Ectopic Expression of *Tsi1* in Transgenic Hot Pepper Plants Enhances Host Resistance to Viral, Bacterial, and Oomycete Pathogens

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In many plants, including hot pepper plants, productivity is greatly affected by pathogen attack. We reported previously that tobacco stress-induced gene 1 (*Tsi1*) may play an important role in regulating stress responsive genes and pathogenesis-related (PR) genes. In this study, we demonstrated that overexpression of *Tsi1* gene in transgenic hot pepper plants induced constitutive expression of several PR genes in the absence of stress or pathogen treatment. The transgenic hot pepper plants expressing *Tsi1* exhibited resistance to *Pepper mild mottle virus* (PMMV) and *Cucumber mosaic virus* (CMV). Furthermore, these transgenic plants showed increased resistance to a bacterial pathogen, *Xanthomonas campestris* pv. *vesicatoria* and also an oomycete pathogen, *Phytophthora capsici*. These results suggested that ectopic expression of *Tsi1* in transgenic hot pepper plants enhanced the resistance of the plants to various pathogens, including viruses, bacteria, and oomycete. These results suggest that using transcriptional regulatory protein genes may contribute to developing broad-spectrum resistance in crop plants.

Additional keywords: pathogen resistance, transcription factor.

To survive, plants must defend themselves against numerous pathogens. Plant-microbial pathogen interactions can be divided broadly into three categories, depending on the outcome. Most frequently, a nonhost interaction occurs, in which the plant usually exhibits no response to the pathogen and the pathogen fails to be colonized in the plant. Alternatively, the plant may actively resist infection by activating numerous defense systems that prevent pathogen replication or spread. These induced resistance responses include the development of a hypersensitive response (HR), in which the pathogen is restricted to the cells within and surrounding necrotic lesions that form at the sites of infection (Dempsey et al. 1998). Often associated with the HR are the increased synthesis of phytoalexins, the deposition of compounds that increase cell wall strength, and the expression of several families of pathogenesis-related (PR) genes. Subsequent to HR development, the upper uninoculated portions of the plant frequently develop a long-lasting, broad-spectrum resistance to even unrelated

pathogen infection known as systemic acquired resistance (SAR) (Dempsey et al. 1998; Ward et al. 1991).

The PR proteins are classified into 14 families. PR proteins include fungal cell wall-degrading enzymes (β -glucanase [PR-2], and chitinases [PR-3, -7, -8, and -11]), hevein-like protein (PR-4), osmotin (PR-5), the protein with RNase activity (PR-10), proteinase inhibitor (PR-6), and antimicrobial peptides (defensin [PR-12], thionin [PR-13], and lipid transfer proteins [PR-14]) (van Loon and van Strien 1999). Overexpression of some PR proteins in plants has been reported to enhance plant resistance against fungal or bacterial disease (Alexander et al. 1993; Broglie et al. 1991; Datta et al. 1999). However, Linthorst and associates (1989) reported that constitutive expression of each of the PR proteins in transgenic plants did not affect the susceptibility of the plants to infection with various viruses. In addition, no report has shown development of broad-spectrum resistance using PR proteins. To check the feasibility of broad spectrum resistance, we tried the ectopic expression of tobacco stress-induced gene 1 (*Tsi1*) in hot pepper because our previous result showed overexpression of the gene in transgenic tobacco plants enhanced host resistance to pathogens (Park et al. 2001b).

Tsi1, a tobacco ethylene-responsive element-binding protein/APETALA 2 (EREBP/AP2)-type transcription factor gene showing sequence similarity to *Pti6* gene, has been previously cloned. The induced transcription of the EREBP/AP2 subfamily genes appears to be necessary for the plant's adaptation to a variety of stresses. The transcriptional activation of *AtERF1* is most likely responsible for the ethylene-induced expression of a subset of *Arabidopsis* genes with the GCC box (Solano et al. 1998), whereas those of CRT/DRE binding-factor proteins/dehydration-responsive element-binding proteins (CBFs/DREBs) contribute to the cold-induced expression of genes containing the dehydration-responsive element/C-repeated element (DRE/CRT) (Liu et al. 1998). Ectopic expression of *AtERF1* under the control of the CaMV 35S promoter results in constitutive expression of a chitinase gene and the *PDF1.2* gene, and both contain the GCC box in the promoter (Solano et al. 1998). The bacterial pathogen-induced expression of *Pti4* and *Pti5* suggested that the two genes play a role in plant defense signaling (Thara et al. 1999). The *Tsi1* gene is induced by SA, ethylene, methyl jasmonate, salt, and wound treatments. Transgenic tobacco plants carrying the 35S::*Tsi1* fusion gene showed tolerance to high salt stress and bacterial inoculation (Park et al. 2001b). Jaglo-Ottosen and associates (1998) and Kasuga and associates (1999) generated transgenic *Arabidopsis* plants overexpressing genes for stress-responsive transcription factor *CBF-1* and *DREB1A*, respectively. These plants showed increased expression of stress-response genes and enhanced freezing and drought tolerance in *Arabidopsis* spp.

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In this study, 35S::*Tsi1* transgenic hot pepper plants were generated and it was demonstrated that these plants exhibited enhanced resistance to a wide spectrum of pathogens, including viruses, a bacterium, and an oomycete. Tobamoviruses are among the most destructive pathogens of pepper crops (Boukema 1980). Bacterial spot disease of tomato and pepper, caused by *Xanthomonas campestris* pv. *vesicatoria*, can be devastating to commercial production of these crops in areas of the world with high humidity and heavy rainfall (Tai et al. 1999). Late blight caused by the oomycete *Phytophthora* is the most devastating disease of potato (*Solanum tuberosum*) and hot pepper (*Capsicum annuum* L.) worldwide (Ross 1986).

RESULTS

Overexpression of *Tsi1* in transgenic hot pepper plants triggers constitutive expression of multiple PR genes.

Previously, we constructed pMBP2-*Tsi1* for constitutive expression of *Tsi1* in plants (Fig. 1A) (Park et al. 2001b).

Fig. 1. Construction of *Tsi1* expression vector and expression of *Tsi1* and pathogenesis-related (PR) genes in transgenic hot pepper plants. **A**, Schematic diagram of pMBP2-*Tsi1*. *Tsi1*, the coding sequence of the *Tsi1* gene; 35S-Pro, the promoter sequence of the *Cauliflower mosaic virus* 35S transcript; NPTII, coding sequence of the neomycin phosphotransferase II gene; Nos-ter, the terminator sequence of nopaline synthase gene. **B**, Expression of the *Tsi1* gene and PR genes in the pMBP2-*Tsi1* transgenic plants. Total RNA was isolated from the plants and analyzed by RNA gel blot hybridization using the *Tsi1* and PR gene cDNAs as probes; numbers indicate independent lines of transgenic T₁ plants. *CaPR-1*, *ntPR-2*, *CaPR-4*, *ntPR-5*, *CaPinII*, *CaPR-10*, *CaLTP1*, or *CaSAR8.2* indicate various cDNA clones used to measure each PR gene expression. N indicates empty vector-transformed hot pepper plant.

pMBP2-*Tsi1* was transformed into hot pepper plants using *Agrobacterium tumefaciens* (Kim et al. 1997). Fifteen independent T₀ lines were selected based on kanamycin resistance and polymerase chain reaction (PCR) with specific primers for neomycin phosphotransferase II gene (Kim et al. 1997). The expression of the transgene in the transgenic hot pepper T₁ plants was confirmed by RNA gel blot analysis using the *Tsi1* cDNA as a probe (data not shown).

Park and associates (2001b) demonstrated that several PR genes were constitutively expressed in transgenic tobacco plants overexpressing the *Tsi1* gene. We chose eight independent T₁ lines which expressed *Tsi1*, and expression of several PR genes was monitored under normal conditions by RNA gel blot analysis (Fig. 1B). *Tsi1* transgenic hot pepper plants constitutively expressed various families of PR genes as well as the *Tsi1* gene itself. *Tsi1* homologues exist in hot pepper plants; however, these homologues were barely expressed under normal conditions (lane N of Fig. 1B). The expression level of a number of PR families was not exactly in proportion to the expression level of *Tsi1*; however, line 8 was shown to be expressing *Tsi1* and most PR genes tested at much higher levels than the other lines.

Enhanced resistance against *Pepper mild mottle virus* and *Cucumber mosaic virus* infection in transgenic plants overexpressing *Tsi1*.

The *Pepper mild mottle virus* (PMMV) resistance of each transgenic line compared with the control plants through the enzyme-linked immunosorbent assay (ELISA) tests is shown in Figure 2A. Two or three leaves of 10 four- to six-leaf stage transgenic T₁ plants per eight independent lines and 10 empty vector (pMBP2) transformed transgenic plants (control plants) were inoculated with PMMV or *Cucumber mosaic virus* Y (CMV-Y) for determination of enhanced virus resistance. Starting at 14 days post inoculation (dpi), newly emerging upper leaves were taken for infection analysis. At 14 dpi to PMMV, the ELISA reading absorbance of the control plants was two times that of the transgenic plants. Line 7 and especially line 8 showed a very good resistance. Moreover, virus accumulation was not increased in lines 7 and 8 plants up to 56 dpi. Typical resistance and growth difference between a transgenic line 8 hot pepper plant and a control plant 56 dpi of PMMV is shown in Figure 3A (transgenic pepper, left; control, right). In spite of the existence of PMMV in transgenic plants, transgenic plants showed normal growth and reproduction, but control plants showed abnormality. We tested further whether these *Tsi1* transgenic plants would show resistance to other kinds of virus infection. The *Tsi1* transgenic plants showed similar resistance to CMV infection as to PMMV infection. However, unlike the case of PMMV inoculation, the absorbance increased continuously as time went by, meaning that CMV population was increased, except in line 8, after inoculation. Still, *Tsi1* transgenic plants exhibited over 22% resistance to CMV compared with the control plants 56 dpi (Fig. 2B). Again, line 8 showed outstanding resistance to CMV infection, similar to that of a negative uninoculated control (Figs. 2B and 3B).

Enhanced resistance against *P. capsici* infection in *Tsi1* transgenic plants.

Twenty plants of each T₁ transgenic line expressing the *Tsi1* and control plants were raised up to the six-leaf stage and divided into two groups for inoculation with two different *P. capsici* isolates. One *P. capsici* isolate, Pa-23, was known to cause rapid, severe symptoms and the other, *P. capsici* isolate youngyangsubi, was known to cause delayed mild symptoms on the hot pepper plants. At 3 dpi of Pa-23, control plants became slenderized in the stem; then, after 7 days, approximately

90% of the control plants underwent damping-off. However, *Tsi1* transgenic T₁ plants of line 3, line 5, and line 7 were less than 50% infected, and showed only slightly slenderized stem and minor damping-off compared with the control plants. Moreover, among the T₁ transgenic plants of line 8, no single plant showed damping-off symptoms (Table 1). A representative control plant exhibiting damping-off symptoms and a transgenic plant of line 8 showing no detectable disease symptoms are shown in Figure 3C (upper picture).

In the case of the *P. capsici* isolate youngangsubi inoculation, even the control plants did not show damping-off symptoms initially. However, at 14 dpi, the control plants displayed a brown, vertical-split symptom in the stem (Fig 3C-b and -c). We measured the length of the brown split symptom in hot pepper stem after 10 dpi. The transgenic plants expressing the *Tsi1* displayed less than 30% infection symptom; however, the control plants exhibited more than 90% infection. In addition, approximate brown lesion lengths of control plants were two or three times longer than those of transgenic plants (Table 1).

Reduced growth of *X. campestris* in *Tsi1* transgenic plants.

To determine whether the *Tsi1* transgenic plants would exhibit resistance against bacterial infection, we measured *X. campestris* pv. *vesicatoria* race 1 and race 3 population levels in transgenic plants of line 8 and control plants after infiltration of bacteria. When race 1 was infiltrated into the control plants, bacterial populations increased to approximately 3×10^8 CFU/ml 7 days after infiltration. However, in line 8, bacterial populations increased only up to approximately 2×10^6 CFU/ml (Fig. 4). What is more, *Tsi1* transgenic plants of line 8 had a few visible symptoms; however, control plants showed browning, withering, and yellowing of a large area (Fig. 3D). However, when race 3 was infiltrated into line 8 transgenic plants, the transgenic plants showed only slightly reduced bacterial growth compared with the control plants (data not shown).

DISCUSSION

Tsi1, which contains a highly conserved EREBP/AP2-type DNA binding domain, binds specifically to the GCC box sequence containing the core sequence GCCGCC in vitro (Park et al. 2001b), which is present in the promoter region of a large number of ethylene-inducible genes, including PR genes (Ohme-Takagi and Shinshi 1995). In addition, TSI1 also binds to the DRE/CRT element (Park et al. 2001b), which exists in the promoter region of drought-, salt-, and freezing-related genes (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). TSI1 actually might be able to bind to the promoters of several PR genes in vivo and then lead to the expressions of several PR genes. In fact, *Tsi1* overexpressing transgenic plants which were expressing several PR genes showed enhanced tolerance to high-salt stress and bacterial inoculation (Park et al. 2001b). However, the relative quantity of PR gene expression was variable between the different lines and did not always coincide with that of *Tsi1* expression in transgenic plants (Fig. 1B). The resistance level was not exactly correlated to the PR gene expression level. For example, even though *Tsi1* transgenic line 7 also showed resistance as good as that of line 8, at least in the case of PMMV resistance, its PR gene expression profile was rather different from that seen in line 8.

The DRE/CRT motifs are known to be important to low-temperature-induced *LT178* (Mantyla et al. 1995), cold-regulated *COR78* (Horvath et al. 1993), *RD29A* (Yamaguchi-Shinozaki and Shinozaki 1994), and *SFR* ("sensitive to freezing") (Knight et al. 1999) gene expression, and these gene expression levels depend on the binding affinity between the transcription factors and the element. Thus, abiotic stress-induced gene or PR gene

expression levels may rely on binding strength of the transcription factor and the available number of DRE/CRT motif or GCC box in each gene. The difference between the expression levels of each PR gene in *Tsi1* transgenic plants may reflect different TSI1 binding strength to the promoters of PR genes, and the difference between the lines in terms of PR gene expression profiles may be caused by the positional variation of the *Tsi1* expression.

Invasion of plants by pathogens or application of pathogen-derived elicitors results in rapid evolution of ethylene (Yang and Hoffman 1984) and is followed by the increased expression of genes encoding PR proteins such as PR-1, β -1,3-glucanase (PR-2), chitinase (PR-3), and osmotin (PR-5) (Liu et al. 1994; Yang et al. 1997). These PR genes also are induced by exogenous application of ethylene (Raghothama et al. 1997; Ward et al. 1991; Yang et al. 1997; Yun et al. 1997). Ethylene, therefore, is considered to be a component of the signal transduction pathway leading from pathogen infection to defense responses. However, ethylene-insensitive mutants of *Arabidopsis* are not compromised for certain disease-resistance responses (Bent et al. 1992; Lawton et al. 1994), indicating that signal transduction pathways from exogenous ethylene and pathogen infection share overlapping components but are not identical. The osmotin gene (*PR-5*) is induced by fungal infection (Liu et al. 1994), as well as by numerous developmental, hormonal, and environmental signals (Kononowicz et al. 1994).

Fig. 2. Virus resistance of transgenic plants expressing the *Tsi1*. Relative absorbance of transgenic T₁ plants compared with control plants showing infection on successive days after inoculation with a strain of **A**, *Pepper mild mottle virus* (PMMV) and **B**, *Cucumber mosaic virus Y* (CMV-Y). Samples were taken at 14, 28, or 56 days post inoculation (dpi). Each virus was detected by indirect enzyme-linked immunosorbent assay using anti-PMMV immunoglobulin G (IgG) (2 µg/ml) or anti-CMV IgG (3 µg/ml). Lanes 1 to 8: independent T₁ transgenic lines. The mean number of three experiments was shown on the graph. V indicates inoculated empty vector transformed control plants and C indicates noninoculated control plants. Error bars indicate standard deviations.

A 140-bp minimal promoter fragment that was responsive to the same signals as the full-length osmotin promoter was found to contain two GCC boxes that were required for ethylene responsiveness but were not adequate for maximal ethylene response (Raghothama et al. 1997). Superinduction of the osmotin gene by combinations of inducers has been reported (Chang et al. 1997). Like the osmotin gene, other PR genes also have

GCC-boxes that are interacting with *Tsi1*. Not only many of PR genes but also EREBP/AP-2 type transcription factors, including *Tsi1*, are regulated by ethylene; thus, it is possible that ethylene may regulate *Tsi1* expression directly and then *Tsi1* may bind to GCC-box of PR genes' promoters and regulate PR gene expression (Park et al. 2001b; Raghothama et al. 1997; Ward et al. 1991; Yang et al. 1997; Yun et al. 1997).

Fig. 3. Enhanced disease resistance of transgenic hot pepper plants constitutively expressing the *Tsi1*. Enhanced resistance to **A**, *Pepper mild mottle virus* or **B**, *Cucumber mosaic virus* Y. At 56 days after inoculation, plants were examined for disease symptom and plant growth. Left plants are *Tsi1* transgenic plants and right plants are control plants. **C**, Disease response to the oomycete pathogen, *Phytophthora capsici* isolate Pa-23 (upper picture and d and e of lower picture) and youngyangsubi (b and c of lower picture). At 7 days after inoculation for Pa-23 and 10 days after inoculation for youngyangsubi, plants were examined for disease severity: a, healthy stem; b, brown vertical-split symptom stem and slight damping-off; c, brown vertical-split symptom stem; d and e; damping-off. **D**, Comparison of symptom to *Xanthomonas campestris* pv. *vesicatoria* race 1 at 7 days after inoculation. Representative result of *Tsi1* transgenic line 8 T₁ plant (left) or control plant (right) is shown here. The arrows indicate diseased areas.

In tomato, the *R* gene *Pto* confers resistance to *Pseudomonas syringae* pv. *tomato* strains expressing the *avrPto* gene. The molecular cloning of the *Pto* gene and the corresponding avirulence gene, *avrPto*, permitted extensive investigation of the mechanism of *R* gene-mediated defense responses. *Pto* encodes an active serine/threonine protein kinase (Loh and Martin 1995; Martin et al. 1993), and *avrPto* encodes a small hydrophilic protein that probably is delivered into the plant cell by way of a bacterial type III secretion system (Alfano and Collmer 1996; Ronald et al. 1992). To identify components involved in *Pto*-mediated signal transduction pathways leading to disease resistance, Zhou and associates (1997) previously used PTO as a bait in the yeast two-hybrid system to search for Pto-interacting (Pti) proteins. Among them, *Pti4*, *Pti5*, and *Pti6* encode transcription factors which are strikingly similar to those of the EREBPs both in structure and GCC-box binding properties. The *Tsi1* gene showed a similar expression pattern and sequence homology with *Pti6*, and their functions may be similar (Park et al. 2001b). We also cloned hot pepper *Tsi1* homologues and their expression profile resembles that of tobacco *Tsi1* (data not shown). The *Tsi1* homologues in hot pepper may function in similar signal pathways, and similar genes may be regulated.

The resistance of *Tsi1* transgenic plants to bacterial infection was relatively weak compared with the other pathogen infections (Fig. 4). This observation may be related to the fact that infection attempts of various microbial pathogens on plants trigger a complex set of defense responses requiring activation of distinct signaling pathways (Thomma et al. 1998). In *Arabidopsis* spp., induction of the antimicrobial protein genes *PR-1*, *PR-2*, and *PR-5* follow an SA-dependent pathway, whereas the induction of the plant defensin *PDF1.2*, the basic chitinase *PR-3*, and the *PR-4* depends on a pathway involving at least jasmonic acid (JA) as a signal molecule. The availability of different *Arabidopsis* mutants affected in these response pathways allows assessment of the significance of these pathways in resistance against pathogens (Thomma et al. 1998; Uknes et al. 1992). The level of *Tsi1* transcripts was various in each transgenic plant (Fig. 1B). The discrepancy in levels of diverse PR gene transcripts in this case may reveal the delicate tuning requirement of plant resistance upon various pathogen infections. Even though *Tsi1* transgenic plants showed resistance to *X. campestris* pv. *vesicatoria* race 1, when race 3 was infiltrated into line 8 transgenic plants, the transgenic plants showed only slightly reduced bacterial growth compared with the control plants (data not shown). Thus, even though *Tsi1* overexpressing transgenic plants basically showed the broad-spectrum resistance, in this case there were subtle differences depending on the race. This phenomenon also may be related

to the differences of pathogenesis factor of race 1 and race 3 per se (Cook and Sequeira 1991; Gonzales et al. 1998).

Previous results showed that overexpressing one or two PR genes in transgenic plants had a minor effect on pathogen resistance (Linthorst et al. 1989), whereas simultaneous introduction of many PR genes had obvious technical limitations. However, transgenic hot pepper plants expressing the *Tsi1* gene showed viral, oomycete, and bacterial resistance. Such a broad-spectrum disease resistance using transcriptional regulatory protein gene in many crop plants may contribute to solving the practical agricultural problems of multiple infections.

MATERIALS AND METHODS

Construction of plasmid and plant transformation.

A plasmid used in the transformation of hot pepper was constructed with *Tsi1* full-length cDNA that was cloned into a polylinker site of a binary vector, pMBP2 (Han et al. 1999), in the sense orientation. After pMBP2 was digested with *Bam*HI and ligated with the *Bam*HI fragment of the *Tsi1* cDNA, the orientation of the recombinant plasmids was determined by *Eco*RI digestion (Park et al. 2001b). The constructs and pMBP2 empty vector were introduced into *C. annuum* cv. Nockwang using *Agrobacterium*-mediated transformation. The cotyledons and hypocotyls, with tip and petiole ends removed from the seedlings, were transformed as described (Kim et al. 1997; Lee et al. 1993). The explants then were planted on the basal Murashige-Skoog medium supplemented with kanamycin at 200 mg/liter, sucrose at 30 g/liter, zeatin at 2 mg/liter, and naphthalene acetic acid at 0.1 mg/liter, and grown at 25°C in a 16-h photoperiod under fluorescent light (20,000 lux) for 4 weeks.

RNA blot analysis.

Total RNA was prepared from 4-week-old leaves as described by Ausubel and associates (1995). Total RNA (20 µg) was electrophoresed on a 1.5% agarose gel containing 6% formaldehyde in 3-(*N*-morpholino)-propanesulfonic acid buffer, pH 7.0, and transferred to a Nytran Plus membrane. Hybridization was performed according to a published protocol (Church and Gilbert 1984), and the membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 10 min twice, with 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min, and with 0.1× SSC containing 0.1% SDS

Table 1. Enhanced resistance against *Phytophthora capsici* isolates in control and *Tsi1* transgenic T₁ hot pepper plants^a

T ₁ line	Isolate Pa-23		Isolate youngyangsubi	
	Infected/tested plants (%)	Disease severity ^b	Infected/tested plants (%)	Lesion dev. (cm) ^c
3	33.3 ± 5.8	++	23.3 ± 5.8	1.1 ± 0.3
5	53.3 ± 15.3	++	30.0 ± 10.0	1.5 ± 0.2
7	20.0 ± 0.0	+	16.7 ± 5.8	0.8 ± 0.1
8	3.3 ± 5.8	–	13.3 ± 5.8	0.7 ± 0.1
Control	93.3 ± 5.8	+++	90.0 ± 10	3.3 ± 0.4

^a Experiments were carried out three times, with 10 plants used for infection each time.

^b Disease severity in plants after 7 days post inoculation (dpi): – = absence of symptoms, + = slenderized lower stem, ++ = severely slenderized and slight damping-off, +++ = complete damping-off.

^c Lesion development: length of brown split symptom in the hot pepper stem 10 dpi.

Fig. 4. Growth of *Xanthomonas campestris* pv. *vesicatoria* race 1 in leaves of control plants and *Tsi1* transgenic line 8 T₁ plants. Plants were infiltrated with a syringe containing bacterial inoculum at 1×10^5 CFU/ml. The mean number of four experiments was plotted on the graph. Error bars indicate standard deviations.

at 65°C for 5 min. The membranes were dried and exposed to X-ray film or visualized directly with the BAS-2500 phosphorimager (Fuji Photo Film, Tokyo). The *Tsi1* probe and other probes (*CaPR-1*, *ntPR-2*, *CaPR-4*, *ntPR-5*, *CaPinII*, *CaPR-10*, *CaLTP1*, and *CaSAR8.2* cDNA clones isolated previously from hot pepper and tobacco) (Kang et al. 1998; Park et al. 1999, 2001a; Shin et al. 2001), were ³²P-radiolabeled by PCR.

Viruses and antisera.

CMV-Y and its antiserum were kindly supplied by E.-K. Park (Korea Ginseng and Tobacco Research Institute, Taejeon, Korea). PMMV showing P_{1,2} pathotype on pepper plants was obtained from S.-H. Nam (Seminis Korea Co. LTD., Chochiwon, Korea). The antisera of PMMV were produced in our laboratory. CMV and PMMV were maintained in infected leaves of tobacco (cv. Samsun) on CaCl₂-containing petri dishes. Plant sap containing PMMV and CMV was prepared by grinding the infected leaves in phosphate buffer (0.25 M Na₂HPO₄ and 5 mM EDTA). To inoculate plants, sap was applied to the surface of two or three fully expanded young leaves of hot pepper seedlings (four- to six-leaf stage) and rubbed with carborundum (mesh 600) (Hayashi Chemical, Osaka, Japan). Mock-inoculated plants were rubbed with phosphate buffer and carborundum only.

Indirect ELISA.

Indirect ELISA was used for detection of CMV-Y and PMMV. All reagents were used at 150 µl per well in microtiter plate except the blocking steps, which were 200 µl per well. A leaf disk (8 mm in diameter) was put in a microcentrifuge tube and ground with a disposable grinder in 200 µl of extraction buffer (0.1 M Tris-HCl with 1% sodium sulfite, pH 7.4). Each leaf disk was sampled at 14, 28, or 56 days after virus inoculation. The extracts diluted with threefold 0.15 M carbonate buffer, pH 9.8, were incubated in the wells at 37°C for 3 h. After three washes with 0.01 M Tris-HCl containing 0.85% sodium chloride and 0.05% Tween-20 (TBST), pH 7.4, the plates were blocked for 40 min with 1% bovine serum albumen in 0.1 M Tris-HCl with 0.85% sodium chloride (TBS), pH 7.4. After being incubated with antigens, plates were washed with TBST and then incubated with CMV-Y (3 µg/ml) or PMMV (2 µg/ml) antibodies for 2 h. The plates were washed again and incubated with secondary antibody at 1 µg/ml. After three washes in TBST, substrate solution containing *p*-nitrophenyl phosphate (1 mg/ml) in 0.2 M carbonate buffer, pH 9.8, was added and a reading was done at A₄₀₅ nm with an ELISA reader (Bio-Rad, Hercules, CA, U.S.A.) 30 min after substrate addition.

Inoculation with *P. capsici*.

To investigate the resistance to *P. capsici* infection, two isolates were used: Pa-23 with severe symptoms and youngyangsubi with mild symptoms. *P. capsici* was fully grown in oat meal agar plate at 28°C for 7 days and then cultured in a 16-h photoperiod under fluorescent light (20,000 lux) at 25°C for 3 days. After light treatment, sterile tap water was added to the plate and then the plate was kept at 4°C for 30 min. After that, the plate was put at room temperature for 30 min, and then the numbers of zoospores were scored. The soaked cotton wool in a suspension of zoospores (5 × 10⁴ ml⁻¹) was wrapped around the hot pepper stem between cotyledon and real leaves of six-leaf stage hot pepper plants.

X. campestris pv. *vesicatoria* inoculation.

Bacterial cultures for plant inoculations were grown in nutrient broth for 18 h at 28°C with shaking incubator (Vision, Seoul, Korea). Cells were pelleted by centrifugation (4,000 × g, 15 min) and resuspended in sterile tap water. The concentra-

tion of bacterial cells was adjusted to an optical density₆₀₀ = 0.3 (2 to 5 × 10⁸ CFU/ml) with a spectrophotometer (WPA, Cambridge, U.K.). Bacterial suspensions were diluted to a concentration of 1 × 10⁵ CFU/ml in sterile tap water, and then leaves of pepper were infiltrated with a hypodermic syringe as previously described (Hibberd et al. 1987).

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