

The Photorespiratory Arabidopsis *shm1* Mutant Is Deficient in *SHM1*^{[W][OA]}

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Mitochondrial serine hydroxymethyltransferase (SHMT), combined with glycine decarboxylase, catalyzes an essential sequence of the photorespiratory C₂ cycle, namely, the conversion of two molecules of glycine into one molecule each of CO₂, NH₄⁺, and serine. The Arabidopsis (*Arabidopsis thaliana*) mutant *shm* (now designated *shm1-1*) is defective in mitochondrial SHMT activity and displays a lethal photorespiratory phenotype when grown at ambient CO₂, but is virtually unaffected at elevated CO₂. The Arabidopsis genome harbors seven putative *SHM* genes, two of which (*SHM1* and *SHM2*) feature predicted mitochondrial targeting signals. We have mapped *shm1-1* to the position of the *SHM1* gene (At4g37930). The mutation is due to a G → A transition at the 5' splice site of intron 6 of *SHM1*, causing aberrant splicing and a premature termination of translation. A T-DNA insertion allele of *SHM1*, *shm1-2*, and the F₁ progeny of a genetic cross between *shm1-1* and *shm1-2* displayed the same conditional lethal phenotype as *shm1-1*. Expression of wild-type *SHM1* under the control of either the cauliflower mosaic virus 35S or the *SHM1* promoter in *shm1-1* abrogated the photorespiratory phenotype of the *shm* mutant, whereas overexpression of *SHM2* or expression of *SHM1* under the control of the *SHM2* promoter did not rescue the mutant phenotype. Promoter-β-glucuronidase analyses revealed that *SHM1* is predominantly expressed in leaves, whereas *SHM2* is mainly transcribed in the shoot apical meristem and roots. Our findings establish *SHM1* as the defective gene in the Arabidopsis *shm1-1* mutant.

Photorespiration is caused by the dual affinity of Rubisco for both CO₂ and molecular oxygen (Bowes et al., 1971; Ogren and Bowes, 1971; Bowes and Ogren, 1972; Ogren, 1984). Whereas the carboxylation of the acceptor ribulose 1,5-bisphosphate (RuBP) leads to the production of two molecules of 3-phosphoglycerate (two C₃ moieties) that can both be reconverted into RuBP by the Calvin cycle, the addition of molecular oxygen to RuBP (oxygenation) yields one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate (a C₂ unit). The regeneration of 2-phosphoglycolate to 3-phosphoglycerate involves a reaction sequence that is known as the oxidative C₂ cycle or the photorespiratory carbon cycle (termed C₂ cycle here-

after). This cycle involves three organelles (chloroplasts, peroxisomes, and mitochondria) and one molecule each of CO₂ and NH₄⁺ are liberated during the conversion of two molecules of 2-phosphoglycolate into one molecule of 3-phosphoglycerate. These CO₂ and NH₄⁺ molecules have to be refixed by Rubisco and the glutamine synthase/GOGAT system, respectively.

The C₂ cycle was elucidated in the 1970s and the enzymatic steps involved as well as some salvage pathways are well established (Leegood et al., 1995; Douce and Neuburger, 1999; Wingler et al., 1999, 2000). Mutants in the C₂ cycle have contributed much to our understanding of this important biochemical pathway (Somerville and Ogren, 1982; Leegood et al., 1995; Somerville, 2001). These photorespiratory mutants display a conditional lethal phenotype, which means they are unable to thrive at ambient conditions whereas apparently they are not affected in conditions that suppress photorespiration, such as high CO₂ (Somerville and Ogren, 1982; Blackwell et al., 1988).

The Arabidopsis (*Arabidopsis thaliana*) Ser hydroxymethyltransferase (SHMT) mutant *stm* was one of the first photorespiratory mutants described by Somerville and Ogren (1981) and was later renamed *shm* to avoid confusion with the mutant *shoot meristemless* (Barton and Poethig, 1993). The defective gene in *shm*, hereafter termed *shm1-1*, has not been identified until now.

In the initial study of this Arabidopsis mutant, no SHMT activity could be determined in *shm1-1*

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leaf mitochondria and foliar Gly levels under photorespiratory conditions were 40-fold higher in *shm1-1* in comparison to the wild type (Somerville and Ogren, 1981). These two observations indicated that a defective mitochondrial SHMT gene accounts for the photorespiratory phenotype of the *shm1-1* mutant.

Together with the Gly decarboxylase complex, SHMT is involved in the reversible interconversion of Ser and Gly and both enzymes are closely associated with each other. During the operation of the C₂ cycle, one molecule of Gly is first decarboxylated and subsequently deaminated in the Gly decarboxylase complex yielding CO₂, NH₄⁺, and the C₁ donor, 5,10-methylene tetrahydrofolate (THF), which is used by SHMT to transfer the activated C₁ unit onto another molecule of Gly (Douce and Neuburger, 1999). In leaves of C₃ plants, mitochondrial SHMT is predominantly involved in the C₂ cycle, as evidenced by the conditional lethal photorespiratory phenotype of *shm1-1* (Somerville and Ogren, 1981). Very recently, however, a weak *shm1* allele was isolated, which we will address as *shm1-3* (Moreno et al., 2005). Homozygous *shm1-3* mutants exhibit chlorotic lesions but are viable in ambient conditions (Moreno et al., 2005). Because of its compromised C₂ cycle, *shm1-3* overproduces reactive oxygen species and is thus more susceptible to salt stress and pathogens (Moreno et al., 2005).

Molecular studies of the *shm1-1* mutant revealed that *SHM* transcripts of apparently normal length accumulated in the mutant, although these transcripts

were more abundant at elevated CO₂ conditions in the mutant than in the wild type (Beckmann et al., 1997). In silico analyses showed that the Arabidopsis genome harbors seven *SHM* genes, two of which encode gene products that are predicted to be targeted to the mitochondria (McClung et al., 2000; Bauwe and Kolukisaoglu, 2003). *AtSHM1* appears to encode the major SHMT isozyme in Arabidopsis leaves and its transcript accumulation is controlled by light and the circadian clock (McClung et al., 2000). Thus, *AtSHM1* was considered a good candidate for the defective gene in the *shm1-1* mutant (McClung et al., 2000), but this hypothesis has not been directly tested.

In this study, we report on the positional cloning and the molecular characterization of the defective gene in *shm1-1*, on the isolation of a new allele, *shm1-2*, and on the complementation of the *shm1-1* mutant with the wild-type *SHM1* allele. In addition, we show that the gene encoding the second putatively mitochondrial-targeted SHM isozyme in Arabidopsis, *SHM2*, is predominantly expressed in roots and the shoot apical meristem (SAM), whereas *SHM1* encodes the major isoform in leaves. Surprisingly, expression of *SHM2* in *shm1-1* under the control of either the 35S or the *SHM1* promoter failed to complement the photorespiratory *shm* phenotype, indicating that either *SHM2* does not encode a fully functional SHMT protein or the protein is not targeted to mitochondria. Our findings unequivocally demonstrate that At4g37930 (*AtSHM1*) is crucial for plant growth in ambient air and for proper function of the C₂ cycle.

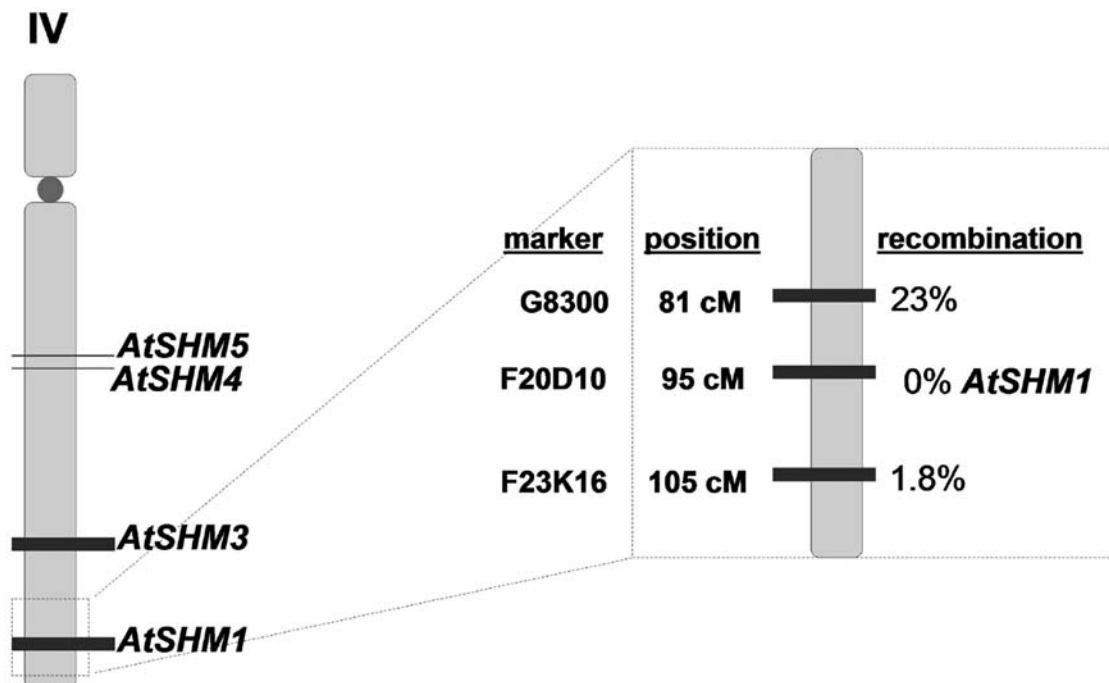


Figure 1. Cartoon depicting the map position of the *shm1-1* locus on the recombinant inbred map (Lister and Dean, 1993) according to our mapping data. A total of 186 F₂ individuals of a cross between the *shm1-1* mutant (background Col-0) and *Ler* showing a photorespiratory phenotype were scored for the cosegregation of 23 CAPS markers (see Supplemental Table II) with the photorespiratory phenotype as described in "Materials and Methods."

RESULTS

Positional Cloning of the Defective Gene in the *shm1-1* Mutant

Somerville and Ogren (1981) have demonstrated that the Arabidopsis mutant *shm1-1* lacks mitochondrial SHMT activity and therefore displays a photorespiratory phenotype. The Arabidopsis genome encodes seven putative SHMT proteins, two of which

(AtSHM1 and AtSHM2) are presumably localized in the mitochondrial matrix as indicated by the presence of a putative mitochondrial targeting signal (McClung et al., 2000). The subcellular localization was predicted by computer algorithms and not further supported by experimental evidence (McClung et al., 2000; Bauwe and Kolukisaoglu, 2003). In addition, conceptual translations of genes in the Arabidopsis genomes are frequently hampered by the inclusion or omission of

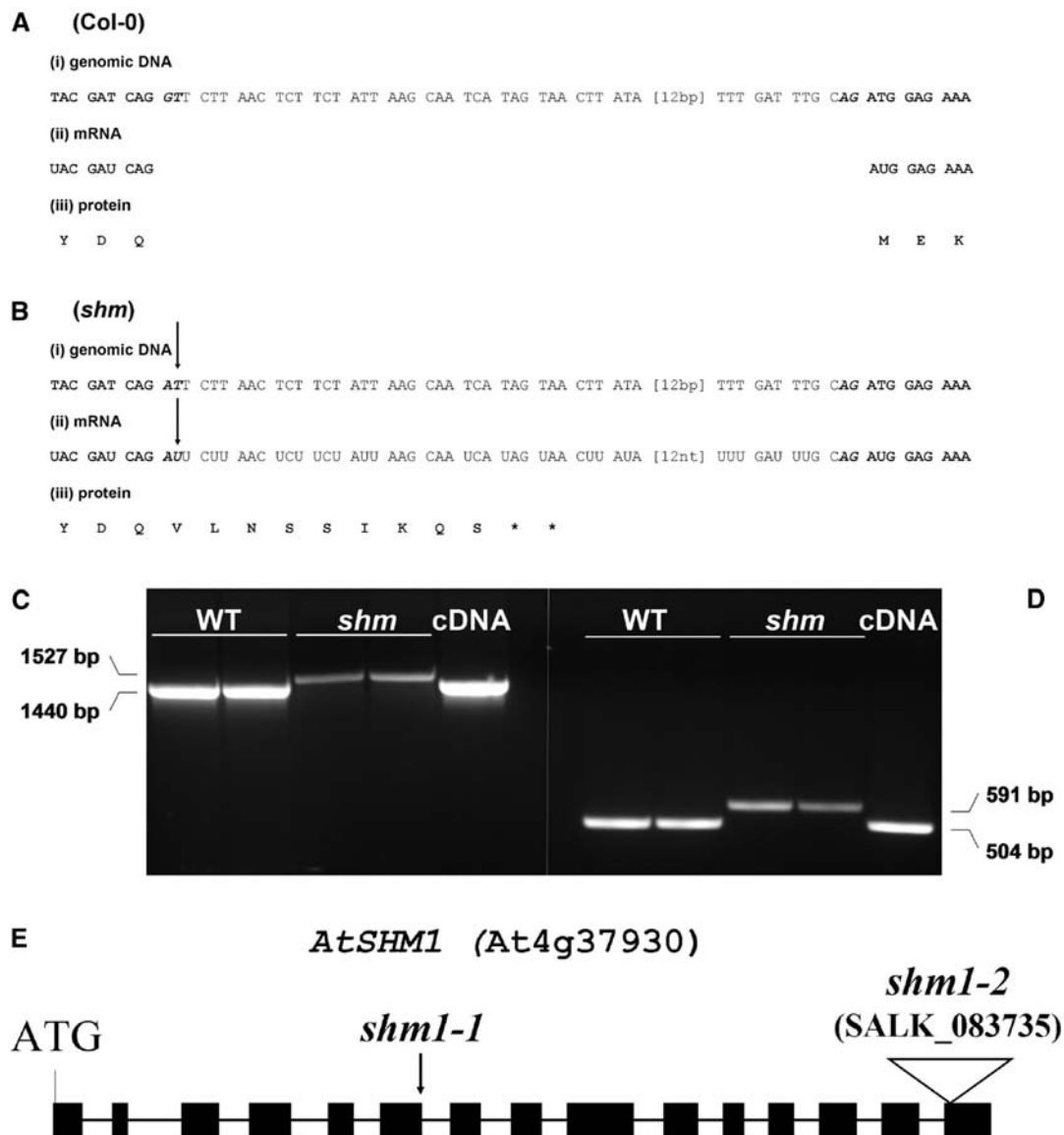


Figure 2. Comparison between the genomic DNA (i), the mRNA (ii), and the derived protein sequence (iii) of wild-type (A) and *shm1-1* mutants (B). Only the sequence between the end of exon 6 (start-ATG + 1,272 bp) and the beginning of exon 7 (start-ATG + 1,376 bp) is shown. Bold, Exon; italics, intron; bold italics, conserved splice motif. The arrows indicate the point mutation in *shm1-1*. Asterisk (*), Translational stop. C, RT-PCR products from wild-type and *shm1-1* mRNA obtained with primers prSHM4 and prSHM9 (see Table I). D, Primers prSHM5 and prSHM6 (see Table I). Two independent RNA preparations of each line were taken for the RT-PCR reactions. From left to right, Two lanes wild-type (Col-0); two lanes *shm1-1* mutant; one lane cDNA. The RT-PCR fragment sizes are indicated beside the image. Plasmid containing the SHM1 expressed sequence tag clone 148C5T7 was used as a positive control in the PCR reaction. E, Cartoon showing *AtSHM1* gene structure and the *shm1-2* T-DNA insertion site in the last of the 15 *SHM1* exons. As a reference, the point mutation in *shm1-1* is indicated by an arrowhead.

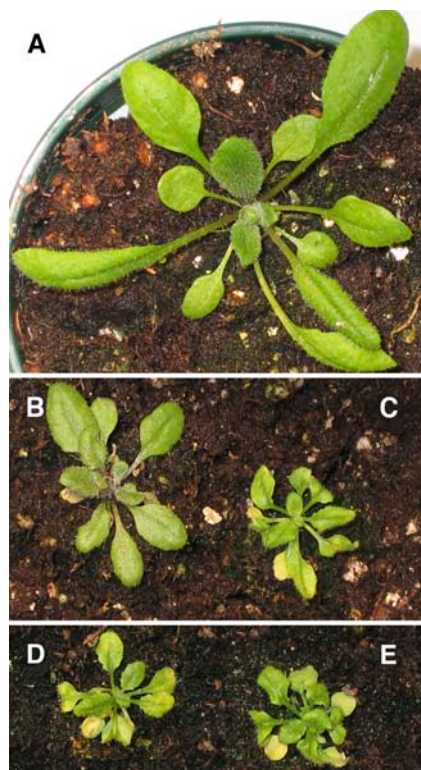


Figure 3. Phenotypes of representative Col-0 wild-type (A), *shm1-2* (C), *shm1-1* (D), and *shm1-1* × *shm1-2* F₁ (E) plants after 20 d in ambient air. Plants had been grown at 3% CO₂ for 1 week before they were shifted to air. B, Representative *shm1-2* individual that was constantly grown at 3% CO₂. Please note that all images represent the same scale.

exon sequences, thus raising the possibility that additional, unrecognized mitochondrial SHMT isozymes are encoded by the Arabidopsis genome. To narrow down the number of candidates for the defective gene in *shm1-1*, the mutation was mapped using a cleaved amplified polymorphic sequence (CAPS) marker approach (Konieczny and Ausubel, 1993). To this end, a mapping population was developed from a genetic cross between *shm1-1*, which is in the ecotype Columbia (Col-0) background, and Landsberg *erecta* (*Ler*). For F₂ individuals, 186 of 837 (22.1%) displayed a photorespiratory phenotype, and these plants were selected for the mapping procedure. Using all 186 F₂ individuals showing the mutant phenotype, we mapped the *shm1* locus to 95 cM on chromosome IV (Fig. 1) of the recombinant inbred map (Lister and Dean, 1993). *AtSHM1* (At4g37930) is located at this map position and a CAPS marker (F20D10) was developed for this locus. No recombination of the marker F20D10 and the *shm1* locus was observed (Fig. 1), indicating that *AtSHM1* is deficient in *shm1-1*.

A Mutation in the 5' Splice Site of Intron 6 of *AtSHM1* Causes Aberrant Splicing of the *SHM1* mRNA in *shm1-1*

Genetic mapping strongly indicated that *AtSHM1* is the affected gene in *shm1-1*. Therefore, the *SHM1* gene

was sequenced in the mutant and a G → A transition was detected in the consensus sequence of the 5' splice donor site of intron 6 in the mutant allele (Fig. 2B). To check whether the mutation of the consensus splice site would cause mis-splicing of the mRNA, the corresponding mRNA from the *shm1-1* mutant was amplified by reverse transcription (RT)-PCR and sequenced. Sequencing demonstrated that the mutation led to aberrant splicing of intron 6 during *shm1-1* mRNA maturation (Fig. 2B), producing a slightly longer *shm1-1* mRNA (Fig. 2, C and D). Although intron 6 spans 87 bp and would thus not cause a translational frameshift in the mutant, it also contains several in-frame translational stop codons (Fig. 2B). Hence, a premature termination of *AtSHM1* mRNA translation is to be expected, which would account for the absence of mitochondrial SHMT activity in *shm1-1*.

A Presumptive Loss-of-Function T-DNA Allele *shm1-2* Also Exhibits a Photorespiratory Phenotype

We have isolated a T-DNA insertion allele for *AtSHM1* from the SALK collection (SALK_083735), hereafter termed *shm1-2*, which was tested for allelism with *shm1-1*. The T-DNA insertion in *shm1-2* is located in the last of the 15 exons of the *SHM1* gene (Fig. 2E). The *shm1-2* T-DNA allele has recently been used for an allelism test with a third, weak *shm* allele (referred to as *shm1-3* in this study; originally designated *shmt1-1* by Moreno et al. (2005). According to Moreno et al. (2005), *shm1-2* never reached maturity and exhibited a chlorotic and dwarf phenotype. In our hands, *shm1-2* lines homozygous for the T-DNA insertion uniformly showed a photorespiratory phenotype of chlorosis at ambient CO₂ levels that was fully rescued at 3% CO₂ (Fig. 3, B and C), which could also be inferred from the description by Moreno et al. (2005). SHMT activity in crude leaf extracts of both *shm1-1* and *shm1-2* was approximately 10% of the wild type (Table I). This indicates that (1) *AtSHM1* is the predominant SHMT isoform in leaves and (2) *shm1-1* and *shm1-2* are loss-of-function alleles. Homozygous *shm1-2* was crossed

Table I. SHMT activity in *Arabidopsis* leaf extracts

Total SHMT activity was assayed in leaf extracts from the wild type (Col-0), the *shm1-1* mutant, two independent primary *shm1-1-35S:SHM1* transformants (2T1 and 3T1), homozygous *shm1-2*, and F₁ progeny from a cross between *shm1-1* and *shm1-2*. Results represent the means of 12 replicates ± se that were obtained in four independent experiments.

Genetic Background	SHMT Activity
	nmol mg ⁻¹ min ⁻¹
Col-0	1.64 ± 0.07
<i>shm1-1</i>	0.18 ± 0.01
2T1	1.60 ± 0.06
3T1	1.49 ± 0.08
<i>shm1-2</i>	0.20 ± 0.02
<i>shm1-2</i> × <i>shm1-1</i>	0.22 ± 0.02



Figure 4. Complementation analysis of *shm1-1* with chimeric *SHM* constructs and by constitutive overexpression of *SHM1* and *SHM2*. A, Col-0 control, *shm1-1* mutant plants were transformed with CaMV 35S:*SHM1* (B), *pSHM1:SHM1* (C), *pSHM1:SHM2* (D), empty-vector pH2GW7.0 vector only (E), CaMV 35S:*SHM2* (F), *pSHM2:SHM1* (G), and *pSHM2:SHM2* (H). Representative T₂ individuals are shown after growth for 28 d in ambient air. Please note that the images in A and B are presented at 1.5 × lower magnification.

to *shm1-1* and the resulting F₁ progeny uniformly exhibited a photorespiratory phenotype (Fig. 3E) and similar total SHMT activity to both mutant parental lines (Table I). A T-DNA loss-of-function mutant of *SHM2* (SALK_095881) that lacked detectable *SHM2* mRNA and presumably lacked *SHM2* protein function did not display the conditional lethal photorespiratory phenotype at ambient conditions (data not shown), indicating that *SHM2* is not functionally equivalent to *SHM1*, although the *SHM2* gene product is predicted to be targeted to the mitochondrial matrix (McClung et al., 2000; Bauwe and Kolukisaoglu, 2003).

SHM1 and *SHM2* Are Not Redundant

To assess whether the failure of *SHM2* to complement a lack of *SHM1* function in *shm1-1* is due to different expression patterns of *SHM1* and *SHM2*, we overexpressed both genes in *shm1-1* under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter. In addition, we performed a promoter-swap experiment. The wild-type full-length cDNAs encoded by the *SHM1* (expressed sequence tag 148C5T7) and *SHM2* (C104687; Arabidopsis Biological Resource Center [ABRC]), were expressed either under the control of the constitutive CaMV 35S promoter or approximately 1 kb of their own proximal promoters, or the promoter of the respective other isoform (promoter swap) in stably transformed *shm1-1* mutants. Figure 4 shows representative individuals of all transformants (Fig. 4, B–D and F–H), as well as empty-vector (Fig. 4E) and wild-type (Fig. 4A) controls after growth for 28 d in ambient air. *SHM2* expression failed to rescue the conditional lethal phenotype, regardless of the promoter employed (Fig. 4, D and F). In contrast, expression of *SHM1* under the control of the constitutive CaMV 35S promoter or its endogenous promoter re-

stored growth (Fig. 4, B and C) and total foliar SHMT activity to wild-type levels (Table I). However, transformation of *shm1-1* with *pSHM2:SHM1* did not complement the mutant (Fig. 4G), indicating that the expression pattern and/or strength of the *SHM2* promoter are not sufficient to permit complementation of the mutant phenotype.

To further test this hypothesis, the *Escherichia coli uidA* reporter gene, encoding β-glucuronidase (GUS), was fused to the *SHM1* and *SHM2* promoter fragments used in the complementation study described above and the reporter gene constructs were transformed into Arabidopsis. While *pSHM1* mediated strong GUS activity in the entire shoot, including leaves (Fig. 5A), little GUS activity was detected in the shoots of plants carrying *pSHM2:GUS*, in which GUS activity was restricted to the roots, the SAM, and the first true leaf (Fig. 5, B and C). GUS expression from *pSHM2* was not observed in mature, fully expanded leaves. The reporter gene data are supported by *AtSHM2* transcript profiles generated using the digital northern tool of GENEVESTIGATOR (Zimmermann et al., 2004), indicating that *AtSHM2* is expressed only at very low levels in photosynthetic tissues (data not shown). In addition, transcript corespondence analysis using the Arabidopsis Co-Response Database (Steinhauser



Figure 5. Localization of *SHM1* and *SHM2* promoter activity. T₂ progeny of stably transformed wild-type plants carrying *pSHM1:GUS* (A) or *pSHM2:GUS* (B and C) were stained for GUS activity as described in “Materials and Methods.”

et al., 2004a, 2004b) showed that only *AtSHM1* transcripts, but not those of other putative *SHM* genes in Arabidopsis, show transcriptional corespondence with genes encoding enzymes of the photorespiratory pathway, such as phosphoglycolate phosphatase or hydroxypyruvate reductase (data not shown).

DISCUSSION

The Arabidopsis mutant *shm1-1* was isolated in the early 1980s and since then it has been clear that insufficient mitochondrial SHMT activity accounted for the photorespiratory phenotype of the mutant (Somerville and Ogren, 1981). However, the molecular identity of the *shm1-1* mutation has remained elusive and it had been hypothesized that a locus required for SHMT activity rather than an SHMT structural gene was affected in *shm1-1* (Beckmann et al., 1997; McClung et al., 2000).

This hypothesis seemed evident, since *SHM1* steady-state transcript levels were increased in the *shm1-1* mutant under nonphotorespiratory conditions (Beckmann et al., 1997) and because no apparent difference in transcript size between the mutant and the wild type was observed (Beckmann et al., 1997). Furthermore, the products of at least two *SHM* genes, *SHM1* and *SHM2*, were predicted to be targeted to the mitochondria (McClung et al., 2000). Thus, both genes could potentially play a role in the C_2 cycle, making functional redundancy of the two mitochondrial SHMT isoforms likely.

This study provides unequivocal evidence that mutation in the *shm1-1* mutant indeed affects *SHM1* and that a second putative mitochondrial SHMT encoded by *SHM2* cannot complement loss of *SHM1* function. *SHM1* and *SHM2* are highly similar at the nucleotide and amino acid levels (McClung et al., 2000; Bauwe and Kolukisaoglu, 2003) and are likely to represent the products of a duplication event: Arabidopsis has undergone multiple rounds of polyploidization and a recent estimate is that 27% of the gene pairs formed by polyploidization persist in the genome (Blanc and Wolfe, 2003). However, the majority of these gene pairs have undergone functional divergence (Blanc and Wolfe, 2004), as is apparently the case for *SHM1* and *SHM2*.

Whereas the conditional lethal photorespiratory phenotype of the *shm1-1* mutant could be cured by expression of wild-type *SHM1* under the control of either its own or the constitutive viral CaMV 35S promoter, *SHM1* expression from the *SHM2* promoter failed to rescue the *shm1-1* mutant phenotype (Fig. 4). *SHM2* expression in the mutant background was not able to rescue the mutant, regardless of the promoter used to drive *SHM2* expression (Fig. 4). In addition, strong GUS activity was detected in leaves when GUS expression was driven by the *SHM1* promoter, whereas GUS expression driven by the *SHM2* promoter was restricted to roots, the SAM, and the first true leaves

(Fig. 5). Probing *SHM1* and *SHM2* promoter activities provides a straightforward explanation as to why *SHM1* expression driven by the *SHM2* promoter could not cure the photorespiratory phenotype of *shm1-1* (Fig. 4): The *SHM2* promoter lacks activity in rosette leaves, where photorespiration takes place. Thus, in the case of *SHM1* and *SHM2*, functional divergence has apparently occurred at the promoter level. It can thus be unambiguously concluded that *AtSHM1* is the SHMT coding gene involved in the C_2 cycle. Surprisingly, however, *SHM2* was not able to complement the *shm1-1* phenotype even under the control of a strong promoter. Apparently, functional divergence of *SHM1* and *SHM2* has also occurred at the level of enzymatic activity or subcellular targeting; either *SHM2* does not encode a fully functional SHMT protein or the *SHM2* gene product is not targeted to the mitochondrial matrix. Further studies on the subcellular localization and activity of the SHMT2 protein are in progress to resolve this question.

Together with the recent identification of the peroxisomal Ala:glyoxylate aminotransferase (Liepman and Olsen, 2001, 2003) and D-glycerate 3-kinase (Boldt et al., 2005), the molecular identification of the defective gene in *shm1-1* completes the identification of all genes encoding enzymes known to be involved in the photorespiratory C_2 cycle. However, the C_2 cycle also requires transporters that catalyze the transport of photorespiratory intermediates across the membranes of chloroplasts, peroxisomes, and mitochondria. With the exception of the plastidic oxoglutarate/malate and Glu/malate translocators (Weber et al., 1995; Weber and Flügge, 2002; Renné et al., 2003) none of these transporters are known (Linka and Weber, 2005). It remains a challenge for the future to identify all genes involved in the C_2 cycle.

MATERIALS AND METHODS

Seed Material

Seeds of the *shm1-1* mutant (CS8010) and of the SALK T-DNA insertion line SALK_083735 (*shm1-2*) were obtained from the ABRC.

Plant Growth

Seeds were sterilized as described by Clough and Bent (1998) and germinated at room temperature in a 12-h/12-h light/dark cycle on one-half-strength Murashige and Skoog medium in 3% CO_2 at a photon flux density of approximately $100 \mu mol m^{-2} s^{-1}$. Plantlets were transferred to soil after the first four primary leaves had emerged and the growth cycle was allowed to complete under the same conditions.

Mapping of the *shm1-1* Locus

The *shm1-1* mutant (Col-0) was crossed to *Ler*, the F_1 was self-fertilized, and the resulting F_2 mapping population (837 F_2 individuals) was grown for 7 weeks at $1,300 \mu L mL^{-1} CO_2$. The population was transferred to ambient conditions, the photorespiratory phenotype was scored 4 d after the transfer, and the cosegregation of the *shm1-1* phenotype with 23 CAPS markers (obtained from The Arabidopsis Information Resource [TAIR]; see also www.arabidopsis.org, unless stated otherwise; see Supplemental Table II) was determined.

Constitutive Overexpression of *SHM1* and *SHM2* and Complementation Studies with Chimeric *SHM1* and *SHM2* Expression Constructs

For constitutive overexpression of wild-type *SHM1* in the *shM1-1* mutant background, a *Bam*HI-*Kpn*I fragment of expressed sequence tag 148C5T7 (GenBank accession no. T75910; ABRC), encoding the full-length *SHM1* cDNA, was cloned into a modified pGREENII bar vector (Hellens et al., 2000) in which the multiple cloning site was replaced by the *Eco*RI-*Hind*III fragment of the 35S promoter/nopaline synthase terminator cassette derived from pBIN-AR (Höfgen and Willmitzer, 1990). The RT-PCR product corresponding to the full-length *SHM2* cDNA was cloned in pGEMT easy, the resulting *Eco*RI fragment was cloned in pENTR-A vector, and then moved to the modified pB7GWIWG2I destination vector. *shM1-1* mutants were transformed with the construct by the floral-dip method (Clough and Bent, 1998). The T₁ progeny were grown at 0.3% CO₂ for 14 d before the plantlets were selected for the bar marker by spraying them with a Basta solution (0.025% [w/v] phosphinotricine, 0.1% [v/v] Tween 20) twice a week. The Basta-resistant plants were then surveyed for the absence of the photorespiratory phenotype after transfer to ambient air.

Chimeric constructs were generated by Gateway technology (Invitrogen). A *Pst*I and a *No*I restriction site were added to the N and C terminus of the *SHM1* cDNA; a *Pst*I and an *Eco*RI site were added to the N and C terminus of the *SHM2* cDNA, respectively, using the primers: SHM1*Pst*I Fwd (5'-CCATTTGGTATTCTCGAGTCTCTCTCTCGTTCATG), SHM1*No*I Rev (5'-ATATCTCGAGTGC GGCCGCCCTTAGTCTTGTACTTCATGGTTTC), SHM2*Pst*I Fwd (5'-AATCGCAGTCTCTCGAGAGAAACAGAGAAGACGATAGAT), and SHM2*No*I Rev (5'-ATATCTCGAGTGC GGCCGCCGCCGCTACTCTTGATCTCATCGTCTCTTC).

Upstream regions of 925 bp from the *SHM1* and 1,234 bp of the *SHM2* gene were amplified by PCR on Col-0 genomic DNA using the primers: pSHM1*Bam*HI (5'-CTTTTAAATTGATCTGGATCCTTACAAAACATGCATGCACCATT-3'), pSHM1*Pst*I (5'-CATGAACGAGAGAGAAGACATGCAGAAATAACAAAATTGG-3'), pSHM2*Pst*I (5'-TCTTCTCTGTTTCTCTGCA-GTGAGTGC GATTA-3'), and pSHM2*Eco*RI (5'-AATTGCTTCATTTTCGGA-ATCCACAAGCTTCTCTTTTTTAA-3').

Following restriction digestion with the appropriate endonucleases, different combinations were ligated into the pENTR vector, transformed into *Escherichia coli*, and transferred to the modified pH2GW7.0 gateway vector (without 35S promoter) by LR clone reactions. *Agrobacterium tumefaciens* strain AGL1 was transformed with the resulting plasmids by floral dip as described above. The T₁ generation was grown at 3% CO₂ for 14 d during selection for hygromycin-resistant individuals and Hyg plantlets were then assessed for the presence of the transgene by PCR (as described below) and for the photorespiratory phenotype as described above.

Screening of Transgenic Plant Populations by PCR

Genomic DNA was extracted from Arabidopsis (*Arabidopsis thaliana*) leaves as described by Edwards et al. (1991) and PCR analysis of transgenic progeny was conducted according to standard protocols using appropriate primer pairs.

To identify *SHM1* T-DNA insertion mutants, we used the gene-specific primer Shmt1-TDNA rvs (5'-GTTACAGCTTTCATCATCCACAC-3') together with the T-DNA left-border-specific primer LBb1 (5'-GCGTGGACCG-CTTGCTGCAACT-3').

Promoter-uidA Fusions

For promoter-uidA fusions, the promoter regions (925 bp for pSHM1, 1,234 bp for pSHM2) were amplified using two-step PCR reactions: The first step was performed with specific primers containing 12 nucleotides of the *attB* sites (in capitals), as well as gene-specific nucleotides (lowercase): SHM1-B1guspro (5'-AAAAAGCAGGCTCCcttgatgttcacaaactgc-3'), SHM1-B2guspro (5'-AGAAAGCTGGGTCttctgtaaacctctctct-3'), SHM2-B1guspro (5'-AAAAAGCAGGCTCCctgagattacaagctctct-3'), and SHM2-B2guspro (5'-AGAAAGCTGGGTCttctctatctatctctctct-3').

In the second PCR step, the universal *attB* adapter primers were used to amplify the product produced in step 1. The resulting PCR products were moved into pDONR207 by BP clone reactions (Invitrogen). The promoters were then transferred to the pBGWFS7 destination vector using the LR clone reaction. T₁ populations were selected with Basta, as described above for the

chimeric constructs and bar plants were examined for the presence of the transgene as described below.

Histochemical Analysis of GUS Activity

Plant tissues were incubated in GUS assay solution (50 mM sodium phosphate, pH 7.2, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% methanol, and 2 mM 5-bromo-4-chloro- β -glucuronide) at 37°C for 12 to 16 h, essentially as described by Jefferson et al. (1987; Jefferson, 1989). Slight vacuum was applied to facilitate substrate infiltration. Chlorophyll-containing tissue was cleared in 70% ethanol for photographic analysis.

Sequencing of Genomic DNA

Genomic DNA from wild-type and *shM1-1* inflorescences was isolated using a urea-based buffer (Liu et al., 1995), 20 ng of the genomic DNA preparations were subjected to PCR amplification with different sets of *AtSHM1*-specific primers (see Supplemental Table I) according to standard protocols and the obtained fragments were subcloned into pGEMT-Easy (Promega). Standard dye-termination sequencing reactions containing 1 μ g of vector with subcloned fragments and 30 pmol of primer were resolved on an ABI Prism 3100 sequencer.

Sequencing of *SHM1* mRNA

RNA from wild-type and *shM1-1* mutant Arabidopsis leaves was isolated by the Z6 buffer method (Logemann et al., 1987) and aliquots of the RNA preparations were reverse transcribed using the ImProm reverse transcriptase kit (Promega) following the manufacturer's instructions. The *SHM1* cDNAs were amplified by PCR with two different primer sets, prSHM4-prSHM9 and prSHM5-prSHM6 (see Table I) and sequenced as described previously.

Assay of SHMT Activity

Crude extracts were prepared by grinding approximately 400 mg of leaf tissue in 300 μ L of extraction buffer (50 mM phosphate buffer, 1 mM β -mercaptoethanol, and 2.5 mM EDTA) and the extracts were clarified by centrifugation at 20,000g for 10 min. SHMT activity was tested by following the conversion of radioactive carbon from Ser to methylene THF (Geller and Kotb, 1989). The assay was performed with 0.25 mM pyridoxal 5' phosphate, 2 mM THE, 0.4 mM Ser [³H] Ser (33 Ci mmol⁻¹), and the crude extract in a final volume of 100 μ L. The enzyme assays were performed at 37°C for 20 min. Twenty-five microliters of the reaction mixture were streaked onto Whatman DE.81 paper. After drying the filter, unreacted Ser was removed by washing the filter three times for 20 min with 20 mL of water. The radioactivity associated with methylene THF was measured by liquid scintillation counting.

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