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ORIGINAL ARTICLE

Light and sucrose up-regulate the expression level of Arabidopsis cystathionine γ-synthase, the key enzyme of methionine biosynthesis pathway

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Abstract The sulfur-containing essential amino acid methionine controls the level of important metabolites and processes in plants. In addition, methionine levels limit the nutritional quality of many crop plants. The level of methionine is regulated mainly by cystathionine γ-synthase (CGS), the first enzyme committed to its biosynthesis. Within our efforts to reveal factors that regulate CGS and methionine content in plants, we have analyzed how light regulates the transcript and protein level of Arabidopsis CGS (AtCGS). The expression of AtCGS is up-regulated in the light and reduced in the dark, independent of the diurnal cycle. Using tobacco plants overexpressing AtCGS, we have found that the light sensitive motives of the At-CGS gene are found within the coding sequence of AtCGS and not in its promoter, terminator or the untranslated regions of the gene. Sucrose can partially mimic the effect of light in dark grown plants while the addition of nitrogen and sulfur sources does not have any effect. The kinetics of the changes in the expression level of AtCGS suggest that its level can be maintained during extended darkness, or even when the sucrose content is reduced, such as during abiotic stresses. The up-regulation of AtCGS by light is in agreement with previous studies showing that other enzymes regulating the level of the carbon/amino skeleton and the sulfur group of Met, are up-regulated by light. The results indicate that light and dark participate in the regulation of the carbon/amino skeleton flux in the synthesis of amino acids of the aspartate family.

Keywords Cystathionine γ-synthase · Methionine · Light regulation · Sucrose regulation · Aspartate family · Threonine synthase · Threonine

Introduction

Methionine (Met) is a fundamental metabolite in plant cells. Apart from its role as a protein constituent and its central role in the initiation of mRNA translation, Met indirectly regulates a variety of cellular processes as the precursor of S-adenosylMet (SAM). SAM is the primary biological methyl group donor for a number of essential cellular processes, including DNA methylation, cell division, cell wall biosynthesis and chlorophyll biosynthesis. SAM is also the precursor of ethylene, polyamines, nicotinamide, biotin and phytosiderophores (Amir 2008, 2010; Hesse et al. 2004; Roje 2006). Met itself also serves as a donor for secondary metabolites mainly through S-methylMet (SMM), which also serves as a mobile and storage form of Met (Ranocha et al. 2001), and also as the precursor for the synthesis of glucosinolates (Falk et al. 2007). In addition to Met's metabolic role in plants, it also plays a major role in human and monogastric livestock nutrition as a sulfur-containing essential amino acid that exists in very low levels in plants, thus limiting the nutritional quality of crop plants (Amir et al. 2012; Hesse and Hoefgen 2003; Hesse et al. 2004; Galili and Amir 2013; Ravanel et al. 1995).

Y. Hacham and I. Matityahu contributed equally to this work.

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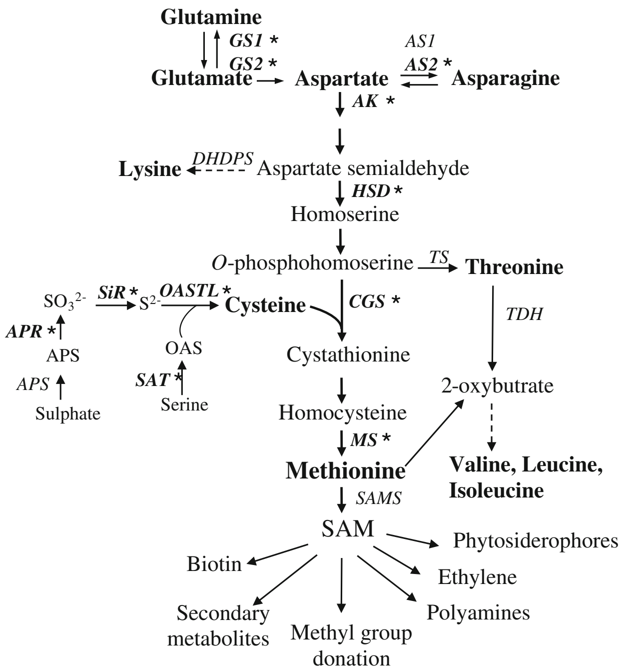

Fig. 1 Scheme of the aspartate family biosynthesis pathway and Met metabolism. Only some of the enzymes and metabolites are indicated. *Broken arrows* represent more than one enzymatic step. *SAM* S-adenosylMet, *APS* adenosine 5′-phosphosulfate, *OAS* *O*-acetyl serine. *Italics letter* indicates the name of enzymes: *GS* glutamine synthase, *AS* asparagine synthase, *AK* aspartate kinase, *HSD* homoserine dehydrogenase, *TS* threonine synthase, *CGS* cystathionine γ-synthase, *MS* Met synthase, *SAMS* SAM synthase, *DHDPS* dihydrodipicolinate synthase, *APS* ATP sulfurylase, *APR* adenosine 5′-phosphosulfate reductase, *SiR* sulfite reductase, *OASTL* *O*-acetylserine (thiol) lyase, *SAT* serine acetyltransferase. The enzymes that according to the literature are up-regulated by light are indicated in *bold letters* and *asterisks*

Despite the major biological and nutritional importance of Met, our understanding of its regulation is far from complete. It was proposed that in Arabidopsis, Met level is regulated mainly by the expression level of cystathionine γ-synthase (CGS), the first committed enzyme of the Met biosynthesis pathway (Kim et al. 2002; Ravanel et al. 1995), which combines the carbon/amino skeleton derived from aspartate, with the sulfur moiety derived from cysteine (Fig. 1). Indeed, over-expression of Arabidopsis CGS (AtCGS) in Arabidopsis, tobacco, potato and alfalfa leads to a significant increase in Met content (Avraham et al. 2005; Di et al. 2003; Hacham et al. 2008; Kim et al. 2002; Kreft et al. 2003). Studies performed with AtCGS have shown that its expression level is tightly regulated, as expected by the major role that Met has in plant metabolism. First, it was found that the transcript level of AtCGS is negatively regulated by the Met downstream product, SAM, via a post-transcriptional mechanism (Chiba et al. 1999, 2003; Onouchi et al. 2005). This regulation occurs in the MTO1 domain located in the *N*-terminal region of AtCGS (after the plastid transit peptide), which is comprised of ~100 amino acids, that does not exist in bacterial enzymes. This region is not essential for the catalytic activity, but influences the levels of Met and its downstream metabolites in plants (Hacham et al. 2002). Another regulatory domain comprised of 30 amino acids was also found in the *N*-terminal region close to the MTO1 domain (Hacham et al. 2006) (supplementary Fig. 1A). Transgenic tobacco plants that overexpress AtCGS lacking this 30 aa domain show that this form of AtCGS is not subjected to feedback regulation by Met (Hacham et al. 2006), as reported for the full-length transcript (Onouchi et al. 2005). As a result, the level of Met significantly increased in these plants (Hacham et al. 2008, 2006).

In addition to the down regulation by SAM, the level of CGS mRNA is enhanced by the application of threonine in Arabidopsis (Avraham and Amir 2005) and by the SAM-derived hormone, ethylene, in tomato (Katz et al. 2006). Other metabolites may also regulate the CGS level or activity, since it was shown that in cells starved for folates for a prolonged period, the regulatory *N*-terminal region of AtCGS was removed by proteolytic cleavage, leaving the enzyme active (Loizeau et al. 2007). The level of CGS is also regulated by environmental factors, as was reported by (Less and Galili 2009) through the use of bioinformatics tools. In addition, studies performed on potato CGS (StCGS) show that its expression level is up-regulated by light (Riedel et al. 1999). Light plays an essential role in plant development, growth and reproduction. Correspondingly, many enzymes in plants are light-inducible. These include not only the carbon assimilation enzymes but also those that contribute to the assimilation of nitrogen and sulfur (Kopriva et al. 1999). Consequently, many genes in the pathways leading to the synthesis of amino acids are regulated by light/dark transition (Oliveira et al. 2001).

In the current study we have examined the role of light/dark transition on the expression level of AtCGS, and on the level of Met. The results show that light, most probably through sucrose, which is one of the main photosynthesis products, up-regulates the transcript level of AtCGS. The results also indicate that the domains transducing this regulation are located in the coding sequence of AtCGS.

Materials and methods

Plant and growth conditions

For the dark\light experiments, Arabidopsis C24 ecotype plants were grown in soil at day/night cycles (16/8) (19–22 °C) for 20 days, and then exposed to continuous light or darkness for 48 h. The plants were subsequently exposed to light or dark for different times as referred in the text.

For experiments in which plants were transferred to different media, the plants were grown in MS agar plates [MS salt (DUCHEFA), pH adjusted to 5.8 with KOH 0.9 % (w/v) containing 3 % sucrose (90 mM)], at day/light cycles (16/8) for 12 days and then transferred for 2 days to MS medium supplemented with 3 % (90 mM) sucrose; MS lacking any carbon source; MS supplemented with mannitol (3 %); or to MS with or without 3.4 mM glutamine or cysteine. The plants were then transferred to continuous darkness or light for 24 h and tested as described in the text.

Transgenic tobacco plants overexpressing three different types of Arabidopsis Colombia AtCGS [GenBank- u43709, (Hacham et al. 2002)] (supplementary Fig. 1B) were grown in soil in a growth camber kept at 26–30 °C for 16/8 light/dark cycle for 10 weeks. The plants were then transferred to continuous light for 48 h, and then to darkness or light for additional 24 h.

Western blot analysis

Leaves from Arabidopsis and tobacco plants were homogenized in a mortar and pestle in a solution containing 25 mM phosphate buffer pH 7.8, 2 mM EDTA, 1 mM DTT, 1 mM PMSF and plant protease cocktail (Sigma) at 4 °C. After 20 min of centrifugation (14,000 g at 4 °C), the supernatant was collected. Protein samples (40 μg) were fractionated on 10 % SDS-PAGE and transferred to a PVDF membrane using Bio-Rad Protein Trans-Blot apparatus (Bio-Rad, http://www.bio-rad.com). The membranes were blocked overnight at 4 °C with 5 % (v/v) non-fat dried milk in phosphate buffer pH 7.8, incubated for 2 h in the presence of antibodies against AtCGS (Hacham et al. 2002) at room temperature, followed by incubation with anti-mouse IgG conjugated to horseradish-peroxidase under the same conditions (Hacham et al. 2002). Immunodetection was conducted with an enhanced chemiluminescence kit (Pierce, http://www.piercenet.com) according to the manufacturer's instructions.

RNA extraction, northern analysis and quantitative real-time PCR analysis

For northern blot analysis, the total RNA was extracted from the frozen material using the Tri reagent (Sigma–Aldrich) according to the manufacturer's instructions. RNA samples (20 μg) were subjected to electrophoresis in 1 % agarose gel containing 2.2 M formaldehyde and 50 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS), pH 7.0, and were then transferred onto a nitrocellulose Hybond N membrane (Amersham, http://www.amersham.com). The blots were hybridized for 12 h at 65 °C with probes labeled with [α-^{32}P]dCTP (redivue deoxycytidine 5′[α-^{32}P]triphosphate triethylammonium) using the Rediprime kit (Amersham). The probe was a SacI and EcoRI fragment of the Arabidopsis CGS. The quantity of 18S rRNAs was visualized by staining the membrane with methylene-blue in 0.5 M sodium acetate, pH 5.3.

For quantitative real-time PCR (qRT-PCR), the RNA was extracted from the leaves using Spectrum$^{\mathrm{TM}}$ plant total RNA kit (Sigma) according to the manufacturer instructions. RNA samples were treated with DNase I (Promega) for 15 min. First strand DNA was synthesized at 42 °C with 0.5 μg total RNA as a template, using Verso cDNA Kit (Thermo Scientific) with oligo(dT) primer. qRT-PCR assays were performed in the Corbett Rotor-Gene 6,000 real-time PCR system using Kapa SYBR fast universal qPCR kit (Kapa biosystems). The conditions were set as the following: an initial polymerase activation step for 3 min at 95 °C, followed by 42 cycles of 95 °C for 3 s, 60 °C for 25 s, 72 °C for 10 s. The expression of AtCGS was detected using Forward primer 5′ GGTTTGCATTG ATGGCACCT 3′ and Revers primer 5′ GCGTTTGGGTT AAGTGTTCCTC 3′. To normalize the variance among samples, the PP2A-A3 transcript level was used as endogenous control (Czechowski et al. 2005) using Forward primer 5′ TGTTGGAGCCCCAGGACTGT 3′ and Reverse primer 5′ CGTCCTAGTTGGCTCCGGTC 3′ (At1g13320). According to the Genevestigator data base (https://www.genevestigator.com/gv/), the gene of the PP2A-A3 is not regulated by light/dark transition or by sucrose application. Relative expression values are given in arbitrary units. The values presented are the mean of three to five biological replicates, each with three technical replicates.

Amino acids analysis

Amino acids analysis was performed by GC–MS as previously described (Golan et al. 2005).

Statistical analysis

Significance is marked in the text only when the *P* value was found to be <0.05.

For statistical analysis of quantitative data, Microsoft Excel was used to perform two-tailed Student's *t* tests assuming equal variances.

Results

The expression level of AtCGS is up-regulated by light

To assess the effect of light on the expression level of AtCGS, 20-day-old Arabidopsis wild type plants were transferred from continuous light to dark or left in the light for 24 h. In the dark, the protein and transcript levels of AtCGS were reduced to a similar extent, while such reduction was not observed in plants growing at continuous light (supplementary Fig. 2). Next, the kinetics of the changes in mRNA and protein levels in response to the transfer to darkness were studied. To this end, northern and western blot analyses were performed on 20-day-old Arabidopsis that grew in light/dark cycles (16/8). The plants were exposed to continuous light for 48 h followed by dark conditions for 4–52 h. The transcript level of AtCGS had significantly decreased after 8 h in the dark and continued to decline for up to 52 h. The reduction in the protein level was significantly delayed and could be detected after 24 h, after which it gradually continued to drop for up to 52 h (Fig. 2a, upper left panel). Samples collected at the same times from plants left under continuous light conditions did not demonstrate significant changes in AtCGS transcript and protein levels (Fig. 2a, upper right panel).

To determine the kinetics of AtCGS expression during light exposure after a dark period, a second time-course experiment was performed. The plants were grown at light/dark cycles (16/8) for 20 days and then transferred to continuous darkness for 48 h. After which they were exposed to light for 1 h up to 48 h. AtCGS mRNA levels were very low after 48 h in the dark (Fig. 2b, lower left panel; lane 1, time 0 h), and were rapidly induced by light within 1 h, achieving maximal elevation after 2 h, although this rapid and significant elevation, elevation of the protein level was delayed and detected only after 16 h of exposure to light. Samples taken at the same times from plants left in the dark show that the transcript levels were very low and could barely be detected (Fig. 2b, lower right panel; lane 5, time 8 h). However, the protein level of AtCGS, although very low, could still be detected by immuno-blot assay (Fig. 2b, lower right panel). This suggests that the protein levels in the dark are controlled by factors that cause stabilization of AtCGS, or it may still be produced from the very low levels of mRNA remaining.

The expression levels of many genes are regulated by circadian rhythm, which usually has very similar pattern to that obtained by the transition from light to dark (Kopriva et al. 1999). However, the relatively fast response of

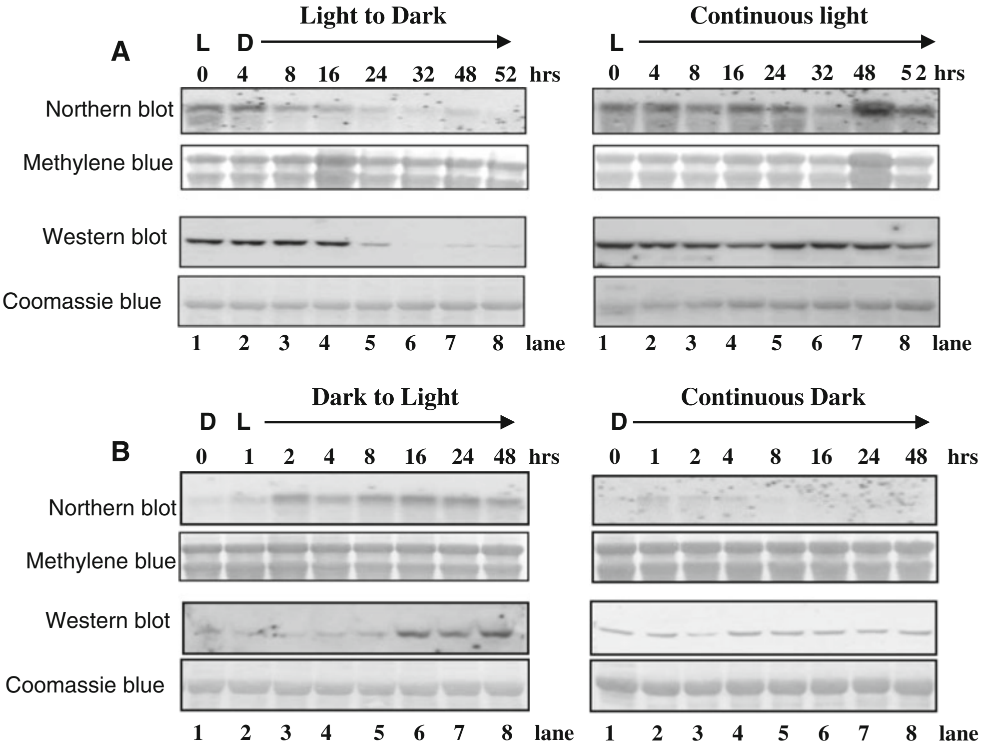

Fig. 2 Light induction of AtCGS mRNA and protein accumulation. **a** *Dark* kinetics—Arabidopsis plants that were grown in a 16/8 h light/dark cycle were adapted to light for 48 h and subsequently transferred to darkness for 0–52 h (*left*). As a control the plants were kept under continuous light (*right*); (**b**) *Light* kinetics—plants grown at 16/8 h light/dark cycle were adapted to the dark for 48 h and subsequently exposed to light for 0–48 h (*left*). As a control the plants were grown under continuous darkness (*right*). Representatives of three different western and northern blots analyses are shown. Samples were collected following the time indicated from the transfer to darkness (**a**) or light (**b**). *Upper panels*: RNA blot analysis of total RNA isolated from leaves hybridized with AtCGS cDNA as a probe. *Methylene-blue* staining of the 18S rRNA was used as an internal loading control. *Lower panels*: immunoblot analysis using antibodies against the AtCGS. *Coomassie-blue* staining of the rubisco band was used to verify equal loading. *L* refers to light and *D* to darkness

AtCGS to light and dark, and the observation that the diurnal rhythms disappeared when the plants were subjected to continuous light or darkness indicate that AtCGS is not under the control of an endogenous circadian rhythm.

Taken together the results show that light induces the transcript expression level of AtCGS within 2 h after transfer from darkness, while the expression level of AtCGS in plants remaining in the dark decrease within the first 8 h, and continue to decrease for up to 16 h. Unlike the induction by light, there is little relevance of extended darkness to physiological conditions, since extended darkness is rare in nature. The results also show that after long period of darkness, the protein level of AtCGS can still be detected although in very low levels, suggesting that its levels are regulated by a yet unknown mechanism.

The regulation of AtCGS level by light is mediated by its coding sequence

Studies have shown that the expression of many nuclear-encoded plastid localized proteins are regulated by light and dark. Most of the studies have shown that in plants, light- or dark-responsive elements are located within the promoter or within the 5′ or 3′ untranslated regions (UTR) (Tang et al. 2003). In order to determine if the expression level of AtCGS is controlled by elements present in these regions of AtCGS, we have used tobacco transgenic plants that overexpress the full-length AtCGS (Hacham et al. 2002) (F- AtCGS, supplementary Fig. 1b). These plants express only the cDNA of the coding sequence of the mature AtCGS, in which the original 5′ and 3′ UTRs were removed, as well as the terminator (supplementary Fig. 1b). In addition, the AtCGS original promoter was replaced by the constitutive promoter of 35S CaMV, which is not regulated by light or by photoreceptor (Cheng et al. 1992).

Ten-week-old transgenic tobacco plants overexpressing the full-length AtCGS that were grown in a 16/8 day/night cycle were transferred to 48 h continuous light followed by 24 h darkness. Again, the expression level of AtCGS was reduced significantly in plants transferred to the dark (Fig. 3a) compared to those remaining in continuous light (Fig. 3b). These results suggest that the light-responsive elements of AtCGS are located within its coding region.

To assess whether the light-responsive elements are part of the *N*-terminal region of AtCGS, that is considered to be the regulatory region of AtCGS (Amir 2010), we have used transgenic tobacco plants overexpressing the AtCGS that lack the *N*-terminal region (T- AtCGS, supplementary Fig. 1b); (Hacham et al. 2002), or the 30 aa domain (D- AtCGS, supplementary Fig. 1b); (Hacham et al. 2006); (supplementary Fig. 1b). When these plants were transferred from light to dark, the transcript level of AtCGS was reduced in the dark to a similar extent as in the plant expressing the full-length AtCGS (Fig. 3c, d). These results indicate that the light-responsive element is not located within the *N*-terminal regulatory region of AtCGS and thus it must be located in the conserved region of AtCGS where the catalytic domain is also found (Hacham et al. 2002). While the transcript levels of all three forms of AtCGS were reduced in the dark, as were the protein level of the full-length and the form that lacks 30 aa domain, the protein level of AtCGS that lacks the entire *N*-terminal region was not significantly altered (Fig. 3c). This indicates that this form of AtCGS is more stable as was previously demonstrated (Loizeau et al. 2007).

Plants growing in light accumulate more Met than plants transferred to darkness

The expression level of AtCGS is generally correlated with the level of Met in plants (Hacham et al. 2008; Amir 2008,

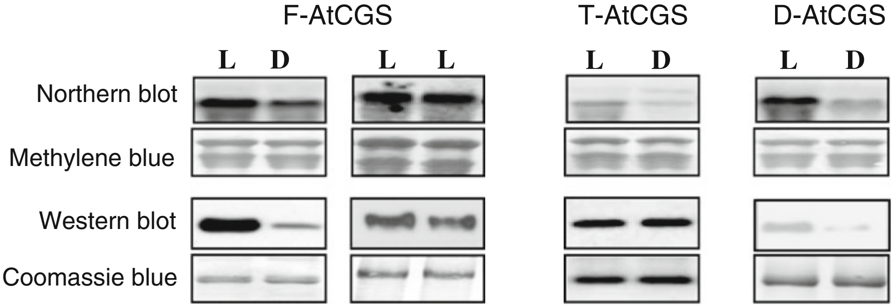

Fig. 3 The effect of light on AtCGS level is mediated by its coding sequence. mRNA expression and protein levels of three forms of AtCGS in transgenic tobacco plants overexpressing the full-length (*F*-AtCGS), the truncated form that lacks the *N*-terminal region (*T*-AtCGS), and the form that lacks the 30 aa in the *N*-terminal region (*D*-AtCGS). Ten-week-old plants were grown in 16/8 light/dark cycle, light adapted for 48 h, and then transferred to darkness for 24 h. Samples were collected before (*L*) and after the transfer to darkness (*D*). As a control (*C*) the plants were left to grow at continuous light (shown for the full-length AtCGS expressing plants). *Upper panels*: RNA blot analysis of total RNA isolated from leaves hybridized with AtCGS cDNA as a probe. *Methylene-blue* staining of the 18S rRNA was used as an internal loading control. *Lower panels*: immunoblot analysis using antibodies against the AtCGS. *Coomassie-blue* staining of the rubisco band was used to verify equal loading. *L* refers to light and *D* to darkness

Table 1 The content of soluble amino acids in transgenic tobacco plants overexpressing the full-length AtCGS

Amino acids	Light	Dark
Methionine	41.4 ± 10.0	17.6 ± 9.5*
Homocysteine	6.4 ± 2.3	2.4 ± 0.5*
Proline	9.7 ± 3.0	11.1 ± 3.9
Alanine	29.6 ± 12.9	8.8 ± 1.0*
Glutamine	59.7 ± 22.7	34.0 ± 4.1
Glycine	51.5 ± 18.8	64.9 ± 19.4
Serine	24.4 ± 2.5	38.1 ± 11.3
Aspartate	39.9 ± 2.0	45.6 ± 6.5
Threonine	16.9 ± 3.9	19.2 ± 2.6
Glutamate	157.6 ± 12.3	130.5 ± 18.8
Lysine	4.6 ± 2.2	23.3 ± 8.7*
Leucine	5.2 ± 1.7	20.8 ± 4.2*
Isoleucine	4.2 ± 2.0	18.4 ± 3.9*
Valine	4.8 ± 1.3	20.5 ± 3.3*
Tryptophan	4.5 ± 0.9	8.6 ± 2.0*
Tyrosine	8.5 ± 3.5	27.9 ± 2.5*
Phenylalanine	22.1 ± 3.2	39.1 ± 7.3*

Two experimental sets of ten-week-old plants grown in 16/8 light/dark cycles were transferred to continuous light for 48 h and then to darkness for 24 h. Samples were removed from the plants before and after the transfer to the dark. The quantities of amino acids were analyzed using GC–MS, and their levels were normalized to norleucine and calculated as *n* mole/g fresh weight. The data is presented as the mean ± SD obtained from four independent plants. Microsoft Excel was used to perform two-tailed Student's *t* tests and the statistically significant differences ($P < 0.05$) are marked by asterisk. Amino acids that their levels were significantly higher in the light are marked by bold letters, while those that were significantly higher in the dark were marked by underline

2010). Therefore, we have studied whether transferring plants to the dark for 24 h, which reduces the expression level of AtCGS, will also affect the level of Met. Since the content of Met is very low in leaves of Arabidopsis and in tobacco wild type plants, it is difficult to measure changes in its content. Therefore, we have studied this effect in tobacco plants overexpressing the full-length AtCGS that have elevated Met levels (Hacham et al. 2008). Ten-week-old plants that were grown at 16/8 light cycle were transferred to 48 h continuous light and then to the dark for 24 h. Samples for amino acids analysis by GCMS were taken before and after the transition to the dark. The results show that the levels of Met were significantly higher in the light compared to dark (2.3-fold, Table 1). The level of the Met intermediate metabolite, homocysteine, has also significantly increased in light.

The levels of the three branched amino acids (valine, leucine and isoleucine) and the three aromatic amino acids (tryptophan, tyrosine and phenylalanine) were significantly lower in the light compared to darkness (Table 1). The levels of threonine and aspartate that belong to the aspartate family were not altered significantly.

AtCGS expression is induced by sucrose in the absence of light

Light can trigger direct modulation of gene expression via photoreceptor (phytochrome, cryptochrome) activities, or through photosynthesis-related signals, originating from the photosynthesis process (Oliveira et al. 2001). Since light affects the AtCGS level in transgenic tobacco plants expressing AtCGS under the control of the 35S CaMV promoter, which is not regulated by light (Cheng et al. 1992), it is unlikely that the photoreceptors that operate through activity of the promoter (Oliveira et al. 2001) or the UTRs are involved in AtCGS regulation. Thus, the effects of signals derived from the photosynthesis process were studied. The most obvious signalling compound derived from photosynthesis is sucrose, which is known to regulate gene expression in plants (Pego et al. 2000).

Thus, next we have investigated whether sucrose can induce the expression of AtCGS mRNA in the absence of light. Twelve-day-old Arabidopsis plants grown on MS medium with 3 % (90 mM) sucrose were transferred to MS lacking any carbon source, to MS supplemented with a non-metabolizable carbon source, mannitol (3 %), and to the same medium as before. Mannitol was used to determine whether the effect of sucrose on AtCGS is caused by a change in the osmotic level. The plants were kept for 2 days under 16/8 day/night cycles and then transferred to continuous light for 48 h. Light-adapted plants were then transferred to darkness for 24 h, or left under continuous light as a control.

The protein level of AtCGS in the dark was relatively high in plants grown in medium supplemented with 3 % sucrose, unlike plants grown on medium with mannitol and medium without sucrose (Fig. 4a). This suggests that light regulates the transcript level of AtCGS through sucrose. qRT-PCR was used to measure AtCGS transcript levels in these plants. The transcript levels of AtCGS in plants grown on 3 % sucrose in the dark were significantly higher than those detected in plants grown on medium without sucrose, or with 3 % mannitol in the dark (Fig. 4b). This again suggests that sucrose can mimic the effect of light. Plants exposed to light and 3 % sucrose had the highest AtCGS transcript levels compared to plants grown in medium without sucrose, with 3 % mannitol, or that were transferred to the dark (Fig. 4b). However, plants that were grown in 3 % sucrose in the dark had about twofold lower level of

AtCGS than those grown at 3 % sucrose in light. This suggests that light together with sucrose can significantly enhance the transcript expression level of AtCGS.

The elevation of AtCGS transcript levels by sucrose can be explained by the induction of expression, or by stabilization of the transcript. To distinguish between these two possibilities and to determine the kinetics of the regulation of AtCGS expression by sucrose, a time-course experiment was performed. To this end, 12-day-old Arabidopsis plants grown on MS with 3 % sucrose at 16/8 light/dark cycles were transferred to MS medium without sucrose for 48 h. Then the plants were transferred to continuous darkness for additional 48 h, at that point (time 0 h), 3 % sucrose was applied to the medium. The plants were sampled at various times after sucrose application as described in Fig. 5. Plants remaining in the dark for 8, 24 and 48 h without the addition of sucrose served as a control. The transcript levels of AtCGS were low after 48 h in darkness (Fig. 5, time 0 h), and were rapidly induced when sucrose was applied, achieving their maximal elevation after 4 h.

Taken together, these results show us that sucrose regulates the transcript level of AtCGS in the dark and can partially mimic the effect of light. Thus, the results support the assumption that light regulates the level of AtCGS through sucrose. In addition, the results described in Fig. 5 imply that sucrose affects AtCGS levels through the induction of expression, and not through transcript stabilization.

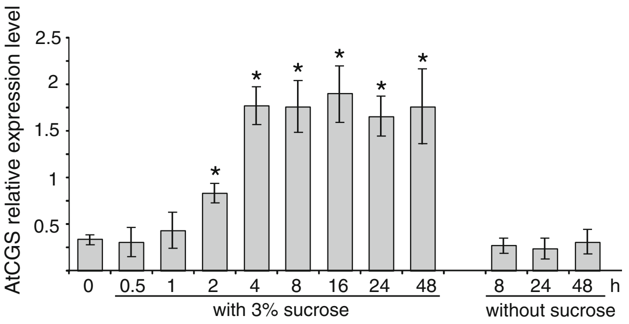

Fig. 5 The effect of sucrose on the transcript level of AtCGS. Arabidopsis plants grown in 16/8 day/night cycle were transferred to medium without sucrose for 48 h, and then transferred to the dark for 48 h. 3 % sucrose was then added to the growth media and the plants were *left* to grow under continuous darkness for 0.5, 1, 2, 4, 8, 16, 24, 48 h. Plants remaining in the *dark* for 8, 24 and 48 h without the addition of sucrose served as a control. The transcript levels of AtCGS were normalized to the transcript level of PP2A-A3. The data represent the mean ± standard deviation of five independent samples; each sample is a pool of 20 plants. Statistically significant changes ($P < 0.05$) from the treatment without sucrose, time 0, are identified by *asterisks*

The amino and sulfate group donors do not regulate the levels of AtCGS

Light and the metabolic status of the cells are two critical factors controlling the conversion of inorganic nitrogen into amino acids in plants (Oliveira and Coruzzi 1999). Thus, we have studied whether exogenous supply

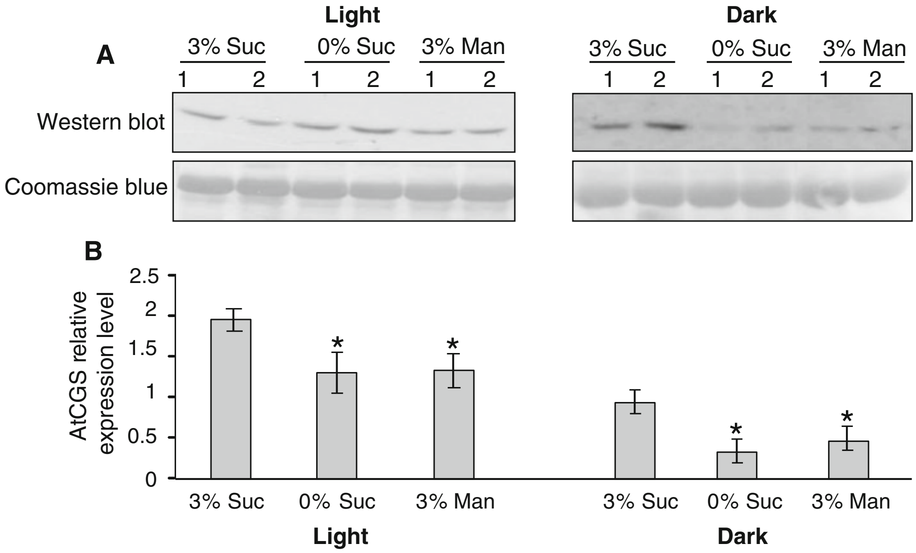

Fig. 4 Light and sucrose affect the protein and transcript expression levels of AtCGS. Twelve-days-old Arabidopsis plants grown in 16/8 day/night cycle were exposed to various metabolites as follows: 0, 3 % sucrose or 3 % mannitol. The plants were light adapted for 48 h and then placed in the light or in the dark for 24 h. **a** immunoblot analysis using antibodies against AtCGS. *Coomassie-blue* staining of the rubisco band was used for equal loading. The figure shows two samples that are representatives of eight repeats. **b** transcript levels of AtCGS were analyzed by qRT-PCR. Expression was normalized to the transcript level of PP2A-A3. The data represent the mean ± standard deviation of five independent samples; each is a pool of 20 plants. Statistically significant changes ($P < 0.05$) from the sucrose treatment are identified by *asterisk*

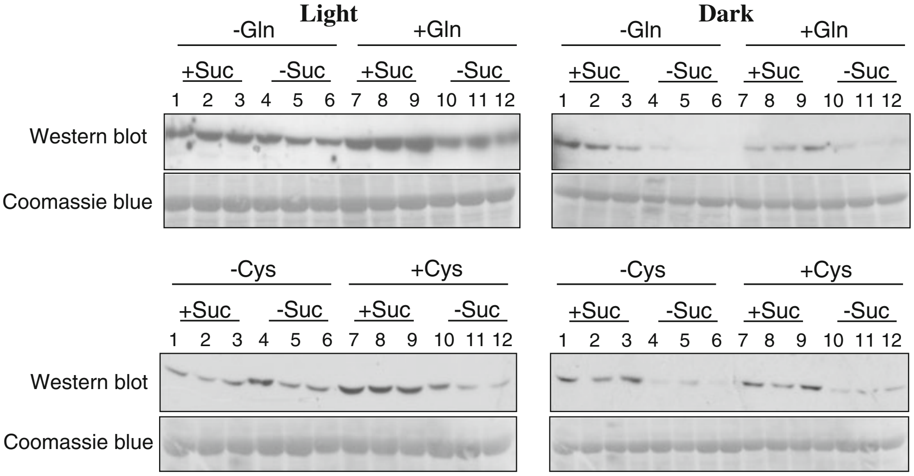

Fig. 6 Nitrogen or sulfur sources do not affect AtCGS protein level in Arabidopsis plants. Twenty-day-old plants grown in 16/8 day/night cycle were light adapted for 48 h and then exposed to 0 or 3 % sucrose with or without 3.4 mM glutamine or 3.4 mM cysteine, in the light or in the dark for 24 h. Total soluble proteins were separated by SDS-PAGE and subjected to immunoblot analysis using AtCGS antiserum. *Coomassie blue* staining of the rubisco band was used for relative gel loading. The figure shows three samples that are representative of eight repeats

of glutamine (as a source of amino group) with or without sucrose affects the level of AtCGS. Arabidopsis plants were transferred either to MS medium supplemented with 3 % sucrose; or to MS lacking any carbon source, with or without glutamine (3.4 mM) ("Materials and methods"). The results show that application of glutamine does not significantly alter the level of AtCGS in the dark (Fig. 6, right upper panel), while sucrose with or without glutamine can mimic the effect of light in dark grown plants (Fig. 6, right upper panel), as shown in Fig. 4 for sucrose supplementations. This result is different from previous results describing the effect of nitrogen source on the induction of other enzymes participating in amino acid synthesis: Both asparagine synthase and glutamine synthase levels are induced by the application of sucrose, and this effect is reversed by the addition of a nitrogen source (Lam et al. 1998; Oliveira and Coruzzi 1999).

Cysteine is the thiol group donor for Met synthesis and is also a substrate of AtCGS. To assess whether cysteine with or without sucrose affects the expression level of AtCGS, we have added cysteine to the growth medium. The results were similar to those obtained with glutamine (Fig. 6, right lower panel), suggesting that cysteine content does not regulate the expression level of AtCGS in the dark. Notably the expression level of AtCGS in plants grown in light has increased when sucrose plus glutamine, or sucrose plus cysteine were added to the medium, compared to those supplemented only with sucrose (Fig. 6, left upper and lower panels). The reason for such increases is not clear yet, but it suggests that AtCGS can respond to higher flux of carbon/amino skeleton and thiol group in light.

Threonine synthase levels are not regulated by light

Previous studies have shown that CGS and threonine synthase (TS), the last enzyme in the threonine biosynthesis pathway, compete for their common substrate, O-phosphohomoserine (Fig. 1); (Amir et al. 2002; Hesse et al. 2004). This competition controls the flux of the carbon/amino skeleton between the biosynthesis pathways of Met and threonine (Lee et al. 2005). To gain more knowledge whether light/dark transition regulates this branching point, AtTS protein expression levels were measured in Arabidopsis plants that were grown in 16/8 light/dark cycle, light adapted for 48 h and then transferred to darkness for 24 h; or dark adapted for 48 h and then transferred to light for 24 h.

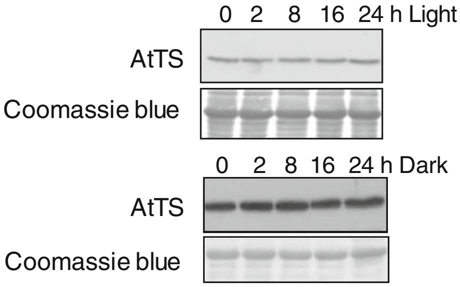

Fig. 7 The protein expression levels of Arabidopsis threonine synthase (*AtTS*) are not affected by light–dark transition. 20-day-old Arabidopsis plants were grown in 16/8 light/dark cycle, and (**a**) *light* adapted for 48 h and then transferred to darkness for 24 h, or (**b**) *dark* adapted for 48 h and then transferred to light for 24 h. Total soluble proteins were separated by SDS-PAGE and subjected to immunoblot analysis using AtTS antiserum. *Coomassie blue* staining of the rubisco band was used for relative gel loading

The protein level of AtTS did not change significantly when the plants were transferred from light to dark or from dark to light (Fig. 7). These results show that the expression levels of AtTS and AtCGS are differently regulated by light/dark transition. This suggests that light/dark transition regulates the flux of *O*-phosphohomoserine between these two biosynthesis pathways.

Discussion

The results of the current study show that the expression of AtCGS is enhanced by light and reduced in the dark (Fig. 2). The higher levels of AtCGS in the light, cause an elevation to Met content in plants grown in light compared to those transferred to darkness for 24 h (Table 1), showing the importance of this regulation for Met levels and most probably on other Met-metabolites. The light responsiveness was partially mimicked by the addition of sucrose (Fig. 4), one of the major products of photosynthesis, accumulating in the light. The kinetics of AtCGS expression induction by light and sucrose are quite similar (Figs. 2b, 5). The results show that the effect of sucrose application is delayed by about 2 h compared to the effect of light. This delay is most probably caused by the time required for the absorption of the sucrose by the roots and its transfer to the rosettes leaves. Sucrose is well known to be involved in the regulation of the expression of genes involved in the synthesis of amino acids, where it mimics the effect of light (Oliveira et al. 2001), as was found in the current study (Figs. 4, 5, 6). However, further studies are required to reveal whether light together with sucrose have synergistic effect on the expression level of AtCGS as can be suggested from the results described in Fig. 4. In addition, further studies are required to study the effect of sucrose plus glutamine or cysteine on AtCGS in plants growing in the light, as we have seen that under these conditions AtCGS protein expression level is elevated (Fig. 6).

The kinetics of the response to light and sucrose are very similar to those found for asparagine synthase (Asn) in Arabidopsis, which is considered very rapid (Lam et al. 1998). While the response of AtCGS transcript level to transition from darkness to light is rapid, the response of the protein level is significantly delayed and is manifested only after 16 h (Fig. 2a). Thus, the reduction in AtCGS levels in response to the dark is not part of the diurnal cycle, but a response to prolonged periods of darkness that might often challenge a small easily covered plant as Arabidopsis. In addition, the results indicate that other yet unknown regulatory mechanisms might be involved in AtCGS translational control. The regulatory effect of sucrose on the level of AtCGS is physiologically relevant independently of the effect of light, since the content of sucrose can be reduced even under light conditions when the plants grow under various abiotic stresses (Pego et al. 2000). Thus, we assume that depletion of sucrose under these stresses may reduce AtCGS levels.

The kinetics of the changes in the protein expression level of AtCGS suggest that its level can be maintained, even when the sucrose content is reduced, such as during abiotic stresses or during extended darkness. However, further studies are required to assess the significance of AtCGS regulation by sucrose and light under different stresses.

Light and sucrose can affect the expression of genes at different levels, including mRNA transcription, mRNA stability, translation and post-translational (Tjaden et al. 1995; Casal and Yanovsky 2005). According to the results obtained in the current study, we suggest that the regulation of AtCGS occurs mostly at the post-transcriptional level, most probably through the stability of the mRNA. This assumption is based on the observation that the transcript level of AtCGS is affected by light and sucrose not only in wild type Arabidopsis plants but also in transgenic tobacco plants expressing AtCGC under the control of heterologous promoter and terminator, meaning that the regulation does not involve promoter or terminator sequences. Moreover, the results suggest that the regulatory sequences are part of the coding region of the AtCGS gene (Fig. 3). The results also suggest that the *N*-terminal region of AtCGS, which is considered to be a regulatory region (Hacham et al. 2002) is not involved in this regulation (Fig. 3). One explanation to the stability of AtCGS mRNA in plants grown in light or with sucrose is that it is mediated through the polysomes occupying the mRNA's coding sequence. It was reported that the transcript level of several nuclear-encoded mRNAs coding for proteins that take part in photosynthesis is protected from degradation in the light by an association with ribosomes (Hansen et al. 2001; Tang et al. 2003). However, further studies are required to reveal if this is the case for AtCGS. In addition to the post-transcriptional regulation, AtCGS is apparently also regulated in the dark through a post-translational process, since the protein content of AtCGS remains stable in continuous darkness, while the transcript levels are reduced (Fig. 2B).

The changes in the expression level of AtCGS significantly affect the level of Met, which was found to decrease significantly after 24 h in darkness compared to plants remaining in the light (Table 1). Met belongs together with threonine, lysine and isoleucine to the aspartate family of amino acids that obtain their carbon/amino skeleton from aspartate (Fig. 1). Thus, it is important to assess whether other genes coding for enzymes participating in the Met pathway, upstream to CGS, are also up-regulated by light enhancing the flux towards Met synthesis. Aspartate is synthesized from glutamate, which together with glutamine is the first assimilated nitrogen product (Champigny and Foyer 1992; Lam et al. 1995). Several studies have shown that the expression levels of nitrate reductase and nitrite reductase in the nitrogen assimilation pathway, and glutamine synthase (Gln) are up-regulated by light and sucrose (Cheng et al. 1992; Oliveira and Coruzzi 1999; Small and Gray 1984). Aspartate kinase (AK, Fig. 1), the first enzyme of the aspartate family biosynthesis pathway, which appears as a monofunctional and bifunctional enzyme together with homoserine dehydrogenase (HSD), is stimulated by light at the levels of its activity and at the transcript level in barley and Arabidopsis (Rao et al. 1999; Zhu-Shimoni and Galili 1998). This strongly suggests that the flux towards aspartate family increases in the light.

The first branch point within the aspartate family is the branch that leads to either lysine synthesis that is regulated by the first committed enzyme of the lysine pathway, dihydrodipicolinate synthase (DHDPS), or to the synthesis of Met, threonine and isoleucine that is regulated by HSD (Fig. 1). The expression of HSD that competes with DHDPS for the same substrate is up-regulated by light together with AK (Zhu-Shimoni and Galili 1998), pulling the flux of the carbon/amino skeleton towards the Met/threonine branch in the light. The next branch point in the aspartate family biosynthesis pathway separates between threonine and Met syntheses. This branch point is regulated by CGS and TS (Fig. 1). The transcript level of CGS in potatoes (Riedel et al. 1999) and in Arabidopsis (Fig. 2) is significantly up-regulated by light. In addition to CGS, Met synthase, the last enzyme of the Met biosynthesis pathway, is also up-regulated by light and sucrose in potato plants (Zeh et al. 2002). However, while the expression levels of CGS increase in light, the expression levels of TS in Arabidopsis (Fig. 7) and in potato (Casazza et al. 2000) were not affected by light. This suggests that dark/light transition might regulate the flux of the carbon/amino skeleton towards Met and threonine biosynthesis pathways. It is assumed that since the expression level of AtCGS is reduced in the dark, more of the carbon/amino skeleton is channeled towards threonine synthesis. However, the level of threonine does not significantly increase in the dark (Table 1). Since threonine is the main precursor for the synthesis of the branched amino acids, valine, leucine and isoleucine (Joshi and Jander 2009), one possible explanation for this observation is that threonine is used during the night for the synthesis of these amino acids. Indeed the levels of these branched amino acids increase significantly in response to transfer to darkness (Table 1). However, since the protein level of AtCGS decreases only after 16 h in the dark, further studies are required to assess whether light and sucrose regulate the partitioning of the carbon/amino skeleton between the Met and threonine biosynthesis pathways under normal growth conditions. Taken together, these studies suggest that light up-regulates the major enzymes leading to Met biosynthesis pathway, starting with nitrate and nitric reductases, followed by AK, HSD and CGS.

In addition to the carbon/amino skeleton, Met synthesis requires the thiol group from cysteine (Fig. 1). Thus, it is also important to reveal whether the expression levels of enzymes in the sulfur assimilation pathway and the cysteine biosynthesis pathway, which is the donor of the thiol group for Met synthesis, increase in light. Several studies have shown that genes coding for enzymes in these pathways are up-regulated by light (Kopriva et al. 1999; Lee et al. 2011). The expression level of adenosyne 5′-phosphosulfate reductase (APR, Fig. 1) that is considered to be the key enzyme of sulfate assimilation is up-regulated by light and sucrose, and is decreased in darkness (Kopriva et al. 1999; Lee et al. 2011). Similarly, the expression of four rice genes coding to *O*-acetylserine (thiol) lyase (OASTL), the last enzyme in the cysteine biosynthesis pathway, is up-regulated by light and reduced by darkness (Nakamura et al. 1999). In Arabidopsis the mRNA levels of APS kinase, sulphite reductase (SiR), OASTL, and serine acetyl-transferase were several times higher in green leaves than in etiolated tissues (Hell et al. 1997). In addition, transfer to darkness for 38 h significantly reduced the mRNA level of ATP sulfurylase, APR and SiR (Hesse et al. 2003). The results of these studies suggest that light up-regulates the cysteine biosynthesis enzymes. Indeed 24 h after Lemna plants were transferred to darkness, the assimilation of ^{35}S into sulfur-organic compounds had decreased by 80 % (Neuenschwander et al. 1991). These studies suggest that light up-regulates the enzymes in the aspartate family leading to Met synthesis (AK and HSD) as well as the enzymes that produce cysteine (the enzymes that are up-regulated by light are indicated in Fig. 1 in bold and asterisk). This up-regulation of both substrates enhances the flux of carbon/amino skeleton and the thiol group towards CGS in the light.

All in all, the results of this study enhance our knowledge on roles of light and sucrose in the regulation of AtCGS. However, further studies are required to assess the physiological relevance of this regulation, especially under conditions in which the sucrose content is reduced, such as during abiotic stresses.

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Conflict of interest The authors declare that they have no conflict of interest.

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