Cloning and Expression of Calmodulin Genes Regulating Phytoalexin Production in Carrot Cells

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A homology-based cloning strategy yielded four cDNA clones encoding the open reading frame of carrot calmodulin, designated *cam-4* and *cam-8* from an oligogalacturonide elicitor-treated cell culture and *cam-11* and *cam-16* from cells exposed to ethylene, respectively. Reverse-transcription polymerase chain reaction analyses revealed that the expression of the *cam-4* gene increased transiently when carrot seedlings were treated with oligogalacturonides, although, the cells incubated with fungal mycelial walls or ethylene did not show a significant change in the expression level. In contrast, marked and transient expression was observed for either *cam-11* or *cam-16* only when carrot cells were treated with ethylene. These results suggest that two classes of stimuli which are capable of triggering phytoalexin production in carrot cells, oligogalacturonides and ethylene, evoke the activation of the Ca^{2+} cascade in the cells by expressing distinct calmodulin genes to initiate the biosynthesis of the compound.

Key words calmodulin gene; phytoalexin biosynthesis; calcium cascade; gene cloning; carrot cell

Calmodulin (CAM) is a well known Ca^{2+}-binding protein which functions in various cellular processes by regulating the activities of numerous CAM-dependent proteins.[1,2] CAM had been considered to be a "constitutive" protein. However, a series of recent studies clearly demonstrated[3—5] that plant CAMs are composed of several isoforms and the expression of specific CAM genes is sometimes induced by appropriate stimuli or physiological stresses. For example, eight genes encoding two CAM isoforms are contained in potato plants, and expression levels of these genes were shown to be different in various developmental stages.[3] In tobacco cells, it was demonstrated[5] that specific CAM genes are transcriptionally activated against bacterial infection. We showed previously[6—8] that CAM is a key component to evoke the production of phytoalexin, 6-methoxymellein, in carrot cell culture treated with oligogalacturonide elicitor or ethylene. This modulator protein regulates the activation of Ca^{2+}/CAM-dependent protein kinases, which results in the expression of the genes encoding biosynthetic enzymes of carrot phytoalexin.[6] However, we have also shown[7,9] that this protein functions in the series of response-decay processes of the cells as well as the activation of the Ca^{2+} cascade. We have reported that certain CAM isoforms participate in the closing of cation channels located at the plasma membrane[7] and in the efflux of cytoplasmic Ca^{2+} to the extracellular space[9] to lead the cells from the excitatory state to the resting state again. These results suggest the possibility that multiple isoforms of CAM occur in carrot cells, and they might play distinct physiologic roles in phytoalexin production. However, only one gene encoding carrot CAM, *cam-1*, was reported to date,[10] and therefore in the present study, we attempted to isolate new clones of the CAM gene from carrot cells. Possible changes in the expression levels of these genes in response to various elicitor-active substances were also examined to elucidate the functions of the multiple CAM genes in the induction of defense-related secondary metabolism in carrot cells.

MATERALS AND METHODS

Materials Cultured carrot (*Daucus carota* L.) cells (ft-2525) were grown in 70 ml of Murashige and Skoog's liquid medium[11] on an Innova 2300 rotary shaker (200 rounds per min) at 26 °C as described previously in detail.[12] Oligogalacturonide elicitor was prepared by partial hydrolysis of the pectic fraction extracted from cell walls of cultured carrot according to the method described previously.[12] Mycelial walls of the fungus *Chaetomium globosum* were prepared from 3-d-old culture by the method reported previously.[13] 2-Chloroethylphosphonic acid (2-CEPA) was purchased from Sigma.

Cloning of CAM Genes Cultured carrot cells (10 d old) were treated with oligogalacturonide elicitor for 2 h,[12] and total RNA was isolated from the cells with an RNeasy Plant Mini Kit (Qiagen). The RNA obtained (approximately 5 μg) was added to a reverse-transcriptase (RT) mixture (25 μl, cDNA Synthesis Kit, Life Sciences Inc.) for first-strand cDNA synthesis. A primer pair for polymerase chain reaction (PCR) amplification (5′-ATG GCN GAY CAR YTN CAN GAY GAY CAR ATH-3′ as the forward and 5′-NGC CAT CAT NAC YTT NAC RAA YTC YTC-3′ as the reverse) was developed from conserved structures in the DNA sequences of plant CAMs in databases, and PCR products amplified from the template were cloned into the pCR2.1-TOPO vector (Invitrogen). Their sequences were determined on both strands using the dye-terminator method with M13-20 and RV-P (Takara) as the sequencing primers on a PRISM 3100 Genetic Analyzer (Applied Biosystems). An amplification product (250 basepairs) bearing 98.8% identity with a CAM gene from *Arabidopsis thaliana*[14] was employed as the probe to screen the cDNA library in pBluescript II SK derived from oligouronide-treated carrot cells using the cDNA Library Construction Kit (Stratagene). Primary screening of the library was performed employing a biotin-labeled probe complexed with streptavidin-coated paramagnetic particles using the RecActive Gene Enrichment Kit (Active Motif), and the second screening was carried out using DNA blot analysis

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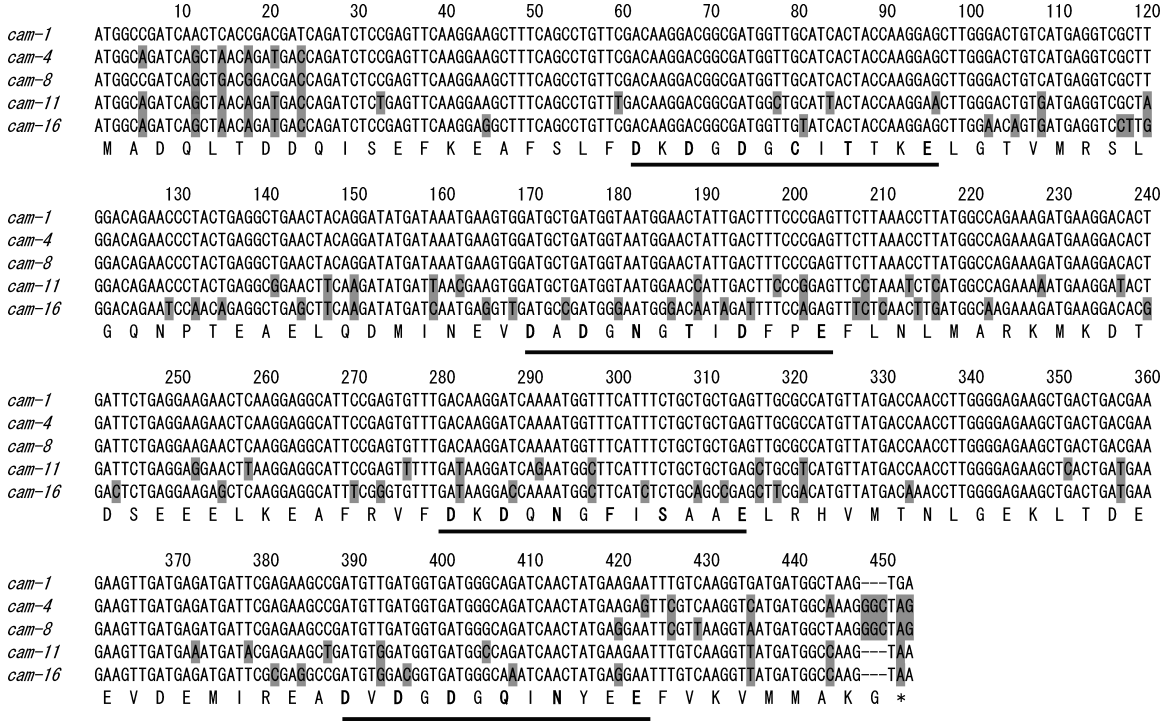

Fig. 1. Nucleotides and Predicted Amino Acids Sequences of Carrot CAM Genes, *cam-1*, *cam-4*, *cam-8*, *cam-11* and *cam-16*

Four novel CAM genes were isolated from oligogalacturonide-treated carrot (*cam-4* and *cam-8*) or from the cells exposed to ethylene (*cam-11* and *cam-16*). Nucleotides different from those of *cam-1* are shaded, and four Ca^{2+}-binding sites and the conservative amino acids in the domains are underlined and in bold, respectively.

after digestion with *Eco*RI with a digoxigenin-labeled probe (Roche Molecular Biochemicals). Nucleotide sequences of the positive clones in pBluescript were determined for both strands, and two new clones designated *cam-4* and *cam-8* were obtained. In a separate experiment, a cDNA library was also prepared from 2-CEPA-treated carrot, and after appropriate screening and sequencing, the two clones, *cam-11* and *cam-16* were isolated.

Expression of CAM Genes The expression level of CAM genes was analyzed semiquantitatively by RT-PCR. Oligogalacturonides and 2-CEPA solutions and a suspension of the insoluble fungal mycelial walls (10 mg/ml water) were carefully sprinkled over carrot seedlings (20 d old) germinated under sterilized conditions in test tubes (25 mm in diameter, 400 μl per tube). Controls received autoclaved water instead of the elicitor-active substances. They were incubated at 26 °C, and at the regular intervals, total RNA was isolated from 100 mg of tissue as described above. Aliquots (1 μl each) of RNA solutions were added to the RT-PCR mixture prepared from the OneStep RT-PCR Kit (Qiagen), and after the RT reaction, PCR was carried out with the combination of appropriate primers using 25 cycles of fragment amplification. For the analysis of *cam-4* and *cam-8*, specific primer pairs (5′-GCA GAT CAG CTA ACA GAT GAC CAG ATC-3′ as the forward and 5′-TGC CAT CAT GAC CTT GAC GAA CTC TTC-3′ as the reverse for *cam-4*, and 5′-GCC GAT CAG CTG ACG GAC GAC CAG ATC-3′ as the forward and 5′-AGC CAT CAT TAC CTT AAC GAA TTC CTC-3′ as the reverse for *cam-8*) were employed from which 440 mer fragments were expected to be the products, respectively. A primer pair designed for the common core structures of many plant CAM genes (*cam*-common, 5′-AGA TCT CCG AGT TCA AGG AAG CTT TC-3′ and 5′-GAT CTG CCC ATC ACC ATC AAC ATC GG-3′, 385 mer as the product) was also employed to assess the "bulk" expression of the genes. Participation of *cam-11* and *cam-16* in ethylene-induced responses in carrots was also examined with RT-PCR employing two primer pairs, *cam-11* specific (5′-AAG GAA CTT GGG ACT GTG ATG AGG TCG CTA-3′ as the forward and 5′-TTA CTT GGC CAT CAT AAC CTT GAC AAA TTC-3′ as the reverse, 360 mer as the product) and *cam-16* specific (5′-AAG GAG CTT GGA ACA GTG ATG AGG TCC TTG-3′ and 5′-TTA CTT GGC CAT CAT AAC CTT GAC AAA TTC-3′ as the forward and the reverse, respectively, 360 mer as the product), respectively.

RESULTS AND DISCUSSION

CAM Genes from Oligogalacturonides-Treated Carrot Two new genes encoding the open reading frame of CAM, designated *cam-4* (GenBank accession number AY364011) and *cam-8* (AY364012), were isolated from cultured carrot cells treated with oligogalacturonide elicitor (Fig. 1). Several differences were observed in the nucleotide sequences near the 5′ and 3′ terminals, although the predicted amino acid sequences for these two clones were identical (150 amino acids). The products of both genes contained four Ca^{2+}-binding sites, which are highly conservative in CAM proteins from animal and plant sources.[1,2] Although most plant CAMs involving the carrot *cam-1* product are composed of 149 amino acids,[10,14,15—18] an additional glycine is attached to the *C*-terminals of the proteins encoded by *cam-4* and *cam-8*. The nucleotide sequences of *cam-4* and *cam-8* share 97.8% and 98.2% identities with *cam-1* within 1—447 regions, respectively (Fig. 1).

To elucidate the possible participation of *cam-4* and *cam-8*

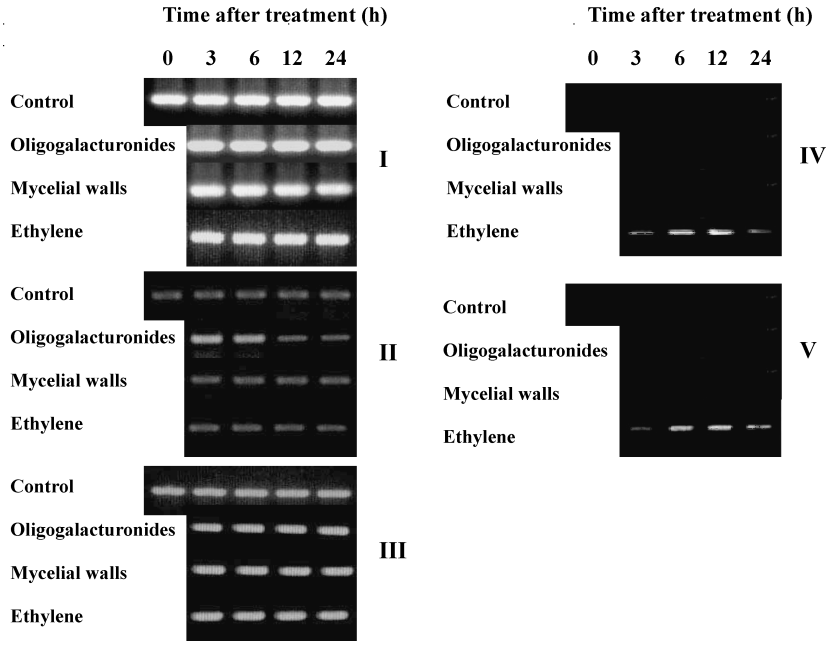

Fig. 2. Semiquantitative Analyses of the Expression of Carrot CAM Genes Using RT-PCR

Carrot seedlings were treated with oligogalacturonides, fungal mycelial walls, or 2-CEPA, and controls received only water instead of these elicitor-active chemicals. They were harvested at regular intervals, and RT-PCR was performed employing five primer pairs: *cam*-common expected to amplify bulk CAM (I), *cam-4* specific (II), *cam-8* specific (III), *cam-11* specific (IV), and *cam-16* specific (V) primers, respectively.

in oligouronide-induced phytoalexin production in carrot cells, changes in the expression levels of these genes upon contact with elicitor-active chemicals were examined using RT-PCR. Carrot seedlings were treated with three types of elicitors: i) oligogalacturonides, specific inducers of phytoalexin biosynthesis in carrot[12]; ii) fungal mycelial walls, inducers of chitinase production and hypersensitive cell death[13]; and iii) 2-CEPA, an ethylene-generating reagent that is capable of inducing both phytoalexin production and chitinase activities.[7,13,19] Total RNA was prepared from these plants at regular intervals. As shown in Fig. 2, CAM genes appeared to be expressed constantly in control carrot cells during the experimental period (0—24 h) when PCR amplification was carried out with the *cam*-common primer pairs, and this was also the case in the cells treated with oligogalacturonides, fungal mycelial walls, or 2-CEPA. These observations also imply that the amounts of RNA subjected to RT-PCR analyses were almost comparable in the series of these experiments. It appeared that *cam-4* expression was maintained at a very low level in the controls, although a marked and transient increase (approximately 2.5- to 5.2-fold increase in repeated experiments as determined by densitometric scan) was observed when carrot seedlings were incubated with oligouronides for 3—6 h. This phenomenon was specifically observed in oligouronide-treated carrots, and the plants incubated with mycelial walls and ethylene did not show any notable changes in *cam-4* expression. In contrast to *cam-4*, the *cam-8* gene showed no significant change in the expression level even if carrot seedlings were treated with either oligouronides or other elicitor-active substances, as was the bulk expression of CAM genes examined with the *cam*-common primers. These results suggest that *cam-4* plays an important role(s) in oligogalacturonide elicitor-induced activation of the Ca^{2+} cascade in carrots, which leads the cells to induce phytoalexin biosynthetic activity. We attempted to repeat this set of RT-PCR experiments using cultured carrot cells. However, unlike in the seedlings, the increase in the amplified product for *cam-4* was rather low (1.2- to 1.7-fold increase as compared with controls). At present, it is not clear whether this difference depends on the inherent nature of carrot seedlings and cell cultures or merely reflects the difference in the experimental systems. It might be also possible that the transient increase in *cam-4* expression is very sharp in carrot cell cultures, and therefore the maximal response would be observed only in a very short period after treatment with oligogalacturonides.

CAM Genes from Ethylene-Treated Carrot We demonstrated previously that, as well as oligogalacturonides, phytoalexin production in carrot cells is induced by the addition of ethylene-generating reagents such as 2-CEPA.[7,19] However, although the expression level of *cam-4* increased markedly with uronide treatment, it did not show any notable change upon incubation with ethylene as far as determined (Fig. 2). This observation suggests the possibility that another CAM gene(s) would be expressed upon the exposure of carrot to ethylene, and the produced CAM protein functions in the signal transduction mechanisms in ethylene-induced phytoalexin biosynthesis instead of the *cam-4* product. We therefore attempted to isolate new CAM genes from ethylene-treated carrot cells, and two clones, *cam-11* and *cam-16* (GenBank accession number AY543014 and AY543015, respectively), were obtained. The predicted amino acid sequences of both clones (149 amino acids) were identical to that of the product of *cam-1* (Fig. 1). However, unlike *cam-4* and *cam-8*, the nucleotide sequences of *cam-11* and *16* were rather varied (90.4% and 89.9% identities with *cam-1*, respectively), and replacements of the nucleotides were distributed almost equally within the open reading frames of these two genes.

The possible participation of CAM proteins produced by

cam-11 and *cam-16* in ethylene-induced responses in carrot was also examined using RT-PCR, and, as shown in Fig. 2, the expression levels of *cam-11* and *cam-16* were negligible at time 0, although both genes were transiently expressed upon exposure of carrot seedlings to ethylene for 3 to 24 h. In sharp contrast, treatment of the cells with neither oligouronides nor fungal mycelial walls triggered the expression of these two genes. These observations strongly suggest that *cam-11* and *cam-16* are specifically expressed in carrot cells in response to ethylene. At present, it is uncertain whether *cam-11* and *cam-16* similarly play roles in the ethylene-induced multiple responses in carrot, such as phytoalexin biosynthesis and chitinase induction, *etc.*, or independently function in the distinct cellular processes evoked by the plant hormone.

In the present study, coding regions of four novel CAM genes were isolated from oligogalacturonide- and ethylene-treated carrot cells. Although phytoalexin biosynthesis is similarly induced in carrot upon the contact with oligouronides and ethylene, the expression level of *cam-4* was specifically elevated by oligouronide treatment while *cam-11* and *cam-16* were expressed only when carrot cells were treated with ethylene. These results suggest that at least two independent signal transduction mechanisms function in the elicitation of phytoalexin production: a pathway activated in response to oligogalacturonides in which *cam-4* is expressed as an important component; and another pathway triggered by the exposure to ethylene involving the products of *cam-11* and/or *cam-16* as the mediator proteins. We are now attempting to isolate the full-length cDNAs involving noncoding regions of these CAM genes and other isoforms of CAM from carrot cells to understand the physiologic roles of the multiple CAM genes in the induction of secondary metabolism involved in plant defense responses.

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