Journal of Experimental Botany, Vol. 64, No. 4, pp. 1039–1048, 2013
doi:10.1093/jxb/ers378 Advance Access publication 10 January, 2013
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in *Arabidopsis*

Stine Huseby[1,2,*], Anna Koprivova[1], Bok-Rye Lee[1,†], Shikha Saha[3], Richard Mithen[3], Anne-Berit Wold[2], Gunnar B. Bengtsson[4] and Stanislav Kopriva[1]

[1] Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK
[2] Department of Plant- and Environmental Sciences, Norwegian University of Life Sciences, PO Box 5003, NO-1432 Aas, Norway
[3] Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK
[4] Nofima-Norwegian Institute of Food, Fisheries and Aquaculture Research, Osloveien 1, NO-1430 Aas, Norway
[†] Present address: College of Agriculture & Life Science, Chonnam National University, Buk-Gwangju, Gwangju, 500–600, Korea.

* To whom correspondence should be addressed. E-mail: stine_huseby@yahoo.no

Received 28 September 2012; Revised 11 December 2012; Accepted 13 December 2012

Abstract

Glucosinolates are a major class of sulphur-containing secondary metabolites involved in plant defence against pathogens. Recently many regulatory links between glucosinolate biosynthesis and sulphate assimilation were established. Since sulphate assimilation undergoes diurnal rhythm and is light regulated, this study analysed whether the same is true for glucosinolate biosynthesis. The levels of glucosinolates and glutathione were found to be higher during the day than during the night. This agreed with variation in sulphate uptake as well as activity of the key enzyme of the sulphate assimilation pathway, adenosine 5'-phosphosulphate reductase. Correspondingly, the flux through sulphate assimilation was higher during the day than during the night, with the maximum flux through primary assimilation preceding maximal incorporation into glucosinolates. Prolonged darkness resulted in a strong reduction in glucosinolate content. Re-illumination of such dark-adapted plants induced accumulation of mRNA for many genes of glucosinolate biosynthesis, leading to increased glucosinolate biosynthesis. The light regulation of the glucosinolate synthesis genes as well as many genes of primary sulphate assimilation was controlled at least partly by the LONG HYPOCOTYL5 (HY5) transcription regulator. Thus, glucosinolate biosynthesis is highly co-regulated with sulphate assimilation.

Key words: Diurnal, glucosinolates, HY5, light, primary metabolism, secondary metabolism, sulphate assimilation.

Introduction

Glucosinolates (GLS) are sulphur-rich compounds found in the Brassicaceae family. GLS and their breakdown products (thiocyanates, isothiocyanates, and nitriles) play an important role in plant defence against pathogens and herbivores, while humans may gain protection against cancer through the consumption of vegetables containing GLS, such as broccoli, kale, and Brussels sprouts (Traka and Mithen, 2009).

The GLS core structure is an *S*-glucose moiety and a sulphoxime group with an attached amino acid-derived side chain (Fig. 1). GLS are classified based on the origin of their side chain, and the main groups are aliphatic, indolic, and benzenic GLS derived from the amino acids methionine, tryptophan, and phenylalanine, respectively (Brown *et al.*, 2003; Agerbirk and Olsen, 2012). The biosynthesis of GLS starts with chain elongation of the amino acids followed by oxidation, to form an aldoxime (Fig. 1). Glutathione donates a sulphur atom to the aldoxime, resulting in a thiohydroxymate, which quickly undergoes glycosylation by UDP-glucose to give desulpho-GLS. GLS are formed when the desulpho-GLS are sulphated by sulphotransferases using

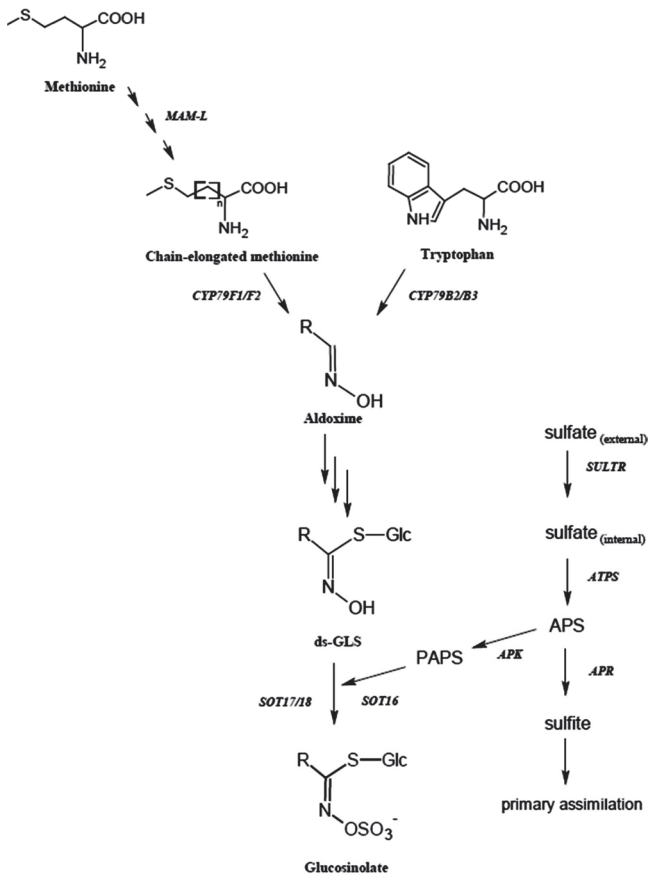

Fig. 1. The biosynthesis of glucosinolates linked with the sulphate assimilation pathway. APS, adenosine 5'-phosphosulphate; PAPS, 3'-phosphoadenosine 3'-phosphosulphate.

3'-phosphoadenosine 3'-phosphosulphate (PAPS), linking GLS biosynthesis to primary sulphate assimilation (Mugford *et al.*, 2009). The GLS can further undergo secondary modifications, such as hydroxylations, methylations, or oxidations, which give rise to a large variation of structures (Grubb and Abel, 2006; Sønderby *et al.*, 2010).

Glucosinolates contain at least two sulphur atoms in their core structure, and aliphatic GLS may have additional sulphur incorporated in their side chains (Fig. 1). The sulphur status of the plant therefore affects the biosynthesis of GLS (Falk *et al.*, 2007), which is co-regulated with sulphate assimilation, both positively controlled by the same group of MYB transcription factors (Hirai *et al.*, 2004, 2005; Yatusevich *et al.*, 2010). The availability of other nutrients such as nitrogen and potassium can also affect production of GLS (Omirou *et al.*, 2009; Troufflard *et al.*, 2010).

GLS content and the expression of GLS biosynthetic genes are regulated by a range of environmental factors. In line with their function in biotic stress defence, the genes are inducible by wounding, jasmonate, or pathogens (Mikkelsen *et al.*, 2003; Jost *et al.*, 2005; Kusnierczyk *et al.*, 2007). In addition, GLS and the mRNA levels of biosynthetic genes have been shown to change during a 24 h light/dark cycle; the metabolic fluctuations are affected by temperature in cultivars of *Brassica oleracea* (Rosa *et al.*, 1994; Rosa, 1997; Schuster *et al.*, 2006). Branched-chain aminotransferase (BCAT4) and methylthioalkylmalate synthase (MAM1), both involved in the early side chain elongation process, show higher expression levels under light compared with darkness, and the expression levels remain high when the plants are kept under continuous light (Schuster *et al.*, 2006). The three sulphotransferases involved in core GLS biosynthesis, SOT17 in particular, show higher expression levels during the light period followed by a gradual decrease during the dark period (Klein *et al.*, 2006). AOP2, involved in secondary modification of the side chain, has a high expression level under continuous light, while it is not detectable in continuous darkness (Neal *et al.*, 2010).

Despite the link that seems to exist between light and the biosynthesis of GLS, their total content often fluctuates more than the gene expression, and elevated levels can be seen during the dark period when the genes have low expression levels (Rosa, 1997; Klein *et al.*, 2006; Schuster *et al.*, 2006). In addition, the closely related sulphate assimilation is also regulated by light. The key enzyme of sulphate assimilation, adenosine 5'-phosphosulphate reductase (APR), undergoes diurnal rhythm in *Arabidopsis* and maize, with a maximum during the light period (Kocsy *et al.*, 1997; Kopriva *et al.*, 1999). On the other hand, no diurnal changes in cysteine or glutathione contents were observed in poplar (Noctor *et al.*, 1997), although the capacity for glutathione synthesis is higher in light than in the dark (Buwalda *et al.*, 1988). Other components of sulphate assimilation are regulated by light: ATP-sulphurylase activity was higher in irradiated oat, barley, and maize (Passera *et al.*, 1989). In *Arabidopsis*, mRNA levels of genes of sulphate assimilation were several times higher in green leaves than in etiolated tissues (Hell *et al.*, 1997). APR activity is repressed in dark-adapted plants, and induced rapidly by re-illumination (Neuenschwander *et al.*, 1991; Kopriva *et al.*, 1999). The transcription factor LONG HYPOCOTYL5 (HY5) has been shown to be involved in the regulation of APR light response (Lee *et al.*, 2011). HY5 is a bZIP transcription factor and a positive regulator of photomorphogenesis (Ang *et al.*, 1998). Mutation in HY5 causes defects in the inhibition of hypocotyl elongation in all light conditions, suggesting that HY5 acts downstream of phytochromes A and B, cryptochromes, and UV-B (Ang *et al.*, 1998; Ulm *et al.*, 2004). HY5 directly binds to the promoters of >1000 light-inducible genes, including APR (Lee *et al.*, 2007, 2011).

However, while the light and diurnal regulation of sulphate assimilation is well established, the data have been obtained in different species in various growth conditions. In addition, particularly little is known about such regulation of GLS biosynthesis and the coordination with sulphate assimilation in *Arabidopsis*. The aims of this study were, therefore, to analyse light induction and diurnal regulation of GLS biosynthesis genes and to provide a comprehensive study of diurnal variation of sulphur-containing metabolites, sulphate uptake, flux through sulphate assimilation, and the rate of GLS biosynthesis, in the same plant material.

Materials and methods

Plant material and growth conditions

For all experiments, *Arabidopsis thaliana* ecotype Col-0 or the *hy5* mutant [T-DNA insertion line SALK_056405 (Lee *et al*., 2011)] were grown on vertical plates with Murashige and Skoog (MS) medium, without sucrose supplement, and 0.5% phytagel. The seeds were stratified for 72 h at 4 °C before being transferred to a controlled environment room at 20 °C and with a 16 h light/8 h dark cycle. For the diurnal experiments, samples of 15-day-old seedlings were collected every fourth hour, for 24 h. For the light induction experiments, 21-day-old seedlings were either kept under a normal 16 h/8 h day–night rhythm or transferred to continuous darkness. After 44 h, half of the dark-treated plants were re-illuminated while the remainder were kept in the dark. Sampling was done 3 h (metabolites and RNA) or 4 h (^{35}S incorporation) after re-illumination. For the experiments with *hy5*, the seedlings were grown for 7 d, transferred to dark for 38 h, and re-illuminated for 90 min before sampling. The control group were kept in the dark. This allowed direct comparison of these results with the previous analysis of *hy5* (Lee *et al*., 2011) and of regulation of the GLS synthesis genes at different developmental stages.

GLS analysis

The content of GLS and desulpho-GLS was determined using ~50 mg of frozen plant material. The extraction and quantification of GLS followed the protocol described in Mugford *et al*. (2009). Quantification was based on UV absorption at 229 nm and response factors relating to the internal standard. Identification was done by liquid chromatography–mass spectrometry (LC-MS), using atmospheric pressure chemical ionization and the $+H^+$ molecular ion. The following GLS were identified: 3MSOP (3-methylsulphinylpropyl GLS), 4MSOB (4-methylsulphinylbutyl GLS), I3M (indol-3-ylmethyl GLS), 4OHI3M (4-hydroxy-indolyl-3-methyl GLS), 4MOI3M (4-methoxyindol-3-methyl GLS), and 1MOI3M (1-methoxyindol-3-ylmethyl GLS).

RNA extraction and expression analysis

RNA was isolated by phenol–chloroform–isoamyl mixture (25:24:1) extraction and LiCl precipitation. cDNA was synthesized from 1 µg of total RNA with a QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK), which includes a DNase step to remove possible DNA contamination. Quantitative real-time PCR was performed using gene-specific primers (Supplementary Table S1 available at *JXB* online) and the fluorescent intercalating dye SYBR Green (Sigma, Dorset, UK) as described by Lee *et al*. (2011). All quantifications were normalized to the *TIP41* gene, and relative quantification was performed using the comparative *Ct*-method. The real-time PCRs were performed in duplicate for each of the three independent biological replicates.

Sulphate uptake

Every fourth hour plants were placed in 24-well plates with 1 ml of MS nutrient solution {adjusted to contain 0.2 mM sulphate and supplemented with 5.6 µCi of [^{35}S]sulphate (Hartmann Analytic, Braunschweig, Germany)}. Plants were incubated in light or dark according to the light period previously described. When collecting samples in the dark, the plants were transferred using green light. After 4 h incubation the plants were washed in sterile water, blotted in paper tissue, weighed, and placed in scintillation vials. A 4 ml aliquot of tissue solubilizer (Soluene-350, Perkin Elmer) was added and the samples were left overnight to dissolve. After addition of 10 ml of Optiphase HiSafe3 scintillation cocktail (Perkin Elmer), the radioactivity of the samples was measured in a scintillation counter (Beckman, High Wycombe, UK).

Determination of flux through sulphate assimilation and rate of GLS biosynthesis

The flux through primary sulphate assimilation, measured as incorporation of ^{35}S from [^{35}S]sulphate to thiols and proteins, and the GLS biosynthesis rate were determined essentially as described by Mugford *et al*. (2011). The plants were transferred into 24-well plates containing 1 ml of MS nutrient solution adjusted to a sulphate concentration of 0.2 mM and supplemented with 5.6 µCi of [^{35}S]sulphate (Hartmann Analytic), and incubated for 4 h in the corresponding light/dark regime. After the incubation, the seedlings were extensively washed in water, carefully blotted in paper tissue, and the shoots were cut, weighed, transferred, into 1.5 ml tubes, and frozen in liquid nitrogen. The quantification of ^{35}S in proteins and thiols followed extraction in 0.1 M HCl, while for the [^{35}S]GLS analysis the shoots were extracted in 70% methanol. The incorporation of ^{35}S in desulpho-GLS was determined by scintillation counting of the flow-through and wash of the DEAE-Sephadex used to isolate GLS.

APR activity

APR activity was determined as the production of [^{35}S]sulphite, assayed as acid volatile radioactivity formed in the presence of [^{35}S]APS and dithioerythritol as reductant (Lee *et al*., 2011). The protein concentrations were determined with a Bio-Rad protein kit (Bio-Rad, Hemel Hempstead, UK) with bovine serum albumin as a standard.

Glutathione measurements

Total glutathione levels were determined by HPLC as described by Lee *et al*. (2011) from 20–30 mg of plant material.

Statistical analysis

The results were analysed for variance by the Genstat software using ANOVA and a significance level of P=0.05. Where only two genotypes were compared, Student's t-test was used.

Results

Diurnal variation in sulphur metabolism and GLS biosynthesis

To obtain a more detailed understanding of the diurnal variation of sulphur metabolism, [^{35}S]sulphate was used to determine sulphate uptake and fluxes in both primary and secondary sulphate assimilation. Sulphate uptake was measured in six 4 h intervals over a 24 h period, starting at light onset. The uptake increased from the start of the day to a higher level during the rest of the light period, followed by a decrease, until reaching the same level as at the beginning of the light period (Fig. 2A). The flux through primary assimilation was determined as incorporation of radioactivity into thiols (cysteine and glutathione) and proteins, while GLS represented the secondary metabolites (Mugford *et al*., 2011). The incorporation of ^{35}S into thiols was higher in the light than in the dark, with maximal values between 8 h and 12 h (Fig. 2B). The incorporation of ^{35}S into proteins, however, had a different pattern, showing an increase only late in the day and in the first part of the dark period (Fig. 2C). There was a clear minimum in incorporation of sulphate into thiols and proteins in the second part of the night. The GLS biosynthesis rate showed a very similar pattern, with a minimum

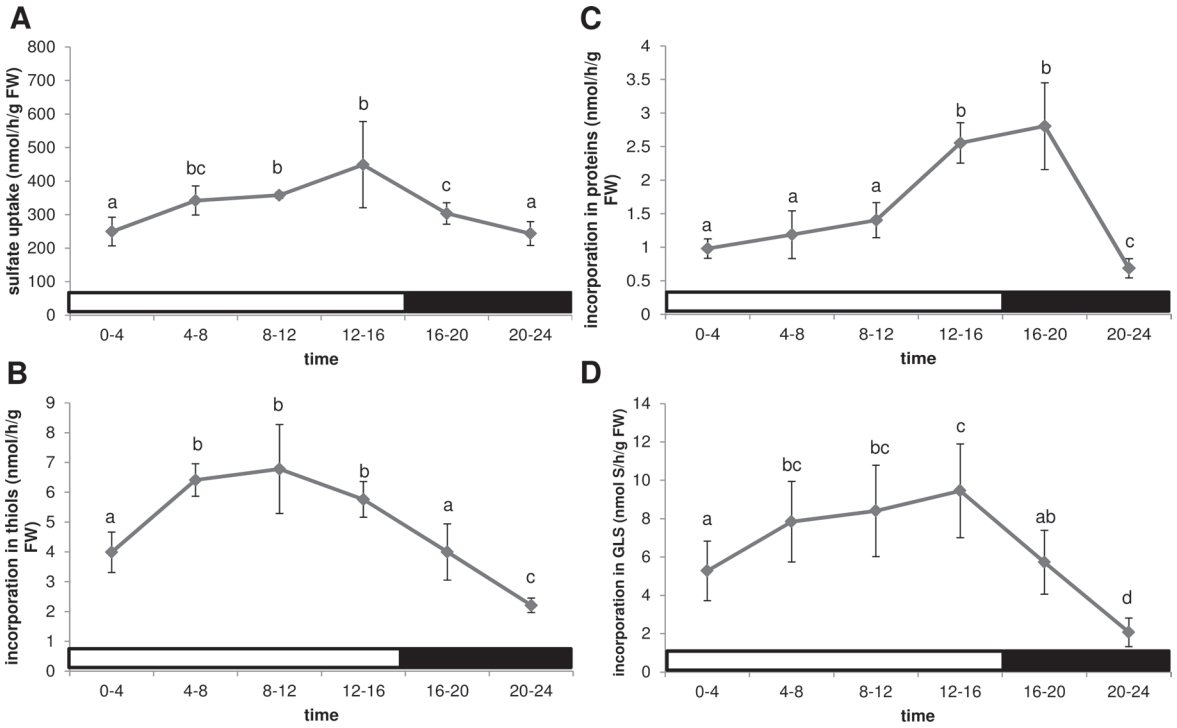

Fig. 2. Diurnal variation in sulphate uptake and fluxes through primary and secondary sulphate assimilation in *Arabidopsis* (Col-0) grown on MS-agarose plates for 2 weeks in long days (16 h light/8 h dark). Sulphate uptake (A) and incorporation of ^{35}S into thiols (B), proteins (C), and GLS (D) was determined. The light regime is indicated by white or black bars. Data are presented as means ±SD. Different letters mark significantly different values from four biological replicates ($P < 0.05$).

at the end of the night (Fig. 2D). Thus, sulphur metabolism seems to be well coordinated throughout the day, with higher activity during the light period than during the night.

The levels of two major sulphur-containing metabolites, glutathione and GLS, were compared as representatives of primary and secondary sulphate assimilation pathways. Glutathione levels were higher during the light period than in the dark, without a clear maximum (Fig. 3A). GLS levels were relatively stable except for a peak 8 h after light onset (Fig. 3B; Supplementary Table S2 at *JXB* online). APR, which controls flux through the sulphate assimilation pathway (Vauclare *et al*., 2002), had previously only been shown to undergo diurnal rhythm in plants adapted to short days (Kopriva *et al*., 2009); therefore, it was necessary to confirm the same regulation in plants that were grown in long days. APR activity was again higher during the light period than in the dark, but without the strong maximum observed under short days (Fig. 3C).

Regulation of GLS biosynthesis by light

The direct effect of dark–light transitions on GLS biosynthesis was analysed in more detail through a light induction experiment testing both the biosynthetic rate and gene expression. Plants kept under the normal light/dark rhythm contained the highest GLS levels (Fig. 4A; Supplementary Table S3 at *JXB* online). Forty-four hours of darkness resulted in an ~25% decrease in GLS levels. Re-illumination for 3 h was not sufficient to increase the GLS content significantly, and this stayed significantly lower than in control plants (Fig. 4A). However, 44 h of darkness affect many processes in plants, and the lack of difference in total GLS content between the dark-treated and re-illuminated plants does not preclude that light directly regulates GLS biosynthesis.

To measure the effect of light on the GLS biosynthesis rate, the incorporation rate of ^{35}S into GLS and desulpho-GLS was determined for the different light regimes. While there was some basal incorporation of ^{35}S in dark-incubated plants, the rate of biosynthesis was greatly increased by light (Fig. 4B). Labelling was found in both GLS and desulpho-GLS, with the former being labelled to a higher degree, reflecting the nature of desulpho-GLS as pathway intermediates. Thus, biosynthesis of GLS is indeed a light-regulated process.

Among the sulphate assimilation genes, *ATPS1–ATPS3* isoforms of ATP sulphurylase and *APK1–APK3* genes encoding APS kinase were down-regulated after 44 h in the dark, while *ATPS4* and *APK4* were not affected (Fig. 5). The three *ATPS* genes were induced by light, but only *ATPS2* reached the levels in control plants after 3 h re-illumination. All *APK* isoforms were up-regulated by light; in particular, *APK1* and *APK4* transcript levels were increased very strongly, ~15-fold compared with the levels in dark-adapted plants. The increase led to mRNA levels for *APK3* and *APK4* being higher than in control plants, but not for *APK2* which was most strongly repressed (Fig. 5). The six genes directly associated with the GLS biosynthesis pathway (*SOT16*, *SOT17*, *SOT18*, *MAM-L*, *CYP79F1*, and *CYP79B2*) all followed the same pattern of down-regulation in the dark and up-regulation after light

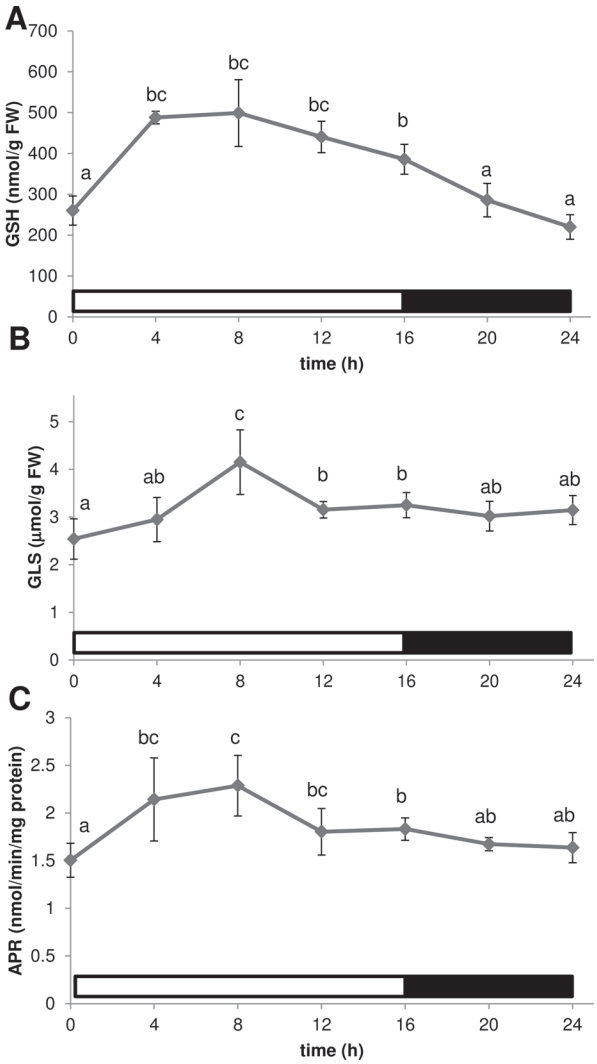

Fig. 3. Diurnal variation in contents of glutathione (GSH) (A), total GLS (B), and APS reductase activity (C). The light regime is indicated by white or black bars. Data are presented as means ±SD. Different letters mark significantly different values from four biological replicates ($P < 0.05$).

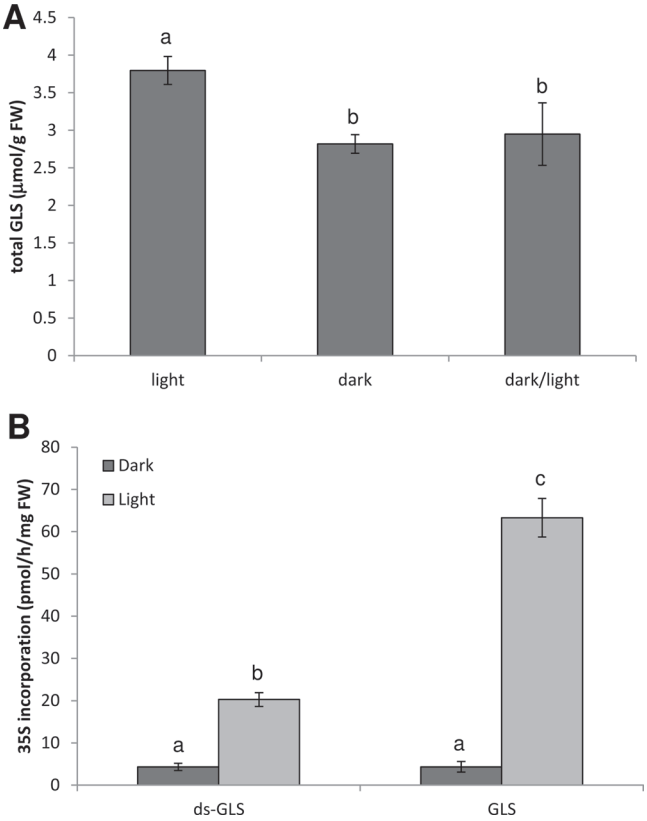

Fig. 4. GLS synthesis in *Arabidopsis* seedlings under different light regimes. Three-week-old *Arabidopsis* seedlings on MS-agarose plates were transferred to darkness for 44 h. One part of these seedlings was re-illuminated by white light for 3 h (dark/light), while the other part remained in darkness (dark). (A) Total GLS levels in these plants were compared with those of plants continuing to grow in the normal day/night cycle (light). (B) ^{35}S incorporation in desulpho-GLS and GLS during 4 h was determined. Data are presented as means ±SD from four pools of three seedlings. Different letters mark significantly different values from four biological replicates ($P < 0.05$).

induction, with both *CYP* genes being repressed to the highest degree. The same expression pattern can be seen in regulation of mRNAs for transcription factors *MYB28*, *MYB29*, and *MYB76*, which have been shown to control biosynthesis of aliphatic GLS (Hirai *et al*., 2007). For the transcription factors associated with indolic GLS (*MYB51*, *MYB34*, and *MYB122*), the results are more varied. *MYB51* was down-regulated in the dark, but showed no response to light induction. *MYB34* was regulated in an opposite manner, namely it did not respond to dark treatment but was still induced by light. *MYB122*, on the other hand, was the only gene that showed a strong up-regulation under dark conditions and no response after light induction (Fig. 5). Thus, the genes of GLS metabolism are highly regulated by light but not in a completely coordinated manner.

Having established that the GLS biosynthetic genes are light regulated, tests were conducted to determine whether they may also be under HY5 control (Fig. 6). Although 1-week-old plants were analysed, compared with 3-week-old plants in a previous experiment, in Col-0 most genes showed the same response to re-illumination. The major difference seems to be the regulation of *MAM-L*, which was repressed by 90 min re-illumination of 1-week-old plants but induced in 3-week-old plants. The regulation of several genes of GLS biosynthesis was strongly affected in the *hy5* mutant. The most pronounced difference was the opposite effect of light on the two genes involved in core GLS biosynthesis (*CYP79F1* and *CYP79B2*), which were induced in Col-0 but repressed in *hy5* (Fig. 6). Also *SOT18* was up-regulated to a much lower degree in *hy5* compared with Col-0, whereas the other *SOT* genes and *MAM-L* were regulated in the same way in the two genotypes. The regulation of all ATPS isoforms and *APK1* and *APK3* was not affected by HY5 disruption. However, strikingly, the MYB factors that are associated with aliphatic GLS biosynthesis were more strongly up-regulated in *hy5* than in Col-0. The same was true for *APK2* and *APK4*, indicating that HY5 may act as a transcription repressor as well

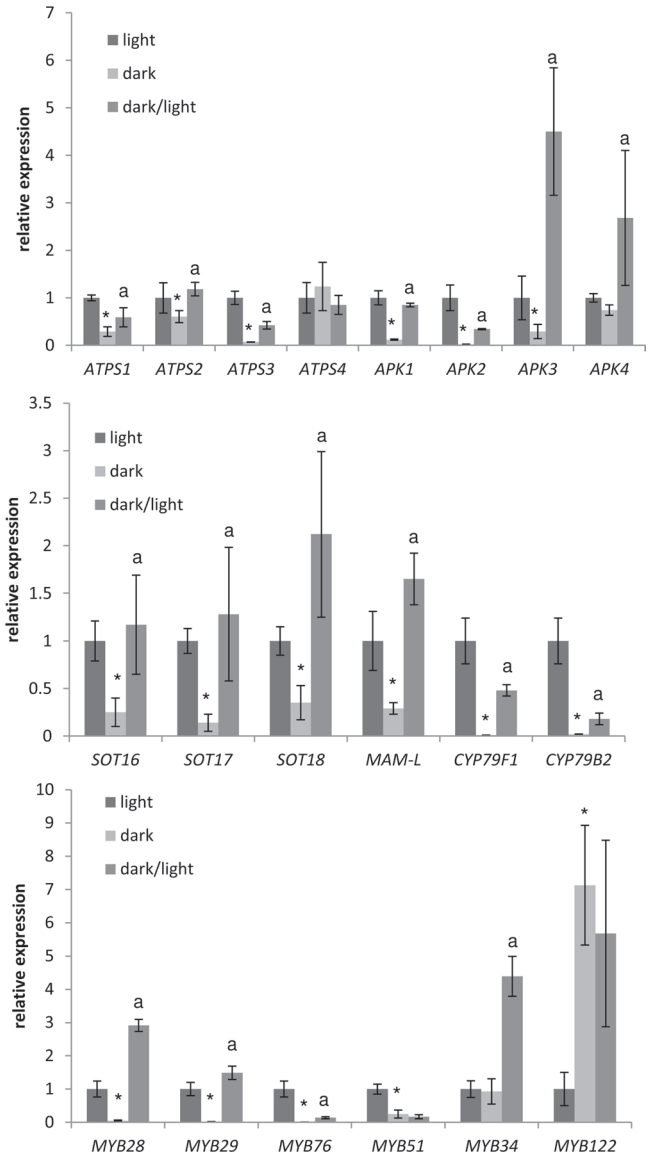

Fig. 5. Light regulation of genes of sulphate assimilation and GLS biosynthesis. Three-week-old *Arabidopsis* seedlings on MS-agarose plates were transferred to darkness for 44 h. One part of these seedlings was re-illuminated by white light for 3 h (dark/light), while the other part remained in darkness (dark). These plants were compared with plants continuing to grow in the normal day/night cycle (light). Transcript levels of genes involved in sulphur metabolism and GLS biosynthesis were determined by quantitative real-time PCR. The values for control plants (light) were set to 1. Data are presented as means ±SD from three pools of three seedlings. Asterisks mark significant differences between dark and light transcript levels, while the letter 'a' marks values significantly different between dark/light and dark samples ($P < 0.05$).

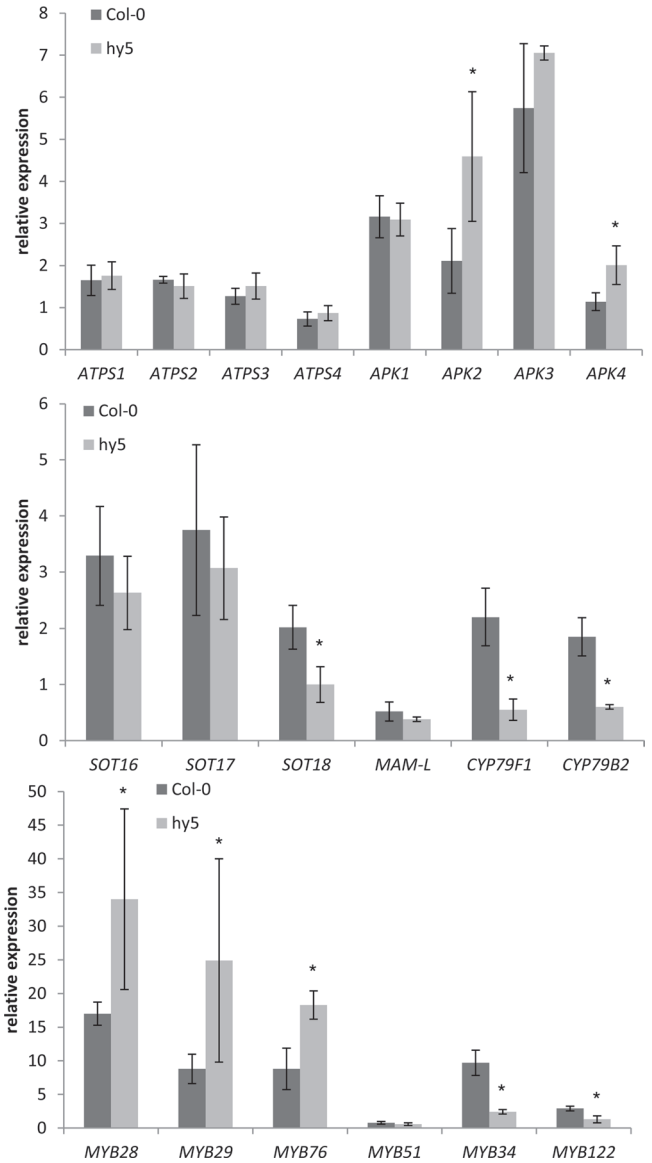

Fig. 6. HY5 controls light regulation of genes of sulphate assimilation and GLS biosynthesis. One-week-old *Arabidopsis* Col-0 and *hy5* seedlings on MS-agarose plates were transferred to darkness for 38 h. One part of these seedlings was re-illuminated by white light for 90 min, while the other part remained in darkness. Transcript levels of genes involved in sulphur metabolism and GLS biosynthesis were determined by quantitative real-time PCR. The values in dark-grown plants were set to 1. Data are presented as means ±SD of the relative expression in re-illuminated plants compared with dark-grown plants determined in three pools of three seedlings. Asterisks mark values significantly different between the two genotypes ($P < 0.05$).

as an activator. *MYB51* was not regulated by light in either genotype, whereas both *MYB34* and *MYB122* were induced to a lower degree in *hy5*. Thus, most genes of the GLS biosynthetic network, but not genes of sulphate assimilation involved in the biosynthesis of PAPS, are light regulated in a HY5-dependent manner.

Discussion

Diurnal variation of sulphur metabolism and GLS biosynthesis in Arabidopsis

Plant physiology and the life cycle are under strong control of diurnal and circadian rhythms. For example, more than a third of *Arabidopsis* genes are under circadian control

(Pruneda-Paz and Kay, 2010). Key genes of the major pathways of primary metabolism, carbon, nitrate, and sulphate assimilation show clear diurnal and/or circadian rhythmicity (Pilgrim *et al*., 1993; Kopriva *et al*., 1999; Zeeman *et al*., 2007). Much less is known about circadian regulation of secondary metabolism, even though a large number of cytochrome P-450 genes were found to be controlled by the clock (Pan *et al*., 2009). There is, however, evidence for diurnal variation in levels of GLS in field-grown *Brassica oleracea* plants (Rosa *et al*., 1994) which was confirmed under controlled conditions (Rosa, 1997). In the results presented here, a variation in GLS content in *Arabidopsis* grown under control conditions has been detected, with greater accumulation in the day than in the night. This is surprisingly in contrast to the results with *B. oleracea*, which showed a significant decrease of GLS content during the day both in field and in controlled conditions (Rosa *et al*., 1994; Rosa, 1997). On the other hand, the GLS levels agree well with the variation in sulphate uptake, APR activity, sulphate reduction rate, and, most importantly, the GLS biosynthesis rate measured in the same plants. The difference compared with *B. oleracea* may thus be due to the different developmental stages of plants analysed or due to species-specific variation, possibly linked to the different nature of the major herbivores associated with these species. Diurnal rhythms for a large range of secondary compounds involved in plant–herbivore interactions have been described, some showing higher foliar accumulation in the light, but others, importantly for nocturnal insects, more abundant in the night (De Moraes *et al*., 2001; Kim *et al*., 2011).

The various components of sulphur metabolism were to a large extent coordinated throughout the 24 h day cycle. Only subtle differences were found between, for example, accumulation of glutathione or GLS and their rates of synthesis (Figs 2, 3). This is confirmed by a high level of correlation between individual components, particularly APR activity and incorporation into thiols, with glutathione and GLS levels (Supplementary Table S4 at *JXB* online). Only the incorporation in proteins shows a very different pattern and only a weak correlation with sulphate uptake. Interestingly, for both GLS and glutathione, the highest metabolite accumulation seems to be achieved before the period of the highest synthesis rate. This may seem contra-intuitive; however, metabolite levels are not dependent solely on the biosynthesis rate but also on the breakdown of the compound. This has been demonstrated, for example, in the *apr2* mutant lacking the major isoform of APR, which shows lower flux through sulphate assimilation without affecting glutathione levels or in *apk1 apk2* mutants which showed an increased rate of incorporation into GLS despite much lower GLS levels (Mugford *et al*., 2011). Interestingly, reduced sulphur seems to be utilized first for synthesis of glutathione and GLS and only in later stages incorporated into proteins (Fig. 2). This corresponds to the results of Koprivova *et al*. (2000) who showed that after resupply of nitrogen to N-starved plants the glutathione pool was filled before proteins. The diurnal variation of APR (Fig. 3C) agrees with a previous report (Kopriva *et al*., 1999); however, the amplitude of the cycle was much less in the present study. This is probably caused by the difference in light regime between the studies, short days (10 h light) in Kopriva *et al*. (1999) versus long days (16 h light) reported here. Thus, although the transcript level of *APR2* is under circadian control (Harmer *et al*., 2000), the control of APR variation also has to include a component dependent on daylength. The daylength also seem to affect the variation in fluxes, as the significant drop at the end of the night (Fig. 2) was not detected in plants grown in short days (Kopriva *et al*., 1999).

Sulphate uptake showed a diurnal variation, with the highest rate measured in the second half of the day (Fig. 2A). The maximum rate of sulphate uptake seems to follow after the maximal flux through the pathway. The trigger for such regulation thus could be depletion of sulphate due to increased reduction, as the maximum APR activity was measured in the first half of the day. Indeed, low internal sulphate levels in mutants of the sulphate transporter *SULTR1;2* and a *FIERY1* gene trigger sulphate deficiency responses, which include induction of sulphate transporters (Lee *et al*., 2012; Matthewman *et al*., 2012). The gradual increase in sulphate uptake rate during the light period is very similar to the diurnal regulation of nitrate uptake (Lejay *et al*., 1999). The variation in nitrate uptake can be explained by changes in expression of the nitrate transporters *Nrt1* and *Nrt2;1* (Lejay *et al*., 1999). Also the transcripts of the PHT4 group of phosphate transporters undergo circadian regulation, with a maximum during the day (Guo *et al*., 2008). Whether the same is true for sulphate transporters still has to be established.

GLS biosynthesis is controlled by light

Diurnal regulation is often connected with light regulation. In sulphur metabolism, APR activity and mRNA levels were shown to decrease strongly when plants were kept in continuous darkness and increased rapidly after re-illumination (Kopriva *et al*., 1999). The present results show that other components of the pathway are also light regulated. With the exception of *ATPS4* and *APK4*, genes encoding the two enzymes necessary for PAPS biosynthesis (ATP sulphurylase and APS kinase) were down-regulated upon incubation of plants in prolonged darkness. *ATPS4* is the major isoform of ATPS in the roots, while *APK4* is a minor plastidic form of APS kinase, so that the lack of light regulation is not entirely surprising (Kopriva *et al*., 2009). The *ATPS* and *APK* genes, including *APK4* but not *ATPS4*, were induced upon re-illumination of dark-adapted plants. This regulation is consistent with regulation of APR and shows a well-coordinated response of the whole pathway to changes in light regime.

Similarly coordinated was the regulation of GLS biosynthetic genes (Fig. 5). It has been shown previously that the expression of genes of GLS biosynthesis is well coordinated, for example repressed by sulphur deficiency (Hirai *et al*., 2005) or induced in *apk1 apk2* mutants (Mugford *et al*., 2009). Similar coordination of the transcript accumulation is seen in plants with modulated expression of the two groups of MYB factors (Gigolashvili *et al*., 2007*a*, *b*, 2008; Sønderby *et al*., 2007; Malitsky *et al*., 2008). The MYB factors themselves are regulated in the same way (Hirai *et al*., 2005; Mugford *et al*., 2009), so it appears to be the changes

in the expression of MYB factors driving the regulation of the downstream transcript abundance. This seems to be true for the light regulation of the aliphatic GLS subset of the network, as the biosynthetic genes were regulated in the same way as the genes for MYB28, MYB29, and MYB76 factors. The genes involved in biosynthesis of indolic GLS, *CYP79B2* and *SOT16*, were co-regulated with the other biosynthetic genes; however, the MYB factors responded to light in a different manner. Nevertheless, mRNA for the main indolic MYB factor, MYB51, was reduced in dark-grown plants, resulting in repression of *CYP79B2* and *SOT16*. This decrease was probably not compensated by the presence of *MYB34* and *MYB122*, which were not affected or were even induced by darkness, respectively, which agrees with the finding that disruption of MYB51 severely reduces transcription of genes for biosynthesis of the indolic GLS (Gigolashvili *et al*., 2007a). On the other hand, *MYB34* was induced by re-illumination and shown previously to induce expression of genes of biosynthesis of indolic GLS (Celanza *et al*., 2005). It seems, therefore, that unlike the aliphatic group of MYBs, the MYB factors controlling indolic GLS biosynthesis have a specific function in light regulation of their target genes.

The effect of light on GLS biosynthesis, however, was not confined to regulation of gene expression. In agreement with the low transcript levels of GLS biosynthetic genes, the total GLS contents were reduced in plants kept in darkness. The decrease in GLS was not as strong as the reduction in mRNA levels, probably since the turnover of GLS is slower. In line with the induction of gene expression, re-illumination induced GLS biosynthesis that was very low in the dark-incubated plants. The down-regulation of GLS biosynthesis in prolonged darkness is consistent with a decrease in the synthesis rate in the night, particularly in the second half (Fig. 2D). The light induction also seems to be similar to that seen during the day; however, it has to be noted that in the first 4 h of re-illumination, the GLS biosynthesis rate was still almost 100-fold lower than during the day (cf. Figs 2D and 4). This is similar to the light regulation of the primary sulphate assimilation. The sulphate reduction rate also decreases in prolonged darkness and is induced by light (Lee *et al*., 2011). The flux during the first 4 h of re-illumination (Lee *et al*., 2011) was ~8-fold lower than the flux during the day determined in this study. This is partly caused by averaging the flux over the 4 h experimental period, with initial rates expected to be particularly low because the necessary enzymes need to be synthesized. The lower rate of GLS biosynthesis compared with sulphate reduction presumably reflects the plants' needs for cysteine for protein biosynthesis and thus represents further evidence that primary and secondary sulphur metabolism are highly coordinated.

HY5 contributes to regulation of GLS synthesis by light

The induction of *APR* mRNA by light is at least partly dependent on HY5. HY5 also controls the regulation of *SULTR1;2*, the major sulphate transporter in the roots. Thus, it was intriguing to test whether the pathway of sulphate assimilation is regulated by HY5 coordinately, particularly as the regulation of *ATPS1–ATPS3* (Fig. 5) was very similar to light regulation of APR (Lee *et al*., 2011). This, however, was not the case; ATPS was regulated by light in the same way in Col-0 and the *hy5* mutant (Fig. 6). Different mechanisms of regulation between ATPS and APR, despite catalysing the subsequent steps in the pathway, are not surprising. For example, APR is highly induced by sulphate deficiency but ATPS is repressed (Takahashi *et al*., 1997; Kawashima *et al*., 2011). Also the mechanisms of this regulation are different. APR is transcriptionally regulated, whereas ATPS is a target of a sulphur limitation-inducible microRNA, miR395, and is thus down-regulated post-transcriptionally (Kawashima *et al*., 2011). Given the different mechanisms of regulation of APR and ATPS by sulphate deficiency, it is not surprising that the mechanism of their light regulation differs.

On the other hand, many genes of the GLS biosynthesis network are regulated in a HY5-dependent manner and remarkably several of the MYB factors, too (Fig. 6). Again, the aliphatic and indolic MYB groups were regulated differently; whereas all three aliphatic MYB factors were more strongly induced by light in *hy5*, the light induction of *MYB34* and *MYB122* was almost abolished in the mutant. Thus, HY5 acts as a repressor of the aliphatic MYB factors and an activator for the two indolic ones. This is consistent with the influence of HY5 on many regulatory networks not through direct binding but through controlling other transcriptional regulators (Zhang *et al*., 2011). It seems that GLS biosynthesis is another example of such networks, even though the mechanism of HY5 action is less than clear. While the *MYB28*, *MYB29*, and *MYB76* genes are induced by light to a higher degree in the *hy5* mutant than in Col-0, the target genes *SOT18* and *CYP79F1* are less strongly up-regulated. The hierarchy of HY5 and the MYB factors in regulation of GLS biosynthesis by light thus still needs to be established.

Altogether, these results show that GLS biosynthesis is regulated by light and shows diurnal variation that is well coordinated with general sulphur metabolism. The transcription factor HY5 seems to be involved in regulating the GLS biosynthetic network. These new data complement and expand the available knowledge on the coordination of primary and secondary sulphur metabolism in *Arabidopsis*.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primer sequences used for quantitative real-time PCR.

Table S2. Diurnal variations of individual GLS.

Table S3. Light regulation of individual GLS.

Table S4. Correlation analysis of diurnal variations in levels of sulphur-containing metabolites, fluxes, and APR activity.

Acknowledgements

The work of SHs is supported by the Research Council of Norway (project no. 185017/O10). The work of SK is supported by British Biotechnology and Biological Sciences Research Council (BBSRC).

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