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REVIEW ARTICLE

The relevance of compartmentation for cysteine synthesis in phototrophic organisms

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Abstract In the vascular plant *Arabidopsis thaliana*, synthesis of cysteine and its precursors *O*-acetylserine and sulfide is distributed between the cytosol, chloroplasts, and mitochondria. This compartmentation contributes to regulation of cysteine synthesis. In contrast to *Arabidopsis*, cysteine synthesis is exclusively restricted to chloroplasts in the unicellular green alga *Chlamydomonas reinhardtii*. Thus, the question arises, whether specification of compartmentation was driven by multicellularity and specified organs and tissues. The moss *Physcomitrella patens* colonizes land but is still characterized by a simple morphology compared to vascular plants. It was therefore used as model organism to study evolution of compartmented cysteine synthesis. The presence of *O*-acetylserine (thiol)lyase (OAS-TL) proteins, which catalyze the final step of cysteine synthesis, in different compartments was applied as criterion. Purification and characterization of native OAS-TL proteins demonstrated the presence of five OAS-TL protein species encoded by two genes in *Physcomitrella*. At least one of the gene products is dual targeted to plastids and cytosol, as shown by combination of GFP fusion localization studies, purification of chloroplasts, and identification of N termini from native proteins. The bulk of OAS-TL protein is targeted to plastids, whereas there is no evidence for a mitochondrial OAS-TL isoform and only a minor part of OAS-TL protein is localized in the cytosol. This demonstrates that subcellular diversification of cysteine synthesis is already initialized in *Physcomitrella* but appears to gain relevance later during evolution of vascular plants.

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Abbreviations

ac	Acetyl
ARP	APS reductase
APS	Adenosine 5′phosphosulfate
ASRP	Assimilatory sulfate reduction pathway
ATP	Adenosine triphosphate
ATPS	ATP sulfurylase
CLSM	Confocal laser scanning microscopy
CSC	Cysteine synthase complex
GFP	Green fluorescence protein
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
mRNA	Messenger ribonucleic acid
OAS	*O*-acetylserine
OAS-TL	*O*-acetylserine(thiol)lyase
PAGE	Polyacrylamide gel electrophoresis
SAP	SAT-affinity purification
SAT	Serine acetyltransferase
SDS	Sodium dodecyl sulfate
SiR	Sulfite reductase
SULTR	Sulfate transporter

Introduction

Sulfur is an essential macronutrient for animals and plants. Total sulfur content in plant tissues varies depending on the plant species. In *Arabidopsis thaliana*, it accounts for approximately 1 % of dry weight (Khan et al. 2010). Due to its presence in cysteine and methionine sulfur is crucial for protein structure and functionality (Takahashi et al. 2011). Glutathione, a sulfur-containing tripeptide, is the key player in the oxidative stress response and essential for detoxification of heavy metals in vascular plants and mosses (Takahashi et al. 2011; Rother et al. 2006). It is the most abundant low molecular weight thiol and thus the major storage compound for soluble reduced sulfur in almost all eukaryotic cells and in most Gram-negative bacteria including cyanobacteria (Meyer and Rausch 2008). In addition, several cofactors like iron–sulfur clusters and vitamins contain sulfur as functional constituent. Notably, sulfur is bound in its reduced form in all of these compounds, whereas in the environment it is almost exclusively available as oxidized sulfate. However, in contrast to animals, plants, fungi, and many bacteria are able to reduce sulfate to sulfide in the assimilatory sulfate-reduction pathway (ASRP). The final product of this multistep pathway is cysteine, which is the direct or indirect precursor for all organic compounds containing reduced sulfur in plants and animals (for review, see Takahashi et al. (2011)).

Sulfur metabolism and cysteine synthesis in *Arabidopsis*

Sulfur assimilation in vascular plants is probably best characterized in the model organism *A. thaliana*. Initially, sulfate is taken up from the soil by SULTR and temporarily stored in the vacuoles, if sufficient reduced sulfur is available in the plant. In the reducing phase of the ASRP, ATPS activates sulfate by adenylation with adenosine triphosphate (ATP) to adenosine 5′phosphosulfate (APS). APS is reduced in a two step process by APS reductase (APR) and SiR producing sulfide. Finally, sulfide is incorporated into the amino acid backbone *O*-acetylserine (OAS) by *O*-acetylserine(thiol)lyase (OAS-TL) to produce cysteine. OAS is provided by serine acetyltransferase (SAT), which uses serine and acetyl-CoA as substrates. Sulfate reduction is exclusively localized in plastids and mainly regulated via APR expression and activity (Koprivova et al. 2000; Vauclare et al. 2002; Hesse et al. 2003; Koprivova et al. 2008). In contrast, OAS and cysteine are synthesized in the cytosol, the plastids, and the mitochondria by compartment-specific SAT and OAS-TL isoforms. AtSAT5, AtSAT2, and AtSAT4 are localized in the cytosol, whereas AtSAT1 and AtSAT3 are targeted to the plastids and mitochondria, respectively (Kawashima et al. 2005). AtOAS-TL A represents the cytosolic OAS-TL isoform, AtOAS-TL B is targeted to the plastids, and AtOAS-TL C is localized in the mitochondria (Hell and Wirtz 2011). All SAT and OAS-TL isoforms are encoded by the nuclear genome. However, in contrast to SAT, a cyanobacterial origin of the OAS-TL genes is most likely (Kopriva et al. 2008). For a long time, the presence of compartment-specific SAT and OAS-TL isoforms was explained by the demand of the protein biosynthesis machinery in these compartments for cysteine (Lunn et al. 1990). Recent transgenic approaches, however, revealed that OAS and cysteine are exchangeable between the compartments and the compartment-specific isoforms contribute to a different extent to total OAS and cysteine production (Watanabe et al. 2008; Heeg et al. 2008; Haas et al. 2008; Krüger et al. 2009). Mitochondrial AtSAT3 contributes to approximately 80 % of total SAT activity in the leaf cell, demonstrating the significant role of mitochondria for OAS production (Haas et al. 2008). In contrast to the role of mitochondrial AtSAT3, contribution of mitochondrial AtOAS-TL C to total OAS-TL activity in leaves is very low (<5 %). Cytosolic AtOAS-TL A and plastidic AtOAS-TL B contribute most, each accounting for more than 45 % of total OAS-TL activity (Heeg et al. 2008). Interestingly, only loss of AtOAS-TL A results in a significantly decreased flux of sulfur into cysteine, which might indicate a

predominant role of cytosolic cysteine production compared to plastids and mitochondria (Heeg et al. 2008). Viability of all single loss-of-function mutants for SAT and OAS-TL on the other hand indicates functional redundancy (Heeg et al. 2008; Watanabe et al. 2008). However, in *Arabidopsis* generation of substrates for cysteine synthesis and cysteine synthesis itself are spatially separated from each other: sulfate is reduced to sulfide in the plastids, OAS is mainly synthesized in the mitochondria, and both are finally combined to form cysteine in the cytosol. This spatial separation is also reflected by the subcellular concentrations of these metabolites (Krüger et al. 2009).

In contrast to the loss-of-function mutants for AtOAS-TL A and AtOAS-TL B, the AtOAS-TL C loss-of-function mutant shows a retarded growth phenotype, although AtOAS-TL C accounts for less than 5 % of total OAS-TL activity (Heeg et al. 2008). Thus, AtOAS-TL C seems to have an additional function different from cysteine synthesis in mitochondria. Indeed, AtOAS-TL C is part of the cysteine synthase complex (CSC), whose formation regulates SAT activity to adjust OAS synthesis to the demand of the cell (Wirtz et al. 2010a; Wirtz and Hell 2006; Droux et al. 1998). The CSC consists of one SAT hexamer interacting with two OAS-TL dimers and is present not only in the mitochondria, but also in the cytosol and plastids. Function of CSC formation is not substrate channeling since OAS-TL is inactive within the complex due to binding of the SAT C terminus within the active site of OAS-TL (Francois et al. 2006). In the presence of sulfide, the CSC is stabilized and OAS is produced by SAT. OAS is released and used together with sulfide by free and thus active OAS-TL to produce cysteine. In contrast, excess of OAS in the absence of sulfide dissociates the CSC, releasing SAT. Free SAT is now inhibited by cysteine, which prevents further OAS synthesis (Wirtz et al. 2010a). Thus, mitochondrial AtOAS-TL C prevents AtSAT3 inhibition through complex formation and in the end allows efficient OAS production in mitochondria if sulfide is present. The importance of AtOAS-TL C for regulation of SAT activity but not for cysteine synthesis is further supported by the OAS-TL/SAT activity ratios in the different compartments. In the cytosol and plastids, the OAS-TL/SAT activity ratio is high (200:1 and 300:1, respectively), which results in a high excess of free and thus active OAS-TL. In contrast, in mitochondria the OAS-TL/SAT ratio is low (~4:1), which allows efficient complex formation but not cysteine synthesis (Heeg et al. 2008). In fact, a 200 times excess of OAS-TL over SAT activity, as realized in cytosol and plastids, is needed for efficient cysteine synthesis from OAS and sulfide (Droux et al. 1998; Ruffet et al. 1994).

The presence of SAT and OAS-TL isoforms in cytosol, plastids, and mitochondria along with the unequal distribution of their activities in these compartments, seem to be conserved among vascular plants, e.g., *Brassica oleracea* (Rolland et al. 1992; Lunn et al. 1990) and *Pisum sativum* (Ruffet et al. 1995). In spinach (*Spinacea oleracea*), however, purified mitochondrial extract lacks SAT activity (Brunold and Suter 1982) and it remains uncertain whether a true OAS-TL is present in the mitochodrion. Although mitochondrial OAS-TL activity could be detected in spinach (Lunn et al. 1990), it was later identified to be a side activity of β-cyanoalanine synthase (Warrilow and Hawkesford 2000). Taken together, these results indicate that, in contrast to *Arabidopsis*, there is no mitochondrial CSC in spinach. Nevertheless, to our knowledge, spinach is the only vascular plant in which no mitochondrial SAT and OAS-TL were identified.

Cysteine synthesis in *Chlamydomonas* and *Physcomitrella*

Our knowledge about the subcellular localization of SAT and OAS-TL isoforms and their contribution to total cysteine synthesis is mainly restricted to vascular plants. However, the genome of the unicellular green alga *Chlamydomonas reinhardtii* encodes at least one isoform of each of the enzymes of ASRP. The proteins show high similarity to the proteins from vascular plants and are supposed to be localized exclusively to the plastids (Shibagaki and Grossman 2008; Giordano et al. 2008; Patron et al. 2008), which would be a fundamental difference compared with vascular plants. The presence of compartment-specific isoforms as basis to separate the syntheses of cysteine and its precursors might be an achievement consistent with the evolution of vascular plants. From an evolutionary point of view, mosses represent a transition stage from algae to vascular plants (Lang et al. 2008). The moss *Physcomitrella patens* is commonly used as model organism to study developmental and physiological aspects in an evolutionary perspective (Reski 1998; Cove et al. 2006; Prigge and Bezanilla 2010). With respect to sulfur assimilation in *Physcomitrella*, research was almost exclusively focused on the reduction of APS to sulfite. Besides the vascular plant type PhypaAPR, *Physcomitrella* possesses a novel type of APS reductase, PhypaAPR-B, which shows no iron-sulfur cluster and is restricted to primitive land plants (Kopriva et al. 2007; Wiedemann et al. 2007). This result indicates differences between the ASRP of mosses and vascular plants.

A homology search against the *Physcomitrella* proteome (www.cosmoss.org) with *Arabidopsis* AtOAS-TL A, AtOAS-TL B, and AtOAS-TL C as template revealed five putative OAS-TL-like proteins. Phylogenetic analysis of OAS-TL-like proteins from photosynthetic organisms representing different evolutionary levels suggests that Phypa116788 (Pp1s17_59V2.1) and Phypa164579 (Pp1s71_187V2.1)

cluster together with the OAS-TLs and OAS-TL-like proteins from *Arabidopsis* (Fig. 1). Phypa116788 and Phypa164579 are highly similar (89 % sequence identity) and the very few differences are almost completely restricted to the first 100 amino acid residues. Both proteins share approximately 60 % identity to the *Arabidopsis* major OAS-TL isoform AtOAS-TL A according to pairwise alignments (www.ebi.ac.uk/Tools; Needleman–Wunsch global alignment algorithm). The third OAS-TL-like protein, Phypa230402 (Pp1s105_118V2.1), is more distant to the OAS-TLs from *Arabidopsis*. Amino acid residues known to be crucial for enzymatic activity are present (Francois et al. 2006). However, three out of 14 amino acid residues differ in the highly conserved β8A/β9A loop of OAS-TL (Francois et al. 2006), which is responsible for interaction with SAT (Supplemental Fig. 1). The two remaining OAS-TL-like proteins, Phypa199268 (Pp1s359_40V2.1) and Phypa168723 (Pp1s186_44F4.1), differ significantly from the AtOAS-TLs and completely lack the β8A/β9A loop. Consequently, they do not cluster together with the *Arabidopsis* OAS-TLs. The function of these proteins as OAS-TLs for cysteine synthesis is thus questionable.

Functional assessment of OAS-TL proteins in *Physcomitrella*

True OAS-TLs were discriminated from OAS-TL-like proteins in *Physcomitrella* performing a SAT-affinity purification (SAP) experiment as described by Heeg et al. (2008). Interaction of OAS-TL with SAT is one of the accepted criteria for true OAS-TLs and mediated by the SAT C terminus, which is highly conserved among plant SATs (Hell and Wirtz 2011). To this end, AtSAT5 was used as bait to purify native OAS-TLs from a soluble protein extract containing both cytosolic and organellar proteins isolated from *Physcomitrella* protonemata. Five protein species with a molecular weight around 30 to 40 kD could be identified (Fig. 2a). Sequencing of all protein species using LC-MS/MS (Supplemental Text 1) revealed that each protein species actually represented a mixture of two true OAS-TLs, Phypa116788, and Phypa164579, which will therefore be named PpOAS-TL 1 and PpOAS-TL 2 in the following. Phypa199268, Phypa168723, and Phypa230402 could not be purified by SAP which is in agreement with the amino acid changes in the conserved β8A/β9A loop. In conclusion, the SAP experiment clearly demonstrates that only two of the five genes encoding OAS-TL-like proteins actually code for true OAS-TLs. However, expression of the two genes results in five protein species. This might be explained by the presence of four and three methionine residues in the N-terminal part of PpOAS-TL 1 and PpOAS-TL 2, respectively (Fig. 2b). In principle, each of

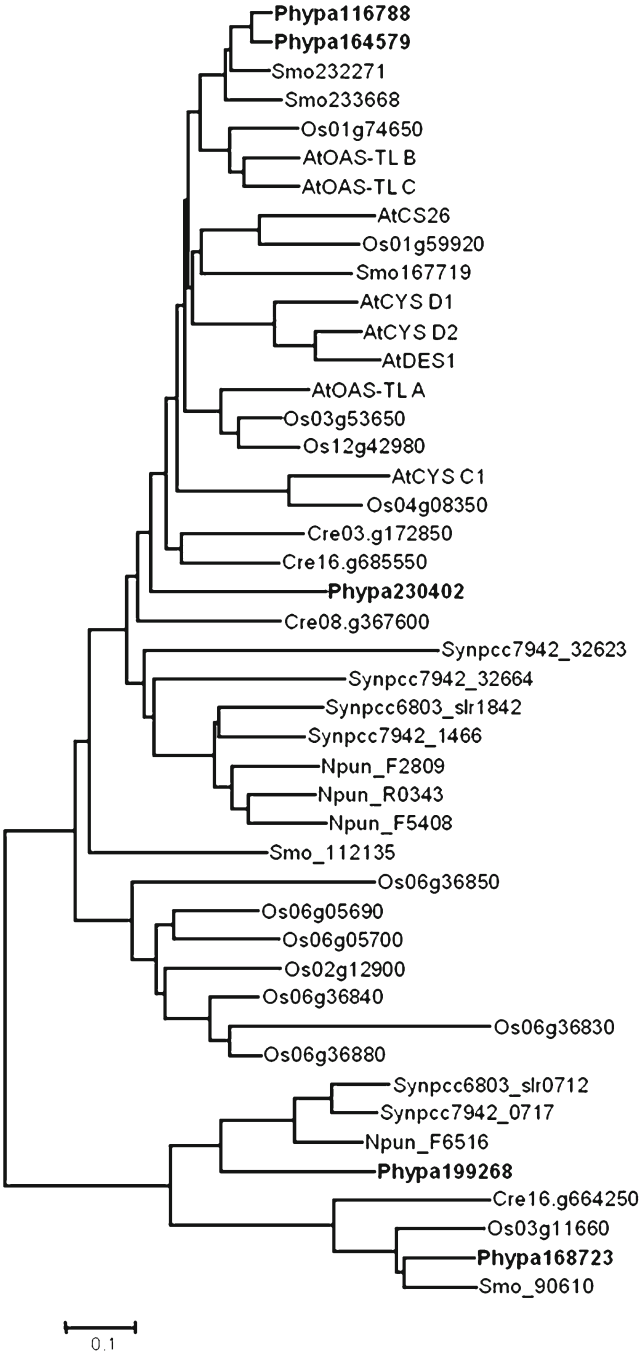

Fig. 1 Phylogenetic analysis of OAS-TL-like proteins of phototrophic organisms Primary sequence of OAS-TL-like proteins from the cyanobacteria *Nostoc punctiforme*, *Synechococcus PCC7942* and *Synechocystis PCC6803*, the green alga *C. reinhardtii*, the moss *P. patens*, the lycophyte *Selaginella moellendorffii*, and the monocot *Oryza sativa* (Nipponbare) were obtained from the NCBI (www.ncbi.nlm.nih.gov) or JGI (www.jgi.doe.gov) database by homology search using *A. thaliana* OAS-TL A, OAS-TL B, and OAS-TL C as template. Phylogenetic analysis was done using the MEGA5 software (Tamura et al. 2011). The neighbor-joining method was conducted for reconstruction of the phylogenetic tree (Saitou and Nei 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated

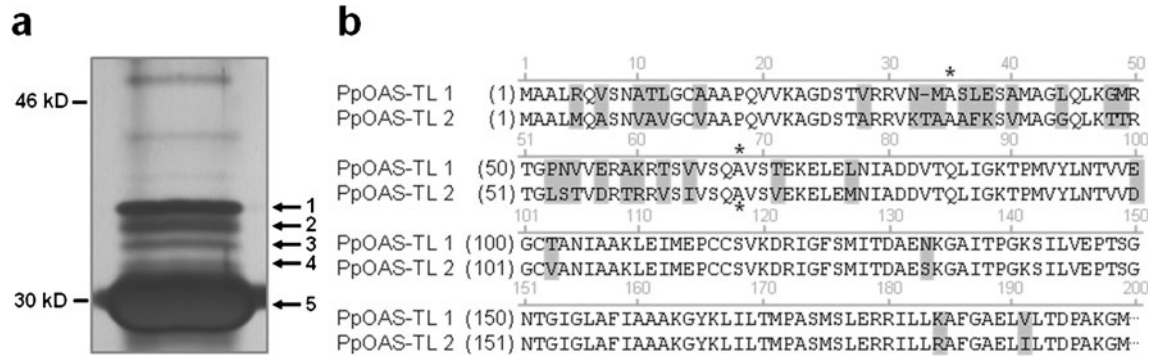

Fig. 2 Purification and identification of native *Physcomitrella* OAS-TLs Native OAS-TL were purified from a soluble protein extract isolated from 150 g *Physcomitrella* protonemata by SAT-affinity purification (Heeg et al. 2008). Purified OAS-TLs (35 μg) were separated by one dimensional SDS gel electrophoresis and visualized by silver staining according to Rabilloud (1999) (**a**). Protein species 1 to 5 were sequenced by LC-MS/MS (Supplemental Text 1). At least three peptides were identified per protein. The first 200 amino acid residues of the identified proteins were aligned using the ClustalW algorithm (**b**). Sequence differences are colored in *grey*, amino acid residues identified to be N^{α}-terminally acetylated were marked by *asterisks*

these methionines could represent a translational initiation site. Met^{48} and Met^{5} of PpOAS-TL 1 and PpOAS-TL 2, respectively, are less likely functional translational initiation sites since the context of the AUG start codon neither fits the Kozak consensus sequence of eukaryotic mRNAs (Kozak 1987) nor the context sequence for lower plants described by Joshi et al. (1997) (Supplemental Fig. 2). The context of the AUG start codon, however, is critical for the strength of the translation initiation site. Nevertheless, usage of the remaining translational initiation sites would result in several protein species, which only differ in the length of the N terminus. Additionally, processing of the OAS-TLs upon targeting to organelles could contribute to the generation of different protein species. Indeed, identification of several N^{α}-terminally acetylated peptides by LC-MS/MS and analysis of the data using the Mascot algorithm supports both explanations for the generation of different protein species (Supplemental Table 1; Supplemental Fig. 3). For PpOAS-TL 1, two alanine residues were identified to be N^{α}-terminally acetylated: Ala^{34} and Ala^{67}. Acetyl (ac)-Ala^{34} was only found in protein species 1, whereas ac-Ala^{67} could be detected in protein species 1 and 5. The cytosolic N^{α}-terminal acetyltransferase complex A acetylates the α-amino group of the adjacent amino acid residue upon cleavage of methionine at position 1 by methionine aminopeptidase (Polevoda and Sherman 2003). Ala^{34} is located next to Met^{33}, which thus probably represents the translational start of a first PpOAS-TL 1 species, which will be referred as Met^{33}-PpOAS-TL 1. The calculated molecular weight of processed Ala^{34}-PpOAS-TL 1 is 38.2 kD, which would fit the size of the observed protein species 1. The internal amino acid residue Ala^{67} can only be N^{α}-terminally acetylated, if the protein is processed, leaving Ala^{67} at the N terminus. This indicates organellar targeting of the PpOAS-TL 1, cleavage between Gln^{66} and Ala^{67}, and N^{α}-terminal acetylation by an organellar N^{α}-terminal acetyltransferase (Ala^{67}-PpOAS-TL 1). Identity of the organellar N^{α}-terminal acetyltransferase is not known. The processed Ala^{67}-PpOAS-TL 1 has a calculated molecular weight of 34.7 kD, which would fit the size of protein species 5. However, ac-Ala^{67} was also found in protein species 1, which might be explained by additional posttranslational modification resulting in a higher molecular weight. In contrast to PpOAS-TL 1, only one N^{α}-terminally acetylated amino acid residue was identified for PpOAS-TL 2: ac-Ala^{68}, detected in protein species 5. The calculated molecular weight for Ala^{68}-PpOAS-TL 2 of 34.6 kD would be again in good agreement with protein species 5. Similar to Ala^{67}-PpOAS-TL 1, Ala^{68}-PpOAS-TL 2 is probably also targeted to organelles. Interestingly, N^{α}-terminal acetylation of OAS-TL proteins is conserved between *Physcomitrella* and *Arabidopsis*. The native cytosolic AtOAS-TL A is also N^{α}-terminally acetylated at Ala^{2} upon removal of the initiation methionine as it was found for Met^{33}-PpOAS-TL 1. The plastidic AtOAS-TL B is acetylated at Ala^{61} upon targeting and removal of the transit peptide (Wirtz et al. 2010b). Ala^{61} of AtOAS-TL B corresponds to Ala^{67} and Ala^{68} of PpOAS-TL 1 and PpOAS-TL 2, respectively.

Subcellular distribution of OAS-TL proteins in *Physcomitrella*

Analysis of the N termini of PpOAS-TL 1 and PpOAS-TL 2 proves the existence of at least three protein species, two of which are probably targeted to organelles. Target signal prediction is not very accurate for *Physcomitrella* proteins (Lang et al. 2011) since the available algorithms mainly work on proteins from *Arabidopsis* or other higher plants. Nevertheless, using the full length protein sequences of PpOAS-TL 1 and PpOAS-TL 2 for prediction, a plastidic localization of both proteins was favored by several

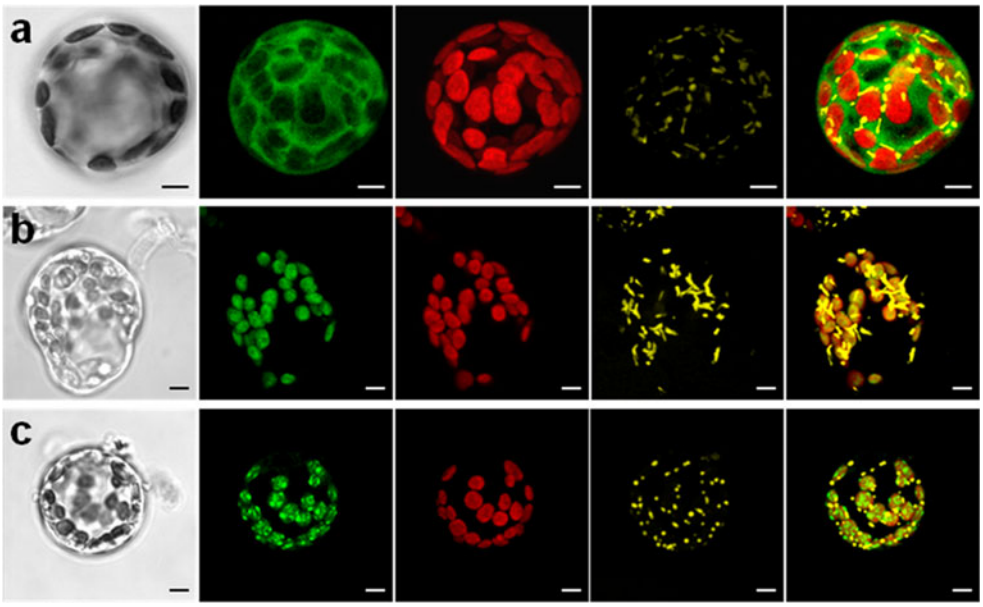

Fig. 3 Subcellular localization of OAS-TL:GFP fusion proteins in *Physcomitrella* protoplasts The coding sequence of Met^{33}-PpOAS-TL 1 (**a**), Met^{1}-PpOAS-TL 1 (**b**), and Met^{1}-PpOAS-TL 2 (**c**) was cloned into the mAV4 vector (Kircher et al. 1999) using the primers given in Supplemental Table 3. Constructs were transiently transfected into *Physcomitrella* protoplasts according to Hohe et al. (2004) and localization of GFP fluorescence was analyzed by CLSM upon two days incubation in the dark using a LSM510 META/Axiovert 200 M microscope (Zeiss). GFP emission was collected upon excitation with 488 nm using a band pass filter of 505–530 nm. Autofluorescence of chloroplasts was detected upon excitation with 488 nm at >560 nm. Mitochondria were specifically stained using Mitotracker® Orange CMTMRos (Invitrogen) and the Mitotracker® signal was recorded upon excitation with 543 nm at >560 nm. From *left* to *right*, transmission light, GFP, autofluorescence chloroplasts, Mitotracker® Orange, merge. Picture analysis was done using ImageJ 1.44p (http://imagej.nih.gov/ij). *Scale bar*, 5 μm

prediction programs included in the ARAMEMNON plant membrane protein database (http://aramemnon.uni-koeln.de/index.ep; Supplemental Table 2). The predicted cleavage sites for the transit peptides are close to Ala^{67} and Ala^{68}, which were identified to be N^{α}-terminally acetylated in PpOAS-TL 1 and PpOAS-TL 2, respectively. To address the subcellular localization, green fluorescence protein (GFP) fusion constructs were designed based on all identified N termini (Supplemental Table 3) and transiently transfected into *Physcomitrella* protoplasts. The localization of the GFP signal was detected by confocal laser scanning microscopy (CLSM) analysis (Fig. 3). The GFP signal was detected in the cytosol, when the truncated Met^{33}-PpOAS-TL 1/GFP was expressed (Fig. 3a). In contrast, expression of the full-length protein Met^{1}-PpOAS-TL 1/GFP resulted in a distinct GFP signal in the chloroplasts, which is demonstrated by the perfect overlap of the GFP signal in green and the autofluorescence of the chloroplasts in red (Fig. 3b). Taken together, our results evidence that PpOAS-TL 1 is dual targeted to both cytosol and plastids, depending on the length of the N terminus. Met^{1}-PpOAS-TL 2/GFP is also targeted to the plastids, as it was shown for Met^{1}-PpOAS-TL 1/GFP (Fig. 3c). A mitochondrial localization of PpOAS-TL 1 and PpOAS-TL 2 can be excluded since there was no overlap of the GFP signal and the Mitotracker® Orange signal for any of the tested GFP fusion proteins (Fig. 3a–c). The GFP localization experiments together with the identified N^{α}-terminally acetylated amino acids explain the localization of at least three OAS-TL species in *Physcomitrella*. Ala^{34}-PpOAS-TL 1 is localized in the cytosol, whereas Ala^{67}-PpOAS-TL 1 and Ala^{68}-PpOAS-TL 2 are localized in plastids. However, the SAP experiment suggested the presence of five protein species (Fig. 2a), indicating that not all N termini were identified so far. It is therefore reasonable that, despite the tested methionines, additional methionine residues encoded by the PpOAS-TL 1 and PpOAS-TL 2 genes are used as translation initiation site in *Physcomitrella*. Additionally, posttranslational modifications could contribute to the generation of different protein species.

These observations so far show no indications for mitochondrial OAS-TL isoforms. To further analyze whether there is a mitochondrial OAS-TL in *Physcomitrella*, intact and highly pure mitochondria were isolated from *Physcomitrella* protonemata according to Lang et al. (2011). A photometric OAS-TL activity test according to Heeg et al. (2008) did not reveal any detectable OAS-TL activity for soluble proteins from purified mitochondria. Applicability of the OAS-TL activity test for *Physcomitrella* protein was proven using a soluble protein extract isolated from 100 mg of protonemata. The specific OAS-TL activity was determined to be 266±56 nmol min^{-1} mg^{-1} protein, which is in

the range of the activity found for *Arabidopsis* (Heeg et al. 2008). Additionally, no OAS-TL protein could be detected in mitochondrial fractions using an antibody against AtOAS-TL C, which also detects other *Arabidopsis* (Heeg et al. 2008) and *Physcomitrella* OAS-TLs (Fig. 4). However, two OAS-TL species could be detected in the soluble protein extract isolated from protonemata which correspond to protein species 1 and 5 found in the SAP experiment as verified by direct comparison of the signals using the protonemata soluble proteins and the purified OAS-TL proteins for immunological detection (Fig. 4, lane 2 and 7, respectively). In contrast, when soluble proteins of purified chloroplasts were used for immunological detection of OAS-TL proteins, only protein species 5 but not protein species 1 was detected (Fig. 4, lane 3). This confirms that Ala^{67}-OAS-TL 1 and Ala^{68}-OAS-TL 2 are indeed localized in the plastids. Importantly, protein species 5 is at least ten times more abundant than protein species 1 according to densitometric measurements, implicating that OAS-TL activity in *Physcomitrella* is almost exclusively localized to plastids. It can be concluded that cysteine synthesis in the moss *Physcomitrella* differs from cysteine synthesis in *Arabidopsis* as representative of vascular plants with regard to localization of different OAS-TL isoforms. In *Arabidopsis*, the bulk of cysteine is produced in the cytosol, although there is also a plastidic and a mitochondrial OAS-TL isoform (Heeg et al. 2008; Hell and Wirtz 2011). In *Physcomitrella*, not only the reducing phase of ASRP is localized in plastids (Wiedemann et al. 2010; Kopriva et al. 2007) but also cysteine synthesis predominantly occurs in this compartment. A cytosolic OAS-TL is present but of minor importance regarding total OAS-TL protein amount in the cell. Remarkably, in *Arabidopsis* the compartment-specific OAS-TL isoforms are encoded by different genes, whereas *Physcomitrella* OAS-TL 1 is dual targeted. As a result PpOAS-TL 1 in plastids and cytosol differs only by the length of the N terminus. Additionally, there is no specific function of mitochondria regarding cysteine synthesis in *Physcomitrella*, since the respective enzymatic activity seems to be absent in this compartment. However, cysteine is needed in mitochondria for protein translation and iron-sulfur cluster biosynthesis. This implies that cysteine needs to be imported into *Physcomitrella* mitochondria. The responsible cysteine importer system is not identified yet (Linka and Weber 2010).

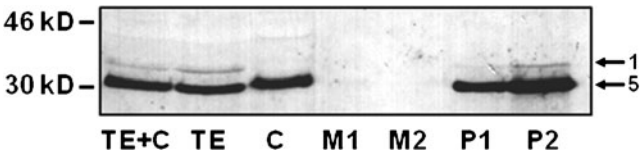

Fig. 4 Immunological detection of OAS-TL proteins in total extract and isolated organelles from *Physcomitrella* Chloroplasts (*C*) and mitochondria (subpopulation M1+M2) were isolated from *Physcomitrella* protonemata according to Lang et al. (2011) and soluble proteins were extracted using 50 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA. Mitochondria isolated in this way are essentially free of contamination by other compartments (Lang et al. 2011). Total extract (*TE*) was obtained by extraction of soluble proteins from 100 mg protonemata by ultrasound sonification for 5 min on ice using the same extraction buffer. Forty microliters of total extract and organellar extracts were used for SDS-PAGE and immunological detection of OAS-TL proteins using an antibody raised against AtOAS-TL C according to Heeg et al. (2008). To verify that the shift for the OAS-TL signal detected in the chloroplast extract results from contamination of the sample by thylakoid membrane lipids, a 1:1 mixture of total extract and chloroplast extract was used for immunological detection (TE+C). Additionally, 0.2 and 1 μg of the purified OAS-TL protein from the SAT-affinity purification experiment were used for immunological detection (P1 and P2, respectively). Position of protein species 1 and 5 were marked by *arrows*

Interestingly, one of the putative SAT-like proteins identified by a homology search using AtSAT3 and AtSAT5 as bait, Phypa220060 (Pp1s187_23V6.2), seems to be localized in mitochondria according to target signal prediction (Supplemental Table 4). The protein shows the highest similarity to AtSAT2 and AtSAT4 (43 and 49 %, respectively) and displays a significantly different C terminus compared with AtSAT1, AtSAT3, and AtSAT5 (Supplemental Fig. 4). The SAT C terminus, however, is responsible for interaction with OAS-TL, a major characteristic of true SAT proteins. AtSAT2 and AtSAT4 probably possess only little in vivo SAT activity and seem to function predominantly during specific stress conditions (Kawashima et al. 2005). The remaining three PpSAT-like proteins, Phypa169562 (Pp1s212_107V6.1), Phypa169849 (Pp1s222_99V6.1), and Phypa153861 (Pp1s403_26V6.1) exhibit highest similarity to the three true AtSAT proteins AtSAT1, AtSAT3, and AtSAT5 (50–66 %) and share the conserved C terminus (Supplemental Fig. 4). CSC formation between one of the PpOAS-TLs and these PpSATs is therefore very likely. Prediction of localization for Phypa169562 is not conclusive (Supplementary Table 4). Phypa169849 and Phypa153861, on the other hand, are predicted to be localized in chloroplasts. However, as for the PpOAS-TLs, several methionine residues following the initial Met^1 suggest dual targeting (Supplemental Fig. 4). To ultimately locate the PpSAT proteins, localization studies are necessary.

Concluding remarks

The presence of compartment-specific SAT and OAS-TL isoforms in the vascular plant *A. thaliana* allows spatial separation of OAS, sulfide, and cysteine synthesis, which contributes to regulation of cysteine homeostasis of the cell. In contrast, in the unicellular alga *C. reinhardtii* reduction of sulfate to sulfide and cysteine synthesis are exclusively restricted to chloroplasts. The predominant role of chloroplasts for cysteine synthesis is still realized in the moss *P. patens* but, like in vascular plants, cysteine can also be

synthesized in the cytosol. However, mitochondrial cysteine synthesis seems not to be present, which is different from vascular plants. Thus, *Physcomitrella* exhibits features from both algae and vascular plants regarding cysteine synthesis. Compartmentation of cysteine synthesis is already initialized but localization of sulfur reduction and cysteine synthesis in one compartment is still favored.

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