

Palmitoylation-dependent Membrane Localization of the Rice Resistance Protein Pit Is Critical for the Activation of the Small GTPase OsRac1*

Received for publication, April 3, 2014, and in revised form, May 1, 2014. Published, JBC Papers in Press, May 19, 2014, DOI 10.1074/jbc.M114.569756

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Background: Mechanisms and significance of immune receptor R protein localization to specific compartments remain unknown.

Results: Palmitoylation-deficient R protein Pit fails to induce GTPase OsRac1 activation at the plasma membrane, thereby compromising Pit-mediated disease resistance.

Conclusion: Palmitoylation-dependent membrane localization of Pit contributes to Pit-mediated disease resistance through OsRac1 activation.

Significance: Elucidating relationships between R protein localization and activation of downstream molecules improve our understanding of plant immunity.

Nucleotide binding domain and leucine-rich repeat (NLR)-containing family proteins function as intracellular immune sensors in both plants and animals. In plants, the downstream components activated by NLR family proteins and the immune response mechanisms induced by these downstream molecules are largely unknown. We have previously found that the small GTPase OsRac1, which acts as a molecular switch in rice immunity, is activated by Pit, an NLR-type resistance (R) protein to rice blast fungus, and this activation plays critical roles in Pit-mediated immunity. However, the sites and mechanisms of activation of Pit *in vivo* remain unknown. To clarify the mechanisms involved in the localization of Pit, we searched for consensus sequences in Pit that specify membrane localization and found a pair of potential palmitoylation sites in the N-terminal coiled-coil region. Although wild-type Pit was localized mainly to the plasma membrane, this membrane localization was compromised in a palmitoylation-deficient mutant of Pit. The palmitoylation-deficient Pit displayed significantly lower affinity for OsRac1 on the plasma membrane, thereby resulting in failures of the Pit-mediated cell death, the production of reactive oxygen species, and disease resistance to rice blast fungus. These results indicate that palmitoylation-dependent membrane localization of Pit is required for the interaction with and the activation of OsRac1 and that OsRac1 activation by Pit is vital for Pit-mediated disease resistance to rice blast fungus.

Plants have developed a two-branched system of innate immunity to prevent the invasion of pathogens. The first line of defense involves receptors that detect nonspecific microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) (1–3) and is termed MAMP-triggered immunity. To promote pathogen virulence, pathogen effectors target plant proteins and thus perturb host cell physiology and immunity. On that occasion, a second line of plant defense counterattacks pathogens. This defense system is called effector-triggered immunity and provides a high level of disease resistance (1–3). Disease resistance (R)² proteins act as intracellular receptors for the direct or indirect recognition of specific pathogen effectors. Effector-triggered immunity triggered by R proteins results in strong host responses accompanied by cell death (4). Most R genes encode members of the nucleotide binding (NB) domain and leucine-rich repeat (LRR) domain (NLR, also called NB-LRR) family, which often displays a tripartite domain architecture and is subdivided according to its N-terminal domains into coiled-coil (CC-NB-LRR) and Toll/interleukin-1 receptor homology (TIR-NB-LRR) subclasses. Although sharing broad structural similarities, many NLR proteins also show unique structural variations that are important for their function and subcellular localization (5).

Evidence is accumulating that the subcellular localization of R proteins is important for their function; the nuclear accumulation of MLA10 and both cytosolic and nuclear pools of Rx1 and RPS4 are required for proper regulation of defense signaling (6–8). Recognition of the fungal effector A10 by MLA10 promotes a nuclear interaction between MLA10 and HvWRKY1

* This work was supported by MAFF Genomics for Agricultural Innovation Grant PM10007 (to K. S.) and by Japan Society for the Promotion of Science KAKENHI Grants 19108005 (to K. S.) and 2377044, 24113515, 26113712, and 26450055 (to Y. K.).

The work is dedicated to Ko Shimamoto, who passed away on September 28, 2013.

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² The abbreviations used are: R protein, resistance protein; NLR, nucleotide binding domain and leucine-rich repeat; ROS, reactive oxygen species; NB, nucleotide binding; LRR, leucine-rich repeat; CC, coiled-coil domain; 2-BP, 2-bromopalmitate; DAB, diaminobenzidine tetrahydrochloride; Nip, Nipponbare; dpi, days post-inoculation; BIFC, bimolecular fluorescence complementation; CFP, cyan fluorescent protein.

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and HvWRKY2 transcription factors (6). The suppression of these WRKY proteins by MLA10 induces defense responses. In contrast, Rx1 is activated in the cytoplasm and cannot be activated in the nucleus (7). Perturbation of Rx1 function by mutating the ATP binding site in the NB domain or by silencing the cochaperone SGT1 impaired the accumulation of Rx1 protein in the nucleus, whereas an Rx1 mutant lacking the LRR domain is not affected in this respect. It appears that interdomain interactions and folding states determine the nucleocytoplasmic distribution of Rx1. Although our knowledge of R protein localization has improved in recent years, the mechanisms and significance of the dynamic nature of R protein localization to different subcellular compartments remain largely unknown.

Proteins belonging to the Rac family small GTPase (also called the Rop family) act as a molecular switch by cycling between a GDP-bound inactive and a GTP-bound active form in cells. The Rac family participates in innate immunity in rice, barley, and other species (9–11). We have shown that the *Oryza sativa* Rac family protein OsRac1 forms protein networks, collectively termed the defensome, with various downstream molecules including the signaling protein OsMAPK6, the lignin biosynthesis protein OsCCR1, the scaffolding protein OsRACK1, and the (co)chaperone proteins RAR1, SGT1, Hsp90, and Hop/Sti1 and thereby regulates phytoalexin production, lignin biosynthesis, and the transcription of pathogenesis-related genes (9, 12–17). OsRac1 plays a dual role in the induction of ROS production via direct interactions with NADPH oxidases and the suppression of the ROS scavenger OsMT2b (9, 18–21). OsRac1, therefore, appears to be one of the key regulators in rice immunity. Moreover, we have found that OsRac1 forms a complex with Pit, an NLR family R protein that confers resistance to rice blast fungus at the plasma membrane and is required for Pit-mediated resistance to rice blast fungus (22).

Here we revealed that Pit is a palmitoylated protein and localized at the plasma membrane in a palmitoylation-dependent manner. A palmitoylation-deficient Pit mutant displayed decreased binding to OsRac1, resulting in reductions of OsRac1 activation as well as of Pit-mediated cell death, ROS production, and disease resistance to rice blast fungus. These results suggest that palmitoylation of Pit is required for its localization and interaction with OsRac1 on the plasma membrane and thus plays a critical role in Pit-mediated disease resistance.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The cDNAs of *Pit* and *OsRac1* were described previously (12, 22). Mutant *Pit* genes were generated by a QuikChange II site-directed mutagenesis kit (Agilent Technologies) and then transferred into various vectors depending on the experiment. These included pGWB2, -5, and -6 (provided by Dr. T. Nakagawa (Shimane University)), pBI221-Vn-Gateway, pBI221-Gateway-Vc (provided by Dr. S. Takayama (Nara Institute of Science and Technology)), 35S-Gateway-Venus, 35S-Vn, and pUbq-Gateway.

Subcellular Localization—Venus was fused to either the C or N terminus of Pit using the Gateway system (Invitrogen). The Pit-Venus, mCherry, and Cerulean-NLS constructs were controlled by the cauliflower mosaic virus 35S promoter. Proto-

plast isolation from rice *O. sativa* L. C5924 suspension cultures and protoplast transformations were performed as described previously (23). Some of the transfected cells were treated with the palmitoylation inhibitor 2-bromopalmitate (2-BP) (Sigma) at 100 μ M (24). After incubation for 12–36 h at 30 °C, the protoplasts were observed with a Leica TCS-SP5 microscope.

Agroinfiltration into *Nicotiana benthamiana* Leaves—Agroinfiltration of *N. benthamiana* was performed as described previously (25). *Agrobacterium tumefaciens* strain GV3010, harboring the helper plasmid pSoup and binary plasmids carrying the cDNAs of Pit WT and mutants, was used to infiltrate leaves of 5-week-old *N. benthamiana* plants. In some experiments we used the p19 silencing suppressor to enhance gene expression (26). Each *Agrobacterium* culture was resuspended in a buffer containing 10 mM MgCl₂, 10 mM MES, pH 5.6, and 150 μ M acetosyringone and incubated at 23 °C for 2–3 h before infiltration. The plants were kept in a growth chamber at 23 °C after agroinfiltration. To visualize hydrogen peroxide, a major endogenous ROS, *in situ*, the agroinfiltrated leaves were detached and incubated in 1 μ g/ml DAB solution for 2–8 h, after which they were decolorized in boiling ethanol. Photographs were taken at 7 days post-inoculation (dpi) for cell death and at 3 dpi for ROS production.

Pit WT and mutants fused with HA were agroinfiltrated into *N. benthamiana* leaves. Infiltrated leaves were homogenized and extracted with SDS sample buffer (0.2 M Tris-HCl (pH 6.8), 2% SDS, 15% sucrose, 0.01% bromophenol blue, 15% 2-mercaptoethanol), and the homogenized samples were further solubilized by boiling, cleared by centrifugation at 20,000 \times g for 10 min at 4 °C, and then subjected to immunoblot analysis with anti-HA antibody (3F10, Roche Applied Science).

Plant Cultivation and Pathogen Infection—The *japonica* rice cultivar Nipponbare (Nip) carrying the exogenous *Pit* WT or mutants was generated using *Agrobacterium*-mediated transformation of rice calli, as previously described (27). Growth of *Magnaporthe grisea* strain Ina86–137 (Race 007.0) and punch infections of leaf blades were performed as described previously (13, 28). Lesion length was measured at 5 dpi.

To measure the growth of *M. grisea* at 6 dpi, specific primers against *M. grisea* *Pot2* (*MgPot2*) (5'-ACGACCCGTCTT-TACTTATTTGG and 5'-AAGTAGCGTTGGTTTTGTG-GAT) and *O. sativa* ubiquitin (*OsUbq*) (5'-AACCAGCTGAG-GCCCAAGA and 5'-ACGATTGATTTAACCAGTCCATGA) were used for real-time PCR (22, 29). The sequence used for quantifying rice DNA is a single-copy sequence from the rice ubiquitin gene, which has no homologous sequences in the *M. grisea* genome. The *Pot2* transposon sequence used to quantify the fungus has ~100 copies in the *M. grisea* genome. An infection ratio (number: *MgPot2*/(number: *OsUbq* \times 100)) was calculated from the determined numbers of target sequences of *MgPot2* and *OsUbq* in each sample. An infection ratio that represents the relative number of fungus cells and plant cells was calculated. Each data point represents the average and S.E. of 12 DNA samples taken from 72 infected areas at 6 dpi (the same samples shown in Fig. 4, A and B).

Bimolecular Fluorescence Complementation—For use in bimolecular fluorescence complementation (BiFC) experiments, vectors containing sequences encoding the N- and

C-terminal halves of the Venus protein were kindly provided by Dr. S. Takayama (Nara Institute of Science and Technology) (30). *Pit* and *OsRac1* were cloned into BiFC vectors, which were then purified using the Purelink Plasmid Midiprep Kit (Invitrogen) and introduced into rice protoplasts as described previously (22, 23). The mCherry expression plasmid was introduced simultaneously as a marker for transformed cells.

Förster Resonance Energy Transfer (FRET) Analysis—To analyze the activation of OsRac1 by Pit *in vivo*, we previously established the Raichu intramolecular FRET system (22). 10–12 h after transfection of Raichu-OsRac1 into rice protoplasts, the cells were imaged using an Olympus IX-81 inverted microscope with a Yokogawa CSU22 confocal scanner equipped with the cooled charge-coupled device camera EM-CCD C9100–02 (Hamamatsu Photonics). Raichu-OsRac1 was excited using a 440-nm diode laser (iFLEX 2000, Point Source). The CFP and YFP filters were 480 ± 15 and 535 ± 20 nm, respectively. Background fluorescence was subtracted, fluorescence bleed-through was normalized, and FRET efficiency was calculated according to published procedures (31).

RT-PCR—To check the expression level of exogenous *Pit* and *OsRac1*, three sets of specific primers against exogenous *Pit* (5'-ATGTTCCAAAAGGAGTTGCA and 5'-ACTTTGTACAA-GAAAGCTGGGTCG), *OsRac1* (5'-CAATCAAGCTCCAAT-CGGCCAGT and 5'-CATCCAGAAGAGCACATCTTATTACC), and *O. sativa* ubiquitin (5'-CCAGGACAAGATGATCTGCC and 5'-AAGAAGCTGAAGCATCCAGC) were used for RT-PCR.

RESULTS

The N-terminal-fused Fluorescent Tag Interferes with Pit Activity—As a first step toward elucidating the subcellular distribution of the R protein Pit in living rice protoplasts, we constructed two fluorescent proteins in which a variant yellow fluorescent protein (Venus) was fused at either the N or the C terminus of wild-type Pit (Pit WT). Rice protoplasts were co-transfected with one of these fluorescent constructs and the control fluorescent protein mCherry, and fluorescence in the protoplasts was observed under a microscope. Interestingly, the two Pit fusion proteins showed different localizations: Pit WT-Venus accumulated mainly at the plasma membrane, whereas Venus-Pit WT was distributed diffusely throughout the cytoplasm (Fig. 1A). Next, we examined cell death and ROS production to determine whether Pit proteins fused N- or C-terminally to GFP are functional in defense responses (Fig. 1B). We previously reported that Pit D485V behaves as a constitutively active form of Pit and induces cell death as well as ROS production after agroinfiltration of *N. benthamiana* leaves (22). The C-terminal-fused fluorescent tag did not interfere with Pit activity because Pit D485V-GFP was able to efficiently induce cell death and ROS production. On the other hand, GFP-Pit D485V failed to trigger cell death. ROS production was observed in some leaves expressing GFP-Pit D485V, but the amount of ROS production induced by GFP-Pit D485V is much lower than that by Pit D485V-GFP, indicating that only C-terminal-tagged Pit is functional. There were no detectable effects on leaves expressing either N- or C-terminally-tagged Pit WT in the absence of pathogen effector. Based on these data, it is likely that

the localization of Pit is closely related to its function; thus, we next tried to identify factors contributing to Pit localization.

Palmitoylation Plays a Critical Role in the Membrane Localization of Pit—To gain a deeper understanding of the localization of Pit, we searched for features in its primary sequence using Myristoylator, CSS-Palm, etc. that are known to be associated with plasma membrane localization and found three potential residues for lipid modification in the N-terminal CC domain of Pit. Glycine 2 is a potential myristoylation site, and both cysteine 97 and 98 constitute a consensus sequence for palmitoylation. To examine the importance of these sites on Pit localization, we constructed alanine mutants of these sites and looked at their localization in rice protoplasts (Fig. 2A). Similar to Pit WT, the Pit G2A mutant was predominantly localized at the plasma membrane. On the other hand, plasma membrane localization was severely compromised in the Pit C97A C98A mutant, which was localized mainly in the cytoplasm and the nucleus. Although 70% of Pit WT- and G2A-expressing cells exhibited membrane localization, <30% of Pit C97A C98A-expressing cells showed membrane localization (Fig. 2, A and B). The combination of G2A and C97A C98A mutations had no additive effect on perturbation of plasma membrane localization, indicating that palmitoylation, rather than myristoylation, participates in the membrane localization of Pit. We could not detect the efficient membrane localization in Pit WT fused with the nuclear localization signal (NLS). It is known that some R proteins such as MLA10 and RPS4 are localized in the nucleus after pathogen effector recognition (6, 8). Thus, using the nuclear marker Cerulean-NLS, we examined whether Pit C97A C98A really exists in the nucleus. The fluorescence of Pit C97A C98A overlapped with that of Cerulean-NLS, showing that Pit C97A C98A occurs not only in the cytoplasm but also in the nucleus (Fig. 2C). Palmitoylation, a common lipid modification, generally involves covalent attachment of palmitic acid to cysteine residues. Palmitoylation promotes protein hydrophobicity and consequently membrane association and also contributes to the subcellular trafficking of proteins to specific compartments and to regulating protein-protein interactions (32, 33). We further tested the involvement of palmitoylation in Pit localization using the palmitoylation inhibitor 2-BP (Fig. 2, D and E). 2-BP treatment decreased the percentage of the membrane localization of Pit WT-Venus to 43%, and Pit WT-Venus was accordingly observed mainly in the cytoplasm and the nucleus. The intracellular distribution of Pit WT in the presence of 2-BP is thus similar to that of Pit C97A C98A. Taken together, these results indicate that Pit is a palmitoylated protein and that palmitoylation plays a critical role in the membrane localization of Pit.

Palmitoylation Contributes to Pit-mediated Disease Resistance to Rice Blast Fungus—To assess the importance of palmitoylation and the membrane localization of Pit in defense responses, we constructed new Pit mutants that combine the activating D485V mutation with C97A C98A or NLS and performed an agroinfiltration assay using *N. benthamiana* leaves (Fig. 3A). There were no detectable effects in leaves expressing Pit WT, C97A C98A, or NLS. Pit D485V strongly induced cell death, whereas this effect was abolished by the addition of the palmitoylation-deficient mutation or NLS, indicating that

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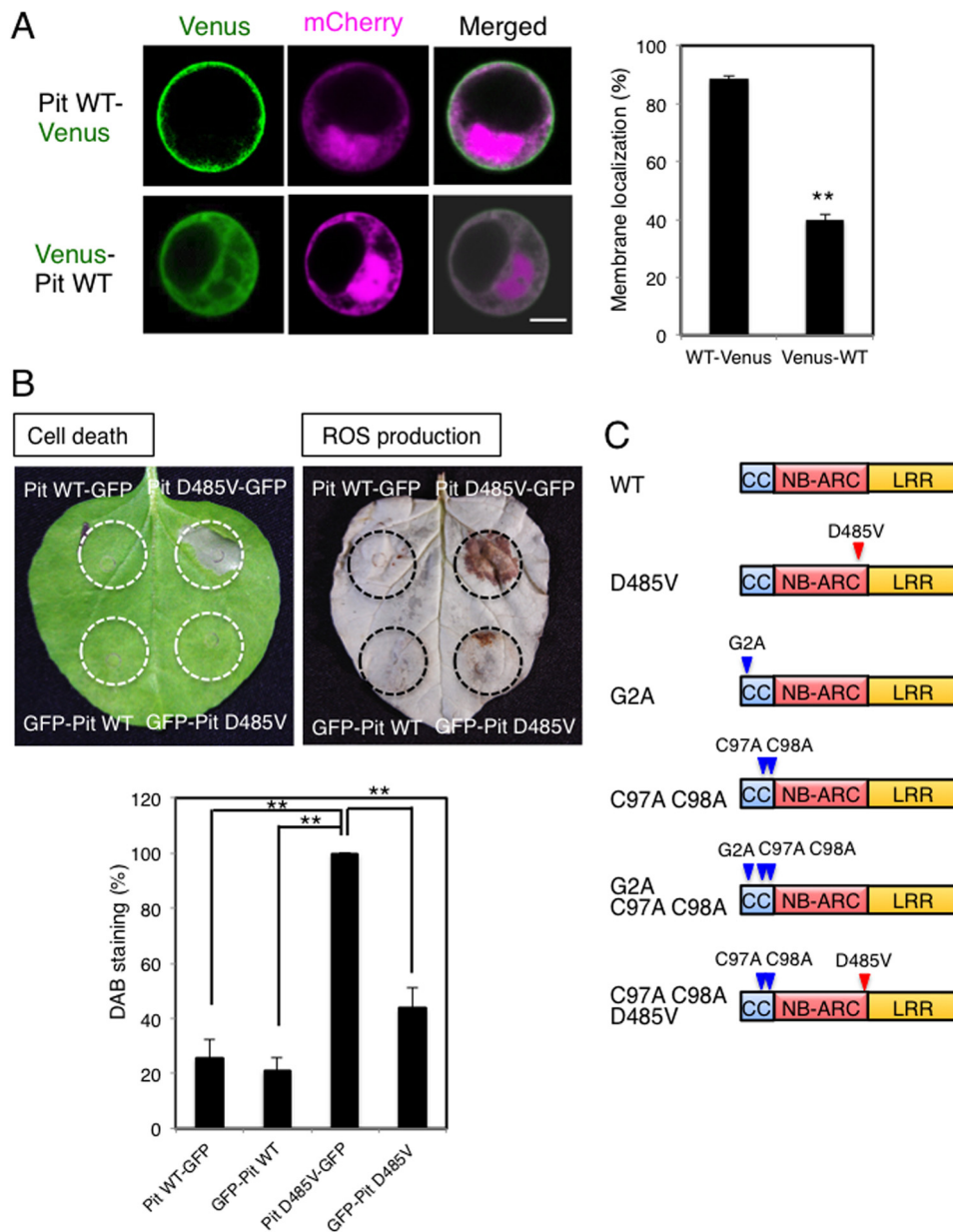


FIGURE 1. Localization of Pit is closely related to its function. *A*, localization of Pit in rice protoplasts. Rice protoplasts were co-transfected with the fluorescent constructs Pit WT-Venus and Venus-Pit WT (green) and the control fluorescent protein mCherry (magenta). The scale bar represents 5 μm . Error bars indicate S.E. ($n = 90$). Double asterisks indicates significant differences when compared with WT ($p < 0.01$). *B*, C-terminal-tagged Pit is functional. Cell death and ROS production in *N. benthamiana* leaves expressing various constructs of Pit, as indicated on the figure. Leaves were photographed at 7 dpi for cell death (right). ROS production at 3 dpi (left) was detected by DAB staining. Bars indicate the DAB staining intensity relative to that observed after infiltration with Pit D485V. The double asterisk indicates a significant difference ($p < 0.01$). Error bars indicate S.E. ($n = 4$). *C*, schematic representation of Pit mutants. Blue box, coiled-coil domain. Red box, nucleotide binding domain and APAF-1, certain R gene products, and CED-4 domain (NB-ARC). Yellow box, leucine-rich repeat. The arrowheads indicate the position of the mutation sites.

palmitoylation is required for Pit-induced immune responses. Consistent results were obtained when we tested for ROS production. This difference cannot be explained by different expression levels, because we compared the expression levels of Pit mutants in *N. benthamiana* leaves and found that the most active Pit D485V was the lowest expression among the Pit mutants tested (Fig. 3B). Interestingly, insertion of the activating mutation D485V into Pit significantly decreased its expression for an unknown reason. Next, we looked at the localization of these Pit mutants. We tried to determine the localization of Pit D485V-Venus in living cells, but we were unable to observe

Pit D485V-Venus fluorescence due to Pit D485V-induced cell death (data not shown). Pit C97A C98A D485V was distributed diffusely in both the cytoplasm and the nucleus, and Pit D485V-NLS was localized mainly in the nucleus and moderately in the cytoplasm (Fig. 3C).

To examine whether palmitoylation participates in Pit-mediated disease resistance against rice blast fungus, we generated transgenic plants of the susceptible rice cultivar Nipponbare (Nip) carrying the exogenous Pit resistance gene and chose the rice blast fungus *M. grisea* race 007.0 because Pit-dependent disease resistance has been established between Pit and *M. gri-*

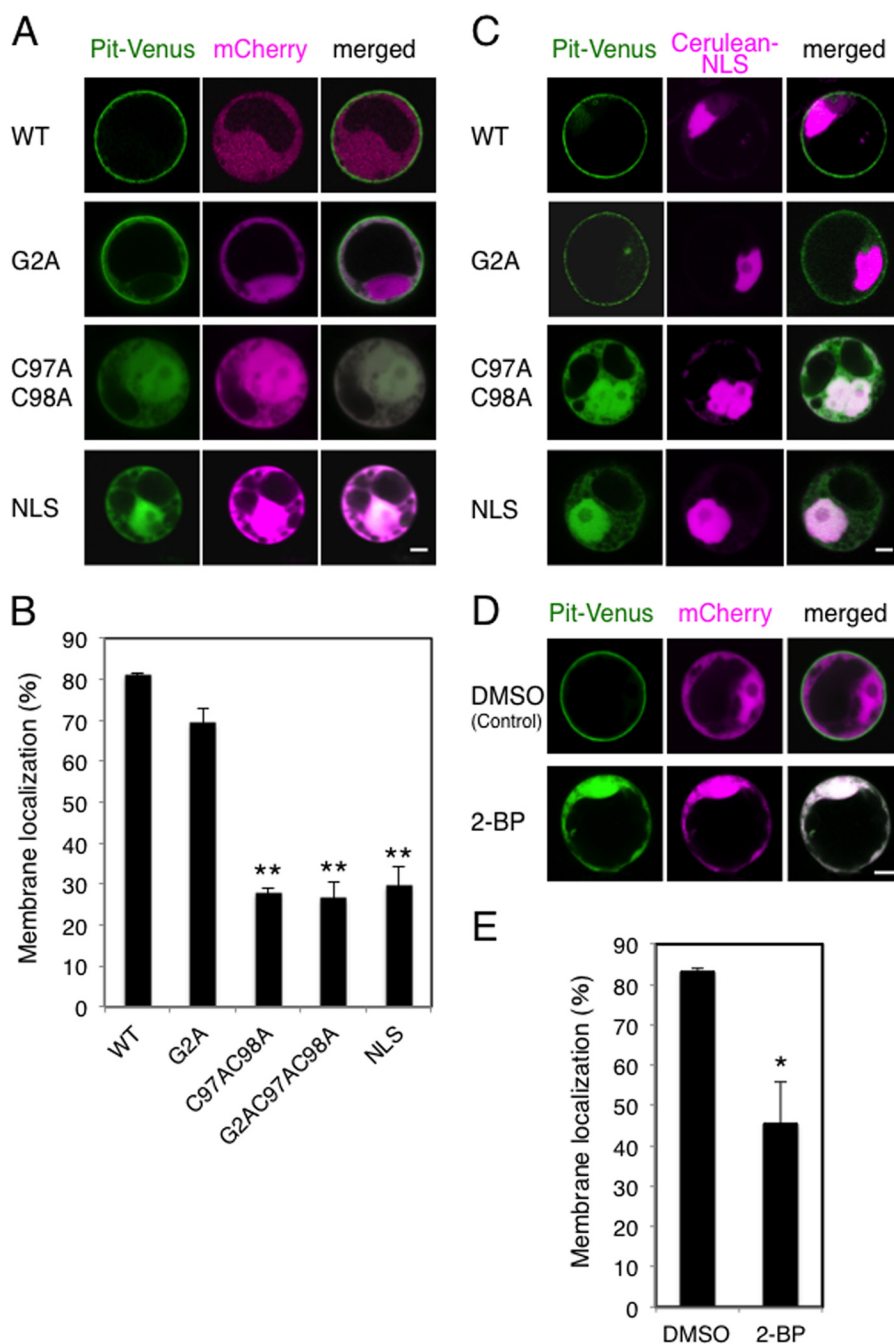


FIGURE 2. Palmitoylation is important for the membrane localization of Pit. *A* and *B*, localization of palmitoylation-deficient mutants of Pit. Rice protoplasts were co-transfected with the indicated fluorescent Pit-Venus constructs (green) and the control fluorescent protein mCherry (magenta). Error bars indicate S.E. ($n = 90$). Double asterisks indicate significant differences when compared with WT ($p < 0.01$). *C*, localization of palmitoylation-deficient Pit mutants and of the nuclear marker Cerulean-NLS. *D* and *E*, effect of the palmitoylation inhibitor 2-BP on the localization of Pit. Rice protoplasts expressing Pit WT-Venus were treated with $100 \mu\text{M}$ 2-BP. Error bars indicate S.E. ($n = 90$). Scale bars represent $5 \mu\text{m}$. Asterisks and double asterisks indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively).

sea race 007.0 (Fig. 4A) (28). There are no obvious differences in the expression of *OsRac1* between Pit WT and mutants (Fig. 4D). The lesion lengths caused by *M. grisea* race 007.0 in Nip expressing Pit WT were much shorter than those in Nip at 5 dpi (Fig. 4, A and B), and this Pit-dependent disease resistance was abolished by the addition of either the palmitoylation-deficient mutation or the NLS. To accurately measure fungal growth in plants, we used DNA-based real-time PCR to quantify *M. grisea* with two sets of specific primers against *M. grisea* *Pot2* and *O. sativa* ubiquitin (Fig. 4C). For DNA samples from infected

rice leaf blades, the real-time PCR analysis showed that the infection ratio ($MgPot2/OsUbq \times 100$) in Nip expressing Pit WT was remarkably lower than that in both Nip and Nip expressing either Pit C97AC98A or Pit-NLS, indicating that palmitoylation and membrane localization of Pit are required for Pit-mediated disease resistance against rice blast fungus.

Palmitoylation of Pit Is Important for the Interaction with and the Activation of OsRac1—Because OsRac1 binds to Pit at the plasma membrane in rice protoplasts (22), we examined the interaction between palmitoylation-deficient Pit and OsRac1

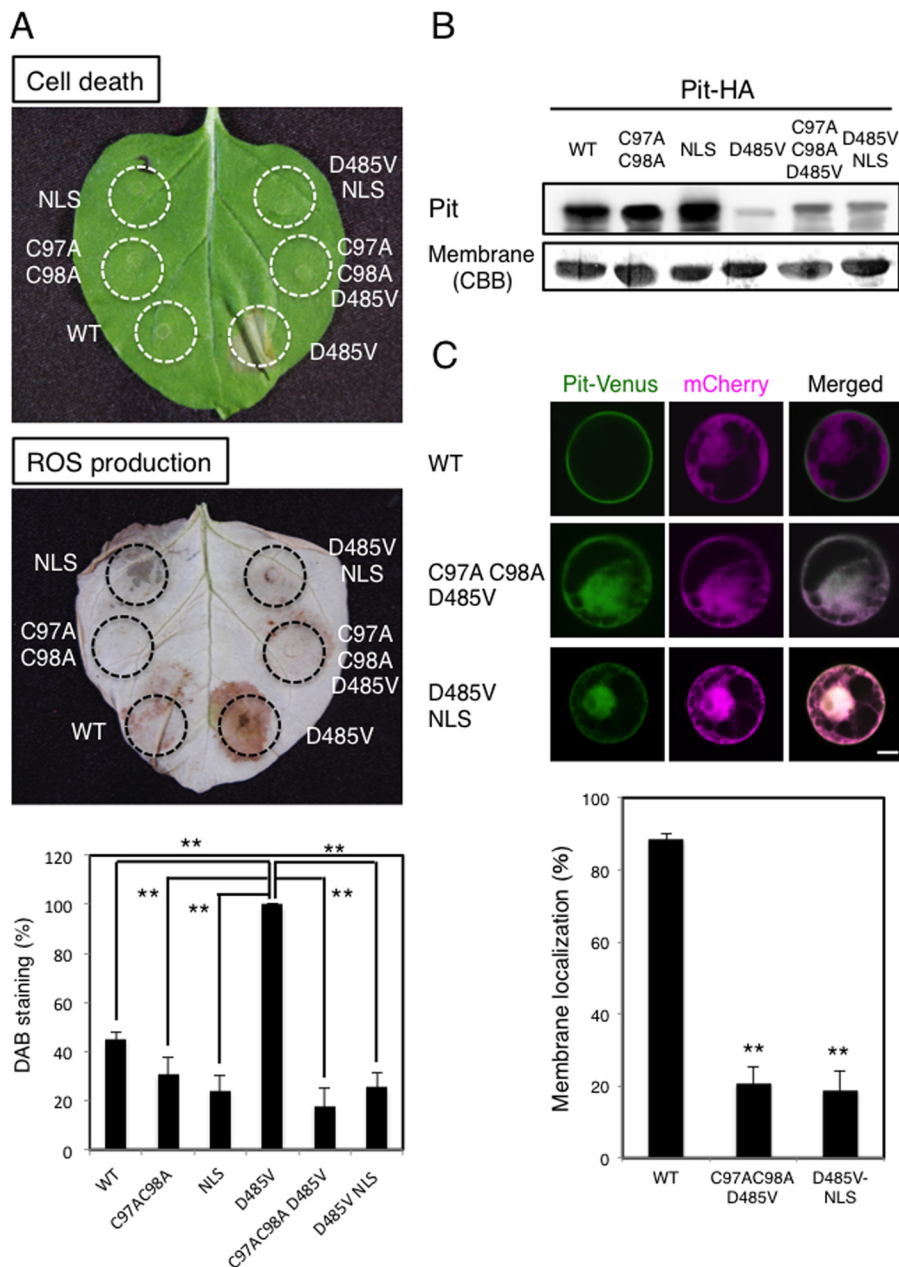


FIGURE 3. Palmitoylation is required for Pit-induced immune responses. *A*, effect of palmitoylation-deficient Pit mutants on immune responses. Cell death (*upper*) and ROS production (*middle*) in *N. benthamiana* leaves expressing various constructs of Pit, as indicated on the figure. Leaves were photographed at 7 dpi for cell death and at 3 dpi for ROS production. ROS production was detected by DAB staining. Bars indicate the DAB staining intensity relative to that observed after infiltration with Pit D485V. Double asterisks indicate a significant difference ($p < 0.01$). Error bars indicate S.E. ($n = 4$). *B*, detection of Pit proteins by immunoblot analysis. HA-tagged Pit mutants were transiently expressed in *N. benthamiana* leaves. At 2 dpi the infiltrated leaves were homogenized with sample buffer for SDS-PAGE. The resultant samples were immunoblotted with anti-HA antibody (*upper*). The transferred membrane was stained with Coomassie Brilliant Blue (CBB) to visualize Rubisco to check the amounts of loading samples (*lower*). *C*, localization of Pit mutants. Rice protoplasts were co-transfected with the indicated fluorescent Pit constructs (*green*) and the control fluorescent protein mCherry construct (*magenta*). The scale bar represents 5 μm . Error bars indicate S.E. ($n = 90$). Double asterisks indicates significant differences when compared with WT ($p < 0.01$).

using a BiFC assay. Pit WT associated with OsRac1 WT at the plasma membrane as reported previously, and this interaction was significantly decreased when Pit WT was replaced with the palmitoylation-deficient or the NLS mutant (Fig. 5A). Approximately 68% of the Pit WT-expressing cells formed BiFC complexes, whereas only 29% of Pit C97A C98A-expressing cells and 43% of Pit NLS-expressing cells demonstrated this effect (Fig. 5B). These results indicate that palmitoylation and membrane localization are important for the interaction between OsRac1 and Pit.

We previously established the FRET probe Raichu-OsRac1 for monitoring the activation of OsRac1 in living cells. Raichu-OsRac1 is composed of OsRac1, the CRIB motif of human PAK1, which binds specifically to the GTP-bound form of OsRac1, and YFP and CFP, as FRET donor and acceptor (Fig. 5C). Intramolecular binding of the active GTP-OsRac1 to CRIB brings CFP closer to YFP, enabling FRET from CFP to YFP to occur. The resulting YFP fluorescence provides an estimate of the activation state of OsRac1 *in vivo*, with low and high ratios of YFP/CFP fluorescence corresponding to low and high levels

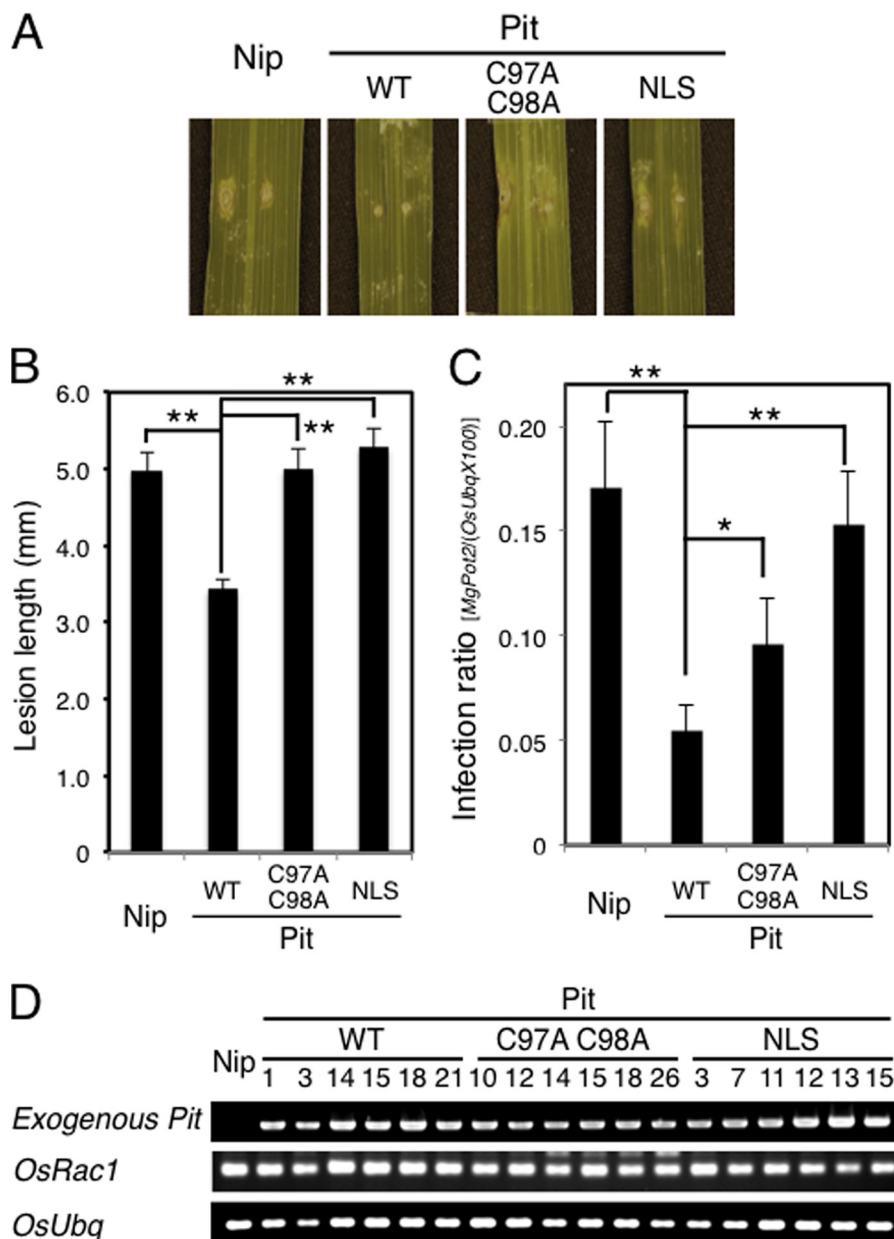


FIGURE 4. Palmitoylation contributes to Pit-mediated disease resistance to rice blast fungus. *A*, response of Nip expressing *Pit* mutants to infection with the incompatible *M. grisea* race 007.0. The photographs were taken at 5 dpi. *B*, quantitative analysis of lesion length induced by the blast fungus was performed at 5 dpi. Each data point represents the average and S.E. of about 72 infected areas. *C*, growth of *M. grisea* in Nip-expressing *Pit* mutants. The infection level of *M. grisea* race 007.0 at 6 dpi was evaluated by quantifying the numbers of the *O. sativa* ubiquitin and the *M. grisea* transposon *Pot2*. From these data, an infection ratio that represents the relative numbers of fungus cells and plant cells was calculated. Error bars indicate S.E. Asterisks and double asterisks indicate a significant difference from the control vector ($p < 0.05$ and $p < 0.01$, respectively). *D*, transcript levels of exogenous *Pit* measured by RT-PCR. The numbers indicate the line number of the transgenic plants.

of OsRac1 activation, respectively. Using Raichu-OsRac1, we previously found that Pit D485V activates OsRac1 at the plasma membrane in rice protoplasts (22). Thus, we tried to monitor the activation level of OsRac1 *in vivo* in the presence of Pit C97A C98A D485V using Raichu-OsRac1. The ratio of YFP/CFP fluorescence was higher in cells expressing Pit D485V than in cells expressing the control GUS vector (Fig. 5, *D* and *E*). Because activation signals were detected mainly at the plasma membrane, our results indicate that Pit D485V activates OsRac1 at the plasma membrane in rice protoplasts. In contrast, the ratios of YFP/CFP fluorescence in cells expressing Pit C97A/C98A/D485V or D485V NLS were comparable with

those in cells expressing the control vector, indicating that Pit C97A/C98A/D485V and Pit D485V NLS both fail to trigger the activation of OsRac1 at the plasma membrane. Taken together these results provide evidence that palmitoylation and membrane localization of Pit are involved in the activation of OsRac1 by Pit at the plasma membrane.

DISCUSSION

Palmitoylation Is a Key Factor for Membrane Localization and Functions of R Proteins—Known palmitoylation sites are classified into three clusters, Type I (sites display a -CC- pattern), Type II (-CXXC-), and Type III (other sites) (34). In this

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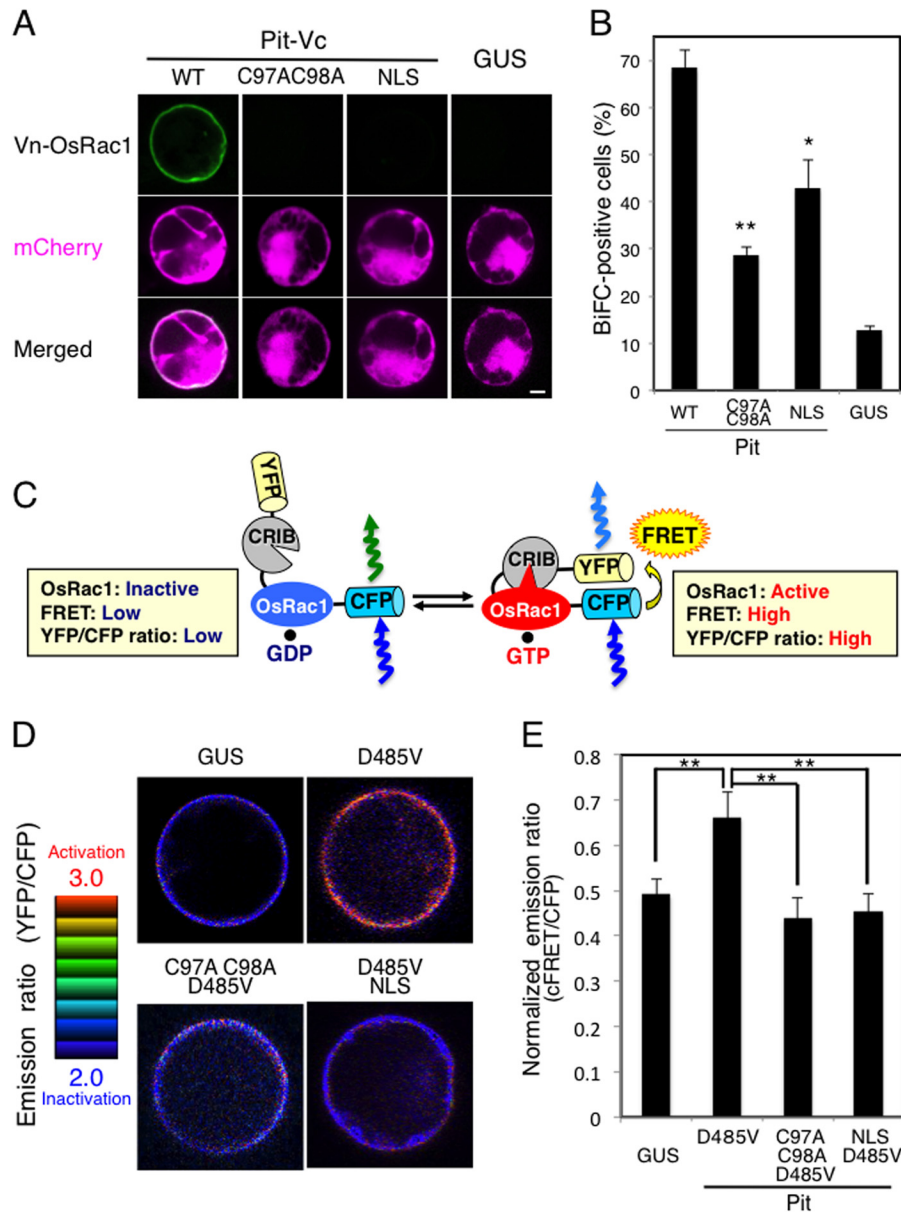


FIGURE 5. Palmitoylation is important for the interaction with and activation of OsRac1 by Pit. *A* and *B*, BiFC analysis of the interaction between OsRac1 and Pit mutants in rice protoplasts. OsRac1 tagged with the N-terminal fragment of Venus (Vn-OsRac1) and Pit tagged with the C-terminal fragment of Venus (Pit WT-Vc) were co-expressed in rice protoplasts. Venus fluorescence indicates an interaction between OsRac1 and Pit. Asterisks and double asterisks indicate significant differences from the data for Pit WT ($p < 0.05$ and $p < 0.01$, respectively). Error bars indicate S.E. ($n = 90$). *C*, schematic representation of Raichu-OsRac1 bound to GDP or GTP. *D* and *E*, emission ratio images of confocal laser-scanning micrographs of rice protoplasts expressing the Raichu-OsRac1 constructs. Rice protoplasts were co-transfected with constructs expressing Raichu-OsRac1 and individual Pit mutants. The FRET images are shown in intensity-modulated display mode, which associates color hue with emission ratio values and the intensity of each hue with the brightness of the source image. Asterisks and double asterisks indicate significant differences from the data for Pit D485V ($p < 0.05$ and $p < 0.01$, respectively). Error bars indicate S.E. ($n = 30$).

study we found that Pit has a predicted myristoylation site at glycine 2 and one pair of potential Type I palmitoylation sites at cysteines 97 and 98 in the N-terminal CC region. Alanine substitution of these predicted palmitoylation sites remarkably compromised both plasma membrane localization and Pit-induced resistance, whereas there was no obvious effect of Pit G2A on its membrane localization (Fig. 2). Because the combination of myristoylation- and palmitoylation-deficient mutations did not have an additive effect on perturbation of plasma membrane localization, palmitoylation rather than myristoylation is essential for the plasma membrane localization of Pit (Fig. 3). Supporting this idea, the results obtained with 2-BP, a

palmitoylation inhibitor, are consistent with those for the palmitoylation-deficient mutant of Pit (Fig. 2). Palmitoylation is the only reversible lipid modification of proteins and has thus unique features (32). Palmitoylation is catalyzed by DHHC (Asp-His-His-Cys) domain-containing protein S-acyl transferases, which are integral membrane proteins, whereas the reversal of palmitoylation is catalyzed by acyl protein thioesterases. Reversible palmitoylation is important for the cycling on and off membranes or in and out of microdomains, which has been observed in many palmitoylated proteins. The N-terminal-fused fluorescent tag inhibited the plasma membrane localization and immune responses of Pit (Fig. 1). This N-ter-

minal fluorescent tag might interfere with the interaction of palmitoylation enzyme toward the N-terminal CC domain of Pit and thereby repress Pit palmitoylation.

Evidence is accumulating that plasma membrane localization of R proteins plays a critical role in their function. Myristoylation and palmitoylation of R protein RPS5 have an additive effect on its localization and the induction of immune responses but do not contribute to pathogen effector recognition (35). Mutation of either myristoylation or the palmitoylation site alone reduces cell death and the accumulation of RPS5 at the plasma membrane, whereas the double mutant completely abolishes cell death induction and plasma membrane accumulation. Interestingly, the protein level of the double mutant is significantly reduced as compared with RPS5 WT, suggesting that lipid modification-dependent membrane localization enhances stabilization of RPS5 or avoids degradation of RPS5. The *Arabidopsis* resistance protein RPM1 is reported to be localized at the plasma membrane (36, 37), although its sequence appears to lack potential lipid modification sites. RPM1 interacts with a plant guard cell protein RIN4, which is localized to the membrane by palmitoylation (24). RPM1 recognizes modifications of RIN4 mediated by the bacterial effector proteins AvrRpm1 or AvrB and thus appears to associate indirectly with the plasma membrane through its interaction with RIN4 (38). The CC-NB-LRR protein RPS2 also interacts with RIN4 at the membrane, and the corresponding pathogen effector, AvrRpt2, is likely to be acylated and localized to the plasma membrane, implying that the activation of RPS2 initiates on the plasma membrane (37, 39). Effector-activated RPS2 is stable and localized to a microsomal compartment, although it is not clear whether this is actually the plasma membrane.

Why Is Pit Attached to the Plasma Membrane?—Recently, Bhattacharjee *et al.* (41) and Heidrich *et al.* (40) have provided important clues to the mystery of how the R protein RPS4 perceives pathogen effectors and activates downstream responses (40, 41). The nucleocytoplasmic basal resistance regulator EDS1 is indispensable for immunity mediated by the Toll/interleukin-1 receptor (TIR)-NB-LRR receptor RPS4. Although the intranuclear activities of RPS4 restrict bacterial growth, programmed cell death and transcriptional resistance reinforcement require nucleocytoplasmic coordination. Thus, the functions of the R proteins are closely associated with their subcellular distributions. RPM1 is a plasma membrane-localized R protein, and its autoactive mutant, which mimics the activated state of wild-type RPM1, remains localized to the plasma membrane (42). Further experiments showed that nuclear exclusion or direct membrane tethering does not compromise RPM1 activity, suggesting that the nuclear localization of RPM1 is not required for its function and that activated RPM1 remains on the plasma membrane and induces effector-triggered immunity.

Why is Pit attached to the plasma membrane? We have previously shown that OsRac1 is targeted to the plasma membrane through a C-terminal lipid modification (13). Here, we found that the failure of the palmitoylation-deficient Pit mutant to localize to the plasma membrane substantially reduced its interaction with OsRac1 and the activation of OsRac1 (Fig. 2 and 5), indicating that the palmitoylation-mediated plasma

membrane localization of Pit is required for the efficient activation of downstream molecules. Localization to the two-dimensional plasma membrane may effectively increase the local concentration of signaling proteins relative to their concentration in the three-dimensional cytosol to promote interactions of signaling molecules.

Plant NADPH oxidase, also known as RBOH, is an important generator of ROS, has six transmembrane domains, and localizes in the plasma membrane (43). Using *atrbohD* and *atrbohF* mutants, Torres and Dangl (44) have shown that NADPH oxidases may be important for R protein RPM1-mediated apoplastic ROS production. Rac/Rop family proteins directly interact with NADPH oxidases during effector-triggered immunity, MTI (microbe/pathogen-associated molecular pattern-triggered immunity), and root hair development (13, 20, 22, 45) and may thus be among the major regulators of NADPH oxidase activities (46). The dominant negative form of OsRac1 suppresses both Pit- and tobacco R protein N-induced ROS production (10, 21). The Raichu-OsRac1 probe revealed that Pit interacts with and activates OsRac1 at the plasma membrane where NADPH oxidases are located (Fig. 5). Therefore, it seems likely that OsRac1 acts as a signal transducer from Pit to NADPH oxidases at the plasma membrane during the oxidative burst. Overall, plasma membrane is a good platform for Pit to increase the local concentration of itself and downstream molecules such as OsRac1 and NADPH oxidases and to efficiently interact with and activate them.

Acknowledgments—We thank Dr. S. Takayama (Nara Institute of Science and Technology) for BiFC vectors and Dr. T. Nakagawa (Shimane University) for pGWB2, -5, and -6. We also appreciate the technical assistance of T. Aoi, Y. Konomi, J. Naritomi, and Y. Tamaki (Nara Institute of Science and Technology).

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