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Signal transduction and metabolic flux of β-thujaplicin and monoterpene biosynthesis in elicited *Cupressus lusitanica* cell cultures

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Abstract

β-Thujaplicin is an antimicrobial tropolone derived from geranyl pyrophosphate(GPP) and monoterpene intermediate. Yeast elicitor-treated *Cupressus lusitanica* cell cultures accumulate high levels of β-thujaplicin at early stages and other monoterpenes at later stages post-elicitation. The different regulation of β-thujaplicin and monoterpene biosynthesis and signal transduction directing metabolic flux to β-thujaplicin firstly and then shifting metabolic flow from β-thujaplicin to other monoterpene biosynthesis were investigated. The earlier rapid induction of β-thujaplicin accumulation and a later stimulation of monoterpene biosynthesis by yeast elicitor are in well agreement with elicitor-induced changes in activity of three monoterpene biosynthetic enzymes including isopentenyl pyrophosphate isomerase, GPP synthase, and monoterpene synthase. Yeast elicitor induces an earlier and stronger β-thujaplicin production and monoterpene biosynthetic enzyme activity than methyl jasmonate (MeJA) does. Profiling all monoterpenes produced by *C. lusitanica* cell cultures under different conditions reveals that β-thujaplicin biosynthesis parallels with other monoterpenes and competes for common precursor pools. Yet β-thujaplicin is produced pre-dominantly at early stage of elicitation whereas other monoterpenes are mainly accumulated at late stage while β-thujaplicin is metabolized. It is suggested that yeast elicitor-treated *C. lusitanica* cells preferentially accumulate β-thujaplicin as a primary defense and other monoterpenes as a secondary defense. Inhibitor treatments suggest that immediate production of β-thujaplicin post-elicitation largely depends on pre-existing enzymes and translation of pre-existing transcripts as well as recruitment of precursor pools from both the cytosol and plastids. The later β-thujaplicin and other monoterpene accumulation strictly depends on active transcription and translation. Induction of β-thujaplicin production and activation of monoterpene biosynthetic enzymes by elicitor involves similar signaling pathways, which may activate early β-thujaplicin production and later monoterpene biosynthesis and induce a metabolic flux shift from β-thujaplicin to monoterpene accumulation.

Keywords: Biosynthetic enzyme; *Cupressus lusitanica* cell culture; Metabolic flux; Monoterpene profile; Signal transduction; β-thujaplicin

1. Introduction

The terpenoids compose the largest family of natural products, including several important biological molecules chlorophyll, abscisic acid, carotenoids, and gibberellins in plants; but the majority of terpenoids are secondary metabolites that play various roles in plant defense responses against microbial pathogens, insects, and herbivores (Chappell, 1995; Croteau et al., 2000). Terpenoids are synthesized from isopentenyl pyrophosphate (IPP), an universal precursor for all isoprenoids. IPP can be synthesized either via acetate/mevalonate (MVA) pathway from acetyl-CoA in the cytosol or endoplasmic reticulum

Abbreviation: AD, Actinomycin D; CH, cycloheximide; DMAPP, dimethylallyl pyrophosphate; DPI, diphenylene iodonium; EGTA, ethylene glycol-bis-β-aminoethylether-*N*, *N*, *N*′, *N*′-tetraacetic acid; GPP, geranyl pyrophosphate; G-proteins, GTP-binding proteins; IP_3, inositol-1, 4, 5-trisphosphate; IPP, isopentenyl pyrophosphate; MeJA, methyl jasmonate; NaPP, sodium pyrophosphate

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compartment, by which sesquiterpenes and triterpenes are produced, or via 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway from pyruvate and glyceraldehydes-3-phosphate in the plastid, by which monoterpenes, diterpenes, and tetraterpenes are generated (Lichtenthaler, 1999). Isomerization by IPP isomerase of IPP to dimethylallyl pyrophosphate (DMAPP) and then condensation of DMAPP with one unit of IPP by the action of prenyltransferases generates geranyl pyrophosphate (GPP). All these pyrophosphates are the common precursors for all monoterpenes and their derivatives. Because these pyrophosphate pools are also required for phytohormone biosynthesis, modification of proteins, lipids, and other biomolecules, the sizes and distribution of these precursor pools are tightly controlled (Manzano et al., 2004). Although these precursor pools and various terpenoid products markedly increase in plant response to biotic or abiotic stresses (Bohlmann et al., 1998), imbalance in these precursor pools and metabolic flux may cause impaired defects in development and defense responses (Stephanopoulos, 1999; Manzano et al., 2004).

Cupressaceae trees produce a large number of terpenoids, including mono-, di- and tri-terpenes as well as tropolone compounds that most often accumulate in the heartwood resin (Haluk and Roussel, 2000). Among these secondary metabolites, β-thujaplicin is regarded as a major phytoalexin in Cupressaceae trees, such as *Cupressus lusitanica* (Mexican cypress), *Thuja occidentalis*, and *Calocedrus formosana*, conferring to long-term wood preservation and durability against fungi, bacteria, and insects (Witte et al., 1983; Ono et al., 1998; Haluk and Roussel, 2000; Sakai, 2004). Because β-thujaplicin has strong and broad-spectrum antimicrobial activity (Sakai, 2004), and many other biological activities of β-thujaplicin were also revealed recently, there is an increasing demand for the β-thujaplicin in the industry (Sakai, 2004). Cell cultures derived from several Cupressaceae trees can produce β-thujaplicin and a number of monoterpenes (Witte et al., 1983; Ono et al., 1998; Matsunaga et al., 2003), and have been studied as a way to produce this valuable compound (Sakai, 2004). In *C. lusitanica* cell cultures, de novo biosynthesis of β-thujaplicin can be stimulated by a yeast elicitor, methyl jasmonate (MeJA), and other stresses (Itose and Sakai, 1997; Zhao et al., 2001a). Fujita et al. (2000) demonstrated in tracer experiments that geraniol and glucose were efficiently incorporated into β-thujaplicin via a MEP pathway through an intermediate with menthane-type skeleton, suggesting that MEP pathway, rather than MVA pathway, acts as a main synthesis pathway for β-thujaplicin, although a MVA pathway also contributes a minor portion to β-thujaplicin biosynthesis (Yamaguchi et al., 1997). Up to date little is known about enzymes and genes that are involved in β-thujaplicin formation from GPP, and the monoterpene intermediates after GPP cyclization and before β-thujaplicin formation. Previous studies have demonstrated that multiple signaling pathways are involved in elicitor-induced production of β-thujaplicin; these signaling pathways include Ca^{2+}, GTP-binding proteins, H_2O_2, JA, inositol trisphosphate, and ethylene (Zhao and Sakai, 2003a, b; Zhao et al., 2004a, b, 2005a). Recent work also suggests that both methylation and oxidation of β-thujaplicin are responsible for β-thujaplicin transformation in *C. lusitanica* cell cultures since a high level of β-thujaplicin is toxic to plant cells (Yamada et al., 2002; Zhao and Sakai, 2003b). All these data show that biosynthesis of β-thujaplicin in *C. lusitanica* cell culture is highly regulated and that its metabolism is also tightly controlled. It is believed that elicitor-induced de novo biosynthesis of β-thujaplicin is through signal transduction and transcription factors that target β-thujaplicin-biosynthesis genes (Davies and Schwinn, 2003; Zhao et al., 2005b). Recently, various transcriptional factors have been found to bind to *cis*-acting elements in promoter regions of genes involved in biosynthesis and regulation of plant secondary metabolites, including monoterpene biosynthetic genes (Chiron et al., 2000; Hahlbrock et al., 2003; Xu et al., 2004; Zhao et al., 2005b).

Since β-thujaplicin is derived from monoterpene and *C. lusitanica* cell cultures synthesize many monoterpenes, finding the possible monoterpene precursors for β-thujaplicin biosynthesis and the relationship between β-thujaplicin and other monoterpene biosynthesis, as well as their regulation, will be of great interest. We compared the biosynthesis and regulation of monoterpene and β-thujaplicin in *C. lusitanica* cell culture and found different stimulation of monoterpenes and β-thujaplicin biosynthesis in the cell culture upon on yeast elicitor and MeJA treatment. Elicitor preferentially stimulates monoterpene precursors flow to β-thujaplicin production first and then shifts the metabolic flux to biosynthesis of other monoterpenes. These changes in metabolic flux are mediated by signal transduction pathways that similarly regulate monoterpenes and β-thujaplicin biosynthesis in *C. lusitanica* cell culture. These results indicate that *C. lusitanica* regulates biosynthesis of defensive secondary metabolites by using toxic β-thujaplicin as the first phase of defense and other monoterpenes as a secondary one. This study provides new insights into the biosynthesis and regulation of monoterpenes and β-thujaplicin in *C. lusitanica* cell cultures, which will significantly facilitate the production of β-thujaplicin or other useful secondary metabolites by metabolic engineering the cell cultures.

2. Materials and methods

2.1. Plant cell culture and elicitor treatment

C. lusitanica suspension cultures from callus were established as previously described (Zhao et al., 2001a). About 4 g of fresh cells was inoculated into 20 ml production medium in 100-ml flasks and incubated on a rotary shaker (120 rpm) at $23 \pm 2\,^{\circ}C$ in the dark. For the time-course study, an autoclave-sterilized yeast elicitor

(YE, 1 mg/ml) or water (control) was added to 5-day-old cell cultures. The cell cultures were collected at certain intervals for analysis of β-thujaplicin and enzyme activity. The yeast elicitor is a 70–80% ethanol-insoluble polysaccharide fraction of yeast extract prepared as described previously (Sakai et al., 1994). Five-day-old suspension cultures were treated with yeast elicitor and MeJA for 24 h, and then were collected for analysis.

2.2. Reagents and treatment profiles

Calcium ionophore A23187, melittin, cholera toxin and mastoparan were obtained from Sigma (St. Louis, MO, USA) and were prepared in 0.05% DMSO or sterilized water. Lanthanum chloride, verapamil hydrochloride, neomycin sulfate and lithium chloride were obtained from Wako Pure Chemicals (Osaka, Japan), and these reagents were dissolved in water or 0.05% DMSO. Actinomycin D (AD) was in 2 mM methanol stock solution (treatment concentration 1.5–10 μM), and cycloheximide (CH) was in 20 mM methanol stock (the final treatment concentration 5–10 μM). Diphenylene iodonium (DPI), sodium pyrophosphate (NaPP), and Ethylene glycol-*bis*-β-aminoethylether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were obtained from ICN (Tokyo, Japan), these reagents were dissolved in water or 0.05% DMSO solution. Unlabeled DMAPP, IPP, and GPP were obtained from Sigma St. Louis, USA. [4-^{14}C] IPP was from Sigma (St. Louis, MO, USA) and [1-^{3}H] GPP was from American Radiolabeled Chemicals (St. Louis, MO, USA).

Five-day-old *C. lusitanica* suspension cells were used in all treatment experiments. In single treatment experiments, filter-sterilized A23187, melittin, cholera toxin, and mastoparan solutions were added to cell cultures to desired final concentrations alone or 20 min prior to yeast elicitor treatment. The control cells received the same volume of 0.05% DMSO solution. Cell cultures were harvested at intervals for β-thujaplicin and enzyme assay. For effects of CH and AD on β-thujaplicin and monoterpene biosynthetic enzyme activity, AD and CH were added to the cell cultures 20 min before elicitor addition and cells cultures were collected after 12 and 24 h for β-thujaplicin measurement and assay of monoterpene biosynthetic enzymes. Control cells received vehicle solution only. For effect of NaPP on β-thujaplicin biosynthesis, NaPP was added to the cell cultures at various concentrations 20 min before elicitor treatment for 12 h of treatment. NaPP was added to the cell cultures (final concentration 3.3 mM) before elicitation, or 12, and 36 h after elicitor treatment; cell cultures were treated for 12 h and collected at 12, and 24 and 48 h. Controls received water and non-elicited cultures were also treated with NaPP or water, as another set of controls to check side effect of NaPP.

2.3. Extraction and determination of β-thujaplicin by HPLC

β-Thujaplicin was extracted and determined as previously described (Zhao et al., 2001a). Briefly, fresh cells were collected by vacuum filtration through Miracloth, then weighed and quickly frozen. After homogenization, 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 dried under vacuum, and the residues were then treated with boron trifluoride methanol solution to form a β-thujaplicin–BF_2 complex, which were analyzed with HPLC. A Waters 996 system with a PDA detector monitoring at 320 nm, an Inertsil ODS-3 column (150 mm long, 4.6 mm i.d., 5 μm), and a mobile phase composed of water/methanol (55:45) at a flow rate of 1 ml min^{-1} were used with vanillin as an internal standard. Identification of β-thujaplicin by ^{13}C-NMR was as reported elsewhere (Fujita et al., 2000).

2.4. Extraction and determination of monoterpene by GC-MS

Extraction and identification of monoterpenes with GC-MS was done as previously described (Matsunaga et al., 2003). *C. lusitanica* cells were incubated for 8 days in the production medium with yeast elicitor. After incubation, cells and medium were extracted with diethylether separately. Each extract was fractionated into the acidic and the neutral fractions by using 1 M HCl and NaOH. Each fraction was partially purified with silica-gel CC and then analyzed by GC-MS (QP-5000, Shimadzu, Kyoto, Japan). Analysis conditions: GC Column DB-5 (0.25 mm id × 30 m), the carrier gas He at inlet pressure 100 kPa in a split mode. The injector temperature was set at 220 °C, and the column initial temperature was 70 °C for 1 min then increased 10 °C/min. MS source was at 70 eV; scanning at an interval of 2 scans/s, and scanning speed of 1000 amu/s. Monoterpenes were detected in the neutral fraction from the medium extract and identification of monoterpene structures was based on MS spectra as compared with data from authentic samples. Alternatively, these monoterpenes without authentic samples were isolated by normal or reverse-phase HPLC and identified by NMR spectroscopy (AL-400, JEOL, Akishima, Japan). The elicited cells were cultivated in the production medium containing Mygliol that traps volatile compounds. After an 8-day incubation and extraction with diethylether, Mygliol was removed from the concentrated ether layer by washing through silica–gel CC with pentane. Fractions were also analyzed by GC-MS. Quantification was performed by GC-MS. All data were expressed as a ratio of peak area to internal standard, camphoer.

2.5. Enzyme extraction and activity assay

Cells were collected by filtration under vacuum, and then frozen immediately at −80 °C. For monoterpene-synthesis related enzyme assay, the cells were homogenized in liquid nitrogen into powder, and extracted with an extraction

buffer containing 50 mM Hepes buffer (pH 7.9), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM sodium ascorbate, 5 mM $Na_2S_2O_3$, 5 mM dithiothreitol, 10% glycerol, 0.1 mM PMSF, 10 μg/ml leupeptin and aprotinin, 1% polyvinylpyrrolidone (PVP Mr. 3000). Homogenate was centrifuged at 11000*g* for 20 min at 4 °C, and the supernatant was used as crude enzyme extract. Protein content was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. To characterize the requirement of these enzymes for divalent metals (Mg^{2+} and Mn^{2+} in the chloride salts), a concentrated enzyme extraction was dialyzed overnight against assay buffer containing 50 mM EDTA. The dialysate was desalted and exchanged into the assay buffer (50 mM Hepes buffer, pH 7.8, 10 mM KCl, 5 mM dithiothreitol, 10% glycerol, 10 μg ml^{-1} each of leupeptin and aprotinin) by using PD-10 column (Pharmacia, Econo-Pak 10 DG). Mg^{2+} requirement (0–20 mM) for the enzyme activities was assayed with Mn^{2+} at 10 mM, and Mn^{2+} requirement (0–20 mM) with Mg^{2+} at 5 mM.

GPP synthase and IPP isomerase activities were assayed according to Burke et al. (1999) with minor modifications. GPP synthase assay reaction (100 μl) was composed of 50 mM Hepes buffer (pH 7.8) containing 10% glycerol, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM DTT, 10 μM DMAPP and 7.5 μM [4-^{14}C] IPP (57.5 mCi /mmol). IPP was added to start the reaction at 31 °C for 30 min. Then 10 μl of 6 N HCl was added for incubation at 31 °C for 30 min. The reaction solution was extracted twice with pentane, and then the remaining aqueous phase was extracted twice with 1 ml diethyl ether. The radioactive products in the diethyl ether fraction were measured by scintillation counting. IPP isomerase activity was assayed in a 100 μl reaction mixture containing 50 mM Hepes buffer (pH 7.8), 10 mM KCl, 10 μM [4-^{14}C] IPP (57.5 mCi / mmol) and cold IPP, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM DTT, and 10% glycerol. Reactions were kept at 31 °C for 30 min, and then 10 μl of 6 N HCl was added for acid hydrolysis of reaction products at 31 °C for another 20 min. The aqueous phase was firstly extracted twice with 1 ml of pentane; then, the remaining aqueous phase was extracted with 2 × 1 ml diethyl ether. The radioactive products in diethyl ether fraction were measured by scintillation counting. Monoterpene synthase (cyclase) activity was assayed according to Lewinsohn et al. (1991, 1994) with slight modifications. The monoterpene synthase reaction mixture (100 μl) was in 50 mM Hepes buffer (pH 7.8) containing 10 mM KCl, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 7.3 μM (1-^{3}H) GPP (100 mCi / mmol) and cold GPP. The aqueous solutions were overlaid with 1 ml pentane, and kept at 31 °C for 1 h. After reaction, the mixture was extracted twice with 1 ml pentane and the combined extractions were transferred to a $MgSO_2$–silica gel column to provide terpene hydrocarbon fraction. The reaction mixture was extracted with 1 ml diethyl ether again and passed through the same column to provide the oxygenated terpenoids.

2.6. Statistics analysis

All data were generated in two- or three independent experiments. Data were analyzed with Students *t*-test. The difference between treatments was considered as significant when $P<0.05$ in a two tail analysis.

3. Results

3.1. Differences in elicited β-thujaplicin biosynthesis by yeast elicitor and MeJA

Effects of MeJA and yeast elicitor on β-thujaplicin production measured by using HPLC were compared. Both MeJA and elicitor stimulated β-thujaplicin biosynthesis (Zhao et al., 2001a). However, elicitor treatment initiated a more rapid and stronger induction of β-thujaplicin biosynthesis (maximal 60 mg L^{-1} at 72 h) than MeJA did. MeJA treatment resulted in a slower and lower accumulation of β-thujaplicin (maximal 52 mg L^{-1} at 96 h) (Fig. 1). The difference in regulation of β-thujaplicin biosynthesis by elicitor and MeJA can be seen more clearly in an inset in Fig. 1. Examining elicitor- or MeJA-induction of β-thujaplicin production at the early period from 0 to 12 h shows that an increased β-thujaplicin accumulation (35 mg L^{-1}) was found at 12 h post-elicitation whereas MeJA-treated cell cultures almost kept in a basic level. This suggests that elicitor initiates a signal transduction leading to β-thujaplicin biosynthesis in a manner different from that of MeJA.

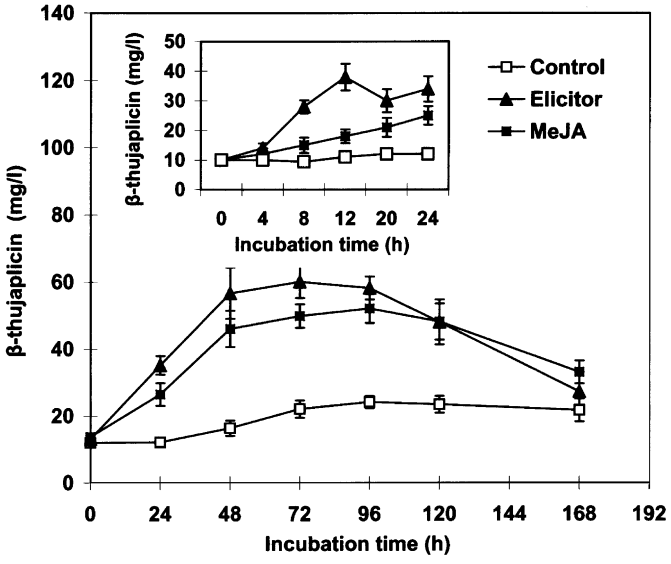

Fig. 1. Differential induction of β-thujaplicin accumulation in *C. lusitanica* cell cultures by yeast elicitor and MeJA. The cells were incubated in the production medium and treated with yeast elicitor (Elicitor, 1 mg ml^{-1}) or methyl jasmonate (MeJA, 100 μM). β-thujaplicin includes that from both cells and the medium. Cells receiving water was used as a control. The inset shows that a short-run production of β-thujaplicin. Data represent means ± standard deviations from three independent experiments.

3.2. Elicitor stimulates an earlier β-thujaplicin but a late monoterpene biosynthesis

More than 10 monoterpenes, including β-ocimene, mycene, limonene, α-terpineol, terpinolene, sabinene, 4-terpineol, *p*-ment-4(8)-en-1,2-diol (*p*-MED), 1,6-epoxy-4(8)-*p*-menthene-2-ol (1,6-EPMO), 4-hydroxyphellandric acid methyl ester (4-HAME), as well as β-thujaplicin and its methyl ether (Me-β-thujaplicin) were isolated and identified from *C. lusitanica* cell cultures (Fig. 10) (Matsunaga et al., 2003). These monoterpenes were analyzed using GC-MS to reveal biosynthesis and regulations of these monoterpenes, particularly β-thujaplicin, in the cell cultures. Fig. 2 shows GC-MS profile of monoterpene products in *C. lusitanica* cell cultures. The volatile monoterpenes were only detected when Mygliol was added to the cell cultures as an extracellular sink, and most oxygenated monoterpenes were found in the medium and easily extracted and detected. The production medium was initially designed for improving the production of β-thujaplicin based on observations that decreased major elements and increased Fe^{2+} concentration in B5 medium resulted in a higher β-thujaplicin production (Itose and Sakai, 1997). As shown in Fig. 3A, *C. lusitanica* cell cultures in the production medium synthesize β-thujaplicin and other monoterpenes: more β-thujaplicin was produced than monoterpenes. Moreover, elicitor treatment further enhances β-thujaplicin and monoterpene production markedly, 10-fold for β-thujaplicin and 5–7-fold for oxygenated monoterpene production at day 4 (Fig. 3B). The β-thujaplicin biosynthesis was enhanced by elicitor earlier and more significantly than the other monoterpenes. An increase in β-thujaplicin level was found at 12 h after elicitation whereas that for monoterpenes was at 48 h post-elicitation. In the same experiments, the maximum β-thujaplicin production was about 3-fold over that of monoterpenes. In addition, most β-thujaplicin (about 70%) and oxygenated monoterpene (about 80%) were secreted to the medium, and only a minor portion of methyl β-thujaplicin was excreted into the medium (Fig. 3E). Only trace amount of monoterpenes were found in the *C. lusitanica* cell (Matsunaga et al., 2003).

We also compared effects of [Fe^{2+}] and major elements on β-thujaplicin and monoterpene production. Removing Fe^{2+} from the production medium (0.25 mM) to the standard level of B5 medium (0.1 mM) obviously decreased the elicitor-induced monoterpene and β-thujaplicin production (Fig. 3C). Particularly, most monoterpenes, except for 4-HAME, decreased dramatically, almost back to levels in non-elicited cell cultures. Nevertheless, β-thujaplicin decreased only about 40% by day 4 while 4-HAME production even showed a little increase (Fig. 3C). Increasing major elements to level of standard B5 medium almost eliminated all elicitor-induced β-thujaplicin production, and decreased about 30% of 4-HAME production (Fig. 3D). However, changing the major elements did not obviously affect the production of other monoterpenes

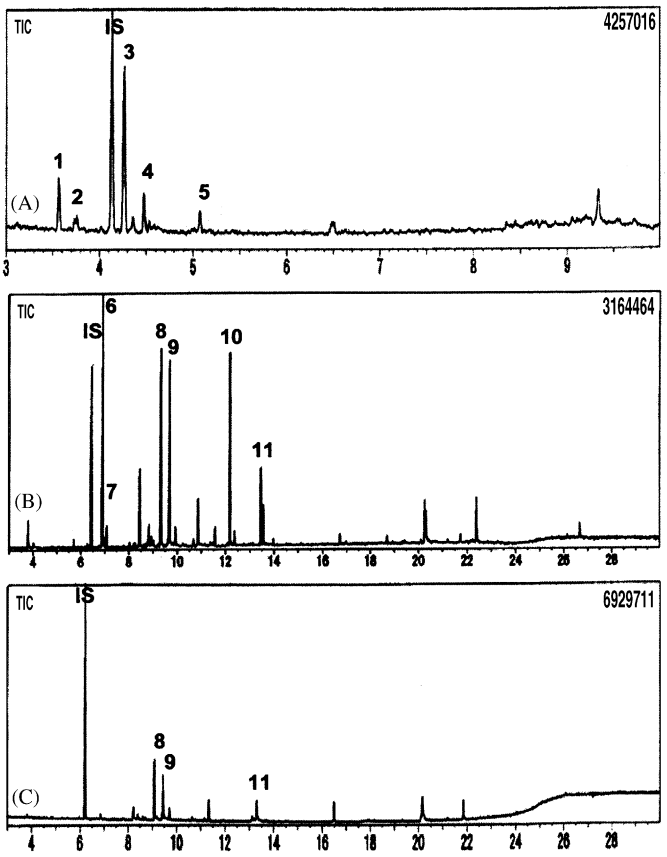

Fig. 2. GC/MS (TIC) profiles of monoterpenes from *C. lusitanica* suspension cell cultures. *C. lusitanica* cells were incubated in the production medium and treated with yeast elicitor. After 8 days of incubation, culture medium, together with Mygliol, was extracted with ether for monoterpene determination by the method described in Section 2. (A) Profile of volatile monoterpenes in *C. lusitanica* cell cultures. Mygliol was added to the elicitor-treated cell cultures to trap monoterpenes. Peaks are: IS, internal standard; 1, β-ocimene; 2, myrcene; 3, limonene; 4, sabinene; 5, terpinolene. (B) Oxygenated monoterpens accumulated in the culture medium from elicited *C. lusitanica* cell culture. Peaks are: IS, internal standard; 6, 4-teroineol; 7, β-terpineol; 8, 1,6-epoxy-4(8)-*p*-menthene-2-ol; 9, *p*-ment-4(8)-en-1,2-diol; 10, 4-hydroxyphellandric acid methylester; 11, β-thujaplicin methylester. (C) Monoterpenes in the culture medium from water-treated (control) *C. lusitanica* cell culture. Peaks are: IS, internal standard; 8, 1,6-epoxy-4(8)-*p*-menthene-2-ol; 9, 4(8)-menthen-1,2-diol; 11; β-thujaplicin methylester. Y-axis in figures shows m/z intensity, and X-axis shows rentention time in minutes.

such as 4-terpineol (Fig. 3D). It is clearly shown that high concentration of Fe^{2+} contributes to more production of β-thujaplicin and monoterpene biosynthesis (Itose and Sakai, 1997; Zhao et al., 2001a, 2005a), major inorganic elements play more important role in elicitor-induced β-thujaplicin and monoterpene production.

3.3. Different regulation of monoterpene biosynthesis enzymes by yeast elicitor and MeJA

To gain details of differential regulation of monoterpene and β-thujaplicin production by elicitor and MeJA, activity of three enzymes involved in early steps of monoterpene

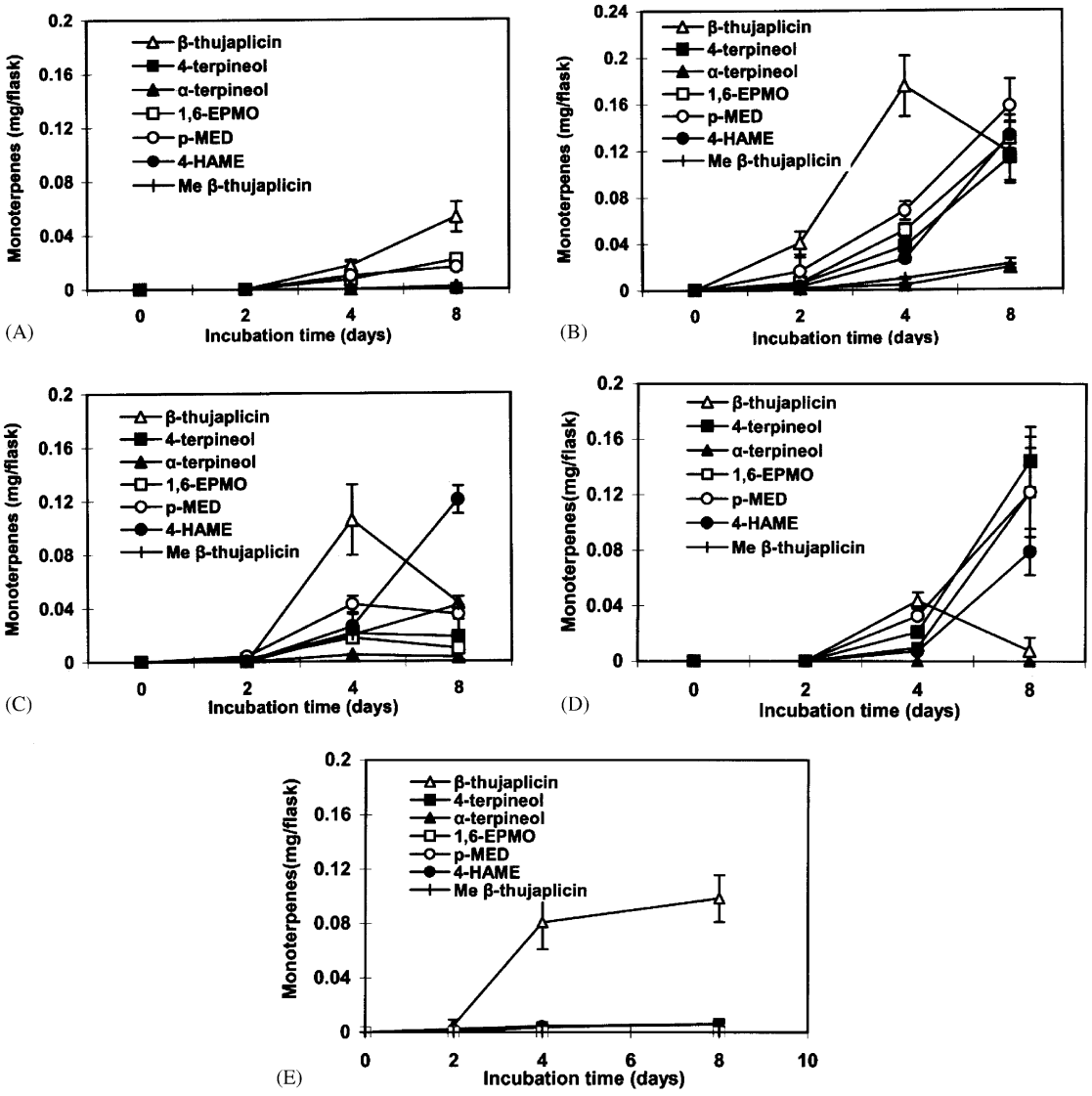

Fig. 3. Monoterpene accumulation in *Cupressus lusitanica* cell cultures. (A) Non-treated cells were incubated in the production medium in which major inorganics are reduced to 10% of standard B5 medium, and $FeSO_4$ and EDTA were increased to 250% of standard B5 medium. (B) Cells incubated in the production medium were treated with yeast elicitor (1 mg ml^{-1}). (C) Cells incubated in the modified production medium by decreasing Fe^{3+} to normal level were treated with yeast elicitor (1 mg ml^{-1}). (D) Cells were incubated in the modified production medium by increasing major inorganics to normal level of standard B5 medium. All β-thujaplicin and monoterpenes were recovered from the production medium. (E) Production of β-thujaplicin from elicitor-treated cells.

biosynthesis were analyzed in *C. lusitanica* cell cultures. We first examined the metal requirement for activity of IPP isomerase, GPP synthase, and monoterpene synthase to optimize their assay conditions. Steady-state kinetic assay showed that 15–20 mM Mn^{2+} and 5 mM Mg^{2+} are required by monoterpene synthase for maximal activity (Fig. 4A and B). The IPP isomerase and GPP synthase activity only requires 5 mM Mn^{2+} and 3 mM Mg^{2+}. These properties are similar to corresponding enzymes from other gymnosperm plants (Stofer Vogel et al., 1996; Bohlmann et al., 1997, 1998).

Yeast elicitor induced a quicker and higher increase in activity of all three enzymes than MeJA did. IPP isomerase activity was induced by MeJA to a peak at about 36–48 h, and by elicitor to a peak at 24 h after treatment (Fig. 5A). Moreover, elicitor stimulated a higher level of IPP isomerase activity than MeJA did. Fig. 5B shows that GPP synthase activity was stimulated by MeJA to a peak at 48 h and by elicitor to a higher peak at 24 h. Monoterpene synthase activity was significantly induced by both elicitor and MeJA (Fig. 5C). The elicitor caused a peak at 36 h and MeJA stimulate a later and lower peak at 48 h for terpene synthase. All enzymes show a steadily increasing activity at late stage of culture, which is consistent with increased accumulation of monoterpenes. The time and strength differences in activation of

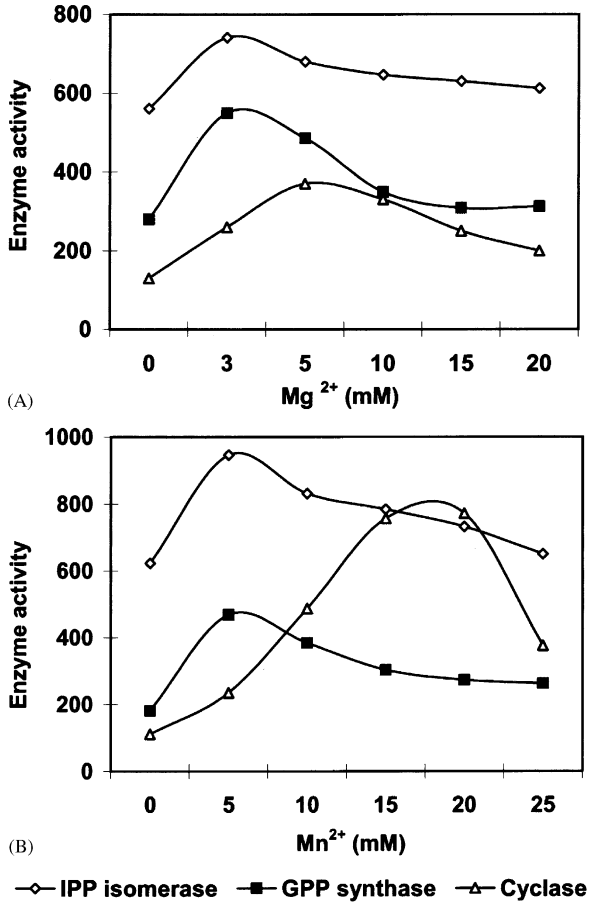

Fig. 4. Requirement of divalent cations by monoterpene biosynthetic enzymes for activity. Monoterpene biosynthetic enzymes were assayed in steady-state kinetics as shown in Section 2. Mg^{2+} requirement at 0–20 mM for each of enzyme activities was assayed with Mn^{2+} at 10 mM; and Mn^{2+} requirement at 0–20 mM for each of three enzyme activities was assayed when Mg^{2+} concentration in the assay buffer was at 5 mM. IPP isomerase and GPP synthase activity is expressed as cpm/μg protein 30 min, and monoterpene synthase activity is expressed as cpm × 100/μg protein h.

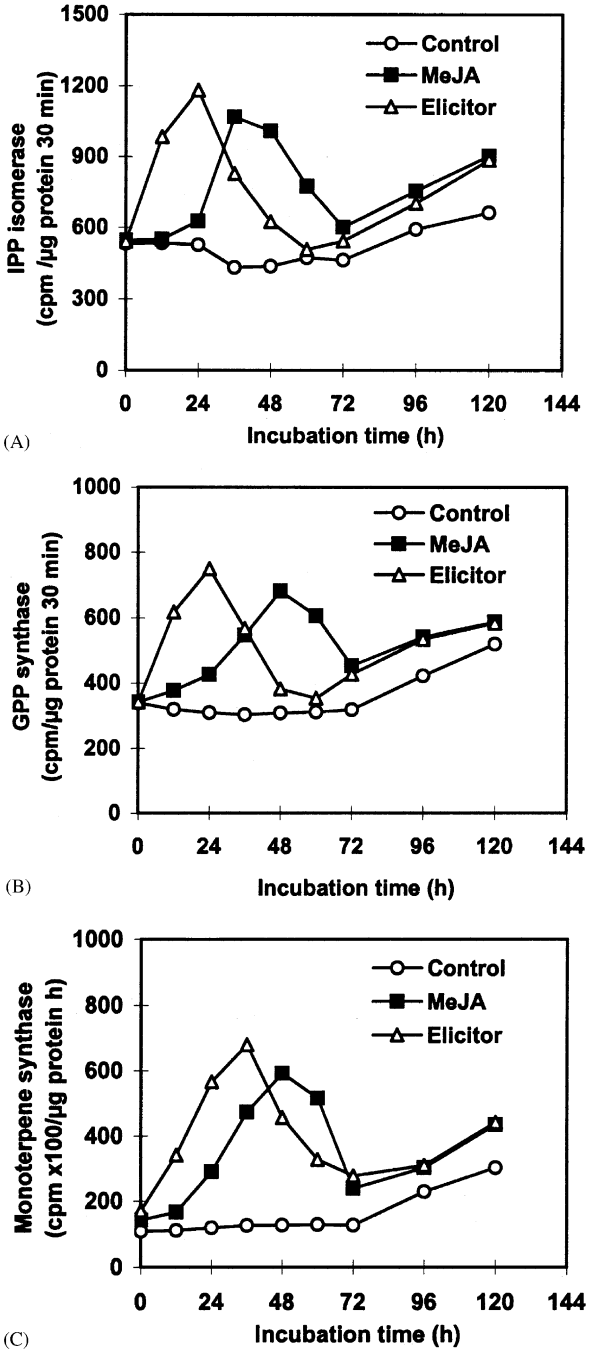

Fig. 5. Time-course activation of monoterpene biosynthetic enzymes by yeast elicitor and MeJA. The cells were incubated in the production medium and treated with yeast elicitor (Elicitor, 1 mg ml^{-1}), methyl jasmonate (MeJA, 100 μM), or water (Control). Cells were taken out at intervals for enzyme activity assay. Data represent means from three independent experiments.

monoterpene biosynthesis enzymes by MeJA and elicitor suggest they initiated two different pathways leading to activation of gene expression and enzyme activity.

3.4. Early accumulation of β-thujaplicin mainly depends on pre-existing transcripts and enzymes

Since yeast elicitor induces a quick β-thujaplicin accumulation even at 12 h after elicitation, and the cellular process from elicitor perception, gene transcription, and enzyme activation, to accumulation of secondary metabolites through signal transduction generally takes several hours, immediate biosynthesis of β-thujaplicin post-elicitation might start with activation of existing enzymes or translation from transcripts of GPP synthase, IPP isomerase, and monoterpene synthase, which are already accumulated in cells inoculated in the production medium. To verify this hypothesis, we tried RNA and protein biosynthesis inhibitors, AD and CH, to probe effect of transcription and translation activity on elicitor-induced β-thujaplicin biosynthesis. AD and CH are often used to inhibit transcriptional and translation activity in plant and animal cells. As shown in Fig. 6A and B, CH inhibited about 20–40% of β-thujaplicin accumulation in the production medium at 12 h post-elicitation, whereas AD had almost no effect on immediate β-thujaplicin accumulation induced by

elicitor. But at 24 and 48 h after elicitation, CH and AD similarly inhibited much more β-thujaplicin biosynthesis (Fig. 6). However, compared with less effective inhibition of β-thujaplicin accumulation by CH and AD at 12 h, activity of three biosynthetic enzymes was more significantly inhibited by CH than by AD (Fig. 7). AD and CH application correspondingly inhibited the increased soluble proteins that were induced by elicitor (results not shown). These results suggest that immediate β-thujaplicin biosynthesis largely relies on pre-existing enzymes or translation of such pre-existing transcripts, while later production of β-thujaplicin and monoterpenes mainly relied on de novo transcription and translation activity. Although AD and CH application might have non-specific effects on culture cells, they effectively inhibited protein biosynthesis, β-thujaplicin accumulation, and enzyme activation induced by elicitor even at low concentrations. Compared with their effects with controls, AD and CH showed specific effects to a high degree.

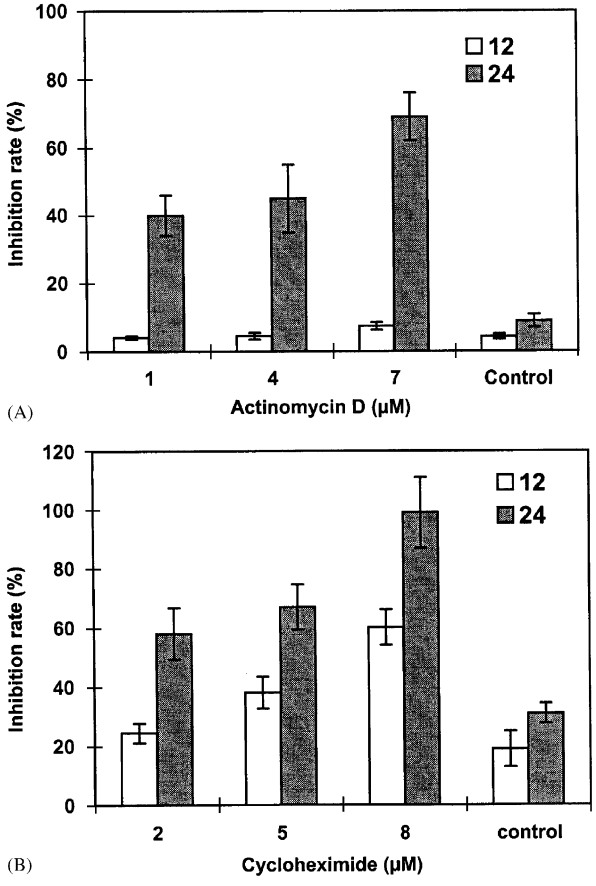

Fig. 6. Effects of RNA and protein biosynthesis inhibitors on elicitor-induced β-thujaplicin production. AD or CH at different concentrations (AD, 1, 4, 7 μM; CH, 2, 5, 8 μM) was added to the cell cultures 20 min prior to elicitor treatment for 12 h incubation (12 h) or 12 h after elicitor treatment for incubation for additional 12 h (24 h) before cultures were collected for β-thujaplicin determination. The elicited cells with only 0.05% DMSO pretreatment was positive control, from which inhibition rate of AD and CH on β-thujaplicin production was calculated. Data represent means ± standard deviations from three independent experiments.

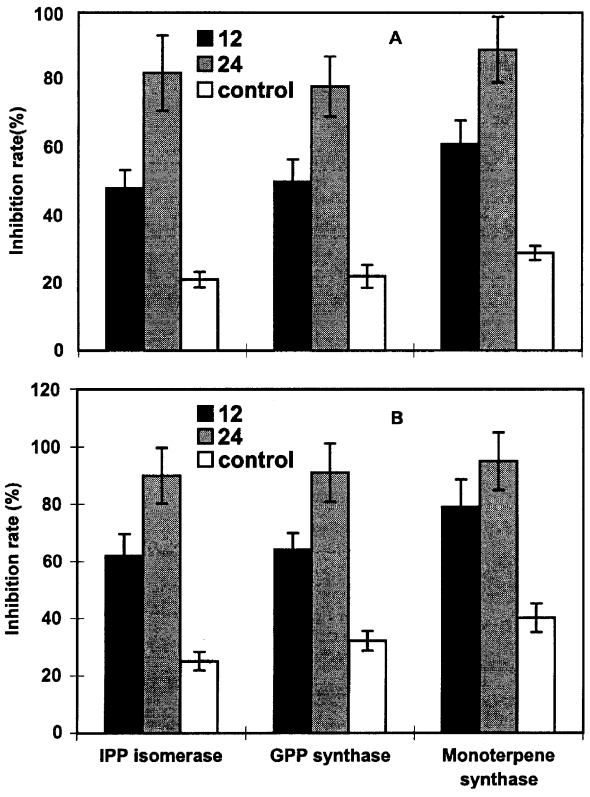

Fig. 7. Effects of RNA (A) and protein biosynthesis (B) inhibitors on elicitor-induced activation of monoterpene biosynthetic enzymes. AD (1 μM) or CH (2 μM) was added to the cell cultures 20 min prior to elicitor treatment for 12 h incubation (12 h) or 12 h after elicitor treatment for incubation for additional 12 h (24 h) before cells were collected for enzyme activity assay. The elicited cells with only 0.05% DMSO pre-treatment as positive control, from which inhibition rate of AD (A) and CH (B) on enzyme activities was calculated. Data represent means ± standard deviations from three independent experiments.

3.5. *Translocation of monoterpene precursors is important for immediate production of β-thujaplicin*

It is clear that biosynthesis and storage of plant secondary metabolites involves several different organelles, such as the plastid, cytosol, and vacuole; Subcellular trafficking is one important means of regulation for biosynthesis of plant secondary metabolites (Chappell, 1995; Trapp and Croteau, 2001). Biosynthesis of monoterpenes has been suggested to involve the plastid and cytosol, as well as vacuole for storage of some toxic derivatives (Bohlmann et al., 1998). The biosynthetic precursors IPP or GPP can be derived either from cytoplasmic and mitochondrial pools or from the plastids independently. Cytosolic and mitochondrial isoprenoids are both formed from IPP produced through the MVA pathway, whereas plastid isoprenoids are synthesized from IPP provided by the MEP pathway (Fig. 11). The different subcellular localization allows either working independently or cross talking between each other. It has been increasingly evident that in some situations IPP/DMAPP

or downstream common precursors such as GPP can be exchanged between the cytoplasmic MVA and the plastid MEP pathways (Hemmerlin et al., 2003; Laule et al., 2003; Rodríguez-Concepción et al., 2004). To gain more information about exchanges of these precursors during β-thujaplicin production, as well as the possible role of such precursor pool exchange on regulation of β-thujaplicin and monoterpene production, we used an inhibitor of IPP or GPP exchange between subcellular compartments to examine its effect on β-thujaplicin production. NaPP can act as a substrate analogue to interfere with the uptake of IPP by the plastids (Soler et al., 1993). Obviously, NaPP inhibited immediate production of β-thujaplicin induced by elicitor in a dose-dependent manner, as shown in inset figure (Fig. 8). When adding NaPP at low concentration (3.3 mM) to the elicited cell cultures at different time of elicitation, the most obvious inhibition on β-thujaplicin production was only observed at 12 h of treatment (Fig. 8). The treatment at 24 and 48 h had less effect on β-thujaplicin production. This might suggest that immediate production of β-thujaplicin largely depends on recruitment of precursors, IPP or GPP, most probably from cytosol to plastids. Even NaPP treatment may cause other effects; NaPP at low concentration indeed only inhibited β-thujaplicin biosynthesis at 12 h, which could suggest there are active cellular processes important for β-thujaplicin biosynthesis at the moment.

3.6. Elicitor signaling pathways regulates terpenoid-biosynthesis enzymes

Our previous studies have shown that multiple signal transduction pathways, such as G-proteins, IP_3, Ca^{2+}, H_2O_2, and jasmonate signaling, are involved in YE-induced β-thujaplicin accumulation (Zhao and Sakai, 2003a, b; Zhao et al., 2004a, b). We tested whether these signaling pathways are also involved in elicitor-regulated activation of monoterpene biosynthetic enzymes, GPP synthase, IPP isomerase, and monoterpene synthase. As shown in Fig. 8, activities of all three enzymes rose at 24–48 h post-elicitation. However, pre-treatment with calcium signaling inhibitors, EGTA and verapamil, or polyphosphoinositide metabolism inhibitors, LiCl and neomycin, or the NADPH oxidase inhibitor DPI, partially blocked elicitor activation of all three enzymes. H_2O_2 and G-protein activator alone markedly stimulated the three enzymes after 24 h of treatment. MeJA and A23187 also up-regulated these enzymes. However, the stimulating effects of MeJA and A23187 on these enzyme activities became evident only after a longer treatment (48 h) (Fig. 9). These results suggest that H_2O_2, G-proteins, Ca^{2+}, IP_3, and MeJA signaling pathways were involved in the regulation of elicitor activation of these enzymes. The observations in delayed activation of these enzymes by MeJA and A23187 as compared with H_2O_2, yeast elicitor and G-protein activator treatments seemed to be in agreement with these results about β-thujaplicin accumulation, in which these corresponding signaling pathways are involved differently (Zhao and Sakai, 2003a).

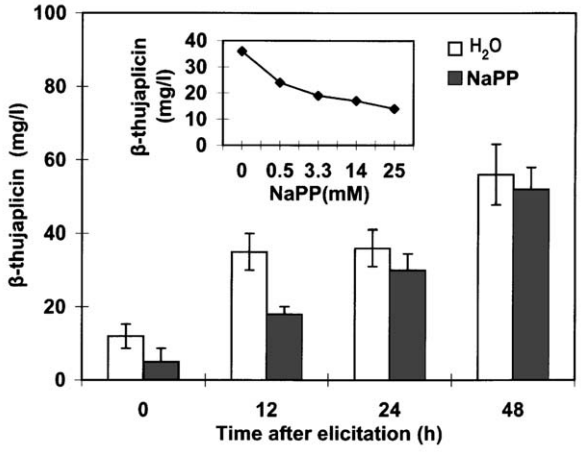

Fig. 8. Effects of inhibitor of IPP/GPP translocation on elicitor-induced β-thujaplicin production. NaPP at 0.5 mM was added to cells at various intervals after elicitor treatment. Cells were collected at day 4 after elicitation for β-thujaplicin analysis. Data represent means or means ± standard deviations from two–three independent experiments.

4. Discussion

C. lusitanica cell cultures can synthesize various hydrocarbon monoterpenes and their derivatives including highly modified ones such as β-thujaplicin and other oxygenated monoterpenes (here all called monoterpenes). These monoterpenes in heartwood are responsible for a wide range of defense against fungi, bacteria, and insects since most of these monoterpenes have antimicrobial/insecticide activity (Haluk and Roussel, 2000). One of these monoterpenes, β-thujaplicin, has special interest. On the basis of previous studies on the biosynthetic pathway (Fujita et al., 2000), identification of monoterpenes (Matsunaga et al., 2003), and elicitor signal transduction pathways (Zhao and Sakai 2003a, b; Zhao et al., 2004a, b), the present work demonstrated different regulation on biosynthesis of β-thujaplicin and monoterpenes and the mechanism in elicitor-treated *C. lusitanica* cell cultures by assessing signal transduction and metabolic flux. Combined data from profiles of metabolic intermediates and products with GC-MS, activity assay of biosynthetic enzymes, probing and analysis of signal transduction have provided comprehensive new insights into monoterpene biosynthesis and regulation.

4.1. Elicitor-induced β-thujaplicin and monoterpene biosynthesis are all mediated by Ca^{2+}, G-proteins, H_2O_2, IP_3, and JA pathways in a similar pattern

Elicitor signaling pathway consists of several parallel or cross-talking signaling pathways. These pathways finally are converged into transcription factors to regulate defense responses (Hahlbrock et al., 2003). Many secondary

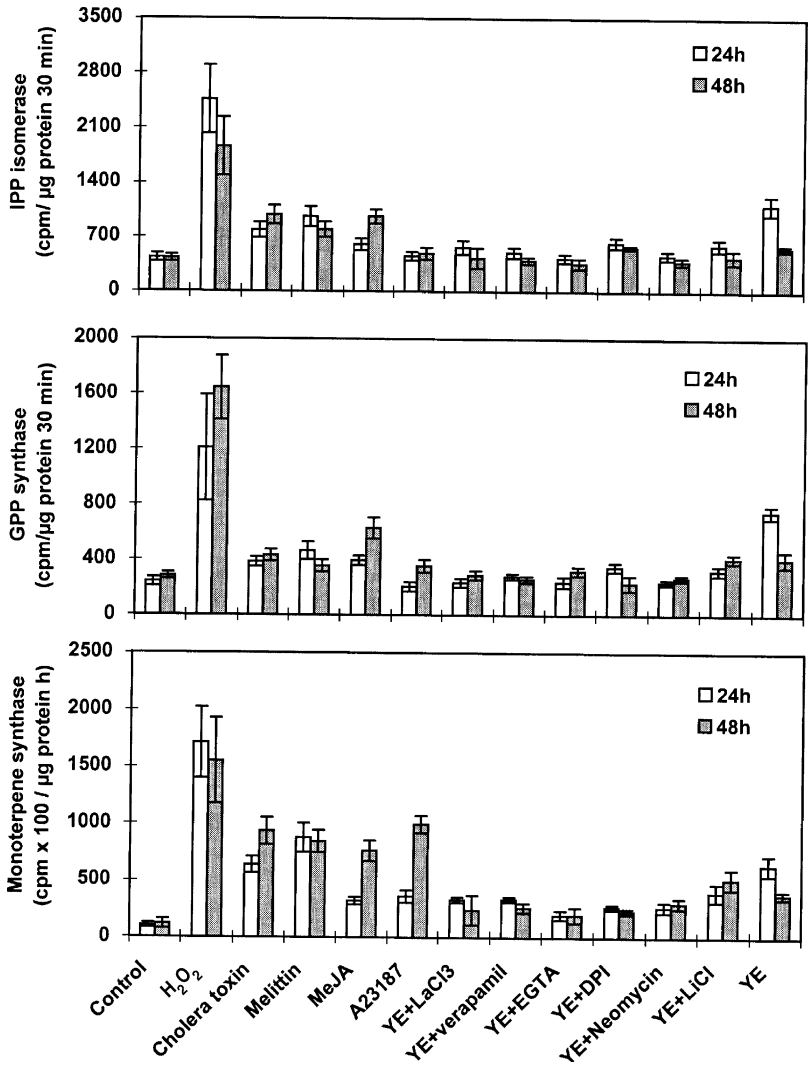

Fig. 9. Effects of elicitor signaling components on elicitor-induced activation of monoterpene biosynthetic enzymes, prenyltransferase, IPP isomerase and monoterpene synthase in *C. lusitanica* cell cultures. Yeast elicitor (1 mg ml^{-1}), MeJA (100 µM), H_2O_2(25 mM), A23187 (10 µM), melittin (10 µg/ml) or cholera toxin (4 µg/ml) were added separately to the 5-day-old cell culture in the production medium for 24 and 48 h of incubation before harvest. On other experiments, 20 min before the cell cultures were treated with the yeast elicitor (1 mg ml^{-1}), LiCl (10 mM), neomycin (200 µM), EGTA(10 mM), verapamil (100 µM), $LaCl_3$ (1 mM), or DPI (100 µM) were added to the cell cultures. Data were expressed as means ± standard deviations from three independent experiments.

metabolism genes have particular *cis*-element sequences in their promoter regions, which can be bound by different transcription factors (Hahlbrock et al., 2003; Zhao et al., 2005b). Transcription factors can either suppress or activate expression of target genes (Davies and Schwinn, 2003). It has been reported that fungal elicitors, H_2O_2 or MeJA regulate terpenoid biosynthesis and related enzymes or gene expression (Lewinsohn et al., 1991, 1994; Yin et al., 1997; Burke et al., 1999; Mandujano-Chavez et al., 2000). Elicitor-induced β-thujaplicin accumulation involves multiple signaling pathways (Zhao and Sakai, 2003a, b; Zhao et al., 2004a, b). Comparison of previous results with the present data about effects of activators/inhibitors of these signaling pathways on monoterpene biosynthetic enzyme activity strongly suggests that both monoterpene biosynthetic enzyme activity and β-thujaplicin production are regulated through similar signaling pathways, including G-protein, Ca^{2+}, H_2O_2, JA, elicitor, and phosphoinositide signaling. This supports the notion that β-thujaplicin is synthesized by action of common monoterpene-biosynthetic enzymes. Activity changes in these three monoterpene biosynthetic enzymes most often reflect their gene expression and enzyme protein levels (Steele et al., 1998; McConkey et al., 2000). Therefore, it is believed that these signals most probably regulate three enzymes and their encoding genes, as well as other β-thujaplicin and monoterpene biosynthesis at transcription and translation levels. Several lines of evidence show that monoterpene biosynthesis is regulated by wounding, elicitor, or MeJA at transcription and translation levels in different plants

(Steele et al., 1998; Yin et al., 1997; Mandujano-Chavez et al., 2000; Martin et al., 2002). Although monoterpene accumulation in cell cultures under these treatments was not analyzed in this study, coordinate regulation of three enzymes, particularly the rate-limiting monoterpene synthase, by these signaling pathways strongly suggests that accumulation of the enzymatic products monoterpenes are also regulated in the same way.

Both β-thujaplicin production and activity of three monoterpene biosynthetic enzymes are differently regulated by elicitor and MeJA. The coincidental time-courses for production of monoterpenes with activation of three monoterpene biosynthetic enzymes in elicitor-treated *C. lusitanica* cell cultures further supports the idea that β-thujaplicin biosynthesis is via isoprenoid pathway. These data also help to dissect elicitor signaling pathway that MeJA and elicitor initiate two different pathways leading to activation of monoterpene-biosynthetic enzymes and biosynthesis of monoterpenes and β-thujaplicin. Although MeJA-induced production of monoterpenes is not profiled in this study, but adequate data have shown that monoterpene production is induced differentially by MeJA (Mandujano-Chavez et al., 2000; Martin et al., 2002). Many studies have shown that elicitor-induced downstream events are two-phase processes, such as Ca^{2+} spiking and H_2O_2 production (Lecourieux et al., 2002). It is thus not surprising for a two-phase enzyme activation and monoterpene biosynthesis in elicitor-treated *C. lusitanica* cell cultures.

4.2. Elicitor induces an immediate biosynthesis of β-thujaplicin and late monoterpene production by a recruitment of precursors and selective activation of β-thujaplicin branch pathway

Data from profiling of elicitor-induced monoterpene and β-thujaplicin accumulation and from elicitor activation of monoterpene-biosynthetic enzymes indicate that elicitor-induced monoterpene accumulated at 48 h after elicitation, which is consistent with activation of monoterpene biosynthetic enzymes. However, β-thujaplicin started accumulation much earlier (at 8–12 h after elicitor treatment) than monoterpene accumulation. In addition, monoterpene biosynthetic enzymes were reactivated after 5 days of elicitation, which is consistent with high monoterpene production at day 8 after elicitation, but opposite to the decreasing β-thujaplicin accumulation. These results, together with different time-courses of monoterpene and β-thujaplicin accumulation, suggest that β-thujaplicin and other monoterpene biosynthesis are subjected to differential regulation, on which metabolic flux shifted from β-thujaplicin biosynthesis to other monoterpenes.

Several studies have shown that differential regulation of two secondary metabolites on two branches derived from the same biosynthetic pathway is accomplished by activation of a key rate-limiting enzyme at one branch but suppression of the other rate-limiting enzyme at another branch (Chappell, 1995; Clive Lo and Nicholson, 1998). One important reason for this regulation is to keep balances of precursor pools and metabolic fluxes. β-thujaplicin and other monoterpenes may lie at different branches derived from a common MEP/MVA isoprenoid pathway and IPP/GPP precursor pools (Fig. 10). Various monoterpene synthases that use GPP to produce monoterpenes with different types of skeleton are regarded as the rate-limiting enzymes for monoterpene biosynthesis (Chappell, 1995; Davis and Croteau, 2000). It is proposed that elicited *C. lusitanica* culture cells also have different isoforms of active monoterpene synthase that are responsible for biosynthesis of different skeleton types of hydrocarbon monoterpenes, such as β-ocimene, limonene, and terpinolene (Fig. 10). These rate-limiting enzymes and their encoding genes are strictly regulated by post-translation or various transcription factors, which in turn are regulated by upstream signaling pathways (Chappell, 1995; Davis and Croteau, 2000). Among the three elicitor and MeJA-induced enzymes examined here, monoterpene synthase is the most significantly activated, with the peak being more than 3-fold over the control. It is likely that monoterpene synthase activity at the early stage of elicitation mainly catalyze the immediate production of β-thujaplicin (at 8–24 h after elicitation). The later peak of monoterpene synthase activity (36 h after elicitation) should be responsible for biosynthesis of both some β-thujaplicin and most other monoterpenes. This is similar to wounding-induced grand fir monoterpene synthases: the synthases induced at the initial stage of the response are different from these that appeared later (7 day) (Steele et al., 1998).

Results from treatments with protein and RNA synthesis inhibitor and IPP/DMAPP/GPP translocation inhibitor suggest that the rapidly increased β-thujaplicin biosynthesis requires both activation of biosynthetic enzymes and recruitments of substrate like IPP and GPP, most probably from the cytosol to plastids (Figs. 5–7). Whether IPP/DMAPP or GPP is corresponding substance for crossover of MVA/MEP pathways is not completely determined, an active uptake of IPP into isolated plastids was inhibited by NaPP (Soler et al., 1993), implying that at least IPP may be involved in the precursor exchange between the MVA and MEP pathways. Several other recent reports consistently support the cross talk between MVA and MEP pathways at IPP/DMAPP or GPP translocation (Hemmerlin et al., 2003; Laule et al., 2003). The present study further supports this idea about elicitor-induced biosynthesis of β-thujaplicin, which is also consistent with previous results showing that β-thujaplicin production partly attributes to MVA pathway (Yamaguchi et al., 1997). Therefore, results suggest that elicitor activates a recruitment of precursors and directs precursor flow or metabolic flux to biosynthesis of β-thujaplicin at early stage of elicitation.

It was proposed that the early biosynthesis of β-thujaplicin in elicited cell cultures require more protein

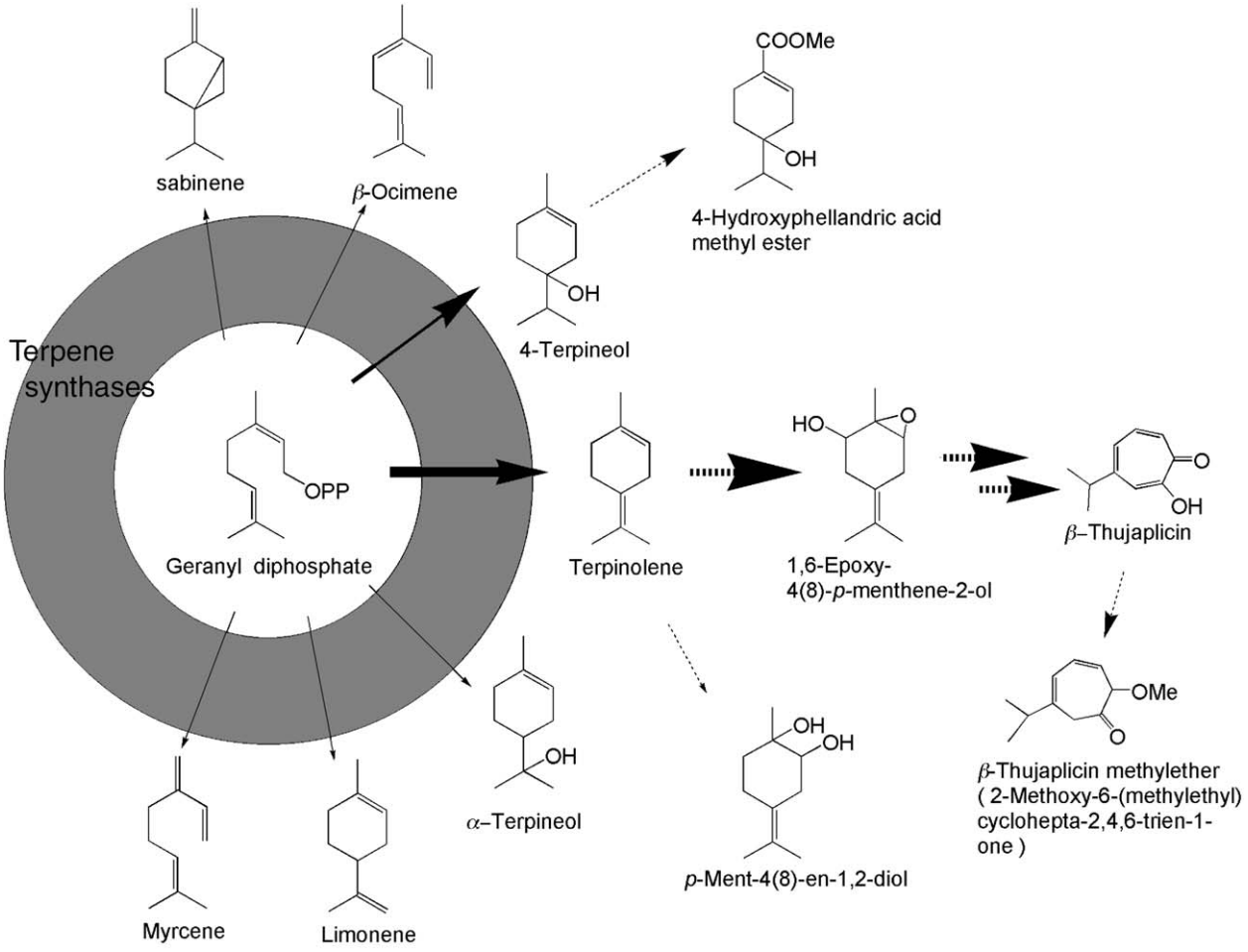

Fig. 10. Proposed biosynthetic pathways for monoterpenes in *Cupressus lusitanica* cell cultures. Solid arrow, terpene synthase reactions; Dotted arrow, putative multi-step reactions; Width of arrows expresses the strength of the metabolic flow.

biosynthesis than RNA biosynthesis since inhibition of protein biosynthesis blocked more β-thujaplicin accumulation than inhibition of RNA biosynthesis did. Because there were basal levels of β-thujaplicin and other monoterpene accumulation (Fig. 3), as well as activities for monoterpene biosynthetic enzymes in *C. lusitanica* cells incubated in the production medium (Fig. 5), a basal level of enzymes and transcripts for all genes involved in biosynthesis β-thujaplicin definitely continuously accumulates in the cell cultures. We proposed that these pre-existing enzymes and transcripts of biosynthetic genes might be quickly translated into enzymes upon elicitation to catalyze the immediate production of β-thujaplicin. It is reported that monoterpene biosynthesis genes, such as monoterpene synthase gene, have relatively long half-life (Steele et al., 1998), particularly upon elicitor treatment, which could regulate stability of transcripts of target genes (Mehdy, 1994). It is also interesting that transcripts for monoterpene synthases can be detected as early as 2 h after wounding for oleoresinosis in grand fir (Steele et al., 1998). Yeast elicitor may induce a rapid expression of monoterpene synthase for elicitor-induced β-thujaplicin biosynthesis; however, we have not detected such an early induction of monoterpene synthase transcript.

4.3. Analysis of metabolic intermediate and products shows an earlier activation of β-thujaplicin biosynthesis and a later shift of metabolic flux from β-thujaplicin to other monoterpenes

Since β-thujaplicin was synthesized pre-dominantly at early stage of elicitation (from day 0 to 4), and only minor portion of GPP was directed into other monoterpenes, it is reasoned that elicitor signaling preferentially activated β-thujaplicin-biosynthesis specific genes or enzymes but temporally suppressed the genes or enzymes for biosynthesis of other monoterpenes (from day 0 and 2 after elicitation). Also, because of the decreased biosynthetic speed and increased metabolism of β-thujaplicin and accumulation of other monoterpenes after day 4, we conclude that elicitor signaling directed a metabolic flux shift from β-thujaplicin to other monoterpenes. In addition, β-thujaplicin accumulation in culture medium from day 4 to 8 significantly decreased by about 30%, but Me-β-

thujaplicin increased after day 4, suggesting that the culture cells controlled β-thujaplicin level by both slowing down the biosynthesis rate and increasing metabolism of β-thujaplicin.

How the precursor pools are directed to β-thujaplicin production in a major portion and privilege by elicitor signaling is an important and interesting question. A proposed metabolic net for biosynthesis of all these monoterpenes in *C. lusitanica* cell culture shows that all these monoterpenes are derived from GPP by actions of different monoterpene synthases (Fig. 10). These proposed biosynthetic pathways are based on their structures and similar pathways occurred in other plants (Bohlmann et al., 1998; Davis and Croteau, 2000). These oxygenated monoterpenes including β-thujaplicin might be derived from hydrocarbon monoterpene intermediates, such as terpinolene and 4-terpineol. Therefore, biosynthesis of these monoterpenes, including *p*-MED, 1,6-EPMO, 4-HAME, and β-thujaplicin, competes for IPP and GPP precursor pools. Analysis of monoterpene profiles under various elicitation conditions provided such insights into metabolic flux and regulation on biosynthesis of these monoterpenes. Elicitor activated metabolic flux to all monoterpene branches, but most of precursor pools were directed to biosynthesis of β-thujaplicin within first 4 days post-elicitation. In the modified production medium with normal Fe^{2+} level, elicitor directed most of early metabolic flux to β-thujaplicin, and later to 4-HAME. In the modified production medium with normal inorganic elements, elicitor directs most of flux to other monoterpenes such as 4-terpineol, *p*-MED, and 1,6-EPMO, rather than β-thujaplicin. In this study, 4-terpineol often occupies one of the major portions in monoterpenes; as soon as β-thujaplicin biosynthesis increased, 4-terpineol level decreased. Together with a likely competitive relationship between β-thujaplcin and 4-HAME, these results suggests that GPP→4-terpineol→4-HAME pathway may be one of the major pathways to compete with β-thujaplicin production. An in vitro assay using cell free extracts from *C. lusitanica* cell culture suggested that terpinolene is the major portion of reaction products and a putative intermediate of β-thujaplicin synthesis (Fujita et al., 2003). It is therefore reasoned that GPP→terpinolene→β-thujaplcin pathway is one major metabolic flux in elicited *C. lusitanica* cell cultures, in which 1,6-EPMO might be one of the intermediates. When β-thujaplicin biosynthesis was blocked 1,6-EPMO rapidly accumulated (Fig. 3B and E); if metabolic flux to β-thujaplicin biosynthesis is smoothly channeled, 1,6-EPMO keeps a basal level. Another major monoterpene pathway might be GPP→terpinolene→*p*-MED pathway, which also competed with 4-terpineol→4-HAME and terpinolene→β-thujaplcin pathways for precursor flows. While β-thujaplicin biosynthesis rate slowed down and metabolic flux shifted to biosynthesis of other monoterpenes, β-thujaplicin metabolism through methylation and oxidation was also activated to regulate its level (Yamada et al., 2002; Zhao and Sakai, 2003b). This study shows that Me-β-thujaplicin is induced at late stage of elicitation, particularly when Fe^{2+} concentration decreased (Fig. 3A), suggesting that methylation of β-thujaplicin might be suppressed by Fe^{2+}. All these analyses suggest that monoterpene synthases as well as modification enzymes for later biosynthesis steps towards biosynthesis of β-thujaplicin and other monoterpenes might be the main regulatory switches for controlling whether monoterpenes or β-thujaplicin should be accumulated (Chappell, 1995). Previous feeding study showed that terpinyl acetate and 2-carene feeding could enhance elicitor-induced β-thujaplicin production (Zhao et al., 2001b), probably through a feed-forward effect since these two monoterpenes were not detected in the cell culture yet.

4.4. Elicitor-induced differential accumulation of β-thujaplicin and monoterpenes consists of a two-phase defense strategy, in which toxic β-thujaplicin accumulates at the early stage of elicitation while less toxic monoterpenes were synthesized as a second defense

The present study shows that elicitor-induced biosynthesis of β-thujaplicin at first phase of defense during day 1–4 after elicitation and then later biosynthesis of other monoterpenes as a second phase of defense after day 4. It is often observed that one plant is able to produce multiple terpenoids by actions of several different monoterpene synthases in response to pathogenic microbes, insect, or herbivores (Trapp and Croteau, 2001; Hahlbrock et al., 2003). Accumulation of these secondary metabolites at different time consists of a multiple-phase defense due to their different biological functions, usually with phytoalexin as first defense, followed by other antimicrobial compounds as second defenses (Mandujano-Chavez et al., 2000; Hahlbrock et al., 2003). β-Thujaplicin biosynthesis is the first priority for the plant defense because of its strong antimicrobial activity; production of other monoterpene derivatives may act as the second phase of defenses. The time frame for β-thujaplicin biosynthesis is actually comparable to that of elicitor-induced terpenoid phytoalexin production in other plants (Yin et al., 1997; Chappell, 1995; Martin et al., 2002). Plants use this multiple defense strategies for efficient and economical accomplishment of various defenses. Elicitation also causes increased membrane permeability and transport activity (Fig. 11), which causes more β-thujaplicin and monoterpenes to be excreted into extracellular parts to inhibit bacteria, insect, or herbivores.

5. Conclusion

Studies on shift of metabolic fluxes and changes in metabolic genes or enzymes, precursor pools and flow, metabolic intermediates and products in elicited plants or in vitro cell cultures by comparing elicited with non-treated ones can provide numerous invaluable information about plant metabolic flux and signaling net work (Goossens

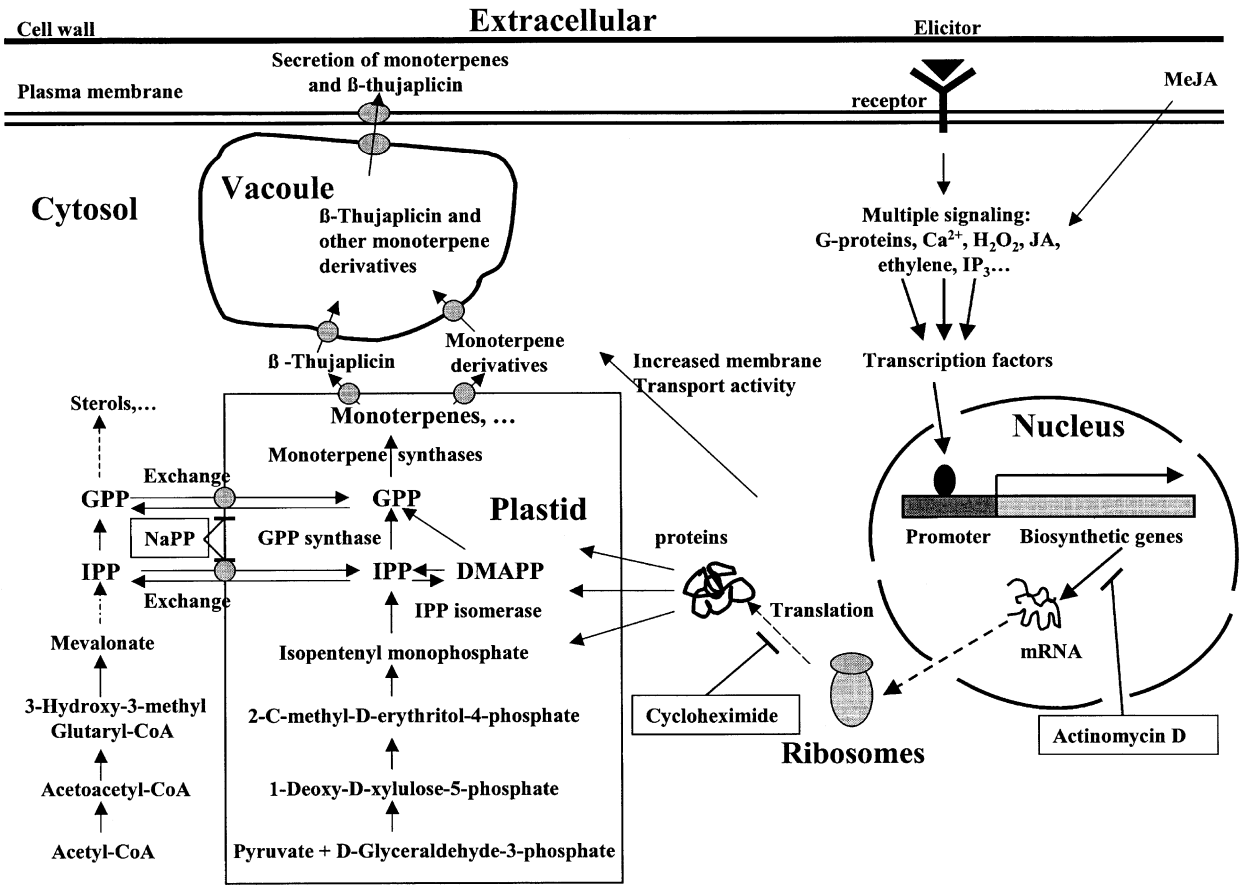

Fig. 11. Schematic illustration of signal transduction and metabolic flux of elicitors-induced β-thujaplicin and other monoterpenes in *Cupressus lusitanica* cell cultures. Multiple elicitor signals are incorporated into transcriptional factors, which selectively activate or inactivate metabolic gene expression and downstream metabolic pathways. β-thujaplicin biosynthesis involves two separate pathways and compartmentation regulation by the plastid, cytosol, and vacuole. Transport of metabolic substrates and products across through these compartments may require different transporters (marked with gray circles), which could also be regulated by elicitor. Metabolic inhibitors this study used are indicated in frames near their action sites.

et al., 2003; Walker et al., 2003). The present work systematically studied different regulation of β-thujaplicin and monoterpene biosynthesis by MeJA and yeast elicitor by analyzing metabolic intermediates and products, signaling pathways, and biosynthetic enzymes. As concluded in Fig. 11, this study indicates that elicitor-induced production of both β-thujaplicin and monoterpenes are mediated by similar signaling pathways. This transcriptional and translational regulation of monoterpene biosynthetic enzymes by elicitor signaling pathways results in a preferable induction of β-thujaplicin production over other monoterpenes by elicitor, reflected by a quick requirement for translation activity and recruitments of GPP and IPP precursors. The temporal and spatial changes in accumulation and secretion of β-thujaplicin and other monoterpene into extracellular environments reflect a multiple phase of plant defense induced by elicitor. The present study provides important clues for metabolic engineering of these desired secondary metabolites in *C. lusitanica* cell cultures. Engineering β-thujaplicin production could be archived through directing most metabolic fluxes to biosynthesis of β-thujaplicin by either over expressing β-thujaplicin biosynthesis pathway or suppressing other pathways leading to biosynthesis of other monoterpenes that may compete for precursors and against β-thujaplicin biosynthesis.

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