

Cytokinin regulates the expression of a soybean β -expansin gene by a post-transcriptional mechanism

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Received 26 August 1997; accepted in revised form 16 December 1997

Key words: cytokinin, expansins, mRNA stability, pollen allergen, protein phosphatase, soybean

Abstract

The cytokinin-inducible soybean mRNA Cim1 accumulates 20–60-fold upon cytokinin addition to cytokinin-starved soybean suspension cultures. In this report, we demonstrate that cytokinin-induced stability of the Cim1 mRNA plays an important role in the accumulation of the message. We also present evidence that cytokinin-induced Cim1 stability is blocked by the addition of the protein phosphatase inhibitor okadaic acid. Thus, we suggest that protein phosphatase activity is required for the cytokinin-induced stability and subsequent accumulation of Cim1 in soybean cells. The deduced amino acid sequence of the Cim1 protein product is similar to the group I pollen allergens from various plants, which constitute a subfamily of expansin proteins. The relatedness between Cim1 and the expansins supports our hypothesis that the protein product of Cim1 is localized to the cell wall and suggests a role for Cim1 in cytokinin-regulated cell wall expansion. Thus, post-transcriptional regulation of Cim1 by cytokinin may represent a molecular link between cytokinin and changes in cell shape and size.

Introduction

Cytokinin is a growth substance that is involved in a multitude of physiological and developmental processes in plants, such as chloroplast development [35], shoot organogenesis [34], lateral bud release [29], delayed tissue senescence [28] and cell division [27]. Despite the wide range of physiological effects ascribed to cytokinin, a detailed understanding of the molecular basis of cytokinin action is lacking.

Cytokinin induces the accumulation of certain plant mRNAs and proteins and causes a decline in the levels of others [6]. Cytokinin-inducible mRNAs include those encoding an early nodulin (SrEnod2) from *Sesbania rostrata* [8], the light-harvesting chlorophyll *a/b*-binding protein [15, 24, 38], the small subunit of Rubisco [12, 15], a 'multiple stimulus response' protein (*msr1*) from *Nicotiana glauca* [11], and nitrate reductase [9, 10, 21, 23].

The roles of both transcriptional and post-transcriptional mechanisms in cytokinin-induced mRNA accumulation have been examined. For

example, cytokinin-induced accumulation of *msr1* mRNA [11] and cytokinin-enhanced light induction of nitrate reductase mRNA [25] have been shown to be the result of increased rates of transcription. Cytokinin-responsive promoters have provided further evidence of cytokinin-regulated transcription of plant genes [2, 22]. However, it has become evident that post-transcriptional mechanisms are also involved in cytokinin regulation of plant gene expression [1]. These conclusions are based on comparative analyses of rates of mRNA synthesis (nuclear run-on transcription) and mRNA accumulation (northern analysis) or on direct measurements of mRNA half-lives in the presence of potent inhibitors of transcription (cordycepin or actinomycin D) [17, 37]. Flores and Tobin have shown that the cytokinin-induced accumulation of the mRNAs encoding the light-harvesting chlorophyll *a/b*-binding protein and the small subunit of Rubisco occurs primarily by a post-transcriptional mechanism in *Lemna gibba* [15, 16]. Recently, mRNA stability was also found to be involved in cytokinin-induced accumulation of SrEnod2 mRNA [33].

Changes in protein phosphorylation have been shown to be involved in various cytokinin signal transduction processes. Inhibitors of protein phosphatases (okadaic acid) and protein kinases (staurosporine) [13, 19, 36] have provided pharmacological evidence of a role for protein phosphorylation/dephosphorylation in a variety of molecular responses to cytokinin. For example, okadaic acid stimulated the accumulation of *msr1* mRNA, whereas staurosporine blocked cytokinin- and auxin-induced *msr1* mRNA accumulation [11]. In contrast, cytokinin induction of *SrEnod2* mRNA accumulation was blocked by both okadaic acid and staurosporine [33]. The involvement of protein phosphorylation/dephosphorylation in cytokinin-mediated phenomena is supported by the observation that changes in the levels of various protein kinases have been observed in cytokinin-treated plants and plant cells. For example, the addition of cytokinin and auxin to *Nicotiana tabacum* pith parenchyma caused the accumulation of a p34^{cdc2}-like protein with histone H1 kinase activity [41]. However, the addition of auxin alone induced the accumulation of a highly phosphorylated, inactive form of the enzyme. Treatment of auxin-induced cells with cytokinin, or addition of yeast protein phosphatase *cdc25* *in vitro*, resulted in the dephosphorylation and activation of the p34^{cdc2}-like protein [41]. In addition, cytokinin-independent shoots from *Arabidopsis thaliana* were recently generated by activation T-DNA tagging and shown to over-express a gene (*CKI1*) encoding a protein with similarities to bacterial two component system proteins, including a region with homology to histidine kinases [20]. The transcript encoding a wheat protein kinase, P58^{wpk4}, was also shown to accumulate in response to cytokinin [30]. These observations suggest that protein phosphorylation/dephosphorylation is intimately involved in multiple aspects of cytokinin signal transduction.

We are investigating the cytokinin-induced soybean mRNA *Cim1* [7]. *Cim1* abundance increases 20–60-fold within four hours of cytokinin addition to cytokinin-starved soybean suspension cultures. *Cim1* accumulation is stimulated in the absence of cytokinin by staurosporine and inhibited in the presence of cytokinin by okadaic acid, suggesting a role for protein dephosphorylation in cytokinin regulation of *Cim1* abundance [5]. The predicted *Cim1* protein product is related to the group I pollen allergens from various plants, which were recently shown to be related to expansin proteins [4]. Indeed, the *Zea m* pollen allergen from maize was shown to possess expansin activity [4]. These findings suggest that pollen allergens and

their vegetative homologues (e.g., *Cim1*), now called β -expansins, are involved in cell wall expansion during the vegetative and/or reproductive phases of plant development. In order to better understand cytokinin regulation of plant gene expression and its role in plant development, we have investigated the mechanism of cytokinin-induced *Cim1* accumulation.

Materials and methods

Plant materials

Suspension cultures of soybean (*Glycine max* cv. Mandarin) were grown in Murashige-Skoog liquid medium containing 10 μ M α -naphthaleneacetic acid (NAA) (2 mg/l) and 4 μ M benzyladenine (BA) (1 mg/l) at 26 ± 1 °C in continuous fluorescent light on a rotary shaker set to 100 rpm. These cultures were propagated every 14 days by transferring 10 ml of stationary phase cells into 30 ml of fresh medium. Cytokinin starvation was performed by washing stationary phase cells with 1 liter of cytokinin-free Murashige-Skoog liquid medium containing 10 μ M α -NAA (5 washes of 200 ml each) on a sterile Buchner funnel and then culturing the cells in 40 ml of fresh cytokinin-free medium for 6 days. Cytokinin-starved cells were either harvested directly or were harvested after various treatments. Cells were harvested by suction filtration, frozen in liquid nitrogen, and then stored at -80 °C prior to RNA isolation.

Chemical treatments

Cytokinin-starved soybean suspension cultures were treated with 1 μ M benzyladenine (BA) in double-distilled H₂O (Sigma, St. Louis, MO), 1 μ M staurosporine in 0.1% DMSO (Sigma), 1 μ M okadaic acid (OKA) in 0.1% DMSO or 0.15% ethanol (LC Labs, Woburn, MA), 1 or 0.1 μ M Calyculin A (Cal A) in 0.1% DMSO (LC Labs), and/or 50 μ M actinomycin D (ActD) in ddH₂O (Sigma). Combinations and durations of treatments varied and are described in Results and figure legends. Appropriate solvent controls were included for each treatment. All experiments were replicated at least twice.

RNA isolations

Frozen soybean suspension cultures were ground to a fine powder in liquid nitrogen. The powders were then

mixed with 1.0 ml phenol per gram fresh weight (pre-equilibrated with 1 M Tris-HCl, pH 8.0) and 0.7 ml extraction buffer per gram fresh weight (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS), 0.5 M NaCl, 0.5% (v/v) 2-mercaptoethanol) at 60 °C for 1 min. Chloroform (0.7 ml per gram fresh weight) was mixed into the samples and the resulting emulsions were centrifuged at $12\,000 \times g$ for 10 min. Aqueous phases were removed and extracted with an equal volume of chloroform, after which RNAs were precipitated in the presence of 2.5 M lithium chloride at 4 °C. The precipitated RNAs were dissolved in 3 ml of CE buffer (10 mM citric acid, pH 7.0, 1 mM EDTA, 0.1% (v/v) diethylpyrocarbonate), precipitated in the presence of 2 M ammonium acetate, 70% ethanol and reprecipitated in the presence of 0.3 M sodium acetate, pH 7.5, 70% ethanol. The final RNA precipitates were dissolved in CE buffer and quantitated by measuring absorbance at 260 nm.

RNA blot analyses

RNA (10 μ g total RNA per lane) from soybean suspension cultures was size-fractionated by 7.2% (v/v) formaldehyde, 1% (w/v) agarose gel electrophoresis, transferred onto nylon membranes (Nytran Plus, Schleicher and Schuell, Keene, NH) by capillary blotting, and hybridized to antisense RNA probes, which were prepared *in vitro* by T3 RNA polymerase-mediated transcription of linearized Pbluescript SK+ plasmids containing Cim1 or control (Sam45) cDNAs. Prehybridizations were carried out in sealed trays at 70 °C for a minimum of 2 h on an orbital shaker in hybridization buffer (250 mM NaCl, 20 mM sodium phosphate pH 7.0, 5% (w/v) SDS, 2.5 mM EDTA, 0.5% (w/v) non-fat dry milk, 0.5 mg/ml yeast RNA, and 0.5 mg/ml herring sperm DNA, 0.1% (v/v) diethylpyrocarbonate). Probes were added to 10^6 cpm/ml and allowed to hybridize for a minimum of 18 h. Blots were then washed in low salt buffer (40 mM sodium phosphate pH 7.0, 1% (w/v) SDS, 1 mM EDTA) at 70 °C until areas of specific hybridization were detected. Blots were typically exposed to Kodak X-Omat AR5 film with an intensifying screen for 12 to 16 h at -80 °C. Band intensities were quantitated by liquid scintillation of excised bands or by densitometry using the Image Analysis System and Molecular Analyst version 2.0 software from BioRad (Hercules, CA). Densitometric analyses of multiple autoradiographic exposures insured accurate quantitation.

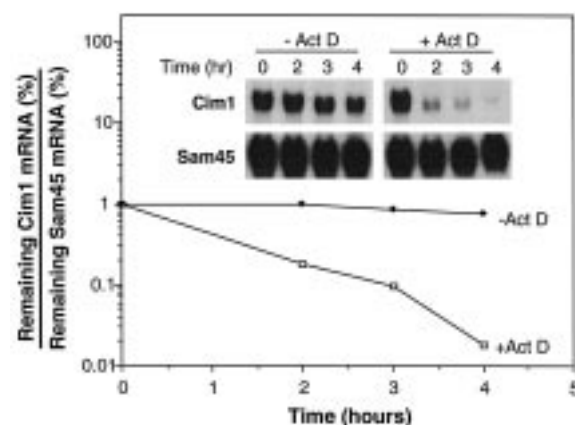


Figure 1. Actinomycin D affects Cim1 abundance. Cytokinin-starved soybean suspension cultures were treated with or without actinomycin D (50 μ M, Act D). At various times, total RNA was isolated and RNA blot analysis was performed with probes specific to Cim1 or a control RNA (Sam45). The ratio of remaining Cim1 mRNA (%) to remaining Sam45 mRNA (%) is plotted versus time in hours.

Results

Post-transcriptional regulation of Cim1 accumulation

To determine whether post-transcriptional mechanism(s) contribute to cytokinin induction of Cim1 accumulation, we first examined the rate of Cim1 decay in cytokinin-starved soybean suspension cultures. Actinomycin D, a potent inhibitor of transcription, was used to examine mRNA decay in the absence of transcription. As shown in Figure 1, actinomycin D caused basal Cim1 levels to decline in cytokinin-starved cells. This result suggests that the rate of Cim1 synthesis is balanced by an equivalent rate of Cim1 decay in untreated (cytokinin-starved) cells, and that actinomycin D blocks synthesis. The resulting Cim1 decay was exponential, suggesting pseudo-first order kinetics. Thus, if cytokinin treatment stabilizes the Cim1 mRNA, a less rapid decline in Cim1 abundance would be expected in the presence of 1 μ M BA and actinomycin D. As shown in Figure 2, 1 μ M BA stabilized the Cim1 mRNA in the presence of actinomycin D. In Figure 2A, cytokinin was added 30 min prior to actinomycin D, whereas in Figure 2B, cytokinin and actinomycin D were added simultaneously. In both cases, the half-life of Cim1 was increased more than 4-fold in the presence of cytokinin, while the rate of control mRNA decay was unaffected by cytokinin.

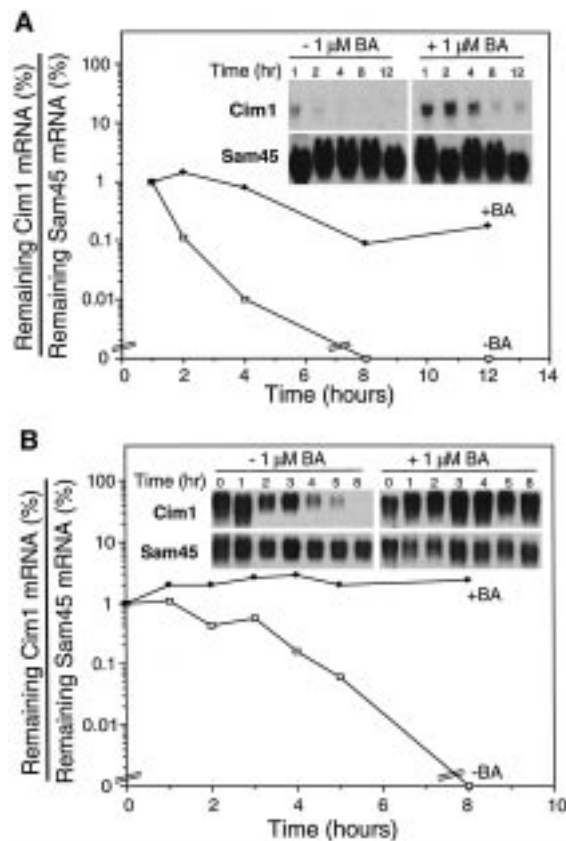


Figure 2. Cim1 stability *in vivo*. Actinomycin D (50 μ M) was added at t_0 to cytokinin-starved soybean suspension cultures treated with or without benzyladenine (1 μ M, BA). Benzyladenine was added (A) 30 min prior to actinomycin D addition, or (B) at t_0 . At various times, total RNA was isolated and RNA blot analysis was performed with probes specific to Cim1 or a control RNA (Sam45). The ratio of remaining Cim1 mRNA (%) to remaining Sam45 mRNA (%) is plotted versus time in hours.

Role of protein dephosphorylation in cytokinin induction of Cim1 accumulation

Cim1 accumulation has been shown to be stimulated in the absence of cytokinin by 1 μ M staurosporine and moderately inhibited in the presence of cytokinin by 0.5 μ M okadaic acid [5], suggesting a role for protein dephosphorylation in cytokinin induction of Cim1 accumulation. To further investigate these effects and, ultimately, to determine whether protein dephosphorylation is involved in cytokinin-induced Cim1 stability, we examined cytokinin regulation of Cim1 accumulation in the presence of higher doses of okadaic acid (1 μ M) or in the presence of calyculin A, a structurally unrelated inhibitor of protein phosphatases 1

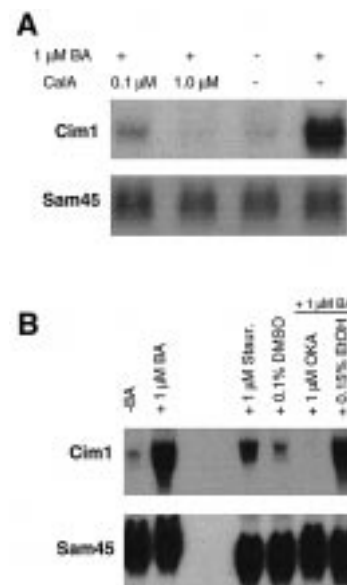


Figure 3. Protein dephosphorylation and Cim1 accumulation. Cytokinin-starved soybean suspension cultures were treated with or without 1 μ M benzyladenine (BA) for four hours as indicated. After all treatments, total RNA was isolated and RNA blot analysis was performed with probes specific to Cim1 or a control RNA (Sam45). A. The protein phosphatase inhibitor calyculin A (0.1 μ M or 1.0 μ M, CalA) was added to cells 45 min prior to cytokinin addition. All samples shown in panel A contained 0.1% dimethylsulfoxide. B. The protein kinase inhibitor staurosporine (1 μ M, Staur.) or dimethylsulfoxide (0.1%, DMSO) were added to cells for 24 h in the absence of cytokinin. The protein phosphatase inhibitor okadaic acid (1 μ M, OKA) or ethanol (0.15%, EtOH) were added 45 min prior to benzyladenine addition.

and 2A. As shown in Figure 3A, calyculin A (0.1 μ M or 1.0 μ M) strongly inhibited cytokinin induction of Cim1 accumulation. Furthermore, 1 μ M okadaic acid completely blocked cytokinin-induced Cim1 accumulation, whereas 1 μ M staurosporine stimulated Cim1 accumulation in the absence of cytokinin (Figure 3B). These results are consistent with the hypothesis that protein phosphatase activity is involved in cytokinin regulation of Cim1 mRNA levels. A control mRNA (Sam 45) was not affected by these treatments.

The role of protein dephosphorylation in cytokinin-induced Cim1 stability

To determine whether protein phosphatase activity is required for cytokinin-induced Cim1 stability, the effect of 1 μ M okadaic acid on cytokinin-induced Cim1 stability was examined in the presence of actinomycin D. In Figure 4A, actinomycin D was added 30 min

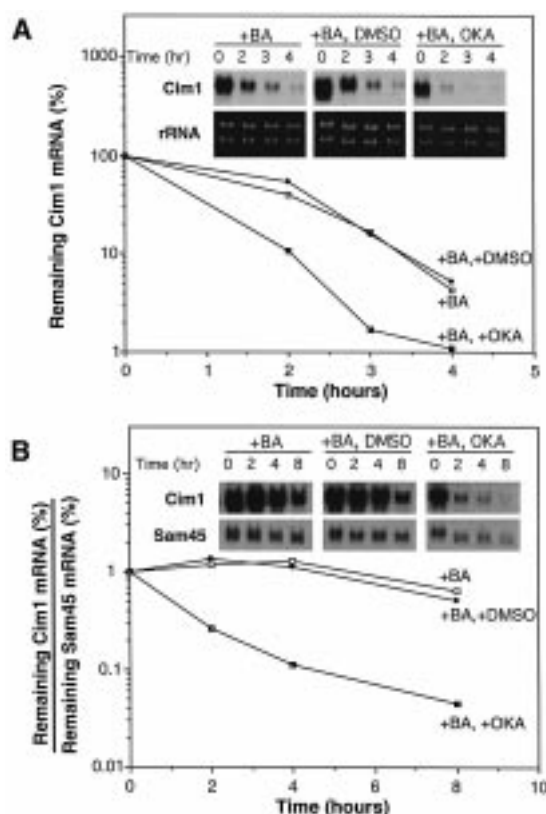


Figure 4. Protein dephosphorylation and Cim1 stability. Cytokinin-starved soybean suspension cultures were treated with benzyladenine ($1 \mu\text{M}$ BA) at t_0 . In panel A, actinomycin D ($50 \mu\text{M}$) was added 30 min prior to benzyladenine treatment, and okadaic acid ($1 \mu\text{M}$, OKA) or dimethylsulfoxide (0.1%, DMSO) was added 45 min prior to benzyladenine treatment. Ribosomal RNAs are shown and remaining Cim1 mRNA (%) is plotted versus time in hours (one of the control Sam45 lanes was lost during the experiment, so ratios were not computed). In panel B, actinomycin D ($50 \mu\text{M}$) and benzyladenine were added simultaneously, and okadaic acid ($1 \mu\text{M}$, OKA) or dimethylsulfoxide (0.1%, DMSO) was added 45 min prior to benzyladenine treatment. At various times, total RNA was isolated and RNA blot analysis was performed. The ratio of remaining Cim1 mRNA (%) to remaining Sam45 mRNA (%) is plotted versus time in hours.

prior to cytokinin, which was added at t_0 . Thus, Cim1 levels were in decline prior to cytokinin addition. In Figure 4B, on the other hand, actinomycin D and cytokinin were added simultaneously. In both cases, okadaic acid inhibited cytokinin-induced Cim1 stability when added 45 min prior to cytokinin treatment. In contrast, the abundance of the control mRNA (Sam45) was not similarly affected. These results indicate a requirement for the activity of type 1 and/or 2A protein phosphatase(s) in the post-transcriptional regulation of Cim1 by cytokinin.

Discussion

The results reported here demonstrate that Cim1 accumulation in response to cytokinin occurs, at least in part, by a post-transcriptional mechanism that involves protein dephosphorylation. Our observations have led us to propose the model shown in Figure 5. We suggest that cytokinin directly or indirectly leads to an increase in type 1 or 2A protein phosphatase activity. This phosphatase activity rapidly shifts the equilibrium of one or more protein(s) from a predominantly phosphorylated to a predominantly dephosphorylated state. Protein(s) thus dephosphorylated then perpetuate the cytokinin signal, ultimately leading to the stabilization of Cim1. Consistent with this model, staurosporine induces Cim1 accumulation in the absence of cytokinin, presumably by blocking protein phosphorylation and causing a gradual dephosphorylation of the protein(s) that promote Cim1 stabilization, and okadaic acid inhibits cytokinin-induced Cim1 stability by blocking protein dephosphorylation. In contrast, staurosporine blocks cytokinin-induced accumulation of *msr1* mRNA and *SrEnod2* mRNA, and okadaic acid induces *msr1* mRNA accumulation but blocks cytokinin-induced accumulation of *SrEnod2* [11, 33]. The differences in the effects of protein phosphatase and kinase inhibitors on Cim1, *msr1*, and *SrEnod2* mRNA levels may reflect differences in the complex cytokinin signal transduction pathways functioning in these different organisms. In some cases, physiological responses to cytokinin may lead to changes in both protein kinase and protein phosphatase activities. Indeed, both activities are relevant to cytokinin-mediated dephosphorylation and activation of a $p34^{cd2}$ -like protein from *Nicotiana tabacum* which exhibits histone H1 kinase activity [41]. The observation that protein phosphatase activity is required for cytokinin-induced accumulation of Cim1 contributes to an emerging model of cytokinin signal transduction, which appears to rely heavily on protein phosphorylation and dephosphorylation.

Cim1 is stabilized in response to cytokinin treatment of cytokinin-starved soybean cells. Direct analysis of Cim1 stability revealed a greater than 4-fold increase in the half-life of the mRNA in response to cytokinin. While the precise mechanism of cytokinin-induced Cim1 stability is not well understood (i.e., it is not clear whether cytokinin inhibits the activity of a ribonuclease, recruits the binding of a Cim1 mRNA stabilizing protein or stabilizes the Cim1 mRNA through regulated translation [18, 40]), our

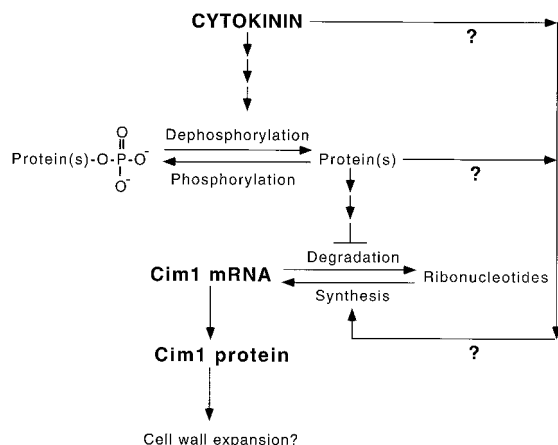


Figure 5. A model of Cim1 regulation and function.

data suggest that this mechanism is dependent on protein phosphatase 1 and/or 2A activity. The increased stability of Cim1 in response to cytokinin is similar to the 4-fold light-induced increase in the stability of the ribulose-1,5-bisphosphate carboxylase small subunit mRNA [32]. Regulated mRNA stability has also been described for the proline-rich-protein (PvPRP1) mRNA of *Phaseolus vulgaris*, which exhibits at least a three fold decrease in stability in response to fungal elicitors [42]. This decreased stability is caused by a fungal elicitor-induced protein that binds specifically to a 27 nucleotide sequence in the 3'-untranslated region of the PvPRP1 mRNA. Our results do not, however, exclude the possibility of transcriptional regulation of Cim1 accumulation by cytokinin. Indeed, both transcriptional and post-transcriptional regulatory mechanisms have been proposed for other cytokinin-regulated genes [2, 11, 15, 16, 22, 25, 33]. For example, both transcriptional and post-transcriptional mechanisms control the abundance of the Rubisco small subunit mRNA [15, 16, 39].

Group I pollen allergens and their vegetative homologs, including Cim1, have been classified as a subfamily of expansin proteins and are now collectively called β -expansins [4, 5]. Interestingly, more than one β -expansin protein is believed to be regulated by cytokinin [26]. Sherban *et al.* [31] have described three groups of amino acids that are highly conserved among expansins and the group I pollen allergens from *Lolium* and *Phleum*, including six cysteine, four tryptophan, and four glutamic acid residues. The cysteine residues are confined to the amino-terminal half of these proteins and are arranged in a pattern similar to that of the conserved cysteines found in the chitin-binding

domain of wheat germ agglutinin. These cysteines have been shown to form disulfide bonds and to stabilize the chitin binding domain of wheat germ agglutinin. Thus, the conserved cysteines found in expansins and pollen allergens may have a similar function. The pattern of conserved tryptophan residues in expansins and pollen allergens resembles the pattern of tryptophan residues in the cellulose binding domains of cellulase. These tryptophan residues, which are located within the carboxyl terminal third of these proteins, may interact with components of the cell wall. Finally, the glutamic acid residues conserved amongst expansins and pollen allergens may mediate sensitivity to pH, which has been demonstrated for expansin activity [3, 31]. This relatedness to the expansins suggests a role for Cim1 in cytokinin-mediated changes in cell wall morphology.

Cytokinin has long been known to stimulate plant cell division and to promote shoot organogenesis. Changes in the cell wall are critically involved in cytokinesis and cell plate formation and control the size and shape of plant cells. Therefore, it is perhaps not surprising that cytokinin regulates the expression of genes involved in cell wall extensibility. Consistent with the hypothesis that cell wall expansion plays a crucial role in organ development, Fleming *et al.* recently reported that beads loaded with expansin protein could induce the ectopic formation of leaf primordia when placed onto the apical meristem of tomato plants [14]. This observation suggests that regulation of expansin synthesis may be an essential step in the growth and development of plant organs. Since the Cim1 gene is a β -expansin gene, it is reasonable to propose that the Cim1 protein may govern cell wall dynamics in response to cytokinin and thus may be an important determinant of plant growth and development.

Acknowledgements

The authors would like to thank C. Ryan Steinbaker for technical assistance and Drs Stephen K. Randall and John C. Watson for helpful discussions. This work was supported by USDA grant AMD-9402330.

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