C-Terminal Region of Plant Ferredoxin-Like Protein Is Required to Enhance Resistance to Bacterial Disease in *Arabidopsis thaliana*

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ABSTRACT

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Protein phosphorylation is an important biological process associated with elicitor-induced defense responses in plants. In a previous report, we described how plant ferredoxin-like protein (PFLP) in transgenic plants enhances resistance to bacterial pathogens associated with the hypersensitive response (HR). PFLP possesses a putative casein kinase II phosphorylation (CK2P) site at the C-terminal in which phosphorylation occurs rapidly during defense response. However, the contribution of this site to the enhancement of disease resistance and the intensity of HR has not been clearly demonstrated. In this study, we generated two versions of

truncated PFLP, PEC (extant CK2P site) and PDC (deleted CK2P site), and assessed their ability to trigger HR through harpin (HrpZ) derived from *Pseudomonas syringae* as well as their resistance to *Ralstonia solanacearum*. In an infiltration assay of HrpZ, PEC intensified harpin-mediated HR; however, PDC negated this effect. Transgenic plants expressing these versions indicate that nonphosphorylated PFLP loses its ability to induce HR or enhance disease resistance against *R. solanacearum*. Interestingly, the CK2P site of PFLP is required to induce the expression of the NADPH oxidase gene, *AtrbohD*, which is a reactive oxygen species producing enzyme. This was further confirmed by evaluating the HR on NADPH oxidase in mutants of *Arabidopsis*. As a result, we have concluded that the CK2P site is required for the phosphorylation of PFLP to enhance disease resistance.

Elicitor-induced defense responses enable plants to prevent or restrict the growth and proliferation of pathogens. Several sequential events are involved in the perception, induction, and expression of elicitors in the process of such defensive tactics. The production of reactive oxygen species (ROS) triggered by pathogen-associated molecular patterns is a well-known defensive strategy employed by plants to resist pathogenic infection (10,23,24,26,30). ROS are responsible for hypersensitive response (HR) involving localized cell death at the site of the infection (25). Many transgenic plants use this strategy to increase resistance to plant pathogens (16,38,40) through processes such as the ectopic expression of plant ferredoxin-like protein (PFLP). Our laboratory reported that PFLP in transgenic plants provides resistance against a broad spectrum of bacterial pathogens (6,16,17,21,22,39).

PFLP is a leaf-type ferredoxin isolated from sweet pepper with three functional domains, including the N-terminal signal peptide, the 2Fe-2S domain, and the casein kinase II phosphorylation (CK2P) site at C-terminal (6). The functional domains of PFLP that confer disease resistance can be confirmed through replacement or truncation techniques. Previous research has shown that conferring disease resistance against bacterial pathogens requires PFLP targeting to extra-chloroplasts, indicating that PFLP-mediated disease resistance depends on subcellular localization (22). The 2Fe-2S domain has also shown antimicrobial activity, as reported in a previous study (15). In our previous study, an unknown harpin-activated kinase phosphorylated the CK2P site at

the C-terminal of PFLP (14). The CK2P site was phosphorylated by a casein kinase II (CK2), which phosphorylates a variety of proteins in the regulation of nuclear transport, plant development, and flowering time (19,27,29). Whether the CK2 phosphorylates PFLP is still unknown, it is clear that PFLP intensifies harpin-mediated HR (6,14,16,22). Because HR is an effective defense response during pathogenic infection (23), determining the precise means by which the CK2P site of PFLP enhances disease resistance warrants further examination.

In this study, we generated two truncated versions of PFLP to determine the importance of the CK2P site in enhancing disease resistance and discuss the possible role played by the phosphorylation of PFLP in intensifying harpin-triggered HR.

MATERIALS AND METHODS

Plants and bacteria growth conditions. Seeds of *Arabidopsis thaliana* ecotype Columbia were sown in sterilized peat moss, and the seedlings were transferred to individual pots for further experiments. The plants were grown in a growth chamber at 22°C (16 h light/8 h dark). The 5-week-old plants were used for *Agrobacterium*-mediated transformation. *Escherichia coli* DH5 α , BL21 (DE3) and *Agrobacterium tumefaciens* GV3101 were grown in Luria-Bertani (LB) broth or on LB agar plates at 37 and 28°C, respectively. Antibiotics were used in the following concentrations: kanamycin at 50 μ g/ml for recombinant plasmids in *E. coli*, and gentamycin at 50 μ g/ml for *Agrobacterium tumefaciens*. For pathogen inoculation, *Ralstonia solanacearum* Rd15 was cultured at 28°C for 48 h on a 2,3,5-triphenyl tetrazolium chloride (TZC) medium (18).

Preparation of recombinant proteins. To prepare recombinant proteins of PFLP and two truncated versions, PDC (CK2P-deleted PFLP) and PEC (CK2P-extant PFLP) (Fig. 1A), three kinds of DNA fragments were amplified from the *pflp* gene

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(AF039662) with primers PFLP-N16F/PFLP-B16RM, PFLP-N16F/PDC-B16R, and PFLP-N16F/PEC-B16R, respectively (Table 1). Three distinct DNA fragments were subcloned in an expression vector, pET16b, to generate pET-PFLP, pET-PDC, and pET-PEC, respectively. The *E. coli* BL21 (DE3) containing each recombinant plasmid was cultured in LB broth in the presence of 0.1 M isopropylthio- β -D-galactoside. To produce a recombinant protein, the bacterial culture was sonicated in 25 mM Lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM Imidazole, pH 7.5) for 30 s, and centrifuged at $10,000 \times g$ for 10 min. The recombinant proteins were then purified using a Ni-NTA Spin Kit (Qiagen). Purified protein extracts of 0.5 μ g were fractionated into 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were then transferred to an Immobilon-P PVDF membrane (Millipore). This membrane was stained with Ponceau S (Sigma-Aldrich) prior to immunoblotting, and western blot was performed by using Anti-6x-His rabbit polyclonal antiserum (1:2,000; Rockland) specific for 6x-His tag and HRP-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:2,000; Rockland). The immuno-signals were then colored with a metal-enhanced DAB substrate kit (Thermo Scientific).

Detection of HR and H_2O_2 accumulation. The preparation of protein of HrpZ was carried out according to the procedure adopted in previous research (9). The HrpZ-induced HR ratio and ROS accumulation of all of the *Arabidopsis* plants used in this study were determined following the protocol previously described (22). Briefly, the mature leaves were punctured with a 25-gauge needle to form tiny holes and then infiltrated with HrpZ at 0.5 mg/ml by pressing a 1-ml blunt syringe through the holes. After treatment, plants were grown in a growth chamber for 24 h. The HR ratio of one plant was calculated as $(Nn/6) \times 100\%$, where Nn is the number of inoculation sites with necrosis and 6 is the total number of inoculation sites. For H_2O_2 observation, leaves were cut 16 h after treatment according to the established protocol (22) using 5 mM of 3,3'-diaminobenzidine (DAB). Brown precipitates indicated H_2O_2 accumulation in the leaves (7).

Phosphorylation assay. The experiments in this study were designed to determine whether the CK2P site is required for the phosphorylation of PFLP in response to harpin. The concentration of HrpZ solution was adjusted to avoid protein denaturing when PFLP intensified the acute response of HR necrosis (22). Thus, each purified protein was mixed with HrpZ at 0.3 μ g/ml of the final concentration and infiltrated into the leaves of Col-0. Protein

was extracted from the leaves after 1 h infiltration using an extraction buffer (100 mM NaCl, 50 mM Tris-HCl, and 1 mM PMSF at pH 7.5) containing 1 $\times$ of PhosSTOP (Roche, Germany) to avoid protein dephosphorylation. Fifty micrograms of total protein extract was fractionated through electrophoresis in 15% SDS-PAGE and transferred to an Immobilon-P PVDF membrane (Millipore). Western blot testing was performed using 3 μ g (1:2,000) of rabbit polyclonal anti-PFLP-P and anti-PFLP-NP antisera specific to the phosphorylated and total purified protein, respectively (LTK BioLaboratories, Taiwan). The reactions of secondary antibodies and color visualization were performed in accordance with the process described above.

Construction of plasmids for plant transformation. One recombinant plasmid pBI-ESF harboring the *es-pflp*, which encodes full-length PFLP for extracellular secretion, was constructed in pBI121 (Clontech) (22). To produce two truncated versions derived from *es-pflp*, DNA fragments were amplified from *es-pflp* using primers PFLP-ESX-F/PFLP-PECS-R and PFLP-ESX-F/PFLP-PDCS-R, respectively (Table 1). The amplified fragments *es-pec* and *es-pdc* were further subcloned in pBI121 to generate the plasmids pBI-ESFEC and pBI-ESFDC, respectively. These two plasmids were transformed into *Agrobacterium tumefaciens* GV3101, and the inserted fragments of *es-pec* and *es-pdc* were sequenced and compared with the sequences on Genbank.

Generation and molecular characterization of transgenic *Arabidopsis*. The transformation of the *Arabidopsis thaliana* ecotype Col-0 was performed using *Agrobacterium tumefaciens* (5). Transgenic seeds were screened using a rapid screening protocol (12) using 1/2 Murashige and Skoogs medium (1/2 MS) containing kanamycin, whereupon the seedlings were transplanted into pots containing sterilized soil. Over 10 T_1 transgenic lines

TABLE 1. Primers used in this study

Primer	Sequence
PFLP-N16F	5'-TCCGCATATGGCTTCATACAAAGTG-3'
PFLP-B16RM	5'-GAGAGGATCCTTAGCCCACGAG-3'
PEC-B16R	5'-CAGCCGGATCCTTATTCTTTG-3'
PDC-B16R	5'-CTTCGGATCCTTACTCAATAGTAAC-3'
PFLP-ESX-F	5'-TCTAGAAACCATGTCTCTTAGCCAGAAC-3'
PFLP-PECS-R	5'-ACGAGCTCTCACTCTTTG-3'
PFLP-PDCS-R	5'-CTGAGCTCAAACATCAG-3'
LS-ap1-RTF	5'-CACACCTGACGGACCAAT-3'
LS-ap1-RTR2	5'-CAGACTGTGGATAAGCAAC-3'

Fig. 1. Expression of full-length and truncated versions of plant ferredoxin-like protein (PFLP) in *Escherichia coli*. **A**, The amino acid sequence of PFLP and arrows show the deletion site at C-terminal of each truncated version (PEC and PDC). **B**, A western blot with 0.5 μ g of purified proteins that is detected by anti-histidine antibody (Anti-His) and stained by Ponceau S staining (S). Arrows indicate the protein size at 17 kDa.

were selected using polymerase chain reaction (PCR). The generation of T₂ and T₃ plants for each line was performed through self-pollination, followed by collection of the seeds. Over 100 T₃ transgenic plants were identified with PCR to determine whether the T₂ transgenic plant was a homozygous line. Two homozygous lines of T₂ plants were selected, and their seeds were prepared to generate T₃ plants for the succeeding experiments.

Total RNA was isolated from fresh leaf tissue of 4-week-old *Arabidopsis* plants. The first-strand of cDNA was synthesized using first-strand cDNA synthesis kit for RT-PCR [AMV] (Roche, Germany) from 1 µg of total RNA. Then, 200 ng of cDNA was subjected to PCR with primers LS-ap1-RTF (22) and LS-ap1-RTR2 (Table 1). The *tubulin* gene served as a positive control using primers Tub2AF and Tub2AR (20).

For western blot analysis, total protein was extracted from 4-week-old leaf tissue using extraction buffer as described above. Three micrograms of protein extract was fractionated and transferred for immunoblotting. The anti-PFLP antiserum was used as a primary antibody (1:2,000) (15), and an HRP-conjugated goat anti-rabbit IgG (Rockland) was used as a secondary antibody. The relative quantity of protein compared with nontransgenic Col-0 plant was calculated using AlphaEase FC StandAlone software (Alpha Innotech).

Assessment of disease severity. The disease index was evaluated using the protocol described previously (22). Four-week-old *Arabidopsis* plants were grown in a growth chamber at 25°C to assess the severity of disease according to bacterial wilt. The disease index of *Arabidopsis* plants challenged with *R. solanacearum* Rd15 was calculated every 2 weeks postinoculation. The scale of wilting symptoms spanned from 0 to 6 (0, no wilting; 1, one to two leaves wilting; 2, three to four leaves

wilting; 3, five to six leaves wilting; 4, seven to eight leaves wilting; 5, over nine leaves wilting; and 6, death) was scored for the total number (N) of plants. The disease index for each trial was calculated using the following formula: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6) / (6 \times N)] \times 100\%$ (37). This assay tested 12 *Arabidopsis* plants for replicates.

Expression of *Atrboh* genes in transgenic plants. To determine whether transgenic plants with truncated versions of PFLP influenced the expression levels of *Atrboh* genes during harpin-mediated HR, 4-week-old transgenic plants were infiltrated with HrpZ at 0.5 mg/ml. At 0 and 24 h postinfiltration, first-strand cDNA was prepared and amplified with specific primers (22). The intensity of PCR products in 1.5% agarose gel of *Atrboh* genes was compared with that of the *tubulin* gene using AlphaEase FC StandAlone software (Alpha Innotech). The relative expression level was normalized by the mean of Col-0 at 0 h.

RESULTS

Full-length and truncated versions of PFLP on harpin-mediated HR. To generate the truncated versions of PFLP, DNA fragments were constructed and expressed in *E. coli*. The PEC had five amino acids (from residue 94 to 98) deleted to reveal or uncover the CK2P site, while the PDC had nine amino acids (from residue 90 to 98) deleted to remove CK2P site (Fig. 1A). Purified PFLP and its truncated versions were detected using Anti-6x-His rabbit polyclonal antiserum with distinct sizes in SDS-PAGE (Fig. 1B).

The recombinant protein of PFLP purified from *E. coli* intensifies harpin-mediated HR (6). During this response, harpin-activated kinase phosphorylates the PFLP in the tobacco plants

Fig. 2. Phosphorylation and hypersensitive response (HR) assay of purified proteins in *Arabidopsis thaliana* Col-0 plants. Each purified protein (B, blank; P, plant ferredoxin-like protein [PFLP]; E, PEC; and D, PDC) was mixed with HrpZ (H) at a final concentration of 0.3 mg/ml to obtain their mixtures (PH, EH, and DH). The mixtures were infiltrated into the leaves, and samples were collected and extracted at 1 h postinfiltration. **A to C,** Purified proteins (**A**, PFLP; **B**, PEC; and **C**, PDC) were detected by anti-PFLP-P antiserum (P) or anti-PFLP-NP antiserum (NP) to detect the phosphorylated or total purified proteins, respectively. **D,** HR ratios induced by distinct mixtures at 24 h after infiltration, respectively. Letters indicate significant differences based on Duncan's multiple range test ($P < 0.05$).

(14). To determine how the CK2P site of PFLP contributes to the enhancement of this HR, purified proteins were co-infiltrated with harpin in *Arabidopsis* Col-0 plant, whereupon the phosphorylation signal was checked. Blotting images using antiserum specific to phosphorylated PFLP (anti-PFLP-P) revealed the immunosignals of phosphorylated proteins, while the total purified proteins were detected using antiserum specific to PFLP (anti-PFLP-NP) as a control. Treatment without PFLP or its truncated versions revealed no signal in the leaf extract, indicating that the antisera were specific to PFLP (Fig. 2). In the treatment involving full-length PFLP, the PFLP with HrpZ produced a stronger phosphorylated signal than the sample without HrpZ did (Fig. 2A). In addition, during the treatment with PEC, the PEC with HrpZ showed a phosphorylated signal, whereas in PEC without HrpZ disappeared (Fig. 2B). For PDC, no phosphorylated signals were detected with or without HrpZ (Fig. 2C). These results demonstrate that the CK2P site of PFLP is necessary for phosphorylation in response to HrpZ.

To determine whether the PFLP or its truncated versions intensify harpin-mediated HR, purified proteins were co-infiltrated with HrpZ in Col-0 plants to evaluate the ratio of harpin-mediated HR. Because the HrpZ solution at 0.5 mg/ml did not induce HR necrosis in all of the inoculation sites on *Arabidopsis* Col-0

plants, the ratio of HR was calculated by counting the number of inoculation sites with necrosis and dividing this by six inoculation sites in one plant, at 24 h postinfiltration. The HR ratio on Col-0 plants initially induced by HrpZ was 14.5%. When HrpZ was added, the ratio with PFLP or PEC increased to 65.4 and 53.3%, respectively. However, PDC with HrpZ was similar to that of HrpZ alone (Fig. 2D). These results indicate that the full-length PFLP and PEC can be phosphorylated and its harpin-mediated HR intensified, while PDC cannot.

Molecular characterization of transgenic lines. To estimate the actual contribution of the CK2P site of PFLP to the enhancement of disease resistance in transgenic plants, DNA fragments harboring two truncated versions of PFLP were constructed in the plasmid pBI121. The plasmid pBI-ESPEC contained the *es-pec*, encoding PEC for extracellular secretion, and pBI-ESPDC contained *es-pdc*, encoding PDC for extracellular secretion (Fig. 3A). Transgenic *Arabidopsis* plants, secreting extracellular PEC (ESFEC) and PDC (ESFDC), were generated with no apparent morphological differences between transgenic and nontransgenic Col-0 plants (data not shown). Two homozygous lines of each transgenic *Arabidopsis* plant were selected for further experiments. All lines of ESFEC (line 2-4 and line 3-2) and ESFDC (line 6-2 and line 7-5) T₃ transgenic plants showed the presence of

Fig. 3. Molecular characterizations of ESFEC and ESFDC transgenic *Arabidopsis* lines. **A**, Schematic diagrams of T-DNA region in plasmids pBI-ESPFLP, pBI-ESPEC, and pBI-ESPDC harboring *es-pflp*, *es-pec*, and *es-pdc* fragments, respectively. NOS promoter (NP) was used to drive kanamycin resistance gene (NPT II) as a selection maker for transformation, and CaMV 35S promoter (35P) was used to drive the target genes. **B** and **C**, Reverse transcription-polymerase chain reaction revealed the presence of mRNA transcripts in ESFEC and ESFDC lines. Letter P in upper panels of B and C shows the presence of *es-pec* and *es-pdc* transcripts. Letter T in the lower panels indicates the presence of a 0.5-kb band for the positive control of *tubulin* transcripts in all samples. **D** and **E**, Western blots with 3.0 µg of total proteins detected by anti-plant ferredoxin-like protein [PFLP]-NP antiserum (P) and stained by Ponceau S staining (S). Arrows indicate the protein size at 17 kDa.

es-pec or *es-pdc* transcripts by RT-PCR, respectively (Fig. 3B and C). Neither recombinant DNA of PFLP nor transcripts of its truncated versions appeared in nontransgenic Col-0 plants. The protein expression levels of T₃ transgenic plants and Col-0 plants were examined using anti-PFLP antiserum in western blot analysis. The endogenous ferredoxin at 17 kDa was visualized in Col-0 and all transgenic plants, but their intensities varied. Compared with the signal in Col-0 plants (as 1.00), the signal intensity increased 1.57-fold in ESFEC line 2-4, 1.79-fold in ESFEC line 3-2, 1.47-fold in ESFDC line 6-2, and 1.51-fold in ESFDC line 7-5 (Fig. 3D and E). These results indicate that the transgenic *Arabidopsis* plants expressed the transgenes at the protein level in T₃ plants.

Harpin-mediated HR in *Arabidopsis* plants. Because the CK2P site is required for PFLP to intensify harpin-mediated HR, its effect could be confirmed by evaluating the degree of harpin-mediated HR in transgenic plants. The infiltration of HrpZ in Col-0 plants did not induce significant H₂O₂ generation or HR necrosis (Fig. 4). ESF 1-19, which is another homozygous line capable of expressing the full length of PFLP for extracellular secretion, was also used as a positive control (22). The accumulation of H₂O₂, which appeared in dark brown precipitate, was displayed in the leaves of ESF 1-19 and ESFEC plants. However, ESFDC plants showed no obvious H₂O₂ accumulation at 16 h postinfiltration (Fig. 4A). The ratios of HR in ESF 1-19 plants were 67.5%, whereas the ESFEC lines were 61.8 and 59.3%. These plants exhibited higher HR ratios than ESFDC (18.6 and 15.2%) and Col-0 plants (15.2%) (Fig. 4B). These results indicate that transgenic plants expressing PFLP with CK2P site have intensified harpin-mediated HR.

Bacterial wilt disease on *Arabidopsis* plants. Transgenic plants harboring PFLP confer resistance to bacterial pathogens. To verify whether transgenic plants with truncated versions of

PFLP confer resistance to bacterial pathogens, transgenic plants in this study were challenged with *R. solanacearum* Rd15. A lower disease index means the plants have greater disease resistance. The disease indexes of ESF 1-19 and ESFEC plants were lower than that of Col-0 (32.4%) at 2 weeks postinoculation (wpi) (Fig. 5A). The disease indexes of ESF 1-19 and ESFEC plants were approximately half (38.5 to 55.3%) those in Col-0 plants (95.2%) at 4 wpi. Wilting symptoms in the transgenic plants with PFLP or PEC indicated that they remained resistant to the challenge by Rd15. The degree to which PEC enhanced disease resistance by did not reach that of PFLP (Fig. 5B); however, two lines of ESFDC plants did not decrease their disease indexes (91.6 and 91.7%) at 4 wpi (Fig. 5A). These results demonstrate that PFLP requires the CK2P site to enhance disease resistance against pathogens causing bacterial wilt.

Expression of *AtrbohD* and *AtrbohF* genes during harpin-mediated HR. PFLP-enhanced disease resistance and the intensification of harpin-mediated HR were consistent in this study. The level of HR enhancement achieved by PFLP is associated with the expression of the *AtrbohD* gene (22). Both *AtrbohD* and *AtrbohF* play a major role in the production of ROS, triggering HR following interaction with a pathogenic infection (32,33). For this reason, we monitored the expression of *AtrbohD* and *AtrbohF* genes in *Arabidopsis* plants in this study. The ESF 1-19 and ESFEC plants exhibited higher levels of the *AtrbohD* gene at 24 h postinfiltration (Fig. 6); while the ESFDC plants showed almost no enhancement in the *AtrbohD* gene (Fig. 6). All of the transgenic plants exhibited similar expression levels of *AtrbohF* gene when compared with Col-0 plants (Fig. 6). These results indicate that the CK2P site is required to induce the expression of the *AtrbohD* gene and trigger HR.

Harpin-mediated HR in *Atrboh* mutants. Because PFLP with the CK2P site is associated with the expression of the *AtrbohD*

Fig. 4. Harpin-mediated hypersensitive response (HR) in *Arabidopsis* plants. The HrpZ prepared from *Escherichia coli* was infiltrated into the leaves of transgenic *Arabidopsis* plants. Reactive oxygen species accumulation was observed at 16 h postinoculation and HR necrosis was observed to calculate the HR ratio at 24 h postinoculation. Left panel shows the H₂O₂ accumulation revealed by DAB staining. Right panel indicates the ratio of HR necrosis on leaves of ESF 1-19, ESFEC, and ESFDC plants, respectively. Letters indicate significant differences based on Duncan's multiple range test ($P < 0.05$).

gene in *Arabidopsis* plants, harpin-mediated HR in *Arabidopsis* plants with *Atrboh* mutated genes could be used to confirm their influence on HR. Figure 7 shows that the full-length PFLP intensified the ratios of harpin-mediated HR in nontransgenic Col-0 plants. This effect decreased in *AtrbohF* mutants and disappeared in *AtrbohD* and *AtrbohD/F* double mutants. Treatment with PEC had a similar effect on all of the plants; however, treatment with PDC did not intensify the HR in any plants. These results indicate that the *AtrbohD* mutant eliminates the majority of harpin-mediated HR.

DISCUSSION

PFLP mediated-disease resistance can protect plants against a broad range of bacterial diseases through HR. The ratio of HR necrosis induced by harpin in the leaves of transgenic *Arabidopsis* plants acted as an index describing disease resistance (13,16,22).

Previous research has suggested that sustained resistance to disease can be achieved by apoplastic PFLP through the replacement of the N-terminal signal peptides (22). The 2Fe-2S domain of PFLP also demonstrates antimicrobial activity inhibiting the growth of bacterial pathogens (15). This study used two truncated versions of PFLP to determine the importance of the CK2P site in intensifying harpin-triggered HR. Purified PEC from *E. coli* was phosphorylated and found to increase the ratio of harpin-mediated HR; however, PDC did not. The detection of phosphorylated proteins indicates that full-length PFLP can be phosphorylated rapidly, and this phosphorylation requires the CK2P site. This implies that the CK2P site is necessary for the phosphorylation of PFLP to increase the intensity of harpin-mediated HR. The CK2P site is phosphorylated by a casein kinase II (CK2), and this phosphorylation regulates physiological pathways in plants (19,27,29). However, induction of innate immune responses related to CK2 has only been discussed in animal systems (4). The mitogen-

Fig. 5. Disease indexes and bacterial wilt symptoms of *Arabidopsis* plants. Four-week old *Arabidopsis* plants were inoculated with *Ralstonia solanacearum* strain Rd15. Disease indexes exhibit the appearance of wilting leaves in inoculated plants at 2 and 4 weeks postinoculation. **A**, White bars represent the Col-0, black bars represent ESF 1-19, light gray bars represent ESFEC lines, and dark gray bars represent ESFDC lines. Error bars indicate standard deviation of the mean for 12 individual plants. Letters indicate significant differences based on Duncan's multiple range test ($P < 0.05$). **B**, Bacterial wilt symptoms caused by Rd15 on Col-0, ESF 1-19, ESFEC, and ESFDC *Arabidopsis* plants at 4 weeks postinoculation.

associated protein kinase cascade is a key pathway in plant immunity triggered by pathogen-associated molecular patterns (1,2). Evidence has shown that the phosphorylation of CK2P site in the PFLP is clearly associated with intensified harpin-mediated HR, but the components involved in this reaction remain unknown and worthy of further investigation.

Transgenic *Arabidopsis* plants ESF, ESFEC, and ESFDC were generated to evaluate the actual contribution of PFLP and its truncated versions on disease resistance. The *pflp* gene does not exist in nontransgenic Col-0 plants, but we detected a signal at 17 kDa using anti-PFLP antiserum similar to results in previous reports (22). The signal was identified as an endogenous leaf type

Fig. 6. Relative expression level of *Atrboh* genes in *Arabidopsis* plants during harpin-mediated hypersensitive response. The transcripts of *Atrboh* genes were monitored at 0 and 24 h after the protein solution of HrpZ was infiltrated into 4-week-old Col-0 (white columns), ESF 1-19 (black columns), ESFEC (dark gray columns), and ESFDC (light gray columns) plants. The relative values of *Atrboh* transcripts in all of the samples were divided by the mean values of Col-0 at 0 h to obtain the relative quantity. Each column represents the mean value of six individual leaves postinfiltration. Letters above each column indicate significant differences based on Duncan's multiple range test ($P < 0.05$).

Fig. 7. The harpin-mediated hypersensitive response (HR) in *Atrboh* mutants. The purified proteins (P, plant ferredoxin-like protein [PFLP]; E, PEC; and D, PDC) were mixed with HrpZ (H) at the final concentration of 0.5 mg/ml (PH, EH, and DH). Each mixture was infiltrated into the leaves of *AtrbohD*, *AtrbohF*, and *AtrbohD/F* double mutants. HR necrosis was observed to calculate the HR ratio at 24 h postinoculation. Letters indicate significant differences based on Duncan's multiple range test ($P < 0.05$).

ferredoxins, *Atfd2* (At1g60950) or *Atfd1* (At1g10960) (11,35). Our results show that expressed truncated proteins and endogenous ferredoxins extracted from transgenic plants cannot be separated in SDS-PAGE. The anomalous migration in SDS-PAGE of truncated proteins and endogenous ferredoxin is associated with similarities in the tertiary structure and working conditions involved in electrophoresis (28,36). However, a comparison of the intensity of immuno-signals in transgenic and Col-0 plants identified differences of approximately 1.5- to 1.8-fold. In addition, mRNA transcripts of engineered transgenes in transgenic plants were made during transcription. Therefore, we propose that the transgenes were expressed at the protein level in transgenic plants, and these transgenic plants could be used for further analysis.

Harpin-mediated HR is an indicator of disease resistance against bacterial pathogens, and the results of this study show the HR was enhanced in transgenic plants with PFLP and PEC, but not plants with PDC. This suggests that the CK2P site of PFLP in transgenic plants is required to intensify harpin-mediated HR. Transgenic plants were challenged with a bacterial pathogen, *R. solanacearum*, to evaluate their resistance to disease. Results indicate that the enhancement of resistance to disease decreased slightly in ESFEC plants, but this effect disappeared in ESFDC plants. Interestingly, an in vitro study of CK2P with truncated PFLP maintains its antimicrobial activity against a soft-rot pathogen (15). However, our results suggest that the resistance to disease is primarily due to the PFLP-intensified HR pathway rather than antimicrobial activity. In addition, transgenic plants with PEC, an extant CK2P site, also exhibit HR and disease resistance when compared with plants with full-length PFLP. Although harpin treatment can phosphorylate PFLP and PEC, evidence has shown that PFLP is more effective than PEC in enhancing HR and resistance to disease. The efficiency of an engrailed protein phosphorylated by casein kinase II decreases following truncation of its domain (3). The last five amino acids from the C-terminal may regulate the recognition or biochemical activity of PFLP and confer disease resistance. In addition to the nature of PFLP as an electron transfer protein, the redox potential of PFLP and its truncated proteins in plants are also important issues requiring further investigation.

The generation of ROS in plants during an attack of bacterial pathogens is associated with the membrane-bound NADPH oxidase homologues encoded by the *rboh* genes (31,40). The *AtrbohD* and *AtrbohF* genes are essential for the accumulation of ROS in the defense response of *Arabidopsis* (32). Disease resistance intensified by PFLP is also associated with the *AtrbohD* gene (22). The expression level of *AtrbohD* increased by approximately threefold in transgenic plants with PEC compared with the levels in nontransgenic Col-0 plants. However, this increase in intensity did not appear in transgenic plants with PDC. These results indicate that the CK2P site of PFLP is associated with the induction of the *AtrbohD* gene in transgenic plants. The *AtrbohD* and *AtrbohD/F* double mutants eliminated PFLP-intensified HR, indicating that the membrane protein RBOHD must play a role in increasing the intensity of HR. This implies that PFLP phosphorylation and *AtrbohD* gene induction occurred after the recognition of bacterial elicitors triggering resistance to disease in plants. Similarly, *Nbrboh* genes in tobacco contribute to the generation of ROS and resistance to *Phytophthora infestans* (40). The *AtrbohD*-mediated generation of ROS is also triggered by oligogalacturonide from *Arabidopsis*, but this reaction is not required to provide resistance against the fungal pathogen *Botrytis cinerea* (8). Moreover, the RBOHA of barley participates in the penetration of powdery mildew fungus (34). Thus, whether PFLP enhances disease resistance against fungal pathogens is worthy of further investigation. This study demonstrates that the CK2P site of PFLP is required for phosphorylation to further activate the generation of ROS, and this effect is crucial to the enhancement of disease resistance against bacterial pathogens.

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