Gene Note

Cloning and transcriptional regulation of *Sdrac* encoding a Rac/Rop small guanosine 5′-triphosphate-binding protein gene from *Scoparia dulcis*

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Abstract   A cDNA clone designated *Sdrac* (965 bp) was isolated from the seedlings of *Scoparia dulcis*. This gene contains an open reading frame encoding the protein of 196 amino acid residues with high homology to Rac/Rop small GTPases from various plant sources. In Southern hybridization analysis, the partial hydrolysates of genomic DNA of *S. dulcis* prepared by the digestion with *Xba*I, *Xho*I, or *Eco*RI showed one main band together with a few weakly hybridized signals. The change in the transcriptional activity of *Sdrac* was analyzed by RT-PCR under various conditions, and it showed a marked increase by the treatment of the leaf tissues of the plant with methyl jasmonate and 2-chloroethylphosphonic acid, an ethylene-generating reagent. However, no significant change in the expression activity was observed upon the treatment of the leaves with Ca^{2+}-ionophore A23187. Treatment of the leaves with high concentration of NaCl also did not affect the expression level of the gene. These results suggest the possibility that *Sdrac* product plays roles in a certain cellular event in the signal transduction processes evoked by methyl jasmonate and ethylene accompanying the change in the transcriptional activity.

Key words: Ca^{2+}-ionophore, ethylene, methyl jasmonate, Rac/Rop GTPase, *Scoparia dulcis*.

Rac/Rop proteins constitute a small GTPase family of plants that corresponds to Rho family in animal cells (Valster et al. 2000; Winge et al. 2000; Zheng & Yang 2001). These monomeric GTPases have been shown to regulate various cellular processes of plants, such as growth, differentiation, hormone signaling and defense responses (Yang 2002; Vernoud et al. 2003; Gu et al. 2004; Berken 2006). Rac/Rop GTPase genes are usually organized as multigene family in plant cells, and seven Rac/Rop genes were reported for rice and eleven genes were for *Arabidopsis thaliana* (Yang 2002; Miki et al. 2005). It has been assumed that each member of Rap/Rop subfamily is functionally distinct and plays specific roles, respectively (Li et al. 1998; Yang 2002; Miki et al. 2005). As well as Rac/Rop GTPases, plant calmodulin (CAM), an important modulator protein in the signal transduction processes, are usually encoded by more than 10 genes, and expression of specific gene is induced upon the contact of plant cells with various stimuli (Takezawa et al. 1995; Yamakawa et al. 2001). It is assumed, therefore, that CAM and Rac/Rop GTPase genes are organized as multigene family, and selective expression of specific genes of these functional proteins

is sometimes essential for the proper responses of plant cells toward various external stimuli and environmental stresses.

It has been shown (Creelman and Mullet 1997; Fujiwara et al. 2006) that methyl jasmonate (MJ) functions as the messenger molecule in various plant defense responses, and Rac/Rop GTPase plays predominant role in the defense-related secondary metabolism in plants. On the other hand, we demonstrated previously (Kurosaki et al. 1992; Kurosaki & Nishi 1993; Kurosaki 1997) that the activation of Ca^{2+}-cascade in plant cells is the essential event to induce the defensive secondary metabolism, and CAM functions as the key component in these processes. Therefore, it might be expected that both CAM and Rac/Rop GTPases play important roles in the defense-related signal transduction processes triggered by MJ. We showed previously (Kasidimoko et al. 2005) that the biosynthetic activity of scopadulcic acid, a tetracyclic diterpenoid in *Scoparia dulcis*, is markedly increased in a CAM-dependent manner when the plant was stimulated by MJ. However, we have recently found (Saitoh et al. 2007) that, unlike in many other plants,

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CAM in *S. dulcis* occurs as a sole copy gene in the genome. Therefore, it is interesting to investigate how *S. dulcis* properly responds to various stimuli with only one CAM gene. Primary aim of the present study was the elucidation of genomic organization and transcriptional regulation of Rac/Rop small GTPase gene(s) as another candidate for the key modulator for proper responses in the signal transducing processes of *S. dulcis*, a unique 'sole CAM gene' plant. An attempt was made to isolate Rac/Rop gene from *S. dulcis*, and Southern blot analysis was performed to examine the genomic organization of the gene. Possible change in the expression level of the Rac/Rop GTPase gene toward various stimuli was also examined.

The cDNA fragments presumably encoding the small

GTPases of *S. dulcis* were isolated from the seedlings employing the degenerate primer pair, 5′-AAR TGY GTN CAN GTN GGN GAY-3′ as the forward and 5′-NGC NGC RTN RAA NAG NGC YTT NAC-3′ as the reverse primer, respectively. Total RNA was isolated with RNeasy Plant Mini Kit (Qiagen), and cDNA templates were generated by reverse-transcription (RT) reaction. After the amplification of cDNA fragments by polymerase chain reaction (PCR), the DNA fragments were subcloned into the pCR2.1-TOPO vector (Invitrogen). The nucleotide 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), and two cDNA fragments (fragment 1 and fragment 2)
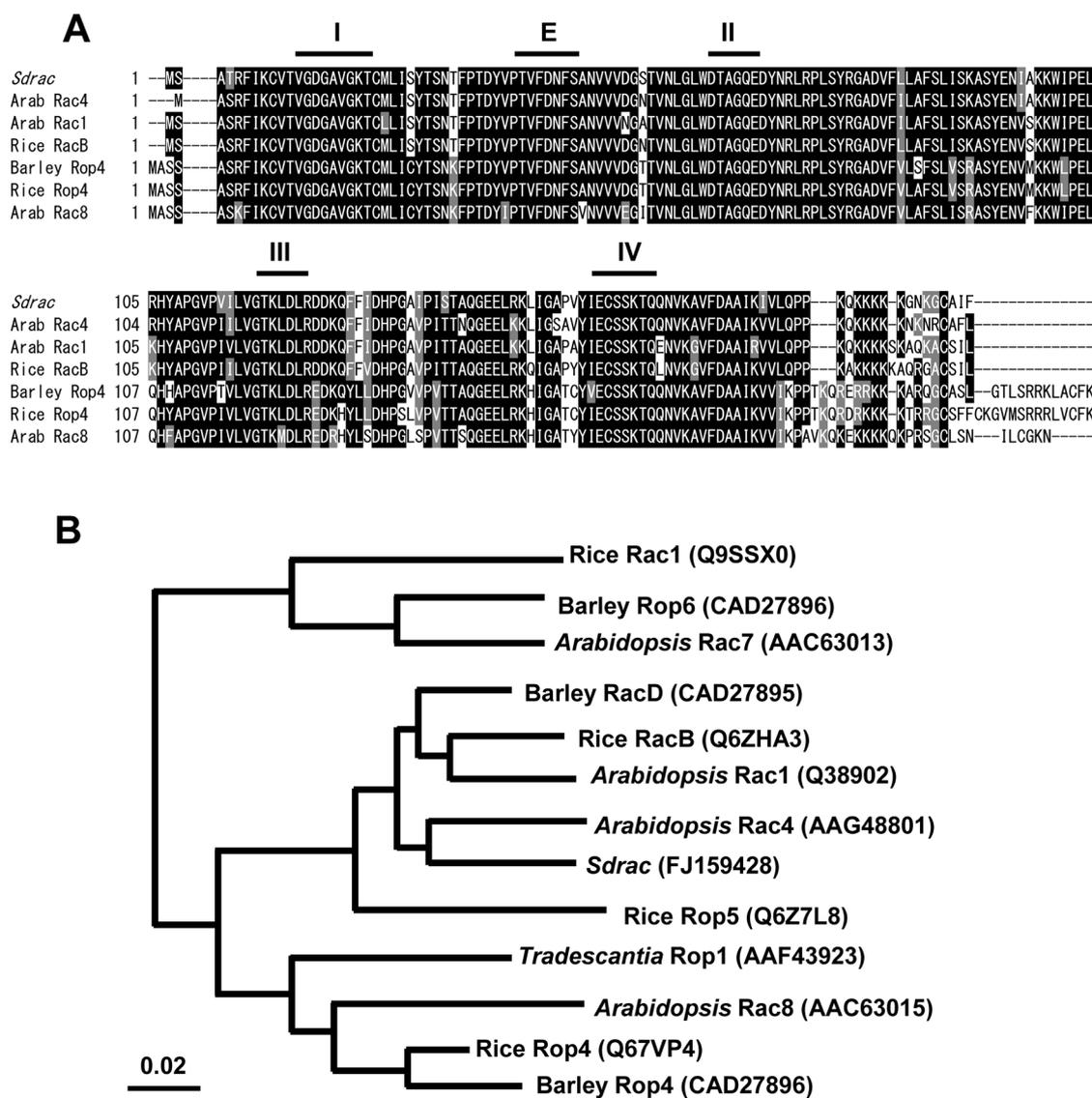

Figure 1. A, Alignment of the deduced amino acid sequences of Rac/Rop proteins. The amino acid sequences of the small GTPases from various plant sources were aligned by CLUSTALW, and their similarity was compared and analyzed by BOXSHADE. Four conserved structures for GTP-binding and hydrolysis were presented as bars I–IV, and E denotes the effector region. 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*. B, Phylogenetic tree analysis of Rac/Rop proteins from various plant sources. Phylogenetic tree for the amino acid sequences of the small GTPases from various plants was constructed by NJplot, and GenBank accession numbers of the corresponding genes were shown in the parentheses.

which showed high homology with plant Rac/Rop GTPases were isolated. The full length sequencess of these cDNA fragments were determined by means of rapid amplification of cDNA ends (RACE) method using the GeneRacer Kit (Invitrogen). The 3′-RACE was performed with GeneRacer Oligo dT as the reverse primer, and 5′-TGG GAC AAA GCT CGA TCT TAG AGA TGA CAA G-3′ (for fragment 1) and 5′-GTC CCA ACC GTG TTC GAC AAT TTC-3′ (for fragment 2) as the gene-specific forward primers for PCR amplification of the DNA fragments. The 5′-RACE was carried out with GeneRacer RNA Oligo as the forward, and 5′-GTG GGA TAC TGC AGG ACA AGA GGA TTA C-3′ (for the fragment 1) and 5′-GGA ACT CCA GGA GCA TAA TGT CTC-3′ (for fragment 2) as the reverse primers, respectively. The gene isolated from fragment 1 (designated *Sdrac*, GenBank accession number FJ159428, 965 bp) contains an open reading frame of 196 amino acid residues, and the primary sequence of *Sdrac* translate showed high homology with those of Rac/Rop proteins from various plant sources (Figure 1A). Another clone obtained from fragment 2 (designated *psd-Sdrac*, GenBank accession number FJ159427, 804 bp) also showed high homology with plant Rac/Rop GTPases. However, this gene is lacking in the stop codon in the structure, and therefore, we concluded that *psd-Sdrac* is a pseudo gene resembling Rac gene(s) of *S. dulcis*. In *Sdrac* product, the four characteristic motifs for GTP-binding and hydrolysis, the unique structures of small GTPases, are well conserved (Kawasaki et al. 1999) and it also contains an effector-binding domain as the common structures of Rac/Rop proteins. The deduced amino acid sequence of the translate product of *Sdrac* was further compared with other Rac/Rop proteins by molecular phylogenetic tree analysis. As shown in Figure 1 B, plant Rac/Rop including *Sdrac* product appeared to be close each other, and *Sdrac* was found to be most close to Rac4 isolated from *A. thaliana*.

In order to characterize the genomic organization of *Sdrac* in *S. dulcis*, Southern blot hybridization analysis was carried out employing the nucleotides corresponding to the translatable region of the gene as a probe. Genomic DNA was isolated from leaves of *S. dulcis* using OmniPrep (G-Biosciences) according to the instruction manuals, and the restriction digests were prepared using *Xba*I, *Xho*I, or *Eco*RI (Takara). They were electrophoresed on a 0.8% agarose gel, and the separated DNA fragments were transferred onto an Immobilon-NY$^+$ (Millipore). The translatable region of *Sdrac* including stop codon (591 nucleotides) was amplified by PCR and was directly labeled with AlkPhos Direct Labeling and Detection System (GE Healthcare Bio-Science) to be used as the probe. The membrane was hybridized with the probe for 24 h at 55°C in a solution

containing 6×SSC, and, after several washings, the filters were dried and exposed to an X-ray film for 6 h at room temperature as describe previously in detail (Asakura and Kurosaki 2007). As shown in Figure 2, the labeled probe hybridized to the restriction digests of genomic DNA prepared from *S. dulcis*, and one main band and a few weakly hybridized signals were observed in the DNA fragments hydrolyzed with either *Xba*I, *Xho*I or *Eco*RI. These results suggest that, unlike in several plants examined (Yang 2002; Miki et al. 2005), Rac/Rop small GTPase subfamily of *S. dulcis* would be composed of a limited number of homologues.

The tissue specificity and possible change in the transcriptional levels of *Sdrac* upon the treatment of *S. dulcis* with various external stimuli were semi-quantitatively examined by RT-PCR analysis. The leaves of *S. dulcis* were detached from the middle part of the stem, and they were incubated with MJ (100 μM water, Wako Pure Chemicals), 2-chloroethylphosphonic acid (2-CEPA), an ethylene generating reagent (10 mg ml^{-1}, Sigma), or Ca^{2+}-ionophore A23187 (1 μM, Sigma) at 26°C according to the method described previously (Kasidimoko et al., 2005; Asakura and Kurosaki, 2007). Control treatments received only water instead of these chemicals, and, in some experiments, the leaf tissues were incubated at 42°C or in 400 mM NaCl solution. At regular intervals, the treated leaves were harvested and total RNA was isolated according to the
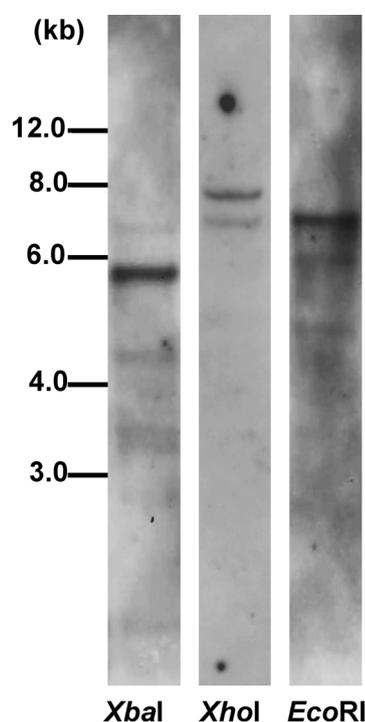

Figure 2. Genomic DNA gel blot hybridization analysis of *Sdrac*. Genomic DNA isolated from *S. dulcis* was digested with *Xba*I, *Xho*I and *Eco*RI, and, after the separation by electrophoresis, the DNA fragments were probed with the labeled nucleotides corresponding to the coding region of *Sdrac*.

method described above. Aliquots of RNA solutions (approximately 0.5 µg RNA equivalent) were subjected to RT reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche), and then, PCR was carried out with the primer pair, 5′-ATG AGT GCA ACA AGG TTC ATT AAG TG-3′ as the forward and 5′-TTA AAA AAT AGC GCA GCC TTT GTT CC-3′ as the reverse primer (591 mer as the product). In the parallel experiments, 5′-TCT ACA ATG AGC TCC GTG TTG C-3′′ and 5′-TGC CAC GAC CTT AAT CTT CAT GC-

3′ were used as the primers for the amplification of actin gene fragments as the controls (GenBank accession number AB290348, 725 mer as the product). Significant expression of *Sdrac* was detected in roots, stems and leaves, and the levels in these tissues seemed to be almost comparable (Figure 3A). Transcriptional activity of *Sdrac* slightly decreased after 3 h of MJ treatment, and then, it turned to appreciable increase (Figure 3B). The relatively high expression level of *Sdrac* was maintained from 6 to 24 h of the incubation in the presence of MJ. In
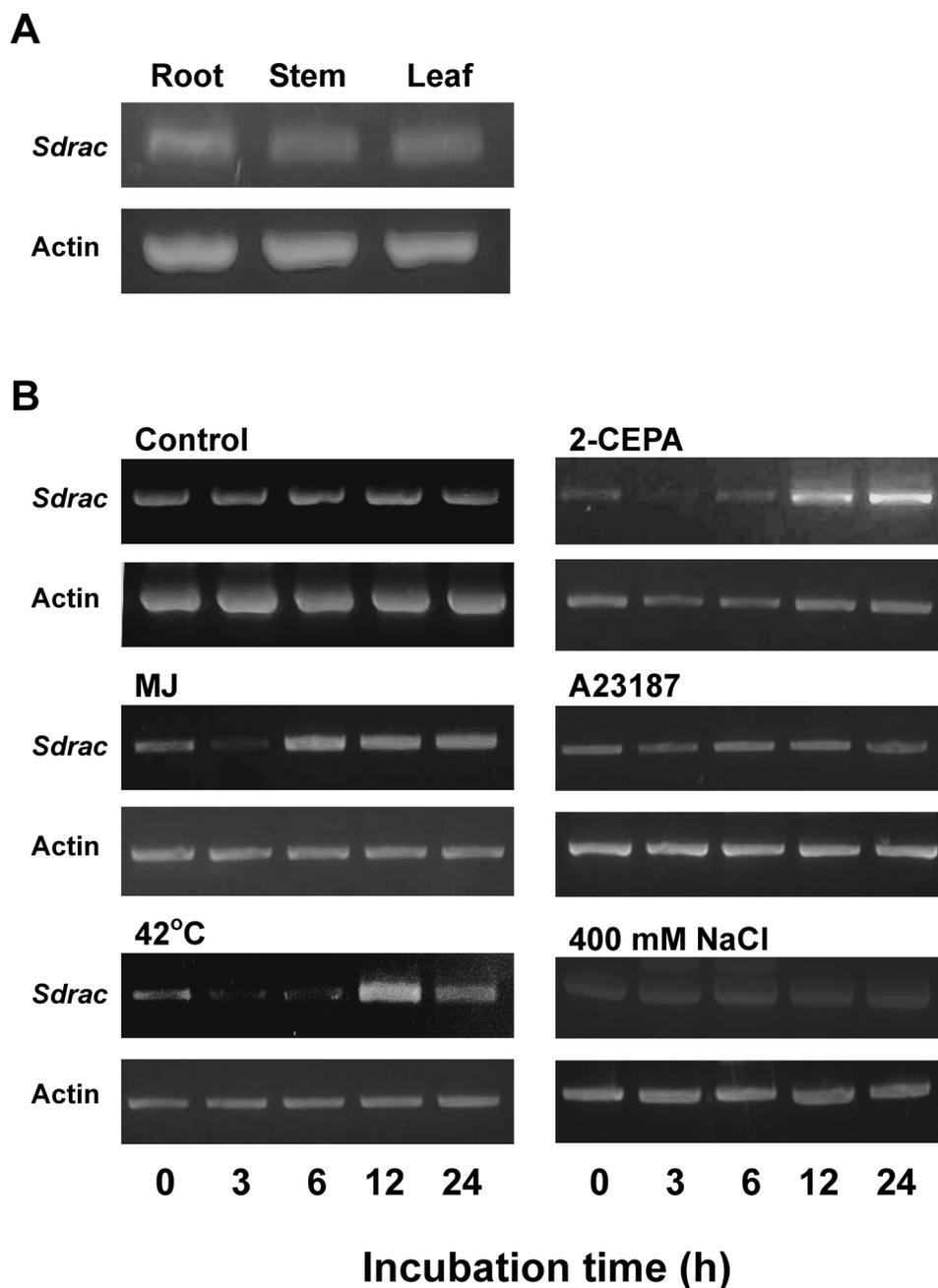

Figure 3.   A, Tissue specific expression of *Sdrac* gene. RNA samples were prepared from root, stem and leaf tissues of *S. dulcis*, and the expression levels of *Sdrac* were tested by RT-PCR. Actin gene of *S. dulcis* was also amplified as the control. B, Changes in the transcriptional activities of *Sdrac* after stimulation or under stress conditions. Expression levels of *Sdrac* were examined after the treatment of the leaf tissues of *S. dulcis* with various stimuli, or under temperature- and salt-stress conditions. At regular intervals, RNAs were prepared from the treated leaf tissues, and, after reverse-transcription reaction, the transcriptional levels of *Sdrac* and actin genes were determined by PCR amplification.

contrast, the control treatment did not show the significant change as far as tested. We showed previously (Kurosaki et al. 1992) that the induction of defense-related responses accompanying the activation of Ca^{2+}-cascade could be mimicked by the treatment of plant cells with an ethylene-generating reagent, 2-chloroethylphosphonic acid (2-CEPA). In 2-CEPA-treated tissues, as was in MJ, the expression of *Sdrac* transiently decreased after 3–6 h of the treatment, and then, the marked increase in the transcriptional level was observed. In sharp contrast, however, expression activity of *Sdrac* showed only very slight change, if any, in the presence of Ca^{2+}-ionophore A23187, and it was maintained at almost constant level within the experimental period. The elevation of *Sdrac* expression was also observed by the incubation of the leaf tissues at high temperature (42°C), while no obvious change was observed in the presence of 400 mM NaCl (Figure 3 B). In repeated experiments, a similar set of the results was reproducibly observed for the expression of *Sdrac* toward these stimuli and stresses.

In the present study, we have isolated a cDNA clones, *Sdrac*, as a Rac/Rop GTPase gene from *S. dulcis* (Figure 1A), and shown that, unlike in many other plants, Rac/Rop subfamily of *S. dulcis* would consist of a few homologous genes (Figure 2). We have previously reported (Saitoh et al. 2007) that CAM gene of *S. dulcis* occurs as a one copy gene in the genome, and therefore, this plant is very unique in which some of the signal transduction-relating events would be mediated by only one or a few isoforms of theses functional proteins. The expression levels of *Sdrac* appreciably elevate in response to MJ- and 2-CEPA-treatment (Figure 3B). These observations suggest that *Sdrac* product plays roles in MJ- and 2-CEPA-induced signal transduction processes accompanying the change in the transcriptional activity of the gene.

It is well known (Yang 2002; Berken 2006; Yang and Fu 2007) that plasma membrane targeting by prenylation, especially protein geranylgeranylation, is usually a prerequisite for the proper activation of Rac/Rop GTPases in plants. Although *Sdrac* product is apparently lacking in the post-translational modification site for prenylation near the C-terminal (CXXL, Figure 1A), heterogeneous prenylation sites of Rac/Rop proteins, the putative motif CAA in maize (Ivanchenko et al. 2000) and CTAA and CGKN in *Arabidopsis* (Li et al. 2001), have been recently reported. Moreover, it was demonstrated that *Arabidopsis* Rop10 localizes to the plasma membrane in a prenylation-independent manner (Lavy et al. 2002). Further studies on the activation mechanisms of the Rac/Rop GTPase in *S. dulcis* and isolation of the other GTPase genes are in progress in our laboratory.

References

Asakura Y, Kurosaki F (2006) Cloning and expression of *Dcga* gene encoding α subunit of GTP-binding protein in carrot seedlings. *Biol Pharm Bull* 30: 1800–1804

Berken A (2006) Rho GTPases in plants—Molecular mechanisms and signaling pathways in the process of fertilization. *Cell Mol Life Sci* 63: 2446–2459

Creelman RA, Mullet ME (1997) Biosynthesis and action of jasmonates in plants. *Ann Rev Plant Physiol Plant Mol Biol* 48: 355–381

Fujiwara M, Umemura K, Kawasaki T, Shimamoto K (2006) Proteomics of Rac GTPase signaling reveals its predominant role in elicitor-induced defense response of cultured rice cells. *Plant Physiol* 140: 734–745

Gu Y, Wang Z, Yang Z (2004) ROP/RAC GTPase: an old new master regulator for plant signaling. *Curr Opin Plant Biol* 7: 527–536

Ivanchenko M, Vejlupkova Z, Quatrano RS, Fowler JE (2000) Maize ROP7 GTPase contains a unique, CaaX box-independent plasma membrane targeting signal. *Plant J* 24: 79–90

Kasidimoko NM, Kurosaki F, Lee JB, Hayashi T (2005) Stimulation of calcium signal transduction results in enhancement of production of scopadulcic acid B by methyl jasmonate in the cultured tissues of *Scoparia dulcis*. *Plant Biotechnol* 22: 333–337

Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H, Shimamoto K (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. *Proc Natl Acad Sci USA* 96: 10922–10926

Kurosaki F (1997) Role of inward K^+ channel located at carrot plasma membrane in signal cross-talking of cAMP with Ca^{2+} cascade. *FEBS Lett* 408: 115–119

Kurosaki F, Nagase H, Nishi A (1992) Stimulation of anthraquinone production in rhubarb tissue culture by an ethylene-generating reagent. *Phytochemistry* 31: 2735–2738

Kurosaki F, Nishi A (1993) Stimulation of calcium influx and calcium cascade by cyclic AMP in cultured carrot cells. *Arch Biochem Biophys* 302: 144–151

Lavy M, Bracha-Drori K, Sternberg H, Yalovsky S (2002) A cell-specific, prenylation-independent mechanism regulates targeting of type II RACs. *Plant Cell* 14: 2431–2450

Li H, Shen J, Zheng Z, Lin Y, Yang Z (2001) The Rop GTPase switch controls multiple developmental processes in *Arabidopsis*. *Plant Physiol* 126: 670–684

Li H, Wu G, Ware D, Davis R, Yang Z (1998) *Arabidopsis* Rho-related GTPases: Differential gene expression in pollen and polar localization in fission yeast. *Plant Physiol* 118: 407–417

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. *Plant Physiol* 138: 1903–1913

Saitoh D, Asakura Y, Kasidimoko NK, Shite M, Sugiyama R, Lee JB, Hayashi T, Kurosaki F (2007) Cloning and expression of calmodulin gene in *Scoparia dulcis*. *Biol Pharm Bull* 30: 1161–1163

Takezawa D, Liu ZH, An G, Poovaiah BW (1995) Calmodulin gene family in potato: developmental and touch-induced expression of the mRNA encoding a novel isoform. *Plant Mol Biol* 27: 693–703

Valster AH, Hepler PK, Chernoff J (2000) Plant GTPases: the Rhos in bloom. *Trends Cell Biol* 10: 141–146

Vernoud V, Horton AC, Yang Z, Nielsen E (2003) Analysis of the

small GTPase gene superfamily of *Arabidopsis*. *Plant Physiol* 131: 1191–1208

Winge P, Brembu T, Kristensen R, Bones AM (2000) Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. *Genetics* 156: 1959–1971

Yamakawa H, Mitsuhara I, Ito N, Seo S, Kamada H, Ohashi Y (2001) Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plants. *Eur J Biochem* 268: 3916–3929

Yang Z (2002) Small GTPases, versatile signaling switches in plants. *Plant Cell* 14: S375–S388

Yang Z, Fu Y (2007) ROP/RAC GTPase signaling. *Curr Opin Plant Biol* 10: 490–494

Zheng ZL, Yang Z (2001) The Rop GTPase: an emerging signaling switch in plants. *Plant Mol Biol* 44: 1–9