AtOSA1 a member of Abc1-like family as a new factor in cadmium and oxidative stress response.

Michal Jasinski[1,5*], Damien Sudre[1,2*], Gert Schansker[3], Maya Schellenberg[1], Signarbieux Constant[2] Enrico Martinoia[1], Lucien Bovet[2,4*]

[1] University of Zurich, Institute of Plant Biology, Zollikerstrasse 107, 8008 Zürich, Switzerland; [2] University of Bern, IPS-Plant nutrition, Altenbergrain 21, 3012 Bern, Switzerland; [3] University of Geneva, Bioenergetics Laboratory, Chemin des Embrouchis 10, 1254 Jussy/Lullier, Switzerland; [4] University of Fribourg, Department of Biology - Plant Biology, rue Albert Gockel 3, 1700 Fribourg, Switzerland. [5] Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznan, Poland.

* These authors equally contributed to this work

Current address of Lucien Bovet: Philip Morris Products S.A., Research & Development, Quai Jeanrenaud 56, 2000 Neuchâtel, Switzerland.

Corresponding author: e-mail lucien.bovet@pmintl.com; fax +41 32 888 76 03

3

Abstract

The analysis of gene expression in *Arabidopsis thaliana* using cDNA-microarrays and RT-PCR showed that *AtOSA1* (Arabidopsis thaliana Oxidative Stress related Abc1 like protein) transcript levels are influenced by Cd^{2+} treatment. The comparison of protein sequences revealed that AtOSA1 belongs to the family of Abc1 proteins. Up to now, Abc1-like proteins have been identified in prokaryotes and in the mitochondria of eukaryotes. AtOSA1 is the first member of this family to be localized in the chloroplasts. However, despite sharing homology to the mitochondrial ABC1 of *Saccharomyces cerevisiae*, AtOSA1 was not able to complement yeast strains deleted in endogenous ABC1 gene, thereby suggesting different function between AtOSA1 and the yeast ABC1.

The *atosa1-1* and *atosa1-2* T-DNA insertion mutants were more affected than wild type plants by Cd^{2+} and revealed an increased sensitivity towards oxidative stress (H_2O_2) and high light. The mutants exhibited higher superoxide dismutase activities and differences in the expression of genes involved in the antioxidant pathway.

In addition to the conserved Abc1 region in the AtOSA1 protein sequence, putative kinase domains were found. Protein kinase assays *in gelo* using myelin basic protein as a kinase substrate revealed that chloroplast envelope membrane fractions from the *AtOSA1* mutant lacked a 70 kD phosphorylated protein compared to the wild type. Our data suggest that the chloroplast AtOSA1 protein is a new factor playing a role in a balance of oxidative stress.

Introduction

Heavy metals like Cu^{2+}, Zn^{2+}, Mn^{2+} in trace amounts play an essential role in many physiological processes but can be toxic if accumulated at high concentrations. In contrast, other heavy metals such as Cd^{2+} and Pb^{2+} have no biological functions and can be extremely toxic. Cadmium is a non-essential heavy metal widespread in the environment being an important pollutant, known to be toxic for plants not only at the root level where Cd^{2+} is taken up, but also in the aerial part. It can be transported from root-to-shoot via the xylem (Salt et al., 1995; Verret et al., 2004). Cadmium has been reported to interfere with micronutrient homeostasis (Clemens, 2001; Cobbett and Goldsbrough, 2002). It might replace Zn^{2+} in the active site of some enzymes, resulting in the inactivation of the enzymatic activity. Cadmium also strongly reacts with protein thiols potentially inactivating the corresponding enzymes. To overcome this problem, cells produce excess quantities of chelating compounds containing thiols, such as small proteins called metallothioneins (Cobbett and Goldsbrough, 2002), or peptides like glutathione and phytochelatins (Clemens et al., 2002), which limit the damage induced by Cd^{2+}. In addition, several types of transport systems have been shown to contribute to heavy metal resistance including P-type ATPases and ABC transporters. They transport either free or ligand-bound heavy metals across biological membranes extruding them into the apoplast or into the vacuole (Kim et. al., 2007).

In response to heavy metals diverse signal transduction pathways are activated including mitogen–activated protein kinase, transcription factors, stress induced proteins (Jonak et. al., 2004). Our knowledge concerning components of these pathways is growing but still incomplete.

The Abc1 protein family originates from the *S. cerevisiae ABC1* gene (Activity of bc$_1$ complex) which has been isolated as a suppressor of a cytochrome b mRNA translation defect (Bousquet et al., 1991). The mitochondrial ABC1 in yeast was suggested to have a chaperone-like activity essential for a proper conformation of cytochrome b complex III (Brasseur et al., 1997). However, more recent data suggest that the ABC1 protein might be implicated in the regulation of coenzyme Q biosynthesis (Hsieh et al., 2004). The Abc1 family has also been described as a new family of putative kinases (Leonard et al., 1998) and it has been suggested that the putative kinase function of Abc1-like proteins is related to the regulation of the synthesis of ubiquinone (Poon et. al., 2000). Homologues of yeast ABC1 have been isolated in higher eukaryotes. In Arabidopsis, the only studied ABC1-like protein has been predicted to be localized in the mitochondria. It partially restored the respiratory complex deficiency when expressed in *S. cerevisiae* (Cardazzo et al., 1998). In humans, a homologue of the Abc1 proteins (CABC1) has been identified and it is possibly involved in apoptosis (Iiizumi et al., 2002). The human CABC1 protein has 47 and 46% similarity to ABC1 of *Arabidopsis thaliana* and *Schizosaccharomyces pombe*, respectively.

The data presented in this study suggest that the chloroplast AtOSA1, an Arabidopsis protein belonging to the Abc1 protein family, is implicated in the plant response to oxidative stress that can be generated by Cd^{2+}, H_2O_2 and light. Our results show that AtOSA1 is functioning differently from Abc1, hence the proteins of the Abc1 family can fulfil diverse functions.

Results

AtOSA1 transcript levels change in response to cadmium exposure.

The elucidation of the physiological functions of gene products which transcript levels are up- or down-regulated by Cd^{2+} in the model plant *Arabidopsis thaliana* is of major interest to understand response of plants to Cd^{2+}. Several transcriptomic analyses have been performed using a sub-array spotted with a large number of different cDNA sequences. cDNA microarrays from two independent experiments revealed that transcript levels of *AtOSA1* (*At5g64940*) were down-regulated after the treatment with 0.2 μM $CdCl_2$ for 21 days (Fig. 1A). The microarray data were confirmed by RT-PCR using the same mRNA template used for the microarray analyses and RNA isolated from plants exposed to 0.5 and 1 μM $CdCl_2$. After 1 μM $CdCl_2$ treatment the transcript level of *AtOSA1* was up-regulated (Fig. 1B). Additionally a time-course experiment was carried out with one-week-old plants exposed to 0.5 μM $CdCl_2$ (Fig. 1C). The data showed that *AtOSA1* was up-regulated in the leaves after 5 days of Cd^{2+} exposure, was then stably expressed until day 12 and, finally, down-regulated. In the absence of Cd^{2+}, an increase in the expression of *AtOSA1* was found to be correlated with plant aging. The analysis of *AtOSA1* transcript levels in the major plant organs of 6 week-old flowering plants revealed that this gene is expressed particularly in leaves, but also in flowers and slightly in stems (Fig. 1D). Under normal growth conditions, we found only a very low level of *AtOSA1* transcripts in roots. Expression of *AtOSA1* is in all likelihood related to the green tissues, since in this experiment the flowers were not dissected and still contained green sepals. However, we cannot exclude that *AtOSA1* is also expressed in petals, stamen and pistils. The data collected for the *At5g64940* entry in the digital Northern program Genevestigator (www.genevestigator.ethz.ch; Zimmermann et al., 2004) confirm predominant expression of *AtOSA1* in leaves and flowers and that the transcript level of *AtOSA1,* which is age-dependent (Fig.1C), is also down-regulated in the night (circadian rhythm dependencies) and senescent leaves. We confirmed these two last results experimentally (data not shown).

AtOSA1 has homology to the Abc1-like protein family

The protein sequence of AtOSA1 possesses a conserved region of around 120-130 aa (according to CDD: a Conserved Domain Database for protein classification, Marchler-Bauer et al., 2005) that is characteristic for the so-called Abc1 protein family (Fig 2 and Supplementary 1). Using the Conserved Domain Database (CDD) search engine at NCBI (Marchler-Bauer et al., 2003), putative kinase domains were detected within the AtOSA1 protein sequence (Fig. 2). Similar domains were found in Phosphoinositide 4-kinase (PI4K) and Mn^{2+}-dependent serine/threonine protein kinase.

The hydropathy plot made with TMpred (Hofmann and Stoffel, 1993) revealed the presence of two transmembrane spans within the C-terminal part of AtOSA1 (Supplementary 1). Similar results were obtained using the DAS transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS/).

The members of Abc1 protein family have been identified in both pro- and eukaryota e.g. AarF from *E. coli* (Macinga et al., 1998) and ABC1 from yeast (Bousquet et. al., 1991). It is worth to underline that the Abc1 protein family is not related to ABC transporters despite the fact that AtOSA1, has been previously described as an ABC transporter belonging to the ATH (ABC2) subfamily (Sanchez Fernandez et al., 2001). AtOSA1 does not possess any typical features including for instance the most characteristic sequence of ABC transporters known as signature motif [LIVMFY]S[SG]GX$_3$[RKA][LIVMYA]X[LIVFM][AG] (Bairoch, 1992).

In Arabidopsis, the sole ABC1-like protein (*At4g01660*) studied so far has been predicted to be localized in mitochondria and can partially restore the respiratory complex deficiency when expressed in *S. cerevisiae* (Cardazzo et al., 1998). This protein has 32% amino acid identity with AtOSA1. The Arabidopsis genome contains 17 putative Abc1-like genes. Based on the aligned translated products, a phylogenetic tree has been drawn (Fig. 3). The closest Arabidopsis homologue is *At3g07700* which shares 45% amino acid identity with AtOSA1. To date, nothing is known about the localization and potential function of both gene products, although the expression of an apparent homologue of *At3g07700* in *Brassica juncea* (DT317667) has also been found to be regulated by cadmium (Fusco et al., 2005). Two translated gene products from rice *Os02g0575500* and *Os09g0250700* share high homologies with AtOSA1. In prokaryotes, the closest homologues of AtOSA1 are the members of the Abc1 family found in different Cyanobacteria like *Nostoc* (*NP_4885555*) and *Synechocystis* sp. (*P73627*) sharing, respectively, 45 and 44% identity at the amino acid level. Prokaryotic Abc1 proteins have been detected also in *E.coli* and *Clostridium*. Interestingly, these organisms lack complex III (Trumpower, 1990; Unden and Bongaerts, 1997), suggesting that the possible function for Abc1-like proteins may not be exclusively linked to the transfer of electrons in membranes.

Identification of the Abc1 domain within the AtOSA1 sequence prompted us to determine the functional homology of AtOSA1 with Abc1 proteins. For this purpose, we used the yeast *S. cerevisiae*

deletion mutant W303-1A *abc1::HIS3* deficient in the endogenous ABC1 activity (Hsieh et al., 2004). Deletion of the *ABC1* gene in yeast disturbs the function of the respiratory chain and prevents growth of this mutant strain on media containing non-fermentable carbon sources such as glycerol (Bousquet et al., 1991). The expression of the entire *AtOSA1* gene including its targeting pre-sequence (TP) in W303-1A *abc1::HIS3* strain did not restore growth of this mutant on glycerol containing media. Neither AtOSA1-EYFP nor AtOSA1 restored growth. As a control, the growth of the same strain was restored after complementation with yeast *ABC1* gene (Fig. 4A/B) suggesting functional divergence between AtOSA1 and the yeast ABC1. We included the targeting pre-sequence, because chloroplast proteins tend to be targeted to the mitochondria when expressed in fungal cells (Pfaller et al., 1989; Brink et al., 1994). We monitored the expression of AtOSA1-EYFP by confocal microscopy. The signal emitted by the strains expressing TPAtOSA1-YFP (Fig. 4C) was similar to that of the Rhodamine HexylB used for staining mitochondria (Fig. 4D), confirming localization of AtOSA1 in yeast mitochondria or mitochondrion in the presence of the chloroplast targeting pre-sequence.

AtOSA1 is localized in the chloroplast

Sequence analysis of the AtOSA1 protein with Target P (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., (2000)) used for proteomic analyses and theoretical predictions of protein localization (Koo and Ohlrogge, 2002; Peltier et al., 2002), revealed the presence of a twenty eight amino acid N-terminal chloroplast targeting pre-sequence (Supplementary1). Both rice sequences Os02g0575500 and Os09g0250700 also have such putative chloroplast transit peptide regions of 56 and 39 aa, respectively. To verify its sub-cellular localization, AtOSA1 was fused (C-terminal) with EYFP and transiently expressed under the control of the CaMV 35S promoter in Arabidopsis suspension cell culture (Fig. 5A). The signal was visualized by confocal microscopy. The observed localization was identical with that obtained for the Tic110-GFP (Fig. 5B), an integral inner envelope membrane protein of the chloroplast import machinery (Inaba et al., 2003). Our results confirm in silico and proteomic data suggesting a localisation of the AtOSA1 protein in the chloroplast envelope of Arabidopsis (Froehlich et al., 2003).

Cadmium effect on *AtOSA1* mutants

The identification of mutants for *AtOSA1* was possible from T-DNA insertion lines of SALK-institute (SALK 045739) and GABI Kat (GABI, 132G06). To find the homozygote lines for both mutants, we screened the F3-F4 generation by PCR using RP, LP and LB T-DNA primers designed by SIGnAL "T-DNA Express" (http://signal.salk.edu). The mutants were named *atosa1-1* (SALK 045739) and *atosa1-2* (GABI, 132G06), respectively (Supplementary 2). In both mutants T–DNA insertions are located towards the 3'end, thereby excluded membrane anchor to be present in case truncated

transcripts are translated (Supplementary 3). Seedlings of both mutants accumulated less cadmium than the wild type at 10 and 20 μM CdCl$_2$ (Fig. 6A). Therefore we investigated cadmium tolerance in *AtOSA1* T-DNA insertion mutants in one-week-old seedlings grown on bactoagar plates containing 20 μM CdCl$_2$. Interestingly roots of *atosa1-1* and *atosa1-2* mutant seedlings were longer than that of wild-type seedlings (Fig. 6B), thereby suggesting that root growth is less affected by Cd toxicity in *AtOSA1* than in wild-type. Under hydroponic culture conditions, leaves from wild-type Arabidopsis plants took up significantly more cadmium than *atosa1-1*, confirming the data obtained in seedlings (Fig. 6C). A similar picture could be observed in the autoradiograms from four week-old plants exposed to 0.04 MBq ^{109}CdCl$_2$ for 4h (Fig. 6D) where higher radioactivity was detected in wild-type plant. Surprisingly, despite the fact that the mutant plants took up less cadmium, they exhibited a marked chlorotic phenotype when exposed to 0.5 μM CdCl$_2$ for 7 days (Fig 6E).

Superoxide dismutase activity and gene expression in the *AtOSA1* T-DNA mutants

Leaf chlorosis observed in the *AtOSA1* T-DNA insertion mutants but not in wild type (WT) plants after Cd treatment prompted us to determine whether *atosa1-1* is more sensitive to oxidative stress than WT and whether some of the genes involved in reactive oxygen species (ROS) scavenging are differently regulated in mutants. A suitable approach to determine sensitivity to ROS is measurement of superoxide dismutase (SOD) activity, an essential enzyme to attenuate plant oxidative stress. In the first approach, we determined the overall SOD-activity in the leaves of WT and *atosa1-1* exposed or not to 1 μM CdCl$_2$. The *AtOSA1* mutant plants showed increased SOD-activity compared to wild type plants, both in absence and presence of Cd^{2+}. The effect was particularly marked in the absence of Cd^{2+} treatment (Fig. 7A). To determine whether chloroplasts also exhibit an increased SOD-activity, we isolated chloroplasts from plants grown in the presence or absence of Cd^{2+}. The data showed that chloroplasts isolated from the *AtOSA1* deletion mutant displayed a slight but consistently higher SOD-activity, compared to the wild type chloroplasts. This effect was independent of whether the plants were exposed to Cd^{2+} or not (Fig. 7B).

Transcript levels of genes (*AtAPX1*, *At1g07890*; *AtFSD1*, *At4g25100*; *AtFSD2*, *At5g51100*) responding to oxidative stress (Ball et al., 2004, Kliebenstein et al., 1998) were investigated. *AtAPX1*, *AtFSD1, AtFSD2*, as well as *AtOSA1* were found to be up-regulated in WT after 1 μM Cd treatment. In *atosa1-1*, only *AtFSD2* was comparatively induced by Cd. Interestingly, *AtFSD1* was more expressed in *atosa1.1* under control conditions when compared to WT and induction of *AtFSD2* was stronger in the mutant (Fig. 7C).

H_2O_2, known as an ROS inducer, reduced the growth of the seedling roots more in the mutants than in the wild type (Fig. 8A). The effect of H_2O_2 was also more pronounced in *atosa1-1* leaves compared to the wild type leaves. Indeed, after spraying leaves of wild type and mutant plants with 300 μM H_2O_2, in 0.2 % (v/v) Tween 20, we observed a rapid appearance of necrotic spots in the mutant, already one day after spraying (Fig. 8B/C). In contrast, only very few or no spots were found in the wild type plants, 4 days after spraying with H_2O_2 (Fig. 8B). No necrotic spots were detected when both the wild type and *AtOSA1* T-DNA inserted mutant were sprayed with 0.2 % (v/v) Tween 20, only (data not shown).

The effect of light on *AtOSA1* T-DNA inserted mutants

Light has a complex effect on *AtOSA1* mutants depending on light intensities. At low light regime (50 μmol m^{-2} s^{-1}) for 8 h during 4 weeks, the shoot growth of *atosa1-1* and *atosa1-2* was significantly altered compared to the WT (Fig. 9A/B). After additional 4 weeks of growth in the same experimental conditions, leaf sizes were still different and based on fresh weight chlorophyll a, chlorophyll b and carotenoid contents were higher in the mutants compared to the WT (Fig.9C/D). Under a light regime of 120-150 μmol m^{-2} s^{-1} for 8 h and 16 h no visible phenotypes were found in the *AtOSA1* mutants. Surprisingly, under 16 h high light (350 μmol m^{-2} s^{-1}), *atosa1-1* exhibited a pale green phenotype (Fig. 9E). In this case, the analyses of pigments showed slightly less chlorophyll and carotenoids in *atosa1-1* compared to the wild type (Fig. 9F).

Analysis of photosynthetic activities in terms of net CO_2 assimilation rate also revealed differences between *Atosa1* mutants and the wild type depending on the light intensities. Under higher light intensities mutants were more affected than WT (Fig. 10A). Increasing light intensities from 50 to 150 μmol m^{-2} s^{-1} led to a reduction of *AtOSA1*, *AtAPX1*, *AtFSD1* and *AtFSD2* transcript levels in wild-type plants. A similar reduction of *AtAPX1*, *AtFSD1* and *AtFSD2* could be observed in the *atosa1-1* mutant, but this effect was visible only under higher light intensities (Fig. 10B).

No significant differences were found by the electron microscopic analysis in chloroplast structures (stroma lamellae, grana stacks and envelope membranes) between the *atosa1-1* and wild type. In addition, the ICP-MS data showed that the content in essential metals and heavy metals was not changed by the *AtOSA1* T-DNA insertion (data not shown).

Because possible connections between Abc1 proteins, electron transport and ubiquinone (plastoquinone and phylloquinone) synthesis have been postulated (Poon et al., 2000) we performed analysis of electron transport in *AtOSA1* mutants. The kinetic measurements of Chl *a* fluorescence probing the redox state of Q_A, the primary quinone acceptor of photosystem II (PSII) and 820 nm transmission probing the redox state of mainly plastocyanin (PC) and P700 (reaction center chlorophylls of photosystem I (PSI)) revealed no differences between *atosa1-1* and wild type (data not

shown). This indicates that the electron transport functioned well in *atosa1-1* and that the number of oxidized electron acceptors per chain at the beginning of the measurement was similar to the wild-type at "standard" light regime (120 µmol m^{-2} s^{-1}).

Detection of protein kinase activities *in gelo*

In addition to the Abc1 protein family, AtOSA1 contains motifs found in eukaryotic-type protein kinases. Therefore, we decided to examine protein kinase activities in the *AtOSA1* mutant by *in gelo* phosphorylation assays using MBP (myelin basic protein) as a substrate. Since we localized AtOSA1 in chloroplasts and the proteomic analysis identified AtOSA1 in the envelope fraction (Froehlich et al., 2003), we isolated and used this fraction for the assay. In gel protein kinase assay allowed us to detect one chloroplast envelope protein kinase of about 70-kDa in the Col 0 ecotype of *A. thaliana* (Fig. 11A). Interestingly, this labelled band was not present in the envelope membranes isolated from the *AtOSA1* T-DNA inserted plants. This might indicate that the *AtOSA1* mutant lacks this protein kinase. The labelled bands with a similar M_r were not detected in thylakoid membranes and a more complex phosphorylation pattern, which, however, did not show the absence of a labelled band, was obtained with Histone III-S as a substrate (data not shown). The envelope protein profile after Coomassie blue staining of the SDS gel did not show marked differences between the mutant and the wild-type (Fig. 11B).

Discussion

We performed microarray chip analyses to identify genes up- and down-regulated in response to cadmium stress. Among the genes exhibiting an altered transcript level in response to Cd^{2+}, we identified *AtOSA1* (*At5g64940*), as a member of the Abc1 family. In Arabidopsis, 17 genes contain a typical Abc1 motif and hence constitute a small gene family. The sole Abc1 representative described so far in plants (*At4g01660*) is a homologue to the yeast ABC1 (Cardazzo et al., 1998). Both are localized in mitochondria (Bousquet et. al., 1991; Cardazzo et al., 1998), in contrast to AtOSA1 which is targeted to the chloroplast and does not sub-cluster with them. *AtOSA1* transcript-level followed a complex kinetics in response to Cd^{2+} during dose-dependent and time-course experiments. In the absence of cadmium treatment, its expression in leaves increased during the life of Arabidopsis and it has been reported that plant aging increases oxidative stress in chloroplasts (Munne-Bosch and Alegre, 2002).

Two independent T-DNA insertion mutants, lacking functional AtOSA1, exhibited a complex behaviour towards cadmium. Indeed, the seedling roots of *AtOSA1* deletion mutants were less affected by Cd^{2+} than those of the wild type plants, possibly due to a reduced Cd^{2+} uptake.

The increased Cd tolerance of wild-type compared to *atosa1* mutants is very likely not supported by the direct binding of Cd to AtOSA1. Indeed AtOSA1 lacks of sequence motifs containing cysteins, involved in the binding of heavy metal ions (Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Ag^+, or Cu^+), like CXXC and CPC. Such motifs have been found, for example, in members of the subclass of heavy-metal-transporting P-type ATPases (P_{1B}-type ATPases, Eren and Argüello, 2004). In addition, AtOSA1 is likely not a heavy-metal (Cd) transporter, since vesicles isolated from YMK2 yeast (Klein et al., 2002) transformed with *AtOSA1* did not show any Cd transport (data not shown).

The pale phenotype of leaves was more pronounced in case of mutant plants exposed even to low dose of Cd^{2+} despite the fact that lack of *AtOSA1* results in lower Cd^{2+} uptake rates in shoots. Such a chlorotic phenotype of leaves was not correlated with an elevated accumulation of cadmium and was also observed under high light conditions. This pale phenotype might be a consequence of Cd^{2+} toxic effect due to a modification of the cellular cadmium distribution (Ranieri et al., 2001) and an increased Cd^{2+} sensitivity related to an increased production of ROS in the *AtOSA1* mutants, similarly to those described in *Euglena gracilis* (Watanabe and Suzuki, 2002), or yeast (Brennan and Schiestl, 1996).

Although the mechanism of oxidative stress induction by Cd^{2+} is still obscure, Cd^{2+} can inhibit electron transfer and induces ROS formation (Wang et al., 2004). It has been also suggested that Cd^{2+} can interfere in living cells with cellular redox reactions and displaces or releases other active metal ions (e.g. Zn^{2+}) from various biological complexes, thereby causing a reduction of the capacity of the antioxidant system (Jonak et. al., 2004).

Beside cadmium, the *AtOSA1* T-DNA inserted mutants showed actually a phenotype illustrated by a reduced tolerance to H_2O_2 and light. At 150 µmol $m^{-2}s^{-1}$ we observed the same transpiration rate for WT, *atosa1-1* and *atosa1-2*. Nevertheless stomatal conductance and CO_2 assimilation were higher in WT than in mutants (data not shown). This observation suggests that, at this light intensity (150 µmol $m^{-2}s^{-1}$), transpiration occurs not only at the stomatal level, but also directly through the epidermis. This hypothesis is supported by the experiments showing increased sensitivity of *atosa1* towards H_2O_2 (Fig. 8B). Indeed it is still possible that the AtOSA1 mutation also affects the epidermal cell wall and the cuticle. At low light intensity and period, *atosa1* exhibited retardation in growth correlated with an increase in pigment production (Chla, Chlb and carotenoids). Under higher light intensity and period, a pale green phenotype correlated with a decrease in pigment contents when compared with the wild type. In addition changes of light intensities influenced photosynthetic activities. This data suggest participation of the chloroplast AtOSA1 in light generated stress (ROS) and pigment response. Obtained results suggest that *AtOSA1* mutants have a hypersensitivity to broad abiotic stresses, including photooxidative stress. RT-PCR analyses in *atosa1* plants showed different behaviour for transcripts of genes responding to oxidative stress. For instance it was shown that *AtFSD1* transcript in

Arabidopsis is high at 60 µmol m^{-2} s^{-1} and then down regulated under increasing light fluences (Kliebenstein et al., 1998). A similar tendency could be observed for *atosa1* but under higher light intensity. The lack of *AtOSA1* caused a global shift under increasing light conditions. This might indicate necessity to compensate increased oxidative stress level in the mutant by the expression of components of the antioxidant network like *AtAPX1* and *AtFSD1* and permanent SOD activities (Ball et al., 2004). Interestingly, the increased SOD activity detected in the isolated chloroplasts was not enhanced by Cd^{2+} treatments, thereby confirming the data reported by Fornazier et al. (2002), showing that the Cd^{2+} treatment did not enhance SOD activities, possibly by displacing Fe^{2+}, Zn^{2+} or Cu^{2+} required for the SOD activity. Most presumably, these results indicate that *AtOSA1* deletion mutants permanently suffer from oxidative stress and compensate it to a certain level under controlled growth conditions, however, these plants are apparently not able to do it when environmental parameters like ROS inducers, light regime or nutrient supply vary.

Probably, AtOSA1 is not directly induced by external oxidative stress but acts in a more complex manner for example as a part of a signal transduction pathway related to oxidative stress. Indeed, the Abc1 family has been described as a family of putative kinases (Leonard et al., 1998) and it is possible that AtOSA1 exhibits protein kinase activity, since the predicted molecular mass of mature AtOSA1 (83 kD) is close to the phosphorylated polypeptide detected in the autoradiography (~70 kD) after in-gel assay. The phosphorylated polypeptide is not present in the envelope membranes derived from *AtOSA1* mutant. Nevertheless, we cannot exclude that the protein kinase detected within the gel matrix is a member of a signal transduction cascade, which is not active in the *AtOSA1* mutant. Indeed, it would not be surprising since another protein kinase (NADK2) may play a vital role in chlorophyll synthesis and chloroplast protection against oxidative damage (Chai et al., 2005). Further studies are required to elucidate the role of this protein kinase within the chloroplast.

Based on the phylogenetic tree, cell localization and involvement in oxidative stress response, AtOSA1 is rather not a functional homologue of the yeast ABC1 and *At4g01660* (Cardazzo et al., 1998). As a chloroplast protein, AtOSA1 is more closely related to prokaryotic Abc1 proteins from Cyanobacteria like *Synechocystis,* or *Nostoc* than to those of mitochondria. These ABC1 proteins have not been characterized so far. Therefore, our data are in agreement with the studies on evolutionary relations between different Abc1 proteins, which led to the conclusion that Abc1-proteins from Cyanobacteria and chloroplasts, on the one hand, and from mitochondria on the other have independent origins (Leonard et al., 1998). To date, it has been suggested that Abc1 proteins control the biogenesis of respiratory complexes in mitochondria. The yeast ABC1 knock-out mutants are

unable to grow on glycerol, making the exact molecular functions of these proteins still a matter of debate (Do et al., 2001).

In Arabidopsis *AtOSA1* (*At5g64940*) cluster together with Abc1 like gene *At3g07700*, interestingly homologue of this gene in *Brassica juncea* is also cadmium regulated and possibly localized in the chloroplast (Fusco et al., 2005). Concerning the other Abc1 sequence-related genes in Arabidopsis, four of them (*At5g5200*, *At4g31390*, *At1g79600* and *At1g71810*) have been recently found to be localized in plastoglobules in a proteomic study and are possibly involved in the regulation of quinine monooxygenases (Ytterberg et al., 2006). As illustrated by the pleiotropic effect and permanent oxidative stress caused by deletion of AtOSA1, despite the fact that our knowledge about Abc1 related proteins is still scarce, our results indicate this gene family triggers essential regulatory functions.

Material and methods

cDNA-microarrays

The mRNAs were isolated as described at http://www.unil/ibpv. Fluorescent labeling of cDNAs, hybridization on home-made DNA-microarray slides spotted with ESTs and 3' ends coding sequences (corresponding to putative ABC transporter proteins (124 from 127) and other protein families), and fluorescence analyses (Scanarray 4000) were performed as described in Bovet et al., 2005.

Semi-quantitative PCR

For semi-quantitative RT-PCR, the housekeeping genes Actin2 (*AtACT2*) gene (*At3g18780*) and S16 (*AtS16*) gene (*At5g18380*) were amplified using the primers actin2-S (5'-TGGAATCCACGAGACAACCTA-3') and actin2-AS (5'-TTCTGTGAACGATTCCTGGAC-3') and S16-S: GGCGACTCAACCAGCTACTGA and S16-AS: CGGTAACTCTTCTGGTAACGA, respectively. For the ascorbate peroxidase 1 (*AtAPX1*) gene (*At1g07890*), Fe-superoxide dismutase 1 (*AtFSD1*) gene (*At4g25100*) and Fe-superoxide dismutase 2 (*AtFSD2*) gene (*At5g51100*), we designed the following primers: APX1-S (5'-GCATGGACATCAAACCCTCTA-3') and APX1-AS (5'-TTAAGCATCAGCAAACCCAAG-3'); FSD1-S (5'-GGAGGAAAACCATCAGGAGAG-3') and FSD1-AS (5'-TCCCAGACATCAATGGTAAGC-3'), FSD2-S (5'-CCACTCCCTCGTCTCTCTTG-3') and FSD2-AS (5'- CCACCTCCAGGTTGGATAGA-3'). The primers for *AtOSA1* were AtOSA1-S (5'-GACAGGCAATCACAAGCATTC-3') and AtOSA1-AS (5'-CGATTAGAACTTGGAGGCTGA-3'), respectively. For the selection of the *atosa1-1* T-DNA insertion homozygote lines (SALK 045739), the primers were: RP (5'-AACGCGTTGAAATGCCCTCTC-3'), LP (5'-CTTGCTTCTTATCCATCGAGC-3') and LB T-DNA (5'-GCGTGGACCGCTTGCTGCAACT-3'). For the selection of the *atosa1-2* T-DNA insertion

homozygote lines (GABI 132G06), the primers were: RP (5'-TTTGTTGGAGGCATTTTATGG-3'), LP (5'-GAATGCTTGTGATTGCCTGTC-3') and LB T-DNA (5'-ATTTGGACGTGAATGTAGACA-3'). The primers for the verification of truncated transcript were: 1-S (5'-AATCGCCGGGATCTTCTTAC-3') and 1-AS (5'-TTGTCACTTCCTCCGTTTCC-3'), 2-S (5'-TTTGTTGGAGGCATTTTATGG-3') and 2-AS (5'-AACGCGTTGAAATGCCCTCTC-3'), 3-S (5'-GACAGGCAATCACAAGCATTC-3') and 3-AS (5'-CGATTAGAACTTGGAGGCTGA-3'). The PCR reactions were performed in a final volume of 25 μL containing the following mixture: PCR buffer, 0.2 mM dNTPs, 0.5 μM of both 5' and 3' primers, 1 U Taq DNA polymerase (Promega, Madison, WI, USA), and adjusted amounts of cDNA. DNA was isolated using NUCLOSPIN plant (Macherey-Nagel, Oensingen, Switzerland). Total RNA was purified from the plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and stored at -80°C following quantification by spectrophotometry. After DNAse treatment (DNase, RQ1, RNase free, Promega, Madison, WI, USA), cDNAs were prepared using M-MLV reverse transcriptase, RNaseH minus, point mutant (Promega, Madison, WI, USA) as indicated by the manufacturer and stored at -20°C. cDNAs were diluted approximately 10 times for the PCR reaction. After denaturation at 95°C for 3 min, 35 PCR cycles (94°C for 45s, 58°C for 45s and 72°C for 1 min) were run.

Complementation of yeast *S. cerevisiae*

For the complementation of the W303-1A *abc1::HIS3* (Hsieh et al., 2004) deficient in the endogenous Abc1 gene we used AtOSA1 sequence with the chloroplast targeting presequence. Two constructs were tested with and without EYFP. The construct with EYFP was obtained by recloning of AtOSA1-EYFP from pRT vector into pNEV (Sauer and Stolz, 1994) via NOTI site. The construct without YFP and with targeting presequence was obtained by PCR (5NOTAtOSA1-S 5'TGCTACCGGTGCGGCCGCATGGCGACTTCTTCTTCTTCATCG 3' and 3NOTAtOSA1-AS 5' ATAAGAATGCGGCCGCTTAAGCTGTTCCAGTGATTAGTTTTTCC 3') using pRT-AtOSA1-EYFP as a template. PCR product was sequenced to avoid errors. Yeast transformation was performed using standard protocols. Transformants were growing on the SD medium (2% (w/v) glucose, 0.7% (w/v) yeast nitrogen base and required amino acids) with glucose or glycerol as a source of carbon. Cells were analyzed by confocal laser scanning microscopy (TCS SP2 Leica).

Localization of AtOSA1

The *AtOSA1* cDNA was PCR amplified (AtOSA1-S 5'TGCTACCGGTGCGGCCGCATGGCGACT-TCTTCTTCTTCATCG3' and AtOSA1-AS 5'TCGTCCATGGAAGCTGTTCCAGTGATTAGTTTTT-CC 3') to introduce appropriate restriction sites and cloned into AgeI/NcoI from vector pEYFP (BD Biosciences) in order to fuse it with EYFP.

We used cDNA prepared as described above as a template for the PCR. The resulting AtOSA1-EYFP was cut off by Not I and cloned into vector pRT (Überlacker and Werr, 1996), resulting in pRT-AtOSA1-EYFP. The entire gene fusion product was sequenced to verify the absence of PCR errors. The Tic110-GFP construct was kindly provided by F. Kessler, University of Neuchatel.

Arabidopsis suspension cell cultures were grown as described in Millar et al. (2001). Three days after culture dilution, the cells were transferred onto solid medium and 48 h later the plants were transfected with appropriate constructs using a particle inflow gun (PDS1000He; Bio-Rad) with 0.6 µm particles and 1300 psi pressure. The transfected Arabidopsis cells were analyzed by confocal laser scanning microscopy (TCS SP2 Leica) 24 and 48 hours after bombardment.

Chloroplast and Envelope Membrane preparation

Firstly, the mesophyll protoplasts were prepared from leaves according to the protocol described in Cosio et al. (2004) and subsequently, the intact chloroplasts were obtained according to the method of Fitzpatrick and Keegstra (2001). The collected protoplast pellet was resuspended briefly in 300 mM sorbitol, 20 mM Tricine−KOH, pH 8.4, 10 mM EDTA, 10 mM NaHCO₃, 0.1% (w/v) BSA and forced twice through 20 and 11 µm nylon mesh. Released chloroplasts were immediately purified on an 85%/45% (v/v) Percoll gradient, and collected by centrifugation at 250 *g*. The chloroplast envelope membranes were isolated from purified chloroplasts as described in Froehlich et al. (2003).

Plant growth

Arabidopsis thaliana (ecotype Columbia 0) called above wild type and *AtOSA1* T-DNA inserted mutant (SALK 045739, GABI 132G06) plants were grown on soil in a growth chamber (8 h light period, 22°C; 16 h dark period, 21°C; relative humidity 70 %) and at light intensity of 140−160 µmol $m^{-2}\,s^{-1}$.

For sterile growth after sterilization the seeds (~20) were placed on 0.8 % (w/v) agar plates containing 1/2 MS (Duchefa) or MAMI and 1 % (w/v) sucrose. MAMI medium is: KH_2PO_4 (200 mg/L); $MgSO_4.7H_2O$ (187.5 mg/L); $Ca(NO_3).4H_2O$ (79.25 mg/L); KNO_3 (22 mg/L); Fe-EDDHA sequestren (17.5 mg/L); $MnCl_2.4H_2O$ (48.75 µg/L); H_3BO_3 (76.25 µg/L); $ZnSO_4.7H_2O$ (12.25 µg/L); $CuSO_4.5H_2O$ (6.875 µg/L); $NaNoO_4.2H_2O$ (12.5 µg/L); $Ni(NO_3)_2.6H_2O$ (3.75 µg/L). The plates were stored at 4°C for 24 h for synchronization of seed germination and then, placed vertically in the phytotron (25°C, 16 h light and 70 % humidity) at light intensity of 80−120 µmol $m^{-2}\ s^{-1}$. For treatments, seeds were germinated and grown vertically on 1/2 MS bactoagar plates in the presence or absence of 1, 10 ,20 µM $CdCl_2$ or 1 mM H_2O_2 at 16 h light for 7 days. Hydroponic culture: seeds were first germinated and grown vertically on 1/2 MAMI bactoagar plates at 8 h light for 2 weeks.

Seedlings were then transferred in 1/2 MAMI liquid medium under the same growth conditions for 2 weeks. Plants were finally cultivated for additional 3 weeks in the presence or absence of 0.2, 0.5 or 1 μM $CdCl_2$ in 1/2 MAMI. Cd was desorbed after 10 min of roots incubation in 1mM $CaCl_2$ cold solution. The Cd content was determined by AAS in shoots and roots.

Plant labelling

The plants were root labelled with 0.04 MBq [109]$CdCl_2$ in 1/8 MAMI for 4 h. After washing with cold distilled water, plants were grown in 1/2 MS for additional 3 days, dried and subjected to autoradiography

Determination of superoxide dismutase activity

For the superoxide dismutase (SOD) activity measurements without any treatment we used 4 week-old plants grown on soil. For measurement following a Cd^{2+} application plants were germinated on 0.8 % (w/v) agar plates containing 1/2 MS (Duchefa) and 1 % (w/v) sucrose. The plates were stored at 4°C for 16 h for synchronization of seed germination, then, placed vertically in the phytotron (22°C, 8 h light and 70 % humidity). Two-week-old seedlings were transferred to liquid medium and cultivated under hydroponic conditions for three weeks on MAMI-medium. $CdCl_2$ was added to the medium to a final concentration of 1 μM and the samples were taken 24 hours later. The activity of superoxide dismutase (SOD) was measured as described in Hacisalihoglu et al. (2003). Leaves were homogenized briefly with 50 mM HEPES buffer (pH 7.6) containing 0.1 mM Na_2EDTA, 1 mM PMSF, 1 % (w/v) PEG4000, 1 % (w/v) Polyvinylpolypyrrolidon (Sigma) and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was desalted on a Biospin column P6 (BioRad) according to the supplier's protocol and used for protein and SOD assays. The assay determines the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (1977). The 1 mL reaction mixture for the SOD assay, contained 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.4), 13 mM Met, 75 μM NBT, 0.5 mL of enzyme extract, and 2 μM riboflavin. The reaction mixtures were illuminated for 15 min at 250 μmol m^{-2} s^{-1} light intensity or kept in the dark (negative control). One unit (U) of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT measured at 560 nm. Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

Gas exchange

Photosynthetic gas exchange measurements were performed on attached leaves before plants flowering using an open infrared gas analyzer (IRGA) system (CIRAS-1; PP-Systems, Hitchin, UK).

Measurements were made on plants grown at 8 h light at a PPFD (photosynthetic photon flux density) of 50, 100 or 150 µmol m^{-2} s^{-1}, and CO_2 concentration of 350 µmol. Leaf temperature (LT) was adjusted to the desired level using the internal heating/cooling system of the analyser.

Detection of protein kinase activity in gelo

The method for detecting protein kinases (PK) *in gelo* was adapted from Mori and Muto (1997). Chloroplast envelope membranes were isolated from wild type and the *AtOSA1* mutant and separated by SDS-PAGE. In this experiment, 350 µg of myelin basic protein (MBP, M1891, Sigma) were used as a PK-substrate and incorporated in the running gel solution before polymerisation. After electrophoresis, polypeptides were renatured for 12 h in 50 mM MOPS-KOH pH 7.6, changing the buffer 4 times during this period. The gel was then labelled with 1.6 MBq (γ^{32}P)ATP (AA0068, Amersham-Bioscience) in 5 ml 50 mM MOPS-KOH pH 7.6, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$ for 3 h, following a 45 min preincubation in the same buffer without the labelled ATP. The gel was then rapidly washed with deionized water and incubated in 100 ml of 50 mM MOPS-KOH pH 7.6 containing 10 g of a strong basic anion exchanger (Amberlit IRA-410, Sigma) for 3 h. The removal of unbound ^{32}P was terminated by incubation of the gel in 50 mM MOPS-KOH pH 7.6 supplemented with 1 % (w/v) sodium pyrophosphate for 3 h. The polypeptides were then fixed in the gel in 10 % (v/v) 2-propanol, 5 % (v/v) acetic acid and 1 % (w/v) sodium pyrophosphate. The gel was finally dried and subjected to autoradiography.

Pigments analyses

The plants were grown at 8 h light (50 µmol m^{-2} s^{-1}) for 8 weeks. From these plants, leaf samples (50 mg) were collected and analysed for the content of chlorophyll a (Chla) and b (Chlb), as well as carotenoids (car) (n=10). Plants were grown at 16 h light (350 µmol m^{-2} s^{-1}) for 5 weeks. From these plants, leaf samples (50 mg) were collected and analysed for the content of chlorophyll a (Chla) and b (Chlb), as well as carotenoids (car) (D). Pigments were measured using the method described in Pruzinska et al. (2005).

Statistics

Each value represents the mean of n replicates. Error bars represent SE: Significant differences from wild-type (WT) as determined by Student's t-test are indicated * P< 0.1, ** P<0.05, *** P<0.001 respectively.

Acknowledgements

References:

Bairoch A. (1992) PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res **20:** 2013-2018.

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A,Kular B, Leyland N, Mejia-Carranza J, Reynolds H, Karpinski S, Mullineaux PM (2004) Evidence for a Direct Link between Glutathione Biosynthesis and Stress Defense Gene Expression in Arabidopsis. Plant Cell **16:** 2448–2462

Baroli I, Niyogi KK (2000) Molecular genetics of xanthophyll-dependent photoprotection in green algae and plants. Philos Trans R Soc Lond B Biol Sci **355:** 1385-1394

Bousquet I, Dujardin G, Slonimski PP (1991) ABC1, a novel yeast nuclear gene has a dual function in mitochondria: it suppresses a cytochrome b mRNA translation defect and is essential for the electron transfer in the bc 1 complex. EMBO J **10:** 2023-2031

Bovet L, Feller U, Martinoia E (2005) Possible involvement of plant ABC transporters in cadmium detoxification: a c-DNA sub-microarray approach. Env Int **31:** 263-267

Brasseur G, Tron G, Dujardin G, Slonimski PP, Brivet-Chevillotte P (1997) The nuclear ABC1 gene is essential for the correct conformation and functioning of the cytochrome bc1 complex and the neighbouring complexes II and IV in the mitochondrial respiratory chain. Eur J Biochem. **15:** 103-111

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248‒254

Brennan RJ, Schiestl RH (1996) Cadmium is an inducer of oxidative stress in yeast. Mutat Res **356:** 171–178

Brink S, Flugge UI, Chaumont F, Boutry M, Emmermann M, Schmitz U, Becker K, Pfanner N (1994) Preproteins of chloroplast envelope inner membrane contain targeting information for receptor-dependent import into fungal mitochondria. J Biol Chem. **269:**16478-16485

20

Cardazzo B, Hamel P, Sakamoto W, Wintz H, Dujardin G (1998) Isolation of an Arabidopsis thaliana cDNA by complementation of a yeast abc1 deletion mutant deficient in complex III respiratory activity. Gene **221:** 117-125

Chai MF, Chen QJ, An R, Chen YM, Chen J, Wang XC (2005) NADK2, an Arabidopsis chloroplastic NAD kinase, plays a vital role in both chlorophyll synthesis and chloroplast protection. Plant Mol Biol. **59:** 553-64

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis**.** Planta **212:** 475-486

Clemens S, Palmgren MG, Kramer U (2002) A long way ahead: understanding and engineering plant metal accumulation.Trends Plant Sci **7:** 309-315

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis**.** Annu Rev Plant Biol **53:** 159-182

Cosio C, Martinoia E, Keller C (2004) Hyperaccumulation of cadmium and zinc in Thlaspi caerulescens and Arabidopsis halleri at the leaf cellular level. Plant Physiol **134:** 716-725

Do TQ, Hsu AY, Jonassen T, Lee PT, Clarke CF (2001) A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in Saccharomyces cerevisiae abc1 mutants. J Biol Chem **276:** 18161-18168

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol **300:** 1005-1016

Eren E, Argüello JM (2004) Arabidopsis HMA2, a divalent heavy metal-transporting P(IB)-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. Plant Physiol **136:** 3712-3723

Fitzpatrick LM, Keegstra K (2001) A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. Plant J **27:** 59-65

Froehlich JE, Wilkerson CG, Ray WK, McAndrew RS, Osteryoung KW, Gage DA, Phinney BS (2003) Proteomic study of the Arabidopsis thaliana chloroplastic envelope membrane utilizing alternatives to traditional two-dimensional electrophoresis. J Proteome Res **2:** 413-425

Fornazier RF, Ferreira RR, Vitoria AP, Molina SMG, Lea PJ, Azevedo RA (2002) Effects of cadmium on antioxidant enzyme activities in sugar cane**.** Biol Plant **45:** 91-97

Fusco N, Micheletto L, Dal Corso G, Borgato L, Furini A (2005) Identification of cadmium-regulated genes by cDNA-AFLP in the heavy metal accumulator Brassica juncea L. J Exp Bot **56:** 3017–3027

Giannopolitis CN, Ries SK (1977) Superoxide dismutases-occurrence in higher plants. Plant Physiol **59**: 309–314

Hacisalihoglu G, Hart JJ, Wang YH, Cakmak I, Kochian LV (2003) Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiol **131:** 595-602

Hofmann K, Stoffel W (1993) TMbase - A database of membrane spanning proteins segments**.** Biol. Chem. Hoppe-Seyler **374:** 166

Hsieh EJ, Dinoso JB, Clarke CF (2004) A tRNA(TRP) gene mediates the suppression of cbs2-223 previously attributed to ABC1/COQ8. Biochem Biophys Res Commun **317:** 648-653

Iiizumi M, Arakawa H, Mori T, Ando A, Nakamura Y (2002) Isolation of a novel gene, CABC1, encoding a mitochondrial protein that is highly homologous to yeast activity of bc1 complex. Cancer Res **62:** 1246-1250

Inaba T, Li M, Alvarez-Huerta M, Kessler F, Schnell DJ (2003) atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. J Biol Chem **278:** 38617-38627

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress. Activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. Plant Physiol **136:** 3276-3283

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J. **50** (2):207-18.

Klein M, Mamnun YM, Eggmann T, Schuller C, Wolfger H, Martinoia E, Kuchler K (2002) The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast. FEBS Lett **520:** 63-67

Kliebenstein DJ, Monde RA, Last RL (1998) Superoxide Dismutase in Arabidopsis: An Eclectic Enzyme Family with Disparate Regulation and Protein Localization Plant Physiol. **118:**637-50

Koo AJK, Ohlrogge JB (2002) The Predicted Candidates of Arabidopsis Plastid Inner Envelope Membrane Proteins and Their Expression Profiles. Plant Physiol **130:** 823-836

Leonard CJ, Aravind L, Koonin EV (1998) Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily. Genome Res **8:** 1038-1047

Macinga DR, Cook GM, Poole RK, Rather PN (1998) Identification and characterization of aarF, a locus required for production of ubiquinone in Providencia stuartii and Escherichia coli and for expression of 2'-N-acetyltransferase in P. stuartii. J Bacteriol **180:** 128-35

Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, and Bryant SH (2003) CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res **31:** 383-387

Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Marchler GH, Mullokandov M, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Yamashita RA, Yin JJ, Zhang D, Bryant SH (2005), "CDD: a Conserved Domain Database for protein classification.", Nucleic Acids Res **33:** D192-6

Millar AH, Sweetlove LJ, Giege P, Leaver CJ (2001) Analysis of the Arabidopsis mitochondrial proteome. Plant Physiol **127:** 1711-1727

Mori IC, Muto S (1997) Abcisic acid activates a 48-kDa protein kinase in guard cell protoplasts, Plant Physiol **113:** 833-839

Morgenstern B (2004) DIALIGN: multiple DNA and protein sequence alignment at BiBiServ. Nucl. Acids Res. **32:** W33-W36

Munne-Bosch S, Alegre L (2002) Plant aging increases oxidative stress in chloroplasts. Planta **214:** 608-15

Peltier JB, Emanuelsson O, Kalume DE, Ytterberg J, Friso G, Rudella A, Liberles DA, Soderberg L, Roepstorff P, von Heijne G, van Wijk KJ (2002) Central functions of the lumenal and peripheral thylakoid proteome of Arabidopsis determined by experimentation and genome-wide prediction. Plant Cell **14:** 211-236

Pfaller R, Pfanner N, Neupert W (1989) Mitochondrial protein import. Bypass of proteinaceous surface receptors can occur with low specificity and efficiency. J Biol Chem **264:** 34-39

Poon WW, Davis DE, Ha HT, Jonassen T, Rather PN, Clarke CF (2000) Identification of Escherichia coli ubiB, a gene required for the first monooxygenase step in ubiquinone biosynthesis. J Bacteriol **182:** 5139-5146

Pruzinska A, Tanner G, Aubry S, Anders I, Moser S, Müller T, Ongania KH, Kräutler B, Youn JY, Liljegren SJ, Hörtensteiner S (2005) Chlorophyll Breakdown in Senescent Arabidopsis Leaves. Characterization of Chlorophyll Catabolites and of Chlorophyll Catabolic Enzymes Involved in the Degreening Reaction1. Plant Physiol **139:** 52–63

Ranieri A, Castagna A, Baldan B, Soldatini GF (2001) Iron deficiency differently affects peroxidase isoforms in sunflower. J Exp Bot **52:** 25-35

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of Cadmium Mobility and Accumulation in Indian Mustard. Plant Physiol **109:** 1427-1433

Sanchez-Fernandez R, Davies TG, Coleman JO, Rea PA (2001) The Arabidopsis thaliana ABC protein superfamily, a complete inventory. J Biol Chem **276:** 30231-30244

Sauer N, Stolz J (1994) SUC1 and SUC2: two sucrose transporters from Arabidopsis thaliana; expression and characterization in baker's yeast and identification of the histidine-tagged protein. Plant J **6:** 67-77

Trumpower BL (1990) Cytochrome bc1 complexes of microorganisms. Microbiol Rev **54:** 101-129

Überlacker B, Werr W (1996) Vectors with rare-cutter restriction enzyme sites for expression of open reading frames in transgenic plants. Mol Breed **2:** 293-295

Unden G, Bongaerts J (1997) Alternative respiratory pathways of Escherichia coli: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta **1320:** 217-234

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett **576:** 306-312

Wang Y, Fang J, Leonard SS, Rao KMK (2004) cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Rad Biol Med **36:** 1434-1443

Watanabe M, Suzuki T. (2002) Involvement of reactive oxygen stress in cadmium-induced cellular damage in Euglena gracilis. Comp Biochem Phys **131:** 491-500

Ytterberg AJ, Peltier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol **140:** 984-997

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621-2632

Legends:

Figure 1.

AtOSA1 gene expression in Arabidopsis. (A) Analysis of the transcript levels of *AtOSA1* in leaves after exposure to 0.2 μM $CdCl_2$ for 3 weeks under hydroponic growth conditions using cDNA spotted arrays. The data presented show the +Cd/-Cd ratio obtained from spotted array replicates. (B) Confirmation of the chip data and Cd dose dependent experiment using semi quantitative RT-PCR (35 cycles). (C) Time dependent (days) regulation of *AtOSA1* in leaves of Arabidopsis in the presence (+) or absence (-) of 0.5 μM $CdCl_2$. (D) RT-PCR analyse of *AtOSA1* in plant organs, leaf (L), root (R), flower (F), stem (St), silique (Si).

Figure 2

Schematic illustration of the AtOSA1 protein topology. Identified domains are depicted as follows: white box represents a chloroplast targeting peptide, black box the ABC1 like domain (ABC1), a horizontal stripe box Mn^{2+}-dependent serine/threonine protein kinase (S/T K), a dot box Phosphoinositide 4-kinase (PI4K), shaded barrel a region with predicted transmembrane spans.

Figure 3.

Phylogenetic tree of Arabidopsis Abc1 proteins (Accession numbers according to TAIR http://www.arabidopsis.org/) and *Oryza sativa* Os02g0575500, Os09g0250700, *Saccharomyces cerevisiae* ABC1 (CAA41759), YLR253W, *Ostreococcus tauri* Q00Y32, *Crocosphaera watsonii* ZP_00517317, *Trichodesmium erythraeum* YP_722994, *Anabaena variabilis* YP_323883, *Nostoc* NP4885555 and *Synechocystis* P73627. Protein sequences were aligned using the program DIALIGN (Morgenstern, 2004), and the phylogenetic tree was drawn with the TreeView32 software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Scale bar indicates distance values of 0.01 substitutions per site.

Figure 4.

Complementation test of the *S. cerevisiae* mutant W303-1A *abc1::HIS3*. Yeast strains *S. cerevisae* ABC1 (pRS316 harbouring *S.cerevisiae* ABC1), pNEV (vector only), AtOSA1 (pNEV harbouring AtOSA1), AtOSA1YFP (pNEV harbouring AtOSA1 with YFP) were streaked on plates containing minimal medium lacking uracil for selection (A) and onto minimal medium containing glycerol as a sole carbon source (B). Plates were incubated for 4 d at 28°C. (C) Superposition of a confocal and a bright field image of W303-1A *abc1::HIS3* expressing AtOSA1YFP. (D) Superposition of a confocal image and a bright field image of the same cell stained with Rhodamine HexylB.

Figure 5.

Confocal laser scanning microscopic analysis of an Arabidopsis suspension cell culture transiently expressing EYFP-tagged AtOSA1 (A) and Tic110-GFP (B) and the corresponding bright field images (C, D).

Figure 6.

Cadmium tolerance and accumulation in *atosa1*. (A) Determination of Cd accumulation by AAS in 10 seedlings exposed to 0 (1/2 MS), 1, 10 or 20 μM $CdCl_2$ on agar plates (n=5). (B) The effect of Cd on root growth of *atosa1-1*, *atosa1-2* and Col 0 (WT) in the absence (1/2 MS) or presence of 20 μM $CdCl_2$. Root length of 8 days old seedlings (10<n<20, representative results from 4 independent experiments). (C) Determination of Cd content in leaves and roots in WT (Col 0) and *atosa1-1* grown under hydroponic conditions (n=8) (mean +/- SE; t test:*.p=0.1, ** p=0.05, *** p=0.01). (D) Autoradiography of plant roots labelled with 0.04 MBq $^{109}CdCl_2$ in 1/8 MAMI for 4h. (E) Phenotype of *atosa1* grown in the absence or presence of 0.5 μM $CdCl_2$.

Figure 7.

SOD activity and *AtOSA1* expression. (A) Comparison of the total SOD activities between Arabidopsis wild type Col 0 (WT) and *atosa1-1* under normal growth conditions (-) and after treatment with 1 μM $CdCl_2$ (+) (n=4). (B) Measurement of SOD activity in intact chloroplasts obtained from wild type Col 0 (WT) and the *AtOSA1* T-DNA inserted mutant (*atosa1-1*) treated (+) or not (-) with 1 μM $CdCl_2$ (n=4) (mean +/- SE; t test:*.p=0.1, ** p=0.05, *** p=0.01). (C) Analyses of the expression of *AtOSA1*, *AtAPX1, AtFSD1* and *AtFSD2*, in wild type Col 0 (WT) and *atosa1-1* by RT-PCR in the absence (-) or presence (+) of 1 μM $CdCl_2$ under light superior to 100 μmol m^{-2} s^{-1}. *AtS16* was used as control (30 cycles).

Figure 8.

Effect of oxidative stress. (A) After treatment with 1 mM H_2O_2 on agar plates, root length of 8 days old seedlings was measured (10<n<20, representative results from 4 independent experiments; mean +/- SE; t test:*.p=0.1, ** p=0.05, *** p=0.01). (B) Five week-old Col 0 (WT) and *atosa1-1* plants were sprayed with 300 μM H_2O_2, 0.2% (v/v) Tween 20 at day 0. Plants were photographed at days 0, 1 and 4 following the treatment. (C) Magnification of *atosa1* leaves 4 days after treatment with H_2O_2.

Figure 9.

Effects of light on pigments and shoot growth of *atosa1-1* and *atosa1-2*. Plants were grown at 8h light (50 µmol m^{-2} s^{-1}) for 4 weeks (A) or 8 weeks (C). (B) Shoot weight of 4 weeks old plants (n=10). (D) Contents of chlorophyll a (Chla) and b (Chlb) and carotenoids (car) of 8 weeks old plants (n=10). (E) Plants grown at 16 h light (350 µmol m^{-2} s^{-1}) for 5 weeks: (F) Contents of chlorophyll a (Chla) and b (Chlb) and (car) in plants depicted in (E) (n=10) (mean +/- SE; t test:*.p=0.1, ** p=0.05, *** p=0.01).

Figure 10.

Effect of light intensity on gas exchange and expression of oxidative-stress related genes. Analyses of CO_2 assimilation rate (A) of Col 0 (WT), *atosa1-1* and *atosa1-2*. Measurements were performed in plants grown at 8 h light at a PPFD of 50, 100 or 150 µmol m^{-2} s^{-1} (n=10) (mean +/- SE; t test:*.p=0.1, ** p=0.05, *** p=0.01). (B) RT-PCR expression analysis of *AtS16* (house-keeping gene), *AtOSA1*, *AtAPX1, AtFSD1* and *AtFSD2* in plants used for the determination of gas exchange measurements (A) (28 cycles).

Figure 11.

Protein kinase activity. (A) Detection of protein kinase activity in chloroplast envelope membranes isolated from leaves of wild type and *atosa1-1*. The arrow indicates the position of the phosphorylated myelin basic protein at around 70 kDa in the WT. (B) Coomassie blue staining of the gel shown in panel A. For details see material and methods.

Supplementary material:

Figure 1

Alignment of predicted Abc1 proteins related to AtOSA1: *Arabidopsis,* AtOSA1 (NP_201299), *Oryza*, (Os02g0575500), *Oryza*, (Os09g0250700), *Nostoc* NP_488555 Nos (NP_488555), *Synechocystis,* P73627 Syn (P73627), *Arabidopsis,* ABC1 A.th. (CAA16542), and *Saccharomyces cerevisiae,* ABC1 S. cerev. (CAA41759). Conserved amino acids within the Abc1 domain are indicated with an asterisk. Chloroplast targeting pre-sequences within AtOSA1 proteins as predicted with TargetP are presented in bold. Putative trans-membrane spans are presented in bold italic. The Abc1 region is underlined in AtOSA1.

Figure 2

AtOSA1 T-DNA insertion mutants. (A) Schematic representation of the T-DNA insertion sites in *atosa1-1* (SALK 045739) and *atosa1-2* (GABI 132G06). For PCR screening of homozygote lines, combinations of DNAg left and right border primers and a T-DNA left border primer were used.

Figure 3

Verification of a truncated transcript in *atosa1-1* (SALK 045739) and *atosa1-2* (GABI 132G06). We have designed 3 sets of primers in different position of the transcript. A transcript could be only detected with primers 1 (close to the 5' end).

A

Transcripts	Signal ratio (+Cd/-Cd)	
AtACT2 (At3g18780)	1.1	0.95
AtS16 (At5g18380)	1.1	1.05
AtOSA1 (At5g64940)	0.26	0.1

B

$CdCl_2$ (µM)

0   0.2   0.5   1

AtOSA1

AtS16

C

$CdCl_2$ $CdCl_2$

−   +   **d**   −   +

0

5

7

12

19

AtS16   AtOSA1

D

L   R   F   St   Si

AtOSA1

AtACT2

NH2　　ABC1　　PI4K　　S/T K　　COOH

A — Cd content (µg/g FW) vs CdCl$_2$ (µM): 0, 1, 10, 20

B — Root length (mm): WT, atosa1-1, atosa1-2; ½ MS and 20 µM CdCl$_2$

C — Cd content (µg/g DW): Leaf and Root; CdCl$_2$ (µM): 0, 0.2, 0.5, 1

D — WT, atosa1-1

E — 0.5 µM CdCl$_2$ − / +; WT, atosa1-1

A

SOD activity (U/mg protein)

120

90

60

30

0

***

WT

atosa1-1

−   +   1 M CdCl$_2$

B

SOD activity (U/mg protein)

250

200

150

100

50

0

***   ***

−   +   1 M CdCl$_2$

C

1 M CdCl$_2$ −   +

wt   atosa1-1   wt   atosa1-1

AtS16

AtOSA1

AtAPX1

AtFSD1

AtFSD2

A

Root length (% of control)

WT

atosa1-1

atosa1-2

*** ***

B

WT   *atosa1-1*

Day 0

Day 1

Day 4

C

A

Assimilation rate (μmol CO_2 m^{-2} s^{-1})

5

4

3

2

1

0

50   100   150

PPFD (μmol.m^{-2}.s^{-1})

* **

□ WT
▨ *atosa1-1*
■ *atosa1-2*

B

wt   *atosa1-1*

50 100 150 50 100 150

AtS16

AtOSA1

AtAPX1

AtFSD1

AtFSD2

A

B

WT   *atosa1-1*   WT   *atosa 1-1*

172 —
112 —
86 —

62 —
53 —

37 —
31 —

```
                        10        20        30        40        50        60
AtOSA1        -MATSSSSSSLLLPNINFNSRQSPITR-SVSIAGIFLPRNRLSYNHNLRIRTRLIRAS
Os02g0575500  --MSAAAGAATIVASSASLSVPDHLRLRRFRLHLHPRFPPHPQLRSRSLRQRRRFVLAV
Os09g0250700  MAMMMADLSFSSSISNVKLQRFRASSNETSLIKRSPVFRAEARSETEKFRNGRAIKMV
NP_488555 Nos ----------------------------------------------------------
P73627 Syn    ----------------------------------------------------------
ABC1 A.th     -MSSWSKSFTRLVNGVSLVAKEIYNQSPELQRARNGDLEGLITSSGKKALVAATDLVGL
ABC1 S.cerev  ----------------------------------------------------------

                        70        80        90       100       110       120
AtOSA1        KDDNVAVEDRDNAVKINGDYNGSARLNGNGSARKSVNGDF--NGSARLNGNGSLVKYV-
Os02g0575500  LQEDRSPSAPDEEARRYG-LNGSAPSSGVGYDDAAVEAYIGTNGNGRGNGAAAVVKPAAE
Os09g0250700  PTTELKRSKSGVRVRPDT-VNGSPSGAVNGSTKVAING---SPNAAVNGSMKAVTNGSV
NP_488555 Nos ----------------------------------------------------------
P73627 Syn    ----------------------------------------------------------
ABC1 A.th     TSGKLRELSIRRSKEPSVVYFDEGDNKDNVATPEVRSV--NTKPSEKIVDSKVLRNEF
ABC1 S.cerev  -------------------------------------MVTNMVKLRNLRRLYC

                       130       140       150       160       170       180
AtOSA1        -NGSVTVETEEVTKKRKEVRKKRVEDIGQEDAWFKNNTQQKQVEVSVTPGGWNRFKTY
Os02g0575500  SRSSAALVSAGPGPGDDERRRKERVEEIGREDAWFKQSGGDSKPEVSVAPGGWNRFKTY
Os09g0250700  -NGTSLVKGSNMSALVKTQKRMRENDDPFEEELKVLPSDE-------GFSWAK-DNY
NP_488555 Nos -----MAIYANNLGIRVSELPGDSVITQRYAQMETNYSDK-------AQARWNR-ENY
P73627 Syn    -----MSALSTKLEPTNSYESLPPQNRSPLEAEG------RYRWNR-GNY
ABC1 A.th     SDEVKNSNREGEIK-VSGVEAGEIIGVRAVESGTVTEASSPSEVVPPVKRRPRERKVPS
ABC1 S.cerev  SSRLRTIQNGRISSVSSISLSKKYTTKSARGEENVERKHEEE---KKDTIKSSSVPT

                       190       200       210       220       230       240
AtOSA1        STIQRTLEIWGFVVQFIFRTWLSNKKFSYKGMTEEK------KVLRRKVLAKWLKENILR
Os02g0575500  STIQRTLEIWGFVFKFIFRSWLNNQKFTYRGMTEEK------RVMRRKVLAKWLKESILR
Os09g0250700  NSWQRSADIWSFVLSFIRVLFDNAKWAYAGGFSEEK------QKVRRRTASWLREQVLQ
NP_488555 Nos SSKRRFVDIWGFVSELPGDSVITQRYAQMETNYSDK------QAARRAQVSTTAFGLRRE
P73627 Syn    SITRRRIDIWGFVLTLLYQFWLNGKKWSWSYPGGVTEAK------LQQRRRQAKWIRENLLS
ABC1 A.th     TPMARAYGEFNLGAALAWGAVKEETYRMVNGTPMTPDNQPALSSMSKENARLALGLCE
ABC1 S.cerev  SRISRLFHYGSLAAGVGMNNAAAKGISEVAKGNSPTWK----SLIISDSNIDRITNKFSK

                       250       260       270       280       290       300
AtOSA1        LGPTFIKIGQQFSTRVD-ILPQEYVDQISELQDQVPFFSATALSIVEEELGSSVEDIFD
Os02g0575500  LGPTFIKIGQQFSTRVD-ILPQEYVDQISELQDQVPFFPFSETAVSIIEEELGASVNKIFD
Os09g0250700  LGPTFIKLGQLSSTRSD-LFPREFVDELAKLQDKVPAFSPEKAKAFIEKEMGCPIEVVYK
NP_488555 Nos LGPTFIKVGQLFSTRAD-IFPAEYVEELSKLQDKVPAFSYEQAAGIEEQLGKPIAKLVH
P73627 Syn    LGPTFIKVGQLFSTRAD-IFPAEYVEELSKLQDEVPAFSYEQAAGIEEQLGKPIAKLVH
ABC1 A.th     MRGAALKVGQMLSIQDESLVPAPILNALEYVRQGADVMPRSQLNPVLDAELSNWQSKLT
ABC1 S.cerev  MRGVALKIGQLLSFQDEKVLPKELVEILSRVQNSANHMPQRQLEKVMAKELGANWKTKFS

                       310       320       330       340       350       360
AtOSA1        RFDYEPIAAASLGQVHRARL-KGQEVVLKVQRPGLKLFDIDLKNLRVIAEYLQKVDPKS
Os02g0575500  RFDFEPIAAASLGQVHRACL-NGKEVVIKVQRPGLKELFDIDLKNLRVIAEYLQKVDPKS
Os09g0250700  EFDNRPIAAASLGQVHRAVLHNGESVVKVAVKVQRPGLKKLFDIDLQIKGIARYFQSHEFKWG
NP_488555 Nos SFEPIPLAAASLGQVHRAVLHSGESVVKVQRPGLKKLFEIDLQILKGIARYQSHPKWG
P73627 Syn    SFDPVPLAAASLGQVHKAQLHTGEDVVVKVQRPGLKKLFTIDLAILKKIAQYFQNHPKWG
ABC1 A.th     SFDYEPIAAASLGQVHRAVTKDGLEVAMKIQYPGVANSIEDSDIENVRLLNYTNLIFPK---
ABC1 S.cerev  KFDKIPMAAASIGQVHAAELPSGQVVVKIQYPGVKESIDSINSLLMLLTASSLLPK--

                       370       380       390       400       410       420
AtOSA1        DGAKRDWVAIYDECASVLYQEIDYTKEAANSELFANNFKDLEYVKVPSIYWEYTTPQVLT
Os02g0575500  DGAKRDWVAIYDECASVLYQEIDYTKEAFNAEKFSENFKNMDYVKVPEILWEYTTPVLT
Os09g0250700  -GESRDWIGIYEECSKILYEEIDYLNEGRNADTFRRNFRGYDWVKVPEVIWEYAKRVLT
NP_488555 Nos RG--RDWMGIYEECRIIWEEIDYLNEGRNADTFRRNFPGYDWKVPRVVWEYTTASPRVLT
P73627 Syn    RG--RDWNGIYEECKIIWQETDYLREGRSADTFRRNFREGEDWKVPRVYWRYTSTQILT
ABC1 A.th     -GLFLD--RAIKVAKELAQECDYIEEAVSQKRFRDLLSDTPGFYVPLVVDESSKKILT
ABC1 S.cerev  -GLFLD--KTIANARTELKWECDYNREARALQKFEALLKDDPAFEVPHVFFPEYTTDNIIT
```

```
                       430       440       450       460       470       480
AtOSA1        MEYVPGIKNKIQALDQLGVDRKRLGRYAVESYLEQILSHGFFHADP----HPGNIAVDD
Os02g0575500  MEYVPGIKINRIKQLDKLGVDRKRLGRYAVESYLEQILSHGFFHADPISLQHPGNIAVDD
Os09g0250700  LEYVPGIKINNLTLDNRGYRSLIASRSIESYLIQILKTGFFHADP----HPGNIAID-
NP_488555 Nos LEYLPGIKISQYEAIEAAGLDRKVLARQGAQAYLQLINNGFFHADP----HPGNIAVS-
P73627 Syn    LEYLPGIKISHYDALEAAGLERKELAQLGARAYIFQLINHGFFHADP----HPGNIAVS-
ABC1 A.th     TELISGIPIDKVALLDQK--TRDYVGRKMLELTIKELFVFRMQTDP----NWGNFLYNE
ABC1 S.cerev  MTRMEGTFIMKKLPKASQE--TRNFISENIMRICLEBIATFKIVMQTDP----NWANFIYNG

                       490       500       510       520       530       540
AtOSA1        VNGGRLIFYDFGMMGSISPNIREGLLEAFYGVYEKDPDKVLQAMVQMGVLVPTGDLTAVR
Os02g0575500  VNGGRLIFYDFGMMGSISPNIREGLLEAFYGVYEKDPDKVLQSMIQMGVLVPTGDMTAVR
Os09g0250700  -TDGSLIYYDFGMMGEIKSFTRERLLSIFYAVYEKDANKVMRALIDLEALQATGDLSPVR
NP_488555 Nos AD-GALIFYDFGMMGQIKSNIREGLMQDTFGIAQKDGDRVVQSLIDLGAIAPVDDMGPVR
P73627 Syn    PEAGELIFYDFGMMGEITFDTNKIMDTFGVAEKNAERIVNSLVALGALKETEDMGPIR
ABC1 A.th     AT-KTINLIDFGAARDYPKKFVDDYLRMVMACAEKDSEGVIEMSRRLGFL-TGDESDVM
ABC1 S.cerev  RT--KKIELLDFGASRPFAEDFIIKYRKLLTYATLRDRKGAYEMSVQLGYL-TGLSQSM

                       550       560       570       580       590       600
AtOSA1        RTALFFLNSFEERLAAQRKEKEIAAAAELGFKKPLSKEEKQEKKKQRLAAIGEDLLAIA
Os02g0575500  RTAQFFLDSFEERLAAQRKERE--MATELGFKKQLTKEEKFEKKKQRLAAIGEDLLSIA
Os09g0250700  RSIQFFLDNLLSQTFDQQ--------------------------QSVAAISDDLFAIS
NP_488555 Nos RSVQYMLDNFMDKPFEN--------------------------QSITKISDDLYEIA
P73627 Syn    LDAHVQAGFIVGLPFAEP--GG------------YAFRTNNIASSINLGATVP
ABC1 A.th     ----------------------------------YAFRTNNIASSINLGATMLKHRLSPPDEA
ABC1 S.cerev  KDAHVDSVITLGEFFRGDVDKS---------FDFKDQTVSDRIRGNIGLMNERLCFPEET

                       610       620       630       640       650       660
AtOSA1        ADQPFRFPATFTFVVRAFSVLDGIGKGLDPRFDITEIAKPYALELLRFREAGVEVVVK-
Os02g0575500  ADQPFRFPATFTFVVRAFSVLDGIGKGLDPRFDITEIAKPYAMELLRFNEAGVEVIVKAN
Os09g0250700  QDQPFRFPSTFTFVIRAFSTLEGIGYIIDPDFSFVKVAQPYAQELLDIKQRRTSA---
NP_488555 Nos YQPFRFPATFTFVMRAFSTLGVGKGLDPENFMEVAQPYAMQLMTDMNG-SDS---
P73627 Syn    YDQPFRFPATFTFVMRAFSTLEGVGKGLDPENFMAVAQPFALQIMNSNGFNPA--
ABC1 A.th     YSLHRKLSGAFLACIKLGATVPCRDLLQVYNKYQFDDEPQDPVVATSSVSS------
ABC1 S.cerev  YSLHRKFSGIFLLCARMGASVHCAKLFKEIFAYKV-----------------------

                       670       680       690       700       710       720
AtOSA1        ---------------DLRKRWDRQSQAFYNLFRQADRVEKLAVVIERLEQGLKLRVRALES
Os02g0575500  LRVLSALGFILPQDARKRWERQSRAFYNLFRQPDRVEKLAQIIERLEQGDIKLRVRTLES
Os09g0250700  ---------NSFLNELSRQAVQVSTTAFGLPRRLE---DTLEKLERGDMRLRVRSIES
NP_488555 Nos ---------NSFLNELSRQAVQVSTTAFGLPRRLE---DTLEKLERGDMRLRVRSIES
P73627 Syn    ----------GNIMDELGRQAVQVGNSAIGLPRRIE---DSDRLDRGDIRVVRSTET
ABC1 A.th     ----------------------------------------------------------
ABC1 S.cerev  ----------------------------------------------------------

                       730       740       750       760       770       780
AtOSA1        ERAFQRVRAVQKTVGSAVAAGSLVNLATIIYLNSIKTPATIAYTVCAFFSLQVLIGIKV
Os02g0575500  ERAFQRVRAAVQKTIGVVAAGSLVNLATVLYLNSIRLPATIAYSLCAFFGLQVLVGLKV
Os09g0250700  ERAARKRRQGNIQIGIGYALIISGFTLSATILVNHYVWLALLAGLIAAAVSVILIRLLRI
NP_488555 Nos ERLLRRQGNIQIGIGYALIISGFTLSATILVNHYVWLALLAGLIAAAVSVILIRLLRL
P73627 Syn    DRLLRRMGTMQMGTNYVLFTCALVLSATLLFVNNIFWAAAAVLLMSLVPAFALWLLLKRL
ABC1 A.th     ----------------------------------------------------------
ABC1 S.cerev  ----------------------------------------------------------

                       790
AtOSA1        KKFDQREKLITGTA
Os02g0575500  KKLDQQERLITGTA
Os09g0250700  KKLDKFETMI----
NP_488555 Nos DRYDRMY-------
P73627 Syn    ERQDRMF-------
ABC1 A.th     --------------
ABC1 S.cerev  --------------
```

A

gDNA

atosa1-1

LB T-DNA

LP → ← RP

LB T-DNA

atosa1-2

B

WT   *atosa1-1*

900 ■

LP

300 ■

RP

900 ■

LB T-DNA

300 ■

RP

WT   *atosa1-2*
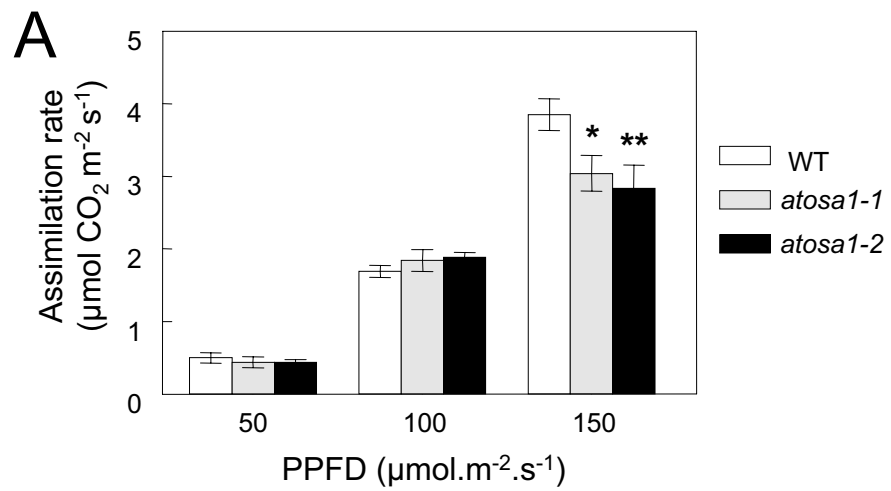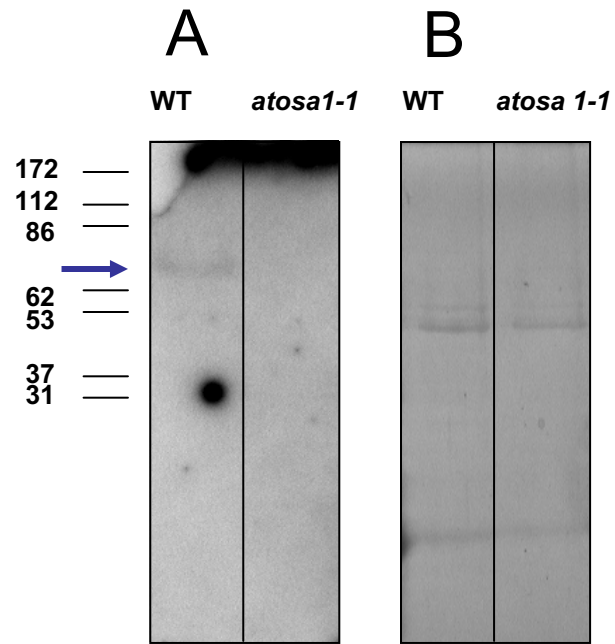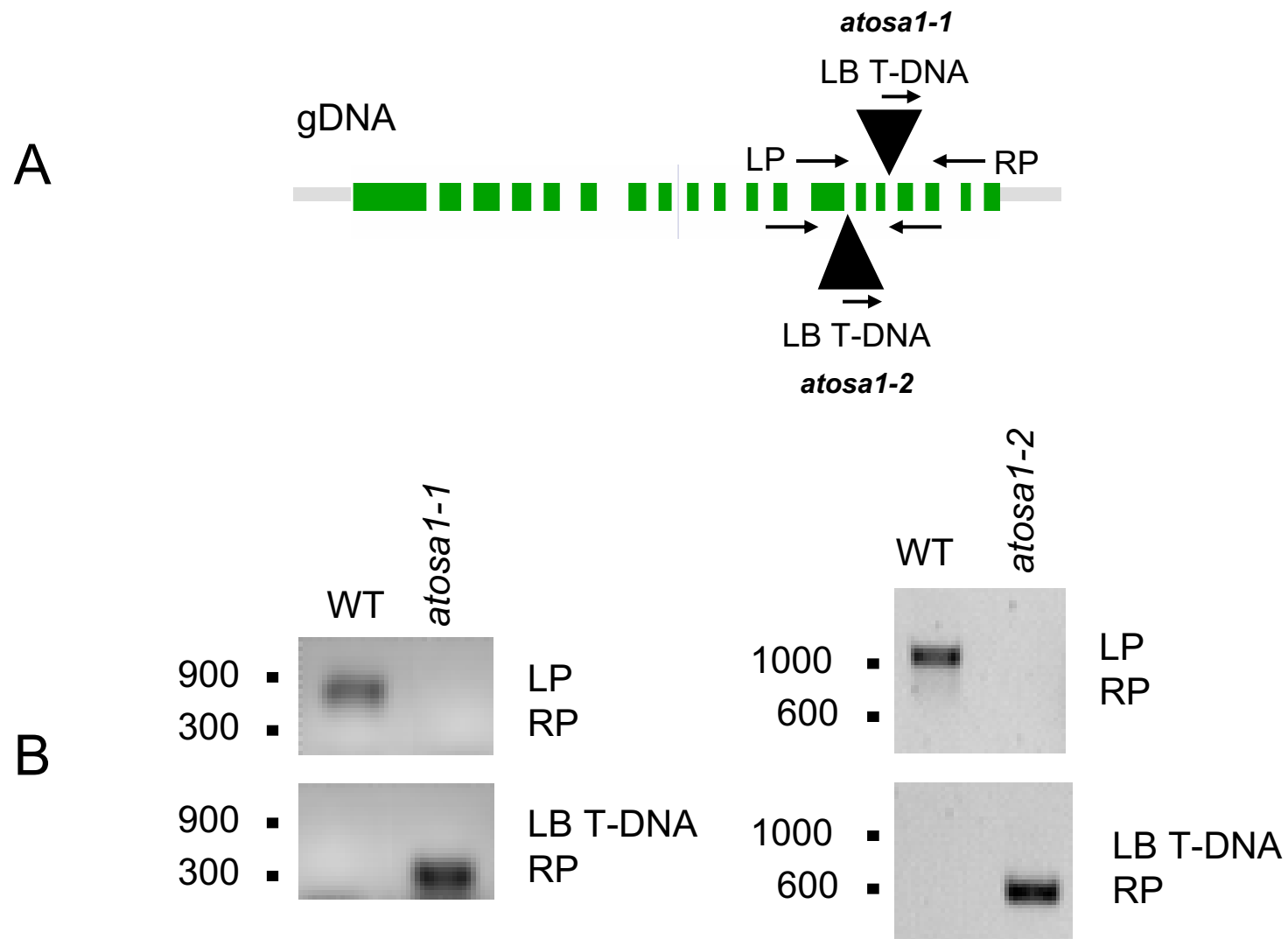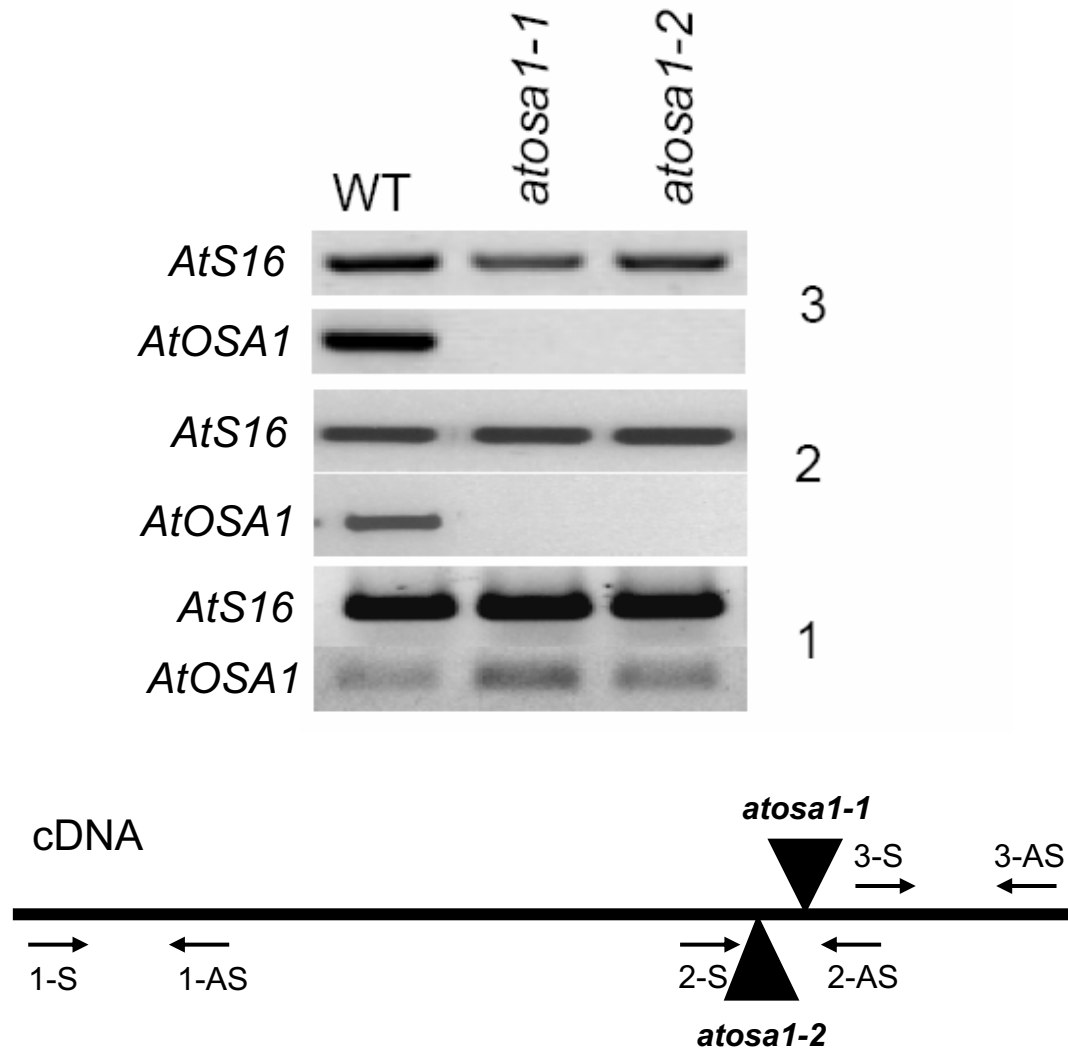
1000 ■

LP

600 ■

RP

1000 ■

LB T-DNA

600 ■

RP

Supplementary material:

Figure 1

Alignment of predicted Abc1 proteins related to AtOSA1: *Arabidopsis,* AtOSA1 (NP_201299), *Oryza*, (Os02g0575500), *Oryza*, (Os09g0250700), *Nostoc* NP_488555 Nos (NP_488555), *Synechocystis,* P73627 Syn (P73627), *Arabidopsis,* ABC1 A.th. (CAA16542), and *Saccharomyces cerevisiae,* ABC1 S. cerev. (CAA41759). Conserved amino acids within the Abc1 domain are indicated with an asterisk. Chloroplast targeting pre-sequences within AtOSA1 proteins as predicted with TargetP are presented in bold. Putative trans-membrane spans are presented in bold italic. The Abc1 region is underlined in AtOSA1.

Figure 2

AtOSA1 T-DNA insertion mutants. (A) Schematic representation of the T-DNA insertion sites in *atosa1-1* (SALK 045739) and *atosa1-2* (GABI 132G06). For PCR screening of homozygote lines, combinations of DNAg left and right border primers and a T-DNA left border primer were used.

Figure 3

Verification of a truncated transcript in *atosa1-1* (SALK 045739) and *atosa1-2* (GABI 132G06). We have designed 3 sets of primers in different position of the transcript. A transcript could be only detected with primers 1 (close to the 5' end).