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Mini-Review

Functional Characterization of PR-1 Protein, β-1,3-Glucanase and Chitinase Genes During Defense Response to Biotic and Abiotic Stresses in *Capsicum annuum*

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Spatial and temporal expression of pathogenesis-related (PR) gene and proteins has been recognized as inducible defense response in pepper plants. Gene expression and/or protein accumulation of PR-1, β-1,3-glucanase and chitinase was predominantly found in pepper plants during the inoculations by *Xanthomonas campestris* pv. *vesicatoria*, *Phytophthora capsici* and *Colletotrichum coccodes*. PR-1 and chitinase genes were also induced in pepper plants in response to environmental stresses, such as high salinity and drought. PR-1 and chitinase gene expressions by biotic and abiotic stresses were regulated by their own promoter regions containing several stress-related *cis*-acting elements. Overexpression of pepper PR-1 or chitinase genes in heterogeneous transgenic plants showed enhanced disease resistance as well as environmental stress tolerances. In this review, we focused on the putative function of pepper PR-1, β-1,3-glucanase and chitinase proteins and/or genes at the biochemical, molecular and cytological aspects.

Keywords : chitinase, disease resistance, environmental stress, β-1,3-glucanase, PR-1

Plants exhibit constitutive and inducible defenses against pathogen attacks. Basal defenses, R gene-mediated resistance and non-host resistance systems were triggered in plant cells by pathogens. Differential responses of plants to invading pathogens were found in many plant-pathogen interactions, which led to initiation of complex defense signal transductions within plant cells. Compatible and incompatible interactions also have been found in the pepper plants infected by *Xanthomonas campestris* pv. *vesicatoria* and *Phythophthora capsici*. Physiological and molecular responses of pepper plants such as pathogenesis-related (PR) protein accumulation and cytological alterations differentially occurred during compatible and incompatible interactions with these pathogens. Recently, resistance of pepper plant to Phythophthora blight was extensively reviewed at the cytological, physiological and molecular aspects (Hwang, 2001).

The PR proteins are induced in plant tissues in response to pathogen infections, especially during the hypersensitive response (HR). The PR proteins are classified into 14 groups (PR-1 to PR-14) based on their structural homologies within the groups, which were PR-1, glucanase, chitinase, thaumatin-like protein, peroxidase, ribonuclease-like protein, thionin, defensin and lipid transfer proteins (van Loon and van Strien, 1999). Induction of PR proteins was one of the active defense responses against pathogen infections. PR-proteins have been found in several plant species following infection with various pathogens including viroids, viruses, bacteria and fungi. Several PR-proteins are likely to play a key role in establishing systemic acquired resistance (SAR) against pathogen attacks in pepper and tobacco (Lee and Hwang, 2005). PR-1 mRNA and/or proteins strongly accumulate in numerous plant species of monocots and dicots upon infection by bacterial (Hoegen et al., 2002), oomycete (Hoegen et al., 2002) and fungal pathogens (van Kan et al., 1992). Overexpression of the PR-1 gene in transgenic plants provided critical evidence for the *in vivo* function of the PR-1 proteins. The ectopic expression of PR-1a in the transgenic tobacco plants conferred increased tolerance to infections by *Peronospora tabacina* and *P. parasitica* var. *nicotianae* (Alexander et al., 1993). In contrast, the overexpression of the PR-1a or PR-1b proteins in tobacco plants was not effective in triggering resistance to the tobacco mosaic or alfalfa mosaic viruses, which suggests that the PR-1 proteins may not function as unique antiviral factors (Cutt et al., 1989; Linthorst et al., 1989). Biological and biochemical functions of PR-1 protein during the defense reactions and developmental processes are still unclear.

β-1,3-Glucanase hydrolyses the β-1,3-linked glucans, major components of cell wall of oomycetes and synergistically acts with chitinase to inhibit fungal growth *in vitro* (Kim and Hwang, 1997; Mauch et al., 1988). The enzyme may also mediate plant defense by releasing glucan

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fragments from fungal or plant cell walls, as signal molecules that can activate a variety of plant defenses (Keen and Yoshikawa, 1983; Takeuchi et al., 1990). Expression of β-1,3-glucanases has shown to be induced by abiotic elicitors such as ethylene, salicylic acid, and methyl jasmonate as well as pathogen attack. Chitinase is a catalytic enzyme which is responsible for the hydrolysis of the chitin polymer (a β-1,4-linked N-acetylglucosamine), a structural component found in fungal cell wall, insects, a variety of crustaceans, and nematode eggs (Punja and Zhang, 1993). Earlier studies of the role of the chitinase focused on its involvement in plant defense responses to fungal pathogen infection (Daugrois et al., 1990; Nielsen et al., 1993; Wubben et al., 1992). Transgenic plants constitutively expressing a basic chitinase showed increased resistance against the fungal pathogen *Rhizoctonia solani* (Broglie et al., 1991). However, chitinase expression in plant tissues is also strongly induced by infection with viruses, bacteria and oomycetes, which do not have chitin or related structures as a component of their cell wall (Métraux et al., 1988). Environmental stresses such as high salinity, drought, wounding and ozone (O_3) also stimulate the accumulation of chitinase in plants, such as tomato and winged bean (Chen et al., 1994; Ernst et al., 1992; Wu et al., 1999). Chitinase expression is prevalently found in the diverse plants infected by different pathogens. However, the diverse biological functions of chitinase in the abiotic stress signaling and other cellular adaptation are poorly understood.

Accumulation of PR-1 protein, β-1,3-glucanase and chitinase in infected pepper plants. PR-1 proteins were accumulated in the pepper stems 2-4 days after inoculation with virulent isolate of *P. capsici* (Hwang et al., 1997). The PR-1 homologues were not found in the healthy stem tissues.

Total β-1,3-glucanase and chitinase activities may be coordinately induced and accumulated in pepper leaf tissues infected by *X. campestris pv. vesicatoria*. Higher β-1,3-glucanase and chitinase activities were induced in the leaf tissues infected by avirulent strain of *X. campestris pv. vesicatoria*. Multiple acidic and basic isoforms of β-1,3-glucanase and chitinase were detected in pepper leaf and stem tissues infected by *X. campestris pv. vesicatoria* and *P. capsici*, respectively (Hwang et al., 1997; Kim and Hwang, 1994; Lee and Hwang, 1996). Synthesis and accumulation of various isoforms of β-1,3-glucanase and chitinase may be differentially regulated in pepper leaf tissues during pathogenesis and defense responses. Some isoforms present in the healthy leaf tissues disappeared in the infected leaf tissues, whereas other isoforms were induced in the leaf tissues during compatible interaction with *X. campestris pv. vesicatoria*. *P. capsici* infection also induced synthesis and accumulation of β-1,3-glucanases and chitinases in the stem tissues. During Phytophthora blight symptom development on the pepper stems, accumulation of β-1,3-glucanases became much pronounced in the incompatible interaction. However, induction of chitinases by *P. capsici* infection was very similar in both compatible and incompatible interactions. Most basic isoforms of β-1,3-glucanases were induced and accumulated in both the compatible and incompatible interactions, suggesting a possible involvement of the hydrolases in the infection process rather than in the resistance expression (Lee and Hwang, 1996).

Purified β-1,3-glucanase and chitinase protein from pepper have antimicrobial activities against pathogenic fungi *in vitro* (Kim and Hwang, 1994, 1997). The 34 kDa β-1,3-glucanase inhibited hyphal growth of the oomycete *P. capsici*, but did not show antifungal activity against the chitin-containing fungi *Alternaria mali*, *Colletotrichum gloeosporioides*, *Magnaporthe grisea*, and *Fusarium oxysporum* f.sp. *cucumerinum*. Treatment with high amounts of the β-1,3-glucanase caused lysis of the hyphae and zoospores of *P. capsici*. Basic chitinase isoforms exhibit antifungal activities against *C. gloeosporioides*, *F. oxysporum* f.sp. *cucumerinum*, *M. grisea*, and *Trichoderma viridae in vitro*. Chitinases inhibited hyphal growth of *P.*

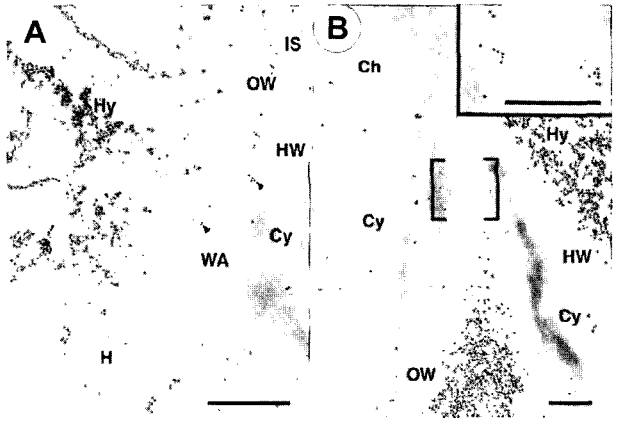

Fig. 1. Transmission electron micrographs of pepper stem tissues at 24 h after inoculation with virulent and avirulent isolates of *Phytophthora capsici*. Immunogold labeling was performed with tomato anti-chitinase antiserum and with goat anti-rabbit gold (10 nm) antibodies. (**A**) The invading oomycete hyphae forming haustoria. Gold particles are deposited over oomycete cell wall (arrowheads) and in the host cytoplasm. However, the oomycete cytoplasm is not specifically labeled and the wall opposition is nearly free of labeling. (**B**) The gold particles are usually visible over oomycete cell wall. Labeling occurs also in host cytoplasm. bars represent 500 nm. Ch, chloroplast; Cy, cytoplasm; Hy, hyphae; OW, oomycete wall; HW, host wall; IS, intercellular space; H, haustorium; WA, wall apposition (Lee et al., 2000a). (Bars = 500 nm).

capsici lacking chitin in their cell wall. Zoospore germination and germ tube elongation of *P. capsici* were effectively inhibited by treatment with chitinases. Moreover, the 34 kDa β-1,3-glucanase acted synergistically with a pepper basic 32 kDa chitinase in inhibiting hyphal growth of *F. oxysporum* f.sp. *cucumerinum* and *P. capsici*.

The *in situ* localization of PR-1 proteins and chitinase during the compatible and incompatible interactions of pepper stems with *P. capsici* through immunocytochemical studies. Immunogold labeling of PR-1 proteins showed that PR-1 protein was predominantly accumulated in the intercellular spaces of stem tissue infected by *P. capsici*, whereas chitinase deposited on the cell wall of the oomycete in both compatible and incompatible interactions (Fig. 1; Lee et al., 2000a, b). This indicates that PR-1 and chitinase functionally play roles in spatially different area within pepper stem tissues. Interestingly, hyphal cell walls of oomycetes are mainly constituted by β-glucans, but hydrolysis also yields small amounts of glucosamine which could be derived from chitin or glycoproteins (Sietsma et al., 1969). The presence of chitin in the cell wall of an oomycete pathogen *Pythium ultimum* was earlier demonstrated (Chérif et al., 1992). Kim and Hwang (1996) reported that purified chitinases from pepper stems inhibited zoospore germination and germ tube elongation of *P. capsici*. These results suggest that chitinases alone or in combination with other types of antifungal proteins, i.e. β-1,3-glucanase, may be significant in limiting *P. capsici* infection in pepper stem tissues.

Differential expression of PR-1 protein, β-1,3-glucanase and chitinase genes in infected pepper plants. A variety of genes encoding putative PR-1, β-1,3-glucanase and chitinase has been cloned from pepper plants (Table 1). In particular, pepper genes encoding basic PR-1 protein, basic β-1,3-glucanase and class II basic chitinase were isolated and functionally characterized from leaves infected by avirulent strain of *X. campestris* pv. *vesicatoria* (Hong et al., 2000; Jung and Hwang, 2000b; Kim and Hwang, 2000).

Constitutive expression of pepper PR genes was found in different organ of healthy pepper plants. The basic PR-1 gene, *CABPR1*, was highly expressed in roots, flowers and green (unripe) fruits, but not in leaves, stems and red (ripe) fruits (Kim and Hwang, 2000). Basic β-1,3-glucanase gene, *CABGLU*, was not expressed in healthy leaf and fruit tissues, whereas expression of the *CABGLU* gene was strongly expressed in roots (Hwang and Jung, 2000b). The abundance of *CAChi2* mRNA was also much greater in root and flower tissues than leaf, stem, and green and red fruit, in which *CAChi2* mRNA was hardly detected (Hong and Hwang, 2002). These indicate that expressions of the *CABPR1*, *CABGLU* and *CAChi2* genes vary throughout a healthy pepper plant in a developmental and organ-specific manner.

It was shown previously that the *CABPR1*, *CABGLU and CAChi2* genes are differentially expressed in leaf tissues with *X. campestris* pv. *vesicatoria* during compatible and incompatible interactions (Hong et al., 2000; Jung and Hwang, 2000b; Kim and Hwang, 2000). The rapid and strong induction of the *CABPR1*, *CABGLU and CAChi2* gene expression during the incompatible interactions indicated that *CABPR1*, *CABGLU and CAChi2* genes might play a critical role coordinately in the active defense responses to bacterial pathogen infections (Fig. 2A).

Table 1. PR-1 protein, β-1,3-glucanase and chitinase genes from pepper plants

GenBank Accession no.	Gene	Origin	Reference
AF053343	basic PR-1 gene (*CABPR1*)	*Capsicum annuum* cv. Hanbyul	Kim and Hwang (2000)
AF348141	basic PR-1 gene	*Capsicum annuum*	–
AY284926	PR-1 gene (sHSP), cytoplasmic small heat shock protein class I	*Capsicum frutescens*	–
AY560589	genomic DNA of *CABPR1* gene (gCABPR1)	*Capsicum annuum* cv. Hanbyul	Hong et al. (2005)
AF082724	β-1,3-glucanse, partial cDNA	*Capsicum annuum* cv. Hanbyul	Jung and Hwang (2000a)
AF227953	basic β-1,3-glucanse-like protein gene (*CABGLU*)	*Capsicum annuum* cv. Hanbyul	Jung and Hwang (2000b)
AF294849	β-1,3-glucanse-like protein gene	*Capsicum annuum*	–
AY683478	β-1,3-glucanse-like protein gene	*Piper nigrum*	–
AA840648	endochitinase	*Capsicum annuum*	–
AF082713	class I chitinase, partial cDNA	*Capsicum annuum* cv. Hanbyul	–
AF091235	class II basic chitinase (*CAChi2*)	*Capsicum annuum* cv. Hanbyul	Hong et al. (2000)
AF108893	class V chitinase	*Capsicum annuum*	–
AY775335	genomic DNA of *CAChi2* gene (gCAChi2)	*Capsicum annuum* cv. Hanbyul	–

CABPR1, CABGLU and CAChi2 genes are also differentially expressed in stem tissues with *P. capsici* during compatible and incompatible interactions. *CABPR1* expression was found in the pepper stem tissues at 1 day after inoculation with both virulent and avirulent isolates of *P. capsici*. The dramatic increase in *CABPR1* mRNA occurred in the inoculated stems with both isolates at 2 days after inoculation (DAI), especially being more apparent in the compatible than the incompatible interactions. *CABPR1* expression gradually declined at 3 and 4 DAI of the virulent isolate, whereas remained at high levels by 4 days during the incompatible interaction (Hwang and Kim, 2000). *CABGLU* mRNA was slightly detected in healthy stems (Fig. 2B). In the compatible and incompatible interactions with *P. capsici*, induction of *CABGLU* mRNA was found at 1 DAI. The highest level of the *CABGLU* transcripts was detected in the compatible response at 2 DAI, but the mRNA levels gradually declined from 2 to 4 d after inoculation. In contrast, the mRNA levels of *CABGLU* gene in the incompatible response gradually increased from 1 to 3 DAI (Fig. 2B). *P. capsici*-infected stems also showed different accumulation of *CAChi2* transcripts in the compatible and incompatible interactions. Induction of *CAChi2* appeared in both interactions 1 DAI. In the compatible interactions, *CAChi2* slightly increased 2 DAI and then diminished after 3 DAI when pepper plants began to die due to severe *P. capsici* infection. In the incompatible interaction, however, drastic increase of *CAChi2* mRNA occurred 2-3 DAI. After 4 days, the transcripts decreased to the level of 1 DAI (Hong et al., 2000).

The disease response of pepper plants to *Colletotrichum coccodes* infection is dependent on plant growth stage, as previously observed (Hong and Hwang, 1998). Distinctly different lesions of the anthracnose disease occurred in pepper plants at the 4- and 8-leaf stages. Characteristic leaf blight was produced in pepper plants at the 4-leaf stage, whereas restricted leaf spots occurred on upper leaves 8 DAI at the 8-leaf stage (Hong and Hwang, 2002). To understand the effect of *C. coccodes* infection on expression of PR genes, RNA gel blot analyses were performed using the total RNA of leaf tissues at various time intervals after inoculation with *C. coccodes*. The time course of the mRNA accumulation of *CABPR1*, *CABGLU* and *CAChi2* after *C. coccodes* inoculation on pepper plants at the 4- and 8-leaf stages was examined. During age-related resistance of pepper plants to *C. coccodes* inoculation, differential inductions of *CABPR1*, *CABGLU and CAChi2* genes were found in leaf tissues (Hong and Hwang, 2002). Slight induction of *CABPR1* gene also occurred in the leaf tissues at the two growth stages 12 h after inoculation (HAI). A continuous increase in the *CABPR1* transcript was found to 72 HAI at the 4-leaf stage.

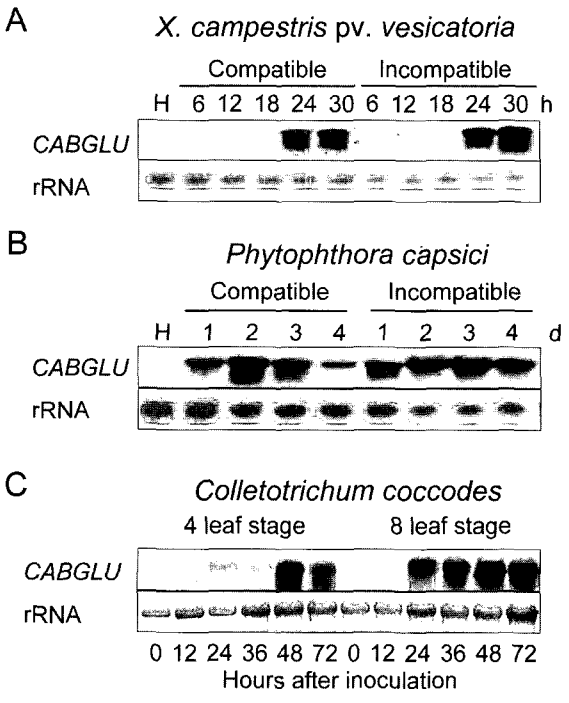

Fig. 2. Time course of mRNA expression of *CABGLU* gene in leaf and stem tissues of pepper plants by pathogen inoculation. (**A**) Induction of *CABGLU* mRNA at various times after inoculation with virulent and avirulent strains of *Xanthomonas campestris* pv. *vesicatoria*, (**B**) induction of *CABGLU* mRNA at various times after inoculation with virulent and avirulent isolates of *Phytophthora capsici*. (**C**) *CABGLU* expression in pepper leaf tissues infected with *Colletotrichum coccodes* at 4- and 8-leaf stages. Twenty micrograms of total RNA were loaded per lane and hybridized with ^{32}P-labelled cDNAs. A *Capsicum annuum* 25S rRNA probe was used as an internal standard. The Northern blot analysis was repeated three times with similar results (Hong and Hwang, 2002; Jung and Hwang, 2000b).

At the 8-leaf stage, however, higher amount of the transcript was detected at 24 HAI compared to the 4-leaf stage, and the transcript accumulation was remarkably elevated to 72 HAI. *CABGLU* mRNAs were first visible in leaf tissue at 12 HAI at the two growth stages (Fig. 2C). At the 4-leaf stage, accumulation of *CABGLU* mRNA was strongly induced at 48 h and then decreased at 72 HAI. In contrast, an earlier strong induction of the *CABGLU* mRNA occurred 24 HAI at the 8-leaf stage. The abundance of the transcripts remained at high levels during the anthracnose development. *CAChi2* mRNAs was found 24 HAI. Maximum level of the *CAChi2* mRNAs was reached at 48 HAI. At the 8-leaf stage, the *CAChi2* transcripts were detected at 12 HAI and their increase was more retarded than induction of the chitinase gene at the 4-leaf stage (Hong and Hwang, 2002). These results indicate that *CABPR1* and *CABGLU* genes may be involved in age-related resistance, and *CAChi2* gene may be involved in

anthracnose development in the pepper seedlings.

CAChi2 transcripts were clearly detectable as a purple color *in situ* in flower and root tissues (Fig. 3, panels A, B, and C), whereas there was no transcript signal in leaf and stem tissues of healthy pepper plants (Fig. 3, panels D and F). The *CAChi2* transcripts were also detected in endodermis and protoxylem of root tissue. Strong accumulation of the *CAChi2* mRNA was also detected at phloem cells of vascular bundles in the major and minor veins of leaf tissues infected by *C. coccodes*. The *CAChi2* mRNAs were detected along the vascular strands (Fig. 3E; Hong and Hwang, 2002). The *CABPR1* and *CAChi2* mRNA was spatially detected in the pepper stem tissues infected by virulent and avirulent isolates of *P. capsici*. *In situ* mRNA hybridization in stem tissues infected by *P. capsici* demonstrated that the predominant accumulation of *CABPR1* and *CAChi2* mRNA occurred in the vascular bundle of infected stem tissues, especially in confined area of phloem cells (Fig. 3G; Lee et al., 2000a, b). Expression of some PR genes and their protein products in host plants by pathogens or abiotic stresses has been demonstrated to be localized in vascular bundles of plant tissues (Breda et al., 1996; Constabel and Brisson, 1995; Eyal et al., 1993; Jacinto et al., 1997; Lee et al., 2000a, b). Predominant *CABPR1* and *CAChi2* mRNA accumulation occurred in the phloem cells of leaf and stem tissues may be a common response to some pathogen invasion. However, regulation for the spatial expression of pepper *CABPR1* and *CAChi2* genes in pepper leaf and stem tissues remains elucidated.

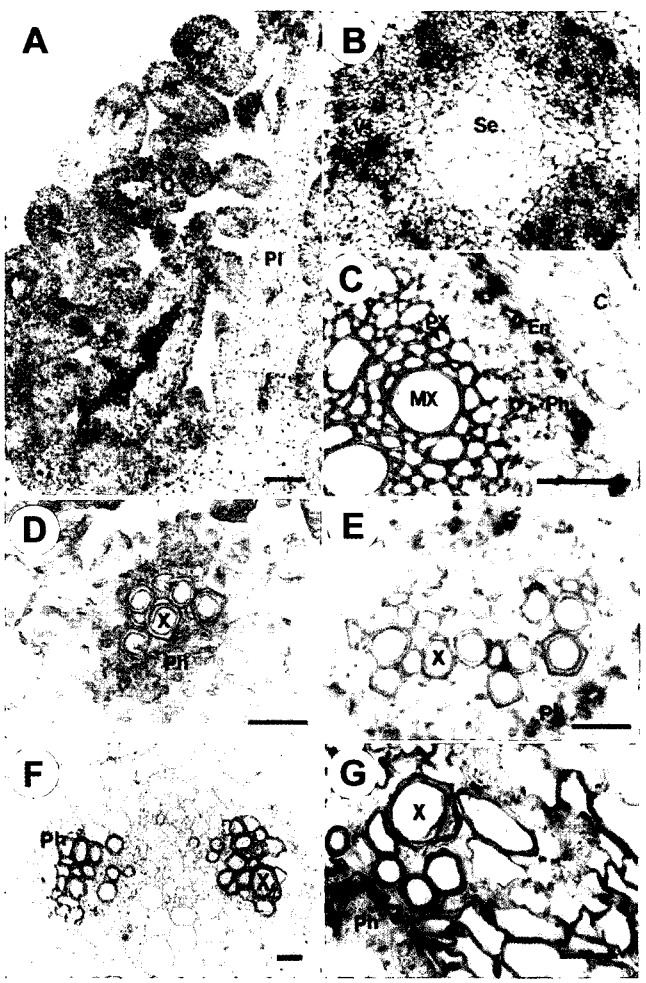

Fig. 3. *in situ* localization of *CAChi2* in pepper leaf, stem, root and flower tissues. (**A**) Longitudinal section of pepper flower (bar = 100 μm), (**B**) Transverse section of sepal (bar = 100 μm) (**C**) Transverse section of root tissue (bar = 100 μm), (**D**) Healthy leaf tissues (bar = 30 μm), (**E**) Leaf tissues of pepper plants infected with *Colletotrichum coccodes* at 48 h after inoculation (bar = 30 μm). (**F**) Healthy pepper stem tissue (bar = 50 μm), and (**G**) Pepper stem tissues infected by avirulent isolate of *Phytophthora capsici* at 24 h after infection (bar = 25 μm). More labeling of chitinase mRNA is visible in the phloem cells, but the xylem cells are nearly free of labeling. (Hong and Hwang, 2002; Lee et al., 2000a). C, cortical layer; En, endodermis; MX, metaxylem; O, ovule; Ph, phloem; Pl, placenta; PX, protoxylem; Se, sepal; Vs, vascular bundle; X, xylem. (bar = 100 μm)

Induction of pepper PR-1 protein, β-1,3-glucanase and chitinase genes by abiotic elicitors and environmental stresses. Ethylene-inducible expression of *CABPR1*, *CABGLU and CAChi2* genes was predominantly found in leaf tissues, indicating that ethylene is a putative signal for the pepper gene activation (Hong et al., 2000; Jung and Hwang, 2000b; Kim and Hwang, 2000). In particular, the treatment with L-α-(2-aminoethoxyvinyl)glycine (AVG), an inhibitor of ethylene biosynthesis, distinctly suppressed *CABPR1* expression in leaves inoculated with *X. campestris* pv. *vesicatoria*, which suggest that ethylene may function as a strong elicitor for the activation of *CABPR1* gene, eventually mediating the defense response of pepper plants. Ethylene-inducible genes in other plant species contain ethylene-responsive *cis*-acting elements, GCC-boxes, in their promoter regions (Eyal et al., 1993), indicating that *CABPR1*, *CABGLU* and *CAChi2* gene expressions may be regulated by putative GCC-box or GCC-box-like elements in their promoters.

Interestingly, *CABPR1 and CAChi2* gene expressions were induced in pepper plants by environmental stresses such as high salinity, drought as well as pathogen infections (Hong and Hwang, 2002, 2005a, b; Hong et al., 2005). It is well supported by previous studies reporting that PR-1 and/or chitinase genes were induced in plant tissues by environmental stresses such as UV-light, high salinity (A-H-Mackerness et al., 2001; Chen et al., 1994; Ernst et al., 1992; Pasqualini et al., 2003; Wu et al., 1999). *CABPR1* expression was strongly induced in the pepper leaves by exogenous NaCl and mannitol treatments. Rapid induction of the *CABPR1* gene was detected in the leaf tissues within

1 h after the 400 mM NaCl treatment, and the transcription strongly increased at 12-24 h after the treatment (Hong and Hwang, 2005a). Drought-mimicking mannitol also induced *CABPR1* expression in pepper leaf tissues 12-24 h after treatment (Hong and Hwang, 2005a). Low temperature at 4°C was enough to induce *CABPR1* transcription in the leaf and stem tissues (Hong et al., 2005). Treatment with methyl viologen (MV), causing elevation of cellular reactive oxygen species (ROS) in plant tissues, increased *CABPR1* transcript levels in the pepper leaf tissues. The induction of *CABPR1* expression by MV treatment was more pronounced than those of the *CAPOA1* and *CAPO1* genes, which encode the antioxidant enzymes of the putative pepper ascorbate peroxidase and peroxidase, respectively (Do et al., 2003). Treatment with 200 mM NaCl induced *CAChi2* mRNA in both the leaf and stem tissues. The *CAChi2* mRNA induction in the leaf tissue was slightly lower than in the stem tissue. Dose-dependent increase of *CAChi2* expression in pepper plants treated with NaCl was also found on leaf or stem tissues. In the leaf tissue with drought treatment, a slight activation of *CAChi2* gene was induced 4 days after wilting. In contrast, in the stem tissue, the *CAChi2* transcripts were detected 1 day after wilting and continuously increased up to 4 days after wilting (Hong and Hwang, 2002).

Promoter activation of pepper PR-1 protein and chitinase genes by pathogen infection. To gain an insight into the functional regulatory mechanisms of *CABPR1* and *CAChi2* gene expression, putative promoter regions of the two genes were isolated from pepper genome. A variety of stress-related or phytohormone-responsive *cis*-acting elements were revealed in the promoter sequences of *CABPR1* and *CAChi2* genes (Abe et al., 1997; Eulgem et al., 1999; Eyal et al., 1993; Shinozaki et al., 2003; Sutoh and Yamauchi, 2003). 5′-Deletion constructs of the *CABPR1* and *CAChi2* promoters fused to the β-glucuronidase (GUS) reporter gene were differentially activated during a bacterial pathogenesis, application of abiotic elicitors and environmental stresses (Hong and Hwang, 2005b; Hong et al., 2005).

GUS induction was detected in tobacco leaves harboring the -2162, -1670 and -1157 regions of the *CABPR1* promoter 24 HAI with *P. syringae* pv. *tabaci* (Fig. 4). The -2162 region of the *CABPR1* promoter directed a relatively low level of GUS activity, compared with the highest GUS activity in the tobacco leaves harboring the -1670 regions of the promoter. The GUS expression level was significantly lower in the tobacco leaves harboring the -1157 region of the promoter, but was abolished in the -603 and -369 regions of the promoter. This suggests that the minimal *cis*-regulatory sequences necessary for the molecular response to *P. syringae* pv. *tabaci* inoculation may be located in the

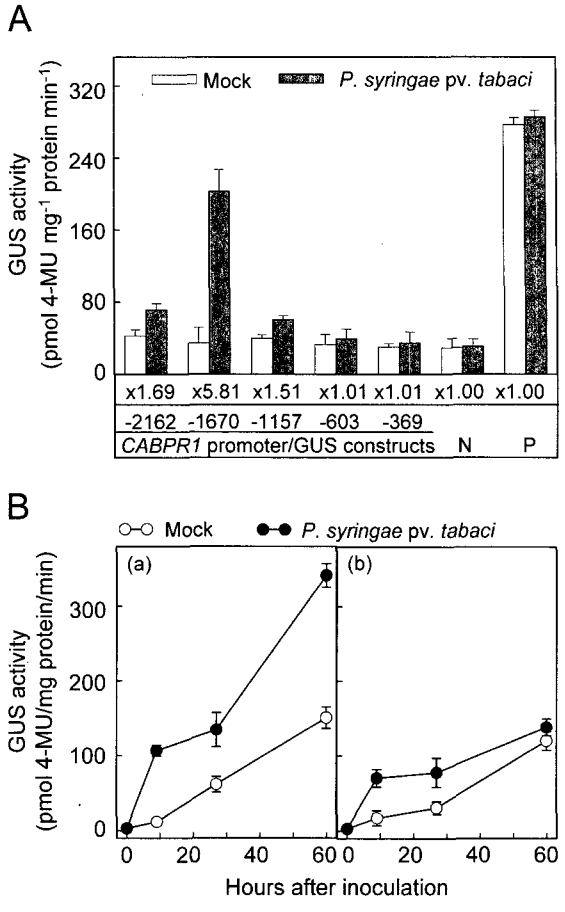

Fig. 4. GUS activity driven by the *CABPR1* promoters in the tobacco leaf tissues in response to a *Pseudomonas syringae* pv. *tabaci* inoculation. The GUS activity, which was analyzed fluorometrically, is expressed as pmole 4-methylumelliferone (MU)/mg protein/min. The averages of the GUS activity and the standard deviations of the experimental data are from independent assays of the extracts from tobacco leaf tissues. (**A**) Induction of GUS activity by the five 5′-*CABPR1* promoter deletion-GUS chimeric constructs. The tobacco leaves were infiltrated with either a bacterial suspension of *P. syringae* pv. *tabaci* (2×10^8 cfu/mL in 10 mM $MgCl_2$) or 10 mM $MgCl_2$ as mocks. The numbers under the bars indicate the fold induction of GUS activity after a pathogen infection versus a mock inoculation. N: negative control, P: positive control. (**B**) Time courses of (a) local and (b) systemic activation of the *CABPR1* (-1670) promoter by *P. syringae* pv. *tabaci* infection (Hong et al., 2005).

554-bp region between -1157 and -603 of the *CABPR1* promoter. The 513-bp region between -1670 and -1157 also was responsible for the drastic activation of the *CABPR1* promoter. Two W-boxes (TTGACC) were located within the 513-bp region between -1670 and -1157 and the 554-bp region between -1157 and -603 of the *CABPR1* promoter. The W-box is known to be a *cis*-acting element that can bind the WRKY transcription factors unique to plants, which are involved in the pathogen defense, senescence and

trichome development (Eulgem et al., 2000). The *CABPR1* promoter activity induced by *P. syringae* pv. *tabaci* inoculation was significantly reduced in the presence of the two W-boxes, suggesting that other *cis*-acting elements may be involved in *CABPR1* promoter activation by *P. syringae* pv. *tabaci* inoculation. The increase in GUS expression by the 5'-deletion derivative of -1670 bp provides evidence for the presence of negative regulatory sequences of the *CABPR1* promoter at the region between -2162 and -1670. The bacterial inoculation drastically induced local and systemic -1670::GUS expression in the inoculated leaves. Mock-inoculation also activated the *CABPR1* promoter activity in the tobacco leaves.

GUS activity was directed by a series of deleted *CAChi2* promoters in tobacco leaf tissues at 24 HAI with *P. syringae* pv. *tabaci*. Tobacco leaf tissues with constructs containing the promoter fragments -1365 bp and -878 bp exhibited 1.89- and 1.7-fold higher GUS activity in response to *P. syringae* pv. *tabaci* inoculation, as compared to the mock-inoculation levels. The -878 *CAChi2* promoter region proved sufficient to trigger the expression of *CAChi2-GUS* due to the bacterial inoculation. These findings indicate that the *cis*-regulatory sequences which confer responsiveness to *P. syringae* pv. *tabaci* inoculation reside in the 218-bp region located between -878 and -661 of the *CAChi2* promoter. GUS activity in the tobacco leaves containing the *CAChi2* promoter -878-GUS construct were temporally increased after inoculation of *P. syringae* pv. *tabaci* (Hong and Hwang, 2005b).

Promoter activation of pepper PR-1 protein and chitinase genes by abiotic elicitors. Abiotic elicitors and environmental stresses induced *CABPR1* and *CAChi2* gene expression in pepper leaves, which may result from activation of *CABPR1* and *CAChi2* promoter. We determined the effects of abiotic elicitors and environmental stresses on *CABPR1* and *CAChi2* promoter activation.

Treatment with ethylene induced GUS expression in the leaves harboring the -2162, -1670, -603 and -369 regions of the *CABPR1* promoter, but not in the leaves harboring the -1157 region of the promoter. Treatment with methyl jasmonate (MeJA) induced high GUS expression in the leaves harboring the -1670 and -1157 regions of the *CABPR1* promoter. After treatment for 48 h, salicylic acid (SA) induced the GUS expression driven by the three promoter regions of -1670, -1157 and -603 bp, whereas the full-length -2162 region and the shortest -369 region of the promoter did not respond to the SA treatment. The nitric oxide (NO)-induced GUS expression level was relatively high in the leaves harboring the -2162 and -1670 regions of the *CABPR1* promoter 24 h after sodium nitroprusside (SNP) treatment. However, the -1157 sequence of the *CABPR1* promoter was not sufficient for GUS induction by NO treatment. Differential GUS induction by the various environmental stresses was observed in the tobacco leaves harboring the -1670 region of the *CABPR1* promoter. A high salinity of 400 mM NaCl, a low temperature of 4°C and a mannitol-induced drought resulted in GUS activation in the treated leaves, whereas treatment with 1 mM $CuCl_2$ and mechanical wounding were ineffective in inducing GUS expression (Hong et al., 2005). These indicated that specific region is responsible for differential activation *CABPR1* promoter by a variety of biotic and abiotic elicitors.

Treatments with mannitol or NaCl for 24 h proved sufficient to trigger activation of GUS expression driven by the four *CAChi2* promoter constructs. Treatment with mannitol induced 5.32- and 5.95-fold increases in GUS activity in the tobacco leaf tissues harboring the -1365 and -878 promoter constructs, respectively. However, no significant activation of the GUS activity was driven by *CAChi2* promoter regions -661 and -378 in the tobacco leaves treated with mannitol. In contrast, the tobacco leaf tissues which harbored all constructs of the *CAChi2* promoter-GUS exhibited 2.43-4.56-fold increase in the induction of GUS activity 24 h after treatment with NaCl. Substantial dose-dependent increases in the -878 *CAChi2* promoter-GUS expressions were induced by mannitol and NaCl treatments (Hong and Hwang, 2005b).

Overexpression of pepper PR-1 protein, and chitinase increased disease resistance. To study the functional effects of the *CABPR1* and *CAChi2* genes on disease resistance and environmental stress tolerances *in vivo*, transgenic plants overexpressing *CABPR1* and *CAChi2* genes were generated and their tolerance to bacterial inoculation, and osmotic and oxidative stresses were analyzed.

The transgenic Arabidopsis plants overexpressing *CABPR1* exhibited enhanced resistance to *P. syrinage* pv. *tomato* (*Pst*) inoculation (Fig. 5A; Hong and Hwang, 2005). All four-week-old transgenic plants inoculated with the virulent bacterial strain were highly resistant, showing 100-fold greater retardation in bacterial multiplication, compared with that in the wild-type plants. The wild-type plants and transgenic plants containing pBIN35S empty vector showed progressive chlorotic symptoms on the inoculated leaves. However, no visible symptoms appeared on the leaves of the transgenic plants 5 DAI (Fig. 5B). The *CABPR1* gene was also introduced into tobacco plants, and disease responses of *CABPR1* transgenic plants were evaluated against *P. nicotianae,* tobacco black shank (Sarowar et al., 2005). Disease symptoms were visible within 3 DAI in both transgenic and non-transgenic tobacco

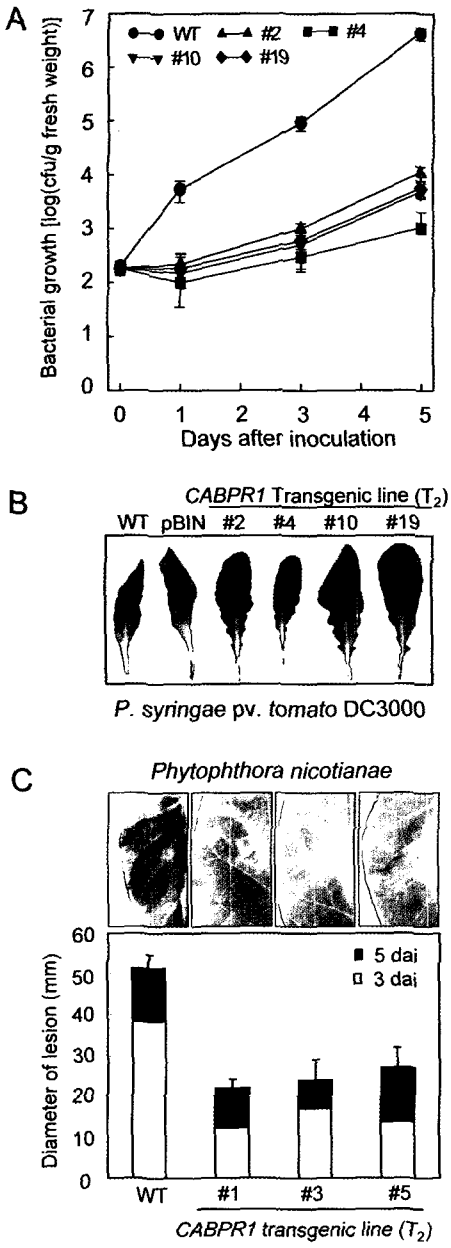

Fig. 5. Enhanced disease resistance of transgenic Arabidopsis and tobacco plants overexpressing pepper *CABPR1* gene. (**A**) Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 in wild-type and transgenic Arabidopsis plants. The data points are the mean bacterial cell numbers [$\log_{10}$(cfu/g fresh weight)]. Error bars indicated standard deviations. (**B**) Differential disease responses to *Pseudomonas syringae* pv. *tomato* strain DC3000 infection of the wild-type and transgenic Arabidopsis plants (#2, #4, #10 and #19). The photograph was taken 5 days after inoculation. WT, Col-0 wild-type plants; pBIN, Col-0 plants transformed with pBIN35S vector only (Hong and Hwang, 2005a). (**C**) Inhibition of disease development on transgenic plants infected by *Phytophthora nicotianae* KACC40906. Photos were taken 5 days after infection (upper panel) and data (size of lesion in millimeters) were recorded 3 and 5 days after inoculation (*dai*) of tobacco leaves (lower panel) (Sarowar et al., 2005).

plants. Symptom development was significantly delayed in transgenic tobacco lines compared to wild type plants. Five DAI, symptom development was found to be significantly retarded in the transgenic plants (Fig. 5C), indicating that ectopic expression of *CABPR1* in tobacco plants enhanced resistance to *P. nicotianae*. Typical bacterial wilt symptoms were observed on tobacco leaves inoculated with *R. solanacearum* and *P. syringae* pv. *tabaci*. Bacterial growths from the transgenic plants were significantly lower than those recovered from the lesions of wild type plants. All three transgenic plants tested showed significantly higher resistance against *P. nicotianae, R. solanacearum* and *P. syringae* pv. *tabaci*. To determine whether or not the constitutive expression of the *CAChi2* gene induced disease resistance, wild type and transgenic Arabidopsis plants were inoculated with the virulent strain DC3000 of *P. syringae* pv. *tomato*. There was a 4.5-8.0-fold more reduction of bacterial growth in the infected transgenic plants 2 DAI than that in the wild type plants. However, no drastic increase in disease resistance was observed in the transgenic plants 4 DAI (Hong and Hwang, 2005b). Direct antimicrobial activity was observed in the purified PR-1 proteins and chitinases from the tomato and broad bean (Kim and Hwang, 1996; Niderman et al., 1995; Rauscher et al., 1999). However, direct *in vitro* anti-fungal activity *in vitro* against *F. solani* has been identified only found in the specific tobacco chitinase isoform (Sela-Buurlage et al., 1993). Further studies will be needed to better understand the functional roles of the CABPR1 and CAChi2 proteins in the enhanced disease resistance in plants.

Overexpression of pepper PR-1 protein, and chitinase increased tolerance to environmental stresses. To determine if the *CABPR1* transgenic Arabidopsis plants exhibited enhanced salt tolerance during early seedling development, the seeds from the four independent T_2 lines were germinated on the MS media containing 250 mM NaCl. No significant differences in seed germination were observed between the wild-type and transgenic plants in the basal MS medium without the addition of NaCl (Hong and Hwang, 2005b). The NaCl treatment strongly inhibited germination in the wild-type plants, but the transgenic plants were much less sensitive to high concentration of NaCl. None of the wild-type seeds germinate in 250 mM NaCl for 10 days after sowing, whereas 20-35% of the seeds from the transgenic plants germinated. The seedlings of transgenic plants also exhibited a 40-55% reduction in their chlorophyll content after 150 mM NaCl treatment. However, wild-type plans showed a drastic reduction in chlorophyll content after treatment with 150 mM NaCl. Treatment with 600 mM mannitol significantly inhibited the seed germination of wild-type plants, but did not

markedly affect the germination of all the transgenic Arabidopsis plants. However, the seedling of transgenic plants did not show any tolerance to the mannitol treatment. MV, which is an herbicide that elevates the cellular ROS levels by inhibiting photosynthesis and photorespiration, was used to evaluate the effects of oxidative stresses on the tolerance levels of the transgenic Arabidopsis plants to oxidative stress (Hong and Hwang, 2005a). The *CABPR1*-overexpressing transgenic plants were more tolerant to the MV-mediated oxidative stress than the wild-type plants in all plant developmental stages, including germination, early seedling development and mature plants, some wild-type plants became bleached and died. In the presence of MV, the fresh weight and chlorophyll content was higher in the transgenic plants than in the wild-type plants. Treatment with 0.5 μM MV caused necrotic and bleached lesions on the leaves of the wild-type plants, but had little or no effect on the transgenic plants (Hong and Hwang, 2005a). Pathogen inoculation triggered oxidative burst and induced expression of antioxidant enzymes in various plant species (Apel and Hirt, 2004; Kuzniak and Sklodowska, 2005). The defensive role of CABPR1 protein against oxidative stresses may also contribute to the increased resistance of the transgenic plants to a *Pst* inoculation.

Transgenic tobacco plants overexpressing *CABPR1* were evaluated for heavy metal tolerance by germinating seeds on medium containing $CdCl_2$ or $HgCl_2$. The *CABPR1* transgenic tobacco plants were found to be tolerant to cadmium and mercury, whereas germination of control plants was severely inhibited by all treatments. At 250 μM $CdCl_2$, the transgenic plants produced well-developed broad leaves and regular roots with normal lateral roots, whereas control plants developed spindly shoots and relatively few lateral roots. Roots of the transgenic plants were approximately 10-40% longer than those of control plants at all four $CdCl_2$ concentrations. In particular, at 100 μM $HgCl_2$, the transgenic plants produced well-developed root hairs on root surfaces as compared to the control plants. The tolerance to heavy metal in the adult stage was observed when 30-day-old *in vitro* grown shoots were sub-cultured on medium containing 100 μM $HgCl_2$ (Sarowar et al., 2005). The results suggest that enhanced resistance to heavy metals may be induced by overexpression of *CABPR1* gene in tobacco plants (Mithofer et al., 2004). Interestingly, *CABPR1* overexpression in transgenic tobacco plants was found to confer partial resistance to heavy metal toxicity *in vitro*, but not to high concentration of salt and non-ionic osmotic stresses, suggesting that CABPR1 protein have potentially functional to play roles in stress tolerances differently in various plant species.

The germination of *CAChi2*-overexpressing plants was compared with that of wild type plants under high concentration of NaCl. Treatment with high concentrations of NaCl did not significantly inhibit the germination of transgenic plant seeds. The *CAChi2*-overexpressing plants exhibited enhanced tolerance to NaCl stress at the seedling stage. Seedlings of transgenic plants were markedly tolerant to higher concentration of NaCl. The fresh weight, chlorophyll content and root growth of the seedling of transgenic plants grown at 150 and 200 mM NaCl were significantly higher than those of the wild type plants. The mature leaves of the *CAChi2*-overexpressing plants were also more tolerant to high concentrations of NaCl relative to wild type leaves (Hong and Hwang, 2005b). After treatment with 300 mM NaCl for 5 days, severe bleaching of the leaves as the result of chlorophyll degradation was observed in the wild type plants. *CAChi2* gene may be useful genetic materials for improving salt tolerance of crops (Apse and Blumwald, 2002).

Abscisic acid (ABA) is a phytohormone involved in diverse physiological processes including osmotic stress responses in seeds and seedlings (Finkelstein et al., 2002). To examine whether or not the transgenic Arabidopsis plants were sensitive to ABA, the seeds of transgenic lines were germinated on the MS media containing 1 mM and 2 mM of ABA. Two mM ABA was sufficient to inhibit the germination of the *CABPR1* transgenic plants by 40% or more, whereas the wild-type plants retained 85% germination under the same conditions. Germination of transgenic plant seeds was strongly inhibited, as compared to that of the wild type seeds. Overexpression of *CABPR1* and *CAChi2* genes in the *Arabidopsis* plants altered ABA response during seed germination (Hong and Hwang, 2005a, b).

Concluding remarks. Pepper genes encoding PR-1 protein, β-1,3-glucanase and chitinase, were differentially activated and regulated in pepper plants against pathogen inoculation and environmental stresses. Overexpressing the *CABPR1* and *CAChi2* genes in transgenic plants conferred enhanced resistance to bacterial inoculation as well as to higher tolerance to osmotic stresses and oxidative stress. This suggests that CABPR1, CABGLU and CAChi2 proteins may play important roles as defense proteins against biotic and/or abiotic stresses. More precisely defensive mechanisms driven by CABPR1 and CAChi2 proteins in the plant cell under stress conditions remains elucidated. Increasing knowledge on the regulation of PR-gene promoters and mode-of-actions of PR-proteins during enhanced tolerance to biotic and abiotic stresses in plants will lead to novel and integrated approaches toward engineering disease resistance and environmental stress tolerances of economic plants, including pepper plants.

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