PCP
PLANT & CELL PHYSIOLOGY

Functional Identification of Arabidopsis *ATSIP2* (At3g57520) as an Alkaline α-Galactosidase with a Substrate Specificity for Raffinose and an Apparent Sink-Specific Expression Pattern

Shaun Peters[1,3], Aurélie Egert[1,3], Bruno Stieger[2] and Felix Keller[1,*]

[1]Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland
[2]Division of Clinical Pharmacology and Toxicology, University Hospital Zürich, Rämistrasse 100, 8091 Zürich, Switzerland
[3]These authors contributed equally to this work
*Corresponding author: E-mail, fkel@botinst.uzh.ch; Fax, +41-44-634-82-04
(Received June 30, 2010; Accepted August 16, 2010)

Short Communication

Arabidopsis *ATSIP2* has recently been suggested to be a raffinose synthase gene. However, it has high amino acid identity to functionally characterized alkaline α-galactosidases from *Cucumis melo* and *Zea mays*. Using the *Sf9* insect cell expression system, we demonstrate that recombinant ATSIP2 is a genuine alkaline α-galactosidase with a distinct substrate specificity for raffinose, and not a raffinose synthase. A β-glucuronidase reporter construct using the *ATSIP2* promoter shows that *ATSIP2* is strongly expressed in sink tissues of Arabidopsis, i.e. sink leaves and non-xylem parts of the root stele, suggesting a physiological function in raffinose phloem unloading.

Keywords: Alkaline α-galactosidase • Phloem unloading • Raffinose • *Sf9* insect cells • Sink metabolism.

Abbreviations: DGJ, 1-deoxygalactonojirimycin; α-Gal, α-galactosidase; Gal, galactose; Gol, galactinol; GUS, β-glucuronidase; Mel, melibiose; pNPαGal, *p*-nitrophenyl α-D-galactopyranoside; pNPβGal, *p*-nitrophenyl β-D-galactopyranoside; PAD, pulsed amperometric detection; Raf, raffinose; RafS, raffinose synthase; SIP, seed imbibition protein; sqPCR, semi-quantitative PCR; Sta, stachyose; Suc, sucrose; Ver, verbascose.

The Arabidopsis gene, *ATSIP2* (At3g57520), presently has controversial annotations and functions. It has been annotated as a putative raffinose synthase (RafS) or a seed imbibition protein (SIP) with *O*-glycosyl hydrolase (e.g. α-D-galactoside hydrolase, α-Gal) activity and suggested to function accordingly in both the biosynthetic and hydrolytic pathways of raffinose (Raf) metabolism, especially under certain abiotic stress conditions (drought, high salinity or high temperature; Nishizawa et al. 2008, Maruyama et al. 2009). The aim of this study was (i) to functionally express and characterize ATSIP2 to determine if it encodes a RafS or an α-Gal; and (ii) to identify a possible physiological function in sink tissues.

Interestingly, ATSIP2 shares 76% amino acid similarity with CmAGA1 and 67% with CmAGA2, both functionally identified as alkaline α-Gals from melon fruit with distinct substrate preferences for stachyose (Sta) and Raf, respectively (Gao and Schaffer 1999, Carmi et al. 2003). Numerous higher plant α-Gals have been identified and described from a variety of species (for reviews, see Keller and Pharr 1996, Peterbauer and Richter 2001). Most studies have dealt with acidic isoforms, which appear to play important roles in seed development and germination and in sprouting of Sta-containing tubers (see Keller and Pharr 1996, Peterbauer and Richter 2001). Alkaline α-Gals, however, have been mostly associated with sink activities, hydrolyzing phloem-translocated Raf and Sta in sink leaves and developing fruits (Gaudreault and Webb 1986, Bachmann et al. 1994, Carmi et al. 2003) as well as with thylakoid galactolipid breakdown during leaf senescence (Lee et al. 2009).

The cloning and functional expression of two cDNAs from melon fruit (*CmAGA1* and *CmAGA2*) showed that they displayed distinct α-Gal activity at alkaline pH. Most importantly, these genes showed the highest homology to *SIP* genes, suggesting that SIPs are likely to represent alkaline α-Gals in plants and revealing a hitherto unknown subfamily of glycosyl hydrolases (Carmi et al. 2003). On the basis of sequence homology, *SIP* genes have been identified in at least four other plant families including Poacea (barley, SIP: M77475), Leguminoseae (Cicer, SIP: X95875), Solanaceae (tomato, SIP: TC94379) and Brassicaceae (Arabidopsis, SIPs: AAC83062, CAB66109). We here provide clear evidence that *ATSIP2* encodes a Raf-specific alkaline α-Gal (and not a RafS) and suggest that it is involved in sink metabolism, most probably in phloem unloading of Raf.

ATSIP2 was heterologously expressed in *Sf9* insect cells. Crude extracts from *Sf9* cells infected with a baculovirus carrying the *ATSIP2* cDNA were clearly able to hydrolyze Raf to sucrose (Suc) and galactose (Gal) at pH 7.5, in contrast to crude extracts from uninfected *Sf9* cells (**Fig. 1A**). Furthermore, this hydrolase activity was completely abolished when the

Plant Cell Physiol. 51(10): 1815–1819 (2010) doi:10.1093/pcp/pcq127, available online at www.pcp.oxfordjournals.org

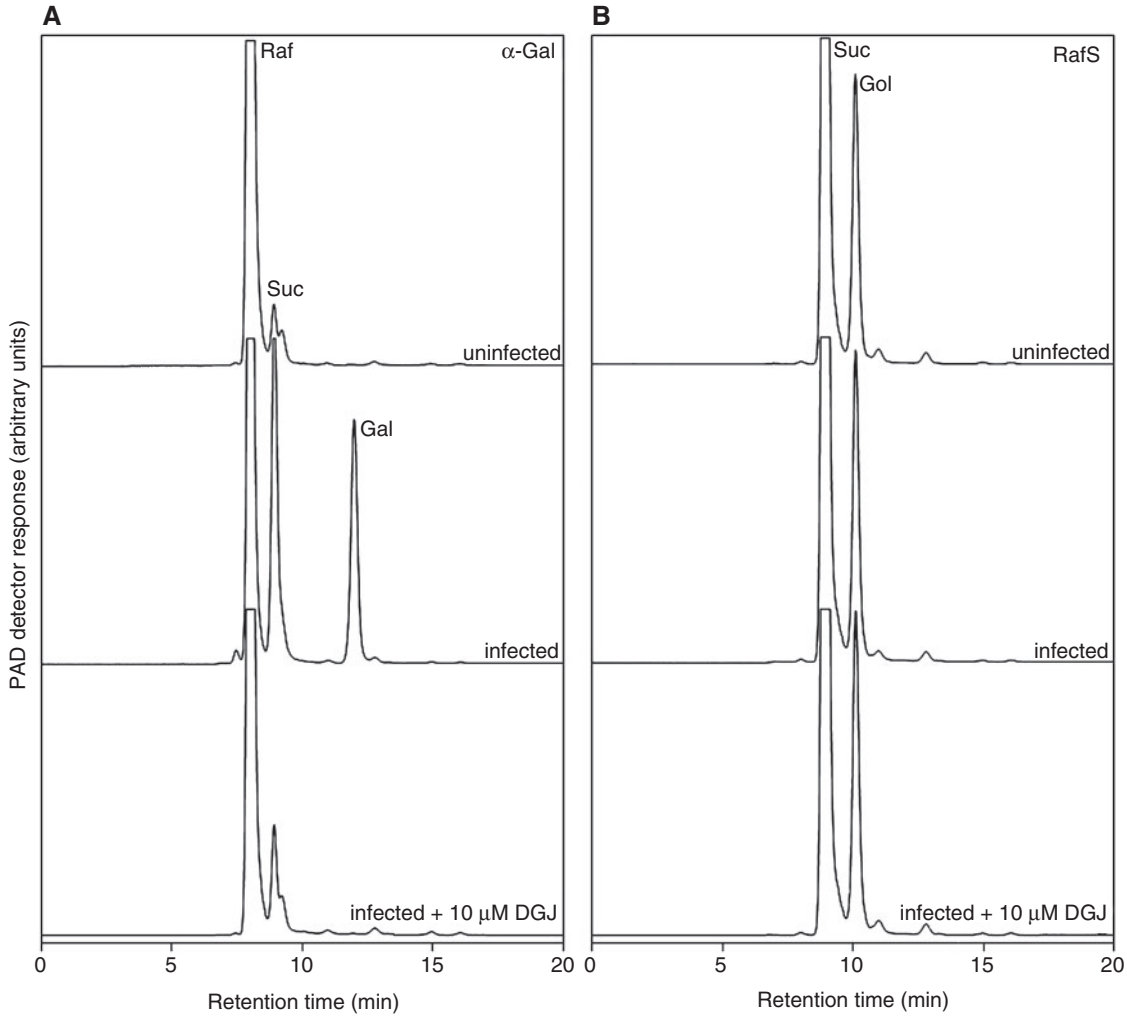

Fig. 1 Enzyme activities of *Sf9* cells infected or uninfected (controls) with an *ATSIP2*-carrying baculovirus. Crude cell lysates were incubated at pH 7.5 either with 50 mM Raf to assay for alkaline α-Gal activity (A) or with 10 mM Gol and 100 mM Suc to assay for RafS activity (B). DGJ, 1-deoxygalactonojirimycin, is a potent inhibitor of α-Gals (Butters et al. 2005, Blöchl et al. 2007).

enzyme assay was performed in the presence of 10 μM 1-deoxygalactonojirimycin (DGJ), a potent α-Gal inhibitor (Butters et al. 2005, Blöchl et al. 2007; **Fig. 1A**). The recombinant ATSIP2 activity showed Michaelis–Menten-type kinetics with apparent K_m and V_{max} values of 105 ± 18 mM and 1.80 ± 0.16 nkat mg^{-1} protein, respectively (**Fig. 2A**), with a pH optimum around pH 7.5–8.0 (**Fig. 2B**) for Raf as substrate. The α-Gal activity was end product-inhibited by Gal, displaying a 50% inhibition at 7.4 mM Gal (with 50 mM Raf as substrate). It was also very specific. When tested with the natural α-galactosyl substrates, Raf, Sta, verbascose (Ver), galactinol (Gol) and melibiose (Mel), only Raf was recognized as an efficient substrate (**Fig. 2C**). When tested with the artificial substrate, *p*-nitrophenyl α-D-galactopyranoside (pNPαGal), a very high activity was observed (432 μkat mg^{-1} protein), in contrast to the β-linked variant of pNPGal, *p*-nitrophenyl β-D-galactopyranoside (pNPβGal; activity not detectable), confirming that it is indeed an α-Gal (and not a β-Gal). Using Suc and Gol as substrates, recombinant ATSIP2 exhibited no ability to produce Raf, clearly excluding it from being a functional RafS (**Fig. 1B**).

Collectively, these observations unambiguously identify ATSIP2 as an alkaline α-Gal in Arabidopsis with a substrate preference for Raf, and not a RafS as recently reported (Nishizawa et al. 2008, Maruyama et al. 2009, Wu et al. 2009).

To investigate ATSIP2's putative physiological function in sink metabolism, an *ATSIP2* promoter:β-glucuronidase (GUS) fusion was created, using the Gateway-compatible vector, pMDC163 (Curtis and Grossniklaus 2003), and a 0.5 kb fragment of genomic DNA, upstream of the start codon of *ATSIP2* (−500 bp). A second fusion included this 0.5 kb plus an additional 1 kb of upstream DNA. Arabidopsis (Col-0) transformed with these constructs showed strong GUS activity in sink leaves of 5-week-old soil-grown plants (**Fig. 3A, B**), suggesting that *ATSIP2* is expressed in these tissues. *ATSIP2* promoter expression was also found in steles of lateral roots (**Fig. 3C, D**).

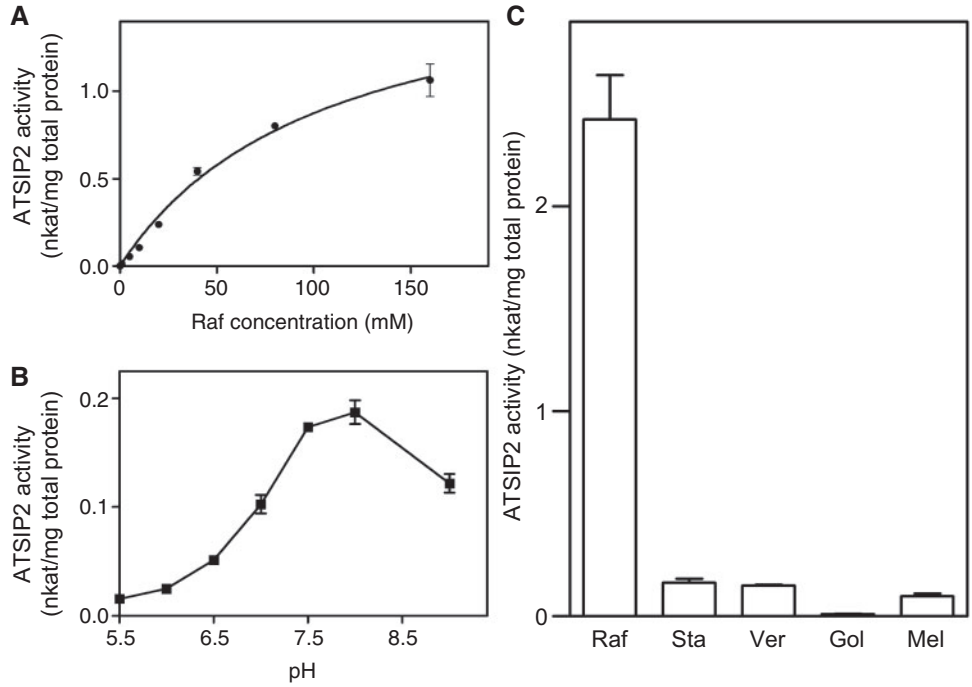

Fig. 2 Biochemical characterization of the recombinant ATSIP2 enzyme. (A) The Raf concentration dependence shows Michaelis–Menten-type kinetics with apparent K_m and V_{max} values of 105 ± 18 mM and 1.80 ± 0.16 nkat mg^{-1} protein, respectively. (B) The pH dependence shows a pH optimum at around pH 7.5–8.0 with 50 mM Raf as substrate. (C) The ATSIP2 activity shows clear Raf specificity when different natural substrates are compared (measured at pH 7.5 with 50 mM each of Raf, Sta, Ver, Gol and Mel). Data are means ± SE of 3–6 replicates.

Cross-sections of young roots further revealed that this expression is located in the non-xylem parts of the stele, including the phloem (**Fig. 3E, F**). Finally, we were able to correlate this GUS expression pattern to in vivo alkaline α-Gal activity. Using Raf as substrate at pH 7.5, the α-Gal activity was significantly higher in sink tissues (roots and young leaves) than in source tissues (old leaves) (**Fig. 3G**). Using semi-quantitative reverse–transcription PCR (sqPCR), these α-Gal activities were positively correlated to the presence of *ATSIP2* transcripts in all tissue types described (**Fig. 3H**). Such an expression pattern for alkaline α-Gal is reminiscent of a putative function in phloem unloading (Gaudreault and Webb 1986, Bachmann et al. 1994, Carmi et al. 2003). Although Suc has been reported to be the primary phloem-mobile carbohydrate in Arabidopsis, there is also good evidence that some Raf is additionally transported in the phloem (Haritatos et al. 2000). In that study, following exposure of Arabidopsis source leaves to $^{14}CO_2$ and light, radiolabel was clearly found in [^{14}C]Raf in sink leaves (in addition to the predominant [^{14}C]Suc). In this study, we have shown ATSIP2 to be a Raf-specific alkaline α-Gal with a promoter active exclusively in sink tissues, suggesting that it may legitimately be involved in the unloading of phloem-mobile Raf in sink tissues. This finding does not rule out the possibility of additional putative physiological functions for ATSIP2 in Arabidopsis, for instance in abiotic stress tolerance or seed germination, and these are currently being investigated using a reverse genetic approach.

Materials and Methods

Following stratification (48 h, 4°C), Arabidopsis Col-0 ecotype seeds were propagated on soil (Einheitserde, type ED73, Gebr. Patzer GmbH & Co. KG, Schopfheim, Germany) in a controlled-environment chamber (8 h light, 120 µmol photons m^{-2} s^{-1}, 22°C, 16 h dark, 60% relative humidity).

ATSIP2 (At3g57520) was obtained as a full-length cDNA from the Riken Arabidopsis full-length clone database (pda02775, www.brc.riken.jp). This cDNA was amplified using a high fidelity PCR (Expand High Fidelity PCR System, Roche) according to the manufacturer's instructions, using open reading frame-specific primers (*ATSIP2*$_{fwd}$ 5′-ATGACGATTA CATCAAATATCTCTG and *ATSIP2*$_{rev}$ 5′-CTAGACCAGAATC TCAACATG). *ATSIP2* was subcloned by standard restriction digest and ligation reactions, from pGEM-T Easy into the pFastBac HTc vector (Invitrogen AG), using the *Not*I restriction endonuclease. Bacmid preparation, insect cell transfection and recombinant protein expression were conducted as outlined in the bac-to-bac manual (Invitrogen), using *Sf*9 cells grown in monolayer cultures.

*Sf*9 cells were collected by centrifugation (500 × *g*, room temperature, 5 min) 72 h after baculovirus infection. Cell pellets were re-suspended in 2 ml of extraction buffer [100 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% (v/v) Triton X-100] and homogenized on

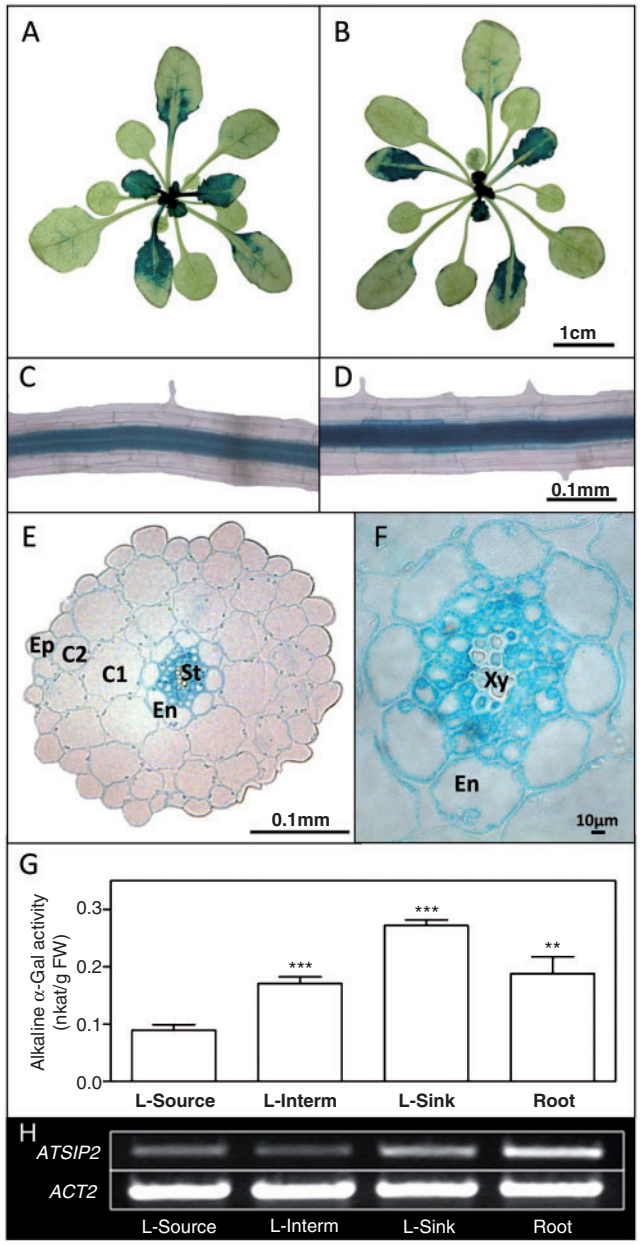

Fig. 3 The *ATSIP2* promoter is active in sink leaves and the non-xylem parts of the root stele (blue GUS staining). (A) Sink leaf-specific expression pattern in the 1.14 stage rosettes of pATSIP2$_{500}$::pMDC163 and (B) of pATSIP2$_{1500}$::pMDC163. (C) Root stele-specific expression of pATSIP2$_{500}$::pMDC163 and (D) of pATSIP2$_{1500}$::pMDC163. (E and F) Cross-sections of roots of young pATSIP2$_{500}$::pMDC163 plants showing promoter activity in the non-xylem parts of the root stele. (G) The extractable alkaline α-Gal activity of wild-type plants is highest in the sink tissues. α-Gal activity was measured at pH 7.5 with 50 mM Raf. All plants were of the same age (5 weeks; soil-grown), except for E and F (10-day-old, MS agar-grown). (H) SqPCR of cDNA from the four tissues described shows that the *ATSIP2* transcripts are most abundant in sink tissues. The *ACTIN2* gene was used as a constitutively expressed control. Ep, epidermis; C1-C2, cortex; En, endodermis, St, stele; Xy, xylem vessels; L-Source, source leaves; L-Interm, intermediate leaves; L-Sink, sink leaves. The statistical probabilities represented by asterisks reflect an unpaired *t*-test (***$P < 0.0001$; **$P < 0.006$).

ice using a Potter homogenizer connected to an electric drill. After centrifugation (12,000×*g*, 4°C, 10 min), 30 µl aliquots of clarified crude extracts were incubated with 30 µl of Raf assay buffer (100 mM HEPES-KOH, pH 7.5, 100 mM Raf) at 30°C for 1 h. Samples were desalted and analyzed by HPLC with pulsed amperometric detection (HPLC-PAD), using a Ca^{2+}/Na^{+}-moderated ion partitioning carbohydrate column (Peters et al. 2007, Peters and Keller 2009). The pH optimum of recombinant ATSIP2 was determined using 50 mM Raf in the following buffers: full-strength McIlvaine buffer (pH 5.0, 5.5 and 6.0), 100 mM MES-KOH buffer (pH 6.0, 6.5 and 7.0) and 100 mM HEPES-KOH buffer (pH 7.0, 7.5, 8.0 and 9.0).

The specificity of recombinant ATSIP2 for α- or β-galactosides was tested using the artificial substrate, *p*-nitrophenyl-D-galactopyranoside (pNPGal) as previously described (Gao and Schaffer 1999). Briefly, 10 µl of clarified crude extract was incubated with 90 µl of assay buffer (100 mM HEPES-KOH pH 7.5) containing 3 mM *p*NPαGal or pNPβGal. The assay mixture was incubated in a 96-well microtiter plate at 30°C and the reaction stopped by the addition of 160 µl of 1 M Na_2CO_3. Absorbance was read at 405 nm. The specificity for the natural substrates, Raf, Sta, Ver, Gol and Mel, was tested at 50 mM final concentrations using the HPLC-PAD method as described above.

For the determination of α-Gal activities in crude leaf extracts, 5-week-old soil-grown Arabidopsis plants were separated into source, intermediary and sink leaves, as well as roots. Tissue (100 mg) was homogenized in 200 µl of an alkaline extraction buffer as previously described (Peters et al. 2007, Peters and Keller 2009). Aliquots (10 µl) of clarified crude extracts were incubated with 10 µl of Raf assay buffer at 30°C for 1 h and the activity was determined as described above.

Total RNA was extracted from source, intermediary and sink leaves as well as roots using the Plant RNeasy kit (Qiagen AG). The cDNA template for sqPCR was obtained by reverse transcribing 1 µg of total RNA with an oligo(dT_{15}) primer and M-MLV (H^-) reverse transcriptase (Promega AG) according to the manufacturer's protocol. The sqPCR was carried out in 50 µl containing 5 µl of cDNA, 1.25 U of GoTaq DNA polymerase (Promega), 1× PCR buffer, 0.5 mM of each dNTP and 0.5 µmol of each primer, at a primer annealing temperature of 58°C for 23 cycles. The number of cycles chosen for the sqPCR was determined to occur in the linear range of the constitutively expressed *ACTIN2* gene (*ACT2*, At3g18780). The *ACT2* primer pair (*ACT2*$_{fwd}$ 5′-ATGGCTGAGGCTGATGATAT and *ACT2*$_{rev}$ 5′-TTAGAAACATTTTCTGTGAACGAT) amplified a 1.1 kb fragment of the cDNA. The *ATSIP2* primer pair (*ATSIP2*$_{fwd}$ 5′-ATGACGATTACATCAAATATCTCTG and *ATSIP2*$_{rev}$ 5′-TGAACTGGGTATGCTAATGC) amplified a 1.0 kb fragment of the cDNA.

A 0.5 kb fragment of Arabidopsis genomic DNA, upstream from the ATSIP2 start codon, was amplified using a high fidelity PCR (Expand High Fidelity PCR System, Roche), following the manufacturer's instructions. This fragment was cloned into the pCR8/GW/TOPO vector system (Invitrogen) and subcloned into the Gateway destination vector pMDC163 (Curtis and

Grossniklaus 2003) using a conventional LR clonase reaction (Invitrogen). This ATSIP2–promoter–GUS reporter construct was transformed into *Agrobacterium tumefaciens* (GV3101) by electroporation, using a Genepulser (2.5 kV; 100 Ω; 25 μF; Bio-Rad). A second reporter construct included the 0.5 kb described above and an additional 1 kb of upstream sequence, containing a putative TATA consensus sequence. Col-0 Arabidopsis plants were transformed using a floral dip method (Clough and Bent 1998). Hygromycin B-resistant plants were selected as previously described (Harrison et al. 2006). Transgenic plants (T_3) were used to assay for GUS activity.

T_3 Arabidopsis seeds transformed with the reporter construct described above were sown onto MS agar supplemented with Suc (5%, w/v) and hygromycin B (25 μg ml^{-1}). One week after germination, plants were transferred onto soil and used to stain for GUS activity (Parcy et al. 1998) 4 weeks later.

For the preparation of root cross-sections, roots of 10-day-old plants grown on MS agar were used. They were stained for GUS activity, fixed for 3 min under vacuum in 4% (v/v) glutaraldehyde and incubated for 4 h at room temperature. The tissue was washed three times with ddH_2O and dehydrated using an ethanol series (70%, 30 min; 90%, 30 min; 100%, 1 h). The final dehydration step using 100% ethanol was repeated once. Embedding of the tissue was conducted using Technovit 7100 (Heraeus Kulzer), following the manufacturer's instructions, and root cross-sections (2–3 μm) were cut using a hand-operated microtome.

Funding

This work was supported by the Swiss National Foundation [grant number 31-116599].

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