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Biosynthesis of Sesquiterpene in Hairy Root and Cell Suspension Cultures of *Hyoscyamus muticus* by Elicitation Using *Rhizoctonia solani* Extracts

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Rhizoctonia solani 추출액 첨가에 의한 *Hyoscyamus muticus*의 현탁세포배양 및 모상근배양에서 Sesquiterpene 생합성

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The extracellular sesquiterpenoids were accumulated in cell and hairy root cultures of *Hyoscyamus muticus* by elicitation using extracts of *Rhizoctonia solani*. The vetispiradiene synthase (VS) which is the first committed step in biosynthetic pathway leading to formation of solavetivone, lubimin, and rishitin from isoprenoid intermediate farnesyl pyrophosphate was induced upon elicitation, whereas no sesquiterpenoids and VS activity were detected in both control cell and hairy root cultures. VS activity increased rapidly and reached its maximum 12 h in both cell and hairy root cultures upon elicitor treatment. VS activities were paralleled with the absolute levels of VS polypeptide(s). Interestingly, the profiles of sesquiterpenoid accumulation in hairy root cultures were different from those in cell cultures. The hairy root culture seemed to fail to metabolize solavetivone further to lubimin.

Key words: Elicitation, phytoalexin, lubimin, solavetivone, vetispiradiene synthase

The production of secondary metabolites in cell and hairy root cultures with elicitor treatments has broadly been performed (Eilert, 1988). It is well known that Solanaceae produces many different kinds of antifungal phytoalexins and stress compounds under elicitor treatments and various conditions of stress (Stoessl et al., 1976). One of Solanaceae species, *Hyoscyamus muticus* secretes large amounts of the fungitoxic sesquiterpenes, lubimin and solavetivone, into the liquid medium upon elicitation with a mycerial extract from the soil pathogen *Rhizoctonia solani* (Signs et al., 1989: Signs and Flores, 1989). Solavetivone was first isolated as a stress metabolites of potato tubers infected with *Phytophthora infestans* or *Erwinia cartovora* (Coxon et al., 1974). Also, solavetivone was isolated from the leaves of *Nicotiana tabacum* cv Xanthi-nc infected with tobacco mosaic virus but it could not detected in extracts of uninoculated leaves (Fujimori et al., 1979). These antimicrobial compounds comprising of bicyclic, carbon 15 bearing skeleton are collectively termed as sesquiterpenoids or sesquiterpene compounds which have roles as mediators of plant-plant, plant-insect and plant-microbe interaction (Elakovich, 1987: Mabry and Gill, 1979: Stoessl, 1976). Within the Solanaceae including *Nicotiana tabacum*, *Capsicum annum*, and *H. muticus*, the accumulation of species-specific, anti-microbial, bicyclic sesquiterpenoids has been demonstrated for pathogen- or elicitor-challenged plants and cell cultures (Guedes et al., 1982: Watson and Brooks, 1984: Corry et al., 1993: Whitehead et al., 1990).

The isoprenoid intermediate farnesyl pyrophosphate (FPP) is the precursor to the biosynthesis of sesquiterpenoids and other isoprenoids including sterols, dolichols, ubiquinone and growth regulators (Figure 1). The initial step in the

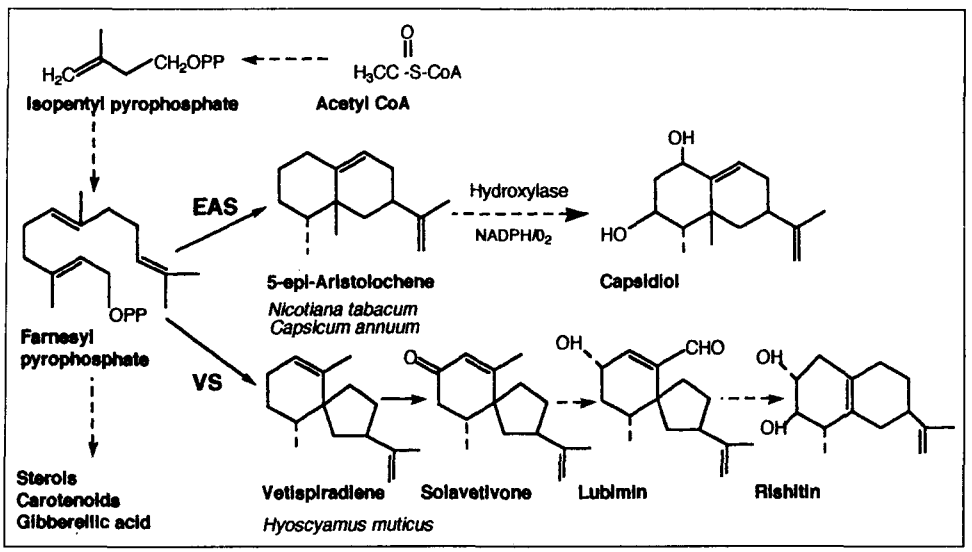

Figure 1. The proposed reactions catalyzed by sesquiterpene cyclases from tobacco, pepper and *Hyoscyamus muticus* leading to the formation of the major accumulating sesquiterpenoids in each species. EAS and VS stand for 5-epi aristolochene synthase and vetispiradiene synthase, respectively.

conversion of FPP to sesquiterpenoids is catalyzed by an inducible sesquiterpene cyclases. The direct products of these cyclases in tobacco and henbane are structurally different bicyclic sesquiterpenoids, 5-epi aristolochene and vetispiradiene, respectively (Back et al., 1994: Back and Chappell, 1995). The elicitor-induced 5-epi aristolochene synthase (EAS) was first assayed and purified in tobacco cell cultures (Vogeli et al., 1990). In tobacco, EAS activity was absent from control cell cultures but induced rapidly with addition of elicitor to the cell cultures, and that its enzymatic product converted further into antifungal phytoalexin capsidiol (Vogeli and Chappell, 1988). In contrast, there has not been reported any regulation of vetispiradiene synthase (VS) which is the first committed enzyme for the production of sesquiterpene phytoalexins, lubimin and solavetivone, in *H. muticus*, even vetipiradiene synthase genes were cloned, sequenced and theirs transcripts seemed to be regulated in transcriptional level after elicitor treatment to cell cultures (Back and Chappell, 1995).

To date, the secondary metabolism has been focused on the opportunities for the improvement of the productivity of plant secondary metabolites in cultures emphasizing on the section of explant, medium constituents and physical environment of the culture (Rhodes, 1989). Therefore, knowledge of secondary metabolite regulations is limited. The present study was performed to investigate the regulation between the production of sesquiterpenoids and vetispiradiene synthase in elicitor treated hairy root and cell cultures of *H. muticus* in order to provide fundamental biochemical basis for the production of secondary metabolism.

MATERIALS AND METHODS

Cell Suspension and Hairy Root Cultures

Cell cultures of *Hyoscyamus muticus* were maintained as described previously (Signs et al., 1989). Cell cultures in their rapid phase of growth were used for the induction studies. The cell cultures were incubated with 2 mL's of homogenate of *Rhizoctonia solani* per 50 mL of cell cultures for indicated lengths of time before harvesting cells by light suction filtration onto Whatman filter paper. Root cultures of *H. muticus* were maintained as before (Signs and Flores, 1989). The root cultures were elicited with 2 mL's of above elicitor for indicated lengths of time. Each sample of time point was done with four replicates.

Cyclase Assay

Cells or roots (fr wt 0.5 g) were homogenized in a mortar and pestle with 1 mL of 80 mM potassium phosphate buffer (pH 7.0), 20% (w/v) glycerol, 10 mM sodium metabisulfate, 10 mM sodium ascorbate, 15 mM $MgCl_2$, 1% (w/v) of PVP and 5 mM β-mercapthoethanol. The homogenate was centrifuged in an Eppendorf centrifuge tube for 10 min at 12,000 g. For the cyclase assay, 5 to 10 μL of the supernatant, corresponding to 5 to 10 μg protein, was incubated in a total volume of 75 μL containing 5 mM β-mercaptorthanol, 150 mM Tris/HCl (pH 7.0), 30 mM $MgCl_2$, and 5% (w/v) glycerol. The reaction was initiated by the addition of 3 nM of ^{3}[H]FPP. After incubation for 5 to 10 min at 35° C, the reaction was chilled in an ice bath, and the

reaction products were partitioned into 150 μL of *n*-hexane. After a brief centrifugation, an aliquot of the *n*-hexane (100 μL) was reacted with 50 mg of silica powder to remove any contaminating FPP of farnesol generated by phosphatase activity. The radioactivity remaining in the *n*-hexane was then determined by scintillation counting and taken as the measure of the product. Cyclase activity is expressed as nmol of product/mg protein · h.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970) in an 11.5% (w/v) acrylamide/bis gel. 100 μg proteins from each time course of cell and hairy root cultures were loaded per lane. Proteins separated by SDS-PAGE were transferred to nitrocellulose as described by Towbin and Gordon (1984). After blocking with 5% low fat milk in TBS (20 mM Tris/HCl (pH 7.5), 500 mM NaCl), the blots were incubated overnight with blocking solution containing 3 μL EAS-specific monoclonal antibody ascites fluids/mL. Goat anti-mouse antibodies coupled to alkaline phosphatase and the appropriate chromogenic system served for visual detection of polypeptides reacting with cyclase antibodies (Leary et al., 1983). Protein concentration was determined by the Bradford method (Bradford, 1976).

Analysis of Sesquiterpenes

Sesquiterpenes were recovered from the media by extracting 5 mL of culture medium on a Water C-18 Sep-PAC column. HPLC procedures were carried out (Corry et al., 1993) with a slight modification as follow: calculation of solavetivone levels was based on calibration curves generated with purified solavetivone as described more recently (Reddy et al., 1993).

RESULTS

Induction of Vetispiradiene Synthase Followed by Sesquiterpenoid Accumulation in *Hyoscyamus muticus* Cell Cultures

Time courses for the production of sesquiterpenoids, the induction of cyclase activities, and the absolute level of the cyclase protein in elicitor-treated *Hyoscyamus muticus* cell cultures are shown in Figure 2. When *H. muticus* was incubated with elicitor, the sesquiterpene cyclase activity increased rapidly and reached its maximum 12 h after elicitor treatment. Thereafter the cyclase activity declined. No sesquiterpene cyclase activity was detected in the control cell cultures. Sesquiterpenoid accumulation in culture media was followed after a rapid induction of cyclase activities and reached maximum level after 50 h and that the level of sesquiterpene compounds began to diminish suggesting conversion of other product or degradation. Also no sesquiterpenoids were detected in control cell cultures.

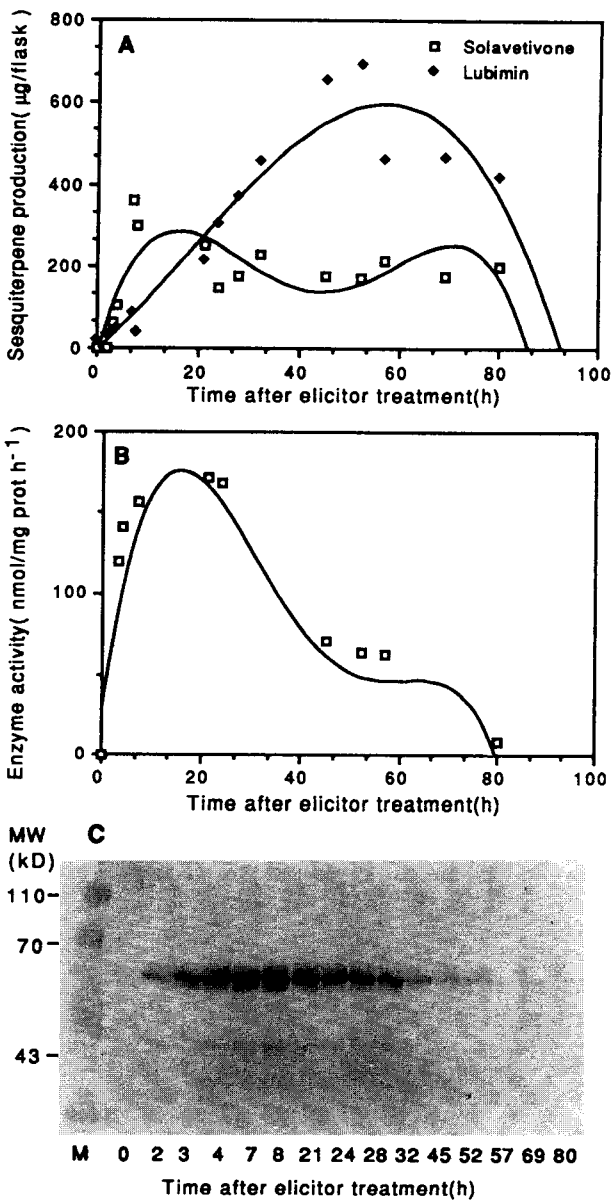

Figure 2. The accumulation patterns of sesquiterpenoid phytoalexins (A), the induction of vetispiradiene synthase activity (B), and the immunodetection of the cyclase protein (C) in cell cultures of *Hyoscyamus muticus* by elicitation using extracts of *Rhizoctonia solani*.

Interestingly, the rapid and maximum synthesis of solavetivone, a precursor for the synthesis of lubimin, in 8 h after elicitation preceded the slow, gradual increasing synthesis of lubimin. After reaching the maximum synthesis of solavetivone at 8 h, the level of solavetivone synthesis keeps maintaining 50-60% of its highest level until 80 h. The rest portion of solavetivone synthesized seemed to directly metabolized into lubimin that is accumulated in culture medium with further enzymatic degradation or modification later. The accumulation of the cyclase polypeptide(s) was paralleled by the induction of the cyclase enzyme activity. The induction of cyclase protein (62-63 kD) was seen very rapidly in 2 h and reached its maximum level around 21 h and decreased gradually until it could barely seen at 80 h. However, in tobacco cell challenged with cellulase, cyclase activity and absolute level of the cyclase protein increased sharply and reached a maximum 12 h to 14 h, and remained at those level for the next 60 h (Vogeli and Chappell, 1990). In contrast, cyclase activity and cyclase protein in *H. muticus* did not sustain longer than those in tobacco cell cultures.

Induction of Vetispiradiene Synthase Followed by The Accumulation of Different Profiles of Sesquiterpenoids in *Hyoscyamus muticus* Hairy Root Cultures

As shown in Figure 3, relative production of sesquiterpenoids is two fold higher that of cell cultures. The cyclase activity increased sharply and reached maximum at 12 h. The overall enzyme activity still remains higher to the end of time course. The induction pattern of cyclase activity did well correlate with the absolute amount of cyclase polypeptide(s). The absolute level of cyclase polypeptide(s) has risen its maximum at 12 h, decreased slowly until 32 h, and increased again from 35 h. The synthesis of sesquiterpenoids in hairy root cultures had shown different pattern from those in cell cultures. The root cultures favored to produce and accumulate solavetivone, suggestive of disturbance of further metabolization into lubimin. Even the small amount of lubimin synthesis was followed by the synthesis of solavetivone, but remained in a very low level during the entire root culture period.

DISCUSSION

It has been reported that sesquiterpene cyclase was an inducible enzyme which could be stimulated only with elicitor

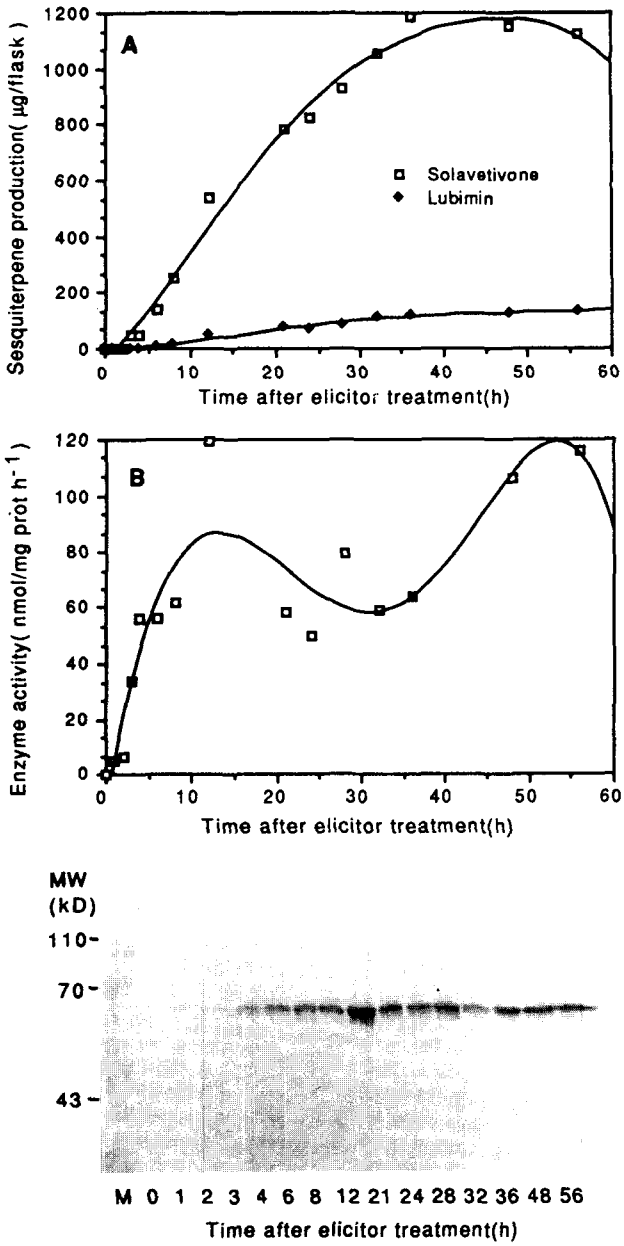

Figure 3. The accumulation patterns of sesquiterpenoid phytoalexins (A), the induction of vetispiradiene synthase activity (B), and the immunodetection of the cyclase protein (C) in hairy root cultures of *Hyoscyamus muticus* by elicitation using extracts of *Rhizoctonia solani*.

treatment in tobacco suspension cultures (Vogeli et al., 1988). Therefore, sesquiterpene cyclase activity was absent in control tobacco cell cultures. Accordingly, tobacco cells do not produce extracellular sesquiterpenoids in the absence of elicitor treatment, in which the carbon flow of FPP is to supply for the production of sterols and synthesis of geranyl geranyl pyrophosphate (GGPP), an isoprenoid intermediate which is the common substrate for diterpene biosynthesis and

carotenoid biosynthesis (Figure 1).

Elicited hairy root cultures produced and released into the medium of the sesquiterpene compounds such as lubimin and solavetivone. The amount of compound produced represents nearly 0.5% of the root dry weight. These elicited mediums were reported to cause an ~25% inhibition of *Phytophthora infestans* germ tube growth (Signs and Flores, 1990).

Our results demonstrated that the production of sesquiterpenoids is highly correlated with the induction of sesquiterpene cyclase in elicited cell and hairy root cultures of *H. muticus*. Also cyclase enzyme activities are highly paralleled with the absolute level of cyclase polypeptide(s) during the entire elicitation period of cultures. Interestingly, cell suspension and hairy root cultures of *H. muticus* upon elicitation had shown the different profiles of sesquiterpenoid accumulation into medium.

In cell suspension cultures, solavetivone, the precursor metabolite in sesquiterpenoid biosynthesis pathway was accumulated in early time course and further metabolized into lubimin by next step enzyme. The relative conversion ratio from solavetivone into lubimin was around 75% according to the relative percentage of lubimin synthesis out of total amount of lubimin and solavetivone production. In contrast, hairy root cultures dominated to produce solavetivone, and that the accumulated solavetivone was unable to metabolize into lubimin synthesis efficiently. These distinct profiles of sesquiterpenoid synthesis can be simply explained by the induction of enzymes, differential expression of enzyme which are required for the synthesis of solavetivone, lubimin sesquiterpenes.

In hairy root and cell cultures, vetispiradiene synthase and putative solavetivone hydroxylase (catalyzing vetispiradiene to solavetivone) seem to express equally, but putative lubimin hydroxylase catalyzing solavetivone into lubimin is either induced only in cell cultures or absent in hairy root cultures. These can be further elucidated measuring lubimin hydroxylase activity between hairy root and cell cultures of *H. muticus*. The overall enzyme activity and the absolute level of cyclase polypeptide (s) were quite well regulated in cell suspension cultures, but, the differentiated hairy root cultures showed somewhat complicated response, in that the increased enzyme activity and cyclase polypeptide level were accompanied by the second boost. It is unclear whether distinct elicitor perception, signal transduction, activation of response genes for the synthesis of sesquiterpene compounds between hairy root and cell suspension cultures upon elicitation may exist.

To date, secondary metabolite production using *Hyoscyamus* species has been centered on the alkaloids such as hyoscyamine and scopolamine which are the two common tropane alkaloids in the Solanaceae. Hashimoto et al. (1991) reported that hyoscyamine 6β-hydroxylase which catalyzes the first oxidative reaction in the biosynthetic pathway leading from hyoscyamine to scopolamine was localized at the pericycle of root. This enzyme was high activity in cultured roots but very low in cultured cells and cultured shoots. Also they isolated hyoscyamine 6β-hydroxylase cDNA that encodes 344 amino acids for studying molecular mechanism for this tissue and cell specific expression of alkaloid biosynthesis (Matsuda et al., 1991).

The sesquiterpene cyclase plays an essential role in production of sesquiterpenoid phytoalexins from members of the Solanaceae. Especially, these sesquiterpene cyclases catalyze different reaction products using common substrate, FPP, among species. Potato and *Datura stramonium*, for example, produced solavetivone, lubimin, and rishitin which belong to the vetispirane and noreudesmane subclasses of eudesmanoid sesquiterpenes, whereas capsidiol which is a member of the eremophilane subclass of eudesmanoid sesquiterpenes was major product in tobacco and pepper species (Stoessl et al., 1976: Guedes et al., 1982: Watson et al., 1984: Zook et al., 1992).

Impressive progress in molecular biology of sesquiterpenoid biosynthesis has been shown that there are distinct functional amino acid domains among various cyclase enzymes, and that these amino acid domains are responsible for the final product of distinct cyclase enzymes (Back and Chappell, 1996). The molecular, cellular, and enzymatic studies of sesquiterpenoid synthesis among plants will provide a better insight of secondary metabolism, furthermore leading to possible application of a novel secondary metabolite production in different plant species with help of modern molecular biology technique.

적 요

*Hyoscyamus muticus*의 phytoalexin으로 알려진 solavetivone, lubimin, rishitin 생합성에 결정적 역할을 담당하는 isoprenoid pathway의 첫 분지효소인 vetispiradiene synthase의 유도특성과 solavetivone, lubimin phytoalexin 생합성과의 관련성을 구명하기 위하여 *H. muticus* 현탁세포배양 및 모상근배양에 *Rhizoctonia solani* 추출액을 elicitor로 처리하였다. Elicitor을 처리하지 않은 현탁세포 및 모상근배양에서는 효소활성, 효소단백질 및 solavetivone, lubimin 등이 전

혀 검출되지 않았으나, elicitor을 처리한 세포에서는 효소활성이 급속히 유도되어 처리 12시간 후에 최고의 활성을 보였으며 그후 점점 감소하였다. 효소활성의 정도는 면역반응을 이용하여 측정한 효소 단백질의 함량과 밀접한 상관관계를 보였으며, phytoalexin 총 함량은 모상근배양에서 2배 이상 높았다. 특히 solavetivone 및 lubimin 생합성은 현탁 및 모상근배양에서 서로 다른 특이성을 보였는데, 현탁배양에서는 lubimin을, 모상근배양에서는 solavetivone을 선택적으로 다량 생산하였다.

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