

Capsicum annuum Tobacco Mosaic Virus-Induced Clone 1 Expression Perturbation Alters the Plant's Response to Ethylene and Interferes with the Redox Homeostasis¹

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Capsicum annuum tobacco mosaic virus (TMV)-induced clone 1 (*CaTin1*) gene was expressed early during incompatible interaction of hot pepper (*Capsicum annuum*) plants with TMV and *Xanthomonas campestris*. RNA-blot analysis showed that *CaTin1* gene was expressed only in roots in untreated plants and induced mainly in leaf in response to ethylene, NaCl, and methyl viologen but not by salicylic acid and methyl jasmonate. The ethylene dependence of *CaTin1* induction upon TMV inoculation was demonstrated by the decrease of *CaTin1* expression in response to several inhibitors of ethylene biosynthesis or its action. Transgenic tobacco (*Nicotiana tabacum*) plants expressing *CaTin1* gene in sense- or antisense-orientation showed interesting characteristics such as the accelerated growth and the enhanced resistance to biotic as well as abiotic stresses. Such characteristics appear to be caused by the elevated level of ethylene and H₂O₂. Moreover, in transgenic plants expressing antisense *CaTin1* gene, the expression of some pathogenesis-related genes was enhanced constitutively, which may be mainly due to the increased ethylene level. The promoter of *CaTin1* has four GCC-boxes, two AT-rich regions, and an elicitor-inducible W-box. The induction of the promoter activity by ethylene depends on GCC-boxes and by TMV on W-box. Taken together, we propose that the *CaTin1* up-regulation or down-regulation interferes with the redox balance of plants leading to the altered response to ethylene and biotic as well as abiotic stresses.

Multiple preformed antimicrobial compounds contribute to the constitutive defense machinery of plants against pathogenic organisms. In addition, plants can trigger the inducible defense programs upon the perception of invaders. The early signaling molecules leading to *R*-gene-mediated resistance are ion fluxes, GTP-binding proteins, protein kinases, phosphatases, and phospholipases (Hammond-Kosack and Jones, 1996). Plants also respond to external stimuli such as microbial elicitors of cell death and/or defense responses by changing the calcium influx of the cell (Ebel and Scheel, 1997; Higgins et al., 1998). The increase of cytosolic calcium leads to cell death, which seems necessary for hypersensitive cell death triggered by rust fungi (Xu and Heath, 1998). The calcium channel blocker La³⁺, on the other hand, prevents the bacteria-

induced hypersensitive response (HR) in soybean (*Glycine max*) leaves (Levine et al., 1996). The burst in the oxidative metabolism generated within minutes of infection leads to the accumulation of the reactive oxygen intermediates (ROI) such as H₂O₂ and superoxide anion (O₂⁻), involving NADPH-dependent oxidase. ROI serve as signaling molecules, for example in the recognition of the attack by fungal pathogens (Lamb and Dixon, 1997).

Ethylene is widely known to modulate the organ senescence induced by various stress factors such as plant pathogens (Moore et al., 2000), O₃⁻ (Pell et al., 1997), and hypoxia (He et al., 1996). O₃⁻ induces genes involved in the ethylene biosynthesis (Overmyer et al., 2000). The study of O₃⁻-sensitive Arabidopsis *rcd1* mutant demonstrated that lesion-initiation processes in plants were independent of ethylene while lesion-propagation processes were ethylene-dependent (Overmyer et al., 2000). In addition, Arabidopsis *ctr1* mutants exhibit the constitutive activation of the ethylene-response pathway. It is proposed that exposure to ethylene inactivates a *CTR1* MAP kinase cascade, triggering plant ethylene responses (Bleecker and Kende, 2000). The study of the role of ethylene in the disease resistance revealed the interrelationship between ethylene and another stress hormone, methyl jasmonate (MeJA). MeJA appears to stimulate the ethylene biosynthesis and vice versa (Laudert and Weiler, 1998). In general, during the disease resistance

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signal transduction, at least two separate pathways are required for the resistance against different pathogens: salicylic acid (SA)-dependent pathway and MeJA/ethylene-dependent pathway. However, our study demonstrates that the newly isolated gene *Caspsicum annuum* TMV-induced clone 1 (*CaTin1*) expression is dependent only on ethylene but not MeJA or SA. Previous studies showed that ethylene exerted both the positive and the negative effect on its own biosynthesis (Johnson and Ecker, 1998).

The TMV-induced hot pepper (*Caspsicum annuum*) gene, *CaTin1* gene, was isolated as an incompatible interaction-specific gene through the differential screening between TMV inoculated and mock inoculated hot pepper plants (Shin et al., 2001). *CaTin1* was characterized further because *CaTin1* was induced by ethylene but not by SA or MeJA. *CaTin1* gene has the PKD1, lipoxygenase, alphatoxin, and triacylglycerol lipase (PLAT) domain that is known to exist in PKD gene whose function is unknown (Bateman and Sanford, 1998). The three-dimensional structure of the PLAT domain of human pancreatic lipase (van Tibeurgh et al., 1993), rabbit 15-lipoxygenase (Gillmor et al., 1997), and alpha toxin from *Clostridium perfringens* (Naylor et al., 1998) is characterized. In plants, the PLAT domain may interact directly with the membrane in a Ca^{2+} dependent manner. Or the PLAT domain may be involved in the protein-protein and/or protein-lipid interaction (Bateman and Sanford, 1998). An ethylene-induced cDNA encoding a lipase that contains the PLAT domain was expressed at the onset of senescence (Hong et al., 2000). In tobacco, ethylene induces the expression of basic pathogenesis-related (PR) genes by a calcium-dependent pathway that requires protein phosphorylation (Raz and Fluhr, 1992, 1993). Calcium could influence the ethylene biosynthesis by affecting ACC oxidase transcription (Kwak and Lee, 1997) and its activity (Gallardo et al., 1999). Calcium also regulates the ethylene-dependent pathway of the PR gene induction that is required for the ethylene-promoted morphogenic responses in seedling (Raz and Fluhr, 1992). The data suggest that *CaTin1* may function on the ethylene-dependent incompatible interaction between plants and avirulent pathogens.

CaTin1 gene was induced by ethylene and methyl viologen (MV). The promoter of *CaTin1* gene has four GCC-boxes and one W-box. The analysis of PR gene promoters has led to the identification of the 11-bp ethylene-responsive element TAAGAGCCGCC, which has been referred as the GCC-box (Ohme-Takagi and Shinshi, 1995). This element was shown to be required for the regulation of ethylene in plants. Several ethylene-responsive element binding proteins function by binding to the ethylene-responsive GCC-box that is involved in the pathogen-activated transcription of PR genes (Leubner-Metzger et al., 1998; Park et al., 2001b). The elicitor responsive element, W-box (T)TGAC(C), was first identified within the parsley (*Petroselinum crispum*) *PR1-1* and *PR1-2* pro-

motors and the WRKY transcription factor is known to bind to the W-box (Rushton et al., 1996). Another WRKY transcription factor, *TDBA12*, also recognizes a W-box. *TDBA12* is suggested to serve as a component in pathogen-induced signal transduction pathways in plant cells and to regulate the expression of certain plant defense genes (Yang et al., 1999).

RESULTS

The *CaTin1* Gene Has the PLAT Domain and Is Expressed Only in Roots

In the previous study, we isolated *N6-11* gene that was one of the genes induced specifically during the incompatible interaction between hot pepper plants and TMV (Shin et al., 2001). The clone, renamed as *CaTin1*, contains the 775-bp insert encoding a protein of 180 amino acid residues (Fig. 1). The molecular mass of the predicted mature protein is 19.8 kD. The sequence of *CaTin1* is not homologous to any known genes except the PLAT domain (lined in Fig. 1). The function of this domain in plants is not known. However, a few elicitor-induced clones having the PLAT domain were found (AF314810, AB040407, and AC083945).

Based on the high stringent Southern-blot analysis, a few copies of *CaTin1* gene were detected in pepper genome (data not shown). To examine the steady state transcription level of *CaTin1* gene in various organs of pepper plants, the total RNA was extracted from the red ripe fruits, green unripe fruits, young leaves, old leaves, roots, stems, and flowers. As shown in Figure 2A, the transcription of *CaTin1* was detected only in roots and not in any other organs.

The *CaTin1* Expression during the Incompatible Interaction with TMV and *Xanthomonas campestris*

The expression pattern of *CaTin1* gene was monitored in hot pepper cv Bugang plants after TMV-P₀ (avirulent strain) inoculation. A mock inoculation treatment was carried out as a control to rule out any *CaTin1* gene expression that might arise from wounding due to rubbing with carborundum. As shown in Figure 2B, the accumulation of *CaTin1* transcripts was barely detected in mock-inoculated or TMV-P_{1,2} (virulent strain) inoculated leaves. However, *CaTin1* transcripts started to accumulate from 24 h after the inoculation with TMV-P₀ and increased until 72 h after the inoculation. As a positive control, the expression pattern of the *CaPR1* gene, one of the PR genes isolated from hot pepper, was also monitored (Shin et al., 2001). The induction of *CaPR1* gene was observed from 48 h after the inoculation. This indicates that the expression of *CaTin1* gene was induced earlier than *CaPR1* gene.

We also examined whether *CaTin1* could be induced during the systemic acquired resistance (SAR) after TMV inoculation. The transcripts corresponding to *CaTin1* gene were accumulated in the distant

CaTin1	MS...NQFWRLTIILISISVSSISGSE	25
Tobacco	MGVAHQVNFQWFPLIILLISITISSTSGAE	30
Arabidopsis	MA...RRDVLPL.FLLLLATVSAVAFAEDD	26
Rape	MMP...RRDVLFLSLLLVIAVSAVALADDE	28
Rice	MN.....KLSLALASYLILFLAAAATAAT	24
Plexaura	AV.....YTWVQHLRKLKIGSLVPAG	390
Human	FC.....GQR	3105
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CaTin1	LNCVYSVYVQGGQFFGAGDSKITLSLYDA	55
Tobacco	SNCVYTAYIRGPFMEDADDSKISLTLYDA	60
Arabidopsis	PDCVYTFYLRGSIWKAGDSIISARIYDK	56
Rape	ADCVYTFFLRGSTFKAGDSIISARVYDK	58
Rice	TRCTFEIIVVKDGRNAGDARVSLQVRAA	54
Plexaura	QNAIYNVEVEGDREHAGDATITIRITGA	420
Human	GRFKYEILVKGWGRGSGTAHVGMILYGV	3135
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CaTin1	DGYGLRIKSTFAWGGLMGSGYDYFEFGKLD	85
Tobacco	SGYGIIRIKNVAWGGLMGSGYNYFETDHS	90
Arabidopsis	DGDYIGIKNQAWAGLMPDYNFERGNLD	86
Rape	YGDYIGIRNFAWGGLMGPGYNYFERGNLD	88
Rice	RGPTLTVAVEESWG.QMAAGHDYFEKGNLD	83
Plexaura	KG.RTDYLLKDKWF.....HNDFEAGSKE	444
Human	DS.RSGHRHLDGDR.....AFHRNSLD	3157
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CaTin1	LFTGRGPCLN.PVCKMNLTSIGT...QHAG	112
Tobacco	MESGHGPCLT.PICKMVLTSIGT...RQSA	117
Arabidopsis	IFSGRAPCLPSPICALNLTSGSE...DHHG	114
Rape	IFSGKAPCLPSPVCSNLNLTSGSE...DHHG	116
Rice	RFRGAGDCMPSEPCNM.LTSGSE...NKPG	110
Plexaura	QYTVQGFVDG.DIQLIELHSEGGFYWSGPD	474
Human	IFRIATPHSLGSLVVKIRVNHNK...LSPA	3185
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CaTin1	Y.VNYVEVTTSTG.EHKRCNQQLFIVEKQL	140
Tobacco	YFYCSYVEVTTSTG.DHKQCSQQLFNVDQL	146
Arabidopsis	Y.VNYVEITTTAG.VHAQCSTQDFEIEQQL	142
Rape	Y.VNYVEVTTAG.VHAKCSYQSDFVEQQL	144
Rice	Y.VSYVMVTQLGQGRLPSTMTHRWAVDQQL	139
Plexaura	YF.VNRIIISST...QDRVYSFPCFRV	500
Human	YF.LQHVIIVRDQL...TARSAFLVNDQL	3214
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CaTin1	GAGEF.....PDGLTAIKNNCGRKFPEAQ	164
Tobacco	STDRS.....PYQLTATRNNCRMRSGDQQ	170
Arabidopsis	ATDTS.....PYELTAVRNNCPVKLRDSV	166
Rape	ASDTS.....PYELSAVRNNCPVSLRESV	168
Rice	AIDEA.....PHMLTAERRGCGIG.....	158
Plexaura	IKDMVLF...PGEATLPFNEVPAIVSEQR	526
Human	SVTEANGGLVEKEVLAASDAFALLRFRRLL	3244
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CaTin1	LSTYDPQ.....SYHVVDVL.....	179
Tobacco	P.....IVVDVI.....	177
Arabidopsis	SRVGSEI.....RKKLSWVV.....	181
Rape	GRVGSEI.....RKTLRWIV.....	183
RiceAAAP.....	162
Plexaura	QKELEQR.....KLTYQWDY.....	541
Human	LVAELQRGFDFKCKFKERNINGFFS.....	3268

Figure 1. Comparison of the predicted amino acid sequence of *CaTin1* (AF242731) with various PLAT domain-containing genes. Compared genes are tobacco (AB040407), Arabidopsis (NP_195683, chromosome 4), rape (AF314810), rice (AC083945), the PLAT domain part of *Plexaura* lipoxigenase gene (T30903), and the PLAT domain part of human PKD gene (2203412A). The black bold line indicates a PLAT domain and each black, dark gray, and light gray box indicates 100%, 75%, and 50% homology, respectively.

uninoculated leaves 3 d after TMV inoculation (Fig. 2C). The systemic induction of the *CaTin1* gene was earlier than *CaPR1* gene that is known to be induced during SAR (Sticher et al., 1997).

It was investigated whether the TMV-P₀-inducible *CaTin1* gene in plants is also involved in the defense against other pathogens. Pepper leaves were challenged with a pathogenic bacterium *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). When the leaves of the *Xcv* resistant cv ECW-20R were infiltrated with *Xcv*, the leaves became dark purple within 24 h of inoculation and subsequently became necrotic. In contrast, within 48 h after the infiltration, the susceptible cv ECW leaves were damaged severely and exhibited chlorosis (data not shown). Northern-blot analysis carried out with the *CaTin1* probe revealed that the transcripts of *CaTin1* gene accumulated only in ECW-20R. This suggested HR-specific induction of *CaTin1* by viral and bacterial challenges (Fig. 2D). Thus, the *CaTin1* expression upon biotic stresses had a tendency of earlier induction than that of well-known *CaPR1* gene.

The *CaTin1* Gene Expression Was Related to Ethylene but Not to SA or MeJA Signal

To examine whether *CaTin1* gene can be induced by other stimuli, plants were treated with various abiotic inducers and the expression of *CaTin1* gene was examined at the times indicated.

In pepper leaves sprayed with SA, the transcripts of *CaTin1* gene were not detected. This is clearly different from the induction of *CaPR1* and *CaPR4* transcripts. Also *CaTin1* gene was not induced by MeJA treatment but the *CaPR4* transcripts were well induced. *CaPR1* was known to be not induced by MeJA (Park et al., 2001a; Fig. 3A). On the other hand, *CaTin1* gene expression started to increase 2 h after ethylene treatment, and then maintained until 8 h at similar level. Compared to the *CaPR1* gene expression, the *CaTin1* gene expression started very early and quickly disappeared (Fig. 3A).

To see whether other abiotic inducers induce the *CaTin1* expression, ABA at a concentration of 10 μ M, 250 mM NaCl, or 0.5 mM MV was applied to the unrooted pepper plants. MV induces H₂O₂ (Dodge, 1994). NaCl induced the expression of *CaTin1* gene slightly. MV, on the other hand, induced the expression of *CaTin1* gene significantly (Fig. 3B). In pepper plants, MV induced the expression of *CaTin1* gene more efficiently than the expression of *Capsicum annuum* ascorbate peroxidase (*CaAPX1*) gene that was used as the positive control (Yoo et al., 2002).

In regard to the response to various stimuli, the expression of *CaTin1* was different from previously characterized PR genes that are responsive to ethylene and MV only. We thus examined the role of ethylene and H₂O₂ in the induction of the *CaTin1* gene expression in response to biotic stresses. The leaves of hot pepper inoculated with TMV were treated with the

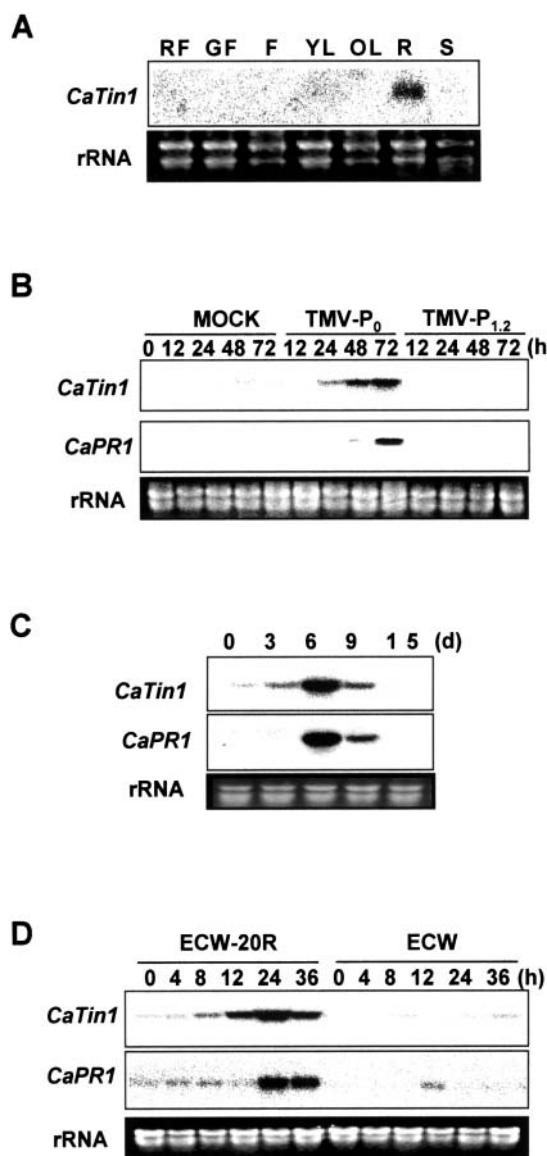


Figure 2. Expression patterns of the *CaTin1* gene in various organs and in response to biotic stresses. **A**, Organ-specific expression of the *CaTin1* gene. In each lane, 15 μ g of total RNAs prepared from various organs were loaded. RF, red ripe fruit; GF, green unripe fruit; F, flower; YL, young leaf; OL, old leaf; R, root; S, stem. **B**, Expression pattern of *CaTin1* gene upon TMV-P₀ or TMV-P_{1,2} inoculation. As a control, leaves were mock-inoculated with phosphate buffer. **C**, Expression pattern of *CaTin1* gene to TMV-P₀ during SAR. To monitor expression of *CaTin1* gene, 2 leaves of hot pepper plant were inoculated with TMV-P₀ sap (approximately 20 μ g/mL) and upper uninoculated leaves were detached at 0, 3, 6, 9, and 15 d postinoculation. **D**, Expression pattern of pepper *CaTin1* gene upon *Xcv* inoculation. A hot pepper cultivar (ECW-20R) resistant to *Xcv* and a susceptible cultivar (ECW) were infiltrated with *Xcv* culture (1×10^8 cells/mL). Total RNA was extracted from the leaves harvested at time points indicated after inoculation. RNA was separated in formaldehyde-agarose gel and transferred onto nylon membrane. The membranes were hybridized with *CaTin1* cDNA probe. *CaPR1* was used as a control for HR. The rRNA bands in ethidium bromide-stained gels are shown as a loading control. Experiments were performed at least three or four times for each treatment and the representative result was shown here.

ethylene biosynthesis inhibitors aminoethoxyvinylglycine (AVG) and α -aminoisobutyric acid (AIB), the ethylene action inhibitor silver thiosulfate (STS), or the H₂O₂ production enzyme NADPH oxidase inhibitor (diphenylene iodonium, DPI) and the induction of the *CaTin1* gene was examined. Treated with various ethylene synthesis inhibitors or ethylene action inhibitors, the *CaTin1* gene expression in the TMV-inoculated plants was reduced. Particularly, the expression was markedly diminished in plants treated with STS (Fig. 3C). Treated with DPI, the *CaTin1* gene expression induced by TMV or MV was reduced in hot pepper plants (Fig. 3C). The data showed that the induction of the *CaTin1* gene expression by TMV is associated with the ethylene-dependent and/or the H₂O₂-dependent signal pathway.

To examine whether the expression of *CaTin1* gene is induced by incision-wounding, leaves were cut randomly with a pair of scissors and their expression of *CaTin1* gene was examined. Control was the untreated upper leaves of the same plant. The proteinase inhibitor II gene (*CaPinII*) that has been reported to be induced by local and systemic wounding was used as the positive control (Shin et al., 2001). Northern-blot analysis using the duplicate RNA samples showed that the transcripts of *CaPinII* were dramatically induced by local as well as by systemic incision-wounding, whereas the transcripts of *CaTin1* gene was not detected (Fig. 3D).

The Altered Gene Expression and the Growth Rate in *CaTin1* Transgenic Tobacco Plants

To understand the biological function of *CaTin1* in planta, we generated transgenic tobacco plants harboring the sense *CaTin1* gene or the anti-sense *CaTin1* gene. Although *CaTin1* gene is a hot pepper gene, we used the tobacco system because of the difficulty of obtaining a large number of transgenic pepper plants (Shin et al., 2002). Tobacco plants have the *CaTin1* homolog whose expression pattern is similar to *CaTin1* under normal conditions as well as in response to TMV or ethylene (data not shown). *CaTin1* gene and its tobacco homolog showed 84% identity at the nucleotide level (data not shown). Tobacco plants were transformed with a binary vector carrying the fusion of the double 35S promoter and *CaTin1* cDNA in the sense or antisense orientation by the *Agrobacterium*-mediated transformation. Transgenic plants were regenerated from the verified transformants and used for the analysis. The expression of *CaTin1* and several other PR genes in transgenic plants was compared with control plants transformed with the empty pMBP2 vector (Han et al., 1999). All experiments of the transgenic plants were performed in triplicates using the T₁ descendants of independent T₀ lines that had single copy of *CaTin1*.

As we observed that ethylene was the key regulator of the *CaTin1* gene expression, we examined specifically whether the expression of *CaTin1* gene is induced

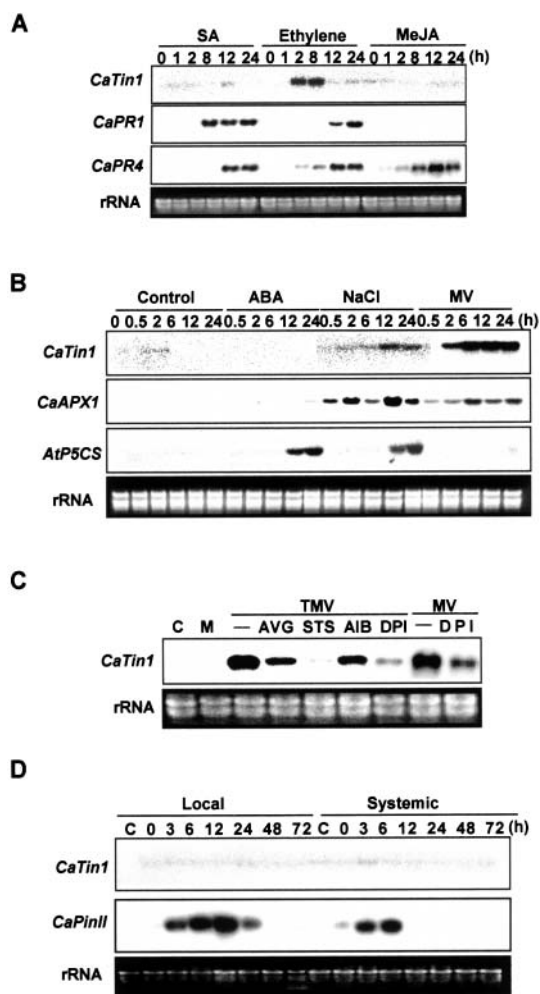


Figure 3. Expression of the *CaTin1* gene in response to abiotic stresses. A, Expression patterns of *CaTin1* gene upon SA, ethylene, or MeJA treatment. Hot pepper leaves were sprayed with 2 mM SA, 50 μ L/L gaseous ethylene, or 10 μ M MeJA and detached at time points designated. The *CaPR1* and *CaPR4* expression was compared as a control for treatment. B, Expression patterns of *CaTin1* gene upon ABA, NaCl, and MV treatments. Hot pepper leaves were sprayed and the unrooted plants were soaked in each solution with 10 μ M ABA, 250 mM NaCl, or 0.5 mM MV and detached at time points designated for total RNA extraction. *CaAPX1* or *AtP5CS* gene was used as a control for MV, salt, or ABA treatment. C, *CaTin1* gene expression in the presence of ethylene synthesis or action inhibitors and H₂O₂ production inhibitor. RNA was isolated from the 2 leaves that were inoculated with TMV-P₀, incubated for 24 h, and then treated for an additional 24 h with water, 2 mM AIB, 1 mM AVG, or 0.5 mM STS. The unrooted plants were soaked in solution with 100 μ M DPI for 4 h, were inoculated with TMV-P₀ and treated with 0.5 mM MV for 24 h. C indicates the uninoculated leaves of a control plant at 0 h and M indicates the mock-inoculated leaves of control plant after 24 h. D, Expression patterns of *CaTin1* gene by incision-wounding. Hot pepper leaves were incised with a pair of scissors and RNA was extracted from the incised leaves (Local) and the upper unincised leaves to detect systemic induction (Systemic). The *CaPinII* gene expression was compared as a control for wounding treatment. The membranes were hybridized with *CaTin1*-specific cDNA probe. The rRNA bands in ethidium bromide-stained gels were shown as a loading control. Experiments were performed at least three times for each treatment and the representative result was shown here.

by the ethylene-dependent but SA-independent signal transduction pathway. Since the expression of *CaTin1* was quite fast, it was possible that this gene might be regulated earlier than other PR genes in defense signal transduction pathway. At first, we compared the level of protein expression in transgenic plants and control plants. The expression level in transgenic plants harboring the sense *CaTin1* gene (sense transgenic plants) was higher than control plants. The expression level in control plants, in turn, was higher than in transgenic plants harboring the antisense gene (antisense transgenic plants). In fact, *CaTin1* protein in antisense transgenic plants was barely detectable (Fig. 4A). And after TMV inoculation, *CaTin1* protein level was high in sense transgenic plants but very low in antisense transgenic plants even though the plants were infected by TMV. We also investigated the expression of PR genes in these transgenic plants. *NtPR1*, *NtPR2*, and *NtPR3* show 100% homology to X06361, M59442, and S44869 at NCBI database and the full-length *NtPR1*, the 3' 1 kb region of *NtPR2*, or *NtPR3* were used as probes. As shown in Figure 4B, *NtPR2* and *NtPR3* but not *NtPR1* gene were expressed in the untreated antisense transgenic plants. Acidic *NtPR1* was known to show SA-dependent gene expression pattern but basic *NtPR2* and *NtPR3* ethylene-dependent expression pattern. The above results suggest the expression of *NtPR2* and *NtPR3* might have been related to ethylene dependent *CaTin1* expression change. To investigate the possible relationship of their expressions with ethylene, transgenic plants were treated with ethylene blocker (STS) and the expression of *CaTin1* was examined (+ signs in Fig. 4B). The expression of *NtPR2* and *NtPR3* gene in the antisense transgenic plants disappeared or decreased. From this result, we speculated that the expression of *NtPR2* and *NtPR3* gene in the antisense transgenic plants is regulated mainly by ethylene. Interestingly, both the sense and antisense *CaTin1* transgenic plants flowered earlier than control plants. Furthermore, the sense transgenic plants were taller than controls, whereas the antisense transgenic plants were shorter than control plants (Fig. 4C). The data suggest that the expression of *CaTin1* may be an important factor of the ethylene signal transduction pathway.

The Alteration of the HR Lesion Formation and Ion Conductivity in *CaTin1* Transgenic Plants after TMV Inoculation

The HR response elicited by avirulent pathogens is rapid cell death. We noticed that the sense transgenic plants showed the irregular and bigger HR lesion formation after TMV inoculation. As shown in Figure 5A, 10 d after TMV inoculation, the HR lesions of the sense transgenic plants were over 40% bigger than control plants (a versus b). The lesion in the antisense plants was smaller than control (b versus c). The correlation between the cell death severity and the ion leakage

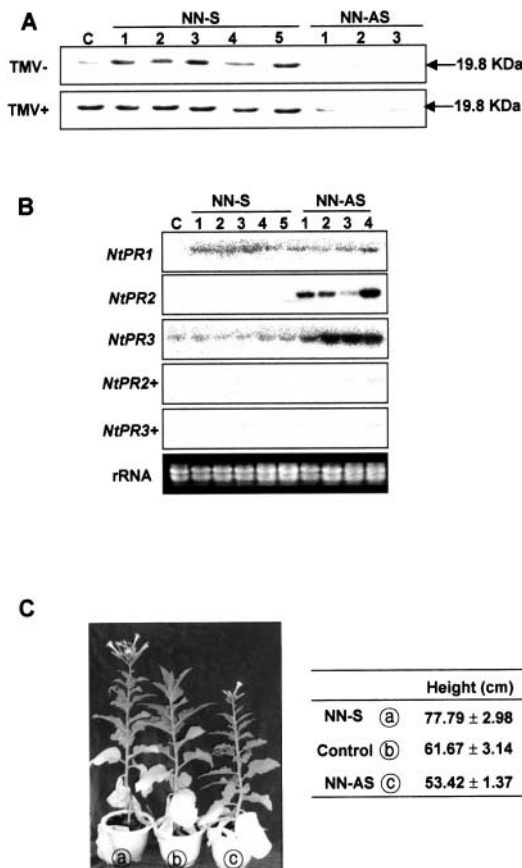


Figure 4. Expression pattern of genes and growth difference in sense- and antisense-oriented *CaTin1* transgenic tobacco plants and control plants. A, Western-blot analysis of *CaTin1* transgenic plants. Western-blot analysis using anti-rat-*CaTin1* antibody (2 µg/mL) was carried out for detection of *CaTin1* in transgenic plants. TMV+ means western-blot analysis performed with TMV-*P*₀-inoculated plants and TMV- means western-blot analysis performed with no TMV-*P*₀-inoculated plants. B, RNA gel blotting was conducted to measure the amount of *NtPR1*, *NtPR2*, or *NtPR3* mRNA in empty-vector transformed control and sense- and antisense-oriented independent transgenic tobacco plants (*T*₁ generation) when treated with ethylene blocker, STS (+), or no treatment. C, Comparison of the height of transgenic plants and control plants. a, sense transgenic plant; b, control plant; c, antisense transgenic plant. Their heights were measured from 100-d-old plants. The heights are the average of experiments performed three times, each time with 10 *T*₁ descendents of typical transgenic line. NN-S designates the *CaTin1* sense transgenic tobacco cv Samsun NN *T*₁ plants and NN-AS designates the *CaTin1* antisense transgenic tobacco cv Samsun NN *T*₁ plants. The numbers indicate independent lines of transgenic *T*₁ plants. C indicates control plants. The rRNA bands in ethidium bromide-stained gels were shown as a loading control. Experiments were performed at least three times for each treatment and the representative result was shown for blot analyses.

level has been reported (Greenberg and Ausubel, 1993). We thus compared the ion leakage level of the transgenic plants with control plants. Two days after TMV inoculation, the ion conductivity level of the sense transgenic plants was lower than control plants (data not shown). The level in the transgenic plants

was increased more rapidly than control plants. Ten days after inoculation, the ion leakage of the sense transgenic plants was slightly but consistently higher than control plants (Fig. 5B). However, the antisense transgenic plants showed lower ion conductivity than control plants, which correlates to the smaller size of the HR lesions.

The Elevated Level of Ethylene and H₂O₂ in *CaTin1* Transgenic Plants

When ethylene blockers were applied to the transgenic plants, the expression of *CaTin1* as well as PR genes was decreased or disappeared (Figs. 3C and 4B). In addition, the sense transgenic plants showed the significant progression of the HR lesion and increased ion conductivity in response to TMV inoculation (Fig. 5). Based on these results, we postulated that the ethylene and/or H₂O₂ level may be elevated in the transgenic plants. Hence, in addition to measuring

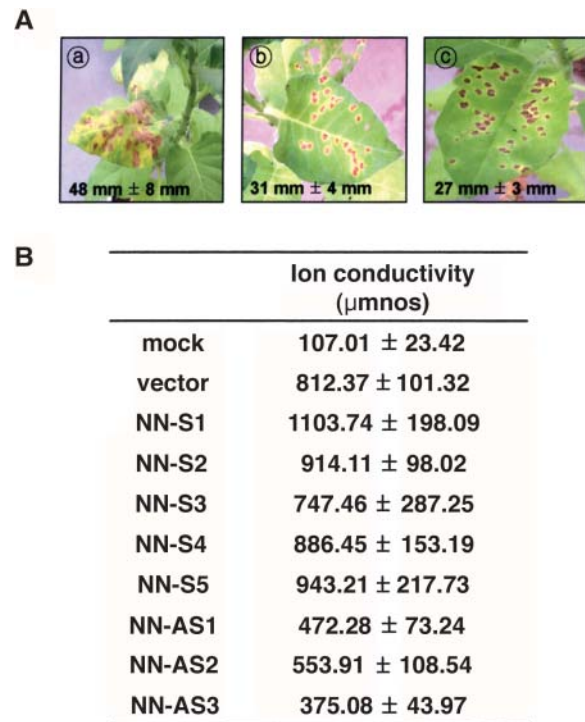


Figure 5. Comparison of HR lesion formation and ion conductivity after TMV inoculation between control plants and *CaTin1* transgenic *T*₁ plants. A, Comparison of HR lesion formation. a, Sense transgenic plant; b, control plant; c, antisense transgenic plant. Each leaf here shows typical HR lesion formation 10 d after TMV inoculation. Each number in the figures indicates the average size of 100 HR lesions' diameter in each case. B, Comparison of ion conductivity during HR lesion formation between control plants and *CaTin1* transgenic *T*₁ plants 10 d after TMV inoculation. The mean number of five experiments was shown on the table. ± indicates SD. NN-S designates the *CaTin1* sense transgenic tobacco cv Samsun NN *T*₁ plants and NN-AS designates the *CaTin1* antisense transgenic tobacco cv Samsun NN *T*₁ plants. The numbers indicate independent lines of transgenic *T*₁ plants.

the in vivo ethylene and H₂O₂ level directly, we evaluated the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene and glutathione-S-transferase (GST) gene in the reverse transcription (RT)-PCR analysis. The former gene is the marker for the ethylene level and the latter gene is the marker for the H₂O₂ level (Fig. 6). In the antisense transgenic plants, the expression of the ACC oxidase gene and the ethylene level were higher than control plants. In the sense transgenic plants, the levels were slightly elevated (Fig. 6A). In contrast, the H₂O₂ level and the GST expression level were higher in the sense transgenic plants than control plants. The levels in the antisense plants were slightly higher than control plants not undergoing HR (Fig. 6B). However, the H₂O₂ level of TMV inoculated control plants undergoing HR was about 5 times higher than that of sense transgenic plants.

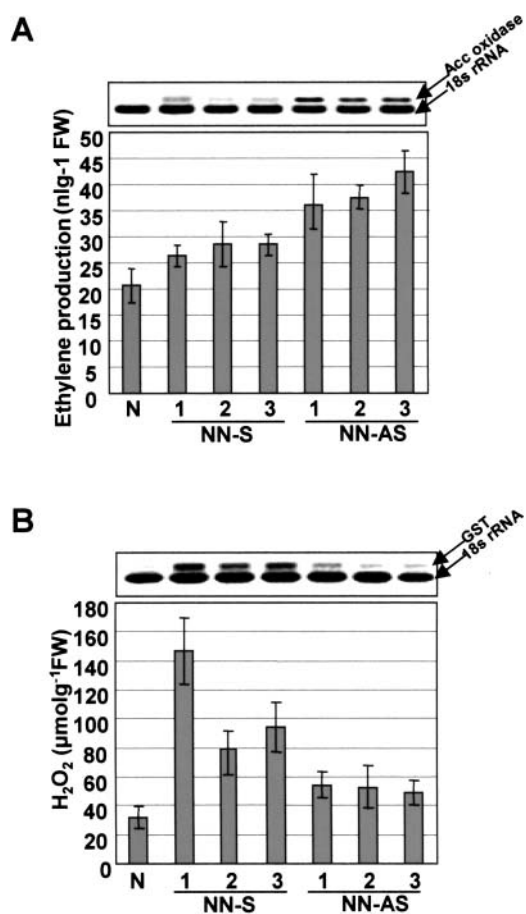


Figure 6. Measurements of ethylene and H₂O₂ levels in plants in transgenic plants and control plants. A, Analysis of ACC oxidase transcripts using RT-PCR and measurement of ethylene level in plants through gas chromatography. B, Analysis of GST transcripts using RT-PCR and measurement of H₂O₂ level in plants. The mean number of five experiments was shown on the graph. Error bars indicate sds. 18S indicates 18S rRNA products.

CaTin1 Transgenic Plants Are Enhanced in Tolerance against Virus Infection, Drought, and Salt Treatments

Our data show that the sense as well as the antisense *CaTin1* transgenic plants have the higher ethylene and H₂O₂ levels than control plants (Fig. 6). We thus examined whether *CaTin1* sense and antisense transgenic plants showed altered tolerance against biotic and/or abiotic stresses. To determine whether *CaTin1* transgenic plants showed enhanced resistance to pathogens, we inoculated control and transgenic plants with the virulent viral pathogen cucumber mosaic virus Y (CMV-Y) that infects hot pepper systemically. Uninoculated upper leaves were taken for infection analysis after 21 d of virus inoculation. As shown in Table I, resistance to CMV-Y of the sense as well as the antisense transgenic plants was increased by 19% to 73%.

We also examined whether the higher levels of ethylene and H₂O₂ in transgenic plants alter their tolerance to drought or salt. The leaf discs of transgenic tobacco plants were immersed in 6% mannitol or 400 mM NaCl for 7 d. Subsequently the chlorophyll content in transgenic tobacco and control plants was compared. As shown in Table I, the tolerance in the sense and the antisense transgenic plants to drought and salt was increased by 2.8 times and from 1.5 times to the maximum 11 times, respectively.

The data demonstrated that the resistance to various stresses in the sense transgenic plants might be caused mainly by the elevated H₂O₂ level. The increased resistance in the antisense transgenic plants may be due to the higher ethylene level resulting in the PR gene expression.

The GCC-Boxes and W-Box Were Important Elements for the *CaTin1* Gene Expression

To characterize the sequences involved in the *CaTin1* transcriptional regulation, we generated the deletions of the full-length promoter region of *CaTin1*, connected them to GUS coding gene, and produced transgenic tobacco plants. The *CaTin1* promoter has four ethylene responsive GCC-boxes, two AT-rich regions known to be responsible for the root-specific expression, and an elicitor-induced WRKY transcription factor-binding W-box. The activity of each deletion construct was measured by using the leaf samples, which showed almost no expression of *CaTin1* without any treatment. The activity was not detected by the histochemical GUS staining in the leaf even when the full-length promoter construct (p-1434Tin1) was used. However, constructed with the AT-rich regions, the GUS activity could be detected specifically in the apical meristem of roots without any treatment (data not shown).

When the transgenic tobacco plants of the deletion constructs of *CaTin1* promoter were inoculated with TMV, they had higher GUS activity compared to the promoterless GUS construct as long as they had a W-box. In contrast, the GUS activity of the deletion

Table I. Analyses of various stress tolerance in transgenic *CaTin1* tobacco plants

NN-S designates the *CaTin1* sense transgenic tobacco cv Samsun NN T₁ plants and NN-AS the *CaTin1* antisense transgenic tobacco cv Samsun NN T₁ plants. The control plants indicated empty vector transgenic plants. Each number represents an independent T₀ transgenic plant. Each mean number is derived from 10 combined T₁ descendents of each T₀ line and experiments were performed 5 times. \pm indicates SD.

	Absorbance of A ₄₀₅ ^a	Chlorophyll $\mu\text{g}/\text{mg}^{\text{b}}$	Chlorophyll $\mu\text{g}/\text{mg}^{\text{c}}$
mock ^d	0.42 \pm 0.17	20.42 \pm 1.42	20.64 \pm 0.42
vecto ^e	1.51 \pm 0.21	7.01 \pm 1.22	1.58 \pm 0.32
NN-S1	0.68 \pm 0.31	10.88 \pm 1.49	6.49 \pm 0.99
NN-S2	1.19 \pm 0.47	16.42 \pm 2.49	3.88 \pm 0.49
NN-S3	0.72 \pm 0.20	11.26 \pm 2.03	2.87 \pm 0.25
NN-S4	0.41 \pm 0.09	20.02 \pm 1.99	5.71 \pm 1.09
NN-S5	0.62 \pm 0.14	16.23 \pm 2.17	12.03 \pm 0.73
NN-AS1	0.98 \pm 0.11	15.84 \pm 2.37	13.21 \pm 1.24
NN-AS2	1.27 \pm 0.32	9.26 \pm 0.94	5.44 \pm 0.54
NN-AS3	1.12 \pm 0.13	16.43 \pm 1.86	11.62 \pm 0.97

^aThe absorbance of transgenic T₁ plants compared to control plants was measured 21 d after inoculation with CMV-Y. Virus was detected by indirect ELISA using anti-CMV IgG (3 $\mu\text{g}/\text{mL}$). ^bLeaf discs from the transgenic plants carrying the *CaTin1* gene in the sense and antisense orientation and the empty vector transgenic tobacco plants were floated in 6% mannitol solution for 1 week under continuous white light at 25°C and chlorophyll content (milligrams per gram fresh weight) was measured. ^cLeaf discs were floated in 400 mM NaCl solution for 1 week under continuous white light at 25°C and chlorophyll content (milligrams per gram fresh weight) was measured. ^dNontreated control plants. ^eCMV, mannitol, or NaCl treated control plants.

promoters treated with ethylene was dependent on the GCC-boxes as expected. The construct with one GCC-box showed weak activity. The constructs with three or four GCC-boxes showed strong activity (Fig. 7).

DISCUSSION

The expression of *CaTin1* gene containing the PLAT domain is induced by avirulent pathogens, ethylene, NaCl, and MV. SA or MeJA is ineffective. Many genes containing the PLAT domain, such as lipoxigenase and lipase, have other functional domains in addition to the PLAT domain. *CaTin1* and its homologs of other plants have only PLAT domain (Fig. 1). Although PKD1 and PKD2 appear to form homo- and heterodimer with each other (Tsiokas et al., 1997), we found that *CaTin1* does not form homodimers based on the yeast-two-hybrid interaction experiment (data not shown). *CaTin1* gene may be different from other known genes containing the PLAT domain in terms of not having other functional domain such as the lipoxigenase domain of lipoxigenase gene. However, our results together with previous reports suggest that the function of *CaTin1* gene may be to help the communication between the ethylene signal pathway

and the abiotic/biotic stress-related pathway through the protein-protein or protein-lipid interaction in a Ca²⁺ dependent fashion (Gillmor et al., 1997; Tsiokas et al., 1997; Bateman and Sanford, 1998).

Several studies confirmed the importance of SA for the establishment of disease resistance (Dempsey et al., 1999). Plants with the defect in the SA-dependent response show the enhanced susceptibility to fungus. SA, however, is not required for the defense against all pathogens. The plant hormones MeJA and ethylene are implicated as mediators in many physiological processes. MeJA and ethylene coregulate a subset of PR genes *PR2*, *PR3*, and *PR12* in Arabidopsis that encode antimicrobial proteins (Penninckx et al., 1998; Thomma et al., 1998). For the expression of *PR12* (*PDF1.2*), the concomitant activation of both the MeJA and ethylene response pathway is required. Although the mechanism of the regulation of *PR2*, *PR3*, and *PR12* by MeJA and ethylene is still obscure, the regulation is clearly distinct from that of SA-dependent PR genes such as *PR1* and *PR5* (Thomma et al., 1998). *CaTin1* is induced only by ethylene and not by SA and MeJA (Fig. 3A). In many cases, the SA-dependent and the SA-independent signals of disease response were divided. The SA-independent signal of disease response was not divided between MeJA-specific signal and ethylene-specific signal, however (Thomma et al., 2001). The ethylene signal pathway of fruit development or biosynthesis is relatively well characterized (Bleecker, 1999). In contrast, the ethylene dependent signaling of disease response is not well characterized. The expression of *CaTin1* was specific to ethylene signal judging from ethylene synthesis or the action inhibitor treatment experiments (Figs. 3C and 4B), in addition to the *CaTin1* induction result upon ethylene treatment, but not SA or MeJA treatment. The *CaTin1* expression was also related to the incompatible plant-pathogen interaction (Fig. 2, B and D). Thus, *CaTin1* gene seemed to be related to the SA/MeJA independent and the ethylene dependent defense signal transduction.

The expression of *CaTin1* was induced during SAR (Fig. 2C). SAR induction was known to require the signal molecule SA. The removal of SA in transgenic plants expressing salicylate hydroxylase (*nahG*) prevents the establishment of SAR (Gaffney et al., 1993). Lawton et al. (1994) reported that the pathogen-induced SAR is ethylene independent. Similarly, many reports have shown that the low expression level of genes and proteins are regulated by ethylene during SAR (Brederode et al., 1991; Ward et al., 1991; Cordelier et al., 2003). However, SA-independent but ethylene-dependent *CaTin1* was induced during SAR (Fig. 2C). Furthermore, *CaTin1* transgenic plants showed tolerance at systemic leaves after CMV inoculation (Table I). A recent report has shown convincingly that ROI generated during HR may serve as signals mediating the SAR (Alvarez et al., 1998). The systemic expression of *CaTin1* showed the possibility that the SA-independent SAR mechanism

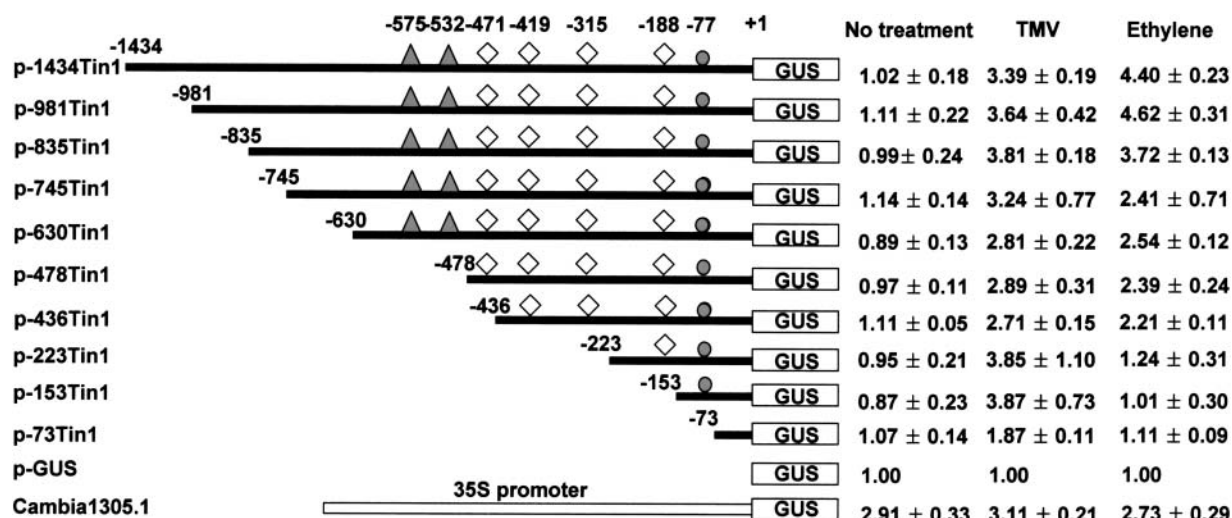


Figure 7. The deletion constructs of *CaTin1* promoter and their GUS activities analysis upon TMV and ethylene treatments. Deletion analysis of the *CaTin1* promoter fused to the promoterless GUS in Cambia 1305.1 vector (p-Gus) was carried out. The numbers of each construct indicate the distance from the *CaTin1* transcription start site. Diamonds indicate the GCC-box, a circle indicates W-box, and triangles indicate AT-rich region. For each construct 10 independent transgenic lines were investigated. Average value of GUS activities (pmol methylumbelliferone/min/mg protein) resulted from five independent experiments were shown. We normalized the activities of deletion constructs results by comparing those to the activity of p-GUS construct of which activity adjusted to 1.00.

exists in plants. As shown in Figure 6, the sense transgenic plants showed the higher H_2O_2 level. The antisense transgenic plants showed the higher ethylene level than control plants. Plants may have to maintain the optimal *CaTin1* level. Once its homeostasis is broken as in the case of transgenic plants, many changes including the elevated ethylene or H_2O_2 levels may occur.

The promoter of *CaTin1* has bidirectional activity. In addition, another homologous gene (*CaTin1-2*) is located in the front of *CaTin1* in the head-to-head fashion. The expression of *CaTin1* and *CaTin1-2* in response to several elicitors was very similar except for the slightly different induction time upon TMV challenge (Shin et al., 2003). Even though the *CaTin1* and its homolog *CaTin1-2* are closely located in hot pepper, the homologs of *CaTin1* in Arabidopsis are located at different chromosomes (2 and 4). Furthermore, so far only one *CaTin1* homolog of tobacco has been reported at the National Center for Biotechnology Information. It is not known how the homologs of *CaTin1* are regulated in other plants. The promoter of *CaTin1* may finely tune up the expression of *CaTin1* and *CaTin1-2* for keeping their homeostasis in hot pepper.

CaTin1 antisense transgenic tobacco plants expressed some basic PR genes constitutively, especially *NtPR2* and *NtPR3*, without any treatment (Fig. 4B). As mentioned earlier, *NtPR2* and *NtPR3* are SA-independent PR genes. On the other hand, *NtPR1* gene belonging to SA-dependent PR genes was not expressed in these transgenic plants under the same conditions. The *CaTin1* protein expression level in the sense transgenic plants was higher than the antisense

transgenic plants (Fig. 4A). The *CaTin1* protein level homeogenesis in planta may be important. If *CaTin1* is more underexpressed than the normal biological level, plants should have to make *CaTin1* and the ethylene level may be increased for producing *CaTin1*. Moreover, because the *CaTin1* level seems to be important in planta, the promoter of *CaTin1* should regulate its homolog in hot pepper at the same time (Shin et al., 2003). Thus, to supply proper amount of *CaTin1*, the *CaTin1* antisense transgenic plants may continuously make the *CaTin1* transcripts. Because of the antisense transcripts, however, they could be unable to make *CaTin1* protein resulting in the higher ethylene level continuously in vivo (Figs. 4A and 6A). The antisense transgenic plants express the ethylene-related PR genes due to the higher ethylene level. The ethylene blockers suppress the PR gene expression. This confirms that the PR gene expression was mainly due to the increased ethylene level in the antisense transgenic plants (Fig. 4B).

The *CaTin1* sense transgenic plants were taller than control plants, whereas the antisense plants were shorter than control plants (Fig. 4C). Both the sense and the antisense transgenic plants flowered earlier than control plants. Although the *CaTin1* sense transgenic plants grew more rapidly than control plants, the number of internodes was not changed (data not shown). After TMV inoculation, the sense transgenic plants showed bigger, and the antisense plants smaller, HR lesion than control plants (Fig. 5A). Furthermore, 10 d after the inoculation, ion conductivity was higher in the sense transgenic plants than control plants (Fig. 5B). Bent et al. (1992) reported that ethylene controls the amplitude and development of

disease symptom after inoculation with virulent pathogen. Overmyer et al. (2000) reported that ethylene acted as a promoting factor during the propagation phase of oxygen radical-dependent lesion development, whereas MeJA plays a role in the lesion containments. In addition, when HR is generated, the activation of ion fluxes and the production of H₂O₂ are the initial response detected in plant cells. Biochemical evidences suggest that the processes that occur prior to the transcriptional activation of defense-related genes appear to be mediated through the regulation of plasma-membrane-bound enzymes. These include changes in Ca²⁺-ATPase and H⁺-ATPase activation of plasma membrane-bound ion channels (Blumwald et al., 1998). As shown in Figure 5, the changes of the size of the HR lesion and ion conductivity may have been due to ethylene as well as the ROI changes. The data demonstrated that *CaTin1* may be affected by the level of ethylene and H₂O₂. Hence, the *CaTin1* transgenic plants may mimic the state of being affected in the signal pathway involving ethylene and H₂O₂ action. Transgenic tobacco plants of reduced catalase showed that although PR-1 protein was not accumulated in these plants, their ion leakage was increased during the HR (Mittler et al., 1999). Transgenic tobacco deficient in the H₂O₂-removing enzyme catalase showed the enhanced tolerance to pathogens and the rapid activation of the defense system (Chamnonpol et al., 1998). Pellinen et al. (2002) reported that during the progression of HR lesion formation, H₂O₂ was detected not only in the area with the undetectable damage but also the necrotic area, so an internal signal that was positively regulated by H₂O₂ induced systemic oxidative burst. Levine et al. (1994) showed that H₂O₂ could play a dual role in the plant defense response depending on its concentration. At high concentrations, it triggers HR, whereas at low concentrations it functions as a diffusible signal that induces cellular protecting genes involved in the blocking of the oxidant-mediated cell death. *CaTin1* sense transgenic plants also seem to show the rapid strong activation of some defense signals because of the elevated H₂O₂ level even though this activation could not induce the expression of PR genes resulting in *CaTin1* sense transgenic plant showing the enhanced stress tolerance (Table I). Figures 4B and 6A show that the increase of ethylene by at least 2-fold may be required to induce PR genes. The slight ethylene increase may not induce the PR gene expression in the sense transgenic plants. The transgenic plants may have experienced some cellular and biochemical changes especially in the ethylene signal pathway(s). Several PR genes may be expressed probably due to the increased ethylene level in the antisense transgenic plants. Due to the elevated ethylene level and/or H₂O₂ level and the expression of PR genes, both the sense and the antisense plants may show the biotic and abiotic stress tolerance (Table I).

Previous studies show that WRKY transcription factors and their downstream genes such as *NPR1* are

associated with the pathogen- and SA-induced pathway (Chen and Chen, 2000; Du and Chen, 2000; Kirsch et al., 2001; Yu et al., 2001). Although the promoter of *CaTin1* has a W-box, *CaTin1* was not related to the SA-dependent signal pathway. Further studies of finding any WRKY transcription factor that binds to this W-box may elucidate the function of W-box more conclusively. The GCC-box has been found in the promoters of various elicitor-responsive genes, such as genes encoding the basic-type pathogenesis proteins PR2, PR3, and the osmotin PR5 (Eval et al., 1993; Ohme-Takagi and Shinshi, 1995). Two GCC-boxes in the osmotin promoter are required, but not sufficient, for the maximal ethylene responsiveness (Xu and Heath, 1998). The GCC-boxes in *CaTin1* promoter are important key elements for the response to ethylene, similarly to other PR genes. Other PR genes have two GCC-boxes on the average (Xu et al., 1998), whereas the promoter of *CaTin1* has four GCC-boxes. This suggests that the *CaTin1* expression is more dependent on ethylene than other PR genes. We have observed that *CaTin1* gene is expressed only in roots under normal condition and the promoter seems to be responsible for root specific expression under normal condition. The reports describing the regulation of the expression of genes in roots are scarce. The cis-acting sequences regulating the root-specific expression have not been identified. Furthermore, only a few root specific promoters were described and whose activity was rather restricted to some parts of the root (Nitz et al., 2001). The known cis-acting sequences specific to roots are *ocs*-elements (Ellis et al., 1987), *as-1*-elements (Lam et al., 1989), and AT-rich region (Reisdorf-Cren et al., 2002). *CaTin1* promoter has two AT-rich regions, which may be related to the root specific expression of *CaTin1*.

The *CaTin1* gene expression was regulated by ethylene and the ethylene level seemed to be controlled by the *CaTin1* expression level. We propose that the perturbation of the *CaTin1* expression alters the response of plants to ethylene and interferes with the redox homeostasis.

MATERIALS AND METHODS

Plant Cultivation and Pathogen Inoculation

Hot pepper (*Capsicum annuum*) L. cv Bugang that is susceptible to the P_{1,2} pathotype of TMV but resistant to the P₀ pathotype was used. Plants were grown in a growth chamber or greenhouse at 25°C with the photoperiod cycle of 16 h light and 8 h dark. Healthy and well-expanded leaves of 2-month-old plants were used. TMV-P₀ and TMV-P_{1,2} strains were maintained in the leaves of tobacco (*Nicotiana tabacum* cv Burley 21) in petri dishes containing CaCl₂. The leaf sap of TMV-P₀ or TMV-P_{1,2} was prepared by grinding infected leaves in 0.25 M phosphate buffer containing 5 mM EDTA (pH 7.4). To inoculate plants, virus-containing sap was applied to the surface of 4 to 5 fully expanded leaves of hot pepper plants and rubbed with carborundum (Hayashi Chemical, Osaka). Mock-inoculated plants were rubbed with phosphate buffer and carborundum only. To assess systemic responses, 2 lower leaves were inoculated with TMV-P₀ sap and the upper leaves were harvested at 0, 3, 6, 9, and 15 d after inoculation. Two cultivars of hot pepper, Early Calwonder (ECW; *bs1/bs1*, *bs2/bs2*, and *bs3/bs3*) and Early Calwonder-20R (ECW-20R;

bs1/bs1, *Bs2/Bs2*, and *bs3/bs3*) were used for infiltration with *Xcv* (*avrBs2*). *Xcv* was cultured in the medium containing yeast extract, dextrose, and calcium carbonate. Cell suspensions (1×10^8 cells/mL) were infiltrated into leaves, and RNA was extracted from the infiltrated leaves.

Treatments with the Inhibitors of Ethylene Synthesis or Its Action, the Inhibitors of H₂O₂ Synthesis, and Other Chemicals

Pepper leaves were sprayed with 5 mM SA or 10 μ M MeJA solution. Control plants were sprayed with distilled water. Leaves thus treated were harvested at the times indicated, quickly frozen in liquid nitrogen, and stored at -80°C . For the treatments of abscisic acid (ABA), NaCl, or MV, unrooted pepper plants were placed in Falcon tubes filled with the chemicals or water for various durations and frozen in liquid nitrogen. The ethylene treatment was performed by keeping pepper plants in a chamber equilibrated with 50 μ L/L gaseous ethylene for the durations indicated. Ethylene concentration was determined by gas chromatography. Control plants were kept in a chamber without ethylene. For the wound treatment, leaves were cut randomly with a pair of scissors. RNA was extracted from the wound-treated leaves and the nontreated upper leaves of the same plant at various times after wounding. *CaPR1* was used as a control for HR, SA, or ethylene treatment. *CaPR4* was used as a control for MeJA treatment. *CaAPX1* was used as a control for MV treatment. *Arabidopsis* pyrroline-5-carboxylate synthase (*AtP5CS*) was used as a control for salt or ABA treatment. *CaPinII* was used as a control for wound treatment (Kasuga et al., 1999; Shin et al., 2001).

Two leaves from hot pepper plants were inoculated with 1 μ g/mL TMV. After 24 h incubation at 22°C, the infected leaves from 2 plants were injected with either water, 2 mM AIB, 1 mM AVG, or 0.5 mM STS. Immediately following the injection, the treated leaves were detached and immersed in the corresponding solution. The unrooted plants were soaked in 100 μ M DPI solution for 4 h and were inoculated with TMV-*P₀* or were treated with 0.5 mM MV for 24 h (Orozco-Cárdenas et al., 2001).

After 24 h incubation in various solutions, the leaves were harvested and used for RNA analysis (Guo et al., 2000).

DNA, RNA, RT-PCR, and Protein Analyses

Genomic DNA and RNA were isolated from the pepper leaves by the method of Ausubel et al. (1995). Southern- and RNA-blot hybridizations were carried out as described by Shin et al. (2001). For RT-PCR, the total RNA (5 μ g) was reverse-transcribed in the total volume of 20 μ L according to Sambrook et al. (1989). One microliter of the 1:10 diluted RT reaction product was used as a template in 40 μ L PCR premix (Bioneer, Taejeon, Korea) with the sense primer (GST; 5'-ttgtcaagtaccagctttg, ACC oxidase; 5'-agtggagaaaatgacaaagg), the antisense primer (GST; 5'-ttgtactactcaagtaatat, ACC oxidase; 5'-ccaaggtta-accacaatagaga) or QuantumRNA Universal 18S (Ambion, Austin, TX). The condition of RT-PCR was 94°C for 2 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min. After RT-PCR, the products were cloned into pGEM Easy Vector (Promega, Madison, WI) and sequenced for their confirmation.

Indirect ELISA was used for the detection of CMV-Y using anti-rabbit-CMV-Y antibody (3 μ g/mL). For the detection of CaTin1 in transgenic plants, western-blot analysis using anti-rat-CaTin1 antibody (2 μ g/mL) was performed.

Analysis of CaTin1 Transgenic Plants

Plasmids used in the transformation of tobacco were prepared with the sense and antisense orientation of the full-length *CaTin1* cDNA cloned into the polylinker site of the binary vector pMBP2 (Han et al., 1999). The constructs were introduced into tobacco cv Samsun NN using *Agrobacterium*-mediated transformation as described (Hoekema et al., 1983).

To investigate the tolerance to the salt and drought stress, healthy and fully expanded leaves of wild-type and transgenic plants were detached and washed briefly in distilled water. The 1-cm diameter leaf disc was cut and immersed in Murashige and Skoog medium containing 400 mM NaCl or 6% mannitol for 7 d (Veena and Sopory, 1999). The treatment was performed in the continuous white light at 25°C. The chlorophyll content was measured as described by Aono et al. (1993).

Ethylene, H₂O₂, and Ion Conductivity Measurements

For the measurement of ethylene evaporation, 3 to 5 plants per transgenic T₁ line were analyzed. Plants were kept in a sealed test bottle for 12 h. Each 1-mL gas sample was withdrawn from the bottle with a hypodermic syringe and ethylene was assayed on a gas chromatography equipped with an aluminum column and flame ionization detector (Yi et al., 1999). For H₂O₂ measurements, frozen leaves (1 g) were ground to powder in liquid nitrogen, homogenized, and H₂O₂ was measured as described by Rao et al. (2000).

The ion leakage was measured with the Orion ion conductivity meter (model 150; Beverly, MA). Leaves of 2-month-old plants were inoculated with TMV. 10 d later, leaf discs obtained by punching with a number 4 cork borer (diameter 7.5 mm) were immersed in 2 mL of distilled water with the abaxial side down. After 2 h immersion at room temperature, the conductivity of the bathing solution was measured (Greenberg and Ausubel, 1993).

The Generation and the Analysis of the Promoter Region of CaTin1

By using the *CaTin1* gene specific primer 1 (5'-AGCCTGAAATAGAAG-AAACGGAGATGGAGATGAGA-3') and 2 (5'-GGAACCAGAATTGGTTAC-TCATGGCTACCTGAAC-3'), *CaTin1* promoter region was obtained from the adaptor-ligated genomic DNA fragments in GenomeWalker libraries according to the manufacturer's instructions (CLONTECH, Palo Alto, CA). The deletion fragments of *CaTin1* promoter were generated by PCR using the primers corresponding to serial 100-bp deletions and containing the *EcoRI* restriction site (forward primers) and the *NcoI* site (reverse primer). Amplified products were cloned into pGEM Easy Vector (Promega, Madison, WI), digested with *EcoRI* and *NcoI*, and cloned into pCambia 1305.1 (CAMBIA, Canberra, Australia) that contains a *gus* reporter gene and the hygromycin selectable marker flanked by T-DNA border sequence. The constructs were introduced into tobacco cv Samsun NN using *Agrobacterium*-mediated transformation.

For the GUS activity assay, 112.5 μ L 4-methylumbelliferyl β -D-glucuronide solution in a microtube was preincubated in a water bath for 5 min at 37°C. Then, 75 μ L of cell extract in GUS/luciferase buffer (0.1 M KPO₄, pH 7.8, 2 mM Na₂-EDTA, 2 mM dithiothreitol, and 5% glycerol) was added and incubated at 37°C. After 30 min or 60 min, 50 μ L was removed from each assay tube and transferred to a test tube containing 1 mL stop solution (0.2 M Na₂CO₃). The progress of the reactions was quantitatively assessed under the emission wavelength of 455 nm. The standard curve was generated by determining the fluorescence of various concentrations of 4-methylumbelliferone standards ranged from 0 to 10 μ M (Maliga et al., 1995).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AF242731 for *CaTin1* and AF480414 for *CaTin1-2*.

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