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Short Communication

Cytochalasin A Inhibits the Binding of Phenylalanine Ammonia-Lyase mRNA to Ribosomes during Induction of Phytoalexin in Pea Seedlings

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Cytochalasin A (CA) blocked the accumulation of phytoalexin and phenylalanine ammonia-lyase (PAL)-protein in pea tissues treated with a fungal elicitor but scarcely affected the PAL-mRNA content. Further analysis showed that CA decreased the PAL-mRNA bound to ribosomes. These results indicate that actin filaments are tightly associated with the translational process of the *PAL* gene.

Key words: Actin filament — Elicitor — Phenylalanine ammonia-lyase (PAL) — Pea (*Pisum sativum* L.) — Phytoalexin — Polysome-bound mRNA.

It is well known that the cytoskeleton is involved in diverse functions in plant cells, including the development of cell (Hardham et al. 1980), cytoplasmic streaming (Kamiya et al. 1981) and organelle movement (Williamson et al. 1986). The cytoskeleton, especially the actin filament, has also been reported to participate in interaction between plants and fungal pathogens. Cytoskeletal reorganization in response to pathogen attacks was observed in interactions of potato-late blight fungus (Tomiyama 1956), barley-powdery mildew fungus (Kobayashi et al. 1997b) and cowpea-rust fungus (Skalamera and Heath 1998). Tomiyama et al. (1982) found that treatment with cytochalasin B, an inhibitor of actin polymerization, delayed hypersensitive cell death in potato tuber tissues inoculated with an incompatible race of *Phytophthora infestans*. Similar results were observed in parsley (Gross et al. 1993), flax (Kobayashi et al. 1994), tobacco (Kobayashi et al. 1997a) and cowpea (Skalamera and Heath 1998). Cytochalasin D delayed the accumulation of PAL-mRNA and inhibited the production of a potato phytoalexin, rishitin, in potato tuber discs induced by an elicitor from *P. infestans* (Furuse et al. 1999, Takemoto et al. 1999). While these reports suggest that the actin filament is tightly associated with plant defense responses, its precise action site during the defense process remains obscure. In this study, to evaluate a role of actin filaments for defense responses, we examined the effects of CA, an inhibitor of actin polymerization, on phytoalexin biosynthesis, one of the most characterized defense responses, by using a system of pea seedlings and an elicitor from a pea pathogen, *Mycosphaerella pinodes*.

Seeds of *Pisum sativum* L., cv. Midoriusui were sown on moistened vermiculite in a plastic container and grown in darkness at 22±2°C. Stem segments prepared from 6-day-old seedlings were divided longitudinally into two parts and treated with test solution as described below. An elicitor preparation was isolated from the germination fluid of pycnospores of *Mycosphaerella pinodes* (Berk. et Blox) Verstergren, strain OMP-1 (IFO-30342, ATCC-42741) as described previously (Yoshioka et al. 1990). CA was obtained from Sigma-Aldrich Co. Ltd. (St. Louis, Mo, U.S.A.) and dissolved in 100% DMF as stock solution. All test solutions included 0.5% (v/v) DMF (final conc.).

A major phytoalexin of pea, pisatin, was extracted from tissues with hot ethanol and quantified by HPLC, as described previously (Masuda et al. 1983).

The activity of PAL, one of key enzymes in the biosynthetic pathway to pisatin, was measured with a spectrophotometer (Beckman DU series 600; Fullerton, CA) according to the method described by Minamikawa and Uritani (1965). In brief, 0.4 g of pea tissues was homogenized with 2 ml of 0.1 M Tris-HCl (pH 8.8) containing 1 mM 2-mercaptoethanol and protease inhibitors (Complete™; Boehringer Mannheim, Mannheim, Germany), and the homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was then precipitated in 80%-saturated ammonium sulfate, and the resultant pellet was used to determine PAL activity.

For immunoblot analysis, the extracted proteins were separated on a 7.5% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). PAL-proteins on the blot were detected with a polyclonal antibody raised against a pea PAL protein and a goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad).

PAL-mRNAs in stem tissues were extracted by a method described previously (Yamada et al. 1992). Total RNA was extracted from 0.4 g of frozen tissues by

Abbreviations: CA, cytochalasin A; DMF, *N,N*-dimethylformamide; EGTA, *O,O'*-bis (*b*-aminoethyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid; PAL, phenylalanine ammonia-lyase; 1 × SSC, 0.015 M sodium citrate with 0.15 M NaCl; Triton X-100, polyethylene glycol mono-*p*-isooctylphenyl ether.

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the single step method of Chomczynski and Sacchi (1987). Denatured RNA was separated on 1% agarose gel containing 0.41 M formaldehyde and blotted onto a Hybond^{TM}-N^{+} membrane (Amersham). The blots were prehybridized in a solution containing 5×SSC, 50% formamide, 2% (w/v) blocking reagent (Boehringer Mannheim), 0.02% (w/v) SDS and 0.1% (w/v) sodium *N*-lauroyl-salcosinate at 68°C for 2 h. This step was followed by hybridization for 12 h in the presence of digoxigenin-labeled gene specific RNA probes. Gene-specific probes were prepared from our previously cloned cDNAs (Yamada et al. 1992), by transcription with RNA polymerase (Stratagene) in the presence of digoxigenin-11-UTP (Boehringer Mannheim), according to the manufacturer's instructions. The membrane was washed twice with 2× SSC and 0.1% SDS for 30 min at 68°C and then washed twice with 0.1×SSC and 0.1% SDS for 30 min at 68°C. Hybridization signal was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and a chemiluminescent substrate, CDP-star^{TM} (Boehringer Mannheim).

Polysomal fractions were prepared from stem tissues according to the method described by Yoshioka et al. (1996) with a slight modification. Three grams of frozen tissues were homogenized in a polysome extraction buffer [0.2 M Tris-HCl (pH 9.0), 0.4 M KCl, 60 mM MgOAc, 50 mM EGTA, 0.25 M sucrose, 0.5% (v/v) Triton X-100 and 7 mM 2-mercaptoethanol; 8 ml per g fresh weight tissue]. After centrifugation at 15,000×*g* for 15 min at 4°C, the supernatant was further put on a 1.5 M sucrose cushion containing 40 mM Tris-HCl (pH 9.0), 0.1 M KCl, 30 mM MgOAc, 5 mM EGTA and 0.1% Triton X-100 and was centrifuged at 150,000×*g* for 4.5 h at 4°C with a Himac CP56GII (Hitachi; Tokyo, Japan). RNAs were isolated from the resultant polysomal pellets and used for northern blot analysis.

The effect of CA on the accumulation of pisatin was shown in Figure 1A and B. The simultaneous presence of CA dose-dependently reduced the accumulation of pisatin in pea tissues treated with the elicitor. Inhibition by 1 mM CA was greater than 90% as compared to treatment with the elicitor alone (Fig. 1A). A time-course study showed that, with the elicitor alone, pisatin was detectable within 9 h and reached greater than 25 μg (g FW)$^{-1}$ at 24 h after the start of treatment; the presence of 0.5 mM CA completely suppressed the pisatin accumulation to the same extent as the water treatment (Fig. 1B). Thus CA was able to remarkably suppress a pea defense response. A similar result was obtained in pea tissues treated with cytochalasin B (data not shown).

Our previous study showed that the elicitor induced the accumulation of PAL-mRNA within 1 h, followed by an increase in extractable PAL activity within 6 h and by the accumulation of pisatin within 9 h (Yamada et al. 1989). To further evaluate the effect of CA on this process, we determined the PAL activity in elicitor-treated tissues with or without CA. As shown in Figure 2, a maximal induction of PAL activity was observed 12 h after the start of the elicitor treatment, whereas such an activation was delayed in the presence of CA. This result coincided with the accumulation of PAL proteins (Fig. 2; inset), indicating that an action site of CA during pea phytoalexin biosynthesis may exist in the process on or before the translation of *PAL* genes.

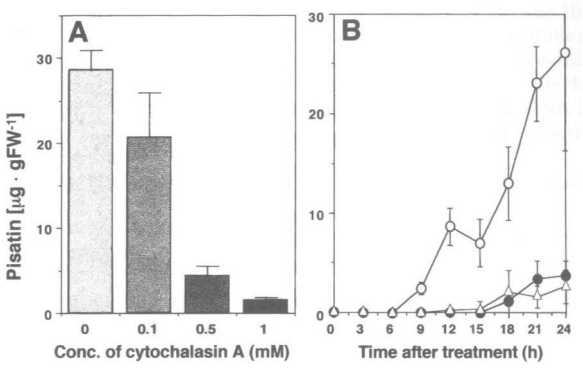

Fig. 1 Inhibitory effect of cytochalasin A on the phytoalexin production in pea stem tissues. (A) Tissues were treated with 500 μg ml^{-1} of the *M. pinodes*-elicitor in the absence or the presence of CA (0.1–1 mM). The amounts of pisatin accumulated in tissues were determined 18 h after the start of treatment. Each value represents a mean with standard deviation (SD) from three epicotyl tissues. (B) Changes in the amount of pisatin accumulated in tissues with respect to time after the start of treatment. Tissues were treated with the elicitor alone (○), the elicitor plus CA (●) or distilled water (△). The concentrations of elicitor and CA used were 500 μg ml^{-1} and 0.5 mM, respectively. Each value shows a mean with (SD) from three tissues.

Northern blot analysis showed that the PAL-mRNA accumulated within 1 h and increased up to 4 h after treatment with the elicitor alone (Fig. 3A). Surprisingly, the simultaneous application of CA scarcely affect the accumulation of transcripts for PAL as compared to treatment with the elicitor alone (Fig. 3A). This result differs from that of Takemoto et al. (1999) with potato tuber discs in which the accumulation of PAL-mRNA was delayed for 1 h by the presence of cytochalasin D. Thus, in potato tuber tissues, cytochalasin D may affect the signal transduction cascade to nuclei or the transcriptional process of *PAL* genes. Presently, the cause of this difference is unknown.

Together with the result in Figure 2, the data from the northern blot analysis with total pea RNAs (Fig. 3A) led us to a hypothesis that CA may mainly affect the translational process on ribosomes. To support this argument, we measured the amount of PAL-mRNA in a polysomal fraction prepared from pea tissues that had been treated with the elicitor in the presence or the absence of CA.

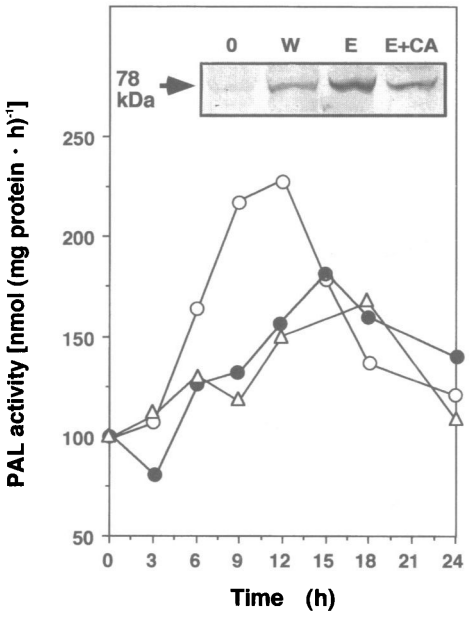

Fig. 2 Time course study of PAL activity in pea stem tissues treated with cytochalasin A. Five stem segments (0.4 g) were treated with the elicitor alone (○), the elicitor plus CA (●) or distilled water (△). Proteins were extracted at the indicated time after the start of treatment. PAL activity was measured in a reaction mixture (100 μl) containing 50 mM borate buffer (pH 8.8), 10 mM L-phenylalanine and 8–10 μg of the extracted proteins. The reaction was carried out at 37°C for 90 min and terminated by addition of 25 μl of 2 M perchloric acid. After centrifugation at 7,500 × *g* for 10 min at 4°C, the amount of *t*-cinnamic acid in the supernatant was determined at A_{280}. Each value was expressed as a specific activity (nmol *t*-cinnamic acid formed [(mg protein) h]$^{-1}$). Inset shows an immunoblot analysis of PAL-proteins with an anti-pea PAL antibody. Proteins were extracted from tissues 12 h after treatment with the elicitor alone (E), elicitor plus CA (E + CA) or distilled water (W). The concentrations of elicitor and CA used were 500 μg ml^{-1} and 0.5 mM, respectively. Zero indicates PAL in the protein fraction extracted from untreated tissues.

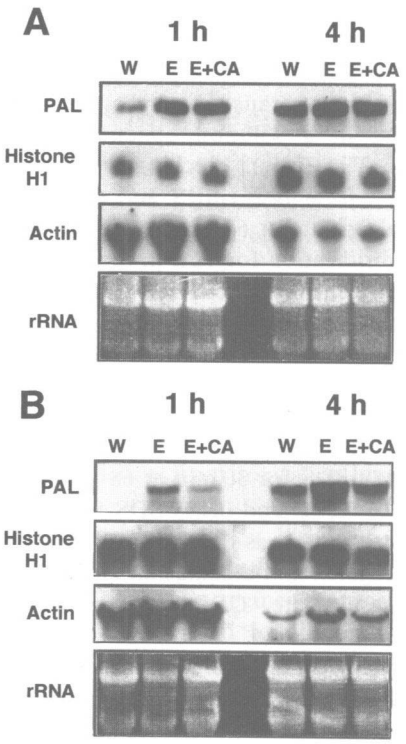

Fig. 3 Northern blot analyses of PAL-mRNA in total RNAs (A) or polysomal RNAs (B) that were prepared from pea tissues treated with the elicitor in the presence or the absence of CA. Total RNA or polysomal RNA was prepared from pea tissue 1 h or 4 h after treatment with the elicitor alone (E), elicitor plus CA (E + CA) or distilled water (W). The concentrations of elicitor and CA used were 500 μg ml^{-1} and 0.5 mM, respectively. Denatured RNA (10 μg) was electrophoresed, blotted onto a nylon membrane, and allowed to hybridize with digoxigenin-labeled RNA probes specific to PAL-, histone H1- or actin-mRNA. The amount of loaded RNA in each lane was verified by staining with an ethidium bromide of 26S and 18S rRNA (respective bottom panels).

Transcripts for PAL were detected in polysomal fraction at 1 h and was increased by 4 h after application of the elicitor alone. However, the presence of CA markedly decreased the amount of PAL-mRNA in the polysomal fraction both 1 h and 4 h after treatment (Fig. 3B). These results indicate that reduction of PAL activity and PAL protein by the CA-treatment may be attributed to a decrease in PAL-mRNA arranged on polysomes. In other words, actin filaments may play a crucial role in organization of polysomes with PAL-mRNA.

On the other hand, the amount of histone H1-mRNA in total and polysomal fractions was not affected 1 h after CA-treatment (Fig. 3A, B). Four h after treatment, histone H1-mRNA was increased in total RNA fraction but was reduced in polysomal RNA fraction. Furthermore, CA slightly decreased the amount of histone H1-mRNA in both fractions.

CA scarcely affected the amount of actin m-RNA in total and polysomal RNA fractions 1 h after treatment. Regardless of respective treatments, actin-mRNA was remarkably reduced in total and polysomal RNA fractions 4 h after the start of treatment. CA-treatment further decreased actin-mRNA as compared to treatment with the elicitor alone. Thus, the treatment with CA for 4 h may markedly affect the binding of actin-mRNA to polysomes.

Actin filaments have been found to be associated with polysomes (Davies et al. 1991, Ito et al. 1994) and other translational components such as elongation factor 1α (Condeelis et al. 1995), plant-specific initiation factor eIFiso4F (Bokros et al. 1995) and eukaryotic elongation factor 2 (Bektas et al. 1994). Therefore, disruption of actin filaments resulted in reduction of protein synthesis (Morelli et al. 1998, Ornelles et al. 1986). In wounded potato tuber tissues, cytochalasin D caused a significant reduction in

polysome abundance with a resultant decrease in translational activity of wound-inducible genes (Morelli et al. 1998). Thus, cytochalasins may interfere with translational competence by disrupting a scaffold for assembly of the translational machinery and/or by inhibiting mRNA-trafficking onto polysomes, as described in several reports (Bassell and Singer 1997, Bassell et al. 1994, Morelli et al. 1998, Ornelles et al. 1986). However, unlike PAL-mRNA, the binding of histone H1- and actin-mRNAs to polysomal fractions was not severely affected by the application of CA for 1 h. From these results, it is thought that CA may preferentially inhibit the binding of PAL-mRNA to polysomes during an early stage of induction of phytoalexin production, but the kind of mechanism that operates in a separate action of CA on the binding of respective mRNAs to polysomes within 1 h after treatment, remains obscure.

In regard to other possibilities, Ornelles et al. (1986) reported that cytochalasin released mRNA from the cytoskeletal fraction and decreased protein synthesis in HeLa cells. According to the results shown in Figure 3B, it is also plausible that PAL-mRNAs may be released specifically from the polysomes if a similar phenomenon occurs in pea tissues. Recent reports suggest the role of cytoskeleton in signal transduction (Lloyd et al. 1996). At present, we do not know whether the acute inhibition of protein synthesis results from attenuation of signaling cascades because the translational machinery could be regulated substantially by phosphorylation/dephosphorylation (Scharf and Nover 1982, Garcia-Hernandez et al. 1996). Further investigation is needed to dissect the exact roles of actin filaments in defense processes accompanied by the expression of defense-responsive genes. Nevertheless, we can conclude that a tight linkage between the protein synthetic machinery and actin filaments may be crucial during induction of defense responses.

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