MPMI Vol. 24, No. 6, 2011, pp. 671–684. doi:10.1094/MPMI-08-10-0183. © 2011 The American Phytopathological Society

e-Xtra*

Use of a Secretion Trap Screen in Pepper Following *Phytophthora capsici* Infection Reveals Novel Functions of Secreted Plant Proteins in Modulating Cell Death

Seon-In Yeom,[1] Hyang-Ku Baek,[1] Sang-Keun Oh,[1] Won-Hee Kang,[1] Sang Jik Lee,[2] Je Min Lee,[1] Eunyoung Seo,[1] Jocelyn K. C. Rose,[2] Byung-Dong Kim,[1] and Doil Choi[1]

[1]Department of Plant Science, Plant Genomics and Breeding Institute, Seoul National University, Seoul, 151-921, Republic of Korea; [2]Department of Plant Biology, Cornell University, Ithaca, NY 14853, U.S.A.

Submitted 11 August 2010. Accepted 22 January 2011.

In plants, the primary defense against pathogens is mostly inducible and associated with cell wall modification and defense-related gene expression, including many secreted proteins. To study the role of secreted proteins, a yeast-based signal-sequence trap screening was conducted with the RNA from *Phytophthora capsici*-inoculated root of *Capsicum annuum* 'Criollo de Morelos 334' (CM334). In total, 101 *Capsicum annuum* secretome (CaS) clones were isolated and identified, of which 92 were predicted to have a secretory signal sequence at their N-terminus. To identify differences in expressed CaS genes between resistant and susceptible cultivars of pepper, reverse Northern blots and real-time reverse-transcription polymerase chain reaction were performed with RNA samples isolated at different time points following *P. capsici* inoculation. In an attempt to assign biological functions to CaS genes, we performed in planta knock-down assays using the *Tobacco rattle virus*-based gene-silencing method. Silencing of eight CaS genes in pepper resulted in suppression of the cell death induced by the non-host bacterial pathogen (*Pseudomonas syringae* pv. *tomato* T1). Three CaS genes induced phenotypic abnormalities in silenced plants and one, *CaS259* (PR4-l), caused both cell death suppression and perturbed phenotypes. These results provide evidence that the CaS genes may play important roles in pathogen defense as well as developmental processes.

In plants, one of the fiercest battlefields of plant–microbe interactions is the apoplastic milieu, which includes the cell wall matrix and associated extracellular environment (Hugot et al. 2004; Schulze-Lefert 2004). Here, secreted protein populations, or "secretomes", play a crucial role in the numerous complex defense responses that are mounted against pathogens, (Birch et al. 2006; Kamoun 2006; Lee et al. 2004, 2006a). During early stages of pathogen infection, plants recognize a pathogen-secreted elicitor, triggering defense responses through signaling pathways. Secreted proteins are known to be associated with cell wall thickening, to resist penetration, through cell polarization and papilla formation at the site of pathogen attack (Schmelzer 2002; Schulze-Lefert 2004). Secreted proteins, such as the classically defined pathogenesis-related (PR) proteins, contribute to the establishment of resistance and systemic acquired resistance in plants (Jones and Takemoto 2004; Stinzi et al. 1993; Wang et al. 2005).

The oomycete *Phytophthora capsici* is a soilborne pathogen of critical food crops, causing root and fruit rot of pepper, tomato, and other solanaceous or cucurbitaceous plants (Hausbeck and Lamour 2004; Walker and Bosland 1999). Recently, the incidence and infection of *P. capsici* has increased, leading to severe economic losses for these crops worldwide (Ristaino and Johnston 1999). To date, no useful management methods have been developed, because chemical and biological controls are limited and ineffective in preventing the spread of *P. capsici* to pepper crops (Oelke et al. 2003). Although many studies have reported that the resistance to *P. capsici* is polygenic and is controlled by quantitative trait loci (Kim et al. 2008; Lefebvre and Palloix 1996; Thabuis et al. 2003), little is known about pepper–oomycete interactions at the molecular and genetic levels. Furthermore, most genetic and molecular studies have focused on *P. infestans*– and *P. sojae*–host interactions (Birch et al. 2006; Kamoun 2006; Moy et al. 2004; Tyler 2007).

The defense response in plants is associated with a change in the repertoire of secreted proteins in response to *Phytophthora* spp. infection (Hugot et al. 2004; Lee et al. 2006a; Mithofer et al. 2002). The pepper–*P. capsici* interaction in resistant plants also occurs as an intercellular rather than intracellular response. For example, growth of *P. capsici* in resistant pepper is restricted to the extracellular environment through cell wall apposition related to secreted proteins; therefore, host cells are not severely damaged. In contrast, in susceptible pepper, *P. capsici* shows rapid intra- and intercellular growth, cell walls are degraded, and cells became plasmolyzed (Hwang et al. 1989; Ilarslan et al. 1996; Lee et al. 2000). A better understanding of defense mechanisms and, in this case, extracellular mechanisms may provide new insights into the interactions between *P. capsici* and pepper.

Because of the importance of secretory proteins, many researchers have developed high-throughput screening methods for their detection (Cutler et al. 2000; Jacobs et al. 1997; Tashiro et al. 1993). Among these, a yeast-based secretion trap

Current address for J. M. Lee: Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, U.S.A.

Corresponding author: D. Choi; Telephone: +1 82-2-880-4568; Fax: +1 82-2-873-2056; E-mail: doil@snu.ac.kr

*The *e*-Xtra logo stands for "electronic extra" and indicates that six supplementary figures and three supplementary tables are published online. Also, Figures 1, 2, and 6 appear in color online.

(YST), using invertase as the reporter gene, has been successfully used for screening the plant secretome (Goo et al. 1999; Klein et al. 1996; Jacobs et al. 1997; Yamane et al. 2005) and for mining secretory proteins related to host–pathogen interactions (Hugot et al. 2004; Lee et al. 2006a; Oh et al. 2005). This technique involves ligating a plant-derived cDNA library into a YST vector fused to the invertase gene (*Suc2*) lacking the N-terminal signal peptide. The heterologous cDNA library is transformed into an invertase-deficient yeast mutant. Any yeast transfected with cDNA encoding a secreted protein could secrete an invertase-fused protein, resulting in growth on medium containing sucrose as a sole carbon source. Rescued mutant yeast transformants are isolated and the genes encoding the secreted proteins can then be identified.

The purpose of this study was to characterize secreted proteins related to the defense response from a pathogen-resistant cultivar of pepper following inoculation with *P. capsici*. We isolated 101 *Capsicum annuum* genes encoding secreted proteins using the YST screen and compared the transcript levels in resistant and susceptible pepper cultivars at different time points following *P. capsici* infection. In addition, *Tobacco rattle virus* (TRV)-based gene silencing assays were performed to help determine the role of the secreted proteins in plant defense and development. Our study will facilitate a better understanding of the molecular functions of those secreted proteins that modulate the host defense response in plants.

RESULTS

Confirmation of compatible and incompatible interactions of pepper against *P. capsici*.

To better understand the spectrum of extracellular defense responses against pathogen attack, we attempted to isolate genes encoding secreted proteins modulated during cell death due to infection by an oomycete pathogen. Specifically, we inoculated the roots of *C. annuum* 'Criollo de Morelos 334' (CM334) and 'Chilsungcho', which are cultivars that are resistant and susceptible, respectively, to *P. capsici*, with *P. capsici* zoospores. Disease symptoms (root rot) were observed in Chilsungcho but not in CM334 within 72 h postinoculation (hpi) (Fig. 1A). To validate these symptoms, we performed a 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay as a cell vitality indicator (Chen et al. 2006). Although no differences were observed above ground during infection until 72 hpi, the vitality of *P. capsici*-infected roots changed dramatically between the two pepper cultivars (Fig. 1B). Differences in TTC reductase activity in the root of both cultivars were observed at 3 hpi but were significant from 24 to 48 hpi, showing two or three times more activity in CM334 than in Chilsungcho.

We also determined biomass of *P. capsici* hyphae in the infected pepper roots and the expression of PR proteins as positive markers for *P. capsici* infection (Silvar et al. 2008). During infection, transcript levels of the *P. capsici elongation factor 1α* (*PcEF1α*) gene were significantly lower in roots of CM334 than in those of Chilsungcho (Fig. 1C). *CaPINII* and *CaBPR1* transcripts were more strongly and rapidly induced in the resistant than the susceptible cultivar (Fig. 1D), confirming that the two cultivars exhibit compatible and incompatible interactions, respectively, with *P. capsici* (Hwang et al. 1989; Ueeda et al. 2006). Root samples were harvested at various time points after inoculation of *P. capsici* and total RNA extracts were isolated for further study.

Isolation of *C. annuum* secretome using YST.

To understand the extracellular events during the defense response, a cDNA library was constructed in pYST 0-2 vector (Lee et al. 2006a,b) using pools of mRNA (3, 6, 12, 24, 48, and 72 hpi) from CM334 pepper roots infected with *P. capsici.* An invertase-deficient yeast strain (DBYα2445) was transformed with the pepper cDNA library constructed in pYST 0-2 vector and plasmid DNA was isolated from all colonies that grew on yeast-peptone-sucrose (YP-Suc) media. To avoid selection of overlapping clones, the cDNAs selected through the first screen were then used to identify redundant clones by Southern blot analysis using amplified inserts as probes, in an iterative step. In total, 600 yeast transformants were selected from the YST screen and we identified 101 unique *Capsicum annuum* secretome (CaS) genes, which we used for further study (Table 1).

The CaS cDNAs were sequenced and the deduced amino acid sequences were analyzed with the SignalP 3.0 (Bendtsen et al. 2004), TargetP (Emanuelsson et al. 2007), and PSORT programs (Nakai and Kanehisa 1992) to confirm the presence of secretory signal peptide and to predict subcellular localization, respectively. Of these, 92 CaS clones were predicted to encode proteins with signal peptides or signal anchors that indicate targeting to the secretory pathway or apoplast by at least one prediction program. The other nine CaS clones were predicted to encode proteins with no signal sequence or to localize to microbodies, the cytosol, or nucleus (Table 1).

Functional classification of CaS genes.

All CaS clones were used to search the GenBank nonredundant public sequence database (Table 1) for similarities to known proteins using the BLASTX program and were classified into eight functional groups (Fig. 2).

Members of the largest group (27%) were annotated as hypothetical proteins or proteins of unknown function in that they showed no or low sequence similarities to any protein with characterized functions. Twenty-six CaS genes (26%) shared high similarities with defense- or stress-related proteins. Several members of PR protein and antifungal protein families were included in this group, which were known to play important roles in biotic or abiotic stresses (Kim et al. 2002; Oh et al. 1999). These included a chitinase (Hong and Hwang 2002), a germin-like protein (Park et al. 2004a), and a ribonuclease (Park et al. 2004b), which were previously characterized as stress-induced genes in pepper. Thus, these proteins likely have roles in apoplastic defense mechanisms.

Approximately 13% of the CaS genes were classified as proteases or protease inhibitors, and included members of the cysteine protease, subtilisin-like protease, and aspartyl protease families. Protease/protease inhibitors have been suggested to play a role in defense and the immune response against biotic stress (Dunaevskii Ia et al. 2005; van der Hoorn 2008); however, their specific modes of action and substrates in the interaction with pathogens are not well understood. A further 14% of the clones were predicted to encode cell wall structural proteins, including proline-rich proteins, glycine-rich proteins, extensin, cell adhesion protein, and U-rim protein. Another group of CaS genes (4%) belonged to a general category of development and growth-related proteins, which includes organ-specific growth protein, ripening-related protein, and expansin-like protein. The remaining CaS clones (12%) were classified in the metabolism group, which includes proteins that contribute to the nutrient reservoir hydrolase, glutamine cyclotransferase, electron transporters, tonoplast intrinsic protein, and NADH dehydrogenase. These results suggested that the isolated CaS genes collectively contribute to both pathogen defense and the normal range of plant developmental process.

Expression of CaS genes in resistant and susceptible plants following *P. capsici* infection.

To address the molecular functions of CaS genes, we performed a reverse Northern blot (dot-blot) assay using RNA

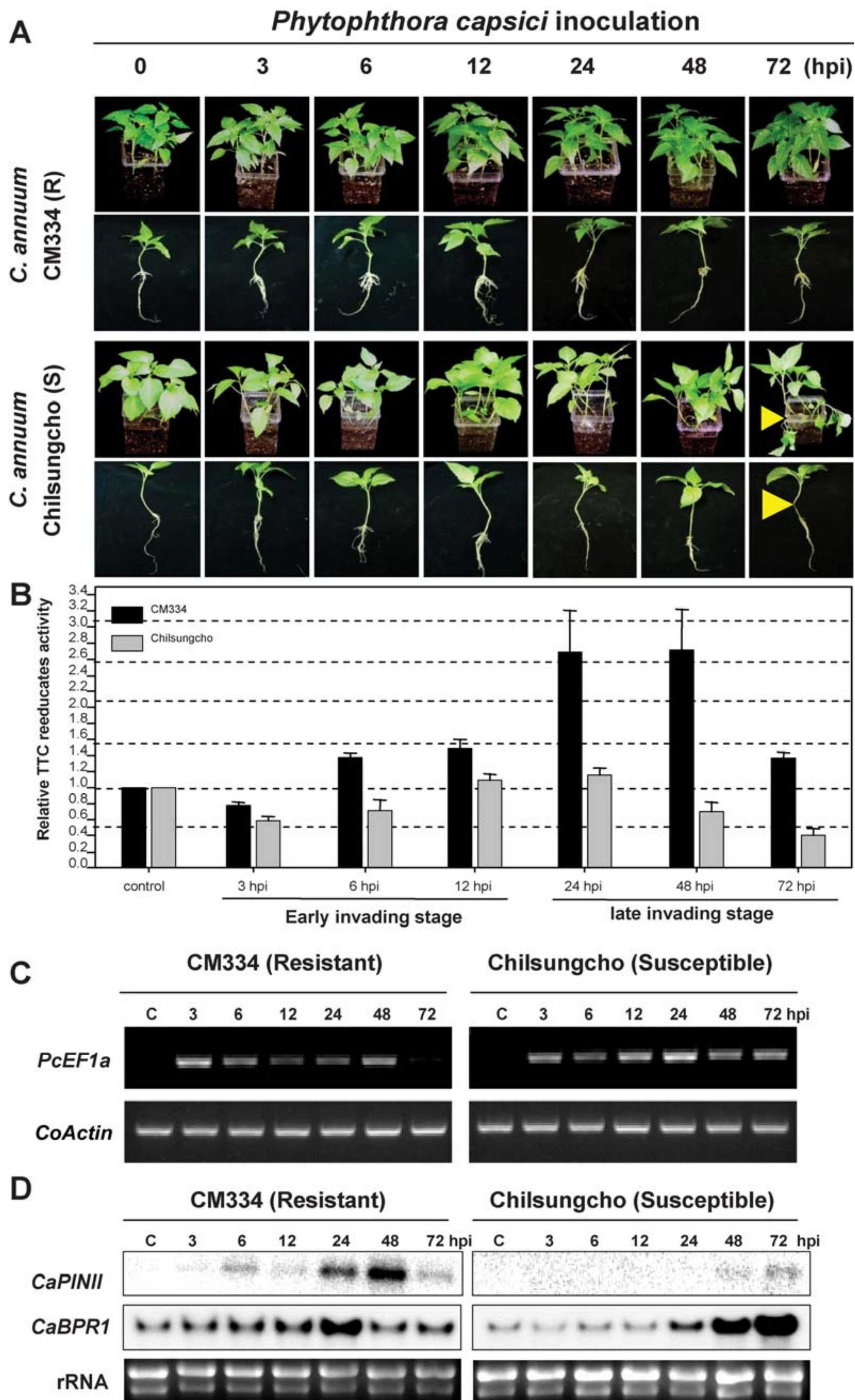

Fig. 1. Phenotype in resistant (*Capsicum annuum* CM334) and susceptible pepper (*C. annuum* Chilsungcho) roots. **A,** Symptoms observed at 72 h postinoculation (hpi) in Chilsungcho (susceptible, S) after drenching inoculation with zoospores of *Phytophthora capsici* (2×10^5 zoospores/ml). **B,** 2,3,5-Triphenyltetrazolium chloride (TTC) reductase activity was also measured in resistant and susceptible pepper roots as cell vitality indicator. TTC reductase activity was normalized to that of noninfected root. Values are the means ± standard deviation. **C,** Biomass of *P. capsici* in CM334 and Chilsungcho was assessed by the expression level of *P. capsici elongation factor 1α* (*PcEF1α*) gene. *CoActin* was used as control. **D,** Expression of pathogenesis-related (PR) genes during *P. capsici* infection in resistant and susceptible pepper cultivars. The *CaBPR1* and *CaPINII* genes were used to confirm the activation of defense-related genes.

probes from different time points of infection (0, 3, 6, 12, 24, 48, and 72 hpi). Based on this analysis, altered expression patterns were observed for 68 CaS genes relative to uninfected controls. The differential expression patterns of CaS mRNAs in resistant and susceptible cultivars of pepper following P. capsici infection are shown in Table 1 and Supplementary Table S1.

Of the 68 CaS genes, 35 were induced in both resistant and susceptible plant interactions. Although these 35 CaS genes showed similar expression patterns, 19 CaS genes showed earlier and higher levels of expression in the resistant than in the susceptible cultivar (Table 1). Conversely, 14 CaS genes showed earlier and higher levels of expression in the susceptible cultivar

Table 1. Yeast secretion trap clones isolated from *Phytophthora capsici*-infected pepper roots

		Database annotation			SignalP 3.0[a]		Predicted localization	
Classification[b]	Pattern[c]	Protein name	Accession[d]	E value[e]	HMM	NN	TargetP[f]	PSOPT[g]
Defense								
CaS1	R+	Ribonuclease T2	ABB73003	2.0E-11	1	0.907	S	Outside
CaS2	IB_R+	Peroxidase	AAL35364	6.0E-116	0.165	0.668	S	PM
CaS4	R+	*Tobacco mosaic virus* (TMV)-induced protein I	AAF63515	1.0E-76	0.934	0.863	S	Outside
CaS8	IB_R+	Class IV chitinase	BAF44533	8.0E-12	0.992	0.822	S	Outside
CaS12	nc	Protein disulfide-isomerase	Q9XF61	9.0E-28	0.997	0.825	S	Outside
CaS17	R+	TMV-induced protein 1-2	AAO49266	7.0E-19	0.987	0.883	S	Outside
CaS22	IB_R+	Putative peroxidase	CAC42086	2.0E-42	1	0.786	S	Outside
CaS25	–	Peroxidase	AAA65637	2.0E-54	0.984	0.906	S	PM
CaS30	R+	Antifungal protein	AAL73184	1.0E-30	1	0.932	S	PM
CaS54	R+	Pathogenesis-related protein osmotin	AAC64171	3.0E-19	0.983	0.925	S	Outside
CaS57	nc	Germin-like protein	NP_198731	6.0E-34	0.972	0.857	S	PM
CaS135	IB_R+	class1 chitinase	BAC81645	5.0E-137	0.989	0.938	S	PM
CaS160	IB_S+	Allergen-like protein BRSn20	AAF16869	1.0E-13	1	0.859	S	Outside
CaS168	IB_R+	SAR8.2A	AAX20029	4.0E-15	0.971	0.889	S	Outside
CaS175	nc	Chitinase	CAA78844	5.0E-89	1	0.928	S	Outside
CaS182	R+	Putative defensin AMP1 protein	AAL36289	3.0E-03	0.997	0.877	S	Outside
CaS220	IB_R+	Defensin protein	AAL35366	2.0E-07	0.985	0.873	S	Outside
CaS221	IB_R+	Wound-induced protein CBP1	AAF18934	7.0E-37	0.999	0.946	S	Outside
CaS259	IB_R+	Pathogenesis-related protein P2	CAA41439	9.0E-36	0.992	0.826	S	Outside
CaS290	IB+	Pleiotropic drug resistance like protein	BAB92011	4.0E-72	0.030[(SA)]	0.551	S	ER
CaS359	IB_R+	Putative peroxidase	cacn37	1.0E-102	1	0.905	S	Outside
CaS388	IB+	Pathogenesis-related protein 4b	BAD11073	1.0E-06	0.997	0.915	S	Outside
CaS390	IB_R+	Putative gamma-thionin precursor	AAD21200	3.0E-32	0.996	0.924	S	Outside
CaS504	R+	CPRD2	BAB33033	9.0E-72	0.933	1	S	Outside
CaS507	R+	Pathogenesis-related protein PR-1	AAK30143	2.0E-75	0.840	0.999	S	Outside
CaS547	nc	Thioredoxin	AAR83852	1.0E-16	0.945	0.516	*	ER
Protease								
CaS102	R+	Cathepsin B-like cysteine	AAR25800	3.0E-72	0.994	0.905	S	Outside
CaS279	nc	Aspartyl protease family protein	NP_191467	3.0E-22	0.998	0.929	S	Outside
CaS450	nc	Matrix metalloprotease 1	ABF58910	2.0E-177	0.002[(SA)]	0.118	*	PM
CaS457	IB_S+	Putative subtilisin-like proteinase	XP_482712	7.0E-05	0.999	0.852	S	Outside
CaS13	R+	Putative proteinase inhibitor II	AAF25496	4.0E-48	0.985	0.904	S	Outside
CaS40	R+	Metallocarboxypeptidase inhibitor	AAZ94183	3.2E-01	0.81	0.743	S	Outside
CaS64	–	Putative invertase inhibitor	AAX63191	1.0E-22	0.507	0.435	C	ER
CaS153	IB_S+	Putative miraculin	CAC40756	5.0E-17	0.999	0.77	S	Outside
CaS333	R+	Ethylene-responsive proteinase inhibitor 1	AAA60745	6.0E-13	1	0.935	S	Outside
CaS360	IB_R+	Cystatin	AAR92224	2.0E-03	0.983	0.782	S	Outside
CaS439	nc	Opening-related protein-like	AAM62643	6.0E-21	0.984	0.786	S	ER
CaS475	IB_S+	Trypsin proteinase inhibitor precursor	ABA42905	3.0E-04	0.995	0.798	S	Outside
CaS551	IB_S+	Putative kunitz-type proteinase inhibitor	AF492358_1	2.0E-47	0.949	0.998	S	Outside
Cell wall								
CaS3	R+	Glycine-rich protein Tfm5	X95262	4.0E-52(n)	0.998	0.862	S	Outside
CaS16	R+	Cell wall protein 3	CAJ13710	4.0E-08	0.997	0.918	S	Outside
CaS58	R+	Fasciclin-like AGP 12	AAT37955	8.0E-61	0.997	0.884	S	Outside
CaS67	–	Extensin	Z21937	2E-100(n)	0.999	0.851	S	Outside
CaS71	nc	Putative membrane protein	CAC37357	3.0E-15	0.998	0.907	S	Outside

(*continued on next page*)

[a] Signal peptide was predicted by SignalP3.0. (SA) = signal anchor and no predicted signal peptide was indicated scores as bold. HMM and NN = hidden Markov model and neural network methods, respectively.

[b] Classification abbreviations: Defense = defense- or resistance-related proteins, Protease = protease or protease inhibitor, Cell wall = cell wall structure protein, Growth = development or growth-related protein, and Hypothetical = hypothetical protein or unknown. *Capsicum annuum* secretome (CaS) names = *C. annuum* 'CM334"–*Phytophthora capsici* interaction secretome.

[c] Expression pattern of each gene by dot-blot assay. IB_R+ = induced in both pepper cultivars (resistant and susceptible) but earlier and higher expression in resistant pepper; IB-S+ = induced in both cultivars but earlier and higher expression in susceptible pepper; IB+ = similar expression in both cultivars; R+ = induced in resistant pepper but no change or reduced in susceptible pepper; – = repressed in both; nc = transcripts >0.5-fold and <2-fold or no changes in expression profiling.

[d] Pepper expressed sequence tag database: cacn or caKS accession, Gene Pool; SGN accession, Sol Genomic Network.

[e] E value from protein blast or blastx; (n) = nucleotide blast.

[f] Prediction of localization by TargetP program. S = secretory pathway, C = chloroplast, – = any other localization, and * = don't know. In this analysis, specificity >0.95 (predefined set of cutoffs that yielded this specificity on the TargetP test sets).

[g] Sublocalization by PSORT program. Outside = cell wall or extracellular space, N = nucleus, Cyto = cytoplasm, PM = plasma membrane, ER = endoreticulum, Mt = mitochondrion, Mb = microbody (peroxisome), Too short = amino acid too short for prediction.

while 2 CaS genes showed similar expression in both cultivars. On the other hand, 27 CaS genes were upregulated in the resistant cultivar but showed no significant changes in the susceptible cultivar. Expression levels of six CaS genes were reduced in both cultivars, compared with the controls (Table 1). The remaining 33 CaS genes showed no significant expression level changes in this comparison.

For more precise monitoring of expression levels of CaS genes between resistant and susceptible cultivars, we selected only the differentially expressed CaS genes for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analyses. Based on representative qRT-PCR, gene expression patterns could be divided into four groups: the first showed early induction before the onset of the hypersensitive

Table 1. (*continued from preceding page*)

		Database annotation			SignalP 3.0[a]		Predicted localization	
Classification[b]	Pattern[c]	Protein name	Accession[d]	E value[e]	HMM	NN	TargetP[f]	PSOPT[g]
CaS81	–	Glycine-rich protein 2	CAA42622	6.0E-26	0.260[(SA)]	0.679	S	Outside
CaS100	R+	Cell wall protein	AAF63514	5.0E-41	0.995	0.907	S	Outside
CaS114	nc	Extensin-like protein	Z46675	7E-46(n)	0	0.178	*	Too short
CaS171	R+	Glycine-rich protein TomR2	AAP83840	3.0E-10	0.996	0.904	S	Outside
CaS203	–	Putative proline-rich protein	AAC49600	5.0E-04	0.995	0.847	S	Outside
CaS216	IB_S+	Extensin	NP_568813	4.0E-16	0.939	0.757	S	ER
CaS236	IB_R+	EXPANSIN-LIKE B1	NP_193436	1.0E-21	0.984	0.891	S	Outside
CaS283	nc	U-Lim protein	AAR83883	3.0E-15	0.998	0.921	S	Outside
CaS319	IB_R+	Fiber protein Fb34	AAR07596	2.0E-13	0.939	0.838	S	PM
Metabolism								
CaS20	nc	Arabinosidase ARA-1	AAL18931	7.0E-24	0.997	0.807	S	Outside
CaS23	IB_R+	Putative high-affinity nitrate transporter	AAF00053	4.0E-54	0.002	0.099	–	ER
CaS44	IB_S+	ARIADNE-like protein	AAR96008	6.E-05	0.006	0.519	*	ER
CaS76	nc	Aquaglyceroporin	CAB40742	2.0E-25	0.413[(SA)]	0.48	S	PM
CaS97	IB_S+	Dihydropyrimidinase/ hydrolase	NP_568258	2.0E-87	0.997	0.811	S	Outside
CaS111	IB_S+	NADH dehydrogenase subunit 5	ABD36074	5.0E-32	0.989	0.84	S	PM
CaS245	IB_S+	Electron transporter	NP_568926	6.0E-64	1	0.811	S	Outside
CaS253	nc	Vacuolar sorting receptor 6	Q9FYH7	2.0E-88	0.791	0.954	S	Outside
CaS256	R+	Glutamine cyclotransferase like	ABB86263	4.0E-17	0.995	0.822	S	Outside
CaS257	R+	Copper ion binding / electron transporter	NP_563820	7.0E-23	0.964	0.928	S	Outside
CaS336	nc	Protein carrier	NP_564256.	5.0E-37	0.904	0.879	S	Outside
CaS389	IB_R+	Hydrolase	NP_177929	3.0E-44	0.988	0.858	*	Outside
Signal								
CaS10	nc	Receptor like kinase	NP_198716	2.0E-46	0.999	0.789	S	Outside
CaS70	nc	Kinase	NP_849788	9.0E-02	0.998	0.756	S	PM
CaS88	nc	Receptor protein kinase-like protein	CAB81062	7.0E-21	0.129	0.517	S	PM
Growth								
CaS5	IB_R+	FH protein NFH1	AAF24496	3.5E-02	0.998	0.916	S	Outside
CaS75	–	Putative ROX1	ABH01084	1.0E-28	0.999	0.85	S	Outside
CaS404	nc	Organ-specific protein P4	P17771	1.0E-04	1	0.91	S	Outside
CaS502	nc	Putative auxin-independent growth promoter	BAD69015.	8.0E-13	0.416[(SA)]	0.869	*	PM
Others								
CaS200	nc	Retrotransposon protein	None	None	0.213	0.135	*	Mb
CaS209	nc	Ribosomal protein PETRP	AAR83848	2.0E-63	0.024	0	*	Cyto
Hypothetical								
CaS11	IB_S+	Hypothetical protein	None	None	0.989	0.814	S	Outside
CaS89	nc	Hypothetical protein	None	None	0.000[(SA)]	0	S	PM
CaS113	IB_R+	Hypothetical protein	None	None	0.997	0.874	S	Outside
CaS148	R+	Hypothetical protein	None	None	0.999	0.758	S	Outside
CaS150	R+	Hypothetical protein	None	None	0.187[(SA)]	0.479	S	Mt
CaS162	IB_R+	Hypothetical protein	None	None	0.801	0.911	S	Outside
CaS163	nc	Hypothetical protein	None	None	0.005[(SA)]	0.223	–	ER
CaS194	Nc	Hypothetical protein	None	None	0.999	0.805	S	Outside
CaS202	nc	Hypothetical protein	None	None	0.998	0.885	S	Outside
CaS238	R+	Hypothetical protein	None	None	1	0.892	S	Outside
CaS260	nc	Hypothetical protein	None	None	0.083	0	C	Cyto
CaS270	IB_S+	Hypothetical protein	None	None	0.903	0.992	S	Outside
CaS284	R+	Hypothetical protein	None	None	0.999	0.929	S	Outside
CaS369	nc	Hypothetical protein	None	None	0	0.342	*	Mb
CaS411	R+	Hypothetical protein	None	None	0.001	0.468	S	Cyto
CaS431	nc	Hypothetical protein	caKS15062F02	6.0E-75	0	0.377	*	Too short
CaS449	nc	Hypothetical protein	None	None	0.867	0.838	S	Outside
CaS463	nc	Hypothetical protein	EAZ07418.1	6.0E-48	0.013	0.279	–	N
CaS464	R+	Hypothetical protein	None	None	0.994	0.883	S	Outside
CaS472	R+	Hypothetical protein	None	None	0.178[(SA)]	0.546	S	ER
CaS501	–	Hypothetical protein	SGN-U330124	1.0E-82	0.949	0.996	S	Outside
CaS503	R+	Hypothetical protein	None	None	0.958	0.981	S	Outside
CaS505	IB_R+	Hypothetical protein	None	None	0.951	0.972	S	PM
CaS226	nc	Unknown	cacn8472	9E-62	1	0.923	S	Outside
CaS229	IB_S+	Unknown	caKS17001A12	4.0E-26	0	0.035	–	Mb
CaS356	IB_S+	Unknown	NP_564344.1	2.0E-09	0.992	0.794	S	Outside
CaS456	nc	Unknown	XP_002326189	4.0E-04	0.351[(SA)]	0.844	S	Outside

response (HR); the second group showed late induction at 12 hpi, and remained at the higher level until 72 hpi; the third group showed stronger induction in the susceptible than the resistant cultivar; and the fourth group showed decreased transcript levels following *P. capsici* infection (Fig. 3). Under the same conditions, the transcripts of *CaS102* and *CaS113* increased earlier, at 3 hpi, in the resistant cultivar. The *CaS290* gene was expressed as early as 3 hpi in both cultivars (Fig. 3A). The time points for appearance of *CaS22*, *CaS148*, and *CaS259* transcripts were late stage, from 24 to 72 hpi in both cultivars (Fig. 3B). Interestingly, some CaS clones (*CaS162*, *CaS475*, and *CaS551*) were much more upregulated in the susceptible cultivar, most notably *CaS551*, whose expression level was eight times higher (Fig. 3C). Finally, the genes of the fourth group showed decreased or abolished expression during *P. capsici* infection (Fig. 3D). This class, which includes *CaS67* (extensin) and *CaS203* (putative proline-rich protein [PRP]), major structural cell wall proteins (Bernhardt and Tierney 2000; Fowler et al. 1999; Niebel et al. 1993), was downregulated but gradually recovered in the resistant cultivar and not in the susceptible one. Extensin and PRP play a role in the plant response to pathogen attack (Hematy et al. 2009) and, therefore, their downregulation may be involved in effective invasion of the pathogen (Wei and Shirsat 2006).

The expression patterns of the qRT-PCR analysis largely agreed with those of the dot-blot assay. Taken together, these results indicated that the YST technique could be efficiently used for isolation of pathogen-responsive secreted proteins in the pepper–*P. capsici* interaction.

Roles of CaS genes in development and cell death in pepper plants.

Expression analysis indicated that many CaS genes might have a role in defense responses (Fig. 3). To test this, a loss-of-function strategy was taken using the TRV-based virus-induced gene silencing (VIGS) method for gene knock-down (Chung et al. 2004; Liu et al. 2002). The VIGS experiments were performed in chili pepper plants with 68 *CaS* genes.

We observed that silencing of only three CaS genes led to phenotypic abnormalities at 3 to 4 weeks after VIGS. CaS17-, CaS221-, and CaS259-silenced pepper plants showed retarded growth compared with green fluorescent protein (GFP)-silenced control plants. We also observed delay of cell death following *Pseudomonas syringae* pv. *tomato* T1 inoculation in 8 of 68 CaS gene-silenced cases: *CaS75*, *CaS113*, *CaS203*, *CaS259*, *CaS270*, *CaS389*, *CaS390*, and *CaS501* (Fig. 4; Supplementary Fig. S3). Among these, *CaS259*, which showed both cell death suppression by *P. syringae* pv. *tomato* T1 and phenotypic abnormalities, was selected for validation of the gene-silencing effects.

We measured plant height in CaS259-silenced pepper plants. The growth of CaS259-silenced pepper showed severe growth retardation (40% of control) (Fig. 5A). RT-PCR was performed to verify the suppression of *CaS259* gene expression in the silenced plant (Fig. 5A). These results indicate that the aberrant phenotype correlates with the suppression of transcript level of gene *CaS259*. To validate the effect of *CaS259* silencing on pathogen-induced cell death, leaves of the *CaS259*-silenced pepper were inoculated with *Xanthomonas axonopodis* pv. *glycines* 8ra (optical density = 0.1), a non-host pathogen of pepper (Oh et al. 2008; Yi et al. 2009). The cell death induced by *X. axonopodis* pv. *glycines* 8ra was significantly delayed in *CaS259*-silenced pepper plants compared with that of control plants (Fig. 5C). Ion leakage was also significantly reduced compared with that of control plants during *X. axonopodis* pv. *glycines* 8ra inoculation (Fig. 5D).

We also determined the effects of silencing of selected *CaS* genes (Fig. 4) in resistant pepper (CM334) following *P. capsici* inoculation. Only CaS259-silenced plant significantly showed reduction in cell death challenged by *Phytophthora capsici*

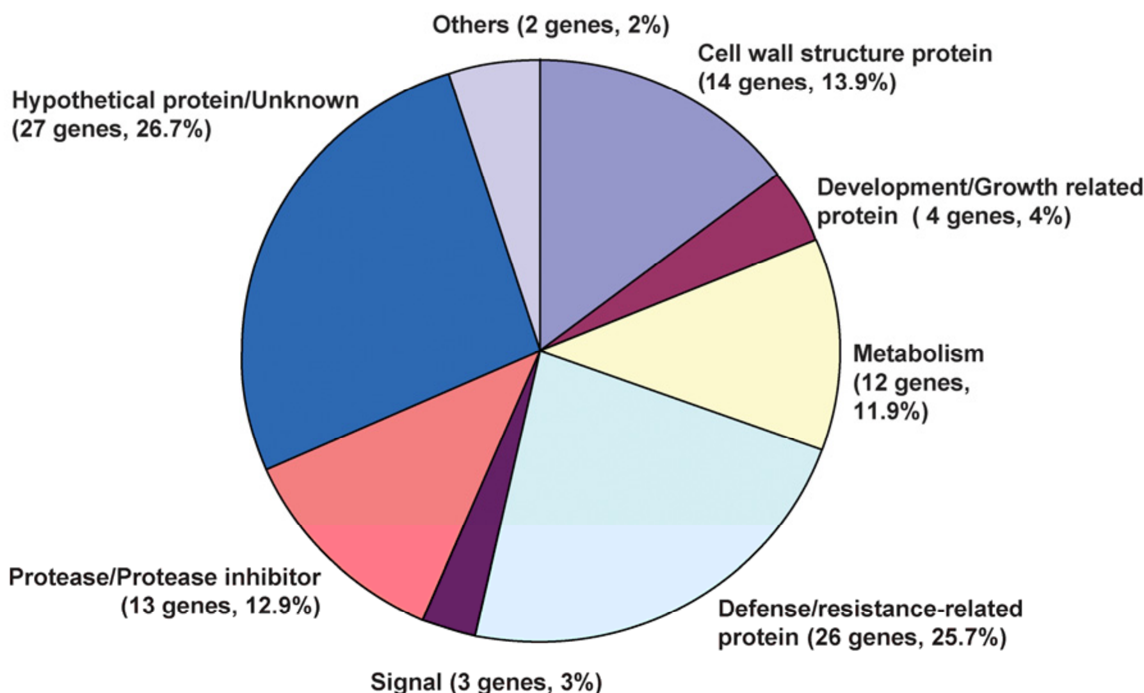

Fig. 2. Functional classification of *Capsicum annuum* secretome (CaS). In all, 101 CaS genes were isolated. Based on the sequence analysis using the BlastX algorithm with the nr database of the National Center for Biotechnology Information, the genes were classified into eight categories: 27 genes of unknown function, 26 genes in defense or resistance, 18 genes in development or growth of cell wall structure, 12 genes in metabolism, and 13 genes in protease or protease inhibitor. The remaining five genes were grouped in signal-related genes and others. Representative clones described in each group are shown in parentheses.

(Supplementary Fig. S4; Fig. 6A). The index of cell death and *P. capsici* colonization in CaS259-silenced resistant pepper were decreased compared with those of control plant (Fig. 6B and C). The expression levels of Ca*Hin1* and Ca*CDM1* genes, cell death markers, were decreased and the Ca*PR1* gene was downregulated in the CaS259-silenced resistant pepper following pathogen infection (Supplementary Fig. S1). We also performed the VIGS assay of the CaS259 gene in susceptible pepper plants following *P. capsici* inoculation. Disease symptoms and *P. capsici* colonization were also decreased in *CaS259*-silenced susceptible pepper compared with those of control (Fig. 7). These data suggested that one of the pathogen-responsive secreted proteins identified, CaS259, has an important role in both normal growth and the modulation of cell death induced by pathogen in pepper plants.

DISCUSSION

The goal of this study was to isolate the secreted proteins of pepper resistant to *P. capsici* infection for comparison of transcript profiles between susceptible and resistant cultivars. We isolated 101 secreted proteins using the YST system from *P. capsici*-infected pepper roots. Of the 101 unique sequences, 92 (92%) contained a predicted secretory signal peptide. The other

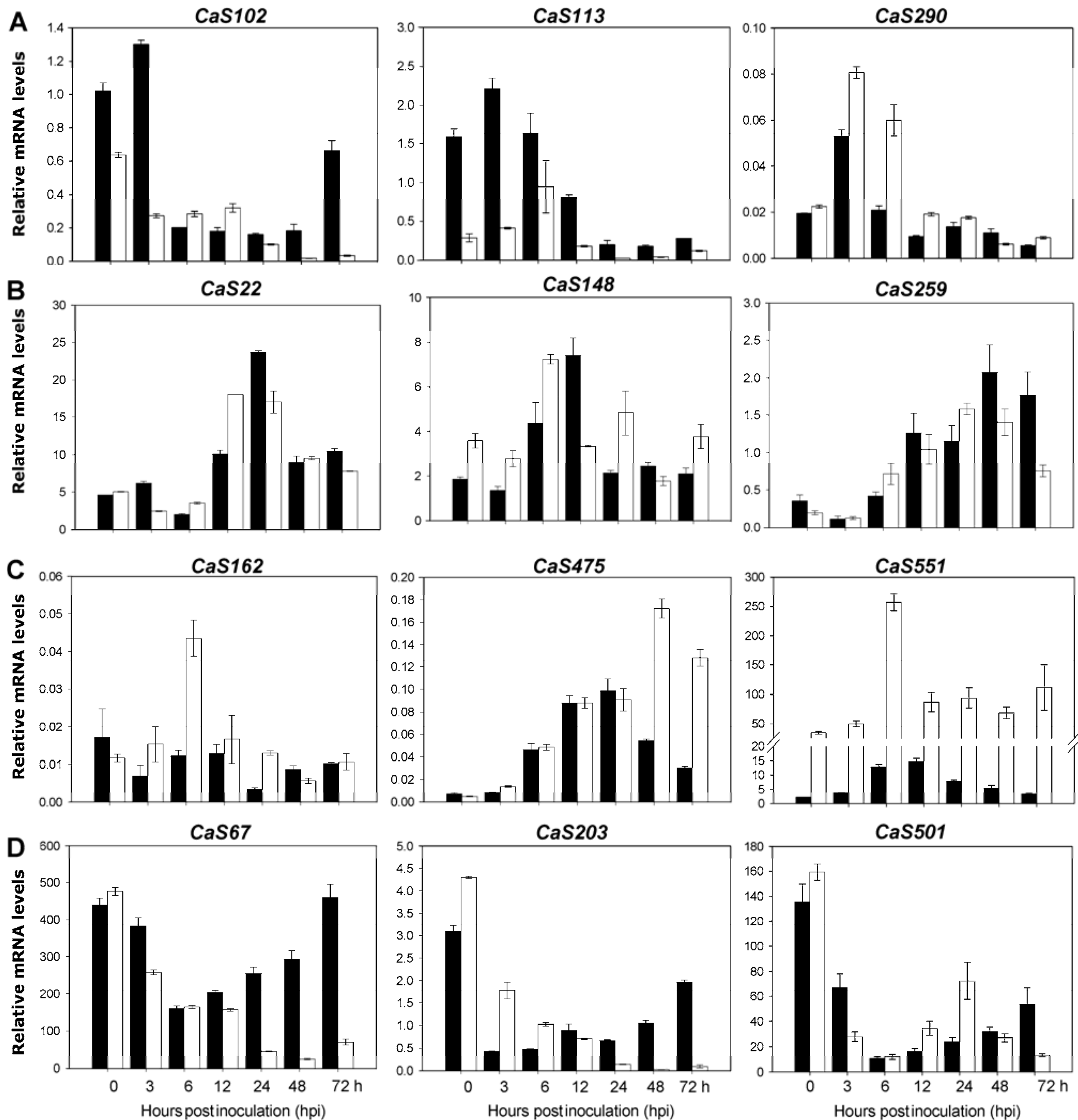

Fig. 3. Representative quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of several *Capsicum annuum* secretome (CaS) clones. Total RNAs were isolated at different time points following inoculation of *Phytophthora capsici* into roots of resistant and susceptible pepper plants. qRT-PCRs were performed on cDNA using gene-specific primers for each CaS clone. Each bar represents the value of relative gene expression at different time points following inoculation of *P. capsici* for indicated CaS gene, between resistant pepper (black bar, CM334) and susceptible pepper (white bar, Chilsungcho) plants. The expression of CaS genes was normalized to the expression of *CoActin*. Values were calculated for CaS genes following three replications and standard deviations are shown. Similar results were obtained from at least two independent experiments. One representative experiment is shown.

nine CaS genes might be "false positives", because some truncated proteins could exhibit unnaturally exposed N-terminal hydrophobic or highly basic regions and, thus, be artificially secreted, or they might be secreted through noncanonical pathways (Rose and Lee 2010). Nevertheless, the rate of signal sequence recovery is high compared with previous studies, showing 76 to 83% using other signal-sequence trap technologies with tobacco or *Arabidopsis* (Goo et al. 1999; Hugot et al. 2004). Several CaS genes (CaS1, CaS2, CaS67, CaS203, CaS388, and CaS507) encode well-known secreted cell wall or extracellular proteins, which confirmed that our experimental system worked for the pepper–*Phytophthora* interaction (Table 1). However, the large proportion of unknown secreted proteins (27 genes) suggests that many functions of secreted proteins still remained to be elucidated.

We did not find any pathogen-derived secreted proteins by computational analysis of these clones after searching numerous DNA databases. In this regard, our results differed from those of Lee and associates (2006a), who reported that the same YST system was valuable for the identification of both host- and pathogen-derived secreted proteins involved in the interaction between tomato (*Solanum lycopersicum*) and the oomycete *P. infestans*. This difference might reflect the susceptible or resistant interaction between pathogen and plant host. In our study, we constructed the YST cDNA library from resistant pepper following *P. capsici* infection, which may have resulted in undetectable levels of pathogen biomass compared with that of the susceptible interaction used by Lee and associates (2006a). It is also possible that the greater abundance of plant cDNA encoding secreted proteins prevented detection of pathogen-derived cDNAs, which are relatively rare.

The transcript levels of a number of CaS genes were significantly changed in resistant or susceptible cultivars following *P. capsici* infection. The differences in CaS transcripts could be grouped by temporal changes and by transcript levels between the susceptible and resistant cultivars (Fig. 3). These results are consistent with the observations of Richins and associates (2010), who showed global gene expression profiles using microarrays in resistant and susceptible pepper cultivars infected by *P. capsici*. The gene expression profiling allowed us to determine differentiation of expression at the molecular level between resistance and susceptibility to pathogen.

To select CaS genes related to the defense response to pathogen, 68 CaS genes affected by pathogen infection were selected for gene silencing. We observed only three CaS genes that induced significant morphological change in silenced pepper plants, which were assumed to be defense-related proteins and not cell wall structure-related proteins (Table 1; Figs. 3 and 4). According to in silico analyses, 18 CaS clones were predicted to be cell wall structural protein or growth- or development-related protein. However, when CaS genes were silenced in *Nicotiana benthamiana*, 31 of 68 CaS genes (approximately 50%) showed morphological abnormalities, such as curly leaves, stunted growth, and severe developmental defects (data not shown). It could be that the efficiency of gene silencing in pepper is less uniform than in *N. benthamiana* (Dong et al. 2007; Liu et al. 2004).

Gene silencing experiments also revealed that eight CaS genes compromised cell death following *Pseudomonas syringae* pv. *tomato* T1 infection, to less than 20% relative to the control (Fig. 4). Of these, three (CaS113, CaS270, and CaS501) are classified in the hypothetical protein/unknown function group. Two CaS genes (CaS259 and CaS390) belong to PR gene families and defensin, which are well known to act on plant defense showing anti-bacterial or antifungal activity, respectively (Pelegrini and Franco 2005; van Loon et al. 2006). The remaining CaS genes can be classified within the groups of cell wall structure (CaS203, putative PRP), metabolism (CaS389, hydrolase), and development- or growth-related proteins (CaS75, putative ROX1) (Cecchetti et al. 2007).

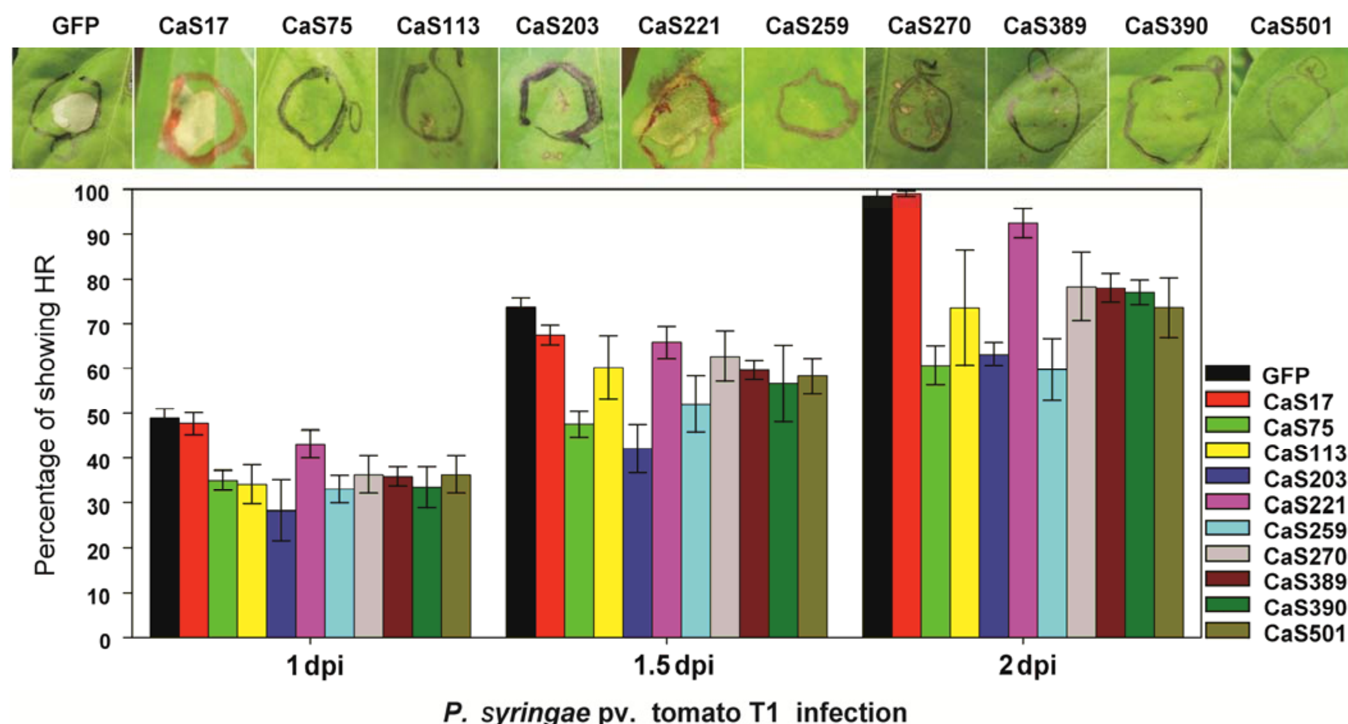

Fig. 4. Hypersensitive response (HR) of representative *Capsicum annuum* secretome (CaS) gene-silenced pepper following bacterial pathogen (*Pseudomonas syringae* pv. *tomato* T1). CaS gene-silenced pepper plants were infiltrated with non-host bacterial pathogen *P. syringae* pv. *tomato* T1 (optical density at 600 nm = 0.05). The HR symptoms were taken at 2 days postinoculation (upper panel). Delay of HR by *P. syringae* pv. *tomato* T1 was scored by the mean percentages of sites showing cell death (lower panel). Standard deviations were scored from 15 infiltration site per line, comprising three leaves from five independent plants. Similar results were obtained from at least two independent experiments. One representative experiment is shown.

To validate these findings, the *CaS259* gene was chosen and the gene-silenced pepper was infected with a non-host pathogen, *X. axonopodis* pv. *glycines* 8ra. The cell-death delay was determined by scoring for ion leakage (Fig. 5D). In addition, the cell death symptoms and *Phytophthora capsici* infection were reduced in both the resistant and susceptible pepper plants following silencing of *CaS259* gene (Figs. 6 and 7). In contrast, biomass of *P. capsici* was increased in CaS259 transient overexpressed *N. benthamiana* compared with that of the control (Supplementary Fig. S6). These data suggested that the function of CaS259 might be related to susceptibility factor for *P. capsici*, or else the pleiotropic effects of CaS259-silencing in pepper have rendered the plant more resistant, showing cell-death delay.

The CaS259 protein showed a high degree of similarity to PR4 (Supplementary Fig. S2). The PR4 family has been known to have potent antifungal and antimicrobial activity in vitro against a wide range of pathogens (Fiocchetti et al. 2008; Li et al. 2010; Zhu et al. 2006). This family of proteins is also modulated by pathogen infection, as well as by defense-signaling molecules (Bertini et al. 2003; Park et al. 2001). Expression of PR4 has also been known to be developmentally controlled in an organ-specific manner in healthy pepper (Park et al. 2001). Recently, PR4 proteins from various plants have been described as having RNase or DNase activity (Caporale et al. 2004; Guevara-Morato et al. 2010; Li et al. 2010), much of which is secreted to the apoplast, resulting in the breakdown of DNA and RNA and consequent HR cell death (Mittler and Lam 1997). In addition,

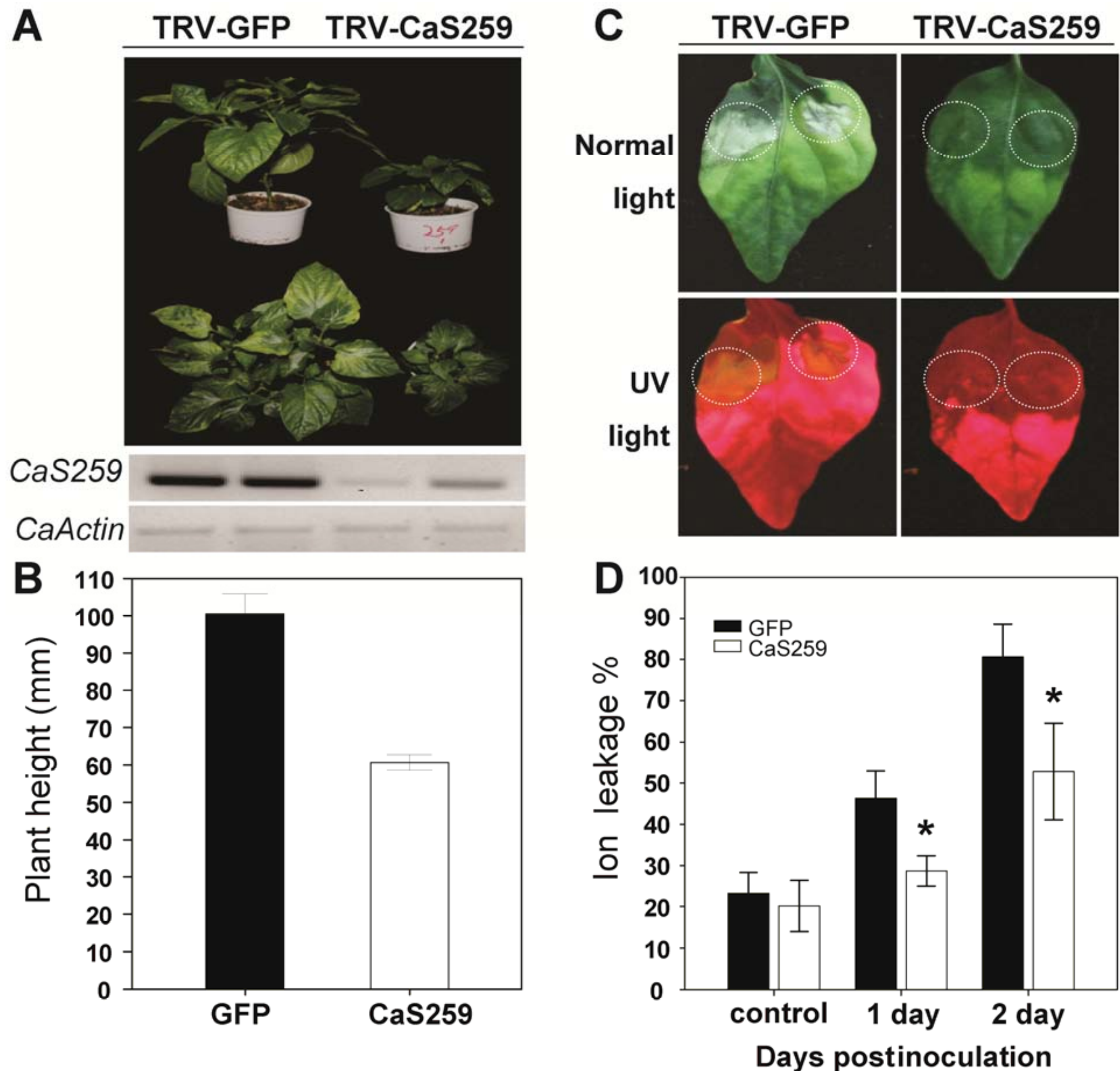

Fig. 5. Effects of *Capsicum annuum* secretome (CaS)259 silencing in pepper. **A,** Phenotype of *Ca*S259-silenced pepper plants (upper panel). The picture was taken at 6 weeks after silencing. Semi-quantitative reverse-transcription polymerase chain reaction analysis for expression analysis of *CaS259* gene in *Tobacco rattle virus* (TRV)-Δgreen fluorescent protein (GFP) and TRV-*Ca*S259 (N-terminal) infiltrated pepper. The level of actin was used as control (lower panel). **B,** Plant height of *CaS259*-silenced plants represented as comparison of plant height. The plant height was measured at 3 weeks after the onset of virus-induced gene silencing. In total, 15 plants were measured and data are indicated as means ± standard deviation. Similar results were obtained from three independent experiments. **C,** Cell death of *Ca*S259-silenced pepper plants following non-host pathogen (*Xanthomonas axonopodis* pv. *glycines* 8ra) inoculation. *Xanthomonas axonopodis* pv. *glycines* 8ra was infiltrated as 1×10^8 CFU/ml (optical density at 600 nm = 0.1). The picture was taken at 2 dpi under normal and UV light. **D,** Cell death by *X. axonopodis* pv. *glycines* 8ra (as in C) was quantified by measuring the ion leakage of inoculated regions. Data represent means of six leaf discs (1 cm in diameter) and error bars represent standard deviations. The experiments were repeated three times with similar results. Asterisks indicate difference of significant level as determined by Student's *t* test ($P < 0.05$).

Guevara-Morato and associates (2010) suggested that the nuclease activity of *C. chinense* PR4 might contribute to the depletion of RNA and DNA fragments during cell death, as in programmed cell death and necrosis.

However, the roles of plant PR4 proteins in relation to cell death and development have not yet been elucidated. The nuclease function of PR4 can reasonably explain cell death delay in *CaS259*-silenced pepper following inoculation with *Pseudomonas syringae* pv. *tomato* T1 and *X. axonopodis* pv. *glycines* 8ra (Figs. 4 and 5C) or with *Phytophthora capsici* (Figs. 6A and 7A). To our knowledge, this is the first report showing that CaS259, a CaPR4-like protein, plays an essential role in the regulation of development and the cell death that can result from pathogen infection (Figs. 5, 6, and 7). These observations provide evidence that CaS genes play an important role in pathogen defense as well as plant development.

Our data strongly suggest that studies using these secreted proteins might contribute to understanding the fundamental basis of plant innate immunity that occurs at the interface of pathogen and host cell. Further investigations into the role of each gene could provide insights into some of the unknown functions of the plant secretome in pathogen defense and normal growth and development.

MATERIALS AND METHODS

Plant materials.

C. annuum CM334 (resistant to *P. capsici*) and *C. annuum* Chilsungcho (susceptible to *P. capsici*) were used for *P. capsici* infection experiments. *C. annuum* 'Bukang' was used for VIGS experiments and followed by bacterial pathogen infection. All plants were grown in a growth chamber at 25°C under a cycle of 16 and 8 h of light and darkness, respectively, and transported in a Magenta box (7.2 by 7.2 by 10 cm^3; SPL Life Science, Gyeonggi-do, Korea) for *P. capsici* infection (Kim et al. 2008).

Pathogen preparation and inoculation.

Preparation of *P. capsici* inocula was described previously (Kim et al. 2008). *P. capsici* Leon, 'Pa23', was grown in 3.9%

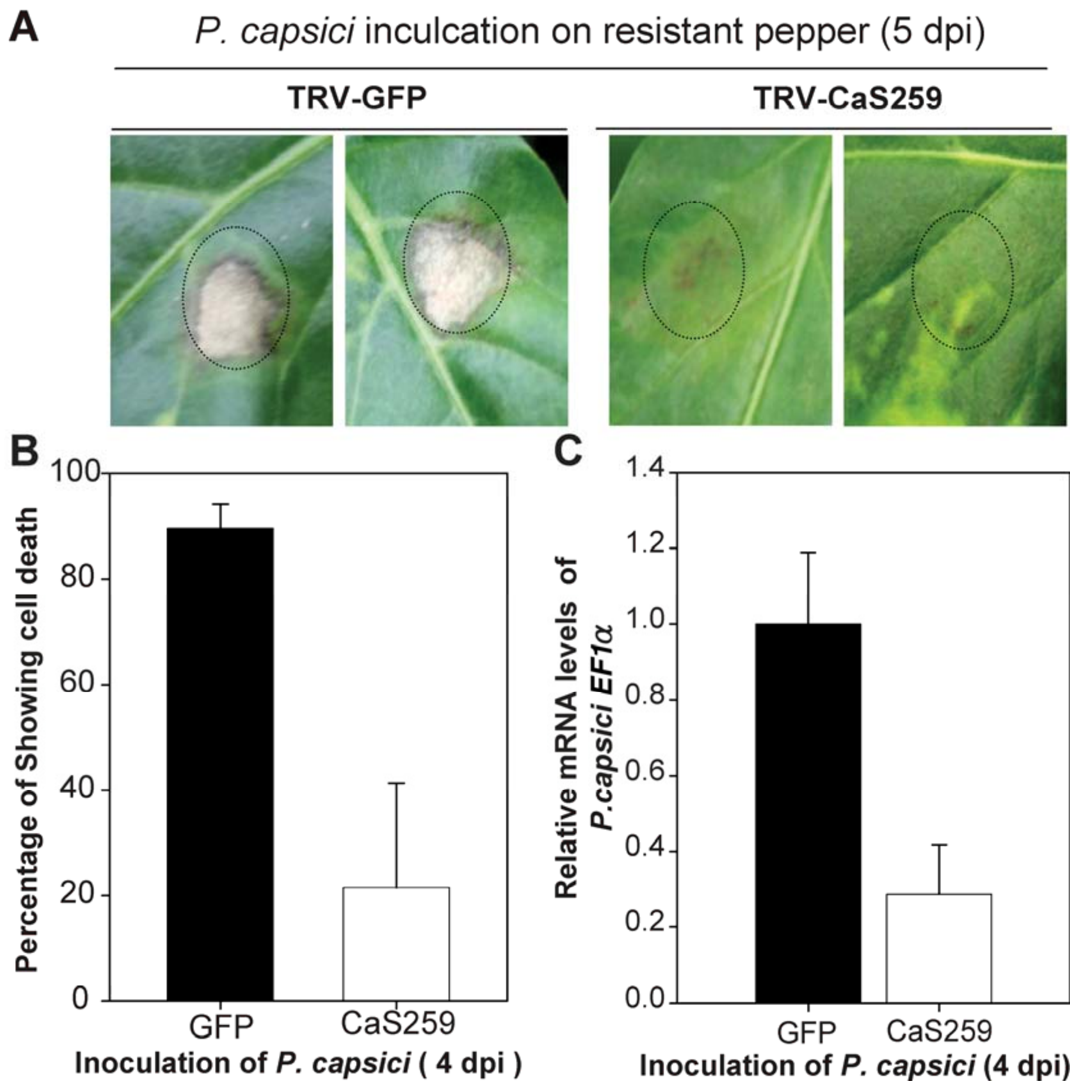

Fig. 6. Effects of *Capsicum annuum* secretome (CaS)259 gene silencing following *Phytophthora capsici* infection in resistant pepper. **A,** CaS259-silenced 'CM334' pepper was infiltrated on leaves by *P. capsici* as 2×10^5 zoospores/ml. The picture was taken at 5 days postinoculation (dpi). **B,** Percentages of sites showing cell death following *P. capsici* infection of leaves of CaS259-silenced CM334. In total, 10 leaves per line, comprising two leaves from five independent plants, were scored at 4 dpi. Data points represent the means ± standard deviation. Similar results were obtained from three independent experiments. One representative experiment is shown. **C,** Quantitative reverse-transcription polymerase chain reaction of *P. capsici* colonization levels in CaS259-silenced CM334 and control pepper cultivars. Total RNA was extracted from *P. capsici*-infected regions at 4 dpi. Expression of the *P. capsici Ef1*α (*PcEf1*α) gene was normalized to the expression of *CaActin*. Values were calculated with biological three replications as standard deviations.

potato dextrose agar medium for 7 days (27°C, in the dark)and mycelial plugs (8 mm in diameter) were cut from the periphery and cultured on V8 juice agar media (20% V8 juice, 0.4% $CaCO_3$, and 1.8% agar) for 5 days. The mycelia were scraped and incubated under light for 2 days to promote sporangium formation. The plate was flooded with sterile water and incubated at 4°C for 1 to 2 h; then, plates were placed at 28°C for 30 min. The released zoospores were counted by

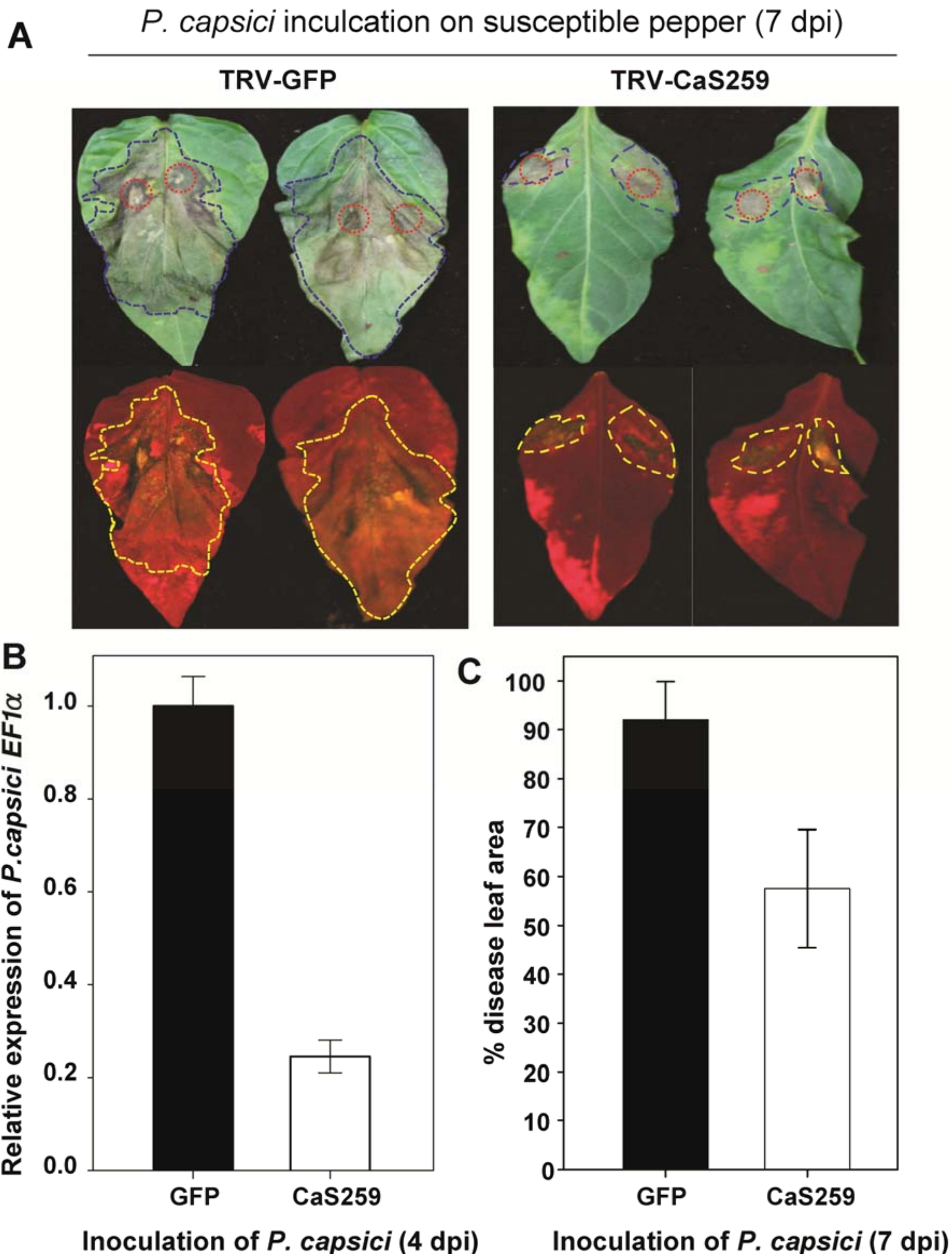

Fig. 7. Effects of *Capsicum annuum* secretome (CaS)259 gene silencing following *Phytophthora capsici* infection in susceptible pepper **A,** Disease symptom development on the leaves after *P. capsici* inoculation (2×10^5 zoospore/ml). The picture was taken at 7 days postinoculation (dpi) under normal (upper panel) and UV light (lower panel). Red circles indicate the site of *P. capsici* inoculation. Colored dotted lines (blue or yellow) indicate disease symptoms. **B,** *P. capsici* colonization in CaS259-silenced susceptible pepper was assessed by quantitative reverse-transcription polymerase chain reaction of the *P. capsici Ef1*α (*PcEf1*α) gene. Total RNA was extracted from *P. capsici*-infected leaves at 4 dpi. Expression of *PcEf1*α gene was normalized to the expression of *CaActin*. Values were calculated with biological three replications as standard deviations. **C,** Leaf area covered with Phytophthora blight lesion (%) in *CaS259*-silenced pepper and control (*Tobacco rattle virus* [TRV]-green fluorescent protein [GFP]-infiltrated pepper) after inoculation with *P. capsici.* Data points represent the means ± standard deviation from disease symptoms of 20 leaves (TRV-GFP) and 32 leaves (TRV-CaS259). Similar results were obtained from two independent experiments. One representative result is shown.

hemacytometer and the concentration adjusted to 2×10^6 zoospores/ml with sterile water.

Five-week-old pepper plants were inoculated with *P. capsici* zoospores. For root inoculation, 10 ml of 10^6 zoospores/ml (final concentration 2×10^5) was introduced into the Magenta box. Growth conditions were maintained at 27°C with cycles of 16 and 8 h of light and darkness, respectively. *P. capsici*-infected pepper roots were collected at 0, 3, 6, 12, 24, 48, and 72 hpi. *Pseudomonas syringae* pv. *tomato* T1 and *X. axonopodis* pv. *glycines* 8ra were grown overnight in liquid Luria-Bertani (LB) medium. The bacterial cultures resuspended in 10 mM $MgCl_2$ were introduced into pepper leaves by pressure infiltration using a needless syringe (Oh et al. 2008; Yi et al. 2009).

TTC reduction assay.

The TTC reduction assay was modified from the methods of Chen and associates (2006). *P. capsici*-infected roots from each time point, including noninfected roots, were washed with sterile water for 10 min before TTC tests. Fresh roots (each 300 mg) were incubated with 5 ml of 0.6% TTC (Sigma-Aldrich, St. Louis) in 50 mM phosphate buffer (pH 7.4) for 22 h at 30°C in the dark. Roots were then washed twice with sterile water. Formazan (reduced TTC) was extracted twice from the roots with 95% EtOH at 80°C for 30 min. Combined extracts were adjusted to a final volume of 15 ml and absorbance was read at 490 nm (model DU 730; Beckman Coulter, Fullerton, CA, U.S.A.). Assays were performed three times in each independent experiment.

Construction of YST cDNA library.

Isolation of total RNAs from resistant and susceptible pepper roots following *Phytophthora capsici* infection were performed by the method of Choi and associates (1996) and mRNAs from resistant pepper roots were purified by the oligotex mRNA minikit (Qiagen, Chatsworth, CA, U.S.A.). The pYST vector system used for library construction was as previously described (Lee et al. 2006b). The HybriZAP cDNA synthesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for random-primed cDNA synthesis and a random primer (5′-GA GAGAGAGAGAGAGACCGCGGCCGCCGCCNNNNNN-3′), including a *Not*I restriction enzyme site (underlined), was used for first-strand synthesis. After second-strand synthesis, ligation with an *Eco*RI adaptor, 5′-end phosphorylation with T4 polynucleotide kinase, digestion with *Not*I, and cDNA size-fractionation was performed using the approximately 300- to 1,000-bp gel elution fraction. The cDNAs were ligated to an equimolecular mixture of the *Eco*RI- and *Not*I-digested pYST 0, 1, and 2 vectors. TOP10 electrocompetent cells (Invitrogen, Carlsbad, CA, U.S.A.) were used for transformation of the ligation mixture. After transformation, the cells were plated on LB agar plates including ampicillin at 50 mg/ml. Plasmid DNA was isolated from a pooled sample of the transformants (YST library) using the Perfect prep plasmid midi kit (Eppendorf, Hamburg, Germany).

Yeast transformation, selection, and sequencing.

YST library plasmids were transformed into an invertase-deficient yeast mutant strain, DBYα2445 (*Saccharomyces cerevisiae*, MATα, *suc*2Δ-9, *lys*2-801, *ura*3-52, *ade*2-101) by the YEASTMAKER Yeast Transformation System2 (BD Bioscience, San Jose, CA, U.S.A.). Transformants were selected on YP-Suc medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% agar), and incubated at 30°C for 8 to 9 days. Colonies were restreaked on an YP-Suc medium. After incubation at 30°C for 2 days, plasmids were isolated from the yeast colonies. The cDNAs selected from the first screen were used for identifying redundant clones using Southern blot assay, in an iterative step. The plasmids were then transformed into DH10b *Escherichia coli* electrocompetent cells, and the isolated plasmids were sequenced using the ADH1 primer (5′-TCCTCGTC ATTGTTCTCGTTCC-3′) (Lee et al. 2006b). All rescued plasmids from *E. coli* were retransformed into yeast to reconfirm the ability to grow on sucrose selection medium at least twice. YST clones were sequenced (NICEM, Korea) and sequence similarity was determined using the National Center for Biotechnology Information and *Phytophthora* Functional Genomics Database. To identify the gene annotation, the sequences of the CaS clones were compared with pepper expressed sequence tag databases (Gene Pool and Sol Genomic Network). DNA sequences were translated into amino acid sequence using the ExPasy translation tool (Appel et al. 1994). Signal peptides were predicted by the SignalP3.0 program (Bendtsen et al. 2004) and subcellular localization was predicted using the TargetP (Emanuelsson et al. 2000) and PSORT programs (Nakai and Horton 1999).

Northern blot analysis.

Total RNA (10 μg) was electrophoresed on 1.2% formaldehyde agarose gels and blotted onto Hybond-N$^+$ membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.). The membranes were hybridized with [α-^{32}P]-labeled partial cDNA fragments of YST clones at 65°C overnight. After hybridization, the membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.2) and 0.1% sodium dodecyl sulfate (SDS) at 65°C for 15 min, with 1× SSC and 0.1% SDS at 65°C for 15 min, and with 0.5× SSC and 0.1% SDS at 65°C for 15 min. Membranes were exposed to X-ray film at –80°C for 3 to 24 h.

Dot-blot assay.

Approximately 2 μg of pYST plasmid DNA containing cDNA-encoding signal peptide (containing approximately 100 ng of each cDNA) was used for reverse Northern blotting. Dot-blot hybridization was performed using the Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA, U.S.A.) following the manufacturer's protocol, with slight modification. Fourteen RNA probes were generated: time course RNA (0, 3, 6, 12, 24, 48, and 72 h) from resistant and susceptible pepper roots after *P. capsici* infection. Total RNA (5 μg each sample) was used for template cDNA synthesis using the SUPERSCRIPT II RNase–Reverse-Transcriptase system (Invitrogen) in a mixture with anchor primer (oligo-dT), dNTP mixture minus dCTP, and [α-^{32}P]-dCTP (3,000 Ci/mmol) (Amersham Biosciences). The labeled probes were separated from unincorporated nucleotides using mini-Quick Spin DNA columns (Roche Applied Science, Mannheim, Germany). Membranes were prehybridized at 65°C for 3 h in hybridization buffer (0.5 M sodium phosphate [pH 7.2], 7% SDS, 1 mM EDTA). Labeled cDNA probe was mixed with fresh buffer and hybridized at 65°C for at least 16 h. After hybridization, blots were washed twice in 2× SSC and 0.1% SDS at 65°C for 5 min, once in 1× SSC and 0.1% SDS at 65°C for 15 min, and twice 0.1× SSC and 0.1% SDS at 65°C for 10 min. The washed membranes were exposed to a BAS imaging plate (Fujifilm, Tokyo) and quantified by scanning the plate with a Fujix BAS2500 (Fujifilm).

qRT-PCR.

For qRT-PCR, total RNA (5 μg) was reverse-transcribed using the SUPERSCRIPT II RNase–Reverse-Transcriptase system (Invitrogen). Triplicate samples were analyzed using a Rotor-Gene 6000 apparatus (Qiagen) with SYBR Green (Invitrogen), according to the manufacturer's instructions. The relative quan-

titation of gene expression was calculated by the relative standard curve method (Larionov et al. 2005). The *CaActin* gene was used to normalize expression levels, and noninoculated pepper plants were used as controls for expression of the target genes (Supplementary Table S2). Expression levels were reported as mean values with standard errors.

Construction of the TRV-CaS vector and VIGS in pepper. CaS genes in the pYST vector were digested with *Eco*RI and *Kpn*I and cloned into a TRV-based gene silencing vector (pTRV2) via the same enzyme sites. Average insert size was approximately 370 nucleotides. The pTRV2 vectors containing CaS genes were transformed into *Agrobacterium* sp. strain GV2260 by the freeze-thaw method (An 1987) and the TRV-based VIGS on pepper was performed as described by Chung and associates (2004).

Measurement of ion leakage. The measurement of ion leakage was performed as described by Lee and associates (2010). Two days after inoculation with bacterial pathogen, three leaf discs (1 cm in diameter) were floated on 5 ml of distilled water for 2 h at room temperature. Electrical conductivity was measured using a conductivity meter (model 455C; Istek, Seoul). To release whole electrolytes from leaf discs, samples were autoclaved, cooled to room temperature, and measured with a conductivity meter. Ion leakage was expressed as percent leakage to conductivity of control sample.

ACKNOWLEDGMENTS

This work was supported by a grant from the Crop Functional Genomic Center (CG1132) and the National Research Foundation (project number 2010-0015105) of MEST of Korean Government. S.-I. Yeom is a scholarship grantee (S2_2009_000_0210_1) from the Korea Student Aid Foundation (KOSAF). J. Rose was supported by a grant from the National Science Foundation (NSF) Plant Genome program (DBI-0606595). B.-D. Kim and D. Choi contributed equally as corresponding authors.

LITERATURE CITED

An, G. 1987. Binary Ti-vectors for plant transformation and promoter analysis. Methods Enzymol. 153:292-305.

Appel, R., Bairoch, A., and Hochstrasser, D. 1994. A new generation of information retrieval tools for biologists: The example of the ExPASy WWW server. Trends Biochem. Sci. 19:258-260.

Bendtsen, J., Nielsen, H., Von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP3.0. J. Mol. Biol. 340:783-759.

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

Bertini, L., Leonardi, L., Caporale, C., Tucci, M., Cascone, N., Di Berardino, I., Buonocore, V., and Caruso, C. 2003. Pathogen-responsive wheat PR4 genes are induced by activators of systemic acquired resistance and wounding. Plant Sci. 164:1067-1078.

Birch, P. R., Rehmany, A. P., Pritchard, L., Kamoun, S., and Beynon, J. L. 2006. Trafficking arms: Oomycete effectors enter host plant cells. Trends Microbiol. 14:8-11.

Caporale, C., Di Berardino, I., Leonardi, L., Bertini, L., Cascone, A., Buonocore, V., and Caruso, C. 2004. Wheat pathogenesis-related proteins of class 4 have ribonuclease activity. FEBS (Fed. Eur. Biochem. Soc.) Lett. 575:71-76.

Cecchetti, V., Altamura, M. M., Serino, G., Pomponi, M., Falasca, G., Costantino, P., and Cardarelli, M. 2007. ROX1, a gene induced by rolB, is involved in procambial cell proliferation and xylem differentiation in tobacco stamen. Plant J. 49:27-37.

Chen, C. W., Yang, Y. W., Lur, H. S., Tsai, Y. G., and Chang, M. C. 2006. A novel function of abscisic acid in the regulation of rice (*Oryza sativa* L.) root growth and development. Plant Cell Physiol. 47:1-13.

Choi, D., Kim, H., Yun, H., Park, J., Kim, W., and Bok, S. 1996. Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. Plant Physiol. 112:353-359.

Chung, E., Seong, E., Kim, Y. C., Chung, E. J., Oh, S. K., Lee, S., Park, J. M., Joung, Y. H., and Choi, D. 2004. A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). Mol. Cells 17:377-380.

Cutler, S. R., Ehrhardt, D. W., Griffitts, J. S., and Somerville, C. R. 2000. Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. Proc. Natl. Acad. Sci. U.S.A. 97:3718-3723.

Dong, Y., Burch-Smith, T. M., Liu, Y., Mamillapalli, P., and Dinesh-Kumar, S. P. 2007. A ligation-independent cloning tobacco rattle virus vector for high-throughput virus-induced gene silencing identifies roles for NbMADS4-1 and -2 in floral development. Plant Physiol. 145:1161-1170.

Dunaevskii Ia, E., Elpidina, E. N., Vinokurov, K. S., and Belozerskii, M. A. 2005. Protease inhibitors: Use to increase plant tolerance to insects and pathogens. Mol. Biol. (Moscow) 39:702-708.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300:1005-1016.

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protocols 2:953.

Fiocchetti, F., D'Amore, R., De Palma, M., Bertini, L., Caruso, C., Caporale, C., Testa, A., Cristinzio, G., Saccardo, F., and Tucci, M. 2008. Constitutive over-expression of two wheat pathogenesis-related genes enhances resistance of tobacco plants to *Phytophthora nicotianae*. Plant Cell Tissue Organ Cult. 92:73-84.

Fowler, T. J., Bernhardt, C., and Tierney, M. L. 1999. Characterization and expression of four proline-rich cell wall protein genes in *Arabidopsis* encoding two distinct subsets of multiple domain proteins. Plant Physiol. 121:1081-1091.

Goo, J. H., Park, A. R., Park, W. J., and Park, O. K. 1999. Selection of *Arabidopsis* genes encoding secreted and plasma membrane proteins. Plant Mol. Biol. 41:415-423.

Guevara-Morato, M. A., Garcia de Lacoba, M., Garcia-Luque, I., and Serra, M. T. 2010. Characterization of a pathogenesis-related protein 4 (PR-4) induced in *Capsicum chinense* L3 plants with dual RNase and DNase activities. J. Exp. Bot. 61:3259-3271.

Hausbeck, M. K., and Lamour, K. H. 2004. *Phytophthora capsici* on vegetable crops: Research progress and management challenges. Plant Dis. 88:1292-1303.

Hematy, K., Cherk, C., and Somerville, S. 2009. Host-pathogen warfare at the plant cell wall. Curr. Opin. Plant Biol. 12:406-413.

Hong, J. K., and Hwang, B. K. 2002. Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its in situ localization in pepper (*Capsicum annuum*). Physiol. Plant. 114:549-558.

Hugot, K., Riviere, M. P., Moreilhon, C., Dayem, M. A., Cozzitorto, J., Arbiol, G., Barbry, P., Weiss, C., and Galiana, E. 2004. Coordinated regulation of genes for secretion in tobacco at late developmental stages: Association with resistance against oomycetes. Plant Physiol. 134:858-870.

Hwang, B. K., Kim, W. B., and Kim, W. K. 1989. Ultrastructure at the host-parasite interface of *Phytophthora capsici* in roots and stems of *Capsicum annuum*. J. Phytopathol. 127:305-315.

Ilarslan, H., Ustun, A. S., and Yilmazer, R. 1996. Ultrastructural changes in crowns of peppers resistant and susceptible to *Phytophthora capsici*. J. Turk Phytopathol. 25:11-22.

Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., LaVallie, E. R., Merberg, D., Spaulding, V., Stover, J., Williamson, M. J., and McCoy, J. M. 1997. A genetic selection for isolating cDNAs encoding secreted proteins. Gene 198:289-296.

Jones, D. A., and Takemoto, D. 2004. Plant innate immunity - direct and indirect recognition of general and specific pathogen-associated molecules. Curr. Opin. Immunol. 16:48-62.

Kamoun, S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. Annu. Rev. Phytopathol. 44:41-60.

Kim, H. J., Nahm, S. H., Lee, H. R., Yoon, G. B., Kim, K. T., Kang, B. C., Choi, D., Kweon, O. Y., Cho, M. C., Kwon, J. K., Han, J. H., Kim, J. H., Park, M., Ahn, J. H., Choi, S. H., Her, N. H., Sung, J. H., and Kim, B. D. 2008. BAC-derived markers converted from RFLP linked to *Phytophthora capsici* resistance in pepper (*Capsicum annuum* L.). Theor. Appl. Genet. 118:15-27.

Kim, Y. S., Park, J. Y., Kim, K. S., Ko, M. K., Cheong, S. J., and Oh, B. J. 2002. A thaumatin-like gene in nonclimacteric pepper fruits used as molecular marker in probing disease resistance, ripening, and sugar accumulation. Plant Mol. Biol. 49:125-135.

Klein, R. D., Gu, Q., Goddard, A., and Rosenthal, A. 1996. Selection for genes encoding secreted proteins and receptors. Proc. Natl. Acad. Sci. U.S.A. 93:7108-7113.

Larionov, A., Krause, A., and Miller, W. 2005. A standard curve based method for relative real time PCR data processing. BMC Bioinf. 6:62.
Lee, S. J., Saravanan, R. S., Damasceno, C. M., Yamane, H., Kim, B. D., and Rose, J. K. 2004. Digging deeper into the plant cell wall proteome. Plant Physiol. Biochem. 42:979-988.
Lee, S. J., Kelley, B. S., Damasceno, C. M., St. John, B., Kim, B. S., Kim, B. D., and Rose, J. K. 2006a. A functional screen to characterize the secretomes of eukaryotic pathogens and their hosts in planta. Mol. Plant-Microbe Interact 19:1368-1377.
Lee, S.-J., Kim, B.-D., and Rose, J. K. C. 2006b. Identification of eukaryotic secreted and cell surface proteins using the yeast secretion trap screen. Nat. Protocols 1:2439.
Lee, Y. K., Hong, J. K., Hippe-Sanwald, S., and Hwang, B. K. 2000. Histological and ultrastructural comparisons of compatible, incompatible and DL-beta-amino-n-butyric acid-induced resistance responses of pepper stems to *Phytophthora capsici*. Physiol. Mol. Plant Pathol. 57:269-280.
Lefebvre, V., and Palloix, A. 1996. Both epistatic and additive effects of QTLs are involved in polygenic induced resistance to disease: A case study, the interaction pepper–*Phytophthora capsici* Leonian. Theor. Appl. Genet. 93:503-511.
Li, X. D., Xia, B., Jiang, Y. M., Wu, Q. S., Wang, C. Y., He, L. S., Peng, F., and Wang, R. 2010. A new pathogenesis-related protein, LrPR4, from *Lycoris radiata*, and its antifungal activity against *Magnaporthe grisea*. Mol. Biol. Rep. 37:995-1001.
Liu, Y. L., Schiff, M., and Dinesh-Kumar, S. P. 2002. Virus-induced gene silencing in tomato. Plant J. 31:777-786.
Liu, Y. L., Schiff, M., and Dinesh-Kumar, S. P. 2004. Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to *Tobacco mosaic virus*. Plant J. 38:800-809.
Mithofer, A., Muller, B., Wanner, G., and Eichacker, L. A. 2002. Identification of defence-related cell wall proteins in *Phytophthora sojae*-infected soybean roots by ESI-MS/MS. Mol. Plant Pathol. 3:163-166.
Mittler, R., and Lam, E. 1997. Characterization of nuclease activities and DNA fragmentation induced upon hypersensitive response cell death and mechanical stress. Plant Mol. Biol. 34:209-221.
Moy, P., Qutob, D., Chapman, B. P., Atkinson, I., and Gijzen, M. 2004. Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. Mol. Plant-Microbe Interact. 17:1051-1062.
Nakai, K., and Horton, P. 1999. PSORT: A program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24:34-36.
Nakai, K., and Kanehisa, M. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14:897-911.
Niebel, A., de Almeida Engler, J., Tire, C., Engler, G., Van Montagu, M., and Gheysen, G. 1993. Induction patterns of an extensin gene in tobacco upon nematode infection. Plant Cell 5:1697.
Oelke, L. M., Bosland, P. W., and Steiner, R. 2003. Differentiation of race specific resistance to Phytophthora root rot and foliar blight in *Capsicum annuum*. J. Am. Soc. Hortic. Sci. 128:213-218.
Oh, B. J., Ko, M. K., Kostenyuk, I., Shin, B., and Kim, K. S. 1999. Coexpression of a defensin gene and a thionin-like via different signal transduction pathways in pepper and *Colletotrichum gloeosporioides* interactions. Plant Mol. Biol. 41:313-319.
Oh, I. S., Park, A. R., Bae, M. S., Kwon, S. J., Kim, Y. S., Lee, J. E., Kang, N. Y., Lee, S., Cheong, H., and Park, O. K. 2005. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. Plant Cell 17:2832-2847.
Oh, S. K., Bek, K. H., Park, J. M., Yi, S. Y., Yu, S. H., Kamoun, S., and Choi, D. 2008. *Capsicum annuum* WRKY protein CaWRKY1 is a negative regulator of pathogen defense. New Phytol. 177:977-989.
Park, C. J., Shin, R., Park, J. M., Lee, G. J., Yoo, T. H., and Paek, K. H. 2001. A hot pepper cDNA encoding a pathogenesis-related protein 4 is induced during the resistance response to *Tobacco mosaic virus*. Mol. Cells 11:122-127.
Park, C. J., An, J. M., Shin, Y. C., Kim, K. J., Lee, B. J., and Paek, K. H. 2004a. Molecular characterization of pepper germin-like protein as the novel PR-16 family of pathogenesis-related proteins isolated during the resistance response to viral and bacterial infection. Planta 219:797-806.
Park, C. J., Kim, K. J., Shin, R., Park, J. M., Shin, Y. C., and Paek, K. H. 2004b. Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J. 37:186-198.
Pelegrini, P. B., and Franco, O. L. 2005. Plant gamma-thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. Int. J. Biochem. Cell Biol. 37:2239-2253.
Richins, R. D., Micheletto, S., and O'Connell, M. A. 2010. Gene expression profiles unique to chili (*Capsicum annuum* L.) resistant to Phytophthora root rot. Plant Sci. 178:192-201.
Ristaino, J. B., and Johnston, S. A. 1999. Ecologically based approaches to management of Phytophthora blight on bell pepper. Plant Dis. 83:1080-1089.
Rose, J. K., and Lee, S. J. 2010. Straying off the highway: Trafficking of secreted plant proteins and complexity in the plant cell wall proteome. Plant Physiol. 153:433-436.
Schmelzer, E. 2002. Cell polarization, a crucial process in fungal defence. Trends Plant Sci. 7:411-415.
Schulze-Lefert, P. 2004. Knocking on heaven's wall: Pathogenesis of and resistance to biotrophic fungi at the cell wall. Curr. Opin. Plant Biol. 7:377-383.
Silvar, C. S., Merino, F., and Díaz, J. 2008. Differential activation of defense-related genes in susceptible and resistant pepper cultivars infected with *Phytophthora capsici*. J. Plant Physiol. 165:1120-1124.
Stinzi, A., Heitz, T., Prasad, V., Wiedeman-Merdinoglu, S., Kauffmann, S., and Geoffroy, P. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75:687-706.
Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. 1993. Signal sequence trap: A cloning strategy for secreted proteins and type I membrane proteins. Science 261:600-603.
Thabuis, A., Palloix, A., Pflieger, S., Daubeze, A. M., Caranta, C., and Lefebvre, V. 2003. Comparative mapping of *Phytophthora* resistance loci in pepper germplasm: Evidence for conserved resistance loci across Solanaceae and for a large genetic diversity. Theor. Appl. Genet. 106:1473-1485.
Tyler, B. M. 2007. *Phytophthora sojae*: Root rot pathogen of soybean and model oomycete. Mol. Plant Pathol. 8:1-8.
Ueeda, M., Kubota, M., and Nishi, K. 2006. Contribution of jasmonic acid to resistance against Phytophthora blight in *Capsicum annuum* cv. SCM334. Physiol. Mol. Plant Pathol. 67:149-154.
van der Hoorn, R. A. L. 2008. Plant proteases: From phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. 59:191-223.
van Loon, L. C., Rep, M., and Pieterse, C. M. 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44:135-162.
Walker, S. J., and Bosland, P. W. 1999. Inheritance of Phytophthora root rot and foliar blight resistance in pepper. J. Am. Soc. Hortic. Sci. 124:14-18.
Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. 2005. Induction of protein secretory pathway is required for systemic acquired resistance. Science 308:1036-1040.
Wei, G., and Shirsat, A. H. 2006. Extensin over-expression in *Arabidopsis* limits pathogen invasiveness. Mol. Plant Pathol. 7:579-592.
Yamane, H., Lee, S. J., Kim, B. D., Tao, R., and Rose, J. K. 2005. A coupled yeast signal sequence trap and transient plant expression strategy to identify genes encoding secreted proteins from peach pistils. J. Exp. Bot. 56:2229-2238.
Yi, S. Y., Lee, D. J., Yeom, S. I., Yoon, J., Kim, Y. H., Kwon, S. Y., and Choi, D. 2009. A novel pepper (*Capsicum annuum*) receptor-like kinase functions as a negative regulator of plant cell death via accumulation of superoxide anions. New Phytol. 185:701-715.
Zhu, T., Song, F., and Zheng, Z. 2006. Molecular characterization of the rice pathogenesis-related protein, OsPR-4b, and its antifungal activity against Rhizoctonia solani. J. Phytopathol. 154:378-384.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Center for Biological Sequence Analysis TargetP prediction server: www.cbs.dtu.dk/services/TargetP
Gene Pool server: genepool.kribb.re.kr
National Center for Biotechnology Information: www.ncbi.nlm.nih.gov
Oomycete Genomic databse: www.oomycete.org/ogd
PSORT WWW server: psort.nibb.ac.jp
SignalP3.0 server: www.cbs.dtu.dk/services/SignalP
Sol Genomics Network website: sgn.cornell.edu