An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response

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Innate immunity is initiated in animals and plants through the recognition of a variety of pathogen-associated molecules that in animals are called pathogen-associated molecular patterns and in plants are called elicitors. Some plant pathogen-derived elicitors have been identified as peptides, but peptide elicitors derived from the plant itself that activate defensive genes against pathogens have not been previously identified. Here, we report the isolation and characterization of a 23-aa peptide from *Arabidopsis*, called *At*Pep1, which activates transcription of the defensive gene *defensin* (*PDF1.2*) and activates the synthesis of H_2O_2, both being components of the innate immune response. The peptide is derived from a 92-aa precursor encoded within a small gene that is inducible by wounding, methyl jasmonate, and ethylene. Constitutive expression of the *At*Pep1 precursor gene *PROPEP1* in transgenic *Arabidopsis* plants causes a constitutive transcription of *PDF1.2*. When grown in soil, the transgenic plants exhibited an increased root development compared with WT plants and an enhanced resistance toward the root pathogen *Pythium irregulare*. Six paralogs of *PROPEP1* are present in *Arabidopsis*, and orthologs have been identified in species of several agriculturally important plant families, where they are of interest for their possible use in crop improvement.

endogenous elicitor | plant defense | defensin | hydrogen peroxide

Similarities have been noted among early signaling components of animal and plant innate immune systems, including leucine-rich repeat receptor-mediated recognition of pathogen-associated molecular patterns and/or elicitors from pathogens and the resulting activation of defense gene transcription involved in early steps of immunity (1–14). Several peptides originating from pathogens can activate the plant innate immune response, including fungal elicitors Pep13, AVR9, and elicitins (1–3), and bacterial elicitors hrpZ, NPP1, flg22, and elf13 (4–7). We report here that a 23-aa peptide, isolated from extracts of *Arabidopsis* leaves and called *At*Pep1, exhibits characteristics of an endogenous elicitor of the innate immune response. Endogenous plant peptides that activate genes specifically for defense against pathogens have not been reported previously to our knowledge, although systemin peptides, which are found only in Solanaceae species, activate antiherbivore defense genes. *At*Pep1 was first identified in soluble extracts of *Arabidopsis* leaves by its ability, at subnanomolar concentrations, to cause an alkalinization of the medium of suspension cultured cells, a typical response of cell cultures to peptide elicitors (15–19). *At*Pep1 is derived from the C terminus of a 92-aa precursor protein *At*proPep1. The peptide activates the transcription of defensin, a gene extensively studied for its role in innate immunity in *Arabidopsis*, the production of H_2O_2, and the expression of *PROPEP1*. Constitutive overexpression of *PROPEP1* confers resistance against a root pathogen *Pythium irregulare*. *PROPEP1* orthologs are found in numerous important agricultural crop species, including both dicots and monocots, and may provide novel genes for investigating crop productivity.

Results and Discussion

*At*Pep1 was purified to homogeneity (Fig. 1 *A* and *B*) and

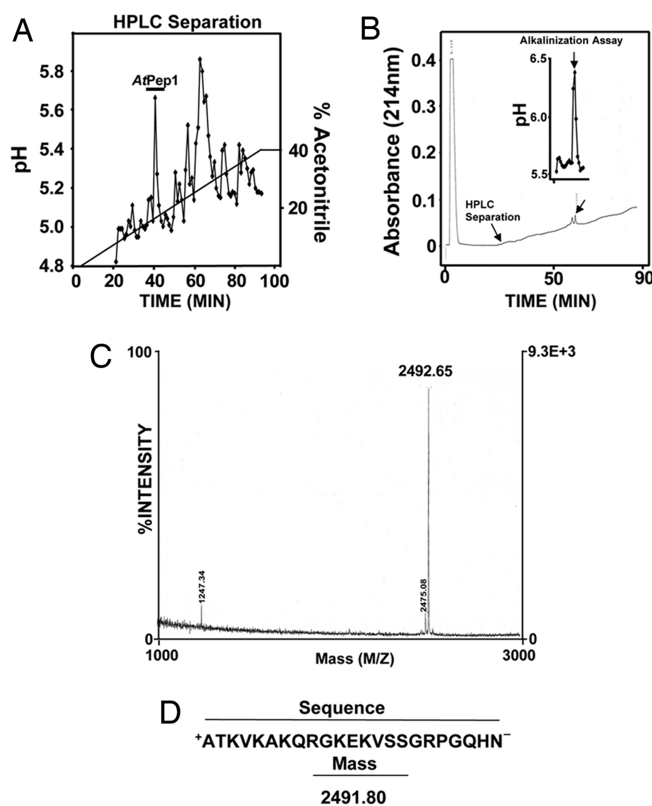

Fig. 1. Isolation of *At*Pep1. (*A*) Peptides present in an 1% trifluoroacetic acid/water extract of *Arabidopsis* tissues were passed through a reverse-phase semipreparative C18 flash chromatography column and separated on a G-25 Sepharose column as described in *Materials and Methods*. The breakthrough peak was applied to a C18 HPLC column, and 10 μl from 2-ml fractions from the column was assayed for alkalinization activity. (*B*) The peak identified in *A* as *At*Pep1 was further purified through two additional chromatography steps and finally purified by narrow-bore HPLC as described in *Materials and Methods*. Fractions were assayed as in *A*. The active peak is identified with arrows. (*C*) Analysis of the biologically active peak by MALDI-MS. (*D*) The amino acid sequence of the purified peptide, determined by Edman degradation. The daltons calculated from the amino acid sequence matched that determined by MS.

characterized as a peptide by its molecular mass (Fig. 1*C*) and amino acid sequence (Fig. 1*D*), which together indicated that the peptide was not posttranslationally modified. Chemically synthesized *At*Pep1 was found to be as active as native *At*Pep1, having a half-maximal activity of ≈0.25 nM in the alkalinization assay.

Conflict of interest statement: No conflicts declared.

Abbreviations: MeJA, methyl jasmonate; DPI, diphenylene iodominium chloride; CaMV, cauliflower mosaic virus.

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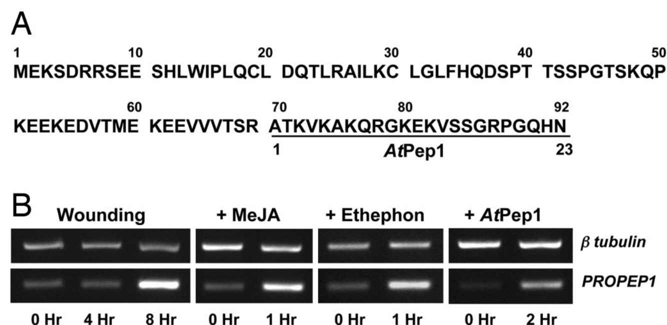

Fig. 2. *At*Pep1 precursor gene expression. (*A*) The amino acid sequence of the *At*Pep1 precursor protein PROPEP1 was encoded by the annotated gene At5g64900. The *At*Pep1 sequence at the carboxyl terminus of the precursor protein is underlined. (*B*) Semiquantitative RT-PCR analysis of *PROPEP1* expression in response to wounding and treatment of leaves with MeJA, ethephon, and *At*Pep1. Relative abundance of the *PROPEP1* transcript was estimated from the expression of the β-tubulin gene as a control. Leaves were wounded by crushing once across the midvein with a hemostat. Plants were sprayed with a 250 μM solution of MeJA in 0.1% Triton X-100, sprayed with a 7 mM solution of ethephon in 0.1% Triton X-100, or supplied throughout petioles with 10 nM *At*Pep1 in water.

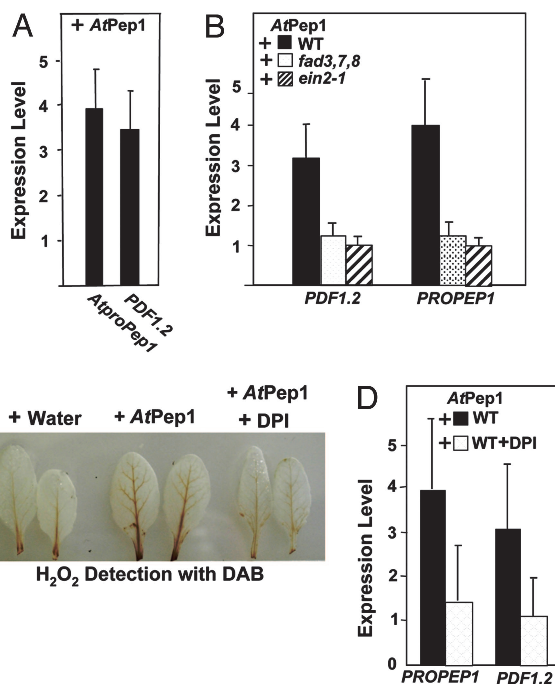

Fig. 3. *At*Pep1 regulates defense gene expression. (*A*) Fold induction of expression of *PROPEP1* and *PDF1.2* in excised *Arabidopsis* leaves supplied for 2 h with 10 nM *At*Pep1 through their cut petioles. Transcript levels were analyzed for expression levels of the two genes relative to their expression in excised leaves supplied with water. Expression was determined by semiquantitative RT-PCR with a β-tubulin gene as a control. (*B*) *At*Pep1-induced expression of *PDF1.2* and *AtproPep1* in leaves of WT plants, jasmonate-deficient *fad3,7,8* triple mutant plants, and ethylene-insensitive *ein2-1* mutant plants. *At*Pep1 was supplied for 2 h at 10 nM, and RNA was isolated and assayed as above. (*C*) Accumulation of H_2O_2 in leaves supplied for 2 h with water, 10 nM *At*Pep1, or 10 nM *At*Pep1 plus 100 μM DPI, an inhibitor of NADPH oxidase. Each treatment contained 1 mg/ml of diaminobenzidine (DAB) to visualize H_2O_2 accumulation. Leaves treated with *At*Pep1 and DAB were cosupplied with 100 μM DPI. (*D*) Expression of *AtproPep1* and *PDF1.2* in excised leaves of WT plants in response to supplying 10 nM *At*Pep1 in the presence or absence of DPI. The expression of each gene was analyzed by semiquantative RT-PCR.

*At*Pep1 was identified in the National Center for Biotechnology Information *Arabidopsis* genome database as being derived from a gene with locus number At5g64900, which encodes a small putative protein of 92 aa, with its C-terminal 23 aa comprising *At*Pep1 (Fig. 2*A*). The full-length amino acid sequence of the precursor protein is highly charged and lacks a signal sequence, indicating that it is not synthesized through the secretory pathway, but on cytoplasmic ribosomes.

As a first step in seeking a possible function for *PROPEP1* and its encoded peptide, the basal expression level of the gene was assessed in leaves, stems, roots, and flowers of *Arabidopsis* plants. The gene was expressed at low levels in all tissues, giving no clues as to its possible function. Monitoring the expression of *PROPEP1* in intact plants exposed to different environmental conditions and chemicals, including drought and cold stress, UV-B irradiation, wounding, methyl jasmonate (MeJA), methyl salicylate, abscisic acid, and ethylene (which is released from ethephon), provided more definitive clues. Whereas most treatments did not cause changes in expression of *PROPEP1*, wounding, MeJA, and ethephon induced expression of the gene (Fig. 2*B*), indicating a possible relationship of the gene and its encoded peptide in plant defense. Supplying excised leaves with 10 nM *At*Pep1 strongly induced the expression of *PROPEP1*, indicating that the *At*Pep1 peptide may be amplified as part of the defense response. Transcription of *PROPEP1* expression in response to wounding was detected within ≈8 h, whereas spraying the plants with MeJA or ethylene induced a strong expression of the gene within 1–2 h (Fig. 2*B*). MeJA and ethylene are known to activate the defense gene *PDF1.2* (*defensin*) through the jasmonic acid (JA)/ethylene pathway (20–25). These data suggested that *At*Pep1 may play a role in the activation of defense genes via the JA/ethylene pathway. Supplying excised *Arabidopsis* leaves with solutions of *At*Pep1 through their cut petioles induced the expression of *PDF1.2* (Fig. 3*A*). The gene was also assayed in the mutants *fad3.7.8* and *ein2-1* in response to supplying *At*Pep1 through their cut petioles. The *fad* mutant is incapable of synthesizing JA (26), and the *ein2-1* mutant is incapable of perceiving ethylene (27). Neither mutant induced the expression of *PDF1.2* or *PROPEP1* (Fig. 3*B*) in response to *At*Pep1, suggesting that *At*Pep1 may act upstream from the JA/ethylene pathway.

*At*Pep1 supplied to leaves caused the production of H_2O_2 that was associated with leaf veins (Fig. 3*C*). The production of active oxygen species has been associated with the signaling pathway of the innate immune response in plants (28). The induction of both H_2O_2 and *PDF1.2* by *At*Pep1 was blocked by first supplying the leaves with diphenylene iodominium chloride (DPI) (Fig. 3 *C* and *D*), an inhibitor of NADPH oxidase in both plant and animal tissues (20, 29). The cumulative results indicated that *At*Pep1 requires H_2O_2 for the transcription of *PDF1.2*, consistent with recently proposed pathways for JA/ethylene signaling (28).

PROPEP1 belongs to a seven-member gene family in *Arabidopsis*, identified in GenBank, of which one gene is unannotated. Three paralogs, At5g64890 (*PROPEP2*), At5g64900 (*PROPEP1*), and At5g64905 (*PROPEP3*), are sequentially encoded in a 5.5-kb region of chromosome V (National Center for Biotechnology Information *Arabidopsis* Genome Database). Paralogs At5g09980 (*PROPEP4*) and At5g09990 (*PROPEP5*) and the unannotated gene (*PROPEP7*) are also found on chromosome V, but in a 3.8-kb region at a distal region on the second arm of the chromosome. At2g22000 (*PROPEP6*) is found on chromosome II. In comparing the amino acid sequences of the ORFs of the paralogs, a low overall amino acid sequence identity was found, but within the C-terminal region of each gene where the putative *At*Pep1 homolog sequences reside the amino acid identities ranged from 35% to 65% (Table 1). All of the putative *At*Pep1

Table 1. Sequence comparisons of amino acids encoded by *PROPEP1* paralogs

Paralogs*	Residues[†]	C-terminal alignments
		1 10 23
At5g64900	92	-ATKVKAKQRGKEKVSSGRPGQHN
At5g64890	109	-DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED
At5g64905	96	-EIKARGKNKTKPTPSSGKGGKHN
At5g09980	81	-GLPGKKNVLKKSRESSGKPGGTNKKPF
At5g09990	86	-SLNVMRKGIRKQPVSSGKRGGVNDYDM
At2g22000	104	-ITAVLRRRPRPPPYSSGRPGQNN
Unannotated	75	-VSGNVAARKGKQQTSSGKGGGTN

The 23-aa sequence of *At*Pep1 is aligned with the C termini deduced from its six known paralogs.
*Gene locus identification numbers.
[†]Total amino acids deduced for each proprotein.

homologs have a conserved glycine at residue 17 (Table 1, numbers aligned with *At*Pep1). Each peptide contains proline, glycine, and serine residues within a 10-aa C-terminal region that may be important for receptor recognition.

Published transcription analyses of *Arabidopsis* genes induced by pathogen attacks and elicitors derived from pathogens (30, 31), including a fungus, *Botrytis cinerea*, an oomycete, *Phythopthera infestans*, and a bacterium, *Pseudomonas syringae*, caused high levels of expression of two paralogs, At5g64890 (*PROPEP2*) and At5g64905 (*PROPEP3*) compared with the other paralogs (Table 2). This finding indicates that a differential expression of the genes is occurring in response to pathogens. In these studies *PROPEP1* was only weakly induced, but the tissue- and cell-specific localizations of the paralogs have not yet been analyzed, and the differential expression may be different among different cells and tissues in response to pathogen attacks and/or wounding.

Arabidopsis plants transformed with a *CaMV-35S-PROPEP1* transgene were assessed for their expression of *PDF1.2*. In previous studies, the overexpression of the tomato prosystemin precursor gene, regulated by the cauliflower mosaic virus (CaMV) 35S promoter (32), caused a constitutive overexpression of $>$20 defensive genes (33). This effect was thought to be caused by the abnormal processing of the constitutively expressed prosystemin in the cytoplasm of cells where it is not normally synthesized and processed, releasing systemin in the absence of wounding. *At*Pep1 resembles systemin in lacking a leader sequence and being synthesized by cytoplasmic ribosomes, and it was considered possible that *At*Pep1 might also be abnormally produced when its gene is overexpressed, inducing the synthesis of *PDF1.2* in the absence of pathogen attacks. Six T2-independent transgenic lines were recovered that overexpressed *PROPEP1*. All six transgenic lines constitutively overexpressed *PROPEP1*, whereas five overexpressed *PDF1.2* (data not shown). The roots of the combined transgenic plants growing in each pot were rinsed with water to remove soil and were found to be generally bulkier than those of the WT plants (Fig. 4*A*). This finding suggested that the overexpression of *PROPEP1* may be providing an advantage to the plants growing in the soil. To assess whether the transgenic plants may exhibit an enhanced resistance to a root pathogen, *P. irregulare* (34, 35) was added to soils of germinating WT and transgenic plants (16 plants each having rosette diameters of $\approx$1.0 cm). The soils were inoculated with either a 250-μl suspension of *P. irregulare* strain 110305 ($\approx$110,000 propagules) or sterile water, and the plants were grown for 25 days after inoculation. The aerial parts and roots from each plant were separated and examined for effects of infection compared with WT plants or transgenic plants inoculated with *Pythium.* A typical result is shown in Fig. 4*B*. Whereas the aerial tissues of the WT and transformed plants showed little visible differences, the roots of individual inoculated WT plants had visibly smaller root masses than those of the inoculated transgenic plants, indicating that overexpression of *PROPEP1* was conferring a growth advantage to the transgenic plants over WT plants in the presence of the pathogen. *Arabidopsis* plants were transformed with *PROPEP2* in the same manner as with *PROPEP1*, and seven of eight *PROPEP2* transformants exhibited increased levels of defensin expression (data not shown). The eight lines were grown in soil, and their aerial and root systems were compared with those of WT plants. The seven transformants strongly expressing *defensin* were visibly larger than WT plants and the transgenic line not expressing *defensin*. Fig. 4*C* shows the aerial tissues and root masses from

Table 2. Changes in expression levels of the six annotated *PROPEP* gene family members in response to elicitors and pathogens

	Average fold change in gene expression					
Treatments	At5g-64900	At5g-64890	At5g-64905	At5g-09980	At5g-09990	At2g-22000
Pathogens						
B. cinerea (48 h)	1.2	27.9	12.6	0.8	0.7	0.6
P. infestans (6 h)	1.1	31.2	62.4	2.7	0.5	0.8
P. syringae (2 h)	0.7	3.2	2.6	2.0	2.4	1.1
Elicitors						
NPP1 (2 μM, 1 h)	1.4	26.9	24.9	1.3	1.9	0.9
HrpZ (1 μM, 1 h)	1.6	40.8	28.1	0.9	0.5	0.8
Flg22 (1 μM, 1 h)	1.5	21.8	14.2	1.2	0.4	0.9

Data were obtained from microarray analyses that were obtained from the Botany Array Resource NASCArray data set. Experiment reference numbers: NASCARRAY-167, NASCARRAY-123, NASCARRAY-120, and NASCARRAY-122 (30, 31).

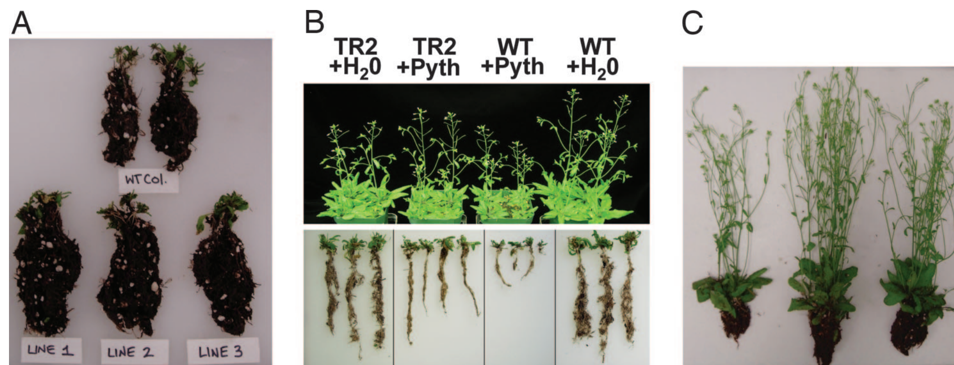

Fig. 4. *Arabidopsis* plants constitutively overexpressing *PROPEP1* and *PROPEP2* exhibit increased root and aerial growth over WT plants grown in potting soil with and without inoculation with the pathogen *P. irregulare*. (*A*) Root masses of WT (WT Col) and lines 1–3 of plants transformed with a *CaMV 35S*:*PROPEP1* chimeric gene. Four plants were grown per pot in soil for 21 days, and the soil was removed by rinsing in a water bath until soil no longer could be washed from the combined root mass. The aerial portions of the plants were excised before photographing them. (*B*) (*Upper*) Rosettes of WT plants and transformed plants as in *A* 3.5 weeks after inoculation with *P. irregulare* strain 110305 or water. (*Lower*) Roots from plants treated as in *Upper* 3.5 weeks after inoculation. Soil was washed from the total root mass, and then the roots of each plant were carefully separated while immersed in water and photographed. (*C*) WT plants (*Left*), transgenic line 3 (*Center*), and transgenic line 8 (*Right*) of plants transformed with *CaMV 35*:*PROPEP2*. Four plants were grown per pot in soil for 4 weeks, and the soil was gently washed from the total root mass as in *A* before photographing them.

soils in which two typical transformed lines, nos. 3 and 8 were grown, compared with WT plants. The transgenic lines have bulkier root systems and nearly twice the numbers of growing inflorescence stems than the WT plants. These results are similar to those of Fig. 4*A* in which the expression of *PROPEP1* in the plants was reflected in the greater root masses of the plants, indicating that the overexpression of two of the *PROPEP* family members in *Arabidopsis* produces growth-enhancing effects.

PROPEP1 orthologs are present in numerous species of dicots and monocots. Fig. 5 shows a cladogram indicating the relationships of known members of this gene family, based on amino acid identities and similarities. Four of the paralogs, *PROPEP1*, *PROPEP2*, *PROPEP3*, and *PROPEP4*, are within a subfamily containing only these genes, whereas *PROPEP5* and *PROPEP6* are in a separate subfamily together with a canola ortholog. The unannotated gene, *PROPEP7*, is in a subfamily with grape that is more closely related to monocot species than with dicot species, which comprise a separate subfamily.

No endogenous plant peptides have been reported previously that have signaling roles in activating genes associated with innate immunity. The data presented here support our hypothesis that the *PROPEP* genes are components of a feedback signaling system that is mediated by the *PEPR1* receptor (36) to amplify the innate immune response of *Arabidopsis*.

The chemical and physiological properties of the *At*Pep1 family members, their precursor proteins, and their genes are strikingly similar to the properties of the 18-aa peptide signal systemin, its precursor prosystemin, and its gene, which are components of the signaling pathway for defense against herbivorous pests of the Solanaceae family (37). Both *At*Pep1 and tomato systemin are cleaved from the C termini of precursor proteins that are induced by MeJA and lack leader peptides. Both precursors are small, highly positively charged proteins, and each produces peptides that activate defense genes. Tomato plants constitutively expressing *prosystemin* exhibited enhanced resistance toward a herbivore (37), whereas *Arabidopsis* plants constitutively expressing the *PROPEP1* were more resistant to a pathogen. However, tomato plants overexpressing the prosystemin gene have not been investigated for resistance against pathogens, and conversely, *Arabidopsis* plants overexpressing *PROPEP1* paralogs have not been assessed for defense against herbivores.

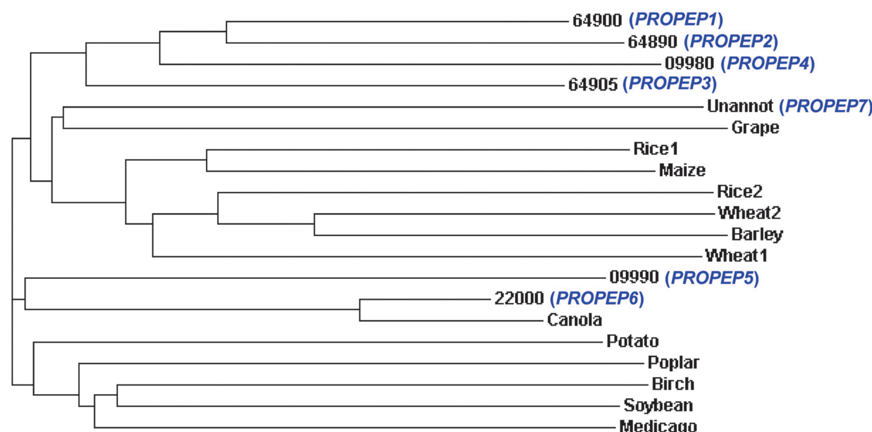

Fig. 5. A cladogram showing the relationships of *PROPEP1* (At5g64900) paralogs and orthologs estimated from their amino acid identities and similarities. GenBank accession numbers are as follows: for dicot genes, canola (*Brassica napus*) CD816645; potato (*Solanum tuberosum*) CV505388; poplar (*Populus balsamifera*) CV23975; medicago (*Medicago sativa*) BI311441; soybean (*Glycine max*) CD401281; and grape (*Vitis vinifera*) CF604664; for monocot genes, rice1 (*Oryza sativa*) CF333408; rice2 AK111113, wheat1 (*Triticum aestivum*) AL809059; wheat2 BF201609, maize (*Zea mays*) DN215793; and barley (*Hordeum vulgare*) BQ763246.

The similarities mentioned above between systemin and *At*-Pep1 support a hypothesis that the major role for receptor-mediated defense-signaling peptides in plants is to amplify signaling that is initiated by wounding and elicitors to mount a rapid, strong defense against invaders (37, 38). If *PROPEP1* orthologs (Fig. 5) similarly induce constitutive expression of defense genes when overexpressed in other plant species they may provide an important approach to enhancing innate immunity in a broad spectrum of agriculturally important crops.

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis thaliana* ecotype Columbia seeds were grown in soil in 4-inch square pots for 6 days under low light at ≈18°C followed by growth in day lengths of 16 h at 21°C. Seeds of the *Arabidopsis fad3-2 fad7-2 fad8* triple mutant were provided by John Browse (Institute of Biological Chemistry, Washington State University). *Ein2-1* mutant seeds were obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus.

Alkalinization Assay. *Arabidopsis* suspension cells were grown with shaking in the dark in 125-ml flasks, using 40 ml of NT (*Nicotiana tabacum*) medium. The cells were transferred weekly (2.5 ml) and used for assays 3–5 days after transfer. The alkalinization assay was performed as reported (17, 18). Aliquots of 1–10 μl from extracts or fractions eluted from HPLC columns were added to cells, and the pH of the medium was monitored after 20 min.

Purification of *At*Pep1. *A. thaliana* (Columbia ecotype), 28 days after planting, consisting of rosettes, flowers, stems, and seed pods, were harvested, frozen in liquid nitrogen, ground to a powder, and stored at −20°C. Peptides were extracted from 600 g of powder as described (18, 19) with 1,200 ml of 1% trifluoroacetic acid. The peptides in the clear extract were separated by using a series of HPLC and ion exchange columns, assaying 10–100 μl of each 0.5- to 2.0-ml fraction for activity in the alkalinization assay.

Identification of *PROPEP1* and Homologous Genes. The gene locus encoding the *At*Pep1 peptide precursor was identified by using the National Center for Biotechnology Information (NCBI) TBLASTN version 2.2.7 algorithm (39) to search genomic sequences from *A. thaliana*. Orthologs to *PROPEP1* were identified by using the NCBI TBLASTN version 2.2.7 and The Institute for Genomic Research (TIGR) TBLASTN 2.0MP algorithms (W. Gish, personal communication). The predicted protein sequence for each was aligned by using CLUSTAL W 1.82 (40), available at the European Molecular Biology Laboratory–European Bioinformatics Institute web site (www.ebi.ac.uk/emb).

Peptide Sequence Analysis and Synthesis. N-terminal sequence analysis was performed by using Edman chemistry on an Applied Biosystems Procise model 492 protein sequencer. MALDI-MS was performed on a PerSeptive Biosystems Voyager time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm) with α-cyano-4-hydroxycinnamic acid as the matrix. Peptide synthesis was performed by using *N*-(9-fluorenyl)methoxycarbonyl chemistry by solid-phase techniques using an Applied Biosystems model 431 synthesizer. Synthetic peptides were purified by reversed-phase C18 HPLC. Peptide stocks (250 μM) were assayed for purity, and the mass was verified with a Finnigan (Breman, Germany) LC/Q mass spectrometer using direct injection.

Plant Stress and Hormone Treatments. To examine effects of cold stress, whole plants were placed in a refrigerated growth chamber set to 2°C. To simulate drought stress conditions, plants grown under standard growth chamber conditions were grown without watering. MeJA (Bedoukian Research, Danbury, CT) was applied as a 625 μM solution in 0.1% Triton X-100 to the upper surface of leaves, and the plants were incubated in Plexiglas boxes. Methyl salicylate (Sigma-Aldrich) was applied to leaf surfaces at 2 mM in a 0.1% Triton X-100 (Sigma-Aldrich) solution. Ethephon (Phytotechnology Laboratories, Shawnee Mission, KS) was sprayed on plants as a 7 mM solution in 0.1% Triton X-100. Abscisic acid effects were analyzed by spraying plants with a 100 μM solution (mixed isomer; Sigma-Aldrich) in 0.1% Triton X-100.

Excised-Leaf Assays. *At*Pep1 was dissolved in double-distilled water, which was supplied to excised leaves of 3- to 4-week-old *Arabidopsis* plants. Leaves were excised, and the petioles were immersed in 800-μl microfuge tubes containing either the peptide solution or distilled water and placed in a closed clear Plexiglas box containing a thin layer of water for humidity and a small opening to allow air to enter. Boxes were incubated in a growth chamber under the plant growth conditions described above and sprayed with a fine mist of distilled water every 30 min to ensure humidity and prevent wilting. To determine variations in basal levels of the *PROPEP1* transcript among assays, four different leaves from four different plants were used for each treatment, and leaves supplied with either water or *At*Pep1 were taken from the same plants. Assays were terminated by immersing the leaves in liquid nitrogen.

Hydrogen peroxide accumulation in excised leaves was detected visually by using diaminobenzidine as described (41) by the method of Thordal-Christensen *et al.* (42).

Semiquantitative RT-PCR Analysis of Relative Gene Expression Levels. RNA was isolated by using Trizol reagent according to the manufacturer's instructions (Invitrogen), and 2 μg of RNA template was reverse-transcribed with a RETROscript kit (Ambion, Austin, TX). PCRs were carried out with ExTaq Hot Start polymerase and reagents (Fisher Scientific). The *AtproPep1* forward and reverse primers with the respective sequences of 5′-CTT ATC AGA TCT CAA TGG AGA AAT C-3′ and 5′-CAA TGT AAC TTA AAG TGC CTA ATT ATG-3′ generated a 310-bp intron-spanning product. Primers to β-tubulin (At5g62690) of 5′-CAA CGC TAC TCT GTC TGT CC-3′ and 5′-TCT GTG AAT TCC ATC TCG TC-3′ generated a 681-bp intron-spanning product. An initial denaturing/polymerase activating step of 5 min at 94°C was followed by 31 repetitions of the following three steps: a 30-s denaturation phase at 94°C, a 30-s annealing period at 55.5°C, and a 1-min elongation step at 72°C. The amplification program was terminated with a 10-min final 72°C elongation phase.

The products of each reaction were separated by electrophoresis and visualized on a Bio Imaging System (SynGene, Frederick MD) by using GENESNAP software (SynGene) version 6.00.26. A high-resolution image of the gel was analyzed by using GENETOOLS analysis software version 3.02.00 (SynGene). Relative band intensities for each band were normalized to the β-tubulin band. A numerical ratio of amplified cDNAs to amplified tubulin cDNA was obtained for every sample. To calculate average values, semiquantitative RT-PCR assays were performed in duplicate, and RNA extractions were performed in triplicate.

Transformation of *Arabidopsis* with a CaMV 35S:proAtPep1 Gene. Genomic DNA was isolated from *Arabidopsis* leaves by using the DNAzol reagent (Invitrogen). The genomic sequence encoding PROPEP1 was amplified by using a forward primer (5′-ATA AAG AGT CAC ACC CAA TAC CG-3′) and a reverse primer (5′-TGA TAC TGG TTA TGA ACT TAT GAT GG-3′) to generate a 1,078-bp product. A 5′ XhoI recognition site and a 3′ BamHI site were amplified onto the genomic fragment for

ligation into the pART-7 vector (43). Both the *PROPEP1* genomic product and the pART-7 vector were digested with BamHI and XhoI enzymes (Promega) and ligated by using the LigaFast rapid DNA ligation system (Promega). The construct was transformed into chemically competent *Escherichia coli* TOP10F′ cells (Invitrogen) that were plated out on LB-ampicillin (50 μg/ml). A plasmid clone containing the full *PROPEP1* genomic DNA insert with no nucleotide errors was used to generate an *PROPEP1*/pBART construct. Both pBART and *PROPEP1*/PART-7 plasmid were digested with NotI (Promega) to enable ligation of the CaMV 35S/*PROPEP1* expression cassette into the digested pBART plasmid by using the Promega LigaFast kit. An empty pART-7 vector was digested with NotI to generate a control pBART construct. TOP10F′ chemically competent cells were transformed with the constructs and grown in LB medium containing 100 μg/ml spectinomycin (Sigma-Aldrich), 40 μl of a 40 mg/ml solution of X-Gal (Sigma-Aldrich), and 40 μl of 100 mM isopropyl β-D-thiogalactoside (Sigma-Aldrich) stock. A pBART clone containing the *CaMV 35S/PROPEP1* construct, and a second clone containing the empty vector, were transformed into *Agrobacterium tumefaciens* strain AGLO (44) cells by electroporation by using a BioRad electroporator. The transformed cells were grown on 2× yeast tryptone (YT) medium containing 100 μg/ml spectinomycin, and viable colonies were screened by using RT-PCR with pART F and pART R primers.

Liquid cultures of *Agrobacterium* carrying the *CaMV 35S:PROPEP1* or empty construct were grown in 2× yeast tryptone medium and used for floral dip transformation of *Arabidopsis* plants (45). Transformed plants were grown to maturity, and the seed was collected and planted. Newly germinated seedlings were treated with a 350 μM solution of the herbicide BASTA (glufosinate ammonium, brand name Finale; Farnam, Phoenix) four times at 3-day intervals, and healthy plants were screened for the *PROPEP1* transgene by PCR. Plants that were both glufosinate-resistant and amplified products of the appropriate size were grown to maturity, and the seeds were planted to recover T_2 progeny.

Growth and Inoculation of Plants with *P. irregulare*. The oomycete root pathogen *P. irregulare* (strain 110305) was grown on water-agar (1%) plates for maintenance of stock cultures and, after growing at room temperature in the dark for 1 week, was stored at 4°C. *Pythium* stocks for infection assays were grown on 1× potato dextrose agar (Sigma-Aldrich) in the dark for 1 week at room temperature.

Week-old *P. irregulare* cultures were scraped from the plates into 20 ml of sterile distilled water, and the mixture was lightly ground with a mortar and pestle to produce a uniform suspension. Aliquots (250 μl) of the suspension or water were pipetted into the soil of plants having a rosette diameter of 2–3 cm.

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