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

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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; Raf, raffinose; RafS, raffinose synthase; semi-quantitative PCR (sqPCR); Sta, stachyose; Suc, sucrose; Ver, verbascose.
Abstract
Arabidopsis *ATSIP2* has recently been suggested to be a raffinose synthase gene. However, it shares 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.
The Arabidopsis gene, \textit{ATSIP2} (At3g57520), presently exhibits 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. \(\alpha\)-D-galactoside hydrolase, \(\alpha\)-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, high temperature; Nishizawa et al. 2008, Maruyama et al. 2009). The aim of this paper was (i) to functionally express and characterize ATSIP2 to determine if it encodes a RafS or an \(\alpha\)-Gal and (ii) to identify a possible physiological function in sink tissues.

Interestingly, ATSIP2 shares 76\% amino acid similarities to CmAGA1 and 67\% to CmAGA2, both functionally identified as alkaline \(\alpha\)-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 \(\alpha\)-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 reviews cited above). Alkaline \(\alpha\)-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 (\textit{CmAGA1} and \textit{CmAGA2}) showed that they displayed distinct \(\alpha\)-Gal activity at alkaline pH. Most
importantly, these genes showed highest homology to SIP genes, suggesting that SIPs are likely to represent alkaline α-Gals in plants and revealing a hitherto unknown sub-family 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 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, contrary to crude extracts from uninfected Sf9 cells (Fig. 1A). Furthermore, this hydrolase activity was completely abolished when the 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), conversely 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 precluding it as 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 (Maruyama et al. 2009, Nishizawa et al. 2008, 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). 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
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 \(^{14}\text{CO}_2\) and light, radiolabel was clearly found in \(^{14}\text{C-Raf}\) in sink leaves (besides the predominant \(^{14}\text{C-Suc}\)). 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

Plant material and growth conditions
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).

Expression of ATSIP2 in Sf9 insect cells
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, Basel, Switzerland) according to the manufacturer’s instructions, using open reading frame-specific primers (ATSIP2_fwd 5’- ATGACGATTACATCAAATATCTCTG, ATSIP2_rev 5’- CTAGACCAGAATCTCAACATG). ATSIP2 was sub-cloned by standard restriction digest and ligation reactions, from pGEM-T Easy into the pFastBac HTc vector (Invitrogen AG, Basel, Switzerland), using the NotI restriction endonuclease. Bacmid preparation, insect cell transfection and recombinant protein expression were conducted as outlined in the bac-to-bac manual (Invitrogen), using Sf9 cells grown in monolayer cultures.

α-Galactosidase activity assays
Sf9 cells were collected by centrifugation (500xg, RT, 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 MgCl₂, 1 mM EDTA, 10 mM DTT, 1 mM benzamidine, 1 mM PMSF, 0.05% (v/v) Triton X-100] and homogenized on ice using a potter connected
to an electric drill. After centrifugation (12,000xg, 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-PAD, using a Ca²⁺/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, 6.0), 100 mM MES-KOH buffer (pH 6.0, 6.5, 7.0), and 100 mM Hepes-KOH buffer (pH 7.0, 7.5, 8.0, 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 pNPαGal or pNPβGal. The assay mixture was incubated in a 96-well micro-titer plate at 30°C and the reaction stopped by the addition of 160 µl of 1 M Na₂CO₃. Absorbance was read at 405 nm. The specificity for the natural substrates, Raf, Sta, Ver, Gol, and melibiose (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.
RNA isolation and semi-quantitative PCR (sqPCR)

Total RNA was extracted from source, intermediary and sink leaves as well as roots using the Plant RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). The cDNA template for sqPCR was obtained by reverse transcribing 1 μg total RNA with an oligo (dT<sub>15</sub>) primer and M-MLV (H<sup>-</sup>) reverse transcriptase (Promega AG, Dübendorf, Switzerland) according to the manufacturer’s protocol. The sqPCR was carried out in 50 μl containing 5 μl cDNA, 1.25 U 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<sub>fwd</sub> 5´- ATGGCTGAGGCTGATGATAT and ACT2<sub>rev</sub> 5´- TTAGAAACATTTTCTGTGAACGAT) amplified a 1.1 kB fragment of the cDNA. The ATSIP2 primer pair (ATSIP2<sub>fwd</sub> 5´- ATGACGATTACATCAAATATCTCTG and ATSIP2<sub>rev</sub> 5´- TGAACGTGGGTATGCTAATGC) amplified a 1.0 kB fragment of the cDNA.

ATSIP2-promoter-β-glucuronidase (GUS) plants

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-β-glucuronidase reporter construct was transformed into Agrobacterium tumefaciens (GV3101) by
electroporation, using a Genepulser (2.5 kV; 100 Ω; 25 μF, Bio-Rad, Reinach, Switzerland). 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 (T3) were used to assay for β-glucuronidase activity.

_Histochemical staining for β-glucuronidase (GUS) activity_

T3 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.

_Preparation of root cross sections for light microscopy_

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 RT. The tissue was washed thrice with ddH₂O 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, Dübendorf, Switzerland), following the manufacturer’s instructions and root cross sections (2-3 μm) were cut using a hand operated microtome.
**Funding**

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**References**


Legends to Figures

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).

Fig. 2. Biochemical characterization of 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 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 three to six replicates.

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 stars reflect an unpaired t-test (***, p<0.0001; **, p<0.006).

**Figures**
A

Raf

α-Gal

Suc

uninfected

Gal

infected

infected + 10 µM DGJ

Retention time (min)

PAD detector response (arbitrary units)

B

Suc

RafS

Gol

uninfected

infected

infected + 10 µM DGJ

Retention time (min)