Induction of a novel cytochrome P450 (CYP93 family) by methyl jasmonate in soybean suspension-cultured cells

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Abstract **We isolated a cDNA encoding a novel cytochrome P450 (CYP93A1) from soybean suspension-cultured cells that had been treated with methyl jasmonate (MeJA). The amino acid sequence of the gene product had 30–40% identity with those of other plant P450s. The protein contained the heme-binding domain which is highly conserved among plant P450s. Transcription of the cytochrome P450 gene in soybean cells was induced by 30 μM MeJA even in the presence of cycloheximide, and reached maximum level 6 h after MeJA treatment. This is the first report of a plant cytochrome P450 gene whose transcription is induced by MeJA even without protein synthesis.**

Key words: Differential display; Cytochrome P450; Methyl jasmonate; Jasmonate-induced protein; Suspension-cultured cell; *Glycine max*

1. Introduction

Jasmonic acid (JA) and its derivatives are cyclopentanone compounds which are ubiquitously distributed in higher plants, and are involved in various physiological events such as tuber formation, fruit ripening, and defense responses to pathogen attack or wounding [1,2].

The biosynthesis of JA that is initiated by lipoxygenation of linolenic acid [3] is comparable with that of eicosanoids which are synthesized by lipoxygenases and lipoxygenase pathway enzymes [4], from arachidonic acid, a major polyunsaturated fatty acid in mammalian cell membranes. JA behaves as a signal molecule in plant cells similarly to eicosanoids in mammalian cells [5]. In fact, treatment of plant cells with pathogenic elicitor transiently induces endogenous levels of JA [6].

It is still unclear how JA promotes sequential physiological events. The treatment of plant tissues with JA or methyl jasmonate (jasmonic acid methyl ester, MeJA) induces the synthesis of many kinds of proteins called jasmonate-induced proteins (JIPs) [2,7]. More than 20 JIPs have been identified in MeJA-treated plant tissues, including proteinase inhibitors [8], ribosome-inactivating proteins [9,10], vegetative storage proteins [11] and lipoxygenases [12,13]. Several JIPs are probably associated with defense responses of plants against pathogen attack or wounding [2].

However, the functions of these JIPs cannot fully explain multiple physiological functions of jasmonates. Therefore, the isolation of JIPs which are associated with senescence, tuber formation, fruits ripening, and induction of secondary metabolites (flavonoids, alkaloids, phenylpropanoids), is an essential step to clarify the mechanism of JA actions.

For isolation of such genes which play crucial roles in subsequent JA-promoted events, we attempted to clone new JIP genes by the differential display method [14,15]. This method enables us to detect gene expression in response to MeJA, even if they express at low levels. Screening of JIPs was performed in the presence of cycloheximide (CHX) to exclude JIPs which require de novo protein synthesis for the induction by MeJA.

By the differential display method, we isolated a novel MeJA-inducible gene, *cyp93A1* that encodes a cytochrome P450. This is the first report that transcription of cytochrome P450 gene is induced by MeJA.

2. Materials and methods

2.1. Materials

Suspension-cultured cells from the photomixotrophic soybean (*Glycine max*) cell line SB-P established by Horn [16] were grown under continuous white light (9.2×10^3 erg/cm^2·s) at 24 ± 1°C in an incubator equipped with a rotary shaker (100 rpm) in 200-ml flasks. Each flask contained 48 ml of KN1 medium [17] supplemented with 0.5% sucrose. Every 10–14 days, 8 ml of cultured cells was transferred into 40 ml of new medium. 10-day-old cultured cells were used in all experiments.

2.2. Screening by the differential display method

Soybean suspension-cultured cells were treated with 30 μM MeJA and 100 μM CHX for 30 min. MeJA was dissolved in ethanol and added to cells at 1/1000 dilutions. Cells treated with 0.1% ethanol were used as control. Single-strand cDNAs were synthesized from each total RNA using four different degenerate anchored dodeca-dT primer sets (T12VG, T12VA, T12VT, T12VC, where V is G, A, or C), and used as templates for PCR. PCR was carried out using dNTPs containing ^{35}S-labeled dATP for 40 cycles of 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s, followed by a 5 min elongation period at 72°C, using an arbitrary decamer and the same dodeca-dT primer which was used for reverse transcription. Each sample was denatured and electrophoresed with 6% denaturing polyacrylamide gel, and exposed to X-ray film. DNA bands of interest were cut out, and the DNAs were extracted from the gel slices, and reamplified with the same primer set. Reamplified PCR products were cloned into the pCR II vector using a TA Cloning Kit (Invitrogen Co., CA, USA).

2.3. Screening of cDNA library and nucleic acid sequencing

A cDNA library was prepared in λ ZAPII from poly-A$^+$ RNA of

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Abbreviations: CHX, cycloheximide; JIP, jasmonate-induced proteins; JA, jasmonic acid; MeJA, methyl jasmonate; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na_3 citrate pH 7.0.

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the SB-P cells which were treated with 30 μM MeJA for 2 h. Approx. 80000 plaques of the library were screened with the ^{32}P-labeled fragment (G9) obtained by the differential display method. Hybridization was carried out at 60°C for 16 h [18]. The membrane was washed twice with 0.2×SSC/0.1% SDS at 60°C for 10 min. Dideoxy sequencing [19] was carried out on both strands of double-strand DNA templates using a *Bca*BEST Dideoxy Sequencing Kit (Takara Shuzo Co., Otsu, Japan). On the basis of the partial sequence determined, oligonucleotides (20-mer) were synthesized and used to sequence the internal regions.

2.4. Northern blot analysis

Total RNAs were isolated using the procedure of guanidine thiocyanate and phenol-chloroform extraction [20]. For Northern blot analysis, total RNAs (20 μg/lane) were denatured, subjected to electrophoresis on 1.2% agarose gel and transferred onto a nylon membrane. The blot was hybridized with the ^{32}P-labeled G9 at 60°C for 16 h. An actin gene was used as an internal control. After hybridization, the blot was washed with 2×SSC/0.1% SDS at 60°C for 30 min.

3. Results and discussion

3.1. Isolation of an MeJA-inducible gene from soybean suspension-cultured cells

To isolate the genes that are transcribed after MeJA treatment without protein synthesis in plants, we prepared the mRNAs from soybean suspension-cultured cells which were treated with MeJA in the presence of CHX and also from untreated cells. These mRNAs were used for differential screening by the differential display method [14,15] (see section 2). Several bands of the PCR products on a polyacrylamide gel appeared specifically on the lane of the MeJA-treated cell's sample, but not on the lane of the control. These DNA bands were cut out from the gel, reamplified by the same primer set used for PCR, and subcloned into the pCR II vector. These clones were used as probes for Northern analysis of mRNA prepared from soybean suspension-cultured cells after MeJA treatment. Although most of probes prepared from these candidate clones displayed no difference in the amounts of mRNA with or without MeJA treatment, the probe from one clone, G9, showed that the amount of the corresponding mRNA increased after MeJA treatment in the presence of CHX (Fig. 1).

Using the G9 fragment as a probe, the full-length cDNA was isolated from a cDNA library prepared from MeJA-treated SB-P cells. As shown in Fig. 2, the longest cDNA, G9-3, had the identical region with the sequence of the G9 fragment

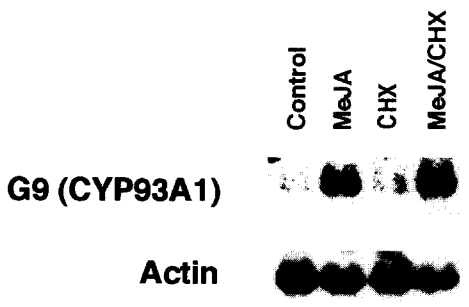

Fig. 1. Induction of transcripts of G9 by treatments with MeJA and CHX. Total RNAs (20 μg) were prepared from SB-P cells treated for 3 h under various conditions as follows: control, 0.1% ethanol; MeJA, 30 μM MeJA; CHX, 100 μM CHX; MeJA/CHX, 30 μM MeJA and 100 μM CHX. Hybridization was performed with the ^{32}P-labeled G9 fragment as probe. Actin gene was used as loading and cell viability control.

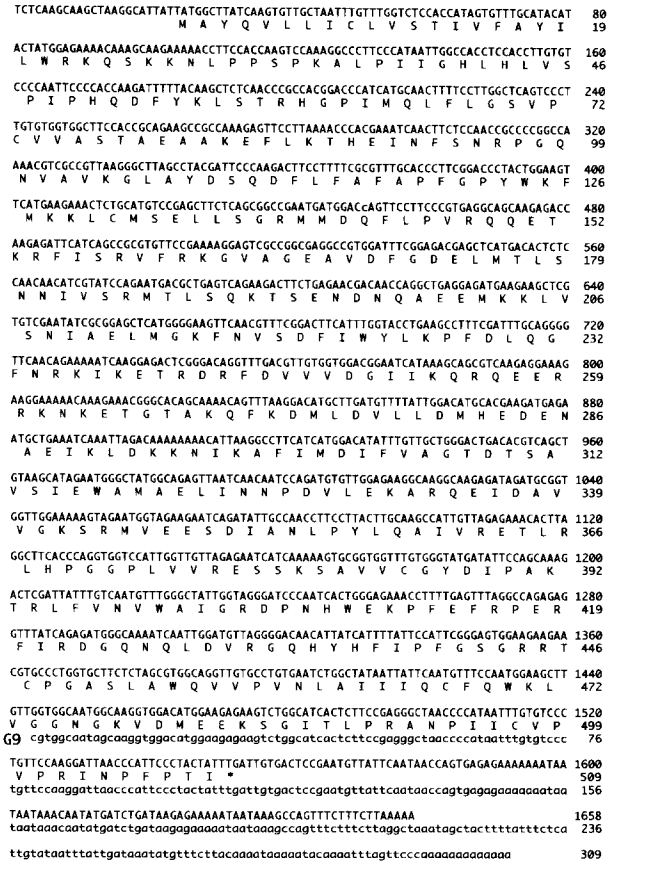

Fig. 2. Nucleotide sequences of the soybean cytochrome P450 (*cyp93A1*) cDNA and the deduced amino acid sequence. The nucleotide sequence of the G9 fragment derived from the differential display is also indicated in lower-case letters.

in its 3′-end. DNA sequencing revealed that the G9-3 cDNA had 24 bp in the 5′-untranslated region, 1527 bp in an open reading frame, and 107 bp in the 3′-untranslated region. However, G9-3 lacked 94 bp sequences of the 3′-end of the G9 fragment, which included the poly-A$^+$ tail. The open reading frame encoded 509 amino acid residues with a predicted molecular mass of 57869 Da. A computer search of databases revealed that the amino acid sequence shared 30–40% homology with those of plant cytochrome P450s. The heme-binding sequence PFGXGRRXCX, which is highly conserved among the P450s of higher plants [21], was also found in the sequence (Fig. 3). These data indicate that the gene encodes an MeJA-inducible cytochrome P450 in soybean.

The gene product of G9-3 shared the highest homology (41%) with that of *cyp71A1* from avocado fruit [22], among other plant P450s, although it shared the least homology with *cyp71*s from maize (33–35%) [23] and from eggplant (35%) [24]. The soybean P450 also shared homology with flavonoid hydroxylases of petunia encoded by *cyp75*s (38%) [25] and eggplant *cyp75* (38%) [26]. Allene oxide, a precursor of JA, is synthesized by a member of plant cytochrome P450s, designated as *cyp74* [27]. Amino acid sequence homology between the gene products of G9-3 and *cyp74* was less than 20%. The low homology between G9-3 and other plant P450s reported so far indicated that the present cytochrome P450 belonged to a novel family in plants. D.R. Nelson and the Committee for Standardized Cytochrome P450 Nomenclature officially named G9-3 as CYP93A1 (personal communication).

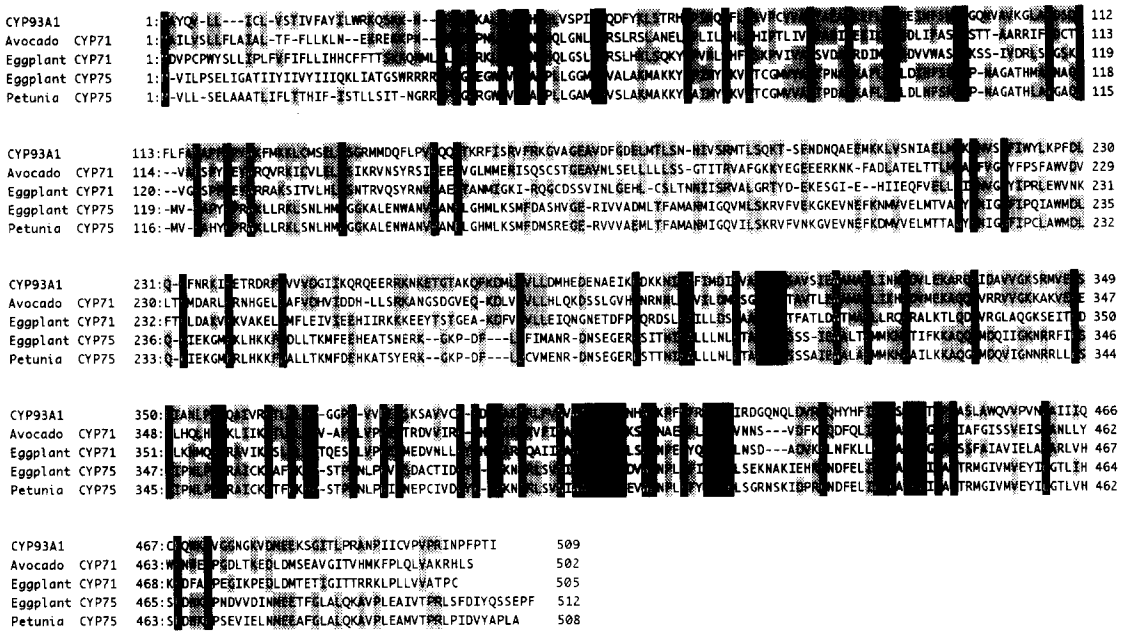

Fig. 3. Multiple alignment of deduced amino acid sequences of CYP93A1 with those of avocado CYP71 [22], eggplant CYP71 [24], eggplant CYP75 [26], and petunia CYP75 [25]. Amino acids are designated using the single-letter code, and gaps indicated by (–) have been inserted to maximize the similarity among sequences. Amino acids which are common to all sequences are indicated by white letters on a black background.

A hydropathy profile (data not shown) revealed that CYP93A1 had a membrane-spanning sequence in the amino-terminus region, similar to other cytochrome P450s.

3.2. *Profiles of the induction of cyp93A1 by MeJA*

The expression of *cyp93A1* was analyzed by Northern blotting (Fig. 4). As all plants investigated previously have several families of cytochrome P450, we used the 3′-end fragment of *cyp93A1*, G9, as a gene-specific probe. The expression of *cyp93A1* appeared 2 h after treatment with MeJA, reached a maximum at 6 h, and gradually declined. As shown in Fig. 4B, maximal expression of *cyp93A1* was observed at 30 μM of MeJA among the various concentrations examined. This is the first report that MeJA induced the transcription of a cytochrome P450 gene in plants. Although some JIPs were also induced by abscisic acid (ABA) [7], this cytochrome P450 was not induced by the hormone.

In plant cells, cytochrome P450s are involved in the biosynthesis of plant secondary metabolites such as flavonoid, terpenoid, and alkaloids [28]. It is also known that some of these metabolites are accumulated in the cells after treatment with JA [6,29]. MeJA induces phenylalanine ammonia lyase [6] and chalcone synthase [30] both of which are involved in the biosynthesis of plant secondary metabolites. It is possible that CYP93A1 induced by MeJA promotes the synthesis of these secondary metabolites. In fact, involvement of two cytochrome P450s, cinnamate 4-hydroxylase (CYP73) [31] and flavonoid 3′,5′-hydroxylase (CYP75) [26] in phenylpropanoid and flavonoid metabolisms has been reported. Although these cytochrome P450s are light-inducible [26,32], CYP93A1 was not induced by light. In animal cells, expression of each cytochrome P450 gene is strictly regulated by the stage of embryogenesis, hormones, and many kinds of drug applied exogenously [33]. Therefore, the fact that CYP93A1 was induced specifically by MeJA is of interest for understanding the physiological significance of cytochrome P450s in plants. It is also possible that CYP93A1 is involved in the synthesis or metabolism of some signal molecule.

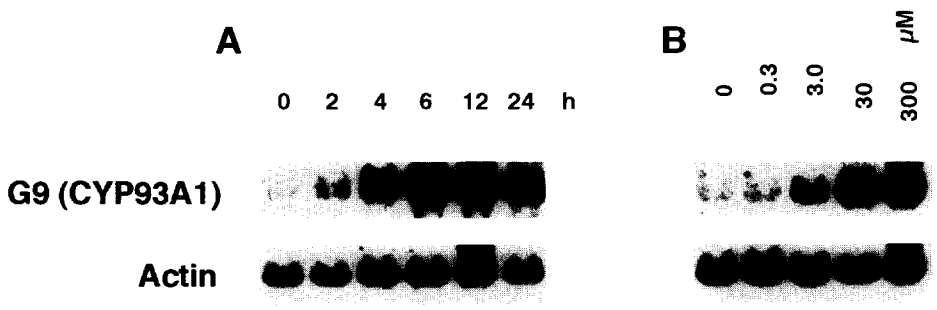

Fig. 4. Profiles of the induction of CYP93A1 mRNA by MeJA. Total RNAs (20 μg) prepared from SB-P cells were electrophoresed, blotted and hybridized with the probes described in Fig. 1. (A) Time course of induction of CYP93A1 by MeJA treatment. SB-P cells were treated with 30 μM MeJA for the times indicated. (B) Effects of concentration of MeJA on induction of CYP93A1. Total RNAs (20 μg) were prepared from SB-P cells treated with MeJA at the concentrations indicated for 6 h.

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