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Variation in structure and activity among elicitins from *Phytophthora sojae*

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SUMMARY

Transcripts encoding elicitin-like protein domains were identified from similarity searches of *Phytophthora sojae* expressed sequence tags and were characterized with regard to molecular structure and elicitor activity. The *P. sojae* elicitin family consists of at least nine genes with products similar to previously described elicitins (SOJA-2, SOJB, SOJ2, SOJ3, SOJ5, SOJ6 and SOJ7) or highly diverged from known sequences (SOJX and SOJY). The predicted structural features of seven (SOJA-2, SOJB, SOJ2, SOJ3, SOJ6, SOJX and SOJY) of the elicitin preproteins were compared. All of the predicted elicitins possess a leader signal sequence and a core elicitin domain. Five (SOJ2, SOJ3, SOJ6, SOJX and SOJY) of the characterized elicitins also contain a variable C-terminal region. In addition, SOJX and SOJY contain a C-terminal hydrophobic membrane-spanning domain. An analysis of expression patterns of the elicitin transcripts showed that SOJA-2, SOJB, SOJ2, SOJ3 and SOJ6 were expressed in axenically grown mycelia and during infection, but not in zoospores. In contrast, SOJX and SOJY were predominantly and specifically expressed in zoospores. Selected elicitin domains were also tested for the induction of the hypersensitive response (HR) in *Nicotiana* spp. All of the elicitin protein domains tested induced the HR, except for SOJX and SOJY. Overall, the results show that the *P. sojae* elicitin gene family is large and diverse, with varying patterns of expression and HR-inducing activity.

The elicitins are a group of extracellular proteins that occur in oomycete species, such as *Phytophthora* and *Pythium* (Ponchet *et al.*, 1999). Elicitins were first characterized on the basis of their ability to induce the hypersensitive response (HR) when infiltrated into tobacco leaves. Although different elicitins may vary in their HR-inducing activity, it is now known that most *Nicotiana* species respond to these proteins. Elicitins also induce the HR in a few *Raphanus* and *Brassica* species. In this regard, elicitins are thought to be determinants of the host range for selected plant–*Phytophthora* interactions and may function as species-specific avirulence genes (Kamoun, 2001; Kamoun *et al.*, 1997, 1998, 1999b).

Analyses of protein sequences, either directly or deduced from cDNA sequences, indicate that elicitins comprise a large family of proteins of at least five different classes (Kamoun *et al.*, 1997, 1999a; Ponchet *et al.*, 1999). The intrinsic function of most elicitins remains unknown, but recent advances have led to new proposals. Certain class I-A and I-B elicitins (Kamoun *et al.*, 1997) bind lipids and sterols, and a role has been suggested for these proteins as sterol carriers and lipid transfer proteins (Blein *et al.*, 2002; Mikes *et al.*, 1997; Osman *et al.*, 2001a,b). In addition, elicitin-like proteins have been reported to exhibit phospholipase activity in *Phytophthora capsici* (Nespoulous *et al.*, 1999), and at least one elicitin gene is induced during mating in *Phytophthora infestans* (Fabritius *et al.*, 2002). Overall, it can be hypothesized that the elicitin domain functions in binding a variety of lipid or lipid-like molecules.

To investigate elicitin expression and sequence diversity in the soybean pathogen *Phytophthora sojae*, we searched databases of expressed sequence tags (ESTs) for transcripts encoding proteins with elicitin-like domains. Several different cDNAs encoding elicitin-like proteins were identified from an original collection of over 3000 ESTs from mycelia, zoospore and *in planta* infection sites (Qutob *et al.*, 2000). Representative transcripts encoding full-length open reading frames were chosen for further analyses. Subsequently, additional elicitin-like sequences were identified by further EST sampling and data analysis, as part of the ongoing Phytophthora Genome Consortium (https://xgi.ncgr.org/pgc). A current summary of the *P. sojae* elicitin gene family is provided in Table 1. At least nine elicitin genes are present in *P. sojae*. Seven of the proteins were similar to elicitin sequences previously described in other *Phytophthora* species, and were named SOJA, SOJB, SOJ2, SOJ3, SOJ5, SOJ6 and SOJ7, based on nomenclature systems proposed for elicitins (Kamoun *et al.*, 1997, 1999a). Two of the *P. sojae* proteins contained elicitin-like core domains, but

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Table 1 Inventory of characterized and predicted elicitins from *Phytophthora sojae*

Proposed name	GenBank accession number*	PGC consensus†	Best match‡	Species of best match	*E*-value§
SOJA	AJ007858, AJ007859, AJ007860, AJ007861	CON_012_04847¶	CI16	*P. cinnamomi*	10^{-32}
SOJB	AY183409	CON_011_04829	MGM-B	*P. megasperma*	10^{-31}
SOJ2	AY183410	CON_014_04882¶	INF2B	*P. infestans*	10^{-32}
SOJ3	AY183411	CON_001_08992	INF2B	*P. infestans*	10^{-17}
SOJ5	—	CON_007_04731	INF5	*P. infestans*	10^{-36}
SOJ6	AY183412	CON_013_04866	INF6	*P. infestans*	10^{-32}
SOJ7	—	CON_003_04223	INF7	*P. infestans*	10^{-14}
SOJX	AY183413	CON_001_05452	meg-alpha	*P. megasperma*	10^{-1}
SOJY	AY183414	CON_018_04904	VEX1	*Pythium vexans*	10^{-1}

*The *SOJA* family includes *SOJA-2* (identical to *Sojein2*; AJ007859), and nearly identical sequences *SOJA-1* (*Sojein1*; AJ007858), *SOJA-3* (*Sojein3*; AJ007860) and *SOJA-4* (*Sojein4*; AJ007858) (Becker *et al.*, 2000).

†The Phytophthora Genome Consortium (PGC) consensus identifier for contigs derived from expressed sequence tag (EST) assemblies (https://xgi.ncgr.org/pgc; Waugh *et al.*, 2000).

‡Nearest protein matches were determined by BlastP analysis, using the deduced amino acid translations corresponding to: SOJB, SOJ2, SOJ3, SOJ6, SOJX, SOJY; and for the PGC consensus sequence of SOJA-2, SOJ5 and SOJ7 against the non-redundant protein database available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

§*E*-value from BlastP search.

¶A representative contig identification number is provided in cases in which several closely related contigs have been recognized.

were unlike any sequences in the public databases. These were named SOJX and SOJY.

A phylogenetic tree of the elicitin domains of seven of the *P. sojae* proteins and representatives from other oomycete species is shown in Fig. 1. The *P. sojae* elicitins SOJA-2, SOJB, SOJ2, SOJ3 and SOJ6 are scattered throughout the tree, and cluster with elicitins from various classes and species. The topology of the tree clearly indicates that the *P. sojae* sequences diverged from a common ancestor prior to speciation of the extant *Phytophthora* genera. The SOJX and SOJY elicitin domain sequences were the most divergent, as they did not cluster together nor with any other known elicitin domain. Orthologous sequences from other species have yet to be described for these two proteins.

The complete domain structure of each of the seven *P. sojae* elicitins from the phylogenetic analysis is shown schematically in Fig. 2. The SOJA-2 and SOJB sequences are class I-A and class I-B elicitins, respectively, and comprise only a signal peptide and an elicitin domain. These proteins are similar to the canonical 10-kDa elicitins that were first described and have been well studied in other species. Class I elicitins are typically abundant, soluble, secreted proteins in mycelia cultures, and have the ability to transfer sterols (Mikes *et al.*, 1997) or bind phospholipids (Osman *et al.*, 2001a). The remaining five elicitin proteins, SOJ2, SOJ3, SOJ6, SOJX and SOJY, possess additional C-terminal domains. The SOJ2, SOJ3 and SOJ6 protein sequences include variable threonine- (Thr-), serine- (Ser-), alanine- (Ala-), proline- (Pro-) and aspartic acid (Asp)-rich domains with C-terminal cysteine (Cys) residues. This domain has been proposed to constitute an O-glycosylated cell wall anchor based on a high density of predicted Ser- and Thr-glycosylation sites within the region (Hansen *et al.*, 1998; Kamoun *et al.*, 1997). In SOJ6, the C-terminal domain includes an extended quadra-peptide [Ala-Pro-Thr-Asp/Ser] reiterated motif. A similar repeat pattern occurs in INF6 from *P. infestans*, and in other proteins, such as the *Haemophilus influenzae* haemoglobin-binding protein ($E = 10^{-10}$).

The SOJX and SOJY protein sequences are most distinct. The elicitin domains of these proteins are variable in the spacing of the six conserved Cys residues. Specifically, the elicitin domains of SOJX and SOJY are compressed at their N-terminal and C-terminal ends, with fewer amino acids interspacing the conserved Cys residues in these regions. These changes would narrow and probably close access to the sterol-binding tunnel that is formed by the tertiary structure of the protein (Boissy *et al.*, 1999). The SOJX and SOJY proteins also possess a variable hydrophilic region and a C-terminal membrane-spanning domain, although the C-terminal hydrophilic region contains fewer potential glycosylation sites than SOJ2, SOJ3 and SOJ6. Taken together, the results from the domain structural analysis suggest that elicitins with extended C-terminal domains are cell surface proteins. A terminal membrane-spanning domain may be necessary to anchor zoospore surface proteins, as these propagules lack a cell wall.

The expression patterns of seven of the elicitin proteins were analysed by a comparative representation analysis of corresponding ESTs from the Phytophthora Genome Consortium website (http://xgi.ncgr.org/pgc/). A total of approximately 17 000 *P. sojae* ESTs from mycelia, zoospores and infection sites were searched for matches to each of the seven cDNA sequences encoding the elicitins. In some instances, it was not possible to

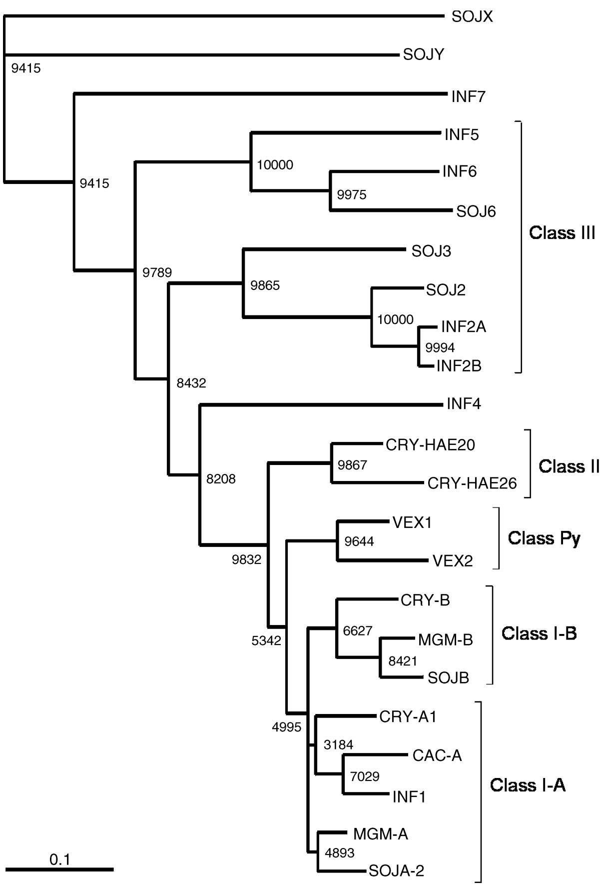
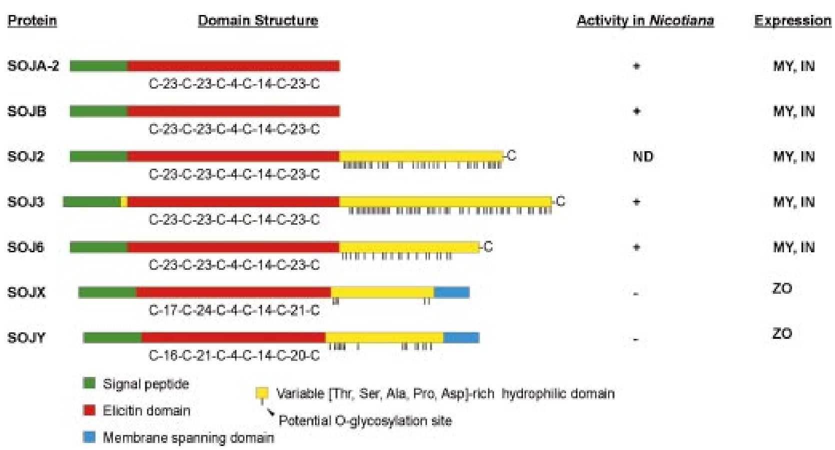


Fig. 1 A phylogenetic tree of elicitin domains from *Phytophthora sojae* and other oomycetes. Protein sequences corresponding to the core elicitin domains without N-terminal transit peptides or C-terminal extensions were compared using the neighbour-joining method of ClustalX 1.81 and were graphically represented using TreeView 1.5.2. Branch lengths represent weighted amino acid substitutions, with the scale bar corresponding to 10% sequence divergence. Bootstrap values from 10 000 replications are shown at branch nodes. Elicitin names correspond to those used in the text and in previous publications (Kamoun *et al.*, 1997, 1999a).

distinguish whether close matches that differed in only a few nucleotides represented identical matches or closely related genes, because ESTs are inherently error prone. Thus, we used an *E*-value cut-off of 10^{-100} as a threshold for this analysis. Two distinct expression patterns were noted and are shown in Fig. 3. The five proteins containing the conserved elicitin domain were exclusively expressed in mycelia and infection sites and were not represented in zoospore ESTs. In contrast, SOJY was predominantly and apparently highly expressed in zoospores. Only 1/25 EST matches to SOJY originated from mycelia transcripts. The one match to SOJX was the original zoospore EST that was identified in the initial elicitin screen. This estimation of expression patterns by EST representation provides clues with regard to tissue specificity and transcript abundance, but requires confirmation by direct measurement of transcript levels by hybridization analysis or reverse transcriptase polymerase chain reaction.

To determine whether the different proteins were active in inducing the HR in *Nicotiana* species, the elicitin domains of SOJB, SOJ3, SOJ6, SOJX and SOJY were cloned into vectors for transient expression in plants. Each tested elicitin domain was first excised from a cDNA template by polymerase chain reaction amplification, fused to an encoded signal peptide sequence from the *Nicotiana tabacum* PR1a protein, and subcloned into binary vectors for the *Agrobacterium*-mediated assays agroinfection and agroinfiltration (Hammond-Kosack *et al.*, 1994; Jones *et al.*, 1999; Qutob *et al.*, 2002; Van der Hoorn *et al.*, 2000). For agroinfection, the PR1a–elicitin constructs were cloned into the potato virus X (PVX) binary vector pGR107, whereas, for agroinfiltration, the constructs were cloned into the cauliflower mosaic virus 35S promoter cassette of pAvr9 (Hammond-Kosak *et al.*, 1994; Jones *et al.*, 1999; Qutob *et al.*, 2002; Van der Hoorn *et al.*, 2000). Representative results are shown in Fig. 4 and summarized in Table 2. The elicitin domains of SOJB, SOJ3 and SOJ6 were active in producing symptoms of HR in leaves of tobacco and *Nicotiana*

Fig. 2 Domain structure of seven different *Phytophthora sojae* elicitin protein sequences. The pattern of spacing between the six conserved cysteine (Cys) residues within the elicitin core domain is shown below each protein. Potential O-glycosylation sites, predicted by NetOGlyc 2.0, are also shown within the C-terminal hydrophilic domain. The activities of the various elicitin domains in inducing hypersensitive response (HR)-like symptoms in *Nicotiana* species are shown as positive (+), negative (–), or not determined (ND). Results are summarized from this investigation or from previous studies for SOJA-2 (Becker *et al.*, 2000; Mao and Tyler, 1996). Expression in mycelia cultures (MY), infected plant tissue (IN), and zoospores (ZO) was determined from the analysis of expressed sequence tags (shown in detail in Fig. 3).



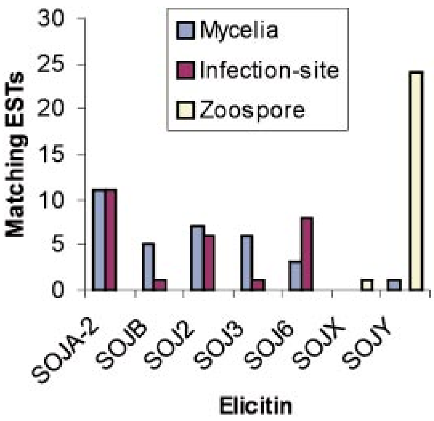


Fig. 3 Expression pattern of *Phytophthora sojae* elicitins determined by comparative expressed sequence tag (EST) representation. A total of 16 931 ESTs from three different cDNA libraries (mycelia, 5851; infected plant, 6121; zoospore, 4959) were analysed for matches ($E = 10^{-100}$) to each of the seven different cDNAs encoding the elicitin proteins shown in Fig. 2.

benthamiana, but the corresponding regions from SOJX and SOJY were not active. It is also known from previous studies that SOJA-2 is active in inducing the HR in tobacco (Becker *et al.*, 2000; Mao and Tyler, 1996). An in-depth comparison of the activities of SOJ3, SOJ6, and SOJX was made on 16 different *Nicotiana* species. The results, summarized in Table 2, demonstrate that all *Nicotiana* species tested consistently responded to the elicitin domains of SOJ3 and SOJ6, but not SOJX. Thus, the activities of the various elicitin domains appeared to depend primarily upon the structural characteristics of the protein and the pattern of spacing of

Table 2 Response of selected *Nicotiana* species to different *Phytophthora sojae* elicitins. Elicitin domains were expressed *in planta* via agroinfiltration and symptoms were scored as: confluent or non-confluent HR (+); no visible HR (–). Control leaves infiltrated with *Agrobacterium tumefaciens* carrying the β-glucuronidase (*GUS*) gene did not display hypersensitive response (HR)-like symptoms

		Response to		
Subgenus/species	Accession*	SOJ3	SOJ6	SOJX
Petuninoides				
N. alata	TW9	+	+	–
N. amplexicaulis	TW10	+	+	–
N. arentsii	TW12	+	+	–
N. bonariensis	TW28	+	+	–
N. cavicola	TW29	+	+	–
N. debneyi	TW44	+	+	–
N. excelsior	TW46	+	+	–
N. pauciflora	TW104	+	+	–
N. rotundifolia	TW114	+	+	–
N. sylvestris	TW136	+	+	–
Rustica				
N. benavidesii	TW15	+	+	–
N. knightiana	TW73	+	+	–
N. raimondii	TW102	+	+	–
Tabacum				
N. glutinosa	TW58	+	+	–
N. otophora	TW97	+	+	–
N. tabacum	TI271	+	+	–

*Based on USDA-ARS National Plant Germplasm System database (http://www.ars-grin.gov/npgs).

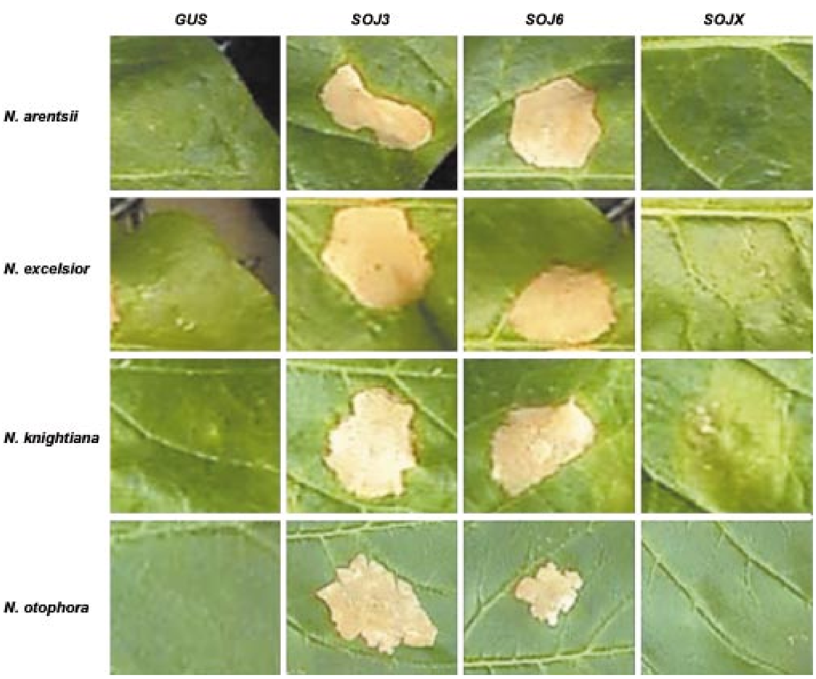


Fig. 4 Agroinfiltration assays of *Phytophthora sojae* elicitins. Fully expanded leaves of *Nicotiana* were infiltrated with *Agrobacterium tumefaciens* strains expressing β-glucuronidase (GUS) (negative control), or the elicitin domains from SOJ3, SOJ6, and SOJX. Photographs were taken 5 days after inoculation. A representative set of *Nicotiana* species is shown. A full list of the tested species is presented in Table 2.

the conserved Cys residues. This result is in accord with recent studies demonstrating that plant recognition of these macromolecules depends upon the formation of an elicitin–sterol complex (Osman *et al*., 2001b). The sterol-binding pockets of SOJX and SOJY are predicted to be constricted or blocked and the elicitin domains of these proteins do not produce any symptoms. Apparently, zoospores have recruited the proteins to contribute to structural or functional roles that may not involve sterol binding.

In general, our observations substantiate past comparisons of elicitins with the plant lipid transfer proteins (LTPs), as LTP-like domains often occur fused to variable Pro-, Ser-, or glycine (Gly)-rich hydrophilic domains in so-called bimodular or hybrid proteins (Castonguay *et al*., 1994). However, in the plant proteins, the variable hydrophilic domain usually occurs in the N-terminal region of the protein, between the signal peptide and the hydrophobic or LTP domain, and may be cleaved off in processing the mature protein (Gijzen *et al*., 1999). Plant LTPs have been implicated in a number of different functions ranging from defence (Broekaert *et al*., 1997; Maldonado *et al*., 2002) to cell adhesion (Park *et al*., 2000). It is also noteworthy that LTP-like proteins with constricted lipid-binding pockets and without any lipid transfer activity have been described (Baud *et al*., 1993; Kader, 1997). Our investigation of *P. sojae* elicitins illustrates the diversity of domain structures present in this family of proteins. Distinct patterns of expression and HR-inducing activity are related to structural characteristics of the proteins. We conclude that the intrinsic functional roles of the various elicitin proteins may be equally diverse, and that a broader view of the gene family will lead to a better understanding of its individual members.

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