The small GTPase AtRAC2/ROP7 is specifically expressed during late stages of xylem differentiation in *Arabidopsis*

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Received 18 January 2005; Accepted 29 May 2005

Abstract

The RAC/ROP family of small GTPases are central regulators of important cellular processes in plants. *AtRAC2/ROP7* is an ancient member of the RAC/ROP gene family in *Arabidopsis thaliana* whose functions are generally unknown. In order to study the spatial expression pattern of the *AtRAC2/ROP7* gene, transgenic plants expressing GUS or GFP under the control of the *AtRAC2/ROP7* promoter were analysed. Functional analysis of *AtRAC2/ROP7* was done using transgenic plants overexpressing wild-type and constitutively activated *AtRAC2/ROP7* (Val15Gly), and an *AtRAC2/ROP7*-DNA insertion mutant. The *AtRAC2/ROP7* promoter directs a highly specific xylem-specific expression in the root, hypocotyl, stem, and leaves. The expression is developmentally limited to the late stages of xylem differentiation, and coincides with the formation of secondary cell walls. Leaf epidermal cells of transgenic plants overexpressing constitutively active *AtRAC2/ROP7* exhibited highly impaired lobe formation, suggesting that *AtRAC2/ROP7* is able to regulate polar cell expansion. Finally, GFP-*AtRAC2/ROP7* fusion proteins were localized to the plasma membrane. The results indicate a role for *AtRAC2/ROP7* in the development of secondary cell walls of xylem vessels.

Key words: GTP-binding protein, Rac, secondary cell wall, xylem.

Introduction

The RHO protein family belongs to the RAS superfamily of small GTPases. These proteins function as molecular switches, being active when binding GTP and inactivated upon hydrolysis of GTP to GDP (Bourne *et al*., 1991). During the last decade, the RHO family of small GTPases (RHO, RAC, and Cdc42) has emerged as key regulators of a number of cellular processes. Among these are organization of the actin cytoskeleton, regulation of superoxide production in defence responses, stress-induced signal transduction, and cell growth and differentiation (Hall, 1998; Aspenström, 1999; Ridley, 2001). In plants, a large family of RAC-like genes has been identified (Winge *et al*., 1997, 2000; Yang, 2002; Christensen *et al*., 2003). This family can be further divided into at least two subfamilies (subgroup I and subgroup II) on the basis of sequence differences and the presence of an additional intron in the members of subgroup II (Winge *et al*., 2000; Christensen *et al*., 2003). The *Arabidopsis thaliana* RAC/ROP proteins ROP1/AtRAC1 and At-Rac2/AtRAC6 have been reported to be involved in polar cell growth through regulation of vesicle secretion to the pollen tube tip and organization of the actin cytoskeleton (Li *et al*., 1999; Kost *et al*., 1999). Recent work on *Arabidopsis thaliana* root hairs and leaf epidermal cells points toward a general role for plant RAC/ROP proteins in polar cell growth (Molendiijk *et al*., 2001; Jones *et al*., 2002; Fu *et al*., 2002). Reports indicate that plant RAC/ROP proteins participate in the regulation of NADPH oxidase activity (Kawasaki *et al*., 1999; Ono *et al*., 2001; Baxter-Burrell *et al*., 2002). The production of oxygen radicals and hydrogen peroxide by NADPH oxidase has been suggested as an important part of the defence response to microbial pathogens in plants (Levine *et al*., 1994), as well as in hormone responses and tolerance to oxygen deprivation (Neill *et al*., 2002; Overmyer *et al*., 2003). RAC/ROP-regulated production of hydrogen peroxide has also been implicated in the differentiation of secondary cell walls of cotton fibres (Delmer *et al*., 1995; Potikha *et al*., 1999). Furthermore, an antibody against Rop1Ps, a pea RAC/ROP protein, cross-reacted with a 25 kDa protein component of the active CLAVATA1

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complex in Arabidopsis, indicating that RAC/ROP proteins are involved in regulation of gene expression during stem cell differentiation in the shoot apical meristem (Trotchoud et al., 1999).

Xylem vessels, the vascular tubes responsible for water transport throughout the plant, are formed by tracheary elements (TEs). The developmental process that leads to the formation of TEs from their procambial progenitors is highly regulated, and includes cell division, elongation and specification, cell wall synthesis, lignification and programmed cell death (Kuriyama and Fukuda, 2001; Milioni et al., 2001). Few proteins have been identified that regulate vascular tissue identity, but the MYB transcription factor ALTERED PHLOEM DEVELOPMENT (APL) appears to play a dual role in vascular tissue development; it both promotes phloem differentiation and inhibits xylem differentiation (Bonke et al., 2003). During the last few years, there has been progress in understanding TE development. A member of the homeodomain-leucine zipper (HD-ZIP) family, ATHB-8, is expressed in the provascular cells and at early stages of vascular cell determination during revascularization (Baima et al., 1995). Overexpression of ATHB-8 in Arabidopsis promotes vascular differentiation, suggesting a role for this gene in regulation of the activity of the vascular meristems. However, ATHB-8 insertional mutants and ATHB-8 antisense plants did not show any phenotypic alterations, probably because of redundancy (Baima et al., 2001). Indeed, a closely related gene, INTERFASCULAR FIBRELESS (IFL)/REVOLUTA (REV), is also expressed in the vascular tissue as well as in interfascicular fibres of Arabidopsis plants. The ifl1 mutant lacks normal interfascicular fibres in stems, and also shows a loss of xylem fibre differentiation and reduced formation of TEs (Zhong et al., 1997; Zhong and Ye, 1999). Mutants have also been described that show defects in later stages of TE development. Three irregular xylem mutants, irx1, irx3, and irx5, all have reduced secondary cell wall formation, resulting in collapsed xylem vessels. IRX1, IRX3, and IRX5 encode the cellulose synthase catalytic subunits AtCesA8, AtCesA7, and AtCesA4, respectively (Taylor et al., 1999, 2000, 2003).

AtRAC2/ROP7 (At5g45970) is an ancient member of subgroup I of the AtRAC/ROP gene family, and may have evolved when vascular plants appeared (Winge et al., 2000; Christensen et al., 2003). In a previous study, the highest expression levels of AtRAC2/ROP7 were found in roots and stems (Winge et al., 1997). In order to study the function of AtRAC2/ROP7, transgenic plants were created that express the reporter genes GUS and GFP under the control of the AtRAC2/ROP7 promoter, as well as plants that overexpress wild-type and constitutively activated AtRAC2/ROP7. A T-DNA insertion mutant of AtRAC2/ROP7 has also been studied. The results indicate that the AtRAC2/ROP7 promoter directs a highly regulated expression, being active only in the primary xylem of roots, cotyledons, stems, and leaves; expression is concomitant with secondary cell wall formation. Leaf pavement cells of transgenic plants that overexpress constitutively active AtRAC2/ROP7 show strongly decreased lobe length, suggesting that AtRAC2/ROP7 has a role in regulation of the actin cytoskeleton.

Materials and methods

Plant material, growth conditions and transformation

All experiments in this study involve Arabidopsis thaliana, ecotype Columbia (Col-0). The atrac2rop7-1 mutant (line 212D04) was obtained from the GABI-Kat T-DNA insertion mutant collection (Rosso et al., 2003). Seeds were sterilized according to standard procedures. Plants were grown asepically on agar medium containing MS basal salt mixture (Sigma), 3% (v/w) sucrose, and 0.7% (v/w) agar (pH 5.8) at 22 °C under a light regime of 16/8 h light/dark cycles and 700 μmol m⁻² s⁻¹ illumination. Plants were also grown in autoclaved soil under the same temperature and light/dark cycles, and 500–600 μmol m⁻² s⁻¹ illumination. Arabidopsis plants were transformed using Agrobacterium vacuum infiltration (Bechtold et al., 1993).

GUS staining, histology and microscopy

Plants were grown on agar medium or in soil as described above. Specimens were fixed in ice-cold acetone for 3 min, incubated in 0.1 M sodium phosphate, pH 7.0 for 30 min, and subsequently submerged in a staining solution (0.1 M sodium phosphate pH 7.0, 1% (v/w) Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM disodium EDTA pH 8.0, and 0.2 mM X-gluc (Duchefa, Haarlem, Netherlands)). The samples were put under vacuum for 3 min and developed at 37 °C for 12 h. Chlorophyll was removed with ethanol (1×70% (v/v), 3×96% (v/v)). Small tissue blocks from 37-d-old plants were GUS-stained, and the tissue blocks were dehydrated and embedded in an acrylic resin (LR white, London Resin Company, London, UK) as described by Peleman et al. (1989). Semi-thin sections (2 μm) were stained with 2.0% (w/v) Safranin O for 20 s. Counter-staining with Mauëre reagent was performed according to standard protocols. For visualization of leaf pavement cells, the fourth rosette leaf from 2-week-old plants grown in vitro was cleared with chloral hydrate as described by Hamada et al. (2000) and observed using phase-contrast microscopy (Nikon S800). Images were captured with a cooled SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI). The perimeter and area of pavement cells larger than 1000 μm² were measured using analySIS 3.1 (Soft Imaging System, Münster, Germany). Scanning electron microscopy (SEM) was performed on a Hitachi 3000N fitted with a cold stage (Deben Research, Suffolk, UK).

GFP fluorescence microscopy

Plant tissue samples were placed on cover slips and mounted in water. Confocal laser scanning microscopy was performed on a Zeiss LSM510 (Zeiss, Jena, Germany). GFP fluorescence excited by Argon laser excitation light (488 nm) and DIC images were captured using LSM510 3.0 software. Digital images were processed in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

Cloning of the AtRAC2/ROP7 promoter and production of the constitutive activated gene construct

DNA was isolated from wild-type Arabidopsis plants with a modified minipreparation procedure adapted from Dellaporta et al. (1983). The atrac2rop7 promoter region was amplified by PCR with primers designed from the known upstream sequence of AtRAC2/ROP7 (GenBank accession no. AB006698), using Turbo Pfu (Stratagene, La Jolla, CA). The sense primer, A2HS (’5′-5′-TAA GCT TTA CGC AGT ATA ACC TC-3′), contained a HindIII site. The antisense

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primer, A2BM (5'-GATAAGTTTAACTTGTTGTCGTC-3') incorporated a BamHI site in the PCR product. Digesting the PCR product with HindIII and BamHI produced a fragment of 1340 bp, which was separated in a low-melt agarose gel (SeaPlaque GTG: Cambrex, East Rutherford, NJ), excised, and low-melt ligated to the HindIII/BamHI sites of pBI101.3 plasmids (Clontech), replacing the 35S promoter. One of the plasmids contained the original GUS gene, while the other contained a modified version of enhanced GFP5-ER (Haseloff et al., 1997). An AtRAC2/ROP7 full-length cDNA was isolated from the Unizap XR cDNA library (Stratagene No. 937010) and cloned into a pBluescript (Stratagene) vector (Winge et al., 1997). The coding region of AtRAC2/ROP7 was amplified from the cDNA clone using the PCR primers A2XM (5'-GAAATGGCATGACTACCAGAAAATGTTAGATGGTGTCGTC-3') and A2SC (5'-GAGAGCTCTGCAGAGAATACGCTG-3'), which contained an XbaI and a SacI site, respectively. Site-directed mutagenesis of AtRAC2/ROP7 was conducted using the QuikChange kit (Stratagene). Two complementary PCR primers were used to introduce a substitution leading to a G15V mutation (the mutation is underlined): 5'-TGCGTGGATGGTGGGATGCTG-3' and 5'-CGCTGCTCATCTCCGAGAATGCCC-3'. The mutation site was confirmed by sequencing. AtRAC2/ROP7 and CA-atrac2/rop7 were then cloned into the XbaI and SacI sites of pBI121, downstream of the CaMV 35S promoter. For creation of GFP fusion proteins, an NcoI site was introduced at the start codon of AtRAC2/ROP7 and CA-atrac2/rop7, using the PCR primers A2Nco (5'-AAGACTGAGGCTGTCGACGAAATGATGGTGTCGTC-3') and A2SC. The introduction of the NcoI site also changed the second amino acid of AtRAC2/ROP7 from serine to glycine, but this mutation was not considered to affect the intracellular localization. AtRAC2/ROP7 and CA-atrac2/rop7 were subsequently cloned into the NcoI and SacI sites of a pBSK vector, in which the modified version of GFP5-ER in which the stop codon was replaced with an NcoI site. The resulting fusion protein construct was cloned into the XbaI and SacI sites of pBI121.

RNA isolation and RT-PCR analysis

mRNA was isolated from 3-week-old plants using Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway) according to the protocol of the manufacturer. Random-primed first-strand cDNA was made, using a first-strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. The reaction mixture was subjected to PCR. For amplification of AtRAC2/ROP7, the primers A2K (5'-GAGAGCTCTGCAGAGAATACGCTG-3') and A2SC (5'-GATAAGTTTAACTTGTTGTCGTC-3') were used. The cytosolic glyceraldehyde-3-phosphate dehydrogenase was co-amplified as a marker for comparison of expression levels using the primers GAPDH1F (5'-ATTARGATGCTGATGGGTAGGCGAC-3') and GAPDH1R (5'-GTAACCCTCTTGTGTTGGC-3'). The PCR profile was 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. For AtRAC2/ROP7 and GAPDH, 45 and 30 cycles of PCR amplification were used, respectively. PCR products were separated on a 1.5% agarose gel.

Results

Construction and verification of transgenic plants expressing AtRAC2/ROP7 promoter constructs and AtRAC2/ROP7 mutant genes

A gene fragment, 1.3 kb of the upstream promoter region of AtRAC2/ROP7 containing the first 24 bp of the coding sequence, was amplified using PCR from genomic DNA. The fragment was cloned into a vector, and the sequence was confirmed to be identical to the sequence published by the A. thaliana genome project (BAC MCL19, GenBank accession no. AB006698). The AtRAC2/ROP7 promoter was then cloned in binary vectors upstream of GUS or GFP5-ER (Fig. 1A, a, b), and the constructs were used for transformation of Arabidopsis plants. Homozygous lines were produced, and the DNA of these plants was demonstrated to contain the correct construct by PCR of isolated DNA with primer pairs specific for each construct (results not shown).

In order to study the functional roles of AtRAC2/ROP7, transgenic plants overexpressing wild-type or constitutively active AtRAC2/ROP7 (CA-atrac2/rop7) were produced. The AtRAC2/ROP7 overexpressing construct encompasses the complete coding sequence, including parts of the 5'- and 3'-UTR. Site-directed mutagenesis (Gly15Val) was used to produce constitutively activated AtRAC2/ROP7. Substitution of glycine with valine in position 15 of AtRAC2/ROP7 inactivates the intrinsic GTPase activity, thereby keeping the mutant protein permanently in its active, GTP-bound state. These constructs were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1A, c, d). Several homozygous transgenic lines were obtained for each construct, and two lines showing similar phenotypic properties were chosen for more detailed analysis. A T-DNA insertion mutant of AtRAC2/ROP7 (atrac2/rop7-1) was obtained from the GABI-Kat collection (Rosso et al., 2003). T-DNA was inserted in the third exon of AtRAC2/ROP7, leading to the truncation of AtRAC2/ROP7 after 91 amino acids. Earlier studies indicated that AtRAC2/ROP7 is expressed at low levels (Winge et al., 1997). RT-PCR analysis showed that AtRAC2/ROP7 expression was absent in atrac2/rop7-1 and strongly increased in two CA-atrac2/rop7-expressing lines (Fig. 1B). DNA microarray experiments verified that CA-atrac2/rop7 expression levels were 20-fold up-regulated in the line CA-atrac2/rop7-4 compared with wild-type AtRAC2/ROP7 expression levels (results not shown).

AtRAC2/ROP7 promoter:GUS expression in Arabidopsis plants

Transgenic AtRAC2/ROP7:GUS Arabidopsis plants were grown on both MS agar medium and soil, and the GUS expression pattern was analysed at all major developmental stages. GUS staining was generally observed to be stronger in plants grown on agar medium than in soil. Figure 2 shows the GUS activity patterns in roots, leaves and stems of AtRAC2/ROP7:GUS plants. In 1-d-old seedlings, GUS staining in roots was limited to two rows of cells along the root axis starting about 400 μm from the root tip, corresponding to the elongation zone. GUS-positive cells were observed in the region between the differentiation zone and
the beginning of the hypocotyl (Fig. 2A). Upon closer inspection, GUS staining was localized to protoxylem. Importantly, AtRAC2/ROP7 expression seemed developmentally to precede the appearance of helical secondary cell wall thickenings in protoxylem (Fig. 2B, arrow), indicating that AtRAC2/ROP7 expression might coincide with the onset of secondary cell wall synthesis. In Arabidopsis roots, the transition from early to later stages of differentiation is characterized by the appearance of metaxylem, which is identified on the basis of reticulate rather than helical secondary cell wall thickenings. The root xylem of 8-d-old Arabidopsis seedlings consists of two protoxylem vessels and two or three metaxylem vessels (Kobayashi et al., 2002). GUS staining in protoxylem and the outer metaxylem vessels usually overlapped (Fig. 2B), but staining in the outer and inner metaxylem was frequently observed to be spatially separated (Fig. 2C, D).

GUS staining appeared in xylem vessels throughout the stem. A weak expression of GUS was also observed in the hypocotyl and petioles, as well as in discontinuous parts of the vascular tissue in young rosette leaves (Fig. 2E). In older leaves, GUS staining was barely detectable. GUS staining was repeatedly seen in vascular tissue at the junction between the hypocotyl and petioles of the most recently developed leaves (results not shown). No GUS staining was observed in the inflorescence, siliques or seeds (results not shown).

Morphology of GUS-positive cells

Semi-thin sections were made from GUS-stained stems of AtRAC2/ROP7::GUS plants at different stages and counter-stained with safranin O to visualize cell structures. As evident from cross-sections, GUS-stained cells are located in the primary xylem bundles of young stem sections (Fig. 2F). The cells have partially thickened secondary cell walls compared with the adjacent mature xylem vessels, which is an indication of developing xylem cells that are in the process of producing secondary cell walls (Fig. 2G). Neighbouring cells also showed weak GUS staining; however, this staining probably resulted from leakage of the GUS enzyme or the X-gluc reaction product from the stained cell. In older, mature stem sections, GUS-positive cells were located adjacent to the procambium, as expected for developing xylem cells (Fig. 2I, arrowheads, and Fig. 2J). AtRAC2/ROP7::GUS stem cross-sections were also counter-stained with Maüle reagent, which stains the free syringyl units of lignin. GUS-positive cells in young stem sections showed weak or no staining with Maüle reagent, whereas mature xylem vessels were strongly stained, indicating that AtRAC2/ROP7 expression mainly precedes lignin synthesis at this stage (Fig. 2H). In older stem sections, GUS-positive cells showed variable Maüle staining (Fig. 2K). Overall, the GUS staining pattern suggests that the AtRAC2/ROP7 promoter is active during primary xylem development.
AtRAC2/ROP7 is developmentally regulated

AtRAC2/ROP7:GUS plants were exposed to a broad array of chemical and physical treatments to see if the AtRAC2/ROP7 promoter region could be induced or repressed. The treatments included exposure to plant hormones [gibberellic acid (GA3), cytokinin (benzylaminopurine), auxin (IAA), brassinosteroid (brassinolide), methyl jasmonate (MJA), and abscisic acid]; oxidative stress [hydrogen peroxide...
(H₂O₂), cadmium chloride (CdCl₂), paraquat, and UV irradiation; osmotic stress (glucose, mannitol, and NaCl); elicitors (oligogalacturonic acid and chitosan), and mechanical wounding. None of these treatments led to a notable increase or decrease in GUS staining intensity in AtRAC2/ROP7:GUS plants (results not shown). Thus, AtRAC2/ROP7 gene expression appears to be mainly developmentally regulated.

Confocal fluorescence microscopy of AtRAC2/ROP7 promoter:GFP plants

GFP fluorescence close to the root tip of AtRAC2/ROP7:GFP plants was observed in two rows of cells, exhibiting a striated pattern due to the presence of secondary cell walls in GFP-expressing cells (Fig. 3A). Z-stack analysis of longitudinal optical slices through the root confirmed that the GFP fluorescence was restricted to two narrow files (Fig. 3A, inset). Fluorescence in all of the optical slices showed the same striated pattern, indicative of xylem elements. Similar to the results observed for the AtRAC2/ROP7:GUS construct, GFP positive cells closest to the root tip did not show any striated pattern (Fig. 3B, arrowheads), suggesting that initial AtRAC2/ROP7 expression may precede or coincide with secondary cell wall formation in the development of root xylem. The fluorescence observed in these less-differentiated cells was weak compared with cells with secondary cell walls. In older parts of the root, GFP fluorescence appeared in the central part of the root, corresponding to the metaxylem (results not shown). It was not possible to observe any noticeable GFP fluorescence in leaves.

Overexpression of constitutively active AtRAC2/ROP7 affects leaf elongation and cell morphology

Next atrac2/rop7-1 T-DNA insertion mutant plants, AtRAC2/ROP7 overexpressing plants, and CA-atrac2/rop7 overexpressing plants were investigated in order to gain functional information on AtRAC2/ROP7. Somewhat surprisingly, atrac2/rop7-1 plants did not display visible phenotypes in overall morphology or in composition and appearance of the vascular tissue (results not shown). Plants overexpressing AtRAC2/ROP7 also appeared normal (results not shown). By contrast, leaf elongation was markedly increased in CA-atrac2/rop7 overexpressing plants. The leaf blades of CA-atrac2/rop7 rosette leaves (Fig. 4B) were more oval-shaped compared with wild-type leaves (Fig. 4A). The differences in organ elongation might be the result of cell morphology alterations. Therefore epidermal cells of rosette leaves were examined using scanning electron microscopy (SEM). Mature wild-type pavement cells, positioned towards the tip of rosette leaves, are characterized by extended lobes (Fig. 4C), leading to a highly wavy cell outline. By contrast, mature pavement cells of CA-atrac2/rop7 plants exhibited a more regular cell shape with only a few reduced lobes (Fig. 4D). In order to quantify this property, the ratio of area (µm²) to perimeter (µm) of wild-type and mutant leaf pavement cells (n=185) was measured. pavement cells in CA-atrac2/rop7 plants had significantly (P <0.001) lower area-to-perimeter ratios compared with wild-type and atrac2/rop7-1 plants (Fig. 4E), indicating a less-lobed cell outline of CA-atrac2/rop7 pavement cells. The average cell area of CA-atrac2/rop7 pavement cells was only slightly increased compared with wild-type pavement cells (Fig. 4F).

Subcellular localization of AtRAC2/ROP7-GFP

The subcellular localization of AtRAC2/ROP7 was also examined. To do this, GFP fusions of wild-type and constitutively activated AtRAC2/ROP7 (Fig. 1A, e, f) and generated transgenic plants stably expressing the GFP fusion proteins were constructed. Both GFP-AtRAC2/ROP7
Fig. 5A) and GFP-CA-atrac2/rop7 (Fig. 5B) localized mainly to the plasma membrane of leaf pavement cells. The cell outline was visualized by the GFP fluorescence, and revealed that GFP-CA-atrac2/rop7 expressing pavement cells have less extended lobes, thereby substantiating the observed phenotype of CA-atrac2/rop7 expressing plants. The changed cell morphology indicates that the GFP-CA-atrac2/rop7 fusion protein is functionally active.

Discussion

In order to study the expression and function of the Arabidopsis RAC/ROP GTPase AtRAC2/ROP7, transgenic Arabidopsis plants were constructed expressing the reporter genes GUS and GFP under control of the AtRAC2/ROP7 promoter, as well as plants overexpressing wild-type and constitutively activated AtRAC2/ROP7 (Fig. 1). AtRAC2/ROP7 expression was observed in roots, leaves, and stems, but not in seeds, flowers, and siliques. The AtRAC2/ROP7 promoter directed expression exclusively in vascular tissue, leading to GUS staining or GFP fluorescence in cells corresponding to developing primary xylem elements (Figs 2, 3). The onset of AtRAC2/ROP7 expression appeared to coincide with the formation of secondary cell walls. Overexpression of constitutively activated AtRAC2/ROP7 resulted in reduced lobe length of pavement cells, indicating that AtRAC2/ROP7 is able to regulate...
polar cell expansion (Fig. 4). The results presented suggest that AtRAC2/ROP7 takes part in xylogenesis, one possible mechanism being through the reorganization of the actin cytoskeleton during specific stages of xylem development.

AtRAC2/ROP7 is expressed during the development of tracheary elements

The finding of AtRAC2/ROP7 promoter driven GUS expression in leaves stands in contrast to earlier results, where AtRAC2/ROP7 was found to be absent in leaves (Winge et al., 1997). The level of GUS staining was, however, much weaker in leaves than in roots, likely reflecting a lower level of expression. The possibility cannot be excluded that the upstream region used in the AtRAC2/ROP7 promoter constructs does not include all AtRAC2/ROP7 gene regulatory elements. Additional gene regulatory elements may exist further upstream or in the large (380 bp) first intron. However, these expression data are supported by a recent report by Birnbaum et al. (2003). Combining the DNA microarray technique with tissue-specific isolation of root protoplasts, they were able to create an expression profile of 22 000 genes in 15 subzones of the Arabidopsis root. Investigation of the supplementary and public available dataset of the experiment revealed that AtRAC2/ROP7 expression clearly peaked in the stele of the root differentiation zone (stage 3), similar to AtRAC2/ROP7.

Table 1. Some genes with root expression pattern similar to AtRAC2/ROP7

A selection of the differentially expressed genes in the Arabidopsis root published by Birnbaum et al. (2003) which shows highest expression in the stele of the root differentiation zone (stage 3), similar to AtRAC2/ROP7.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Peak value (stele stage 3)</th>
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<tr>
<td>At5g45970</td>
<td>AtRAC2/ROP7</td>
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</tr>
<tr>
<td>At5g44030</td>
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<td>AtHB-8 HD-zip transcription factor</td>
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<tr>
<td>At5g60690</td>
<td>REVOLUTA or INTERFASCICULAR FIBRELESS, HD-Zip</td>
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AtRAC2/ROP7 and GFP-CA-atrac2/rop7 under control of the CaMV 35S promoter were observed with a confocal microscope. (A) GFP-AtRAC2/ROP7; (B) GFP-CA-atrac2/rop7. Bar=20 μm.

Fig. 5. Subcellular localization of AtRAC2/ROP7. Epidermal leaf cells of transgenic plants expressing GFP-AtRAC2/ROP7 and GFP-CA-atrac2/rop7 under control of the CaMV 35S promoter were observed with a confocal microscope. (A) GFP-AtRAC2/ROP7; (B) GFP-CA-atrac2/rop7. Bar=20 μm.

Recently, Pyo et al. (2004) studied the Zinnia cysteine protease 4 (ZCP4) promoter in Arabidopsis using GUS as a molecular marker. ZCP4 expression increases prior to autolysis of cultured Zinnia tracheary elements, and is believed to take part in this process (Demura et al., 2002). The GUS expression pattern directed by the ZCP4 promoter during seedling development is highly similar to that observed using the AtRAC2/ROP7 promoter, further indicating that AtRAC2/ROP7 might be involved in the late stages of xylem development.

Putative roles of AtRAC2/ROP7 in xylogenesis

The actin cytoskeleton is believed to be important for directed transport of vesicles carrying membrane and cell wall components to sites of cell expansion (Wasteneys and Galway, 2003; Mathur, 2004). Although there is limited knowledge about the role of the actin cytoskeleton in secondary cell wall synthesis, recent results indicate that...
actin may be central to this process. Interfascicular fibre cells of *Arabidopsis* plants grown in the presence of the actin-disrupting drug cytochalasin D developed much thinner cell walls compared with plants grown without cytochalasin D (Hu et al., 2003). Furthermore, the organization of actin filaments was altered in the mutant *fragile fibre 4* (*fra4*), in which interfascicular fibres have reduced cell wall thickness (Hu et al., 2003).

Expression of constitutively active AtRAC2/ROP7 resulted in increased length of rosette leaves. Leaf pavement cells of CA-*atrac2/rop7* plants also displayed a reduction in the number and length of the lobes that characterize these cells in wild-type plants. These experiments suggest that AtRAC2/ROP7 has the potential to participate in the regulation of cell morphology, probably through the regulation of the actin cytoskeleton. However, it should be noticed that these data indicate that AtRAC2/ROP7 is not normally expressed in epidermal tissues. Since AtRAC2/ROP7 does not appear to be expressed in epidermal tissue, the CA-*atrac2/rop7* phenotypes could be the result of misexpressing CA-*atrac2/rop7* rather than a reflecting a functional role for AtRAC2/ROP7.

The reduced length of pavement cell lobes has earlier been reported for plants expressing constitutively active ROP2/AtRAC4 (Fu et al., 2002). A similar phenotype has also been observed in *Arabidopsis* mutants defective in subunits of the ARP2/3 complex (Mathur et al., 2003; Li et al., 2003) and the upstream WAVE regulatory complex, which interacts with one or several AtRACs (Basu et al., 2003; Brembu et al., 2004). Formation of pavement cell lobes is achieved by polar growth, with a population of fine cortical F-actin localized to the site of growth. In cells expressing constitutively active ROP2/AtRAC4, fine cortical F-actin was evenly distributed in the cell cortex. This change in distribution was hypothesized to be the result of ectopic plasma membrane localization of active ROP2/AtRAC4, suggesting that ROP2/AtRAC4 control polar expansion through regulation of fine cortical F-actin (Fu et al., 2002). A recent publication indicates that the RAC/ROP effectors RIC1 and RIC4 take part in ROP2/AtRAC4-mediated regulation of pavement cell morphogenesis through promotion of cortical microtubules and cortical actin filaments, respectively (Fu et al., 2005). The highly similar phenotypes observed in CA-*atrac2/rop7* transgenic plants indicate that AtRAC2/ROP7 may perform its functions through regulation of the actin cytoskeleton. It is interesting to note that RIC4 shows a highly similar expression pattern to AtRAC2/ROP7 in the root expression dataset published by Bimbaum et al. (2003) (Table 1).

Another putative role for AtRAC2/ROP7 in xylogenesis is regulating NADPH oxidase activity. Several lines of evidence indicate that RAC/ROP GTPases participate in the activation of NADPH oxidase as a part of the plant’s defence response to microbial elicitors (Kawasaki et al., 1999; Ono et al., 2001; Park et al., 2004). Reactive oxygen species produced by NADPH oxidase in cells challenged by microbial elicitors trigger apoptosis (Levine et al., 1994). Studies of the development of cotton fibres indicate that the transition from primary to secondary cell wall synthesis involves production of hydrogen peroxide (Potikha et al., 1999). Furthermore, the results suggest that NADPH oxidase is responsible for generation of H$_2$O$_2$ in this process, and that a RAC/ROP GTPase might act as a regulator of the NADPH oxidase. Expression of the cotton Rac13, which is a homologue of AtRAC2/ROP7, is highly induced at the transition from primary to secondary wall synthesis in cotton fibres (Delmer et al., 1995). Recent experiments indicate that cellulose synthase monomers can interact through their N-terminal RING-finger domains, and that this interaction is regulated by the redox state (Kurek et al., 2002). Dimerization is thought to be induced by oxidation of cysteine residues in the RING-finger domains, and H$_2$O$_2$ generated by NADPH oxidase could be central in this process during the switch to secondary cell wall formation. The mechanism for RAC-regulated activation of the NADPH oxidase in plants has not been resolved. RAC/ROP proteins have been implicated in regulation of Ca$^{2+}$ influx during the growth of pollen tubes (Li et al., 1999) and root hairs (Molendijk et al., 2001). Plant NADPH oxidases contain two Ca$^{2+}$-binding EF-hand motifs (Keller et al., 1998; Torres et al., 1998) and appear to be activated directly by Ca$^{2+}$ (Sagi and Fluhr, 2001). AtRAC2/ROP7 could possibly take part in secondary cell wall deposition in *Arabidopsis* tracheary elements through Ca$^{2+}$-mediated activation of NADPH oxidase, leading to the activation of cellulose synthases. H$_2$O$_2$ produced by an AtRAC2/ROP7-activated NADPH oxidase could also be involved in the induction of programmed cell death (PCD) of tracheary elements. However, addition of the NADPH oxidase inhibitor diphenyleneiodonium to differentiating *Zinnia* cell culture did not inhibit PCD, indicating that NADPH oxidases are not involved in this phase of tracheary development (Groover et al., 1997).

The plasma membrane-specific localization of AtRAC2/ROP7 is in accordance both with a role in actin cytoskeleton regulation (Fu et al., 2001, 2002) and in the activation of NADPH oxidases through calcium influx (Li et al., 1999; Kost et al., 1999; Baxter-Burrell et al., 2002). AtRAC/ROP GTPases from both subgroups have been shown to be localized to the plasma membrane (Bischoff et al., 2000; Ivanenko et al., 2000; Fu et al., 2002; Lavy et al., 2002). Furthermore, the less-lobed phenotype of pavement cells expressing GFP-CA-*atrac2/rop7* implies that AtRAC2/ROP7 is functionally active at the plasma membrane.

**Redundancy of AtRAC/ROP proteins/genes in the development of vascular tissue**

The expression of AtRAC2/ROP7 is predominantly restricted to xylem cells in roots, hypocotyls, stems, and...
leaves. As xylem is also found in other tissues, such as flowers and siliques, AtRAC2/ROP7 is probably not the only RAC/ROP GTPase involved in this process. The RAC/ROP gene family in Arabidopsis consists of 11 members (Winge et al., 2000), and a high degree of redundancy is likely to exist within this family in plants. Expression of the Zinnia elegans RAC/ROP homologue ZeRAC2, which shows the closest similarity to AtRAC1/ROP3, AtRAC6/ROP5, and AtRAC11/ROP1, is induced during differentiation of in vitro-cultured tracheary elements (Nakanomyo et al., 2002). In situ hybridization experiments indicated that ZeRAC2 is mainly expressed in xylem precursor cells and xylem parenchyma cells. Furthermore, the ZeRac2 mRNA transcripts preferentially accumulated at the side facing a developing TE; a possible role for ZeRac2 could be production of hydrogen peroxide for lignin synthesis through the activation of NADPH oxidases. Preliminary results suggest that at least one RAC/ROP gene besides AtRAC2/ROP7 is expressed during xylem development in Arabidopsis (T Brembu et al., unpublished results). Functional redundancy is therefore a likely explanation for the lack of phenotypes observed in the T-DNA insertional mutant atrac2/rop7-1. A more detailed examination of atrac2/rop7-1 may reveal subtle alterations of the vascular tissue. The lack of phenotype observed for plants overexpressing wild-type AtRAC2/ROP7 may be caused by increased expression of RAC/ROP GAPs and/or RhoGDI, which could keep AtRAC2/ROP7 activity at a normal level despite increased expression. Curiously, consistent changes in vascular tissue morphology of CA-attrac2/rop7 plants were not observed. A subset of the CA-attrac2/rop7 plants displayed abnormal organization of the root xylem, with the formation of additional, discontinuous tracheary elements (results not shown). Although these results could imply a role for AtRAC2/ROP7 in deciding the destiny of cells with respect to xylem, there are not sufficient data to support such conclusions. Transgenic plants overexpressing dominant negative AtRAC2/ROP7 has not yet been made; these plants could be helpful in resolving the function of AtRAC2/ROP7. Studies of AtRAC2/ROP7:GUS expression in different mutant backgrounds, generation of T-DNA insertion mutants for multiple AtRAC/ROP genes and identification of AtRAC2/ROP7 interacting proteins will hopefully elucidate the role of AtRAC2/ROP7 in xylogenesis.

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Acknowledgements

We are grateful to Per Olav Johnsen at the Paper and Fibre Research Institute, Trondheim for assistance with scanning electron microscopy, Kjartan Egeberg at the Department of Cancer Research and Molecular Medicine, NTNU for assistance with confocal microscopy, Kjell Evjen for help with light microscopy, and Nancy Bazilchuk for critical reading of the manuscript. This work was supported by grants NFR 159959/I10 and NFR 151991/S10 from the Biotechnology and Functional Genomics (FUGE) programmes of the Norwegian Research Council, respectively.


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