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cry1 and GPA1 signaling genetically interact in hook opening and anthocyanin synthesis in Arabidopsis

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Abstract While studying blue light-independent effects of cryptochrome 1 (cry1) photoreceptor, we observed premature opening of the hook in *cry1* mutants grown in complete darkness, a phenotype that resembles the one described for the heterotrimeric G-protein α subunit (GPA1) null mutant *gpa1*. Both *cry1* and *gpa1* also showed reduced accumulation of anthocyanin under blue light. These convergent *gpa1* and *cry1* phenotypes required the presence of sucrose in the growth media and were not additive in the *cry1 gpa1* double mutant, suggesting context-dependent signaling convergence between cry1 and GPA1 signaling pathways. Both, *gpa1* and *cry1* mutants showed reduced GTP-binding activity. The *cry1* mutant showed wild-type levels of *GPA1* mRNA or GPA1 protein. However, an anti-transducin antibody (AS/7) typically used for plant Gα proteins, recognized a 54 kDa band in the wild type but not in *gpa1* and *cry1* mutants. We propose a model where cry1-mediated post-translational modification of GPA1 alters its GTP-binding activity.

Keywords *Arabidopsis thaliana* · cry1 · Heterotrimeric G-proteins · GPA1 · Signal transduction

Abbreviations

cry	Cryptochrome
GPA1	Heterotrimeric G-protein α subunit of *Arabidopsis thaliana*
RL	Red light
FRL	Far red light
BL	Blue light
phy	Phytochrome
WT	Wild type
MS	Murashige and Skoog basal medium

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Introduction

For plants, light is a major source of information about the surrounding environment. Dark-grown seedlings follow the skotomorphogenic or etiolated pattern of development, which in *Arabidopsis thaliana* is characterized by an elongated hypocotyl, the presence of an apical hook and folded and unexpanded cotyledons. Upon light exposure,

hypocotyl growth is reduced, the apical hook opens and cotyledons unfold and expand. This transition from skotomorphogenesis to photomorphogenesis, also called de-etiolation, depends on the coordinated action of the red (RL) and far-red (FRL) light photoreceptors phytochromes (phyA–phyE) (Fankhauser 2001) and the UV-A blue light (BL) photoreceptors cryptochromes (cry1 and cry2) and phototroprins (phot1 and phot2) (Wang 2005). Cryptochromes are soluble flavoprotein photoreceptors (Ahmad and Cashmore 1993; Cashmore et al. 1999). The chromophore-binding domain bears similarity to DNA repair photolyases, but lacks the DNA repair activity (Brautigam et al. 2004). *cry1* mutants are impaired in several BL-mediated de-etiolation responses including the inhibition of hypocotyl elongation, the promotion of cotyledon unfolding and opening (Lin 2002) and anthocyanin accumulation (Ahmad et al. 1995). The *cry1* mutant is also defective in the entrainment of circadian rhythms (Devlin and Kay 2000; Yanovsky and Kay 2001), membrane depolarization in response to BL (Folta and Spalding 2001), root elongation (Canamero et al. 2006), defense against pathogens (Wu and Yang 2010), regulation of stomatal index (Kang et al. 2009) and light-induced stomatal opening (Mao et al. 2005; Boccalandro et al. 2011).

A large number of genes change their expression in response to BL perceived by crys (Jiao et al. 2003; Folta et al. 2003). cry1 controls gene expression through two different molecular mechanisms. One mechanism involves BL-dependent direct interaction of cry1 with transcription factors (Liu et al. 2011b). The other mechanism involves BL-dependent physical interaction of cry1 with SPA1 (SUPPRESSOR OF PHYA), which causes the dissociation of the COP1 (CONSTITUTVE OF PHOTOMORPHOGENIC 1)-SPA1 complex (Yang et al. 2001; Wang et al. 2001; Liu et al. 2011a). This reduces COP1 E3-ligase activity and allows transcription factors such as HY5 (ELONGATED HYPOCOTYL 5) to accumulate, favoring de-etiolation (Liu et al. 2011b).

Although crys work mainly under BL, BL-independent phenotypes of *cry* alleles have been described. For instance, the gain of function *CRY2* allele of *Cvi* enhances cotyledon unfolding under hourly FRL pulses (Botto et al. 2003). The *cry1 cry2* double mutant shows altered gene expression and protein-level responses to RL (Yang et al. 2008), and reduced stomatal conductance in response to RL (Boccalandro et al. 2011).

While studying the phenotype of cry mutants in darkness, we observed that the *cry1* mutation increases the angle of the apical hook, a phenotype that resembles the Arabidopsis heterotrimeric G *alpha* subunit protein (A*t*GPA1, herein referred as to GPA1) mutant *gpa1* (Ullah et al. 2001). It has been described that BL activates a 40-kDa protein with Gα characteristics (Warpeha et al. 1991) and GPA1 had been implicated in at least two BL responses; the synthesis of phenylalanine and the expression of the *light-harvesting chlorophyll a/b-binding protein* (Warpeha et al. 2006, 2007), but cry is not involved in any of the latter responses (Warpeha et al. 2006, 2007). These results prompted us to investigate the genetic and biochemical links between cry1 and GPA1 in Arabidopsis.

Materials and methods

Plant material and growth conditions

Null *cry1-304* (Bruggemann et al. 1996), *cry1* (SALK_069292) and *gpa1-3* (SALK_066823) mutants are in the Columbia (Col) background of *Arabidopsis thaliana*. *cry1-1*, (Ahmad and Cashmore 1993; Koornneef et al. 1980), *phyA-201* (Nagatani et al. 1993), *phyB-5* (Reed et al. 1993) and *fha-1* (Guo et al. 1998; Koornneef et al. 1991) are in the Landsberg *erecta* (Ler) background. The *cry1 gpa1* double mutant was obtained by crossing *cry1-304* and *gpa1-3* mutants and tested in the segregating population by using PCR with allele-specific primers. The forward 5′-TACCAAGGACATCGCTGAGG-3′ and reverse 5′-TGTCCACTCTATCCGGCGC-3′ primers were used for *GPA1* and the same forward primer was used in combination with T-DNA specific primer 5′-TGGTTCACGTAGTGGGCCATCG-3′ for *gpa1*. The 5′-ATGTCTGGTTCTGTATCTG-3′ and 5′-TTACCCGGTTTGTGAAAGCC-3′ primers were used for *cry1-304*.

Seeds were surface sterilized (4 h of exposure to the fumes produced by 1.25 % HCl/NaClO) and sown on clear plastic boxes containing either plain 0.8 % agar with half-strength Murashige and Skoog basal medium (MS) supplemented with 1 % (w/v) sucrose or 0.5 % (w/v) glucose when indicated. After 3 days at 4 °C in darkness, the seeds were exposed to a white-light pulse for 2 h to promote germination and returned to darkness. When indicated, the seedlings were transferred to specific light conditions for 3 days at 22 °C, starting 24 h after the light pulse. For anthocyanin and hypocotyl length experiments, seedlings were exposed to 9 or 3 μmol m^{-2} s^{-1} of continuous blue light respectively.

Hook opening and hypocotyl growth

Seedlings were photographed under safe green light at different time points between 55 and 120 h after the light pulse used to promote germination. The images were analyzed with the ImageJ program (Abramoff et al. 2004). Hook opening was calculated as the angle “a” formed between hypocotyl and cotyledons as illustrated in Fig. 1a, inset. We used safe dim green light to photograph dark-

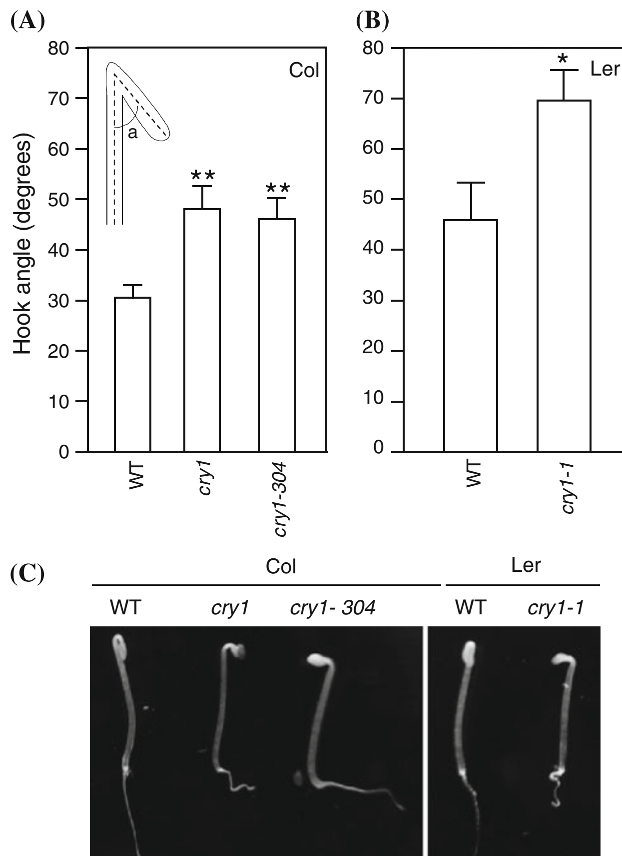

Fig. 1 Dark-grown *cry1* mutants display open hooks in 1 % sucrose. Seedlings were grown for 90 h on MS supplemented with 1 % sucrose in complete darkness. **a** Apical hook opening in the WT, *cry1 and cry1-304*, mutants in the Col background. The *inset* describes how angle "a" was measured. **b** Apical hook opening in WT and *cry1-1* mutants in the Ler background. **c** Representative seedlings were photographed. Data are media ± SEM of 13 replicates (15 seedlings each replicate) $*P < 0.05$, $**P < 0.01$

grown seedlings. Hypocotyl length was measured with a ruler to the nearest 0.5 mm.

GTP binding assays

Seedlings were grown for 3 days in complete darkness. Approximately 300 mg of seedlings were harvested under green safe light in liquid nitrogen and homogenized. Proteins were extracted in 500 µl of extraction buffer [50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 % Triton X-100, and 1× Roche complete protease inhibitor (Roche, Molecular Biochemicals)], centrifuged 15 min at 10,000 rpm. Protein concentration was measured in the supernatant as described (Bradford 1976). For GTP binding, 100 µg of crude protein extract in 100 µl of extraction buffer was added to 80 µl of reaction buffer (50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1 % Triton X-100), followed by 20 µl of initiation buffer (GTP γ^{35}S, 300,000 c.p.m., 10 mM $MgCl_2$, 0.1 % Triton X-100). The reaction was incubated in darkness for 10 min at room temperature and stopped with 2 ml of ice-cold stop buffer (20 mM Tris–HCl pH 8, 100 mM NaCl, 25 mM $MgCl_2$ and 1 mM phosphate buffer). Reactions were filtered under vacuum using 0.45 µm nitrocellulose filters, and filter discs were washed 5 times with cold stop buffer. Filters containing the membranes were dried 15 min at 75 °C and placed in a plastic vial with 2 ml of scintillation solution. Radioactivity in the ^{35}S energy range was determined using scintillation radiometry. Non specific GTP binding was estimated using protein extraction buffer (without proteins) and using 100 µg of protein sample preheated to 95 °C for 10 min. No differences in GTP binding were observed between both controls. The background counts were typically 300 c.p.m. The experiment was repeated 6 times.

RT-PCR

RNA was isolated from 3 days-old, dark-grown, seedlings grown on 0.8 % agar plates. Total RNA was isolated using the guanidinium thiocyanate method (Logemann et al. 1987). Two micrograms of total RNA was reverse transcribed into cDNA using MLV-RT^{TM} (Promega, Madison, WI, USA) following the manufacturer's instructions. *GPA1* expression was determined using the specific primers described above. *ACTIN8* (At1g49240) was used as internal loading control. The primers for *ACTIN8* were 5′-ATG AAGATTAAGGTCGTGGCA-3′ and 5′-GTTTTTATCCG AGTTTGAAGAGGC-3′. Amplification was performed with a thermal cycler (PTC-100, MJ Research, Waltham, MA, USA) using the following protocol: 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C; and a 10-min elongation at 72 °C. PCR products were resolved on 1 % agarose gels in 1× Tris acetic EDTA buffer containing 0.5 mg/ml ethidium bromide.

Immuno detection of GPA1

Seedlings were grown for 3 days in darkness as described above for GTP-binding assays and flash frozen in liquid nitrogen. Total protein was extracted as described for GTP binding assays. Sixty µg of protein were electrophoresed (20 mA for 2 h) on a 10 % SDS PAGE and transferred onto a nitrocellulose membrane using a wet-transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The blots were probed with polyclonal (NC9572) antibodies against GPA1 (Chen et al. 2006a) or an anti transducin (AS/7) antibody as previously described (Muschietti et al. 1993) both diluted 1:1,000 in blocking buffer (1× Tris-buffered saline [TBS], 0.2 % Triton X-100, 4 % nonfat milk and 2 % glycine). Then the blots were washed in 1× TBS 1 % Tween 20 and probed with anti rabbit IgG

(secondary antibody) (GE Healthcare, Piscataway, NJ, USA) conjugated with the horseradish peroxidase diluted 1:4,000. Afterwards, the membranes were washed and developed using an enhanced chemiluminescence kit (GE Healthcare Life Sciences).

Coimmunoprecipitation assays

The full length *GPA1* cDNA was cloned into pGEX-4T-3. GST-GPA1 was expressed in BL21 cells and purified using glutathione-Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocol. CRY1 was expressed using the TNT Quick Coupled Transcription/Translation Kit^{TM} (Promega, Madison, WI, USA) in the presence of $^{[35]}$S-Methionine. For co-immunoprecipitation, 30 µg of GST-GPA was incubated with 20 µl of TNT reaction, 2 µl Anti-GST antibody (GE Healthcare, Piscataway, NJ, USA) and 100 µl Binding Buffer 2× [2× PBS supplemented with 0.2 % v/v NP-40, 0.1 % w/v BSA, and 1 tablet/25 ml Roche complete protease inhibitor (Roche, Molecular Biochemicals)] in a final volume of 200 µl for 2 h at 4 °C. The antibody-antigen complex was then precipitated with Protein G beads (GE Healthcare, Piscataway, NJ, USA). Immunoprecipitates were washed three times with Washing Buffer (1× binding buffer without BSA) and proteins were separated in a 10 % SDS gel electrophoresis. GPA1 protein was monitored in westerns blots using anti-GST antibodies and CRY1 $^{[35]}$S-labeled protein by scintillation radiometry.

Anthocyanin measurements

For anthocyanin measurements seedlings were grown on 0.8 % agar with half-strength MS medium supplemented with 1 % (w/v) sucrose when indicated, and placed under continuous BL (9 µmol m^{-2} s^{-1}). After 3 days 50 seedlings were harvested and pigments were extracted in 600 µl in 1 % HCl in methanol for 48 h at 4 °C. Absorbance at 530 and 657 nm were recorded and anthocyanin content was calculated as A530- 0.25 A657 as described (Mancinelli et al. 1991).

Results

Dark-grown *cry1* mutants exhibit open hook phenotypes in sucrose

In 3.75 days-old seedlings grown in darkness on MS supplemented with 1 % sucrose, three different *cry1* mutant alleles showed significantly more open hooks than the wild type (WT) (Fig. 1). Cryptochromes can absorb green light and activate or antagonize hypocotyl inhibition (Bouly et al. 2007; Sellaro et al. 2010) but apical hook opening was not induced by the very dim green light used here as *cry1* mutant dark-grown seedlings showed opened hooks even though they had never received green light (Fig. 1). Hypocotyl length (mm, mean ± SEM, n = 15; WT: 5.8 ± 0.3, *cry1*: 5.7 ± 0.2; *cry1-304*: 6.0 ± 0.3), seed germination (%, mean ± SEM, n = 5; WT: 82.4 ± 1.4, *cry1*: 87.1 ± 3.9; *cry1-304*: 89.0 ± 8.9), and root length (mm, mean ± SEM, n = 8; WT: 4.8 ± 0.3, *cry1*: 5.6 ± 0.1; *cry1-304*: 5.2 ± 0.1) were similar between WT and *cry1*-mutant seedlings.

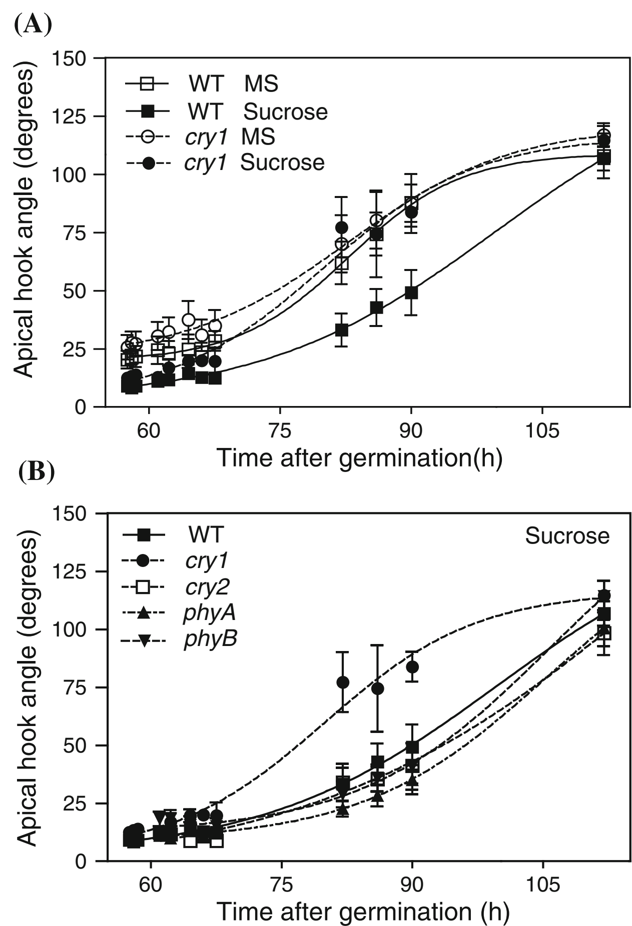

Fig. 2 Apical hook opening is specific to *cry1* mutant. Time course of apical hook opening in WT, *phyA*, *phyB*, *cry1* and *cry2* mutant seedlings in the Ler background. **a** Seedlings were grown on MS in complete darkness with or without 1 % sucrose, or **b** with 1 % sucrose. Data are media ± SEM of 5 replicates (at least 5 seedlings each replicate)

The kinetics of apical hook development includes three phases: formation, maintenance and opening (Raz and Ecker 1999). In dark-grown WT seedlings, addition of 1 % sucrose to the MS substrate delayed apical hook opening (Fig. 2a). In the *cry1* mutant, hook opening followed the WT pattern in the absence of supplementary sucrose. However, sucrose failed to delay hook opening in *cry1* as

observed for the WT seedlings (Fig. 2a). The *phyA*, *phyB* and *cry2* mutants showed normal hook opening in darkness on MS supplemented with 1 % sucrose (Fig. 2b).

cry1 and GPA1 genetic interaction during hook opening

The open-hook phenotype observed in dark-grown *cry1* mutant seedlings grown on MS supplemented with 1 % sucrose resembles the behavior of the *gpa1* null mutant in Arabidopsis (Ullah et al. 2001). Compared to the WT, hook opening was faster in the *cry1-304* null mutant but even faster in the *gpa1-3* null mutant (Fig. 3). We generated the *cry1-304 gpa1-3* double mutant, which was indistinguishable from *gpa1* at any point of the time course (Fig. 3). This indicates that the effects of the *cry1* and *gpa1* mutations are not additive, a result that is consistent with a signaling convergence between cry1 and GPA1. No differences in hook opening for *cry1* mutants were observed on MS or on MS supplemented with 0.5 % glucose, while the effects of *gpa1* and *cry1 gpa1* mutants were also observed in 0.5 % glucose media but not on MS (Supplementary Fig. 1).

GTP binding is reduced in *cry1* mutants

Prompted by the phenotypic convergence and genetic interaction of *cry1* and *gpa1* mutants, we carried on GTP-binding assays in extracts of 3 days-old dark-grown seedlings using the non hydrolysable analog GTP-γ^{35}S. As expected, GTP binding was significantly reduced in the *gpa1* mutant compared to WT seedlings (Colucci et al. 2002; Fig. 4a). The residual levels of GTP-γ^{35}S bound in the *gpa1* mutant could be attributed to other GTP binding proteins (Bischoff et al. 1999; Moshkov et al. 2003; Pandey et al. 2009). Strikingly, *cry1* seedlings showed significantly reduced GTP binding when compared to WT seedlings and similar to *gpa1* levels (Fig. 4a). This reduction was specific to *cry1* as *phyA*, *phyB* and *cry2* mutants showed normal GTP binding levels (Fig. 4b).

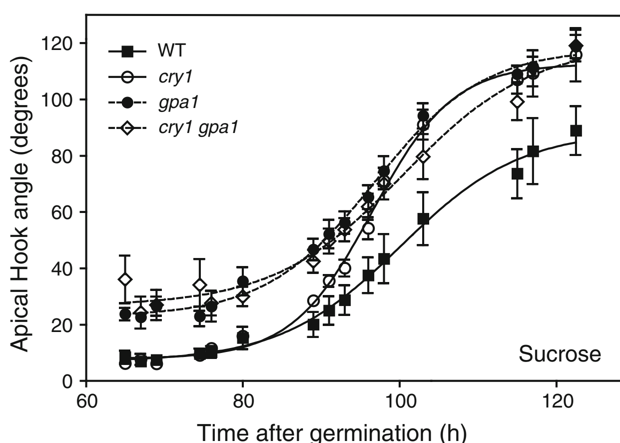

Fig. 3 cry1 and GPA1 show interaction during apical hook opening. Time course of apical hook opening in WT, *cry1-304*, *gpa1-3* and *cry1 gpa1* mutant seedlings in the Col background. Seedlings were grown on MS in complete darkness supplemented with 1 % sucrose. Data are media ± SEM of 6 replicates (at least 5 seedlings each replicate)

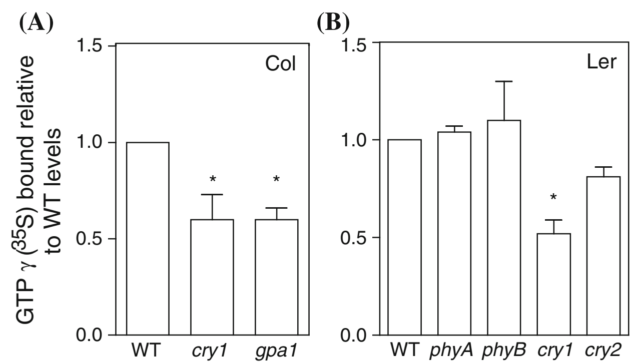

Fig. 4 *cry1* mutants show reduced GTP-γ^{35}S binding in darkness. Three days-old dark-grown seedlings were harvested in safe green light, total proteins were extracted and GTP-γ^{35}S binding was measured. **a** GTP-γ^{35}S binding in WT, *cry1* and *gpa1* mutant seedlings in the Col background. **b** GTP-γ^{35}S binding in WT, *phyA*, *phyB*, *cry1* and *cry2* mutant seedlings in the Ler background. Data are media ± SEM relative to the WT controls of at least 4 replicates. $*P < 0.05$

Distorted GPA1 protein blots in *cry1* mutants

We further analyzed if the reduction of GTP-γ^{35}S bound to *cry1* mutant was a consequence of changes in *GPA1* gene expression or GPA1 protein levels when cry1 is absent. RT-PCR showed that *GPA1* gene expression was similar in WT and *cry1* mutants (Supplementary Fig. 2a). To investigate GPA1 protein levels and to compare GPA1 signal intensity we used two different anti-Gα antibodies. One was developed specifically against the C-terminus of GPA1 (Chen et al. 2006a) and the other (AS/7) was raised against the C-terminus of Gα subunit of bovine transducin (Goldsmith et al. 1987). AS/7 antibody has been widely used to identify plant Gα proteins in species such as *Pisum sativum* (Warpeha et al. 1991), *Avena sativa* (Romero et al. 1991) *Cucumis melo* (Borochov-Neori et al. 1997) and *Medicago sativa* (Muschietti et al. 1993). GPA1-specific antibody showed a band of approximately 46 kDa in WT seedlings, corresponding to the expected size of GPA1 (Fig. 5a). This 46 kDa band was present at similar levels in the *cry1* mutant and, as expected, was absent in the *gpa1* null mutant. Using AS/7 antibody we detected a band of approximately 54 kDa in the WT and *phyB* mutant (used as an additional positive control, Fig. 5c) that was absent not only in *gpa1* mutant but also in the three different *cry1* alleles used (*cry1*, *cry1-304* and *cry1-1*) (Fig. 5b, c). Both, the specific anti-GPA1 and the AS/7 antibodies recognized a GPA1-GST protein fusion expressed in E. coli (Supplementary Fig. 3).

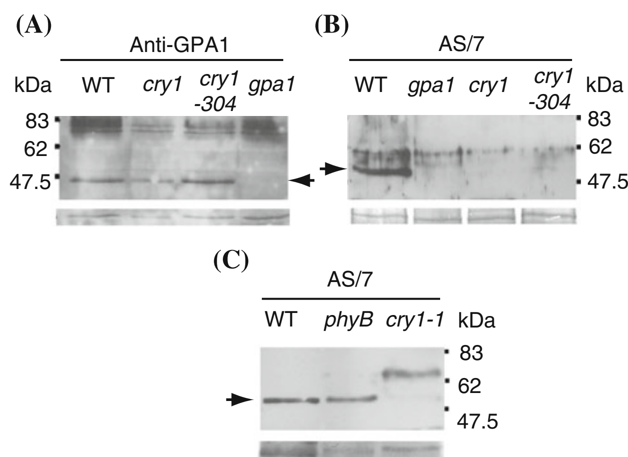

Fig. 5 GPA1 protein levels in *cry1* mutants. Western blots of total protein extracts of 3 days dark-grown seedlings. *Bottom* panels show china ink staining membranes after antibody detection as loading control. **a** Western blot probed with Arabidopsis specific anti-GPA1 antibody. *Arrow* indicates the position of the 46 kDa band. **b** and **c** Western blot probed with anti transducin (AS/7) antibody in Col ecotype (**b**) or in Ler ecotype (**c**). *Arrow* indicates the position of the 54 kDa band. The 46 kDa band detected with the specific anti-GPA1 antibody was not recognized by the AS/7 antibody, and the 54 kDa band detected by the AS/7 antibody was not recognized by the specific anti-GPA1 antibody

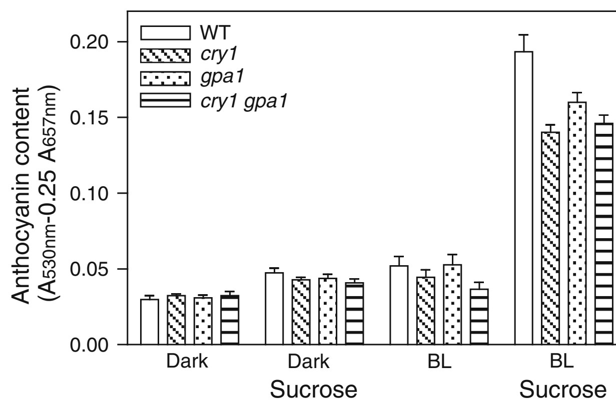

Fig. 6 cry1 and GPA1 are involved in anthocyanin accumulation under BL. Anthocyanin accumulation in *cry1*, *gpa1* single mutants and *cry1 gpa1* double mutant exposed for 3 days to 0 or 9 μmol m^{-2} $s^{-1}$of continuous BL with or without sucrose. Data are media ± SEM relative to the WT controls of at least 8 replicates

Genetic interaction between cry1 and GPA1 during de-etiolation under BL

cry1 is the main photoreceptor involved in anthocyanin accumulation and inhibition of hypocotyl elongation under BL. GPA1 is involved in BL-induction of phenylalanine, a precursor for phenylpropanoid biosynthesis (Warpeha et al. 2007) and many of the GPA1 effects have been observed in sugar signaling pathway (Ullah et al. 2001; Chen et al. 2006b; Wang et al. 2006). These observations prompted us to analyze if anthocyanin levels in seedlings grown in darkness or under BL with or without sucrose were altered in *cry1*, *gpa1* and *cry1 gpa1* mutants. After 3 days in darkness, WT, single and the double mutant seedlings grown on MS with or without 1 % sucrose showed similar anthocyanin content (Fig. 6). In WT seedlings, BL or sucrose increased anthocyanin levels significantly ($P < 0.01$). However, when WT seedlings were grown under BL with MS supplemented with 1 % sucrose, anthocyanin levels were 3.9 fold larger than the addition of each individual effect, indicating a synergistic interaction (interaction between BL and sucrose in two way ANOVA: $P < 0.0001$). Under BL on MS without sucrose, only the *cry1* mutation significantly reduced anthocyanin levels compared to WT ($P < 0.05$), while *gpa1* mutant accumulated anthocyanin at WT levels ($P > 0.05$) (Fig. 6). Interestingly, under BL and sucrose, anthocyanin content in *cry1*, *gpa1* and *cry1 gpa1* mutants was significantly reduced compared to the WT ($P < 0.001$ for *cry1* mutant, $P < 0.05$ for *gpa1* mutant, $P < 0.01$ for *cry1 gpa1* mutant) (Fig. 6). The effect of the reduced accumulation of anthocyanin in the *cry1 gpa1* double mutant was not additive with respect to the single mutant levels, indicating genetic interaction. No involvement of GPA1 was observed for hypocotyl inhibition under BL (Supplementary Fig. 4a), which is a typical cry1-mediated response (Ahmad et al. 1995). We additionally examined apical hook opening under BL, however neither cry1 nor GPA1 are involved in hook opening under BL (Supplementary Fig. 4b). As expected, under BL and sucrose, GPA1 protein was present in WT and *cry1* mutant at the same level (Supplementary Fig. 5). We conclude that both GPA1 and cry1 operate in sugar-regulated, BL-dependent anthocyanin synthesis pathway.

Discussion

While pharmacological experiments suggested a general role of the heterotrimeric Gα subunit in phytochrome signaling (Neuhaus et al. 1993; Bowler et al. 1994), the current view is that GPA1 affects selected phytochrome signaling pathways. The *gpa1* mutant shows normal phytochrome-mediated hypocotyl-growth inhibition under RL or FRL, indicating that GPA1 is dispensable for these responses (Jones et al. 2003). However, the *gpa1* mutant shows alterations in phyA-induced seed germination (Botto et al. 2009) and hypocotyl cell death (Wei et al. 2008). In addition to these phyA-mediated effects, an still unidentified BL photoreceptor different from cryptochromes, phytochromes or phototropins was proposed to activate a 40 kDa protein with Gα characteristics (Warpeha et al. 1991). GPA1 pathway has been also implicated in at least

two BL responses independent of cryptochromes in etiolated seedlings (Warpeha et al. 2006, 2007). Here we describe the occurrence of phenotypic convergence and genetic interaction between *cry1* and *gpa1* mutants.

cry1 and/or *cry2* mutant alleles often have phenotypes in the absence of BL, which include altered gene expression in response to RL (Yang et al. 2008), reduced stomatal opening in response to RL (Boccalandro et al. 2011) and distorted cotyledon unfolding in response to FRL (Botto et al. 2003). These BL-independent phenotypes could be the indirect result of persistent effects of previous BL activation of cryptochromes, which for instance, reduce abscisic acid levels in Arabidopsis plants at the rosette stage and thus favor stomatal opening in response to subsequent RL (Boccalandro et al. 2011). Here we show enhanced opening of the apical hook in three different *cry1* mutant alleles (*cry1* and *cry1-304* in Columbia and *cry1-1* in Landsberg *erecta*) grown in darkness on MS supplemented with 1 % sucrose (Fig. 1), a phenotype that resembles the one described for the *gpa1* mutant (Ullah et al. 2001). The effect was specific as *cry2*, *phyA* and *phyB* mutants showed WT hook opening in darkness (Fig. 2b). The exaggerated hook opening of both *gpa1* (Ullah et al. 2001, 2003; Chen et al. 2003) and *cry1* mutant alleles (Fig. 2a) is strictly sucrose dependent (Supplementary Fig. 1). The *cry1 gpa1* double mutant was indistinguishable from *gpa1*, indicating a genetic interaction consistent with the occurrence of signaling convergence.

The genetic interaction between *GPA1* and *CRY1* is not limited to hook opening in etiolated seedlings. The levels of anthocyanin in seedlings grown under BL on MS supplemented with 1 % sucrose were similarly reduced in *cry1* and *gpa1* mutants, and in the *cry1 gpa1* double mutant (Fig. 6). However, some phenotypes were not shared. For instance, the *gpa1* mutant has a short hypocotyl in darkness (Ullah et al. 2001), a feature not observed in *cry1* mutants; in addition, *cry1* but not *gpa1* mutant displayed elongated hypocotyls under BL (Supplementary Fig. 4a).

From the analysis of the *cry1 gpa1* double mutant phenotypes we observed that the effects of *cry1* and *gpa1* mutations in hook opening in darkness and anthocyanin accumulation in BL in the presence of sucrose are not additive, concluding there is genetic interaction. The occurrence of genetic interaction between cry1 and GPA1 can be explained by the following facts: (a) the *cry1* mutant showed opened hooks in darkness and sucrose, a phenotype characteristic of the *gpa1* mutant; (b) the *gpa1* mutant showed reduced anthocyanin accumulation in BL and sucrose, a phenotype characteristic of *cry1* mutants and (c) reduced GTP binding activity of GPA1 in *cry1* mutants. The genetic interaction is observed only in the presence of sucrose providing further strength to the relationship between cry1 and GPA1. The fact that cry1 and GPA1 interact only in hook opening and anthocyanin accumulation but not in hypocotyl elongation suggests that this interaction depends on developmental context.

GPA1 effects on germination, hook development, hypocotyl growth and root elongation have been shown to be sensitive to sucrose (Ullah et al. 2001, 2003; Chen et al. 2003). RGS1 (REGULATOR OF G SIGNALING 1) accelerates the GTPase activity of GPA1 in a D-glucose dependent manner (Johnston et al. 2007). Here we extend the requirement of sucrose to the effects of *cry1* shared with *gpa1*. In non photosynthetic tissues, sucrose is metabolized via glycolysis and the tricarboxylic acid cycle to produce ATP. ATP binding to CRY1 fractions in darkness is able to induce a conformational changes of the molecule (Burney et al. 2009).

In the search for a molecular mechanism connecting cry1 and GPA1 we observed that GTP binding is reduced in etiolated *cry1* mutant (Fig. 4). As it was reported (Colucci et al. 2002), the *gpa1* mutant also showed reduced GTP binding levels, when compared to WT. As for the hook-opening phenotype, the differential GTP binding did not require BL, and it was specific for cry1 since it was not observed for the *phyA*, *phyB* or *cry2* mutants.

The reduced GTP-binding found in the *cry1* mutant was not caused by reduced levels of *GPA1* mRNA (Supplementary Fig. 2a). Protein blots using a specific GPA1 antibody, showed WT levels of GPA1 in the *cry1* mutant (Fig. 5a and Supplementary Fig. 5), suggesting that the effect of *cry1* on GTP binding could be postranslational. The AS/7 antibody developed against the Gα subunit of bovine transducin and typically used in the literature to detect Gα in different plants, showed a 54 kDa band absent in both *gpa1* and *cry1* mutants (Fig. 5b, c). This band is 8 kDa heavier than the expected for GPA1 (Fig. 5a, b). A trivial explanation could be based on a cross reactivity of the AS/7 antibody with a 54 kDa Arabidopsis protein different from GPA1. However, the 54 kDa protein is absent in *gpa1* and *cry1*, but not in *phyB* mutant. Also, AS/7 antibody recognized a GPA1-GST protein fusion expressed in *E. coli* (Supplementary Fig. 3). There are three extra-large GTP binding proteins that have a carboxyl-terminal half with significant homology to Gα proteins but with molecular masses much greater than 54 kDa which also do not likely bind GTP given that the conserved GTP-binding pocket lacks critical residues (Lee and Assmann 1999; Ding et al. 2008; Assmann 2002). A suitable alternative is that GPA1 and AS/7 antibodies recognize in WT seedlings a different version of the GPA1 protein which could result from a cry1-mediated post-translational modification. The GPA1 antibody is directed to a short sequence at the C-terminus of GPA1 shown to be antigenic whereas AS/7 is directed to the last ten C-terminus aminoacids of a bovine transducin (Goldsmith et al. 1987). This suggests that the possible cry1-dependent post-

translational modification of GPA1 would map to the C-terminus of GPA. The CRY1-GPA1 interaction for modification either is indirect or it would require additional players present in vivo since in vitro physical interaction between GPA1 and CRY1 was not detected (Supplementary Fig. 2b). The cry1-dependent increase in GPA1 molecular mass suggests mono-ubiquitination, as it was reported for yeast heterotrimeric Gα subunit (Marotti et al. 2002; Wang et al. 2005). The mono-ubiquitination of yeast heterotrimeric Gα subunit is revealed by a 63 kDa band compared to the native band of 54 kDa (Marotti et al. 2002; Wang et al. 2005). We can speculate that GPA1 is mono-ubiquitinated and this step is a means for CRY1-regulation of GPA1 protein level or activity. Following this line of argumentation, the AS/7 antibody would be unable to detect non ubiquitinated GPA1.

New models of plant heterotrimeric G-proteins mechanisms propose that most GPA1 molecules are bound to GTP (Temple and Jones 2007; Johnston et al. 2007). Taking into account that *gpa1* and *cry1* mutant seedlings showed similar GTP binding phenotypes, cry1 would be favoring the GTP-binding state (rather than inhibiting the GTPase activity increased by RGS1) regulating its binding capacity and/or steady state levels.

In summary, we provide genetic evidence for the convergence of cry1 and GPA1 signaling involved in hook opening and anthocyanin synthesis. cry1 would operate by controlling the GTP-binding capacity of GPA1, via post-translational modifications.

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