Rac-type small GTPases are known to function in some cellular processes in plants. To further understand the involvement of Rac type GTPases in plant development, we isolated from cultured Zinnia cells a gene (ZeRAC2) encoding a new Rac-type small GTPase. ZeRAC2 mRNA accumulates preferentially in xylem parenchyma and tracheary element precursor cells, and surprisingly the accumulation is restricted to the site facing developing tracheary elements.

Keywords: Rac/Rho subfamily — Small GTPase — Xylem differentiation — Zinnia elegans.

Introduction

Small GTPases act as molecular switches that regulate a variety of cellular processes by transducing signals in the GDP-bound form, which reverts to an inactive GDP-binding form after GTP hydrolysis. The Ras superfamily of small GTPases are normally categorized into five subfamilies; Ras, Rab/ypt, ARF, Ran and Rho (Hall 1998, Ridley 2001). In plants, many members of the Rho subfamily have been found to be expressed specifically during the deposition of the secondary wall in cotton fiber formation (Delmer et al. 1995, Potikha et al. 1999). Like the oxidative burst in the mammalian neutrophils (Abo et al. 1991, Knaus et al. 1991), in plants reactive oxygen species (ROS) are thought to be produced by an NADPH oxidase complex, although molecular evidence for this is still poor. In tobacco and tomato cells, a Rac homologue was identified immunologically as a component of the NADPH oxidase complex associated with ROS production (Kieffer et al. 1997, Xing et al. 1997). A series of experiments with rice, exploiting constitutive active and dominant negative mutants of OsRac1, revealed that OsRac1 regulates ROS production and then induces cell death with apoptotic characteristics (Kawasaki et al. 1999, Ono et al. 2001).

Another physiological function of plant Rac GTPases has been established in the regulation of the elicitor-induced oxidative burst in the hypersensitive response of plants (Park et al. 2000, Xing et al. 1997). Like the oxidative burst in the mammalian neutrophils (Abo et al. 1991, Knaus et al. 1991), in tobacco and tomato cells, Rac homologues are expressed only in mature pollen and hypocotyls (Winge et al. 1997). Arabidopsis and soy bean cell cultures that were transformed with the constitutive active GhRac13 produced H₂O₂, whereas transformation with the dominant negative GhRac13 resulted in a decreased H₂O₂ level. Initiation of secondary wall formation accompanied with the increased level of H₂O₂ was also reported by Potikha et al. (1999). These observations suggest that a specific Rac regulates ROS production, which triggers the process of secondary wall formation.

Plant Rac proteins are also involved in the developmental process of secondary wall formation. In cotton, GhRac13 was found to be expressed specifically during the deposition of the secondary wall in cotton fiber formation (Delmer et al. 1995, Potikha et al. 1999). Arabidopsis and soy bean cell cultures that were transformed with the constitutive active GhRac13 produced H₂O₂, whereas transformation with the dominant negative GhRac13 resulted in a decreased H₂O₂ level. Initiation of secondary wall formation accompanied with the increased level of H₂O₂ was also reported by Potikha et al. (1999). These observations suggest that a specific Rac regulates ROS production, which triggers the process of secondary wall formation.

Plant Rac GTPases are often expressed in a tissue or developmental stage specific manner. For example, AtRAC6/AtRac2 and PsRop1 are expressed only in mature pollen and pollen tube, respectively (Kost et al. 1999, Lin et al. 1996), whereas AtRac2 is expressed exclusively in stems, roots and hypocotyls (Winge et al. 1997). AtRAC3/At-Rac1 is ubiquitously expressed but at a much higher level in guard cells than in the surrounding epidermal cells (Lemichez et al. 2001). GsRac13 is expressed specifically in cotton fibers undergoing transition from primary to secondary wall synthesis (Delmer et
Xylem specific rac GTPase from *Zinnia* al. 1995). These results suggest that some Rac GTPase genes that play specific developmental roles are expressed in a developmental stage specific manner.

Xylem differentiation is a well-studied plant cell differentiation. In vitro experiments with cultured *Zinnia* cells have displayed key events of xylem differentiation (Fukuda 1997) including induction by a combination of auxin and cytokinin (Fukuda and Komamine 1980), final initiation of differentiation by endogenous brassinosteroids (Yamamoto et al. 1997, Yamamoto et al. 2001), dramatic rearrangement of actin filaments leading, eventually, to patterned secondary wall thickenings (Kobayashi et al. 1987, Kobayashi et al. 1988, Fukuda and Kobayashi 1989), biosynthesis of lignin (one of the substrates for which, H$_2$O$_2$ is supplied by NADPH oxidase) (Sato et al. 1993, Sato et al. 1995, Sato et al. 1997, Ogawa et al. 1997, Barceló 1998), and programmed cell death accompanied with the vacuole collapse and nuclear degradation (Minami and Fukuda 1995, Ye and Varner 1996, Aoyagi et al. 1998, Kuriyama 1999, Obara et al. 2001). However, the molecular mechanism governing such events is still unknown. Interestingly, the xylem cell differentiation process involves putative Rac-controlling events such as actin rearrangement, secondary wall thickenings, programmed cell death and NADPH oxidase-dependent H$_2$O$_2$ production. Therefore we suspected that Rac type GTPases might somehow regulate xylem differentiation process. Thus, using the *Zinnia* cell culture system, we attempted to isolate Rac genes that are expressed specifically in developing xylem cells, and these genes are expected to be involved in xylem differentiation.

In this paper, we report a Rac GTPase gene, *ZeRAC2*, whose mRNA accumulates preferentially in xylem cells. Its gene product has GTP-binding and GTPase activity, and is localized to the plasma membrane. We also found that *ZeRAC2* mRNA accumulates in xylem parenchyma cells and tracheary element (TE) precursor cells in an asymmetric manner such that the accumulation site of *ZeRAC2* mRNA faces the developing TEs.

**Results**

**Isolation of cDNA clones of Rac/Rho GTPase from *Zinnia elegans***

We planned to isolate Rho/Rac small GTPases that function specifically in vascular differentiation. Based on conserved protein motifs in the N-terminal part of the Rho/Rac small GTPases, we isolated two full-length cDNA for Rho/Rac Fig. 1 (A) A neighbor-joining tree of plant Rho/Rac GTPases from various species created with Prodist program. Yeast CDC42, Rho1 and mouse Rac1 were selected as outgroups. The protein region analyzed corresponds to amino acids 11–187 of *ZeRAC2*. To evaluate the confidence limits of the internal branches of the tree, a bootstrap analysis with 1,000 replications was performed on the data set. The scale bar indicates the number of amino acid substitutions per site. Abbreviations: At, *Arabidopsis thaliana*; Bv, *Beta vulgaris*; Gh, *Gossypium hirsutum*; Lj, *Lotus japonicus*; Mm, *Mus musculus*; Os, *Oryza sativa*; Ps, *Pisum sativum*; Sc, *Saccharomyces cerevisiae*. (B) Amino acid sequence alignment of *ZeRAC2*, *Pisum sativum* Rop1, *Arabidopsis thaliana* AtRAC1, AtRAC6 and *Beta vulgaris* Rac1, which belong to the same subgroup.
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We performed further investigation on one of the clones designated ZeRAC2 (Fig. 1). The deduced protein sequence of ZeRAC2 was composed of 197 amino acid residues with a calculated molecular mass of 21.4 kDa. Phylogenetic analysis showed that ZeRAC2 belongs to a sub-group including PsRop1, AtRAC1, AtRAC6 and BvRho1 (Fig. 1B). The deduced amino acid sequence of ZeRAC2 has 93.4%, 94.4%, 93.4%, 94.4% identity with that of PsRop1, AtRAC1, AtRAC6 and BvRho1, respectively (Fig. 1B). GTP/GDP-binding and GTPase domains (residues 13–21, 60–65, 118–122, 157–162), effector domain (residues 38–44) and geranylgeranylation site in C-termini (residues 194–197) were all conserved (Fig. 1B).

Developing TE-specific accumulation of ZeRAC2 mRNA

Accumulation of ZeRAC2 mRNA was examined in Zinnia cells cultured for varying periods in D medium. In this culture, TEs with obvious secondary walls began to appear at 48 h, and approximately 40% of the cells differentiated into TEs by 72 h (Fig. 2). Total RNAs were blotted, and hybridized with probes transcribed from 3′-RACE product of ZeRAC2, which has low homology with other Rac GTPase genes. We confirmed that this probe does not hybridize other GTPase genes with Southern hybridization. The accumulation pattern of ZeRAC2 mRNA in the culture is highly dependent on differentiation stages. In D culture, a transient increase in ZeRAC2 mRNA was observed at 48 h, immediately before the onset of secondary wall formation linked to TE differentiation (Fig. 2A, B). Thereafter, ZeRAC2 mRNA decreased rapidly. In contrast, ZeRAC2 mRNA was not significantly accumulated throughout the culture period in C medium where cells divide but rarely differentiate into TEs (Fig. 2A, B). These results suggest that ZeRAC2 is expressed specifically in developing TEs.

Localization of transcripts of ZeRAC2 in planta

To reveal cell-specific accumulation of ZeRAC2 mRNA in planta, in situ hybridization was carried out with a DIG-labeled single-stranded antisense RNA probe of ZeRAC2. Apical regions of 14-day-old Zinnia seedlings which contain two fully developed first leaves and several juvenile leaves were cross-sectioned. ZeRAC2 mRNA was localized predominantly to vascular bundles (Fig. 3). Control hybridization with sense probe gave no signal (data not shown). In the vascular bundles of second leaves in which TEs with lignified cell walls developed, a strong signal was detected in xylem and phloem cells (Fig. 3B, C, D). As vascular bundles developed, the signals were more restricted to specific xylem and phloem cells. In particular, the signal was restricted to xylem and phloem cells. In particular, the signal was detected in developing TEs which had not yet lignified (Fig. 3E, F, G asterisks). Signal was also detected in TE precursor cells (Fig. 3I) and xylem parenchyma cells adjacent to developing TEs (Fig. 3H, I, K). Localization of the signal in cells adjacent to TEs was confirmed by longitudinal section (Fig. 3L, M). The vascular tissue-specific accumulation of ZeRAC2 mRNA was also observed in the cotyledons, hypocotyl and root of 1-day-old Zinnia seedlings (data not shown). Interestingly, the signal of ZeRAC2 transcripts within these cells was localized to the site facing the developing TEs. These polarized signals were not observed when other probes such as antisense constructs of TED2, TED3, TED4, ZCP4, ZeH83 and ZEN1 were used (Demura and Fukuda 1993, Demura and Fukuda 1994, Nishitani et al. 2001, Sassa et al. unpublished data).

ZeRAC2 protein as a small GTPase

To examine whether the encoded protein product of ZeRAC2 displays characteristics of small GTPases, ZeRAC2 protein fused to glutathione S-transferase (GST) was expressed in E. coli and purified. Fig. 4A shows a time course of GTP

Fig. 2 Temporal pattern of accumulation of the ZeRAC2 mRNA during in vitro TE differentiation. (A) Total RNA was extracted from Zinnia cells cultured for indicated periods in C and D media. The RNA was blotted on nylon membrane and hybridized with an antisense probe of ZeRAC2. Ethidium bromide-stained gel shows equal loading of RNA. The ethidium bromide-stained gel is shown below. (B) Time course of TE differentiation. The rate of TEs was defined as the percentage of TEs against total living cells plus TEs. Note that accumulation of ZeRAC2 mRNA occurs before a rapid increase in the rate of TEs. Each point represents the mean result from three samples and vertical lines show standard deviations.
Fig. 3  Tissue-specific expression of ZeRAC2. In situ hybridization of antisense ZeRAC2 to the shoot apical region of a 14-day-old seedling. (A) A cross-section of the shoot apical region of a 14-day-old seedling hybridized with the antisense ZeRAC2 probe. (B and C) Images featuring the same immature vascular bundle of a lateral vein of the second leaf. (B) A cross-section hybridized with the antisense ZeRAC2 probe. (C) Lignified TEs visualized by autofluorescence under UV light. The boxed part in B is magnified in D. (E and F) Images featuring the same mature vascular bundle of lateral vein of first leaf. (E) A cross-section hybridized with the antisense ZeRAC2 probe. (F) Lignified TEs visualized by autofluorescence of lignin under UV light. The boxed part in E is magnified in G. (H and I) Images featuring the same mature vascular bundle of a mid vein of the first leaf. (H) A cross-section hybridized with the antisense ZeRAC2 probe. (I) Lignified TEs visualized by autofluorescence under UV light. The boxed parts in H are magnified in J and K. (L) An image of a longitudinal section of vascular bundle of a lateral vein of the first leaf. The boxed parts in L are magnified in M. Arrows indicate signals detected on xylem cells. Arrowheads indicate signals detected on phloem cells. Asterisks in E, F and G indicate the same cell. Bars: A, 500 μm; B, C, E, F, H, I and L, 50 μm; D, G, J, K and M, 10 μm.
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binding to the GST–ZeRAC2 fusion protein. To minimize complications from GTP hydrolysis during the assay of GTP binding, GTP/S, a nonhydrolyzable analog, was incubated with purified GST–ZeRAC2 or GST protein. The result indicated that ZeRAC2 stoichiometrically bound to GTP, while GST did not bind to GTP. The intrinsic GTPase activity of ZeRAC2 was also assayed (Fig. 4B). GST–ZeRAC2 was preloaded with [γ-32P]GTP (open square) or [35S]GTP/S (open circle), and GTP hydrolysis by GST–ZeRAC2 was measured at the different time points. Each point represents the mean result from three samples and vertical lines show standard deviations.

Subcellular localization of ZeRAC2

Next, we examined the subcellular localization of the ZeRAC2 proteins. For this purpose, we made cDNA constructs encoding ZeRAC2 and its mutant that are fused to GFP at the N-terminus of ZeRAC2, and introduced them by particle bombardment into Zinnia cells cultured for 40 h in D medium. After subsequent culture for 20 h, subcellular localization of the GFP–ZeRAC2 was observed under a confocal microscope. In most cells, GFP–ZeRAC2 was clearly localized to the plasma membrane (Fig. 5A–D). It was also localized to the newly formed septum (Fig. 5A, B). In contrast, GFP fusion of a mutant ZeRAC2 whose CSIL motif in the C-terminus was deleted failed to be localized to the plasma membrane and stayed in the cytoplasm (Fig. 5E, F). It is known that the CXXL motif at the C-terminus directs geranylgeranylation and subsequent methylation of the cystein residue with accompanying cleavage of the XXL terminal residues, and the geranylgeranyl side chain anchors small GTPases to the plasma membrane (Glomset and Farnsworth 1994). Therefore, unlike animal Rac GTPases which reside in the cytoplasm and shuttle to the plasma membrane in response to stimuli, ZeRAC2 seems to be localized constitutively to the plasma membrane.

Discussion

In this study, we cloned the cDNA of a Rac-type small GTPase from Z. elegans that is expressed specifically in xylem differentiation in Zinnia cultured cells and plants. The deduced amino acid sequence of ZeRAC2 revealed that it has four GTP/GDP binding and GTP hydrolysis domains and an effector domain, all of which are highly conserved in Rac GTPases (Valencia et al. 1991). As expected from these conserved sequence motifs, the recombinant GST–ZeRAC2 protein had both GTP binding and hydrolysis activity, indicating that ZeRAC2 encodes a GTPase similar to those in animals. ZeRAC2 also has a C-terminal amino acid sequence (CSIL) that is expected to direct geranylgeranylation for membrane anchoring (Glomset and Farnsworth 1994, Schafer and Rine 1992). A different sub-group of plant Rac GTPases has a distinct sequence in a polybasic domain proximal to the isoprenylation site at the C-terminus and shows a distinct subcellular localization (Bischoff et al. 2000). AtRAC6/At-Rac2, AtRAC11/Rop1At and AtRAC3/Rop6At are localized to the plasma membrane, whereas AtRAC4/Rop2At and AtRAC5/Rop4At are preferentially localized to the perinuclear region (Kost et al. 1999, Bischoff et al. 2000). An anti-PsRop1 antibody recognized the Rop GTPase at the tonoplast of pea tapetal cells (Lin et al. 2001). ZeRAC2 phylogenetically belongs to the subgroup composed of AtRAC1, AtRAC6, AtRAC11, PsRop1 and BvRac1, and ZeRAC2 has a C-terminal sequence homologous to those of AtRAC1, 6 and 11. Our experiment with a GFP fusion protein demonstrated that ZeRAC2 was localized to the plasma membrane. These observations suggest that this subgroup of GTPases may function at the plasma...
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AtRAC6/At-Rac2, AtRAC11/AtRop1 and PsRop1 are expressed preferentially in the pollen tube, and their products are localized on the plasma membrane of the growing pollen tube tip to function in the tip growth. However, we demonstrated that ZeRAC2 mRNA accumulates only under xylogenesis-inducing conditions in vitro, in temporal association with TE differentiation. In plants, the preferential accumulation of ZeRAC2 mRNA was observed in TE precursor cells and xylem parenchyma cells as well as phloem cells. The results from in vitro and in vivo experiments are well correlated with each other and strongly suggest that ZeRAC2 functions in xylem development.

Interestingly, ZeRAC2 mRNA was localized unequally in the cytoplasm. The mRNA was enriched at the site facing developing TEs. Because mRNA for vascular tissue-specific genes tested so far were localized evenly in the cytoplasm (Demura and Fukuda 1994, Domingo et al. 1998, Nishitani et al. 2001, Nishitani et al. 2002), this asymmetric subcellular localization may be specific to ZeRAC2 mRNA. The asymmetric RNA localization within a cell has been reported in a variety of organisms including animals (Berleth et al. 1988, Oleynikov and Singer 1998), yeast (Takizawa et al. 1997) and even plants (Bouget et al. 1996, Im et al. 2000). Such an asymmetric localization of a specific mRNA often results in an asymmetric localization of its corresponding protein. Therefore, the ZeRac2 protein might be localized asymmetrically in xylem parenchyma and TE precursor cells.

Although its mRNA was asymmetrically localized, the GFP–ZeRAC2 fusion protein was evenly localized in the plasma membrane in cultured Zinnia cells. RNA localization mechanisms involve a specific sequence in 3' UTR of the localized RNA and cytoskeletal element upon which the RNA travels (Oleynikov and Singer 1998). However, our GFP–ZeRAC2 construct does not have the 3'UTR region of ZeRAC2. In addition, overproduction of proteins expressed from a cauliflower mosaic virus (CaMV) 35S promoter may perturb their original subcellular localization. Therefore, GFP–ZeRAC2 may not display the original asymmetric subcellular localization of ZeRAC2 protein in the plasma membrane. Further analysis is needed to reveal the original subcellular localization of ZeRAC2, for example, with a specific antibody.

What is the function of ZeRac2 GTPase? Our preliminary experiment in which an antisense ZeRAC2 gene is introduced transiently into differentiating xylem cells did not uncover much information about its function. However, the fact that...
ZeRAC2 mRNA in xylem parenchyma and TE precursor cells is localized at the site facing developing TEs suggests a cell-cell communication between developing TEs and its neighboring xylem cells. For example, xylem parenchyma cells are known to supply substrates for lignin synthesis to the cell walls of developing TEs (Hosokawa et al. 2001) and peroxide for the polymerization of lignin precursors is produced by NADPH oxidases (Ogawa et al. 1997). Therefore if ZeRAC2 GTPase might activate a NADPH oxidase supplying H$_2$O$_2$ to cell walls of developing TEs, the GTPase should be located on the plane facing developing TEs. This speculation is consistent with the fact that ZeRAC2 protein is localized to the plasma membrane. Further analysis about ZeRAC2 GTPase function in xylem differentiation is needed to verify this hypothesis.

In conclusion, we demonstrated that mRNAs for a Rac-type small GTPase, ZeRAC2 accumulated preferentially in xylem cells and unevenly in their cytoplasm. Our findings of the xylem cell-specific and asymmetric subcellular localization of ZeRAC2 mRNA cast a new light on understanding Rac function in xylem differentiation.

Materials and Methods

Plant materials and culture methods

Z. elegans cv Canary Bird (Takii Shubyco, Kyoto) was used in all experiments. The isolation and culture of Zinnia mesophyll cells were performed as described in Fukuda and Konomine 1980. We used two culture media, D (differentiation) medium containing 0.1 mg liter$^{-1}$ 1-naphthaleneacetic acid (NAA) and 0.2 mg liter$^{-1}$ BA as a non-inductive medium (Sugiyama and Fukuda 1995).

Molecular cloning of full-length ZeRac2 cDNA

For 3'-rapid amplification of cDNA end (RACE), a first-strand cDNA fragment was reverse-transcribed from total RNA prepared from Zinnia cells cultured for 48 h in D medium with poly A adapter primer (5'-GGCCACCGCTGACAGTACTT-3') and subjected to PCR amplification with UAP primer (5'-CUACUACACUGCACCGCTGACAGTACT-3') and R1 degenerate primer (5'-GTGGNGAYGGGIGGIGTGNNARAC-3') (Yang and Watson 1993). For 5'-RACE, first-strand cDNA was synthesized with 3481-9-1 specific primer (5'-TGGAAACGTTCTCATGGCTAGCCTT-3') designed from the nucleotide sequence of 3'-RACE clone and tailed by deoxyadenine. First PCR amplification was performed with this cDNA as a template and with 3481-9-1 and dTadp2 (GAGTCGACTGCAGA-AGCTG-3') as primers. Booster PCR amplification was performed with the first PCR product as a template and with 3481-9-2 (5'-GGAATTCCTCCGATAACTCAACCCGTCGCT-3') and adp2 (GAGTCGACTGCAGA-AGCTG-3') as primers. To obtain a full-length ZeRAC2 cDNA clone, a first-stand cDNA was synthesized with dTadp2 primer and subjected to PCR amplification with specific primers designed from the nucleotide sequence of 3'- and 5'-RACE clone. A PCR fragment for the full-length ZeRAC2 cDNA was cloned into pBluescript II TSK (Ichihara and Kurosawa 1993).

RNA gel blot analysis

Total RNA was isolated as described in Ozeki et al. (1990). Ten µg of total RNA was separated electrophoretically on 1.2% agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim/Roche, Basel, Switzerland), and hybridized under highly stringent conditions with DIG-labeled antisense/sense RNA probes that were prepared from the cDNA clone of the 3'-RACE. Hybridization signals were visualized immunologically with an anti-DIG Fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim/Roche) according to the manufacturer’s instructions.

In situ hybridization

Fourteen-day-old plants were used for in situ hybridization. Sections (8 µm) were prepared as described in Demura and Fukuda (1994). Hybridization was performed according to the method of Nishitani et al. (2001). Probes were the same as those used in RNA gel blot analysis.

Sequence alignment and construction of a phylogenetic tree

ZeRAC2 cDNAs were sequenced with ABI PRISM$^\text{TM}$310 Genetic Analyzer (The Perkin-Elmer Corporation, Foster City, CA, U.S.A.). The deduced amino acid sequence of ZeRAC2 was aligned manually with the corresponding sequences taken from the GenBank database. The phylogenetic analysis of the genes was done using PAUP ver.4.0 beta (Swofford 1998). The tree was generated by neighbor-joining method by pairwise distance matrix using Kimura-2-parameters method (Kimura 1980). Bootstrap analysis were calculated by 1,000 replications.

GTP binding and GTPase assay

A full length ZeRAC2 cDNA was cloned in the expression vector, pGEX-4T-2 (Amersham, Pharmacia, Upsala, Sweden), and transformed into Escherichia coli, BL21. From E. coli extracts, GST-fusion protein was purified by glutathione Sepharose beads (Amersham, Pharmacia) and used for assays of GTP-binding and GTPase activities according to published protocols (Kikuchi et al. 1995). For the assay of the GTP-binding activity, the binding of the [32P]GTP to GST-ZeRAC2 was measured. For the assay of the GTPase activity, GST-ZeRAC2 was incubated with [γ-32P]GTP and the release of 32P was measured at different time points.

Particle bombardment

Particle bombardment was performed as described in Kost et al. (1998) with some modifications. Zinnia cells cultured in D medium for 40 h were filtrated onto 10 µm nylon mesh and transferred upside-up onto D medium solidified with 0.5% agarose in 5.5 cm Petri dishes. Particle bombardment was performed using a helium-driven particle accelerator (PDS-1000/He; BIO-RAD, Hercules, CA, U.S.A.) with all basic adjustments set according to the manufacturer's recommendations. ZeRAC2 was incubated with [γ-32P]GTP and the release of 32P was measured at different time points.

Confocal microscopy

Confocal imaging of GFP-fused ZeRAC2 in Zinnia cells was performed using an LSM 410 inverted confocal microscope (Carl Zeiss Inc., Thornwood, NY, U.S.A.) as described in Kost et al. (1998).
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References


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