

Functional investigation of two *1-aminocyclopropane-1-carboxylate (ACC) synthase-like* genes in the moss *Physcomitrella patens*

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Abstract

Key message Two ACC synthase-like (ACL) proteins in the moss *Physcomitrella patens* have no ACS activity, and PpACL1 functions as an L-cystine/L-cysteine C-S lyase.

Abstract The ethylene biosynthetic pathway has been well characterized in higher plants, and homologs of a key enzyme in this pathway, ACS, have been reported in several algae and mosses, including *Physcomitrella patens*. However, the function of the ACS homologs in *P. patens* has not been investigated. In this research, we cloned two putative ACS genes from the *P. patens* genome, namely *PpACS-Like 1* and *2*, and investigated whether their encoded proteins had in vitro and in vivo ACS activity. In vitro biochemical assays using purified PpACL1 and PpACL2 showed that neither protein had ACS activity.

Subsequently, we generated transgenic *Arabidopsis* lines expressing *35S:PpACL1* and *35S:PpACL2*, and found that the transgenic etiolated seedlings that overexpressed either of these proteins lacked the constitutive triple response phenotype and did not emit excess levels of ethylene, indicating that neither of the PpACS-Like proteins had in vivo ACS activity. Furthermore, we found that PpACL1 functions as a C-S lyase that uses L-cystine and L-cysteine as substrates, rather than as an aminotransferase. Together, these results indicated that *PpACL1* and *PpACL2* are not true ACS genes as those found in higher plants.

Keywords Ethylene · *Physcomitrella patens* · ACS · *PpACS-Like* · C-S lyase

Abbreviations

ACS	1-aminocyclopropane-1-carboxylate synthase
C-S lyase	Carbon–sulfur lyase
SAM	S-adenosyl-L-methionine
ACC	1-aminocyclopropane-1-carboxylate
PLP	Pyridoxal-5'-phosphate
AATase	Aspartate aminotransferase

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Introduction

Gaseous ethylene regulates many aspects of growth and development throughout the life cycle of flowering plants, including seed germination, cell elongation, root initiation, flower development, sexual development, fruit ripening, senescence, and responses to biotic and abiotic stresses (Abeles et al. 1992; Argueso et al. 2007; Bleecker et al. 1988; Lin et al. 2009). The rate-limiting step of ethylene

synthesis in flowering plants is catalyzed by 1-aminocyclopropane-1-carboxylate synthase (ACS). Elimination of the entire ACS gene family in *Arabidopsis* resulted in embryonic lethality, indicating that ethylene is essential for *Arabidopsis* viability (Tsuchisaka et al. 2009).

The number of sequenced lower plant genomes has increased extensively in the past few years, providing opportunities to probe the evolutionary origins of biosynthetic and signaling pathways of plant hormones (Rensing et al. 2008; Banks et al. 2011; Ju et al. 2015; Ross and Reid 2010). The ethylene biosynthetic pathway, which includes three key enzymatic reactions, has been well-documented in higher plants. First, methionine is converted to *S*-adenosyl-L-methionine (SAM) by SAM synthase. Then, SAM is converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACS, and finally ethylene is formed from ACC in a reaction catalyzed by ACC oxidase (ACO; Kende 1993; Lin et al. 2009; Adams and Yang 1979). Homologs of the two key enzymes, ACS and ACO, have been reported in some algae and mosses (Ju et al. 2015; Rensing et al. 2008). However, the function of these homologs is unclear. So far, no functionally characterized ACC synthase genes have been reported in gymnosperms except for in two species of *Coniferales* (Barnes et al. 2008; Ralph et al. 2007). The ACO genes are notably absent in the genomes of the early-diverging plant lineages *Physcomitrella patens* and *Selaginella moellendorffii* (Banks et al. 2011; Kawai et al. 2014; Rensing et al. 2008); however, two putative ACS genes were identified in the genome of *P. patens* (Rensing et al. 2008). A homolog of higher plant ethylene receptor genes has been reported in cyanobacteria (Bleecker 1999; Ju et al. 2015) and fresh water algal species (Gallie 2015). Further characterization of these homologs is needed to determine their enzymatic and metabolic roles in lower plants.

Physiological studies in lower plants did not provide a consistent mechanistic view of the ethylene biosynthesis pathway in non-seed plants. There have been several reports that the lower plants, including the chlorophytes green algae *Haematococcus pluvialis* and unicellular alga *Acetabularia* (Maillard et al. 1993; Driessche et al. 1988), charophyte *Spirogyra pratensis* (Ju et al. 2015), moss *Funaria hygrometrica* (Rohwer and Bopp 1985), and ferns *Pteridium* (gametophytes), *Mateuccia* and *Polystichum* (sporophytes; Tittle 1987), convert ACC to ethylene via the same pathway as in higher plants. By contrast, other studies concluded that lower plants, including the liverwort *Riella helicophylla* (Stange and Osborne 1989) and ferns *Regnellidium diphyllum* and *Marsilea quadrifolia* (Chernys and Kende 1996), produce ethylene via an unknown biosynthetic pathway. The most detailed study was performed by Osborne et al. (1996), and they found that representatives of the major groups of lower plants,

including liverworts, mosses (*Sphagnum cuspidatum* and *Polytrichum commune*) and ferns produced ethylene at detectable levels, but that they were unable to convert exogenously added ACC into ethylene. Similar findings were reported for bryophytes and pteridophytes (Chernys and Kende 1996; Cookson and Osborne 1978; Kwa et al. 1995).

Bryophytes, which are non-vascular plants, are the closest extant relatives of early land plants, and the two lineages began to diverge about 450 million years ago (Rensing et al. 2008). *P. patens*, a species of the *Funariaceae*, is the first bryophyte whose genome was fully sequenced and has emerged as a model plant (Cove et al. 2009; Rensing et al. 2008) that is ideal for studying the evolution of mechanisms underlying the complexity of modern plants (Liu et al. 2013). Two putative ACS genes have been identified in *P. patens*, as have seven putative ethylene receptors with amino acid sequence similarity to angiosperm ethylene receptors (Ishida et al. 2010). In addition, the ethylene receptors in *P. patens* were found to have ethylene-binding activity comparable to that of ethylene receptors in other land plants (Wang et al. 2006), and *P. patens* was found to exhibit a characteristic response to exogenous ethylene (Yasumura et al. 2012). However, it is unknown whether one or both of the putative ACS genes in the *P. patens* genome encode true ACS enzymes, and establishing this would provide insight into the evolution of the ethylene biosynthetic pathway.

In this study, we cloned both *PpACS-Like* genes in *P. patens* and investigated whether the two encoded PpACL proteins have ACS activity. We found that neither of the PpACLs had in vitro or in vivo ACS activity. Additionally, we found that PpACL1 functions as a C-S lyase with L-cysteine and L-cysteine as substrates. Together, our findings indicate that neither of the putative ACS genes is true ACS gene, as those found in higher plants, and also that *P. patens* may synthesize ethylene via an unknown biosynthetic pathway.

Materials and methods

Plant materials and growth conditions

Physcomitrella patens (kindly provided by Professor Hongwei Guo of Peking University) was cultured in liquid BCD medium [0.1 mM MgSO₄, 10 mM KNO₃, 45 μM FeSO₄, 1.84 mM KH₂PO₄ (pH 6.5 adjusted with KOH), a trace element solution (alternative Tes: 0.22 μM CuSO₄, 0.19 μM ZnSO₄, 10 μM H₃BO₃, 0.10 μM Na₂MoO₄, 2 μM MnCl₂, 0.23 μM CoCl₂, and 0.17 μM KI) and 1 mM CaCl₂] in a plant growth chamber [22/19 °C, 16/8 h

light/dark, with a photosynthetic photon flux density (PPFD) of $90 \mu\text{E m}^{-2} \text{s}^{-1}$].

Wild-type *Arabidopsis thaliana* (ecotype Columbia-0) was obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH, USA). Seeds were surface-sterilized in 10 % (v/v) sodium hypochlorite for 2 min, washed 10 times with sterilized water, sown on a plate of half-strength Murashige and Skoog (1/2 MS) medium [0.8 % (w/v) agar, pH 5.7, 1 % (w/v) sucrose], stratified at 4 °C for 2 days in darkness, germinated and grown in the plant growth chamber under the same conditions described above.

Construction and RT-PCR analysis

Total RNA isolated from the leafy tissues of *P. patens* cultured in BCD medium was used to synthesize cDNA. *PpACL1* was amplified from the synthesized cDNA by PCR using primers vivoTA-ACL1-L and vivoTA-ACL1-R, inserted into *Spe* I and *Bgl* II sites with 5' and 3' ends, respectively, and *PpACL2* was amplified by PCR using primers PpACL2-L and PpACL2-R, inserted into *Bgl* II and *BstE* II sites with 5' and 3' ends, respectively. Both constructs were cloned into pMD18-T simple vector, respectively to generate *vivo TA-ACL1* and *vivo TA-ACL2*. *Vivo TA-ACL2* was then fused with the *eGFP* reporter gene. Plasmids harboring *vivo TA-ACL1*, *vivo TA-ACL2-eGFP* were then digested with *Spe* I and *Bgl* II, *Bgl* II, and *BstE* II, respectively, and ligated together into modified pCAMBIA3301 digested with the same pairs of enzymes to generate recombinant plasmids harboring *35S:ACL1* and *35S:ACL2-eGFP*. The recombinant plasmids *35S:ACL1* and *35S:ACL2-eGFP* were sequenced and then transformed into *Agrobacterium tumefaciens* strain GV3101 and transgenic *Arabidopsis* plants were obtained by floral dip transformation method (Clough and Bent 1998).

RNA extraction and cDNA synthesis were performed as described previously (Liu et al. 2010). For semi-quantitative RT-PCR analysis, gene-specific primers RT-ACL1L and RT-ACL1R for *35S:ACL1* and PpACL2-1-rt and PpACL2-R for *35S:ACL2-eGFP* were used to amplify the *35S:ACL1* and *35S:ACL2-eGFP* transcripts, respectively. As an internal control, a fragment of TIP41-like, a recommended quantitative RT-PCR standard (Czechowski et al. 2005; Udvardi et al. 2008), was amplified under the same conditions with the primers rtTIP-F and rtTIP-R. The sequences of all primers used are listed in Supplementary Table 1 online.

Expression and purification of PpACL proteins

PpACL1 was amplified from the plasmid *vivoTA-ACL1* by PCR using primers vivoTA-ACL1-L and vitroTA-ACL1-

R28a for His-ACL1, vivoTA-ACL1-L and vitroTA-ACL1-R22b for ACL1-His, inserted into *Bam*H I and *Not* I sites with 5' and 3' ends, respectively, and cloned into pMD18-T simple vector to generate *vitro TA-ACL1-R28a* and *vitro TA-ACL1-22b*. Plasmids of *vitro TA-ACL1-R28a* and *pET28a*, *vitro TA-ACL1-22b* and *pET22b* were then digested with *Bam*H I and *Not* I restriction enzymes and ligated together to generate recombinant plasmid *T7:His-ACL1* and *T7:ACL1-His*. *PpACL2* was amplified from the plasmid *TA-ACL2* by PCR using primers vitroTA-ACL2-L and vitroTA-ACL2-R28a, inserted into *Sac* I and *Hind* III sites with 5' and 3' ends, respectively, and cloned into pMD18-T simple vector to generate *vitro TA-ACL2-28a*. Plasmids of *vitro TA-ACL2-28a* and *pET28a* were then digested with *Bam*H I and *Not* I restriction enzymes and ligated together to generate recombinant plasmid *T7:His-ACL2*. The primers used are listed in Supplementary Table 1 online.

The recombinant plasmids *T7:His-ACL1*, *T7:ACL1-His* and *T7:His-ACL2* were sequenced and then transformed into expression host *Escherichia coli* BL21 [RosettaTM2 (DE3) plysS]. The ACL1-His, His-ACL1 and His-ACL2 fusion proteins were expressed in BL21, induced with 0.5 mM IPTG for 3 h, and purified by affinity chromatography using a His-Trap FF column (GE Healthcare) according to the manufacturer's recommendations.

In vitro ACS activity assay

ACS activity was assayed using purified recombinant ACL1-His/His-ACL1 and His-ACL2 fusion proteins as described previously (Li and Mattoo 1994) with the following modifications: 50–100 μg of the purified protein was placed into 16 ml GC vials containing 475 μl ACS assay buffer [50 mM EPPS (Sigma), pH 8.5, 10 μM PLP (Sigma), 2 mM DTT] and 20 μl of 10 mM SAM (Sigma). The mixture (total volume, 0.5 ml) was incubated at 30 °C for 30 min, and the reaction was terminated with 10 μl 100 mM HgCl_2 . The ACC formed was converted into ethylene with the addition of ten drops of fresh cold mixture of saturated NaOH and bleach (NaClO ; 2:1) by 10 ml syringe. The vials were capped immediately after addition of NaOH:bleach and incubated on ice for 5 min. The ethylene formed was measured using a gas chromatograph (Agilent 7890A) fitted with a GS-Alumina column (50 m \times 0.53 mm ID, J and W Scientific) and a flame ionization detector. Five-hundred microliters of headspace from each sample were injected onto the column. The ethylene peaks were quantified using an Open LAB CDS Chem Station Workstation (Agilent). The enzyme activity of the purified protein was calculated as the amount of ACC converted from SAM per 30 min and per microgram protein based on an ACC standard curve. All experiments

were conducted at least in three replicates, and each experiment was repeated at least twice with comparable results.

Aminotransferase activity assay

Aminotransferase activity was assayed as follows: a reaction mixture of 1200 μ l in total volume was prepared, which contained 0.1 M potassium phosphate buffer, pH 7.8; 10 mM α -ketoglutarate, 12.5 mM pyruvic acid or 5 mM glyoxylic acid as an amino receptor; 20 different natural amino acids (Sigma) as possible amino donors, including 0.3 μ M L-alanine, 0.25 μ M L-arginine, 0.6 μ M L-asparagine, 0.3 μ M L-aspartic acid, 0.5 μ M L-glutamine, 0.3 μ M L-glycine, 0.4 μ M L-histidine, 0.5 μ M L-isoleucine, 0.4 μ M L-leucine, 0.25 μ M L-lysine, 0.4 μ M L-methionine, 0.25 μ M L-phenylalanine, 0.4 μ M L-proline, 0.4 μ M L-serine, 0.5 μ M L-threonine, 0.3 μ M L-tryptophan, 0.2 μ M L-tyrosine, 0.3 μ M L-valine, 3 μ M L-cysteine, and 0.3 μ M L-glutamic acid separately; 10 mM PLP; and 2 mM DTT. Then, a sample of 50–100 μ g purified PpACL1 protein was added, but ddH₂O for the control. The mixed solution was incubated at 30 °C for 30 min, and then 200 μ l chloroform was added to denature and remove the protein. A sample of 1000 μ l supernatant was recovered after centrifugation (4 °C, 12,000 rpm, 10 min), purified by suction filtration (Luer syringe filter, PES 0.22 μ m; syringe, 2.5 ml), and subjected to determine amino acid contents by an amino acid analyzer (A300, MembraPure GmbH).

C-S lyase activity assays

C-S lyase activity was assayed as follows: a reaction mixture of 1200 μ l in total volume was prepared, which contained 0.1 M potassium phosphate buffer, pH 7.8; one of following amino acids as a substrate (L-cystine, L-cysteine, L-cystathionine at 4 mM, or S-methyl-L-cysteine at 14 mM); 10 mM PLP; and 2 mM DTT. Then, a sample of 50–100 μ g purified PpACL1 protein was added, but ddH₂O for the control. The mixed solution was incubated at 30 °C for 30 min, and then 200 μ l chloroform was added to denature and remove proteins. Amino acid contents in the reaction mixture were determined as described above. The content of produced pyruvic acid was determined as follows: a reaction mixture of 300 μ l in total volume was prepared, which contained 0.1 M potassium phosphate buffer, pH 7.8; 4 mM L-cystine or 4 mM L-cysteine (the substrates); 10 mM PLP and 2 mM DTT. A sample of 50–100 μ g purified PpACL1 protein was added, but an equivalent amount of protein inactivated by heating at 100 °C for 10 min for the control. The mixed solution was kept in a shaker incubator for 30 min at 30 °C, and 200 μ l chloroform was then added to denature and remove

proteins. A sample of 200 μ l supernatant was recovered after centrifugation (4 °C, 12000 rpm, 10 min). H₂O (200 μ l) and 2,4-dinitrophenylhydrazine [0.1 % (w/v) in 2 M HCl, 400 μ l] were added to the supernatant, then the solution was mixed and left at room temperature for 10 min. Then, 2 ml of 1.5 M NaOH was added, and the reaction was left at room temperature for 10 min. Absorbance at 520 nm was determined using a spectrophotometer. The content of produced pyruvic acid was calibrated using a standard curve.

Ethylene measurements

Ethylene emissions from 3-day-old etiolated *Arabidopsis* seedlings of wild type plant and transgenic plants transformed with *35S:PpACL1* and *35S:PpACL2* were measured by gas chromatography (Agilent 6890 N) as described by Li and Mattoo (1994). The gametophores of *P. patens* were incubated in 12-ml vials with 5 ml liquid BCD media with or without 100 μ M ACC and 50 μ M AVG. The vials were sealed with caps and left in a plant growth chamber for 24 h (22/19 °C, 16/8 h light/dark). Nine-day-old wild type *Arabidopsis* seedlings grown under the same conditions with *P. patens* were used as the positive control. Ethylene accumulated in the vials was measured by gas chromatography. All treatments were performed in triplicate. The ethylene measured in each vial was then divided by the fresh weight and the rate of ethylene production was expressed as nl g⁻¹ FW 24 h⁻¹.

Results

Two ACS-like genes in the moss *P. patens* belong to AAT-like family

Two putative ACS genes, *PpACS-like1* and 2 were previously identified in the genome of *P. patens* by sequence alignment analysis (Rensing et al. 2008). Now, we conducted an analysis using the NCBI Conserved Domains Database and showed that both the PpACLs contain an AAT-like domain, which suggests that they belong to AAT-like family. The AAT-like family belongs to the pyridoxal phosphate (PLP)-dependent enzyme superfamily, which includes ACS (ACC synthase), AspAT (aspartate aminotransferase), TyrAT (tyrosine aminotransferase), AroAT (aromatic amino acid aminotransferase), and C-S lyase (carbon–sulfur lyase). All these proteins contain an AAT-like domain and share a high degree of amino acid sequence similarity (Alexander et al. 1994).

We then performed a phylogenetic analysis of the two PpACLs and ACS, AspAT, TyrAT and C-S lyase members of the AAT-like family from *A. thaliana*, *Solanum*

lycopersicum, *Oryza sativa*, *Nicotiana tabacum*, *Solanum tuberosum*, *Phaseolus vulgaris*, *Medicago sativa*, *Panicum miliaceum*, *Glycine max*, *Medicago truncatula*, *Zea mays*, *Papaver somniferum*, *Salvia miltiorrhiza*, *Solenostemon scutellarioides*, *Taraxacum brevicorniculatum*, and *Brassica oleracea* (L.) using the neighbor-joining method implemented in Clustal-X2 and MEGA 4.1. BLAST searches and sequence alignment indicated that the amino acid sequences of PpACL1 and PpACL2 showed 25–41 % identity with those of ACS, AspAT, TyrAT and C-S lyase in the above-mentioned species. Phylogenetic analysis showed that neither of the PpACLs belonged to the ACS family (Fig. 1). However, PpACL1 is evolutionarily close to C-S lyase, and PpACL2 to the aminotransferases AtACS10 and AtACS12. The functions of proteins with similar origins tend to be conserved (Rorick and Wagner 2009; Sleator 2014). Thus, phylogenetic analysis suggests that PpACLs may have similar functions with ACS or other AAT-like family members.

PpACL1 has no in vivo or in vitro ACC synthase activity

To investigate the biochemical characteristics of PpACL1, we examined if PpACL1 has the in vitro and in vivo activity of ACS, AspAT, TyrAT, or C-S lyase.

ACC synthase catalyzes the conversion of SAM to ACC in the ethylene biosynthetic pathway in higher plants (Wang et al. 2002). To test whether PpACL1 has ACS activity, we first performed an in vitro experiment. We generated a plasmid encoding PpACL1 protein fused to a His-Tag, expressed this plasmid in *E. coli* and purified the tagged PpACL1. We examined whether the purified PpACL1 protein could convert SAM to ACC by testing for the ethylene production. AtACS7, a confirmed ACS in *Arabidopsis*, was used as a positive control. We found that purified PpACL1 was unable to convert SAM to ACC (Fig. 2), suggesting that PpACL1 has no ACS activity in vitro.

To further investigate whether PpACL1 has ACS activity in vivo, we transgenically expressed *35S:PpACL1* in *Arabidopsis*. The etiolated *Arabidopsis* seedlings overexpressing *PpACL1* did not exhibit a constitutive triple response and had no difference with their wild type control in hypocotyls and roots (Fig. 3a). In addition, the etiolated *Arabidopsis* seedlings overexpressing *PpACL1* did not produce excess amounts of ethylene (Fig. 3b). Taken together, these in vitro and in vivo results indicate that PpACL1 has no ACS activity.

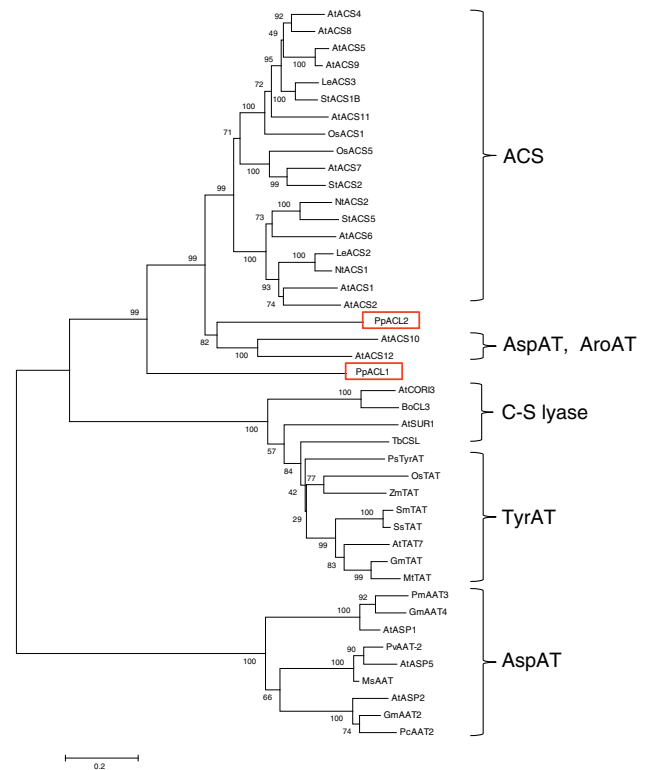


Fig. 1 Phylogenetic relationships between PpACLs and other AAT-like proteins. The phylogenetic tree was constructed by use of Clustal-X2 and MEGA 4.1 software with the neighbor-joining method based on an amino acid sequence alignment. Sequence accession number, ACS-like: *Physcomitrella patens* (PpACL1, Pp1s376_33V6.1; PpACL2, Pp1s235_83V6.1), ACS: *Arabidopsis thaliana* (AtACS1, NP_191710.1; AtACS2, NP_171655.1; AtACS4, NP_179866.1; AtACS5, NP_201381.1; AtACS6, NP_192867.1; AtACS7, NP_194350.1; AtACS8, NP_195491.1; AtACS9, NP_190539.1; AtACS10, NP_564804.1; AtACS11, NP_567330.1; AtACS12, NP_199982.2), *S. lycopersicum* (LeACS2, CAA41855.1; LeACS3, AAB48945.1), *O. sativa* (OsACS1, NP_001051142.1; OsACS5, CAA65776.1), *N. tabacum* (NtACS1, Q07262.1; NtACS2, CAA06288.1), *S. tuberosum* (StACS1B, CAA81748.1; StACS2, CAA81749.1; StACS5, AAB96880.1), C-S lyase: *T. brevicorniculatum* (TbCSL, doi:10.1016/j.jplph.2012.08.018), *B. oleracea* (L.) (BoCL3, AAO27362.1), *Arabidopsis thaliana* (AtCOR13, NP_194091.1; AtSUR1, NP_179650.1), AspAT: *Arabidopsis thaliana* (AtASP1, NP_180654.1; AtASP2, NP_197456.1; AtASP5, NP_001031767.1), *P. vulgaris* (PvAAT-2, AAN76499.1), *P. miliaceum* (PcAAT2, CAA45023.1), *M. sativa* (MsAAT, 1908424A), *P. miliaceum* (PmAAT3, CAA45024.1), *G. max* (GmAAT2, AAC50015.1; GmAAT4 AAA98603.1), TyrAT: *Arabidopsis thaliana* (AtTAT7, NP_200208.1), *G. max* (GmTAT, NP_001238408.1), *M. truncatula* (MtTAT, AAY85183.1), *O. sativa* (OsTAT, NP_001046628.1), *Z. mays* (ZmTAT, NP_001145701.1), *P. somniferum* (PsTyrAT, ADC33123.1), *S. miltiorrhiza* (SmTAT, ABC60050.1), *S. scutellarioides* (SsTAT, CAD30341.1). The number presented in the branch indicates the bootstrap values supporting the evolution. The scale bar indicates amino acid substitutions per site

Fig. 2 Measurement of in vitro ACS activity of PpACL1.

a Purified His-ACL1 fusion proteins were used in the ACS activity. **b** Purified ACL1-His fusion proteins were used in the ACS activity. His-tagged ACS7 fusion protein was used as the positive control, empty vector pET28a or pET22b as the negative control. The results presented show average values of three independent experiments ($n = 9$). Error bars show standard error

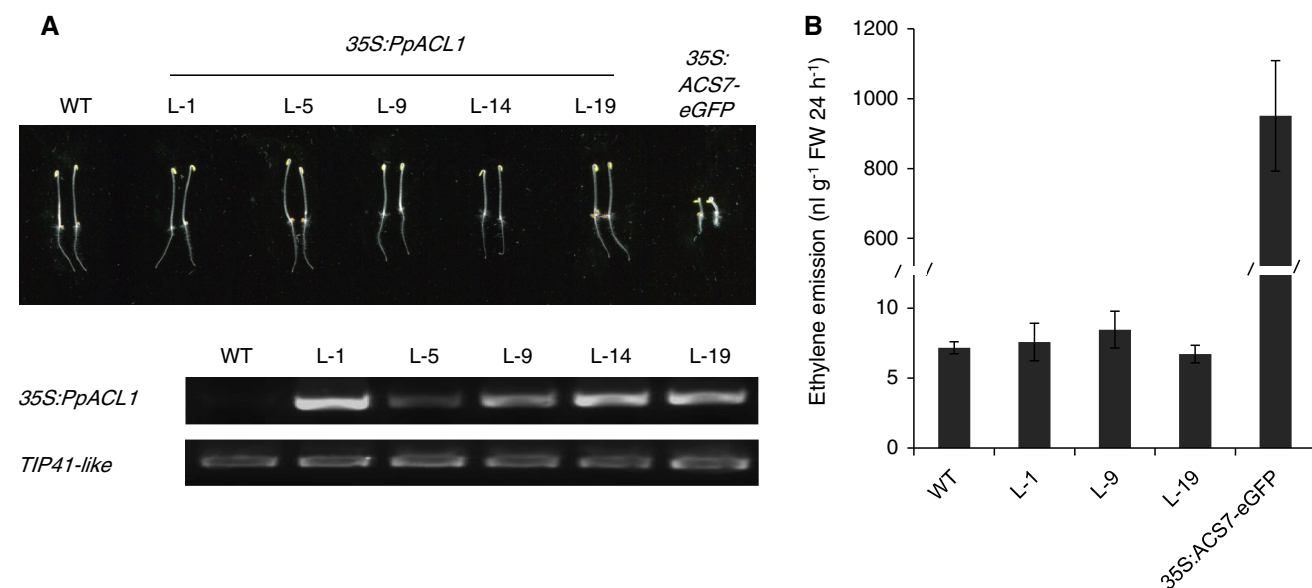
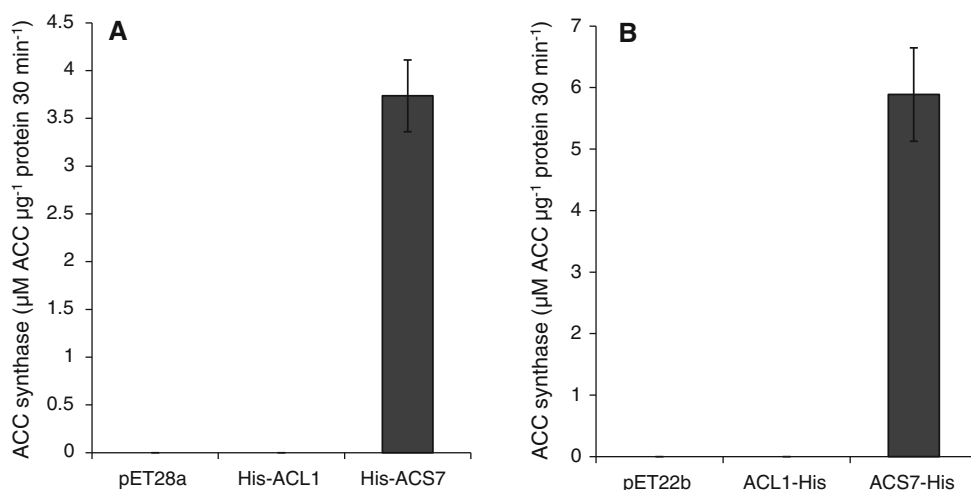


Fig. 3 Phenotypes of and ethylene emission from the etiolated *Arabidopsis* seedlings overexpressing 35S:PpACL1. **a** Phenotypes of the 3-day-old 35S:PpACL1 etiolated seedlings. The transcript level of PpACL1 accumulated in the 35S:PpACL1 transgenic *Arabidopsis* was determined by semi-quantitative RT-PCR. Total RNA was isolated

from the 7th rosette leaf of 33-day-old 35S:PpACL1 transgenic *Arabidopsis* plants. **b** Ethylene emission from the 3-day-old 35S:PpACL1 etiolated seedlings. Error bars show standard error ($n = 9$)

PpACL1 does not function as an aminotransferase

ACC synthase is probably evolutionarily related to subgroup I aminotransferases, another member of the AAT-like family, which includes aspartate, alanine, histidinol-phosphate, tyrosine and phenylalanine aminotransferases (Feng et al. 2000; Jakubowicz 2002). Having established that PpACL1 has no ACS activity, we next investigated whether it has aminotransferase activity.

To examine the aminotransferase activity of PpACL1, α -ketoglutarate was used as an amino receptor and 18 different natural amino acids as possible amino donors, including L-alanine, L-arginine, L-asparagine, L-aspartic

acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. A His fusion of AtASP2, which has AspAT activity, was used as the positive control. Aminotransferase reactions catalyzed by AspAT are expected to generate L-glutamic acid from α -ketoglutarate (Ward et al. 2002). The addition of purified PpACL1 did not result in a significant reduction in the contents of any of the amino acids used as amino donor or in the production of glutamic acid, whereas addition of the well characterized AspAT AtASP2 did (Fig. 4; see Supplementary Fig. 1 online), demonstrating that PpACL1 has no aminotransferase activity.

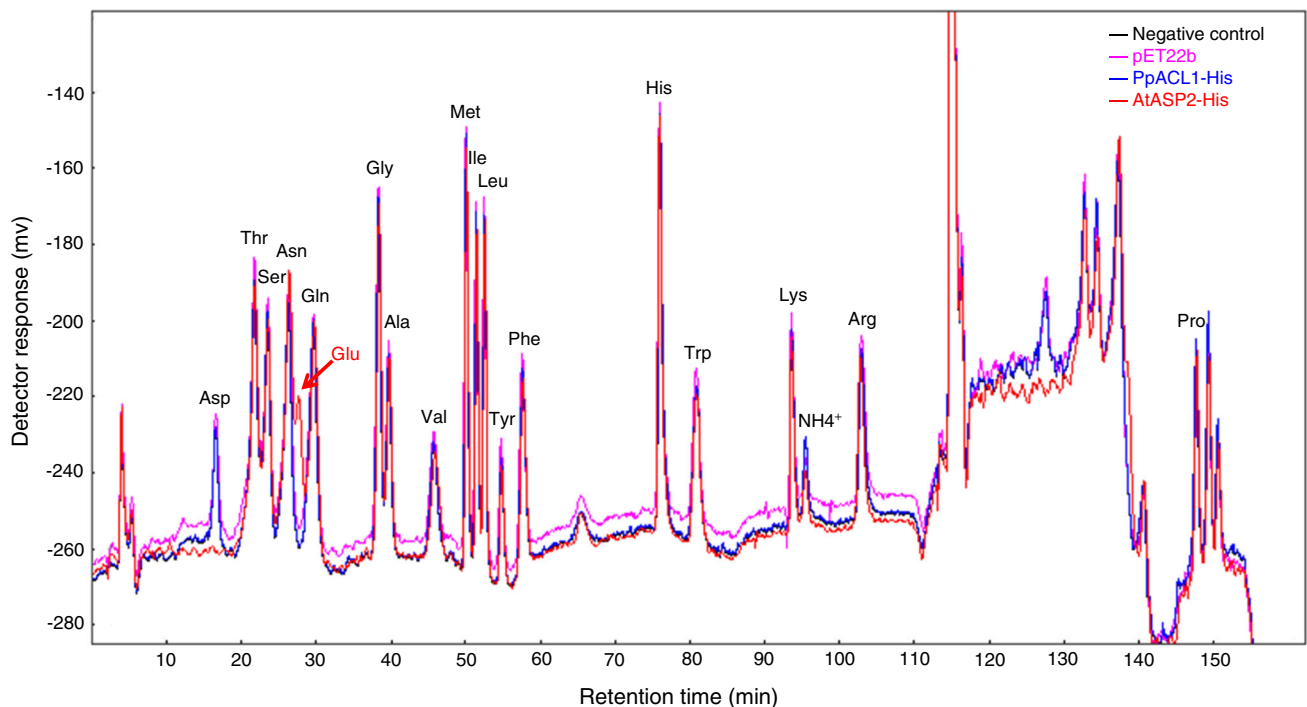


Fig. 4 Chromatogram of amino acids after transamination reactions catalyzed by PpACL1. α -Ketoglutarate was used as an amino acceptor in the reaction, 18 different natural amino acids were used as amino donors. Control: AtASP2 purified protein was used as the

positive control, empty vector pET22b was used as a negative control, and H₂O was also used as negative control instead of purified proteins. L-Glutamic acid was marked with *red arrow*

To further prove that PpACL1 is not an aminotransferase, we then used pyruvic acid and glyoxylic acid as amino receptors, and the same 18 amino acids shown above as amino donors (except that L-alanine was not used in combination with pyruvic acid and L-glycine was not used in combination with glyoxylic acid). Similarly, we found that the addition of purified PpACL1 protein did not significantly reduce the amino acid content or result in the production of L-alanine and L-glycine when pyruvic acid or glyoxylic acid, respectively, was used as an amino receptor (see Supplementary Fig. 2, 3 online). This further supports the conclusion that PpACL1 has no aminotransferase activity.

By contrast, when only cysteine was used as the amino donor and α -ketoglutarate as the amino receptor in the aminotransferase reaction, we found that total cysteine decreased and NH₄⁺ was markedly increased with the addition of purified PpACL1 (Fig. 5). Based on these findings we inferred that PpACL1 may have carbon–sulfur lyase (C-S lyase) activity.

PpACL1 exhibits C-S lyase activity

In the aminotransferase reaction, we found that PpACL1 exhibited features of a C-S lyase. We therefore subjected PpACL1 to C-S lyase activity assays.

C-S lyase catalyzes the cleavage of carbon–sulfur bonds (Munt et al. 2013). To identify the C-S lyase activity of PpACL1, we used L-cysteine, L-cystine, L-cystathionine and S-methyl-L-cysteine as substrates, respectively. As ammonia and pyruvate are common products of the reactions catalyzed by C-S lyase (Yoshida et al. 2002), C-S lyase can be identified by testing for the presence of both products. We tested for generated ammonia using an amino acid analyzer (A300), and found the production of NH₄⁺ with L-cystine and L-cysteine (Fig. 6a, b), but not with L-cystathionine and S-methyl-L-cysteine (Fig. 6c, d), which suggested that PpACL1 catalyzed the cleavage of carbon–sulfur bonds in the former two amino acids. To confirm this result, we tested for generated pyruvate using the 2,4-dinitrobenzene-hydrazine method. We found the reddish brown coloration of 2,4-dinitrobenzene-hydrazone which was generated from pyruvate and 2,4-dinitrobenzene (see Supplementary Fig. 4a, b online), but not in L-cystathionine and S-methyl-L-cysteine (see Supplementary Fig. 4c, d online). In addition, PpACL1 showed higher levels of lyase activity when combined with L-cysteine than with L-cystine (Fig. 7a, b). Based on the above results, we conclude that PpACL1 is a C-S lyase that uses L-cystine and L-cysteine as substrates.

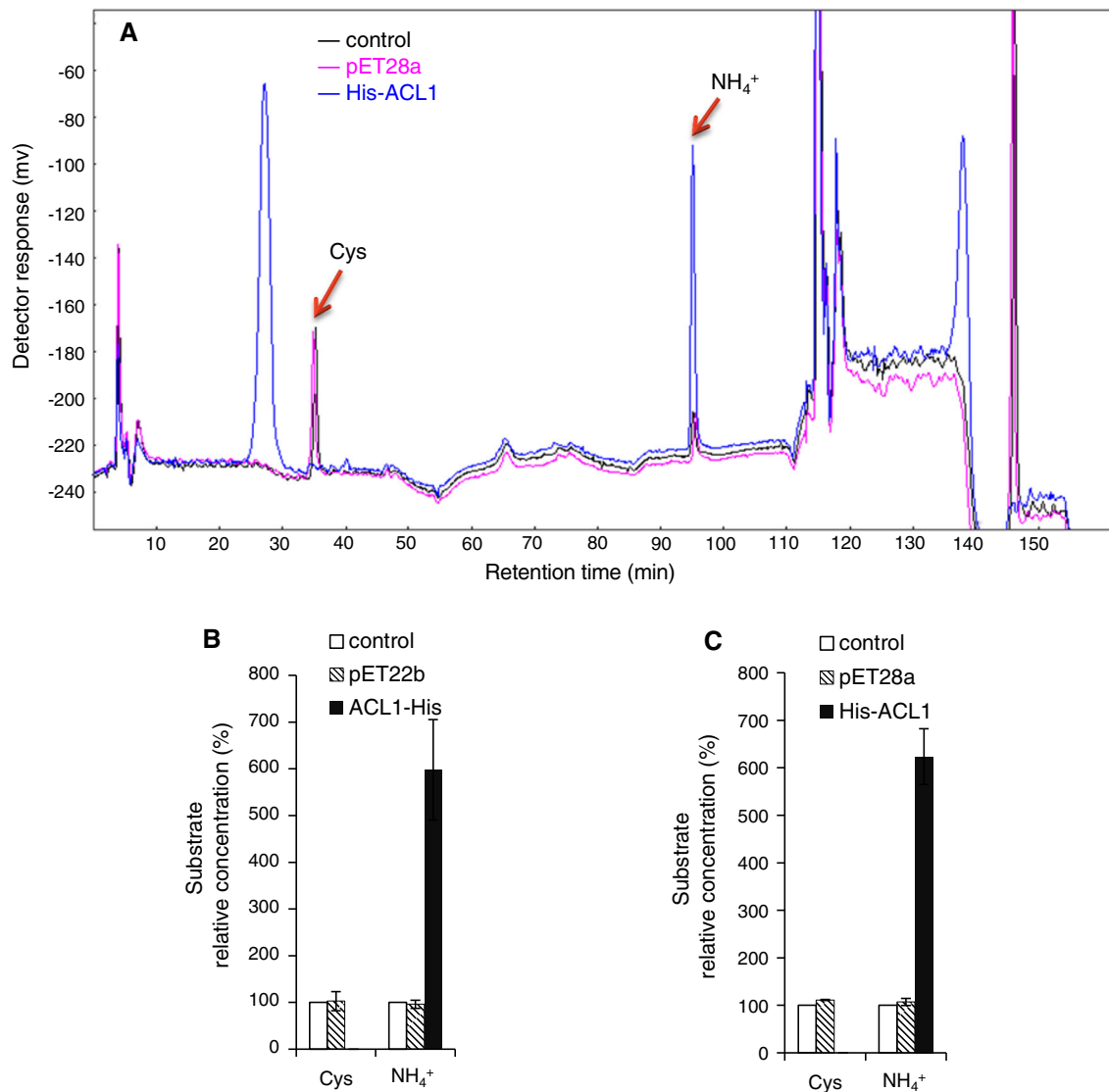


Fig. 5 Chromatogram and relative concentrations of cysteine and ammonium ion after transamination reaction catalyzed by PpACL1. Cysteine was used as the amino donor and glyoxylic acid as the amino acceptor. **a** Chromatogram of cysteine and ammonium ion after transamination reaction. **b, c** Relative concentrations of cysteine and

ammonium ion after transamination reaction catalyzed by ACL1-His or His-ACL1 fusion proteins, respectively. Control: H₂O was added instead of purified proteins. The concentrations of cysteine and ammonium ion were set to 100 in the control

PpACL2 has no in vitro and in vivo ACS activity

We next investigated whether PpACL2 has ACS activity. Similarly, we generated recombinant PpACL2 in *E. coli* and then tested for the ability of the purified PpACL2 protein to generate ACC from SAM. We found that there was no formation of ACC (Fig. 8c), which indicated that PpACL2 has no ACS activity in vitro, either. We next examined phenotypes of the *35S:PpACL2* transgenic etiolated *Arabidopsis* seedlings, and found that these transgenic plants did not exhibit the constitutive triple response (Fig. 8a) and did not emit excess level of ethylene (Fig. 8b). These results suggest that PpACL2 does not have

in vitro and in vivo ACS activity. In addition, we used a mixture of purified PpACL1 and PpACL2 proteins to investigate whether the two proteins functioned as heterodimers, and found that they did not form functional heterodimers with ACS activity in vitro (data not shown).

Additionally, to investigate if PpACL2 functions as a C-S lyase or an aminotransferase, we next performed C-S lyase and aminotransferase activity assays. However, we found that PpACL2 has no C-S lyase activity with the substrates L-cystine (see Supplementary Fig. 5a, b online), L-cysteine (see Supplementary Fig. 5c, d online), L-cystathionine (see Supplementary Fig. 5e online), or S-methyl-L-cysteine (see Supplementary Fig. 5f online). Similarly,

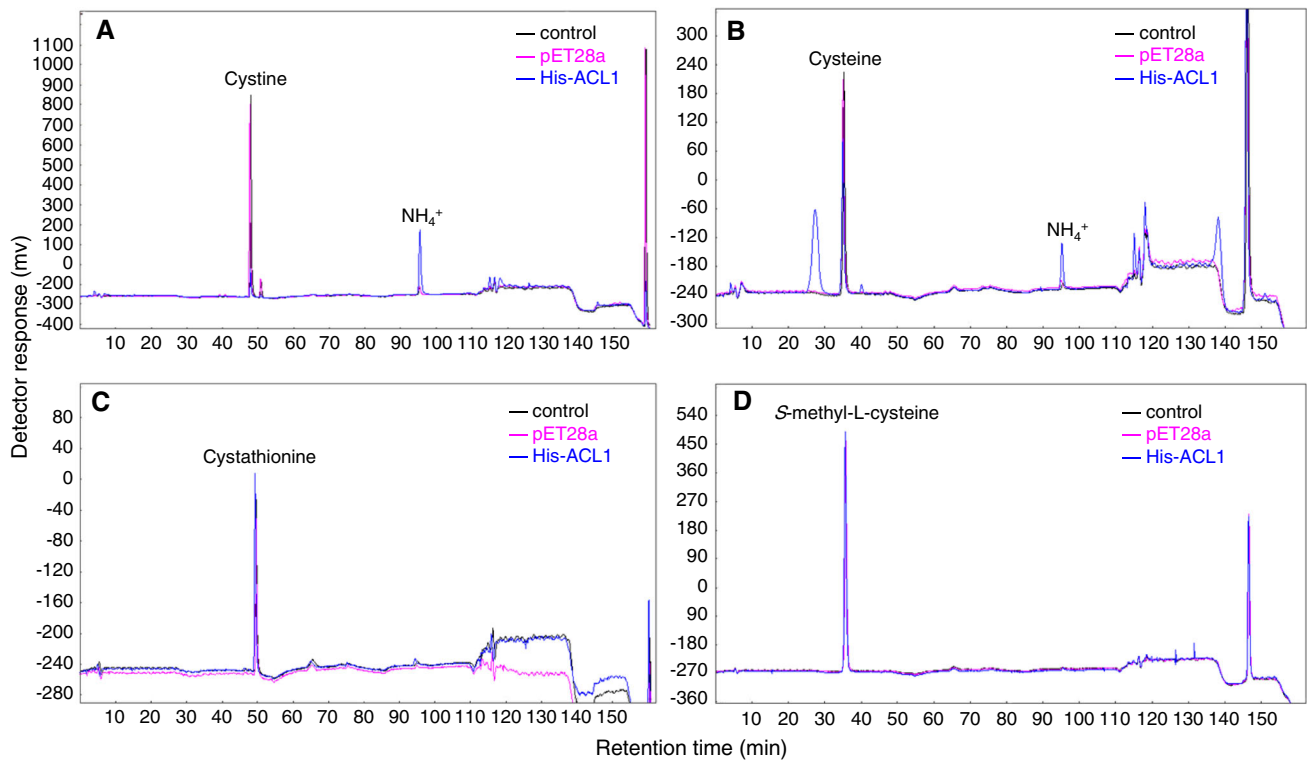
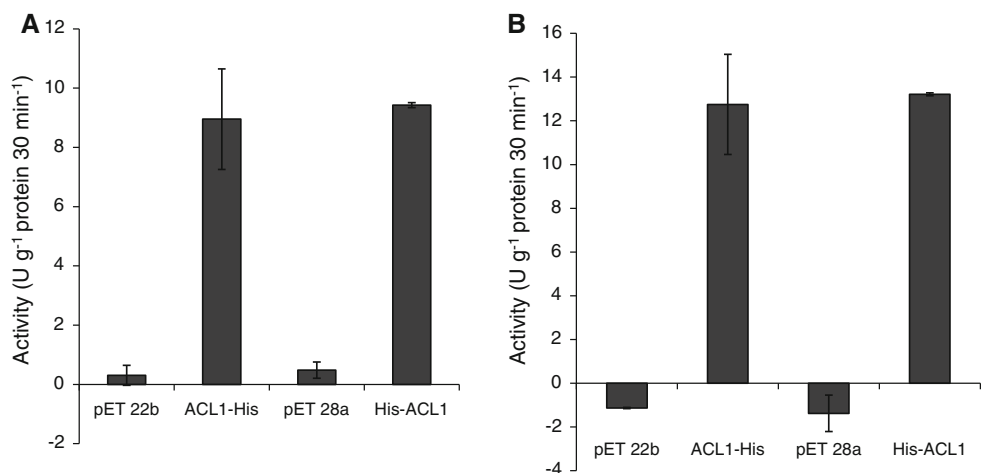


Fig. 6 Chromatogram of products after C-S lyase reaction catalyzed by PpACL1. Cystine (a), cysteine (b), cystathionine (c) or S-methyl-L-cysteine (d) was used separately as a substrate. Control: empty

vector pET28a was used as a control, and H₂O was also used as negative control instead of purified proteins

Fig. 7 Measurement of C-S lyase activity of PpACL1. Cystine (a) or cysteine (b) was used as a substrate. One unit of activity was defined as the amount of enzyme necessary to form 1 μmol 30 min^{-1} of pyruvate at 30 °C. Error bars show standard error ($n = 9$)



PpACL2 also lacks aminotransferase activity (see Supplementary Fig. 6 online).

Ethylene production by the *P. patens* gametophore

The present investigation revealed that neither of the PpACLs has in vitro or in vivo ACS activity, and provided biochemical and biological evidences demonstrating that the two putative ACS genes in *P. patens* do not encode true ACS proteins. We then tested whether *P.*

patens gametophores emitted ethylene. It was found that *P. patens* indeed produced ethylene, but at much lower level than *Arabidopsis* did (Table 1). However, treatment with exogenous ACC (the precursor of ethylene biosynthesis in higher plants, 100 μM) and AVG (an ethylene biosynthesis inhibitor, 50 μM) did not affect the ethylene production in *P. patens* (Table 1). By contrast, ACC treatment significantly enhanced ethylene production in *Arabidopsis* and AVG treatment inhibited ethylene production (Table 1).

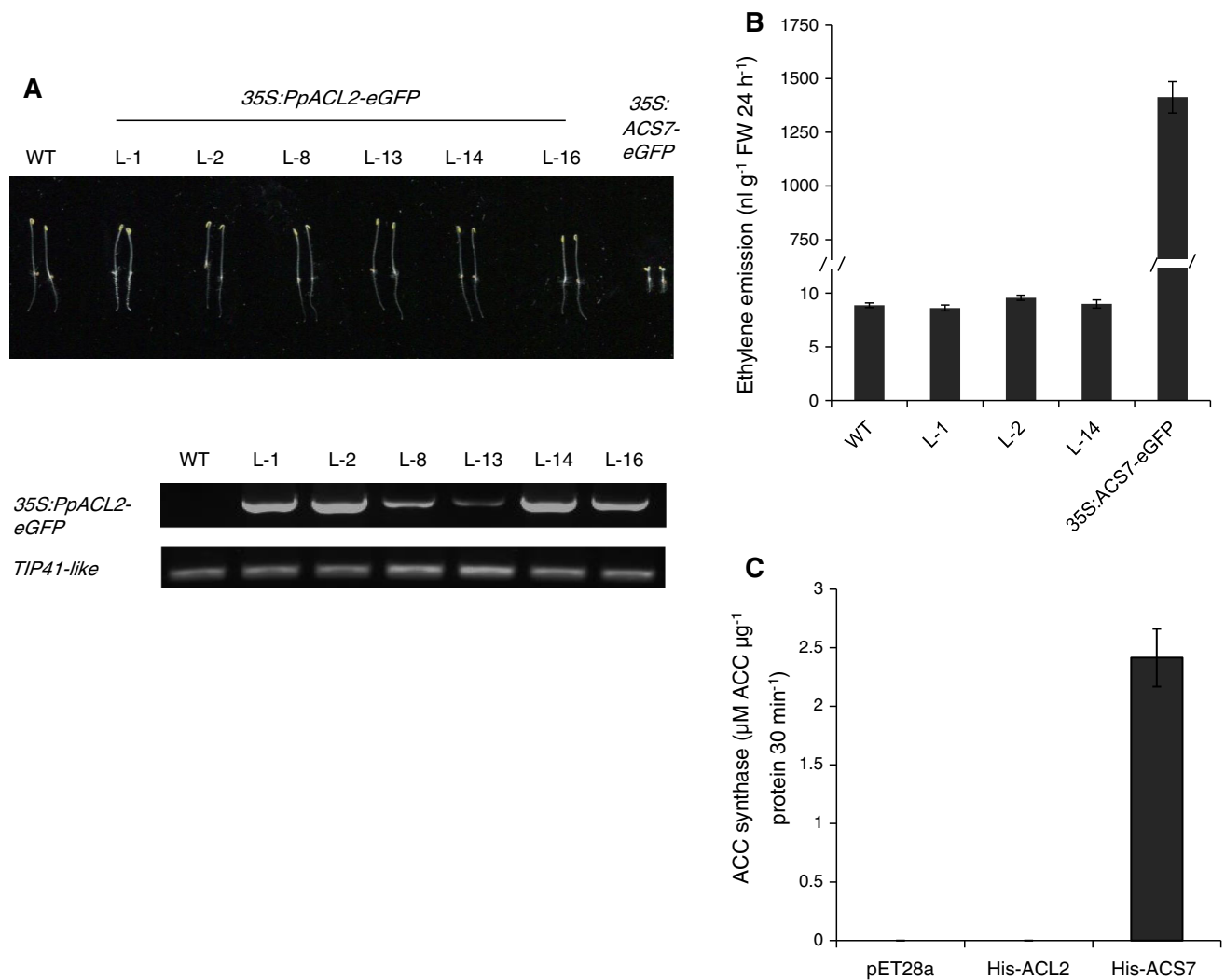


Fig. 8 Measurement of in vivo and in vitro ACS activity of PpACL2. **a** Phenotypes of the 3-day-old 35S:PpACL2 etiolated seedlings. The transcript level of PpACL2 accumulated in the 35S:PpACL2 transgenic *Arabidopsis* was determined by semi-quantitative RT-PCR method. Total RNA was isolated from the 7th rosette leaf of 33-day-

old 35S:PpACL2 transgenic *Arabidopsis*. **b** Ethylene emission from the 3-day-old 35S:PpACL2 etiolated seedlings. **c** Measurement of in vitro ACS activity of PpACL2. Error bars show standard error ($n = 9$)

Table 1 Ethylene emission from gametophores of *P. patens*

Plant	Ethylene emission (nl g ⁻¹ FW 24 h ⁻¹)			
	Control	No ACC/AVG	100 μM ACC	50 μM AVG
<i>P. patens</i>	0	14.58 ^a ± 0.25	19.89 ^a ± 2.58	17.06 ^a ± 1.14
<i>Arabidopsis</i>	0	87.13 ^b ± 21.8	3660.56 ^a ± 424.5	18.51 ^c ± 3.6

Data represent mean ± SE of ethylene emission per gram of plant fresh weight in 24 h of three independent experiments ($n = 9$). Same letters in the respective rows show insignificant differences between treatments (Tukey's multiple range test, $P < 0.05$)

Discussion

PpACL1 functions as a C-S lyase in *P. patens*

C-S lyase catalyzes the cleavage of carbon-sulfur bonds, and, together with ACS and ATase (aminotransferase),

belongs to the family of PLP-dependent enzymes. Some C-S lyases have been identified in lower plants such as *Synechocystis* (Leibrecht and Kessler 1997), and in many higher plants. Plant C-S lyases are involved in a range of biological processes including the synthesis and degradation of natural products, amino acid metabolism, and the

generation of precursors for the synthesis of ethylene, polyamines, and specific proteins (Kiddle et al. 1999; Munt et al. 2013). For example, C-S lyases catalyze the conversion of L-cystathionine to homocysteine in methionine biosynthesis (Ramirez and Whitaker 1998; Kiddle et al. 1999) and the release of reducing sulfur compounds from L-cysteine and its conjugates for use in detoxification reactions (Ramirez and Whitaker 1998; Rose et al. 2005). In the lower plant *Synechocystis* and the plastids of higher plants, C-S lyases may provide sulfur that is incorporated into the Fe-S clusters of photosynthetic protein complexes (Abdel-Ghany et al. 2005; Balk and Pilon 2011; Leibrecht and Kessler 1997). Plant C-S lyases also produce precursors of sulfur-containing secondary metabolites such as S-alkyl-L-cysteine sulfoxides in *Allium* spp. (Lancaster et al. 2000; Bennett and Wallsgrove 1994), and C-S lyases in glucosinolate-containing plants are involved in glucosinolate biosynthesis (Grubb and Abel 2006; Kiddle et al. 1999; Mikkelsen et al. 2004).

In this study, we found that an ACS-like gene in *P. patens*, *PpACL1*, encodes a C-S lyase that uses L-cystine and L-cysteine as substrates. However, the physiological function of the C-S lyase activity of *PpACL1* in *P. patens* remains unknown. Under normal growth conditions, the transgenic *Arabidopsis* seedlings overexpressing *PpACL1* showed no morphological difference from their wild type control. However, cysteine and cystine are the metabolic precursors of essential biomolecules, such as vitamins, cofactors, antioxidants, and many defense compounds (Alvarez et al. 2012; García et al. 2015). Additionally, H₂S, which is produced when *PpACL1* is combined with cysteine, has also been documented to be involved in the antioxidative response against numerous environmental stimuli, including copper, aluminum, heat, drought, and osmotic stresses (Lai et al. 2014; Yu et al. 2013; Zhang et al. 2008, 2010). Therefore, *PpACL1* may have important roles for *P. patens* under biotic and abiotic stress conditions, and if so, might have facilitated terrestrialization. This possibility remains to be tested using molecular biology and genetics methods.

There may be other genes encoding the key enzymes of ethylene biosynthetic pathway in *P. patens*

P. patens is an ideal model for plant evolutionary studies (Mishler and Oliver 2009; Liu et al. 2013), and an analysis of its sequenced genome may provide insight into the evolution of the ACS gene. Two putative ACS-like genes were found in *P. patens* genome (Rensing et al. 2008), but nothing was hitherto known about their functions. However, the sequences of these two putative genes were reported in several recent studies of the evolution of the ethylene biosynthetic pathway (Ju et al. 2015; Wang et al.

2015; Zhang et al. 2012). In this research, we investigated the function of these two putative ACS genes in *P. patens*, and found that the two genes did not encode true ACS proteins, and that *PpACL1* is a C-S lyase. By contrast, *PpACL2* showed no C-S lyase or aminotransferase activity, indicating that it may have other functions. Together these results indicate that the two putative genes are not true ACS genes. In addition, exogenous ACC and AVG treatments did not affect ethylene formation in *P. patens*. These results also indicate that ethylene is not produced in *P. patens* by the same pathway present in higher plants. These findings are in agreement with previous studies (Stange and Osborne 1989; Osborne et al. 1996), which showed that two mosses (*Sphagnum cuspidatum* and *Polytrichum commune*) and two liverworts (*Marchantia polymorpha* and *Riella helicophylla*) were unable to convert exogenously added ACC to ethylene, indicating that a different ethylene biosynthetic pathway may exist in these organisms. However, our results are not in agreement with those reported in other studies. For instance, Rohwer and Bopp (1985) reported that ethylene production in the moss *Funaria hygrometrica* protonema was promoted by the addition of ACC and IAA, suggesting that ethylene was produced in *Funaria hygrometrica* via the same pathway as in higher plants. Additionally, similar results were reported in studies of other lower plants (Osborne et al. 1996; Plettner et al. 2005; Chernys and Kende 1996; Stange and Osborne 1989; Cookson and Osborne 1978; Tittle 1987). While we have demonstrated that the two putative genes in *P. patens* are not true ACS genes, there may be other genes that function in the ethylene biosynthetic pathway in *P. patens*.

Evolution of the key enzyme ACS in the ethylene biosynthetic pathway in higher plants

ACS is the rate-limiting enzyme in the ethylene biosynthetic pathway in higher plants, and its origin and evolution remain unclear. Homologous sequences of ACS were identified in many lower plants, such as algae, mosses, and ferns, and ethylene production was also detected in these plants (Osborne et al. 1996; Maillard et al. 1993; Timme and Delwiche 2010; Driessche et al. 1988). However, little is known about the function of these ACS homologs. It has been proposed that ACS-like enzymes in moss and algae may convert SAM to ACC, or they may catalyze a different, but chemically similar reaction (DeLong and Booker 2015). Sequence and structural alignments indicated that ACC synthases are most closely related to the subgroup I of aminotransferases, which include aspartate, alanine, histidinol-phosphate, tyrosine, and phenylalanine aminotransferases (Alexander et al. 1994; Feng et al. 2000; Mehta and Christen 1994). Phylogenetic analysis, motif

identification, and adaptive selection analysis based on an updated dataset including 107 plant *ACS* genes and eight *ACS-like* genes from animals as well as six *AATase* genes from both plants and animals indicated that all plant *ACS* genes originated from *plant-ACS-like* genes which were derived from *AATase* genes (Zhang et al. 2012). It was also suggested that true *ACS* enzymes and a separate group of *ACS-like* aminotransferases diverged after the split of seed plants from mosses (DeLong and Booker 2015).

Based on sequence homology and structural analyses, as well as the fact that C-S lyases and aminotransferases generate similar products that are involved in amino acid metabolism, C-S lyases have been proposed to be evolutionarily related to aminotransferases (Mehta and Christen 1998; Mikkelsen et al. 2004). The C-S lyases in plants were initially annotated as aminotransferases based on sequence and structural homologies (Jones et al. 2003; Seo et al. 1998; Lin et al. 1999), and a C-S lyase displaying both aminotransferase and C-S lyase activities in *T. brevicorniculatum* was also reported (Munt et al. 2013). Since both *ACS* and C-S lyase belong to AAT-like family, it would be worth investigating whether *ACS* is evolutionarily related to C-S lyase. Both aminotransferase and C-S lyase enzymes have important roles in amino acid metabolism in plants. During plant evolution, the *aminotransferase* and *C-S lyase* genes might appear earlier than the *ACS* gene, and then, the higher plants evolved the ability to produce ethylene, which served important regulatory functions as plants adapted to their complex environment. *P. patens*, a lower land plant, is placed phylogenetically between algae and vascular plants (Reski 1998), and its C-S lyase is therefore likely to provide important clues as to the evolution of AAT-like gene family in plants.

In conclusion, we have shown that two PpACL proteins have no in vitro and in vivo *ACS* activity. Moreover, we found that PpACL1 functions as a C-S lyase that uses cystine and cysteine as substrates, whereas PpACL2 shows neither C-S lyase nor aminotransferase activity. These results imply that neither of the putative *ACS* genes is true *ACS* gene as those found in higher plants.

Author contribution statement NNW conceived and designed the experiments. LFS and HD performed the experiments. LFS, HD and NNW analyzed the data. LFS wrote the manuscript. LFS, Nasrullah, YYM and NNW revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest.

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