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Induction and Activation of Plant Secondary Metabolism by External Stimuli

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1. Introduction

It is widely recognized that plant cells are potentially rich sources of commercially important secondary metabolites. The production of secondary metabolites in plants would be mainly controlled by transcriptional activities of a series of genes which encode the specific enzymes in the biosynthetic pathway of desired products. It is likely, however, that the catalytic activities of these enzymes are sometimes repressed or maintained at very low levels, and the mechanism involved in 'switch on' of the genetic information on secondary metabolism in plants is, at present, only very poorly understood. The response of plant cells to 'elicitors' was first studied from the phytopathological point of view to elucidate the regulation mechanism of phytoalexin production. However, extensive investigations clearly indicated (Zhao et al., 2005) that the treatment of plant cells with elicitor-active substances sometimes results in a rapid accumulation of secondary products other than defense-related compounds. Recently, it has been also demonstrated (Gundlach et al., 1992) that the function of elicitors can be sometimes replaced by jasmonic acid and its methyl ester, methyl jasmonate, the plant specific messenger molecules derived from arachidonic acid. Effective application of elicitors and/or jasmonates to the production of useful metabolites in plant cells requires the elucidation of the basic biochemical mechanisms by which these external stimuli regulate the genetic information involved in the biosynthesis of the natural products. Several questions are raised against the external stimuli-induced activation of the secondary metabolites production in plants. 1) How are the external signals recognized by plant cells? 2) How are the signals transduced in the cells? 3) How do the signals alter the expression of biosynthesis-related genes? 4) How are the enzyme activities controlled to produce secondary metabolites? Effective use of elicitors and jasmonates in producing useful metabolites in plants requires the elucidation of these biochemical mechanisms by which these stimuli regulate the genetic information. To answer these questions, we first attempted to elucidate the possible participation of second messengers in the secondary metabolism activation stimulated by exogenous signals.

2. Elicitation and transmembrane signalling mechanisms of phytoalexin production

6-Methoxymellein, an antifungal isocoumarin (Fig. 1), was first isolated as the metabolite that is responsible for the bitter taste in cold-stored carrot roots. It has been shown (Condon

& Kuc, 1960) that this compound accumulates in carrot roots after inoculation with *Ceratocystis fimbriata*, which causes black rot disease in sweet potato but is not pathogenic to carrot. The resulting production of 6-methoxymellein accounted for the resistance of carrot tissue to microbial infection. This compound inhibits the growth of various fungi, yeasts and bacteria in the concentration range of 0.05 - 0.5 mM, *in vitro*, strongly suggesting that the accumulation of this compound in response to fungal invasion is one of the important induced defense mechanisms of the host plant, carrot (Kurosaki & Nishi, 1983).

2.1 Liberation of elicitors during host-pathogen interaction

Preliminary studies indicate that heat-stable and water-soluble substances which show elicitor activity are released during interaction of carrot cells and the fungus. The elicitor was found to lose its activity after digestion with pectinase or proteases, suggesting that oligogalacturonides and/or peptides are essential for the inducing activity. Also, partial hydrolysates of pectic fractions of carrot cell walls prepared with these enzymes showed strong elicitor activity. These results suggest (Kurosaki & Nishi, 1984) that extracellular hydrolases secreted from fungi, including pectinase and proteases, function to liberate oligosaccharides and peptides from carrot cell walls, and the fragments of the extracellular matrix of carrot trigger 6-methoxymellein production (Fig. 1). This was confirmed by an experiment in which filter-sterilized pectinase and trypsin were directly added to carrot cell culture. Biosynthetic activity of 6-methoxymellein was induced in the carrot cells, implying that eliciting substances are released from live carrot cells by the enzymatic action of these hydrolases.

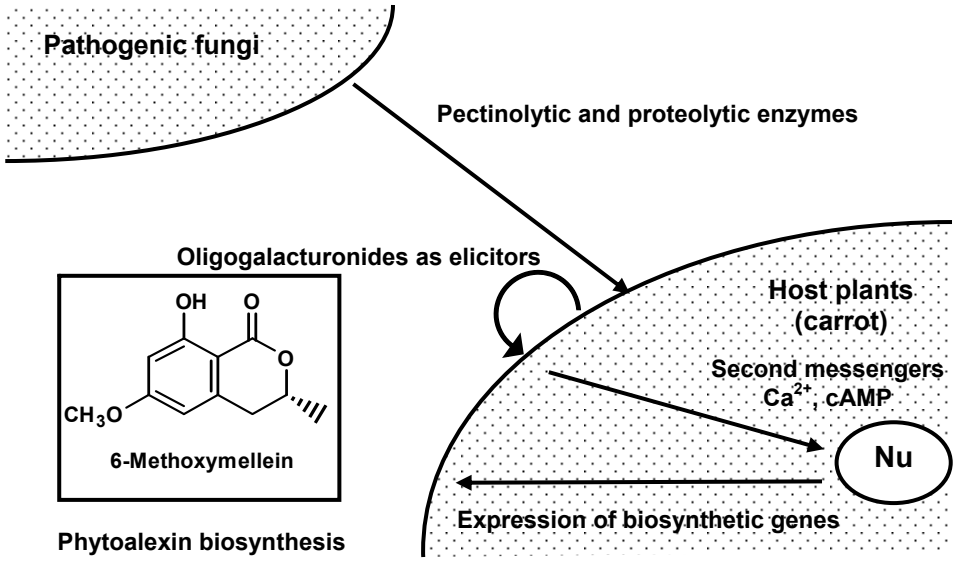

Fig. 1. Elicitation of phytoalexin production in carrot.

2.2 Participation of Ca^{2+} as a second messenger

When elicitor-active pectic fragments were analyzed by ion exchange and gel-filtration chromatography, the activity was found to be distributed in many fractions, suggesting that the elicitor consists not of a single molecule but a mixture of several active substances. This result led us to examine whether or not these elicitors share a common signalling mechanism. Ca^{2+} is an important second messenger in many physiological processes in both animal and higher plant cells, and calmodulin (CAM), a Ca^{2+}-binding protein, plays a central role in many of these systems (Marme & Dieter,1983). 6-Methoxymellein production induced by oligogalacturonides was appreciably inhibited in the presence of the Ca^{2+}-channel blocker verapamil (Kurosaki et al., 1987a). The different class of inhibitors of CAM-dependent reactions, trifluoperazine and W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], also caused marked inhibition. In addition, it was found that appreciable 6-methoxymellein biosynthesis was induced in carrot by treatment with Ca^{2+}-ionophore A23187. These observations strongly suggest that the increase in cytoplasmic Ca^{2+} level is an essential early event in eliciting 6-methoxymellein production. In potato and soybean, phytoalexin production is also a Ca^{2+}-dependent process, and the elicitor-induced responses were significantly inhibited by several Ca^{2+}-inhibitors (Staeb & Ebel, 1987; Zook et al., 1987).

2.3 Activation of phosphatidylinositol cycle

Further support for the hypothesis that Ca^{2+} plays a central role in regulating phytoalexin accumulation is provided by experiments in which the turnover of phosphatidylinositol was measured in the plasma membrane of elicitor-treated carrot cells (Kurosaki et al. 1987b). The carrot cells were labelled with [^{3}H]myo-inositol and, after the addition of elicitors, acid extracts of the cells were analyzed chromatographically for the production of inositol trisphosphate (IP_3). In elicitor-treated cells, the release of radioactive IP_3 increased with time and attained a maximum at 3 - 5 min after the treatment. Phospholipase activity responsible for the degradation of phosphorylated phosphatidylinositol increased correspondingly. Several reports have shown that IP_3 induces rapid release of Ca^{2+} from intracellular stores in animal cells (Morgan et al., 1985). Studies on plant cells have also demonstrated that exogenous IP_3 releases Ca^{2+} from microsomal preparations at micromolar concentrations, although only limited information is available (Drøbak & Ferguson, 1985). Schumaker and Sze (1987) observed IP_3-induced release of Ca^{2+} from intact vacuoles of *Avena* seedlings. Vacuoles are the most prominent organelles in plant cells, and normally contain 0.1 to 10 μM Ca^{2+}; therefore they may serve as the Ca^{2+} store. Diacyl glycerol, another of the hydrolysates of phosphorylated phosphatidylinositol, is a known activator of protein kinase C in animal cells (Michell, 1982). The present experimental results suggest that this protein kinase also participates in the expression of phytoalexin biosynthesis in carrot cells. We found that the synthetic diacylglycerol 1-oleoyl-2-acetyl-rac-glycerol, which has been shown to be intercalated into cell membranes and to activate protein kinase C, induced 6-methoxymellein production even in the absence of elicitor. A similar result was obtained for the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate, another activator of protein kinase C (Kurosaki et al. 1987b). On the other hand the addition of H-7 [1-(5-iso-quinolinesulfonyl) -2-methyl-piperazine], a specific inhibitor for protein kinase C, resulted in suppression of phytoalexin production. These observations strongly suggest that a rapid

breakdown of phosphatidylinositol in the plasma membrane of carrot cells takes place upon contact with elicitor molecules, resulting in the liberation of two types of second messengers, IP_3 and diacyl glycerol.

2.4 Role of cyclic AMP as a second messenger

In contrast to Ca^{2+}, the role of cyclic AMP (cAMP) as a second messenger in plant cells is still obscure, because there is no proof of the presence of cAMP-dependent protein kinase in plant cells. The existence of cAMP itself in plant cells has been confirmed, and, more recently, various works suggest that the cyclic nucleotide is involved in physiological events in plants (Newton & Brown, 1986). We have found (Kurosaki et al., 1987a) that the addition of dibutyryl cAMP (Bt_2cAMP) to carrot cell culture causes 6-methoxymellein production even in the absence of elicitor. Addition of several reagents which are known to change the intracellular level of cAMP, namely cholera toxin, which is an activator of adenylate cyclase, and theophylline, a phosphodiesterase (PDE) inhibitor, also led to production of 6-methoxymellein, suggesting that elevation of the cAMP concentration in carrot triggers phytoalexin production in the cells. In fact, treatment of carrot cells with oligogalacturonide elicitors led to a rapid but transient increase in the concentration of intracellular cAMP. Similar observations have been reported by Bolwell et al. (1991), who tested the effect of various modulators of signal transduction processes on the induction of phenylalanine ammonia-lyase in french bean cell cultures. They found that cholera and pertussis toxins and forskolin all stimulated synthesis of the enzyme. These reagents are known to activate adenylate cyclase, either through interaction with G-protein or directly.

We examined changes in the activity of protein phosphorylation in carrot cells following treatment with either Bt_2cAMP, forskolin or Ca^{2+}-ionophore A23187 (Kurosaki & Nishi, 1993). Addition of cAMP to cell extracts prepared from these treated cells did not cause any change in phosphorylation activity, indicating that cAMP-dependent kinase activity is absent or very low in carrot cells, as well as in most of the other plants. By contrast, the activities of Ca^{2+}- and Ca^{2+}/CAM-dependent protein kinases increased markedly in both cytosolic and microsomal fractions after the treatment. Phosphorylation activity was stimulated not only by Ca^{2+}-ionophore but also by Bt_2cAMP and forskolin. Furthermore, although Bt_2cAMP and forskolin can stimulate phytoalexin production in carrot cells, the effect was severely suppressed by several Ca^{2+} channel blockers and CAM antagonists. These observations suggest that cAMP acts as second messenger by stimulation of the Ca^{2+}-cascade, rather than by activating cAMP-dependent protein kinases. This view is further supported by experimental results in which changes in the concentration of cytosolic Ca^{2+} in carrot cells were measured by a fluorescent Ca^{2+}-indicator (fluo-3) after treatment with the reagents. The Ca^{2+} level in the cytoplasm of untreated carrot cells was found to be about 0.1 μM. A marked increase in the intracellular concentration of Ca^{2+} to 0.6 - 0.8 μM was observed 3 - 6 min after the addition of Bt_2cAMP or forskolin. These results suggest that the increase in cytoplasmic cAMP level leads to the Ca^{2+}-influx into carrot cells. This conclusion was also drawn from an experiment in which the effect of cAMP on the Ca^{2+}-flux was examined using $^{45}Ca^{2+}$-loaded vesicles of plasma membrane (Kurosaki & Nishi, 1993). Plasma membranes prepared by the two-phase partitioning method are generally composed of differently oriented sealed vesicles, rightside-out and inside-out (Graef & Weiler, 1989).

Incubation of these vesicles with $^{45}Ca^{2+}$ in the presence of ATP results in selective incorporation of the radiolabeled ions into the inside-out vesicles by the plasma membrane-located Ca^{2+}-ATPase. When the $^{45}Ca^{2+}$-loaded vesicles were incubated with cAMP, a rapid release of $^{45}Ca^{2+}$ from the vesicles was observed. This discharge was specifically observed with cAMP among the nucleotides tested. These observations are consistent with the hypothesis that the cytoplasmic level of cAMP is raised by an appropriate stimulus, and the nucleotide triggers Ca^{2+}-influx without accompanying cAMP-dependent protein phosphorylation, probably through cAMP-sensitive ion channels.

2.5 Synthesis and degradation of cyclic AMP

Addition of forskolin to carrot cell culture caused an appreciable increase in adenylate cyclase activity. However, the increase was transient although the activator was present throughout the experiment (Kurosaki et al., 1993). The forskolin-stimulated activity of the enzyme in carrot cell extracts was detected only when EGTA was included in the assay mixture, and the addition of exogenous Ca^{2+} strongly inhibited the enzyme activity. The effect of various concentrations of Ca^{2+} on adenylate cyclase activity was therefore studied using buffers with the concentration of free Ca^{2+} adjusted by the EGTA-Ca^{2+} buffer system (Kurosaki et al., 1993). The activity of the cyclase was markedly affected by the free Ca^{2+} concentration, and was maintained at a high level only when the Ca^{2+} concentration was below 0.1 μM. This figure is close to the Ca^{2+} concentration in cytoplasm in the resting state of various plant species.

Constitutive activity of PDE was found in cultured carrot cells, and this activity did not depend on either Ca^{2+} or CAM. In contrast, a CAM-dependent isoform of PDE (CAM-PDE) was induced in the cells by adding forskolin or Bt_2cAMP to the culture (Kurosaki & Kaburaki, 1995). Induction of CAM-PDE activity in Bt_2cAMP-treated carrot cells was markedly inhibited in the presence of verapamil, while addition of Ca^{2+}-ionophore A23187 induced CAM-PDE. These results suggest that increased Ca^{2+}, but not cAMP, in the stimulated carrot cells triggers induction of the PDE isoenzyme. Affinity of CAM-PDE to the substrate was low compared to constitutive PDE (*Km* values, 0.14 and 0.07 μM, respectively); however, *V* for the induced PDE was approximately 2.7 times higher than for the constitutive isoenzyme.

These results suggest that synthesis and degradation of cAMP in cultured carrot cells are both controlled and switched on/off according to the concentration of Ca^{2+} in carrot cytoplasm. Adenylate cyclase activity is induced in the cells only in the resting state, and the enzyme activity is automatically inhibited when the concentration of cytoplasmic Ca^{2+} increases and reaches the level of the excitatory state. The constitutive PDE, which is insensitive to the cytoplasmic Ca^{2+} level, is important in maintenance of the resting state of carrot cells, by keeping cellular cAMP and Ca^{2+} levels very low, while CAM-PDE induced in excited cells hydrolyzes the messenger nucleotide rapidly under conditions of high cAMP and Ca^{2+}, *in vivo*, as a response-decay mechanism.

In animal cells, the cAMP-induced Ca^{2+}-influx through the nucleotide-sensitive channels is terminated by the hydrolysis of cAMP, the ligand of the channels (Ranganathan, 1994). However, in cultured carrot cells, the cytoplasmic Ca^{2+} concentration elevated by the stimulation of cAMP began decreasing even though the level of intracellular cAMP was

high (Kurosaki et al., 1993). Furthermore, when a Ca^{2+}-influx was triggered by treating the cells with Bt_2cAMP, the cytoplasmic concentration of Ca^{2+} returned to its base level after a few minutes, by which time the cAMP analogue was still present at a high concentration. These results clearly indicate that, in contrast to animal cells, degradation of cAMP is not the immediate reason for the response decay of the cAMP-gated cation channel in carrot cells. We found that the discharge of Ca^{2+} from inside-out sealed vesicles of carrot plasma membrane was strongly inhibited when the suspension of the vesicles was supplemented with 1 μM free Ca^{2+}, while Ca^{2+} concentrations lower than 0.1 μM did not affect Ca^{2+}-release. In addition, the inhibited Ca^{2+}-flux across the plasma membrane was restored by the addition of CAM inhibitors and anti-CAM IgG (Kurosaki et al., 1994). These results suggest that the Ca^{2+}-influx initiated by increases in intracellular cAMP in cultured carrot cells is terminated when the cytosolic Ca^{2+} concentration reaches the threshold excitatory level in the cells. It is probable that CAM located in the plasma membrane plays an important role in the decay response of the cyclic nucleotide-gated cation channels. CAM involved in this transmembrane signaling process might be partially embedded in the lipid bilayer as reported in the pea (Collinge & Trewavas, 1989).

2.6 Regulation of Ca^{2+}-ATPase activity

As with other eukaryotic cells, maintenance of low Ca^{2+} concentration in the cytoplasm of non-stimulated higher plant cells is essential. The cytoplasmic Ca^{2+} concentration of plant cells in the resting state, as described above, is generally maintained at approximately 0.1 μM by the action of Ca^{2+}-transporting systems (Poovaiah & Reddy, 1987; Rasi-Caldogno et al., 1989) which sequester the ion into internal organelles, including endoplasmic reticulum, mitochondria, and vacuoles, or mediate its efflux to the cell exterior. It is known that Ca^{2+}-pumping ATPase at the plasma membrane plays a key role in transporting Ca^{2+} to apoplastic spaces. Characteristics of Ca^{2+}-translocating ATPase have been reported from a wide range of plants (Briskin, 1990), although some are highly variable depending on the plant species. One of the most serious controversies over properties of ATPase is the role of CAM in regulation of the enzyme; inconsistent observations on the CAM-dependence of enzyme activity have been reported from several plants (Askerlund & Evans, 1992). It is not yet clear whether this discrepancy represents genuine variation across species or is an experimental artifact. However, it seems that results depend partly on the fact that plasma membrane preparations obtained from higher plant cells sometimes contain the membranes of other organelles. A highly purified plasma membrane fraction from cultured carrot cells was prepared by the aqueous two phase-partition method (Graef & Weiler, 1989), in order to re-evaluate the role of CAM in regulating Ca^{2+}-ATPase at the plasma membrane of the cells. The Ca^{2+}-translocating activity of ATPase was considerably inhibited in the presence of different classes of CAM antagonists or anti-CAM IgG (Kurosaki & Kaburaki, 1994). This Ca^{2+}-pumping activity decreased significantly when the plasma membrane preparation was washed with EGTA-containing buffer; however, it was restored to almost the control level upon adding exogenous CAM. These results suggest that Ca^{2+}-ATPase at the plasma membrane of carrot cells is regulated by CAM, and the modulator protein associates with the enzyme in a manner dependent on the Ca^{2+} concentration. The biochemical basis of CAM-induced stimulation of Ca^{2+}-ATPase activity in carrot cells was studied further by determining the parameters of the Ca^{2+}-translocating reaction of the enzyme in the presence

and absence of exogenous CAM, using EGTA-treated plasma membrane (Kurosaki & Kaburaki, 1994). The affinity of Ca^{2+}-ATPase for Ca^{2+} was considerably increased by association with CAM, and *Km* values decreased from 11.4 μM to 0.7 μM. These figures are close to those of CAM-dependent Ca^{2+}-ATPase at the plasma membrane in animal cells. Affinity of the enzyme for ATP was also increased in the presence of CAM, although the increase was low compared to that for Ca^{2+} (*Km* values of 914 and 670 μM in the absence and presence of CAM). In contrast to the affinities for the substrates, the relative *V* values of the ATPase were similar or slightly decreased by the addition of CAM.

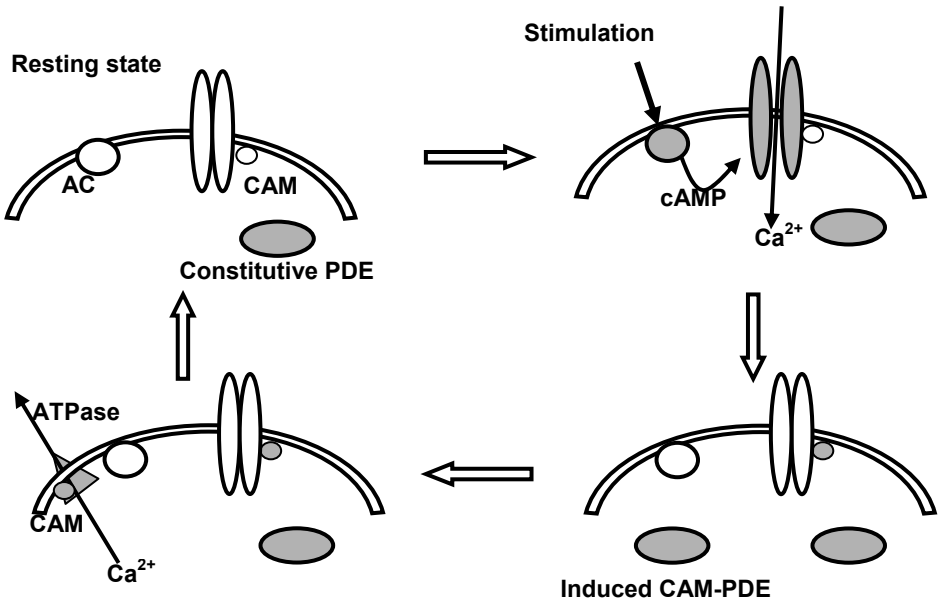

Fig. 2. Signal cross-talking between cAMP and Ca^{2+}.

It is well known that, in the excited plant cells having high Ca^{2+} concentration, CAM is activated by binding to the ion, and is able to associate with various CAM-dependent proteins. The Ca^{2+} concentration in resting plant cells, by contrast, is too low to activate CAM, resulting in the dissociation of the modulator from its target proteins, including Ca^{2+}-ATPase (Malatialy et al., 1988). The *Kca* of the ATPase associated with CAM is similar to that of the cytoplasmic Ca^{2+} level of excited plant cells (0.7 μM), while the *Kca* of the ATPase without CAM increased markedly (11.4 μM) though the cytoplasmic Ca^{2+} concentration in the resting cells is quite low. These observations suggest that Ca^{2+}-ATPase at the carrot plasma membrane plays an important role in the excited cells only as an 'acute' enzyme. However, Rasi-Caldogno et al. (1989) pointed out that *Kca* decreased from about 10 μM to about 0.1 μM if the level of free Ca^{2+} alone is considered. This low *Km* value of CAM-depleted Ca^{2+}-ATPase for Ca^{2+} is consistent with the transport protein involved in

maintaining Ca^{2+} concentration at the submicromolar range as a 'house keeping' enzyme in resting cells. These results strongly suggest that, on binding of CAM, the affinity of the carrot Ca^{2+}-ATPase for Ca^{2+} is markedly increased, and this is the most important biochemical change behind the CAM-induced pumping activity of the enzyme.

The characterization of the functional proteins involved in cAMP-induced cellular events suggests that most components of these signal transduction processes are correlated, and regulate each other. A plausible scheme for signal cross-talking of the messenger nucleotide with the Ca^{2+}-cascade in the early stages of transmembrane signaling processes is as follows (Fig. 2). 1) In the resting state only constitutive PDE is active, and both adenylate cyclase and cAMP-sensitive channels are inactive; therefore cAMP and Ca^{2+} are both maintained at low levels. 2) Upon the arrival of elicitor signals on the receptor protein located at the plasma membrane, adenylate cyclase is activated, and the increased level of cAMP associates with cAMP-sensitive channels as the ligand to open the ion gates. 3) Influx of Ca^{2+} activates the Ca^{2+}-cascade leading to the expression of genes encoding the biosynthetic enzymes of the phytoalexin. In parallel, activity of adenylate cyclase is inhibited by Ca^{2+}, and the ion activates the membrane-embedded CAM to close the cAMP-dependent channels. In addition, CAM-PDE is induced to hydrolyze the messenger molecules rapidly. 4) Finally, Ca^{2+} activates the cytoplasmic CAM to enhance the activity of Ca^{2+}-translocating ATPase, causing the cells to return to the resting state.

2.7 Possible scheme for signal transduction mechanisms of elicitors

These studies all support the hypothesis that external stimuli as elicitors cause an increase in the cytoplasmic Ca^{2+} level via the phosphatidylinositol cycle and/or the adenylate cyclase system. Although an authoritative picture of this process cannot yet be given, possible signal transduction mechanisms are summarized in Fig. 3.

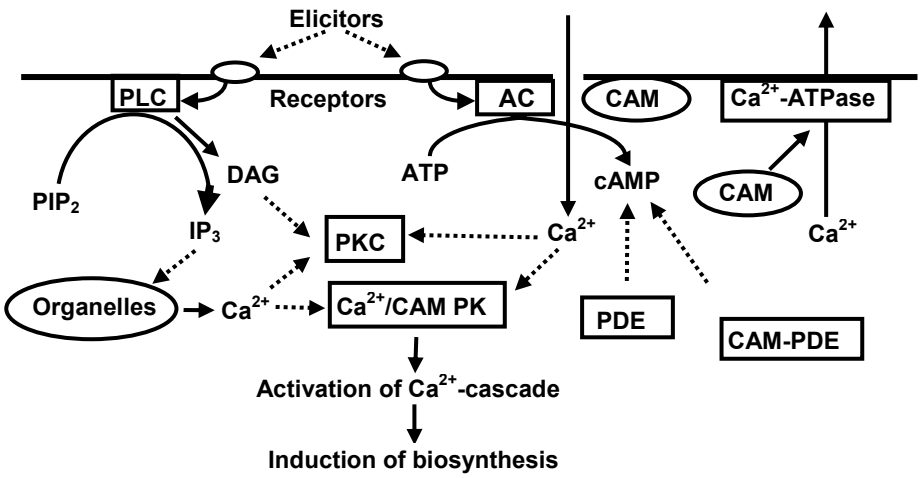

Fig. 3. Schematic presentation of signal transduction mechanisms of elicitors.

At present the data are still fragmentary, so that it is important to learn more about the biochemical nature and function of the components involved in signal transduction process in plants. Evidence has been accumulated suggesting that plant cells contain the major components of the phosphatidylinositol cycle, while the function of cAMP described here is unique. This class of signal transducing mechanism is rare (Kaplan et al., 2007), and is seldom seen in animal and microbial cells. However, a similar gating action of cAMP has been reported in olfactory transduction in animal sensory cells (Nakamura & Gold, 1987). In these cells the cAMP content increases in response to odoriferous substances, and this change induces an influx of Ca^{2+} into the cells without cAMP-dependent protein phosphorylation. Krupinski et al. (1989) have suggested that the amino acid sequence of an adenylate cyclase from the bovine brain is topographically similar to ion channels such as Ca^{2+} and K^{+}. Based on this assumption, Schultz et al. (1992) tested the pore-forming ability of adenylate cyclase from *Paramecium* in an artificial lipid layer, and suggested that the enzyme has a secondary function as a carrier of ions.

3. Jasmonates and plant secondary metabolism

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. It has been also shown (Gundlach et al., 1992; Creelman & Mullet, 1997) that the several cell physiological activities of elicitors can be sometimes replaced by jasmonic acid and MJ, and exogenous application of jasmonates to plant cells enhances accumulation of a variety of secondary metabolites.

Recent studies on the signal transduction mechanisms of jasmonates have demonstrated (Turner et al., 2002) that the active form of jasmonates is an amino acid-conjugate, jasmonoyl-isoleucine, and this adducts would associate with a protein complex that functions as the receptor of this plant hormone. It has been also shown that jasmonates-signalling cascade further links to ubiquitin-proteasome-mediated protein degradation processes, however, only very limited information is available on the detail mechanism by which jasmonates induce the biosynthesis of various secondary metabolites in plant cells.

3.1 Role of Ca^{2+} and CAM in jasmonates signaling

We have recently reported (Kasidimoko et al., 2005) that biosynthesis of a tetracyclic diterpenoid of *Scoparia dulcis*, such as scopadulcic acid A, scopadulciol and scopadulin, is stimulated by the treatment of the plant with MJ, and that this process is triggered by Ca^{2+}-influx into the cytoplasmic space of the plant cells. We also demonstrated that activation of Ca^{2+}-cascade in the signal transduction pathway is an essential requirement for MJ-induced diterpene production in *S. dulcis* (Fig. 4). As is in phytoalexin production in carrot described above, we have demonstrated that CAM plays an important role in the MJ-induced enhancement of the biosynthetic activity. The transcriptional level of the gene(s) encoding CAM had been assumed to be maintained at almost constant level. However, recent studies clearly showed that plant CAMs are composed of several isoforms, and the expression of specific CAM gene(s) is sometimes markedly induced upon the contact with appropriate stimuli or under stress conditions. For example, carrot CAM genes consist of more than fifteen isoforms, and activation of several specific genes was observed by the treatment with

various elicitor-active substances (Ishigaki et al., 2004, 2005). At least eight genes encoding CAM are contained in potato, and these genes were differently expressed during the development of the plant (Takezawa et al., 1995). In tobacco cells, it was demonstrated (Yamakawa et al., 2001) that specific CAM genes are transcriptionally activated against microbial infection.

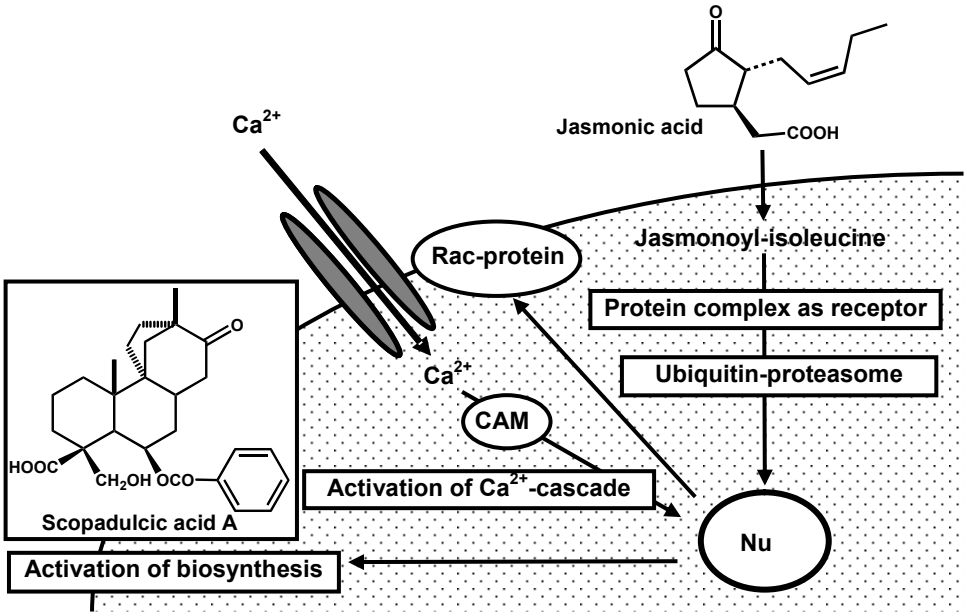

Fig. 4. Schematic presentation of signal transduction mechanisms of jasmonates.

In order to understand MJ-induced activation of diterpene biosynthesis in *S. dulcis* on the molecular basis, we attempted to isolate CAM gene homologues of the plant and examined the possible change in their expression activities (Saitoh et al., 2007). Based on the reported nucleotide sequences of CAM proteins, we isolated and selected sixteen cDNA clones encoding CAM from *S. dulcis* by means of rapid amplification of cDNA end (RACE) method. It was found that the coding regions (149 amino acids) of all of these isolated clones were completely identical, and the deduced amino acid sequence showed very high homology to those of CAMs from various biological sources. In addition, the nucleotide sequences of the 3'-untranslatable regions (UTRs) of these DNA fragments were also identical though a few gaps in the sequences were found in two of these samples. However, even in the cases, the other parts of the nucleotide sequences of either coding regions or UTRs were completely identical. These cDNA fragments were then subjected to 5'RACE, and eight clones were selected. As was in 3'RACE, the nucleotide sequences of these clones were completely identical both in coding regions and in 5'-UTRs. In genomic Southern blot hybridization analyses, only one hybridized band was observed in the DNA fragments digested by either *Eco*RV, *Hin*dIII or *Xho*I. It is well known that the nucleotide sequences of

CAM genes of higher plant cells showed very high identity (for example, 97-87% in *Arabidopsis thaliana* (Ling et al., 1991) and 98-86% in *Daucus carota* (Ishigaki et al., 2004, 2005). Thus, the appropriate probes are capable of hybridizing with the several restriction fragments of plant genomic DNA containing CAM genes, and usually complex patterns with multiple signals were observed. Therefore, the very simple results obtained for CAM gene(s) of *S. dulcis* should be a quite unusual case. For further characterization of CAM gene(s) in *S. dulcis*, genomic DNA of the plant was digested with *Hinf*I which is expected to hydrolyze the CAM gene at the middle point of its coding region (at 241 position of 447 nucleotides). After probing, two hybridized signals with similar intensity were visualized. These results strongly suggest that, unlike many of higher plants, CAM gene of *S. dulcis* occurs as the sole gene that encodes CAM protein in the genome of the plant.

Possible changes in the expression of CAM gene in leaf organ cultures of *S. dulcis* challenged by Ca^{2+}-ionphore A23187 or MJ were examined. The transcription of the CAM gene was transiently activated by the treatment with A23187, and a marked increase in the intensity of the band of the amplified DNA was observed after 3 h of the addition of the ionophore into the culture. After that, the expression of the gene decreased gradually and then returned to the initial level. Addition of MJ showed almost the same results, and the expression level of the CAM gene appreciably elevated after 3 to 6 h of the treatment, and it decreased thereafter. In contrast, the intensity of the band of the DNA fragments appeared to be maintained at the constant level in controls which received ethanol instead of the reagents. These results suggest that the elevation of transcriptional activity of CAM gene of *S. dulcis* is one of the early events of MJ-induced activation of Ca^{2+}-cascade in the cells, which leads to the enhancement of the biosynthetic activity of the tetracyclic diterpene compounds.

As described above, occurrence of several isoforms of CAM and multiple genes encoding the protein has been reported from various plant sources. It has been also demonstrated that a certain specific CAM gene(s) is sometimes expressed under stress conditions or by the stimulation to respond to various environmental change (Takezawa et al., 1995; Yamakawa et al., 2001). In sharp contrast to these reports, it has been shown that *S. dulcis* is a unique plant and only one gene encoding CAM protein should occur in the cells. How does the plant respond to the numerous external stimuli with only one CAM gene? We have shown that regulation of CAM activity by controlling the transcriptional level is, at least, one of the physiological mechanisms of the cells. It might be also possible that intracellular translocation of CAM to the appropriate structures or the spaces in the cells would be another mechanism in the regulation of the activity of the modulator protein (Collinge & Trewavas, 1989).

3.2 Participation of Rac/Rop GTPases in jasmonates signaling

For further elucidation of signal transduction mechanisms of MJ-enhanced diterpene biosynthesis in *S. dulcis*, we examined the possible participation of monomeric GTP-binding proteins in these processes. Monomeric GTPase proteins are involved in regulating the essential functions of eukaryotic cells, such as cell differentiation, intracellular vesicle transport, and cytoskeleton organization (Valster et al., 2000). These small GTPases are classified into several subfamilies, and, among them, Rac/Rop proteins have been shown to regulate auxin-signalling and defense responses in higher plants (Yang, 2002; Gu et al., 2004). Rac/Rop GTPase genes are usually organized as multigene family in plant cells, and

it has been assumed that each member of the subfamily is functionally distinct and plays specific roles, respectively. It has been also shown that these proteins are usually activated by prenylation, and the modification with the hydrophobic groups evokes the translocation of the proteins to plasma membranes to allow the association with the target molecules called effectors. We have isolated two Rac/Rop GTPase genes, *Sdrac-1* (965 bp encoding 196 amino acid residues) and *Sdrac-2* (969 bp encoding 197 amino acid residues), from *S. dulcis,* and found that the transcriptional activities of these genes appreciably increased by the stimulation with MJ, however, in contrast, they did not respond to the treatment with Ca^{2+}-ionophore A23187 (Mitamura et al., 2009; Shite et al., 2009). These observations led us to assume that *Sdrac-1* and *Sdrac-2* might play roles in a certain cellular event in MJ-signalling processes which occurs between reception of the external signal and Ca^{2+}-influx across plasma membranes.

Translocation of small GTPases to membranes is usually initiated by the binding of hydrophobic groups to these proteins (Yang, 2002; Gu et al., 2004). Therefore, we tested the possibility that Rac/Rop proteins of *S. dulcis* are targeted to membrane structures by the stimulation with MJ. We constructed the expression vector harbouring *Sdrac-1* or *Sdrac-2* tagged with glutathione-S-transferase (GST), and young seedlings of *Atropa belladonna* germinated under sterilized conditions were transformed with *Agrobacterium*-mediated methods. The intracellular translocation of Sdrac-1 and Sdrac-2 proteins to cellular membranes was examined by measuring the change in GST activities in microsomal fractions prepared from the transformed belladonna. GST activity in the microsomes of the transformants was maintained at low levels even after being incubated with MJ, and was almost comparable with that of the untreated control. In contrast, enzyme activity in the membrane fraction, which was prepared from belladonna tissues transformed with *GST-Sdrac-1*, was considerably elevated by incubation with MJ for 5 min, and it gradually increased for at least 30 min. However, GST showed low and almost constant activities in the untreated control. A similar set of results was also obtained for belladonna tissues transformed with *GST-Sdrac-2*, and a marked increase in GST activity in the microsomal fraction was specifically observed in MJ-treated cells. These results strongly suggest that both Sdrac-1 and Sdrac-2, Rac/Rop GTPase proteins of *S. dulcis*, rapidly translocate to microsomal fractions in response to MJ stimulation.

We also attempted to elucidate the possible post-translational modifications of these monomeric GTPases with hydrophobic groups which evoke the targeting of the proteins to plant plasma membrane (Mitamura et al., 2011). *In vitro* modifications of Sdrac-1 and Sdrac-2 were studied using the His-tagged recombinant proteins, and the purified Sdrac proteins were incubated with [^{14}C]-isopentenyl diphosphate, geranyl diphosphate and recombinant farnesyl diphosphate synthase protein, the [^{14}C]-farnesyl diphosphate-generating system, in the presence or absence of MJ-treated cell extracts of *S. dulcis*. The radiolabeled prenyl chain appeared to bind to Sdrac-2 protein when the assay mixture was incubated in the presence of MJ-treated cell extracts. In contrast, it was likely that Sdrac-2 did not accept the prenyl group without the cell extracts. Addition of the heat denatured cell extracts also showed no apparent effect on the binding of isoprene units to the protein. These results strongly suggested that Sdrac-2 would bind to prenyl chain, very likely C15 chain, in the presence of prenylation enzyme(s) probably occurring in MJ-treated cell extracts of *S. dulcis*.

In sharp contrast, however, conjugate of Sdrac-1 and the prenyl group was not formed either in the presence or absence of the cell extracts of *S. dulcis*. It was demonstrated (Lavy et al., 2002) that, among the translate products of Rac/Rop GTPase gene homologues of *A. thaliana*, targeting of AtRAC8 to the plasma membrane is initiated by palmitoylation of this protein. Therefore, we examined the possibility whether the palmitoyl group is capable of binding to Sdrac-1 protein in response to MJ stimulation. Considerable radioactivity was exhibited by purified His-tagged Sdrac-1 when it was incubated with [^{14}C]-palmitoyl-CoA in the presence of MJ-treated cell extracts of *S. dulcis*, while much lesser amounts of the radioactivities were found to associate with recombinant Sdrac-1 in the absence of the cell extracts or in the presence of the heat denatured extracts. From these results, we concluded that Sdrac-1 cannot be prenylated, however, it was acylated in response to MJ stimulation.

This set of the experiments strongly suggests that although Sdrac-1 and Sdrac-2 are similarly translocated to the membrane fraction by binding with hydrophobic groups in response to MJ stimulation, translocation of these GTPases is initiated by distinct modification mechanisms, i.e., palmitoylation for Sdrac-1 and prenylation for Sdrac-2. A well known consensus post-translational modification site for prenylation, CXXL, occurs near the C terminal of Sdrac-2. In addition, heterogeneous prenylation sites of Rac/Rop proteins as well as the putative motif CAA in maize (Ivanchenko et al., 2000) and CTAA in *Arabidopsis* (Li et al., 2001) have been recently demonstrated. In contrast, the structural prerequisites for palmitoylation of these GTPases remain obscure. The C-terminal amino acid sequence of palmitoylated *Arabidopsis* AtRAC8 is CGKN (Lavy et al., 2002), while Sdrac-1 with the C terminal of CAIF is also acylated. Therefore, at present, it should be quite difficult to predict the mode of post-translational modification of Rac/Rop GTPase proteins, prenylation or acylation, on the bases of the amino acids sequences near C-terminal.

4. Activation of secondary metabolism by modification of signal transduction processes of plant cells

In this manuscript, we have demonstrated that the increase in cytoplasmic Ca^{2+} level and activation of Ca^{2+}-cascade in higher plant cells would be essential events in the enhancement of plant secondary metabolisms triggered by the treatment with external stimuli such as elicitors and jasmonates. We have also shown that CAM is a key modulator protein to evoke natural products biosynthesis in elicitors- or jasmonates-stimulated plant cells. As mentioned above, a series of recent studies clearly demonstrated (Takezawa et al., 1995; Yamakawa et al., 2001) that, unlike in animal cells, plant CAM proteins are encoded by several homologous genes and the specific gene(s) is sometimes expressed by various physiological stresses. We isolated fifteen clones of CAM genes from carrot, and found that, among them, the transcription level of *cam-4* was markedly increased by the treatment of the cells with oligouronide elicitor (Ishigaki et al., 2004).

We assumed that the introduction and over-expression of appropriate gene(s) encoding key protein in secondary metabolism-related signal transduction mechanism, such as CAM, would activate Ca^{2+}-cascade and maintain the cells in the excitatory state. It might be also possible that the biosynthetic activities of several defensive compounds are significantly activated in these transformed plant cells. At present, only very limited information is

available about the biochemical changes in the plant cells transformed with CAM gene, however, it has been reported (Harding et al., 1997; Cho et al., 1998) that over-expression of the gene in tobacco cells results in the unusual activation of CAM-dependent functional proteins followed by the generation of active oxygen species and NO. In animal cells it was shown (Broillet, 2000) that the elevation of NO concentration appreciably enhances the synthesis of cyclic nucleotides. As described above, we showed that the increase in cytoplasmic level of cyclic nucleotides stimulates Ca^{2+}-cascade in cultured carrot cells by activating the nucleotide-sensitive cation channels. Therefore, we attempted to produce the transgenic plants in which carrot *cam-4* gene was introduced and over-expressed by the infection of transformed *Agrobacterium tumefaciens*. In the preliminary experiments, sesame *(Sesamum schinzianum* Asch.) showed the highest transformation and re-differentiation efficiencies among several plants tested, therefore, we focused on this plant as the target of the transformation.

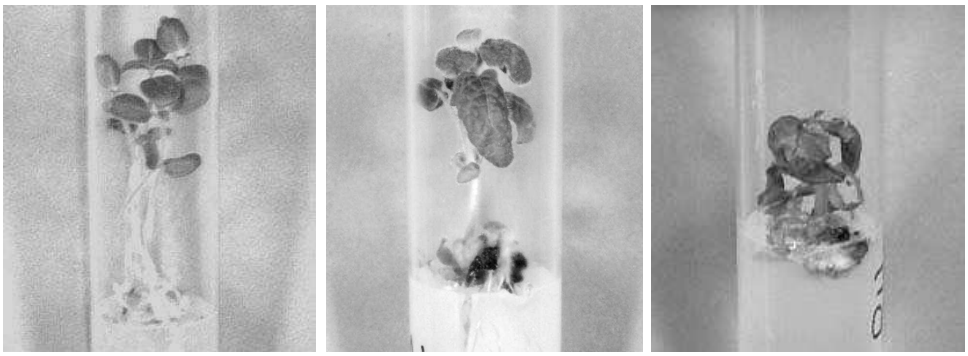

Fig. 5. Control and transformed sesame plants. Wild type (left), transformed with the empty expression vector (middle), and transformed with the vector harbouring *cam-4* gene (right).

Sesame seedlings were infected by *A. tumefaciens* transformed with pMAT vector harbouring cauliflower mosaic virus 35S promoter-*cam-4*, and the formation of crown galls was observed at the cut surfaces of the stems following incubation for 2-3 weeks. The gall tissues were harvested and then transferred onto agar medium containing an antibiotic and cytokinin for the sterilization and the formation of multiple shoots. The re-differentiated sesame plants, transformed with *cam-4* and the control for *Agrobacterium*-infection, were used for further analyses together with the wild sesame seedlings germinated under the sterilized condition (Fig. 5). The appearance of the infection-control of sesame was somewhat different from that of the wild plantlets directly germinated from seeds. However, the shape of the transformed and re-differentiated sesame was quite abnormal as compared with those of the wild and the control plants (Fig. 5). It was also confirmed that the concentration of CAM protein in the transgenic sesame was appreciably higher than those in the wild and the control plants (relative ratio was estimated to be 1: 1.1 : 2.6 as analyzed by immuno-blotting followed by densitometric scan).

In the next experiments, possible changes in the enzyme activities of defense-related secondary metabolisms and the contents of phenolic compounds in the transformed plants were examined. As the key enzymes of defense reactions, we focused on phenylalanine

ammonia-lyase (PAL) and caffeic acid *O*-methyltransferase (COMT), the most important enzymes involved in the biosynthesis of lignin and the related compounds. PAL activity in the transgenic sesame was appreciably higher than those of the wild and the infection-control (1.9- and 2.4-fold higher levels, respectively). This marked stimulation of the catalytic activity in the transformed plant was also found in COMT, and 2.3- and 1.9-fold increases in the activities were observed. The increased ratio of these two enzyme activities in the transgenic sesame were almost comparable to the elevated ratio of the 'bulk' expression levels of CAM genes described above. It was also shown that the contents of phenolic compounds, caffeic acid and ferulic acid, in the *cam-4*-transformed sesame markedly increased (3.0– to 5.8-fold higher levels).

It was demonstrated, therefore, that the CAM gene specifically expressed for the biosynthesis of 6-methoxymellein in carrot, a polyketide compound, is capable of enhancing the production of phenylpropane derivatives in sesame. Is there any specificity in the enhancement of secondary metabolism in higher plants in which CAM gene is over-expressed? Although CAM plays roles in numerous cellular events, the structure of the protein is known to be highly conservative. Therefore, the specificity of the final cellular responses mediated by CAM is considered to be regulated by certain processes in the downstream of the signal transduction cascade. From these facts, we assume that the pathway(s) of secondary metabolism in higher plants which would be activated by the transformation with CAM genes might depend on the inherent natures of the individual plant species, such as the networks of the signalling cascade, the properties of the functional proteins mediating these processes, and the levels and/or timings of the expression of the genes participating in these cellular events.

5. Conclusion

Secondary metabolism of higher plants is appreciably activated by the treatment of the cells with appropriate external stimuli, such as elicitor-active compounds and jasmonates. It is very likely that the increase in cytoplasmic Ca^{2+} concentration followed by the activation of Ca^{2+}-cascade is an essential cellular event for the enhancement of the biosynthesis of natural products in plant cells, and CAM protein appears to play the important cell physiological roles in these processes. Series of the experiments presented in this manuscript suggest the possibility that the elucidation and engineering of signal transduction processes to maintain the plant cells at the excitatory states would be a novel method for molecular breeding of useful medicinal plants.

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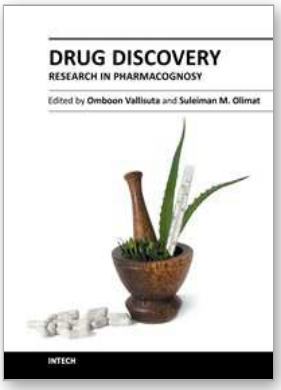

Drug Discovery Research in Pharmacognosy

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This book, Drug Discovery Research in Pharmacognosy provides a full picture of research in the area of pharmacognosy with the goal of drug discovery from natural products based on the traditional knowledge or practices. Several plants that have been used as food show their potential as chemopreventive agents and the claims of many medicinal plants used in traditional medicine are now supported by scientific studies. Drug Discovery Research in Pharmacognosy is a promising road map which will help us find medicine for all!

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