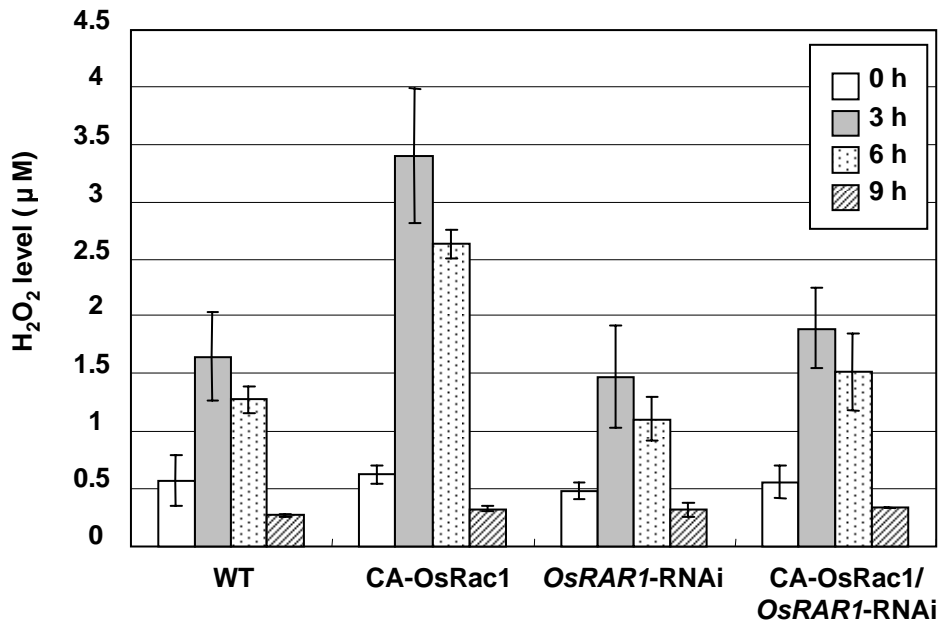
Figure 14. Suppression of SE-induced hydrogen peroxide production by
OsRAR1-**RNAi in the CA-OsRac1 cell culture.**

Levels of ROS were measured 3, 6, or 9 h after SE treatment.
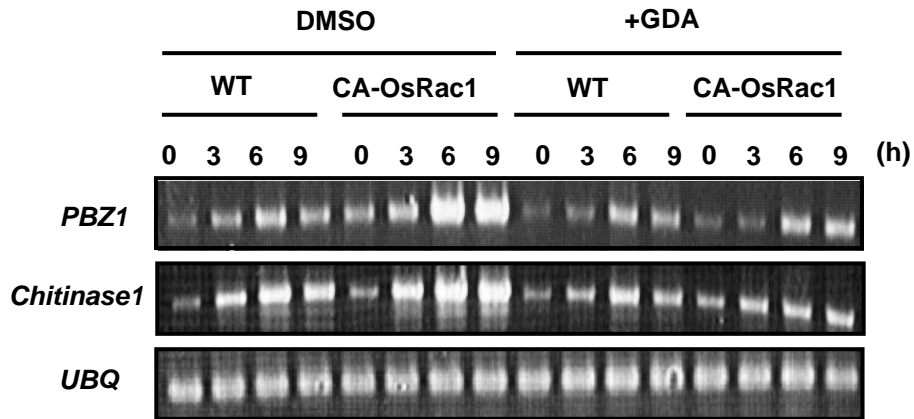
Figure 15. SE-induced *PBZ1* and *Chitinase1* expression in rice cell cultures in   the presence or absence of GDA treatment.

Cell cultures of wild type and CA-OsRac1 were treated with GDA (right) or control DMSO (left) overnight   with a time course of SE treatment. Expression of *PBZ1* and *Chitanase1* was strongly enhanced in CA-OsRac1 cells and reduced by   GDA treatment (right).
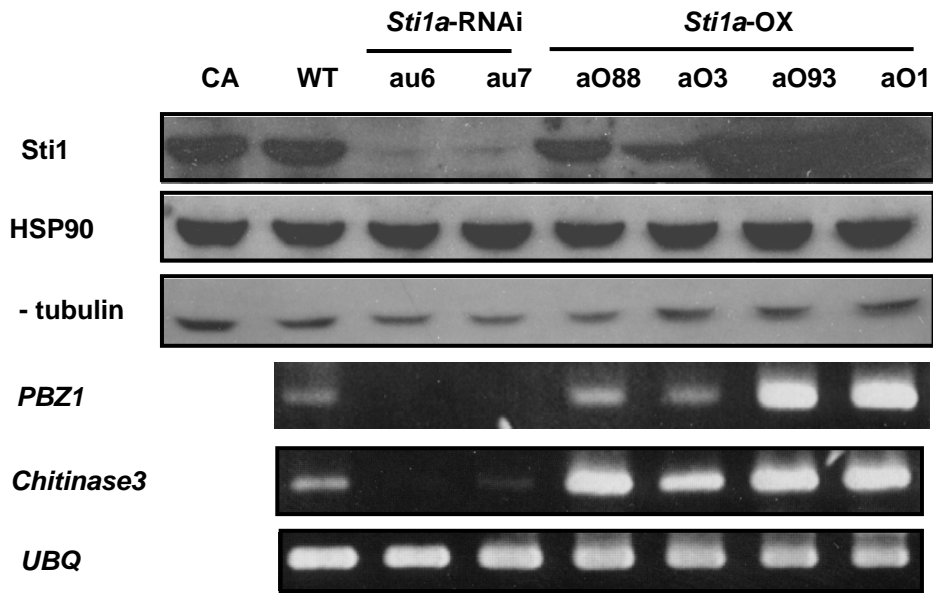
Figure 16. Expression level of Sti1 protein regulates the expression of *PR* genes in cell cultures.

Expression of *PBZ1* and *Chitinase3* was examined in the control nontransgenic (WT), *Sti1a*-RNAi, and Sti1a overexpressing (OX) cultured cells. Lines au6 and au7 are cell lines transformed with *Sti1a*-RNAi. Lines aO88, aO3, aO93, and aO1 are overexpressing lines. Expression of *PBZ1* and *Chitinase3* in the *Sti1a*-RNAi and overexpressing cell cultures was suppressed or strongly induced, respectively. *Ubiquitin* is a loading control for RT-PCR.
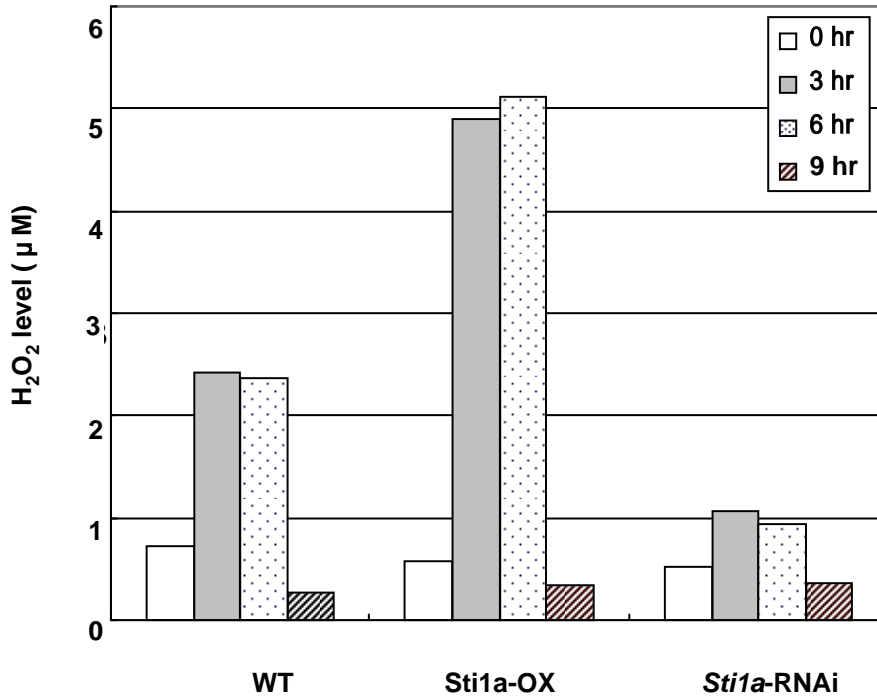
Figure 17. Enhancement and suppression of SE-induced hydrogen peroxide production by *Sti1a*-overexpressing (Sti1a-OX) or *Sti1a*-RNAi respectively in cell cultures.

Levels of ROS were measured 3, 6, or 9 h after SE treatment.
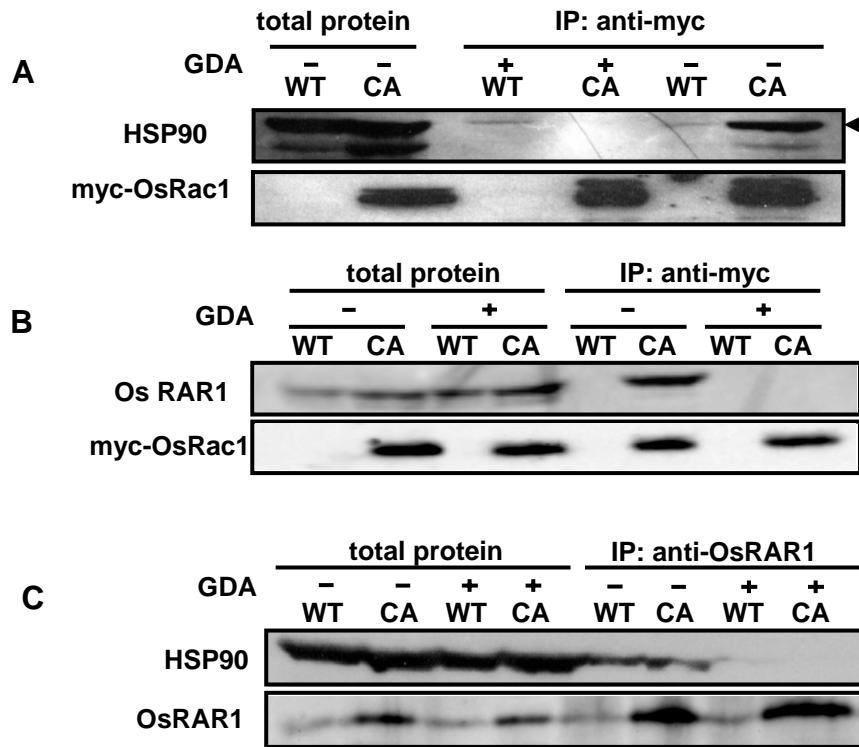
Figure 18. HSP90 function may be essential for the associations of HSP90 and OsRAR1 with the OsRac1 complex

(A) and (B) Wild-type and CA-OsRac1 cell cultures were treated with 10 μ M GDA or DMSO overnight. Total protein extracts were incubated with antimyc antibody and protein A Sepharose beads. Precipitates were washed, collected by centrifugation, and separated by SDS-PAGE. Immunoblot analyses were performed with anti-HSP90 (A) or anti-RAR1 (B) antibody to examine the interactions of OsRac1 with HSP90 and OsRAR1. Signals were detected only from immunocomplexes without GDA treatment. Immunoblots with anti-myc antibody were used as a control ([A], bottom panel).

(C) Immunoprecipitation performed with anti-RAR1 antibody after GDA treatment. Immunoblot analyses were performed with anti-HSP90 antibody. HSP90 was not detected in OsRAR1-precipitated complex after GDA treatment.
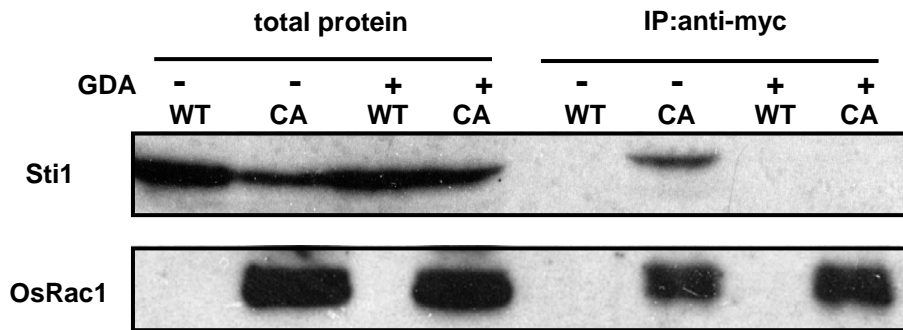
Figure 19. GDA treatment breaks the OsRac1-Sti1 complex formation

Immunoprecipitation performed with anti-myc antibody after GDA treatment. Immunoblot analyses were performed with anti-Sti1 antibody. Sti1 was not detected in OsRac1-precipitated complex after GDA treatment. WT and CA indicate nontransgenic wild-type and CA-OsRac1 cell cultures, respectively.
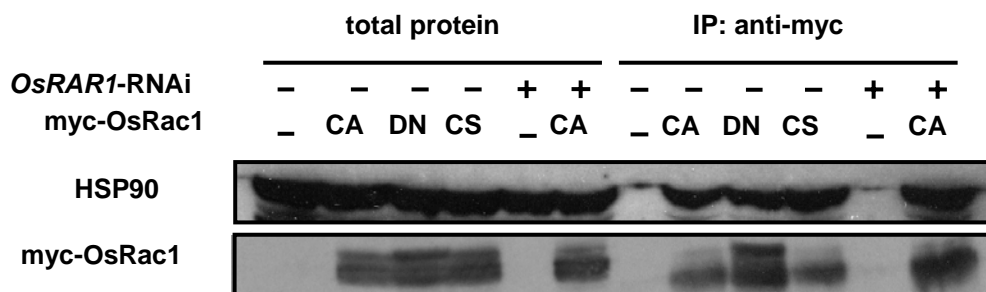
Figure 20. OsRAR1 is not required for association of HSP90 in the OsRac1 complex.

Total protein extracts from the wild type, CA-, DN-, or CS-OsRac1, *OsRAR1*-RNAi, and the CA-OsRac1/*OsRAR1*-RNAi double transgenic mutant were incubated with anti-myc antibody. Precipitates were washed, collected by centrifugation, and separated by SDS-PAGE. Total extracted and immunoprecipitated samples from wild-type cultured cells were used as control. Immunoblot analyses were performed with anti-HSP90 antibody to examine *OsRac1*-HSP90 association. *OsRAR1*-RNAi in the CA-OsRac1/*OsRAR1*-RNAi double transgenic mutant had no effect on OsRac1-HSP90 association (last column).
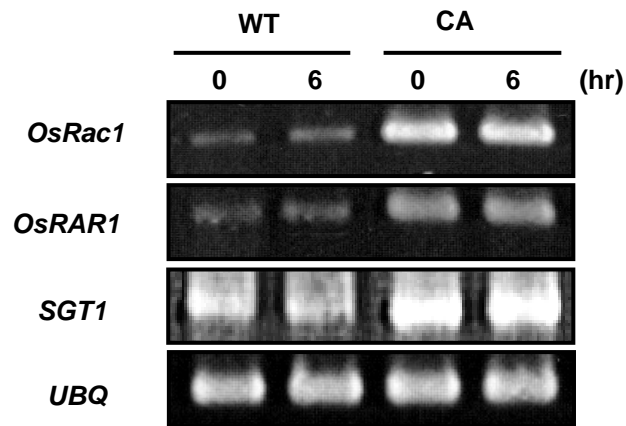
Figure 21. Correlation of mRNA expression levels of *OsRac1*, *OsRAR1*, and *SGT1*.

Overexpression of CA-OsRac1 strongly increased mRNA accumulation of *OsRAR1* and *SGT1* in rice cell cultures. Levels of mRNAs were measured by RT-PCR.
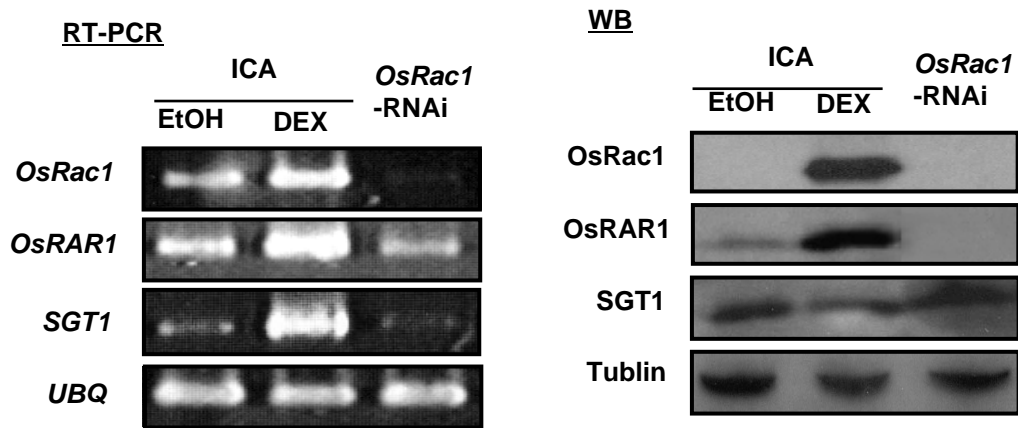
Figure 22. Levels of mRNA (RT-PCR) and protein accumulation (protein gel blot [WB]) for OsRac1, OsRAR1, and SGT1.

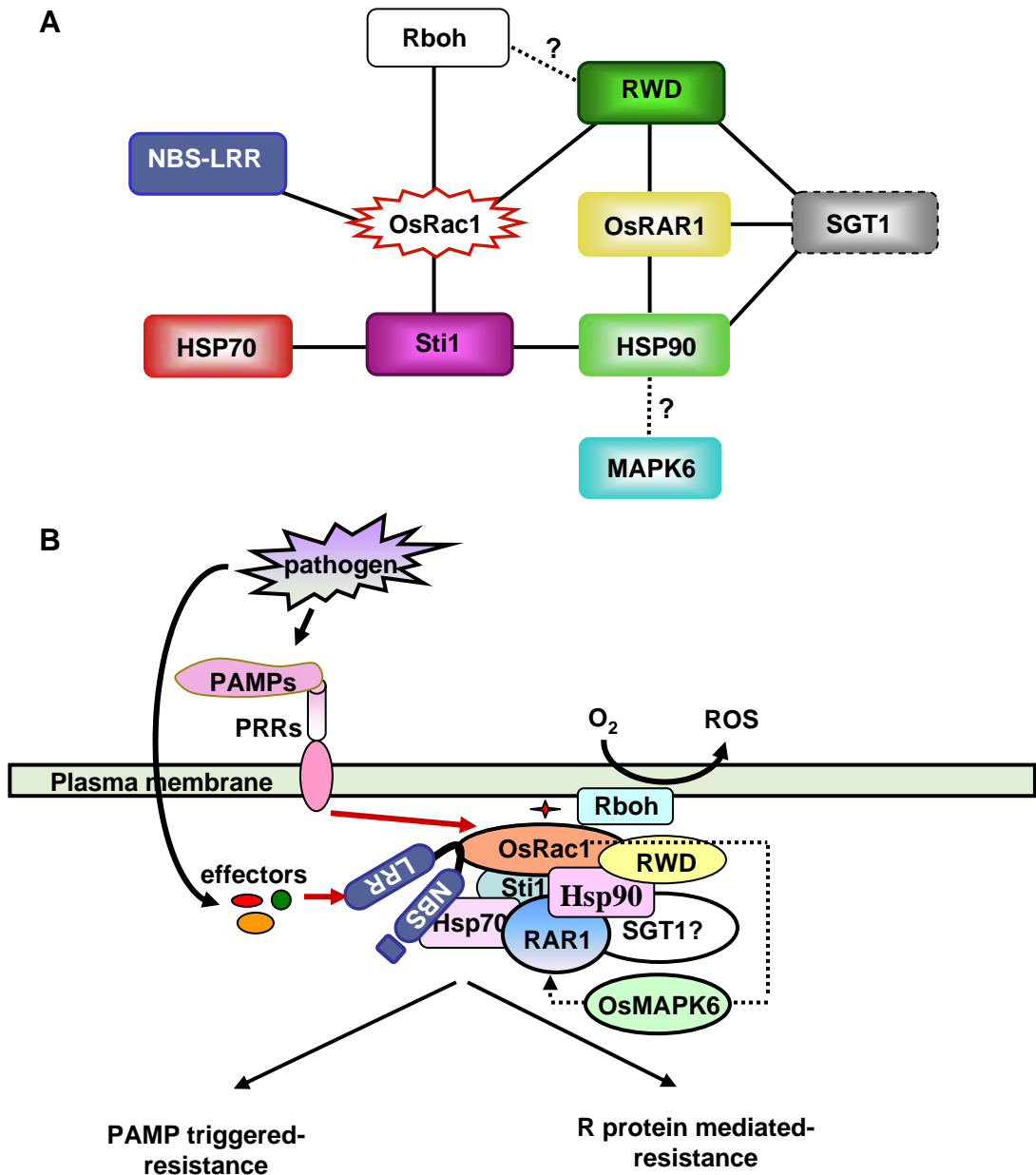DEX-inducible CA-OsRac1 (ICA) and an *OsRac1*-RNAi cell culture were examined by RT-PCR and immunoblotting after DEX induction and ethanol as a control.

Figure 23. Network of proteins involved in rice innate immunity (defensome).

(A) Interactions among proteins are shown by yeast two hybrid assays, and/or *in vitro* binding experiments and co-IP experiments. Dotted lines indicate lack of experimental evidence.

(B) Defensome complex, which contains OsRac1 and other components of rice innate immunity, may localize at the plasma membrane and functions in both basal and R protein mediated resistances.
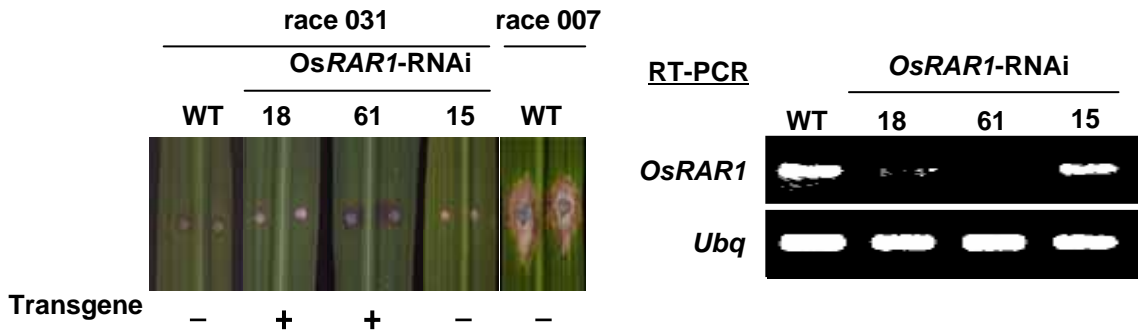
Figure 1. OsRAR1 is not required for the *R* gene *Pi-a* for rice blast resistance

Transgenic plants expressing *OsRAR1*-RNAi were inoculated with an incompatible race 031 of the rice blast fungus *M. grisea*. Lesions are shown on leaf blades 12 days after inoculation. WT indicates untransformed wild type plant, and 18, 61, and 15 are T1 segregants derived from an *OsRAR1*-RNAi plant. Response of leaf blades of the wild type Kinmaze with infection by the compatible race 007 was used as a control for this experiment. Total RNA was extracted from leaves of the wild type Kinmaze and *OsRAR1*-RNAi plants. *OsRAR1* mRNA was amplified by RT-PCR using specific primers. The Ubiquitin primers were included in the PCR reactions as an internal control.
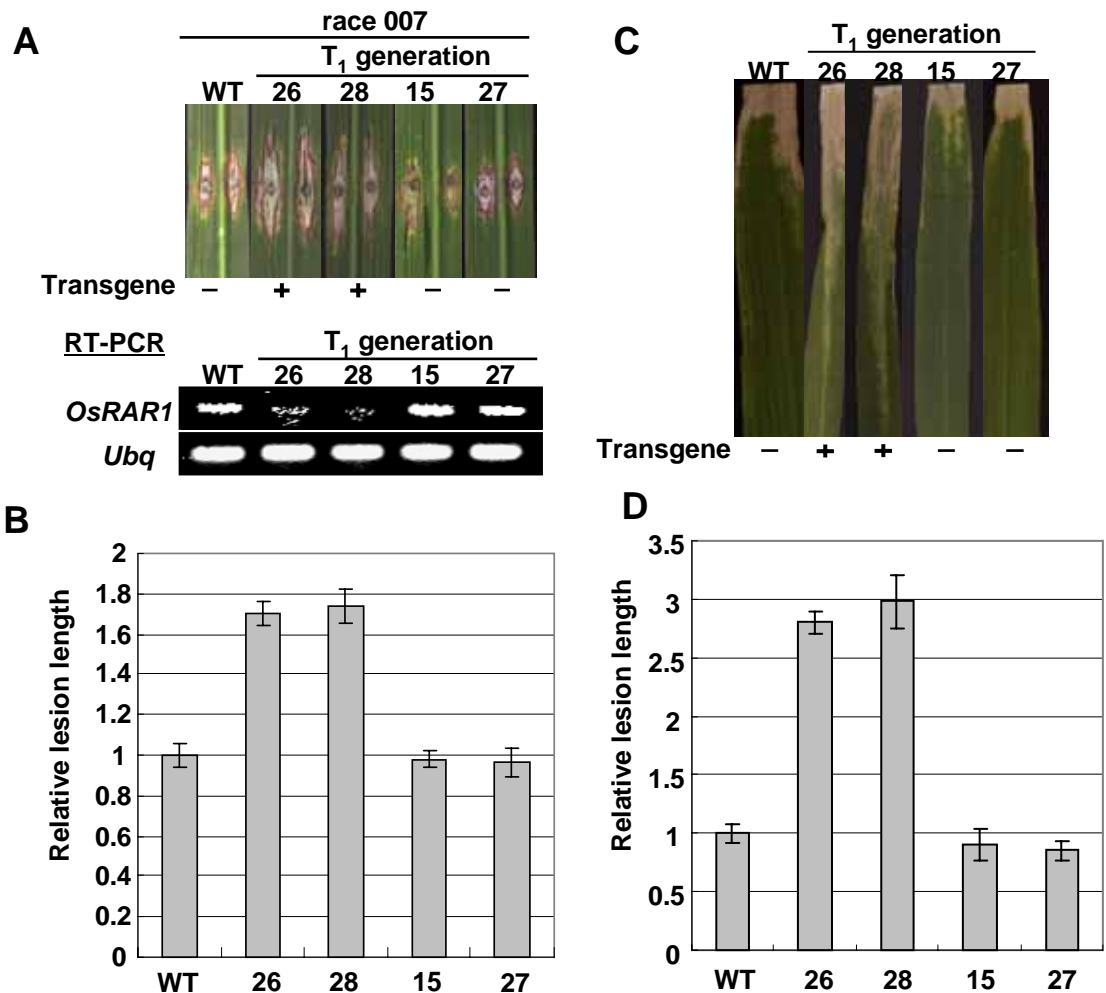
Figure 2. Response of transgenic plants expressing *OsRAR1*-RNAi to infection with the compatible race 007 of *M. grisea*.

WT indicates untransformed control plant; 26, 28, 15, and 27 are T1 segregants derived from an *OsRAR1*-RNAi plant. Photographs were taken 12 days after infection. Presence or absence of the *OsRAR1*-RNAi transgene are indicated by + and -, respectively.

(B) Quantitative analysis of disease lesions induced by infection with a compatible race of rice blast fungus shown in (A). Leaves were inoculated with compatible race 007 of *M. grisea*. Bars indicate SE obtained from 6 to 20 measurements.

(C) Response of transgenic plants expressing *OsRAR1*-RNAi to infection with X. oryzae pv oryzae race 1 (T7174). WT indicates untransformed control plants, and 26, 28, 15, and 27 are T1 segregants derived from an *OsRAR1*-RNAi plant. Photographs were taken 12 days after infection. Presence or absence of the *OsRAR1*-RNAi transgene are indicated by + and -, respectively.

(D) Quantitative analysis of disease lesions induced by infection with a compatible race of bacterial blight shown in (C). Leaves were inoculated with the *X. oryzae* race 1. Bars indicate SE obtained from four to nine measurements.
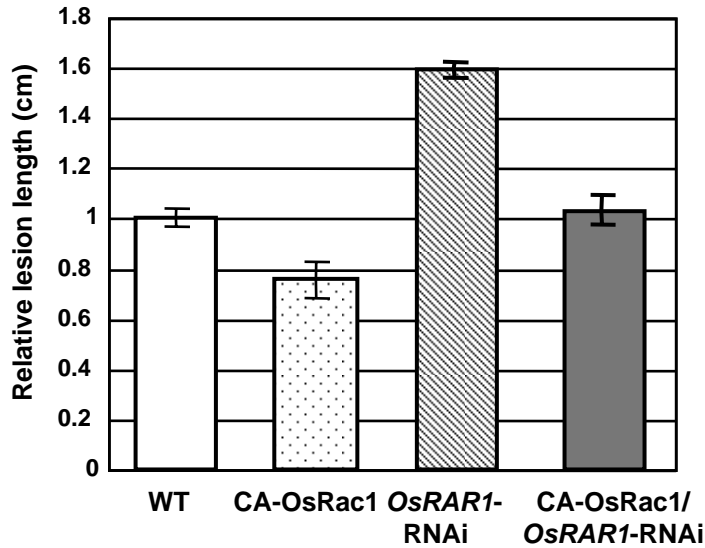
Figure 3. Quantitative analysis of disease lesions induced by infection with a compatible race of *M. grisea*.
Leaves of the nontransgenic wild type (WT), CA-OsRac1, *OsRAR1*-RNAi, and double transgenic CA-OsRac1/*OsRAR1*-RNAi plants were used in three independent experiments (mean 6 SE; n = 12 to 61). Double transgenic plants were made by introducing the O*sRAR1*-RNAi construct into the CA-OsRac1 transgenic line. The double transgenic plants were more resistant to race 007 of *M. grisea* than *OsRAR1*-RNAi plants and more susceptible than CA-OsRac1 plants.
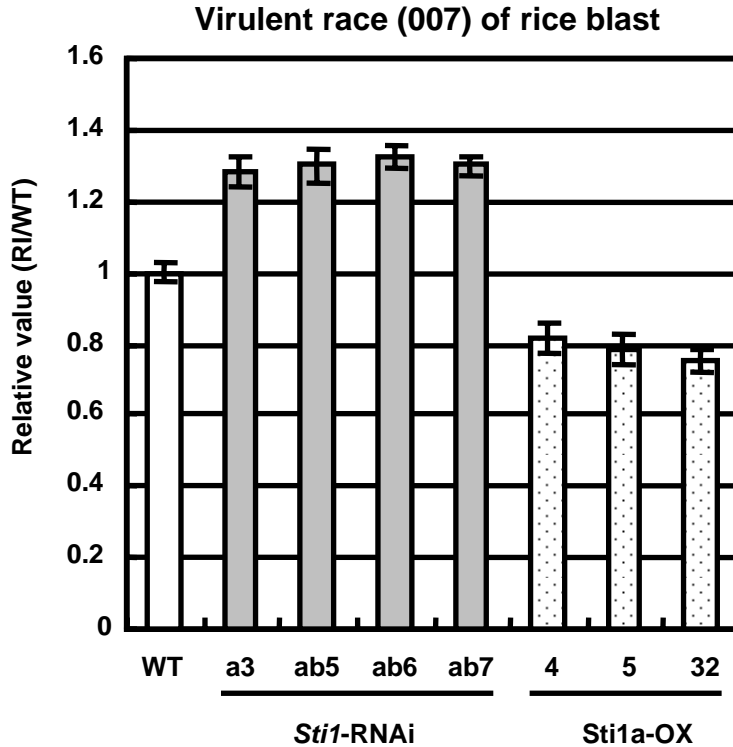
Virulent race (007) of rice blast

Figure 4. Quantitative analysis of disease lesions induced by infection with a compatible race of *M. grisea*.
Leaves of the nontransgenic wild type (WT), *Sti1*-RNAi and overexpressing (OX) plants were were inoculated with a compatible race 007 of the rice blast fungus *M. grisea*. Photographs were taken 12 days after infection. The Sti1-OX plants were more resistant to race 007 of *M. grisea* than the control notransgenic plants WT, and *Sti1*-RNAi plants and more susceptible than WT plants.

Appendix Table 1　Primes used in this study

Primer name	Sequence (5' – 3')
Primers for RT-PCR	
OsRac1-F	AGATAGGGCCTATCTTGCTGATCATC
OsRac1-R	ACAAGCGCTTCCGCAAAAGT
OsRAR1-F	TGCAAAACTGGAAAGCACAC
OsRAR1-R	GGAACTGCAGGCTTCTCAAC
OsSGT1-F	ATGGATCCATATGGCAACCGCCGCCGCG
OsSGT1-R	ATGCGGCCGCTTAGTACTCCCATTTCTTAAGC
Sti1-F	ATG GCC GAC GAG GCG AAG G
Sti1a-R	TATGCC GTCAACGCCGCTG
Ubiquitin-F	CCAGGACAAGATGATCTGCC
Ubiqutin-R	AAGAAGCTGAAGCATCCAGC
Actin-F	ACAGGAGAGAGCAGGCAGAG
Actin-R	TGCAAAGGAGTGAGGCTTTT
PBZ1-F	GGGCACCATCTACACCATGAA
PBZ1-R	GTCGCACACCGCCACC
Chitinase1-F	TCTTAACATCACTGCAACTCAG
Chitinase1-R	CTGCGAGCTCTGGACAC
Primers for analysis of RNAi constructs	
OsRAR1-RNAi-F	TCT GAG TGA GCC TAG GGT TTG
OsRAR1 RNAi-R	GAC CGA AGT CTC CAC ACACA
Sti1a-RNAi-F	CACCTCGACCAAAGCAAGAAGCGCTGTTC
Sti1a-RNAi-R	CTTTCAGCATGTGGCCAGACCCATGACCAAAACCGA
Sti1b-RNAi-R	TAGGTCGTGGTGACTGATTAGGCA

List of publications

Thao, N.P., Chen, L., Nakashima, A., Hara, S., Umemura, K., Takahashi, A., Shirasu, K., Kawasaki, T., and Shimamoto, K. (2007). RAR1 and HSP90 form a complex with Rac/Rop GTPase and function in innate-immune response in rice. Plant Cell **19**, 4035-4045.

Lieberherr, D., Thao, N.P., Nakashima, A., Umemura, K., Kawasaki, T., and Shimamoto, K. (2005). A sphingolipid elicitor-inducible mitogen-activated protein kinase is regulated by the small GTPase OsRac1 and heterotrimeric G-protein in rice. Plant Physiol. **138**, 1644-1652.

Acknowledgements

I would like to express my deepest gratitude for the guidance and support of my great supervisors Prof. Ko Shimamoto and Associate Prof. Tsutomu Kawasaki.

I would like to thank my committee, Prof. Takashi Hashimoto, Prof. Masaaki Umeda and Prof. Seiji Takayama for their time and attention.

I would like to thank Dr. Hann Ling Wong for his helpful advices and support. I would also like to show my great application to Dr. Letian Chen, (NAIST) for allowing me to site his unpublished data and for providing materials in this thesis. I am great thankful to Ayako Nakashima, Shin-ichiro Hara, Mrs. Mika Nobuhara, Yuko Tamaki, and Masako Hamane (NAIST); Drs. Ken Shirasu and Akira Takahashi (John Innes Centre); Dr. Kenji Umemura (Meiji Seika Kaisha Ltd.); and Dr. Priti Krishna (Uniersity of Western Ontario) for providing materials, technical advices and valuable discussions. I am grateful to all the members of this lab for their helps, friendships and discussions. I would like to thank the Japanese Monbukagakuso for supporting me a scholarship for 6 years.

Finally, I would like to dedicate this work to my family for their love, inspiration and moral support throughout my study as well as all my life.