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Photorespiratory serine hydroxymethyltransferase 1 activity impacts abiotic stress tolerance and stomatal closure

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Abstract

The photorespiratory cycle is a crucial pathway in photosynthetic organisms since it removes toxic 2-phosphoglycolate made by the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase and retrieves its carbon as 3-phosphoglycerate. Mitochondrial serine hydroxymethyltransferase 1 (SHMT1) is an essential photorespiratory enzyme converting glycine to serine. SHMT1 regulation remains poorly understood although it could involve the phosphorylation of serine 31. Here we report the complementation of *Arabidopsis thaliana shm1-1* by SHMT1 wild-type, phosphorylation mimetic (S31D) or non-phosphorylatable (S31A) forms. All SHMT1 forms could almost fully complement the photorespiratory growth phenotype of *shm1-1*, however each transgenic line had only 50% of normal SHMT activity. In response to either a salt or drought stress, *Compl-S31D* lines showed a more severe growth deficiency compared to the other transgenic lines. This sensitivity to salt appeared to reflect reduced SHMT1-S31D protein amounts and a lower activity that impacted leaf metabolism leading to proline under-accumulation and over-accumulation of polyamines. The S31D mutation in SHMT1 also led to a reduction in salt-induced and ABA-induced stomatal closure. Taken together, our results highlight the importance of maintaining photorespiratory SHMT1 activity in salt and drought stress conditions and indicate that SHMT1 S31 phosphorylation could be involved in modulating SHMT1 protein stability.

30 **Keywords:** Photorespiration, Protein phosphorylation, SHMT1, Metabolism, Abiotic stress,
31 stomata

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Introduction

Photorespiration is a key primary metabolic pathway that starts with the fixation of O₂ through the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) leading to the synthesis of one 2-phosphoglycolate (2PG) and one 3-phosphoglycerate (3PGA) (Bauwe et al., 2010). However, 2PG, and other intermediates of photorespiration such as glycolate have been shown to reduce photosynthesis by inhibiting triose-phosphate isomerase and RuBisCO, respectively (Anderson, 1971; Kelly and Latzko, 1976; Flügel et al., 2017). Therefore, the photorespiratory cycle is crucial to remove these toxic metabolites and to recover the carbon as 3PGA. It takes place in four cell compartments (cytosol, chloroplasts, peroxisomes and mitochondria) and involves eight core enzymes and several transporters. Together they convert two molecules of 2PG into one molecule of 3PGA, and in doing so consume one molecule of NADH and ATP and produce one molecule of CO₂, NH₃, NADH and ADP (Bauwe et al., 2010; see Figure 1). The photorespiratory cycle interacts with a number of primary metabolic processes such as photosynthesis, nitrate and amino acid metabolism, the tricarboxylic acid (TCA) cycle, and C1-metabolism (Takahashi et al., 2007; Tcherkez et al., 2008; Hanson and Gregory, 2011; Ros et al., 2014; Bloom, 2015). It also impacts pathogen defense (Sørhagen et al., 2013) and more recently it has been proposed to affect stomatal aperture (Eisenhut et al., 2017). The importance of the photorespiratory cycle is highlighted by the strong phenotypes exhibited by air-grown knock-out mutants of photorespiratory enzymes and transporters that include reduced growth, chlorosis, and even lethality. Although the phenotypic severity depends on the affected protein, all photorespiratory mutants can be rescued under high CO₂ growth conditions (Timm and Bauwe, 2013).

Amongst the first photorespiratory cycle mutants identified from ethyl methanesulfonate (EMS)-mutated *Arabidopsis thaliana* were three allelic *stm* (later renamed *shm*) mutants affected in mitochondrial serine hydroxymethyl transferase (EC 2.1.2.1; SHMT) activity (Somerville and Ogren, 1981). In *A. thaliana*, two of the seven putative SHMT genes appear to encode mitochondrial SHMT isoforms (*AtSHMT1*, *At4g37930*; *AtSHMT2*, *At5g26780*) (McClung et al., 2000; Engel et al., 2011). The *shm1-1* (CS8010) mutant of Somerville and co-workers was mapped to the SHMT1 gene (*At4g37930*) (Voll et al., 2006). It displays a strong-to-intermediate photorespiratory phenotype that is rescued under high CO₂ (Timm and Bauwe, 2013). This phenotype shows that SHMT1 has a non-redundant photorespiratory role in which it catalyzes the transfer of a hydroxymethyl group from 5,10-methylene-THF,

67 produced by the glycine decarboxylase complex (GDC), to a glycine to form serine (Figure
68 1). Interestingly, a *shm1-1* x *shm2-1* double mutant lacking both mitochondrial SHMT
69 isoforms was unable to survive even in CO₂-enriched air probably because of the impairment
70 of the additional role of SHMT in C1-metabolism (Engel et al., 2011).

71 Photorespiratory enzymes also appear to play important roles in plant responses to biotic and
72 abiotic stresses including high temperature, drought, salinity and pathogen attack (Moreno et
73 al., 2005; Rojas et al., 2012; Li and Hu, 2015; Noctor et al., 2015; Cui et al., 2016; Xu et al.,
74 2018). In photosynthetic bacteria, the overexpression of SHMT from the halotolerant
75 cyanobacteria *Aphanothece halophytica* either in *E. coli* or in the fresh water cyanobacteria
76 *Synechococcus elongates* conferred a better tolerance to salinity (Waditee-Sirisattha et al.,
77 2012, 2016). In plants, SHMT1 is essential in response to pathogens and it was also suggested
78 to be involved in salt tolerance (Moreno et al., 2005). Recently, a salt-tolerant rice variety was
79 found to have a high SHMT expression associated to a strong accumulation of serine in
80 response to salt stress when compared to a salt-sensitive variety (Mishra et al., 2016).

81 Although SHMT1 has a key role in the photorespiratory cycle and it has been shown to be
82 involved in abiotic and biotic stress responses, the regulation of SHMT1 activity remains to
83 be elucidated. To rapidly respond to changing conditions, enzymes are often post-
84 translationally modified and several reports suggest that this could be the case for SHMT1.
85 Indeed, SHMT1 is a potential target of thioredoxin-dependent redox regulation (Marchand et
86 al., 2004). SHMT1 was also found to be degraded in a 26S proteasome-dependent process,
87 and ubiquitin-specific protease 16 (UBP16) stabilized SHMT1 during a salt stress by
88 removing conjugated ubiquitin (Zhou et al., 2012). Pea mitochondrial SHMT was found to be
89 nitrosylated under control conditions but not during a salt stress (Camejo et al., 2013).
90 According to the Arabidopsis phosphopeptide database PhosPhAt ([http://phosphat.uni-](http://phosphat.uni-hohenheim.de/)
91 [hohenheim.de/](http://phosphat.uni-hohenheim.de/)), 8 residues of SHMT1 are phosphorylated. Previously, we reported that the
92 phosphorylation of serine 31 (S31) of the phosphopeptide (pS)LPSEAVDEKER remained
93 unchanged in Arabidopsis rosettes when treated with varying O₂/CO₂ conditions suggesting
94 that this phosphorylation event was not correlated to leaf photorespiratory activity (Abadie et
95 al., 2016). It should be noted that after removal of the mitochondrial signal peptide and a
96 further processing by intermediate cleavage peptidase 55 (ICP55; Huang et al., 2015), S31
97 become S1 of the mature SHMT1 protein.

98 In this work, the function of *Arabidopsis thaliana* SHMT1 S31 phosphorylation was
99 investigated by studying *shm1-1* plants complemented with either wild-type, phosphorylation

mimetic (S31D) or non-phosphorylatable (S31A) SHMT1. In normal air-grown conditions, no significant differences in growth were observed between the transgenic lines, although they were smaller than WT Col-0 plants. However, when subjected to either a salt or a drought stress, *Compl-S31D* lines exhibited a further reduction in growth. In the case of a salt treatment, the increased sensitivity was associated with altered SHMT1-S31D protein and activity levels. Our data will be discussed in terms of the importance of photorespiratory SHMT1 activity in plant abiotic stress tolerance and ABA-induced stomatal movements, and the possible role of S31 phosphorylation in modulating SHMT1 protein stability.

Materials and methods

Plants material and growth conditions

Arabidopsis thaliana Columbia (Col-0) accession was used as a wild-type control. The *shm1-1* mutant (Somerville and Ogren, 1981; Voll *et al.*, 2006) was provided by Andreas P.M. Weber (University of Dusseldorf, Germany).

For *in vitro* experiments, sterilized seeds were sown on ½MS medium (Murashige and Skoog, 1962), supplemented with 0.8% agar (w/v), and placed at 4°C in the dark for 48 h to promote germination. Plants were grown in short-day conditions (8h light/16h dark, 160 µmol photons.m⁻².s⁻¹, 65% humidity, 20°C day/18°C night, ambient air CO₂ concentration of around 400 µL.L⁻¹). For *in vitro* salt treatments, one-week-old seedlings were grown vertically on ½MS-agar plates before their transfer to new plates with or without 50 mM NaCl or 100 mM mannitol according to Jossier *et al.*, 2010. For soil-grown experiments, *Arabidopsis* seeds were germinated on potting soil for 10 days in short days (8h light/16h dark, 200 µmol of photons.m⁻².s⁻¹, 65% humidity, 20°C day/18°C night, ambient air CO₂). Subsequently, seedlings were transplanted to individual pots and grown further in short day conditions either in ambient air CO₂ or high CO₂ (3000 µL.L⁻¹) enriched air (HC). For the drought treatment, 10-day-old *Arabidopsis* seedlings were transplanted and grown for 2 weeks in ambient air CO₂ short-day-conditions before being subjected to water deprivation for 3 weeks then re-watering for 3 days.

Plasmid construction, site-directed mutagenesis and generation of complemented lines

Genomic DNA was extracted from *A. thaliana* Col-0 leaves according to Edwards *et al.* (1991), and the *SHMT1* promoting region (-1000 to +55) was PCR-amplified using primers (promSHMT1-F/promSHMT1-R). Note, all primers are described in Supplemental Table S4.

The resulting DNA (*SHMT1 promoter*) was cloned into pGEM-T Easy® and sequenced (by MWG OPERON, EUROFINS). The *SHMT1* cDNA in pUNI51 (clone U21423) was obtained from the Arabidopsis Biological Resource Center (ABRC) (<https://abrc.osu.edu/>). *SHMT1* cDNA was PCR-amplified using primers cDNA-SHMT1-F and GW-SHMT1-R and the *SHMT1* promoter region was re-amplified with the primers described above. The two resulting DNA fragments (*SHMT1 cDNA* and *SHMT1 promoter*), with an overlapping region (0 to +55), were used as templates for two consecutive PCR amplifications (using primers GW-SHMT1-F/GW-SHMT1-R followed by Gateway-F/Gateway-R) in order to join the two DNAs and to add attB Gateway® recombination sites to the 5' and 3' ends. The resulting DNA (*promSHMT1::SHMT1*) was further sequenced before being cloned into pDONR207 using the GATEWAY® cloning system to generate *pDONR207-promSHMT1::SHMT1*.

To introduce the required point mutations into *pDONR207-promSHMT1::SHMT1*, primer pairs were selected and used with the QUIKCHANGE® II XL Site-Directed Mutagenesis Kit (AGILENT®), according to the manufacturer's instructions to obtain *pDONR207-promSHMT1::SHMT1-S31A* and *pDONR207-promSHMT1::SHMT1-S31D*. Then, *promSHMT1::SHMT1*, *promSHMT1::SHMT1-S31A* and *promSHMT1::SHMT1-S31D* were subcloned into pH7WG using the GATEWAY® cloning system to generate the plant transformation constructs *pH7WG-promSHMT1::SHMT1*, *pH7WG-promSHMT1::SHMT1-S31A* and *pH7WG-promSHMT1::SHMT1-S31D*. They were used to transform HC-grown *shmt1-1* plants by *Agrobacterium*-mediated floral dip (Clough and Bent, 1999). Hygromycin-resistant seeds were selected and homozygous transgenic lines containing a single insertion were selected by successive rounds of self-crossing and antibiotic selection on ½MS medium agar plates.

Analysis of N-terminal sequence of SHMT1 protein

Arabidopsis rosette leaves (100 mg FW) were rapidly frozen in liquid nitrogen and stored at -80°C until extraction. Protein extraction, LC-MS/MS analysis and identification of SHMT1 peptides were carried out as previously described (Boex-Fontvieille et al., 2013).

Leaf gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence measurements were carried out on 2 cm² of fully expanded leaves of 6-week-old plants in a gas exchange chamber (LCF 6400-40, LiCOR) connected to a portable measuring system (LI 6400XT, LiCOR) (Dellero et al., 2015). Standard measuring conditions were: 200 µmol photons m⁻² s⁻¹ of light intensity, a leaf

temperature of 21°C, 60–70% relative humidity [vapor pressure deficit (VPD) of the leaf approximately 1], 400 $\mu\text{mol mol}^{-1}$ CO₂ and 21% O₂. For chlorophyll fluorescence parameters, plants were adapted for 30 min to darkness before measuring the Fo and Fm levels. Parameters were calculated as follows: $F_v = F_m - F_o$, $NPQ = (F_m - F_m')/F_m'$, $ETR = [(F_m' - F_v)/F_m']0.5 \times I \times \alpha_{\text{leaf}}$, with I corresponding to the irradiance in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and α_{leaf} to the light absorption coefficient of a leaf=0.85.

SHMT activity assays

SHMT activity was determined by following the production of glycine from serine as measured by HPLC. 400 mg of rosette or seedling leaves were harvested and ground to a fine powder in liquid nitrogen. Total soluble protein was extracted in 500 μl buffer containing 50 mM NaH₂PO₄, anti-protease cocktail (Complete-Mini Free-EDTA, ROCHE®), 1 mM β -mercaptoethanol, pH 8.0. Extracts were clarified by centrifugation at 16000xg for 30 min at 4°C, and the resulting supernatant was desalted by NAP-5 (HEALTHCARE) size-exclusion filtration after equilibration with 50 mM NaH₂PO₄, pH 8.0. Protein concentration was determined using Bradford reagent (B6916, SIGMA-ALDRICH). Each reaction was composed of 50 mM NaH₂PO₄, pH 8.0, 0.25 mM pyridoxal-5-phosphate, 2 mM 5,10-methylene tetrahydrofolate, 20 mM serine, 0.15 mM α -amino-n-butyric acid, 400 μg of total soluble protein in a final volume of 500 μl and incubated at 25°C for 5 min (determined to be in the linear phase of the reaction). The reaction was stopped by adding 175 μL of trichloroacetic acid 25% (v/v) followed by incubation on ice for 15 min. After centrifugation at 15000xg for 10 min at 4°C, 480 μL of the supernatant were transferred to a new tube, the pH was neutralized with 50 μL of K₂CO₃ buffer (2.3 M K₂CO₃, 50 mM NaH₂PO₄, pH 8.0) and glycine was quantified by HPLC (see below).

RNA isolation and real-time RT-qPCR

Two-week-old *Arabidopsis* seedlings were transferred to liquid ½MS medium containing 50 mM NaCl for 6 h under light then gene expression was determined by RT-qPCR. Total RNA was extracted with the NucleoSpin RNA Plus Kit then 2 μg RNA was used to make cDNA by reverse transcription (IM-PROMII™), according to the manufacturer's instructions. Quantitative real-time PCR was carried out with a LightCycle-480-Real-Time PCR System and SYBR green (ROCHE DIAGNOSTICS). Relative-expression levels were normalized to Actin-2 (*At3g18780*) and three independent experiments were performed. Primers are given in Supplemental Table S4.

Amino acid analyses by HPLC and metabolite analyses by GC-MS

Seven-day-old *in vitro* grown seedlings were transferred to ½ MS plates with or without 50 mM NaCl for 3 weeks, rosette leaves (100 mg FW) were then collected after 5 hours of light and rapidly frozen in liquid nitrogen and stored at -80°C until extraction. Quantitative glycine amounts were measured by HPLC and relative metabolite levels of whole rosette leaves were analyzed by GC-MS, both after cold-methanol extraction, as described in Noctor et al. (2007).

SDS-PAGE and western-blot

Ten µg of soluble proteins were separated on an SDS-PAGE gel (10% acrylamide) and they were detected by Coomassie blue staining (Laemmli, 1970). For western-blot analyses, SDS-PAGE separated proteins were transferred to nitrocellulose membranes, blocked for 1 h at room temperature in blocking buffer (5% non-fat dry milk in TBST buffer including 50 mM Tris-base, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and incubated overnight at 4°C with either a 1/3500 dilution (in blocking buffer) of potato SHMT1 antibodies (Engel et al., 2011) or actin antibodies (A0480, SIGMA-ALDRICH) diluted 1/5000. Membranes were washed 3-times with TBST buffer, then incubated with either 1/5000 diluted anti-rabbit (A6154, Sigma-Aldrich) or anti-mouse antibodies (A4416, SIGMA-ALDRICH) for 1 h at room temperature in blocking buffer. Proteins were revealed using a chemiluminescent peroxidase substrate-3 kit (CPS350, SIGMA-ALDRICH).

Analysis of H₂O₂ levels

Hydrogen peroxide was measured by two methods. Seedlings were grown on ½MS plates for 14 days in short-day conditions before a 150 mM NaCl treatment (14 h) as in Zhou et al., 2012. For 3,3'-diaminobenzidine (DAB) staining, leaves were incubated in DAB staining buffer (50 mg DAB dissolved in 45 mL sterile H₂O, with 1 M HCl to dissolve the DAB, then 25 µl Tween 20 (v/v) and 2.5 mL of 200 mM Na₂HPO₄ (pH 6.8) were added to adjust the pH to 5.0). DAB staining buffer was removed after 8 h and replaced with a bleaching solution of ethanol:acetic acid:glycerol (v:v:v = 3:1:1). Bleaching was carried out in a boiling water bath for 15 min followed by 30 min under shaking at room-temperature. For H₂O₂ quantification, seedlings were treated as above but H₂O₂ content was measured by a colorimetric assay using the Hydrogen Peroxide Assay Kit according to the manufacturer's instructions (ABCAM®).

Water content and water loss assays

Water content was measured using leaves of 3-week-old seedlings by comparing the fresh and dry weights based on the equation: $\text{water content (\%)} = 100 \times (\text{FW} - \text{DW}) / \text{FW}$. DW was measured after lyophilization in a Christ Alpha 2-4 (PCR-EN7049) for 72 h. Water loss assays were carried out in a growth chamber where rosettes of six-week-old plants were cut and weighed at different times. Water loss is presented as the percentage of the initial FW at the indicated time.

Stomatal aperture assays

Stomatal aperture was measured according to Merlot et al. (2007). Briefly, for ABA-induced stomatal closure, leaves of 5-6 week-old plants were harvested in darkness at the end of the night period. The abaxial side of leaves were glued to a coverslip using non-toxic medical adhesive (Hollister Medical Adhesif (7730 NEWPHARMA)). To obtain isolated epidermal cells, leaves were peeled gently and coverslips with glued epidermis were placed in 4.5 cm-diameter petri-dishes containing 50 mM KCl, 0.1 mM CaCl_2 , 10 mM MES-KOH, pH 6 for 1h in the dark to ensure closed stomata. Then they were incubated under light ($200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 4 h to ensure stomatal opening, then incubated under light for 4 h in the presence/absence of 50 μM ABA. Photographs of stomata were made using a microscope DMI 6000 (LEICA). Stomatal pore area (in μm^2) was measured using ImageJ software. For the NaCl-induced stomatal closure assay, plants were exposed to light ($200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 2.5 h before being watered with or without 300 mM NaCl for 4 h under light according to Chung et al., 2013. Leaves were harvested and stomatal aperture determined using isolated epidermal cells as described above. The same samples were used to determine stomatal index and stomatal density. For the study of stomatal behavior during dark to light and light to dark transitions, leaves were collected from plants two hours before the photoperiod then plants were exposed to light ($200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 4 h before being placed in the dark for 3 h. Photographs were taken under a microscope AxioImager Z2 (ZEISS). Stomatal aperture (width/length) was measured using ImageJ software.

Results

Complementation of the *shm1-1* photorespiratory growth phenotype by S31-mutated SHMT1

To evaluate the impact of SHMT1 S31 phosphorylation on plant physiology and metabolism, site-directed mutagenesis was undertaken using the *Arabidopsis* cDNA of wild-type *SHMT1*, so that S31 was replaced either by a phosphomimetic aspartate (*SHMT1-S31D*) or by a non-phosphorylatable alanine (*SHMT1-S31A*) (Figure 2A). Subsequently, the resulting cDNAs were placed under the control of *AtSHMT1* promoter region and used to transform high CO₂-grown *shm1-1* plants to generate complemented plants named *Compl-SWT*, *Compl-S31A* and *Compl-S31D* (Figure 2B). Homozygous lines containing a single transgene insertion were selected for further analysis. While *shm1-1* was unable to grow in air, this was not the case for the complemented lines although they were slightly smaller compared to wild-type (WT, Col-0) control plants (Figure 2B). After 4 weeks on soil, the development of each transgenic line was similar when compared to each other (Figure 2B).

N-terminal peptide analyses were performed by LC-MS/MS using extracts from rosettes of complemented lines (*Compl-SWT-5*, *Compl-S31A-7* and *Compl-S31D-5*) to determine whether ICP55 processing was modified. In *Compl-SWT-5* and *Compl-S31A-7*, the identified N-terminal peptides showed that ICP55 processing still took place however this was not the case for SHMT1 of *Compl-S31D-5* since only a SDLPSEAVDEK peptide was observed (Supplemental Figure S1).

Western blot analyses were carried out to compare rosette leaf SHMT protein amounts between the different lines (Figure 2C). To allow the *shm1-1* mutant to grow, plants were grown in high CO₂ (3000 µL.L⁻¹). As expected, almost no SHMT protein was detected in *shm1-1* rosettes while complemented lines exhibited different amounts of SHMT protein (between 40% and 70% of Col-0 SHMT protein levels) (Figure 2C and Supplemental Figure S2). Moreover, in accordance with SHMT protein levels in the complemented lines, SHMT activity analysis of high-CO₂-grown rosette leaves showed that they all had a 40-60% reduced activity compared to Col-0 (Figure 2D).

Since a reduced photorespiratory activity has a negative impact on photosynthetic CO₂ assimilation (Somerville and Ogren, 1981; Chastain and Ogren, 1989; Timm et al., 2012) and electron transfer properties (Dellero et al., 2015, 2016), gas exchange and chlorophyll fluorescence parameters of fully expanded leaves were compared between 6-week-old WT plants and complemented lines. In the three transgenic lines *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7*, net CO₂ assimilation rate (A_n) was reduced by 20% compared to control Col-0 plants while stomatal conductance (g_s), transpiration (Tr), photosynthetic electron transfer rate (ETR), non-photochemical chlorophyll fluorescence quenching (NPQ) and

Fv/Fm ratios were not significantly different (Supplemental Table S1). These results suggest that the smaller size of the complemented lines could be due to their reduced capacity to assimilate CO₂ arising from their lower SHMT1 activity leading to an impaired photorespiratory cycle.

***Compl-S31D* lines are hypersensitive to salt stress in air-grown conditions but not in high CO₂-growth conditions**

Since the different complemented lines did not show any distinct growth and photosynthetic differences between them, it was decided to test their response to abiotic stress. Since the *shm1-3* mutant was found to be more sensitive to salt stress (Moreno et al., 2005), *in vitro* grown one-week-old WT, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* seedlings were transferred to 50 mM NaCl or 100 mM mannitol for 3 weeks. In the absence of NaCl, no difference was observed between the complemented lines although they presented a growth deficiency compared to Col-0 seedlings (Figure 3A). This resulted in a 50% decrease of seedling fresh weight (FW) (Figure 3B) and a 20% decrease of primary root length (Supplemental Figure S3A). When transferred to mannitol, no significant differences were observed between the complemented lines but they all exhibited a 40% reduction of FW compared to Col-0 (Figure 3A and 3B). However, in the presence of 50 mM NaCl, primary root length and seedling FW of the *Compl-S31D-5* line showed significant reductions compared to *Compl-S31A-7*, *Compl-SWT-5* and wild-type lines (Figure 3A and Supplemental Figure S3A). While Col-0 seedlings were slightly affected by the salt treatment, the *Compl-S31D-5* FW was decreased by 50% (Figure 3B) and primary root length was decreased by almost 30% (Supplemental Figure S3A) when compared to *Compl-S31A-7* and *Compl-SWT-5* lines. Similar results were obtained for two other independent *Compl-S31D* lines (*S31D-4* and *S31D-7*) when treated with salt, both presenting a larger decrease in seedling FW and primary root length on 50mM NaCl (Supplemental Figure S4A and S4B). To determine whether the observed salt-induced growth phenotypes of *Compl-S31D* lines were associated with photorespiration, experiments were repeated in high CO₂-growth conditions (3000 µL.L⁻¹) and included the *shm1-1* mutant as a control. After 4 weeks, *Compl-S31D-5* seedlings did not display any differences in growth (seedling FW and primary root length) compared to *shm1-1*, *Compl-SWT-5* and *Compl-S31A-7* lines whether grown with or without 50 mM NaCl (Figure 3C and 3D, Supplemental Figure S3B). Again, these observations were confirmed with other *Compl-S31D* lines (Supplemental Figure S4C and S4D). Taken together, our data show that *Compl-S31D* lines are more sensitive to salt stress and this was photorespiration-dependent.

Furthermore, the growth differences between air-grown Col-0 plants and the different transgenic lines also appear to be photorespiration-dependent whether under control or salt-stress conditions (Figure 3).

SHMT1 protein is less stable in the *Compl-S31D-5* line in response to salt stress leading to a lower SHMT activity.

To determine whether the salt-induced *Compl-S31D-5* growth was linked to SHMT activity, this was measured using *in vitro* grown Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* lines under either control or a 3-week salt treatment (Figure 4A). Leaf total soluble proteins were extracted and SHMT activity was measured as the conversion of serine to glycine using an HPLC-based method (Simic et al., 2002). The salt treatment led to a significant 40% decrease in SHMT activity of *Compl-S31D-5* while a much smaller decrease was measured for *Compl-SWT-5* and *Compl-S31A-7* (reduction of 13% and 15%, respectively) (Figure 4A). These activity changes appeared to mirror leaf SHMT protein levels as determined by western blot analyses (Figure 4B and 4C). Indeed, the SHMT protein level of *Compl-S31D-5* was significantly reduced by 35% after a salt stress compared to *Compl-SWT-5* and *Compl-S31A-7* (non-significant 11% and 15% reduction, respectively) (Figure 4B and 4C). Similar results were obtained for the other *Compl-S31D* lines (4 and 7) compared to the *Compl-SWT* (1 and 7) and *Compl-S31A* (4 and 6) lines (Supplemental Table S2). Taken together, it appeared that leaf SHMT1 protein of *Compl-S31D-5* was less stable in response to salt leading to a lower SHMT activity, thus giving rise to a more severe salt-induced growth phenotype (Figure 3).

Metabolic responses of Col-0, *Compl-SWT-5*, *Compl-S31A-7* and *Compl-S31D-5* to salt stress

In order to explore the metabolic consequences of the reduced SHMT activity of *Compl-S31D-5* in response to salt stress, relative metabolite levels of whole rosettes of seedlings grown *in vitro* with or without 50 mM NaCl were analyzed by GC-MS (Supplemental Table S3). In control conditions, a limited number of similar differences were observed in all transgenic lines compared to Col-0. These included certain amino acids, organic acids and sugar levels. Indeed, asparagine, glutamate, glutamine, homoserine and tryptophan as well as the photorespiratory metabolites glycine, serine and glycerate accumulated in complemented lines (Figure 5). TCA cycle-associated organic acids including citrate, malate, and α -ketoglutarate as well as glucose and fructose levels increased while dehydroascorbate

decreased in complemented lines (Figure 5). In response to 50 mM NaCl, while the relative levels of asparagine, glycine, glutamate and glutamine decreased in Col-0, *Compl-SWT-5* and *Compl-S31A-7*, these amino acids remained at a higher level in *Compl-S31D-5*. On the other hand, levels of metabolites associated with stress protection such as proline, galactaric acid, and *myo*-inositol increased in all lines except *Compl-S31D-5* in response to salt stress (Figure 5). Citrate levels did not change in response to salt stress in *Compl-SWT-5* and *Compl-S31A-7* but decreased in *Compl-S31D-5*. Interestingly, major metabolic differences concerned the levels of polyamines (spermidine and putrescine) and their precursors, ornithine and citrulline, that increased 3-fold (2-fold for putrescine) in response to salt only in *Compl-S31D-5* (Figure 5). Hydrogen peroxide is a key element of stress-induced signaling pathways and Arabidopsis lacking SHMT1 exhibit a large accumulation of leaf H₂O₂ in response to salt stress (Moreno et al., 2005). In order to determine if the complemented lines exhibited altered leaf H₂O₂ contents, control and salt stressed Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* seedlings were stained with DAB. The H₂O₂-induced brown deposit in *Compl-S31D-5* was more important than the other plant lines after stress treatment indicating higher H₂O₂ levels while no differences were observed under control conditions (Figure 6A). A quantitative analysis of H₂O₂ content carried out on the same samples showed that all lines exhibited an increase in H₂O₂ after salt treatment, but the increase was 2-fold more important in *Compl-S31D-5* rosettes compared to the increase of other lines (Figure 6B and Supplemental Figure S5).

***Compl-S31D* plants are unable to recover from a drought stress in ambient air**

Since our data indicated that *Compl-S31D-5* plants were more sensitive to a salt stress, it was decided to test their sensitivity to drought. To achieve this, Col-0, *Compl-SWT*, *Compl-S31A* and *Compl-S31D* plants were grown on soil for 3 weeks in well-watered conditions, and then they were subjected to a drought stress by withholding water for 3 weeks before being re-watered. Under such conditions, the *Compl-S31D* lines were unable to recover from the drought stress (Supplemental Figure S6A). Moreover, detached leaves of *Compl-S31D* lines showed a faster initial water loss ratio compared to the other lines (Supplemental Figure S6B). It should be noted that all plant lines had similar initial water contents, (Supplemental Figure S6C).

***Compl-S31D* stomata show a reduced sensitivity to closure in response to NaCl and ABA**

In order to determine whether the observed stress sensitivity of the *Compl-S31D-5* line was linked to impairment of stomatal development, stomata density and stomatal index of five-week-old plants grown on soil were measured, and no differences could be observed between all lines (Figure S7). As photorespiration occurs under light and is implicated in stomatal movements (Eisenhut et al., 2017), stomatal behavior of three-week-old plants grown on soil was tested during transitions from dark to light (after 4 h) and light to dark (after 3 h). Stomata of all lines exhibited a similar behavior for each tested transition (Supplemental Figure S8A). Stomatal closure was measured also in Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* plants in response to salt treatment. Five-week-old soil-grown plants were exposed to a 4 h treatment with or without 300 mM NaCl in the light. After the initial illumination period, stomata of all plant lines were open (Supplemental Figure S8B). After the salt treatment, stomata became closed compared to control conditions (Figure 7A and 7B). However, stomata of *Compl-S31D-5* rosette leaves were less affected by the salt treatment and their stomata remained about 40% more open when compared to other lines (Figure 7A and 7B). As drought and salt-stress induced ABA signaling is involved in stomatal closure, the plant lines were checked for stomatal closure in response to ABA. Leaf epidermal peels of 5 to 6 week-old Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* plants were prepared in darkness at the end of the night period. They were first incubated in stomatal opening buffer under a light for 4 h without ABA, and then incubated for a further 4 h in the presence of 50µM ABA. Stomatal apertures were measured before and after ABA addition. In the absence of ABA, stomata were open in all lines (Figure 7C and 7D) while after ABA treatment, stomata aperture was significantly decreased however *Compl-S31D-5* stomata were less sensitive to ABA since their closure was reduced (by 40%) when compared to other lines (Figure 7C and 7D). These observations suggest that ABA-induced stomatal closure was impaired in *Compl-S31D-5* and this could help explain the differences seen between plant lines after a salt-stress.

***Compl-S31D* plants have altered ABA-induced gene expression under salt treatment**

Since *Compl-S31D* lines were more sensitive to drought and salt stresses and they exhibited a reduced response to NaCl-induced and ABA-induced stomatal closure, it was decided to further explore the ABA signaling responses of the plant lines. Therefore, the expression of several ABA-responsive and ABA-biosynthesis genes was examined by qPCR, before and after a 6 h salt treatment (50 mM) of 2-week-old *in vitro* grown Col-0, *Compl-SWT-5*, *Compl-S31A-7* and *Compl-S31D-5* seedlings. The expression of the ABA-responsive genes *COR15A*

(Wilhelm and Thomshow, 1993), *RAB18* (Lang and Palva, 1992), *RD22* (Yamaguchi-Shinozaki and Shinozaki, 1993b), *RD29A* (Lee et al., 2016), *RD29B* (Nordin et al., 1993) and *P5CS1* (Ábrahám et al., 2003) and of the ABA-biosynthesis gene *NCED3* (Iuchi et al., 2001) increased after the salt treatment in all lines (Figure 8). However, there was a trend for all transgenic lines to show a slightly lower salt-induced expression of *RD29B*, *COR15A* and *NCED3* while this was significant for the salt-induced expression of *RAB18* and *RD22*. However, salt treatment led to only a low induction of *P5CS1* in *Compl-S31D-5* (2-fold) when compared to Col-0, *Compl-SWT-5* and *Compl-S31A-7* (10-fold) while *RD29A* expression in *Compl-S31D-5* was induced more (8-fold) than in Col-0, *Compl-SWT-5* and *Compl-S31A-7* (5-fold) (Figure 8).

Discussion

Reduced SHMT activity of *shm1-1* complemented lines negatively impacts photosynthetic CO₂ assimilation and plant growth

In order to evaluate the impact of SHMT1 S31 phosphorylation on plant metabolism and growth, the *shm1-1* mutant was complemented with SHMT1-S31D (phospho-mimetic), SHMT1-S31A (non-phosphorylatable) or SHMT1-SWT (non-mutated) forms (Figure 2A). In air-grown conditions, each recombinant SHMT1 form reversed the *shm1-1* photorespiratory growth phenotype however they remained smaller than wild-type Col-0 plants (Figure 2B, 3A and 3B). This observation was associated with a 50% lower SHMT activity in all complemented rosettes compared to Col-0 controls and correlated with reduced SHMT1 protein levels (Figure 2C, 2D and Supplemental Figure S2). Therefore, if the phospho-mimetic SHMT1 S31 is indeed equivalent to phosphorylation, it did not appear to regulate maximal SHMT1 activity. As might be expected, the reduced SHMT activity plants also exhibited lower photosynthetic CO₂ assimilation rates when compared with Col-0 (Supplemental Table S1). This suggested that under standard air-grown conditions, SHMT1 activity had become limiting for the photorespiratory cycle requirements of the transgenic plants, probably leading to toxic 2PG accumulation and/or limited C-recycling that negatively impact Calvin Cycle activity and CO₂ assimilation. Indeed, an inhibition of photosynthesis is a common feature of a blocked photorespiratory cycle as seen by transferring photorespiratory mutants, including *shm1-1*, from high CO₂ conditions to air (Somerville and Ogren, 1981; Chastain and Ogren, 1989; Timm *et al.*, 2012). The photorespiratory nature of the observed

growth phenotype of our complemented lines was confirmed by the absence of any growth phenotype under high CO₂ conditions (Figure 3C and 3D). The non-optimal SHMT1 activity also had an impact on rosette metabolite levels in a similar manner (albeit less important) to those reported for *shmt1-1* when transferred from high to normal CO₂ air (Kuhn et al., 2013). Major changes were observed in several photorespiratory metabolite levels, as well as certain sugars, amino and organic acids. Indeed, the 5-to-6 fold increase in glycine is a good indicator of the limiting SHMT1 photorespiratory activity in the complemented lines. The higher serine levels might be due to alternative serine biosynthesis pathways such as the phosphorylated pathway and the glycerate pathway (Ros et al., 2014). Glucose and fructose increased (Figure 5) and this could reflect altered starch metabolism as recently reported in PGLP1 knock-down lines (Flügel et al., 2017) to allow the production of soluble sugars for cell metabolism when photosynthesis is impacted by an inefficient photorespiratory cycle. Surprisingly, the limiting SHMT1 activity had only small effects on amino and organic acid levels although proline content was reduced while α -ketoglutarate amounts were increased (as already reported by Kuhn et al., 2013). Both of these metabolites are associated with glutamate metabolism, however glutamate levels only increased by 40% while glutamine increased by 70-90% thus suggesting a limited impact of reduced SHMT1 activity on GS/GOGAT cycle functioning and nitrogen assimilation.

The sensitivity of *Compl-S31D* lines to salt stress in air-grown conditions is due to a further reduction of SHMT1 protein and activity

Previous studies have shown *shmt1* mutants to be more sensitive to NaCl stress (Moreno et al., 2005; Zhou et al., 2012). Interestingly, *Compl-S31D* lines were more sensitive to salt stress, showing reduced growth and yellow rosettes as well as a lower FW and shorter primary root growth compared to the other lines when grown in air (Figure 3 and S3A). No difference was observed between the complemented lines in response to mannitol-generated osmotic stress. Indeed, transgenic lines remained smaller than Col-0 even though plant growth was severely reduced compared to control conditions (Figure 3A, 3B). Interestingly, this phenotype disappeared when plants were grown under high-CO₂ conditions (Figure 3C, 3D and S3B) thus showing that the salt stress phenotype of *Compl-S31D* lines was linked to photorespiration. Since the transgenic lines had only 50% of normal Col-0 SHMT activity and already exhibited a growth phenotype in air, it was possible that the additional salt-effect on *Compl-S31D* lines reflected a further inhibition of SHMT1 activity. This hypothesis appeared

to be correct since there was a further decrease in SHMT activity (of about 43%) in *Compl-S31D-5* seedlings when salt-stressed compared to control conditions while no significant differences were observed for *Compl-SWT-5* and *Compl-S31A-7* lines (Figure 4A and Table S2). This decrease in activity correlated with a further reduction in SHMT protein (of about 35%) (Figure 4B, C). So after a salt stress, *Compl-S31D* rosettes contained only 33% of WT Col-0 SHMT activity (Figure 4A) and exhibited a more severe growth inhibition phenotype compared to the other lines (Figure 3, S2 and S3). This suggested that a critical SHMT1 activity had been reached that no longer allowed the plants to cope with a salt stress-induced increase of photorespiration. Again, it should be noted that transgenic plants with only 50% SHMT activity already showed a small growth phenotype in non-stressed air-grown conditions. The need to reduce a photorespiratory enzyme activity below a certain level before observing a phenotype is common. Rice glycolate oxidase (GOX) activity had to be reduced below 60% to observe reduced photosynthetic activity (Xu et al., 2009). Arabidopsis *gox1-1* showed no growth phenotype with only 27% of WT leaf GOX activity, while a photorespiratory phenotype required the activity to be reduced to a residual 5-10% (Dellero et al., 2016). So, we conclude that the salt-induced sensitivity phenotype of *Compl-S31D* lines was due to their low photorespiratory SHMT activity brought about by a salt-induced decrease in SHMT1 protein abundance. It was recently proposed that during salt stress, UBP16-dependent SHMT1 stability reduced the accumulation of reactive oxygen species thus increasing the activity of a Na^+/H^+ antiporter (Zhou et al., 2012). Therefore, it is possible that a low SHMT1 activity could reduce cell Na^+ export leading to salt stress sensitivity. Perhaps, the action of UBP16 is altered by phospho-mimetic SHMT1-S31 in *Compl-S31D* lines thus leading to decreased protein stability under salt conditions. In Arabidopsis, ICP55 removes the N-terminal S30 of “mature” SHMT1 thus making it less stable due to an increased turnover rate (Huang et al., 2015). It was found that S30 was correctly removed from SHMT1 in *Compl-SWT-5* and *Compl-S31A-7* rosettes but not in the *Compl-S31D-5* line (Supplemental Figure S1). Surprisingly, SHMT1 S31D protein abundance appeared to be similar to SHMT1 S31A and SHMT SWT proteins in normal growth conditions (soil and *in vitro*) while it decreased in response to salt stress (Figure 2C, 4B, 4C and Supplemental Figure S2). Thus, the presence of S30-containing SHMT1 S31D protein did not appear to impact SHMT1 stability under non-stress conditions. Further evidence suggests that the N-terminal sequence of SHMT1 is important for stability since a deletion of the first six or more amino acids of sheep SHMT decreased the heat-stability of its oligomeric structure (Jagath et al., 1997).

Perhaps the additional negative charges of a phosphate (or an aspartate) at the N-terminus of the protein could result in a less stable SHMT1 under salt stress conditions.

Decreased SHMT1 activity in phospho-mimetic *Compl-S31D* alters metabolic responses to salt stress

In response to salt stress, a number of metabolite levels were altered in all plant lines studied including decreases in glutamine, asparagine, tryptophan, α -ketoglutarate, glucose and fructose while serine and *myo*-inositol increased (Figure 5). The limited number of metabolic changes suggested that our salt treatment was rather mild. Globally, the SHMT1 transgenic lines that maintained 50% of WT Col-0 SHMT activity underwent similar salt-stress metabolic rearrangements as WT Col-0 plants thus indicating that at this low SHMT1 activity additional adverse affects on plant metabolism were not induced by a 50 mM NaCl treatment. This was perhaps unexpected since salt stress led to stomatal closure (Figure 7A, 7B) that should increase photorespiration and a requirement for a high SHMT1 activity. Therefore, it was surprising to observe a salt-stress induced decrease of glycine (Figure 5) that was negatively correlated with SHMT activity (Figure 4A) suggesting that glycine was being mobilized for other processes such as the biosynthesis of glutathione, an important stress-induced molecule involved in ROS-detoxification (Noctor et al., 2012). Indeed, a trend was observed of increased metabolite levels associated with stress responses including proline and *myo*-inositol while galactaric acid increased in all lines except *Compl-S31D-5* (Figure 5). The latter has been reported to have a role in plants under stress conditions (Perlikowski et al., 2016; Sprenger et al., 2018), proline is an essential osmoprotectant in response to salt stress (Szabados and Savouré, 2009) and *myo*-inositol is important for plant tolerance to drought and salt stresses (Kaur et al., 2013). The reduced levels of these three metabolites in salt-stressed *Compl-S31D-5* plants might partially explain their increased sensitivity to salt and drought stresses. *Compl-S31D-5* rosettes were also different compared to other lines with respect to polyamine accumulation (Figure 5) while ornithine also accumulated in *Compl-S31D-5* in response to a NaCl treatment and citrulline remained at a higher level compared to other lines (Figure 5). Therefore, salt-stressed *Compl-S31D-5* plants appeared to modify their metabolism to favor polyamines. The reason for this is unknown but it might reflect the biosynthesis of nitrogen-rich compounds at a lower carbon cost due to limited carbon resources arising from an inability to maintain sufficient photorespiratory carbon recycling and a further reduced photosynthetic capacity.

***Compl-S31D* lines exhibit an altered stomatal response to ABA and salt stress**

Compl-S31D plants were not only more sensitive to salt stress but also to drought stress recovery while *Compl-S31D* detached rosettes exhibited a higher and faster water loss compared to the other lines (Supplemental Figure S6A and S6B). The water loss phenotype was not due to a defect in initial water content (Supplemental Figure S6C) and it probably reflected the altered stress-induced stomatal movements of *Compl-S31D* lines (Figure 7). Indeed, both salt and drought stresses lead to stomatal closure to reduce transpiratory water losses however the reduction of leaf internal CO₂ levels should reduce photosynthesis while increasing photorespiration (Chastain et al., 2014; Fricke et al., 2004). By studying photorespiratory mutants, including *shml*, it was recently shown that photorespiration is important for stomatal movements (Eisenhut et al., 2017). Since no differences in stomatal conductance or aperture were detected between our SHMT1 lines in air-grown conditions (Figure 7 and Supplemental Table S1), it appeared that 50% of WT Col-0 SHMT1 activity was enough to maintain stomatal aperture. However, after a salt stress or an ABA treatment that led to stomatal closure, the stomata of *Compl-S31D-5* remained more open (Figure 7) while SHMT activity was further reduced to only 35% of the Col-0 value. This decreased stomatal closure in response to salt and to ABA (Figure 7) could impact the sensitivity of *Compl-S31D-5* to salt stress and drought recovery. Since mesophyll organic acid metabolism is involved in regulating stomatal aperture (Araújo et al., 2011), metabolic changes in *Compl-S31D* rosettes might help explain the observed differences. However, malate and fumarate levels were not significantly different between *Compl-S31D* and WT Col-0 lines (Figure 5 and Supplemental Table S3). The plant hormone, ABA, is involved in stomatal closure during both salt and drought stresses and *Compl-S31D* plants were less sensitive to ABA (Figure 7C, 7D). This is not the first time that photorespiration has been implicated in ABA signaling. A screen of EMS-mutagenized plants for an altered expression of the ABA and NaCl induced gene *RD29A* (Responsive to Dessication 29A) led to the identification of two glutamate:glyoxylate transferase 1 (GGT1) alleles (Verslues et al., 2007). However, while *ggt1* mutants were ABA desensitized due to a basal H₂O₂ accumulation, this was not the case for *Compl-S31D* lines as their rosettes contained similar amounts of H₂O₂ when compared to the other lines under control conditions and only a small 20% difference in rosette H₂O₂ levels was measured in response to salt stress (Figure 6 and Supplemental Figure S5). To examine whether ABA signaling was involved in the differential stomatal responses, the expression of several stress marker genes, *RD29A*, *RD29B*, *RAB18*, *RD22*, *COR15A* and *P5CS1*, was tested

in response to NaCl treatment. *RD29B*, *RAB18*, *RD22* and *COR15A* expressions are regulated by ABA while *RD29A* expression is regulated by ABA and by an unknown pathway activated by NaCl stress (Lang and Palva, 1992; Sakuma et al., 2002; Wilhelm and Thomshow, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993a; Yamaguchi-Shinozaki and Shinozaki, 1993b) and salt-induced *P5CS1* expression can involve either ABA or an ABA-independent phospholipase C (PLC) pathway (Strizhov et al., 1997; Parre et al., 2007). Since *RD29A*, *RD29B*, *RAB18*, *RD22* and *COR15A* were induced in response to our NaCl treatment in all lines (Figure 8), the ABA signaling pathway appeared unaffected at the whole rosette level. Moreover, ABA biosynthesis does not seem to be affected either as the expression of *NCED3*, encoding a rate-limiting enzyme for stress-induced ABA synthesis (Iuchi et al, 2001; Tan et al, 2003), did not differ between the lines either in control conditions or in response to salt stress (Figure 8). However, a possible role of the PLC pathway was suggested by the lower salt stress induction of *P5CS1* in *Compl-S31D-5* (Figure 8).

In conclusion, we have shown that photorespiration is an important metabolic process when plants undergo salt stress. Indeed, the absence/reduction of photorespiration brought about by high CO₂ conditions almost abolished the salt-induced growth defects. In air, plants must maintain an efficient photorespiratory cycle flux since we observed that a low SHMT1 activity increased salt stress sensitivity. With respect to S31 phosphorylation of SHMT1, based on the position of S31 in the observed phosphopeptides, it is not involved in the removal of the mitochondrial signal peptide nor N-terminal processing by ICP55. Since the phospho-mimetic SHMT1 did not exhibit an altered maximal SHMT activity, S31 phosphorylation does not appear to be directly involved in modulating enzymatic activity. However, the stability of S31 phospho-mimetic SHMT1 appeared to be reduced under mild salt stress conditions, although we would not expect SHMT1 S31 phosphorylation to be induced under such conditions due to its detrimental effect. Finally, photorespiratory cycle flux efficiency appears to influence stomatal movements in a yet to be discovered mechanism that could involve a common metabolic component involved in stomatal closure.

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Figure legends

Figure 1. A simplified scheme of the photorespiratory cycle with SHMT1 highlighted.

In a mitochondrion, the serine hydroxymethyl transferase 1 (SHMT1) catalyzes the conversion of glycine and 5,10-methylene-tetrahydrofolate (5,10-CH₂-THF), produced by the glycine decarboxylase complex (GDC), to serine and THF. Abbreviation: RuBisCO, Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase.

Figure 2. Characterization of *shm1-1* complemented lines *Compl-SWT*, *Compl-S31A* and *Compl-S31D*.

(A) Schematic diagram of T-DNA organization and location of site-directed mutagenesis positions in the SHMT1 sequence. Conversions of phospho-S31 codon (TCT) to alanine (GCT) and aspartic acid (GAT) in *Arabidopsis* SHMT1 wild-type cDNA were performed by oligonucleotide-directed mutagenesis. Mutated nucleotides are underlined. RB and LB, right and left T-DNA border regions; *SHMT1-Pro*, SHMT1 promoter region sequence; T35S, cauliflower mosaic virus 35S terminator; Hyg, hygromycin resistance gene. (B) Growth

phenotype of Col-0 (control), *shm1-1* mutant and complemented lines (*Compl-SWT*, *Compl-S31A*, *Compl-S31D*) grown on soil in ambient air (400 $\mu\text{L.L}^{-1}$ CO_2). The photograph was taken after a 4-week growth period under short-day conditions. (C) and (D) Col-0 (control), *shm1-1* mutant and complemented lines (*Compl-SWT*, *Compl-S31A*, *Compl-S31D*) were grown in short day and in high- CO_2 condition for 4-weeks. Total soluble proteins were extracted from rosette leaves to analysis SHMT1 protein level (C) and SHMT activity (D). SHMT1 protein level was analyzed by western-blot using potato SHMT1 antibodies and actin was used as a loading control. SHMT activity was assayed by following the production of glycine by HPLC. Values are means $\pm$ SD (n = 3 independent experiments including at least 5 plants for each experiment). Statistical significance was determined by a Student's *t*-test ($P < 0.05$).

Figure 3. Salt sensitivity of air-grown complemented lines is reduced under high- CO_2 growth conditions.

Growth phenotype of Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* seedlings in response to salt stress. Seedlings were grown for 1 week on vertical $\frac{1}{2}$ MS plates and transferred to new $\frac{1}{2}$ MS medium with or without either 50 mM NaCl or 100 mM mannitol for a further 21 or 25 days. (A) Air-grown seedlings, (C) HC grown (3000 $\mu\text{L CO}_2.\mu\text{L}^{-1}$) seedlings. (B) and (D) show the FW values of seedlings shown in (A) and (C), respectively. Values are means $\pm$ SD (n>10 seedlings). All experiments were repeated three times. Statistical significance was determined by a Student's *t*-test (* $P < 0.05$; *** $P < 0.001$).

Figure 4. SHMT1 protein stability is altered in *Compl-S31D* plants in response to salt stress.

Seven-day-old *in vitro* grown seedlings of Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* were transferred to $\frac{1}{2}$ MS plates with or without 50 mM NaCl for 3 weeks, then leaf total soluble proteins were extracted. (A) Leaf SHMT activity was measured by following glycine using an HPLC-based method. Values are means $\pm$ SD (n=3 independent experiments). (B) Leaf SHMT1 protein abundance was determined by western-blot analyses using potato SHMT1 antibodies. Coomassie blue stained RuBisCO large subunit was used as a protein loading control. (C) SHMT1 and RuBisCO large-subunit signal intensities were quantified using Image J software. SHMT1 protein levels were normalized to RuBisCO large-subunit amounts, and relative protein levels of *Compl-SWT-5* without NaCl treatment were set to 1. Values are means $\pm$ SD (n = 3 independent experiments). Statistical significance was

determined by a Student's *t*-test. Asterisks indicate significant differences compared to Col-0 under the same condition (**P* < 0.05); black triangles indicate significant differences compared with the same genotype (▲ *P* < 0.05).

Figure 5. Metabolic response to salt stress of *Compl-S31D* line differs from Col-0, *Compl-SWT-5* and *Compl-S31A-7* lines.

Plants were grown as described in Figure 2A on ½ MS with or without 50 mM NaCl. Relative metabolite contents (mean ±SD, n=4 independent samples) are given for whole rosette leaves of Col-0 (black), *Compl-SWT-5* (white), *Compl-S31D-5* (light grey), *Compl-S31A-7* (dark grey) with the corresponding Col-0 control content (without NaCl) set to 1. Histograms show metabolites that were significantly different in control (½MS) and NaCl (NaCl) conditions in *Compl-SWT-5*, *Compl-S31D-5* or *Compl-S31A-7* lines compared to Col-0 under the same condition (marked with an asterisk *) or compared with the initial value of the same genotype (marked with ▲). Statistical significance was determined by a Student's *t*-test (*P*<0.05). See also Table S3.

Figure 6. Salt stress induced H₂O₂ levels.

Two-week-old Col-0, *Compl-SWT-5*, *Compl-S31D-5*, and *Compl-S31A-7* seedlings were treated with or without 150mM NaCl for 14 h, and H₂O₂ levels were (A) observed by DAB staining and (B) quantified using a fluorescent assay. Values are means ±SD (n = 3 independent experiments). Statistical significance was determined by a Student's *t*-test (**P*<0.05).

Figure 7. *Compl-S31D-5* stomata show an altered closure response to ABA and salt treatments.

Five-week-old plants (Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7*) were pretreated under light for 2.5 h to induce stomatal opening and then they were watered with or without 300 mM NaCl for 4 h. Stomatal apertures of epidermal peels were measured before and after the NaCl treatment. (A) Micrographs of NaCl-induced stomatal closure of Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7*. (B) Measured stomatal aperture size (pore width/length) with or without NaCl treatment. Values are means ±SD (n>60). Experiments were repeated at least three times. Statistical significance was determined by a Student's *t*-test (*P*<0.05). Leaf epidermal strips of Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* were incubated in stomatal opening buffer under light for 4 h and then further incubated in the

same buffer containing 50 μ M ABA for another 4 h. Stomatal pore areas were measured before and after ABA treatment. (C) Micrographs of ABA-induced stomatal closure of Col-0, *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7*. (D) Measured stomatal pore area before or after ABA treatment. Values are means $\pm$ SD (n>60) and experiments were repeated at least three times. Statistical significance was determined by a Student's *t*-test (P<0.05).

Figure 8. Relative expression levels of several ABA-responsive genes in *Compl-SWT-5*, *Compl-S31D-5* and *Compl-S31A-7* in response to salt treatment.

Two-week-old Arabidopsis seedlings were transferred to liquid medium lacking or containing 50mM NaCl for 6 h. Gene expression levels were measured by quantitative RT-PCR. Values are means $\pm$ SD (n=3 independent experiments). *Actin2* was used as an internal control, and expression levels were normalized to Col-0 without salt treatment. *P5CS1*, Pyrroline-5-carboxylate synthase 1; *RD29A*, Responsive to desiccation 29A; *RD29B*, Responsive to desiccation 29B; *RAB18*, Responsive to ABA 18; *RD22*, Responsive to desiccation 22; *COR15A*, Cold-regulated 15A; *NCED3*, 9-*cis* epoxycarotenoid dioxygenase 3. Statistical significance was determined by a Student's *t*-test and asterisks indicate significant difference compared to the control line for each condition (*P<0.05).

Amino acids

Organic acids

Sugars

Miscellaneous

Col-0 Compl-SWT-5 Compl-S31D-5 Compl-S31A-7

Relative fold change

A **B**