Hyperphosphorylation of a Mitochondrial Protein, Prohibitin, Is Induced by Calyculin A in a Rice Lesion-Mimic Mutant *cdr1*[1]

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The rice (*Oryza sativa*) lesion-mimic mutants, *cell death and resistance* (*cdr*), show spontaneous cell death on the entire leaf and exhibited significant resistance to the rice blast fungus. Our previous studies showed that *CDR1* and *CDR2* genes negatively regulated the phosphorylation steps leading to the activation of NADPH oxidase, which is associated with oxidative burst. To identify novel factors involved in the phosphorylation steps, the phosphorylation level of total proteins was compared between *cdr* mutants and wild type using two-dimensional gel electrophoresis. Here, we show that the phosphorylation level of four proteins in *cdr1* was increased as compared with the wild type after calyculin A treatment. Partial amino acid sequences revealed that one of the four proteins is homologous to prohibitin (PHB), which has been shown to be associated with senescence and cell death and to function as a chaperone in the assembly of mitochondrial respiratory chain complex in yeast and mammals. Analysis of green fluorescent protein fusions indicated that rice PHB (OsPHB1) was targeted to mitochondria as found in yeast and mammals, suggesting a possibility that PHB is involved in defense response and/or programmed cell death through the mitochondrial function.

Hypersensitive cell death is a major component of defense responses in plants against microbial attack and is associated with restricted pathogen (Dangl and Jones, 2001). The induction of hypersensitive cell death is often triggered by the interaction between race-specific disease resistance (*R*) genes of plants and corresponding avirulence (*Avr*) genes of microbes (Staskawicz et al., 2001). Many *R* genes have been isolated from various species and characterized in the past; however, molecular mechanisms of the signal transduction and regulation of the hypersensitive cell death still remain largely unknown.

Induction of the hypersensitive cell death requires the expression of concerned genes and synthesis of proteins de novo (Dixon et al., 1994; Godiard et al., 1994; He et al., 1994), indicating that the hypersensitive cell death belongs to programmed cell death (PCD). Some of the basic regulatory mechanisms of PCD involved in the response to pathogens have been shown to be conserved in animals and plants (Jacobson et al., 1997; Lam et al., 2001).

Apoptosis is a well-characterized form of PCD in animal cells (Jacobson et al., 1997). A variety of stimuli, including hormones, growth factors, UV irradiation, and reactive oxygen species (ROS), induce apoptotic cell death. Although the recognition of these stimuli is mediated by various receptor molecules, death signals converge on mitochondria to trigger activation of caspase and other molecules required for the execution of cell death (Green and Reed, 1998; Lam et al., 2001). Activation of the caspases requires the release of cytochrome c from mitochondria and the activation of Apaf-1 in the cytosol (Budihardjo et al., 1999). The mitochondrial permeability transition (MPT), which occurs in the inner membrane, causes release of cytochrome c and other activators of cell death such as apoptosis-inducing factor and Smac/Diablo (Susin et al., 1999; Verhagen et al., 2000). Release of various caspase-activating proteins from mitochondria is regulated by the Bcl-2 protein family, which is associated with the mitochondrial outer membrane (Green and Reed, 1998; Lam et al., 2001). This family consists of proapoptotic factors, Bax and Bak, and antiapoptotic factors, Bcl-2 and Bcl-x_L (Tsujimoto et al., 1984; Budihardjo et al., 1999). These studies indicate a central role for mitochondria in signal transduction of animal PCD (Green and Reed, 1998; Green, 2000).

Many of the cell death regulators found in animals are absent from the Arabidopsis genome, suggesting that plants may use other regulators to control this process (The Arabidopsis Genome Initiative, 2000). However, recent studies provide evidence for the conservation of certain regulatory mechanisms underlying PCD in animals and plants. Expression of the murine Bax triggered hypersensitive cell death in

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tobacco (*Nicotiana benthamiana*; Lacomme and Cruz, 1999), and introduction of Arabidopsis *Bax Inhibitor 1* cDNA into Bax-expressing plants caused suppression of cell death (Kawai-Yamada et al., 2001). In tobacco plants expressing mammalian Bcl-x_L, cell death induced by various signals was suppressed, suggesting that Bcl-x_L can function to suppress cell death in plants (Mitsuhara et al., 1999). Furthermore, cell death induced by harpin has been shown to be associated with inhibition of ATP synthesis (Xie and Chen, 2000). More recently, MPT has been implicated in victorin-induced cell death of oat (*Avena sativa*; Curtis and Wolpert, 2002) and cell death induced by oxidative stress in cultured Arabidopsis cells (Tiwari et al., 2002). In the latter case, depletion of ATP and ROS production in mitochondria were also observed. In cultured *Citrus sinensis* cells, MPT has been shown to be associated with nitric oxide-induced cell death (Savian et al., 2002). These results strongly suggest that the mitochondria may have a role in induction of PCD in plants; however, no direct evidence to support this hypothesis has been obtained.

Previously, we have isolated and characterized three lesion-mimic mutants of rice (*Oryza sativa*), designated *cell death and resistance* (*cdr1*, *cdr2*, and *Cdr3*), that exhibited resistance against rice blast fungus, *Magnaporthe grisea* (Takahashi et al., 1999). Transcripts of defense-related genes and antimicrobial compounds accumulated at higher levels in these mutants indicate that these mutations activate the defense-signaling pathway. Calyculin A, an inhibitor of protein phosphatase, induced higher accumulation of ROS in *cdr1* and *cdr2* than in wild type, suggesting that these mutants have alterations in phosphorylation steps leading to the oxidative burst. Because these mutants are thought to have misregulation in the signal transduction steps required for the hypersensitive cell death, they may provide useful tools in identifying the novel factors associated with induction of PCD (Takahashi et al., 1999).

Here, we show by using two-dimensional gel analyses and microsequencing that one of four proteins whose phosphorylation levels were increased in *cdr1* after treatment with calyculin A was prohibitin (PHB). PHB protein has been shown to function as a chaperone in the assembly of mitochondrial respiratory chain complex in yeast and mammalian cells. Rice PHB (OsPHB1) was localized to mitochondria. The result that phosphorylation of OsPHB1 was negatively regulated by *CDR1* suggests that OsPHB1 participates in PCD through mitochondrial function.

RESULTS

Detection of Phosphorylated Proteins after Treatment with Calyculin A

The results of our previous studies suggested that *cdr1* and *cdr2* mutants have alterations in the induction of spontaneous lesion formation and that *CDR1* and *CDR2* genes negatively regulate phosphorylation steps that are required for activation of NADPH oxidase (Takahashi et al., 1999). Therefore, it is possible that phosphorylation levels of certain proteins, which are probably required in the early signaling steps of defense responses upstream of hypersensitive cell death, are increased in the *cdr* mutants as compared with the wild type. To test this idea, we examined phosphorylated proteins using two-dimensional gel electrophoresis. Suspension cell cultures of the wild-type and the *cdr* mutants were incubated in a medium containing ^{32}P orthophosphate for 90 min, and then calyculin A was added in the medium to induce phosphorylation of proteins. Total proteins of the cultured cells were separated using two-dimensional gel electrophoresis, and phosphorylated proteins were detected by autoradiography. Approximately 20 new proteins were detected 30 min after treatment with calyculin A (Fig. 1A), indicating that calyculin A actually induced accumulation of certain phosphorylated proteins in vivo in rice cells. The phosphorylation level of each spot was calculated based on the phosphorylation levels of three separate control spots (Fig. 1B). Comparison of the protein phosphorylation level of the *cdr* mutants with that of the wild type 30 min after calyculin A treatment revealed that four proteins showed higher phosphorylation levels in the *cdr* mutants than in the wild type (Fig. 1, B–D). Spot 1 was phosphorylated strongly in the *cdr1* mutant, but not in the *cdr2* mutant, and its phosphorylation level in *cdr1* was approximately 3-fold higher than that in the wild type. Spot 2, spot 3, and spot 4 were phosphorylated 2- to 3-fold higher in *cdr1* and *cdr2* than in the wild type (Fig. 1D). The enhanced phosphorylation of these four spots in the *cdr* mutants was confirmed in at least three independent experiments. Thus, phosphorylation of four spots was *CDR* gene dependent.

Identification of Highly Phosphorylated Proteins in *cdr* Mutants

To obtain more insights into these phosphorylated proteins, the corresponding spots were isolated from undried gels, digested in the gel with a protease, and sequenced by a peptide sequencer. The database search using the BLAST program revealed that the partial amino acid sequence from spot 1 was part of PHB protein in tobacco (*Nicotiana tabacum*) and Arabidopsis (Fig. 2A). The *PHB* gene was originally identified as a proto-oncogene isolated from rat liver and a negative regulator of cell division in animal cells (McClung et al., 1989; Nuell et al., 1991). PHB protein has been shown to be highly conserved in eukaryotes and its function has been ascribed to various functions such as inhibition of cell cycle, senescence, determination of life span, and apoptosis in diverse organisms and cell types (Dell'Orco et al., 1996; Coates et al., 1997; Welburn and Murphy, 1998).

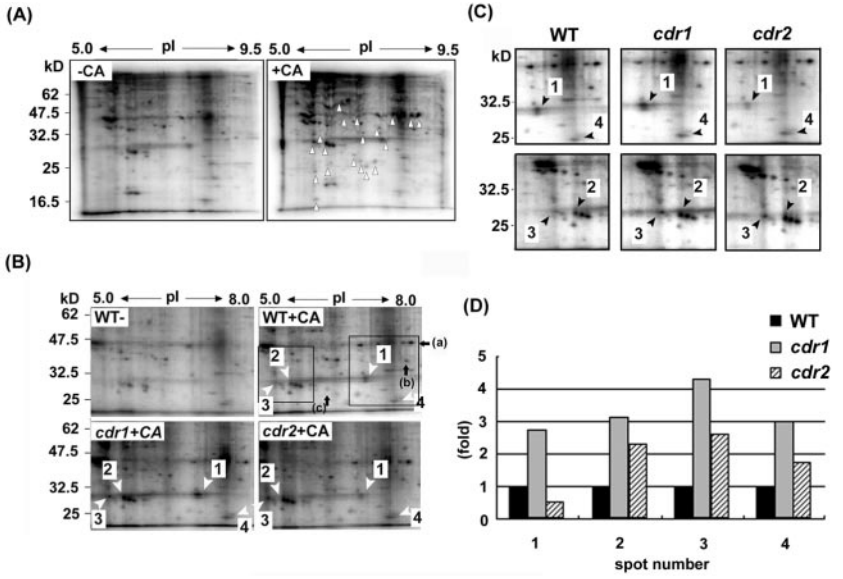

Figure 1. Comparison of protein phosphorylation levels between the wild-type and the *cdr* mutants. A, Phosphoimage of radiolabeled total proteins extracted from the wild-type cells 30 min after treatment with (+CA) or without (-CA) calyculin A. Proteins that were phosphorylated de novo after treatment of calyculin A are indicated by triangles. B, The protein phosphorylation level of the *cdr* mutants was compared with the wild type 30 min after calyculin A treatment. Arrowheads indicate the protein spots that were phosphorylated in the *cdr* mutants with the higher degree in the wild type. Arrows indicate the phosphorylated protein spots (a, b, and c), which were used as the control to standardize magnitude of spots among the different gels. These control spots were not affected by the treatment of calyculin A. C, Close-up of four protein spots. D, The phosphorylation level of spots 1 through 4 relative to the wild type. Spot 1 was highly phosphorylated in the *cdr1* mutant alone. The other three spots were phosphorylated higher in *cdr1* and *cdr2* than in the wild type.

However, recent studies mainly with mammalian and yeast PHB proteins suggest that a primary role of PHB protein is to function as a chaperone in the mitochondrial inner membrane (for review, see Nijtmans et al., 2002). Although homologs of the *PHB* gene have been found in Arabidopsis, tobacco, and maize, their roles in plants are not known (Snedden and Fromm, 1997; Nadimpalli et al., 2000).

We isolated full-length *PHB* cDNA from rice using the tobacco *PHB* sequence as a probe and determined its complete nucleotide sequence (Fig. 2A). It was designated OsPHB1. The predicted *OsPHB1* gene en-

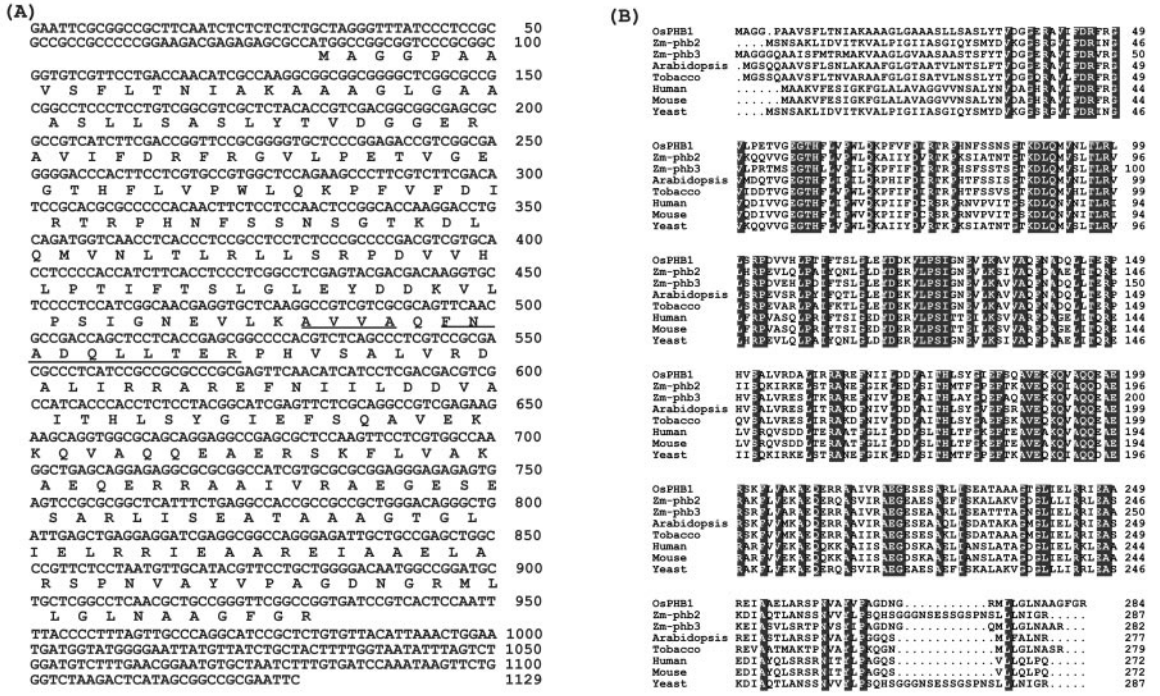

Figure 2. PHB is highly conserved among plants, animals, and yeast. A, Full-lengths of cDNA and deduced amino acid sequence of the *OsPHB1* gene are shown. Underlining indicates amino acid sequences that were derived from peptide sequence from spot 1 protein. B, Alignment of the predicted amino acid sequences of OsPHB1 protein with PHB proteins from various species. The accession numbers for Zm-phb2 and Zm-phb3 maize (*Zea mays*) are AF236369 and AF236370, respectively. The accession numbers for *PHB* cDNAs of Arabidopsis, tobacco, human, mouse, and yeast are NP_198893, AAC49690, AAB21614, NP_032857, and NP_011648, respectively.

codes 284 amino acid resides and the deduced M_r was 30.6 kD. Figure 2B represents the amino acid sequence alignment of deduced PHB proteins from various eukaryotes and revealed a strong conservation of the PHB proteins from yeast to animals and plants. The similarities of OsPHB1 to maize Zm-phb2 and Zm-phb3 are 95% and 81%, respectively. In addition, it shares homology with Arabidopsis PHB (76%), tobacco PHB (79%), yeast (56%), and human and mouse (about 50%).

OsPHB1 Protein Is Phosphorylated in Response to Calyculin A

To test whether *OsPHB1* actually encodes spot 1 protein and the OsPHB1 protein is phosphorylated by calyculin A treatment, we made an antibody against OsPHB1 and performed protein gel-blot analysis. Suspension-cultured cells of the wild type were radiolabeled with ^{32}P orthophospate and were treated with calyculin A to induce protein phosphorylation. The total protein was separated by two-dimensional gel electrophoresis and the OsPHB1 protein was detected by using the specific anti-OsPHB1 antibody. The position of the OsPHB1 signal detected by autoradiography was likely to coincide with that of OsPHB1 detected by immunoblotting using the antibody (Fig. 3A, arrowhead). We performed immunoprecipitation experiments using the same protein sample. The anti-OsPHB1 antibody precipitated a phosphorylated protein of the expected size confirmed by protein gel blotting (Fig. 3B), suggesting that OsPHB1 was most likely phosphorylated. Interestingly, the antibody detected several other signals of the same M_r but with different pIs (Fig. 3A). These results indicated that phosphorylation is one of modifications for OsPHB1 and it may be posttranslationally modified by other mechanisms.

To confirm that the intensity of phosphorylated signal of OsPHB1 was dependent upon the phosphorylation level but not the protein level, we compared the protein levels of OsPHB1 between the wild type and *cdr1* after calyculin A treatment. The calyculin A treatment had no effects on the mRNA and protein levels of OsPHB1 in both the wild type and *cdr1* (Fig. 3, C and D), indicating that the calyculin A enhanced phosphorylation of OsPHB1 in *cdr1* possibly through inhibition of protein phosphatase activity for OsPHB1, or activation of protein kinase for OsPHB1.

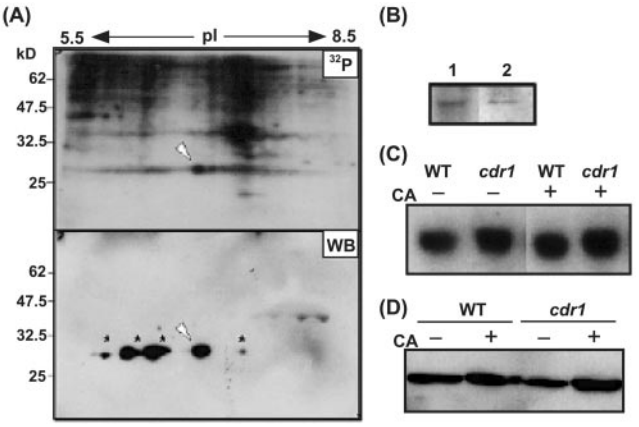

Figure 3. OsPHB1 protein was phosphorylated after calyculin A treatment. A, Total proteins extracted from the wild-type cells that were labeled with [^{32}P] for 30 min after calyculin A treatment were separated by two-dimensional gel electrophoresis. Protein phosphorylation was detected by autoradiography (top: ^{32}P) and the OsPHB1 protein was detected by anti-OsPHB1 antibody (bottom: WB). Arrowheads indicate the protein spot at the same position on the SDS-PAGE gel. Asterisks indicate OsPHB1 proteins with different pIs detected by anti-OsPHB1 antibody. B, Immunoprecipitation of proteins from suspension cell cultures labeled with ^{32}P at 30 min after calyculin A treatment. Lane 1, A radioactive image; lane 2, protein blot detection of immunoprecipitated proteins using anti-OsPHB1 antibody. C, The mRNA levels of *OsPHB1* in *cdr1* mutant. Total RNAs prepared from the wild type and *cdr1* after treatment with or without calyculin A were subjected to RNA-blot analysis using the *OsPHB1*-specific probe. D, Protein gel-blot analysis of OsPHB1 protein. Total proteins extracted from the wild type and *cdr1* after treatment with or without calyculin A were separated by SDS-PAGE. The signals were detected by the anti-OsPHB1 antibody.

OsPHB1 Protein Forms a Complex in Vivo

It has been reported that PHB protein makes a high-molecular-mass complex of approximately 1 MDa in vivo in yeast and human (Berger and Yafee, 1998; Steglich et al., 1999; Nijtmans et al., 2000). To investigate whether OsPHB1 also makes a complex in vivo, we performed native PAGE gel electrophoresis using proteins prepared from suspension cell cultures of the wild type, *cdr1*, and *cdr2*. In all samples, the anti-OsPHB1 antibody detected three bands of approximately 30, 45, and 60 kD (Fig. 4A), although no huge complex was detected in our experimental condition. The three bands were observed in the presence and absence of calyculin A. Because the M_r of the predicted OsPHB1 protein was 30.6 kD, OsPHB1 protein seemed to form a complex with other proteins or dimers in the case of the 60-kD protein. To examine whether OsPHB1 forms dimers in vivo, a carboxyl-terminal c-myc-tagged OsPHB1 was introduced into the wild-type rice cells, and coimmunoprecipitation experiments were performed using anti-c-myc antibody. As a result, we detected two bands, 30 and 34 kD, by protein gel blotting using the anti-OsPHB1 antibody (Fig. 4B). The band of 34 kD was likely to be the size of a fusion protein of c-myc-tag and OsPHB1 because it was also detected by anti-c-myc antibody. This result indicated that c-myc-tagged OsPHB1 could interact with the endogenous OsPHB1 protein, suggesting that OsPHB1 forms dimers in vivo.

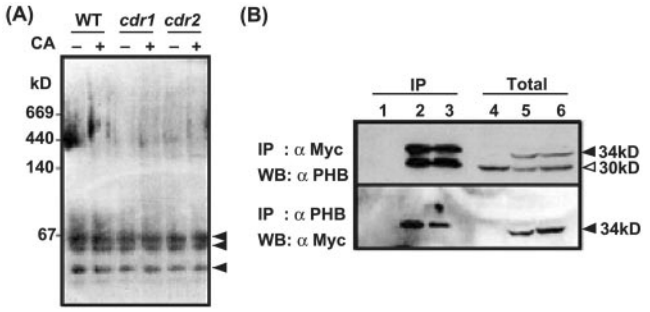

Figure 4. Identification of the OsPHB1 complex. A, Protein gel-blot analysis of the OsPHB1 protein. Total proteins extracted from the wild type, *cdr1*, and *cdr2* after treatment with or without calyculin A were separated by native-gel electrophoresis. The signals were detected by the anti-OsPHB1 antibody. B, Coimmunoprecipitation of c-myc-tagged OsPHB1 with endogenous OsPHB1. Extracts from control cell line (lanes 1 and 4), UPmm3 (lanes 2 and 5), and UPmm4 (lanes 3 and 6) were immunoprecipitated with anti-myc antibody (top panel) or anti-OsPHB1 antibody (bottom panel). The immunoprecipitated proteins (lanes 1–3) and total extracts (lanes 4–6) were separated by SDS-PAGE and analyzed by immunoblotting with anti-PHB (top panel) or anti-myc (bottom panel) antibodies. UPmm3 and UPmm4 are transgenic rice cell lines expressing the C-terminal c-myc-tagged OsPHB1. Transgenic cell overexpressing OsPHB1 without any tag was used as a control.

OsPHB1 Protein Is Localized in Mitochondria

Although various functions of PHB have been studied in animals, molecular mechanisms of its functions are still unclear. However, an important clue for the function of PHB comes from its mitochondrial localization. The PHB protein has been shown to localize in the inner membrane of mitochondria and is concerned with regulation of cell death through the mitochondrial function (Ikonen et al., 1995; Coates et al., 1997; Snedden and Fromm, 1997). In plants, PHB was also detected in the mitochondria-rich fraction (Snedden and Fromm, 1997). Recent studies on the proteome analysis of mitochondrial proteins in Arabidopsis identified the PHB protein as a mitochondrial protein (Kruft et al., 2001; Millar et al., 2001).

To examine whether OsPHB1 is also localized in mitochondria, we made green fluorescent protein (GFP) fusion protein connected to the C terminus of OsPHB1 (OsPHB1::GFP) and transiently introduced it into onion (*Allium cepa*) epidermal cells. Expression and localization of GFP protein were observed by a confocal microscope 12 h after bombardment. The signal of control GFP protein was detected in the cytosol and the nucleus (Fig. 5B). In contrast, the signal of the OsPHB1::GFP showed speckled localization (Fig. 5E), which overlapped with mitochondria stained by Mitotracker (Molecular Probes, Eugene, OR; Fig. 5, D and F). This suggests that OsPHB1 is associated with mitochondria as previously observed in animals and yeast. Although OsPHB1 was shown to be localized in mitochondria, the signal sequence for mitochondrial transport was not found in the OsPHB1 amino acid sequences. It has been reported that a short sequence of hydrophobic amino acids in the N terminus of PHB plays an important role for transport to mitochondria in rat (Ikonen et al., 1995). Although amino acid sequences in the N terminus of PHB were not highly conserved between animals and plants (Fig. 2B), the hydrophobic plot indicated the existence of the hydrophobic sequence in the N terminus of OsPHB1 (data not shown). To examine whether this hydrophobic sequence functions as a signal sequence for mitochondrial localization of OsPHB1, GFP protein was connected to the N terminus of OsPHB1 (GFP::OsPHB1). In this case, N-terminal GFP sequence was predicted to hide the hydrophobic sequence of OsPHB1. The fusion of GFP sequence to

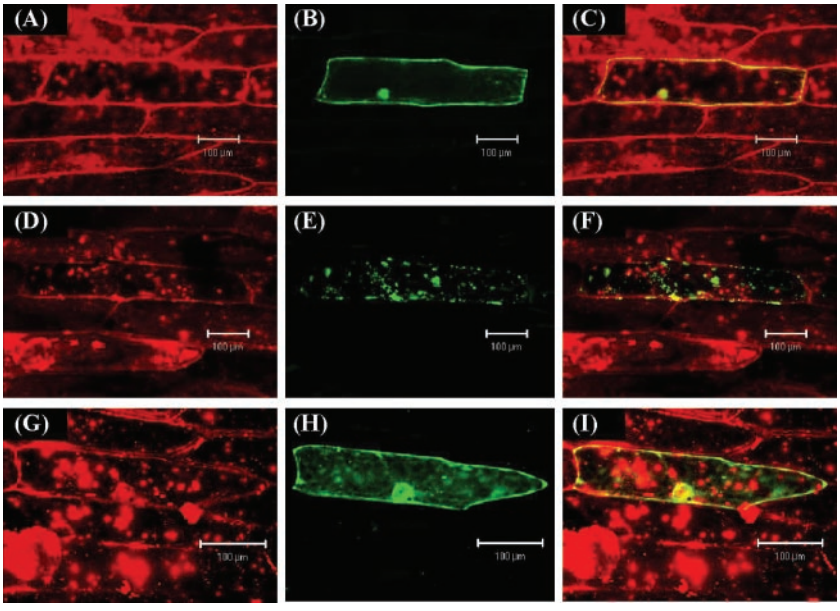

Figure 5. Mitochondrial localization of OsPHB1 protein in onion epidermal cells. Constructs carrying 35S::GFP (A–C), 35S::OsPHB1-GFP (D–F), or 35S::GFP-OsPHB1 (G–I) were bombarded into onion skin epidermal cells. Expression and localization of GFP signal was observed 12 h after bombardment. Mitochondria were specifically stained with MitoTracker Red (A, D, and G). C, F, and I, The merged images of A and B, D and E, and G and H, respectively.

the N terminus of OsPHB1 inhibited localization of OsPHB1 in mitochondria (Fig. 5, H and I), suggesting that the hydrophobic sequence of the N terminus may be important to target OsPHB1 into mitochondria.

DISCUSSION

Use of Calyculin A to Identify Hyperphosphorylated Proteins in *cdr* Mutant Cell Cultures

In this work, we used calyculin A as an inducer of defense responses in rice cells. Calyculin A is an inhibitor of protein phosphatase type 1 and type 2, and the responses induced by calyculin A treatment have been shown to be similar to those stimulated by elicitors in suspension cultured cells of various species (Felix et al., 1994; Lecourieux-Ouaked et al., 2000; Stratmann et al., 2000).

We identified four proteins that were phosphorylated with the higher degree in the *cdr* mutants than in the wild type after calyculin A treatment (Fig. 1). One of the four proteins, spot 1, was phosphorylated strongly in the only *cdr1* mutant, suggesting that it may be regulated by the *CDR1* gene but not by the *CDR2* gene. On the other hand, spot 2, spot 3, and spot 4 proteins were phosphorylated with higher degrees in *cdr1* and *cdr2* than in the wild type. Therefore, they may be regulated differently from OsPHB1. It was previously shown that *cdr1* and *cdr2* mutations lie in the signaling cascade leading to activation of NADPH oxidase and spontaneous lesion formation through the protein phosphorylation/dephosphorylation reaction (Takahashi et al., 1999). Our results suggest that the phosphorylation of these proteins may be associated with induction of the NADPH oxidase activation and/or PCD.

PHB Protein Is Conserved among Yeast, Human, and Plants

The peptide sequence analysis revealed that spot 1 was OsPHB1 protein (Fig. 2A). The amino acid sequences of PHB were highly conserved among various eukaryotes (Fig. 2B). In human and yeast, PHB has been shown to form a complex with PHB-related proteins, BAP37 and Phb2, respectively (Berger and Yafee, 1998; Steglich et al., 1999; Nijtmans et al., 2000). In the screening of *OsPHB*1 cDNA, we isolated a cDNA that was highly homologous with *OsPHB1*. This cDNA encodes a protein of 31.9 kD with different pI (pI 10.28) than OsPHB1 (pI 6.65), and its deduced amino acid sequence showed high homologies with BAP37 and Phb2, and 46% identity to OsPHB1 (data not shown). Although a huge protein complex containing PHB found in yeast was not detected in our experiments, results of native PAGE and coimmunoprecipitation analysis suggested that the OsPHB1 protein forms dimers in vivo (Fig. 4, A and B). It is possible that OsPHB1 also forms dimers with PHB-related proteins. The highly conserved amino acid sequence and complex formation suggest that PHB may have similar functions in plants as in animals and yeast.

The mRNA and protein levels of PHB were almost equal between the wild-type and *cdr* mutants even after calyculin A treatment (Fig. 3, C and D). It was reported that the PHB mRNA was increased in *Trypanosoma brucei* after induction of apoptosis by concanavalin A treatment (Welburn and Murphy, 1998). *PHB* overexpression in *Saccharomyces cerevisiae* showed the reduction of the average life span (Coates et al., 1997). These results might suggest that quantitative control is important for the regulation of PHB functions. However, it was also reported that there was no difference in the *PHB* mRNA levels between young and old Rat1 cells and that PHB protein made multiple forms in two-dimensional gel electrophoresis, suggesting that it was posttranslationally modified (Roskams et al., 1993; Dell'Orco et al., 1996). Our observation that several OsPHB1 signals were detected after two-dimensional gel electrophoresis indicated that it is also posttranslationally modified (Fig. 3A). Phosphorylation of OsPHB1 protein induced by calyculin A was only observed in one of several protein spots detected by immunoblotting using anti-OsPHB1 antibody, suggesting that OsPHB1 protein may be regulated by other modifications than phosphorylation.

Possible Function of PHB Protein in Rice

As a purpose to examine whether OsPHB1 is involved in disease resistance and induction of PCD, we introduced the *OsPHB1* cDNA driven by the ubiquitin promoter into rice cv Kinmaze by *Agrobacterium tumefaciens*-mediated transformation to produce transgenic plants overexpressing OsPHB1. We also tried to suppress *OsPHB1* expression by RNAi by introducing vector constructs transcribing double-strand *OsPHB1* RNA into rice. We produced approximately 50 independent transformed calli for each construct. However, we had difficulties regenerating plants from transformed calli (data not shown). The observed low frequency of plant regeneration from *OsPHB1*-transformed calli may be caused by the property of *OsPHB1* gene; overexpression and reduction of OsPHB1 may be detrimental to plant regeneration from callus, suggesting that OsPHB1 may be associated with cell viability.

Our analysis revealed that the OsPHB1 protein was localized in the mitochondria as seen in the yeast and animal (Fig. 5), suggesting that PHB function is also correlated with mitochondria in rice. Phosphorylation of OsPHB1 was stimulated in *cdr1* by calyculin A, which can trigger defense-related responses in rice, suggesting that *CDR1* negatively regulates phosphorylation of OsPHB1. Thus, OsPHB1 phosphorylation may be involved in disease resistance and PCD, coincident with the observation found in the *cdr1* mutant (Takahashi et al., 1999).

How could OsPHB1 function in disease resistance and PCD in plants? Two possible scenarios can be envisioned. First, because PHB has been shown to function as a chaperone in the assembly of subunits of mitochondrial respiratory chain complex (Nijtmans et al., 2002), phosphorylation of PHB may cause disruption of homeostasis of the proteins in the mitochondrial inner membrane and it may lead to MPT. MPT has been recently shown to be associated with victorin-induced cell death of oat (Curtis and Wolpert, 2002) and cell death induced by oxidative stress in cultured Arabidopsis cells (Tiwari et al., 2002). In these two cases of plant PCD, cytochrome c was shown to be released from mitochondria to the cytosol, although its role in plant PCD remains to be studied. In the case of harpin-induced PCD of tobacco, inhibition of ATP synthesis has been observed (Xie and Chen, 2000). MPT has been shown to be associated with nitric oxide-induced cell death in cultured *Citrus sinensis* cells (Savian et al., 2002). These recent studies strongly suggest the importance of the integrity of the mitochondrial inner membrane proteins and some parallels between animal and plant PCD. Because PHB protein is considered to function to protect the inner membrane proteins, modifications of their properties or their complexes with other proteins may possibly lead to PCD through the function of MPT.

A second scenario originates from the observation that Phb1, Phb2, and mitochondrial-AAA protease form a large protein complex in yeast mitochondria, and that in the *Phb*-deficient mutants, degradation of mitochondrial proteins was accelerated by mitochondrial-AAA protease (Steglich et al., 1999). AAA protease is used for degradation of the membrane proteins of mitochondria (Leonhard et al., 2000). The mRNA level of THE tobacco DS9 gene that encodes a chloroplastic homolog of the bacterial AAA protease, FtsH, was decreased after tobacco mosaic virus inoculation in tobacco carrying the *N* gene. And transgenic tobacco plants in which DS9 was constitutively repressed by the antisense gene showed hypersensitive cell death more quickly than the nontransgenic plants (Seo et al., 2000), suggesting that the decreased expression of chloroplast AAA protease activity leads to accelerated PCD in plants. Recent Arabidopsis DNA database information identified at least one FtsH-like protease in the mitochondria (Adam et al., 2001), therefore, it is possible that PHB protein regulates activity of FtsH-like proteases in mitochondria. When regulation of FtsH-like proteases in mitochondria is disturbed by phosphorylated PHB proteins, it may lead to PCD, possibly through MPT of the inner membrane. This is the main pathway of PCD in animal cells (Green and Reed, 1998; Lam et al., 2001).

Until now, no mitochondrial proteins required for the induction of cell death or disease resistance have been reported in plants. The PHB protein may play an important role in induction of plant hypersensitive cell death through the mitochondrial function.

MATERIALS AND METHODS

Labeling and Two-Dimensional Separation of Proteins

Cell suspension cultures of the *cdr* mutants and the wild type (cv Kinmaze) were grown in 20 mL of R2 liquid medium (Ohira et al., 1973) on a rotary shaker at 30°C. For ^{32}P labeling, suspension cultures were incubated in a medium containing 0.25 mCi ^{32}P orthophosphate at 30°C for 90 min. Calyculin A (1.0 μM) was added in the medium and the cell cultures were incubated for an additional 30 min. After labeling, total protein was extracted with SDS buffer (4% [w/v] SDS, 2% [v/v] mercaptoethanol, 20% [v/v] glycerol, 2 mM PMSF, and 100 mM Tris-HCl, pH 8.5). After centrifugation, trichloroacetic acid was added in the supernatant to bring the trichloroacetic acid concentration to 10% (w/v). The mixture was incubated on ice for 10 min and was centrifuged at 4°C for 10 min. The pellet was resuspended with distilled water, precipitated by acetone to a final concentration of 80% (v/v) at −30°C for 1 h, and then centrifuged. The final protein pellet was resuspended in lysis buffer (9.5 M urea, 2% [v/v] Nonidet P-40, 5% [v/v] mercaptoethanol, and 2% [w/v] ampholines [50% pH 5–7 and 50% pH 3–10]). Two-dimensional gel electrophoresis was performed as described by Matsumoto and Pak (1984). Phosphorylation of proteins was detected by BAS2000 (Fujifilm; Fuji, Tokyo) and quantification of phosphorylation level of each spot was calculated using MacBas software (Fujifilm; Fuji).

Identification of Phosphorylated Proteins

The proteins of interest were identified by staining with Coomassie Brilliant Blue, and they were excised, cleaved with achromobacter protease I in gel, and applied to a gas-phase protein sequencer (model 477A; Perkin-Elmer Applied Biosystems, Foster City, CA).

Isolation of *OsPHB1* cDNA

To isolate *OsPHB1* cDNA from rice (*Oryza sativa*), the *PHB* cDNAs were amplified by PCR from tobacco (*Nicotiana tabacum*) leaf cDNAs using specific primers synthesized based on their nucleotide sequence (accession no. U69154). The *OsPHB1* cDNAs were derived from a rice cDNA library from young panicles (Kyozuka et al., 1998) using *PHB* cDNA of tobacco as a probe.

RNA Isolation and RNA Gel-Blot Analysis

Total cellular RNA was prepared from leaves as described previously (Takahashi et al., 1999). The RNA samples (20 μg lane^{-1}) were separated on a 1% (v/v) formaldehyde gel and were blotted onto nylon membranes (Hybond-N; Amersham Pharmacia Biotech, Piscataway, NJ). Probes were labeled with [α-^{32}P] dCTP using a *Bca*BEST Labeling kit (TaKaRa, Otsu, Japan). Hybridizations with ^{32}P-labeled probes were carried out in hybridization buffer (5× sodium chloride/sodium phosphate/EDTA, 0.1% [w/v] Ficoll, 0.1% [w/v] bovine serum albumin, 0.1% [w/v] polyvinylpyrrolidone, and 0.5% [w/v] SDS) for 16 h at 65°C. The membranes were washed in 0.1× SSC and 0.2% (w/v) SDS wash buffer for 1 h at 65°C with several buffer changes.

Antibody Production, Immunoprecipitation, and Protein Gel-Blot Analysis

Polyclonal anti-OsPHB1 antibodies were generated in rabbits using a recombinant fusion PHB protein fused 6× His tag of pET-15b (Novagen, Madison, WI) as an antigen. Anti-OsPHB1 antibody was antigen purified before use for protein gel-blot analysis with 1:5,000 dilutions. One milligram of His-OsPHB1 fusion protein was bound to 0.3 mg of cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech) in the coupling buffer containing 100 mM $NaHCO_3$ and 500 mM NaCl (pH 8.3), incubated with the anti-serum, washed three times, and eluted with 0.1 M Gly (pH 2.5).

Total protein extracts were prepared from 7-d-old rice cell cultures after extracting in 0.3 M Suc, 50 mM MES-Tris (pH 7.6), 150 mM EGTA, 5 mM EDTA, 20 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 4 mM salicylhydroxamic acid, 2.5 mM $Na_2S_2O_5$, and 1 mM dithiothreitol. For immunoprecipitation reactions, total protein was extracted in the homogenized buffer containing 50 mM MOPS-KOH (pH 7.5), 2.5 mM EDTA, 100 mM NaCl, 10 mM NaF, 1 mM ammonium molybdate, 1 μM calyculin A, 1 mM PMSF, 100 μM leupeptin, 1 mM dithiothreitol, and 1% (v/v) Triton X-100, incubated with 5 μL of PHB antibody, and rotated end-over-end at 4°C overnight. Protein A agarose (Amersham Pharmacia Biotech) was added and the solutions were incubated for an additional 3 h at 4°C. Immunoprecipitation reactions were washed three times with 1 mL of ice-cold 137 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 0.1% [v/v] Triton X-100, resuspended in 40 μL of SDS-PAGE sample buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2% [w/v] SDS, 10% [w/v] Suc, 0.004% [w/v] Coomassie Brilliant Blue R-250, 1 mM PMSF, 1 μg mL^{-1} leupeptin, and 5% [v/v] mercaptoethanol), boiled for 5 min, and run on 10% (w/v) SDS-PAGE gels.

GFP Fusions and Transient Expression

The GFP sequence derived from smRS-*GFP* (Ono et al., 2001) was fused to the N or C terminus of the *OsPHB1* cDNA. Onion (*Allium cepa*) skin epidermal cells were bombarded with 35S-GFP, 35S-GFP-OsPHB1, or 35S-OsPHB1-GFP. After 12 h of incubation at 30°C, expression and localization of the GFP protein were observed by a confocal microscope (LSM510; Zeiss, Jena, Germany) using an excitation filter of 488 nm. For staining mitochondria specifically, samples were stained for 15 min at room temperature with 100 nM MitoTracker (Molecular Probes) and were observed using an excitation filter of 543 nm.

Rice Transformation

To overexpress the *OsPHB1* cDNA in rice, the *OsPHB1* coding sequence was cloned into the Ti-based vector P2K-1 downstream of the maize (*Zea mays*) ubiquitin promoter, and *Agrobacterium tumefaciens*-mediated transformation of rice callus was performed according to a published protocol (Hiei, et al., 1994). Transformed calli were selected using hygromycin, and plants were regenerated from transformed callus cultures. For making c-myc-tagged OsPHB1 (UPmm), four oligonucleotide primers were synthesized as follows; myc1, 5′-GCGGCCGCTACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGACTAGTTGAGGATCCGAGCTC-3′, myc2, 5′-GAGCTCGGATCCTCAACTAGTCAGATCCTCTTCTGAGATGAGTTTTTGTTCCATGGTAGCGGCCGC-3′, myc3, 5′-TCTAGATCCTGCAGGACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGGCGGCCGC-3′, and myc4, 5′-GCGGCCGCCAGATCCTCTTCTGAGATGAGTTTTTGTTCCATGGTCCTGCAGGATCTAGA-3′. Two of them (myc1 and myc2) were annealed, digested by *Xba*I, and ligated with the C terminus of *OsPHB1* coding sequence. Subsequently, myc3 and myc4 were annealed to each other, digested by *Not*I, and ligated to *OsPHB1*, which had already been ligated to myc1 and myc2. The final product, which has a tandem c-myc tag at the C terminus of *OsPHB1*, was cloned into the *Bam*HI site of the P2K-1 vector.

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