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SHORT COMMUNICATION

Arabidopsis cryptochrome-1 restrains lateral roots growth by inhibiting auxin transport

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ABSTRACT

Cryptochromes are blue-light photoreceptors that control many aspects of plant development. In this study, cryptochrome mutants of *Arabidopsis* were examined to assess the role of cryptchrome-1 (CRY1) in lateral roots growth. When grown in blue light for 12 d, mutant seedlings (*cry1*) showed increased growth of lateral roots, while CRY1-overexpressing transgenic seedlings (CRY1ox) exhibited a marked decrease. Lateral roots growth of CRY1ox could be stimulated by auxin, but expression of *PIN1* (efflux carrier of polar auxin transport) was strongly reduced. Contrary, the *cry1* mutation showed the opposite effect, indicating that blue light and the auxin-signaling pathway interact in lateral roots growth of *Arabidopsis*. The free IAA content in CRY1ox roots was half of that in wild type and *cry1* mutant roots. Moreover, the content of flavonoids (quercetin, kaempferol, isorhamnetin), which act as endogenous negative regulators of auxin transport, increased in CRY1ox seedlings. Taken together, these results suggest that *Arabidopsis* CRY1 restrains lateral roots growth by inhibiting auxin transport.

Introduction

Due to their sessile nature, plants have evolved various mechanisms enabling them to adapt to environmental changes, including light adaptation. Cryptochromes (CRY1, CRY2) are blue-light receptors that mediate a number of processes in plants such as inhibition of hypocotyl elongation (Ahmad and Cashmore, 1993; Sancar, 2003), de-etiolation responses (Lin et al., 1996), initiation of flowering (Guo et al., 1998), and maintenance of the plant's endogenous rhythm (Somers et al., 1998; Devlin and Kay, 2000).

Although not exposed to light, evidence indicates that photoreceptors in roots are functional. Phytochromes have been shown to be involved in phototrophic and gravitrophic responses in roots (Ruppel et al., 2001; Correll et al., 2003; Kiss et al., 2003). Molas et al. (2006) demonstrated that red- and blue-light pathways interact in roots and that genes involved in lateral root and root hair formation react to exposure to red light. Phytochromes have also been reported to control red-light mediated shoot and root development (Correll and Kiss, 2005; Salisbury et al., 2007). Since the early studies of phototropism by Darwin (1880), the field has rapidly advanced (reviewed in Briggs, 1963). For example, it has been demonstrated that RPT2 (root phototropism 2) acts as a signal transducer of the phototropic response in *Arabidopsis* (Sakai et al., 2000), and that a low-fluence rate UVB-sensing mechanism in roots is involved in *Arabidopsis* early seedling development (Tong et al., 2008). Cryptochrome photoreceptors CRY1 and CRY2 antagonistically regulate elongation of the primary root in *Arabidopsis* (Canamero et al., 2006).

The principal natural auxin, indole-3-acetic acid (IAA), is a phytohormone that shows polar transported from sites of synthesis to distal sites of activity, mediated by the PIN-FORMED (PIN) efflux carrier proteins (Chen et al., 1998). Polar auxin transport has been shown to play an important role in the development of lateral roots (Casimiro et al., 2001). Removal of apical tissues or application of polar transport inhibitors led to the inhibition of lateral root development (Reed et al., 1998). In this study, cryptochrome mutants of *Arabidopsis* were examined to assess the role of CRY1 in lateral root growth. The results indicate that CRY1 affects the growth of lateral roots through auxin transport.

Materials and methods

Plant material and growth conditions

Wild type (Wt) and mutants (*cry1*, *cry2*, *cry1cry2*) of *Arabidopsis thaliana* shared the Columbia background (Mockler et al., 2003). CRY1ox (CRY1-overexpression, Col) and CRY2ox (CRY2-overexpression, Col) transgenic lines were those described by Yu et al. (2007). Seeds were sown on plates of MS medium containing 1.5% (v/v) sucrose and 0.8% (v/v) agar, stratified at 4 °C in dark for

Abbreviations: CRY1, cryptochrome-1; CRY2, cryptochrome-2; CRY1ox, CRY1-overexpression; IAA, indole-3-acetic acid; NPA, 1-naphthylphthalamic acid; PIN, pin-formed; Q-PCR, quantitative-polymerase chain reaction; Wt, wild type

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4 d, and transferred to a growth room at 22–23 °C. LED-B, 450 ± 20 nm, and LED-R, 650 ± 20 nm, (TOKYD TIKAKIKAI CO., Japan) were used as blue and red light sources, respectively.

RNA isolation and real-time quantitative PCR

Total RNAs (100 mg fresh weight of *Arabidopsis* tissue per sample) were isolated using the Puprep RNA Easy Mini Kit (Ambiogen Life Tech, China). DNA-free RNA was obtained by RQ 1 DNase I treatment carried out according to the manufacturer's instructions (Promega, USA). Real-time Q-PCR was used to quantitatively measure the expression of *PIN1* (AT1g73590) and *PIN2* (AT5g57090) genes. *ACT7* (At5g09810) was used as an internal control. SYBR green-sequence detection reagents (Invitrogen, USA), Taq polymerase, and the sense and anti-sense primers were assayed with an iCycler iQ Detection System (Stratagene, USA). The reaction was performed in Mx3000p (Stratagene, USA) under the following conditions: one cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The primers used for Q-PCR were ACT7F:5′-ATCCCTCAGCACCTTCCAAC-3′, ACT7R:5′-ACAAACTCACCACCA CGAAC-3′; *PIN1*F:5′-TGCGGTGATATTTGG-GATGTTGA-3′, *PIN1*R:5′-CTGCTTCTGATTTAATTTGTGGGTTT-3' and *PIN2*F:5′-TATATTCGGAATGCTGGTTGCTTTG-3′, *PIN2*R:5′-CCATACA-CCTAAGCCTGACCTGGAA-3′.

Immunoblot analysis

The enhanced chemiluminescence immunoblot analysis method used was described by Lin et al. (1996). Briefly, proteins were extracted in a 4 × SDS (sodium dodecyl sulfate) sample buffer, separated (20 μg protein for each sample) in 10% SDS-PAGE gels, blotted to a Polyvinylidene Fluoride (PVDF) membrane (0.45 μm, Millipore Corporation, USA), washed, probed with appropriate primary antibodies (anti-CRY1 antibody) and a secondary antibody conjugated with horseradish peroxidase (Beyotime Institute of Biotechnology, China). Histone1 was used for the blotting to verify equal loading, and the signals developed using the enhanced chemiluminescence method (Whitehead et al., 1983). All other chemicals were acquired from Sigma (St. Louis, MO).

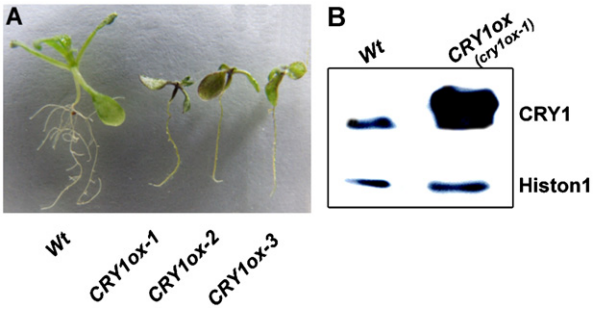

Fig. 1. Root growth in *Arabidopsis* cryptochrome mutants under blue light. Seedlings of Wt and three independent lines of CRY1ox grown under blue light (80 μmol m^{-2} s^{-1}) for 12 d (A) and immunoblot analysis of CRY1 protein in Wt and CRY1ox-1 seedlings (B) are shown.

Determination of endogenous free auxin

Free IAA was purified and quantified using the procedure described by Chen et al. (1988). A 100 mg sample of *Arabidopsis* tissue was used for isolating free IAA. [^{13}C]IAA was used as internal standard at 25 ng/g fresh weight of tissue. [^{3}H]IAA was used as a radiotracer at 50,000 dpm (disintegrations per minute) for each sample. The GC-SIM-MS was used for selected ion measurements to quantify the concentrations of free IAA relative to those of [^{13}C] IAA, which was the internal standard.

HPLC analysis of flavonoids

HPLC analysis was performed following the method of Burbulis et al. (1996). Seedlings were grown under blue light (80 μmol m^{-2} s^{-1}) for 10 d and extracts from 1 g of tissue (80% methanol) used for HPLC analysis. Samples were ground, extracts centrifuged at 14,000*g* for 5 min and 50 μL of the clear supernatant injected. Flavonol aglycones were prepared by mixing 100 μL of the extract with an equal volume of 2 N HCl. The mixture was heated at 70 °C for 40 min and 100 μL of methanol added to the hydrolyzed extract to prevent precipitation of flavonol aglycones. Samples were centrifuged at 14,000*g* for 5 min and 150 μL of the clear supernatant injected. Retention times were compared with those of authentic standards for quercetin, kaempferol, and isorhamnetin (Sigma, USA) at 0.1 mg/mL in 80% (v/v) methanol, of which 40 μL was injected.

Results and discussion

CRY1 restrains lateral roots growth in Arabidopsis thaliana

When seedlings of the Wt and three independent lines of CRY1ox (CRY1ox-1, CRY1ox-2, CRY1ox-3) were grown under blue light (80 μmol m^{-2} s^{-1}) for 12 d, clear differences between the

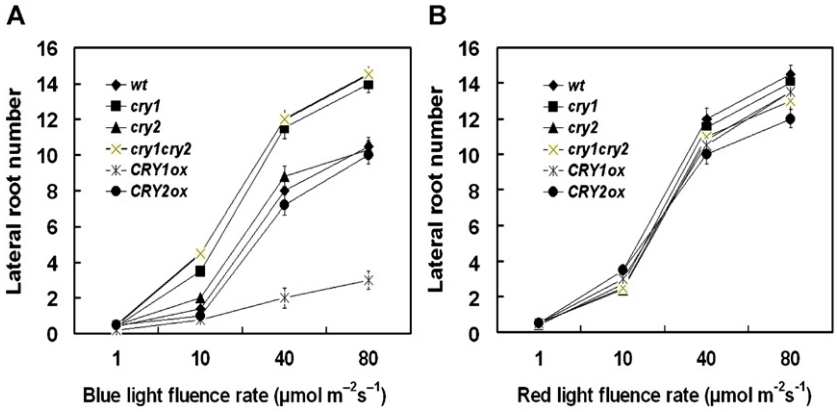

Fig. 2. Effect of cryptochrome photoreceptors on the growth of lateral roots. Seedlings were grown under defined intensities of blue (A) and red light (B) for 12 d. For each mutant, twenty seedlings were measured in three independent trials. Error bars represent the SD.

Table 1
Effect of localized IAA application on growth of lateral roots. Seven-d-old seedlings grown under blue light (80 μmol m^{-2} s^{-1}) were treated with IAA-containing agar at root/shoot junction for 4 d.

Treatment	[IAA] (μM)	Lateral roots no.	Root length (mm)	Lateral roots Density[a]
Wt	0	12.5 ± 1.4	21.8 ± 0.8	0.57 ± 0.03
CRY1ox	0	1 ± 0.2	14.2 ± 0.3	0.07 ± 0.01[b]
CRY1ox	0.1	3.0 ± 0.5	15 ± 0.56	0.2 ± 0.02[b]
CRY1ox	1	6.3 ± 0.45	24 ± 1.2	0.26 ± 0.02[b]
CRY1ox	10	10.1 ± 0.9	19 ± 0.7	0.55 ± 0.04[b]

Twenty seedlings were measured per treatment in three independent trials. Error bars represent the SD.

[a] Root density=number of lateral roots per mm of primary root.
[b] Increased lateral roots density.

Wt and the overexpressors were detectable: the growth of lateral roots was significantly reduced in the CRY1ox seedlings (Fig. 1A), and immunoblot analysis showed accumulation of CRY1 protein in markedly larger quantities in CRY1ox (CRY1ox-1 line) seedlings (Fig. 1B). This suggests that CRY1 restrains lateral roots growth in *Arabidopsis*.

Differences in the growth of lateral roots were further quantified under varying light conditions (Fig. 2). Light enhanced the production of lateral roots in all seedlings, the growth being directly proportional to the intensity of light. Under blue light, root growth was markedly enhanced in the *cry1* and *cry1cry2* double mutants and markedly reduced in CRY1ox (CRY1ox-1 line) seedlings compared to the Wt (Fig. 2A), particularly at high fluence rates. The *cry2* mutant and the CRY2ox transgenic seedlings did not show differences in root growth (Fig. 2A). However, under red light, differences between the cryptochrome mutants and the Wt were not evident (Fig. 2B). The same was found for dark conditions (data not shown). These results further support the view that CRY1 restrains lateral roots growth in *Arabidopsis*.

CRY1 restrain lateral roots growth by inhibiting auxin transport

Growth of lateral roots is highly dependent on auxin transport (Reed et al., 1998; Casimiro et al., 2001). To ascertain whether auxin signaling is involved in CRY1-dependent inhibition of lateral roots growth, the Wt and CRY1ox seedlings were assayed in the presence of exogenous indole-3-acetic acid (IAA) (Table 1). Growth of lateral roots in CRY1ox seedlings was stimulated by IAA. Without IAA, the number and density of lateral roots in CRY1ox seedlings was only approximately one-tenth of the Wt when grown under blue light. This effect was found to be dose-dependent: the higher the concentration of IAA (from 0.1 to 10 μM), the higher the number of roots and the root density, however, without reaching the values for the Wt (Table 1). On the other hand, 1-naphthylphthalamic acid (NPA) suppressed the growth of lateral roots (Fig. 3): Wt, *cry1*, *cry1cry2* and CRY1ox were equally affected. At 10 μM NPA no differences between the genotypes were detectable. These results suggest the involvement of auxin transport in the CRY1-dependent inhibition of lateral root growth.

Transcript expression of efflux carriers involved in polar transport of auxin, namely *PIN1* (*pin-formed1*) and *PIN2* (*pin-formed2*), was quantified using real-time Q-PCR. Compared to the Wt, the expression of *PIN1* in CRY1ox seedlings was reduced by nearly 50%, whereas the expression in *cry1* and *cry1cry2* seedlings increased by 20% (Fig. 4A). Contrary, the cryptochrome mutants and the Wt showed no differences in the

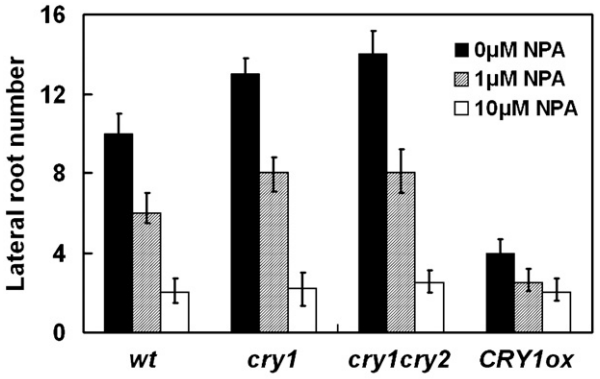

Fig. 3. Inhibition of lateral roots growth by auxin inhibitor NPA. Seedlings were grown under blue light (80 μmol m^{-2} s^{-1}) for 12 d on MS medium supplemented with 0, 1 and 10 μM NPA. Twenty seedlings were measured for each mutant in three independent trials. Error bars represent the SD.

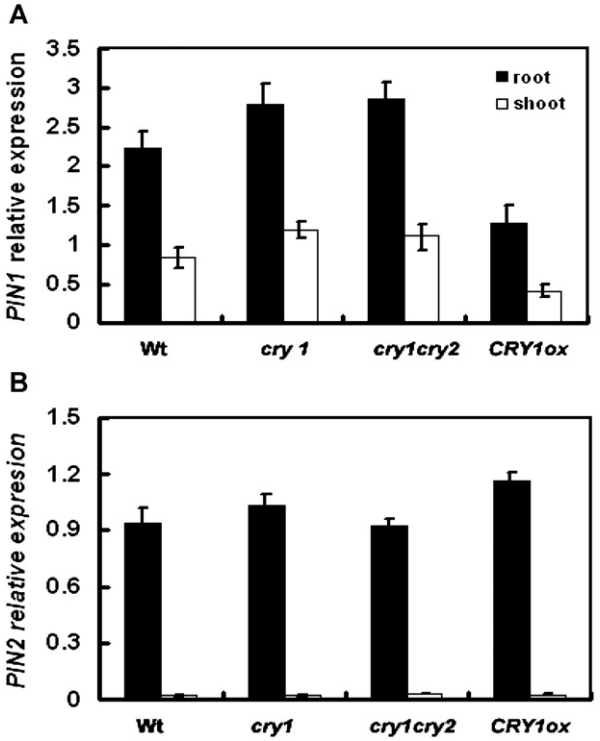

Fig. 4. Expression pattern of *PINs* detected by real-time RT-PCR. Organ-specific expression of *PIN1* (A) and *PIN2* (B) in shoot and root tissues is shown. RNAs were prepared from 12-d-old seedlings grown under blue light (80 μmol m^{-2} s^{-1}). The Q-PCR results are means with SD of three independent sets of RNA analyzed.

expression of *PIN2*, although it was sharply suppressed in shoots (Fig. 4B). These observations are consistent with earlier reports showing that the content of *PIN2* transcript in hypocotyls was either very low or not detectable (Chen et al., 1998; Galweiler et al., 1998).

Further evidence for the involvement of auxin in lateral roots growth in cryptochrome mutants is provided by the fact that the amount of endogenous IAA in shoots of 10-d-old CRY1ox seedlings was not different from those found in the Wt and the *cry1* mutant. Roots of the CRY1ox seedlings contained only half the amount of endogenous IAA compared to Wt and *cry1* when grown under blue light. These results indicate that the decreased levels of IAA in CRY1ox roots were due to a suppressed auxin

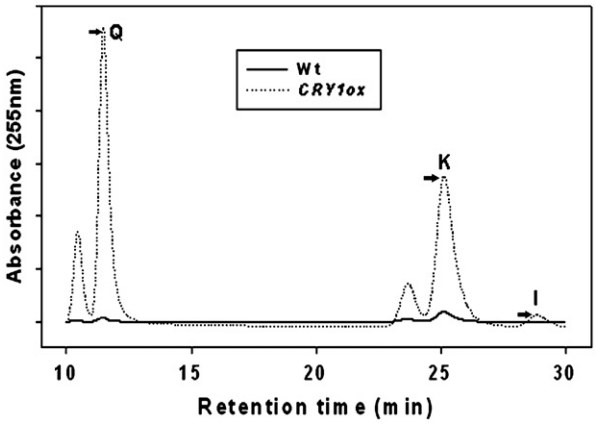

Fig. 5. HPLC analysis of flavonoids in Wt and CRY1ox seedlings. Extracts from whole seedlings were fractionated after hydrolysis. Arrows indicate the predicted positions for the three known flavonoids: Q, quercetin; K, kaempferol; and I, isorhamnetin.

Table 2
Production of endogenous free IAA in 10-d-old seedlings grown under blue light (80 μmol m^{-2} s^{-1}). IAA was determined by GC-SIM-MS.

Line	Free IAA [ng/g FW]	
	Shoots	Roots
Wt	86 ± 9.1	32 ± 8.3
cry1	84 ± 8.3	35 ± 6.2
CRY1ox	94 ± 12.2	15 ± 5.2[a]

[a] Significantly different from Wt and *cry1* mutant; $p < 0.05$.

transport from shoots to roots. The content of flavonoids (quercetin, kaemperol, isorhamnetin) was increased in CRY1ox seedlings (Fig. 5), which is consistent with earlier work showing that flavonoids act as endogenous negative regulators of auxin transport (Brown et al., 2001).

Cryptochromes and auxins are potent regulators of development in plants. Light regulates multiple aspects of plant development that are also controlled by auxins. Earlier reports have demonstrated that NPA intensifies the inhibition of hypocotyl growth under light (Jensen et al., 1998), suggesting the involvement of light in polar auxin transport. We found that CRY1ox roots were sensitive to IAA (Table 1) and that NPA removed the differences in lateral root growth between the Wt, *cry1*, *cry1cry2* and CRY1ox seedlings (Fig. 3). These observations implicate auxin signaling in the inhibition of growth of lateral roots in cryptochrome mutants and are consistent with the view that the elongation of primary roots under blue light is a result of the interaction between cryptochromes and auxin-signaling pathways (Canamero et al., 2006). In our experiments, CRY1ox roots produced only half the amount of endogenous free IAA as found in the Wt and the *cry1* mutant (Table 2). At the same time, the expression of *PIN1* in roots of CRY1ox was only half of the expression in roots of the Wt (Fig. 4A). Earlier reports have demonstrated that shoot-derived auxin controlled by *PIN1* is important for the production of lateral roots (Chen et al., 1998; Marchant et al., 2002; Rishikesh and Bhalerao, 2002). Taken together, these results suggest that in cryptochrome mutants, auxin transport from shoot to roots is affected. Moreover, flavonoids (quercetin, kaempferol, isorhamnetin) (Fig. 5) in CRY1ox seedlings may act as endogenous negative regulators of auxin transport. Thus, the results confirmed that CRY1 restrains lateral roots growth by inhibiting auxin transport.

Acknowledgements

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