RESEARCH PAPER

Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to β-thujaplicin biosynthesis in Cupressus lusitanica cell cultures

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Received 25 November 2003; Accepted 10 February 2004

Abstract

Roles of jasmonate and ethylene signalling and their interaction in yeast elicitor-induced biosynthesis of a phytoalexin, β-thujaplicin, were investigated in Cupressus lusitanica cell cultures. Yeast elicitor, methyl jasmonate, and ethylene all induce the production of β-thujaplicin. Elicitor also stimulates the biosynthesis of jasmonate and ethylene before the induction of β-thujaplicin accumulation. The elicitor-induced β-thujaplicin accumulation can be partly blocked by inhibitors of jasmonate and ethylene biosynthesis or signal transduction. These results indicate that the jasmonate and ethylene signalling pathways are integral parts of the elicitor signal transduction leading to β-thujaplicin accumulation. Methyl jasmonate treatment can induce ethylene production, whereas ethylene does not induce jasmonate biosynthesis; methyl jasmonate-induced β-thujaplicin accumulation can be partly blocked by inhibitors of ethylene biosynthesis and signalling, while blocking jasmonate biosynthesis inhibits almost all ethylene-induced β-thujaplicin accumulation. These results indicate that the ethylene and jasmonate pathways interact in mediating β-thujaplicin production, with the jasmonate pathway working as a main control and the ethylene pathway as a fine modulator for β-thujaplicin accumulation. Both the ethylene and jasmonate signalling pathways can be regulated upstream by Ca²⁺. Ca²⁺ influx negatively regulates ethylene production, and differentially regulates elicitor- or methyl jasmonate-stimulated ethylene production.

Key words: Calcium, Cupressus lusitanica, elicitor, ethylene, jasmonate, phytoalexin, signal transduction.

Introduction

Jasmonate (JA) is widely distributed in the plant kingdom with multiple physiological functions during plant development, growth, and defence responses. It is accumulated in plants in response to wounding, pathogen and herbivore attack, as well as other biotic or abiotic stresses (Creelman and Mullet, 1997). Ethylene (ET) is another well-known plant hormone, whose production is also stimulated by wounding, herbivore and pathogen attack, and other biotic or abiotic stresses (Wang et al., 2002). The production of JA and ET and their consequent signalling pathways mediate various defence responses in plants, either independently or collaboratively to initiate an induced systematic resistance (Dong, 1998). It is believed that crosstalk between JA and ET pathways enables plants...
to optimize their defence strategies more efficiently and economically (Baldwin, 1998).

Methyl jasmonate (MeJA), a methyl ester of JA with the same physiological activity, has been reported to stimulate ET production in many plants such as tomato, Arabidopsis, and tobacco (Xu et al., 1994; O'Donnell et al., 1996; Penninckx et al., 1998). However, ET apparently stimulates JA production in only a few plants such as tomato (O'Donnell et al., 1996). In many cases, JA and ET co-operatively regulate defence responses. For example, JA stimulates proteinase inhibitor gene expression while ET alone does not, but JA and ET induce proteinase inhibitor genes synergistically in tobacco (O'Donnell et al., 1996). MeJA induces gum formation independently, whereas ET can enhance MeJA induction of gum in tulip shoots (Saniewski et al., 1998). A synergistic effect on insect-induced volatile emission in corn was also observed with JA and ET (Schmelz et al., 2003). In other cases, however, ET and MeJA simulate different sets of defence gene expression, and their effects antagonist each other. ET suppresses JA induction of gene expression in nicotine biosynthesis in tobacco leaves (Shoji et al., 2000). Even in the same plant, interaction patterns of JA and ET signalling may vary with different target compounds. While JA and ET acting antagonistically in nicotine biosynthesis in tobacco, no significant interaction can be observed for insect-induced sesquiterpene emission, even though both JA and ET pathways are required (Shoji et al., 2000; Kahl et al., 2000).

The JA pathway is generally regarded as an integral signal or elicitor signal transducer in the induction of a wide range of plant secondary metabolites, such as alkaloids, terpenoids, flavonoids, phenolic compounds, and phytoalexins (Gundlach et al., 1992; Mueller et al., 1993; Mirjalili and Linden, 1996; Brader et al., 2001). This idea has been supported in several plants by elucidating a close relationship between JA biosynthesis and secondary metabolite accumulation induced by elicitors (Gundlach et al., 1992; Mueller et al., 1993; Nojiri et al., 1996; Menke et al., 1999). However, recent studies show that even though octadecanoids can stimulate phytoalexin accumulation in soybean and parsley cell cultures, JA signalling does not mediate the fungal elicitor-induced phytoalexin accumulation. A β-glucan elicitor fails to induce endogenous JA biosynthesis in soybean cell cultures, but it induces the synthesis of phytoalexin via Ca²⁺ influx and oxidative burst (Fliedmann et al., 2003), while Pep-13 elicitor induces JA biosynthesis but it does not lead to a phytoalexin accumulation in parsley cell cultures (Hahlbrock et al., 2003). These results clearly show that not all cases of elicitor-induced phytoalexin accumulation involves JA signalling, and neither MeJA induction of secondary metabolite accumulation nor elicitor induction of JA and secondary metabolite biosynthesis can sufficiently prove that the JA pathway mediates elicitor-induced accumulation of secondary metabolites. Actually JA and elicitor signalling can parallel, or overlap, or interact in even more complicated ways in the induction of different secondary metabolites in plants. For example, both a yeast elicitor and MeJA induce 5-deoxyflavonoid production in Glycrrhiza glabra cell cultures; MeJA also stimulates soyasaponin accumulation but elicitor inhibits it (Hayashi et al., 2003). Both MeJA and cellulase elicitor stimulate capsidiol accumulation and its biosynthetic gene expression in tobacco cell culture, although MeJA induces much weaker and more delayed gene expression than elicitor does. Moreover, MeJA induces expression of at least two sesquiterpene cyclase genes including one that elicitor does not induce (Mandujano-Chavez et al., 2000).

Plant cell cultures derived from Mexican cypress (Cupressus lusitanica) can produce a high level of β-thujaplicin upon elicitor or MeJA treatment (Zhao et al., 2001). β-Thujaplicin is a tropolone compound with a broad spectrum antimicrobial activity, and is used in many areas (Zhao et al., 2001). Therefore, efforts have been made to produce this commercially important compound by C. lusitanica cell cultures. A better understanding of MeJA- or elicitor-induced production of β-thujaplicin will certainly facilitate the production of this compound. Previous studies have demonstrated that elicitor-induced production of β-thujaplicin involves multiple signalling, such as Ca²⁺, GTP binding proteins, and H₂O₂, probably through the JA signalling pathway (Zhao and Sakai, 2003a, b). This paper further investigated the role of JA signalling and its interaction with ET signalling in elicitor-induced production of β-thujaplicin. It is found that both JA and ET signalling pathways are involved in β-thujaplicin production. JA and ET signalling pathways interact in elicitor induction of β-thujaplicin, during which JA signalling works as a main control whereas ET acts as a fine modulator.

Materials and methods

Chemicals

Ibuprofen, diethylidithiocarbamic acid (DETC), pradifen (SKF-525A), aminooxyxynlyglycine (AVG), α-aminoisobutyric acid (AIBA), CoCl₂, silver acetate (AgAC), and 1-aminocyclopropane-1-carboxylic acid (ACC) were obtained from Sigma (St Louis). cis (-)-jasmonic acid, and a mixture of trans and cis (±)-jasmonic acid (JA) were obtained from Sigma. Methyl jasmonate was obtained from Wako Pure Chemicals (Osaka, Japan). Lanthanum chloride, ethylene glycol-bis-(β-aminoethyl) ether-N,N',N'',N'''-tetraacetic acid (EGTA), and ET gas were obtained from ICN (Tokyo, Japan). These reagents other than ET gas were dissolved in 0.05% DMSO solution or in water.

Plant cell culture and time-course study

The C. lusitanica suspension cultures were established from callus as previously described (Zhao et al., 2001). About 25 g of fresh callus were inoculated into 200 ml production medium in 500 ml flasks, and after 5 d of incubation, about 1 g of evenly dispersed fresh cells was inoculated into 20 ml production medium or normal growth
medium in 100 ml flasks. All flasks were incubated on a rotary shaker (120 rpm) at 24 °C in the dark. A yeast elicitor (YE) was a 70–80% ethanol-insoluble oligosaccharide fraction prepared from yeast extract. In time-course studies, the autoclave-sterilized YE (1 mg ml⁻¹), and filter-sterilized MeJA, ACC, or water, as well as ET gas, were added to cell cultures, the cell cultures were collected at desired intervals for analysis of β-thujaplicin, ET, or JA.

Treatment profiles

Five-d-old C. lusitanica suspension cells were used in all treatment experiments. In single treatment experiments, sterile YE, MeJA, (+)-JA, (−)-JA, gas ET, ACC or water were added to 5-d-old cell cultures. In combination treatments, all inhibitors were filter-sterilized, and added to the cell cultures 20 min before the addition of MeJA, ET, ACC, or elicitor. In the ET treatment, a known amount of gas ET from a plastic bag was injected with a syringe into culture flasks through a filter. To the cell cultures, the inhibitors, such as ibuprofen (5–100 μM), DETC (10–200 μM), proadifen (2.5–300 μM), AVG (5–50 μM), AIBA (100–200 μM), CoCl₂ (0.3–9 μM), AgAC (3–18 μM), ACC (100–500 μM), LaCl₃ (100–200 μM), or ETGA (1–3 mM), were added 20 min prior to YE, MeJA, ET, or ACC treatment. In complementary experiments, inhibitors were added to the cell cultures 20 min prior to the addition of YE plus (+)-JA (100 μM) or ET gas (200 ppm), respectively, for further incubation of 24 h. The control received the same volume of water or 0.05% DMSO. Cells were collected at indicated time.

ET determination

For measuring ET in the headspace of cell cultures, flasks were sealed with a plastic plug with a plastic cap-sealed glass tube, through which a syringe needle was inserted for sampling. ET concentration was determined by gas chromatograph (GC-12A, Shimadzu) with a flame ionization detector. Gas in the headspace of flasks was sampled with a tight syringe (1–2 ml), and was injected to the GC. ET was separated on a 3 mm i.d. × 2 m stainless steel column packed with Porapak Q operated isothermally at 80 °C, and using nitrogen as the carrier gas. The limit of detection of this operation was found to be 0.1 ppm, and the ET levels were calculated using a standard curve made under identical conditions as above.

ET production rate is the amount at time T divided by the interval, and is expressed as nl produced by 1 g of fresh cells in 1 h (nl g⁻¹ h⁻¹, at normal atmospheric pressure).

Extraction and measurement of JA

Extraction and measurement of JA was carried out according to Mueller and Brodschelm (1994) with a minor modification. Briefly, after elicitation, suspension cells (about 7 g) were collected by vacuum filtration and frozen in liquid N₂. After homogenization in liquid nitrogen, 10 ml of saturated NaCl solution, 0.5 ml of 1 M citric acid, and 25 ml of diethyl ether containing 0.005% (w/v) butylated hydroxytoluene were added. After further vigorous vortexing, the homogenate was centrifuged at 2000 g for 10 min, the ether phase was removed and the aqueous layer was extracted with 25 ml of ether containing 0.005% butylated hydroxytoluene. The combined ether phase was applied to an aminopropyl glass bead column. The column was washed with 5 ml of chloroform/isopropanol (2:1, v/v), and eluted with 7 ml of ether/acetic acid (98:2, v/v). The sample was dried with a nitrogen stream and dissolved in ether for GC-MS analysis. The GC-MS was set as follows: the injector was set at 280 °C, the carrier gas He is set at linear 23 cm s⁻¹, the column temperature followed a step gradient of 100 °C for 0.5 min, 100–180 °C at 20 °C min⁻¹, 180–300 °C at 10 °C min⁻¹, 300 °C for 5 min; the MS source was set at 140 °C and the electron energy at 70 eV. Retention time of JA was 11 min. The JA in the samples was identified by an MS spectrum compared with authentic JA. Because of the lack of a non-natural JA analogue as an internal standard, an alternative method was used to evaluate the extraction and quantification processes. Known amounts of cis- and trans (+)-JA was added to the harvested cells, and the recovery rate of JA following the above extraction procedure was determined. It was shown that recovery rate is more than 94%. To quantify the amount of JA with GC-MS, a standard curve was drawn by plotting the loading amount of JA against the corresponding peak area. The amount of JA was expressed as the sum of cis-JA and trans-JA on the basis of the method. The JA level was expressed as ng g⁻¹ of fresh cells.

Extraction and determination of β-thujaplicin

β-Thujaplicin was extracted and determined as previously described (Zhao et al., 2001). Cell cultures were collected by vacuum filtration through a Miracloth, and the cells were then weighed and quickly frozen. After homogenization of the cells, the homogenate was extracted twice with the same volume of ethyl acetate. The medium was similarly partitioned twice with ethyl acetate to extract β-thujaplicin. The ethyl acetate extracts from the cell homogenates and the medium were combined and dried under vacuum, and the residues were then treated with boron trifluoride methanol solution to form a β-thujaplicin-BF₃ complex. The samples were analysed by HPLC. A Waters 996 system with a PDA detector monitoring at 254 nm, an Inertsil ODS-3 column (4.6×150 mm, 5 μm), and a mobile phase composed of water/methanol (55:45) at a flow rate of 1 ml min⁻¹ were utilized. Vanillin was used as an internal standard. Biomass was expressed by fresh weight.

Data analysis

Data were generated from three independent triplicate experiments unless otherwise indicated. Data were analysed by one-way analysis of variance (ANOVA). Statistical significance of difference between samples was calculated using the t-test.

Results

YE, MeJA, and ET stimulate β-thujaplicin accumulation

β-Thujaplicin keeps a very low level in C. lusitanica cell cultures when cultivated in normal growth medium and begins to increase after transfer to the production medium containing higher concentration of Fe²⁺ (Zhao et al., 2001). As shown in Fig. 1A, accumulation of β-thujaplicin dramatically increased upon stimulation by YE and MeJA. Different stereoisomers of jasmonate, (+)-7-iso-JA and (−)-trans-JA, accumulated as twofold in the control (Fig. 1A). As previously reported, (+)-7-iso-JA is the physiologically active form in plants and both commercial sources of MeJA and JA contains less than 10% of (+)-7-iso-JA and (+)-trans-JA mixture (Mueller et al., 1993; Creelman and Mullet, 1997). When ET gas was applied to the cell cultures for 48 h, production of β-thujaplicin also increased (Fig. 1B). This effect was dose-dependent since high concentrations of ET (300 ppm) inhibited the accumulation of β-thujaplicin. ACC, an immediate precursor for ET biosynthesis, also stimulated β-thujaplicin accumulation by about 2-fold (Fig. 1B). The optimal dosage of ACC for stimulating β-thujaplicin is 200 μM, and that of ET gas is 200 ppm. These results indicate that
both JA and ET accumulation can stimulate the production of β-thujaplicin.

**YE stimulates JA and ET biosynthesis in the cell cultures**

If the JA or ET signalling pathway indeed mediated YE-induced production of β-thujaplicin, YE treatment should be able to stimulate the biosynthesis of JA or ET. Thus endogenous JA and ET production in the cell cultures were tested before and after elicitor treatment. As shown in Fig. 2A, after 4 h and 10 h of YE treatment, JA levels in *C. lusitanica* cells increased by about 3–5-fold over that in control, but the control cells remained at a basal level (approximately 3.8 ng g⁻¹ fresh cells). This significant increase of JA level in YE-treated *C. lusitanica* cells should be caused by YE treatment only. Elevation of JA occurred before the accumulation of β-thujaplicin, suggesting that biosynthesis of JA may be a signal for β-thujaplicin accumulation.

*C. lusitanica*-cultured cells incubated in the growth medium produce low level of ET. Figure 2B shows that more ET was produced when the cells were cultivated in the production medium. However, elicitor treatment stimulated a rapid and significant ET production (Fig. 2B). ET production rate is stable in both the growth medium and the production medium, while after application of YE, ET production markedly increased within 6 h, which clearly shows that YE stimulated ET biosynthesis.

**Suppression of JA biosynthesis blocks YE-induced β-thujaplicin accumulation**

To verify that the JA signalling pathway mediated YE-induced β-thujaplicin biosynthesis, three inhibitors of JA biosynthesis were tested for any effect on YE-induced β-thujaplicin production. JA biosynthesis, via the octadecanoid pathway, is initiated by lipase-catalysed lipid degradation, which generates the precursor linolenic acid. Linolenic acid is then converted by several enzymes including a lipoxygenase, an allene oxide synthase, and an allene oxide cyclase, into 12-oxo-phytodienoic acid. This intermediate is further converted into JA by a reductase and three rounds of ω-oxidation (Schaller, 2001). Ibuprofen is a potential inhibitor of lipoxygenase and inhibits elicitor-induced JA biosynthesis in rice cell cultures (Nojiri *et al.*, 1996). DETC inhibits JA biosynthesis by reducing the intermediate 13-5-hydroperoxylinolenic acid to 13-hydroxylinolenic acid that is not a precursor of JA biosynthesis (Menke *et al.*, 1999). Proadifen is a potent inhibitor of P450 enzymes, like allene oxide synthase (Rossi *et al.*, 1987). Application of all these inhibitors to the cell cultures inhibited YE-induced β-thujaplicin production (Fig. 3A, B, C). The dosage for inhibition of 50% of YE-induced β-thujaplicin production (*I₅₀*) by ibuprofen is 10 μM (Fig. 3B); *I₅₀* for DETC is 50 μM (Fig. 3A), and *I₅₀* for proadifen is about 20 μM (Fig. 3C). These results indicate that YE-induced biosynthesis of JA is required for YE-induced β-thujaplicin accumulation.

To exclude the side-effects of the three inhibitors on culture cells, loss of cells biomass after elicitor and inhibitor treatment was measured. At low concentrations (equal or lower than *I₅₀*), these inhibitors did not cause more losses in cell biomass than elicitor plus solvent did (results not shown). When 100 μM (±)-JA was added to the elicited cell cultures pretreated with 10 μM of proadifen or ibuprofen or 20 μM DETC, the YE-induced β-thujaplicin accumulation was completely recovered (Fig. 3D). Therefore, it was not the cytotoxic effects, but the suppression of JA biosynthesis by these inhibitors that caused the decreased β-thujaplicin accumulation.
Inhibition of ET biosynthesis and signalling suppresses β-thujaplicin induction by YE

Inhibitors of ET biosynthesis and signalling were used to test whether ET signalling was also involved in YE-induced β-thujaplicin production. ET biosynthesis starts with the conversion of methionine to S-AdoMet by S-AdoMet synthetase. Then S-AdoMet is converted to ACC by the action of ACC synthase, and finally ACC is converted to ET by ACC oxidase (Wang et al., 2002). AVG, a potent inhibitor of ACC synthase, CoCl₂ and AIBA, effective inhibitors of ACC oxidase, and Ag⁺, a potent inhibitor of ET signalling by competitively binding to the ET receptor, are commonly used in studying ET biosynthesis and signalling. Results show that pretreatment of the cultivated cells with these inhibitors suppressed the YE-induced β-thujaplicin production (Fig. 4A, B, C). The IC₅₀ for Ag⁺ inhibition of YE-induced β-thujaplicin production is 9 µM, whereas that for AVG is about 40 µM, and the IC₅₀ for CoCl₂ is 3 mM. These results suggest that YE-induced ET production is also required for YE-induced β-thujaplicin. Complementary experiments also show that inhibition of β-thujaplicin accumulation by AVG and CoCl₂ can be completely recovered by the application of
200 ppm of ET (Fig. 4D), suggesting that ET biosynthesis inhibited by AVG and CoCl₂ and signalling inhibited by Ag⁺ is necessary for the production of β-thujaplicin.

When YE plus ACC was added to the cell cultures, β-thujaplicin accumulation increased further, especially when 200 μM ACC was added (Fig. 4E). The inhibitory effect of AVG on YE-induced β-thujaplicin accumulation was partly restored by the additional application of ACC (100–200 μM). These data further suggest that ET biosynthesis and signalling are an integral part of elicitor signal transduction leading to β-thujaplicin accumulation.

**JA and ET interact in signalling β-thujaplicin accumulation**

How JA and ET signalling interact in YE-induced β-thujaplicin accumulation in *C. lusitanica* cell cultures is an interesting question. One way to answer this question is to test if the production of ET or JA could depend on the other. As shown in Fig. 5A, 100 μM MeJA strongly induced ET production. This ET production is about 1.5–3-fold higher than that in YE-treated cell cultures (Fig. 2). However, ET treatment (200 ppm) had little effect on JA biosynthesis (*P* <0.005) (Fig. 5B), suggesting that the origin of the ET signalling pathway is JA-dependent, whereas the origin of the JA signalling pathway may be ET-independent. On the other hand, the ET- or ACC-induced β-thujaplicin accumulation was almost completely abolished by treatment with DETC or ibuprofen (Fig. 5C), whereas pretreatment of the cells with inhibitors of ET biosynthesis and signalling partly suppressed β-thujaplicin accumulation induced by MeJA (Fig. 5D). Moreover, MeJA promotes β-thujaplicin accumulation
with ACC or ET (Fig. 5D). These data suggest that both JA and ET production is required for \( \beta \)-thujaplicin accumulation; JA signalling is more important than ET signalling.

**Ca\(^{2+}\) fluxes affect ET production in C. lusitanica cell cultures**

Several modulators of Ca\(^{2+}\) signalling were tested with MeJA- and YE-induced ET production to gain further insights into the interaction between the JA and ET signalling pathways. Ca\(^{2+}\) influx has been shown to play important roles in elicitor-induced \( \beta \)-thujaplicin accumulation (Zhao and Sakai, 2003a). As shown in Fig. 6A, application of LaCl\(_3\) and EGTA stimulated ET production in a dose-dependent manner. In the presence of YE or MeJA, the increase of ET caused by EGTA plus YE or MeJA was comparable to that caused by YE or MeJA alone. However, LaCl\(_3\) at 200 \( \mu\)M antagonized YE or MeJA-induced ET production (Fig. 6B, C). These results indicate that Ca\(^{2+}\) influx was involved in MeJA- or YE-induced ET production. All inhibitors decreased MeJA-induced \( \beta \)-thujaplicin accumulation (Fig. 6D), suggesting that Ca\(^{2+}\) influx is an important factor for MeJA- or YE-induced ET and \( \beta \)-thujaplicin production.

**Discussion**

This paper presents solid evidence for the JA signalling pathway acting as an integral signal and elicitor signal transducer for \( \beta \)-thujaplicin accumulation. For the first time, the current study systematically demonstrates that the JA and ET signalling pathways interact as integral parts of elicitor signal transduction leading to phytoalexin accumulation. Together with previous results, this provides a typical example of an elicitor signalling network, in which G-proteins, Ca\(^{2+}\), inositol 1,4,5-trisphosphate, \( \mathrm{H}_2\mathrm{O}_2\), JA, and ET work collaboratively in phytoalexin (\( \beta \)-thujaplicin) production (Zhao and Sakai, 2003a, b; Zhao et al., 2004).

Although the JA signalling pathway has been widely regarded as an integral signal in plant secondary metabolism, and a signal transducer for fungal elicitor-induced accumulation of plant secondary metabolites (Gundlach et al., 1992; Mueller et al., 1993; Nojiri et al., 1996; Menke et al., 1999), recent studies show that the accumulation of some plant phytoalexins is not stimulated by JA/MeJA, elicitor does not induce JA biosynthesis, or elicitor-induced JA biosynthesis does not cause the accumulation of some secondary metabolites (Fliegmann et al., 2003;
It may be necessary to reconsider some previous conclusions based only on octadecanoid treatment or testing elicitor-induced JA accumulation, but without examining the requirement of JA biosynthesis for elicitor induction of plant secondary metabolites. It has been shown that elicitor and MeJA induce the expression of different sets of genes or the accumulation of different groups of secondary metabolites in several plant systems (Hayashi et al., 2003; Mandujano-Chavez et al., 2000). The role of the JA signalling pathway in β-thujaplicin accumulation in C. lusitanica cell cultures has been systematically investigated in this paper. Both MeJA and JA treatment induce β-thujaplicin accumulation and YE treatment induces endogenous JA accumulation. Inhibitor treatment and complementary experiments further show that YE-induced JA biosynthesis is necessary and sufficient for β-thujaplicin accumulation in C. lusitanica cells. These data strongly support the idea that JA is an integral signal in β-thujaplicin production and it also mediates yeast elicitor-induced β-thujaplicin production.

Unlike JA signalling, only a few cases that ET alone induces the biosynthesis of plant secondary metabolites have been reported, such as ET induction of anthocyanin and resveratrol production in grapevine (Chung et al., 2001; El-Kereamy et al., 2003). In addition, ET often affects plant secondary metabolism by interacting with the JA pathway in different patterns. For example, ET enhances JA-induced taxol production in Taxus cell cultures (Miralilili and Linden, 1996). ET and JA together induce volatile emissions during insect-attacked corn leaves (Schmelz et al., 2003). ET also inhibits the JA-induced nicotine production in tobacco (Shoji et al., 2000), and exerts a negative effect on the production of anthocyanin and carotenoid in Vaccinium pahalae cell culture (Shibli et al., 1997).

In C. lusitanica culture cells, the application of ET or ACC induces β-thujaplicin accumulation, and the inhibition of YE-induced ET biosynthesis also decreases β-thujaplicin production, suggesting that ET signalling is also required for β-thujaplicin production. Current results also show that JA and ET signalling pathways interact during the induction of β-thujaplicin accumulation. JA induces significant ET production, whereas ET treatment does not affect the JA level; inhibitors of JA and ET biosynthesis and signalling can suppress ET- and JA-induced β-thujaplicin accumulation, respectively; a combination treatment of MeJA or YE with ET induces more β-thujaplicin accumulation (Fig. 5). These observations suggest that ET signalling interacts with JA in β-thujaplicin accumulation.

However, a comparison of the effects of JA or MeJA and ET or ACC treatment on the induction of β-thujaplicin accumulation shows that JA or MeJA is much more effective than ET or ACC. The more dominant role of JA signalling over ET signalling in the induction of β-thujaplicin is further supported by results from inhibitor treatments. Inhibition of ET production only reduces the MeJA-induced β-thujaplicin accumulation by about 20–
30% while the inhibition of JA biosynthesis can reduce the ET-induced β-thujaplicin accumulation by 70–95% (Fig. 5), suggesting that JA is a major signal whereas ET is a minor regulator for β-thujaplicin accumulation. Furthermore, it is likely that ET or ACC induces β-thujaplicin accumulation through modulating the endogenous JA-induced production of β-thujaplicin, and ET alone may not be able to induce β-thujaplicin accumulation at all. Because inhibition of basal JA biosynthesis significantly decreases the basal β-thujaplicin level in the production medium (Fig. 3D), a basal level of JA biosynthesis in cells cultivated in the production medium may be a direct inducer of ET- or ACC-induced β-thujaplicin accumulation (Fig. 2). JA level in C. lusitanica cells cultivated in normal B5 medium was almost undetectable and ET or ACC treatment of these cells did not induce more β-thujaplicin accumulation (results not shown). Therefore, ET production induced by JA or YE may enhance the effects of MeJA or YE on β-thujaplicin accumulation or increases the sensitivity of cells to MeJA and YE stimulation. This explains why cells in the production medium can produce more ET and β-thujaplicin than in normal B5 medium (Fig. 2B) and inhibition of ET biosynthesis does not substantially reduce the basal β-thujaplicin in the production medium (Fig. 4D). Fe²⁺ stress may be the inducer of a higher basal level of JA in the production medium than in growth medium.

On the other hand, ET signalling is not quantitatively correlated with β-thujaplicin production. More ET or ACC even inhibits β-thujaplicin accumulation (Fig. 1); MeJA induces more ET while less β-thujaplicin production than YE; EGTA does not affect YE-or MeJA-induced ET production but inhibits YE- or MeJA-induced-β-thujaplicin accumulation (Fig. 6). These variations also indicate that the ET pathway regulates the JA or YE effects on β-thujaplicin accumulation as a fine modulator within a certain range; low ET concentrations promote JA and YE-induced β-thujaplicin accumulation while too high a concentration of ET may inhibit them.

How Ca²⁺ signalling negatively regulates ET production in C. lusitanica cell cultures is not clear. In some plants, Ca²⁺ influx enhances ET production and responses (Raz and Fluhr, 1992), whereas in others, EGTA and LaCl₃ enhance ET production and Ca²⁺ influx reduces ET production (Berry et al., 1996). These diverse patterns of interaction between ET and Ca²⁺ signalling seem species-specific.

As summarized in Fig. 7, both JA and ET pathways and their interaction are integral signals for β-thujaplicin production. They are employed by the yeast elicitor as integral parts to mediate elicitor-induced β-thujaplicin accumulation. YE initiates the JA and ET pathways by induction of JA and ET biosynthesis. As a main mediator of YE signal transduction, JA signalling independently induces ET and β-thujaplicin production. As a fine modulator, the ET pathway affects β-thujaplicin production by modulating the JA and YE signalling pathways. It is certain that β-thujaplicin production is a combined result of multiple signalling pathways, including JA-dependent pathways and JA-independent pathways, since the production of β-thujaplicin induced by JA plus ET cannot account for that induced by YE.

The molecular basis of the JA and ET signalling pathways and their interactions has been studied. Several elicitor-, JA- and ET-response elements were found in promoter regions of many plant defence and secondary metabolite biosynthesis-related genes (van der Fits and Memelink, 2000; Fujimoto et al., 2000; Wang et al., 2002). Coexistence of some cis-elements in these genes and cross response of many transcription factors proved a molecular basis for crosstalk of JA and ET signalling (Brown et al., 2003). Moreover, a transcription factor that can integrate JA and ET signalling into a common plant defence response was also found (Lorenzo et al., 2003). Similar mechanisms may be employed in crosstalk of JA and ET pathways in mediating β-thujaplicin accumulation in C. lusitanica cells.

**Acknowledgements**

This work was supported by postdoctoral fellowship grant (No. 12099345) from the Japan Society for the Promotion of Science (JSPS), which is gratefully acknowledged. This work was also partially supported by the scientific research fund (No. 11876040 and No. 13306013) of the Japanese Ministry of Education, Science, and Culture. We are grateful to Dr. Lawrence Davis for critical reading of the manuscript.

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