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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