Mesophyll-localized phytochromes gate stress- and light-inducible anthocyanin accumulation in *Arabidopsis thaliana*

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Abiotic stress and light induce anthocyanin accumulation in *Arabidopsis*. Here, we demonstrate that mesophyll-localized phytochromes regulate nitrogen-, phosphate- and cold-induced anthocyanin accumulation in shoots of *Arabidopsis*. Whereas ecotype-dependent differences result in distinct total levels of anthocyanin accumulation in response to light, cold, or nutrient-deficient treatments, phytochromes generally gate light- and/or stress-induced anthocyanin accumulation in shoots, as plants depleted of mesophyll-localized phytochromes lack or have highly attenuated induction of anthocyanins. Observed interactions between light and stress were found to be wavelength dependent, with red and far-red light stimulating higher total levels of anthocyanin accumulation under cold temperatures, especially in response to nitrogen limitation, whereas blue light did not. The roots of plants depleted of mesophyll-localized phytochromes still respond to nutrient deficiency as determined by elongation of primary roots and root hair elongation when plants are grown under nitrogen- or phosphate-limited conditions. Plants which are constitutively deficient in photoreceptors in both shoots and roots, i.e., *phy* or *cry* mutants, exhibit defects in light- and stress-induced anthocyanin accumulation and defects in root development. Taken together, these results suggest that the response to nutrient limitation in roots and shoots is under distinct control by spatial-specific pools of phytochromes in *Arabidopsis*.

Introduction

Anthocyanins are water-soluble pigments derived from flavonoids, which serve vital roles during plant development and in response to environmental stresses. The accumulation of anthocyanins occurs in specific tissues at discrete developmental stages and is strictly regulated.¹ Among the environmental factors that regulate this process are light, temperature, nutrients, and stress.² The synthesis and accumulation of anthocyanins at distinct times and in distinct tissues can impact specific physiological processes in plants. For example, light-induced accumulation of these compounds has been implicated in protection of cells against damaging light exposure.³

Light regulation of anthocyanin biosynthesis and accumulation in *Arabidopsis* involves phytochromes and cryptochromes.^{4–9} Phytochromes and cryptochromes that are localized in specific tissues and/or organs are able to mediate distinct photoresponses.^{10–13} Anthocyanin levels can also be induced by stress in plants. Low temperature can result in the induction of anthocyanins in *Arabidopsis*, which is light dependent.^{14,15} Photoreceptor-dependent LONG HYPOCOTYL 5 (HY5),

which is a member of the bZIP factor family,¹⁶ and HY5-HOMOLOG (HYH),¹⁷ have roles in low temperature-induced, light-dependent anthocyanin accumulation.¹⁵ Prior results showed that HY5 and HYH were required for light-induced anthocyanin accumulation,^{17,18} thus these factors may serve as central regulators for light- and low temperature-induced anthocyanin accumulation.¹⁵ Nutrient deficiency can also result in increased anthocyanin accumulation. Some plants grown under phosphate-deficient conditions^{19–24} or nitrogen-deficient conditions^{25–28} accumulate significantly higher levels of anthocyanins than when seedlings are grown in nutrient-replete conditions. Notably, low temperature and nitrogen deficiency interact, resulting in an increased anthocyanin accumulation under white light than either stress alone.^{29,30}

The roles of specific and/or spatially distinct photoreceptor pools in the cross-talk between plant responses to light and nutrient deficiency have not been fully investigated. We investigated the roles of phytochromes in stress- and light-induced accumulation of anthocyanins in *Arabidopsis* under distinct wavelengths of light. We explore the functional specialization of spatially-localized pools of phytochromes in regulating a

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Figure 1. Development and anthocyanin content of wild-type and phytochrome-deficient seedlings under continuous far-red light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), and *phyA* (Salk_014575) seedlings were grown at 22 °C for 4 d or 12 °C for 10 d on basal (B), nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium with 1% sucrose under continuous FR (Frc) illumination of 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$. **(A, B)** Images of seedlings. Scale bar represents 1 cm. **(C, D)** Anthocyanin content. Bars represent the mean ($\pm$ SD) of at least 3 independent measurements. Numbers above each bar represent the percent of anthocyanins accumulated relative to WT grown on the same medium. Fold-increase values for anthocyanin levels for seedlings grown on nutrient-deficient medium relative to growth of the same line on basal medium are shown below the bar graph. Two-tailed, unpaired Mann-Whitney U-test analyses were performed to compare the means of anthocyanin contents per mg of fresh weight for a particular line on basal medium relative to respective nutrient limitations. Letters above bars: a, $P < 0.001$; b, $P < 0.01$; c, $P \leq 0.05$.

Table 1. Fold-difference values for anthocyanin levels determined for seedlings grown at 12 °C vs. 22 °C under different light conditions. No-0 wild-type (No-0 WT), CAB3::pBVR1, CAB3::pBVR2, Col-0 wild-type (Col-0 WT), *phyA*, *phyB*, *cry1*, and *cry2* seedlings were grown on basal (B), nitrogen-limited (-N), or phosphate-limited (-P) medium at 12 °C or 22 °C under continuous far-red (Frc), red (Rc) or blue (Bc) illumination. Fold-difference values for anthocyanin content for seedlings grown at 12 °C relative to 22 °C are indicated (based on averages for 3 independent experiments)

Plant line	Fold difference of anthocyanin content (12 °C / 22 °C)								
	Frc			Rc			Bc		
	B	-N	-P	B	-N	-P	B	-N	-P
No-0 WT	0.91	2.70	2.70	2.78	3.19	1.33	0.47	0.63	1.36
CAB3::pBVR1	0.73	0.47	0.50	0.84	1.05	1.09	0.34	0.60	1.07
CAB3::pBVR2	0.78	0.45	0.55	0.56	1.63	0.53	0.45	0.82	2.65
Col-0 WT	0.57	1.91	0.27	6.05	9.15	2.94	0.68	1.13	1.43
<i>phyA</i>	0.71	1.30	0.56	NA ^a	NA	NA	0.48	1.16	1.31
<i>phyB</i>	NA	NA	NA	1.98	26.7	2.10	NA	NA	NA
<i>cry1</i>	NA	NA	NA	NA	NA	NA	0.37	1.06	1.53
<i>cry2</i>	NA	NA	NA	NA	NA	NA	0.17	0.69	0.42

^aNA, not applicable. The indicated line was not grown under the indicated light condition.

plant's ability to respond to nitrogen-limitation, phosphate-limitation, and/or low temperature in the stress-related induction of anthocyanin accumulation.

Materials and Methods

Plant materials and light sources

Mesophyll phytochrome-deficient CAB3::pBVR1 and CAB3::pBVR2 transgenic plant lines were previously isolated.¹³ The remaining *Arabidopsis* ecotypes and genotypes utilized include the following wild-type (WT), mutants and T-DNA insertion lines obtained from the *Arabidopsis* Biological Resource Center: No-0 WT, Col-0 WT, *phyA* (SALK_014575),^{31,32} *phyB* (SALK_022035),^{31,32} *cry1-401*³³ and *cry2-1*.³⁴ Continuous blue (Bc), continuous red (Rc) and continuous far-red (Frc) light sources were described previously.¹³ FR fluence rates were measured with a StellarNet EPP2000 spectroradiometer (Apogee Instruments, Logan, UT). All other light fluence rates were measured using a Li-Cor light meter (model LI-250, Li-Cor, Lincoln, NE) with an attached Li-Cor quantum sensor (model LI-190SA).

Plant growth media and growth conditions

Figure 2. Development and anthocyanin content of wild-type and phytochrome-deficient seedlings under continuous red light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), and *phyB* (Salk_022035) seedlings were grown at 22 °C for 4 d or 12 °C for 10 d on basal (B), nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium with 1% sucrose under continuous R (Rc) illumination of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (A, B) Images of seedlings. Scale bar represents 1 cm. (C, D) Anthocyanin content. Bars represent the mean ($\pm$ SD) of at least 3 independent measurements. Numbers above each bar represent the percent of anthocyanins accumulated relative to WT grown on the same medium. Fold-increase values for anthocyanin levels for seedlings grown on nutrient-deficient medium relative to growth of the same line on basal medium are shown below the bar graph. Two-tailed, unpaired Mann-Whitney U-test analyses were performed to compare the means of anthocyanin contents per mg of fresh weight for a particular line on basal medium relative to respective nutrient limitations. Letters above bars: a, $P < 0.001$; b, $P < 0.01$; c, $P \leq 0.05$.

Basal medium (Basal) consisted of 1X Murashige and Skoog Basal Salt Mixture (PhytoTechnology Laboratories®, KS) with 0.9% (w/v) Phytoblend agar (Caisson Laboratories, UT) and 1% (w/v) Sucrose, adjusted to pH 5.7 with NaOH. For N limitation (-N), media contained 1X Murashige and Skoog Modified Basal Salt Mixture without Nitrogen, Phosphorous, and Potassium (PhytoTechnology Laboratories®, KS) with 0.17 g/L of K_2HPO_4 , 0.9% (w/v) Phytoblend and 1% (w/v) Sucrose, adjusted to pH 5.7 with HCl. For P limitation (-P), media contained 1X Murashige and Skoog Modified Basal Salt Mixture without Nitrogen, Phosphorous, and Potassium with 1.65 g/L of NH_4NO_3 , 1.9 g/L of KNO_3 , 0.9% (w/v) Phytoblend and 1% (w/v) Sucrose, adjusted to pH 5.7 with NaOH.

Seeds were sterilized and planted as previously published.¹³ Sterilized seeds were planted in 100- x 20-mm petri dishes on Basal, -N and -P media and treated with a R pulse at $-75 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 min prior to imbibition. Imbibing seeds were cold-stratified at 4 °C for 3 d in darkness. Plates were grown under FRc illumination of 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Rc illumination of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Bc illumination of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ or in darkness for 4 d at 22 °C or 10 d at 12 °C.

Quantification of Anthocyanin Levels

Anthocyanins were extracted from seedlings using 1% (v/v) HCl in methanol as described³⁵ with prior detailed

modifications.⁶ Two-tailed, unpaired Mann-Whitney U-test analyses were performed to compare the means of anthocyanin contents per mg of fresh weight of transgenic or mutant seedlings relative to cognate WT seedlings and respective nutrient limitations.

Analysis of Root Growth

Seedlings were grown vertically in square 10- x 10-cm petri plates on Basal, -N, or -P media containing 1% (w/v) Phytoblend agar (Caisson Laboratories, UT) under monochromatic light conditions as described previously.¹⁰ Plates containing 7-d-old seedlings were scanned to obtain root images. To quantify root lengths, the roots of scanned seedlings were measured using Image J software (NIH).

Results

Abiotic stress results in a mesophyll phytochrome-dependent enhancement in the accumulation of anthocyanins

Under FRc light, No-0 and Col-0 WT ecotypes exhibited an induction in anthocyanin accumulation under limiting nitrogen and phosphate conditions at 22 °C (Figs. 1A and C, representative seedling images and pigment quantification). Lines in which mesophyll-specific inactivation of phytochromes is achieved by expression of a gene encoding a phytochrome

Figure 3. Development and anthocyanin content of wild-type, phytochrome-deficient and cryptochrome mutant seedlings under continuous blue light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), *phyA*, *cry1*, and *cry2* seedlings were grown at 22 °C for 4 d or 12 °C for 10 d on basal (B), nitrogen-limited (-N), or phosphate-limited (-P) PhytoBlend medium with 1% sucrose under continuous B (Bc) illumination of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (A, B) Images of seedlings. Scale bar represents 1 cm. (C, D) Anthocyanin content. Bars represent the mean ($\pm$ SD) of at least 3 independent measurements. Numbers above each bar represent the percent of anthocyanins accumulated relative to WT grown on the same medium. Fold-increase values for anthocyanin levels for seedlings grown on nutrient-deficient medium relative to growth of the same line on basal medium are shown below the bar graph. Two-tailed, unpaired Mann-Whitney U-test analyses were performed to compare the means of anthocyanin contents per mg of fresh weight for a particular line on basal medium relative to respective nutrient limitations. Letters above bars: a, $P < 0.001$; b, $P < 0.01$; c, $P \leq 0.05$.

chromophore-degrading enzyme biliverdin reductase (*BVR*), i.e., CAB3::pBVR lines (expression in plastids under control of the CAB3 promoter), or a *phyA* mutant showed greatly reduced levels of light-induced anthocyanin accumulation (Fig. 1). These lines also exhibited dampened induction responses to nitrogen-limited and phosphate-limited growth compared with WT ($P < 0.001$, Fig. 1C). Cold treatment (i.e., 12 °C) generally resulted in a greater accumulation of anthocyanins than at ambient temperature (i.e., 22 °C) under nutrient-deficient conditions for the WT ecotypes (Fig. 1B and D; Table 1). Cold induction of anthocyanins on phosphate-limited medium was ecotype specific, with an increased response for No-0 WT that was 4.3-fold higher than on basal medium, whereas there was essentially no response to phosphate-limitation in the cold for Col-0 (Fig. 1D). However, this nutrient limitation-induced anthocyanin accumulation response is phytochrome-dependent as CAB3::pBVR and *phyA* lines largely lacked anthocyanin accumulation under these nutrient-deficient conditions (Fig. 1B and D).

Similar responses were noted for an induction of anthocyanin under nutrient-deficient conditions under Rc at 22 °C (Fig. 2). CAB3::pBVR lines exhibited a more severe phenotype than a *phyB* mutant, especially for phosphate-limited conditions (Fig. 2C). When grown under Rc at lower temperature, the WT ecotypes showed a greater accumulation of anthocyanins than at ambient temperature (Fig. 2, Table 1). At cold

temperatures, the defect of CAB3::pBVR lines was more severe than the *phyB* mutant under all conditions, i.e., basal, nitrogen-limited or phosphate-limited media (Fig. 2D). The latter result suggests a role for phytochromes other than phyB in the induction of anthocyanins under Rc at 12 °C.

Under Bc, the CAB3::pBVR2 line and *cry1* mutant exhibited the most severe defects in the nutrient deficiency-dependent induction of anthocyanins (Fig. 3). Whereas nearly all phytochrome and cryptochrome deficient lines exhibited lower levels of anthocyanin than their WT parents at ambient temperatures, the CAB3::pBVR1 and *cry2* mutant lines accumulated more anthocyanins than their cognate parents on phosphate-limited medium at 22 °C (Fig. 3C). However, at 12 °C all of the photoreceptor deficient mutants exhibited reduced anthocyanin accumulation on all growth media. Different from Rc- and Frc-dependent growth, under Bc there was virtually no low temperature-dependent induction of anthocyanin accumulation for WT lines (Fig. 3; Table 1).

Nutrient deficiencies are sensed and regulated by distinct photoreceptor pools in seedlings

Under Frc, No-0 and Col-0 WT seedlings exhibited significantly longer primary roots on -N medium (Fig. 4A), a response previously noted for *Arabidopsis* grown under white light.^{36,37} CAB3::pBVR lines and *phyA* primary roots were significantly shorter on -N and -P than on basal medium (Fig. 4A). No-0 WT, CAB3::pBVR lines, and Col-0 WT seedlings exhibited

Figure 4. Light-dependent primary root lengths of wild-type, phytochrome chromophore-deficient, phytochrome apoprotein and cryptochrome apoprotein mutants. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), *phyA*, *phyB*, *cry1*, and *cry2* seedlings were grown vertically on basal (B), nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium containing 1% (w/v) Suc for 7 d at 22 °C under (A) far-red continuous (Frc) light of 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$, (B) red continuous (Rc) light of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, or (C) blue continuous (Bc) light of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Bars represent the mean ($\pm$ S.D.) of primary root lengths in mm ($n = 5$ to 10 for 2 independent biological experiments). For statistical significance tests comparisons were made for a particular line on basal medium relative to the respective nutrient limitations: a, $P < 0.001$; b, $P < 0.01$; c, $P \leq 0.05$.

longer root hairs under Frc on nitrogen- and phosphate-limited growth compared with growth on basal medium (Fig. 5). Notably, a *phyA* mutant, which lacks phyA in both shoots and roots, exhibited a shorter primary root on basal medium ($P = 0.017$, Fig. 4) and lacked the elongated root hairs observed under nutrient deficiency (Fig. 5). The shorter primary root for *phyA* mutant under Frc has previously observed.^{10,38,39}

Under Rc, only CAB3::pBVR seedlings exhibited a shorter primary root on -P relative to basal (Fig. 4B). No-0 WT seedlings and CAB3::pBVR lines exhibited increased root hair length under nitrogen- and phosphate-limited growth compared with growth on basal medium in Rc (Fig. 6). The Col-0 WT line exhibited elongated root hairs most notably on

phosphate-limited medium compared with basal (Fig. 6). The phenotype of a *phyB* mutant was distinct in that the root hairs did not significantly elongate in response to phosphate limitation relative to growth on basal medium (Fig. 6). The primary root of *phyB* was also shorter than WT on basal medium under Rc ($P < 0.0001$, Fig. 4B), a phenotype previously observed for Rc^{10,39} and white light.⁴⁰

Under Bc, No-0 WT exhibited a longer primary root length on -N medium (Fig. 4C), which was similar to the phenotype under Frc (Fig. 4A). The roots of all lines, except the *phyA* mutant, were shorter on -P than on basal medium (Fig. 4C). All lines exhibited longer root hairs on N-limited media under Bc illumination (Fig. 7). Under phosphate-limitation, *phyA*

Figure 5. Root development of wild-type and phytochrome-deficient seedlings under continuous far-red light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), and *phyA* seedlings were grown vertically at 22 °C for 7 d on basal, nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium with 1% Suc under far-red continuous (FRC) illumination of 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Representative images of seedlings from 2 independent biological replicates are shown. Scale bars represent 0.5 cm.

and *cry1* mutants did not exhibit stimulation of root hairs as did the other lines under Bc (Fig. 7).

Discussion

Mesophyll-localized phytochromes are required for nitrogen or phosphate limitation-induced accumulation of anthocyanins under FR and R illumination as CAB3::pBVR lines are equivalent to *phyA* mutant under FR (Fig. 1) and similarly deficient in anthocyanin accumulation under Rc (Fig. 2). Similar phenotypes

were previously observed on replete medium at ambient temperatures.^{13,41} The role of mesophyll-localized *phyA*, *phyB* and light-stable phytochromes in addition to *phyB* are shown here to be important additionally for regulating the induction of anthocyanins by low temperature and nutrient limitation (Figs. 1–2; Table 1). The regulatory roles of phytochromes in mediating an increased accumulation of anthocyanins are observed at both ambient and cold temperatures under R and FR light (Figs. 1–2), but not for B (Fig. 3). Notably, though defective relative to cognate WT lines under B, all of the transgenic and mutant lines still

Figure 6. Root development of wild-type and phytochrome-deficient seedlings under continuous red light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), and *phyB* seedlings were grown vertically at 22 °C for 7 d on basal, nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium with 1% sucrose under red continuous (Rc) illumination of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Representative images of seedlings from 2 independent biological replicates are shown. Scale bars represent 0.5 cm.

accumulate significant levels of anthocyanins under Bc (Fig. 3), which provides evidence that the severe defects observed under FRc (Fig. 1) and Rc (Fig. 2) are due to specific disruptions in phytochrome-dependent signaling rather than a complete inability of these lines altogether to accumulate anthocyanins. The lack of an apparent low-temperature induction of anthocyanins under B suggests that low-temperature, light-dependent induction of anthocyanins is regulated by phytochrome perception of R and FR.

Although lines exhibiting a depletion of mesophyll-localized phytochromes were largely impaired in nutrient-deficient and

cold-induced stimulation of anthocyanin levels, the roots of these lines, which still accumulate root-localized phytochromes,¹⁰ exhibit near wild-type responses to nutrient starvation, with the exception of a lack of root elongation in response to nitrogen limitation (Figs. 4–7). In contrast, *phy* mutants, which exhibit phytochrome deficiencies in all tissues including roots, largely lack nutrient limitation-induced changes in root elongation and root hair morphology (Figs. 4–7). These results suggest that photoreceptors in the roots themselves are important for responding to nutrient limitation, which is sensed in the root,

Figure 7. Root development of wild-type, phytochrome-deficient and cryptochrome mutant seedlings under continuous blue light. No-0 wild-type (No-0 WT), CAB3::pBVR1 (CAB-1), CAB3::pBVR2 (CAB-2), Col-0 wild-type (Col-0 WT), *phyA*, *cry1*, and *cry2* seedlings were grown vertically at 22 °C for 7 d on basal, nitrogen-limited (-N), or phosphate-limited (-P) Phytoblend medium with 1% sucrose under blue continuous (Bc) illumination of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Representative images of seedlings from 2 independent biological replicates are shown. Scale bars represent 0.5 cm.

but mesophyll-localized phytochromes primarily regulate nutrient limitation-induced anthocyanin accumulation in shoots.

Our results provide evidence for a role of phytochromes in mesophyll cells in gating the response of plants to nutrient limitation- and low temperature-induced anthocyanin induction, and roles for root-localized photoreceptors in local root responses to nutrient deficiency. These results support a central role for phytochromes in coordinating cross-talk between light and stress signals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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