Myoinositol Oxygenase Controls the Level of Myoinositol in Arabidopsis, But Does Not Increase Ascorbic Acid[1][OA]

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Ascorbic acid (AsA) is a major plant antioxidant. Mutants like *vtc1* show a reduced AsA concentration, which confirmed by genetic evidence the previously proposed AsA pathway via GDP-Man. Here we investigate the role of an animal-like alternative biosynthesis route to AsA, starting from the metabolite D-GlcUA, which is produced in plants by myoinositol oxygenase (Miox). *Miox*-overexpressing lines have a more than 30-fold up-regulated transcript level and higher enzymatic activity as shown by increased incorporation of Miox-derived sugars into cell wall polymers. In addition, *Miox* overexpressors exhibit a lower steady-state level of myoinositol and accumulate less myoinositol in feeding experiments due to an enhanced turnover rate. The AsA concentration remains the same in wild-type and *Miox* overexpressor lines. Even challenging plants with stress, which increases AsA concentration 4-fold, reveals no difference in AsA biosynthesis between wild-type and *Miox*-overexpressing lines. We conclude that D-GlcUA derived from the Miox reaction plays a negligible role for AsA biosynthesis. However, Miox controls the metabolite level of myoinositol in plants.

L-Ascorbate (AsA) is a very important antioxidant in plants protecting especially the chloroplast from oxidative damage (Noctor and Foyer, 1998). In addition, AsA has numerous other functions in plants. It is an important cofactor for enzymes in the xanthophyll cycle (Muller et al., 2001) and the hydroxylation of Pro (Hieta and Myllyharju, 2002). Furthermore, AsA has been suggested to have a role in cell expansion and mitosis (Arrigoni, 1994). Thus, the *vtc1* mutant, which has only approximately 30% AsA of the wild-type plants, grows slowly and shows a late-flowering phenotype (Conklin et al., 1997; Veljovic-Jovanovic et al., 2001). AsA as the major antioxidant in plants strongly influences the redox status of cells.

Plants, fungi, and many animals can synthesize AsA on their own, but humans and many vertebrates lack the terminal biosynthetic enzyme gulonolactone oxidase. In terms of evolution, AsA is very old; plants and animals seem to use distinct, but partially overlapping, routes to AsA biosynthesis (Fig. 1). In rats, the pathway starts from D-GlcUA followed by a reduction to L-gulonic acid and ring formation to gulonolactone (Smirnoff et al., 2001). This product is finally oxidized to L-AsA. The major plant pathway to AsA was first proposed by Wheeler et al. (1998), elucidated and subsequently confirmed by a number of mutants from Arabidopsis (*Arabidopsis thaliana*) obtained in a screen for ozone-sensitive plants (Conklin et al., 1999). The precursor Man-1-P is activated to GDP-Man (*vtc1* mutation) followed by an epimerization to GDP-L-Gal (Fig. 1). After hydrolysis of this nucleotide sugar, the liberated L-Gal is reduced to L-galactono-1,4-lactone, which is eventually oxidized to L-AsA (Wheeler et al., 1998; Smirnoff et al., 2001).

More recently, Radzio et al. (2003), Lorence et al. (2004), and Zhang et al. (2008) presented evidence for an animal-like AsA biosynthesis route in plants. The overexpression of a gene for myoinositol oxygenase (*Miox*) led to an increase of AsA in transgenic Arabidopsis lines by 2- to 3-fold. Because the product of the Miox reaction is D-GlcUA, their data suggest that this metabolite is readily converted into AsA in Arabidopsis. The study by Zhang et al. (2008) overexpressed a purple acid phosphatase that is active on phytate, a hexa-phosphate ester of myoinositol. Again, the increase in phosphatase transcripts could result in an increase of myoinositol, which after ring cleavage to D-GlcUA, would provide a precursor for an alternative AsA pathway in plants.

Miox is a unique monooxygenase that catalyzes a ring cleavage of myoinositol to D-GlcUA. The enzyme was recently purified, sequenced, and cloned from swine kidney (Arner et al., 2001) and *Cryptococcus* yeast (Kanter et al., 2003). The mouse Miox crystal structure is available (Brown et al., 2006). The genes are highly conserved in eukaryotes. Whereas animals use Miox to degrade myoinositol into D-GlcUA, plants make use of this product and activate it into UDP-GlcUA, which is an important precursor for plant cell walls. Miox is encoded by a small gene family in Arabidopsis, all of which are developmentally regulated (Kanter et al., 2005). Labeling studies with ^{3}H-myoinositol clearly showed its incorporation into pectic polymers and hemicelluloses of cell walls (Loewus et al., 1962; Seitz

[1] This work was supported by the German Science Foundation.

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.130948

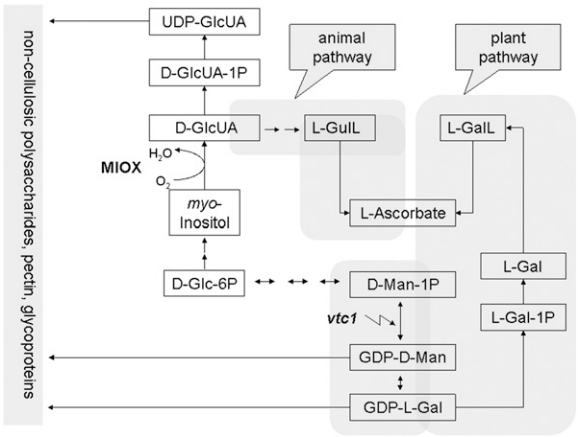

Figure 1. The position of Miox activity and the two discussed routes of ascorbate synthesis in plant hexose metabolism. The shaded areas separate the animal and the plant pathway. Furthermore, the location of the *vtc1* mutation is indicated. For a more comprehensive picture, including participating enzymes, we refer to Davey et al. (1999) and more recently Ishikawa et al. (2006). GulL, Gulono-lactone; GalL, galactono-lactone.

et al., 2000). These findings were supported by the analysis of knockout mutants in *Miox1* and *Miox2*, which showed a strong reduction in the incorporation of ^{3}H-myoinositol into cell wall polymers (Loewus et al., 1962; Kanter et al., 2005). In Arabidopsis, the typical AsA concentration in leaves is approximately 2 to 3 μmol g^{-1} fresh weight provided normal short-day growth conditions at 22°C to 24°C and moderate light intensity (100–150 μE m^{-2} s^{-1}). Abiotic stress by high light and/or cold temperature increases the AsA concentration to counterbalance the elevated levels of reactive oxygen species associated with abiotic stress. Extreme examples from high mountain plants show a circadian rhythm in AsA concentration peaking at about 50 μmol g^{-1} fresh weight in *Soldanella alpine* after a few hours of high light (Streb et al., 2003a, 2003b). This AsA concentration is roughly 25-fold higher than typically found in Arabidopsis leaves. Thus, AsA biosynthesis shows highly dynamic regulation of the biosynthesis rate and an adapted degradation pathway in the apoplast (Pignocchi and Foyer, 2003; Green and Fry, 2005). The plasticity of AsA biosynthesis suggests the possibility that more than one biosynthetic route contributes to the AsA status in plants.

Here we report on the metabolic changes in *Miox*-overexpressing lines and the consequences for AsA biosynthesis in these transgenic Arabidopsis plants.

RESULTS

Transcript Levels

Two independent transgenic Arabidopsis lines produced by the Nessler Lab in Blacksburg, Virginia, overexpressing the *Miox4* gene under the control of the cauliflower mosaic virus 35S promoter were analyzed for the expression level of *Miox4* transcripts in mature leaves. These lines correspond to the lines L2 and L3 in their previous publication (Lorence et al., 2004). Quantitative PCR (qPCR) of leaf tissue shows a drastic increase of *Miox4* transcripts compared to wild-type plants (Fig. 2).

Normally, wild-type Arabidopsis plants express *Miox4* in leaves only at a very low level. Data from public microarray databases analyzed by Genevestigator software (Zimmermann et al., 2004) confirm our qPCR data. The surplus of *Miox4* transcript in the overexpressing lines is even more striking because the regular amount in wild-type leaves is so minute. To impart a better estimation of how much more overall *Miox* transcripts are present in the *35S*::*Miox4* lines, we calculated the transcript level of all four *Miox* genes relative to the expression of the *EF1α* gene. By doing so, we see that the *35S*::*Miox4* lines have a much higher total number of *Miox* transcripts (up to 40 times) than the wild-type plants, the vast majority of it being accounted for by the cauliflower mosaic virus 35S-driven *Miox4*.

Miox Protein Activity

Feeding of ^{3}H-Myoinositol

Miox enzyme activity is difficult to measure, especially in small amounts of samples. To circumvent this problem, we have used several in vivo approaches to show that the Miox-overexpressing plants have elevated enzyme activity for Miox. A first approach used the feeding of leaf discs with ^{3}H-myoinositol.

35S::*Miox4* lines L2 and L3 incorporate more label from myoinositol into polymeric cell wall material (Fig. 3A). This indicates Miox protein activity by means of conversion of myoinositol into D-GlcUA, which is subsequently activated into UDP-GlcUA. The latter is the main precursor for other UDP sugars and a building block for the synthesis of matrix polysaccharides.

Endogenous Level of Myoinositol, and Feeding of Myoinositol

We analyzed metabolite levels for myoinositol by HPLC. The steady-state level in both *35S*::*Miox4* lines

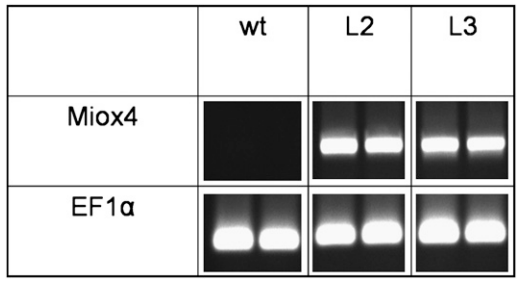

Figure 2. *Miox4* transcript in leaf tissue. Ethidium bromide-stained bands of PCR products of *Miox4* and *EF1α* (control). See "Materials and Methods" section for details.

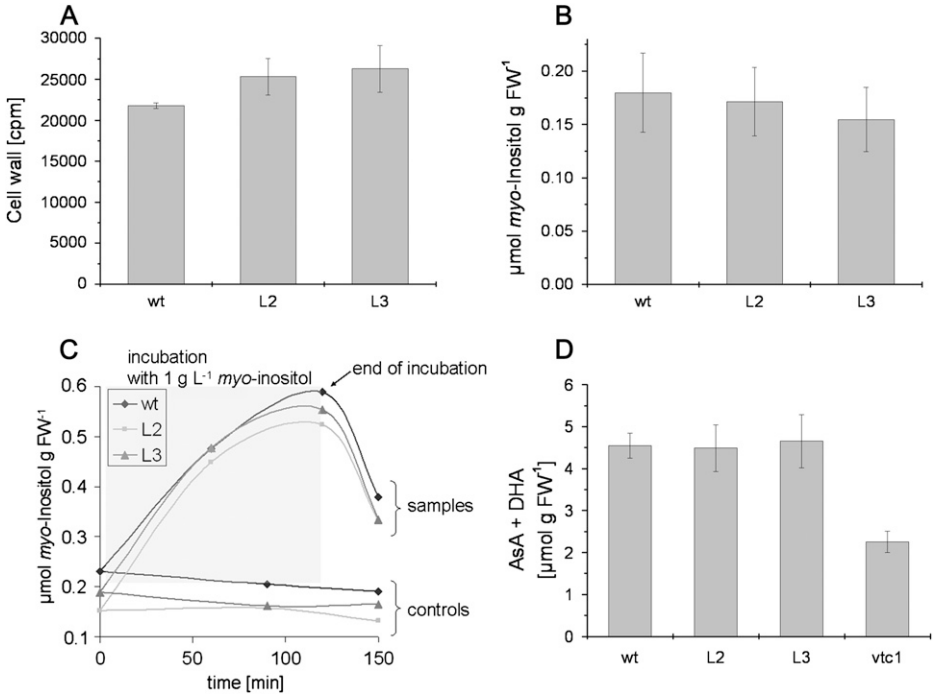

Figure 3. Different approaches to assay Miox activity in vivo. A, Leaf discs were floated for 3 h on one-half-strength Murashige and Skoog basal medium (myoinositol-free) supplied with 1 μM ^{3}H-myoinositol and 500 μM cold myoinositol. Cell wall extraction with subsequent scintillation counting reveals the amount of incorporated label. Shown are means and SDS of three individual experiments. B, Soluble carbohydrates are extracted from plant tissue grown under standard conditions. HPLC and electrochemical detection allow quantification of the intrinsic myoinositol levels. Shown are means and SDS (n = 3). C, Leaf discs were floated on one-half-strength Murashige and Skoog basal medium supplied with 1 g L^{-1} myoinositol (samples). After 2 h, the discs were transferred to myoinositol-free medium. At appropriate time points, the kinetics of myoinositol uptake and metabolization were monitored in the different plant lines. For comparison, leaf discs were kept on myoinositol-free medium over the whole time course (controls). The curves represent the means of three independent experiments. D, Cell extracts of leaf tissues were separated by HPLC and the elution profile at 254 nm was used to determine AsA content. Shown are means and SDS (n = 3).

is lower than in wild-type plants (Fig. 3B), suggesting elevated conversion rates due to increased Miox protein activity.

This prompted us to feed myoinositol to leaf discs (Fig. 3C). All plants show highly increased myoinositol concentrations after feeding for 2 h. In the overexpressing lines, metabolite analysis revealed a lower level of myoinositol to start with (0 min), less accumulation of myoinositol compared to wild-type plants (120 min), and, after termination of the feeding period, a swift decline of the myoinositol pool, all confirming the surmise of rapid myoinositol turnover in the *35S*::*Miox4* lines. The data from all experiments prove that both *35S*::*Miox4* lines have indeed a higher Miox activity than wild-type plants.

Relation between Miox Activity and AsA Levels

To investigate the role of Miox for the biosynthesis of AsA, we adapted a robust and fast HPLC method for quantitative analysis of AsA (Gokmen et al., 2000). The recovery, determined by spiking AsA to the leaf homogenate, is between 99% and 101%. The two *35S*::*Miox4* lines were previously described as AsA hyperaccumulators exhibiting 2- to 3-fold higher AsA levels than wild-type plants (Lorence et al., 2004). Using the HPLC assay, we analyzed several sets of plants. We also included the *vtc1* line, which is compromised in the Man pathway of AsA biosynthesis due to an impairment of the GDP-Man pyrophosphorylase. It serves as an internal control with a published content of approximately 30% of wild-type AsA level (Conklin et al., 1997).

Standard Versus Stress Conditions

In plants grown in standard conditions, we did not measure deviations between AsA levels in young leaves of the *35S*::*Miox4* lines compared to wild-type plants (Fig. 3D).

Because AsA is a typical stress metabolite to counterbalance oxidative damage, we applied stress to the plants to investigate whether this can induce elevated rates of AsA biosynthesis in *35S*::*Miox4* plants.

A set of plants was acclimated to low light (25 μE m^{-2} s^{-1}) for 3 d and afterward exposed to high light conditions (250 μE m^{-2} s^{-1}). There was indeed a rapid response in AsA levels; however, the same approximately 400% increase within 24 h was seen in all lines investigated (Fig. 4A).

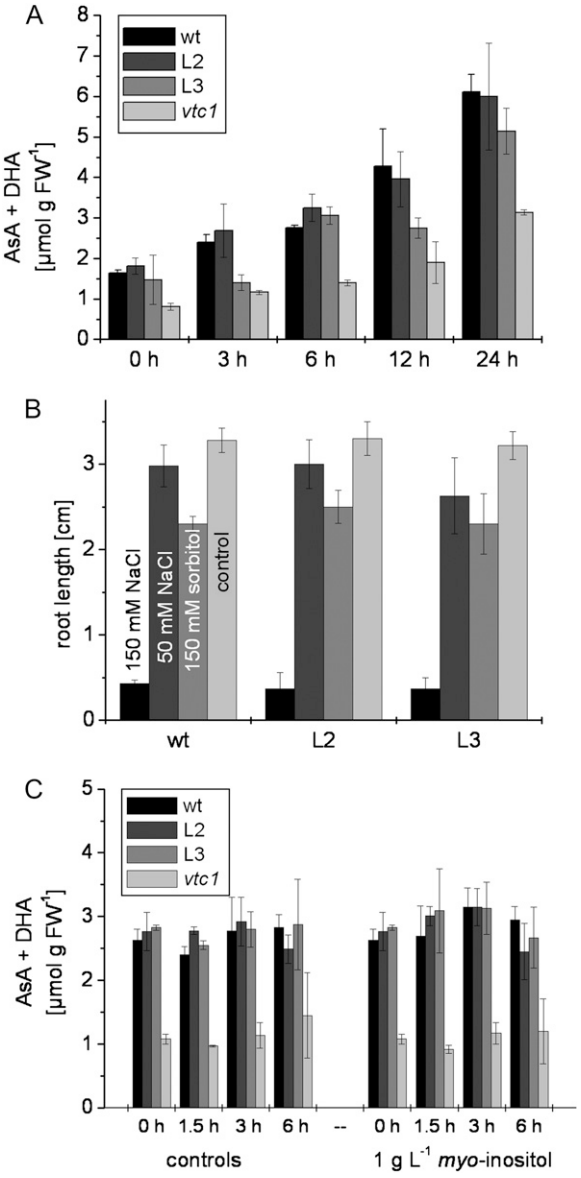

Figure 4. Response to stress or feeding conditions. A, Young plants adapted to low light were exposed to high light conditions (0 h). At appropriate time points, samples were analyzed by HPLC to monitor the amount of AsA in the leaves. Shown are means and SDS (n = 3). B, Surface-sterilized seedlings were grown on one-half-strength Murashige and Skoog/1% Phytagel plates (control), and supplemented with a final concentration of 150 or 50 mM NaCl, or 150 mM sorbitol. Root length after 10 d was measured as an indication of stress tolerance. Shown are the means of three plates and their SDS. C, Leaf discs were floated on one-half-strength Murashige and Skoog medium supplied with 1 g L^{-1} myoinositol and the AsA + DHA content was measured. As a control, leaf discs were floated on myoinositol-free Murashige and Skoog medium. The experiment was repeated three times; shown are means and SE.

Furthermore, we tested the *35S*::*Miox4* lines for their reaction to salt stress.

While the lines do not differ when germinated on regular Murashige and Skoog medium, elevated salt concentrations negatively affect all of the lines tested to a similar degree (Fig. 4B).

Feeding of Cold Myoinositol

To exclude the possibility that the availability of the substrate myoinositol is limiting for potential AsA biosynthesis via Miox, we fed myoinositol to leaf discs (Fig. 4C). During 6-h incubation, none of the lines accumulated excess AsA in the treated samples compared to the control.

In summary, we could not detect an increased AsA level in *35S*::*Miox4*-overexpressing lines under standard or stress conditions.

DISCUSSION

Based on genetic studies, the Smirnoff-Wheeler pathway from Man to AsA is clearly the most important route for AsA synthesis in Arabidopsis and most likely in other plants as well. Nevertheless, there still is a controversy as to whether other pathways to AsA are functional in plants. The study by Agius et al. (2003), in which the strawberry gene for D-GalUA reductase was overexpressed in Arabidopsis, shows a 2- to 3-fold increase in AsA compared to control plants. Our interest is the metabolism of D-GlcUA and UDP-D-GlcUA in plants and the use of these nucleotide sugars for cell wall biosynthesis. Two recent papers suggest the additional role of D-GlcUA in plants as a potential precursor for AsA (Lorence et al., 2004; Zhang et al., 2008). Both studies rely on the conversion of myoinositol into D-GlcUA, followed by an animal-like biosynthetic route to AsA. The two *35S*::*Miox4* lines L2 and L3 analyzed in our study are identical to the plants described by Lorence et al. (2004). To our surprise, we could not detect the strong 2- to 3-fold reported increase in AsA in these transgenic lines. The paper showed a higher transcript level in the *35S*::*Miox4* lines, but did not provide any evidence for higher Miox enzyme activity. We confirmed the high *Miox4* transcript level in leaves, which is normally negligible in wild-type plants (Kanter et al., 2005), ruling out silencing phenomena in the L2 and L3 lines.

Miox is difficult to measure in crude extracts, presumably caused by changes in the di-iron center at the catalytic site (Xing et al., 2006). We therefore tested in vivo the assumed higher activity in L2 and L3. Indeed, the myoinositol concentration in these lines is lower than in wild-type plants, suggesting that higher Miox activity metabolizes most of the available myoinositol. Myoinositol is readily taken up by plant cells using several transporters in the plasma membrane (Schneider et al., 2007). Feeding myoinositol to leaf discs therefore strongly increases the internal concentration. However, the level of myoinositol in the two *35S*::*Miox4* lines remains lower than in wild-type plants. Taken together, we were able to prove that *35S*::*Miox4*-overexpressing plants have indeed a higher Miox activity.

The conflicting data about AsA from the article by Lorence et al. (2004) were analyzed in two further sets of experiments. First, we tested stress conditions. Changes in plant growth stress conditions strongly increase AsA similarly in wild-type and in *35S*::*Miox4* lines. This suggests that stress-provoked increase in AsA is not synthesized predominantly via Miox, but uses the well-established Man to AsA route. Second, we increased the supply of the substrate. Myoinositol is present in low concentrations in plants. Leibowitz et al. (1977) and Farre et al. (2001) determined the metabolite concentration in the range of 60 μM. To synthesize the millimolar concentration of AsA, a very rapid turnover of the metabolite pool would be necessary. To avoid a potentially limiting supply of myoinositol to the AsA biosynthesis pathway, we provided external myoinositol, which increases the equilibrium pool concentration approximately 3-fold in wild-type and *35S*::*Miox4* lines. Nevertheless, no increase in AsA could be detected.

Here, the HPLC method for AsA determination presented in this article differs from the enzymatic one employed in the articles mentioned above. By virtue of its simplicity and unambiguousness, the HPLC measurement of AsA seems favorable to the frequently employed enzymatic assay with AsA oxidase. We obtained recovery rates for AsA around 100% and these values confirm data from Lykkesfeldt et al. (1995), who also reported recovery rates between 99% and 101%. The AsA oxidase assay relies on a drop in absorption at 265 nm due to the conversion of AsA to dehydroascorbic acid (DHA). This wavelength interferes with a variety of other substances in a crude cell extract that absorbs at a similar wavelength. Especially in extracts from high light-treated plants, the assay based on a decrease in UV absorption for AsA might have some problems from interfering plant secondary metabolites.

The rapid metabolism of myoinositol by Miox into D-GlcUA suggests a regulatory role of the successive glucuronokinase (see Fig. 1). At least two competing enzymes could process available D-GlcUA and thereby channel this metabolite into the cell wall polymer pathway or, less likely, into the AsA pathway. Experiments in which ^{3}H-myoinositol labeling was followed in plants showed a preferential conversion into GlcUA, GalUA, Ara, and Xyl residues of cell wall polymers (Seitz et al., 2000). Unfortunately, the glucuronokinase is not biochemically well characterized (Leibowitz et al., 1977) and unknown at the molecular level up to now. The kinase is involved in an anabolic reaction to UDP-GlcUA, which is unique to plants. In animals, UDP-GlcUA is exclusively and in plants predominantly provided by the enzyme UDP-Glc dehydrogenase (S. Endres, R. Reboul, and R. Tenhaken, unpublished data). The still open question is the channeling of D-GlcUA into potential pathways. If D-GlcUA is under certain unknown conditions converted into AsA, then the glucuronokinase is likely the major controlling enzyme of the partitioning of D-GlcUA between the competing pathways.

A second article in support of D-GlcUA derived AsA in plants analyzes the overexpression of a purple acid phosphatase, which can degrade phytate (intermediates) to myoinositol (Zhang et al., 2008). These lines were also reported to have a 2-fold higher AsA level than wild-type plants. Our data strongly argue against a role of Miox and D-GlcUA for AsA in Arabidopsis as outlined before. This is best illustrated by two flux calculations based on numbers from Stevenson-Paulik et al. (2005). Even if all of the phytate of a seed was metabolized within a few days after germination, the liberated myoinositol would account for <5% of the cell wall biomass (1 seed [16.5 μg] contains approximately 0.36 nmol phytate, which releases 66 ng myoinositol; a 6-d-old seedling [200 μg fresh weight] has an approximately 5-μg cell wall, of which 2.5 μg is derived from UDP-D-GlcUA; 66 ng myoinositol of a 2,500-ng cell wall accounts for approximately 2.6%). A similar calculation for AsA suggests that not enough AsA could be theoretically synthesized from the myoinositol of phytate of a seedling. (66 ng myoinositol approximately 360 pmol; 1 seedling [200 μg fresh weight] has a volume of approximately 180 nL; 3 mM AsA in this seedling volume would correspond to 540 pmol AsA). The calculation neglects the rapid turnover of AsA. Alpine plants synthesize more than 50% of their AsA freshly every day with a circadian rhythm (Streb et al., 2003a, 2003b). Thus, the surplus of myoinositol from phytate degradation is very unlikely responsible for much of the carbohydrates in the cell wall as well as for potential AsA. Furthermore, Conklin et al. (2006) showed that a knockout of the L-Gal-1-P phosphatase (VTC4) does not stop AsA biosynthesis, suggesting the existence of other phosphatases. A kiwi phosphatase for L-Gal-1-P was previously character-

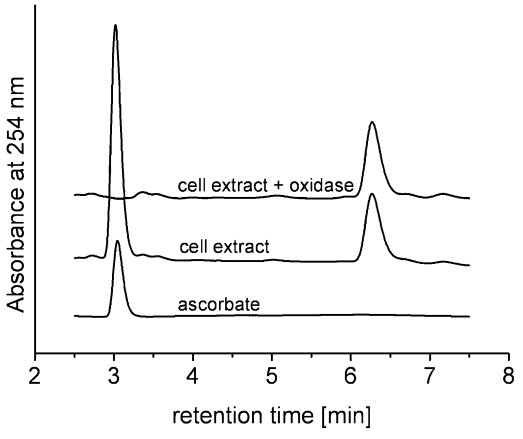

Figure 5. Typical elution profile of cell extracts separated on a C18 column; monitored at 254 nm. Shown is the pure substance (ascorbate), an untreated sample (cell extract), and a sample treated with AsA oxidase (cell extract + oxidase) to prove peak identity. Note that the addition of AsA oxidase completely removes the AsA in the extract.

ized by Laing et al. (2004). Using recombinant kiwi protein, they showed significant enzymatic activity for the alternative substrate myoinositol-1-P. Thus, it seems likely that the purple phosphatase analyzed by Zhang et al. (2008) is also acting on L-Gal-1-P and thereby increases the flux into AsA.

In summary, we could show by several lines of experiments that *35S::Miox4* plants convert myoinositol into D-GlcUA, but that this metabolic route does not lead to an increase of AsA in Arabidopsis. These data are in line with labeling studies, which also found no convincing evidence for a D-GlcUA conversion into AsA (Isherwood et al., 1954; Jang et al., 1956; Loewus, 1999). A recent genetic study in which the two Arabidopsis genes for GDP-L-Gal phosphorylase (*vtc2-1*; *vtc5*) were knocked down is lethal for seedlings, which can only survive in the presence of exogenously applied AsA (Dowdle et al., 2007). This study clearly demonstrates that the GDP-Man pathway to ascorbate is the only significant source for AsA under the tested conditions.

MATERIALS AND METHODS

Plant Growth

Arabidopsis (*Arabidopsis thaliana*) Columbia seeds were obtained from the Nottingham Arabidopsis Stock Centre (order no. N60000). The *35S::Miox4* lines L2 and L3 were kindly provided by the Nessler group (Virginia Tech, Blacksburg, VA) and are described in Lorence et al. (2004). Plants were grown in standard fertilized soil (ED73; Einheitserde) in a growth chamber (23°C; 150 μE m^{-2} s^{-1}). To monitor root growth, surface-sterilized seeds (with 2.5% NaOCl) were sown on square petri dishes and incubated vertically in the growth chamber. The medium was composed of one-half-strength Murashige and Skoog (M0245; Duchefa) with 1% Phytagel, adjusted to pH 5.6 (KOH), and either NaCl in the final concentration of 50 mM or 150 mM, or 150 mM sorbitol.

PCR Techniques

To test for the presence of full-length *Miox4* transcripts, leaf RNA was extracted according to Chomczynski (1993) and transcribed to cDNA using the RevertAid Moloney murine leukemia virus reverse transcriptase kit (Fermentas). The *Miox4* sequence was amplified with TGACGATCTCTGTTGAGAAG forward and TCACCACCTCAAGTTTTCCG reverse primers, 58°C annealing temperature, and 38 cycles. GTAACAAGATGGATGCCACCACC forward and CCTCTTGGGCTCGTTGATCTG reverse were used to amplify the housekeeping gene *EF1α* for internal normalization (58°C annealing temperature; 32 cycles).

For quantification of *Miox* transcripts, qPCR was performed on a Stratagene MX3000 real-time cycler using a SYBR Green method. The reaction (30 μL) consisted of 1× PCR buffer complemented with the PCR additive betaine at a final concentration of 0.6 M, 1:200,000 dilution of SYBR Green stock (Roche), 200 nmol primers each, and 1 unit Taq polymerase (recombinant wild type). Primers were the ones given above for *EF1α*; and for amplicons of the *Miox* isoforms we used for *Miox1*, CATGTACCTTGTTGCGAAGGAG forward, ACCATTTTAGCTTGGACGGA reverse; for *Miox2*, GCTGTCGTTGGCGATACATTTC forward, AGGGTCGTGCCATTCTTCTTAG reverse; for *Miox4*, GGCTGTTGTTGGTGACACATTC forward, CGTGTAGCCACTTCAGATTCTC reverse; and for *Miox5*, GGCTGTTGTTGGTGACACATTTC forward, TAAGCTCCAGCCTTGTGCAATG reverse.

The qPCR program cycles through 30 s at 92°C, 30 s at 59°C, and 15 s at 72°C for 40 times; a conclusive melting curve indicates identity and homogeneity of the product.

For calculations, the reaction efficiencies of the individual wells were computed from the original data employing the LinRegPCR software, version 7.4, and raised to the power of the corresponding threshold cycle value. Results were averaged over triplicates and normalized with the calculated amounts of *EF1α* transcripts. Finally, the results coming from independent RNA extractions were averaged.

HPLC Assay for AsA

For quantification of AsA, the HPLC method introduced by Gokmen et al. (2000) was optimized for plant tissues. The material (usually 50–60 mg fresh weight) was frozen in liquid nitrogen, pulverized using a Retsch ball mill, and extracted following the procedure described in Foyer et al. (1983). The plant material is suspended in 1 mL 1 M $HClO_4$, centrifuged, and the perchloric acid of the supernatant precipitated with K_2CO_3. The resulting extract is either diluted directly with eluent (0.2 M KH_2PO_4, adjusted to pH 2.4, with H_3PO_4) for HPLC characterization (monitoring the amount of AsA) or an aliquot is neutralized with K_2HPO_4, pH 10 (KOH), and reduced with freshly prepared dithiothreitol at a final concentration of 20 mM. The reduction reaction is stopped by addition of eluent and a few microliters of H_3PO_4 to bring the pH back to a value of 2 to 3. In the reduced samples, the total amount of AsA, composed of both the reduced and the previously oxidized form (DHA; lacking A_{254}), can be assayed.

If plant material was scarce, the volumes were scaled down to one-fifth. The samples were kept refrigerated except for the reduction reaction, which is performed at room temperature.

For detection of AsA, the samples are separated on a ProntoSIL 120 C18 AQ Plus column with 0.2 M potassium phosphate (pH 2.4) at a flow rate of 0.5 mL min^{-1} and monitored at 254 nm (Fig. 5).

To estimate the recovery of this method, known amounts of pure AsA were added to a pulverized leaf sample, subjected to the extraction procedure, and AsA as well as AsA + DHA were measured. The experiment was performed in triplicate. Linear correlation was >0.99; the recovery rate was 99% for AsA and 101% for AsA + DHA, which corresponds nicely with results obtained by Lykkesfeldt et al. (1995) for a similar HPLC procedure of AsA quantification.

Precautions must be taken because we observed that AsA is not stable over a longer period of time in the cell extracts. Therefore, we recommend sample preparation to be conducted in a successive manner, adapted to the pace of the HPLC instrument, to ensure comparability of the samples and keep latencies well below 1 h.

HPLC Method for Myoinositol

To determine the myoinositol content of plant material, it was pulverized in liquid nitrogen and extracted with 600 μL of a mixture of MeOH, chloroform, and water (101:4:4 [v/v]), following the protocol for soluble carbohydrate extraction published by Fiehn et al. (2000). After 20-min incubation at 70°C, the sample was centrifuged for 3 min at 18,000*g*; 500 μL of the supernatant were transferred to a fresh Eppendorf tube and extracted with 180 μL chloroform and 460 μL water. The samples were incubated for 5 min at 37°C; phases were separated by 15-min centrifugation at 7,000*g*; 800 μL of the supernatant containing the soluble sugars were dried in a SpeedVac centrifuge, resuspended in 150 μL water, centrifuged 5 min at 18,000*g* to sediment insoluble particles, and aliquots of the supernatant were used for HPLC measurements.

We analyzed the samples on a ICS3000 system (Dionex) using a CarboPac MA1 analytical column (Dionex) with 120 mM NaOH as isocratic eluent at a flow rate of 0.4 mL min^{-1} and electrochemical detection (pulsed Aometric detection; ED50; quadruple waveform recommended for carbohydrate analysis). Concentrations were determined by comparison to authentic standards.

Feeding of Myoinositol/Labeling with ^{3}H-Myoinositol

As a basis for the feeding and metabolization experiments, we needed myoinositol-free Murashige and Skoog medium to begin with. We achieved that by amending the Murashige and Skoog basal salt mixture (M5524; Sigma) with a self-prepared vitamin mix replenishing niacin (0.5 mg L^{-1}), pyridoxine HCl (0.5 mg L^{-1}), thiamine HCl (0.1 mg L^{-1}), and Gly (2.0 g L^{-1}).

For the incubation experiments, discs of 8 mm in diameter were stamped out of fully expanded leaves and immediately floated abaxial side down on this very myoinositol-free medium, complemented with 2.5 mM MES buffered at pH 5.6 (KOH). When feeding cold myoinositol, it was admixed to a final concentration of 1 g L^{-1} (10 times more than in regular Murashige and Skoog medium). After allowing the leaf discs to take up myoinositol at room temperature and low light conditions for 2 h, they were carefully rinsed in

pure water, blotted on filter paper, and transferred to myoinositol-free medium or extracted directly with the procedures described above.

For tritiated myoinositol, myo-[-2-^{3}H]inositol (Amersham/GE Healthcare) and cold myoinositol were added to a final concentration of 1 and 500 μM, respectively. After 3 h of incubation at room temperature and low light conditions, the discs were rinsed in water, blotted dry, followed by the extraction of cell wall as detailed in Kanter et al. (2005). The dry cell wall samples were resuspended in Rotizint Eco Plus cocktail (Roth) and then counted in a liquid scintillation analyzer, TRI-CARB 2100TR (Packard).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_172904.2 (MIOX1), NM_127538.3 (MIOX2), NM_118759.4 (MIOX4), and NM_125047 (MIOX5).

ACKNOWLEDGMENT

We would like to thank Craig L. Nessler for providing *35S::Miox* lines.

Received October 9, 2008; accepted December 16, 2008; published December 17, 2008.

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