Plant Physiology, April 1999, Vol. 119, pp. 1289–1296, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Involvement of the Octadecanoid Pathway and Protein Phosphorylation in Fungal Elicitor-Induced Expression of Terpenoid Indole Alkaloid Biosynthetic Genes in *Catharanthus roseus*

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Two key genes in terpenoid indole alkaloid biosynthesis, *Tdc* and *Str*, encoding tryptophan decarboxylase and strictosidine synthase, respectively, are coordinately induced by fungal elicitors in suspension-cultured *Catharanthus roseus* cells. We have studied the roles of the jasmonate biosynthetic pathway and of protein phosphorylation in signal transduction initiated by a partially purified elicitor from yeast extract. In addition to activating *Tdc* and *Str* gene expression, the elicitor also induced the biosynthesis of jasmonic acid. The jasmonate precursor α-linolenic acid or methyl jasmonate (MeJA) itself induced *Tdc* and *Str* gene expression when added exogenously . Diethyldithiocarbamic acid, an inhibitor of jasmonate biosynthesis, blocked both the elicitor-induced formation of jasmonic acid and the activation of terpenoid indole alkaloid biosynthetic genes. The protein kinase inhibitor K-252a abolished both elicitor-induced jasmonate biosynthesis and MeJA-induced *Tdc* and *Str* gene expression. Analysis of the expression of *Str* promoter/*gusA* fusions in transgenic *C. roseus* cells showed that the elicitor and MeJA act at the transcriptional level. These results demonstrate that the jasmonate biosynthetic pathway is an integral part of the elicitor-triggered signal transduction pathway that results in the coordinate expression of the *Tdc* and *Str* genes and that protein kinases act both upstream and downstream of jasmonates.

The initiation of a plant defense response requires the perception of pathogen-derived (exogenous) or plant-derived (endogenous) signal molecules, collectively referred to as elicitors (for review, see Boller, 1995; Yang et al., 1997). Elicitor-induced defense responses include the biosynthesis of secondary metabolites (Darvill and Albersheim, 1984; Côté and Hahn, 1994) and proteinase inhibitors (Pearce et al., 1991). Protein phosphorylation is an essential component of elicitor-induced signal transduction (Chandra and Low, 1995; Suzuki et al., 1995). The lipid-based octadecanoid pathway leading to JA has also been implicated as an integral part of the signal transduction pathway leading to the activation of defense responses. The octadecanoid pathway was first implicated in wounding-induced biosynthesis of proteinase inhibitors (Farmer and Ryan, 1992). Upon wounding, the octadecanoid pathway is activated by the polypeptide systemin and by oligouronides, resulting in elevated levels of JA (Doares et al., 1995b). JA and its octadecanoid precursors activate the synthesis of wounding-inducible proteinase inhibitors (Farmer and Ryan, 1992). Induction of the synthesis of proteinase inhibitors by wounding, systemin, and oligouronides is blocked by several inhibitors of the jasmonate biosynthetic pathway (Farmer et al., 1994; Doares et al., 1995b). Furthermore, Kim et al. (1992) identified a MeJA-responsive element in the promoter of a proteinase inhibitor II gene, indicating that this gene is transcriptionally regulated in response to MeJA.

How elicitors affect JA biosynthesis and how the JA signal is transduced to effect gene expression is largely unknown. Much more is known about the JA biosynthetic pathway itself. Farmer and Ryan (1992) have proposed that a lipase generates α-linolenic acid, the first precursor in the octadecanoid pathway. α-Linolenic acid is then converted by a lipoxygenase, an allene oxide synthase, and an allene oxide cyclase into the intermediate 12-oxo-phytodienoic acid. This compound is converted into JA through the action of a reductase and three rounds of β-oxidation (Vick and Zimmerman, 1984; Mueller, 1997).

JA and its octadecanoid precursors have also been implicated as intermediate signals in elicitor-induced secondary metabolite accumulation (Gundlach et al., 1992; Mueller et al., 1993; Ellard-Ivey and Douglas, 1996; Nojiri et al., 1996). A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite accumulation was shown in cells of California poppy (Mueller et al., 1993) and rice (Nojiri et al., 1996). In parsley cells phenylpropanoid biosynthetic genes were induced by octadecanoids, and elicitor-induced gene expression was blocked by a lipoxygenase inhibitor (Ellard-Ivey and Douglas, 1996). These reports indicate that in elicitor-induced secondary metabolism JA plays a role that is similar to its role in the accumulation of wound-induced proteinase inhibitors, for which it has been elegantly demonstrated that

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Abbreviations: BH, *Bgl*II/*Hinc*II; DIECA, diethyldithiocarbamic acid; JA, jasmonic acid; MeJA, methyl jasmonate; OA, okadaic acid; PE, partially purified YE elicitor; TIA, terpenoid indole alkaloid; YE, yeast extract.

jasmonates are intermediate signals that transcriptionally activate proteinase inhibitor genes. However, the studies that have been done on various metabolic pathways in different plant species using diverse elicitors lack the integrated approach of measuring jasmonate biosynthesis and studying the effect of JA and octadecanoid pathway inhibitors on gene expression. In general, metabolite accumulation has been studied instead of gene expression. Therefore, in most cases it remains unclear on what regulatory level jasmonates exert their effect on secondary metabolism.

In *Catharanthus roseus* cell suspensions, the expression of two TIA biosynthetic genes, *Tdc* and *Str*, encoding Trp decarboxylase and strictosidine synthase, respectively, is coordinately induced by fungal elicitors such as YE (Pasquali et al., 1992). The enzymes encoded by these genes are important in the TIA biosynthetic pathway (for reviews, see Meijer et al., 1993; Kutchan, 1995). Both genes are present as single copies in the haploid *C. roseus* genome (Pasquali et al., 1992; Goddijn et al., 1994).

Using the integrated approach of measuring JA biosynthesis and using an inhibitor of the octadecanoid pathway, we show conclusively that elicitor-induced expression of the *Tdc* and *Str* genes in *C. roseus* cells is mediated by the octadecanoid pathway. Furthermore, we provide evidence for the existence of protein phosphorylation steps both upstream and downstream of the octadecanoid pathway. We also show that both elicitor-induced and MeJA-induced gene expression are conferred by the *Str* promoter, demonstrating that elicitor and MeJA act at the transcriptional level.

MATERIALS AND METHODS

Cell Culture

Cell-suspension cultures of the *Catharanthus roseus* line MP183L were grown as described by Pasquali et al. (1992). A BH fragment of 396 bp (−339 to +52 relative to the transcriptional start site) was excised from the *Str* promoter (accession no. Y10182) and fused to the *gus*A gene in the vector GusSH digested with *Bam*HI and *Hin*cII (Pasquali et al., 1994). The BH derivative of GusSH was used to make transgenic *C. roseus* MP183L cell lines by particle bombardment according to the method of van der Fits and Memelink (1997).

Elicitor and Jasmonate Treatment

YE (DIFCO Laboratories, Detroit, MI) was dissolved in water, autoclaved, and used as a crude extract at a concentration of 400 g mL^{-1} to elicit *C. roseus* cells. PE was prepared through ultrafiltration and a number of chromatographic steps, including size-exclusion chromatography, anion-exchange chromatography, and reversed-phase HPLC (F.L.H. Menke, M. Harleveld, and J. Memelink, unpublished data). This resulted in a partially purified elicitor preparation of unknown absolute quality. We estimated the active component in PE to be purified at least 1000-fold. Crude YE/PE and induced the alkalinization of the cell culture medium within 5 to 10 min. The kinetics and the amplitude of the alkalinization response were proportional to the amount of elicitor added. Using this semiquantitative alkalinization assay, the amount of PE used for induction experiments was calibrated to give an alkalinization response that was equivalent to the effect of 400 g mL^{-1} crude YE. MeJA (Bedoukian Research, Danbury, CT) and α-linolenic acid and linoleic acid (both from Sigma) were diluted in DMSO.

Inhibitor Treatment

Ibuprofen (Sigma), K-252a (Calbiochem), staurosporine (Sigma), and OA and calyculin A (both from Calbiochem) were dissolved in DMSO. DIECA and acetyl-salicylic acid (both from Sigma) were dissolved in 15 mM KPO_4, pH 6.5. Each inhibitor was added 10 min before the addition of elicitor or MeJA. Cells were incubated in the presence of the elicitor or MeJA for 6 h unless indicated otherwise.

RNA Extraction and Northern Analysis

We extracted RNA and performed northern analysis as described previously by Menke et al. (1996) Unless indicated otherwise, 10-μg RNA samples were loaded onto the gels. All northern blots used ^{32}P-labeled cDNA probes. For *Tdc* we used a cDNA clone that was full length and otherwise homologous to the cDNA cloned by De Luca et al. (1989). The full-length cDNA clone for *Str* (Pasquali et al., 1992) and a cDNA corresponding to *Rps9* (40S ribosomal protein S9) were isolated from *C. roseus* (L. van der Fits and J. Memelink, unpublished data).

JA Extraction and Quantification

Three- or four-day-old *C. roseus* cell suspensions (MP183L) were incubated with YE or PE for 0 to 6 h. The cells were harvested by vacuum filtration using an 80-μm mesh. We divided the samples into portions for RNA extraction and portions for JA extraction. The cells were snap frozen in liquid nitrogen and stored at −80°C. We used 6 to 12 g (fresh weight) of the cells to extract jasmonates and extracted and quantified the jasmonates according to the method described by Mueller and Brodschelm (1994).

RESULTS

Tdc and *Str* gene expression in suspension-cultured *C. roseus* cells is coordinately induced by the addition of fungal elicitors (Pasquali et al., 1992). The crude elicitor preparations used by Pasquali et al. (1992) are less suitable for signal transduction studies because they may consist of multiple components that exert different effects on *C. roseus* cells. Therefore, the purification and characterization of a YE elicitor were undertaken (F.L.H. Menke, M. Harleveld, and J. Memelink, unpublished data), and a PE that induces *Tdc* and *Str* gene expression was obtained (Fig. 1).

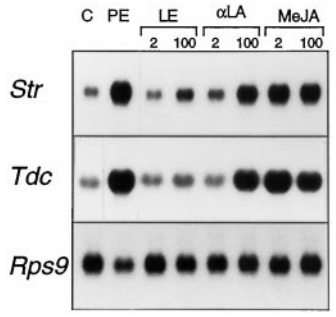

Figure 1. PE and octadecanoids induce the TIA biosynthetic genes *Tdc* and *Str*. *C. roseus* cells were exposed to DMSO as a control (C) or PE, linoleic acid (LE), α-linolenic acid (α-LA), or MeJA for 6 h, after which time cells were harvested and total RNA was isolated. Northern blots were hybridized with *Tdc*, *Str*, and *Rps9* cDNAs. The micromolar concentrations of the octadecanoids are given at the top.

Octadecanoids Induce TIA Biosynthetic Gene Expression

The octadecanoid pathway has been implicated in wounding- and elicitor-induced signaling leading to defense gene expression in plants (Farmer and Ryan, 1992; Gundlach et al., 1992). We tested the ability of octadecanoids to induce TIA biosynthetic gene expression in suspension-cultured *C. roseus* cells. Cultures were treated for 6 h with MeJA, the jasmonate precursor α-linolenic acid, and a related compound, linoleic acid, which cannot function directly as a precursor in the octadecanoid pathway at concentrations between 2 and 200 μM. Figure 1 shows that 2 μM MeJA induced TIA biosynthetic gene expression to a high level. At 2 μM, α-linolenic acid did not induce gene expression over the control level. At 100 μM α-linolenic acid, the levels of *Tdc* and *Str* transcripts were comparable to those induced by PE. Higher concentrations of α-linolenic acid did not further increase the transcript levels (data not shown). The octadecanoid linoleic acid did not significantly induce *Str* or *Tdc* gene expression levels in the concentration range tested (Fig. 1; data for 200 μM not shown). Figure 1 also shows that PE, MeJA, and the two octadecanoids had little effect on the level of *Rps9* mRNA, encoding the 40S ribosomal protein S9. These results indicate that the expression of *Tdc* and *Str* genes in suspension-cultured *C. roseus* cells is induced by MeJA and its precursor, but not by a related octadecanoid that does not form part of the octadecanoid pathway.

PE Induces JA Biosynthesis

The fact that both PE and octadecanoids induced TIA biosynthetic gene expression suggests that the octadecanoid pathway mediated elicitor-induced signaling. If so, then involvement of the octadecanoid pathway should be reflected by an increase in endogenous levels of jasmonates in *C. roseus* cells in response to PE. To test this hypothesis, suspension cultures were exposed for different lengths of time to PE and crude YE. Figure 2A shows a representative experiment. PE induced the accumulation of endogenous JA within 1 h, reached a maximum at 3 h, and then decreased to background level within 5 h. The addition of crude YE induced the accumulation of JA with similar kinetics (Fig. 2A). The corresponding northern blot (Fig. 2B) shows a steady increase in the levels of *Tdc* and *Str* mRNAs, starting at 2 h and continuing up to 5 h for both PE and crude YE, whereas no significant changes were observed in the levels of *Rps9* mRNA. In the noninduced control cultures, neither endogenous JA nor *Tdc* or *Str* transcripts accumulated this time. Four independent experiments within were done and all showed the same pattern, although the maximum amount of JA that was induced upon elicitor treatment varied from 20 to 120 ng g^{-1} (dry weight). These results indicate that the PE induces a transient accumulation of endogenous JA and a concomitant increase in TIA biosynthetic gene expression.

The Octadecanoid Pathway Mediates Elicitor-Induced TIA Biosynthetic Gene Expression

If jasmonates act as intermediates in elicitor-induced signaling, then inhibitors of the octadecanoid pathway should block PE-induced TIA biosynthetic gene expression. To test this hypothesis, we incubated cells with the octadecanoid pathway inhibitor DIECA and then studied the effect on PE-induced responses. DIECA inhibits the octadecanoid pathway by reducing the intermediate 13-*S*-hydroperoxylinolenic acid to 13-hydroxylinolenic acid, which is not an intermediate in the octadecanoid pathway

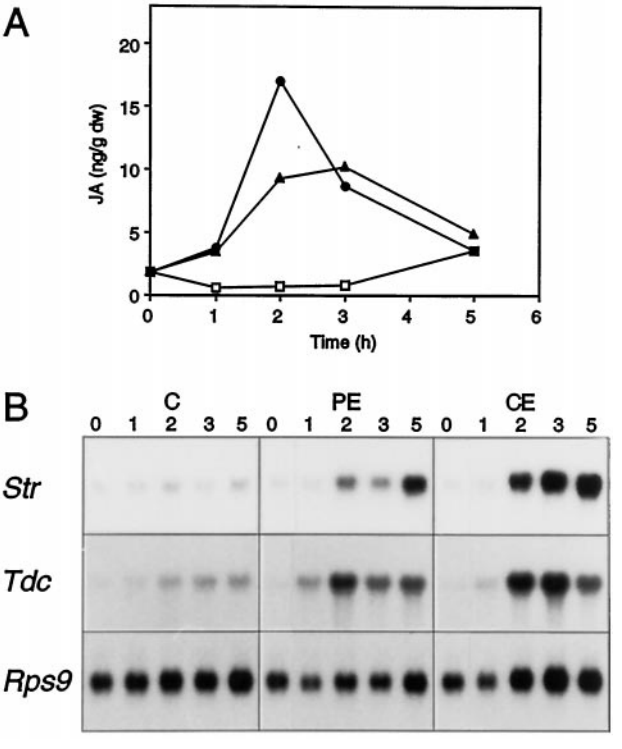

Figure 2. YE elicitor induces JA biosynthesis. A, Amount of JA in *C. roseus* cells at different times after the addition of water as a control (□) or PE (▲) or crude (●) YE. DW, Dry weight. B, *Str*, *Tdc*, and *Rps9* mRNA levels at different times after the addition of water as a control (C) or of PE or crude (CE) YE. RNA was extracted from the same sample of tissue used to extract JA. The times in hours are indicated (top).

(Farmer et al., 1994). DIECA completely blocked the elicitation of *Str* and *Tdc* gene expression in *C. roseus* cells at 500 μM, but the level of *Rps9* mRNA was not affected (Fig. 3A). At 100 μM, DIECA had no effect (Fig. 3A). The two highest concentrations of DIECA tested did not significantly affect MeJA-induced *Str* and *Tdc* gene expression (Fig. 3A). This indicates that DIECA acts on the octadecanoid pathway upstream of (Me)JA, which is in agreement with the results of Farmer et al. (1994).

In a separate experiment to determine the effect of DIECA (500 μM) on JA biosynthesis, we found an inhibition of 95% relative to the PE-induced accumulation of JA (Fig. 3B). The corresponding northern blot showed that DIECA had a clear inhibitory effect on *Tdc* and *Str* mRNA accumulation in this experiment but did not affect the level of *Rps9* mRNA. From Figure 3B, it is clear that strong inhibition of JA accumulation by DIECA resulted in a comparably strong inhibition of *Tdc* and *Str* mRNA accumulation. The results obtained with DIECA show that elicitor-induced *Str* and *Tdc* gene expression in *C. roseus* was mediated through the octadecanoid pathway.

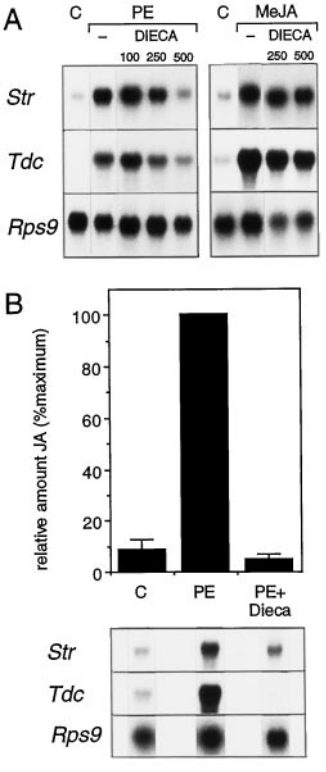

Figure 3. A, Effect of DIECA on TIA biosynthetic gene expression. *C. roseus* cell suspensions were incubated with DIECA for 10 min prior to the addition of water as a control (C), PE, or 50 μM MeJA, and cells were harvested 6 h later. The micromolar concentrations of inhibitors are indicated at the top. B, Effect of octadecanoid inhibitor on PE-induced JA accumulation. *C. roseus* cells were incubated with water or PE for 3 h. DIECA was added 10 min before the addition of PE. Bars represent SE ($n = 3$).

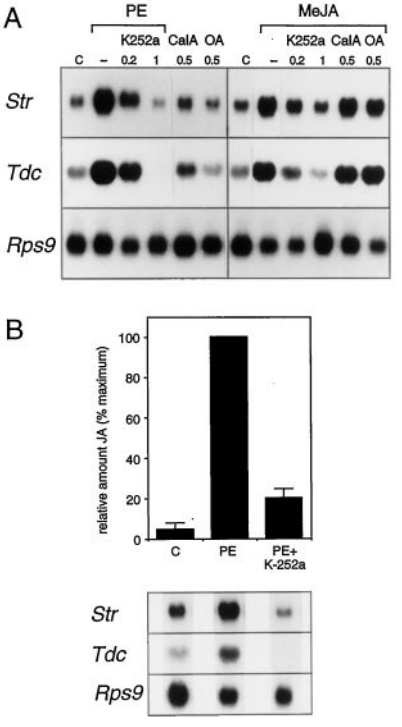

Figure 4. Protein kinases act upstream and downstream of JA. A, Effect of inhibitors on TIA biosynthetic gene expression. *C. roseus* cell suspensions were incubated with DSMO as a control (C), K-252a, calyculin A (CalA), or OA for 10 min before the addition of either PE or 50 μM MeJA, and cells were harvested 6 h later. The micromolar concentrations of the inhibitors are indicated at the top. B, Inhibition of PE-induced JA accumulation by a protein kinase inhibitor. *C. roseus* cells were incubated with 1 μM K-252a for 10 min before the addition of PE, and cells were harvested 3 h later. Bars represent the SE ($n = 3$).

Involvement of Protein Phosphorylation/Dephosphorylation

The mechanisms by which the octadecanoid signal is generated and subsequently transduced to affect gene expression are not known. To begin to address this question, we studied the effect of inhibitors of protein kinases and protein phosphatases on elicitor- and MeJA-induced gene expression. Treatment of *C. roseus* cells with different concentrations of the protein kinase inhibitor K-252a resulted in the inhibition of elicitor-induced TIA biosynthetic gene expression (Fig. 4A). Treatment of the cells with 200 nM K-252a resulted in a slight reduction of the amounts of *Str* and *Tdc* mRNA that accumulated 6 h after the addition of PE. At 1 μM, the mRNA accumulation in response to the elicitor was completely blocked for both *Str* and *Tdc*. The level of *Rps9* mRNA was not affected by incubation with this protein kinase inhibitor, indicating that at these concentrations K-252a does not cause a general down-regulation of gene expression. We obtained similar results using 1 μM of the protein kinase inhibitor staurosporine (data not shown). MeJA-induced TIA biosynthetic gene expression was also sensitive to the protein kinase inhibitors K-252a (Fig. 4A) and staurosporine (data not shown). We found complete inhibition of MeJA-induced expression

at 1 μM K-252a for both the *Str* and the *Tdc* genes (Fig. 4A). The inhibitory effect of the protein kinase inhibitors K-252a and staurosporine on PE- and MeJA-induced *Tdc* and *Str* gene expression shows that downstream of MeJA one or more protein kinases mediate the signaling that leads to TIA biosynthetic gene expression.

To investigate whether a protein kinase is also active upstream of the octadecanoid pathway, we measured the effect of PE on JA levels in the presence of K-252a. In cells that were treated with 1 μM K-252a, the amount of JA that accumulated 3 h after the addition of PE was reduced to 15%, relative to the elicitor-induced level (Fig. 4B). In the same sample the amounts of *Tdc* and *Str* mRNAs were also reduced compared with the PE-induced sample, whereas the level of *Rps9* mRNA was not affected. These results indicate that upstream of the octadecanoid pathway one or more protein kinases mediate the signaling triggered by PE, leading to jasmonate biosynthesis. The fact that K-252a and staurosporine both inhibited a broad range of protein kinases precludes any speculation about their identity.

We tested the effect of the protein phosphatase inhibitors calyculin A and OA on the expression of TIA biosynthetic genes. Addition of the protein phosphatase inhibitor calyculin A at 500 nM induced the accumulation of *Tdc* and *Str* mRNA in the absence of elicitor. The protein phosphatase inhibitor OA did not induce TIA biosynthetic gene expression in the absence of elicitor at the concentrations tested (Fig. 4A). We also tested the effect of the protein phosphatase inhibitors in the presence of MeJA. Treatment with either calyculin A or OA at 500 nM had no effect on the amounts of *Tdc* and *Str* transcripts induced by MeJA. Calyculin A and OA had no effect on the level of *Rps9* mRNA. The induction of gene expression by calyculin A indicates that a protein phosphatase may be involved in the attenuation of TIA biosynthetic gene expression. The results show that protein kinase(s) mediated the elicitor-induced signal both upstream and downstream of the octadecanoid pathway and that protein phosphatase(s) was involved in attenuating the signal.

The *Str* Promoter Responds to Elicitor and MeJA

To test whether elicitor- and MeJA-induced mRNA accumulation is due to transcriptional activation, an *Str* promoter fragment fused to the *gusA* reporter gene was introduced in suspension-cultured *C. roseus* cells through particle bombardment. Each transgenic cell line was a mixed population, estimated to consist of thousands of independent transformants. The transgene expression level therefore reflected an average and can be considered to be independent of the chromosomal position or copy number. Four independent mixed cell populations were generated. Figure 5 shows the expression of the *gusA* gene driven by 396 bp of the *Str* promoter upstream of the translational start codon (fragment BH). In transgenic cells incubated with PE or MeJA, *gusA* mRNA accumulated as compared with the noninduced control (Fig. 5). The responses of the transgene and the endogenous *Str* gene were qualitatively similar in each independent cell line. It can be concluded that elicitor and MeJA induce transcription directed by the BH fragment of the *Str* promoter.

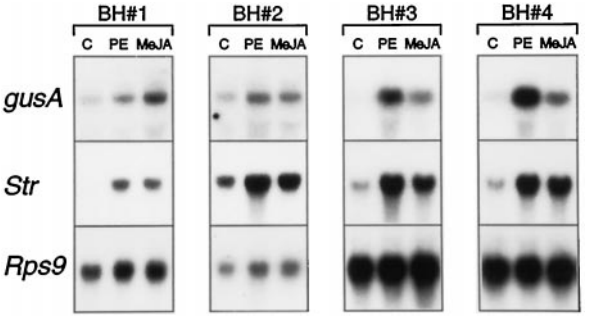

Figure 5. The *Str* promoter responds to elicitor and MeJA mRNA levels of *gusA*, *Str* and *Rps9* in independent transgenic cell lines containing the BH construct (BH#1, BH#2, BH#3, and BH#4). Cells were incubated for 6 h with DMSO as a control (C) or with PE or MeJA. RNA (20 μg) was loaded per lane.

DISCUSSION

For wound-inducible proteinase inhibitor synthesis it has been well established that the octadecanoid pathway is involved in signal transduction, leading to transcriptional activation of proteinase inhibitor genes. The involvement of the octadecanoid pathway in elicitor-inducible expression of genes active in secondary metabolism, however, has not been as well characterized. In this study we established the involvement of the octadecanoid pathway in elicitor-induced transcriptional activation of genes of the TIA biosynthetic pathway in *C. roseus* cells.

Both *Str* and *Tdc* were up-regulated in response to low levels of exogenous MeJA and higher levels of the jasmonate precursor α-linolenic acid. These results are in agreement with the observation that *C. roseus* seedlings (Aerts et al., 1994) and suspension-cultured cells (Gantet et al., 1998) can accumulate TIAs in response to MeJA and imply that octadecanoids stimulate TIA metabolism via induction of the biosynthetic genes. Furthermore, we observed a transient stimulation of endogenous jasmonate accumulation by the PE, which corresponded with the induction of the TIA biosynthetic genes *Tdc* and *Str*. A similar transient increase in endogenous JA was reported for the *Rauvolfia*, *Agrostis*, *Eschscholtzia*, *Phaseolus*, and *Taxus* species, as well as in rice and tobacco cell suspensions in response to elicitors (Gundlach et al., 1992; Mueller et al., 1993; Nojiri et al., 1996; Rickauer et al., 1997). However, it is not just elicitors that can trigger a transient increase in endogenous JA in plants; wounding (Peña-Cortés et al., 1995; O'Donnell et al., 1996) and UV radiation (Conconi et al., 1996) can also cause this increase. Together with our results, these data suggest a general role for the octadecanoid pathway in induced defense responses. Elevation of the level of JA in the plant cell seems to be sufficient to induce defense responses. Alternatively, elevated jasmonate levels could serve to lower the threshold for induction of a specific defense response by a pathogenic signal (Wasternack and Parthier, 1997). According to the

latter scenario, a defined signal leads to a distinct response output due to a specific signal transduction cascade in combination with general, obligatory signaling. In both cases, inhibition of the octadecanoid pathway would leave plant cells unresponsive to the stress signal, thus inhibiting the induction of defense responses.

Inhibitors of jasmonate biosynthesis are useful for determining a direct involvement of the octadecanoid pathway in response to external stress. The induction of proteinase inhibitor genes in tomato by wounding and systemin can be blocked by DIECA (Farmer et al., 1994) and by acetylsalicylic acid (Doares et al., 1995a). Nojiri et al. (1996) showed ibuprofen to be a potent inhibitor of elicitor-induced phytoalexin production in rice suspension cultures. In the current study in which suspension-cultured *C. roseus* cells were used, DIECA inhibited elicitor-induced TIA biosynthetic gene expression. This octadecanoid pathway inhibitor acts on the octadecanoid intermediate 13-*S*-hydroperoxylinolenic acid, and inhibited only elicitor-induced *Tdc* and *Str* gene expression; it did not affect the levels of *Rsp9* mRNA (which encodes the 40S ribosomal protein S9). The results obtained using JA biosynthesis inhibitors should be interpreted with caution because these inhibitors may not be very specific. DIECA, for example, is also used as a free radical scavenger (Jabs et al., 1997). When we used other known inhibitors of the octadecanoid pathway, we found that they were not specific in our system. The lipoxygenase inhibitors ibuprofen and acetylsalicylic acid inhibited elicitor- and MeJA-induced *Tdc* and *Str* mRNA accumulation but also had a strong negative effect on the level of *Rps9* transcript. This indicates that these substances may be phytotoxic or that they may down-regulate transcription in general. These observations point out that it is important to evaluate the effect of these inhibitors on so-called housekeeping genes such as *Rsp9*.

Relatively little is known about the activation of jasmonate biosynthesis in response to stress. There is some evidence for protein kinases acting upstream of the octadecanoid pathway in elicitor- and wounding-induced responses (Blechert et al., 1995; Seo et al., 1995). The inhibition of PE-induced accumulation of endogenous JA in *C. roseus* cells by the protein kinase inhibitor K-252a suggests the need for protein phosphorylation in elicitor-induced signaling leading to stimulation of JA biosynthesis. Because K-252a inhibits a broad range of protein kinases, no conclusion can be made about the nature of the kinase(s) involved. However, Seo et al. (1995) suggested that a mitogen-activated protein kinase in tobacco may be a mediator of wound signal transduction upstream of the octadecanoid pathway. More recently, Stratmann and Ryan, (1997) showed that wounding and systemin in tomato plants can activate a mitogen-activated protein kinase-related kinase upstream of the octadecanoid pathway. Based on these results one might speculate that the kinase(s) acting upstream of the octadecanoid pathway in *C. roseus* is a member of the mitogen-activated protein kinase family. Downstream of the octadecanoid pathway one or more protein kinases are involved in transducing the JA signal. A protein kinase (cascade) ultimately changes the activity of transcription factors that regulate the expression of genes through the recognition of specific sequences in the promoter regions.

A number of *cis*-acting elements in the promoters of defense-related genes in diverse plant species that mediate responses to elicitors (Raventós et al., 1995; Rushton et al., 1996) or to MeJA (Kim et al., 1992; Xiang et al., 1996; Rouster et al., 1997) have been identified. Using transgenic *C. roseus* suspensions harboring *Str* promoter/*gusA* fusions we have demonstrated that elicitor- and MeJA-induced activation of *Str* gene expression occurs at the transcriptional level. In the *Str* promoter a region of 396 bp directly upstream of the translational start codon (BH) was sufficient to confer elicitor- and MeJA-responsive expression. This region of the *Str* promoter contains a G-box, an element that Kim et al. (1992) implicated in MeJA-responsive proteinase inhibitor II gene expression in potato. Detailed analysis of the *Str* promoter using 5′ and internal deletion mutants is required to reveal whether the G-box is an important element in the *Str* promoter.

Figure 6 shows a hypothetical model that is consistent with the results described in this article. Elicitation of TIA biosynthetic gene expression is mediated through protein phosphorylation and the octadecanoid pathway leading to jasmonate. We have shown here that in *C. roseus* cells

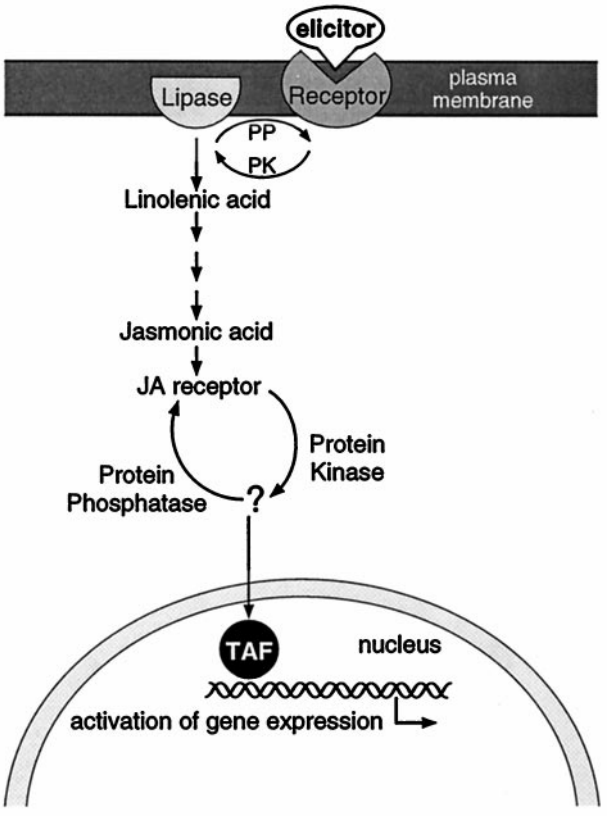

Figure 6. Model for elicitor signal transduction leading to TIA biosynthetic gene expression. The model shows the involvement of protein phosphorylation and the octadecanoid pathway in elicitor-induced TIA biosynthetic gene expression. Figure was adapted from Farmer and Ryan (1992). PK, Protein kinase; PP, protein phosphatase; TAF, transcription-activating factor.

upstream of the octadecanoid pathway and downstream of jasmonate, one or more protein kinases are involved in mediating the signal toward *Tdc* and *Str* gene expression.

ACKNOWLEDGMENTS

The authors acknowledge Marga Harteveld for excellent assistance with purification of the PE and Leslie van der Fits for help with the transformation of *C. roseus* suspension-cultured cells.

Received September 21, 1998; accepted December 8, 1998.

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