Transcriptional Activation of Putative Calmodulin Genes *Am-cam-1* and *Am-cam-2* from *Aquilaria microcarpa*, in Response to External Stimuli

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A homology-based cloning strategy yielded two cDNA clones designated *Am-cam-1* and *Am-cam-2*, presumably encoding calmodulin protein from a callus culture derived from the leaf tissues of *Aquilaria microcarpa*. An appreciable increase in the transcriptional activity of *Am-cam-1* was reproducibly observed by exposure of the cell culture to methyl jasmonate, as analyzed by a reverse-transcription polymerase chain reaction. The expression level of the gene also increased when the cells were treated with yeast extract. The transcription of *Am-cam-2* was similarly stimulated by the treatment with methyl jasmonate and yeast extract, however, the intensities of the enhanced expression appeared to be lower as compared with that of *Am-cam-1*. In contrast, Ca^{2+} -ionophore A23187 did not show inducing activity for the expression of these two calmodulin genes. These results suggest that *Am-cam-1* and *Am-cam-2* and their products play important roles in signal transduction processes in methyl jasmonate- and yeast extract-treated cells of *A. microcarpa*, accompanying the change in the transcriptional activities.

Key words methyl jasmonate; calmodulin; gene expression; agarwood; *Aquilaria microcarpa*

Jasmonates, jasmonic acid and methyl jasmonate (MJ), are essential plant hormones that regulate defense responses against environmental stressors, such as drought, wounding, and microbial infection. In addition, exogenous application of jasmonates to plant cells enhance accumulation of a large number of secondary metabolites belonging to structurally diverse groups.^{1,2)} The jasmonate-mediated signal transduction pathway is intertwined with other hormonal pathways and positively or negatively alters the expression of a variety of target genes. Recent studies have demonstrated^{2,3)} that the active form of jasmonates is an amino acid-conjugate, jasmonoyl-isoleucine. This adduct functions through interaction with a protein complex that acts as the receptor of this plant hormone,^{1,3)} and this jasmonate-mediated signaling further binds to ubiquitin-proteasome-mediated protein degradation (Fig. 1). We have recently reported^{4,5)} that biosynthesis of a tetracyclic diterpenoid of *Scoparia dulcis*, scopadulcic acid, is stimulated by the treatment of the plant with MJ, and that this process is triggered by a Ca^{2+} -influx into the cytoplasmic

space of the cells. We also demonstrated that activation of a Ca^{2+} -cascade in the signal transduction pathway is an essential requirement for MJ-induced diterpene production in *S. dulcis* (Fig. 1). However, very limited information is available on the detailed mechanisms by which jasmonates induce the biosynthesis of various secondary metabolites in plant cells.

Agarwood is generated in woody tissues of *Aquilaria* or some other plant species in response to either microbial infection or mechanical wounding, but is not generated under healthy conditions.^{6,7)} Although agarwood produces a variety of sesquiterpene compounds used as the scent, artificial transformation of agarwood-producing plants to agarwood has proved quite difficult. Okudera and Ito have recently reported⁸⁾ that biosynthesis of a series of sesquiterpenoids, such as α -guaiene, α -humulene and δ -guaiene, is induced in cell cultures of *A. crassuna* and *A. sinensis* by treatment with MJ.

In the present study, we established a callus culture of *A. microcarpa*, the tissues of which accumulate a unique spirovetivane-type sesquiterpene when the plant is transformed to agarwood.⁹⁾ Two cDNA clones presumably encoding calmodulin (CAM), a key mediator protein of the Ca^{2+} -cascade in eukaryotic cells,^{10,11)} were isolated from the culture to investigate the biochemical and molecular biological processes of MJ-induced activation of the sesquiterpene biosynthesis in *A. microcarpa*. A possible change in the transcriptional activities of these genes in *A. microcarpa* cells in response to various external stimuli was also examined.

MATERIALS AND METHODS

Plant Material and Callus Induction *A. microcarpa* was grown in a green house at Experimental Station for Medicinal Plant Research in University of Toyama. Sterilized young leaf tissues of the plant were placed on Murashige and

Fig. 1. Hypothetical Signal Transduction Processes in Jasmonate-Stimulated Plant Cells

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Skoog's agar medium¹²⁾ supplemented with 3 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), and callus formation was observed in the leaf veins after 4 weeks. The dedifferentiated cells were then incubated on the medium in the presence of 3 μM of 2,4-D and 3 μM of *N*⁶-benzyladenine at 26 °C under darkness, and, after establishment of the callus culture, the cells were transferred onto a fresh medium every 4 weeks.

Cloning of *Am-cam-1* and *Am-cam-2* from *A. microcarpa* Total RNA was isolated from the callus culture of *A. microcarpa* with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and cDNA templates were generated by reverse-transcription (RT) reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA fragments presumably encoding the translatable region of CAM gene(s) of *A. microcarpa* were isolated by polymerase chain reaction (PCR) amplification employing the primer pair, 5'-ATG GCC GAT CAG CTG ACG GAC GAC CAG ATC-3' as the forward and 5'-TYA CTT GGC CAT CAT AAC CTT GAC AAA YTC-3' as the reverse primer. The 3'-untranslatable region (UTR) of the CAM gene was obtained by means of 3'-rapid amplification of cDNA end (RACE) method using 5'/3' RACE Kit 2nd Generation (Roche). RT reaction was performed with an oligo dT-anchor primer, and PCR was carried out with 5'-GAT GTG GAT GGT GAT GGG CAG ATC AAC T-3' as the gene specific forward and PCR-anchor as the reverse primer. The amplified DNA fragments were subcloned into the pGEM-T vector (Promega, WI, U.S.A.), and the nucleotide sequences were determined on both strands using the dye-terminator method on a PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, U.S.A.).

Over-Expression and Immunoblot Analysis Over-expression of *Am-cam-1* and *Am-cam-2* genes in *Escherichia coli* was performed using pBAD TOPO TA Expression Kit (Invitrogen, CA, U.S.A.) according to the instruction manual. The translatable regions of *Am-cam-1* and *Am-cam-2* were amplified by PCR and were subcloned into pBAD-TOPO. *E. coli* BL21/DE3 cells were transformed with the constructed expression vectors harboring the coding regions of *Am-cam* genes. The cultures were grown at 37 °C overnight, and then inoculated into 2 ml of fresh medium. Arabinose (final concentration 0.002%, w/v) was added to the cultures as the inducer when they reached an optical density of 0.6 at 590 nm, and the incubation was further continued for 3 h. The bacterial cells were harvested by centrifugation at 8000 *g* for 5 min, and the proteins were extracted by boiling the cells in 200 μl of the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were subjected to SDS-PAGE (12% gel), and the separated proteins were blotted onto a nitrocellulose membrane (0.2 μm) on a semi-dry transfer cell (Bio-Rad, Transblot SD, U.S.A.). After blocking with 1% (w/v) dry fat milk, the membrane was incubated with anti-CAM (*Dictyostelium discoideum* CAM, produced in mouse, Sigma-Aldrich, MO, U.S.A.) in 5 ml Tris-buffered saline at 4 °C overnight. The membrane was then incubated with Protein G conjugated with horseradish peroxidase (Bio-Rad, CA, U.S.A.) at 25 °C for 2 h, and was washed several times with the buffered saline. Primary antibody bound to CAM protein was visualized by color development reaction (Bio-Rad).

RT-PCR Analysis of *Am-cam-1* and *Am-cam-2* Semi-quantitative analysis of transcriptional activities of *Am-cam-1* and *Am-cam-2* was performed using RT-PCR. According to the method described previously,¹³⁾ 50 μl -aqueous solutions of MJ (100 μM , Wako, Osaka, Japan), yeast extract (10 mg/ml, Difco, MI, U.S.A.) or Ca^{2+} -ionophore A23187 (10 μM , Sigma) were added to cultured cells of *A. microcarpa* (approximately 100 mg), and control treatments received only water instead of these solutions. The cells were incubated in Petri dishes at 26 °C under darkness, and at regular intervals, total RNA was isolated from the cells according to the method described above. Aliquots of RNA solutions (approximately 0.5 μg RNA equivalent) were subjected to RT reaction, and then PCR was carried out with the appropriate primer pairs, 5'-GAT GTG GAT GGT GAT GGG CAG ATC AAC T-3' as the common forward primer, and 5'-CAC CAA CAA GCC AAG CTT CTA GGG-3' for *Am-cam-1* and 5'-GAT CAG AAG CAA ATG AAA TCG C-3' for *Am-cam-2* (270 mer and 298 mer as the products), as the gene specific reverse primers.

RESULTS AND DISCUSSION

Based on the reported amino acids sequences of plant CAMs, we isolated two cDNA 450 bp clones presumably encoding the entire translatable region of the protein from *A. microcarpa*. The nucleotide sequences of these cDNA fragments were very similar, and only 6 nucleotides were found to be replaced. The putative amino acids sequences of the translational products of the two genes were identical (149 amino acid residues), and showed high homology with those of CAMs from various plant sources (Fig. 2).^{14–18)} They contained four highly conserved Asp-rich motifs characteristic of proteins that correspond to Ca^{2+} -binding domains. To obtain more information about the structure of these CAM genes from *A. microcarpa*, we attempted to determine the nucleotide sequences of 3'-UTRs in the next experiment. Two cDNA fragments obtained by 3'-RACE method consisted of significantly different 3'-UTR structures, and these two gene regions including the CAM-coding region and 3'-UTR were designated as *Am-cam-1* (450 plus 282 nucleotides, GenBank accession no. HM237343) and *Am-cam-2* (450 plus 251 nucleotides, GenBank accession no. HM237344), respectively. The translatable regions of these two genes were subcloned into pBAD-TOPO, and *E. coli* cells were transformed with the recombinant expression vectors. After the treatment of the cultures with arabinose, the proteins were extracted from the bacterial cells and were analyzed by SDS-PAGE (Fig. 3A). No obvious differences were observed between the control and the arabinose-induced samples after staining with Coomassie Brilliant Blue, however, the signals cross-reacted with anti-CAM were detected at the expected position (approximately 17 kDa) only in the arabinose-treated cells transformed with either *Am-cam-1* or *Am-cam-2* (Fig. 3B). These results strongly suggest that both *Am-cam-1* and *Am-cam-2* genes encode CAM protein in *A. microcarpa*.

The possible change in the transcriptional activity of *Am-cam-1* and *Am-cam-2* in *A. microcarpa* cultures in response to various external stimuli was examined by RT-PCR (Fig. 4). Although the nucleotide sequences of the translational re-

Fig. 2. Predicted Amino Acids Sequence of CAM Genes from *A. microcarpa*

Two CAM genes designated *Am-cam-1* and *Am-cam-2* were isolated from callus culture of *A. microcarpa*. The deduced amino acid sequences of the translational products of these genes were identical, and were presented with those of CAM proteins from various plant sources (GenBank accession numbers; poplar, AAX63770; cedar, BAF32041; Arabidopsis-2, M38380; carrot-11, AY543014; alfalfa, S10533; barley, P13565; potato, GU014578). Four conserved domains for Ca^{2+} -binding were underlined.

Fig. 3. Over-Expression of CAM Genes from *A. microcarpa* in *E. coli* Cells

Coding regions of *Am-cam-1* and *Am-cam-2* were ligated into pBAD-TOPO, and *E. coli* cells were transformed by the constructed expression vectors. After the addition of arabinose as the inducer (+) or H_2O as the control (-), the proteins were extracted from the cultures and were subjected to SDS-PAGE (A). The separated proteins were transferred onto a nitrocellulose membrane and were probed with anti-CAM (B).

gions of *Am-cam-1* and *Am-cam-2* were very similar, their 3'-UTR structures were quite different. We also confirmed that PCR performed with the combination of the common forward and gene specific reverse primers, as described above, yielded desired DNA fragments corresponding to the partial structures of *Am-cam-1* and *Am-cam-2*. This allowed selective monitoring of the transcriptional activities of the two CAM genes in *A. microcarpa* under various conditions (Fig. 4). The expression activity of *Am-cam-1* was maintained at a low level at time zero, however, it appreciably increased when the cell culture was treated with MJ. A marked increase in the gene transcription was observed after 3 h of treatment, and the enhanced activity continued for at least 24 h. Expression of CAM gene, *Am-cam-2*, was also stimulated by incubation with MJ. The intensity of the enhance-

Fig. 4. Semi-Quantitative Analyses of the Expression of *Am-cam-1* and *Am-cam-2* in *A. microcarpa* by RT-PCR

Changes in the expression levels of *Am-cam-1* and *Am-cam-2* in callus culture of *A. microcarpa* were examined after the treatment with MJ, yeast extract or Ca^{2+} -ionophore A23187. The cultures were incubated with these stimuli, and at regular intervals, the cells were harvested and subjected to RT-PCR analyses. Control treatment received only water instead of these reagents.

ment, however, seemed to be appreciably lower than that of *Am-cam-1*. In the control treatment, no notable change in the expression activities was observed either in *Am-cam-1* or *Am-cam-2*. A similar set of results was obtained for an *A. microcarpa* culture treated with yeast extract that has been known to induce biosynthesis of various types of secondary metabolites in many plant species.^{19,20} The transcriptional activity of *Am-cam-1* was greatly enhanced in yeast extract treated cultures, however, the level of induced activation of *Am-cam-2* expression was noted to be somewhat lower than that in *Am-cam-1*. In sharp contrast to incubation with MJ and yeast extract, the expression of both *Am-cam-1* and *Am-cam-2* showed only a negligible change, if any, on incubation with Ca^{2+} -ionophore A23187, and the transcription of these two CAM genes was maintained at an almost constant level over the testing procedure. In repeated experiments, a similar set of results was reproducibly observed, though the relative

levels of the expression activities of *Am-cam-1* and *Am-cam-2* varied to some extent.

In the present study, we isolated two cDNA clones, *Am-cam-1* and *Am-cam-2*, as CAM genes from tissue cultures of *A. microcarpa* (Fig. 2). The expression levels of both *Am-cam-1* and *Am-cam-2* increased in response to stimulation with either MJ or yeast extract, suggesting that these two CAM genes and their products play important roles in the signal transduction processes accompanying the change in the transcriptional activities when the cells are stimulated with these solutions. We have reported previously⁵⁾ that CAM functions as the key mediator protein in MJ-induced diterpene accumulation in *S. dulcis*. The results obtained in the present study are, therefore, consistent with our previous finding that activation of Ca^{2+} -cascade is prerequisite for the enhancement of secondary metabolites biosynthesis triggered by MJ in plant cells. Although expression activities of both *Am-cam-1* and *Am-cam-2* respond positively to stimulation by MJ and yeast extract, it is likely that *Am-cam-1* is more sensitive to such external stimuli (Fig. 4). A series of recent studies^{18,21,22)} has demonstrated that plant CAMs, unlike in animals, are composed of several isoforms and the expression of specific CAM genes is sometimes induced by appropriate stimuli or environmental stressors. The different responses of the two CAM genes of *A. microcarpa* to various external stimuli would be, therefore, in agreement with the hypothetical properties of plant CAMs. The expression levels of *Am-cam-1* and *Am-cam-2* appreciably increased in the presence of MJ and yeast extract, but not in the presence of Ca^{2+} -ionophore A23187 (Fig. 4). This 'selective sensitivity' of *Am-cam-1* and *Am-cam-2*, i.e., increased sensitivity to the primary stimuli (MJ and yeast extract) but negligible sensitivity to Ca^{2+} -ionophore A23187, leads us to assume that the induced expression of these two CAM genes might be evoked by a certain signal generated by MJ reception, but not by the increased concentration of cytoplasmic Ca^{2+} (Fig. 1). However, at present, it is not clear whether or not the apparent insensitivity of CAM genes transcription toward Ca^{2+} -influx is shared as the common mechanism in a wide range of plant species upon the stimulation with MJ.

Further studies on the biochemical and molecular biological mechanisms of MJ-induced activation of secondary me-

tabolism in *A. microcarpa* are in progress in our laboratory.

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