PEPR2 Is a Second Receptor for the Pep1 and Pep2 Peptides and Contributes to Defense Responses in Arabidopsis

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INTRODUCTION

Plants are sessile organisms surrounded by a wide range of microorganisms, including plant pathogens. However, infection is established between a limited combination of plants and pathogens because plants can detect the presence of many potential pathogens through common pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns and subsequently mount a defense response called PAMP-triggered immunity (PTI) (He et al., 2007; Schwessinger and Zipfel, 2008). PAMPs include lipopolysaccharides, flagellin, and its homologs, Pep2-7, are endogenous amplifiers of innate immunity of Arabidopsis thaliana that induce the transcription of defense-related genes and bind to PEPR1, a plasma membrane leucine-rich repeat (LRR) receptor kinase. Here, we identify a plasma membrane LRR receptor kinase, designated PEPR2, that has 76% amino acid similarity to PEPR1, and we characterize its role in the perception of Pep peptides and defense responses. Both PEPR1 and PEPR2 were transcriptionally induced by wounding, treatment with methyl jasmonate, Pep peptides, and pathogen-associated molecular patterns. The effects of Pep1 application on defense-related gene induction and enhancement of resistance to Pseudomonas syringae pv tomato DC3000 were partially reduced in single mutants of PEPR1 and PEPR2 and abolished completely in double mutants. Photoaffinity labeling and binding assays using transgenic tobacco (Nicotiana tabacum) cells expressing PEPR1 and PEPR2 clearly demonstrated that PEPR1 is a receptor for Pep1-6 and that PEPR2 is a receptor for Pep1 and Pep2. Our analysis demonstrates differential binding affinities of two receptors with a family of peptide ligands and the corresponding physiological effects of the specific receptor–ligand interactions. Therefore, we demonstrate that, through perception of Peps, PEPR1 and PEPR2 contribute to defense responses in Arabidopsis.
gene in seedlings or leaf tissue from Arabidopsis plants. Transcripts of the PROPEP1-3 genes were differentially induced by the defense-related hormones methyl salicylate (MeSA) and methyl jasmonate (MeJA) by pathogen infection, application of PAMPs, and by treatment with synthetic Pep peptides (Huffaker et al., 2006; Huffaker and Ryan, 2007). Transcription of a pathogenesis-related protein-1 (PR-1) gene was dramatically induced by Pep1-3 and Pep5-6, and transcription of an antimicrobial peptide gene, defensin (PDF1.2), was induced by Pep1 and Pep2 (Huffaker and Ryan, 2007). Furthermore, transgenic Arabidopsis overexpressing the PROPEP1 and PROPEP2 genes exhibited higher PDF1.2 and PR-1 expression and increased resistance to the oomycete pathogen Pythium irregulare (Huffaker et al., 2006; Huffaker and Ryan, 2007). Because Pep peptides induced the transcription of their own precursor genes in addition to defense genes, it is likely that Pep peptides, which are initially induced by PAMPs, feed back into the signaling pathways to generate additional processed peptides to further upregulate downstream defense responses (Ryan et al., 2007).

The Pep1 receptor, PEPR1, was isolated from Arabidopsis suspension cultured cells using a photoaffinity labeling technique (Yamaguchi et al., 2006). PEPR1 is a typical LRR receptor kinase, having an extracellular LRR domain and an intracellular protein kinase domain, and belongs to the LRR XI subfamily of the 15 LRR-RLK subfamilies (Shiu et al., 2004). Based on the effects of Pep peptides on defense responses, and on the similarity of the receptors between PEPR1 and PRRs, some researchers classify Pep peptides as damage-associated molecular patterns (Boiler and Felix, 2009). However, the specific mechanisms through which Pep peptides and PEPR1 influence defense response are largely unknown.

Since T-DNA insertional mutants of PEPR1 did not show any obvious difference from wild-type plants upon pathogen infection, it was speculated that there is another receptor for Pep peptides. In this study, we selected At1g17750 (PEPR2) as a candidate for a second receptor for Pep peptides based on its phylogenetic relationship with PEPR1. We show that both PEPR1 and PEPR2 are transcriptionally induced by wounding of plants and by treatment with MeJA, Pep peptides, and specific PAMPs. Functional analysis of PEPR1 and PEPR2 using SALK T-DNA insertional mutants demonstrate that the double mutants do not activate transcription of defense-related genes when plants were treated with Pep1. Pretreatment of double mutant plants with Pep1 was not able to inhibit bacterial growth as much as it did in wild-type controls. Binding assays with Pep peptides and PEPR1 and PEPR2 demonstrated that PEPR1 can recognize Pep1-6 and that PEPR2 only recognizes Pep1 and Pep2. These and other results provide evidence that PEPR1 and PEPR2 have differential responses to the Pep peptides and play a role in defense response signaling.

RESULTS

Phylogenetic Analysis of LRR XI Subfamily of LRR Receptor Kinases

Phylogenetic analysis among the LRR RLK XI subfamily of Arabidopsis (Shiu et al., 2004) was conducted (Figure 1A) to identify candidates that share recent common ancestry with PEPR1. The result showed that At1g17750 was the most closely related gene to PEPR1. Other known receptors in this subfamily are involved in development and differentiation (Figure 1A), CLAVATA1 (CLV1), BAM1 (for barely any meristem 1), BAM2, and BAM3 are required for meristem function (Clark, et al., 1997; DeYoung, et al., 2006). HAESA (Jinn et al., 2000) and HSL2 (for HAESA like 2) regulate floral organ abscission (Cho, et al., 2006; Stenvik et al., 2008). PXY/TDR is a receptor of TDIF (for tracheary element differentiation inhibitory factor) (Fisher and Turner, 2007; Hirakawa et al., 2008). IKU2 regulates seed size (Luo et al., 2005). GSO1 and GSO2 are required for formation of a normal epidermal surface during embryogenesis (Tsuwamoto et al., 2008).

The At1g17750 gene encodes a predicted protein with 1088 amino acid residues (119 kD) and all the characteristic domains of an LRR-RLK (Figure 1B). The N terminus contains a hydrophobic secretion signal followed by an extracellular domain with 25 tandem copies of a 24-residue LRR (residues 101 to 699). The LRR domain is flanked by two pairs of Cys residues. A single transmembrane domain (residues 741 to 761) is predicted to separate the extracellular domain from an intracellular Ser-Thr kinase domain (residues 794 to 1080) in which all important subdomains, including guaninyl cyclase catalytic domain (Kwezi et al., 2007) and residues for catalysis, are conserved (see Supplemental Figure 1 online). At1g17750 and PEPR1 are 64% identical and 76% similar at the amino acid level across their entire lengths with one LRR domain fewer in At1g17750 (Figure 1B).

In this study, we designated At1g17750 as PEPR2 and further analyzed PEPR2 in pathogen response pathways and Pep perception and response.

Induction of PEPR1 and PEPR2 Gene Expression by Wounding and MeJA

Since the Pep1 precursor gene PROPEP1 was induced by wounding (Huffaker et al., 2006), quantitative RT-PCR (qRT-PCR) was conducted using total RNA extracted from wounded and unwounded upper leaves of 4-week-old soil-grown Arabidopsis plants to analyze PEPR1 and PEPR2 gene expression (Figures 2A and 2B). Both PEPR1 and PEPR2 mRNA accumulated within 15 min in wounded leaves. The maximum mRNA accumulation occurred 0.5 to 1 h after wounding and then decreased to basal levels at 4 h. In the unwounded upper leaves from the wounded plant, the induction of PEPR1 and PEPR2 was not observed (Figures 2A and 2B).

PROPEP genes were shown to be differentially induced by several plant hormones involved in defense responses, including MeJA and MeSA, and ethephon, an ethylene releaser (Huffaker et al., 2006; Huffaker and Ryan, 2007). To determine whether any of these compounds also induce the PEPR genes, we sprayed 4-week-old Arabidopsis plants with MeJA, MeSA, or 1-aminoacyclopropan-1-carboxylic acid, an ethylene precursor, and total RNA was subjected to qRT-PCR to detect PEPR1 and PEPR2 expression. MeJA induced transcription of both PEPR1 and PEPR2 within 30 min (Figures 2C and 2D), whereas MeSA and 1-aminoacyclopropan-1-carboxylic acid did not induce either PEPR1 or PEPR2 (see Supplemental Figure 2 online). These results were consistent with the data from the AtGenExpress
Figure 1. Phylogenetic Analysis of the LRR XI Subfamily of Arabidopsis LRR Receptor Protein Kinases.

(A) The phylogenetic relationships of the LRR XI subfamily of Arabidopsis LRR receptor protein kinases. The phylogenetic relationships (unrooted) were
Pep Peptide- and PAMP-Induced Transcription of PEPR1 and PEPR2

Treatment with Pep1 has been shown to induce transcription of its precursor gene, PROPEP1, when Pep1 is supplied through the petiole (Huffaker et al., 2006). In order to test the effects of Pep1 on PEPR1 and PEPR2 expression in the absence of wounding, 2-week-old Arabidopsis seedlings grown in liquid medium were incubated with Pep1 (10 nM) and then subjected to qRT-PCR analysis in comparison with PROPEP1 gene expression (Figures 3A to 3C). The levels of induction of PROPEP1, PEPR1, and PEPR2 gene expression by Pep1 were very similar, with rapid induction within 30 min and maximal accumulation (10-fold) observed 0.5 to 1 h after supplying Pep1 (Figures 3B and 3C). PROPEP1 mRNA remained at an elevated level compared with PEPR1 and PEPR2 mRNA and did not decrease to a twofold level compared with controls until 12 h (Figure 3A).

The effects of all of the Pep peptides (Pep1-6) on PEPR1 and PEPR2 transcript levels were also analyzed by qRT-PCR (Figures 3D to 3F). The peptides were supplied at a final concentration of 10 nM for 1 h to 2-week-old Arabidopsis seedlings grown in liquid medium. Among the Pep peptides, Pep1, Pep2, and Pep3 were strong inducers of PROPEP1 (6- to 12-fold), PEPR1 (6- to 10-fold), and PEPR2 (4- to 7-fold) transcript levels. Pep4 and Pep5 caused a weaker induction (2- to 4-fold) of transcript levels for all three genes compared with Pep1-3. Pep6 induced an increase in the levels of PROPEP1 and PEPR1 transcripts weakly (3-fold) but failed to affect PEPR2. Tomato systemin (Sys), an 18–amino acid peptide involved in defense responses to herbivores in Solanaceae plants (Ryan and Pearce, 2003; Schilmiller and Howe, 2005), did not cause any accumulation of transcripts of PROPEP1, PEPR1, or PEPR2, indicating that the response to Pep peptides was not an artifact of our experimental procedure (Figures 3D to 3F). Pep peptides are involved in innate immunity in Arabidopsis, and PROPEP1-3 genes were differentially induced by PAMPs (Huffaker et al., 2006). Analysis of previously published microarray data revealed that PEPR1 and PEPR2 were also induced by fig22 and elf18 (data not shown) (Zipfel et al., 2004, 2006). These results were confirmed by qRT-PCR analysis. A fivefold induction of PROPEP1 and PEPR1 was observed 1 h after supplying fig22 and elf18 (10 nM each), while twofold induction of PEPR2 was observed after supplying elf18 (Figures 3D to 3F). Together, these results indicate that PEPR1 and PEPR2 transcript levels are sensitive to both Pep peptide and PAMP signaling pathways.

pepr1 pepr2 Double Mutants Are Unable to Respond to Pep1

Since the expression patterns of PEPR2 are correlated with those of PROPEP1 and PEPR1 in the experiments described above, it is possible that PEPR2 is also involved in Pep1 perception. To clarify the involvement of PEPR2 in Pep1 signaling, T-DNA insertional mutants, SALK_059281 (pepr1-1) and SALK_014538 (pepr2-1) for PROPEP1 and SALK_036564 (pepr2-1) and SALK_004447 (pepr2-2) for PEPR2, were obtained from the ABRC. Both pepr1-2 and pepr1-1 have T-DNA insertions in regions that encode the extracellular LRRs of PEPR1 (Figure 4A). The pepr2-1 and pepr2-2 have T-DNA insertions in regions that encode the cytoplasmic juxtamembrane and the extracellular LRR domains of PEPR2, respectively (Figure 4A). After selecting homozygous single lines, two sets of double mutants were generated by crossing. RT-PCR was performed to confirm that the T-DNA insertions prevented accumulation of full-length transcripts in all cases (Figure 4B). The pepr1-1 and pepr1-2 mutant lines did not express PEPR1, whereas PEPR2 expression was normal. The pepr2-1 and pepr2-2 mutant lines did not express PEPR2, whereas PEPR1 expression was normal. In double mutant plants, pepr1-1 pepr1-2 and pepr1-2 pepr2-2, no expression was observed for either PEPR1 or PEPR2. The T-DNA single and double mutants showed normal growth and fertility phenotypes (data not shown).

When wild-type Arabidopsis seedlings were supplied with Pep1, the expression of the PROPEP1 gene was induced (Figure 3A). If both PEPR1 and PEPR2 were to perceive Pep1 and lead to the induction of downstream genes, the induction of PROPEP1 gene expression by Pep1 would be partially reduced in the single mutants and lost in the double mutants. Two-week-old seedlings grown in liquid medium were incubated with 10 nM Pep1 for 30 min and then subjected to qRT-PCR analysis using PROPEP1 gene-specific primers (Figure 4C). In wild-type Arabidopsis seedlings, Pep1 induced PROPEP1 gene expression by 7- to 8 fold (Figure 4C). In pepr1-1 and pepr1-2 seedlings, only a 50% average induction was observed compared with the wild type, suggesting the presence of other receptors for Pep1 in addition to PEPR1. In pepr2-1 and pepr2-2 seedlings, the reduction of PROPEP1 induction was not as evident. However, the induction of PROPEP1 by Pep1 was completely abolished in both double mutant lines, suggesting that PEPR2 also perceived Pep1.

Pep peptides are thought to amplify PAMP signals to induce defense-related genes (Huffaker and Ryan, 2007; Ryan et al., 2007; Boller and Felix, 2009). To examine the effect of Pep1 on early response gene expression in the PEPR mutants, we chose mitogen-activated protein kinase-3 (MPK3) and WRKY transcription factor genes, WRKY22, WRKY29, WRKY33, WRKY53, and WRKY55, which were induced by the fungal PAMP chitin.
(Wan et al., 2008), and the bacterial PAMPs flg22 and elf18 (Zipfel et al., 2006). Induction of these genes was sometimes reduced in the single mutants but remained high (50 to 100% of the induction seen in the wild type). However, in the pepr1 pepr2 double mutant plants, induction of all the genes was reduced to levels close to those seen in water controls (Figures 4D and 4E; see Supplemental Figures 3A to 3D online). Since Pep peptides are known to induce several defense-related genes (Huffaker and Ryan, 2007), pepr1 pepr2 double mutant plants treated with Pep1 were also assayed for the expression of the gene encoding a defensin (PDF1.2) (Figure 4F). In this study, 4-week-old Arabidopsis plants grown in soil were sprayed with 1 mM Pep1 and subjected to qRT-PCR analysis after 6 h. The PDF1.2 gene was induced by 30-fold in wild-type plants and was not induced by Pep1 in the double mutants (Figure 4F). A similar induction pattern was observed for a pathogenesis-related protein (PR-1) gene (see Supplemental Figure 3E online).

**Preinfiltration of Pep Peptides Reduces Symptom Development by P. syringae**

Plants overexpressing PROPEP1 showed increased resistance to the root pathogen *P. irregulare* (Huffaker et al., 2006). To elucidate the importance of PEPR1 and PEPR2 in defense responses, pepr1 and pepr2 single and double mutants were infected with *P. irregularare* and the necrotrophic pathogen, *Alternaria brassicicola*. A clear difference in symptom development between wild-type and mutant lines was not observed (data not shown). Infection with the biotrophic pathogen, *Pseudomonas syringae* pv tomato DC3000 (Pst DC3000), of 5-week-old wild-type and mutant plants produced necrotic regions of the same size. However, we found that preinfiltration of a 10 nM solution of Pep1 reduced Pst DC3000 growth in leaves of wild-type plants and that this reduction was concentration dependent with a maximal effect at 1 mM (Figure 5A). This result was comparable to leaves preinfiltrated with flg22, which was reported to cause growth reduction of Pst DC3000 (Zipfel et al., 2004) (Figure 5A). The reduction of Pst DC3000 growth was not observed when [A17] Pep1(9-23), an inactive derivative of Pep1 (Pearce et al., 2008), was preinfiltrated (Figure 5B). Other Pep peptides also enhanced the resistance to Pst DC3000 with differential intensities (Figure 5C). Pep4 and Pep6 treatment produced slightly weaker effects on Pst DC3000 proliferation compared with other Pep peptides.

To assess the relative contributions of PEPR1 and PEPR2 in the resistance to Pst DC3000 after infiltration of Pep1, the T-DNA insertional mutants were inoculated. When 1 mM Pep1 was infiltrated into leaves 1 d before Pst DC3000 inoculation, the size of the necrotic regions was reduced in both wild-type and single mutant lines, pepr1-1 and pepr2-1 (Figure 5D). On the other
hand, Pep1 preinfiltration did not affect symptom development by Pst DC3000 infection in double mutants, pepr1-1 pepr2-1 (Figure 5D). Bacterial growth was reduced to 1/100 in the wild type and pepr2-1 and to 1/25 in pepr1-1 by Pep1 preinfiltration, but no decrease in growth was observed with the double mutants (Figure 5E). Similar results were obtained when another set of mutant lines, pepr1-2, pepr2-2, and pepr1-2 pepr2-2, was used (see Supplemental Figure 4 online). Taken together, these results indicate that Pep1 induces defense against Pst DC3000 as strong as flg22 and that Pep1-mediated defense (but not flg22-mediated defense) is dependent on presence of the PEPR1 or PEPR2 receptors.

PEPR2 Binds to Pep1

The results shown above strongly suggested that PEPR2 also could be a receptor for Pep1. In previous experiments, photo-affinity labeling using microsomal fractions of the T-DNA insertional mutants of PEPR1, pepr1-1, and pepr1-2 did not show any additional Pep1 binding proteins (Yamaguchi et al., 2006). However, if the protein level of PEPR2 or binding capacity of PEPR2 to Pep1 is much lower than PEPR1, it might be difficult to detect binding between PEPR2 and 125I-azido-Cys-pep1. To clarify whether PEPR2 binds to Pep1, the PEPR2 coding region was fused to the cauliflower mosaic virus 35S promoter and transformed into tobacco suspension-cultured cells (Nicotiana tabacum). Tobacco cells expressing PEPR1 and β-glucuronidase (GUS) genes were also created as positive and negative controls, respectively. RT-PCR analysis revealed that transgenic cells selected on kanamycin-containing medium expressed each transgene (Figure 6A). The transgenic cells were incubated with 0.25 nM 125I-azido-Cys-Pep1 (Yamaguchi et al., 2006) and irradiated with UV-B to cross-link the Pep1 binding proteins. After separation of extracted proteins by SDS-PAGE, the labeled proteins were detected on x-ray film (Figure 6B). A major protein band of 170 kD, consistent with the size of PEPR1 in Arabidopsis (Yamaguchi et al., 2006), and a 150-kD band was labeled in PEPR1- and PEPR2-expressing cells, respectively, but not in the

Figure 3. The Effect of Supplying Various Peptides to Wild-Type Arabidopsis Plants on Transcription of Target Genes.

(A) to (C) Two-week-old Arabidopsis seedlings grown in liquid medium were incubated with either Pep1 (10 nM) or water for the indicated time period, and the expression patterns of PROPEP1 (A), PEPR1 (B), and PEPR2 (C) were analyzed by qRT-PCR. Expression levels are indicated relative to the expression at 0 h.

(D) to (F) Two-week-old Arabidopsis seedlings grown in liquid medium were incubated with 10 nM Pep1, Pep2, Pep3, Pep4, Pep5, Pep6, tomato systemin (Sys), flg22, or elf18, and the expression of PROPEP1 (D), PEPR1 (E), and PEPR2 (F) was analyzed after 1 h by qRT-PCR. Expression levels are indicated relative to the expression in water supplying seedlings.

Error bars indicate SE from three different experiments. The number of asterisks indicates samples that are significantly different from corresponding samples at 0 h or supplied with water (t test: one asterisk, P < 0.05; two asterisks, P < 0.02; three asterisks, P < 0.005).
cells incubated with 50 nM unlabeled Pep1 as a competitor of \( ^{125} \text{I-Tyr-Cys-Pep1} \) (Figure 6B). Minor radioactive bands at lower molecular masses probably represent partial degradation of the PEPR1 and PEPR2 proteins (Figure 6B).

To elucidate the different binding properties with Pep1 between PEPR1 and PEPR2, substrate saturation analysis was conducted using radiolabeled Pep1 \( (^{125} \text{I-Tyr-Pep1}) \) and transgenic tobacco cells (Figure 6C). The binding between \( ^{125} \text{I-Tyr-Pep1} \) and PEPR1-expressing cells was almost saturated at 1.5 nM, whereas the binding between \( ^{125} \text{I-Tyr-Pep1} \) and PEPR2-expressing cells was saturated at \( \sim 2 \) to 3 nM (Figure 6C).

Scatchard analysis of these data revealed that dissociation constants between \( ^{125} \text{I-Tyr-Pep1} \) and PEPR1- and PEPR2-expressing cells were 0.56 and 1.25 nM, respectively, suggesting that PEPR1 has a slightly higher affinity for Pep1 than does PEPR2 (Figure 6D).

**PEPR1 Is a Receptor for Pep1-6 and PEPR2 for Pep1 and Pep2**

The binding properties of each Pep peptide to PEPR1 and PEPR2 were analyzed by competition assays with \( ^{125} \text{I-Tyr-Pep1} \) (Figure 6B). Minor radioactive bands at lower molecular masses probably represent partial degradation of the PEPR1 and PEPR2 proteins (Figure 6B).

(A) T-DNA insertion sites in the PEPR1 and PEPR2 genes are indicated by black triangles. Black regions represent the signal peptides, dark-gray regions represent transmembrane domains, light-gray regions represent the kinase domains, and striped regions represent the LRR domain.

(B) RT-PCR analysis of the PEPR1 and PEPR2 transcripts in wild-type and T-DNA insertional mutants. The T-DNA mutants from (A) were analyzed along with two double mutants (pepr1-1 pepr2-1 and pepr1-2 pepr2-2). \( \beta \)-TUBULIN (TUB2) gene was amplified as an internal control.

(C) to (E) The effect of Pep1 on the expression patterns of the early response genes PROPEP1, MPK3, and WRKY33, respectively, for the T-DNA mutants. Two-week-old Arabidopsis seedlings grown in liquid medium were incubated with 10 nM Pep1 for 30 min, and the expression was analyzed by qRT-PCR.

(F) The effect of Pep1 on the expression pattern of the late response gene PDF1.2 for the T-DNA mutants. Four-week-old Arabidopsis plants grown in soil were sprayed with 1 mM Pep1 in 0.01% Silwet L-77, and total RNA was extracted after 6 h. The expression was analyzed by qRT-PCR. Expression levels are indicated relative to expression in wild-type seedlings supplied with water. Error bars indicate SE from three different experiments. The letters indicate groupings by the one-way analysis of variance with Tukey multiple comparison test (P < 0.05).
Just before addition of 0.5 nM $^{125}$I-Tyr-Pep1 to the transgenic tobacco cells, 10 nM unlabeled Pep1-6 was added, and the remaining radioactivity was measured. Unlabeled Pep1, Pep2, Pep5, and Pep6 reduced binding of $^{125}$I-Tyr-Pep1 to PEPR1-expressing cells to 30 to 40% of water control, Pep3 reduced binding to 70% of control levels, and Pep4 did not reduce binding (Figure 7A). Whereas unlabeled Pep1 and Pep2 reduced binding of $^{125}$I-Tyr-Pep1 to PEPR2-expressing cells to 10 and 50% of control levels, respectively, Pep3-6 did not reduce binding (Figure 7B). PEPR1- and PEPR2-expressing tobacco cells responded in the alkalinization assay to each of the Peps in a manner consistent with the results obtained from the competition assay (Figures 7C and 7D). All Pep peptides caused alkalinization of the medium in PEPR1-expressing cells with the weakest
activity found for Pep4 (Figure 7C). These results were comparable to the results obtained using wild-type Arabidopsis cells (Yamaguchi et al., 2006). On the other hand, only Pep1 and Pep2 caused medium alkalinization to PEPR2-expressing tobacco cells (Figure 7D). These results obtained from competition and alkalinization assays using transgenic tobacco cells indicated that PEPR1 binds to Pep1-6 and that PEPR2 binds to Pep1 and Pep2. These results also revealed that the levels of alkalinization of the medium caused by each Pep peptide reflect the binding properties of PEPR1 and PEPR2 to each Pep peptide.

The preferences for each Pep peptide for PEPR1 and PEPR2 were confirmed in Arabidopsis T-DNA mutant lines by monitoring the transcript levels of MPK3 and WRKY33 as marker genes. Two-week-old seedlings grown in liquid medium were supplied with Pep1-6 for 30 min, and gene induction was analyzed by qRT-PCR (Figure 7E; see Supplemental Figure 5 online). The MPK3 and WRKY33 genes were induced by Pep1-6 in wild-type and pep2-1 seedlings with the lowest induction by Pep4. On the other hand, these genes were induced only weakly by Pep1 and Pep2 in pep1-1 seedlings and were not induced by any Pep peptides in pep1-1 pep2-1 seedlings.

DISCUSSION

PEPR1 and PEPR2 Together Perceive Peps

In this study, we found that PEPR1 and PEPR2 together contribute to the perception of Pep1-6 and elicitation of the defense responses induced by these peptides. However, only PEPR1 was purified previously as the Pep1 binding protein from Arabidopsis suspension cultured cells, and other Pep1 binding proteins had not been found by photoaffinity labeling using microsomal proteins of the T-DNA insertional lines of PEPR1, pep1-1, and pep1-2 (Yamaguchi et al., 2006). These results implicated PEPR1 as the primary receptor for Pep1, and this conclusion is further supported by the results obtained in this study. The induction of early responsive genes, such as PROPEP1 and MPK3, by Pep1 was reduced to 50% in the PEPR1 single mutants, whereas this induction was affected less in the PEPR2 single mutants (Figures 4D and 4E). The suppression of the Pst DC3000 growth by Pep1 preinfiltration in wild-type Arabidopsis was reduced to a greater degree in the pep1 single mutants than in the pep2 single mutants (Figure 5D). A plausible
explanation for the activity difference between PEPR1 and PEPR2 was the difference in their binding affinities for Pep1. However, the difference in binding affinities might not be the sole reason, since the calculated Kds of PEPR1 and PEPR2 for Pep1 are within the same range (Figure 6D). Further investigations, such as comparisons of their relative protein levels and their intracellular kinase activities in Arabidopsis, will be needed to understand how these two receptors have different roles. However, PEPR2 contributes significantly to the perception of Peps and to downstream responses. The specific contributions of PEPR2 are revealed through studies that emphasize the overlapping functions of PEPR1 and PEPR2. For example, either

Figure 7. Binding Preference of PEPR1 and PEPR2 for Pep1-6.

(A) and (B) Competition assay of Pep peptides with 125I-Y-Pep1 (0.5 nM) for binding to transgenic tobacco cells expressing PEPR1 (A) and PEPR2 (B). Remaining specific binding of 125I-Y-Pep1 to cells in the presence of unlabeled competitor peptide (Pep1-6) (10 nM) is indicated as a percentage of specific binding of 125I-Y-Pep1 to the cells without competitor.

(C) and (D) Medium alkalinization of transgenic tobacco cells expressing PEPR1 (C) and PEPR2 (D) by the Pep peptides, assayed at the five concentrations shown.

(E) The effect of Pep peptides on the expression pattern of MPK3 gene for the T-DNA mutants. Two-week-old Arabidopsis seedlings grown in liquid medium were incubated with 10 nM peptide for 30 min, and the expression was analyzed by qRT-PCR. Expression levels are indicated relative to the expression in wild-type seedlings supplied with water. Error bars indicate SE for three (A) to (D) and five (E) different experiments. The number of asterisks indicates samples that are significantly different from corresponding samples supplied with water (A), (B), and (E) and with Pep1 (C) and (D) (t test: one asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001).
receptor will provide for transcriptional responses of both early- and late-acting defense response genes (Figures 4C to 4F). Both single mutants were capable of demonstrating resistance to bacterial proliferation in Pep1 preinfiltrated leaves. Both receptors bind strongly to Pep1-2, but PEPR1 shows a higher affinity to ep3-6.

Pep1 Shares Some Signaling Components with the PAMPs flg22, elf18, and Chitin to Amplify Innate Immune Response

Pep1 induces the defense-related genes PR-1 and PDF1.2 (Huffaker et al., 2006; Huffaker and Ryan, 2007). Here, we also found transcriptional induction of MPK3, WRKY22, WRKY29, WRKY33, and WRKY53 by Pep1 (Figures 5D and 5E; see Supplemental Figures 3A to 3D online), which are important in defense signaling (Eulgem and Somssich, 2007; Colcombet and Hirt, 2008) and have been reported to be induced by a fungal PAMP, chitin (Wan et al., 2004; Wan et al., 2008), and bacterial PAMPs, flg22 and elf18 (Zipfel et al., 2004, 2006). Based on the comparative analysis of microarray data using Arabidopsis supplied with flg22, elf18, and chitin (Zipfel et al., 2004, 2006; Wan et al., 2008), Wan et al. (2008) concluded that flg22, elf18, and chitin signaling share a conserved downstream pathway leading to basal resistance. Similarly, Pep/PEPR signaling likely works through some of the same signaling components as PAMPs because Pep1 both induced transcription of MPK3, PDF1.2, PR-1, and WRKY genes, which are also induced by flg22, elf18, and chitin, and enhanced resistance to fungal and bacterial pathogens (Figures 4 and 5; Huffaker et al., 2006).

Like Pep1 receptors, the receptors for flg22 and elf18, FLS2 and EFR, respectively, are plasma membrane LRR receptor kinases (Chinchilla et al., 2006; Zipfel et al., 2006). After perception by FLS2, flg22 induces WRKY22 and WRKY29 through activation of a MAPK cascade composed of MEKK, MKK4/5K3, and MPK3/MPK6 (Asai et al., 2002; Valerie et al., 2009). Chitin also activates MPK3 and MPK6 activity (Wan et al., 2004), and the receptor for chitin is probably an RLK, CERK1/LysM-RLK1, with an extracellular chitooligosaccharide binding motif (LysM) and an intercellular kinase domain (Miyata et al., 2007; Wan et al., 2008). It is possible that the induction of defense-related genes and enhancement of basal resistance by Pep1 occurs through activating the same MAPK cascade after perception by PEPR1 and PEPR2. There is precedence for MAPK signaling downstream of LRR RLKs from studies of the roles of HAESA and HAESA LIKE2, which also belong to LRR XI subfamily (Cho et al., 2008; Stenvik et al., 2008).

Possible Contribution of WRKYs to Pep-PEPR System

In Arabidopsis, there are 72 expressed WRKY genes (http://www.Arabidopsis.org/browse/genefamily/WRKY.jsp), and many of them are implicated in the regulation of the plant immune response positively and negatively via modulation of the JA/SA signaling pathways (Eulgem and Somssich, 2007). WRKY29, WRKY33, and WRKY53, which are induced by Pep1, are reported to be positive regulators of defense responses for bacterial and/or fungal pathogens, such as P. syringae, Botritis cinerea, and A. brassicicola (Asai et al., 2002; Zheng et al., 2006; Murray et al., 2007). WRKY transcription factors bind to W-box DNA elements (C/TTGACC/T) that are found in the promoters of many defense-related genes, including PR-1 and NPR1 (Maleck et al., 2001; Yu et al., 2001; Eulgem and Somssich, 2007). WRKY transcription factors also regulate the expression of their own genes and/or other WRKY genes in addition to the defense-related genes, composing the positive and negative feedback loops and feed-forward modules (Eulgem and Somssich, 2007). Interestingly, multiple W-box DNA elements were predicted in the promoter region of PEPR1 and PROPEP1-5 genes in the AtcisDB (Arabidopsis thaliana cis-regulatory database; http://Arabidopsis.med ohio-state.edu) (Palaniswamy et al., 2006). Therefore, the WRKY transcription factors may play an important role in the amplification of the Pep peptide signal.

The Pep-PEPR System Is One Component of Multiple Amplification Mechanisms

Pep peptides are considered to be endogenous amplifiers of innate immunity after perception of PAMPs by PRRs based on the following results: (1) overexpression of PROPEP1 and PROPEP2 enhanced resistance to P. irregulare; (2) supplying Pep peptides differentially induced defense related genes, such as PDF1.2 and PR-1; (3) supplying Pep peptides also induced the expression of their own precursor genes, except Pep6; and (4) supplying PAMPs and inoculation with pathogens both dramatically induced the PROPEP2 and PROPEP3 genes (Huffaker et al., 2006; Huffaker and Ryan, 2007; Ryan et al., 2007). In this study, we confirmed the Pep1 effects on defense responses, such as transcriptional induction of MPK3 and WRKY gene and enhancement of resistance to Pst DC3000 upon Pep1 application. However, wild-type Arabidopsis and the pepr1 pepr2 double mutants did not show obvious differences upon inoculation with several pathogens without Pep1 preinfiltration in our experimental conditions. The plant defense response is a very sophisticated mechanism and affected by many factors, including specific pathogen-plant combinations, pathogen concentrations, and environmental factors, such as temperature, light, and humidity. Therefore, it is possible that PEPR1 and PEPR2 are important for the resistance to specific pathogens or specific inoculation conditions that we have not identified yet.

Considering the multilayered amplification mechanisms of PTI, it is also not surprising that pepr1 pepr2 double mutants did not show any obvious difference in their resistance to pathogens compared with wild-type plants because other amplification mechanisms still exist in Arabidopsis, including the salicylic acid, jasmonic acid, and ethylene signaling pathways. However, overexpression of PROPEP1 and PROPEP2 and application of Pep peptides can enhance defense responses, including H₂O₂ generation and transcriptional induction of defense-related genes (Huffaker et al., 2006; Huffaker and Ryan, 2007; this study). It will be interesting to determine how many endogenous elicitors and receptors, as well as additional amplification loops and crosstalk, will be identified that protect plants from surrounding microorganisms. It will also be interesting to determine the relative contribution of these endogenous regulators to PTI.
**METHODS**

**Plant Material and Growth Conditions**

*Arabidopsis thaliana* plants, ecotype Columbia, were grown on twice autoclaved soil (120°C, 20 min) with four plants per pot (8 x 8 x 7 cm) at 22 to 25°C with a 9–h photoperiod for 4 to 5 weeks. For experiments conducted under sterile conditions, *Arabidopsis* seedlings were grown in Petri dishes containing half-strength Murashige and Skoog (MS) salts (Sigma-Aldrich), 1% sucrose, and 0.6% agar for a week at 25°C under constant light. Three seedlings were transferred into a flat-bottom glass tube (10 x 2.5 cm diameter) containing 3 mL of liquid medium (half-strength MS salts and 1% sucrose) and incubated on an orbital shaker at 160 rpm for a week at 25°C under constant light. T-DNA insertional lines (see Accession Numbers section) were obtained from the ABRC at Ohio State University. The double mutants pepr1-1 pepr2-1 and pepr1-2 pepr2-2 were obtained by crossing, with homozygous lines screened by two sets of PCR analyses, one using the gene-specific primer pair and the other using the gene-specific primer and the T-DNA left border of the vector as a primer. The primers used in this study are listed in Supplemental Table 1 online.

**Computer Analyses**

Full-length amino acid sequences of all members in the subfamily LRR XI (Shiu et al., 2004) were aligned using the ClustaW program (Thompson et al., 1994) using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Phylogenetic analysis (unrooted) was performed using the PHYLIP program (PHYLIP 3.68; http://evolution.genetics.washington.edu/phylip.html). In this phylogenetic program, SEQBOOT, PROTPARS, and CONSENSE programs were used for making the phylogenetic tree (unrooted). The resulting tree was drawn with the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treepview.html) (Page, 1996). Domain predictions were performed using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004) for the signal sequence and the TopPred program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred) (von Heijne, 2004) for the transmembrane region. LRR and protein kinase domains were based on the data from The Arabidopsis Information Resource (http://www.Arabidopsis.org/).

**Plant Treatments**

The leaves of 4-week-old *Arabidopsis* grown in soil were mechanically wounded across the main vein with a hemostat. At the indicated time points, the wounded leaves and the unwounded upper leaves were collected for extraction of total RNA by the method of de Vries et al. (1988). Four-week-old *Arabidopsis* plants grown in soil were sprayed with MeJA (625 μM in 0.1% Triton X-100) and kept in a closed plexiglas box under light conditions until harvesting. For expression analysis of the PROPE1, PEPR1, PEPR2, MPK3, WRKY22, WRKY29, WRKY33, WRKY53, and WRKY55 genes, 2-week-old *Arabidopsis* plants grown in liquid medium were incubated with 10 nM peptides, and whole plants were harvested. For expression analysis of PDF1.2 and PR-1 genes, 4-week-old *Arabidopsis* grown in soil was sprayed with At Pep1 (1 μM in 0.01% Silwet L-77), and the leaves were collected after 6 h because background expression of PDF1.2 and PR-1 was high in the seedlings grown in liquid medium.

**RT-PCR**

Total RNA was isolated from *Arabidopsis* as described by de Vries et al. (1988). For analysis of expression of PEPR1 and PEPR2 in the *Arabidopsis* T-DNA insertion lines and the transgenic tobacco (Nicotiana tabacum) cells, total RNA was treated with DNase I (New England Biolabs), and 2 μg of total RNA was reverse transcribed by Superscript III (Invitrogen) using an oligo(dT) primer (Invitrogen). Subsequently, 0.2 μL of the reverse transcription reaction was used as a template for PCR amplification. *Arabidopsis* β-tubulin (TUB2) gene and the tobacco elongation factor 1α (EF-1α) gene were amplified as internal controls. The number of PCR cycles was 35 for PEPR1 and PEPR2 and 30 for TUB2 and EF-1α. For the quantitative analysis of gene expression, total RNA treated with DNase was reverse transcribed using the DyNaMo cDNA synthesis kit (Finzyme) with random hexamer, and qPCR was performed using the DyNaMo HS SYBR Green qPCR kit (Finzyme) and Mx3005P QPCR systems (Stratagene). Ubiquitin 5 (UBQ5) was amplified as an internal control. The primers used in this study are listed in Supplemental Table 1 online.

**Pseudomonas syringae Infection**

Twenty four hours prior to bacterial inoculation, leaves were infiltrated with peptides or water (1 μL). Syringe inoculation of *P. syringae* pv tomato DC3000 (Pst DC3000) was performed as described (Zipfel et al., 2004). Pst DC3000 was grown at 28°C on low-salt Luria-Bertani (1 g of NaCl/L) agar medium containing 100 mg/L of rifampicin (Sigma-Aldrich) for 24 h, resuspended in sterile water to 5 x 10⁶ colony-forming units/mL, and pressure infiltrated into leaves of 5-week-old *Arabidopsis* plants with a needleless syringe. The infiltrated area was ~5 mm in diameter. The plants were covered with a clear plastic lid after bacterial solution was completely absorbed. Leaf discs (0.28 cm²) from two different leaves were ground in 100 μL of 10 mM MgCl₂ in a 1.5-mL tube. The samples were thoroughly vortex mixed with 900 μL of water and diluted 1:10 serially. The samples (8 μL) were plated on low-salt Luria-Bertani agar medium containing 100 mg/L of rifampicin. Plates were placed at room temperature for 2 d, after which the colony-forming units were counted.

**Transgenic Tobacco Cells**

The expression vector for PEPR1 was previously created (Yamaguchi et al., 2006). The coding region of PEPR2 was amplified by PCR using the primers attached by Smal sites, ligated into Smal sites of modified pBl121 vector (Yamaguchi et al., 2006) between the 3SS promoter and the nos terminator. The expression vectors for PEPR1 and PEPR2, and the original pBl121 vector, which contains the GUS gene between the 3SS promoter and the nos terminator, were introduced into tobacco BY2 suspension-cultured cells using *Agrobacterium tumefaciens* by the method of Nakayama et al. (2000). The transgenic cells were assayed using the alkalination assay as previously described (Pearce et al., 2001).

**Photoaffinity Labeling**

Photoaffinity labeling using radiiodinated Pep1 was performed as previously described (Scheer and Ryan, 1999; Yamaguchi et al., 2006). Briefly, Cys-Pep1 was coupled to the azido-photoaffinity cross-linker, N-(4-azidosalicylamido)butyl)-3’-pyridyldithio)propionamide (Pierce Biotechnology) and iodinated by Na¹²⁵I using an IODO-GEN iodination tube (Pierce Biotechnology) to create ¹²⁵I-azido-Cys-Pep1. One milliliter of tobacco cells was incubated with ¹²⁵I-azido-Cys-Pep1 (0.25 nM) for 10 min under red light at room temperature and then irradiated with a UV-B lamp (F15T8.UV-B, 15 W) for 10 min on ice. After washing the cells with MS medium, the cells were sedimented by centrifugation at 12,000g and disrupted in 500 μL of 5% SDS by boiling for 30 min. The cell debris was removed by centrifugation at 12,000g. Proteins in the supernatant were
precipitated by addition of 400 μL of methanol and 200 μL of chloroform and precipitated in a microfuge. The pellet was dissolved in 100 μL of Laemmli sample buffer containing 5% SDS at 65°C for 1 h and 1 or 10 μL was separated by 8% SDS-PAGE. The gels were dried and exposed to x-ray film for 50 h.

**Binding and Competition Assays Using Radiolabeled-Pep1**

Radioiodination was performed using 2 mCi of Na\(^{125}\)I with 12.5 nmol of Tyr-Pep1, followed by purification by HPLC as previously described (Scheer and Ryan, 1999; Yamaguchi et al., 2006). The specific radioactivity of \(^{125}\)I-Tyr-Pep1 was calculated to be 2 mCi/nmol. Radioligand binding assays were performed as previously described (Yamaguchi et al., 2006) with minor modifications. Suspension-cultured transgenic tobacco cells were used for assays 5 to 6 d after subculturing. The cells were separated from the medium using Miracloth (Calbiochem), washed twice with 40 mL of culture medium, and adjusted to a fresh weight of 0.2 g/mL with fresh medium. A 2-mL aliquot of cells was pipetted into a well of a 12-well culture plate and allowed to equilibrate for 1 h at room temperature while agitated on an orbital shaker (160 rpm).

**Supplemental Figure 1.** Expression Patterns of PEPR1, PEPR2, CLV1, and BRI1.

**Supplemental Figure 2.** The Effect of MeSA and ACC on the Expression of PEPR1 and PEPR2.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PEPR1, At1g73080; PEPR2, At1g17750; PROPEP1, At5g64900; PROPEP2, At5g64890; PROPEP3, At5g64905; PROPEP4, At5g09980; PROPEP5, At5g09990; PROPEP6, At2g22000; IKU2, At1g09970; HSL2, At5g65710; HSL1, At1g28440; HAESA, At4g28490; GSO1, At4g20140; GSO2, At5g44700; PXY/TDR, At5g61480; BAM1, At5g65700; BAM2, At3g49670; BAM3, At4g20270; BR1, At4g39400; CLV1, At1g75820; MPK3, At3g45640; WRKY22, At4g01250; WRKY29, At4g39400; WRKY33, At2g38470; WRKY53, At4g23810; WRKY55, At2g40740; PDF1.2, At5g44420; PR1, At2g14610; TUB2, At1g62690; UBQ5, At3g62250; At1g08590; At1g17230; At1g34110; At1g72180; At2g33170; At3g19700; At3g24240; At4g26540; At4g28650; At5g68940; At5g69660; At5g56040; At5g63930; EF-1α, D63396; and pBI121, AF485783. The following accession numbers: WRKY22, WRKY29, WRKY33, WRKY55, and PR-1 Genes for the T-DNA Insertional Mutants.

**Supplemental Figure 4.** P. syringae pv Tomato DC3000 (Pst DC3000) Infection Assay of T-DNA Insertional Mutants Pretreated with Either Water or Pep1 (1 μM).

**Supplemental Figure 5.** The Effect of Pep Peptides on the Expression Pattern of the WRKY33 Gene in the T-DNA Mutants.

**Supplemental Table 1.** Primers Used in This Study.

**Supplemental Data Set 1.** Alignment of Arabidopsis LRR-XI Subfamily Proteins Used for Phylogenetic Analysis in Figure 1A (FASTA Format).

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