Modification and Translocation of Rac/Rop Guanosine 5'-Triphosphate-Binding Proteins of Scoparia dulcis in Response to Stimulation with Methyl Jasmonate

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Translocation of two Rac/Rop guanosine 5'-triphosphate-binding proteins from Scoparia dulcis, Sdrac-1 and Sdrac-2, was examined employing transformed belladonna which overproduces these proteins as glutathione-S-transferase-tagged forms. The transferase activities of the fused proteins in microsomal fraction of belladonna markedly increased by the incubation with methyl jasmonate either in Sdrac-1 or Sdrac-2 transformant, while low and constant activities were observed in the untreated control. Recombinant Sdrac-2 protein was found to bind to prenyl chain in the presence of cell extracts prepared from methyl jasmonate-treated S. dulcis, however, Sdrac-1 was palmitoylated by the addition of the cell extracts. These results suggest that both Sdrac-1 and Sdrac-2 translocate to plant membranes by the stimulation with methyl jasmonate, however, targeting of these proteins is triggered by the independent modification mechanisms, palmitoylation for Sdrac-1 and prenylation for Sdrac-2.

Key words methyl jasmonate; Rac protein; plant signal transduction; intracellular translocation; prenylation; palmitoylation

Jasmonates, including 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 enhances the accumulation of various secondary metabolites. Recent studies on signal transduction mechanisms of jasmonate have demonstrated that the active form of jasmonates is an amino acid-conjugate, jasmonoyl-isoleucine, and this adduct associates with a protein complex that functions as the receptor of this plant hormone. The MJ signaling cascade has been found to be associated with ubiquitin-proteasome-mediated protein degradation processes; however, limited information is available on the detailed mechanism by which jasmonates induce various cellular responses in higher plants. We recently reported that biosynthesis of diterpene compounds in Scoparia dulcis is stimulated by treating the cells with MJ. We also demonstrated that MJ-induced enhancement of this biosynthesis is triggered by Ca\(^{2+}\) influx into the cytoplasmic space of the cells, and that activation of the calmodulin-mediated Ca\(^{2+}\) cascade is an essential requirement for MJ-induced diterpene production in S. dulcis. However, cellular events that function between degradation of ubiquitin-tagged proteins and gating of the Ca\(^{2+}\) channel at the plasma membrane remain to be elucidated.

Monomeric guanosine 5'-triphosphatase (GTPase) proteins are involved in the regulation of essential functions of eukaryotic cells, such as cell differentiation, intracellular vesicle transport, and cytoskeleton organization. These small GTPases are classified into several subfamilies among which, Rac/Rop proteins have been shown to regulate auxin signaling and defense responses in higher plants. Rac/Rop GTase genes are usually organized as a multigene family in plant cells, and each member of the subfamily is assumed to be functionally distinct and to play specific roles. These proteins are usually activated by prenylation. Modification with hydrophobic groups promotes translocation of the proteins to plasma membranes and further their association with target molecules called effectors. We recently isolated two Rac/Rop GTPase genes, Sdrac-1 and Sdrac-2, from S. dulcis (Fig. 1), and found that their transcriptional activities were considerably increased by MJ stimulation. However, the genes did not respond to treatment with the Ca\(^{2+}\) ionophore A23187. These observations suggest that Sdrac-1 and Sdrac-2 might play a role in a certain cellular event in the MJ signaling cascade that occurs between the reception of the external signal and the Ca\(^{2+}\) influx across plasma membranes. In the present study, we tested a possibility that Rac/Rop proteins of S. dulcis are targeted to the membranes by MJ stimulation. We also elucidated the mechanisms by which in vitro modifications of these monomeric GTPases with hydrophobic groups promote targeting of proteins to the plant membranes.

MATERIALS AND METHODS

Materials S. dulcis was grown in a greenhouse of the Experimental Station for Medicinal Plant Research, University of Toyama. Seedlings of Atropa belladonna, which were germinated under sterile conditions, were used for the transformation experiments. Isopentenyl diphosphate (IPP), geranyl diphosphate (GPP), coenzyme A (CoA), palmitoyl-CoA, ATP, and acyl-CoA synthetase were purchased from Sigma-Aldrich (MO, U.S.A.), while MJ and isopropyl-thio-β-D-galactopyranoside (IPTG) were from Wako Pure Chemicals (Osaka, Japan). \(^{[14}C\)-IPP and \(^{[14}C\)-palmitic acid (specific activity, 2.04 GBq/mmol) were obtained from American Radiolabeled Chemicals (MO, U.S.A.). All other chemicals were of reagent grade.

Transformation of Belladonna The coding regions of Sdrac-1 and Sdrac-2 (GenBank accession nos. FJ159428 and FJ550362) were amplified by polymerase chain reaction

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The transformed belladonna tissues (approximately 1 g) were incubated with 100 mJ for GST amplification. The RNA samples were immediately subjected to reverse transcription (RT) using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). PCR was performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Leaf segments of belladonna seedlings were infected with Agrobacterium tumefaciens LBA4404 (Invitrogen), which had been transformed with these expression vectors, and placed on Murashige–Skoog agar medium. After incubation for 3—5 d at 26 °C, the segments were transferred onto the medium containing 1 mM cefotaxime, 0.1 mM kanamycine, and 3 mM MgCl2. They were maintained on this medium for 5—8 passages and then transferred onto the same medium, but without antibiotics.

**Preparation of Recombinant Proteins** Overexpression of Sdrac-1, Sdrac-2, and the farnesyl diphosphate (FPP) synthase gene isolated from Aquilaria microcarpa (Am-FPPS-1, GenBank accession no. HM067872) in Escherichia coli was performed using the E. coli expression system with Gateway Technology (Invitrogen). Translatable regions of Sdrac and FPP synthase genes were amplified by PCR and subsequently subcloned into pENTR and then into pDEST17 (Invitrogen). E. coli BL21/DE3 cells were transformed with the constructed expression vectors, and the cell cultures were grown in LB medium overnight at 37 °C. After transfer into the fresh medium, IPTG (final concentration, 0.4 mM) was added to the cultures at an optical density of 0.6 at 590 nm. The cultures were further incubated for 3 h at 37 °C, and the His-tagged proteins were recovered and purified using the MagneHis protein purification system (Promega, CA, U.S.A.). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel) according to the method of Laemmli, and separated proteins were stained with Coomassie brilliant blue. As analyzed by the Image J software, purities of the recombinant products of these two genes as glutathione-S-transferase (GST) fusion proteins. Entry vectors for GST-Sdrac-1 and GST-Sdrac-2 were prepared using the pENTR/D-TOPO Cloning Kit (Invitrogen), and they were inserted into the Gateway cassette downstream of the cauliflower mosaic virus 35S promoter in pBI-OX-GW (Inplanta Innovations, Kanagawa, Japan) using LR clonase (Invitrogen). Leaf segments of belladonna seedlings were infected with Agrobacterium tumefaciens LBA4404 (Invitrogen), which had been transformed with these expression vectors, and placed on Murashige–Skoog agar medium. After incubation for 3—5 d at 26 °C, the segments were transferred onto the medium containing 1 mM cefotaxime, 0.1 mM kanamycine, and 3 mM N3-benzyladenine. They were maintained on this medium for 5—8 passages and then transferred onto the same medium, but without antibiotics.

**Sdrac Expression in Transformed Belladonna** Total RNA was prepared from 100 mg of belladonna tissues, which were transformed with GST, GST-Sdrac-1, and GST-Sdrac-2 using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA samples were immediately subjected to reverse transcription (RT) using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). PCR was performed using appropriate combinations of the primers described above, and 5′-ATG TCC CCT ATA CTA GGT TAT TGG-3′ (forward) and 5′-ACG CCG AAC CAG ATC CTA TTT TGG AGG ATG-3′ (reverse) were used as the primer pair for GST amplification.

**Assay of GST Activity in Transformed Belladonna** The transformed belladonna tissues (approximately 1 g) were incubated with 100 μM of MJ according to the method described previously, and in control treatments, the tissues were treated with water and not MJ. They were harvested after incubation for 5—30 min and homogenized in the presence of quartz sand using a mortar and pestle. The homogenates were transferred into 4 ml of 100 mM K-phosphate buffer (pH 6.5), and after vigorous mixing, they were filtered through a double-layered gauze. The filtrates were centrifuged at 10000×g for 10 min, and the resultant supernatants were further centrifuged at 100000×g for 1 h. The precipitated microsomal fractions were dissolved in 2 ml of K-phosphate buffer, and 4 ml of saturated ammonium sulfate solution was added to the sample. The precipitated proteins were recovered by centrifugation (10000×g, 10 min) and dissolved in 500 μl of the phosphate buffer for the GST assay. GST activity was determined using the GST detection module (GE Healthcare, Buckinghamshire, U.K.) according to the instruction manual, and the amount of enzyme capable of increasing 0.1 absorbance per minute was defined as one unit. Protein concentration was determined by the Bradford method.

**Fig. 1. Alignment of the Deduced Amino Acid Sequences of Rac/Rop GTPase Proteins**

The amino acid sequences of the small GTPases from various plant sources were aligned by CLUSTAL W, and their similarity was compared by BOXSHADE. The back ground of the identical amino acid residues was black and that of similar residues was gray. Arab is the abbreviation of A. thaliana. GenBank accession numbers of the corresponding genes are as follows. Arabrac1, Q38902; RiceracB, Q6ZHA3; BarleyracD, CAD27895; Ricerop5, Q6Z7L8; Barleyrop4, CAD27896; Ricerop4, 067VP4; Arabrac8, AAC63015.
Sdrac-1 and Sdrac-2 proteins were approximately 69% and 92%, respectively. If necessary, the results obtained in the protein modification experiments were normalized considering the purities of the recombinant proteins.

**Prenylation of Sdrac-1 and Sdrac-2 Proteins**

Prenylation of Sdrac proteins was performed by the method of Trainin et al. The leaf tissues of *S. dulcis* (approximately 500 mg) were incubated with 100 μM MJ for 30 min and homogenized in 5 ml of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (pH 7.8). The homogenates were centrifuged at 10000×g for 10 min, and the resultant supernatants were used as MJ-treated cell extracts. The reaction mixture for prenylation of Sdrac proteins consisted of, in a total volume of 50 μl, 50 mM HEPES-KOH, 2 mM MgCl₂, 5 mM dithiothreitol, 1 μM GPP, 0.1 μM [¹⁴C]-IPP (3.7 kBq/assay), and recombinant Sdrac-1 or Sdrac-2 and MJ-treated cell extracts of *S. dulcis* (10 μg protein). The prenylation assay was also performed in the presence of the heat denatured cell extracts or without the addition of the extracts. They were incubated for 1 h at 37 °C, and then, 500 μl aliquot of ethanol : HCl (9 : 1, v/v) was added to hydrolyze the unbound materials. The mixtures were further incubated for 30 min, and the proteins were precipitated by centrifugation (15000×g, 5 min). The samples were subjected to SDS-PAGE after washing with 100% ethanol, twice. The protein bands corresponding to His-tagged Sdrac-1 and Sdrac-2 (approximately 23 kDa) were excised using a blade, and the radioactivities co-migrated with these proteins were determined according to the method described previously.

**Palmitoylation of Sdrac-1 and Sdrac-2 Proteins**

Palmitoylation of the Sdrac-1 and Sdrac-2 proteins was performed according to the method of Veit. [¹⁴C]-Palmitate was converted to [¹⁴C]-palmitoyl-CoA by reaction with CoA, ATP, and acyl-CoA synthetase, and recombinant Sdrac-1 or Sdrac-2 (1 μg protein) were incubated with [¹⁴C]-palmitoyl-CoA (3.7 kBq/assay) in 50 μl of 50 mM HEPES-KOH buffer (pH 7.8) at 37°C for 1 h in the presence or absence of MJ-treated cell extracts of *S. dulcis*. The assay was also carried out with the heat denatured cell extracts. The reaction was terminated by the addition of 500 μl of chloroform : methanol (1 : 1, v/v), and the mixtures were centrifuged at 10000×g for 10 min. The recovered proteins were washed with methanol and denatured in SDS-PAGE sample buffer without a reducing agent. The samples were separated by SDS-PAGE, and the radioactivity of the band corresponding to His-tagged Sdrac proteins was determined.

**RESULTS**

**Transformation of Belladonna**

In the preliminary stage of the present study, we had attempted to examine if plasma membrane-targeted translocation of Sdrac-1 and Sdrac-2 would take place upon the stimulation with MJ, employing regenerated *S. dulcis* leaf tissues transformed with the GST-tagged Rac/Rop GTPase genes. However, serious browning and death had been reproducibly observed in all leaf segments of the plant after the treatment with *A. tumefaciens*, and therefore, the bacteria-mediated transformation was performed using young seedlings of *A. belladonna* as the host plant.

Belladonna seeds were germinated under sterile conditions (Fig. 2a), and leaf segments prepared from the young seedlings were infected with *A. tumefaciens*, which was transformed with expression vectors harboring GST, GST-Sdrac-1, or GST-Sdrac-2 (Fig. 2b). After incubation for 4—6 weeks, shoots regenerated at the cut surfaces of the leaves (Fig. 2c), and transformed plantlets were obtained after a further incubation for 10—15 weeks (Fig. 2d). Expression of...
the desired genes in these transformants was confirmed by RT-PCR analyses (Fig. 3a), and DNA fragments of expected sizes (GST, 669 bp; Sdrac-1, 591 bp; GST-Sdrac-1, 1293 bp; Sdrac-2, 594 bp; and GST-Sdrac-2, 1296 bp) were amplified in each of the cell lines (Fig. 3b). These results strongly suggested that GST, GST-Sdrac-1, and GST-Sdrac-2 were appropriately introduced into the leaf segments of belladonna by Agrobacterium-mediated transformation, and they were properly expressed in these plantlet tissues.

Translocation of Rac/Rop GTPases Possible intracellular translocation of Sdrac-1 and Sdrac-2 to cellular membranes triggered by MJ stimulation was examined by measuring the change in GST activities in microsomal fractions prepared from the transformed belladonna (Fig. 4). 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 the microsomal fractions in response to MJ stimulation. Although MJ-induced translocation of Sdrac-1 and Sdrac-2 in transgenic belladonna was reproducible, majority of the GST-fused proteins remained in cytoplasmic fractions. In repeated experiments, the ratio of membrane- to cytoplasm-located GTPases fluctuated significantly, probably because of the continuous translation of Sdrac genes and overaccumulation of the proteins in transgenic belladonna.

Modification of Rac/Rop GTPases Translocation of small GTPases to membranes is usually initiated by the binding of hydrophobic groups to these proteins. Therefore, in subsequent experiments, in vitro modifications of Sdrac-1 and Sdrac-2 were studied using the His-tagged recombinant proteins (Fig. 5a). Purified Sdrac proteins were incubated with [14C]-IPP, GPP, recombinant FPP synthase, the [14C]-FPP-generating system, in the presence or absence of MJ-treated cell extracts of S. dulcis. As shown in Fig. 5b, 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. It is however very 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. In contrast, it seemed that the conjugate of Sdrac-1 and the prenyl group was not formed either in the presence or absence of the cell extracts of S. dulcis (Fig. 5b).

Some isoforms of Rac/Rop GTPases in Arabidopsis thaliana, including AtRAC7, AtRAC8, and AtRAC10, have been recently demonstrated to localize to the plasma membrane in a prenylation-independent manner. Several lines of evidence strongly suggest that, among these AtRACs, targeting of AtRAC8 (Fig. 1) 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 proteins in response to MJ stimulation. Considerable radioactivity was exhibited by purified His-tagged Sdrac-1 when it was incubated with [14C]-palmitoyl-CoA in the presence of MJ-treated cell extracts of S. dulcis. However, Sdrac-1 also incorporated significant levels of radioactivity even in the absence of the cell extracts (Fig. 5c), although the efficiency of the acyl group binding was considerably lower than that of the cell extract-mediated reaction. A similar result was also obtained when the assay was carried out in the presence of the heat denatured cell extracts. In repeated experiments, Sdrac-1 incubated with [14C]-palmitoyl-CoA in the presence of MJ-treated cell extracts usually showed approximately 3- to 4-fold higher radioactivity levels than the protein assayed without the cell extracts or with the denatured extracts. It appeared that the comparable levels of the radioactivities observed for control treatments of Sdrac-1 were also accompanied with Sdrac-2 incubated with either the native or the denatured cell extracts, or without the addition of the extracts (Fig. 5c). Resh reported that palmitoyl-CoA simultaneously reacts with Cys residues of Rac/Rop
GTPases to some extent; therefore, the apparent binding of the [14C]-palmitoyl group to Sdrac-2 and to the controls for Sdrac-1 could be a result of the non-enzymatic reaction. From these observation and results, we concluded that Sdrac-1 cannot be prenylated, however, it was acylated in response to MJ stimulation.

**DISCUSSION**

In the present study, it has been demonstrated that two Rac/Rop GTPases from *S. dulcis*, Sdrac-1 and Sdrac-2, were targeted to membranes in response to MJ stimulation (Fig. 4). Whether MJ-induced translocation of Rac/Rop GTPase is a common mechanism in a wide range of plant species remains unclear; however, this class of monomeric GTP-binding proteins may play an important role as key mediators in the signal transduction cascade of jasmonates. *In vitro* modification experiments showed that Sdrac-2 was significantly prenylated in the presence of MJ-treated cell extracts (Fig. 5b), while Sdrac-1 was not modified by isoprene units under the present experimental condition. However, this protein was palmitoylated, instead of being prenylated, by the addition of the cell extracts (Fig. 5c). Therefore, 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 consensus post-translational modification site for prenylation, CXXL, occurs near the C-terminal of Sdrac-2 (Fig. 1). In addition, heterogeneous prenylation sites of Rac/Rop proteins as well as the putative motif CAAX in maize and CTAA in *Arabidopsis* 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, while Sdrac-1 with the C-terminal of CAIF is also acylated (Figs. 1, 5).

As discussed above, we have previously reported that the transcriptional activities of Sdrac-1 and Sdrac-2 were considerably increased in response to MJ stimulation. However, elevations in expression of the two small GTPase genes were observed within 3—12 h of MJ treatment. In contrast, translocation of these proteins was initiated 5 min after MJ stimulation (Fig. 4). Therefore, the following two separate events of Rac/Rop GTPase may function in MJ-treated plant cells: rapid modification of the proteins followed by their translocation to the plasma membrane, and some other relatively slow processes accompanying the considerable change in gene expression.

Lavy *et al.* reported that Rac/Rop GTPase proteins in *A. thaliana* were spontaneously modified and translocated to plasma membranes without any external stimuli. In contrast, Park *et al.* demonstrated that the membrane-targeted translocation of Rac-like GTPases in soybean cultures was triggered by hypo-osmotic shock. These GTPases were usually found in the cytoplasmic space of soybean cells, and their translocation was triggered by dilution of the cell suspension with water. Similar to that in soybean, but not in *A. thaliana*, translocation of Sdrac-1 and Sdrac-2, Rac/Rop GTPase proteins from *S. dulcis*, in transformed belladonna was triggered in response to MJ stimulation. However, although majority of the GTPases were translocated to the membrane fraction in stimulated soybean cells, only limited number of the proteins (usually less than 10%) was localized at the membrane in the MJ-treated transgenic belladonna. Currently, no explanation is available regarding these diverse modes of action of Rac/Rop GTPases in plant cells. The apparent variation in the behaviors of Rac/Rop GTPases may be due to the inherent nature of each plant. Alternatively, the efficiency of post-translational modification and transport of Rac/Rop GTPases may depend on the cellular abilities of the respective transgenic plants that overproduce these proteins. Therefore, it still remains to be elucidated how MJ signal is transmitted to evoke the modification and the translocation of Sdrac proteins.

Further studies on physiological functions of Sdrac-1 and Sdrac-2 in MJ signaling and the target effector protein(s) of these membrane-associated small GTPases are in progress in our laboratory.

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