Do PFT1 and HY5 interact in regulation of sulfate assimilation by light in Arabidopsis?

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Abbreviations

APS, adenosine-5'-phosphosulfate; APR, adenosine-5'-phosphosulfate reductase; PFT1, Phytochrome and Flowering Time 1, HY5, Long Hypocotyl 5.

Abstract

Sulfate assimilation pathway is highly responsive to changes in environment, but the mechanisms of such regulation are only slowly beginning to unravel. Here we show evidence that PHYTOCHROME AND FLOWERING TIME 1 (PFT1) may be another component of the sulfate assimilation regulatory circuit. Transcriptional regulation by light of the key enzyme of sulfate assimilation, adenosine 5'phosphosulfate (APS) reductase, is disturbed in *pft1-2* mutants. PFT1, however, affects also APS reductase enzyme activity, flux through the sulfate assimilation pathway and accumulation of glutathione. In addition, our data suggest a possible interplay of PFT1 with another transcription factor, HY5, in regulation of APS reductase by light.

Highlights

- We show that PFT1 may play a role in light regulation of APS reductase.
- PFT1 affects transcript levels of APS reductase in an isoform specific way as activator and repressor.
- Light regulation of APS reductase seems to require a complex interplay of PFT1 and HY5.

1. Introduction

Sulfur is an essential macronutrient for synthesis of amino acids cysteine and methionine and a range of cellular metabolites. It is acquired as sulfate by the roots, reduced and incorporated into the bio-organic compounds by the sulfate assimilation pathway [1, 2]. Adenosine 5'-phosphosulfate reductase (APR) catalyzes the reduction of activated sulfate, adenosine 5'-phosphosulfate, to sulfite. APR is the key enzyme of the pathway; it controls the flux and the synthesis rate of reduced sulfur compounds [3, 4] and the accumulation of sulfate and other S-containing metabolites [5, 6]. APR is highly regulated at the transcriptional as well as post-translational levels according to the demand for reduced sulfur. The enzyme activity is induced by sulfate deficiency, exposure to heavy metals, or inhibition of glutathione synthesis, and repressed by reduced sulfur containing compounds or nitrogen deficiency [1, 7-10]. However, knowledge of the molecular mechanisms of APR regulation is still very limited. The enzyme undergoes redox regulation, resulting in higher activity in oxidising conditions [9] and is regulated by a complex signalling network in response to salt stress [11]. APR is transcriptionally regulated by a group of six MYB factors that control synthesis of glucosinolates, S-containing secondary metabolites [12]. Two of the three *APR* genes, *APR1* and *APR2*, are under direct transcriptional control by LONG HYPOCOTYL5 (HY5), which is important for regulation of the pathway by light, nitrogen availability and reaction intermediates [13].

Given the large number of environmental perturbations affecting APR activity, plants must possess a mechanism for integration of these signals and fine-tuning the transcriptional and other responses. A good candidate for such integrative function in plants is PHYTOCHROME AND FLOWERING TIME 1 (PFT1) previously shown to integrate environmental signals to control plant development [14]. PFT1 was identified initially as an inducer of flowering in suboptimal light conditions [15]. PFT1 affects both CONSTANS-dependent and independent mechanisms of flowering induction and affects FLOWERING LOCUS T transcription [16, 17]. Loss of function of the *PFT1* gene, however, disturbs many aspects of plant life beyond flowering time. PFT1 regulates jasmonate dependent gene expression [18, 19] and the cross talk between jasmonate and abscisic acid signalling [20]. PFT1 also contributes to control of cell growth [21]. Importantly, PFT1 interacts with HY5 in light signalling [22].

Since APR is regulated by light [8] and since HY5 directly controls *APR* transcription [13], we hypothesised that PFT1 may be another component of the sulfate assimilation

regulatory circuit. Here we show that indeed, *APR* regulation by light is altered in *pft1* mutant. In addition, analysis of *hy5 pft1* double mutant suggests an interplay of HY5 and PFT1 in light regulation of sulfate assimilation.

2. Material and Methods

Plant material and growth conditions

In this study, *Arabidopsis thaliana* (ecotype Col-0) were used as wild type. The *pft1-2* mutant was obtained from NASC Arabidopsis stock centre (SALK_129555.20.45.x), genotyped by PCR to ensure that the insertions were homozygous and the lack of *PFT1* transcript was verified by RT-PCR. The *hy5* mutant was described previously [13].

Plants were grown on plates with Murashige Skoog media without sucrose (MS) supplemented with 0.8% agarose. The plates were placed horizontally in a controlled environment room at 20°C under 16 h light/8 h dark cycle and light intensity of 160 µmol m^{-2} s^{-1}. For light induction treatments, plants were grown for 7 days and transferred into darkness for 38 hours before half of the plates were re-illuminated by white light and half remained in darkness. For each experiment, three individual biological replicates were collected from three separate plates and the experiments were independently replicated. Mature plants were grown in controlled environment room for 5 weeks in short days (10 h light/14 h dark).

Expression analysis

Total RNA was isolated by standard phenol/chlorophorm extraction and LiCl precipitation. First-strand cDNA was synthesized from 1 µg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen), which includes a DNAse step to remove possible DNA contamination. Quantitative real-time RT-PCR (qPCR) was performed using gene-specific primers (Supplemental Table S1) and the fluorescent intercalating dye SYBR Green (Applied Biosystems) in a DNA engine OPTICON2 with continuous fluorescence detector (Bio-Rad) for 2 min at 95°C and then 40 cycles consisting of 15 s at 95°C, 15 s at 60°C, and 30 s at 72°C, 10 min at 72°C, followed by a subsequent standard dissociation protocol to ensure that each amplicon was a single product. All quantifications were normalized to ubiquitin UBQ10. The RT-PCR reactions were performed in duplicate for each of the three independent samples.

Enzyme Assays

APS reductase activity was determined as the production of [^{35}S]sulfite, assayed as acid volatile radioactivity formed in the presence of [^{35}S]APS and dithioerythritol as reductant [11]. ATP sulfurylase was measured as the APS and pyrophosphate-dependent formation of ATP [23]. Protein concentration was determined with a Bio-Rad protein kit (Bio-Rad) with bovine serum albumin as a standard.

Determination of flux through sulfate assimilation

Flux through sulfate assimilation pathway was measured as incorporation of ^{35}S from [^{35}S]sulfate to thiols and proteins essentially as described in [24]. The plants were grown for 1 week on vertical MS-agarose plates and transferred to dark for 38 hours. The plants were transferred in darkness into 48-well plates containing 1 mL of MS nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with [^{35}S]sulfate (Hartmann Analytic) to specific activity of 420 Bq nmol sulfate^{-1} and incubated either in darkness or in light for 4 hours. After incubation, the seedlings were washed extensively with water, carefully blotted with paper tissue, weighed, transferred into 1.5 mL tubes, and frozen in liquid nitrogen. The quantification of ^{35}S in different S-containing compounds was performed exactly as in [24]. The uptake and flux data thus represent an average over the first 4 hours of re-illumination and target the early responses of the mutants.

Determination of sulfur-containing metabolites

Sulfate was measured in 50 mg of leaf material by HPLC as described in [25]. The analysis of cysteine and GSH was performed from 10-20 mg of plant material as described [11]. Glucosinolates were extracted from 60-80 mg frozen leaf material and quantified following [25].

3. Results

PFT1 has been shown to affect the function of HY5 in light signaling [22]. Since *APR* is light regulated in an HY5-dependent manner [8, 13], we hypothesized that PFT1 may also be part of this regulatory circuit. An *A. thaliana* T-DNA line, corresponding to the *pft1-2* loss of function allele [15], was therefore tested for disruption in light induction of *APR*. Col-0 and *pft1-2* seedlings were adapted to dark for 38 hours, exposed to white light, and transcript levels of the three *APR* isoforms were determined by qRT-PCR (Figure 1). Light increased steady state mRNA levels for all three *APR*

isoforms in Col-0, but to different extents. Interestingly, in *pft1-2* seedlings *APR1* mRNA was induced by light to a much higher degree than in Col-0. On the other hand, the level of *APR2* induction in *pft1-2* was slightly but significantly lower than in Col-0. Also for transcript levels of *APR3* the degree of induction was higher in the mutant, similar to *APR1* (Fig. 1). These results indicate that PFT1 participates in regulation of *APR* expression by light, in an isoform-specific manner as an activator or a repressor.

To test whether PFT1 and HY5 interact in the light regulation of *APR* we crossed the *pft1-2* and *hy5* mutants. Introduction of the *pft1* mutation into *hy5* resulted in an attenuation of the distinguishing morphological feature of *hy5* mutant, the elongated hypocotyl (Supplemental Figure S1). The *hy5 pft1* mutant grown in white light showed hypocotyl length between that of *hy5* and *pft1-2* or Col-0. Similarly, the late flowering of *pft1-2* was attenuated in *hy5 pft1* plants that, however, set flowers later than Col-0 or *hy5* and at maturity were slightly smaller than either of the parental mutants (Supplemental Figure S1). The double mutant and as a control also the parental mutants, *hy5* and *pft1-2*, were tested for induction of *APR* in dark-adapted seedlings. Light induction of *APR1* was compromised in the *hy5 pft1* mutant in the same way as in *hy5*, i.e., the mRNA levels were not significantly increased in re-illuminated seedlings (Figure 2). On the other hand, the mutations in *HY5* and *PFT1* seemed to have an additive negative effect in regulation of *APR2*. Despite *APR3* not being targeted by HY5 [13], in the *hy5 pft1* mutant the additional disruption of *HY5* abolished the effect of *pft1* mutation (Figure 2). Thus, PFT1 and HY5 seem to cooperate in regulation of *APR* mRNA levels by light in a complex and isoform-dependent manner.

We therefore asked whether the loss of PFT1 and HY5 affects the light regulation of sulfate assimilation beyond the *APR* transcript levels, and measured sulfate uptake and the flux through the pathway during the first four hours of re-illumination. Sulfate uptake was not affected by re-illumination of the dark-adapted seedlings of any genotype (Figure 3A). However, when absolute values were compared, the double mutant *hy5 pft1*, unexpectedly, took up more sulfate in the light then other genotypes, despite no alterations in the individual mutants and only a marginal disturbance of *APR1* and *APR3* regulation. The flux through sulfate assimilation, determined as incorporation of ^{35}S from [^{35}S]sulfate into cysteine, GSH and proteins, increased upon re-illumination in Col-0 and *pft1-2*, but was not different between dark-adapted and re-illuminated plants with disrupted *HY5* (Figure 3B). Interestingly, the flux was higher in *pft1-2* than in Col-0 in both light conditions. The increased flux through sulfate

assimilation resulted in an increase in GSH content in re-illuminated Col-0 and *pft1-2* compared to dark-adapted plants (Figure 3C). Surprisingly, however, despite the higher flux in *pft1-2* compared to Col-0, its GSH levels were actually lower than in the wildtype. In agreement with the results of flux analysis, GSH levels were not increased by re-illumination in *hy5* and *hy5 pft1* mutants (Figure 3C).

However, as these experiments were performed with seedlings we asked whether mature plants also show differences in sulfur metabolism. The levels of sulfur-containing metabolites in *pft1-2* did not differ from wild type Col-0, but enzyme activities of both APR and ATP sulfurylase were significantly higher and lower, respectively (Table 1). APR activity in *hy5* and *hy5 pft1*was also higher than in Col-0, but only in *hy5* was glutathione content elevated. Interestingly, sulfate accumulated in *hy5 pft1* plants despite the higher APR activity (Table 1). Thus, PFT1 appears to contribute to regulation of sulfate assimilation beyond the transcriptional regulation of *APR* and, possibly via a complex interplay with HY5, to be involved in light regulation of the pathway and general fine tuning of sulfur metabolism.

4. Discussion

PFT1 has been an attractive candidate for new components of sulfate assimilation regulatory networks, because this protein was shown to interact with HY5, a known regulator of APR [13], and to be important for control of many cellular processes in plants [15, 18, 22]. Indeed, disruption of *PFT1* resulted in altered transcriptional regulation of *APR* by light, in an isoform-specific manner. Interestingly, the consequences of disruption of *PFT1* for light regulation of *APR* are very different from the effects of *HY5* mutation. Firstly, whereas in *hy5* mutant *APR1* was not induced within the first 90 min of re-illumination of dark-adapted plants [13], in *pft1-2* the level of *APR1* induction was much higher than in wild type plants (Figure 1). Secondly, in *hy5* only *APR1* and *APR2* were affected, while in *pft1-2* all three isoforms are regulated in a different manner to Col-0. In the double mutant *hy5 pft1 APR1* and *APR3* are regulated similarly to *hy5*, while the effects of the two mutations are additive for regulation of *APR2*. Different regulation of *APR2* compared to *APR1* and *APR3*, has been observed before, e.g., in plants treated with ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) [11] or in plants overexpressing MYB51 [12] and reflects the degree of similarity between nucleotide sequences of the three genes. The isoform specific differences are, however, remarkable since unlike other

gene families of sulfate assimilation, the three APR isoforms are all localised in plastids, but they have different kinetic properties and somewhat altered spatial expression pattern [7].

APR1 regulation is consistent with the model of interaction between HY5 and PFT1 in light regulation proposed by [22], in which PFT1 inhibits the PhyB mediated repression of phytochrome-interacting proteins (PIF) that in turn inhibit HY5. Thus, in *pft1-2*, the PIFs are prevented from reducing HY5 activity and *APR1* is de-repressed (Figure 4). Since in *hy5 pft1 APR1* is not up-regulated by light in the first 120 min, HY5 appears to be the main factor responsible for the initial increase in *APR1* transcript levels after re-illumination. For light regulation of *APR2* on the other hand, HY5 is not the main effector, as its loss reduces the transcript increase only by ca. 40% and therefore PFT1 may act mainly through positive interaction with the alternative transcription factor. Thus, while the loss of PFT1 relieves inhibition of HY5, it reduces the activity of the alternative factor which together results in a slightly lower induction of *APR2*. Indeed, five HY5 binding sites were detected in *APR1* promoter compared to two in *APR2* [26] supporting the observed difference in dependence of these two genes on HY5. *APR3* is not under direct control of HY5 [13, 26], therefore the positive effect of PFT1 loss is probably mediated through an unknown negative regulator. It should be noted, however, that while the transcriptional changes in *APR* were triggered by light, they might not be caused by light signalling directly. The actual signal(s) may be derived from metabolic pathway(s) induced by light, e.g. carbohydrates or ammonium, which are known regulators of *APR* [10, 27].

Importantly, the involvement of PFT1, as well as HY5, is not limited to transcriptional regulation of *APR*. In *pft1-2* mutant, the flux through sulfate assimilation is higher than in Col-0. This corroborates the key role of APR in control of the flux, as *APR1* and *APR3* were up-regulated to a greater extent in *pft1-2*. The increased flux, surprisingly, did not result in higher glutathione levels, in fact, they were lower than in Col-0. This demonstrates again that the regulation of glutathione homeostasis is complex and glutathione levels not always directly proportional to the flux; e.g. in *apr2* mutants the flux is reduced compared to Col-0 but the glutathione levels are unchanged [24]. Interestingly, disruption of PFT1 had an opposite effect on APR and ATP sulfurylase activities in mature plants without affecting metabolite levels. It is possible that the increased induction of *APR1* and *APR3* is retained and results in elevated transcript levels and consequently increased APR activity in mature plants as well. *ATPS1*, the

major isoform of ATP sulfurylase, has been shown to be down-regulated in leaves of *pft1* mutant (supplemental data in [18]), which agrees with the reduced enzyme activity. As both APR and ATPS contribute to control of flux through sulfate assimilation [28], the changed activities in *pft1-2* possibly neutralise each other and metabolite levels are not affected. Contrasting regulation of APR and ATP sulfurylase in *pft1-2* is reminiscent of the opposite regulation of these enzymes by sulfate starvation, while APR is induced in these conditions, ATP sulfurylase activity is repressed [1].

On the other hand, the lower extent of *APR* transcript induction in *hy5* mutant was not able to support the light-induced increase in flux and GSH content seen in wild type plants (Figure 3). As in the regulation of *APR* mRNA levels, the double *hy5 pft1* mutant resembled *hy5*, indicating that HY5 is more important for the early phase of *APR* regulation by light than PFT1. Interestingly, the isoform most affected by the disruption of PFT1 and HY5 is APR1, the transcript of which is the least abundant of the three. Nevertheless, the APR1 isoform contributes ca. 20% to total APR activity [29], and clearly, the changes in regulation of *APR1* in the mutants are more strongly translated to changes in APR activity than those of *APR2*. This confirms previous conclusions that APR activity is regulated at multiple levels and is not directly proportional to mRNA accumulation [11].

While single *pft1* or *hy5* mutations had no effect on sulfate uptake in the light, disruption of both genes led to significant increase in uptake capacity. This was reflected in high sulfate content of mature *hy5 pft1* plants, despite a higher APR activity (Table 1). The high sulfate levels are in accordance with higher sulfate uptake rate in the re-illuminated seedlings (Figure 3A). It is possible that the uptake is affected by the simultaneous disruptions of *HY5* and *PFT1* not only during the first phase of re-illumination, but remains de-repressed in the light, so that even the additional APR activity does not prevent sulfate accumulation. HY5 binds to the promoter of *SULTR1;2* [13], which encodes a high affinity sulfate transporter responsible for sulfate uptake from the soil [30]. Thus, HY5 may act as repressor of *SULTR1;2* expression. However as no changes in uptake were observed in single *hy5* mutants, another repressor of sulfate transporter(s) dependent on PFT1 has to be postulated. The uptake would thus be increased only if function of both repressors is disturbed. The role of HY5, PFT1, and generally light in regulation of sulfate uptake thus deserves a more detailed and focused study in the future. Indeed, control of sulfate uptake and homeostasis is complex, including alternative transporters, metabolic signals, or regulation by microRNAs [1],

so that a number of new effectors, activators as well as repressors, is awaiting discovery.

Evidently, PFT1 plays a role in *APR* regulation by light. However, as it is not a transcription factor the effect of PFT1 on *APR* transcription must be indirect, e.g. through its function in the Mediator complex, which facilitates gene transcription by bridging transcription factors with RNA polymerase II complex [31]. As part of the Mediator, PFT1 interacts with a number of transcription factors and modulates so their activity [32], which might be another mechanism of the interplay with HY5. Dissection of the role of Mediator in control of APR regulation and control of the pathway will thus be an exciting topic for further research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplemental Figure 1. Phenotypes of *pft1-2*, *hy5*, and *hy5 pft1* mutants.

Supplemental Table S1. Primer sequences for expression analysis by qPCR.

Figure legends

Figure 1. Regulation of APR expression by light in Col-0 and *pft1-2* plants.
Transcript levels of *APR* isoforms were determined by qPCR in one week-old Col-0 and *pft1-2* seedlings pre-incubated in darkness for 38 h and exposed to light for 30-120 min. The qPCR reactions were performed in duplicate for each of the three independent biological samples. The expression level of *APR1* in Col-0 in the dark at time=0 was set to 1. Full symbols represent measurements in plants kept in darkness for an additional 120 min. Results are presented as means ± SD from three pools of 10 seedlings, asterisks mark *pft1-2* values significantly different from Col-0 at $P<0.05$, a marks values that significantly ($P<0.05$) differ when transcript levels are set to 1 at time=0 in each genotype separately

Figure 2. Regulation of *APR* expression by light in *hy5* and *hy5 pft1* mutants.
Transcript levels of *APR* isoforms were determined by qPCR in one week-old Col-0, *hy5* and *hy5 pft1* seedlings pre-incubated in darkness for 38 h and exposed to light for 60-120 min. The values at time=0 were set to 1 for each genotype. Results are presented as means ± SD from three independent pools of 10 seedlings. Different letters mark significantly ($P<0.05$) different values; n.s. means not significantly different. For direct comparison, the values of *APR2* and *APR3* in *pft1-2* from Fig. 1 are shown again.

Figure 3. Regulation of sulfate assimilation by light in *pft1-2*, *hy5* and *hy5 pft1* mutants.
One week-old Col-0, *pft1-2*, *hy5* and *hy5 pft1* seedlings were pre-incubated in darkness for 38 h, transferred to nutrient solution containing [^{35}S]sulfate and incubated in the dark or in white light for 4 hours. **A** Sulfate uptake. **B** Relative flux through sulfate assimilation, determined as percentage of ^{35}S incorporated in thiols and proteins from ^{35}S taken up. **C** GSH levels. Results are presented as means ± SD from three pools of three seedlings. Different letters mark significantly ($P<0.05$) different values; asterisks mark values significantly different between dark-adapted and re-illuminated plants at $P<0.05$.

Figure 4. Schematic representation of a possible mechanism of short term light regulation of *APR1* and *APR2* by HY5 and PFT1.
The weight of the arrows marks the level of increase in the corresponding transcripts. *APR1* is mainly controlled by HY5, which is under negative regulation by PIFs. Loss of PFT1 unblocks inhibition of PIFs by PhyB and HY5 activity increases, leading to accumulation of *APR1* transcript. When HY5 is disrupted *APR1* is not induced. Light

regulation of *APR2* relies on at least one other transcription factor (TF) besides HY5, which is positively affected by PFT1 and therefore the effects of *pft1* and *hy5* mutations are additive.

Table 1. Contents of sulfur-containing metabolites and activities of enzymes of the sulfate assimilation pathway in rosette leaves of 5 week old Arabidopsis mutants in genes connected with PFT1. Data are shown as means ± standard deviation from at least 3 independent rosettes. Values significantly different from Col-0 at $P<0.05$ are printed bold.

	Sulfate (µmol/g FW)	Cysteine (µmol/g FW)	GSH (µmol/g FW)	glucosinolates (µmol/g FW)	APR (nmol/min/ mg protein)	ATPS (nmol/min/ mg protein)
Col-0	12.2±0.3	9.8±0.3	76.3±3.8	3.41±0.35	2.13±0.31	72.1±4.2
pft1-2	12.5±1.0	11.2±1.5	89.3±10.9	2.97±0.33	**2.82±0.32**	**52.4±2.6**
hy5	12.6±2.9	10.6±1.0	**90.2±2.8**	3.31±0.51	**4.55±0.10**	69.6±5.5
hy5 pft1	**16±0.8**	10.7±1.0	76.6±0.9	**2.73±0.34**	**2.7±0.30**	63.8±9.6

Figure 1

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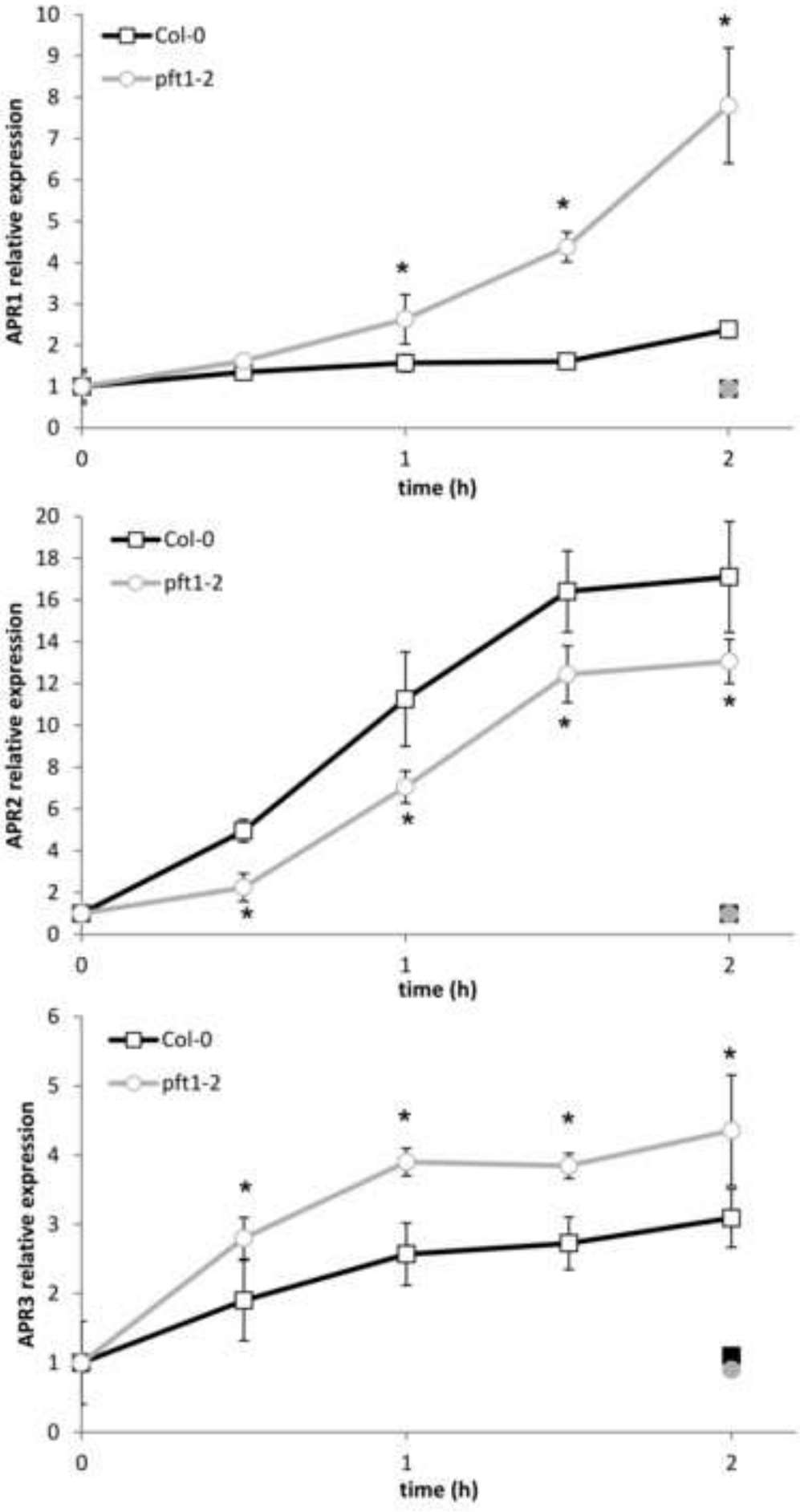

Figure 2

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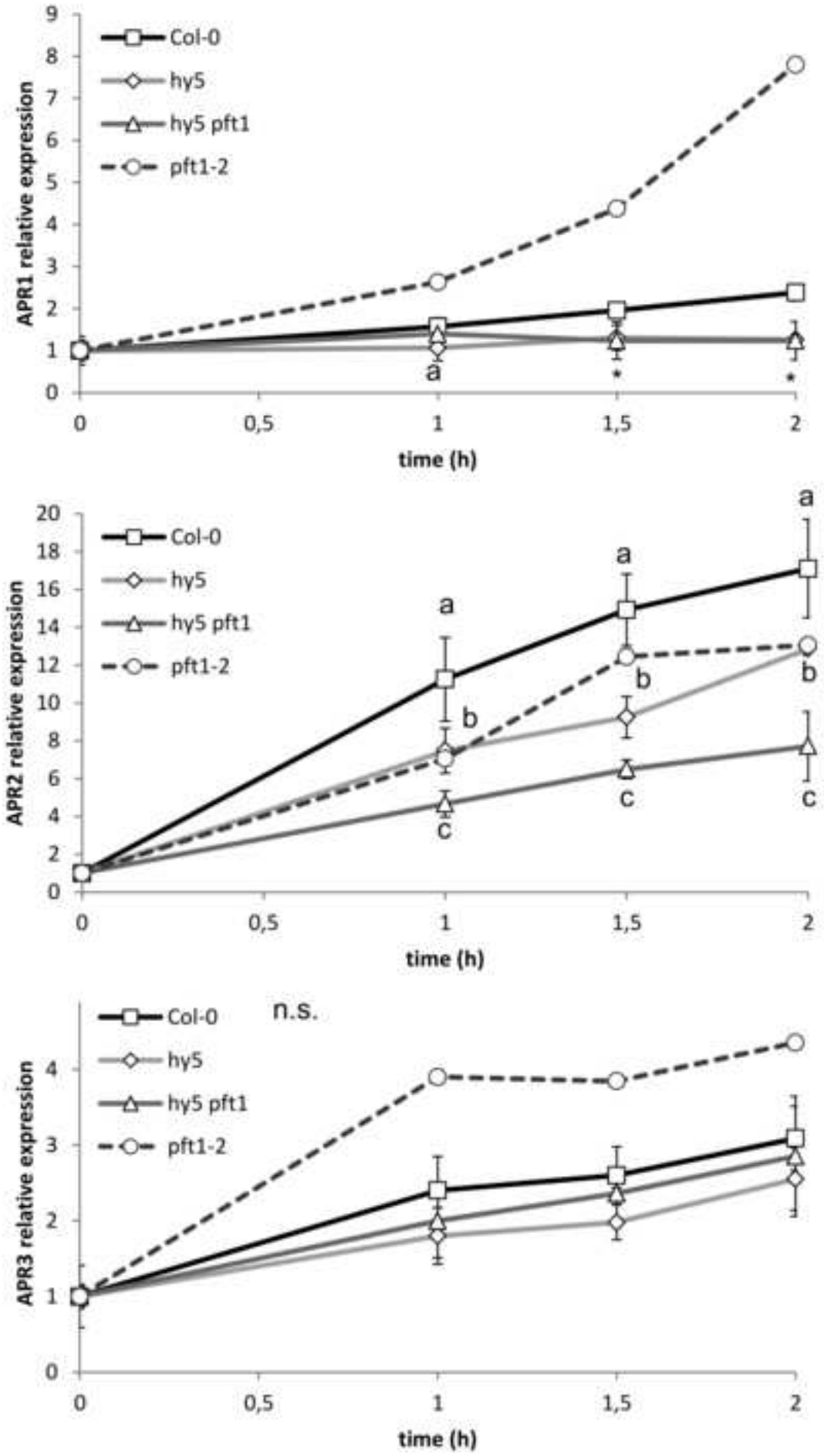

Figure 3

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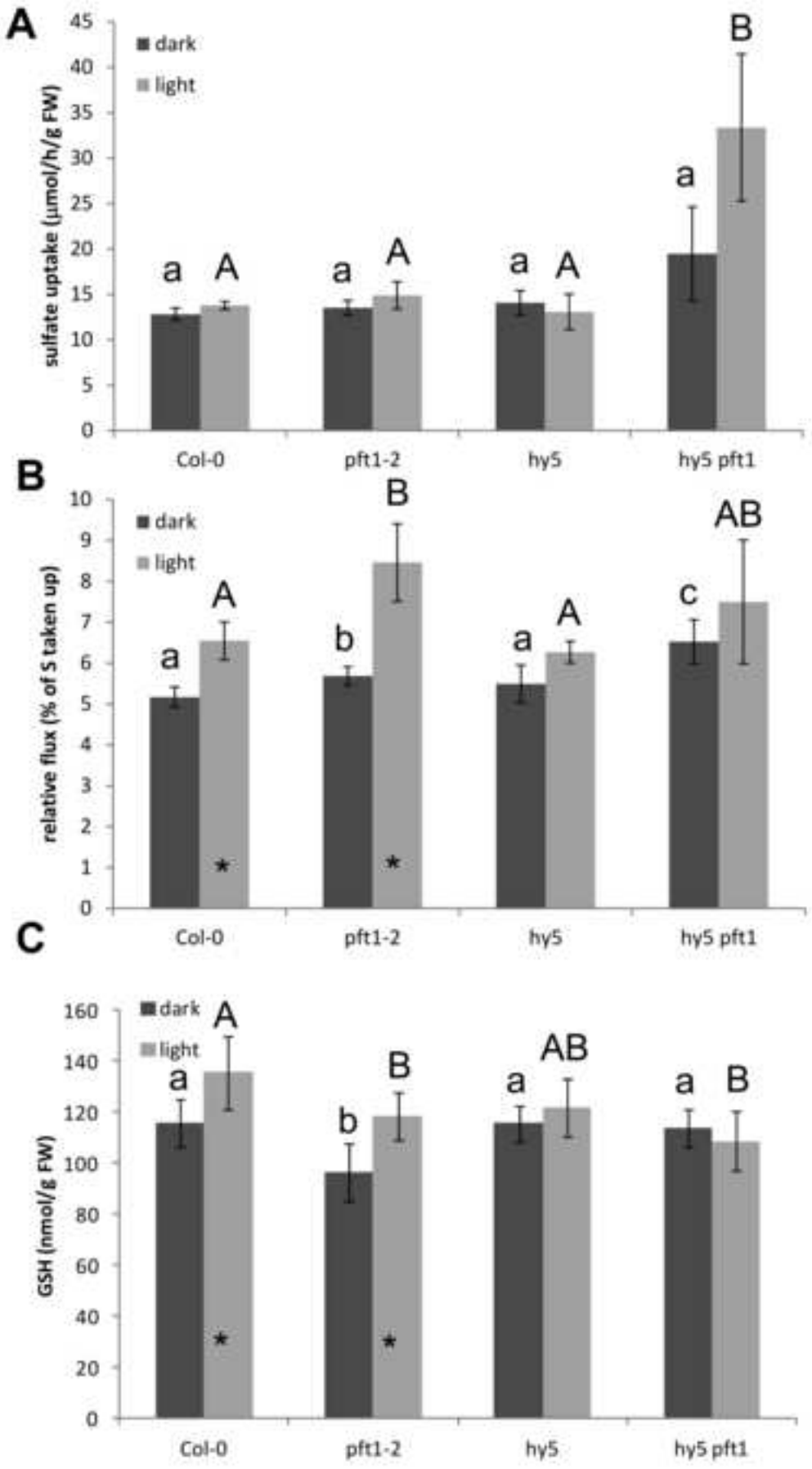

Figure 4

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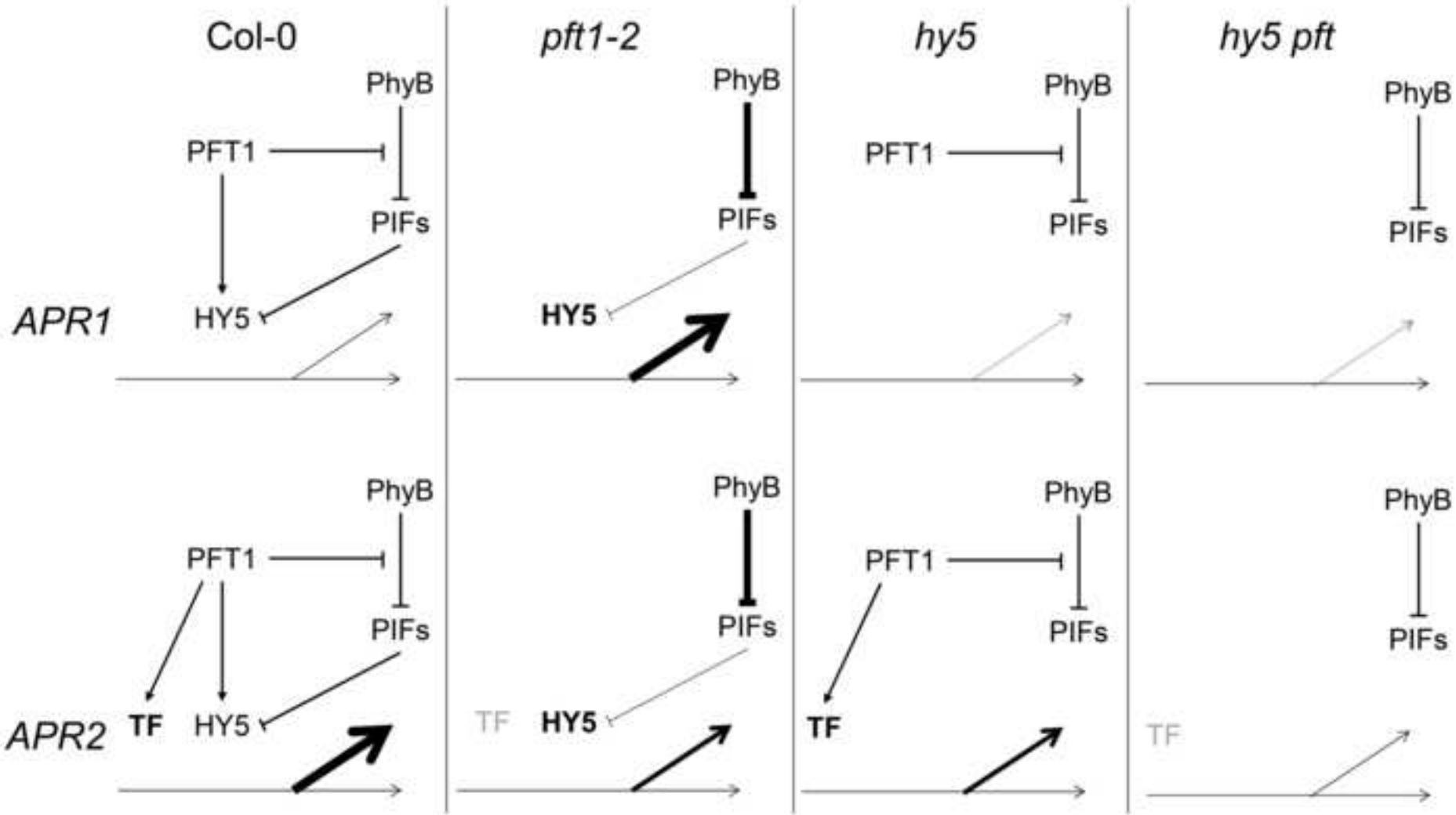

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