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(54) Title: EXTRACELLULAR PLANT FERREDOXIN-LIKE PROTEIN AND USES THEREOF

(57) Abstract: Disclosed are polypeptides, nucleic acids,
and related compositions that render plants resistant to bac-
terial pathogens. Also disclosed are transgenic plants hav-
ing the nucleic acids and resistant to bacterial pathogens.

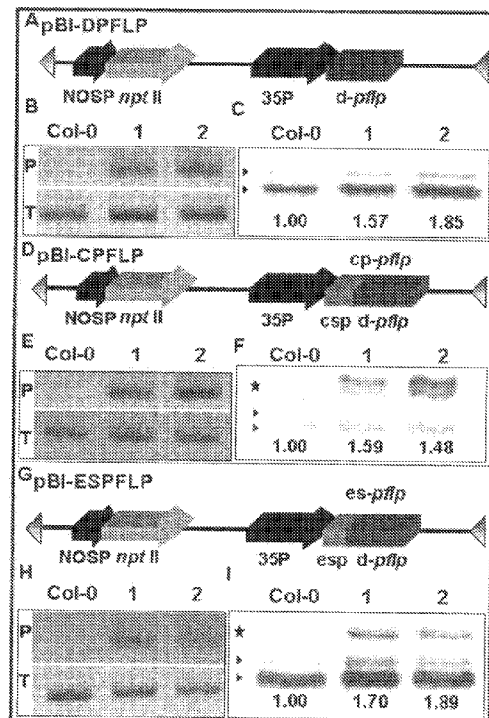


FIG. 1



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NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR,
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Extracellular Plant Ferredoxin-Like Protein and Uses thereof

RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 61/180,563, filed on May 22, 2009, the content of which is hereby incorporated by reference in its entirety.

BACKGROUND

Plant ferredoxin-like protein (PFLP), a photosynthetic type ferredoxin, is a protein associated with the production of active oxygen species and hypersensitive reaction in plants. Over-expression of PFLP renders plants resistant to bacterial pathogens. However, such a resistance is still not sufficient. There is a need for a further enhanced resistance.

SUMMARY

This invention relates to a mutant form of PFLP that enhances plants' resistant to bacterial pathogens. Shown below is the sequence of the full length wild type PFLP (SEQ ID NO: 1) and the nucleotide sequence encoding it (SEQ ID NO: 2).

MASVSATMISTSFMPRKPAVTSLKPIPNVGEALFGLKSANGGKVTCTMASYKV
KLITPDGPIEFDCPDNVYILDQAEFAGHDLPYSCRAGSCSSCAGKIAGGAVD
QTDGNFLDDDQLEEGWVLTCVAYPQSDVTIETHKEAELVG (SEQ ID NO: 1)

atggctagtgtctcagctaccatgattagtagctccttcattgccaagaaaac
cagctgtgacaagccttaaacccatcccaaacggtggggaagcactgtttgg
gcttaaatcagcaaatggtggcaaagtcacttgcattggcttcatacaaagtg
aaacttatcacacctgacggaccaatagaatttgattgcccagataatgtgt
acattcttgatcaagctgaggaagcaggacatgatcttccttattcgtgcag
ggcagggttcttgctcatcttgctgctggtaaaattgctggtggagctgttgat
caaactgatggcaactttcttgatgatgaccaattagaggagggatgggtgc
taacttggtgttgcttatccacagtctgatgttactattgagactcacaaga
ggcagaactcgtgggctaa (SEQ ID NO: 2)

The above PFLP has a chloroplast targeting signal polypeptide (csp) sequence. This csp sequence and corresponding nucleotide sequences are:

MASVSATMISTSFMPRKPAV TSLKPIPNVGEALFGLKSANGGKVTC

(SEQ ID NO: 3) and atggctagtgtctcagctaccatg
 attagtacctcttcatgccaagaaaaccagctgtgacaagccttaaacccatcccaaacgttggggaagcactg
 ttgggcttaaatcagcaaatgggtggcaaagtcacttgc (SEQ ID NO:4). The underlined
 sequences are those that lack the csp, i.e., d-pflp polypeptide and coding
 sequences (SEQ ID NOs: 5 and 6, respectively).

One aspect of this invention features an isolated, fusion polypeptide
 comprising a first segment that contains SEQ ID NO: 5, and a second segment
 that contains the sequence of an extracellular secretion signal polypeptide
 (esp). The polypeptide can be one from a plant, such as a cotton, pepper,
 tobacco, or tomato plant. Once present in a plant, the above-described
 polypeptide enhances disease resistance of the plant against a bacterial
 pathogen. Examples of the pathogen include *Xanthomonas oryzae* pv. *oryzae*,
Erwinia carotovora subsp. *carotovora*, *Ralstonia solanacearum*. Examples of
 diseases caused by these pathogens include bacterial leaf blight in rice,
 bacterial soft rot in tobacco, tomato, calla lily and orchid, and bacterial wilt in
 tomato.

The sequence of the extracellular secretion signal polypeptide can be
 one from any secretory protein. For example, the extracellular secretion signal
 polypeptide can be an esp sequence from sea anemone, such as
 MSLSQNQAKFSKGFVVMWVLFIAACA ITSTEASPM DPR (SEQ ID NO:
 9), which can be encoded by
 atgtctcttagccagaaccaggccaagtttccaagggattcgctcgatgattgggtactattcattgctt
 gtgctatcacttcaactgaagctagtcccatggatccgcgc (SEQ ID NO: 10). Shown below
 are the polypeptide and nucleotide sequences of an exemplary fusion
 polypeptide, es-pflp, having SEQ ID NO: 9 or 10:

MSLSQNQAKFSKGFVVMWVLFIACAITSTEASPM DPR MASYKVKLITPDGP
IEFDPCDDVYILDQAE EAGHDLPYSCRAGSCSSCAGKIAGGAVDQTDGNFLD
DDQLEEGWVLTCVAYPQSDVTIETHKEAELVG (SEQ ID NO: 7)

atgtctcttagccagaaccaggccaagtttccaagggattcgctcgatga
 ttgggtactattcattgcttgtgctatcacttcaactgaagctagtcctcat
 ggatccgcgcgatggcttcatacaaaagtgaacttatcacacctgacggacca
 atagaatttgattgcccagatgatgtgtacattcttgatcaagctgaggaag
 caggacatgatcttccttattcgtgcagggcaggttcttgctcatcttgctgc
 tggtaaaattgctggtggagctggtgatcaactgatggcaactttcttgat
 gatgaccaattagaggagggatgggtgctaacttggtgttgcttatccacagt
 ctgatgttactattgagactcacaagaagcagaactcgtgggctaaggaag
 atctccaataa (SEQ ID NO: 8)

Examples of the above-described polypeptide also include one encoded by a nucleic acid that, under a stringent condition, hybridize to the complement of a reference nucleic acid consisting of SEQ ID NO: 6 or 8.

5 An “isolated polypeptide” refers to a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it is naturally associated. It can be a preparation that contains at least 10% (i.e., any number between 10% and 100%, inclusive) by dry weight the pure polypeptide. Purity can be measured by any appropriate standard method, for
10 example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. An isolated polypeptide of the invention can be purified from a natural source, produced by recombinant DNA techniques, or by chemical methods.

 The invention also features an isolated nucleic acid that contains a
15 sequence encoding one of the above-mentioned polypeptides or a complement thereof. Examples of the nucleic acid include those having SEQ ID NO: 8 as well as those that are at least 70% (e.g., 75%, 80%, 85%, 90%, 95%, 99%, or 100%) identical to SEQ ID NO: 8. Examples also include a nucleic acid that hybridizes to the complement of a reference nucleic acid consisting of SEQ ID
20 NO: 6 or 8 under a stringent condition. This nucleic acid encodes a polypeptide that is expressed extracellularly in a plant and the presence of the polypeptide in the plant enhances disease resistance of the plant against a bacterial pathogen. Such an isolated nucleic acid can encode a polypeptide having an amino acid sequence at least 70- 99% identical to SEQ ID NO:7.

25 The “percent identity” of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci. USA* 87:2264-68, 1990, modified as in Karlin and Altschul *Proc. Natl. Acad. Sci. USA* 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al. J. Mol. Biol.* 215:403-10, 1990. BLAST nucleotide searches can be performed
30 with the NBLAST program, score=100, wordlength-12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50,

wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the invention. Where gaps exist between two sequences, Gapped BLAST can be utilized in the manner described in Altschul *et al.*, *Nucleic Acids Res.* 25(17):3389-3402, 1997. When utilizing BLAST and Gapped
5 BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Stringent hybridization conditions for obtaining nucleic acids encoding functionally equivalent proteins of the above-described polypeptide can be suitably selected by one skilled in the art, and for example, low-stringent
10 conditions can be given. Low-stringent conditions are, for example, 42°C, 2x SSC, and 0.1% SDS, and preferably, 50°C, 2x SSC, and 0.1% SDS. Highly stringent conditions are more preferable and include, for example, 65°C, 2x SSC, and 0.1% SDS. Under these conditions, the higher the temperature, the higher the homology of the obtained nucleic acids will be. However, several
15 factors other than temperature, such as salt concentration, can influence the stringency of hybridization and one skilled in the art can suitably select the factors to accomplish a similar stringency.

A nucleic acid refers to a DNA molecule (e.g., a cDNA or genomic DNA), an RNA molecule (e.g., an mRNA), or a DNA or RNA analog. A
20 DNA or RNA analog can be synthesized from nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic
25 nucleic acid. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a
30 prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a

recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. The nucleic acid described above can be used to express a polypeptide of this invention. For this purpose, one can operatively link the nucleic acid to suitable regulatory sequences to generate an expression
5 vector.

A vector refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. The vector can be capable of autonomous replication or integrate into a host DNA. Examples of the vector include a plasmid, cosmid, or viral vector. The vector of this invention
10 includes a nucleic acid in a form suitable for expression of the nucleic acid in a host cell. Preferably the vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed. A “regulatory sequence” includes promoters, enhancers, and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those
15 that direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vector can be introduced into host cells to produce the polypeptide
20 of this invention.

Also within the scope of this invention is a host cell that contains the above-described nucleic acid. Examples include *E. coli* cells, insect cells (e.g., using baculovirus expression vectors), yeast cells, plant cells, or mammalian cells. To produce a polypeptide of this invention, one can culture
25 a host cell in a medium under conditions permitting expression of the polypeptide encoded by a nucleic acid of this invention, and purify the polypeptide from the cultured cell or the medium of the cell. Alternatively, the nucleic acid of this invention can be transcribed and translated in vitro, for example, using T7 promoter regulatory sequences and T7 polymerase.

30 In another aspect, this invention features a transformed cell containing a heterologous polynucleotide containing a nucleic acid encoding one of the above-described polypeptides. The transformed cell can be made by conventional methods and used to generate a transgenic plant whose genome

comprises a heterologous polynucleotide containing the nucleic acid.

Transgenic plants thus-generated express the above-described polypeptide extracellularly and are resistant to various plant pathogens, such as a bacterial pathogen, including a pathogenic Gram-negative bacterium.

A heterologous polypeptide, nucleic acid, or gene is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. Two fused domains or sequences are heterologous to each other if they are not adjacent to each other in a naturally occurring protein or nucleic acid. In preferred examples, the transgenic plants have genomic DNA comprises the above-described nucleic acids.

The above-described polypeptide can be used in a composition, e.g., a composition which includes agriculturally acceptable carriers, or other agents, to protect plants from pathogens, insects, and other pests. The composition can be formulated and applied by methods described herein or those known in the art protect a plant.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

20 **DESCRIPTION OF DRAWINGS**

FIGs. 1A -1F are diagrams and photographs showing expression constructs and related Western blot results.

FIGS. 2A-2L are photographs showing immuno-localization of PFLP in leaves of transgenic *Arabidopsis* plants.

FIGs. 3A and 3B are diagrams and photographs showing results from Col-0 *Arabidopsis* plants.

FiG. 4 is a group of photographs showing plants having bacterial symptoms.

FIGS. 5C-5D are diagrams and photographs showing results from
30 AtDPFLP transgenic *Arabidopsis* plants.

FIGS. 6C-6D are diagrams and photographs showing results from AtCPFLP transgenic *Arabidopsis* plants.

FIGs. 7C-7D are diagrams and photographs showing results from AtESPFLP transgenic *Arabidopsis* plants.

FIGs. 8C-8D are diagrams and photographs showing results of HrpZ-mediated hypersensitive response (HR) in leaves of transgenic and wild-type *Arabidopsis*.

FIG. 9 is a diagram showing disease index of *Arabidopsis thaliana* plants caused by *Ralstonia solanacearum* Rd4.

FIG 10 is a photograph showing Western blot results of total protein extracted from AtSAP1 (SAP1) and AtESPFLP #1 (ESP1).

DETAILED DESCRIPTION

The present invention relates to plant ferredoxin-like protein (PFLP), a photosynthetic type ferredoxin that is associated with the production of active oxygen species and hypersensitive reaction in plants. As ferredoxin is a protein mediating electron transfer in a range of metabolic reactions, such as those taking place in plant chloroplasts, it was believed that PFLP's function requires chloroplast localization or at least an intracellular localization. Indeed, PFLP contains a signal-peptide at its N-terminal region for targeting to chloroplasts.

Unexpectedly, as will be disclosed below, PFLP induces diseases resistance without being in a chloroplast localization or even inside a cell. This invention is based, at least in part, on this unexpected discovery.

PFLP-dependent disease defense might be mediated by harpin triggered hypersensitive response (HR). The HR is an effective mechanism associated with plant disease resistance during pathogens infection (Mehdy, 1994, *Plant Physiol.* 105:467-472). Harpin is an elicitor protein exported by type III pathway of plant pathogenic *Erwinia*, *Pseudomonas*, *Xanthomonas* spp. and *Ralstonia* spp. (Chen *et al.*, 2008, *Phytopathol.* 98:781-791). Moreover, bacterial harpins are able to trigger plant defense responses (Chen *et al.*, 2008, *Phytopathol.* 98 (7):792-802; Degrave *et al.*, 2008, *Mol. Plant-Microbe Interact.* 21:1076-1086). The HrpZ encoded by *hrpZ* gene which is a harpin from *Pseudomonas syringae* pv. *syringae* can elicit HR in tobacco plants (He *et al.*, 1993, *Cell* 73:1255-1266). In *pflp*-transgenic tobacco plants, PFLP can further enhance the HrpZ-mediated HR (Dayakar *et al.*, 2003, *Plant*

Mol. Biol. 51:913-924). In *pflp*-transgenic tobacco suspension cells, reactive oxygen species (ROS) generation is respond to HrpZ and dependent on membrane bound NADPH oxidase.

As shown below in the example, three different versions of PFLP: a PFLP lacking csp (*d-pflp*), an extracellularly secreted PFLP (*es-pflp*), and a chloroplast-targeting PFLP (*cp-pflp*) were expressed in transgenic Arabidopsis plants. They were targeted to different cellular compartments in the cells of the plants. It was found that transgenic Arabidopsis plants having *d-pflp* and *es-pflp*, but not that with *cp-pflp*, showed disease resistance against *R. solanacearum* strains, and the disease resistance of the transgenic plants are also correlated with the harpin-mediated HR. Most importantly, higher disease resistance was found when PFLP was outside the chloroplast, not inside.

The invention provides nucleic acids and polypeptides, such as *es-pflp*, that render plants enhanced disease resistance against bacterial pathogens. In particular, the nucleic acids can be used to generate transgenic plants.

Polypeptides of this invention include functional variants or functional equivalents of the above-described *es-pflp*, e.g., SEQ ID NO: 8. A functional equivalent of SEQ ID NO: 8 refers to a polypeptide derived from SEQ ID NO: 8, e.g., a fusion polypeptide or a polypeptide having one or more point mutations, insertions, deletions, truncations, or a combination thereof. It is at least 70% (e.g., 75%, 80%, 85%, 90%, 95%, 99%, or 100%) identical to SEQ ID NO: 8. The variants include biologically active fragments whose sequences differ from the *es-pflp* described herein by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions that do not abolish the activity. All of the functional equivalents have substantially the *es-pflp* activity, i.e., the ability to render a plant resistant to a bacterial pathogen. This activity can be determined by the assays described in the examples below or any analogous assays.

The amino acid composition of a polypeptide of the invention may vary without disrupting its activity. For example, such a variant can contain one or more conservative amino acid substitutions. A “conservative amino

acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a polypeptide is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a polypeptide of this invention, such as by saturation mutagenesis, and the resultant mutants can be screened for the activity to identify variants of this invention.

A nucleic acid construct of the present invention can be transformed into a plant cell to produce a desired transgenic plant or plant cell. Methods for transforming plant cells with nucleic acid are routine in the art. Further, the plant cells can be transformed with multiple constructs, e.g., sequentially or concurrently. Depending on the desired physiological and agronomic properties of a plant species, and the nucleic acid construct of the present invention, a target plant or plant cell for transformation can include a species from maize, wheat, rice, soybean, tomato, broccoli, tobacco, sweet pepper, calla lily, orchid, carrots, peanut, potato, sugar beets, sunflower, yam, *Arabidopsis*, rape seed, sunflower, and petunia.

One implementation of the current invention utilizes *Agrobacterium* to introduce the desired construct into plant cells in the manner described in, e.g., U.S. Patent Nos. 5,177,010, 5,104,310, 5,149,645, 5,469,976, 5,464,763, 4,940,838, 4,693,976, 5,591,616, 5,231,019, 5,463,174, 4,762,785, 5,004,863, and 5,159,135; and European Patent Applications 116718, 290799, 320500, 604662, 627752, 0267159, and 0292435). The method can be used with both dicotyledonous plants cells (Bevan *et al.* (1982) *Ann. Rev. Genet.* 16:357-384; Rogers *et al.* (1986) *Methods Enzymol.* 118:627-641), and monocotyledonous

plant cells. (Hernalsteen *et al.* (1984) *EMBO J* 3:3039-3041; Hooykass-Van Slogteren *et al.* (1984) *Nature* 311:763-764; Grimsley *et al.* (1987) *Nature* 325:1677-179; Boulton *et al.* (1989) *Plant Mol. Biol.* 12:31-40.; Gould *et al.* (1991) *Plant Physiol.* 95:426-434). The method can employ binary

5 Agrobacterium T-DNA vectors (Hoekema *et al.* (1983) *Nature* 03:179; Bevan, 1984, *Nuc. Acid Res.* 12:8711-8721), and the co-cultivation procedure (Horsch *et al.*, 1985, *Science* 227:1229-1231).

Additional steps may be required to prepare a desired nucleic acid sequence for plant transformation. For example, in order to utilize T-DNA

10 mediated transformation, the thionin coding sequence, operably linked to a heterologous promoter, is ligated into a binary vector, between the left and right border sequences of T-DNA. The binary vector can further include a gene encoding a selective marker, e.g., an Hph gene coding for hygromycin resistance. The binary vector containing the desired construction is

15 transformed into an *E. coli* strain, e.g., DH5 α . Subsequently, the binary plasmid is transferred into an *Agrobacterium*, e.g., *Agrobacterium* strain LBA4404, using a tri-parental mating.

Other methods for transforming plant cells are available. Of particular utility for transforming monocotyledonous plants or plant cells are methods of

20 protoplast transformation which include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski *et al.*, 1984, *EMBO J* 3:2717-2722, Potrykus *et al.* 1985, *Molec. Gen. Genet.* 199:169-177; Fromm *et al.*, 1985, *Proc. Nat. Acad. Sci. USA* 82:5824-5828; Shimamoto,

25 1989, *Nature* 338:274-276), microinjection, silicon carbide mediated DNA uptake (Kaeppeler *et al.*, 1990, *Plant Cell Reporter* 9:415-418), and microprojectile bombardment (see Klein *et al.*, 1988, *Proc. Nat. Acad. Sci. USA* 85:4305-4309; Gordon-Kamm *et al.*, 1990, *Plant Cell* 2:603-618), whiskers technology (see U. S. Patents Nos. 5,302,523 and 5,464,765), and

30 viral vector systems (see, U.S. Patent Nos. 5,316,931, 5,589,367, 5,811,653, and 5,866,785). A transformed plant or transformed plant tissue can be assayed for resistance to pathogens.

The above-described polypeptides, e.g., es-pflp, d-pflp, and cp-pflp, can be formulated as a composition which is applied to plants in order to confer pathogen resistance. The composition can be prepared in a solution, e.g., an aqueous solution, at a concentration from about 0.005% to 10%, or about 0.01% to 1%, or about 0.1% to 0.5% by weight of polypeptide content. The solution can comprise an organic solvent, e.g., glycerol or ethanol. Alternatively, the composition can be formulated with one or more agriculturally acceptable carriers. Agricultural carriers can include: clay, talc, bentonite, diatomaceous earth, kaolin, silica, benzene, xylene, toluene, kerosene, N-methylpyrrolidone, alcohols (methanol, ethanol, isopropanol, n-butanol, ethylene glycol, propylene glycol, and the like), and ketones (acetone, methylethyl ketone, cyclohexanone, and the like). The formulation can optionally further include a stabilizer, spreading agent, wetting extenders, dispersing agents, sticking agents, disintegrators, and other additives, and can be prepared as a liquid, a water-soluble solid (e.g., tablet, powder or granule), or a paste. The composition can also include other desirable compounds, e.g., protease inhibitors, endotoxins, and the like.

Prior to application, the composition can be combined with another desired composition such as an insecticide, germicide, fertilizer, plant growth regulator and the like. The solution may be applied to the plant tissue, for example, by spraying, e.g., with an atomizer, by drenching, by pasting, or by manual application, e.g., with a sponge. The solution can also be distributed from an airborne source, e.g., an aircraft or other aerial object, e.g., a fixture mounted with an apparatus for spraying the solution, the fixture being of sufficient height to distribute the solution to the desired plant tissues. Alternatively, the composition can be applied to plant tissue from a volatile or airborne source. The source is placed in the vicinity of the plant tissue and the composition is dispersed by diffusion through the atmosphere. The source and the plant tissue to be contacted can be enclosed in an incubator, growth chamber, or greenhouse, or can be in sufficient proximity that they can outdoors.

In another implementation, if the composition is distributed systemically thorough the plant, the composition can be applied to tissues

other than the leaves, e.g., to the stems or roots. Thus, the composition can be distributed by irrigation. The composition can also be injected directly into roots or stems.

5 The specific examples below are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. All publications cited herein are hereby incorporated by reference in their entirety. Further, any mechanism proposed below does not in any way restrict the scope
10 of the claimed invention.

Example 1

Materials and Methods

Plants and bacteria growth condition

15 *Arabidopsis thaliana* ecotype Columbia plants were planted in sterilized soil and grown in a growth chamber at 22°C (16 hour light/8 hour dark). The *Arabidopsis* plants of 4-5 week-old were then used for *Agrobacteria* inoculation. *Escherichia coli* DH5α and *A. tumefaciens* GV3101 were grown in Luria-Bertani (LB) broth or on LB agar plates at 37 °C.
20 Antibiotics were used for selection at the following concentrations: 50 µg/ mL of kanamycin for recombinant plasmids in *E. coli*, and gentamycin for *A. tumefaciens*. All strains of *R. solanacearum* were cultured at 28 °C for 48 hours on a TZC medium containing 1% dextrose, 1% peptone, 0.1% casein hydrolysate, and 0.1% 2,3,5-triphenyl tetrazolium chloride (Kelman 1954,
25 *Phytopathology* 44:693-695).

Construction of the transformation plasmids

One recombinant plasmid pBI-DPFLP (Fig. 1 A), harboring a d-*pflp* DNA fragment in pBI121 (Clontech, U.S.A.), was described in Huang *et al.*, 2006, *Plant Sci.* 171:17-23. In order to make a replacement for the signal
30 peptide of *pflp*, the pBI-DPFLP was used for further construction. The coding sequence of the d-*pflp* DNA fragment was amplified from pBI-DPFLP by polymerase chain reaction (PCR) with the following primers: LS-ap1-sBamHI-F, 5'- CGCGGATCCGCGCATGGCTTCATACAAAGTG -3' and

LS-ap1-SacI-R, 5'- CCATCGGGCTTATGAGATCAGAGCTC -3'. PCR was carried out under the following conditions: 94 °C, 10 minutes for initial denaturation; followed by 30 cycles of 94 °C, 1 minute; 60 °C, 45 seconds; and 72 °C, 1 minute with 72 °C, 10 minutes for final extension. The PCR product was digested with *Bam*HI and *Sac*I and purified after agarose gel electrophoresis. The PCR product was digested with *Bam*HI and *Sac*I, and purified from the gel after agarose gel electrophoresis. The purified *d-pflp* fragment was constructed into ImpactVectorTM 1.2 Secreted expression and 1.4 Chloroplast expression (Wageningen UR, Netherlands) harbouring a chloroplast signal peptide (*csp*) from chrysanthemum and an extracellular secretion signal peptide (*esp*) from sea anemone (Outchkourov *et al.*, 2003, *Plant Physiol.* 133:379-390; Outchkourov *et al.*, 2003, *Planta* 216:1003-1012), respectively. Two constructions were performed by in-frame fusion of *d-pflp* fragment to the *csp* and *esp* to generate a *cp-pflp* and *es-pflp* fragment in recombinant plasmids pIVC-PFLP and pIVES-PFLP.

The *cp-pflp* DNA fragment was then amplified with the following primers: LS-ap1-CP-XbaI-F, 5'- TCTAGAATGGCCTCGATCTC -3' and LS-ap1-SacI-R. The PCR product was digested with *Xba*I and *Sac*I, and the *cp-pflp* DNA fragment was inserted into pBI121 to generate the plasmid pBI-CPFLP (Fig. 1 D). Similarly, the *es-pflp* DNA fragment was amplified with the primer LS-ap1-ES-XbaI-F, 5'- TCTAGAAACCATGTCTCTTAGCCAGAAC -3' and LS-ap1-SacI-R. The *es-pflp* DNA fragment was constructed into pBI121 to generate the plasmid pBI-ESPFLP (Fig. 1 G). These three plasmid vectors, pBI-DPFLP, pBICPFLP and pBI-ESPFLP, were transformed into *A. tumefaciens* GV3101. The inserted fragments of *d-pflp*, *cp-pflp* and *es-pflp* were sequenced and compared to the sequences on Genebank (AF039662).

Generation and selection of transgenic Arabidopsis

Transformation of *Arabidopsis thaliana* ecotype Columbia (Col-0) was performed using *Agrobacterium tumefaciens* containing the recombinant plasmid pBI-DPFLP, pBI-CPFLP and pBI-ESPFLP described above. The *nptII* gene in the vector allows the kanamycin selection of transgenic plants.

Transgenic seeds were screened according to a rapid screening method for transgenic *Arabidopsis* seeds (Harrison *et al.*, 2006, *Plant Methods* 2:19) on 1/2 Murashige and Skoogs medium (1/2 MS) containing kanamycin. Seedlings were then transplanted in the pots containing sterilized soil. The T₁ transgenic lines were confirmed by PCR with a forward primer for 35S (Huang *et al.*, 2007, *Phytopathol.* 97:900-906), and a reverse primer PBI-IndR (5'-CCCAGTCACGACGTTGTAAA-3'). Individual T₂ plants from each line were self-pollinated, and seeds collected. T₃ transgenic plants were identified with PCR to define whether T₂ transgenic plant is a homozygous line.

10 *Molecular characterizations of transgenic Arabidopsis lines*

Genomic DNA was isolated from leaf tissue of each transgenic *Arabidopsis* T₁ plant using a plant genomic DNA purification kit (Geneaid, Taiwan). The coding sequence of *d-pflp*, *cp-pflp* and *es-pflp* were amplified by PCR in the manner described above using with a 35S and PBI-IndR. Total RNA was isolated from fresh leaf tissue of four-week-old tobacco plants using a plant total RNA kit (Viogene, Taiwan). For RT-PCR analysis, first-strand cDNA was synthesized using 1st Strand cDNA synthesis Kit for RT-PCR [AMV] (Roche, Germany) from 1 µg of total RNA, and 1 µl of the cDNA was subjected to PCR in a 20 µl reaction volume with primers LS-ap1-RTF (5'-CACACCTGACGGACCAAT-3') and LS-ap1-RTR (5'-CGAGTTCTGCTTCTTTGTG-3'). The *tubulin* gene was used as a positive control using primers Tub2AF and Tub2AR (Lee *et al.*, 2000, *Arabidopsis. Genes Dev.* 14:2366-2376). The subsequent PCR amplification used the same conditions described above, and the RT-PCR products were analyzed by electrophoresis on a 1.5 % agarose gel. For Western-blot analysis, the total protein was extracted from four-week-old leaf tissues. The tissue (100 mg) was homogenized with a steel ball in 500 µL of an extraction buffer (100 mM NaCl, 50 mM Tris-HCl and 1 mM PMSF at pH7.5), and the amount of total protein was quantified with a BCA protein assay kit (Thermo, U.S.A.). Protein extracts at 3µg of total protein were fractionated on 15% SDS-polyacrylamide gels, and the separated proteins were then transferred to Immobilon-P PVDF membrane (Millipore, USA). Western blot was performed using a rabbit polyclonal antiserum specific for the PFLP protein as

primary antibody (1:2000) and HRP-conjugated goat anti-rabbit IgG (Rockland, USA) as secondary antibody.

Disease severity assay

A disease soil method was used to evaluate wilt symptom on Col-0 plants after inoculation with four *R. solanacearum* strains isolated from radishes (Rd4 and Rd15) and tomatoes (Ps95 and Ps152). Four-week old Arabidopsis (each plant had eight leaves) were grown in growth chambers at 25 °C for disease severity assay of bacterial wilt disease. The colony of each *R. solanacearum* strain tested in this study was picked and enriched on a TZC basal medium for 48hours at 28 °C, and then the cells were washed from plates in distill water and adjusted the bacterial suspension to OD₆₀₀ of 0.3. The bacterial suspension of each *R. solanacearum* strain was mixed with 10-folds volume of soil mixture for disease soil preparation. Arabidopsis plants were then planted in pots containing disease soil, and the wilt symptoms were recorded for four weeks after inoculation. Disease index of wilt disease on Arabidopsis was exhibited by weeks post-inoculation and rated the scales 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, 6: death). And, then the numbers of plants with different scales of wilt disease were obtained as N₀-N₆. Disease index for each trial was calculated by the following formula: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6) / (6 \times N)] \times 100\%$ (Winstead *et al.*, 1952, *Pseudomonas solanacearum*. *Phytopathology* 42:628-634). In this assay, twelve Arabidopsis plants were tested of each *R. solanacearum* strain or each transgenic line.

Confocal Microscopy

Localization of PFLP expressed in transgenic plants was performed by confocal laser microscopy observation as described (Padham *et al.*, 2007, *Arabidopsis*. *Plant Physiol.* 143:1372-1384). Leaves were cut from 3-4 week-old Arabidopsis plants, and the leaf strips (2 mm width) were soaked for 2 hr in 50% ethanol at 4 °C, washed for 30 min with phosphate buffered saline (PBS) twice at room temperature, treated with 1 % Cellulase R10 (Yakult,

Japan) for 1 hr and followed by 1 % Triton X-100 in PBS at room temperature for 1 hr to permeabilize the tissue. For immuno-staining, of leaf strips were then gently shaken overnight at room temperature in the presence of the primary antibody (1:50) in PBS containing 1% bovine serum albumin (BSA).

5 After leaf strips were washed twice in PBS, strips were probed with FITC-conjugated goat anti-rabbit antibody (KPL, USA; 1:80 in PBS containing 1% BSA) for 2 h in the dark at room temperature. Leaf strips were washed twice with PBS and placed on slides for observation under a Zeiss LSM 510 confocal laser-scanning microscope.

10 *HrpZ preparation and HR assay*

A recombinant plasmid pSY10 harbouring the *hrpZ* gene (HrpZ) was maintained in *Escherichia coli* DH5 α . Protein extraction and infiltration of HrpZ were carried out as described in Ger *et al.*, 2002, *Mol. Plant-Microbe Interact.* 15:764-773. *E. coli* strain was cultured overnight in LB broth

15 containing ampicillin (50 μ g/ ml) at 37 °C with shaking in the presence of isopropylthio- β -D-galactoside. To obtain HrpZ, the bacteria were washed and sonicated in a 25 mM phosphate buffer (pH 7.5) for 30 seconds, and then boiled for 10 minutes. After boiling, the protein extracts were centrifuged at 10,000 $\times$ g for 10 minutes, and the supernatants were stored at 4 °C.

20 Arabidopsis leaves were punctured with a 25-gauge needle to form tiny holes on the leaf surface and the then infiltrated with HrpZ by pressing a 1 ml blunt syringe through the holes. For ROS observation, leaves were cut and further infiltrated with 5 mM 3,3'-diaminobenzidine (DAB) at pH3.8 by vacuum for 20 minutes, and then incubated at room temperature for 4 hours. Chlorophyll

25 was removed with ethanol immersion followed by boiling the leaves in water for 10 minutes. The brown product was detected after reactions of DAB, indicating the H₂O₂ accumulation (Fryer *et al.*, 2002, *J. Exp. Botany* 53:1249-1254). After infiltrating for over 24 hours, HR necrosis could also be

30 observed. We calculated the HR ratio in percentage by observation of the ratio of necrosis appeared around six inoculation sites in three independent leaves of one plant, and ten plants of transgenic Arabidopsis lines were tested for replicates in this assay.

Results

Characterization of transgenic lines

To analyze where PFLP functions in Arabidopsis cells to enhance disease resistance, we constructed three different versions of PFLP in pBI121-derived recombinant plasmid, the *pflp* gene with signal peptide deleted (*d-pflp*), with signal peptide replaced with chloroplast targeting peptide (*cp-pflp*), and with signal peptide replaced an extracellular secreted peptide (*es-pflp*) (Fig. 1). Each construct was then transformed into individual Arabidopsis by *Agrobacterium*-mediated transformation to generate transgenic Arabidopsis plants, AtDPFLP, AtCPFLP and AtESPFLP. No apparent morphological differences were observed between transgenic and non-transgenic plants. AtDPFLP (#1-8 and #6-7), AtCPFLP (#2-1 and #11-3) and AtESPFLP (#1-19 and #2-9) transgenic lines were selected to detect the presence of *d-pflp*, *cp-pflp* or *es-pflp* transcripts by RT-PCR, respectively (Fig. 1 B, E, H). Neither recombinant DNA nor its transcripts of *d-pflp*, *cp-pflp* or *es-pflp* were detected in non-transgenic Arabidopsis plant (Col-0).

Protein from leaves of T₂ transgenic plants and Col-0 plant were then extracted and probed with anti-PFLP antibody by Western blot. In Col-0 plants, two endogenous ferredoxin isoproteins with 17 and 19 KDa respectively were detected by PFLP antibody. In all selected transgenic plants, the same immunological signals at 17 and 19 KDa were also detected, with intensities increased by 157 % in AtDPFLP line # 1-8, 185 % in AtDPFLP line # 6-7, 159 % in AtCPFLP line # 2-1, 148 % in AtCPFLP line # 11-3, 170 % in AtESPLP line # 1-19, and 189 % in AtESPLP line # 2-7, when compared to the signal from Col-0 plants (Fig. 1 C, F, I). Two additional signals at 23 and 24 KDa translated from *cp-pflp* were also detected in AtCPFLP lines # 2-1 and # 11-3 (Fig. 1 F), and one extra immunological signal at 22 KDa translated from *es-pflp* was detected in both AtESPFLP lines # 1-19 and # 2-7 (Fig. 1 I). These results demonstrated that engineered *d-pflp*, *cp-pflp* and *es-pflp* genes were all successfully expressed in the T₂ generation of these transgenic Arabidopsis plants. The mRNA and protein of *d-pflp*, *cp-pflp* and *es-pflp* genes were also detected in T₃ transgenic Arabidopsis plants.

Immuno-localization of PFLP

We examined the localization of PFLP in different cellular compartments with FITC-conjugated antibodies which recognized PFLP. We found in the cells of Col-0 plants (as control materials), the FITC signals in the chloroplast were weak, indicating that the endogenous ferredoxins in the chloroplast could be detected by PFLP antibody (Fig. 2 I). In the cells of AtDPFLP plants, the FITC signals appeared outside the chloroplasts besides the endogenous signals (Fig. 2 J). The result indicated that PFLP was existed in the cytoplasm. In the AtCPFLP cells, the FITC fluorescent images superimposed on the chlorophyll autofluorescence were stronger than that in Col-0 plant, indicating that the PFLP was localized in the chloroplasts (Fig. 2 K). In AtESPFLP plants, obvious FITC signals were detected in the extracellular space, indicating that the PFLP was secreted outside the cells (Fig. 2 L). These results demonstrated that the engineered PFLP targeted to designated cellular compartments.

Bacterial wilt disease on Arabidopsis plants

To evaluate the efficacy of engineered PFLP against bacterial wilt diseases on Arabidopsis, a bioassay system was developed. When Arabidopsis was inoculated with *R. solanacearum*, bacterial wilt disease would appear two weeks post inoculation (wpi). Four different strains, Rd4, Rd15, Ps95 and Ps152, to Col-0 and all AtDPFLP, AtCPFLP and AtESPFLP transgenic plants were inoculated, and then the numbers of plants with bacterial wilt symptoms during 4 wpi were obtained. The disease index was then calculated by dividing the number of wilting leaves to total inoculated plants. The higher the index, the less resistant the plants have against the bacterial pathogens.

The disease index on Col-0 wild type plants was examined first. After having inoculated Rd4, Rd15 and Ps152 on Col-0 plants, we obtained the disease index of 80.1 %, 93.9 % and 93.3 %, respectively at 4 wpi. However, we observed a slower development of disease caused by Ps95 and also calculated a lower disease index 58.5 % at 4 wpi (Fig. 3). We then challenged the transgenic plants, and observed that the developments of wilt symptom caused by these strains tested were varied. We compared two strains, Rd4 and Rd15, and found that both of these two strains could cause whole plant death at 4 wpi. Rd15 caused wilt symptom on non-transgenic Col-0 plant at 2wpi

and plant death rapidly (Fig. 4). Then, we obtained the disease indexes of two T₃ AtDPFLP transgenic plants, #1-8 and #6-7, which retain PFLP in the cytoplasm. When these lines were inoculated with Ps95, the disease indexes were 34.8 %, 26.2 % at 4 wpi, respectively, which were lower than that of Col-0 wild type plants (56.1 %) (Fig. 5 A). The disease indexes caused by Rd4 and Ps152 were around 20 % at 2 wpi which was lower than that of Col-0 40 % to 60 % at 2 wpi. At 4 wpi, the disease indexes of AtDPFLP #1-8 and #6-7 lines were only approximately half (43.1 % to 52.6 %) of those in Col-0 plants (81.0 % to 95.2 %) (Fig. 5 B, C). These results demonstrated the resistance of AtDPFLP lines was sustained upon challenges by these three *R. solanacearum* strains Rd4, PS95 and Ps152. However, AtDPFLP lines only exhibited resistance in the first two weeks after inoculation of strain Rd15 (#1-8, 19.5 %; #6-7, 26.3 %; and Col-0, 39.2 % at 2 wpi), and then the wilt symptom developed in the following two weeks rapidly (#1-8, 76.4%; #6-7, 90.2 %; and Col-0, 95.1 % at 4 wpi) (Fig. 5 D). Rd15 could cause lethality to AtDPFLP and Col-0 plants at 4 wpi (Fig. 4).

In contrast, the two AtCPFLP T₃ plants #2-1 and #11-3 which import PFLP to the chloroplast did not show lower disease indexes when compared to Col-0 plants: caused by Ps95, #2-1, 38.9 % and 11-3, 43.0 % (Fig. 6 A); by Rd4, #2-1, 86.1 % and #11-3, 77.8 % (Fig. 6 B); by Ps152, #2-1, 88.9 % and #11-3, 87.5 % (Fig. 6 C); and by Rd15, #2-1, 98.1 % and #11-3, 98.1 % (Fig. 6 D). Notably, the wilt symptoms on transgenic AtCPFLP plants caused by Rd4 or Rd15 developed as rapidly as those on Col-0 plants at 4 wpi (Fig. 4).

We then test if PFLP is functioning as a secreted protein in the transgenic AtESPFLP T₃ plants (#1-19 and #2-9) which are transformed with *es-pflp*. It was found that both AtESPFLP plants had lower diseases indexes compared to those of Col-0 plants at 4 wpi, caused by Ps95, #1-19, 21.2 % and #2-9, 25.0 % (Fig. 7 A); by Rd4, #1-19, 51.5 % and #2-9, 43.9 % (Fig. 7 B); by Ps152, #1-19, 35.4 % and 2-9, 44.4 % (Fig. 7 C); and by Rd15 #1-19, 43.3 %; #2-9, 50.0 % (Fig. 7 D). These results indicated the AtESPFLP plants that secreted PFLP to extracellular matrix had strong resistance against bacterial wilt pathogens.

Hypersensitive response induced by HrpZ in transgenic plants

Disease resistance against pathogens can be activated by harpin-mediated HR, and this effect was intensified in *pflp*-transgenic tobacco plants. The effects of different targeting PFLP in pathogen resistance can thus be measured by the degree of HR induced by HrpZ. We infiltrated the wild type Col-0 plants and the AtDPFLP, AtESPFLP and AtCPFLP transgenic plants with HrpZ and calculated the HR ratio by measuring the level of H₂O₂ accumulation produced by NADPH oxidase. We first used DAB as substrates to trace the level of H₂O₂. We found that dark brown colored areas were displayed in the leaves of AtDPFLP and AtESPFLP plants while no obvious appearance could be detected in those of AtCPFLP and Col-0. These results showed the strong H₂O₂ accumulation in AtDPFLP and AtESPFLP plants, but not AtCPFLP and Col-0 plants. We then measured the HR ratio by counting the necrosis appeared around inoculation sites of the plant leaves. The HR ratios of AtDPFLP lines were 65.1 % to 69.4 %, and AtESPFLP lines were 82.1 % to 80.9 %, respectively, compared to those in the AtCPFLP lines (23.8 % to 31.6 %) and Col-0 (26.3 %) at 24 hours after infiltration (Fig. 8 B). These results showed the HR was intensified in AtDPFLP and AtESPFLP plants, but not AtCPFLP and Col-0 plants.

The bacterial wilt disease, caused by *Ralstonia solanacearum*, is an important disease of many crops and other plants. The ecotype Col-0 was susceptible to strains Ps95, Rd4 and Rd15. By employing the same rating procedure we found that the virulence of three strains of *R. solanacearum* were classified as strong virulent strains (Rd4, Rd15 and Ps152), and one (Ps95) was a weak strain. These four strains could be used to evaluate different levels of resistance obtained from transgene. The AtDPFLP and AtESPFLP transgenic lines described above were resistant to all of the four *R. solanacearum* strains tested. As PFLP also rendered resistance to bacterial soft rot caused by *E. carotovora* subsp. *carotovora* (Huang *et al.*, 2006, *Plant Sci.* 171:17-23), it is expected that the above-described heterologous *d-pflp* or *es-pflp* gene could also protect plants against a wide spectrum of bacterial pathogens.

The PFLP-enhanced plant disease resistance is associated with harpin-mediated HR. HrpZ elicits ROS generation to induce HR in an NADPH-oxidase-dependent manner in *pflp*-transgenic tobacco plants. In Arabidopsis, this is not only evident in the case that the extracellular secreted PFLP in
5 AtESPFLP plants was able to enhance HrpZ-mediated HR and increase disease resistance, but also in other case that neither HR nor disease resistance was enhanced in AtCPFLP plants. It was unexpected that AtESPFLP lines were more sensitive to HrpZ and had a higher resistance against the strong virulent strain Rd15 than AtDPFLP lines. Besides, the PFLP also exhibits
10 antimicrobial activity against bacterial pathogen (Huang *et al.*, 2006, *Plant Sci.* 171:17-23). We suggested the extracellular PFLP provided antibacterial activity and higher sensitivity response to bacterial elicitor would increase more disease resistance against bacterial pathogens than that of cytoplasmic PFLP.

15 In *pflp*-transgenic tobacco cells, PFLP exists in both chloroplasts and cytoplasm, no matter the transgene containing authentic signal peptide sequence or not (Dayakar *et al.*, 2003, *Plant Mol. Biol.* 51:913-924; Huang *et al.*, 2006, *Plant Sci.* 171:17-23), suggesting that the signal peptide of PFLP from pepper has low efficiency of chloroplast targeting in tobacco. Here we
20 demonstrated that in *pflp*-Arabidopsis the PFLP with no signal peptide would exist in the cytoplasm. While the transgene contains the chloroplast targeting signal peptide (*csp*) of RbcS, the expressing PFLP could be delivered into chloroplasts effectively and faithfully in transgenic Arabidopsis plants (Fig. 2, K). The RbcS precursor protein imports into chloroplast of pea followed by
25 two-step cleavage. This explains why PFLP expressed in transgenic Arabidopsis plants with *cp-pflp* had two additional protein bands with 23 and 24 KDa, respectively (Fig. 1, F). When the signal peptide of PFLP was replaced by a typical eukaryotic-secreted signal peptide, the PFLP was observed in the extracellular space. The *esp* signal peptide sequence helps
30 target protein for extracellular secretion through endoplasmic reticulum.

Leaf type ferredoxins are well known as electron transfer proteins in the photosynthetic system of chloroplast, and PFLP is similar to the leaf type ferredoxin. In addition, PFLP can enhance ROS generation and harpin-

mediated HR. ROS generation is related to the membrane bound NADPH oxidase which is encoded by *rboh* gene. Here, the expression of PFLP outside the chloroplast did enhance disease resistance and intensify the harpin-mediated HR. Since PFLP can enhance resistance against bacterial pathogens, our study provides advanced evidence that it is only achieved while PFLP is outside the chloroplast. The ectopic PFLP outside the chloroplast does not process electron transfer in photosynthesis. Instead, it could be responsible for harpin-mediated ROS generation.

Example 2

In order to evaluate the effect of transgenic plants with ES-PFLP or PFLP on disease resistance, two transgenic plants AtESPFLP and AtSAP were challenged with bacterial wilt pathogen *Ralstonia solanacearum* Rd4. The AtSAP express PFLP with original signal peptide. The disease indexes of AtESPFLP (#1: 51.5 %, #2: 42.2 %) and AtSAP1 (66.7 %) plants indicate that they were more resistance against *R. solanacearum* Rd4 than wild type (Col-0: 80.1 %). Moreover, AtESPFLP plants showed 2.4-folds more resistance than AtSAP1 plants (Fig. 9).

Total protein from AtSAP1 (1.6 and 1.2) and AtESPFLP #1 (2.1 and 1.0) plants were extracted, and subject to Western Blot analysis in the same manner described above. The results indicated the averaged fd signals detected by Western blot were similar (Fig. 10).

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising
a first segment that contains SEQ ID NO: 5, and
a second segment that contains the sequence of an extracellular
secretion signal polypeptide.
2. The polypeptide of claim 1, wherein the polypeptide is derived
from a plant.
3. The polypeptide of claim 2, wherein the plant is a cotton,
pepper, tobacco, or tomato plant.
4. The polypeptide of claim 1, wherein the polypeptide comprises
SEQ ID NO:7.
5. The polypeptide of claim 1, wherein the polypeptide enhances
disease resistance of a plant against a bacterial pathogen.
6. The polypeptide of claim 5, wherein the plant is a cotton,
pepper, tobacco, or tomato plant.
7. The polypeptide of claim 1, wherein the polypeptide is encoded
by a nucleic acid that hybridize to the complement of a reference nucleic acid
consisting of SEQ ID NO: 6 or 8 under a stringent condition.
8. The polypeptide of claim 1, wherein the sequence of an
extracellular secretion signal polypeptide comprises SEQ ID NO: 9.
9. An isolated nucleic acid encoding the polypeptide of claim 1.
10. An isolated nucleic acid that hybridizes to the complement of a
reference nucleic acid consisting of SEQ ID NO: 6 or 8 under a stringent
condition, wherein the nucleic acid encodes a polypeptide expressed

extracellularly in a plant and wherein the presence of the polypeptide in the plant enhances disease resistance of the plant against a bacterial pathogen.

11. An isolated nucleic acid encoding a polypeptide comprising an amino acid sequence at least 70- 99% identical to SEQ ID NO:7, wherein the polypeptide is expressed extracellularly in a plant and wherein the presence of the polypeptide in the plant enhances disease resistance of the plant against a bacterial pathogen.

12. The nucleic acid of claim 9, 10, or 11, wherein the nucleic acid comprises the sequence of SEQ ID NO: 8.

13. A vector containing the nucleic acid of claim 9, 10, or 11.

14. A transformed host cell containing the nucleic acid of claim 9, 10, or 11.

15. A transgenic plant that expresses an extracellular polypeptide, wherein the polypeptide is the polypeptide of claim 1, and expression of the polypeptide in the transgenic plant enhances disease resistance of the plant against a bacterial pathogen in the transgenic plant.

16. A transgenic plant whose genomic DNA comprises the nucleic acid of claim 9, 10, or 11.

17. The transgenic plant of claim 12 or 13, wherein the bacterial pathogen is pathogenic Gram-negative bacterium.

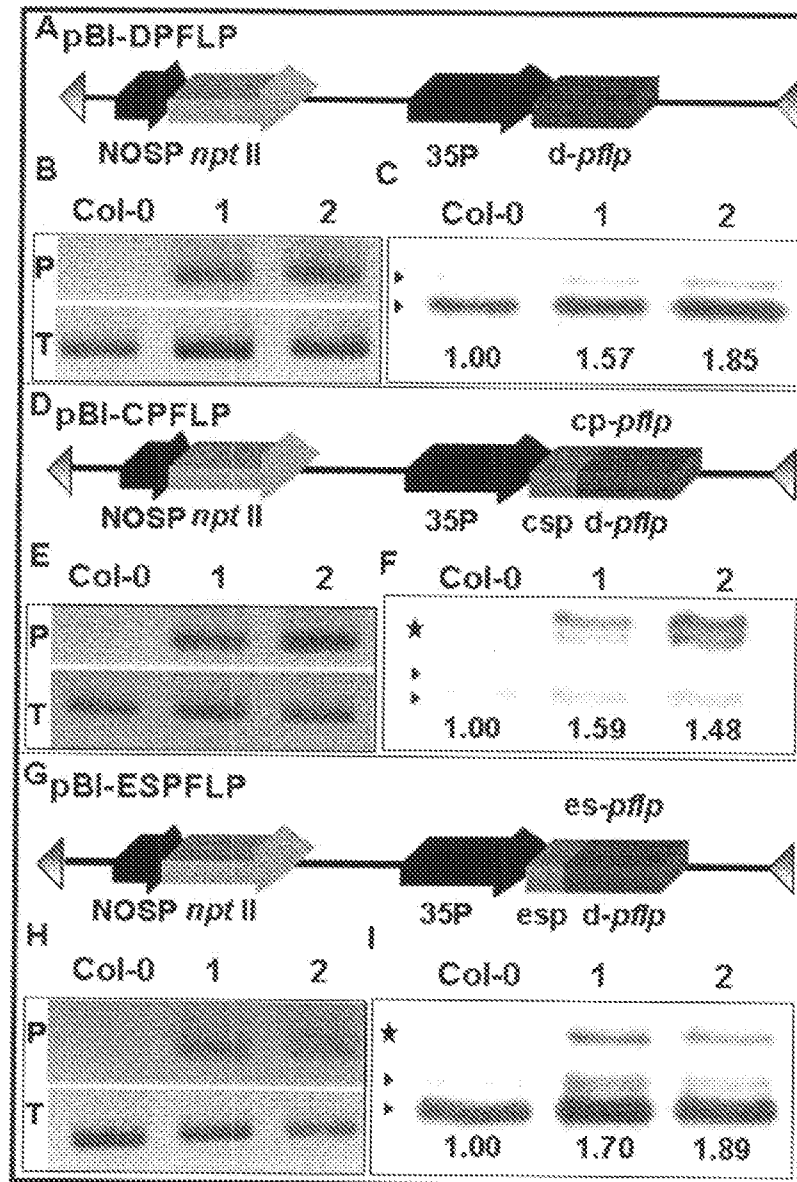


FIG. 1 Molecular characterization of transgenic *Arabidopsis thaliana* lines containing *d-pflp*, *cp-pflp* or *es-pflp* DNA fragment. A, D and G: Schematic diagrams of T-DNA region in plasmids pBI-D-PFLP, pBI-C-PFLP and pBI-E-PFLP harbouring *d-pflp*, *cp-pflp* and *es-pflp* fragments, respectively. NOS promoter (NOSP) was used to drive kanamycin resistance gene (*nptII*) as a selection maker for transformation, and CaMV 35S promoter (35P) was used to drive the target gene (*d-pflp*) or it in-frame fused to a signal peptide sequence (*cp* or *esp*) for chloroplast targeting or extracellular secretion. B, E and H: RT-PCR revealed the presence of mRNA transcripts of two transgenic *Arabidopsis* lines AtDPFLP (1: line #1-8; 2: line #6-7), AtCPFLP (1: line #2-1; 2: line #11-3) and AtESPFLP (1: line #1-19; 2: line #2-9) containing *d-pflp*, *cp-pflp* and *es-pflp* constructs, respectively. Letter P in the upper panels of B, E and H shows the presence of a 0.25 Kb band of the *pflp* gene. Letter T in the lower panels indicates the presence of a 0.5 Kb band for the positive control of a *tubulin* gene in all samples. C, F and I: Western blots using anti-PFLP antibody showing the presence of PFLP at 17 and 19 KDa bands (arrowheads), and extra signals at 22-24 KDa (star) in leaf extracts of AtCPFLP and AtESPFLP *Arabidopsis* lines. The proteins were probed with anti-PFLP antiserum, and the numbers at the bottom indicate folds of fluorescence intensity as compare to the non-transgenic Col-0 plants.

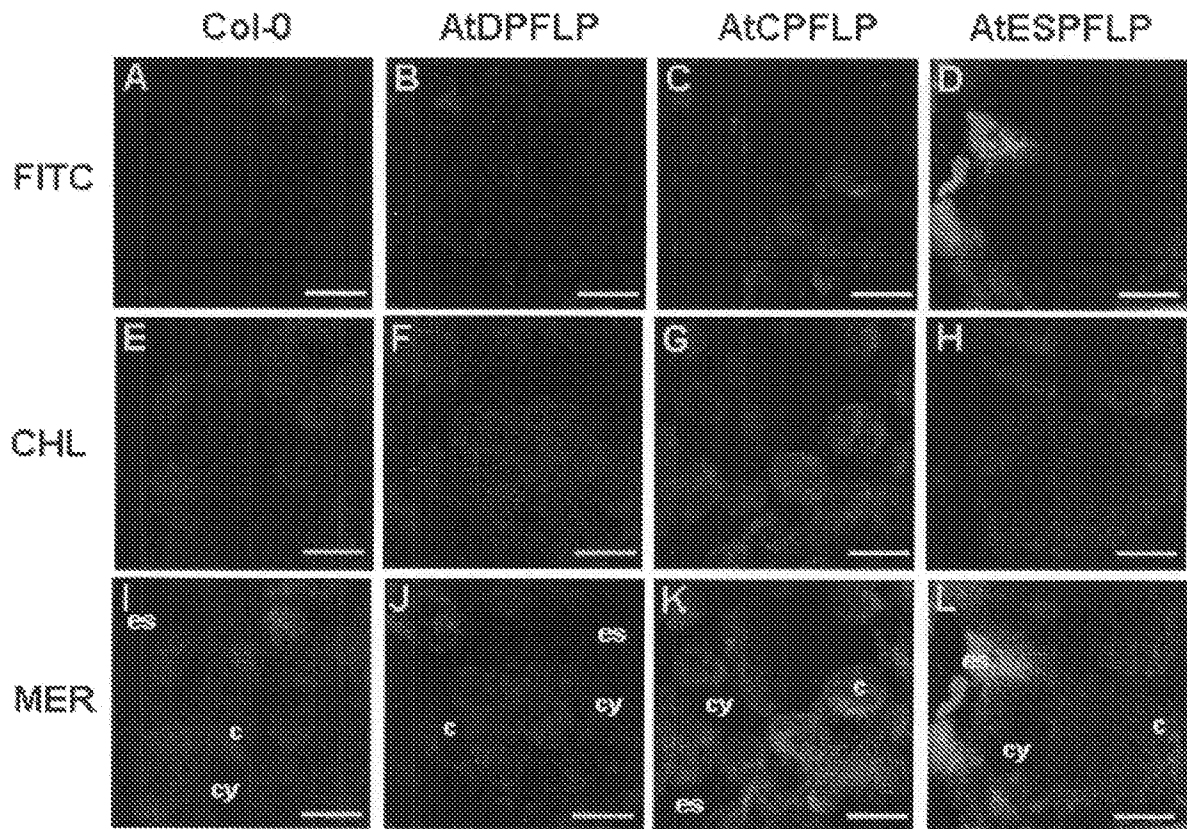


FIG. 2 Immuno-localization of PFLP in the leaves of transgenic *Arabidopsis* plants was observed by confocal laser microscopy. Images of AtDPFLP, AtCPFLP, AtESPFLP and Col-0 were obtained from five-week-old plants. FITC, the green pseudocolor indicates the existence of PFLP. CHL, the red pseudocolor indicates the autofluorescence of chloroplast. MER, the merged image of FITC and CHL shows the localization of PFLP. Different cellular compartments were labeled in merge images (c, chloroplast; cy, cytoplasm; and es, extracellular space). Bar indicates 5 μ m.

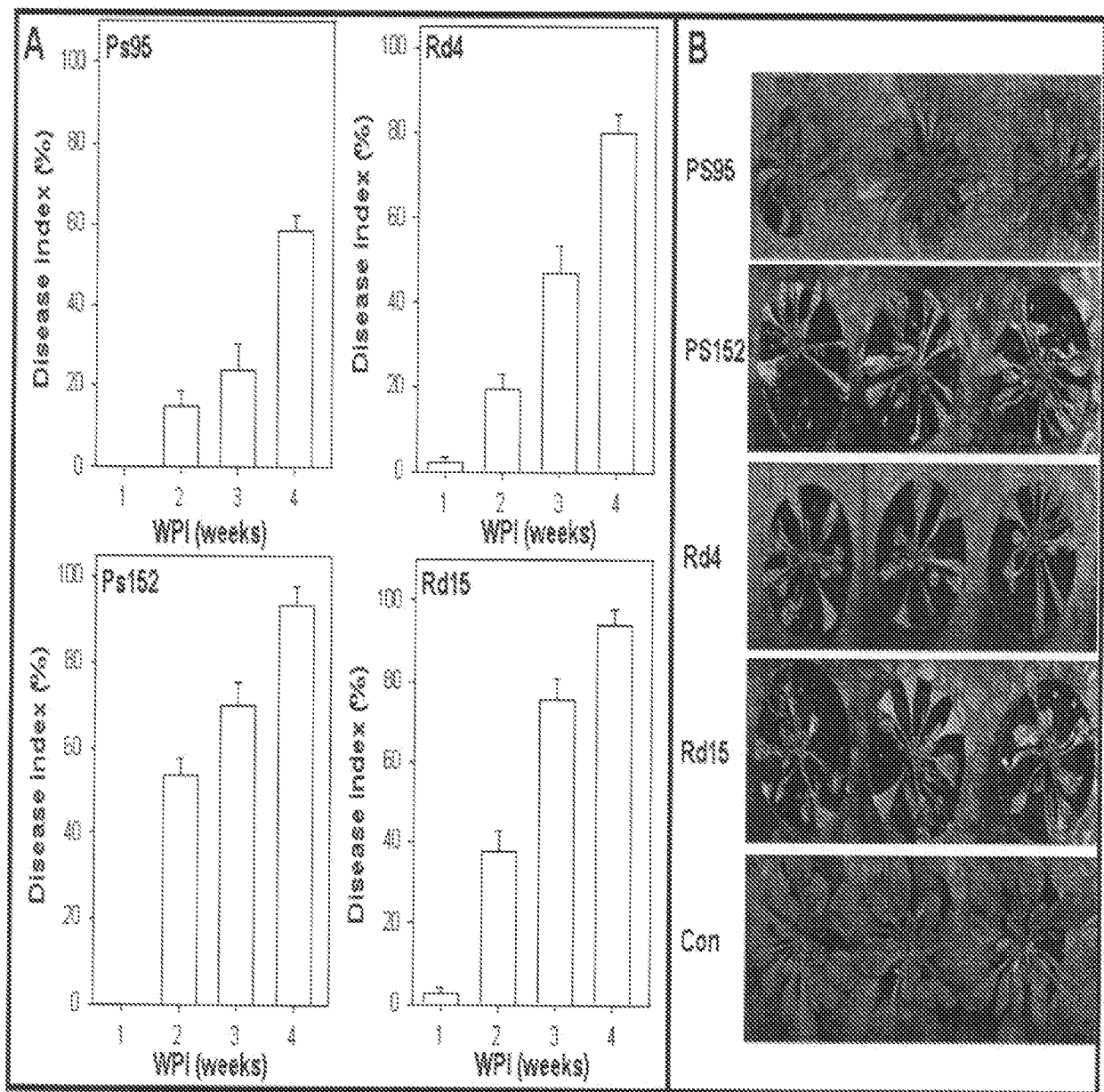


FIG. 3 Disease index and bacterial wilt symptom of Col-0 *Arabidopsis* plants caused by different strains of *Ralstonia solanacearum*. Four-week-old plants were transplanted into soil prepared by mixing with bacterial suspension of different *R. solanacearum* strains (Ps95, Rd4, Ps152 and Rd15). Panel A shows the progressive changes of disease indexes by weekly. Error bars indicate standard deviation of the mean of twelve individual plants. Panel B shows the different severity levels of bacterial wilt symptom four weeks post-inoculation. Distilled water was used as the negative control (Con) in these experiments.

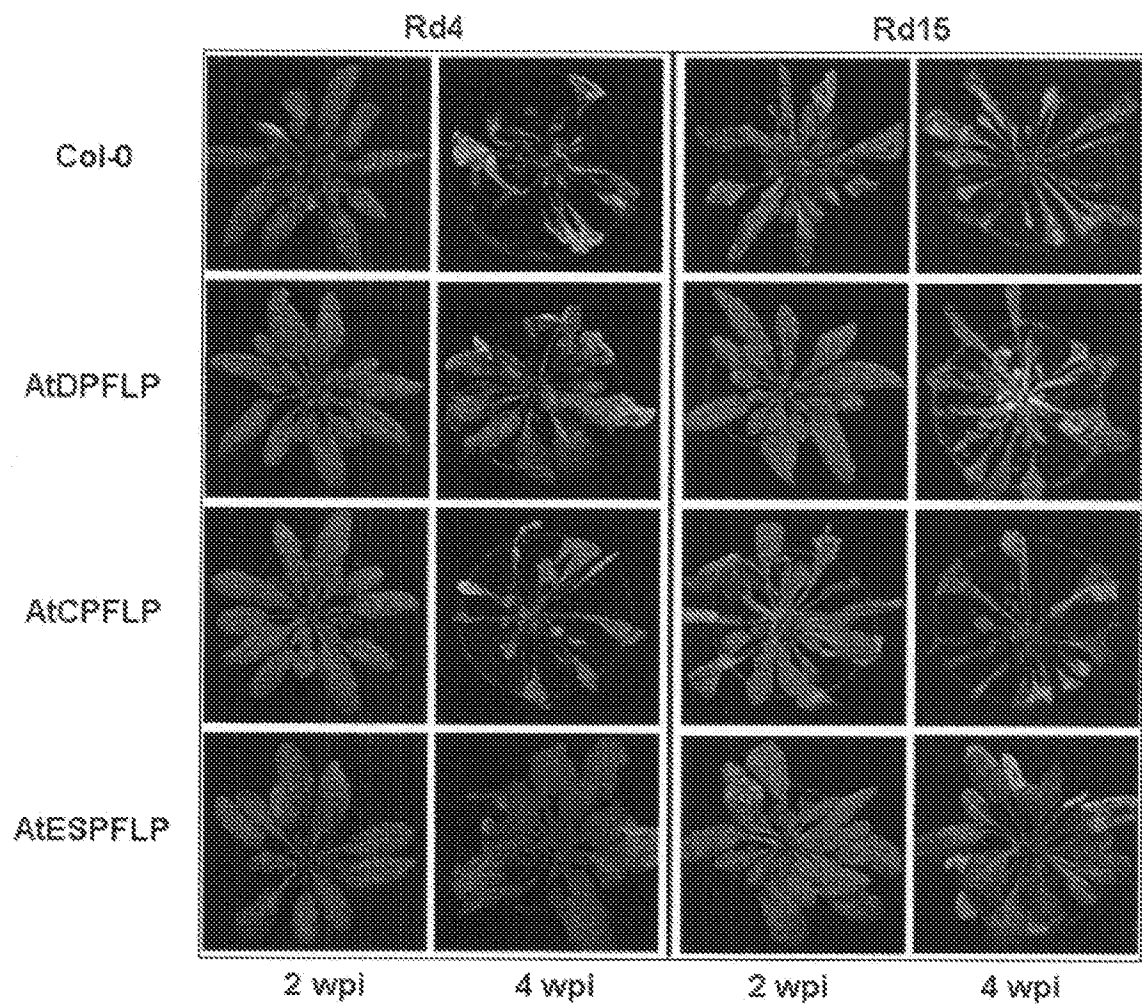


FIG. 4 Bacterial wilt symptoms appeared on transgenic *Arabidopsis* plants caused by two *R. solanacearum* strains. Four-week-old plants were transplanted into soil prepared by mixing with the bacterial suspension. Photos show development of wilt symptom caused by Rd4 and Rd15 strains on AtDPFLP, AtCPFLP and AtESPFLP transgenic and non-transgenic Col-0 *Arabidopsis* plants at two and four weeks post-inoculation.

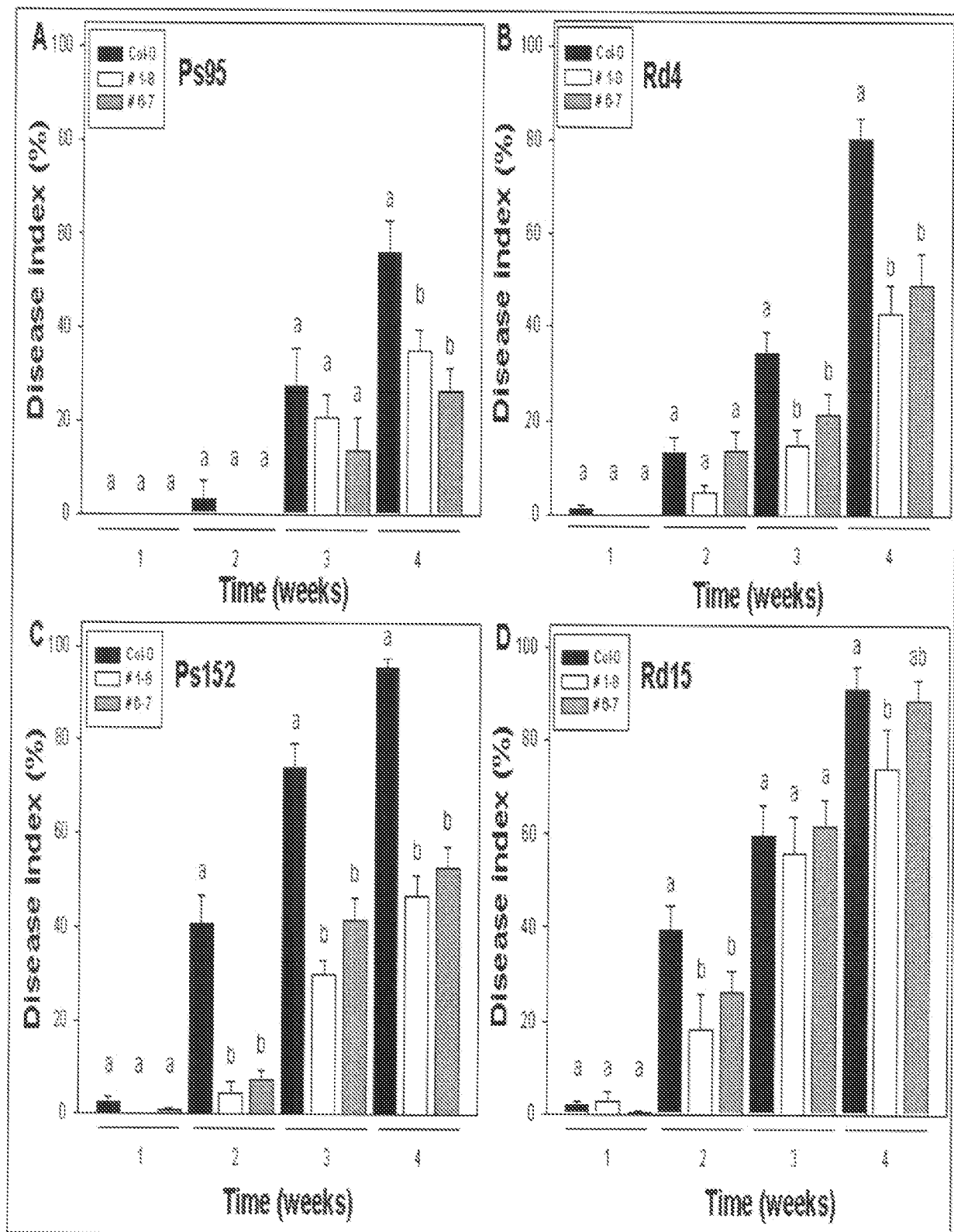


Fig. 5 Disease index of AtDPFLP transgenic Arabidopsis plants. Four-week old Arabidopsis plants were inoculated with different *R. solanacearum* strains (Ps95, Rd4, Ps152 and Rd15). Disease indexes were monitored as described in Fig. 3. Error bars indicate standard deviation of the mean for twelve individual plants inoculated by each strain. Letters in the figures indicate the significant differences based on the Duncan's multiple range test ($P < 0.05$).

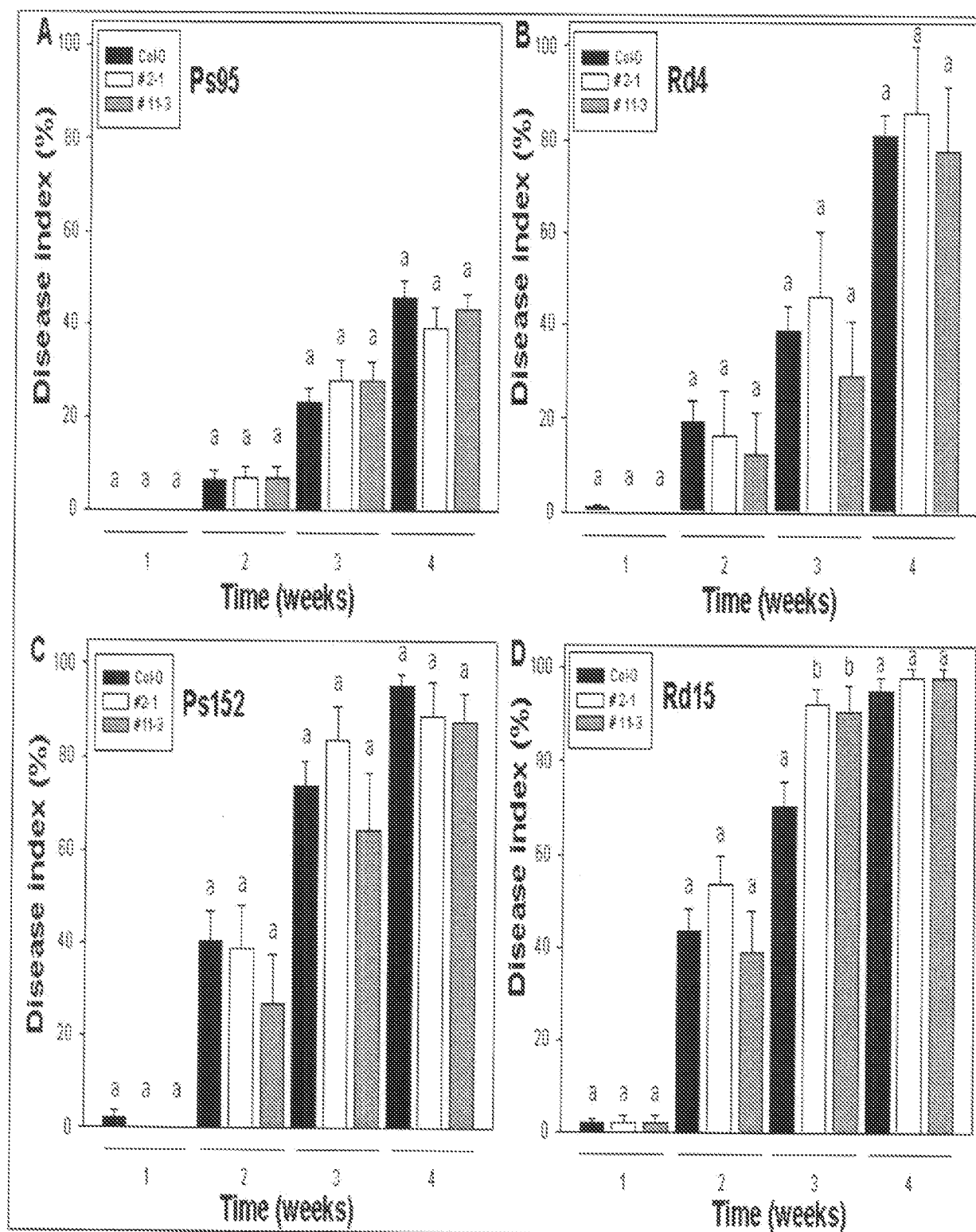


FIG. 6 Disease index of AtCPFLP transgenic Arabidopsis plants. Four-week old Arabidopsis plants were inoculated with different *R. solanacearum* strains (Ps95, Rd4, Ps152 and Rd15) were monitored as described in Fig. 3. White bars represent line #2-1, gray bars represent line #11-3, and black bars represent the Col-0. Error bars indicate standard deviation of the mean for twelve individual plants inoculated by each strain. Letters in the figures indicate the significant differences based on the Duncan's multiple range test ($P < 0.05$).

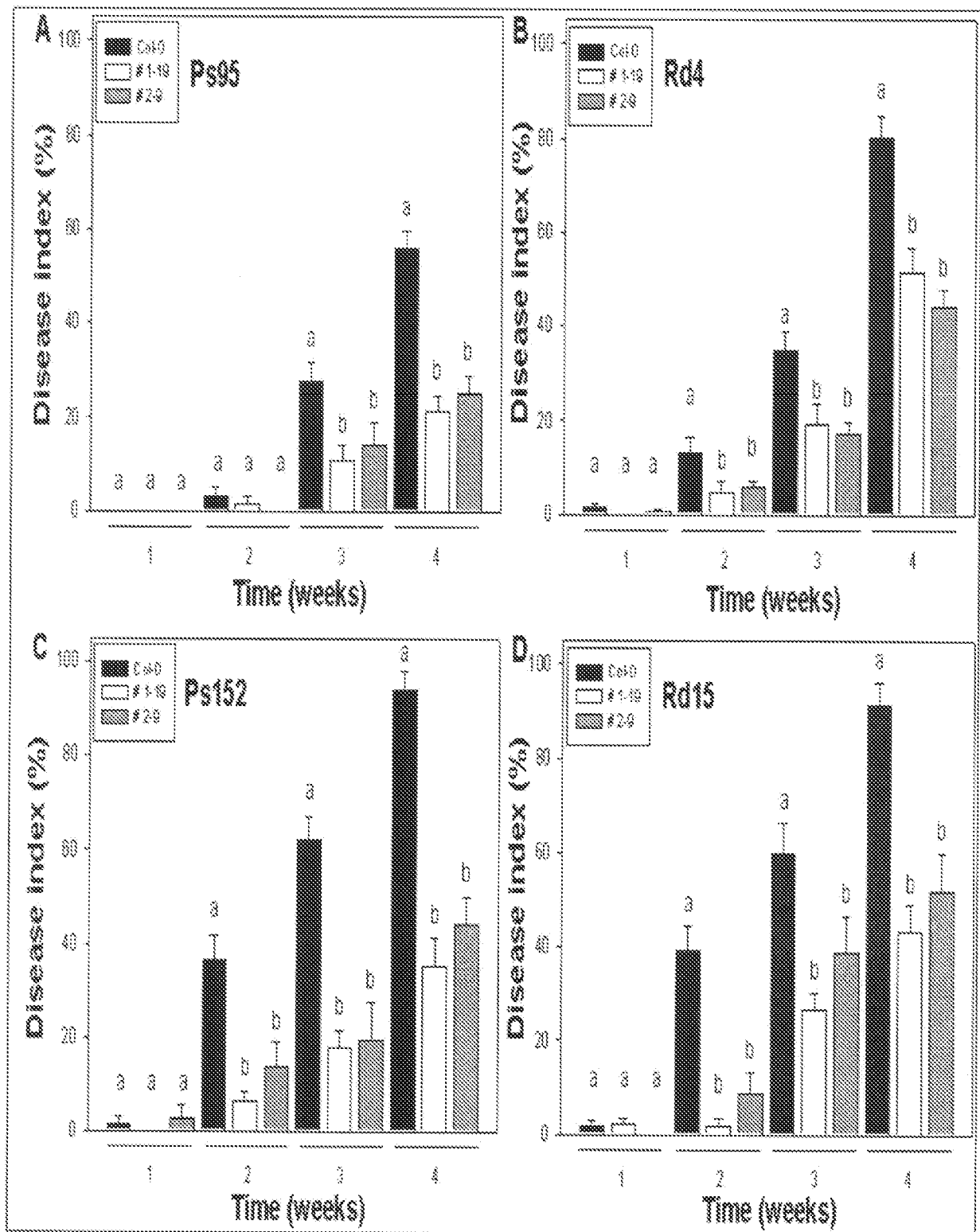


FIG. 7 Disease index of AtESPFLP transgenic Arabidopsis plants. Four-week old Arabidopsis plants were inoculated with different *R. solanacearum* strains (Ps95, Rd4, Ps152 and Rd15) and the progressive changes in bacterial wilt symptom were monitored as described in Fig. 3. White bars represent line #1-19, gray bars represent line #2-9, and black bars represent the Col-0. Error bars indicate standard deviation of the mean for twelve individual plants inoculated by each strain. Letters in the figures indicate the significant differences based on the Duncan's multiple range test ($P < 0.05$).

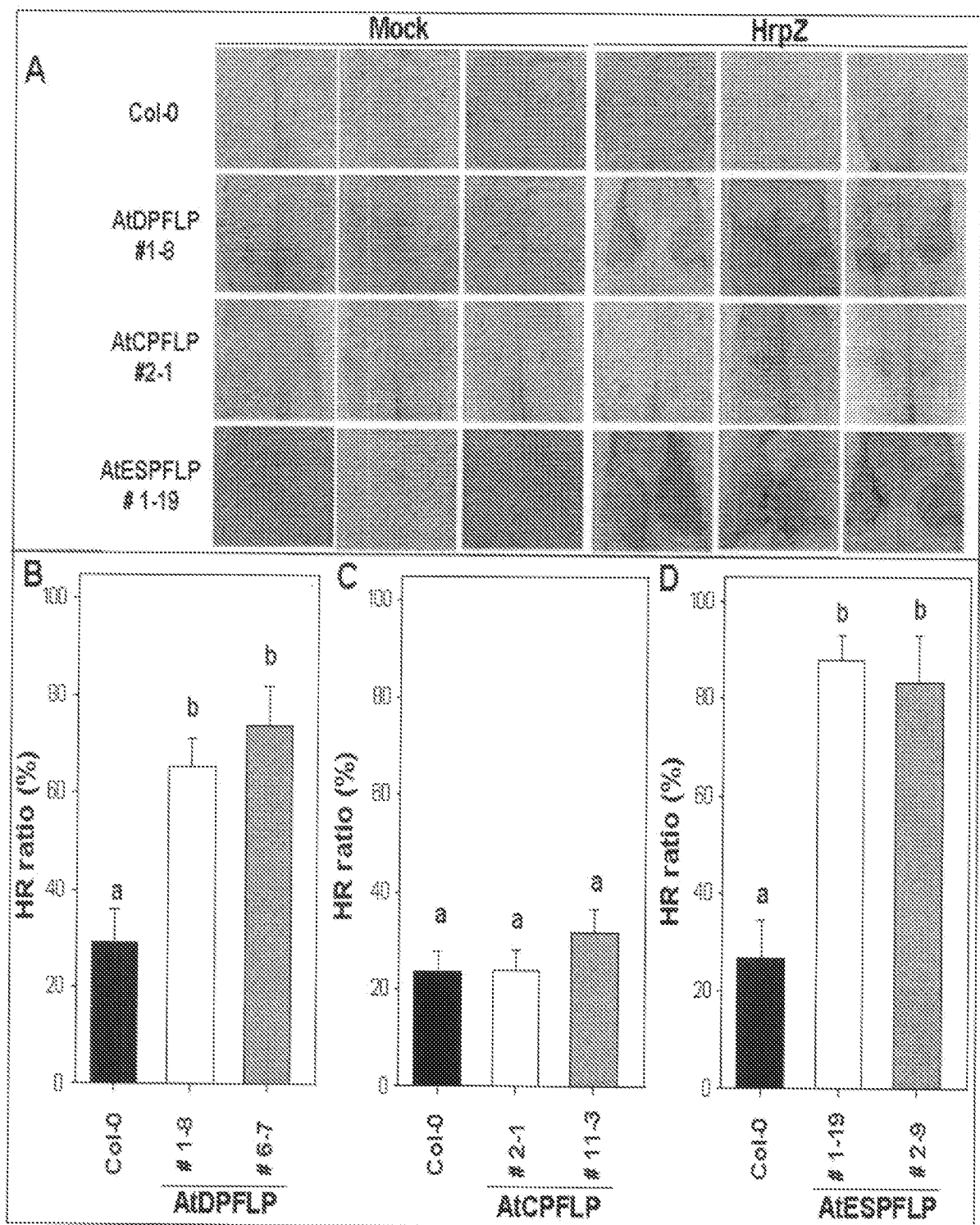


FIG. 8 The HrpZ-mediated hypersensitive response (HR) was tested in the leaves of transgenic and wild-type *Arabidopsis*. The HrpZ recombinant protein prepared from *E. coli* was infiltrated into the leaves of transgenic *Arabidopsis*. HR necrosis was observed after 24 hr post-inoculation and the HR ratio were calculated for each line of the plants. Panel A shows the H₂O₂ accumulation revealed by DAB staining. Panels B, C and D indicate the ratio of HR necrosis appeared on leaves. (Details are described in materials and methods).

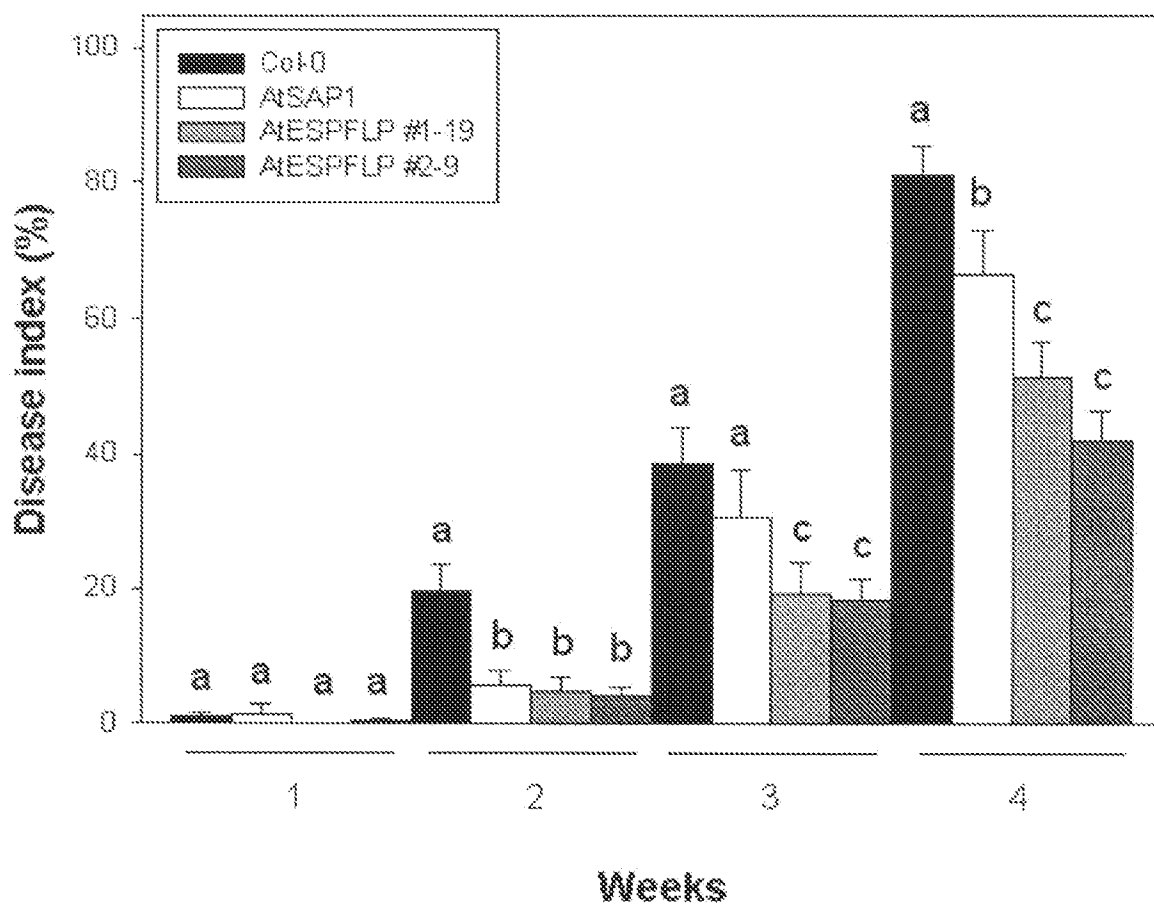


FIG. 9 Disease index of *Arabidopsis thaliana* plants caused by *Ralstonia solanacearum* Rd4. Four-week-old *Arabidopsis* plants were transplanted into a soil that was prepared by mixing at 1:10 ratio (v/v) of bacterial suspension ($OD_{600} = 0.3$) of Rd4. White bars represent AtSAP1, gray and twilled gray bars represent AtESPFLP line #1 and #2, and black bars represent the Col-0. Error bars indicate standard deviation of the mean for twelve individual plants inoculated by each strain. Letters in the figures indicate the significant differences based on the Duncan's multiple range test ($P < 0.05$).

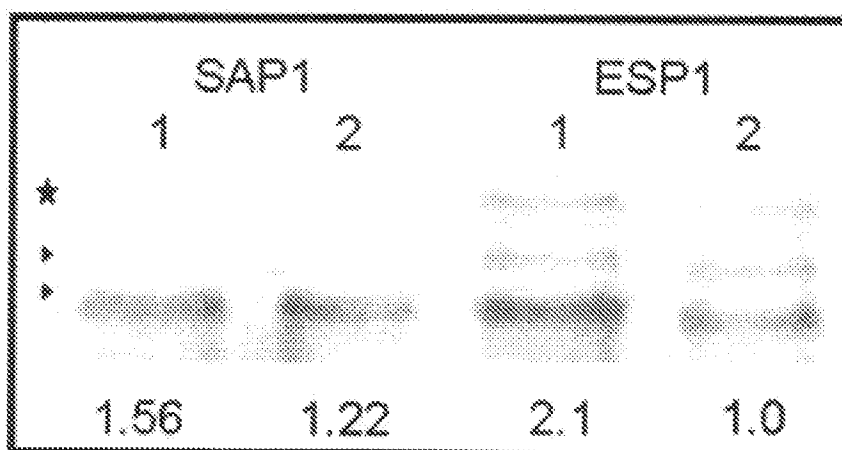


FIG. 10 Total protein extracted from AtSAP1 (SAP1) and AtESPFLP #1 (ESP1) were detected by Western blots. The proteins were probed with anti-PFLP antiserum showed the presence of PFLP at 17 and 19 KDa bands (arrowheads), and extra signals at 22 KDa (star) in leaf extracts of AtESPFLP Arabidopsis lines.