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Enhanced Resistance against Soil-borne Diseases by Root-Specific Expression of J1-1 in Transgenic Tobacco Plant

조선대학교 대학원

생명과학과

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J1-1이 뿌리특이적으로 과발현된 담배 형질전환 식물체의 토양전염병에 대한 저항성 증진

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ABSTRACT

Enhanced Resistance against Soil-borne Diseases by Root-Specific Expression of J1-1 in Transgenic Tobacco Plant

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Rhizoctonia, Phytophthora and *Pythium* species are soil-borne pathogens that infect mainly plant roots and stems and damage plants in both agro- and natural ecosystems. To suppress the devastating pathogen, we generated a root-specific expression system using a root-specific promoter (pPRP3) conferring elevated expression of the target gene in roots that are very susceptible to soil-borne pathogens. To verify root-specific expression, we compared β-glucuronidase (*GUS*) expression driven by a constitutive or root-specific promoters in shoots and roots. In histochemical and fluorometric assays, GUS activity was detected in whole tobacco plants when GUS expression was driven by p35S, but was detected only in the roots by pPRP3. We then expressed a *J1-1* (pepper defensin) gene in tobacco to elucidate its effect on plant resistance. The accumulation of J1-1 was also tissue-specific in transgenic tobacco plants.

Finally, transgenic plants carrying *GUS* or *J1-1* genes under the regulation of p35S or pPRP3 were inoculated with *Rhizoctonia solani, Phytophthora parasitica* and *Pythium aphanidermatum*. Disease symptoms were significantly suppressed in transgenic plants that accumulated J1-1, regardless of the promoter used. Furthermore, the expression of *PR* genes was induced in *J1-1* transgenic plants, exhibiting much higher levels in p35S-driven *J1-1* plants than in pPRP3::*J1-1* plants.

These results demonstrated that *J1-1* transgenic plants were primed for enhanced expression of *PR* genes, which provided synergistic effects with the defensin for disease resistance.

국문초록

J1-1이 뿌리특이적으로 과발현된 담배 형질전환 식물체의 토양전염병에 대한 저항성 증진

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Rhizoctonia, Phytophthora, Pythium 속의 종들은 농업생태계와 자연생태계에서 주로 식물의 뿌리와 줄기를 감염시켜 식물에 손상을 입히는 토양병원균으로 알려져 있다. 본 연구에서는 병저항성 단백질로 알려진 *J1-1* 유전자가 뿌리 특이적으로 과발현된 담배 형질전환체를 제작하여 토양병원균에 대한 저항성을 증진시키고자 하였다. 뿌리 특이적 발현을 확인하기 위하여 비특이적으로 발현하는 35S 프로모터와 뿌리 특이적 프로모터인 PRP3를 이용하여 지상부와 뿌리에서 β-glucuronidase (*GUS*)의 발현을 비교하였다. 식물체 조직에서 단백질 분석 및 형광분석 방법을 이용하여 GUS의 발현을 확인한 결과 35S 프로모터에 의한 GUS 발현은 담배형질전환 식물체 전체에서 활성이 확인되었으며, PRP3 프로모터에 의한 GUS 발현은 뿌리에서만 확인되었다.

J1-1 유전자가 식물 토양균의 저항성에 미치는 영향을 밝히기 위해 담배에서 p35S 와 pPRP3 프로모터로 *J1-1* 유전자를 발현시켰다. 형질전환 담배에서 J1-1 의 축적 역시 조직 특이적으로 확인되었다. *GUS* 또는 *J1-1* 유전자가 35S 또는 PRP3 프로모터의 조절에 의해 발현되는 형질전환 식물체에 토양균인 *Rhizoctonia solani, Phytophthora parasitica* 그리고 *Pythium aphanidermatum* 를 접종한 결과 *J1-1* 이 축적된 형질전환 식물체에서는 프로모터에 상관없이 토양균 감염이 효과적으로 억제되었다. 뿐만 아니라 *J1-1* 형질전환 식물체에서는 병저항성과 관련된 *PR* 유전자의 발현이 유도되었으며, *PR1, 2, 10* 유전자의 발현은 pPRP3::*J1-1* 식물체 보다 p35S::*J1-1* 식물체에서 높게 나타났다. 이 결과는 *J1-1* 형질전환 담배에서 *J1-1* 유전자의 과발현과 *PR* 유전자의 발현이 증가되어 토양병원균에 대한 저항성을 더욱 상승시키는 것으로 보여진다.

I. INTRODUCTION

Defensins are a large class of antimicrobial peptides (AMP) that are widely distributed in various living organisms, from microorganisms to complex eukaryotes (Chen et al. 2012; De Smet and Contreras 2005). Plant defensins are a family of evolutionarily related peptides that possess a cysteine-stabilized α/β motif (CSαβ) (Cornet et al. 1995; Thomma et al. 2002). The main biological function of plant defensins is to inhibit the growth of a broad range of phytopathogenic fungi (Lacerda et al. 2014).

A pepper defensin, J1-1, has been previously isolated from the fruit of bell pepper, *Capsicum annuum* (Meyer et al. 1996). The expression of *J1-1* gene was found to occur during the ripening and after wounding of the fruit, suggesting a role in protecting the fruit against biotic and abiotic stresses. *In vitro* antifungal assay has shown that J1-1 protein effectively suppressed mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea.* Additionally, recombinant J1-1 protein inhibited the appressorium formation of *Colletotrichum gloeosporioides* (Seo et al. 2014).

The expression of defensins is regulated in various tissues in a constitutive, differential, or inducible manner (De Coninck et al. 2013). In particular, the expression of defensins appears to be associated with the defense against biotic stresses as part of the immune response. Plants respond to the onset of pathogen attack by inducing antimicrobial proteins active against the phytopathogen; however, the induction is often too weak to protect the plants from the invading pathogens. In this regard, biotechnological engineering of defensins would be beneficial to enhance plant tolerance toward phytopathogens. A transgenic

approach has been made to produce defensin protein in model and crop plants. Due to selective toxicity of the defensin, the transgenic plants can offer a solution for yielding crops that are resistant to a wide range of phytopathogens (Lacerda et al. 2014). In the first field trial, overexpression of alfalfa defensin (alfAFP) in transgenic potato resulted in field resistance of the plants against *Verticillium dahliae* (Gao et al. 2000). In hot pepper, the constitutive expression of *J1-1* in transgenic plants confers enhanced resistance against *C. gloeosporioides*, resulting in reduced fungal colonization and lesion formation in infected fruits (Seo et al. 2014). However, overexpression of defensins only occasionally has provided a useful level of disease control *in planta*. Indeed, the resistance of engineered plants largely depends on the level and the location of transgene expression in plants (Vain et al. 1999). Fungal behavior also has a strong influence on plant responses during phytopathogen interaction (Rostás et al. 2003). Thus, a detailed analysis of transgene products is necessary to improve disease resistance in transgenic plants. To date, most transgenic plants carrying defensins employed constitutive promoters, such as the cauliflower mosaic virus 35S or ubiquitin promoter. Although constitutive promoters offer high level of gene expression in virtually all plant cells, there is a need for specific expression systems to utilize the defensin more efficiently in the plant cells under pathogen attack.

Root rot diseases are caused by soil-borne pathogens including *Rhizoctonia, Phytophthora,* and *Pythium* species (Adhikari et al. 2013 and Seethapathy et al. 2017). They produce symptoms of a decaying root system, sometimes followed by the wilting and death of leaves as well as the whole plant. *Rhizoctonia solani* is soil-borne basidiomycete fungus and is classified into fourteen anastomosis groups (AGs) based on hyphal fusion (Anderson 1982; Budge et al. 2009; and Rhonda et al. 2016). Because *Rhizoctonia solani* generally do

not produce asexual spores and basidisopore production is rarely seen in nature, these fungi are identified by their hyphae characters (Parmeter 1970; Gonzalez et al. 2011). *R. solani* is known to cause plant diseases that root and stem rot, damping-off, stunting of plant growth and eventually lead to plant death (Tewoldemedhin et al. 2006; Seethapathy et al. 2017). The most of this pathogen initiated adhesion, penetration and colonization by mycelium or sclerotia and activated to produce vegetative hyphae that can attack a wide range of crops (Gonzalez et al. 2011). *Rhizoctonia solani* AG2-1 belong to multinucleate *Rhizoctonia* and has a wide host range (Sneh et al, 1991). *R. solani* AG2-1 was isolated from canola, lupin, medic, and wheat (Tewoldemedhin et al. 2006). In particular, *Phytophthora* and *pythium* species cause destructive diseases in a wide variety of vegetables, fruits, and grains. *Phytophthora parasitica* var. *nicotiana* and *Pythium aphanidermatum* is classified as oomycetes that fungus-like and cause root rot, stem rot, seedling blight of many plants (Mitchell 1978). These pathogens has two life cycles accoding to deffering enviromental conditions (West et al. 2003). The motile zoospores are produced from sporangia and the major infective agents for *P. parasitica* and *P. aphanidermatum* (Kamoun 2003; Raftoyannis et al. 2006; Meng et al. 2014). The zoospores reach leaf or root surface, produce germination tube, form appressorium structures, which can cause infection (Hardham 2001; West et al. 2003).

Previously, a transgenic approach found that constitutive expression of defensins in plants reinforced host resistance to root rot diseases (Anuradha et al. 2008). However, a higher level of root disease resistance may be attained by transgene expression using a root specific promoter. Several organ-specific promoters with root tissue specificity have been characterized (Chen et al. 2014). Members of proline-rich protein (PRP) that appeared to

express preferentially in root were isolated in Arabidopsis and soybean (Chen et al. 2014). The *Arabidopsis* PRP3 promoter drove GUS expression in root hair-bearing epidermal cells of the root, which provides a physical barrier against pathogen invasion (Bernhardt and Tierney 2000). In the present study, to enhance tolerance to the soilborne pathogen in transgenic plants, we compared a root-specific expression system in transgenic tobacco plants with a constitutive ubiquitous expression system. Tobacco plants were generated with four expression cassettes, carrying the β-glucuronidase (*GUS*) or defensin (*J1-1*) gene in combination with the cauliflower mosaic virus 35S promoter or PRP3 promoter. Detailed studies were carried out on a subset of transgenic tobacco plants to characterize the resistance against pathogenic basidiomycete or oomycetes such as *Rhizoctonia solani*, *Phytophthora parasitica* and *Pythium aphanidermatum*.

II. MATERIALS AND METHODS

1. Plant materials and growth conditions

Nicotiana tabacum cv. Havana was subjected to transformation using *Agrobacterium tumefaciens* strain LBA4404. Wild-type (WT) and transgenic seedlings were grown in a growth chamber at 25°C and 50% humidity under a 16-h light/8-h dark cycle. Tobacco plants were transferred to soil and grown in a greenhouse at 24°C day/20°C night for further experiments.

2. Pathogen and inoculation

Rhizoctonia solani AG2-1 (KACC 40124), *Phytophthora parasitica* var. *nicotianae* (KACC 40906) and *Pythium aphanidermatum* (KACC 40156) were obtained from the Korean Agricultural Culture Collection (KACC). Mycelial cultures of *P. parasitica* and *P. aphanidermatum* were grown on V8 juice agar at 25°C for 4 days as described by ŠŠkalamera et al. (2004) and Zhou and Paulitz (1993), respectively and mycelial cultures of *R. solani* was grown on potato dextrose agar (PDA) at 25°C for 3 days as described by Cho et al. (2011) and Kwon et al (2014). For *R. solani*, the mycelial layer from a PDA agar was added 3 mL of sterile water and detached from the plates with a cell scraper. Mycelia were then harvested from the water and diluted with steril water to OD_{600} = 0.1 ~ 0.3 for inoculum, unless otherwise stated. For *P. parasitica*, ten mycelial plugs (10 mm diameter) were cut from the growing margin of the culture and placed upside down on the

surface of 5 mL sterile water in a petri dish (100 mm diameter), and then incubated for 7 days in the dark at 25°C. For *P. aphanidermatum*, the mycelial layer from a V8 juice agar culture was removed and vortexed in 5 mL of sterile water. Zoospores were then harvested from the water and the number was adjusted to 2×10^5 mL^{-1} for inoculum, unless otherwise stated.

3. Plasmid construction and tobacco transformation

We used a pBI121 plant expression vector containing the neomycin phosphotransferase II (*NPTII*) gene. After the vector was digested with two restriction enzymes, *Hind*III and *Xba*I, the cauliflower mosaic virus 35S promoter (p35S) was replaced with the PRP3 promoter (pPRP3) (Fig. 1A). A 1 kb of PRP3 promoter (GenBank accession no. NM_116133) was obtained by PCR amplification using genomic DNA of Arabidopsis. A 0.23 Kb cDNA of *J1-1* (GenBank accession no. XM_016681997) was amplified by PCR as described previously (Seo et al. 2014). The resulting fragment was substituted for the *GUS* gene between *Xba*I and *BamH*I. After the four plasmid constructs confirmed by DNA sequencing, they were transformed into *A. tumefaciens* strain LBA4404 by the freeze-thaw method (Chen et al. 1994). To transform tobacco, the leaf explants were inoculated with *Agrobacterium* suspensions as described by De Block et al. (1987). Regeneration of the transgenic shoots was accomplished on selection medium containing 50 mg mL^{-1} kanamycin. Seeds from self-pollinated primary transgenic (T0) plants were germinated in MS medium containing 50 mg mL^{-1} kanamycin, and the ratios of resistant to sensitive seedlings were analyzed by the chi-square test to screen the transgenic lines carrying a

single copy of T-DNA. The transgenic plants were continuously selected on medium containing kanamycin until the homozygous T3 lines were established that were used for further analysis.

4. Molecular characterization and expression analysis of transgenic lines

Tobacco genomic DNA was isolated from leaves of kanamycin-resistant transgenic lines using a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's procedure. Integration of introduced genes was confirmed by PCR using gDNA as the template. For the *GUS* or *J1-1* gene, a forward primer from the p35S or pPRP3 region was used in combination with a gene-specific reverse primer for the *GUS* or *J1-1* gene. These primer pairs were specific for the *GUS* and *J1-1* genes in the transgenic plants. The primers used are listed in Table 1. To determine the expression level of transgenes, total RNA was isolated from shoots and roots of transgenic tobacco plants and reverse-transcribed from 1 μg RNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The cDNAs were used for reverse transcription PCR and real time RT-RCR of *GUS*, *J1-1*, *LOX*, *PR1*, *PR2*, and *PR10* using AccuPower PCR Premix (Bioneer, Daejeon, Republic of Korea) and HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). The reverse transcription PCR was performed in a Swift Maxi Thermal Cyclers (ESCD Technologies Inc., Pennsylvania, USA). The real time RT-PCR was performed in a Thermal cycler Dice® Realtime system (Takara Bio Inc., Japan) with TaKaRa Dice Real Time software (Ver. 5.11). The actin (*Act*) gene was used as a reference gene for normalization.

Table 1. List of primer sequences used in this study.

Primer	Sequences	Use
HindIII-pPRP3	5'- GCA AGC TTC GCC TCA CAT GAA TCA GAT A -3'	Vector construction
pPRP3-XbaI	5'- GCT CTA GAG CTG CTG AGC GCT TGG CTT -3'	
XbaI-J1-1	5'- GCT CTA GAG CAT GGC TGG CTT TTC CAA A -3'	
J1-1-BamHI	5'- CGG ATC CGT TAA GCA CAG GGC TTC GT -3'	
NPTII-F	5'- ATG ATT GAA CAA GAT GGA TTG CAC G -3'	Confirmation of transgenic lines
NPTII-R	5'- CCA CCA TGA TAT TCG GCA AGC AG -3'	
p35S-F	5'- TCG GAT TCC ATT GCC CAG CT -3'	
GUS-R	5'- TCA TTG TTT GCC TCC CTG CTG C -3'	
PRP3-F	5'- CCT CAC ATG AAT CAG ATA AGT ACT TC -3'	
J1-1-R	5'- TTA AGC ACA GGG CTT CGT GCA -3'	
UBIQ-F	5'- GTT GAT TTT CGC AGG TAA GCA GC -3'	
UBIQ-R	5'- GGT AAA CAT AGG TAA GCC CA -3'	
LOX-F	5'- GGT GCT TCT TTC CTT GAT AG -3'	Real Time PCR
LOX-R	5'- ATT AAA CGT AGC ATC TCC TG -3'	
PR1-F	5'- AAT GGT CGC CGT GAA ATC -3'	
PR1-R	5'- TCC ACG CCT ACA TCT GCA C -3'	
PR2-F	5'- AAC AAT TTA CCA TCA GAC C -3'	
PR2-R	5'- GAC TTC ATT TCC AAC AGC -3'	
PR10-F	5'- AGC TTT GGT TC TTG ATG CAG -3'	
PR10-R	5'- CCT TCG ATT AGT GAG TAT TTG G -3'	
GUS-F	5'- TTA CGT CCT GTA GAA ACC CC -3'	
GUS-R	5'- AGC AGG GAG GCA AAC AAG -3'	
J1-1-F	5'- ATG GCT GGC TTT TCC AAA GTA GTT -3'	
J1-1-R	5'- TTA AGC ACA GGG CTT CGT GC -3'	
ACTIN-F	5'- GGT TAA GGC TGG ATT TGC -3'	
ACTIN-R	5'- ATC TTC TCC ATA TCG TCC CAG -3'	

5. Histochemical and fluorimetric GUS assays

GUS activity was histochemically and fluorometrically assayed in the transgenic tobacco plants. Histochemical staining for GUS activity was conducted based on the method of Jefferson (1987) with 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc) as a substrate. The samples were soaked overnight in X-gluc solution at 37°C. Additionally, fluorometric analysis of GUS activity was carried out in leaves, stems, flower, and roots from transgenic lines as described by Jefferson et al. (1987) with 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate. Enzyme activity was measured using a spectrofluorometer (Farrand Optical, New York, NY, USA) with excitation at 365 nm and emission at 455 nm.

6. Immunohistochemical localization of J1-1 proteins

To observe the accumulation of J1-1 proteins, tobacco leaves, petioles and roots were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.0), dehydrated in ethanol, embedded in paraffin, and sectioned (6 μm thickness). Deparaffinized sections were incubated with anti rabbit J1-1 antibody (1:2000) for 4 h at 12°C, followed by detection with AEC (3-amino-9-ethylcarbazole) chromogen, shown as red (Dako, Glostrup, Denmark). Control experiments using pre-immune serum were not reactive (data not shown).

7. *In vitro* assay of recombinant J1-1 protein against *Rhizoctonia, Phytophthora* and *Pythium*

The J1-1/GST fusion protein was expressed in *E. coli* strain BL21 as described previously (Seo et al. 2014). The *E. coli* (BL21) transformant cell was incubated with 200 mL Luria-Berani (LB) media containing 100 ug mL^{-1} ampicillin at 37°C with 150 rpm shaking up to an OD_{600} = 0.4 ~ 0.5 and then incubated at 28°C with 150 rpm shaking up to an OD_{600} = 0.6. The cells were added to 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C with 150 rpm shaking for 16 hours. The recombinant cells were harvested by centrifugation at 4°C with 3,000 rpm for 15 min and discard culture supernatants. The remaining cell pellets from 200 mL culture were resuspended in 8 mL lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.5) and sonicated with a Ultrasonic Processor (SONICS, USA) on ice (70% amplitude, 10 min total, 20 sec on/30 sec off). The J1-1/GST proteins were purified on glutathione sepharose 4B (GE, Illinois, USA) and eluted with elution buffer (50 mM Tris pH 8.0) including 6.8 mg reduced Glutathione as described by Gozani Lab 2005. The protein concentration was determined using the Bradford method. Following purification, 10 μL of J1-1/GST protein (0.25, 0.5 and 1 mg mL^{-1}) was added to 10 μL of mycelia (OD_{600} = 0.1) of *R. solani* AG2-1 and zoospores (5 × 10^5 mL^{-1}) of *P. parasitica* or *P. aphanidermatum* on a cover glass and kept in a humidified chamber at 26°C for 6 h and 6 day. Then, the mycelia and zoospore were observed under a microscope (Nikon eclipase E200, Tokyo, Japan) and stained with Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes) for 10 min and examined under a fluorescence microscope (Olympus IX-73, Tokyo, Japan) using NIS-elements software (Nikon, Tokyo, Japan).

8. Resistance of transgenic tobacco plants against *Rhizoctonia*, *Phytophthora* and *Pythium*

To test the resistance of transgenic tobacco plants against *R. solani*, two lines of seedlings were chosen from each construct based on the transgene expression. Inoculation experiments were performed in a growth chamber as described previously (Hase et al. 2008) with the following modification. Briefly, roots of one-week-old seedlings were immersed in 200 μL of distilled water containing mycelia (OD_{600} = 0.1) of *R. solani* for 7 days in microtiter plates. The seedlings were incubated at 25°C in 70 ~ 100% relative humidity under a 16-h light/8-h dark cycle. Ten days after infection, the roots of transgenic tobacco were stained with lactophenol cotton blue (Becton Dickinson, New Jersey ,USA) for 3 min at room temperature using a modified Cho et al. 2011 method and examined under a microscopic observation was conducted to examine mycelia growth in infected roots. In the case of *P. parasitica*, one-week-old seedlings were immersed in 200 μL of distilled water containing 1×10^4 mL^{-1} zoospores of *P. parasitica* for 10 days in microtiter plates. The seedlings were incubated at 25°C in 70 ~ 100% relative humidity under a 16-h light/8-h dark cycle. Ten days after infection, microscopic observation was conducted to examine hyphal growth in infected roots. Zoospores in the water were counted by using a hemocytometer. The response of plants to infection was determined by measuring leaf length. In the case of *Pythium*, three-week-old seedlings were used for the assay. Additionally, transgenic plants of a representative line from each construct were grown in soil for 3 or 7 weeks and then inoculated with a 2×10^5 mL^{-1} zoospore of *P. parasitica.* and mycelia (OD_{600} = 0.3) of *R. solani*. For inoculation, 10 mL of zoospore solutions and 3 mL of mycelia solutions were applied to the soil around the base of the stem. The

development of disease symptoms was monitored until day 10 after inoculation. Resistance of the transgenic plants was then determined by measuring the fresh weight and leaf length of the infected plants, compared to control plants carrying the *GUS* gene.

9. Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) using IBM SPSS statistics software. Significant differences between mean values were determined at $P \leq 0.05$ or 0.01. All data were represented as the means ± SD of at least three independent experiments.

III. RESULTS

1. Genetic transformation of tobacco

Transgenic tobacco plants were generated for the overexpression of *GUS* or a pepper defensin (*J1-1*) with either the 35S promoter (p35S) or PRP3 promoter (pPRP3) via *Agrobacterium*-mediated transformation (Fig. 1A). More than two dozen transgenic explants were selected from separate culture dishes to avoid proliferation from the same lines. Independent transgenic plants carrying one copy of the T-DNA were maintained by self-pollination and finally three homozygous T3 plant lines were used for further studies. For genetic and molecular analyses, leaves derived from each transgenic tobacco plant were used (Fig. 1B). The integration of *NPTII* genes into kanamycin-resistant plants was confirmed by PCR. The PCR products revealed clear bands for the *NPTII* genes (0.6 kb), but no bands were observed in non-transgenic control plants. To verify the introduction of *GUS* or *J1-1* gene in transgenic plants, we performed PCR analysis using primers for the detection of the sequence of *GUS* or *J1-1* with its promoter. Agarose gel electrophoresis revealed bands of approximately 2.8 and 1.2 kb that corresponded to the sequences for pPRP3/*GUS* and pPRP3/*J1-1*, respectively. These results indicated that the transgenic plants carried respective T-DNA regions introduced via *Agrobacterium*-mediated transformation.

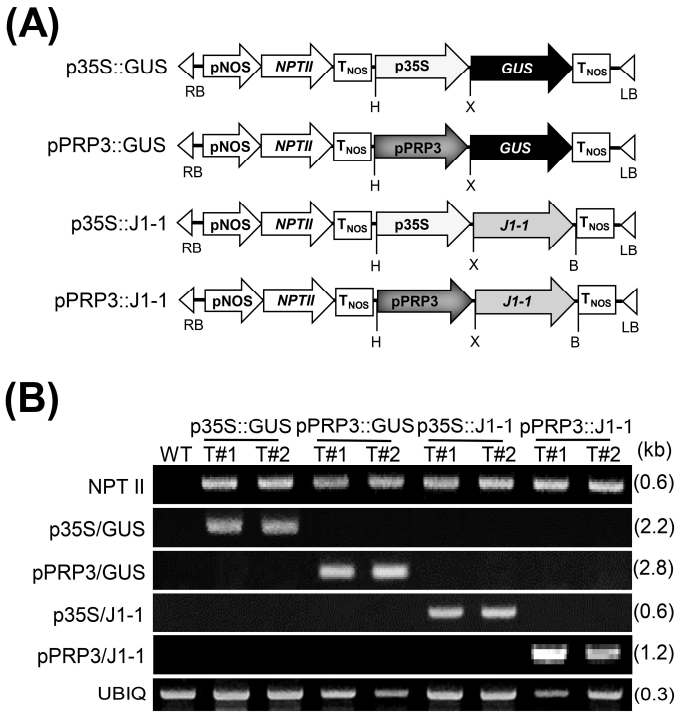

Fig. 1. Detection of introduced genes in transgenic tobacco by polymerase chain reaction (PCR). (A) Schematic diagrams of the T-DNA region of the binary vector, indicating restriction enzyme sites. **p35S**, cauliflower mosaic virus 35S promoter; **pPRP3**, root-specific promoter from the proline-rich protein 3 (*PRP3*) gene; ***GUS***, β-glucuronidase gene; ***J1-1***, pepper defensin gene; **pNOS**, nopaline synthase promoter; **T_{NOS}**, nopaline synthase transcriptional terminator; ***NPTII***, kanamycin resistance gene; **LB**, left border; **RB**, right border; **B**, *Bam*HI; **H**, *Hin*dIII; **X**, *Xba*I. **(B)** PCR products of the *GUS* or *J1-1* genes fused with their respective promoters and the *NPTII* gene from genomic DNA of transgenic tobacco plants. **WT**, wild-type tobacco plant; **T#1-T#2**, individual transgenic plant lines.

2. Expression analysis of transgenic plants

To determine gene expression patterns associated with the promoters, we performed reverse transcription PCR and real time RT-PCR analyses for the *GUS* and *J1-1* genes in the shoots and roots of transgenic plants carrying four different expression cassettes (Fig. 2). The transcripts of *GUS* and *J1-1* were not amplified in non-transgenic plants. Under the control of a constitutive promoter, *GUS* and *J1-1* expression was observed in both leaves and roots in the transgenic plants. However, the expression of transgenes occurred exclusively in the roots of transgenic plants harboring the root-specific PRP3 promoter. Unlike the roots, the shoots of the transgenic plants showed no significant expression of transgenes driven by the pPRP3, suggesting that the PRP3 promoter played a major role in root-specific expression. The results show that the pattern of gene expression differed in the transgenic plants between the 35S promoter and PRP3 promoter. Thus, *J1-1* expression can be restricted to the roots of transgenic plants by using the PRP3 promoter.

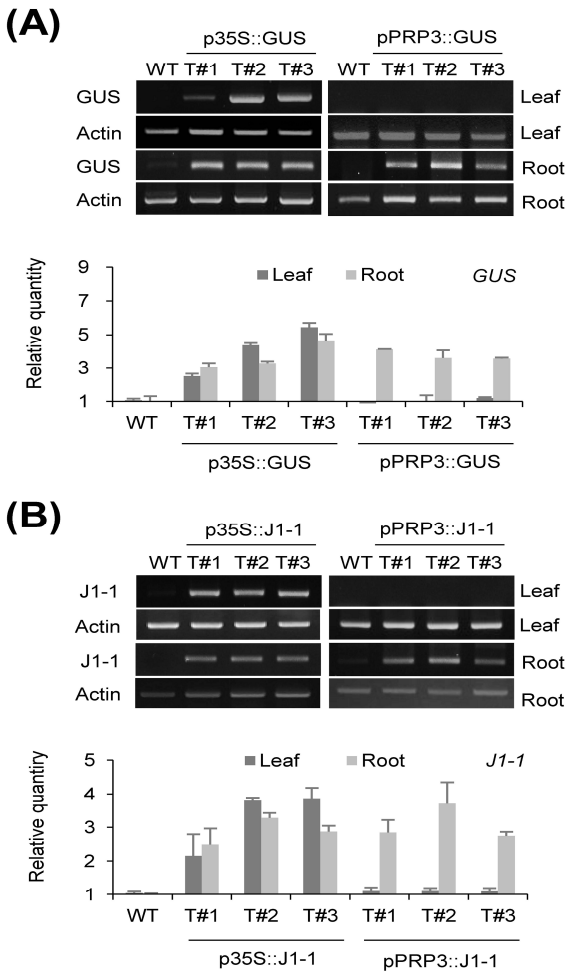

Fig. 2. Detection of *GUS* and *J1-1* expression in the leaves and roots of transgenic tobacco plants using reverse transcription PCR and real time RT-PCR. Reverse transcription PCR and real time RT-PCR were performed for *GUS* and *J1-1* using the total RNA isolated from leaves and roots of 4-weeks-old transgenic tobacco plants. Tobacco actin (*Act*) gene was used for normalization. **(A)** Expression of the *GUS* gene. **(B)** Expression of the *J1-1* gene. **WT**, non-transgenic plant as a control; **T#1-#3**, individual transgenic plant lines. The data are represented as means ± SD of three independent experiments.

3. Quantification of GUS activity in plant organs

To examine the relative levels of transgene expression, we measured the specific activity of GUS in the organs of the transgenic and non-transgenic control plants (Fig. 3). GUS activity was not detected in negative control tobacco plants (data not shown). The histochemical assay showed that the *GUS* gene was constitutively expressed in transgenic plants carrying the 35S promoter (Fig. 3A). However, the degree of GUS activity differed between plant organs and was lower in roots than shoots. In contrast, the expression of *GUS* genes driven by PRP3 promoter was highly root-specific (Fig. 3B). The fluorometric GUS assay showed that GUS activity was highest in the leaf tissues of the transgenic plants carrying the 35S promoter (Fig. 3C). Using the PRP3 promoter, GUS activity was very strong in roots, while the activity was barely detected in leaf, stem, and flower tissues (Fig. 3D). This result indicates that the PRP3 promoter determines the site of gene expression in plant organs, resulting in predominant expression of the gene in the roots of transgenic plants.

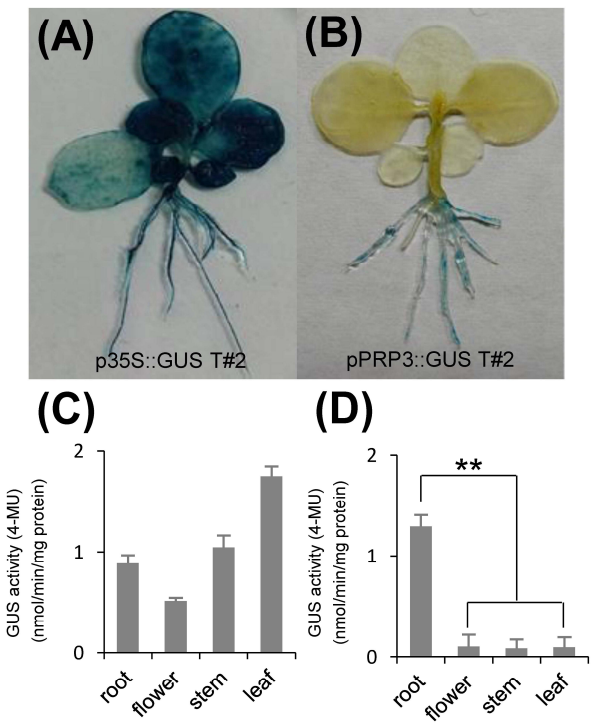

Fig. 3. GUS enzyme assays in transgenic tobacco plants driven by constitutive and root-specific promoters. Histochemical analysis of GUS activity in transgenic plants controlled by p35S **(A)** and pPRP3 **(B)**: 3-week-old. Quantification of GUS activity in various transgenic plant organs with p35S::*GUS* **(C)** and pPRP3::*GUS* construct **(D)**. Specific activity was analyzed fluorometrically and expressed as nmole of 4-methylumbelliferone (MU) min^{-1} mg^{-1} protein. For assays, the samples except flower were taken from 5-week-old plants. Data are expressed as the means ± SD of three independent assays. ** represents statistically significant differences at $P \leq 0.01$.

4. Root-specific expression of J1-1 in transgenic tobacco

Because reverse transcription PCR and real time RT-PCR analysis revealed that *J1-1* expression was regulated in an organ-specific manner depending on the promoter used, we examined the accumulation and localization of the J1-1 protein in various organs of transgenic plants. We performed immunohistochemical assays on the leaves, petioles, and roots of transgenic plants using anti-J1-1 antibody (Fig. 4). This result showed high level of protein accumulation in both leaf and petiole of transgenic plants harboring the 35S promoter, but less in roots. Under the control of the PRP3 promoter, the protein was detected intensively in the roots of the transgenic plants. However, in the leaves and petioles, accumulation of J1-1 protein was negligible. This result further confirmed that the accumulation of J1-1 protein was root-specific in transgenic plants carrying the pPRP3 expression cassette.

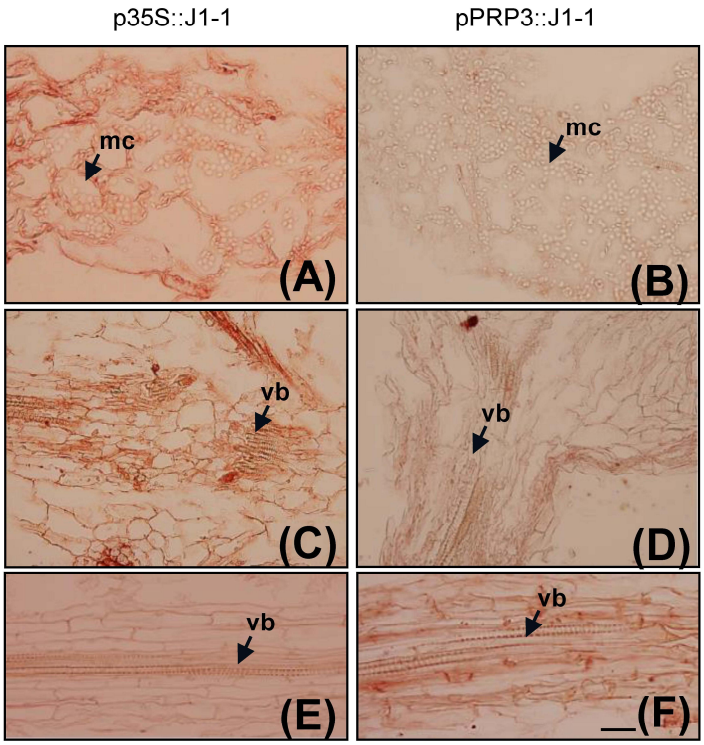

Fig. 4. Immunolocalization of J1-1 protein in transgenic tobacco plants. Deparaffinized sections of p35S::*J1-1* (T#2) and pPRP3::*J1-1* (T#1) lines were incubated with antiserum raised against J1-1 and the protein was detected with peroxidase-labeled secondary antibody. To localize the protein, the antibody was detected with 3-amino-9-ethylcarbazole (AEC) chromogen, shown as red. **(A)-(B)**, leaves; **(C)-(D)**, petioles; **(E)-(F)**, roots. **mc**, mesophyll cell; **vb**, vascular bundle. Bar represents 50 μm.

5. The effect of recombinant J1-1 protein against *Rhizoctonia*, *Phytophtora* and *Pythium*

The potential effects of J1-1 protein were assessed on the root rot pathogens using recombinant J1-1 protein. Its effect was evaluated according to the mycelium development of *R. solani* and the zoospore germination of *P. parasitica* and *P. aphanidermatum*. For microscopic observation, 10 μL of mycelia diluted in sterile water to a density of OD_{600} = 0.1 and zoospores diluted in sterile water to a density of 5×10^5 mL^{-1} was mixed with J1-1/GST protein to yield mixtures of 0.25, 0.5 and 1 mg mL^{-1} on cover glass and kept in a humidified chamber at 26°C for 6 days. The results showed that the 1mg mL^{-1} mixture of J1-1/GST protein had inhibitory effect on hyphal growth and formation of *R. solani* (Fig. 5). As for *P. parasitica* and *P. aphanidermatum,* the 0.25 mg mL^{-1} mixture of J1-1/GST protein had inhibitory effect on hypha formation from the germination tube (Fig. 6). Additionally, the effect of J1-1/GST protein was evaluated according to the loss of mycelia and zoospore viability of *R. solani, P. parasitica* and *P. aphanidermatum* with using a fluorescence microscope (Fig. 7). For fluorescence microscope, the monilioid cells of *Rhizoctonia* and the zoospores of *Phytophthora* or *Pythium* trended toward lower viability in higher protein concentration. When the protein was heated at 90°C, the protein did not affect the viability of mycelia and zoospores. These results indicate that J1-1 is active against root rot pathogens, and also suggest the potential of J1-1 for plant disease control in economically important crops.

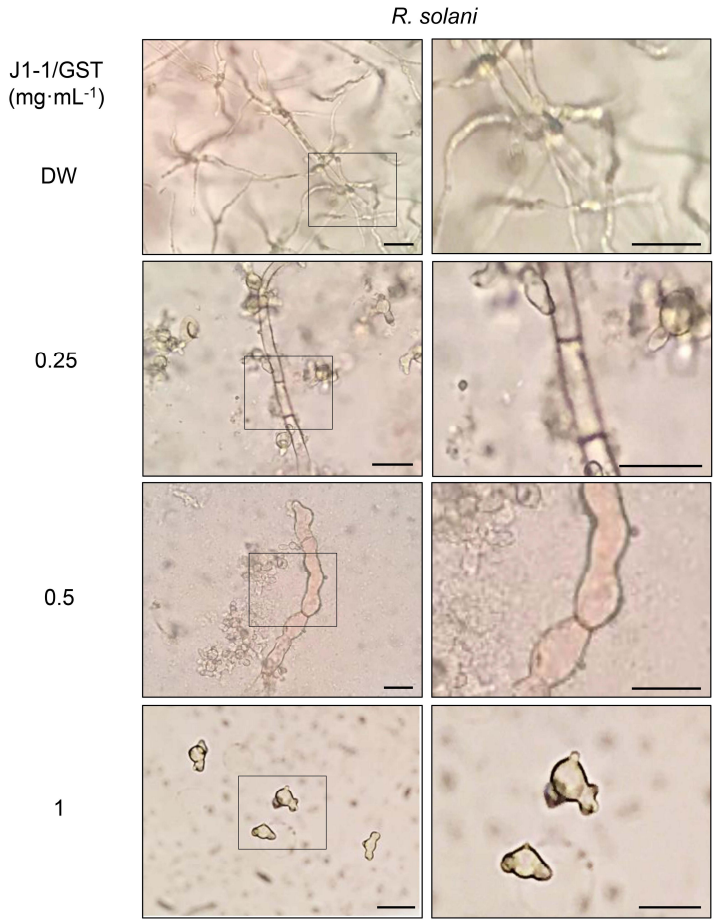

Fig. 5. Effect of J1-1/GST recombinant protein against *Rhizoctonia solani*. Ten microliter of mycelia suspensions (OD_{600} = 0.1) of *Rhizoctonia* was treated with 10 μL of J1-1/GST fusion protein for 6 days at 26℃. After incubation, the mycelia suspensions were then photographed under a microscope. Bar represents 10 μm.

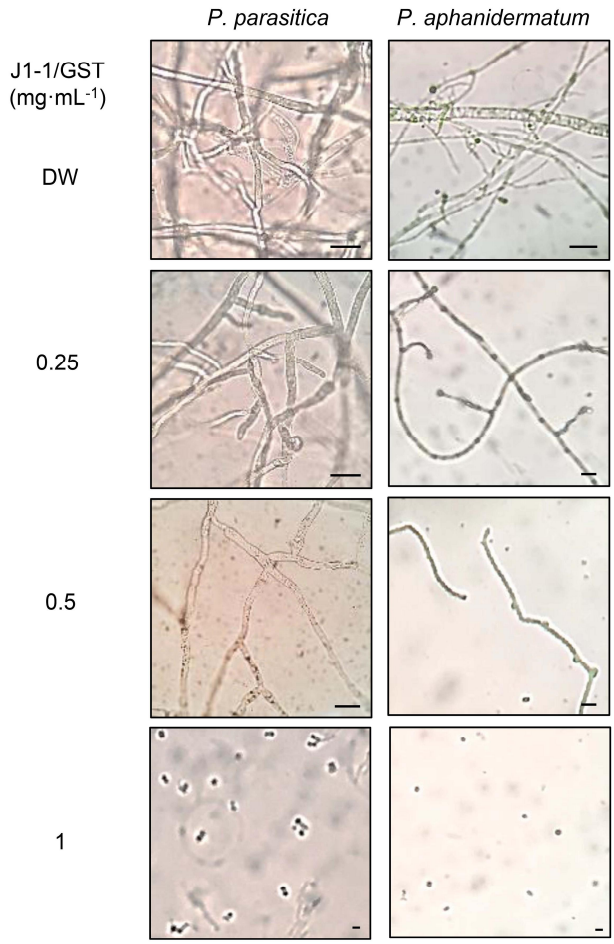

Fig. 6. Effect of J1-1/GST recombinant protein against *Phytophthora parasitica* and *Pythium aphanidermatum*. Ten microliter of zoospore suspensions (5×10^5 mL^{-1}) of *Phytophthora* or *Pythium* were treated with 10 μL of J1-1/GST fusion protein for 6 days at 26℃. After incubation, the zoospore suspensions were then photographed under a microscope. Bar represents 10 μm.

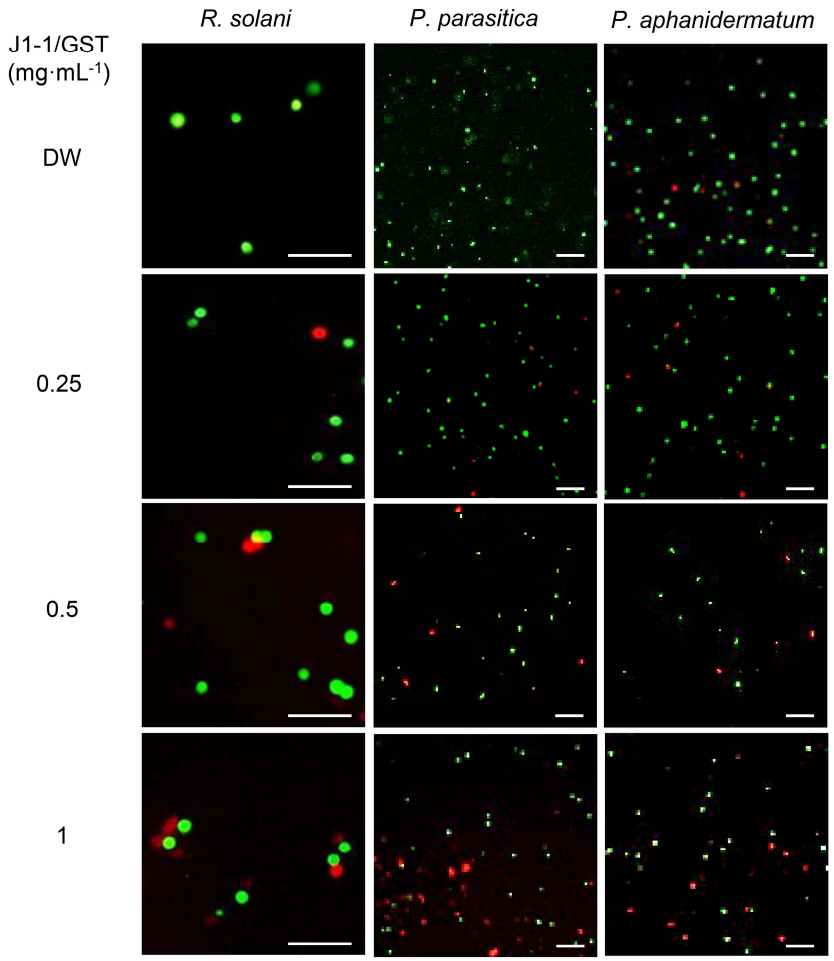

Fig. 7. Effect of J1-1/GST recombinant protein against *Rhizoctonia solani*, *Phytophthora parasitica* and *Pythium aphanidermatum*. Ten microliter of mycelia suspesions (OD_{600} = 0.1) of *Rhizoctonia* and zoospore suspensions (5×10^5 mL^{-1}) of *Phytophthora* or *Pythium* were treated with 10 μL of J1-1/GST fusion protein for 6 hours at 26℃. After incubation, the mycelia and zoospore suspensions were stained with Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes) for 10 min and examined using a fluorescence microscope. Fluorescence images represent viable cells stained with SYTO9 (green) and dead cells stained with propidium iodide (red). Bar represents 20 μm.

6. Suppression of pathogen growth in transgenic roots

To evaluate disease resistance in transgenic plants carrying different promoters, we performed inoculation tests to investigate the growth of *R. solani*, *P. parasitica* and *P. aphanidermatum* on the roots of transgenic plants (Figs. 8, 9 and 10). Transgenic tobacco plants carrying the *GUS* gene were used as negative controls. We inoculated seven-day-old tobacco seedlings with *R. solani* and *P. parasitica* by immersing the roots in a mycelia and zoospore solution (Figs. 8 and 9). In addition, *P. aphanidermatum* was also tested to examine the spectrum of disease resistance of the transgenic plants. Three-week-old seedlings were submerged in zoospore solution of *P. aphanidermatum* (Fig. 10). Ten-days after inoculation, *J1-1* transgenic seedlings remained healthy with normal growth, whereas GUS transgenic seedlings displayed seedling blight symptoms with a retarded growth. The results showed that plant growth suppression had occurred on most of the shoots of the control seedlings carrying *GUS*. However, in seedlings overexpressing *J1-1*, the symptoms were not severe, and the leaves remained green (Figs. 8A, 9A and 10A). The pathogen caused watery rot symptoms in the roots of the inoculated control seedlings at 10 days post-infection. Thus, the infected roots were subjected to microscopic observation to examine pathogen development. Some root cells were colonized and collapsed by *R. solani*, *P. parasitica* and *P. aphanidermatum*. In contrast, tobacco seedlings overexpressing *J1-1* exhibited significantly reduced root colonization by *R. solani*, *P. parasitica* and *P. aphanidermatum* (Figs. 8B, 9B and 10B). Filamentous hypha that produced sporangia filled with motile zoospores grew vigorously along infected roots in both GUS transgenics (Fig. 9B). Hyphal outgrowth on the roots was rarely observed in seedlings with elevated J1-1 accumulation driven by pPRP3. Microscopic observation of the infected roots showed that

the hyphal growth of *R. solani*, *P. parasitica* and *P. aphanidermatum* was easily observed in damaged root tissues of *GUS* transgenic plants but not in the *J1-1* transgenic roots. (Figs. 8B, 9B and 10B). Based on restricted growth of the hypha and normal growth of seedlings, transgenic lines overexpressing J1-1 protein showed significantly enhanced resistance to the root rot pathogens, *R. solani*, *P. parasitica* and *P. aphanidermatum*. These results demonstrated that a reduction of hyphal outgrowth may be associated with the expression of an antimicrobial protein J1-1 in the roots of transgenic seedlings.

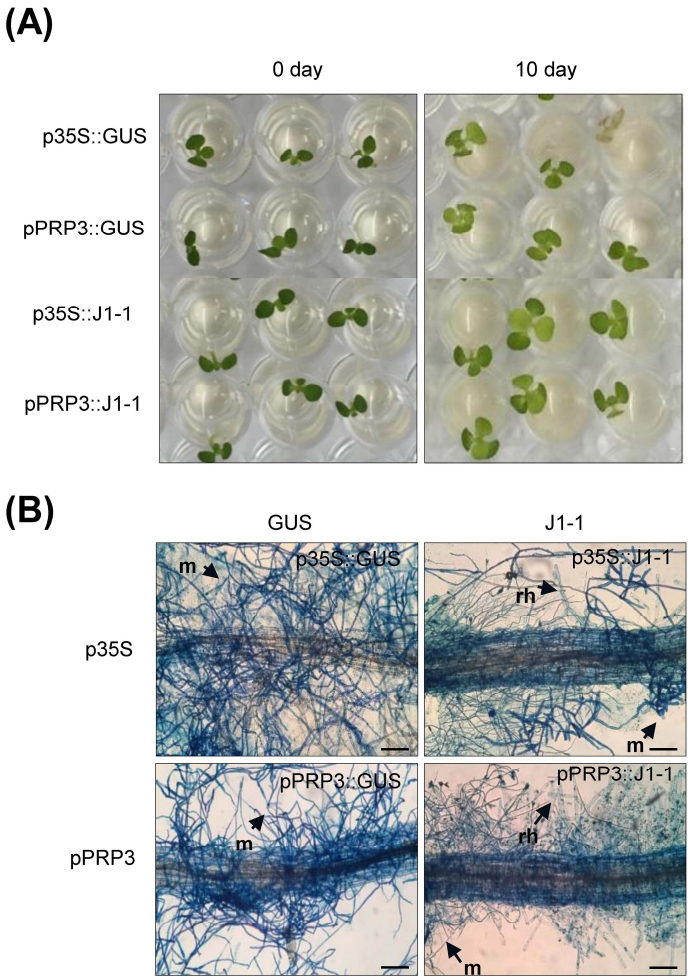

Fig. 8. Development of disease symptoms in transgenic tobacco after infection with *Rhizoctonia solani* AG2-1. (A) One week-old tobacco seedlings of two lines from each construct were immersed in mycelia solution of *R. solani* in distilled water (OD_{600} = 0.1), and then photographed at 10 days post-inoculation. **(B)** Infected roots of transgenic tobacco were stained lactophenol blue and then photographed under a microscope at 10 days post-inoculation. Twenty-five seedlings in a transgenic line were inoculated per experiment. **m**, mycelia; **rh**, root hairs. Bars represents 50 μm.

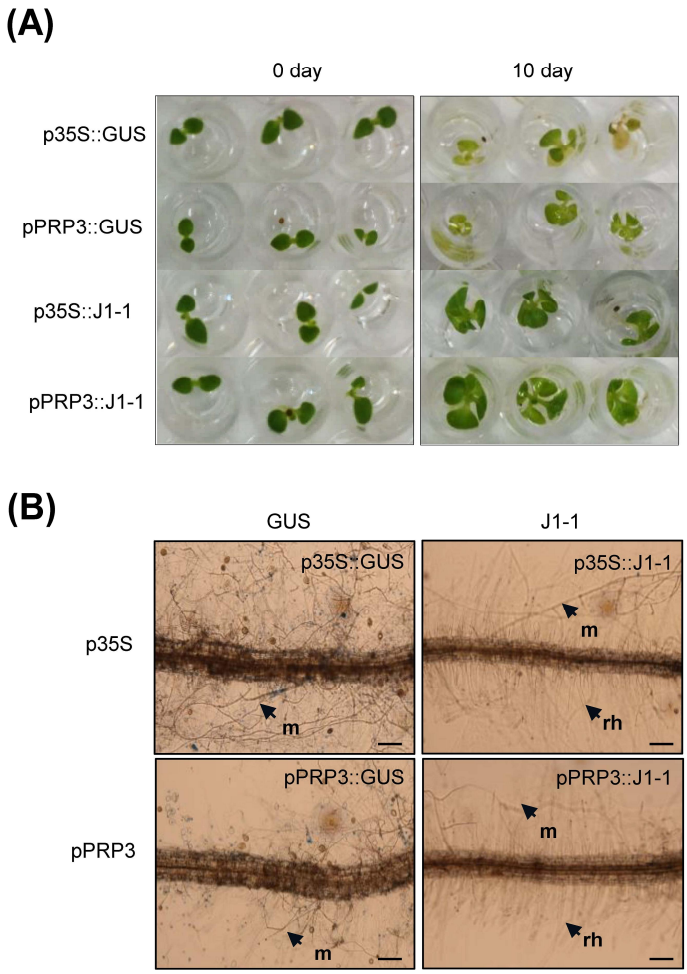

Fig. 9. Development of disease symptoms in transgenic tobacco after infection with *Phytophthora parasitica* var. *nicotianae*. **(A)** One week-old tobacco seedlings of two lines from each construct were immersed in zoospore solution of *P. parasitica* in distilled water at 1×10^4 mL^{-1}, and then photographed at 10 days post-inoculation. **(B)** Infected roots of transgenic tobacco were photographed under a microscope at 10 days post-inoculation. Twenty-five seedlings in a transgenic line were inoculated per experiment. **m**, mycelia; **rh**, root hairs. Bars represents 50 μm.

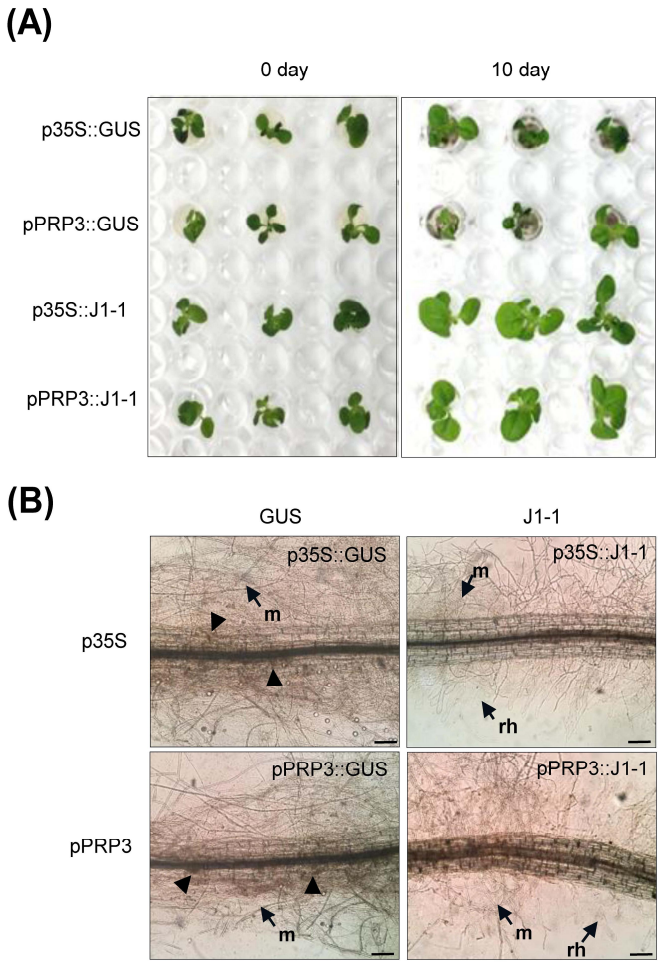

Fig. 10. Development of disease symptoms in transgenic tobacco after infection with *Pythium aphanidermatum*. **(A)** Three week-old tobacco seedlings of two lines from each construct were immersed in zoospore solution of *P. aphanidermatum* in distilled water at 1 × 10^4 mL^{-1}, and then photographed at 10 days post-inoculation. **(B)** Infected roots of the seedlings in a were photographed under a microscope at 10 days post-inoculation. Twenty-five seedlings in a transgenic line were inoculated per experiment. **m**, mycelia; **rh**, root hairs. Bar represents 50 μm.

7. Disease resistance of transgenic plants

To confirm the resistance of plants to root disease, we performed a disease resistance assay in soil-grown transgenic plants. A *R. solani* mycelia and *P. parasitica* zoospore solution were applied by drenching the soil around the base of the plant, followed by measurement of the leaf length and fresh weight of the plants at 10 days post-infection. The growth of the control plants gradually ceased following infection, and the leaves turned yellow (Figs. 11 and 12). In Fig. 11A, some GUS transgenic plants displayed blight symptoms with retarded growth, whereas *J1-1* transgenic plants remained healthy with normal growth. In Fig. 12A, most root tissues were decayed in the control group. The fresh weight of control plants was reduced by half, compare to that of *J1-1* transgenic plants (Figs. 11B and 12B). Leaf length was also shorter in the control transgenic plants compared with the transgenic plants carrying the defensin gene (Figs. 11C and 12C). These results revealed a relationship between reduced disease severity and J1-1 accumulation in the transgenic plants, further evidence that plant growth after infection was enhanced by the activity of the J1-1 protein in the transgenic plants. The infected roots showed massive mycelial growth of *P. parasitica* in the control roots, unlike in *J1-1* transgenic plants. Based on the degree of growth promotion, *J1-1* transgenic lines appeared to have significantly enhanced resistance against *R. solani* and *P. parasitica* the causative agent of black shank disease.

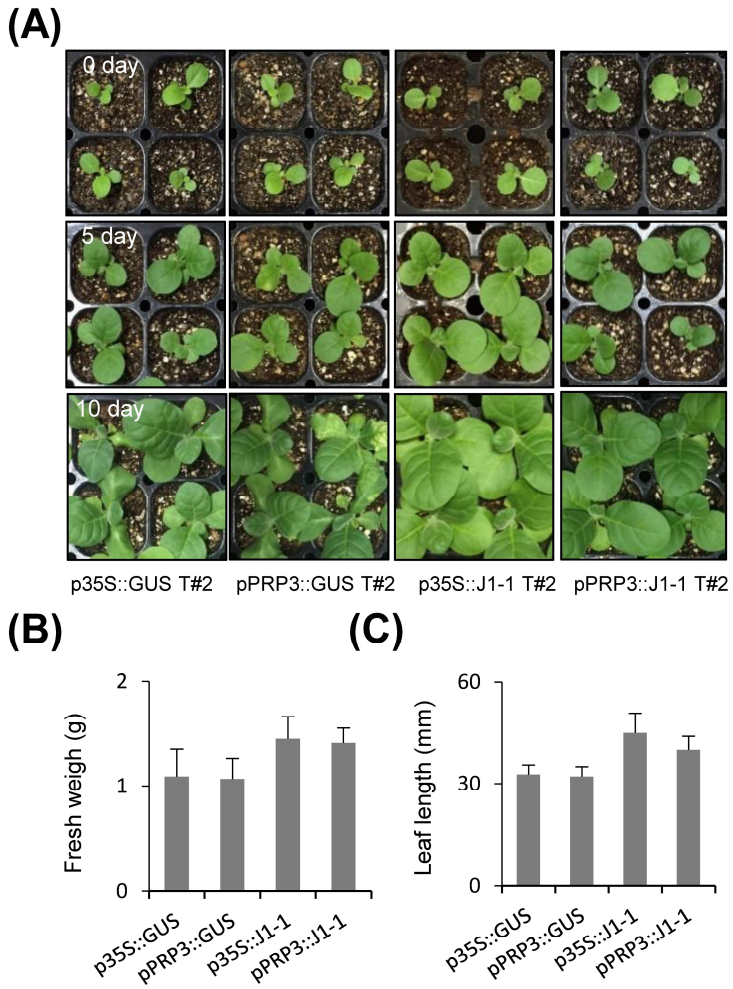

Fig. 11. Enhanced resistance in transgenic tobacco infected with *Rhizoctonia solani*. Three weeks-old tobacco seedlings were inoculated with *R. solani* mycelia (OD_{600} = 0.1), by applying 3 mL of mycelia solution to the soil. The plant phenotypes were scored at 10 days post-inoculation. **(A)** Representative photographs of plants before and at 10 days post-inoculation. Comparison of fresh weight **(B)** and leaf length **(C)** of infected transgenic tobacco plants at 10 days post-inoculation. The experiments were conducted with three replicates of twenty seedlings per transgenic line.

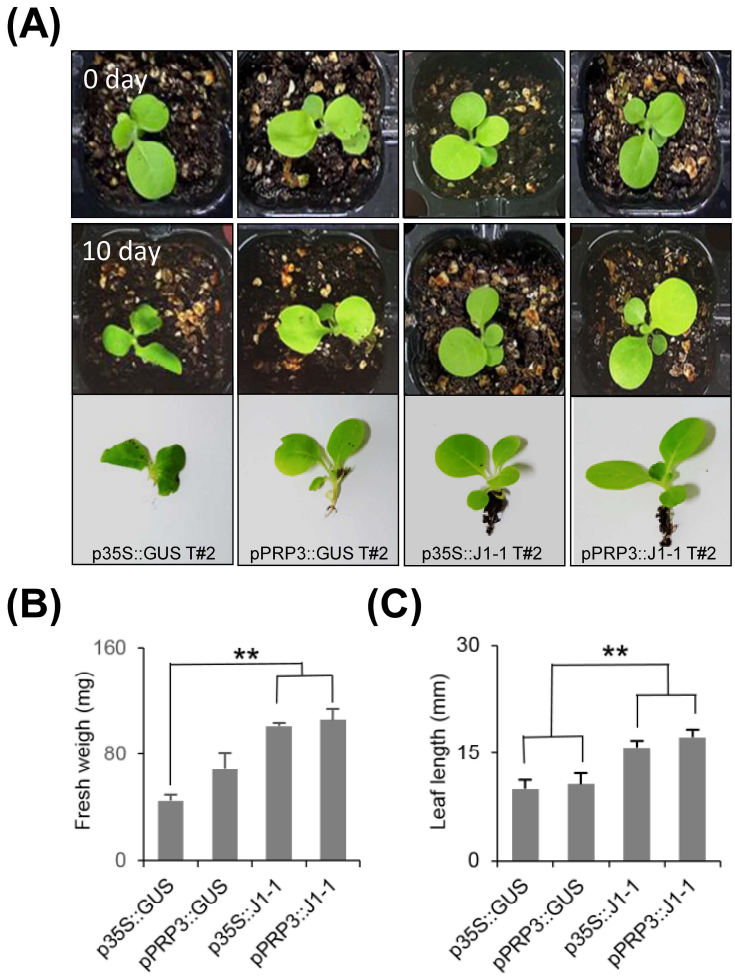

Fig. 12. Enhanced resistance in transgenic tobacco infected with *Phytophthora parasitica*. Three weeks-old tobacco seedlings were inoculated with *P. parasitica* zoospores (2×10^5 mL^{-1}) by applying 10 mL of zoospore solution to the soil. The plant phenotypes were scored at 10 days post-inoculation. **(A)** Representative photographs of plants before and at 10 days post-inoculation. Comparison of fresh weight **(B)** and leaf length **(C)** of infected transgenic tobacco plants at 10 days post-inoculation. The experiments were conducted with three replicates of twenty seedlings per transgenic line. Values are expressed as the means ± SD of three independent experiments. ** $P \leq 0.01$.

8. Differential expression of *PR* genes in the transgenic plants

To further examine disease resistance of the transgenic plants, we analyzed gene expression patterns of defense-related genes, such as *PR1*, *PR2*, and *PR10* in the transgenic lines. Transcript levels of those genes were measured by real time RT-PCR in both shoots and roots of the plants. The expression of *PR* genes was significantly elevated in *J1-1* transgenic plants (Fig. 13). It has been shown that jasmonic acid (JA) plays a key role in certain pathogen-induced defense responses. Thus, the expression of the *LOX* gene that is involved in JA biosynthesis was examined in the plants to understand underlying biochemical relationship between *J1-1* overexpression and enhanced expression of *PR* genes. The *LOX* gene was highly expressed in *J1-1* transgenic pepper fruits compared to that of non-transgenic fruits (Seo et al. 2014). Our results also showed that elevated expression of *LOX* was accompanied with induced expression of *PR* genes, such as *PR1*, *PR2*, and *PR10*. Induced expression of *LOX* was likely involved in the biosynthesis of JA in the shoot. In particular, the expression level of *PR2* and *PR10* was significantly higher in the roots of p35S-driven transgenic plants, while *LOX* expression is much higher in the shoots than in roots. This result suggests that enhanced JA signaling in shoots is mobilized to the root to launch the defense responses in the roots of the *J1-1* transgenic plants. In general, the salicylic acid (SA) pathway that typically induces the activation SA-dependent gene including *PR1*, *PR2* (Riviere et al. 2008; Ali et al. 2018). However, the methyl jasmonate (MeJA) pre-treatment have been reported that induced PR2 mRNA accumulation in tomato fruit (Ding et al. 2002). The expression of *PR10* were reported that regualted by jasmonic acid (JA) signaling pathways response to abiotic and biotic stress (Jain et al. 2015) and the methyl jasmonate (MeJA) pre-treatment induced the expression of *PR10* (Park et al. 2003).

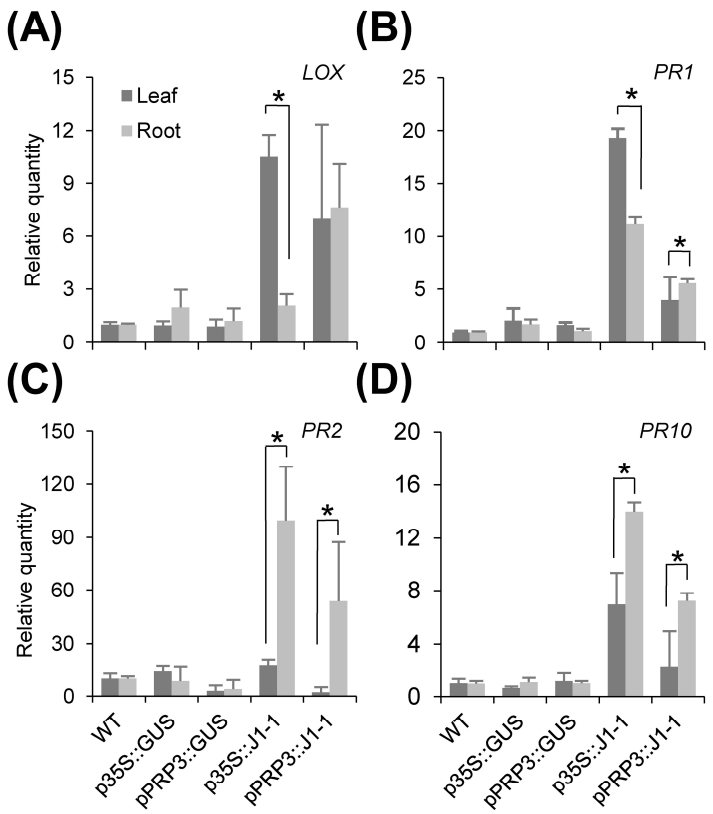

Fig. 13. Expression analysis of lipoxygenase (*LOX*) and pathogenesis-related (*PR*) genes in transgenic tobacco plants using real time RT-PCR. (A) *LOX*, **(B)** *PR1*, **(C)** *PR2*, and **(D)** *PR10*. Total RNA was extracted from the leaves and the roots of 4 weeks-old transgenic tobacco plants. Tobacco actin (*Act*) gene was used for normalization. Error bars indicate the standard deviation from three independent measurements. **WT**, non-transgenic plant as a control. * represents statistically significant differences at $P \leq 0.05$.

IV. DISCUSSION

Our data show that tissue-specific gene expression can be used to increase the expression level of transgenes in specific regions of a plant. We assayed GUS enzyme activity in transgenic plants to localize its expression, as regulated by constitutive or roots specific promoters (Fig. 1). The 35S promoter is a widely used promoter that exhibits a high level of transcriptional activity in a variety of plant specie (Benfey and Chua 1990). Although the 35S promoter is considered a constitutive promoter, the expression pattern of transgenes seem to be spatially and developmentally controlled within a plant. The present result is consistent with previous reports that plants carrying the 35S promoter had higher GUS activity in younger leaves, and much lower expression in the roots (Benfey et al. 1989 and Cornejo et al. 1993). Indeed, the promoter contains several domains with different tissue specificities; the expression in root is controlled by domain A that confers gene expression principally within the root tip (Benfey et al. 1989). Thus, with the aim of evaluating a highly efficient promoter that can drive strong expression in the roots of transgenic plants, we employed the PRP3 promoter and fused to the *GUS* reporter gene or *J1-1* gene (Fig. 2). High levels of gene expression in roots is likely due to the presence of six ATATT motifs in the PRP3 promoter, that have been shown to be involved in root expression (Chen et al. 2014). In transgenic lines carrying the PRP3 promoter, GUS and J1-1 levels were higher in the roots with no detectable accumulation in the aboveground parts of the plants (Figs. 3 and 4). Since the first attempt of overexpression of radish defensin resulting in increased resistance against *Alternaria longipes* in transgenic tobacco, similar results have

been obtained in tobacco plants carrying other defensin genes, such as *Spi*, *DRR230*, and *BSD1* (Elfstrand et al. 2001, Lai et al. 2002 and Park et al. 2002). Additionally, agronomically important plants have been transformed to constitutively express defensin genes including *AlfAFP*, *DRR230*, *J1-1*, *WT1*, and *MsDef1* in potato, canola, pepper, rice, and tomato, respectively (Gao et al. 2000; Wang et al. 1999; Seo et al. 2014; Kanzaki et al. 2002; Abdallah et al. 2010). In this study, the recombinant J1-1/GST fusion protein showed inhibitory activity on the growth and development of the *R. solani*, *P. parasitica* and *P. aphanidermatum* (Figs. 5, 6 and 7). These results suggests that the initial contact of J1-1 with mycelia of *R. solani* and zoospores of *P. parasitic*a and *P. aphanidermatum* can restrict hyphal growth and development Pathogen colonization was suppressed in the *J1-1* transgenic lines, regardless of the promoter used (Figs. 8A, 9A and 10A). Microscopic observation showed that none of the GUS transgenic lines inhibited hyphal growth in the roots, which exhibited massive intracellular and extracellular growth of the pathogen (Figs. 8B, 9B and 10B). In addition, the *J1-1* transgenic lines showed more vigorous growth and minimal root damage compared to control transgenic lines in inoculated soil (Fig. 11 and Fig. 12). These results indicate that the expression of the pepper defensin was also effective in the enhancement of plant resistance to *R. solani*, *P. parasitica* and *P. aphanidermatum*. Meanwhile, J1-1 accumulation was enhanced in the underground parts of transgenic plants using a root-specific promoter while the constitutive promoter resulted in slightly lower expression in the transgenic roots. However, two transgenic plants with two different promoters did not show a significant difference in terms of plant resistance against *R. solani*, *P. parasitica* and *P. aphanidermatum*. Plants may resist a pathogen by restricting its infection processes, which is controlled by multiple signaling molecules. JA is

synthesized from α- linolenic acid by a series of lipoxygenase (LOX) enzymes in chloroplasts (Svyatyna and Riemann 2012). The genes coding for JA biosynthetic enzymes are known to be upregulated when plants are challenged by wounding, insect herbivory or necrotrophic pathogens (Glazebrook 2005). Subsequent recognition of bioactive JA led to the activation of the signaling cascade for the induction of a series of defense genes like *PR* genes including *PR2*, *PR3*, *PR4, PR10* and *PR12* (Thomma et al. 2001; Ding et al. 2002; Park et al. 2003). Enhanced expression of *LOX* in the shoots may resulted in priming of the plant immune system in the roots of transgenic plants (Fig. 13). In this study, the roots of *J1-1* transgenic tobacco driven by p35S suppressed pathogen growth at a level comparable to that of transgenic tobacco with *J1-1* expressed from pPRP3 (Figs. 8 ~ 12). The result suggests that higher expression of *LOX* in the shoots would support induced systemic resistance, resulting activation of *PR* genes in the roots, especially in p35S-driven transgenic plants. Enhanced expression of JA-modulated *PR* genes may provide additional resistance against the root pathogen. Consequently, *J1-1* transgenic plants carrying two different promoters revealed a similar level of resistance to the pathogen. In general, plant growth and defense is essential for plant survival and the activation of plant defense bring to the expense of plant growth (Huot et al. 2014). In this study, the *J1-1* transgenic plant seed germination was delayed by 4 days compared to control transgenic plants. Overexpression of *J1-1* can protect plants from a sudden attack of the pathogen without a lag phase for the activation of disease resistance mechanisms. However, constitutively expressed defense traits can incur a fitness cost to the plant. Additionally, tissue-specific promoters can be used to avert transgene overexpression in non-targeted organs in transgenic plants. We found that root-specific expression of *J1-1* did not induce *PR* gene

expression in stem and leaves, presumably with lower cost for transgene expression. We should consider a trade-off between plant protection and the cost of transgene expression to enhance plant growth. In conclusion, regulation of expression patterns with the currently available promoters would benefit biotechnological applications of antimicrobial proteins to improve plant protection against phytopathogens.

V. REFERENCES

Abdallah, N. A., Shah, D., Abbas, D., & Madkour, M. (2010). Stable integration and expression of a plant defensin in tomato confers resistance to fusarium wilt. *GM Crops, 1*, 344-350.

Adhikari, B. N., Hamilton, J. P., Zerillo, M. M., Tisserat, N., Lévesque, C. A., & Buell, C. R. (2013). Comparative genomics reveals insight into virulence strategies of plant pathogenic oomycetes. *PLoS One, 8*, e75072.

Ali, S., Ganai, B. A., Kamili, A. N., Bhat, A. A., Mir, Z. A., et al. (2018). Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiological Research, 212-213*, 29-37.

Anderson, N. A. (1982). The genetics and pathology of *Rhizoctonia solani*. *Annual Review of Phytopathology*, *20*, 329-347.

Anuradha, T. S., Divya, K., Jami, S. K., & Kirti, P. B. (2008). Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. *Plant Cell Reports, 27*, 1777-1786.

Benfey, P. N., & Chua, N.-H. (1990). The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. *Science, 250*, 959-966.

Benfey, P. N., Ren, L., & Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. *The EMBO Journal, 8*, 2195-2202.

Bernhardt, C., & Tierney, M. L. (2000). Expression of AtPRP3, a proline-rich structural cell wall protein from *Arabidopsis*, is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiology, 122*, 705-714.

Budge, G. E., Shaw, M. W., Colyer, A., Pietravalle, S., Boonham, N. (2009). Molecular tools to investigate *Rhizoctonia solani* distribution in soil. *Plant Pathology*, *58*, 1071-1080.

Chen, H., Nelson, R., & Sherwood, J. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze thaw transformation and drug selection. *BioTechniques, 16*(664-8), 670.

Chen, C., Yadav, P. K., Wang, X., & Liu, Z. (2012). Regulatory role of defensins in inflammatory bowel disease. *Open Journal of Immunology, 2*, 78-84.

Chen, L., Jiang, B., Wu, C., Sun, S., Hou, W., & Han, T. (2014). *GmPRP2* promoter drives root-preferential expression in transgenic *Arabidopsis* and soybean hairy roots. *BMC Plant Biology, 14*, 1-13.

Cho, K.-C., Han, Y.-J., Kim, S.-J., Lee, S.-S., Hwang, O.-J., Song, P.-S., Kim, Y.-S., & Kim, J.-I. (2011). Resistance to *Rhizoctonia solani* AG-2-2 (IIIB) in creeping bentgrass plants transformed with pepper esterase gene *PepEST*. *Plant Pathology, 60*, 631-639.

Cornejo, M.-J., Luth, D., Blankenship, K. M., Anderson, O. D., & Blechl, A. E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology, 23*, 567-581.

Cornet, B., Bonmatin, J.-M., Hetru, C., Hoffmann, J. A., Ptak, M., & Vovelle, F. (1995). Refined three-dimensional solution structure of insect defensin A. *Structure, 3*, 435-

448.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., et al. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *The EMBO Journal, 6*, 2513-2518.

De Coninck, B., Cammue, B. P. A., & Thevissen, K. (2013). Modes of antifungal action and in planta functions of plant defensins and defensin-like peptides. *Fungal Biology Reviews, 26*, 109-120.

De Smet, K., & Contreras, R. (2005). Human antimicrobial peptides: Defensins, cathelicidins and histatins. *Biotechnology Letters, 27*, 1337-1347.

Ding, C.-K., Wang, C. Y., Gross, K. C., Smith, D. L. (2002). Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. *Planta*, *214*, 895-901.

Elfstrand, M., Fossdal, C. G., Swedjemark, G., Clapham, D., Olsson, O., Sitbon, F., et al. (2001). Identification of candidate genes for use in molecular breeding-A case study with the Norway spruce defensin-like gene, Spi 1. *Silvae Genetica, 5*0, 75--81.

Gao, A. G., Hakimi, S. M., Mittanck, C. A., Wu, Y., Woerner, B. M., Stark, D.M., et al. (2000). Fungal pathogen protection in potato by expression of a plant defensin peptide. *Nature Biotechnology, 1*8, 1307-1310.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology, 43*, 205-227.

Gonzalez, M., Pujol, M., Metraux, J.-P., Gonzalez-Garcia, V., et al. (2011). Tobacco leaf spot and root rot caused by *Rhizoctonia solani* Kuhn. *Molecular plant pathology*, *12*, 209-216.

Hardham, A. R. (2001). The cell biology behind *Phytophthora* pathogenicity. *Australasian Plant Pathology*, *30*, 91-98.

Hase, S., Takahashi, S., Takenaka, S., Nakaho, K., Arie, T., Seo, S., et al. (2008). Involvement of jasmonic acid signalling in bacterial wilt disease resistance induced by biocontrol agent *Pythium oligandrum* in tomato. *Plant Pathology, 57*, 870-876.

Huot, B., Yao, J., Montgomery, B. L., He, S. Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Molecular Plant*, *7*, 1267-1287.

Jain, S., Kumar, A. (2015). The pathogenesis related class 10 proteins in plant defense against biotic and abiotic stresses. *Adv Plants Agric Res*, *3*, DOI: 10.15406/apar.2015.03.00077.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter, 5*, 387-405.

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal, 6*, 3901.

Kamoun, S. (2003). Molecular genetics of pathogenic oomycetes. *Eukaryot Cell*, *2*, 191-199.

Kanzaki, H., Nirasawa, S., Saitoh, H., Ito, M., Nishihara, M., Terauchi, R., et al. (2002). Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice. *Theoretical and Applied Genetics, 105*, 809-814.

Kwon, Y-S., Kim, S-G., Chung, W-S., Bae, H., Jeong, S-W., et al. (2014). Proteomic analysis of *Rhizoctonia solani* AG-1 sclerotia maturation. *Fungal biology, 118*, 433-443.

Lacerda, A. F.,Vasconcelos, E.A., Pelegrini, P. B., & Grossi de Sa, M. F. (2014).

Antifungal defensins and their role in plant defense. *Frontiers in Microbiology, 5*, 116.

Lai, F.-M., DeLong, C., Mei, K., Wignes, T., & Fobert, P. R. (2002). Analysis of the DRR230 family of pea defensins: Gene expression pattern and evidence of broad host-range antifungal activity. *Plant Science, 163*, 855-864.

Meng, Y., Zhang, Q., Ding, W., & Shan, W. (2014). *Phytophthora parasitica*: a model oomycete plant pathogen. *Mycology*, *5*, 43-51.

Meyer, B., Houlne, G., Pozueta-Romero, J., Schantz, M.-L., & Schantz, R. (1996). Fruit-specific expression of a defensintype gene family in bell pepper. Upregulation during ripening and upon wounding. *Plant Physiology, 112*, 615-622.

Mitchell, D. J. (1978). Relationships of inoculum levels of several soilborne species of *Phytophthora* and *Pythium* to infection of several hosts. *Phytopathology*, *68*, 1754-1759.

Park, C.-J., Kim, K.-J., Shin, R., Park, J. M., et al. (2003). Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. The plant journal, 37, 186-198.

Park, H. C., Kang, Y. H., Chun, H. J., Koo, J. C., Cheong, Y. H., Kim, C. Y., et al. (2002). Characterization of a stamenspecific cDNA encoding a novel plant defensin in Chinese cabbage. *Plant Molecular Biology, 50*, 57-68.

Parmeter, J. R. (1970). *Rhizoctonia solani*, biology and pathology. *Berkeley : University of California Press.*

Rabhi-Essafi, I., Sadok, A., Khalaf, Noureddine., & Fathallah, D. M. (2007). A strategy for high-level expression of soluble and functional human interferon a as a GST-fusion

protein in *E. coli*. *Protein Eng Des Sel*, *20*, 201-209.

Raftoyannis, Y., Dick, M. W. (2006). Zoospore encystment and pathogenicity of *Phytophthora* and *Pythium* species on plant roots. *Microbiological Research*, *161*, 1-8.

Rostás, M., Simon, M., & Hilker, M. (2003). Ecological cross effects of induced plant responses towards herbivores and phytopathogenic fungi. *Basic and Applied Ecology, 4*, 43‑62.

Rhonda, C. F., Brendan, N. K., James, K. H., Jonathan, P. A., Karam B. S. (2016). Reactive oxygen species play a role in the infection of the necrotrophic fungi, *Rhizoctonia solani* in wheat. *PLOS one*, *11,* e0152548.

Riviere, M. P., Marais, A., Ponchet, M., Willats, W., Galiana, E. (2008). Silencing of acidic pathogenesis-related *PR-1* genes increases extracellular b-(1-3)-glucanase activity at the onset of tobacco defence reactions. *Journal of Experimental Botany*, *59*, 1225-1239.

Seo, H.-H., Park, S., Park, S., Oh, B. J., Back, K. W., Han, O. S., et al. (2014). Overexpression of a defensin enhances resistance to a fruit-specific anthracnose fungus in pepper. *PLoS One, 9*, e97936.

Seethapathy, P., Kurusamy, R., & Kuppusamy, P. (2017). Soil borne diseases of major pulses and their biological management. *Inno. Farm, 2,* 1-11.

Škalamera, D., Wasson, A. P., & Hardham, A. R. (2004). Genes expressed in zoospores of *Phytophthora nicotianae*. *Molecular Genetics, 27*0, 549‑557.

Sneh, B., Burpee, L., Ogoshi, A. (1991). Identification of *Rhizoctonia* species. *American Phytopathological Society*, St. Paul, MN.

Svyatyna, K., & Riemann, M. (2012). Light-dependent regulation of the jasmonate pathway.

Protoplasma, 249, 137-145.

Tewoldemedhin, Y. T., Lamprecht, S. C., Mcleod, A., Mazzola, M. (2006). Characterization of *Rhizoctonia* spp. recovered from crop plants used in rotational cropping systems in the western cape province of south africa. *Plant Dis*, *90,* 1399-1406.

Thomma, B. P., Penninckx, I. A., Cammue, B. P., Broekaert, W. F. (2001). The complexity of disease signaling in Arabidopsis. *Current Opinion in Immunology*, *13*, 63-68.

Thomma, B. P., Cammue, B. P., & Thevissen, K. (2002). Plant defensins. *Planta, 216*, 193-202.

Vain, P., Worland, B., Kohli, A., Snape, J. W., Christou, P., Allen, G. C., & Thompson,W. F. (1999). Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. *The Plant Journal, 18*, 233-242.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L. A., & Fristensky, B. (1999). Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). *Molecular Plant-Microbe Interactions, 12*, 410-418.

West, P. V., Appiah, A. A., Gow, N. A. R. (2003). Advances in research on oomycete root pathogens. *PMPP*, *62*, 99-113.

Zhou, T., & Paulitz, T. C. (1993). In vitro and in vivo effects of *Pseudomonas* spp. on *Pythium aphanidermatum*: Zoospore behavior in exudates and on the rhizoplane of bacteria-treated cucumber roots. *Phytopathology, 83*, 872-876.