

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 (*AS17*) typically used for plant G α 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.

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Abbreviations

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

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 phototropins (*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 (CONSTITUTIVE 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 α subunit protein (*AtGPA1*, herein referred as to *GPA1*) mutant *gpa1* (Ullah et al. 2001). It has been described that BL activates a 40-kDa protein with G α 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'-TACCAA GGACATCGCTGAGG-3' and reverse 5'-TGTCCTACTCT ATCCGGCGC-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'-TTA CCCGGTTTGTGAAAGCC-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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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-

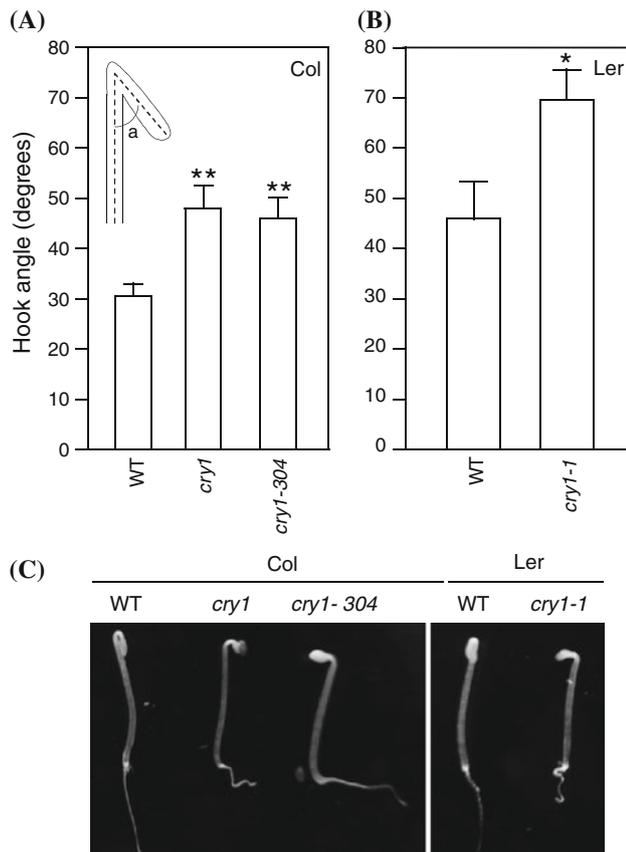


Fig. 1 Dark-grown *cry1* mutants display open hooks in 1 % sucrose. Seedlings were grown for 90 h on MS supplemented with 1 % sucrose in complete darkness. **a** Apical hook opening in the WT, *cry1* and *cry1-304*, mutants in the Col background. The inset describes how angle “a” was measured. **b** Apical hook opening in WT and *cry1-1* mutants in the Ler background. **c** Representative seedlings were photographed. Data are media \pm 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 μ l 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 \times 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 μ g of crude protein extract in 100 μ l of extraction buffer was added to 80 μ l 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 μ l of initiation buffer (GTP γ^{35} S, 300,000 c.p.m., 10 mM

MgCl₂, 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 MgCl₂ and 1 mM phosphate buffer). Reactions were filtered under vacuum using 0.45 μ m nitrocellulose filters, and filter discs were washed 5 times with cold stop buffer. Filters containing the membranes were dried 15 min at 75 $^{\circ}$ 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 μ g of protein sample preheated to 95 $^{\circ}$ 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-RTTM (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′-GTTTTATCCG AGTTTGAAGAGGC-3′. Amplification was performed with a thermal cycler (PTC-100, MJ Research, Waltham, MA, USA) using the following protocol: 5 min at 94 $^{\circ}$ C; 30 cycles of 30 s at 94 $^{\circ}$ C, 30 s at 50 $^{\circ}$ C, and 1 min at 72 $^{\circ}$ C; and a 10-min elongation at 72 $^{\circ}$ C. PCR products were resolved on 1 % agarose gels in 1 \times 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 μ g 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 (Muschiatti et al. 1993) both diluted 1:1,000 in blocking buffer (1 \times Tris-buffered saline [TBS], 0.2 % Triton X-100, 4 % nonfat milk and 2 % glycine). Then the blots were washed in 1 \times 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 KitTM (Promega, Madison, WI, USA) in the presence of [³⁵S]-Methionine. For co-immunoprecipitation, 30 µg of GST-GPA1 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 [³⁵S]-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 ($9 \mu\text{mol m}^{-2} \text{s}^{-1}$). 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 $A_{530} - 0.25 A_{657}$ 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

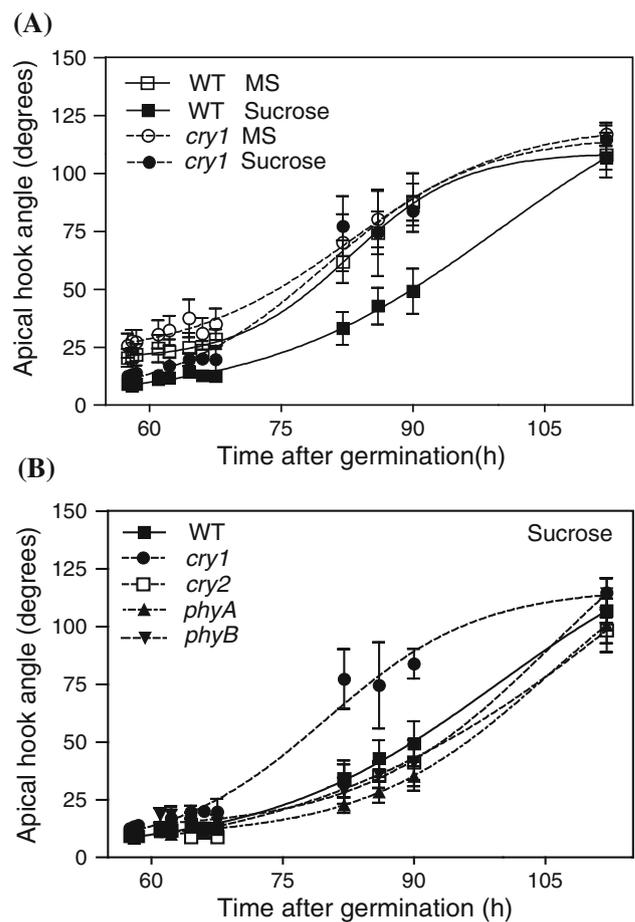


Fig. 2 Apical hook opening is specific to *cry1* mutant. Time course of apical hook opening in WT, *phyA*, *phyB*, *cry1* and *cry2* mutant seedlings in the Ler background. **a** Seedlings were grown on MS in complete darkness with or without 1 % sucrose, or **b** with 1 % sucrose. Data are media \pm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

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 \pm SEM, $n = 15$; WT: 5.8 ± 0.3 , *cry1*: 5.7 ± 0.2 ; *cry1-304*: 6.0 ± 0.3), seed germination (%), mean \pm SEM, $n = 5$; WT: 82.4 ± 1.4 , *cry1*: 87.1 ± 3.9 ; *cry1-304*: 89.0 ± 8.9), and root length (mm, mean \pm 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.

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- $\gamma^{35}\text{S}$. 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- $\gamma^{35}\text{S}$ bound in the *gpa1* mutant could be attributed to other GTP binding proteins (Bischoff et al. 1999; Moshkov et al. 2003; Pandey

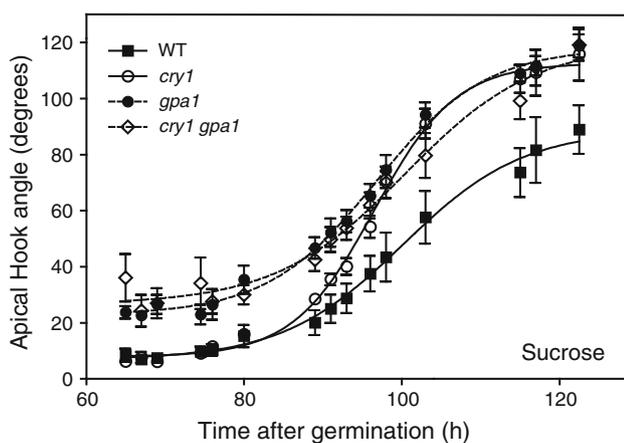


Fig. 3 *cry1* and GPA1 show interaction during apical hook opening. Time course of apical hook opening in WT, *cry1-304*, *gpa1-3* and *cry1 gpa1* mutant seedlings in the Col background. Seedlings were grown on MS in complete darkness supplemented with 1 % sucrose. Data are media \pm SEM of 6 replicates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

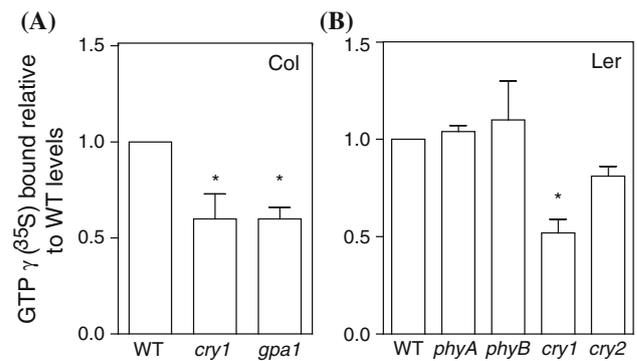


Fig. 4 *cry1* mutants show reduced GTP- $\gamma^{35}\text{S}$ binding in darkness. Three days-old dark-grown seedlings were harvested in safe green light, total proteins were extracted and GTP- $\gamma^{35}\text{S}$ binding was measured. **a** GTP- $\gamma^{35}\text{S}$ binding in WT, *cry1* and *gpa1*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

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).

Distorted GPA1 protein blots in *cry1* mutants

We further analyzed if the reduction of GTP- $\gamma^{35}\text{S}$ 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-G α 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 G α subunit of bovine transducin (Goldsmith et al. 1987). AS/7 antibody has been widely used to identify plant G α 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* (Muschiatti 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).

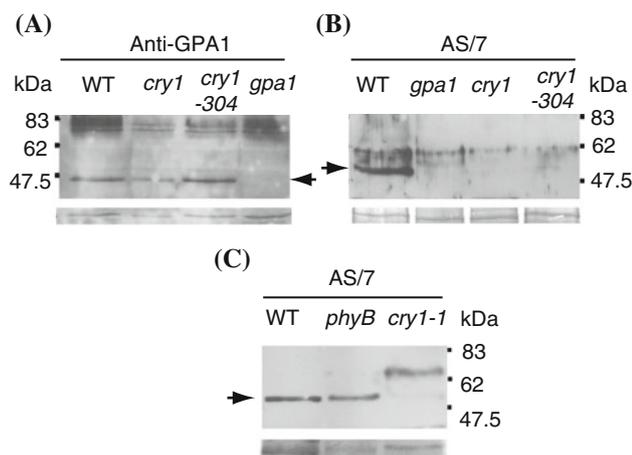


Fig. 5 GPA1 protein levels in *cry1* mutants. Western blots of total protein extracts of 3 days dark-grown seedlings. *Bottom* panels show china ink staining membranes after antibody detection as loading control. **a** Western blot probed with Arabidopsis specific anti-GPA1 antibody. *Arrow* indicates 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

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

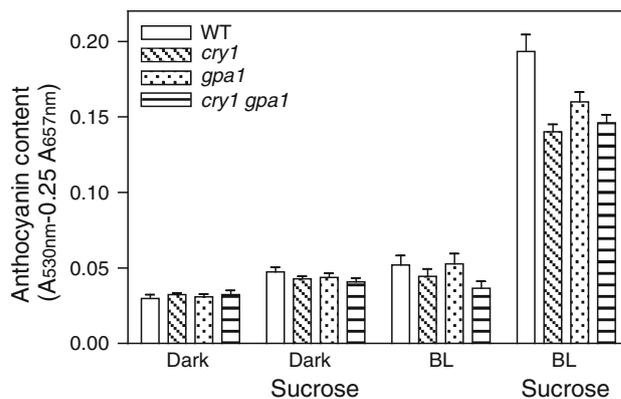


Fig. 6 *cry1* and GPA1 are involved in anthocyanin accumulation under BL. Anthocyanin accumulation in *cry1*, *gpa1* single mutants and *cry1 gpa1* double mutant exposed for 3 days to 0 or $9 \mu\text{mol m}^{-2} \text{s}^{-1}$ of continuous BL with or without sucrose. Data are media \pm SEM relative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

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 G α 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 G α 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 $G\alpha$ subunit of bovine transducin and typically used in the literature to detect $G\alpha$ 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 $G\alpha$ 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 G α subunit (Marotti et al. 2002; Wang et al. 2005). The mono-ubiquitination of yeast heterotrimeric G α 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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References

- Abramoff M, Magelhaes P, Ram S (2004) Image processing with imageJ. *Biophotonics Int* 11:36–42
- Ahmad M, Cashmore AR (1993) HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* 366(6451):162–166
- Ahmad M, Lin C, Cashmore AR (1995) Mutations throughout an Arabidopsis blue-light photoreceptor impair blue-light-responsive anthocyanin accumulation and inhibition of hypocotyl elongation. *Plant J* 8(5):653–658
- Assmann SM (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *Plant Cell* 14(Suppl):S355–S373
- Bischoff F, Molendijk A, Rajendrakumar CS, Palme K (1999) GTP-binding proteins in plants. *Cell Mol Life Sci* 55(2):233–256
- Boccalandro HE, Giordano CV, Ploschuk EL, Piccoli PN, Bottini R, Casal JJ (2011) Phototropins but not cryptochromes mediate the blue light-specific promotion of stomatal conductance, while both enhance photosynthesis and transpiration under full sunlight. *Plant Physiol* 158(3):1475–1484. doi:10.1104/pp.111.187237
- Borochoy-Neori H, Gindin E, Borochoy A (1997) Response of melon plants to salt: 3. Modulation of GTP-binding proteins in root membranes. *J Plant Physiol* 150(3):355–361
- Botto JF, Alonso-Blanco C, Garzaron I, Sanchez RA, Casal JJ (2003) The Cape Verde Islands allele of cryptochrome 2 enhances cotyledon unfolding in the absence of blue light in Arabidopsis. *Plant Physiol* 133(4):1547–1556. doi:10.1104/pp.103.029546
- Botto JF, Ibarra S, Jones AM (2009) The heterotrimeric G-protein complex modulates light sensitivity in *Arabidopsis thaliana* seed germination. *Photochem Photobiol* 85(4):949–954. doi:10.1111/j.1751-1097.2008.00505.x
- Bouly JP, Schleicher E, Dionisio-Sese M, Vandenbussche F, Van Der Straeten D, Bakrim N, Meier S, Batschauer A, Galland P, Bittl R, Ahmad M (2007) Cryptochrome blue light photoreceptors are activated through interconversion of flavin redox states. *J Biol Chem* 282(13):9383–9391. doi:10.1074/jbc.M609842200
- Bowler C, Yamagata H, Neuhaus G, Chua NH (1994) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. *Genes Dev* 8(18):2188–2202
- Bradford M (1976) A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye. *Anal Biochem* 72:248–254
- Brautigam CA, Smith BS, Ma Z, Palnitkar M, Tomchick DR, Machius M, Deisenhofer J (2004) Structure of the photolyase-like domain of cryptochrome 1 from *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 101(33):12142–12147
- Bruggemann E, Handwerker K, Essex C, Storz G (1996) Analysis of fast neutron-generated mutants at the *Arabidopsis thaliana* HY4 locus. *Plant J* 10(4):755–760
- Burney S, Hoang N, Caruso M, Dudkin EA, Ahmad M, Bouly JP (2009) Conformational change induced by ATP binding correlates with enhanced biological function of Arabidopsis cryptochrome. *FEBS Lett* 583(9):1427–1433. doi:10.1016/j.febslet.2009.03.040
- Canamero RC, Bakrim N, Bouly JP, Garay A, Dudkin EE, Habricot Y, Ahmad M (2006) Cryptochrome photoreceptors cry1 and cry2 antagonistically regulate primary root elongation in *Arabidopsis thaliana*. *Planta* 224(5):995–1003
- Cashmore AR, Jarillo JA, Wu YJ, Liu D (1999) Cryptochromes: blue light receptors for plants and animals. *Science* 284(5415):760–765
- Chen JG, Willard FS, Huang J, Liang J, Chasse SA, Jones AM, Siderovski DP (2003) A seven-transmembrane RGS protein that modulates plant cell proliferation. *Science* 301(5640):1728–1731. doi:10.1126/science.1087790
- Chen JG, Gao Y, Jones AM (2006a) Differential roles of Arabidopsis heterotrimeric G-protein subunits in modulating cell division in roots. *Plant Physiol* 141(3):887–897. doi:10.1104/pp.106.079202
- Chen Y, Ji F, Xie H, Liang J, Zhang J (2006b) The regulator of G-protein signaling proteins involved in sugar and abscisic acid signaling in Arabidopsis seed germination. *Plant Physiol* 140(1):302–310. doi:10.1104/pp.105.069872
- Colucci G, Apone F, Alyeshmehi N, Chalmers D, Chrispeels MJ (2002) GCR1, the putative Arabidopsis G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. *Proc Natl Acad Sci USA* 99(7):4736–4741
- Devlin PF, Kay SA (2000) Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. *Plant Cell* 12(12):2499–2510
- Ding L, Pandey S, Assmann SM (2008) Arabidopsis extra-large G proteins (XLGs) regulate root morphogenesis. *Plant J* 53(2):248–263. doi:10.1111/j.1365-313X.2007.03335.x

- Fankhauser C (2001) The phytochromes, a family of red/far-red absorbing photoreceptors. *J Biol Chem* 276(15):11453–11456. doi:10.1074/jbc.R100006200
- Folta KM, Spalding EP (2001) Opposing roles of phytochrome A and phytochrome B in early cryptochrome-mediated growth inhibition. *Plant J* 28(3):333–340
- Folta KM, Pontin MA, Karlin-Neumann G, Bottini R, Spalding EP (2003) Genomic and physiological studies of early cryptochrome 1 action demonstrate roles for auxin and gibberellin in the control of hypocotyl growth by blue light. *Plant J* 36(2):203–214
- Goldsmith P, Gierschik P, Milligan G, Unson CG, Vinitzky R, Malech HL, Spiegel AM (1987) Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J Biol Chem* 262(30):14683–14688
- Guo H, Yang H, Mockler TC, Lin C (1998) Regulation of flowering time by Arabidopsis photoreceptors. *Science* 279(5355):1360–1363
- Jiao Y, Yang H, Ma L, Sun N, Yu H, Liu T, Gao Y, Gu H, Chen Z, Wada M, Gerstein M, Zhao H, Qu LJ, Deng XW (2003) A genome-wide analysis of blue-light regulation of Arabidopsis transcription factor gene expression during seedling development. *Plant Physiol* 133(4):1480–1493. doi:10.1104/pp.103.029439
- Johnston CA, Taylor JP, Gao Y, Kimple AJ, Grigston JC, Chen JG, Siderovski DP, Jones AM, Willard FS (2007) GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. *Proc Natl Acad Sci USA* 104(44):17317–17322. doi:10.1073/pnas.0704751104
- Jones AM, Ecker JR, Chen J-G (2003) A reevaluation of the role of the heterotrimeric G protein in coupling light responses in Arabidopsis. *Plant Physiol* 131(4):1623–1627. doi:10.1104/pp.102.017624
- Kang CY, Lian HL, Wang FF, Huang JR, Yang HQ (2009) Cryptochromes, phytochromes, and COP1 regulate light-controlled stomatal development in Arabidopsis. *Plant Cell* 21(9):2624–2641. doi:10.1105/tpc.109.069765
- Koornneef M, Rolf E, Spruit CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana*. *Heynh Z Pflanzenphysiol* 100:147–160
- Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol Gen Genet* 229(1):57–66
- Lee YR, Assmann SM (1999) *Arabidopsis thaliana* ‘extra-large GTP-binding protein’ (AtXGLG1): a new class of G-protein. *Plant Mol Biol* 40(1):55–64
- Lin C (2002) Blue light receptors and signal transduction. *Plant Cell* 14(Suppl):S207–S225
- Liu B, Zuo Z, Liu H, Liu X, Lin C (2011a) Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. *Genes Dev* 25(10):1029–1034
- Liu H, Liu B, Zhao C, Pepper M, Lin C (2011b) The action mechanisms of plant cryptochromes. *Trends Plant Sci* 16(12):684–691
- Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Anal Biochem* 163(1):16–20. doi:0003-2697(87)90086-8
- Mancinelli AL, Rossi F, Moroni A (1991) Cryptochrome, phytochrome, and anthocyanin production. *Plant Physiol* 96(4):1079–1085
- Mao J, Zhang YC, Sang Y, Li QH, Yang HQ (2005) From the cover: a role for Arabidopsis cryptochromes and COP1 in the regulation of stomatal opening. *Proc Natl Acad Sci USA* 102(34):12270–12275
- Marotti LA Jr, Newitt R, Wang Y, Aebersold R, Dohlman HG (2002) Direct identification of a G protein ubiquitination site by mass spectrometry. *Biochemistry* 41(16):5067–5074
- Moshkov IE, Mur LA, Novikova GV, Smith AR, Hall MA (2003) Ethylene regulates monomeric GTP-binding protein gene expression and activity in Arabidopsis. *Plant Physiol* 131(4):1705–1717. doi:10.1104/pp.014035
- Muschietti JP, Martinetto HE, Coso OA, Farber MD, Torres HN, Flawia MM (1993) G-protein from *Medicago sativa*: functional association to photoreceptors. *Biochem J* 291(Pt 2):383–388
- Nagatani A, Reed JW, Chory J (1993) Isolation and initial characterization of Arabidopsis mutants that are deficient in phytochrome A. *Plant Physiol* 102(1):269–277. doi:102/1/269
- Neuhaus G, Bowler C, Kern R, Chua NH (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* 73(5):937–952. doi:0092-8674(93)90272-R
- Pandey S, Nelson DC, Assmann SM (2009) Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. *Cell* 136(1):136–148. doi:10.1016/j.cell.2008.12.026
- Raz V, Ecker JR (1999) Regulation of differential growth in the apical hook of Arabidopsis. *Development* 126(16):3661–3668
- Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. *Plant Cell* 5(2):147–157. doi:10.1105/tpc.5.2.147
- Romero LC, Sommer D, Gotor C, Song PS (1991) G-proteins in etiolated *Avena* seedlings. Possible phytochrome regulation. *FEBS Lett* 282(2):341–346. doi:0014-5793(91)80509-2
- Sellaro R, Crepy M, Trupkin SA, Karayekov E, Buchovsky AS, Rossi C, Casal JJ (2010) Cryptochrome as a sensor of the blue/green ratio of natural radiation in Arabidopsis. *Plant Physiol* 154(1):401–409. doi:10.1104/pp.110.160820
- Temple BR, Jones AM (2007) The plant heterotrimeric G-protein complex. *Annu Rev Plant Biol* 58:249–266. doi:10.1146/annurev.arplant.58.032806.103827
- Ullah H, Chen JG, Young JC, Im KH, Sussman MR, Jones AM (2001) Modulation of cell proliferation by heterotrimeric G protein in Arabidopsis. *Science* 292(5524):2066–2069. doi:10.1126/science.1059040
- Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM (2003) The beta-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. *Plant Cell* 15(2):393–409
- Wang H (2005) Signaling mechanisms of higher plant photoreceptors: a structure-function perspective. *Curr Top Dev Biol* 68:227–261
- Wang H, Ma LG, Li JM, Zhao HY, Deng XW (2001) Direct interaction of Arabidopsis cryptochromes with COP1 in light control development. *Science* 294(5540):154–158
- Wang Y, Marotti LA Jr, Lee MJ, Dohlman HG (2005) Differential regulation of G protein alpha subunit trafficking by mono- and polyubiquitination. *J Biol Chem* 280(1):284–291
- Wang HX, Weerasinghe RR, Perdue TD, Cakmakci NG, Taylor JP, Marzluff WF, Jones AM (2006) A Golgi-localized hexose transporter is involved in heterotrimeric G protein-mediated early development in Arabidopsis. *Mol Biol Cell* 17(10):4257–4269. doi:10.1091/mbc.E06-01-0046
- Warpeha KM, Hamm HE, Rasenick MM, Kaufman LS (1991) A blue-light-activated GTP-binding protein in the plasma membranes of etiolated peas. *Proc Natl Acad Sci USA* 88(20):8925–8929
- Warpeha KM, Lateef SS, Lapik Y, Anderson M, Lee BS, Kaufman LS (2006) G-protein-coupled receptor 1, G-protein α -subunit 1, and prephenate dehydratase 1 are required for blue light-induced production of phenylalanine in etiolated Arabidopsis. *Plant Physiol* 140(3):844–855. doi:10.1104/pp.105.071282
- Warpeha KM, Upadhyay S, Yeh J, Adamiak J, Hawkins SI, Lapik YR, Anderson MB, Kaufman LS (2007) The GCR1, GPA1,

- PRN1, NF-Y signal chain mediates both blue light and abscisic acid responses in Arabidopsis. *Plant Physiol* 143(4):1590–1600. doi:[10.1104/pp.106.089904](https://doi.org/10.1104/pp.106.089904)
- Wei Q, Zhou W, Hu G, Wei J, Yang H, Huang J (2008) Heterotrimeric G-protein is involved in phytochrome A-mediated cell death of Arabidopsis hypocotyls. *Cell Res* 18(9): 949–960. doi:[10.1038/cr.2008.271](https://doi.org/10.1038/cr.2008.271)
- Wu L, Yang HQ (2010) CRYPTOCHROME 1 is implicated in promoting R protein-mediated plant resistance to *Pseudomonas syringae* in Arabidopsis. *Mol Plant* 3(3):539–548
- Yang HQ, Tang RH, Cashmore AR (2001) The signaling mechanism of Arabidopsis CRY1 involves direct interaction with COP1. *Plant Cell* 13(12):2573–2587
- Yang YJ, Zuo ZC, Zhao XY, Li X, Klejnot J, Li Y, Chen P, Liang SP, Yu XH, Liu XM, Lin CT (2008) Blue-light-independent activity of Arabidopsis cryptochromes in the regulation of steady-state levels of protein and mRNA expression. *Mol Plant* 1(1): 167–177. doi:[10.1093/mp/ssm018](https://doi.org/10.1093/mp/ssm018)
- Yanovsky MJ, Kay SA (2001) Signaling networks in the plant circadian system. *Curr Opin Plant Biol* 4(5):429–435