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Reduced polysome levels and preferential recruitment of a defense gene transcript into polysomes in soybean cells treated with the syringolide elicitor

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Abstract

The bacterial syringolide elicitor induces a hypersensitive defense response (HR) in leaf tissue and cell suspensions of soybean cultivars carrying resistance gene *Rpg*4. To investigate the possible involvement of translational control of gene expression in the syringolide-induced HR, ribosome profiles and polysome-loading of two gene transcripts were investigated. Sucrose density gradient fractionation of soybean cell extracts was used to demonstrate a syringolide-induced, *Rpg*4-specific reduction in large polysomes and a concomitant increase in 80S ribosomes, presumably reflecting an overall inhibition of translation initiation. Syringolide treatment also led to an accumulation of chalcone synthase defense gene mRNA, which remained efficiently loaded into large polysomes in spite of a 22% reduction in cellular large polysome levels. In contrast, while mRNA levels of the housekeeping gene actin remained unchanged following syringolide treatment, the amount of actin mRNA loaded into large polysomes was decreased by 49%. Thus, the translational machinery appeared to selectively exclude a subpopulation of actin transcripts while simultaneously maintaining efficient loading of transcripts for the defense gene chalcone synthase. These data suggest that elicited soybean cells utilize translational control of gene expression during the shift from normal metabolism to defense response activation.

Keywords: Syringolide; HR; Soybean defense response; Elicitor; Translation; Polysome

1. Introduction

Accumulation of defense gene mRNA is a common response of plants to elicitors and pathogen infection (e.g. [1]). However, increased transcription of a gene (or increased mRNA stability) during a stress response does not ensure its translation into a protein product. For example, steady state mRNA levels increased in the absence of protein accumulation for cytosolic ascorbate peroxidase (APX) in TMV-infected tobacco [2], a calmodulin isoform in wounded tomato [3], pyrroline-5-carboxylate reductase in heat and salt stressed Arabidopsis [4], and a glycine-rich RNA-binding protein in water deprived maize [5]. It is therefore important to consider the possibility of translational control when dissecting the components of biotic and abiotic plant stress responses. In fact, many abiotic plant stress responses are likely to involve a substantial cellular inhibition of translation initiation, seen as a reduction in the level of large polysomes and a concomitant increase in 80S ribosomes [6]. Such shifts in the ribosome profile have been seen in plant responses to wounding [3], hypoxia [7,8], and dehydration [9–11]. A reduction in the level of large polysomes in response to abiotic plant stress has also been associated with a shift toward preferential recruitment of stress response gene mRNAs into polysomes, and an associated shift away from the polysome-loading and/or translation of housekeeping genes [4,6,7,9,10], thus

Abbreviations: APX, ascorbate peroxidase; *CHS*, chalcone synthase; HR, hypersensitive defense response

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allowing the cell to focus its resources on mounting an effective and rapid stress response.

The response of soybean cell suspensions to the bacterial syringolide elicitor offers a convenient system to study the possible occurrence of these translational phenomena in a plant defense response. The syringolide elicitor induces an HR specifically in soybean cultivars carrying the resistance gene *Rpg*4. In leaf tissue, syringolide induces hypersensitive cell death [12,13] and the accumulation of chalcone synthase (*CHS*) and phenylalanine ammonia lyase (*PAL*) defense gene mRNAs [14]. In suspension cultures of soybean cells, syringolide induces *Rpg*4-specific hypersensitive cell death [15], ion fluxes (H^+, K^+, and Ca^{2+}) [16], a hydrogen peroxide burst, and protein phosphorylation and dephosphorylation [17]. Furthermore, a cytosolic, specific, high-affinity syringolide-binding protein, P34, has been identified [15,18], and shown to interact with NADH-dependent hydroxypyruvate reductase as part of a proposed light-dependent HR-generating system [19].

Herein, we describe the ability of the syringolide elicitor to induce what is presumably a substantial *Rpg*4-specific inhibition of translation initiation—seen as a reduction in large polysome levels accompanied by an increase in 80 ribosomes. This shift in the ribosome profile occurred independent of hypersensitive cell death. Furthermore, reduced polysome levels were accompanied by both an accumulation of *CHS* mRNA and a preferential maintenance of polysome-association of transcripts for the defense gene *CHS*, in contrast to the simultaneous reduction in polysome-associated mRNA for the housekeeping gene actin. These data suggest a role for translational control in the syringolide-induced soybean defense response, and to our knowledge is the first demonstration of a shift in ribosome profiles and polysome-loading specificity during a plant defense response.

2. Materials and methods

2.1. Plant cell culture and inoculation

Soybean cultivars Harosoy (*Rpg*4) and Acme (*rpg*4) (provided by Dr. R.I. Buzzell) were grown at 21 °C and 90% RH with 16 h per day lighting. Three to five weeks post-planting, stem segments were plated on Schenk and Hildebrandt medium (Sigma–Aldrich Co, St. Louis) with 0.8% agarose, 3% sucrose, and supplemented with 2 mg/l 4-chlorophenoxyacetic acid (pCPA), 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/l kinetin for callus production. Cell suspensions cultures were started with ca. 2 g friable callus in 100 ml of Schenk and Hildebrant liquid media supplemented as above. Liquid cultures were grown in dark at 27 °C with 150 rpm rotation. Initial liquid cultures were transferred 1:1 to fresh medium after 1 week and subsequently transferred 1:1 every 4 days up to 40 days, at which time cultures began to lose vigor and elicitor responsiveness [16].

For all experiments, 3–5 day post-transfer cells were captured over miracloth and rinsed three times with cell assay medium (CAS): 0.5 mM MES (pH 6.0), 0.175 M mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$ [16]. Cells were resuspended in CAS at a concentration of 1.5 ml packed wet cells per 15 ml CAS (ca. 1.2 g wet cells/15 ml) and cells adapted 2 h at 28 °C, 150 rpm, under laboratory lighting before syringolide treatments (added as 1/1000th of the cell assay volume in 95% ethanol—final ethanol concentration 0.1%).

2.2. Cell death assays

Treated assay suspensions were collected over miracloth and resuspended in 0.125% Evan's Blue in CAS, shaken 15 min at room temperature, recollected over miracloth, and ca. 300 μl of packed cells suspended in 1 ml CAS. Forty microlitres of suspension were viewed using a Leitz Wetzlar 6.3× dissecting microscope and six randomly chosen sets of 10 unclumped cells were counted for stained and unstained cells.

2.3. Polysome profile analysis

Treated assay suspensions were collected over miracloth, snap frozen, and ground under liquid nitrogen with mortar and pestle. Packed cell powder (1.25 ml) was suspended in extraction buffer (EB) consisting of 0.2 M Tris–HCl (pH 9.0), 0.2 M KCl, 0.025 M EGTA (pH 8.3), 0.035 M $MgCl_2$, 100 μM β-mercaptoethanol, 50 μg/ml cycloheximide (CH), 50 μg/ml chloramphenicol (CP), and 0.5 ml of 45 °C 20% detergent mix (1%) (for extraction of membrane-associated polysomes). Detergent mix contained 20% Brij-35 (w/v), 20% Triton X-100 (v/v), 20% NP-40 (v/v), 20% Tween 40 (v/v). The suspension was thawed on ice and centrifuged 45 s at high speed in a microcentrifuge. Supernatant (300 μl) was loaded onto a 5 ml semi-continuous 15–60% sucrose gradient prepared in a 13 mm × 51 mm polyallomer tube (Beckman) by thawing from −80 °C to 4 °C overnight a pre-made, frozen sucrose gradient consisting of 0.75 ml 15%, 1.5 ml 30%, 1.5 ml 45%, and 0.75 ml 60% sucrose steps, containing 0.04 M Tris–HCl (pH 8.4), 0.02 M KCl, 0.01 M $MgCl_2$, 50 μg/ml CH, and 50 μg/ml CP. Gradients were spun for 80 min, 4 °C at 50,000 × *g* in a SWTi55.1 rotor (Beckman/Coulter, Fullerton, CA). Polysome profiles were read at UV_{254} on an ISCO gradient fraction collector. Raw data was collected for graphic and quantitative analysis in Excel using a software package created by Alan Williams (Department of Botany and Plant Sciences, University of California, Riverside).

2.4. RNA preparation from enriched large polysomes

Fifty ml of packed suspension cells (ca. 50 g) were placed in 500 ml of CAS, treated, recollected, and snap frozen in

liquid nitrogen (N_2). For enriched large polysome extraction, cells were powdered under liquid N_2 with mortar and pestle and the frozen homogenate stirred into 100 ml of ice-cold EB supplemented with 0.4 M KCl instead of 0.2 M KCl. The suspension was passed through miracloth and the filtrate stirred for 15 min on ice after addition of 5 ml of detergent mix. The resulting solution was centrifuged at 10,000 × *g* for 20 min, 4 °C; this supernatant was collected and spun again at 10,000 × *g* for 10 min, 4 °C. Supernatant (55 ml) was loaded onto each of two 15-ml sucrose cushions in 70 ml polycarbonate bottle assemblies. Sucrose cushions consisted of 0.04 M Tris–HCl (pH 9.0), 0.2 M KCl, 0.005 M EGTA, 0.03 M $MgCl_2$, 1.75 M sucrose (58.85 g/100 ml), 5mM DTT, 50 μg/ml CH, and 50 μg/ml CP. Sucrose cushions overlaid with sample were centrifuged 4 h at 35,000 rpm in a Beckman Type 35 rotor at 4 °C.

Following removal of the supernatant and sucrose cushion by aspiration, the resulting pellets which contained enriched large polysomes were washed quickly with ice-cold sterile distilled water and allowed to loosen for 30 min on ice in 150 μl of resuspension buffer (RB) consisting of 0.04 M Tris–HCl (pH 8.4), 0.2 M KCl, 0.005 M EGTA, 0.03 M $MgCl_2$, 50 μg/ml CH, and 50 μg/ml CP. Collected pellets were pooled with one additional 100 μl RB rinse per tube, and the final pool was centrifuged 1 min in a microcentrifuge. Supernatant (300 μl) was loaded onto 5-ml semi-continuous sucrose gradients which were run as for polysome analysis except that each polysome profile was collected in a series of 13 nine-drop fractions into an equal volume (440 μl) of TES: 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, 1% SDS, 0.5 mg/ml heparin. Fractions with TES were mixed, frozen in dry ice, and stored at −80 °C for up to 1 week before RNA extraction. RNA was extracted from each fraction using the phenol:-chloroform:isoamylalcohol method and total RNA from each fraction was run in formaldehyde agarose gels according to Sambrook et al. [20].

2.5. RNA blots and hybridizations

Total RNA was extracted directly from cell assay suspensions using the Trizol method and separated in formaldehyde agarose gels as for polysomal RNA. Northern blotting, DNA probe synthesis, and hybridizations were according to Sambrook et al. [20]. DNA used for probe synthesis were 1) a 4.0-kb fragment containing the soybean actin genomic clone (*SAc*6), kindly provided by Dr. R.B. Meagher (Department of Genetics, University of Georgia) and 2) a 1.7-kb *Sst*I-*Hind*III cDNA clone of soybean chalcone synthase (*Gmchs*1), kindly provided by Dr. Shyam Dube (Center for Agricultural Biotechnology, University of Maryland). Following hybridization, membranes were washed twice for 10 min at room temperature in 2× SSC, 0.1% SDS, 30 min at 37 °C in 0.2× SSC, 0.1% SDS, and 30 min at 67 °C in 0.2% SSC, 0.1% SDS. All blots were analyzed using the BioRad (Hercules, CA) Personal FX phospoimager and BioRad QuantityOne imaging software.

3. Results

3.1. Syringolide-induced changes in the ribosome profile

The effect of the syringolide elicitor on the ribosome profile was examined using elicitor-sensitive (*Rpg*4 cv. Harosoy) and elicitor-insensitive (*rpg*4 cv. Acme) soybean cell suspensions. Ribosome profiles were obtained by separating crude cell extracts over sucrose density gradients

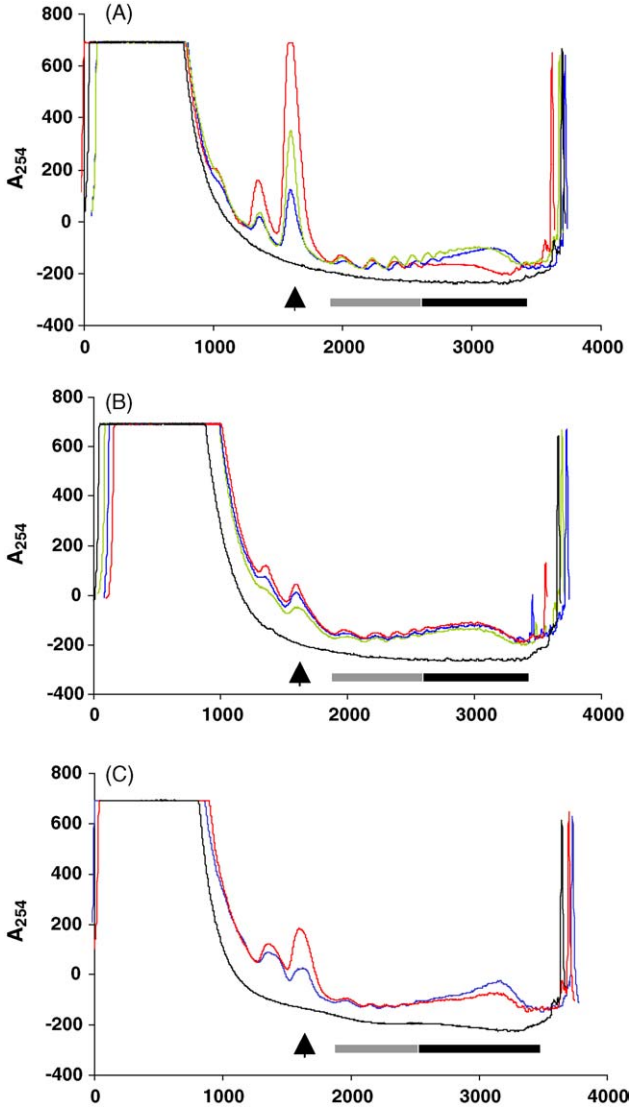

Fig. 1. A_{254} profiles of ribosome pools separated over sucrose density gradients. Arrow denotes 80S ribosomes, grey bar denotes small polysomes, black bar denotes large polysomes. (A) Harosoy cells treated with 200 μM syringolide for 0 h (green) and 3 h (red), or with ethanol (0.1%, v/v) for 3 h (blue). (B) Acme cells treated as in (A). (C) Harosoy cells treated for 3 h with 5 μM syringolide (red) or ethanol alone (blue). Sucrose density gradient blanks run with extraction buffer only are shown in black. Data are representative of three independent experiments. The *x*-axis represents time in 55 ms increments; *y*-axis represents arbitrary absorbance units at 254 nm.

and quantitatively analyzing fractions from these gradients using A_{254} and peak area calculations (Figs. 1 and 2A–C). Treatment of Harosoy cell suspensions with 200 μM syringolide led to a 63% decrease in the level of large polysomes (six or more ribosomes per message) and a corresponding 128% increase in 80S ribosomes 3 h after addition of elicitor, as compared to ethanol treated control cells (0.1%, v/v) (Figs. 1A and 2A). A less dramatic decrease in small polysomes (two to five ribosomes per message) was observed in the majority of experiments (Fig. 2A). In contrast, no shift in the ribosome profile was observed in soybean cv. Acme cell suspensions treated with syringolide, nor with ethanol alone (Figs. 1B and 2B).

The syringolide elicitor also induces hypersensitive cell death in Harosoy cell suspensions [15]. Thus, it is important to demonstrate that the observed inhibition of polysome formation is not the result of cell death per se, particularly since 87% of Harosoy cells showed Evan's Blue staining, an indicator of cell death, 3 h after elicitation with 200 μM syringolide (Fig. 2D). To address the relationship between cell death and polysome levels, Harosoy cells were treated with 5 μM syringolide and assessed for both cell death and for shifts in the ribosome profile. In contrast to the 200 μM treatment, cell viability was not affected 3 h after treatment with 5 μM syringolide (Fig. 2D). However, by 3 h the 5 μM syringolide treatment did induce a 22% reduction in the level of large polysomes, and a concomitant 40% increase in the level of 80S ribosomes (Figs. 1C and 2C). Furthermore, the ribosome profile did not shift beyond the 3 h level even with the onset of cell death that occurred 6 h post-treatment with 5 μM syringolide (Fig. 2D), demonstrating that the 22% reduction in large polysome levels occurred prior to, and independent of, hypersensitive cell death.

3.2. Steady state mRNA accumulation and recruitment of specific gene transcripts into polysomes following syringolide treatment

Following the observation of a defense-associated reduction in large polysomes, we next wanted to determine whether this shift was accompanied by preferential recruitment of defense-gene transcripts into polysomes. To assess this possibility, the steady state mRNA level for chalcone synthase (*CHS*), a known defense response gene, and actin (*SAc6*), a presumed housekeeping gene, were investigated following treatment of Harosoy cells with 5 μM

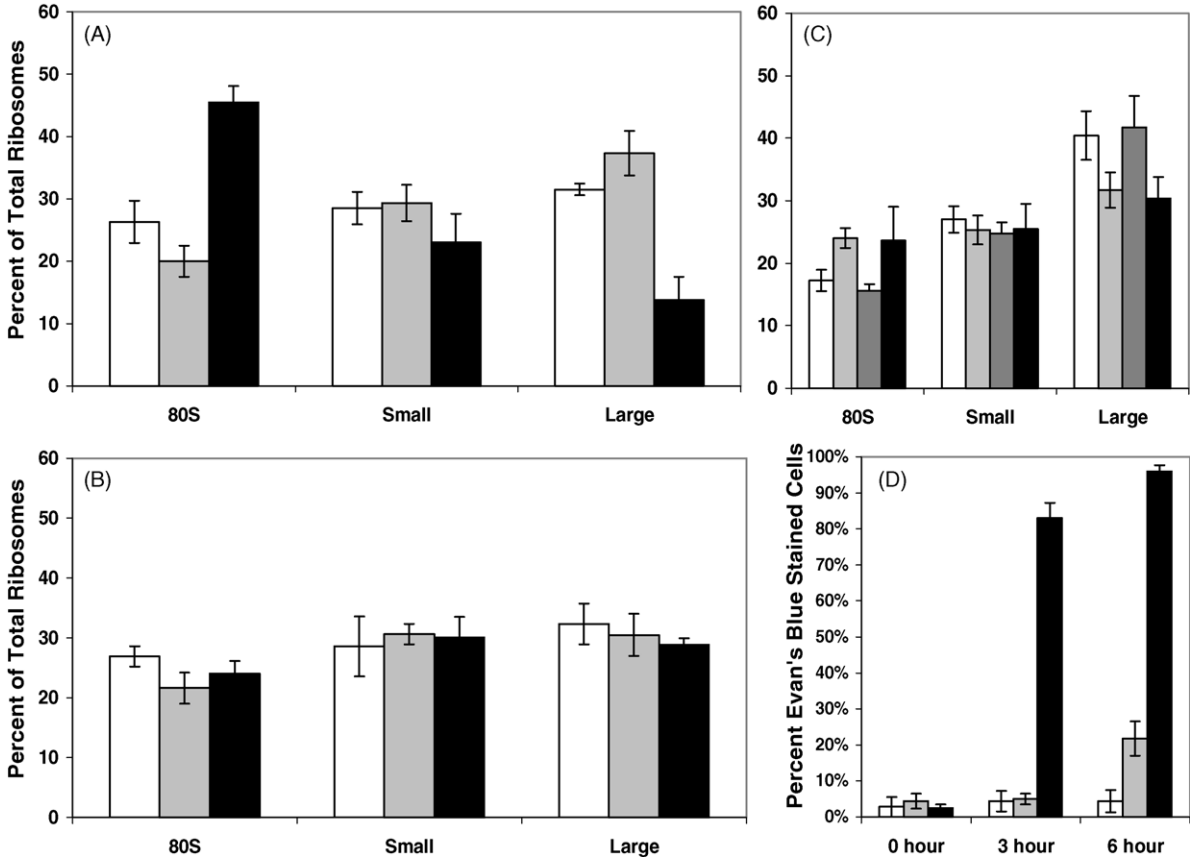

Fig. 2. Quantitative analysis of syringolide's effect on ribosome profiles and soybean cell death. (A–C) *80S* monosome and *small* and *large* polysome levels in Harosoy cells (A) and Acme cells (B) after 0 h (white), 3 h with ethanol (0.1% v/v) (grey), or 3 h with 200 μM syringolide (black). (C) Harosoy cells after 3 h with ethanol (white), 3 h with 5 μM syringolide (light grey), 6 h with ethanol (dark grey), or 6 h with 5 μM syringolide (black). (D) Evan's Blue staining of Harosoy cells as a measure of cell death after treatment with ethanol (0.1%, v/v; white), 5 μM (grey), or 200 μM (black) syringolide. All values represent the mean and standard error of three independent experiments.

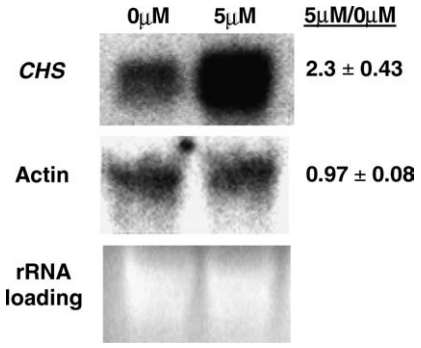

Fig. 3. Northern analysis of total RNA extracted from Harosoy cells treated for 6 h with 0 μM (ethanol alone) or 5 μM syringolide. RNA blots were hybridized with probe for soybean chalcone synthase (*CHS*) and actin. The ratios of steady state mRNA levels in treated over control cells (5 μM/0 μM) represent data from three independent experiments. Equal rRNA loading is shown by parallel ethidium bromide staining. Data were analyzed on a BioRad Personal FX phosphoimager using BioRad Quantity One® image-analysis software.

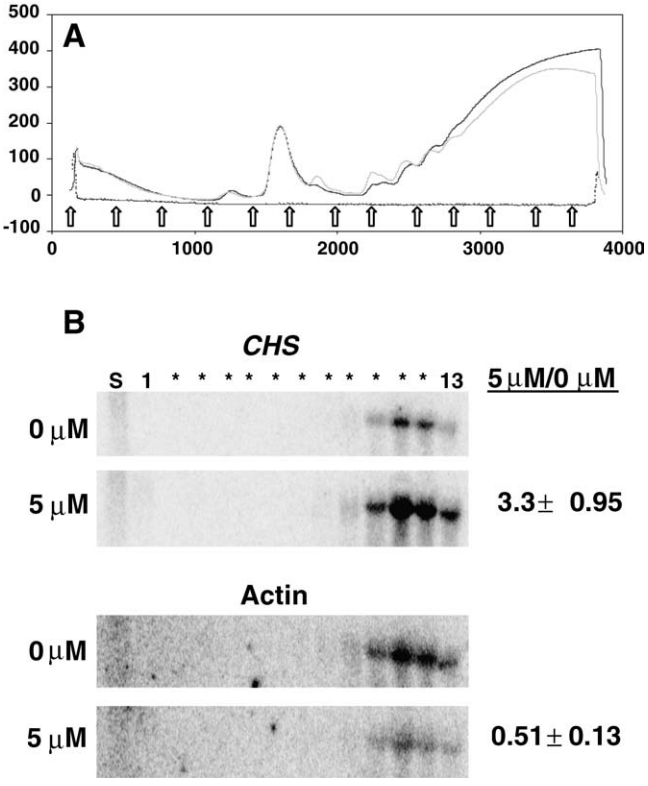

Fig. 4. (A) Representative A_{254} profiles of enriched polysomes fractionated for RNA extraction. Profiles from Harosoy cells treated for 6 h with ethanol alone (black) or with 5 μM syringolide (grey) are shown. Arrows denote approximate start points for collection of fractions 1 (left most arrow) to fraction 13 (right most arrow). Fractions 10–13 contain large polysomes. (B) Northern analysis of RNA extracted from fractions noted in (A). RNA blots were hybridized with probe for soybean chalcone synthase (*CHS*) and actin. "S" represents lane 0 which contained RNA MW markers; lanes 1–13 correspond to consecutive sucrose gradient fractions. Quantitative data represent the ratio of the sum signal density from lanes 10–13 in three independent experiments. Data were analyzed on a BioRad Personal FX phosphoimager using BioRad Quantity One® image-analysis software.

syringolide (Fig. 3). As expected from studies in leaf tissue [14], syringolide treatment led to a 2.3-fold increase in *CHS* mRNA compared to control cells. Actin mRNA levels were unaffected by syringolide treatment (Fig. 3). To investigate the possibility of selective recruitment of these mRNAs into polysomes in response to elicitor, enriched large polysomes were fractionated over a sucrose gradient and total RNA extracted from gradient fractions (Fig. 4A). Specific mRNA levels were then measured by Northern blot analysis (Fig. 4B). In control Harosoy cells treated with ethanol alone, both *CHS* and actin mRNAs were efficiently loaded into large polysomes (Fig. 4B). Following syringolide-treatment, levels of polysome-associated *CHS* mRNA increased 3.3-fold compared to control cells (Fig. 4B), an increase which exceeded the accumulation of *CHS* mRNA. In contrast, while the level of actin mRNA remained constant in syringolide-treated cells (Fig. 3), the level of polysome-associated actin mRNA was reduced by 49% (Fig. 4B), suggesting that approximately half of the actin transcripts present are selectively excluded from polysomes as a result of syringolide treatment. In light of the reduced association of actin mRNA with polysomes, the maintenance of efficient polysome-loading of *CHS* mRNA in the presence of a 22% overall reduction in large polysomes suggests that *CHS* expression in these cells is controlled both by an increase in transcription/mRNA stability (Fig. 3) and by the maintenance of efficient translational initiation/re-initiation evidenced by an increase in polysome-association (Fig. 4B).

4. Discussion

The syringolide elicitor induces a hypersensitive plant defense response specifically in soybean cultivars carrying the *Rpg*4 resistance gene. We have here shown that this defense response includes a substantial, *Rpg*4-specific reduction in large polysome levels, as well as an increase in the level of 80S ribosomes, presumably reflecting an inhibition of translation initiation [6]. Importantly, this shift in the translational machinery occurred independently of hypersensitive cell death induced by the syringolide elicitor. Thus, the shift in the ribosome profile appears to be a bona fide response of *Rpg*4 soybean cells to the syringolide elicitor. To our knowledge this is the first demonstration of shifts in the ribosome profile occurring in association with a plant defense response. Similar shifts, however, have been seen in plant stress responses to wounding [3], hypoxia [7,8], and dehydration [9–11], as well as in non-stress responses to gravistimulation [21] and light:dark transition [22]. Whether the syringolide-induced shifts reflect reduced translation initiation for a broad range of gene transcripts, or for a limited number of abundant transcripts remains unclear.

The shift in translational machinery induced by syringolide was accompanied by an apparent selectivity for maintaining efficient loading of *CHS* defense gene

transcripts into polysomes while a subset of mRNAs for the housekeeping gene actin were excluded from polysomes. Reductions in large polysome levels resulting from abiotic stress have also been accompanied by a preference for the recruitment of stress response-related gene transcripts into the remaining large polysomes [4,7,9,10,21–23]. Because *CHS* is an important rate-determining enzyme in the glyceollin phytoalexin biosynthetic pathway, a preference for polysome-associated *CHS* transcripts over those of actin is not surprising. Elicited cells are expected to gain an advantage by focusing available resources on the immediacy of stress adaptation [24]—the above results reflecting a focus on phytoalexin production to help combat a perceived bacterial pathogen.

Interestingly, actin mRNA levels were reduced in soybean cells following treatment with the elicitor glutathione or with the protein phosphatase inhibitor okadaic acid, both of which induced *CHS* mRNA accumulation [25]. These results, along with our own, may represent a common regulatory relationship between *CHS* and actin in plant cells responding to biotic (or simulated biotic) stress, even if the regulatory mechanism differs in response to distinct stress stimuli. Reduced levels of polysome-associated actin transcripts were also seen in oxygen-deprived maize roots, where polysome-loading of hypoxic stress-related gene transcripts was increased [7]; *CHS*, however, was not observed in that study. Furthermore, while actin is often represented as a housekeeping gene in plant stress response studies (e.g. [7,26]), and was so used in the present study, the rearrangement of actin filaments has been shown to be required for resistance to some fungal pathogens [27,28]. The apparent down-regulation of actin gene expression may play a role in such re-arrangements, and thus be an integral part of the plant defense response. It therefore remains an interesting question whether syringolide's affect on actin expression in soybean is really the presumed down-regulation of house-keeping function, or represents an active component of the defense response.

Finally, increased polysome-loading of mRNA for defense-gene PR1 and the defense-associated gene APX occurred during the TMV-induced defense response in tobacco [2]. As in the syringolide:soybean system, the increase in polysome-associated transcript for both of these genes paralleled steady state mRNA accumulation. However, while PR1 protein did accumulate, APX protein levels fell, suggesting that translation elongation was the rate limiting step in APX synthesis during TMV-induced HR [2]. This also demonstrates that the level of polysome-associated mRNA does not necessarily reflect the level of protein synthesis. In order to gain a more complete picture of the level and breadth at which translational control is involved in the syringolide:soybean system, it will therefore be important to determine how *CHS* and actin protein levels are affected by syringlide, as well as to assess the translational status of additional genes.

In conclusion, a substantial reduction in cellular large polysome levels and the selective maintenance of polysome-recruitment for a defense gene transcript are regulatory events associated with the syringolide-induced soybean defense response. The mechanisms that may be involved in these translational shifts, while not yet defined, are likely complex. For example, in hypoxically stressed maize roots, a drop in large polysome levels and preferential loading of stress–response gene transcripts were accompanied by changes in ribosomal protein quantity, electrophoretic mobility, and protein phosphorylation status [7,8]. Initiation and the specificity of translation could further be affected by changes in the composition of the mRNA 5′-cap-binding complex, the ribosome proper, the complex that mediates 5′-cap:3′-poly(A)-tail interactions, and/or the level or regulatory status of proteins which bind to the 5′ untranslated region of mRNAs [6,29]. How the level and modification-state of such proteins may be affected by the syringolide elicitor in soybean cells, and what role they may play in syringolide's ability to shift the ribosome profile and affect polysome-loading selectivity, remains to be determined.

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