

Syringolide elicitor-induced oxidative burst and protein phosphorylation in soybean cells, and tentative identification of two affected phosphoproteins

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Abstract

The bacterial plant pathogen *Pseudomonas syringae* pv. *glycinea* carrying an active allele of avirulence gene D (*avrD*) elicits a hypersensitive defense response (HR) specifically in soybean cultivars carrying the disease resistance gene *Rpg4*. Expression of *avrD* leads to production of a low molecular weight elicitor known as syringolide. Infiltration of syringolide into soybean leaves elicits an *Rpg4*-specific HR and also induces *Rpg4*-specific Ca²⁺, H⁺, and K⁺ ion fluxes and cell death in soybean cell suspensions. We have demonstrated that syringolide treatment of soybean cell suspensions further induces an *Rpg4*-specific hydrogen peroxide burst and subsequent shifts in protein phosphorylation. Furthermore, proteins co-migrating with two affected phosphoproteins (45 and 20 kDa) were collected from preparative 2D-PAGE gels and internal peptide amino acid sequences were obtained. Partial amino acid sequence from the 45 kDa protein showed strong identity to a family of plant EF-hand calcium-binding proteins and sequences from the 20 kDa protein showed strong identity to a family of plant glycine-rich RNA-binding proteins (GRPs). The soybean GRP cDNA was cloned and sequenced.

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1. Introduction

Induction of host-specific resistance in plants depends on the presence of an avirulence (*avr*) gene in the pathogen and a corresponding resistance gene in the host, a phenomenon known as gene-for-gene resistance. Gene-for-gene resistance involves three stages: pathogen recognition, signal transduction, and defense activation. In recognition, the resistance gene protein (R-protein) may either directly recognize (interact with) the pathogen-derived *avr* protein (elicitor) in the plant cell cytosol [1], or the R-protein may recognize the altered structure of a host plant protein targeted by the pathogen *avr* protein, the latter scenario being known as the “guard hypothesis” [2]. In both cases, the second stage of resistance involves transduction of the

recognition signal to the nucleus and to pre-formed enzyme complexes in the cell [3]. The end result of this signaling process is the generation of active defenses which involve a variety of biochemical events, defense gene activation, phytoalexin biosynthesis, generation of reactive oxygen species (ROS), cell wall fortification, and programmed cell death [4], ultimately leading to pathogen containment and/or death.

The syringolide elicitor is produced by the bacterial plant pathogen *Pseudomonas syringae* pv. *glycinea* (*Psg*) strains carrying an active allele of *avr* gene D (*avrD*). Syringolide is the exception to the pathogen *avr* protein (effector protein) rule in plant defense recognition in that whereas pathogen effector proteins are encoded directly by *avr* genes and injected into plant cells via the bacterial type III secretion system [3], *AvrD* appears to encode an enzyme responsible for the production of syringolide, a low molecular weight acyl glucoside [5]. Furthermore, syringolide is believed to enter plant cells by passive diffusion [6,7], where its recognition depends on the *Rpg4* resistance gene in

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soybean [8]. Syringolide, however, binds to a cytoplasmic 34-kDa protein (p34) that is present in both resistant (*Rpg4*) and susceptible (*rpg4*) soybean cultivars [9], suggesting that p34 is the syringolide's target in the plant cell and that the guard hypothesis is active in this system [6].

In soybean leaf tissue, syringolide induces *Rpg4*-specific hypersensitive cell death, as does infiltration of *Psg* carrying an active *avrD* allele into leaf tissue. However, while a variety of signaling mechanisms have been shown to regulate plant defense responses in general, relatively little is known about signaling in the syringolide/soybean system. In general, plant defense responses include plant-specific signaling molecules such as salicylic acid, jasmonic acid, and/or ethylene [10,11] and signaling mechanisms common to both plant and animal systems such as the generation of reactive oxygen species (oxidative burst), ion fluxes, nitric oxide, and protein phosphorylation/dephosphorylation [3,12–15]. Syringolide, however, has only been shown to induce Ca^{2+} , H^+ , and K^+ ion fluxes in an *Rpg4*-specific manner in soybean cell suspensions [7]. We have therefore investigated the ability of syringolide to induce a *Rpg4*-specific oxidative burst and protein phosphorylation.

The oxidative burst, particularly the generation of extracellular hydrogen peroxide (H_2O_2), has been shown to play an essential signaling role in the development of the hypersensitive defense response (HR) [16]. The plant HR has also been associated with, and often shown to require, the activity of various protein kinases. Cloned and characterized protein kinases involved in plant defense include receptor-like *R*-gene kinases—e.g. Pto, Xa21, Xa21D, and Lr10 [17–19], mitogen-activated protein kinases (MAPKs) [20], and the Pto-interacting serine/threonine protein kinase, Pti1 [21]. The involvement of other protein kinases, such as the calcium-dependent protein kinase (CDPK), have been implied in plant defense via biochemical and/or pharmacological approaches (e.g. [22]). In addition, a growing number of defense-associated protein kinase substrates have been identified [23]. These include transcription factors [24,25], a plasma membrane H^+ -ATPase [26], the Arabidopsis AvrB/AvrRpm1-interacting protein RIN4 [27], and protein kinases themselves [20–22,28]. Finally, Peck et al. [29] have recently used a phosphoproteomics approach to identify AtPhos43, an Arabidopsis ankyrin-repeat containing protein that is rapidly phosphorylated following treatment with the fungal and bacterial elicitors chitin and flagellin, respectively.

Here, we demonstrate a syringolide-induced, *Rpg4*-specific H_2O_2 burst and a syringolide-induced, *Rpg4*-specific shift in the phosphorylation status of a number of soybean proteins. Finally, we have used a conservative phosphoproteomics approach to identify two soybean proteins that co-migrate with phosphoproteins whose status is altered in a syringolide/*Rpg4*-specific manner: a plant EF-hand protein and a glycine-rich RNA-binding protein.

2. Materials and methods

2.1. Plant cell culture and inoculation

Soybean cultivars Harosoy (*Rpg4*) and Acme (*rpg4*) (provided by Dr. R.I. Buzzell, Experiment Station, Harrow, Ont., Canada) were grown at 21 °C, 90% RH, with 16 h per day photoperiod. Three to 5 weeks after planting, surface sterilized stem segments were placed on Schenk and Hildebrandt cell culture medium (Sigma–Aldrich Co., St. Louis, MO) with 0.8% agarose supplemented and 2 mg/l 4-chlorophenoxyacetic acid (pCPA), 0.5 mg/l 2,4-D, and 0.1 mg/l kinetin for callus culture. Cell suspension cultures were started with ca. 2 g friable callus in 100 ml of Schenk and Hildebrandt liquid media, supplemented with hormones as above. Liquid cultures were dark grown at 27 °C, shaking at 150 rpm. Cell cultures were maintained by 1:1 (v/v) transfer to fresh medium every 4 days for 40 days, at which time cultures began to lose vigor and elicitor responsiveness [7].

For all experiments, 3–5-day post-transfer cells were captured on miracloth (CalBiochem, La Jolla, CA) and rinsed thoroughly with cell assay solution (CAS): 0.5 mM 2-(4-morpholino)-ethane sulfonic acid (MES)–Tris (pH 6.0), 0.175 M mannitol, 0.5 mM K_2SO_4 , 0.5 mM CaCl_2 [7]. Rinsed cells were resuspended in CAS at a concentration of 1.5 ml packed wet cells (ca. 1.2 g) per 15 ml of CAS and cell assay suspensions were adapted for 2 h at 28 °C under low fluorescent lighting at 150 rpm. Cell assays were carried out under the same conditions. Syringolide elicitor (syringolide 1) was isolated from culture filtrates of *E. coli* cells expressing *avrD* and purified according to Midland et al. [5]. Syringolide was added at the end of the cell adaptation period in 95% ethanol for a final ethanol concentration of 0.1% (v/v), except for the H_2O_2 assays where DMSO was used as carrier at 0.05% final concentration. Syringolide concentration in all assays was 200 μM .

2.2. Quantification of hydrogen peroxide and cell death

Extracellular H_2O_2 was quantified using the scopoletin-based “Procedure 1” from Corbet [30] with the following modifications: (1) reactions were incubated for 10 min prior to addition of potassium borate and (2) the fluorescence was read with excitation wavelength 365 nm and emission wavelength 460 nm. Samples of cell assay medium were collected as the supernatant from 2 ml aliquots of a 45 ml cell assay suspension by centrifugation. Standard curves for H_2O_2 concentration were created with known concentrations of H_2O_2 (2.5–80 μM) in CAS.

Syringolide-induced cell death was measured as percentage of soybean cells that took up Evan's blue dye. A 1 ml aliquot of a 15 ml cell assay suspension was mixed with 1 ml of 0.2% Evan's blue in CAS and incubated at room temperature with gentle agitation for 15 min. Cells were collected over miracloth, rinsed thoroughly with CAS, and resuspended in 0.5 ml CAS. Wet mounts were observed at 40×

magnification and percent Evan's blue stained cells counted from 3×30 cells in three separate fields of view per sample (90 cells per sample). Only cells that were individual or discernible in small clumps of two to six cells were counted.

2.3. ^{32}P -labeling and protein extraction

For in vivo ^{32}P -labeling, 300 μCi [^{32}P]-orthophosphoric acid (9000 Ci/mM HCl-free) (NEN Research Products, Boston, MA) was added to adapted 3 ml cell assay suspensions in disposable 35×10 mm petri dishes 45 min prior to cell collection. Cells from each cell assay suspension were collected over miracloth, rinsed three times with 15 ml fresh CAS, transferred to eppendorf tubes, and snap frozen in liquid N_2 . Cells were processed the same day as collection.

Total proteins were extracted using a modified version of the method of Hurkman and Tamaka [31]. Cells were ground with mortar and pestle under liquid N_2 and 0.75 ml of packed cell powder was resuspended in 1.0 ml of extraction buffer in a 2 ml microcentrifuge tube and immediately mixed with 0.7 ml of water-saturated phenol by vortexing for 50 s. Extraction buffer consisted of 0.7 M sucrose, 0.5 M Tris-HCl (pH 9.0), 50 mM EDTA, 0.1 M KCl, 2% β -mercaptoethanol, 1 \times protease inhibitor cocktail (CalBiochem, La Jolla, CA), 50 mM sodium fluoride, and 50 mM β -glycerophosphate. Tubes were vigorously shaken for 30 min, then centrifuged for 15 min at $16,000 \times g$ at 4°C . The upper phenol phase was transferred and back-extracted once with 0.7 ml extraction buffer as described above. A 0.6 ml aliquot of the resultant phenol phase was mixed with 5.0 ml of -20°C 0.1 M ammonium acetate in methanol (AmMe) and sat 3 h at -20°C for protein precipitation. Precipitated proteins were collected by centrifugation for 15 min at $24,000 \times g$ at 4°C and the pellet was washed first by resuspension in 5.0 ml of -20°C AmMe and centrifugation as above, followed by transfer to a 1.5 ml eppendorf tube in 1.5 ml of -20°C AmMe and centrifugation for 5 min at 4°C at $16,000 \times g$. The resulting pellet was resuspended by vortexing in 1.0 ml of -20°C acetone to remove major lipid contaminants, and pelleted. The final pellet was dried in a speed-vac for 1 min and solubilized overnight in 70 μl of L2 buffer: 9.5 M urea, 2.0% Triton X-100, 5.0 mM K_2CO_3 , 500 $\mu\text{g/ml}$ L-lysine, 35 mM dithiothreitol, 0.16% pH 5–7 ampholines, 0.04% pH 3–10 ampholines (Serva, Heidelberg, Germany).

2.4. Two-dimensional gel electrophoresis and protein sequencing

IEF tube gels (1.5 mm i.d., 14.5 cm long) consisting of 9.2 M urea, 5% acrylamide (29:1 acrylamide/bis-acrylamide), 2% Triton X-100, 1.6% pH 5–7 ampholines, 0.4% pH 3–10 ampholines were pre-run for 15 min each at 200 and 300 V followed by 30 min each at 500 and 700 V, consecutively. Protein samples (500,000 cpm) were loaded and run for 14 h at 700 V followed by 2 h at 1000 V. Gels were extruded, equilibrated for 15 min in equilibration buffer:

2.3% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% glycerol, 62 mM Tris-HCl (pH 6.8), and electrophoresed on $20 \text{ cm} \times 20 \text{ cm} \times 0.1 \text{ cm}$ SDS-PAGE slab gels (4.5% stacking gel, 12.5% resolving gel). Gels were run according to Laemmli [32] at 30 mA per gel. For autoradiography, gels were fixed, dried, and autoradiographed for 24 h at -80°C on Kodak XOMAT-AR X-ray film.

For preparative purposes, proteins were extracted as above, except the procedure was scaled up $5\times$ for 15 ml of packed cells per extraction. Preparative 4 mm \times 14.5 cm IEF tube-gels were pre-run on ice for 30 min at 100, 200, and 350 V, consecutively. Protein samples (1/8 of the preparation to each of eight IEF gels) were fractionated 36 h on ice at 350 V. Second dimension slab gels were 1 mm thick and were run as above, except that a beveled inner plate was used to facilitate protein movement into the stacking gel. Following Coomassie blue R250 staining, gel slices containing the proteins of interest were collected, washed twice for 10 min in 30 ml sterile, distilled water, blotted dry and stored at -20°C . For internal peptide sequencing, the Coomassie-stained proteins from 24 preparative 2D gels were concentrated into a single band by placing the excised gel pieces into a single expanded well of a SDS-polyacrylamide gel and electrophoresed alongside quantity standards of three different proteins. Quantity standards consisted of bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12 kDa) (Sigma-Aldrich Co.) loaded such that each well contained all three proteins in one of the following amounts: 0.1, 0.3, 0.5, 1.0, 2.0, or 5.0 μg . The resultant Coomassie blue-stained proteins of interest were excised and sent to the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University. Briefly, proteins were digested with trypsin and the resultant tryptic peptides were separated by reverse-phase HPLC. Two peptides from each protein sample were subjected to Edman amino acid sequencing.

2.5. PCR and cDNA cloning of GmGRP

The consensus nucleotide sequence of eight dicot GmGRP homologs corresponding to the region of peptide 2 (Fig. 5) was used to design a forward primer ("HP20,RV": 5'-TC-ATCGATATCGCTTTCTCTCAGTACGGCGA-3') for use with an oligo-dT₂₀ reverse primer in anchored RT-PCR. Sequences encoding *EcoRV* and *KpnI* restriction enzyme sites were added on the 5' and 3' ends of the forward and reverse primers, respectively. The RT-PCR reaction mixture consisted of 0.4 mM dNTPs, 200 ng of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2 mM MgCl_2 , 10 mM dithiothreitol, 10 units RNasin, 15 units AMV reverse-transcriptase, 2.5 units Taq polymerase, 100 ng soybean poly(A+)-RNA. Soybean poly(A+)-RNA was isolated from non-treated soybean (cv. Harosoy) cell suspensions with the Ambion Poly(A)Pure mRNA isolation kit according to the manufacturer's instructions (Ambion, Austin, TX). PCR reactions consisted of 45 min at 42°C for cDNA

synthesis followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C, followed by 5 min at 72 °C. The amplified partial GmGRP cDNA was digested with *EcoRV* and *KpnI* and cloned into *EcoRV/KpnI*-digested pBluescript KS+, resulting in pHP20C(ERV).

The partial GmGRP cDNA was used to screen a pBlue-script plasmid-based soybean cDNA library (from soybean cv. Williams 82 cell suspension) provided by Dr. Pedro Miguel Canovas-Impuesto (Salk Institute, San Diego, CA). Library screening was done according to Sambrook et al. [33]. The partial GmGRP cDNA was labeled using random hexanucleotide primers and [α - 32 P]-dCTP according to the procedure of Tabor and Struhl [34]. After hybridization, filters were washed three times for 10 min in 300 ml of 2 \times SSC, 0.1% SDS at room temperature, followed by two washes of 1.5 h each at 68 °C in 1 \times SSC, 0.1% SDS, and once for 30 min at 68 °C in 0.2 \times SSC, 0.1% SDS. Filters were dried and exposed to Kodak X-OMAT-AR film for 16 h at –80 °C. A secondary screen (performed as above) was required to isolate three positive hybridizing colonies from which plasmid DNA was extracted using discontinuous cesium chloride gradient centrifugation according to Sambrook et al. [33]. This resulted in pHP20(2.1), pHP20(4.2), and pHP20(7.1). Nucleotide sequencing used Stratagene T3 and T7 high-temperature primers (Stratagene, La Jolla, CA) for big dye terminator cycle sequencing with analysis on an ABI PRISM 377 automated DNA sequencer (Perkin-Elmer, Norwalk, CT). Because all three cDNAs encoded for the same, complete reading frame, pHP20(2.1) was arbitrarily chosen as representative.

3. Results

3.1. Syringolide-induced hydrogen peroxide burst and cell death

Because H₂O₂ bursts are a common early response in plant defense responses [16], the induction of H₂O₂ production by syringolide was investigated. Furthermore, the development of syringolide-induced hypersensitive cell death in the soybean cell population was tracked over time for comparison to the timing of H₂O₂ production and protein phosphorylation events (see Section 3.2).

The production of extracellular H₂O₂ was investigated following the addition of syringolide to cell suspensions of the elicitor-sensitive cv. Harosoy (*Rpg4*) and the elicitor-insensitive cv. Acme (*rpg4*). Syringolide was added at a final concentration of 200 μ M in the cell assay medium. Furthermore, the concentration of H₂O₂ in the cell assay suspension medium was determined for each experiment by comparison to a standard curve created in parallel to each set of experiments. Fig. 1 shows that syringolide elicited a *Rpg4*-specific H₂O₂ burst in Harosoy cells. Induction of extracellular H₂O₂ was significant by 20 min after addition of syringolide and was sustained for ca. 1.5 h, dropping

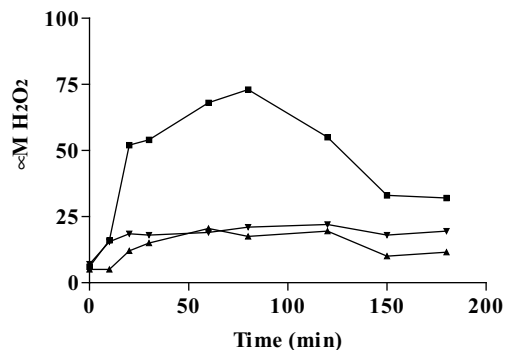


Fig. 1. Syringolide induction of a *Rpg4*-specific oxidative burst in soybean cells. (■) Harosoy cells (*Rpg4*) treated with 200 μ M syringolide, (▼) Acme cells (*rpg4*) treated with 200 μ M syringolide, and (▲) Harosoy cells treated with 0.05% DMSO. Data represent the mean of two independent experiments. The x-axis represents time after addition of syringolide to the cell assay suspension.

to near basal level by 150 min post-treatment. In addition, hypersensitive cell death was also *Rpg4* and syringolide specific, as expected (Fig. 2). Cell death, however, did not become significant until between 1 and 2 h after addition of syringolide. Neither of these responses were observed in syringolide-treated Acme cells (Fig. 1), nor in Harosoy cells treated with ethanol (0.1%) or DMSO (0.05%) carrier alone (data not shown).

3.2. Syringolide-induced, *Rpg4*-specific changes in protein phosphorylation

In order to determine whether or not protein phosphorylation was activated as part of the syringolide-induced defense signaling network, changes in protein phosphorylation were examined in Harosoy and Acme cell suspensions following treatment with the syringolide elicitor. Cells were 32 P-labeled in vivo for 45 min prior to collection, and

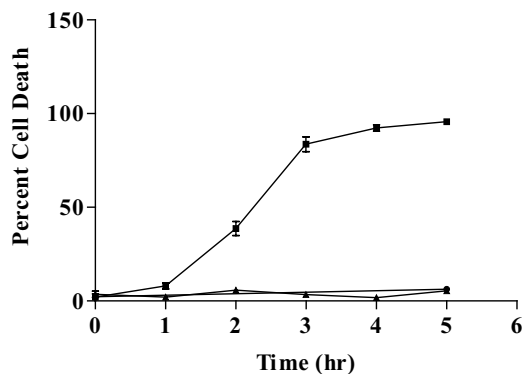


Fig. 2. Syringolide induction of *Rpg4*-specific hypersensitive cell death in soybean cells. (■) Harosoy cells (*Rpg4*) treated with 200 μ M syringolide, (●) Acme cells (*rpg4*) treated with 200 μ M syringolide, and (▲) Harosoy cells treated with 0.1% ethanol. Data represent the mean of three independent experiments; standard error bars are shown. Cell death was determined by cellular uptake of Evan's blue dye. The x-axis represents time after addition of syringolide to the cell assay suspension.

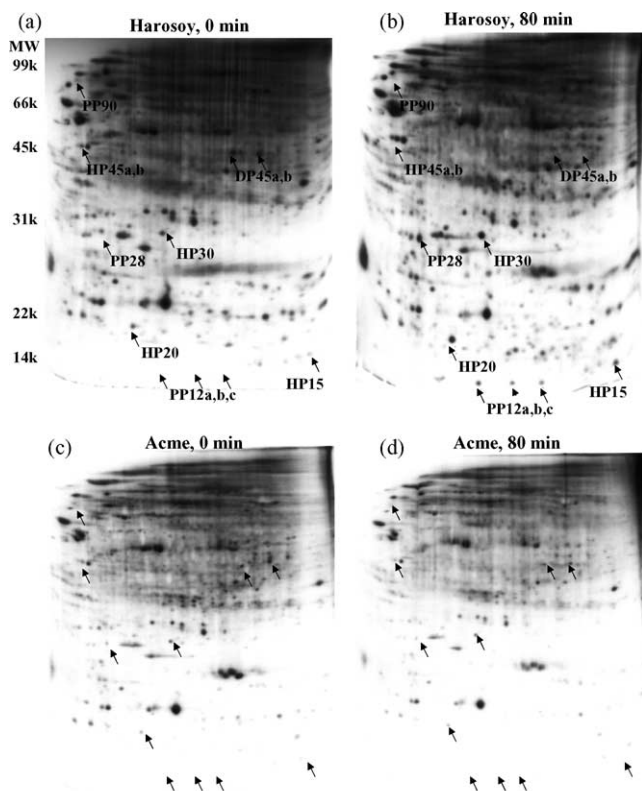


Fig. 3. Autoradiographs of 2D-PAGE ^{32}P -labeled phosphoprotein profiles from soybean cells treated with and without $200\ \mu\text{M}$ syringolide elicitor. The IEF dimension was separated from pH 5.0–7.0. Harosoy cells (*Rpg4*) at 0 min (a) and treated with $200\ \mu\text{M}$ syringolide for 80 min (b). Acme cells (*rpg4*) at 0 min (c) and treated with $200\ \mu\text{M}$ syringolide for 80 min (d). Arrows note phosphoproteins that were consistently affected by syringolide treatment in an *Rpg4*-specific manner. Protein labels in (a) and (b) designate either a phosphorylated protein (PP), hyper-phosphorylated protein (HP), or a dephosphorylated protein (DP) and the approximate M_r of the protein as determined by inclusion of pre-stained M_r standards in the SDS-PAGE dimension (left margin of panel a).

phosphoprotein profiles of total cellular proteins were analyzed by differential two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Syringolide ($200\ \mu\text{M}$) specifically induced the phosphorylation and hyperphosphorylation of ten well resolved phosphoproteins specifically in Harosoy cells (Fig. 3a and b). Following a time course of 5, 10, 15, 20, 40, 60, 80, and 120 min, the phosphorylation and hyperphosphorylation events were first observed at 40 min after addition of elicitor to Harosoy cells, and were sustained for up to 120 min (time course data not shown). Furthermore, syringolide treatment specifically induced the dephosphorylation of two proteins in Harosoy cells (Fig. 3a and b), which were first observed in their dephosphorylated state 60 min after addition of syringolide, and were again sustained in that state up to 120 min post-treatment (time course data not shown). Thus, the phosphorylation and dephosphorylation events occurred after the onset of the H_2O_2 burst, but before the onset of hypersensitive cell death. No consistent changes in protein phosphorylation were observed in elicitor-insensitive (*rpg4*) Acme cells treated with $200\ \mu\text{M}$

syringolide (Fig. 3c and d), nor in Harosoy cells treated with 0.1% (v/v) carrier ethanol (data not shown).

Because reproducibility is a consideration in 2D-PAGE analysis, we wish to note that while the majority of observed phosphoproteins appeared consistently, some inconsistencies were observed among replicated experiments. This is why Fig. 3 shows what appear to be additional, but unnoted, changes in protein phosphorylation in treated Harosoy and Acme cells. However, we have only noted changes in protein phosphorylation that replicated consistently among five independent experiments. Of these reproducible events, proteins that were phosphorylated included ca. 90 kDa (PP90), 28 kDa (PP28), and three 12 kDa (PP12a, b and c) proteins; hyperphosphorylated proteins included two ca. 45 kDa proteins (HP45a and b), a 30 kDa (HP30), 20 kDa (HP20), and 15 kDa (HP15) protein; and the two dephosphorylated proteins were each ca. 45 kDa (DP45a and b). All protein molecular mass estimations were determined by inclusion of protein molecular mass standards in the SDS-PAGE dimension.

3.3. Identification of two soybean defense-associated phosphoproteins

Finally, an important step in understanding the signaling pathways involved in plant defense is the identification of downstream substrate/target proteins. In this regard, we have identified two of the syringolide-affected phosphoproteins as potential, yet unconfirmed, defense-associated protein kinase substrates. Coomassie blue R250-stained preparative 2D-PAGE gels of in vivo labeled soybean cell proteins were aligned with their autoradiographs to identify syringolide-affected phosphoproteins that were both well resolved and of moderate abundance. This conservative approach was chosen in order to simplify the probability of obtaining adequate amino acid (aa) sequence for protein identification. Of the ten phosphorylation/hyper-phosphorylation events noted in Fig. 3, four of the labeled proteins consistently co-migrated with well-resolved Coomassie-staining spots in preparative 2D-PAGE: PP90, HP15, HP45b, and HP20 (data not shown). All four proteins were collected from preparative 2D-PAGE gels, and concentrated by 1D SDS-PAGE into single bands. The final protein sample for HP45b and HP20 were sufficient for in-gel tryptic digestion and Edman aa sequencing of internal peptides.

Following in-gel tryptic digestion, the generated peptides were subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) peptide mass pattern searches. Unfortunately, this approach did not help to identify either of the two submitted proteins. However, two peptide sequences from both HP45b and HP20 showed strong identity to known or predicted plant protein families.

3.3.1. HP45b

Two peptide aa sequences from a protein co-migrating with HP45b (RDPIVILR and RVALDTVAPSAGLPPV-

		<u>Tryptic peptide #1</u>	
GmEFH		RDPIVILR	
Arab.	115	LKRDP	LKRDPIVILRMDGEDLSEFV 134
Pot.	101	LKRDP	LKRDPIVLLRMDGEDLLEFV 120
Rice	127	LKRDP	LKRDPIVILRINGEDLNEFV 146
		<u>Tryptic peptide #2</u>	
GmEFH		RVALD	
Arab.	280	RAVLDT	RVALDTPVAPSAGLPPVGAIEEMDK 303
Pot.	263	RVALD	RVALDVLAPSAGLPPVGAIVDQMDK 286
Rice	319	RVAFDR	RVAFDRMADSINLPPYGAVEQVDA 344

Fig. 4. Line-up of tryptic peptide amino acid sequences from GmEFH with corresponding regions of the predicted Arabidopsis, potato, and rice orthologs. Sequence position within the orthologs is shown numerically. Grey shading designates amino acid identities to GmEFH peptide sequence.

GAIEEMDK) showed strong identity to a family of predicted EF-hand proteins from potato, Arabidopsis, and rice (Fig. 4). Tryptic peptide #1 from the ca. 45 kDa protein showed 100, 88, and 100% aa identity to the corresponding regions of the Arabidopsis, potato, and rice EF-hand proteins, respectively. Peptide #2 showed 67, 75, and 54% identity, respectively. All three predicted proteins contain a pair of EF-hand motifs—which occur functionally in pairs, are involved in calcium binding, and are usually associated with regulation of protein function [35]. Furthermore, the theoretical molecular masses of the three proteins are: Arabidopsis, 41 kDa; potato, 39 kDa; and rice, 46 kDa. Because of the similarity of these three proteins to the submitted protein sample with regard to peptide sequence alignment and estimated molecular mass, the identified protein has tentatively been named GmEFH, for *Glycine max* EF-Hand protein. Because at this stage GmEFH can only be identified as a protein co-migrating with HP45b, however, confirmation that GmEFH is the affected HP45b phosphoprotein needs to be confirmed. It remains possible that GmEFH is co-migrating with a low abundance HP45b phosphoprotein. If the identity of HP45b as GmEFH is confirmed, however,

the identification of a putative Ca²⁺-binding protein as a defense-associated phosphoprotein fits well with the fact that the oxidative burst, other ion fluxes, defense gene transcription, and cell death have all been shown to require an influx of extracellular calcium into elicited cells [3,4].

3.3.2. HP20

Amino acid sequence of two tryptic fragments from a protein co-migrating with HP20 (AFVGGGLA and AF-SQYGEIVETK) showed strong identity to segments of the RNA-binding domain of a large family of plant glycine-rich RNA-binding proteins (GRPs) (Fig. 5). The identified protein has thus been tentatively named GmGRP for *Glycine max* glycine-rich RNA-binding protein. To clone the GmGRP cDNA, a consensus nucleotide sequence corresponding to GmGRP tryptic fragment #2 and based on the eight most closely related dicot homologs, was used as a forward primer for anchored RT-PCR; cDNA derived from poly(A+) mRNA of non-induced Harosoy cell suspensions was used as template. A single ca. 400-bp partial *GmGRP* cDNA was recovered and sequencing confirmed identity to plant GRPs, but the partial cDNA lacked the coding sequence for expected aas 1–26 (data not shown). Using the 400-bp partial *GmGRP* PCR product as a probe, three cDNAs were isolated from a soybean cDNA library. All three cDNAs contained the identical and complete *GmGRP* protein-coding region (GenBank accession no. AF169205) (Fig. 5). Both HP20 peptide amino acid sequences were predicted from the *GmGRP* cDNA sequence.

The *GmGRP* cDNA encodes a protein with significant homology to GRPs from diverse plant species (Fig. 5). In general, GRPs consist of a single RNA recognition motif (RRM) in the N-terminal 85–90 aas, which includes two highly conserved hexa- and octa-meric motifs; the RRM is followed directly by an ca. 70–80 aa glycine-rich C-terminal domain (Fig. 5) [36]. Likewise, GmGRP consists of 160 aas containing a single RRM in the N-terminal 85 aas and a glycine-rich (67% glycine residues) C-terminal domain. Within the RRM, GmGRP shows 80, 80, and 75% aa identity

	<u>Tryptic peptide #1</u>	<u>Tryptic peptide #2</u>	
GmGRP	MASADVEYRCFVGGGLAWATDDHALERAFSQYGEIVETKIINDRETGRSRGFGFVTFASEQ	AFSQYGEIVETK	60
RGP-1c	MAEVEYRCFVGGGLAWATTDRTLGEAFSQYGEVLESKIINDRETGRSRGFGFVTFGDEK		60
GRP7	MASGDVEYRCFVGGGLAWATDDRALETAFQAQYGDVIDSKIINDRETGRSRGFGFVTFKDEK		60
RAB15	MAAADVEYRCFVGGGLAWATSNESENENAFASYGEITLDSKVVITDRETGRSRGFGFVTFSSSEN		60
GmGRP	SMKDAIGAMNGQNLDRNITVNEAQRSGGGGGGGGGGGYNRGGGGYGGRSGGGGGGGGY		120
RGP-1c	SMRDAIEGMNGQDLDRNITVNEAQRSGSGGGGGGGGGFRGGRREGGGYGGGGYGGGRRE		120
GRP7	AMKDAIEGMNGQDLDRSITVNEAQRSGSGGGGGHRRGGGGGGYRSGGGGGYSGGGGSYGG		120
RAB15	SMLDAIENMNGKELDRNITVNEAQRSGGGGGGGGGYGGRRGGGGYGGRRDGGYGGGGGY		120
GmGRP	RSRDGGYGGGGYGGGGGGGGYGGRRDRGYSRGGDGGDGGWRN		160
RGP-1c	GGGGYGGGGYGGRRDRGYSRGGDGGDGGSRYSRGGGDSGDGNWRN		167
GRP7	GGGRREGGGGGYSGGGGGYSSRGGGGGSYGGRRREGGGYGGGEGGGYGGSGGGGGW		176
RAB15	GGRRREGGGGGYGGGGGGYGGRRREGGGGGYGGGGGGWRD		157

Fig. 5. Line-up of the predicted GmGRP cDNA translation product with GRPs from *Nicotiana sylvestris* (RGP-1c), Arabidopsis (GRP7), and maize (RAB15). Grey shading designates amino acid identities to GmGRP. Tryptic peptide sequences from GmGRP are shown at top; the highly conserved hexa- and octa-meric regions of the RRM are underlined.

to GRPs from *Nicotiana sylvestris*, *Arabidopsis thaliana*, and *Zea mays*, respectively. The RRM is highly conserved among species, whereas the glycine-rich domain is more variable [36]. Like HP45b, however, the identity of HP20 as GmGRP remains to be confirmed.

4. Discussion

4.1. Syringolide elicitation of signal transduction events and cell death

We have demonstrated a *Rpg4*-specific syringolide elicitor-induced H_2O_2 burst and shifts in protein phosphorylation in soybean cell suspensions. Furthermore, in this work, two of the syringolide-affected phosphoproteins were tentatively identified as an EF-hand protein and a glycine-rich RNA-binding protein by identification of co-migrating protein spots on 2D-PAGE gels.

As long known for protein phosphorylation, H_2O_2 has recently come of age as a bona fide signaling compound in plant defense and other environmentally-induced plant responses [16]. In this light, the bacterial syringolide elicitor induced a significant and prolonged increase in extracellular H_2O_2 production by 20 min post-treatment that was sustained for ca. 1.5 h. This oxidative burst (OB) is most likely equivalent to the “phase II” *avr* gene-specific OB seen in tobacco and soybean cells treated with avirulent bacteria [37]. While both virulent and avirulent bacteria induce a more transient contact-induced “phase I” OB prior to the phase II burst, the phase I OB is by-passed in our system by use of purified syringolide elicitor. Furthermore, the syringolide-induced OB occurs more rapidly than the bacteria-induced phase II OB because of the requirement for living bacteria to first activate *avr* and *hrp* gene expression following addition to plant cell suspensions [37].

In addition to an oxidative burst, we have shown that following syringolide treatment of soybean cells carrying the *Rpg4* resistance gene (cv. Harosoy), ten proteins were phosphorylated or hyperphosphorylated and two proteins were dephosphorylated (Fig. 3a and b). Soybean cells lacking *Rpg4* (cv. Acme) showed no change in protein phosphorylation in response to syringolide. The phosphorylation and hyperphosphorylation events occurred between 20 and 40 min post-elicitation, and the two dephosphorylation events occurred between 40 and 60 min. The syringolide-induced changes in protein phosphorylation were sustained for at least 1 h in the case of protein dephosphorylation and at least 1.5 h for protein hyperphosphorylation.

At least two further points of interest arise concerning the timing of both the syringolide-induced OB and protein phosphorylation. First, is the timing and sustainability of both signaling events compared to addition of purified elicitor to other plant cell systems. The OB and protein phosphorylation are considered two of the earliest events in response to elicitor, as are ion fluxes, being observed within 1–5 min

after addition of elicitor in some systems [29,38,39]. Therefore, the ca. 20–30 min lag between addition of syringolide to Harosoy cell suspensions and the onset of the OB and protein phosphorylation may sound unusual. This is, however, expected for the syringolide elicitor. Like the induction of the OB and shifts in protein phosphorylation observed in our study, syringolide-induced Ca^{2+} , H^+ , and K^+ ion fluxes also began ca. 30 min after addition of syringolide to soybean cell suspensions [7]. We believe the discrepancy between our system and others is that while many plant elicitor-recognition systems, especially those involved in non-host resistance, utilize plasma membrane-localized elicitor-receptors [38,40], the soybean syringolide-binding protein, P34, is cytosolic [9]. Furthermore, because syringolide appears to readily cross biological membranes, it is likely to require a period of passive diffusion into the plant cell prior to achieving a defense response-activating concentration [6,7]. Finally, while in most systems, the observed changes in protein phosphorylation are transient, those induced by syringolide are sustained for at least 1 h and 20 min. This, however, is also in agreement with the nature of syringolide-induced responses observed by Atkinson et al. [7] where syringolide-induced Ca^{2+} , H^+ , and K^+ ion fluxes were sustained for at least 1.5 h (and in the case of H^+ fluxes, at least 2.5 h) in syringolide-treated Harosoy cells. Both the syringolide-induced OB and protein phosphorylation events proceed the development of hypersensitive cell death, which becomes significant between 1 and 2 h post-elicitation. Cell death likely accounts for the lack of reversal (transience) seen in protein phosphorylation in the syringolide/soybean cell system.

Second, is that the OB proceeds the observed changes in protein phosphorylation in our system. For one, it is known that the OB can induce downstream mitogen-activated protein kinase activity [16]. However, there is a noticeable absence of syringolide-induced protein phosphorylation corresponding to the 38–55 kDa size of MAPKs (HP45b is thought to be an EF-hand protein). In this regard, we have carried out replicated in-gel kinase assays using myelin basic protein (MBP), as well as histone H1 and dephosphorylated casein, as substrate and syringolide-induced and uninduced soybean cell suspensions and leaf tissue extracts as a kinase source (Slaymaker and Keen, unpublished). While this method has successfully demonstrated the activation of MAPKs in other systems [20,41], various time courses covering from 3 min to 3 h after syringolide addition failed to show syringolide-induction of an MBP-, H1-, or casein-kinase activity. Constitutive kinase activities within the expected MAPK molecular mass range of 38–55 kDa, however, were observed.

In addition, the OB in other systems has been shown to require protein phosphorylation and Ca^{2+} influx where pharmacological data using protein kinase inhibitors and calcium channel blockers were used to inhibit an elicitor-induced OB [16,37]. In our system, however, the OB proceeds the observed protein phosphorylation events,

as well as proceeds the timing of syringolide-induced Ca^{2+} influx [7]. It is of course possible that a transient phosphorylation event(s) occurred between the earlier time points in our protein phosphorylation study (5, 10, 15, and 20 min). Furthermore, Atkinson et al. [7] only measured Ca^{2+} influx from the cell assay medium, which is currently the only established means of cytosolic Ca^{2+} regulation in plant defense [3]. It remains possible again, however, that a more rapid intracellular-sourced Ca^{2+} flux could be responsible for the syringolide OB if this OB is indeed Ca^{2+} -dependent [16]. Establishing causal relationships among these syringolide-induced events using pharmacological agents will be an important next step in understanding the syringolide: soybean system.

4.2. Tentative identification of defense-associated phosphoproteins

While fairly extensive experiments are currently analyzing the transcriptome of the plant defense response, the use of proteomics to identify defense-response-induced synthesis or post-translational modifications of proteins has only begun [23]. Prior to this report, only Peck et al. [29] have reported using a proteomics approach to identify phosphoproteins affected during a plant defense response. Thus, to begin to more fully understand the role of protein phosphorylation in the syringolide-induced defense response, we have used a phosphoproteomics approach to tentatively identify two of the syringolide-affected phosphoproteins. Partial amino acid sequence for proteins co-migrating with two affected phosphoproteins were obtained. One set of peptide sequences identified a protein co-migrating with an ca. 45-kDa phosphoprotein (HP45b) as belonging to a family of predicted EF-hand Ca^{2+} -binding proteins from Arabidopsis, potato, and rice (Fig. 4). The gene encoding these proteins appears to occur in single copy in the Arabidopsis and rice genomes and the function of this family of plant-specific EF-hand proteins is not known. Therefore, if GmEFH is confirmed as the syringolide-affected HP45b phosphoprotein, it will be the first suggestion of a role for this class of EF-Hand proteins in plants, and will fit well with the known role of Ca^{2+} in plant defense response signaling.

The presence of a pair of EF-hand motifs and the lack of any searchable enzymatic homology domains in the members of this protein family suggests a regulatory function for these proteins through their presumed ability to bind calcium [35]. It is well known that calcium influxes are an essential component of plant defense signaling and that blocking calcium influx or chelating calcium in the cell suspension medium blocks or mitigates downstream events such the OB, PR gene expression, and other ion fluxes [4,7,42]. Other EF-hand proteins implicated in plant defense signaling include calmodulin isoforms induced in soybean in response to fungal pathogen or elicitor [43], the plant NAPDH oxidase gp91(phox) subunit [44], and the calcium-dependent

protein kinase [22]. It will be interesting to determine if GmEFH and its orthologs in other plant species act independently like calmodulin as a calcium signal-carrier protein, or if these proteins serve as regulatory subunits for one or more multi-protein enzyme complexes. Current work is being carried out to address this and other questions concerning the function of these proteins in plants in general and in plant defense.

The second set of peptide amino acid sequences, from a protein co-migrating with an ca. 20 kDa hyper-phosphorylated protein (HP20), showed strong identity to plant glycine-rich RNA binding proteins. The identity of the co-migrating protein as a soybean GRP was confirmed by subsequent cloning and sequencing of the *GmGRP* cDNA (Fig. 5). In accordance with previously cloned GRPs, the N-terminal 85 amino acids of GmGRP contain a conserved RNA-recognition motif (RRM) followed by a glycine-rich C-terminal 75 amino acids. This class of GRPs are nucleolar proteins which occur in both dicots and monocots, mouse and human cells, and cyanobacteria [36].

The function of GRPs, however, also remain unknown. Other plant proteins that contain one or more RRM include splicing factors, a poly(A)-binding protein, a mitochondrial ribosomal protein, and nucleolin [36]. Nucleolin also contains a glycine-rich C-terminal domain. Based on the shared domains between these proteins and GRPs, and because of GRPs' nucleolar localization, GRPs have been suggested to play a role in RNA processing and/or ribosome assembly [36]. Interestingly, genes encoding GRPs are transcriptionally activated in response to a number of external plant-stress stimuli. Increased GRP transcript levels have been reported during plant responses to chilling, wounding, drought, and pathogenesis, as well as following exogenous application of salicylic acid to tobacco [36]. However, in none of these cases was an increase in GRP protein levels reported. In fact, while transcript levels of the *maize* GRP, MA16, increased following water-deficit, the level of MA16 protein showed no significant change [45]. It would be interesting to know if this pattern consistently occurs in other plant systems where GRP gene transcription is activated.

Finally, a barley GRP (Blt801) is capable of being phosphorylated *in vitro* by the catalytic subunit of protein kinase A [46], and the *maize* GRP, MA16, is basally phosphorylated *in vivo* [47]. Similarly to MA16, HP20 also showed basal level phosphorylation in soybean (Fig. 3a). *In vivo* changes in GRP phosphorylation-status have not been previously reported. However, confirmation that GmGRP is indeed the syringolide-affected HP20 phosphoprotein will be required to definitely demonstrate such a change in our system. In addition, *in vitro* dephosphorylation had no effect on purified MA16's ability to bind poly(G). The latter observation with purified MA16 should be interpreted with caution, however, since MA16 was also shown to occur as part of a heterogeneous multiprotein complex *in vivo* [47]. Thus, it is possible that if GRP protein levels do not change concomitant with increases in gene transcription, GRP activity may

be regulated through stress-induced post-translational modification, and any affect GRP phosphorylation status may have on RNA-binding be mediated through the GRP protein complex. While the potential for a role of GRPs in biotic and abiotic stress responses remains high, a function for these proteins, whether it be in plants, animals, or cyanobacteria, needs to be demonstrated.

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