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Constitutive expression of two pathogenesis-related genes in tomato plants enhanced resistance to oomycete pathogen *Phytophthora capsici*

Sujon Sarowar[1], Young Jin Kim[1], Eui Nam Kim[2], Ki Deok Kim[2], Jun Young Choi[3], Nam In Hyung[3] & Jeong Sheop Shin[1,*]

[1]*School of Life Sciences and Biotechnology, Korea University, 136-701 Seoul, Korea;* [2]*College of Life and Environmental Sciences, Korea University, 136-701 Seoul, Korea;* [3]*Department of Plant Science and Technology, Sangmyung University, 330-720 Cheonan, Korea (*requests for offprints: Phone: +82-2-3290-3430; Fax: +82-2-927-9028; E-mail: jsshin@korea.ac.kr)*

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Abstract

The potential role of two pathogen-induced pepper genes, encoding basic pathogenesis-related protein 1 (*CABPR1*) and ascorbate peroxidase-like 1 (*CAPOA1*), in tolerance against phytopathogens was examined in transgenic tomato (*Lycopersicum esculentum* cv. House Momotaro) plants. Polymerase chain reaction and reverse transcription-polymerase chain reaction analyses using gene-specific primers revealed that the pepper *CABPR1* and *CAPOA1* genes were integrated into the tomato genome. The constitutive expression of *CABPR1* and *CAPOA1* in the tomato did not exhibit any morphological abnormalities. However, these transgenic tomato plants showed enhanced tolerance to the oomycete pathogen *Phytophthora capsici*, and very weak resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. These results suggest that overexpression of *CABPR1* and *CAPOA1* in tomato plants altered their resistance responses to pathogenic attack.

Introduction

Plants have their own defense systems that can be induced upon infection by various microorganisms. These defense responses of higher plants during plant-pathogen interaction, through rapid changes in gene expression, can result in the *de novo* syntheses of specific proteins. Most of these plant proteins are inducible pathogenesis-related (PR) proteins, which accumulate in host plants as a result of pathogen infections or abiotic stress conditions (Kim and Hwang, 2000). Some of the genes associated with PR proteins encode metabolic enzymes and others encode proteins with unknown biological functions and activities. These proteins are assumed to function in inhibition of pathogen growth and spread. Of these PR proteins, basic pathogenesis-related protein 1 (BPR1) and ascorbate peroxidase (POA) are the most dominant and accumulate the most rapidly after pathogen infection.

PR proteins are induced by various types of pathogens (viroids, viruses, bacteria, and fungi), by diverse environmental stresses, and by treatment with salicylic acid or ethylene. PR-1 is a dominant group of PRs and is commonly used as a marker for systemic acquired resistance (SAR). In our previous study, pepper basic PR-1 protein (*CABPR-1*) was strongly expressed after ethephon treatment, rather than after wounding or infection

by the bacterial pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Kim and Hwang, 2000). Several biological functions have been proposed for PR1 protein but its precise role is still unknown. Specific PR-1 members of tobacco and tomato have been known to have anti-oomycete activity, but the mechanisms underlying these effects remain unknown. More recently, we have reported that overexpression of the *CABPR1* gene in tobacco plants enhanced their resistance to *Phytophthora nicotianae*, to the bacterial pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci*, and to stress by heavy metals such as mercury and cadmium (Sarowar et al., 2005b). Overexpression of *CABPR1* in *Arabidopsis* also enhanced host resistance to *Pseudomonas syringae* pv. *tomato* DC3000 and oxidative stress (Hong and Hwang, 2005).

Ascorbate peroxidase (APX) is one of the most important enzymes in scavenging potentially harmful H_2O_2 from the chloroplasts and cytosol of plant cells (Jespersen et al., 1997). It is believed that the peroxidases are also involved in some physiological processes, such as strengthening of the cell wall via lignin deposition or cross-linking of cell wall proteins (Mader and Fussl, 1982; Bradley et al., 1992). These phenomena might constitute a general defense mechanism of plants against a broad range of pathogens. It is also reported to be an important defense enzyme that protects plant cells from disease agents via systemic acquired resistance (Kvaratskhelia et al., 1997). Increased activity of APX has been reported in different plant species in response to such environmental stresses as salinity, chilling, metal toxicity, drought, and heat (Davis and Swanson, 2001; Bueno and Piqueras, 2002). In our previous study, increased expression of a pepper ascorbate peroxidase-like 1 (*CAPOA1*) gene was observed in pepper plants infected with *Xanthomonas campestris* pv. *vesicatoria* (Do et al., 2003). Moreover, constitutive expression of the pepper *CAPOA1* gene in transgenic tobacco plants conferred resistance against the oomycete pathogen *Phytophthora nicotianae*, but not against the bacterial pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci* (Sarowar et al., 2005a). Up-regulated expression of the ascorbate peroxidase gene was also observed in barley leaves after inoculation with *Blumeria graminis* f.sp. *hordei* (Huckelhoven et al., 2001). Pathogenesis-related peroxidases (*Shpx2* and *Shpx6a*) have been reported to enhance resistance against oomycete pathogens (Kazan et al., 1998; Way et al., 2000). There are also several reports that ascorbate peroxidase plays an important role in oxidative stress tolerance (Aono et al., 1995; Camp et al., 1996; Sarowar et al., 2005a).

In this study, we transformed the pepper cDNA coding for basic pathogenesis-related protein 1 (*CABPR1*) and ascorbate peroxidase-like 1 gene (*CAPOA1*) into tomato plants and attempted to identify the potential functions of these genes against phytopathogens. We report that the constitutive expression of the pepper *CABPR1* or *CAPOA1* genes in transgenic tomato plants enhanced resistance against the oomycete pathogen, *Phytophthora capsici* and conferred very weak resistance against the bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000.

Materials and methods

Plant materials

Seeds of a commercial tomato cultivar (*Lycopersicum esculentum* cv. House Momotaro, Takii Seed Co., Japan) were surface sterilized with a diluted commercial bleach solution (Clorox; 0.6% NaOCl (v/v) in final concentration) containing 0.1% (v/v) Tween-20 for 10–12 min. Sterilization was followed by washing three times with sterilized distilled water. Seeds were germinated on MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.8% (w/v) phytoagar (DUCHEFA, Netherlands). Seven days after germination, cotyledons were excised and used to generate transgenic plants.

Plant expression vector construction, tomato transformation and plant regeneration

Two pepper genes were used. One encoded basic pathogenesis-related protein 1 (*CABPR1*) and the other encoded ascorbate peroxidase (*CAPOA1*). The recombinant plasmids pMBP1-*CABPR1* and pMBP1-*CAPOA1* were used to generate transgenic tomato plants in this study (Figure 1A). Both plasmids contained the 35S *Cauliflower mosaic virus* promoter, a nopaline synthase terminator, and a neomycin phosphotransferase gene

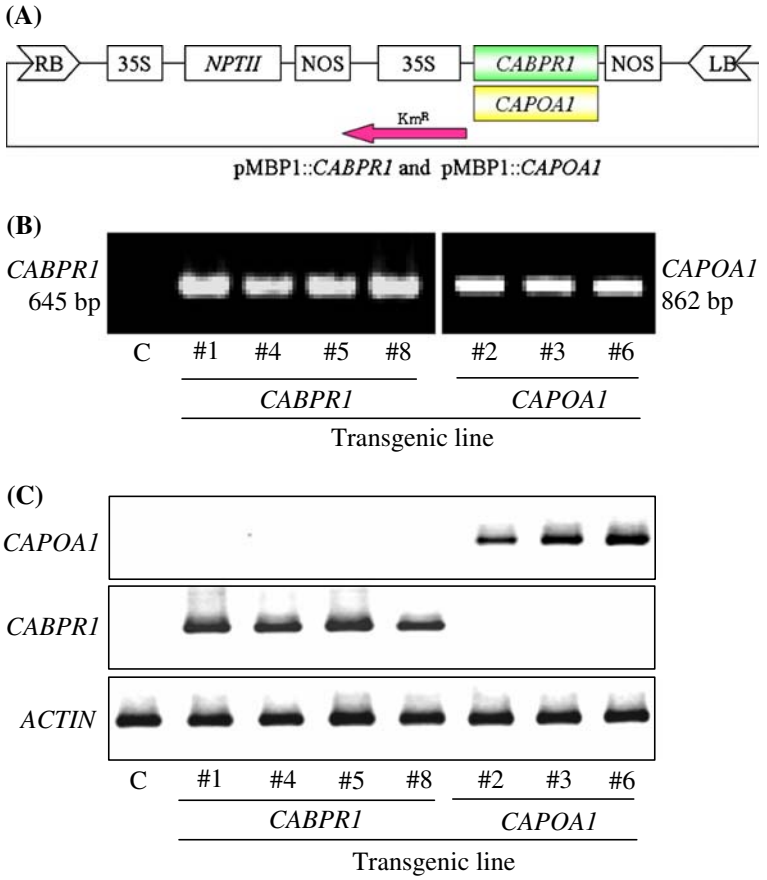


Figure 1. Vector construction and generation of the *CABPR1* and *CAPOA1* transgenic tomato plants. (A) Diagram of the constructed plant expression vector pMBP1-*CABPR1* and pMBP1-*CAPOA1*. RB, right border; LB, left border; *NPT*II, neomycin phosphotransferase; 35S, *Cauliflower mosaic virus* 35S promoter; *NOS*, nopaline synthase terminator. (B) Genomic DNA PCR analysis of regenerated plants. The kanamycin-resistant putative transgenic plants were screened by PCR analysis by using *CABPR1* and *CAPOA1* gene-specific primers. (C) RT-PCR analysis of different transgenic plants for detection of *CABPR1* and *CAPOA1* transcripts. c, control plant; lines #1, #4, #5, #8 represents four lines of *CABPR1* and #2, #3, #6 represents three individual transgenic lines of *CAPOA1*.

(*npt*II, the kanamycin resistance marker gene), as reported in our previous reports (Sarowar et al., 2005a, b). *Agrobacterium tumefaciens* strain LBA4404 was used for tomato transformation according to the procedure described by Park et al. (2003) with some modifications. *A. tumefaciens* was cultured overnight in YEP medium supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin at 28 °C with shaking to mid-log phase ($OD_{600} = 0.8$). Then, the bacterial cells were collected by centrifugation at 1500×g for 5 min and then resuspended in liquid shoot regeneration medium up to a final OD_{600} of 0.8 for infection.

After transformation, the explants were cultured on a selective medium for shoot regeneration (Figure 2A, B). The selection medium consisted of shoot regeneration medium (MS salts and vitamins, 2.0 mg/l BA, 0.01 mg/l IBA and 30 g/l sucrose) supplemented with 200 mg/l kanamycin and 200 mg/l cefotaxime. Regenerated shoots (T_0) were cut and grown in the same medium for shoot elongation (Figure 2C, D). After selection, the kanamycin-resistant shoots were then transferred to a rooting medium (MS salts and vitamins, 0.1 mg/l IBA and 30 g/l sucrose) supplemented with 200 mg/l kanamycin (Figure 2E). Growth conditions such as light intensity and temperature were the same as in the previous report (Sarowar et al., 2003). Ten plantlets with well-developed roots of each construct were transferred to soil,

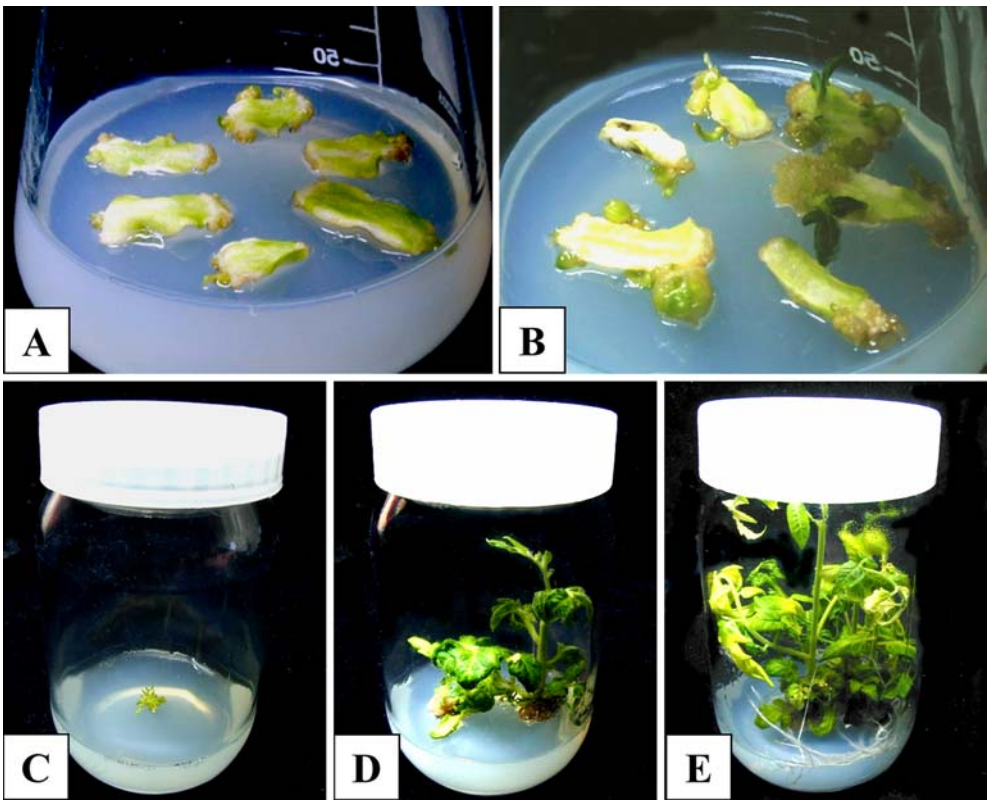


Figure 2. Procedure of *Agrobacterium*-mediated gene transformation of tomato. (A) Initiation of callus formation at the cut end of cotyledon explants 7 days after cocultivation with *Agrobacterium*. (B) Callus formation and shoot regeneration. (C & D) Shoot multiplication and elongation. (E) Root formation in the rooting medium.

and then grown in a greenhouse. Plants regenerated in the same shoot regeneration medium, without cocultivation of *Agrobacterium*, were used for a control.

Molecular detection of transgenic plants by polymerase chain reaction

The presence and integrity of the transgene were verified by PCR amplification using primers specific to *CABPR1* and *CAPOA1*. Genomic DNA was isolated according to the procedure of Edwards et al. (1991). PCR was carried out with the two gene specific primers, *CABPR1-5* (5′-CCGGGATCCGTCATGGGACACTCTAATATT GCC-3′) and *CABPR1-3* (5′-CAAGAGCTCGTAACGTACTCCACAGAAC-3′), and *CAPOA1-5* (5′-GTTCTGGATCCAATGGCGAAGCCAATTGTC-3′) and *CAPOA1-3* (5′-TCGTAGGATCCACTTCATCTTTTTCCGGACTTCATACC-3′). The amplification was performed under the following conditions: 95 °C for 5 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min. Finally, the PCR products were separated on a 1% agarose gel, and visualized by ethidium bromide staining.

RNA isolation and RT-PCR analysis

Total RNAs were extracted from tomato leaves, using TRIzol® Reagent (Gibco-BRL, USA), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using 0.5 μg of oligo (dT) and 200 units of SuperScript™ III (Invitrogen, USA). Tomato actin DNA was amplified as an internal equal-loading control. The specific primers, *CABPR1-5* and *CABPR1-3*, and *CAPOA1-5* and *CAPOA1-3*, were used for amplification. One microliter of the RT reaction and 10 pmol of each oligonucleotide primer were used for semi-quantitative RT-PCR, in a total volume of 30 μl. The PCR conditions

used were: 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min, followed by 7 min of final extension at 72 °C. The amplified products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Pathogen inoculation

Phytophthora capsici, which causes buckeye rot disease in tomato, was grown in the dark at 26 °C on the V8 juice agar medium (200 ml V8 juice, 3 g $CaCO_3$, 800 ml DW, 15 g agar, pH 6.4). For the leaf infection assay, fully expanded leaves of 5 or 6-week-old plants were used for *P. capsici* inoculation. An oomycete plug of five-day-old culture (0.4 mm in diameter) was placed at the center of the abaxial leaf surface. Twenty-one fully expanded leaves from three plants of each line of T_0 generation – approximately seven leaves from one individual plant – were used for *P. capsicum* infection (total 84 leaves from four lines of *CABPR1* and about 60 leaves from three lines of *CAPOA1*), and this experiment was replicated at least twice. The inoculated plants were cultured in a growth chamber at 25 °C and 100% humidity. The infected leaf area was measured 2 days after inoculation with *P. capsici*. Photos were taken 2 days after infection.

The bacterial pathogens, *P. syringae* pv. *tomato* DC3000, were grown at 28 °C in King's B medium (King et al., 1954). Bacterial suspensions (10^4 cfu ml^{-1}) were infiltrated into the leaf mesophyll tissues of intact plants using a hypodermic syringe without a needle. In order to monitor bacterial growth in leaf tissues 5 days after infiltration, the tissue was ground with sterile water in a microfuge tube, and spread out on selective King's B agar media. The bacterial populations were determined based on the number of colonies forming on the rifampcin-selective medium. Data were calculated as the means ± standard errors from three independent experiments.

Results and discussion

Generation of the CABPR1 and CAPOA1 transgenic tomato plants

To generate the transgenic tomatoes expressing *CABPR1* or *CAPOA1*, we constructed two chimeric genes fused to the *CaMV35S* promoter and *nos* terminator sequence with the full length cDNA sequences of these genes from pepper (Figure 1A). Of 240 explants transformed with *CABPR1* or *CAPOA1*, 76 shoots were regenerated after 8 weeks of culture on selection medium containing 200 mg/l kanamycin. Generally, single shoot, with the occasional double or triple shoot(s), was produced from the explants.

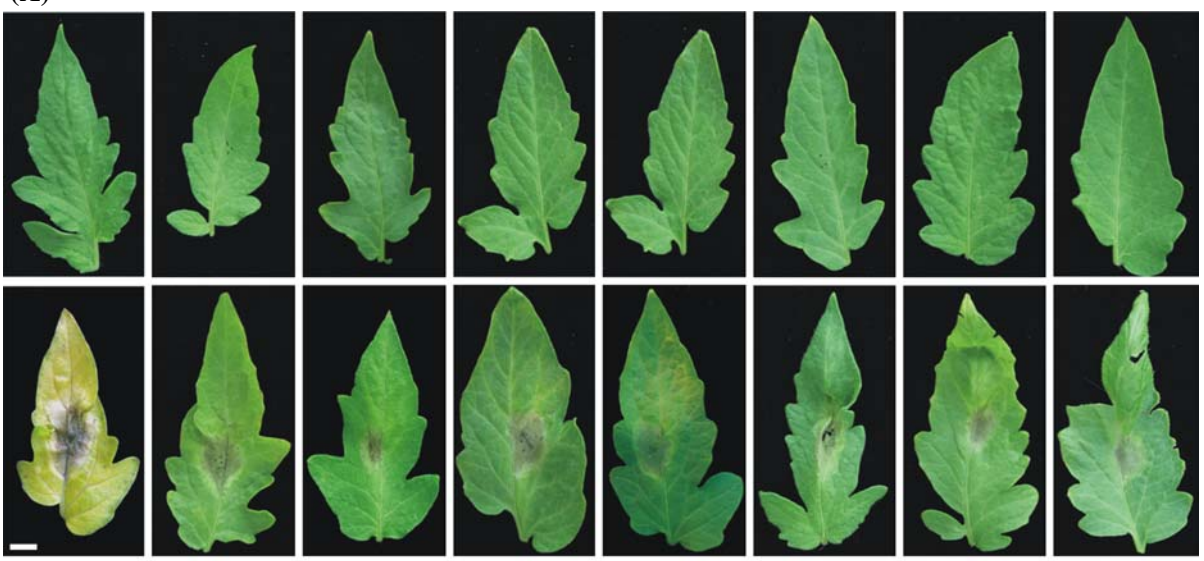
After screening for kanamycin-resistant shoots on selection medium containing kanamycin, the constitutive expression of the *CABPR1* and *CAPOA1* genes in transformed plant tissues was examined using PCR and RT-PCR. The results shown in Figure 1(B, C) confirmed that *CABPR1* and *CAPOA1* genes were successfully integrated into the tomato genomic DNA and that those genes were constitutively expressed in transgenic tomato T_0 lines. Four lines of *CABPR1* and 3 lines of *CAPOA1* were finally selected and micropropagated (Figure 2). *In vitro* grown microshoots were then moved to rooting medium for root induction. Well-developed rooted plantlets were transferred to soil to evaluate disease resistance.

Disease evaluation in CABPR1 and CAPOA1 transgenic plants

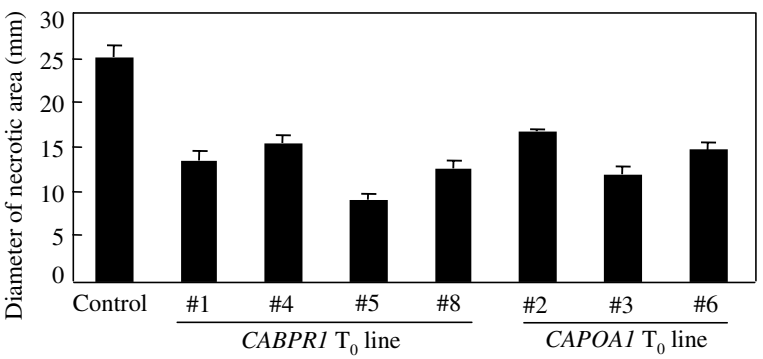
To determine whether or not *CABPR1* or *CAPOA1* transgenes conferred resistance to tomato buckeye caused by *P. capsici*, 6-week-old control and transgenic tomato plants were inoculated by using the agar plug method. Significant differences in symptom development between transgenic lines and wild type tomato were observed 2 days after

Figure 3. Disease evaluation in *CABPR1* and *CAPOA1*-transgenic tobacco plants. (A & B) Inhibition of disease development (mm) on transgenic plants infected by *Phytophthora capsici*. (upper panel) healthy leaves with agar plug inoculation and (lower panel) disease development in the tested leaves. Photos and data recorded 2 days after infection and represented as the mean ± standard errors of three replications, each replication comprising three sub-replications. Bar indicates 0.8 cm. (C) Inhibition of *Pseudomonas syringae* pv. *tomato* DC3000 growth in transgenic tomato leaves. Data were taken 5 days after infiltration of the bacterial pathogen, and represented as the mean ± standard errors of three replications, each replication comprising three sub-replications.

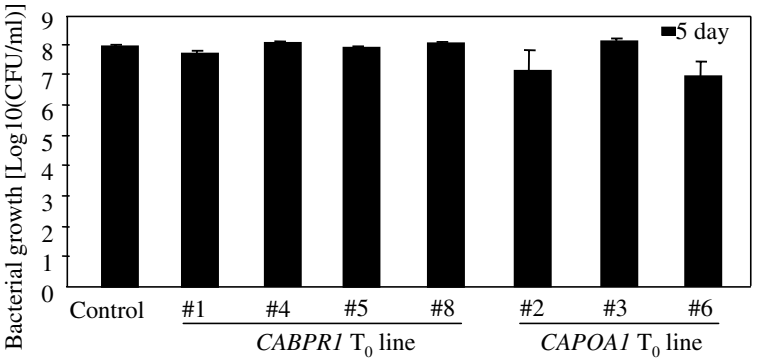
(A)


Control
#1
#4
#5
#8
#2
#3
#6
CABPR1 transgenic T0 line
CAPOA1 transgenic T0 line

(B)


Diameter of necrotic area (mm)
30
25
20
15
10
5
0
Control
#1
#4
#5
#8
#2
#3
#6
CABPR1 T0 line
CAPOA1 T0 line

(C)


Bacterial growth [Log10(CFU/ml)]
9
8
7
6
5
4
3
2
1
0
5 day
Control
#1
#4
#5
#8
#2
#3
#6
CABPR1 T0 line
CAPOA1 T0 line

inoculation (Figure 3A). Two days after inoculation, prominent necrotic areas with severe bleaching symptoms appeared on the leaves of control plants, whereas symptom development was greatly retarded in all transgenic lines. As compared to control, *CABPR1* showed an average of 50% and *CAPOA1* showed 43.5% decreased necrotic area (Figure 3B), while slight chlorotic symptoms were observed in the areas adjacent to infection on transgenic leaves.

Enhanced resistance of *CABPR1* and *CAPOA1* transgenic plants was also observed after inoculation of the 6-week-old plants with bacterial speck disease. Typical bacterial speck symptoms were observed on wild-type tomato leaves inoculated with *P. syringae* pv. *tomato* DC3000. The amount of bacterial growth was calculated 5 days after inoculation. The numbers of bacteria recovered from the bacterial specks on leaves were slightly reduced in two lines of *CABPR1* (line #1, #5). High levels of reduction of bacterial growth were observed in two lines of *CAPOA1-2* and *CAPOA1-6* when compared to control plants (Figure 3C). But, there was no correlation between the expression level of *CABPR1* or *CAPOA1* and enhanced resistance. Though a varied resistance response was observed in the transgenic plants, all the tested lines showed enhanced resistance to necrotropic oomycete *P. capsici* and weak resistance to *P. syringae* pv. *tomato* DC3000. The results shown in Figure 3 are similar to those from previous reports on the transformation of *PR1a* and *PR1b* and *APX1* genes in tobacco (Alexander et al., 1993; Kazan et al., 1998; Way et al., 2000; Sarowar et al., 2005a). These results suggest that overexpression of *CABPR1* or *CAPOA1* in tobacco and tomato plants enhance tolerance to oomycete pathogens such as *P. nicotianae* and *P. capsici*. The overexpression of *CABPR1* or *CAPOA1* in tomato plants may lead to expression of defense-related genes by disturbing the homeostasis of the plant cells (Herbers et al., 1996). One interesting finding is that the transgenic lines of *CABPR1* or *CAPOA1* differed in their defense responses to challenge-infection by the necrotrophic oomycete *P. capsici* and the virulent pathogen *P. syringae* pv. *tomato* DC3000. Both transgenic lines were highly resistant to *P. capsici* infection, but they were not resistant to *P. syringae* pv. *tomato* DC3000. In our previous results, we observed enhanced tolerance to bacterial pathogen such as, *R. solanacearum* and *P. syringae* pv. *tabaci* in *CABPR1* transformed tobacco plants (Sarowar et al., 2005b) and weak resistance in *CAPOA1* transformed plants (Sarowar et al., 2005a). CABPR1 or CAPOA1 proteins are localized in plant cell vacuoles (Kim and Hwang, 2000; Sarowar et al., 2005a). Oomycete *P. capsici* actively destroys host cells prior to and during colonization, usually through the secretion of toxic protein elicitin (Kamoun et al., 1993). Therefore, both proteins may be released to prevent penetration and propagation when *P. capsici* begins to break down the tomato cells. However, CABPR1 and CAPOA1 have little effect on bacterial infection because these biotrophic bacteria reside in intercellular spaces and do not have chance to contact CABPR1 or CAPOA1 proteins directly. Overall, it appears likely that *CABPR1* or *CAPOA1* overexpression is a causal factor for the enhancement of disease resistance in tomato plants.

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