The Arabidopsis ascorbate peroxidase 3 is a peroxisomal membrane-bound antioxidant enzyme and is dispensable for Arabidopsis growth and development

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Abstract

Ascorbate peroxidase (APX) exists as several isoforms that are found in various compartments in plant cells. The cytosolic and chloroplast APXs appear to play important roles in antioxidation metabolism in plant cells, yet the function of peroxisomal APX is not well studied. In this study, the localization of a putative peroxisomal membrane-bound ascorbate peroxidase, APX3 from Arabidopsis, was confirmed by studying the green fluorescent protein (GFP)–APX3 fusion protein in transgenic plants. GFP–APX3 was found to co-localize with a reporter protein that was targeted to peroxisomes by the peroxisomal targeting signal 1. The function of APX3 in Arabidopsis was investigated by analysing an APX3 knockout mutant under normal and several stress conditions. It was found that loss of function in APX3 does not affect Arabidopsis growth and development, suggesting that APX3 may not be an important antioxidant enzyme in Arabidopsis, at least under the conditions that were tested, or the function of APX3 could be compensated by other antioxidant enzymes in plant cells.

Key words: Antioxidation, hydrogen peroxide scavenging, peroxisomal membrane targeting, reactive oxygen species, subcellular localization.

Introduction

Reactive oxygen species (ROS, e.g. O₂ and H₂O₂) are produced as by-products of plant cellular metabolism (Asada and Takahashi, 1987; Foyer and Noctor, 2000). On the one hand, ROS serve as signalling molecules in plant redox signal transduction (Foyer and Noctor, 2005), and on the other hand ROS are harmful to plant cellular metabolism (Mittler, 2002; Apel and Hirt, 2004). Under stress conditions, ROS can be overproduced in plant cells, which creates a condition called oxidative stress that can damage cellular components including DNA, proteins, and membrane lipids (Mittler, 2002; Apel and Hirt, 2004). Plants have developed efficient antioxidant systems that can protect plants from oxidative stress (Asada, 1999; Mittler et al., 2004). These antioxidant systems include antioxidant molecules such as ascorbic acid, glutathione, α-tocopherol, and carotenoids, and antioxidant enzymes such as ascorbate peroxidase (APX), catalase, glutathione reductase (GR), and superoxide dismutase (Noctor and Foyer, 1998; Apel and Hirt, 2004). Antioxidant molecules can interact with ROS directly, thereby removing ROS from cells. However, they are more effective in serving as electron donors to scavenge ROS in reactions carried out by some antioxidant enzymes such as APX and GR (Noctor and Foyer, 1998; Apel and Hirt, 2004). For example, APX uses ascorbic acid as the electron donor to scavenge H₂O₂ efficiently in plant cells (Asada, 1992, 1999; Shigeoka et al., 2002).

APX exists in several isoforms that are found in various compartments of plant cells (Panchuk et al., 2002;
Shigeoka et al., 2002; Chew et al., 2003). The cytosolic APXs play important roles in antioxidant metabolism in plant cells. The loss-of-function mutation in APX1 that encodes a cytosolic APX in Arabidopsis leads to lower photosynthetic rates, slower growth, and delayed flowering under normal growth conditions in comparison with these characteristics of wild-type plants (Pnueli et al., 2003). Under high-intensity light, the chloroplast H$_2$O$_2$-scavenging system is unable to keep pace with H$_2$O$_2$ production, and H$_2$O$_2$ accumulates, causing massive protein oxidation in APX1-deficient cells (Davletova et al., 2005). These data suggest that cytosolic APXs, particularly APX1, play a key role in protecting chloroplasts under light stress conditions. Chloroplast APXs also play important roles in photosynthesis under stress conditions (Asada, 1999). For example, the loss of a thylakoid membrane-bound APX (tAPX) in hexaploid wheat leads to a reduction in CO$_2$ assimilation (Davletova et al., 2005). These data indicate that the increased level of APX in peroxisomal membranes appears to increase stress tolerance in plants. However, these studies did not address the question as to how important is the peroxisomal APX to cellular antioxidation metabolism.

One objective of this research was to obtain evidence that the Arabidopsis APX3 is indeed a peroxisomal membrane-bound antioxidant enzyme. A further objective was to study the possible function of APX3 by analysing the performance of APX3 knockout mutants under conditions that would favour oxidative stress. The APX3-deficient plants did not display any major abnormalities in growth and development under normal growth condition, or under the stress conditions that were tested here, suggesting that perhaps APX3 is not absolutely required for Arabidopsis growth and development.

Materials and methods

Construction of GFP–APX3 fusion construct and Arabidopsis transformation

The green fluorescent protein (GFP) coding sequence was amplified by polymerase chain reaction (PCR) using primers gFP-3 and gFP-5, digested with enzymes BamHI and XbaI, and subcloned into the vector pGEM-3Z (Promega, Madison, WI, USA) to form the intermediate vector pGEM-3Z-GFP-B. The full-length APX3 was amplified by PCR from a cDNA library with primers APX3-18 and APX3-19, digested with BamHI and then subcloned into the C-terminal end of GFP in the intermediate vector pGEM-3Z-GFP-B to form the vector pGEM-3Z-GFP-APX3. The correct orientation of the DNA insert was confirmed by PCR using GFP-specific and APX3-specific oligonucleotides, and sequenced for accuracy. The vector pGEM-3Z-GFP-APX3 was digested with enzymes XbaI and SacI (partial digestion due to an internal SacI site), and the GFP–APX3 fusion fragment was then subcloned into pBI121 (Jefferson et al., 1987) by replacing the ‘GUS’ gene to form the transforming vector that was transformed into the Agrobacterium tumefaciens GV3101. The Arabidopsis transformation was conducted according to the protocol of Clough and Bent (1998). The sequences of oligonucleotides used for cloning are listed as follows: APX3-18, 5’-TAGTCGAGTCGATCCCTACTCCATCTCCCTCTCGAATC-3’; APX3-19, 5’-CAGCTGACGGTCGATCCCTACTCCATCTCCCTCTCGAATC-3’; gFP-3, 5’-CAGCTGACGGTCGATCCCTACTCCATCTCCCTCTCGAATC-3’; and gFP-5, 5’-CGTACTCTAGAGTAAAGGAGAAGAACT-3’.

RNA isolation and hybridization

Total RNAs were isolated from 3-week-old Arabidopsis plants using the TRIZol reagent from Invitrogen (Carlsbad, CA, USA), separated by electrophoresis (10 μg per lane), blotted to a Nylol membrane, and hybridized with various probes. Hybridization was carried out according to the method of Church and Gilbert (1984)
using probes labelled by random priming. The washing conditions were as follows: once for 10 min in 0.5% bovine serum albumin (BSA), 1 mM EDTA, 40 mM NaHPO₄ (pH 7.2), and 5.0% SDS at 64 °C; then four times (10 min each) in 0.1 mM EDTA, 40 mM sodium phosphate (pH 7.2), and 1% SDS at 64 °C. The same filter was used for hybridizations with probes APX3 and PolyUb consecutively. The condition for stripping the filter was as follows: twice (15 min each) in 2 mM TRIS (pH 8.2), 2 mM EDTA (pH 8.0), and 0.08% SDS. The polyubiquitin gene [Arabidopsis Biological Resources Center (ABRC) number ATIS 0348] was obtained from ABRC at Ohio State University, Columbus, OH, USA.

**Immunoblot analysis**

Plants at the 5–6 leaf stage were ground to a fine powder in liquid nitrogen. The powder was transferred to a fresh tube containing 200 μl of 50 mM NaHPO₄ (pH 7.0) and centrifuged at 15 000 g for 10 min at 4 °C. The supernatant that contained mainly cytosolic proteins was discarded, and the pellet was re-suspended in 200 μl of 50 mM sodium phosphate (pH 7.0) and 200 μl of 2× SDS buffer [125 mM TRIS–HCl, 2% SDS, 20% glycerol, 200 mM dithiothreitol (DTT), 0.02% bromophenol blue, pH 6.8]. The resuspended pellet was boiled in a water bath for 10 min and centrifuged at 15 000 g for 10 min at 4 °C. The supernatant was transferred to a fresh tube, and the protein concentration in the supernatant was determined by using the Bradford assay (Bradford, 1976). Proteins from GFP–APX3 transgenic plants, APX3 knockout plants, and wild-type plants were subjected to electrophoresis in a 12% SDS–polyacrylamide gel. The conditions for blotting and colour development were the same as described previously (Yan et al., 2003), except that the antibody used was anti-APX3 antibody.

**Protoplast isolation**

Protoplasts were isolated using a modified method of Sheen (2002). Large rosette leaves from 3-week-old seedlings were rinsed, blotted dry, piled one on top of another, and sliced into 0.5–1 mm strips. The strips were transferred to a 25 ml flask containing the enzyme solution (1% cellulase, 0.25% macerozyme, 0.4 M mannitol, 20 mM KCl, 0.02% bromophenol blue, pH 6.8). After this step, the speed was increased to 80 rpm for 1 min to aid the complete release of protoplasts. The enzyme solution was then passed through a 70 μm nylon mesh and centrifuged at 60 g for 5 min. The pellet was washed twice with wash and incubation solution (0.5 M mannitol, 4 mM MES, 20 mM KCl, pH 5.7) and then resuspended in 100 μl of the wash and incubation solution.

**Creation of transgenic plants that express GFP–APX3 and RFP–PTS1 fusion proteins**

Five independent transgenic lines that express GFP–APX3 fusion protein at high levels (based on western blot analysis) were separately crossed to a line that expresses red fluorescent protein (RFP)–PTS1 fusion protein at high levels (based on western blot analysis) were separately crossed to a line that expresses red fluorescent protein (RFP)–PTS1 fusion protein (Lin et al., 2004), and the F₁ progeny from these crosses were analysed for the presence of both GFP–APX3 and RFP–PTS1 fusion proteins. All five F₁ progeny gave rise to similar results under the fluorescence microscope.

**Arabidopsis growth and stress treatments**

*Arabidopsis* seeds were sown on MS medium and kept at 4 °C for 5 d before being allowed to grow at room temperature for 7 d, and then they were divided into three groups: one for the normal growth condition, one for chilling temperature treatment, and one for heat treatment. For the normal growth condition, plants were grown under white light (60 μE m⁻² s⁻¹) at 22 °C for 21 d before harvesting for biomass analysis. For chilling temperature treatment, plants were put inside a refrigerator with white light (50 μE m⁻² s⁻¹) and a temperature of 8 °C for 33 d before harvesting for biomass analysis. For heat treatment, plants were put in a growth chamber with white light (200 μE m⁻² s⁻¹) and a temperature cycle of 40 °C for 3 h and 22 °C for 21 h d⁻¹ for 25 d before harvesting for biomass analysis.

**Seed germination experiment**

Sterilized seeds of wild-type plants and the APX3 knockout mutant were planted on MS plates that contained either 50 or 100 mM NaCl for germination. The percentage of germination was calculated as the ratio of seeds that germinated to total seeds planted 7 d after stratification.

**Fluorescence microscopy analysis**

The transgenic plants that express the GFP–APX3 fusion protein at a high level selected from western blot analysis were used for fluorescence microscopy analysis. Protoplasts made from GFP–APX3 transgenic and wild-type plants were analysed with an Olympus BX-50 fluorescence microscope. The flower petals and root tissues from F₁ transgenic plants that expressed both GFP–APX3 and RFP–PTS1 fusion proteins were used directly for localization studies using a fluorescence microscope. The GFP images were then taken using a fluorescence microscope equipped with an exciter filter (HQ 470/40), a dichroic mirror (Q495LP), and a barrier filter (HQ 525/50). The red fluorescence images of the RFP–PTS1 fusion protein and chloroplasts were taken using an exciter filter (BP530/ 550), a dichroic mirror (DM570), and a barrier filter (BA590). The overlay images were created using the Simple PCI software (version 4.0). The black and white images for the same fields were obtained using white light on the same microscope.

**Chlorophyll fluorescence measurement**

Detached dark-acclimated leaves were placed in the temperature-controlled chamber of an oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) and illuminated by actinic light (photoin flux density = 350 μmol m⁻² s⁻¹) at room temperature (25 °C) and ambient CO₂. Then the temperature was quickly (within 5 min) increased up to 40 or 38 °C. Chlorophyll fluorescence emission from the leaves was measured with a pulse amplitude-modulated fluorometer (PAM 101/103, Heinz Walz GmbH, Effeltrich, Germany) through a port in the chamber at different times during the light exposure. Saturating light pulses, 2 s in duration, were provided by a KL 1500 light source (Schott, Wiesbaden, Germany). The experimental protocol described by Schreiber et al. (1986) and the nomenclature of van Kooten and Snel (1990) were used. Quantum efficiency of electron transport in photosystem II complexes (Φₚₛₛᵢ) was estimated as described by Genty et al. (1989). The following formula was used:

$$Φₚₛₛᵢ = (Fₘ' - F)/Fₘ'$$

where $Fₘ'$ is maximal chlorophyll fluorescence yield by the saturating pulse light under actinic light, and $F$ is the level of fluorescence just before application of a saturating flash.

**Results**

APX3 is predicted to be a peroxisomal membrane-bound enzyme

Although APX3 from *Arabidopsis* shows the highest sequence similarity to the cotton pAPX and pumpkin pAPX throughout its entire length (Zhang et al., 1997), including the transmembrane domain at the C-terminus
Bunkelmann and Trelease, 1996; Nito et al., 2001), APX3 was never experimentally determined to be localized to glyoxysomal or peroxisomal membranes. The cotton pAPX is localized to the peroxisomal membrane (Mullen et al., 1999), and the sequence that targets cotton pAPX to the peroxisomal membrane is composed of a C-terminal transmembrane domain followed by a few basic amino acid residues (Mullen et al., 2000, 2001). This sequence was defined as mPTS (i.e. the targeting signal of peroxisomal membrane-bound proteins) (Mullen et al., 2000, 2001).

Since the C-terminal sequence of APX3 resembles the mPTS from cotton pAPX, it is predicted that APX3 is peroxisomal membrane-bound.

Compared with other Arabidopsis APXs, APX3 is most similar to APX5 (Panchuk et al., 2002). Like APX3, APX5 also has a C-terminal transmembrane domain followed by a few positively charged amino acid residues (Fig. 1). It appears that there are only two APXs in Arabidopsis that are likely to be peroxisomal membrane-bound. The third APX that contains a C-terminal transmembrane is the

![Fig. 1. Sequence comparison of Arabidopsis APX proteins. Amino acid residues that are completely conserved are in dark blue, those that share ≥75% homology are in red, and those that share ≥50% homology are in light blue. The transmembrane domain found at the C-terminal end of APX3 or APX5 is underlined, and the positively charged amino acid residues following the transmembrane domain are marked with asterisks.](image-url)
thylakoid membrane-bound APX in *Arabidopsis* (i.e. tAPX in Fig. 1). The other five APX isoforms in *Arabidopsis* do not have transmembrane domains (Fig. 1). APX1 and APX2 are cytosolic proteins, APX4 may be a peroxisomal matrix protein (Panchuk et al., 2002) or a chloroplast protein (Chew et al., 2003), APX6 may be a cytosolic protein (Panchuk et al., 2002; Chew et al., 2003), and finally the stomatal APX (i.e. sAPX) can also be targeted to mitochondria (Chew et al., 2003). In addition, two more APXs were found in the *Arabidopsis* genome (Narendra, 2005): APX7 (At1g33660) and an unknown APX (At1g43220). APX7 is a very small APX and its subcellular localization is not known. The unknown APX has no expressed sequence tag (EST) sequences associated with it; therefore it might be a pseudogene (Narendra, 2005).

**APX3 is localized to the peroxisomal membrane**

In order to localize APX3, it was fused to the C-terminal side of the GFP and the subcellular localization of the GFP–APX3 fusion protein was studied. More than 30 transgenic plants were created and it was found that most of them expressed the GFP–APX3 fusion transcript at high levels (Fig. 2A). The steady-state levels of the GFP–APX3 fusion protein were then studied by western blot analysis in lines that contained high levels of the transgene transcript. As expected, all high expression lines at the transcript level also expressed GFP–APX3 fusion protein (Fig. 2B).

The subcellular localization of GFP–APX3 fusion protein was analyzed in those high expression lines. Fluorescence microscopy was used to observe the green fluorescence signal in protoplasts made from high expression lines. The data clearly indicated that the GFP–APX3 fusion protein was found in the membranes of organelles that appeared to be peroxisomes (Fig. 3A). To prove that the organelles to which GFP–APX3 was targeted were indeed peroxisomes, a reference marker line was used that expressed RFP that was tagged with PTS1. The RFP–PTS1 fusion protein was previously shown to be targeted to peroxisomes by Lin et al. (2004). GFP–APX3 lines were crossed to RFP–PTS1 lines to obtain F1 plants that expressed both fusion proteins, and then the localization of these two fusion proteins was determined using fluorescence microscopy. To avoid the red fluorescence signal coming from chlorophylls, cells that did not contain chloroplasts were used. It was found that the two fusion proteins co-localized to the same organelles, namely peroxisomes in petal cells (Fig. 3B) or glyoxysomes in root cells (Fig. 3C). Based on these data, it was concluded that APX3 is localized to the peroxisomal/glyoxysomal membranes.

**APX3 is dispensable for *Arabidopsis* growth and development**

Previously it was demonstrated that overexpression of APX3 increases photosynthetic tolerance to drought stress (Yan et al., 2003) and oxidative stress (Wang et al., 1999) in transgenic plants. Therefore, it was thought that APX3 might play an important role in plant cellular metabolism. To explore the function of APX3 further, an APX3 knockout mutant was studied that was obtained from the ABRC at Ohio State University. This mutant was due to a T-DNA insertion in the seventh exon (Fig. 4A), which leads to a null phenotype at the transcript and protein level (Fig. 4B, C). The size of the T-DNA insert is larger than 5 kb, which might result in highly unstable transcripts and disrupt the translation process. Despite several trials, no APX3 transcript was ever detected from this mutant. Consequently, no APX3 protein could be detected by western blot analysis (Fig. 4B). The phenotypes and growth behaviours of the APX3 knockout mutant were examined under normal and temperature stress conditions. No major morphological and biomass differences were detected between APX3 knockout mutant and wild-type plants under these conditions (Fig. 5A, B). Since H$_2$O$_2$ is produced from peroxisomes during lipid mobilization (via fatty acid β-oxidation) in seed germination, the seed germination rates of APX3 knockout mutant and wild-type plants were compared under salt stress conditions. Again no significant differences were observed between APX3 knockout mutant and wild-type plants (Fig. 5C). The data indicate that APX3 is dispensable for *Arabidopsis* under normal growth conditions and the stress conditions tested.

**APX3 knockout plants display an altered response to heat treatment**

Since peroxisomal APX is probably involved in scavenging H$_2$O$_2$ produced during photorespiratory metabolism
The APX3 knockout mutant may behave differently from wild-type plants under conditions where photorespiration is increased. Therefore, chlorophyll fluorescence analysis was used to study the photosynthetic electron transport in APX3 knockout mutant and wild-type plants under two different conditions that could enhance photorespiration: heat stress and elevated oxygen levels. Dark-acclimated, detached leaves were initially illuminated at a photon flux density of 350 μmol m$^{-2}$ s$^{-1}$ and 25 °C. The level of U$_{PSII}$, which is used to estimate the quantum efficiency of electron transport in PSII complexes, stabilized after 20 min of illumination (Fig. 6A). No statistically significant differences between genotypes were found at this time. Then the leaf temperature was elevated from 25 °C to 40 °C within 5 min without changes in the light intensity. A temporary increase in $\Phi_{PSII}$, presumably due to temporary enhancement of the activity of the enzymes in the Calvin cycle, was followed by a decline in the level of this parameter. This decline represented the damage to the photosynthetic apparatus caused by the high temperature treatment. The decrease in $\Phi_{PSII}$ occurred earlier for the APX3 knockout mutant when compared with wild-type plants (Fig. 6A), although the differences were statistically significant only at the beginning of the decline. Interestingly, this subtle difference in photosynthetic electron transport between APX3 knockout mutant and wild-type plants did not occur at 38 °C (data not shown). The $\Phi_{PSII}$ values did not differ significantly between APX3 knockout mutant and wild-type plants under photorespiratory (i.e. elevated oxygen level) (Fig. 6B) or non-photorespiratory conditions (i.e. reduced oxygen level) (Fig. 6C). The data indicate that there might be a threshold of stress tolerance for which the role of APX3 could be reflected.

**Discussion**

In this study, the earlier prediction that APX3 is targeted to peroxisomes was confirmed (Zhang et al., 1997). In fact, the resolution of the fluorescence microscope allows GFP–APX3 fusion protein to be viewed clearly on peroxisomal membranes (Fig. 3A). Because APX3 co-localizes with a marker protein (i.e. RFP–PTS1) that was shown to be targeted to peroxisomes (Lin et al., 2004), there is no doubt that APX3 is a peroxisomal membrane-bound protein. Occasionally it was observed that GFP–APX3 is localized to a cellular structure that resembles peroxisomal endoplasmic reticulum (ER; data not shown), which is consistent...
with the localization pattern of the cotton pAPX (Mullen et al., 1999; Lisenbee et al., 2003a, b). Compared with the peroxisomal localization, the peroxisomal ER localization of GFP–APX3 was observed much less frequently in this study. Nevertheless, the data are consistent with the localization data from studies of cotton pAPX (Mullen et al., 1999, 2000; Lisenbee et al., 2003a, b). Furthermore, the rice APX3 was recently shown to be targeted to peroxisomes (Teixeira et al., 2006), which is also consistent with the present data.

The function of APX3 was studied by analysing an APX3 null mutant in Arabidopsis, and the result is somewhat surprising. Based on early studies with APX3-overexpressing plants (Wang et al., 1999; Yan et al., 2003), it was thought that APX3 might play an important role in antioxidation metabolism in Arabidopsis. Yet the APX3 knockout mutant performed just as well as wild-type plants did under normal growth conditions (Fig. 5A). Even under the stress conditions tested (e.g. low and high temperatures and low salt treatment), no major morphological and biomass differences could be detected between the APX3 knockout mutant and wild-type plants (Fig. 5B, C). Only a slight genotypic difference in the protection of photosynthesis during heat stress could be detected (Fig. 6A). The analysis with the APX3 knockout mutant implies that APX3 is not absolutely required for Arabidopsis growth and development.

Since the peroxisome is a major site in plant cells where H$_2$O$_2$ is produced (Corpas et al., 2001; del Rio et al., 2002), one would think that APX3 would play a major role in protecting Arabidopsis under conditions that increase photorespiration (consequently increasing H$_2$O$_2$ production).
The present study with the APX3 knockout mutant apparently does not support this assumption. For one thing, the peroxisome has considerable catalase activity that may be sufficient under most conditions to scavenge the H$_2$O$_2$ produced in photorespiratory metabolism. There are three genes in Arabidopsis that encode peroxisomal catalases (Mittler et al., 2004), and their roles in compensating for the loss of APX3 in the APX3 knockout mutant will probably be substantial. Also, perhaps the role of APX3 in the APX3 knockout mutant is compensated by another peroxisomal membrane-bound antioxidant enzyme (i.e. APX5). However, it was noticed that the expression of APX5 appeared to be very low in Arabidopsis, because the transcript level of APX5 was found to be <3% of the transcript level of APX3 (Panchuk et al., 2002, 2005). In fact, APX5 could not be detected in the APX3 knockout mutant using the polyclonal anti-APX3 antibodies in western blot analysis (Fig. 4C), despite the fact that APX3 and APX5 share 67% identity in amino acid sequence, which confirms that APX5 was expressed at a very low level. Therefore, the role of APX5 in compensating for the function of APX3 in the APX3 knockout mutant might be limited.

A thorough in silico analysis of APX3 expression, using the tool provided by the Genevestigator website (www.genevestigator.ethz.ch), showed that the expression patterns of APX3 and APX5 are distinct (Table 1), which suggests that they may function in different metabolic pathways. Interestingly, the expression pattern of APX4 overlaps extensively with that of APX3. APX4 encodes a protein that is probably targeted to the peroxisomal matrix (Mittler et al., 2004), and the transcript level of APX4 is at the same or higher levels of APX3 (Panchuk et al., 2002, 2005), suggesting that APX4 could compensate for the loss of APX3 in the APX3 knockout mutant if they have redundant roles in peroxisomal antioxidation metabolism. Because the catalytic domain of APX3 probably faces the cytoplasm (Yamaguchi et al., 1995), it might play a role in scavenging H$_2$O$_2$ in cytoplasm. If so, the role of cytosolic APXs, especially APX1, in compensating for the function of APX3 in the APX3 knockout mutant should not be ignored. After all, APX1 was shown to play a prominent role in antioxidation metabolism in Arabidopsis (Panchuk et al., 2002; Fryer et al., 2003; Pnueli et al., 2003; Davletova et al., 2005).

One question that remains to be answered is whether peroxisomal APXs are important for plants. The study with the APX3 knockout mutant appears to suggest that APX3 is not essential for Arabidopsis under normal and certain stress conditions. It could be argued that APX3 does play an important role in Arabidopsis, but that the present study did not find the condition in which the role of APX3 would be reflected. To answer the question, more experimental conditions need to be tried to test how APX3 knockout mutants would behave or to create double mutants that lack both APX3 and APX5 or APX3 and APX4, and study the behaviour of those double mutants under normal and stress conditions, which seems to be the next logical approach to understand the roles of peroxisomal APXs in plant cellular metabolism.

**Acknowledgements**

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**Table 1.** In silico analysis of the expression levels of APX3, APX4, and APX5 under various stress conditions

Data were compiled from the GENEVESTIGATOR database (www.genevestigator.ethz.ch).

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