

Research Note

## Decreased Inositol 1,4,5-Trisphosphate Content in Pathogen-Challenged Soybean Cells

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**Phosphoinositide-specific phospholipase C (PI-PLC) has been shown to be transiently activated when plant cells were treated with elicitors. We thus investigated the activity of PI-PLC when soybean cells were infected with the bacterial pathogen *Pseudomonas syringae* pv. *glycinea*, by measuring cellular cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) levels. We observed that IP<sub>3</sub> content decreased in both compatible and incompatible interactions. In vitro phosphatase activities were similar in both water control and infected cells with slightly lower IP<sub>3</sub> degradation observed for infected cells, indicating that the reduced IP<sub>3</sub> content in infected cells most likely results from reduced PI-PLC activity. We hypothesize that reduced IP<sub>3</sub> content following infection may lead to suppression of various housekeeping activities of the cells, thus diverting the cellular resources either to the synthesis of defense-related compounds against pathogens, and/or to the growth of pathogens.**

*Additional keywords:* oxidative burst, signal transduction.

Phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) on the plasma membrane generating cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and plasma membrane-associated diacylglycerol (DAG), both of which serve as second messengers in many animal signal transduction pathways (Dennis 1983; Majerus 1992; Singer et al. 1997). These two molecules are known to modulate intracellular events through the regulation of intracellular free Ca<sup>2+</sup> and protein kinase C isozymes (Singer et al. 1997). Many components of the animal phosphoinositide signaling pathway are also found in plants (Coté and Crain 1993; Munnik et al. 1998). Recently, several plant PI-PLCs have been cloned from soybean, Arabidopsis, and potato (Shi et al. 1995; Hirayama et al. 1995; Kopka et al. 1998; Hartweck et al. 1997).

Physiological roles of PI-PLCs in plants have been proposed in a number of systems. In *Catharanthus roseus* cells,

auxin application relieved cell cycle arrest in G1, which was preceded by transient changes in IP<sub>3</sub> and inositol 4,5-bisphosphate (IP<sub>2</sub>) (Ettliger and Lehle 1988). Abscisic acid treatment of guard cell protoplasts of *Vicia faba* induced a 90% increase in levels of IP<sub>3</sub> within 10 s of administration, suggesting a possible role of PI-PLC in guard cell shrinking and stomatal closure (Lee et al. 1996). In *Samanea saman* (a legume), 15 to 30 s of white light pulse caused an increase in IP<sub>3</sub>, IP<sub>2</sub>, and DAG in the pulvinus (motor organ) (Morse et al. 1987, 1989). Recently, it was proposed that a sustained increase in IP<sub>3</sub> is responsible for pulvinus cell elongation in maize (Perera et al. 1999). IP<sub>3</sub> was also involved in Ca<sup>2+</sup>-mediated pollen tube inhibition in *Papaver rhoeas* (Franklin-Tong et al. 1996).

Plants respond to pathogen attack by activating various defense mechanisms, including hypersensitive cell death. Based on elicitor studies, involvement of elevated levels of IP<sub>3</sub> was suggested to play a role in triggering these responses. For example, oxidative burst, a cause of hypersensitive cell death (Levine et al. 1994), was shown to be regulated in part by the PI-PLC activated by an elicitor (Legendre et al. 1993). A glycoprotein elicitor from the phytopathogenic fungus *Verticillium albo-atrum*, which triggers Ca<sup>2+</sup>-mediated phytoalexin accumulation, induced a 100 to 160% increase of IP<sub>3</sub> over a water control in lucerne (*Medicago sativa*) suspension culture cells (Walton et al. 1993). These observations suggest a possible role of IP<sub>3</sub> signals in the regulation of host defenses following elicitation. However, whether such a regulation plays any role in the activation of plant defense mechanisms in response to pathogen infection is not known.

We investigated the possible role of IP<sub>3</sub> in the expression of plant defenses in the soybean cell suspensions and *Pseudomonas syringae* pv. *glycinea* (*Psg*) interaction. The cell suspensions, prepared from the cultivar Williams 82, carry a resistance gene *Rpg2* that confers resistance against *Psg* carrying the avirulence gene *avrA*. However, Williams 82 does not carry the resistance gene (*Rpg3*) corresponding to *avrC* and is susceptible to the *Psg* strain that carries *avrC* but not *avrA*. This cell culture shows a strong oxidative burst following infection with *Psg(avrA)* but not with *Psg(avrC)* (Levine et al. 1994). We measured IP<sub>3</sub> content in soybean cells following infection with both strains of *Psg* and have observed that IP<sub>3</sub> content decreased after inoculation with either strain of *Psg*.

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Suspension cell culture of soybean (*Glycine max* L.) cultivar Williams 82 was maintained at 25°C in the dark on an orbital shaker (130 rpm; G10 Gyrotory Shaker; New Brunswick Scientific, Edison, NJ) in Murashige-Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2.22 µM 6-benzylaminopurine and 3 mg of picloram and vitamins per liter. Cultures were transferred every 7 days by diluting five-fold in fresh MS medium, and experiments were performed 3 to 5 days after transfer.

*Psg* race 4 containing either the *avrA* or *avrC* gene (Keen and Buzzell 1991) was maintained at 28°C on King's medium B (KMB; King et al. 1954) plates with 50 µg of streptomycin or kanamycin per ml, respectively. Liquid cultures were prepared by inoculating KMB broth containing an appropriate antibiotic with a single colony of plate cultures, and grown for 18 h at 28°C with shaking. The overnight culture was centrifuged, and resuspended in sterile water for inoculation of soybean cell cultures. The final concentration of the bacteria in inoculated suspension cultures was adjusted to optical density (OD)  $A_{600} = 0.1$ .

H<sub>2</sub>O<sub>2</sub> production by the oxidative burst was measured by the quenching of scopoletin fluorescence at 460 nm following excitation at 350 nm (Root et al. 1975). Briefly, 5 µl of 2 mM scopoletin was added to cell culture and incubated for 5 min, and fluorescence was determined spectrophotometrically.

To evaluate the defense activity against the pathogen, phenylalanine ammonia-lyase (PAL) activity was assayed by incubating [<sup>14</sup>C]phenylalanine with the crude cell extracts. Cinnamic acid, a product of the PAL enzymatic activity, was extracted with toluene for scintillation counting (Legrand et al. 1976).

We measured IP<sub>3</sub> content with a commercially available radioreceptor assay kit (TRK 1000; Amersham International, Little Chalfont, Buckinghamshire, U.K.). Crude extract of soybean cells was prepared according to the method of Legendre et al. (1993). In short, 1 ml of suspension culture samples was collected at appropriate sampling times in microtubes, frozen in liquid nitrogen immediately, and stored at -80°C until use. Extraction was done by adding 500 µl of 15% trichloroacetic acid to each sample and vortexing vigorously. The samples were subsequently centrifuged at 10,000 × *g* for 20 min to remove insoluble material and the supernatants were extracted four times with 5 ml of water-saturated ethyl ether. The samples were then neutralized to pH 7.5 by adding an appropriate amount (5 to 8 µl) of 16% Na<sub>2</sub>CO<sub>3</sub>. The radioreceptor assay was performed according to the manufacturer's protocol. The binding protein used in the kit is specific for inositol 1,4,5-trisphosphate, and discriminates against other isoforms of IP<sub>3</sub>, or other inositol phosphates. Cellular IP<sub>3</sub> content was standardized to unit fresh weight.

To confirm the results obtained with the radioreceptor assay, we performed high performance liquid chromatography (HPLC). *myo*-[<sup>3</sup>H]inositol (NEN Life Science Products; Boston) was added to a 3-day-old cell culture to a final concentration of 50 µCi/ml. The cells were then incubated for 2 days. The cell cultures were grown in inositol-free MS medium for 10 days prior to labeling. A filter-sterilized solution of glucuronic acid (100 µg/ml; Aldrich, Milwaukee, WI) was added to prevent incorporation of *myo*-[<sup>3</sup>H]inositol to glucuronic acid (Loewus and Loewus 1980). Treatments were made by adding 100 µl of 10× MS salts solution, MS salts, and *Psg* ( $A_{600} =$

1.0), or water as a control to an aliquot of suspension cells (900 µl). Thirty minutes after the treatment, the samples were harvested in microtubes and immediately frozen in liquid nitrogen. The samples were stored in -80°C until use. A crude extract of soybean cells was prepared in the same manner as for a radioreceptor assay.

The separation of inositol phosphates by HPLC was based on the method of Irvine et al. (1985). The Partisil 10 SAX anion exchange column (Phenomenex, Torrance, CA) was initially washed with water for 8 min, and then the eluant (1.7 M ammonium formate adjusted to pH 3.7 with phosphoric acid) was increased linearly to 100% over 24 min, and the buffer held at this concentration for 10 min. After the elution, the buffer concentration was decreased linearly to water over 2 min. Ninety-five fractions were collected over the elution period, and analyzed by scintillation counting. Peaks were identified by comparison with those of authentic standards.

The change in IP<sub>3</sub> content can be a result of changes either in the rate synthesis or degradation of IP<sub>3</sub>. We examined whether there is any difference in phosphatase activities on IP<sub>3</sub> between the water control and infected cells. The method was based on the report of Joseph et al. (1989). Two milliliters of suspension cells was inoculated with *Psg*(*avrA*) at final OD  $A_{600} = 0.1$ , or water. Cells were centrifuged at 1,000 × *g* for 10 min and the pellets were resuspended in buffer A (120 mM KCl, 20 mM Tris/HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 5 mM EGTA, and 1 mM dithiothreitol, pH 7.2). Samples were ground with a glass homogenizer in buffer A containing 1 µg/ml each of aprotinin, pepstatin, leupeptin and antipain (Sigma, St. Louis, MO). The crude extract was centrifuged at 755 × *g* for 5 min, and the supernatant was centrifuged at 60,000 × *g* for 60 min. The supernatant was desalted on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 35 ml of buffer A, and was loaded with 2.5 ml of the crude extract. The sample was eluted with 3.5 ml of buffer A.

Dephosphorylation was assayed in a buffer consisting of 120 mM KCl, 20 mM Tris/HEPES, and 0.3 mM MgCl<sub>2</sub> (buffer B). The reaction was carried out at 30°C by adding 450 µl of crude extract to a 1-ml reaction mixture containing 0.3 µCi of tritium-labeled IP<sub>3</sub> and 15 µM unlabeled IP<sub>3</sub>. The reaction was stopped after 15 min by adding 500 µl of 15% trichloroacetic acid, followed by extraction with 5 ml of water-saturated ethyl ether four times. The samples were neutralized to pH 7.0 by adding appropriate volumes of 16% Na<sub>2</sub>CO<sub>3</sub>. Phosphatase products were analyzed by HPLC by the same method as for IP<sub>3</sub> analysis. Injection volume was 200 µl.

We examined the change in cellular IP<sub>3</sub> content for a 3-h period following pathogen inoculation. IP<sub>3</sub> content in all treatments including the water control started to show a decrease immediately following treatments. However, in *Psg*(*avrA*)- and *Psg*(*avrC*)-inoculated cells, the decrease was much greater than that of the water control treatment (Fig. 1A). In our suspension culture systems, IP<sub>3</sub> levels fluctuate for unidentified reasons. The fluctuation appears to be synchronous among all treatments. Thus, if we compare the IP<sub>3</sub> levels, in the cells inoculated with the pathogens, with the IP<sub>3</sub> levels in the water control as the background, the depressed IP<sub>3</sub> content in the former was maintained throughout the experiment.

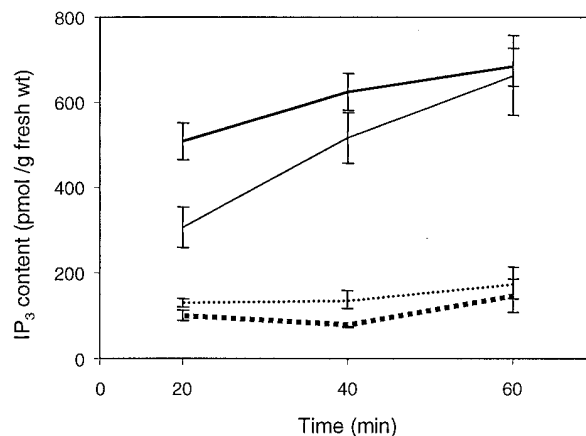
In most PI-PLC studies, the IP<sub>3</sub> peak has been reported to be observed within seconds or minutes following treatment.

However, we did not observe any significant difference in IP<sub>3</sub> content among *Psg(avrA)*- or *Psg(avrC)*-infected cells and the water control within 3 min following treatment (Fig. 1B). An oxidative burst, considered to be *avrA* gene-specific (Levine et al. 1994), was observed in *Psg(avrA)*-inoculated cells 3 to 4 h after inoculation (Fig. 1C). PAL enzyme activity, which is commonly associated with plant defense response to pathogens, was gradually increased in both *Psg(avrA)*- and *Psg(avrC)*-inoculated cells, with higher activities observed for *Psg(avrA)*-inoculated cells (Fig. 1D).

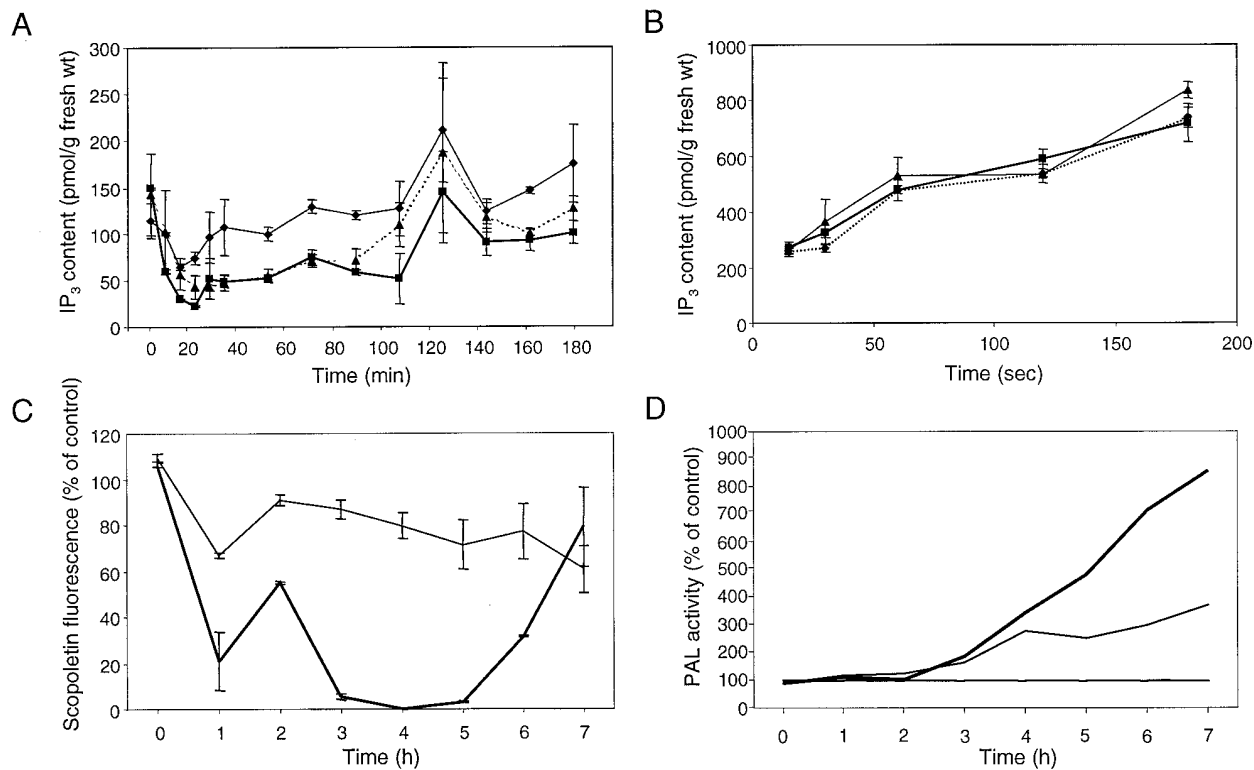
Addition of MS salts in cell suspensions caused an increase in the basal cellular IP<sub>3</sub> levels to several-fold (Fig. 2). When the cells were treated with both MS salts and *Psg(avrA)*, the IP<sub>3</sub> increase was less than that observed for the MS salts-treated cells (Fig. 2).

The decrease in cellular IP<sub>3</sub> content in pathogen-infected cells was also demonstrated by HPLC. At 30 min following infection or treatment with MS salts, the amount of IP<sub>3</sub> in the cells treated with both MS salts and *Psg(avrA)* was significantly smaller, compared with that in cells treated only with MS salts (Fig. 3), confirming the results obtained with the radioreceptor assay.

The decrease in IP<sub>3</sub> levels we observed in pathogen-infected cells appears to be contradictory to the increase in IP<sub>3</sub> content reported previously in elicitor-treated cells (Legendre et al.



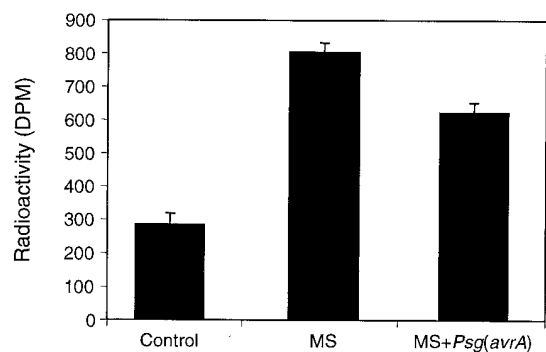
**Fig. 2.** Effect of *Pseudomonas syringae* pv. *glyciniae* (*Psg*) *Psg(avrA)*-inoculation on cellular cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) content of Murashie-Skoog (MS) salts treated cells. Cellular IP<sub>3</sub> content was measured by a commercial radioreceptor assay kit. Data are means of three replications. Bars indicate standard errors of mean. Broken bold line, inoculated with *Psg(avrA)*; broken regular line, water control; solid bold line, MS-treated cells; solid regular line, cells treated with MS salts and *Psg(avrA)*.



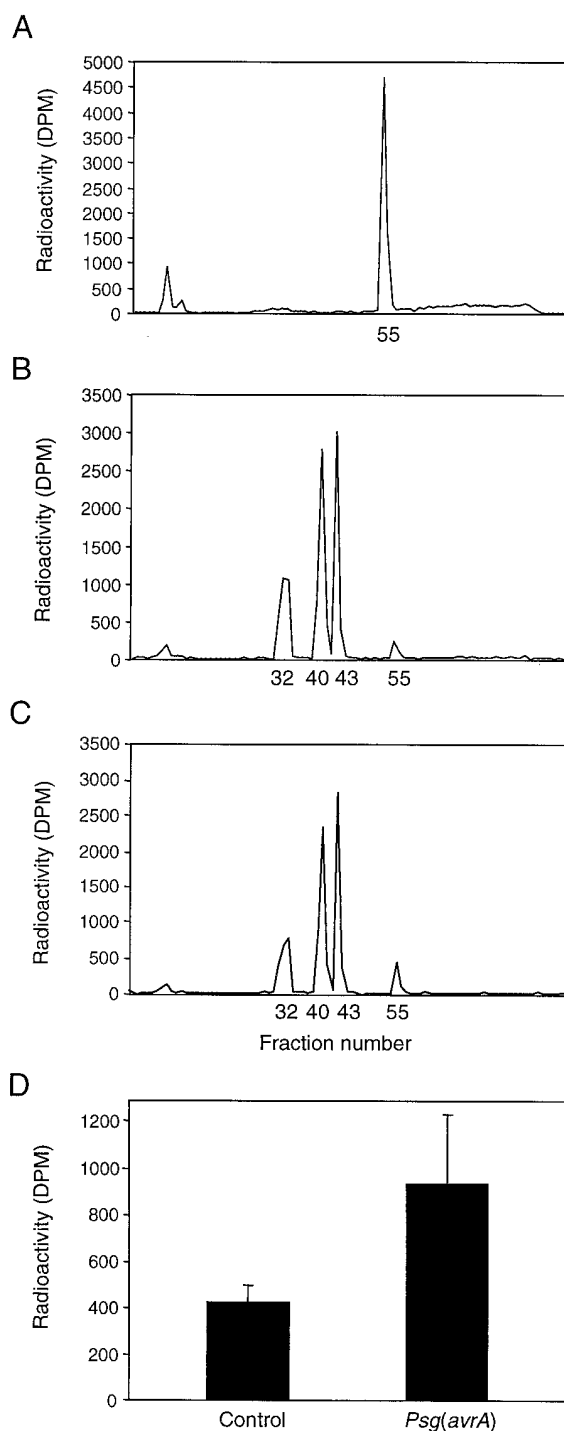
**Fig. 1.** Changes in cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) content, H<sub>2</sub>O<sub>2</sub> generation, and phenylalanine ammonia-lyase (PAL) enzyme activity in cells infected with *Pseudomonas syringae* pv. *glyciniae* (*Psg*) *Psg(avrA)* or *Psg(avrC)*. Regular line, *Psg(avrC)*-infected cells; bold line, *Psg(avrA)*-infected cells; broken line, water control. **A** and **C**, Data are means of two replications. **B**, Data are means of three replications. Bars indicate standard errors of mean. **A**, Changes in cellular IP<sub>3</sub> content over 3 h. Cellular IP<sub>3</sub> content was measured by a commercial radioreceptor assay kit. **B**, Short-term changes in IP<sub>3</sub> content between 15 s and 3 min following addition of water or inoculum. Absolute values of IP<sub>3</sub> appear to vary among experiments. **C**, H<sub>2</sub>O<sub>2</sub> generation measured as quenching of scopoletin fluorescence. Depletion of scopoletin fluorescence in cell culture indicates H<sub>2</sub>O<sub>2</sub> production by the cells. Data are expressed as percentages of those of the water control values. **D**, PAL enzyme activity measured by production of <sup>14</sup>C-labeled cinnamic acid after labeling with [<sup>14</sup>C]phenylalanine. Data are percentage of radioactivity over water control.

1993; Walton et al. 1993). This indicates that the changes in elicitation may not reflect the changes that occur in infected cells. It is conceivable that in a pathogen attack, whether by a virulent or avirulent strain, much of the energy must be utilized in defense, at the expense of the housekeeping cell functions. In parsley (*Petroselinum crispum*) suspension cells, UV irradiation or fungal elicitor treatment causes transcriptional activation of defense-related genes and a deactivation of cell cycle-related genes (Logemann et al. 1995).

The HPLC profiles of inositol phosphates for the water control and infected cells were similar when radiolabeled IP<sub>3</sub> was added to crude extracts to assay phosphatase activities (Fig. 4B,C). However, the amount of undegraded IP<sub>3</sub> in *Psg(avrA)*-infected cells was greater than that in the water control (Fig. 4D), indicating that the rate of IP<sub>3</sub> degradation is faster in uninoculated cells. Therefore, the IP<sub>3</sub> decrease in *Psg(avrA)*-infected cells is attributed to reduction in IP<sub>3</sub> synthesis. While it is possible that IP<sub>3</sub> can be synthesized by pathways other than PI-PLC in other organisms (Drayer et al. 1994; Van Dijken et al. 1996), it was shown that the plant IP<sub>3</sub> is an enzymatic product of PI-PLC (Brearley et al. 1997). Thus, deactivation of PI-PLC is most likely responsible for the infection-specific reduction in IP<sub>3</sub> content. PI-PLC plays an important role in cell growth in yeast and animals (Flick and Thorner 1993; Yoko-o et al. 1993; Berridge 1993). Growth retardation due to aluminum toxicity was attributed to PI-PLC inhibition in plant (Jones and Kochian 1995). A gravitation-stimulated IP<sub>3</sub> increase has been proposed as a signal for pulvinus cell elongation in maize (Perera et al. 1999). PI-PLC also most likely plays a major role in the DNA synthesis in soybean cell suspension following replenishment of cell culture medium (T. Shigaki and M. K. Bhattacharyya, *unpublished*). In plants, most likely the IP<sub>3</sub> signal pathway is constitutive, as shown by the higher resting state IP<sub>3</sub> contents (this study and Perera et al. 1999), and probably plays a major role in cell division, growth, and elongation. In infected cells, such functions are most likely inhibited through the reduction of IP<sub>3</sub> content. This enables the utilization of host energy supply in meeting the new demands of a host-pathogen interaction. We speculate that the signal(s) of either plant or pathogen origin suppresses the PI-PLC following infection so that plant metabolites are utilized (i) in the synthesis of defense-



**Fig. 3.** Reduction of cellular cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) content in *Pseudomonas syringae* pv. *glycinea* (*Psg*) *Psg(avrA)*-infected cells measured by high performance liquid chromatography (HPLC). Samples were collected 30 min after treatment. Radioactivity of the peak corresponding to inositol 1,4,5-trisphosphate was measured. Data are means of three replications. Bars indicate standard errors of the mean.



**Fig. 4.** High performance liquid chromatography (HPLC) profiles of inositol phosphates following incubation of [<sup>3</sup>H]cytosolic inositol 1,4,5-trisphosphate (IP<sub>3</sub>) in crude extracts from *Pseudomonas syringae* pv. *glycinea* (*Psg*) *Psg(avrA)*-infected cells or water control. Fractions number 55 and 40 correspond to IP<sub>3</sub> and I(1,4)P<sub>2</sub>, respectively. Fraction number 32 and 43 are most likely various isoforms of inositol mono-phosphates and I(4,5)P<sub>2</sub>, respectively. **A**, Boiled crude extract as a negative control. **B**, Extract from water control. **C**, Extract from *Psg(avrA)*-infected cells. **D**, Amount of undegraded IP<sub>3</sub>. Data represent means of three replications. Bars indicate standard error of the mean.

related compounds at the expense of housekeeping functions such as cell division and/or (ii) in supplementing the growth of plant pathogens.

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