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REGULAR PAPER

REVEILLE1 promotes *NADPH: protochlorophyllide oxidoreductase A* expression and seedling greening in *Arabidopsis*

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Abstract Chlorophyll biosynthesis plays a crucial role in the greening process and survival of etiolated seedlings and yet the mechanism underlying the regulation of this process is poorly understood. Upon light stimulation, NADPH: protochlorophyllide oxidoreductase (POR) catalyzes the reduction of protochlorophyllide (Pchlide) to chlorophyllide. Whereas this represents a key step in the chlorophyll biosynthetic pathway, the regulation of *POR* remains largely unknown. Three POR isoforms exist in *Arabidopsis thaliana*, i.e., *PORA*, *PORB*, and *PORC*. In this study, we identified a transcription factor, REVEILLE1 (RVE1), that binds directly to the *PORA* promoter through the EE-box cis-regulatory element. Analysis of *PORA* expression in *RVE1* loss-of-function (*rve1*) and overexpression (*RVE1-OX*) *Arabidopsis* plants showed that RVE1 positively regulates the transcription of *PORA*. We found that Pchlide levels were reduced in *RVE1-OX* seedlings. Furthermore, *rve1* etiolated seedlings had lower greening rates than the wild type when exposed to light, whereas *RVE1-OX* seedlings had higher greening rates. In addition, when etiolated seedlings were exposed to light, *RVE1-OX* plants had less reactive oxygen species (ROS) accumulation and cell death than the wild type, and had reduced levels of ROS-responsive gene expression. Taken together, our study reveals an important role for RVE1 in regulating chlorophyll biosynthesis and promoting seedling greening during early plant growth and development.

Keywords Chlorophyll biosynthesis · RVE1 · POR · Seedling greening

Gang Xu and Haiyan Guo have contributed equally to this work.

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Introduction

Chlorophylls are the major pigments that harvest light energy and drive electron transfer during photosynthesis in plants. Abnormal chlorophyll formation affects chloroplast biogenesis (Frick et al. 2003; Pogson and Albrecht 2011). Chlorophylls are biosynthesized in chloroplasts via a branch of the tetrapyrrole metabolic pathway that involves sequential enzymatic conversion by more than a dozen nucleus-encoded plastid-localized enzymes (Tanaka and Tanaka 2007; Tanaka et al. 2011), and chlorophyll biosynthesis is regulated at the transcriptional, post-translational, and redox levels (Tanaka and Tanaka 2007). Miniarray profile analysis demonstrated that light and circadian signals broadly affect the expression of genes involved in the chlorophyll biosynthetic pathway (Matsumoto et al. 2004). Two transcription factors of the light signaling pathway, FAR-RED ELONGATED HYPOCOTYLS3 and FAR-RED-IMPAIRED RESPONSE1, bind to the promoter of *HEMB1*, which encodes 5-aminolevulinic acid dehydratase, and positively regulate its expression (Tang et al. 2012). A regulatory protein, FLU, binds to glutamyl-tRNA reductase and strongly represses 5-aminolevulinic acid synthesis

(Meskauskiene et al. 2001). Furthermore, thioredoxin has been reported to regulate CHLI1 at the redox state level, with the ATPase activity of CHLI1 being reversibly inactivated by oxidation (Ikegami 2007). Misregulation of metabolism can lead to severe photooxidative stress.

Under dark conditions, the chlorophyll biosynthetic pathway is paused at the intermediate protochlorophyllide (Pchlide). Light activates NADPH: protochlorophyllide oxidoreductase (POR) which catalyzes the reduction of Pchlide to produce chlorophyllide and then chlorophyll a and b (Runge et al. 1996; Buhr et al. 2008; Heyes and Hunter 2005). POR has a photoprotective role during plant seedling greening (Sperling et al. 1997; Buhr et al. 2008). Over-accumulation of Pchlide in darkness and/or impairment of POR activity may result in the production of reactive oxygen species (ROS) upon light stimulation and even cause cell death in the cotyledons (op den Camp et al. 2003). Therefore, chlorophyll biosynthesis is critical for plant growth and survival and must be properly regulated.

POR is an important target for transcriptional regulation in chlorophyll biosynthesis. The *Arabidopsis* genome encodes three *POR* homologs, designated as *PORA*, *PORB*, and *PORC*, which are differentially expressed during plant development (Masuda et al. 2003; Frick et al. 2003). *PORA* is highly expressed in etiolated seedlings and its transcript levels decline rapidly after exposure to light, whereas *PORB* and *PORC* are up-regulated in response to light (Su et al. 2001). Several transcription regulators have been shown to mediate the expression of the *POR* genes. PHYTOCHROME-INTERACTING FACTOR1 (PIF1), a bHLH transcription factor of the light signaling pathway, regulates chlorophyll biosynthesis by binding to the promoter of *PORC* and activating its expression (Moon et al. 2008). ETHYLENE INSENSITIVE3 (EIN3), a central component of ethylene signaling, directly activates the expression of *PORA* and *PORB*, and consequently the *ein3* loss-of-function mutant accumulates excess Pchlide in etiolated seedlings and becomes photobleached after light exposure (Zhong et al. 2009). In addition, DELLA proteins up-regulate *POR* expression and limit ROS accumulation and photooxidative damage during seedling de-etiolation (Cheminant et al. 2011). However, the level of *POR* mRNA was moderately reduced in the *pif1*, *ein3*, and *della* mutants, suggesting that other regulator(s) might be involved in regulating the transcription of *POR* genes.

In this study, we focused on the regulation of *PORA* and identified REVEILLE1 (RVE1) as its direct regulator by yeast one-hybrid screening. We demonstrate that RVE1 binds to the promoter region of *PORA* in vitro and in vivo. Moreover, we showed that RVE1 regulates *PORA* expression and etiolated seedling greening. Our results demonstrate that the RVE1 transcription factor is a novel positive regulator of chlorophyll biosynthesis and seedling de-etiolation.

Materials and methods

Plant materials and growth conditions

The *rve1-1* (Salk_057420) mutant and *RVE1-OX* transgenic plants are in the *Arabidopsis thaliana* Columbia (Col) background (Rawat et al. 2009). After sterilization, seeds were sown on MS medium containing 1 % Suc and 0.8 % agar, incubated at 4 °C in darkness for 3 days, and then exposed to white light for 9 h to promote germination. White light was supplied by cool-white fluorescent lamps.

Plasmid construction

To amplify the open reading frame (ORF) of *RVE1*, total RNA was isolated from Col wild-type seedlings, and first-strand cDNA was reverse transcribed using oligo(dT)18 primer. The ORF of *RVE1* was amplified by high-fidelity Pfu DNA polymerase (Invitrogen) and cloned into the pEASY vector (Transgen) to generate pEASY-RVE1. The pEASY-RVE1 plasmid was digested with EcoRI and SalI and the *RVE1* ORF was ligated into the EcoRI/XhoI sites of the JG4-5 vector (Clontech), resulting in pAD-RVE1. The same fragment was cloned into the EcoRI/XhoI sites of pET28a (Novagen), to generate pET-HIS-RVE1.

To generate 35S: Myc-RVE1 transgenic plants, the pEASY-RVE1 plasmid was digested with EcoRI and SalI to release the *RVE1* ORF, which was then ligated into the EcoRI/XhoI sites of pRI101AN-6Myc (Takara), resulting in 35S:Myc-RVE1.

The promoter sequence ($\sim$1.2-kb fragment upstream of the start codon) of *PORA* was amplified from Col genomic DNA and ligated into the pEASY vector, giving rise to pEASY-PORAp. This plasmid was then digested with EcoRI and XhoI to release the *RVE1* fragment, which was then inserted into the EcoRI/XhoI sites of pLacZi2u (Lin et al. 2007) to produce pPORA:LacZ. pEASY-PORAp was digested with EcoRI and XbaI, and the *PORA* fragment was ligated into the EcoRI/XbaI sites of the pHISi-1 vector (Clontech), resulting in pPORA: HIS.

Plant transformation

The 35S: Myc-RVE1 binary vector was electroporated into the *Agrobacterium tumefaciens* strain GV3101 and then introduced into the *rve1* mutant via the floral dip method (Clough and Bent 1998). Transgenic plants were selected on MS plates containing 50 mg/L hygromycin.

Yeast one-hybrid assay

Yeast one-hybrid screening was performed as described in the Yeast Protocol Handbook (Clontech). Briefly, the pPORA: HIS target reporter construct was first integrated into the genome of yeast strain YM4271, and the background expression was tested in the presence of a series of concentrations of 3-amino-1,2,4-triazole (3-AT). The plasmid DNAs were isolated from the *Arabidopsis* cDNA Library (CD4-30, TAIR, ligated in the pAD-GAL4-2.1 vector), which contains about 1×10^6 clones. These plasmid DNAs were then transformed into the yeast strain containing the pPORA: HIS reporter gene, and the transformants were selected on dropout plates supplemented with synthetic dextrose (SD)/-His-Leu plus 45 mM 3-AT. The largest colonies were picked and restreaked on the same medium, and positive colonies were sequenced.

pAD-RVE1 (or pJG4-5 control) and pPORA: LacZ plasmids were co-transformed into yeast strain EGY48 using the method described in the Yeast Protocols Handbook. Transformants were grown on SD/-Trp-Ura dropout plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for color development. Protein-DNA interactions resulted in blue yeast colonies.

EMSA

Electrophoresis mobility shift assay (EMSA) was performed as previously described (Tang et al. 2012). HIS-RVE1 recombinant fusion proteins were expressed in the *Escherichia coli* BL21 (DE3) strain. The proteins were then purified using Ni-NTA Agarose (Qiagen), according to the manufacturer's instructions. The core evening element sequence, AAAATATCT, was mutagenized to AAACTGCAG. The DNA–protein interaction signal was detected on X-ray film.

Chromatin immunoprecipitation assay

The ChIP experiment was carried out as previously described (Tang et al. 2012). The chromatin complexes were isolated from the *35S: Myc-RVE1* and Col wild-type seedlings and precipitated with anti-Myc antibody (Abcam). The precipitated DNA fragments were recovered and quantified by quantitative PCR. The primer sequences are shown in Supplemental Table 1.

Gene expression analysis

Plant total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen), and first-strand cDNA was synthesized using reverse transcriptase (Invitrogen). Quantitative PCR was carried out using the SYBR Premix ExTaq Kit (Takara) following the manufacturer's instructions. The expression levels were normalized to those of *UBIQUITIN1* (*UBQ1*). The primers are listed in Supplemental Table 1.

Measurement of greening rate

Dark-grown seedlings were transferred to different intensities of continuous white light for 2 days at 22 °C as indicated in the text. The phenotypes of the cotyledons were recorded using a digital camera. Greening rate was scored as the percentage of dark-green cotyledons from 50 to 80 seedlings of each genotype. At least three independent biological repeats were performed for each genotype examined.

Determination of pchlide

Fifty etiolated seedlings were collected and homogenized in 500 μL of ice-cold 80 % acetone and incubated in darkness for 4 h. The samples were centrifuged at 5000 g for 5 min and 150 μL of the supernatant was mixed with 350 μL of glycol. Room temperature fluorescence was excited at 440 nm and scanned from 600 to 720 nm using a fluorescence spectrophotometer (Hitachi).

Trypan blue staining, H_2DCFDA fluorescence determination, and analysis of electrolyte leakage

Trypan blue staining, H_2DCFDA fluorescence and electrolyte leakage analyses were carried out as previously indicated (Chen et al. 2013). Dark-grown seedlings were transferred to white light for 2 days before staining. Seedlings were mounted on slides, and the cotyledons were photographed using the camera coupled to a dissecting microscope (Olympus).

Results

RVE1 directly binds to the cognate promoter region of *PORA*

To identify novel upstream regulator(s) of *PORA*, we generated a pPORA:HIS reporter construct in which the HIS reporter gene was driven by a 1.2-kb *PORA* promoter sequence, and screened an *Arabidopsis* GAL4 activation domain fusion library using a yeast one-hybrid approach. We identified five positive colonies that contained sequences from the same gene, *RVE1* (At5g17300). We then fused the full-length open reading frame of *RVE1* to the activation domain of B42 (AD-RVE1) and co-transformed yeast with the resulting construct and *pPORA: LacZ*

(containing the *LacZ* reporter gene driven by the *PORA* promoter). AD-RVE1, but not AD alone, was able to bind *pPORA: LacZ* and activate *LacZ* reporter expression (Fig. 1a), confirming the interaction between RVE1 and the *PORA* promoter in yeast cells.

RVE1 is a Myb-like clock-regulated transcription factor that is homologous to the central clock proteins, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Rawat et al. 2009). These proteins can bind a conserved motif, termed evening element (EE, AAAATATCT), in the promoter of target genes (Alabadi et al. 2001; Rawat et al. 2009). The *PORA* promoter contains a putative EE. To further determine the direct interaction between the *PORA* promoter and RVE1 in vitro, we conducted an EMSA. When His-RVE1 recombinant protein was incubated with ^{32}P-labeled *PORA* fragment, an upshifted band was produced. This specific band was abolished by excess un-labeled wild-type oligonucleotides, but not by excess unlabeled oligonucleotides containing mutations within the EE motif (AAACTGCAG) (Fig. 1b). We then generated transgenic plants that overexpressed the *Myc-RVE1* fusion under the control of the 35S promoter (*35S: Myc-RVE1*), and used these and wild-type plants in a chromatin immunoprecipitation (ChIP) assay with MYC antibody. The precipitated DNA was amplified with primer pairs annealing to either the promoter or coding regions of *PORA*. The promoter fragment containing the EE-box, but not the coding region, was markedly enriched when DNA extracted from the *35S: Myc-RVE1* seedlings was precipitated with Myc antibody compared with the Col wild-type control (Fig. 1c). These results together indicate that *PORA* is a direct target of RVE1.

RVE1 positively regulates *PORA* expression

To test whether RVE1 regulates the expression of *PORA* and its homologous genes, we analyzed the *rve1-1* knockout mutant and *RVE1* overexpression transgenic plants (*RVE1-OX*) (Rawat et al. 2009; Fig. 2a). Seedlings were grown in darkness for 6 days, and the transcript levels of *PORA*, *PORB*, and *PORC* were analyzed by reverse

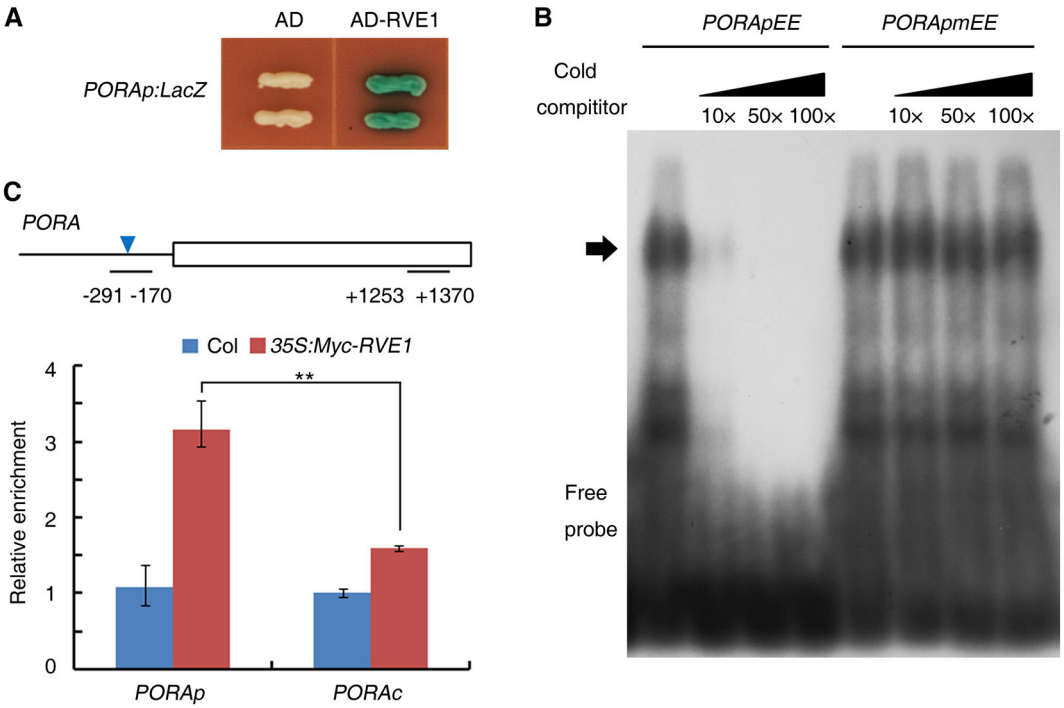

Fig. 1 RVE1 directly binds to the promoter of *PORA*. **a** Yeast one-hybrid assay. The *LacZ* reporter gene driven by the *PORA* promoter was co-expressed with AD-RVE1 or AD alone in the yeast strain EGY48. **b** EMSA in which His-RVE1 recombinant proteins were incubated with ^{32}P-labled *PORA* oligonucleotides in the absence or presence of series of excess amounts of unlabeled wild-type or mutant competitors. *PORApEE*, *PORA* promoter fragment containing EE-box; *PORApmEE*, *PORA* promoter fragment containing mutated EE-box. *Arrow* indicates shifted bands of protein-DNA complexes.**c** ChIP assay. Chromatin samples were isolated from 4-day-old dark-grown Col wild type and *35S: Myc-RVE1* seedlings, and immunoprecipitated with Myc antibody. ChIP DNA was quantified by real-time PCR with primers targeting *PORA* fragments of the promoter (*PORAp*) or coding (*PORAc*) regions. Mean ± SD of three technical replicates. *Asterisks* denote statistically significant differences ($P < 0.01$, Student's *t* test). The experiments were repeated twice with similar results. The *upper panel* shows a diagram of *PORA* and the regions amplified by PCR. The *triangle* denotes the position of the putative evening element (AAAATATCT) and digitals indicate distance (bp) *upstream* or *downstream* of the translation start codon

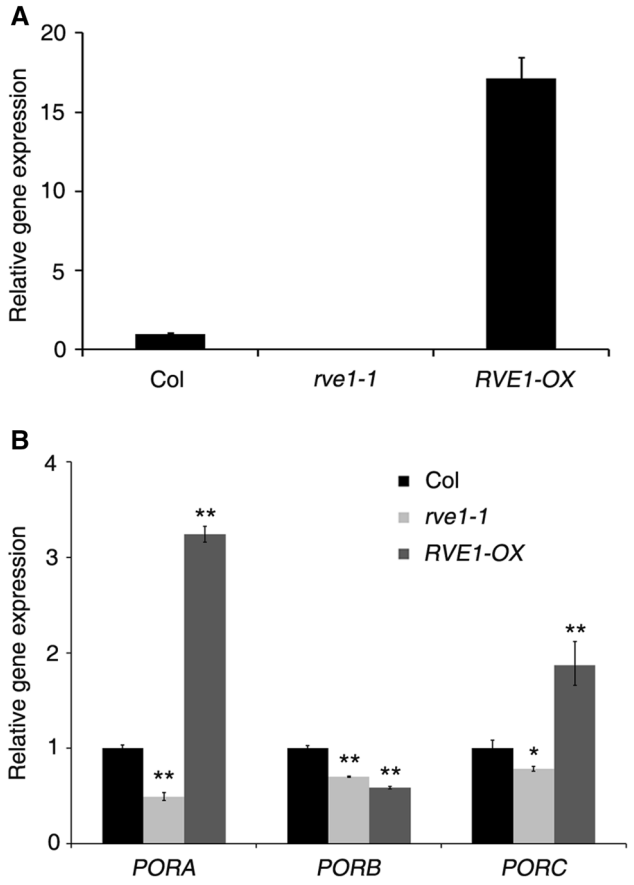

Fig. 2 RVE1 modulates *PORA* expression. **a** *RVE1* expression levels in Col, *rve1*, and *RVE1-OX* seedlings. **b** Col, *rve1*, and *RVE1-OX* seedlings were grown in darkness for 6 days and the expression of *PORA*, *PORB*, and *PORC* was analyzed by qRT-PCR. Relative expression levels were normalized to that of *UBQ1*. Data are Mean ± SD of three technical replicates. *Asterisks* denote statistically significant differences compared with the wild-type control ($P < 0.01$, Student's t test). The experiments were repeated twice with similar results

transcription followed by quantitative polymerase chain reaction (qRT-PCR). We found that the expression levels of *PORA* were drastically decreased in *rve1-1*, but increased in the *RVE1-OX* transgenic plants compared with the Col wild-type control (Fig. 2b). Surprisingly, *PORB* transcript levels were reduced in both the *rve1-1* mutant and *RVE1-OX* plants. The expression of *PORC* was not dramatically affected (Fig. 2b). These data indicate that RVE1 activates *PORA* expression in *Arabidopsis* seedlings.

Overexpression of *RVE1* decreases pchlide accumulation

Pchlide is the substrate of POR in the chlorophyll biosynthesis pathway (Tanaka and Tanaka 2007). We examined whether the level of Pchlide was affected by mutation or overexpression of *RVE1*. We extracted Pchlide from 6-day-old etiolated seedlings exposed or not to white light for 5 min and determined the fluorescence emission of the isolated compounds using a fluorescence spectrophotometer. As shown in Fig. 3a, Pchlide levels (peak at 635 nm) were similar in *rve1-1* and the Col wild type. However, we found that Pchlide levels were much lower in the *RVE1-OX* plants than in the wild-type seedlings when grown in darkness (Fig. 3a). After 5 min of light illumination, Pchlide and chlorophyllide (peak at 672 nm) levels were much lower in *RVE1-OX* than in the wild-type control, whereas those of *rve1* were slightly increased (Fig. 3b). These data suggest that overexpression of *RVE1* decreases Pchlide accumulation.

To determine the cause of the low accumulation of Pchlide in *RVE1-OX* plants, we randomly selected four genes encoding enzymes in the chlorophyll biosynthetic pathway prior to Pchlide formation and examined their transcript levels. These genes includes *GSA2* (encoding glutamate 1-semialdehyde aminotransferase), *HEMA1* (encoding glutamate-tRNA reductase), *CHLH* (encoding a subunit of magnesium chelatase), and *CRD1* (encoding Mg-proto IX monomethyl cyclase). The mRNA levels of *GSA2*, *CHLH* and *CRD1* were decreased in *RVE1-OX* plants compared with the wild type, whereas *HEMA1* expression was not affected (Fig. 3c), indicating that *RVE1* regulates the transcription of other chlorophyll biosynthetic genes in addition to *PORA*.

RVE1 promotes seedling greening

Next, we investigated whether RVE1 is a regulator of the seedling greening process. Etiolated seedlings grown in darkness for a period of time were transferred to white light (150 μmol m^{-2} s^{-1}), and the percentage of green cotyledons (greening rate) was calculated after 2 days of illumination. When the seedlings were grown in darkness for 4 days and then illuminated for 2 days, all of the *rve1* and *RVE1-OX* seedlings turned green, as did wild-type seedlings (Fig. 4a). However, when 6-day-old etiolated seedlings were transferred to light, the greening rate of the *rve1-1* mutant was much lower than that of the wild type, whereas that of *RVE1-OX* was drastically higher. After 8 days of growth in darkness, the *rve1* mutants and wild-type seedlings were unable to turn green upon transfer to light conditions, whereas more than 4 % of *RVE1-OX* seedlings became green after 2 days of illumination (Fig. 4a). We also tested the effect of light intensity on greening ability using 6-day-old etiolated seedlings. The greening rate of *rve1-1* was much lower than that of the wild type, whereas the rate of *RVE1-OX* was higher than that of the control when subjected to a light intensity of 300 μmol m^{-2} s^{-1}. Under high light conditions (500 μmol m^{-2} s^{-1}), more than 20 % of the

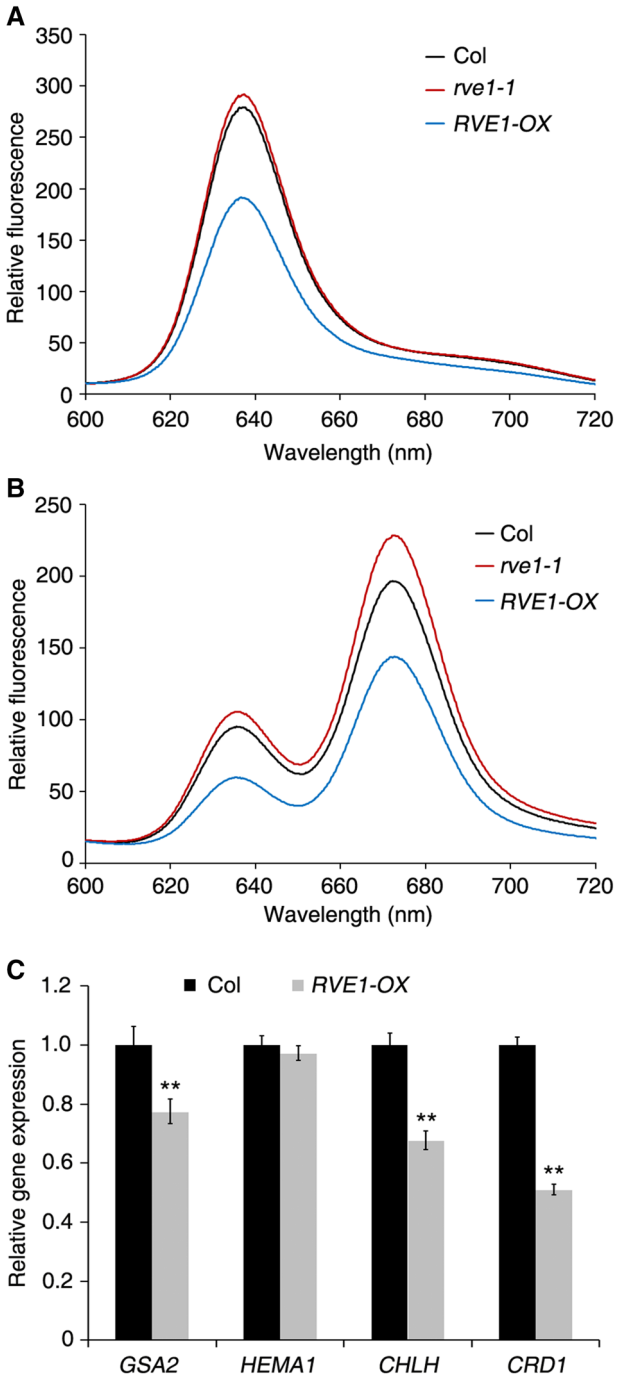

Fig. 3 Pchlide levels in the mutant and transgenic plants of *RVE1*. **a**, **b** Pchlide and chlorophyllide levels in 6-day-old dark-grown seedlings **a** or in 6-day-old dark-grown seedling transferred to light for 5 min **b**. Room temperature fluorescence was excited at 440 nm and detected from 600 to 720 nm by a fluorescence spectrophotometer. **c** Expression of chlorophyll biosynthetic genes in 6-day-old dark-grown Col and *RVE1-OX* seedlings. Relative expression levels were normalized to that of *UBQ1*. Data are Mean ± SD of three technical replicates. *Asterisks* denote statistically significant differences compared with the wild-type control ($P < 0.01$, Student's t test). The experiments were repeated twice with similar results

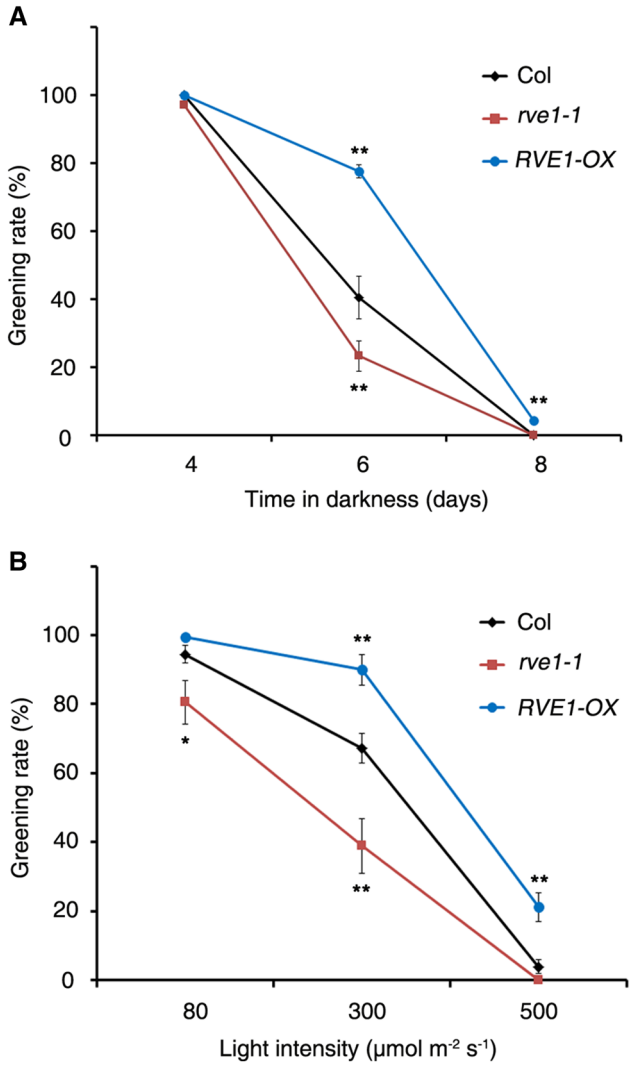

Fig. 4 RVE1 regulates seedling greening. **a** The percentage of green cotyledons of seedlings grown in darkness for the indicated periods of time followed by illumination with 150 μmol m^{-2} s^{-1} white light for 2 days. **b** Percentage of plants with green cotyledons when 6-day-old etiolated seedlings were exposed to the indicated intensities of white light for 2 days. For **a** and **b**, data denote Mean ± SD of four replicates. *Asterisks* denote statistically significant differences compared with the wild-type control (**for $P < 0.01$, *for $P < 0.05$, Student's t test)

RVE1-OX seedlings turned green, while the *rve1* mutants could not survive (Fig. 4b). These results indicate that RVE1 promotes greening during seedling de-etiolation.

RVE1 regulates ROS accumulation and cell death

To test whether the altered greening rate of the *rve1* mutant and *RVE1-OX* seedlings is the result of photobleaching, we investigated total ROS production by detecting the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluores-

cence of the cotyledons (Chen et al. 2013). When 6-day-old dark-grown seedlings were illuminated for 24 h, H_2-DCFDA fluorescence (Fig. 5a, shown in green) was similar between *rve1* and wild type but barely detectable in *RVE1-OX*, whereas chlorophyll auto-fluorescence (shown in red) exhibited the reverse pattern (Fig. 5a). Accumulated ROS within the cell may lead to cell death. After 2 days of light exposure, the seedlings were stained with trypan blue to monitor the extent of cell death. We found that the cotyledons of *rve1* mutant and wild type exhibited strong blue staining, whereas the *RVE1-OX* seedlings were barely stained (Fig. 5b). Consistently, the cell death-induced electrolyte leakage was much reduced in *RVE1-OX* compared with wild type and *rve1* mutant (Fig. 5c). Therefore, overexpression of *RVE1* inhibits photobleaching and cell death during seedling greening.

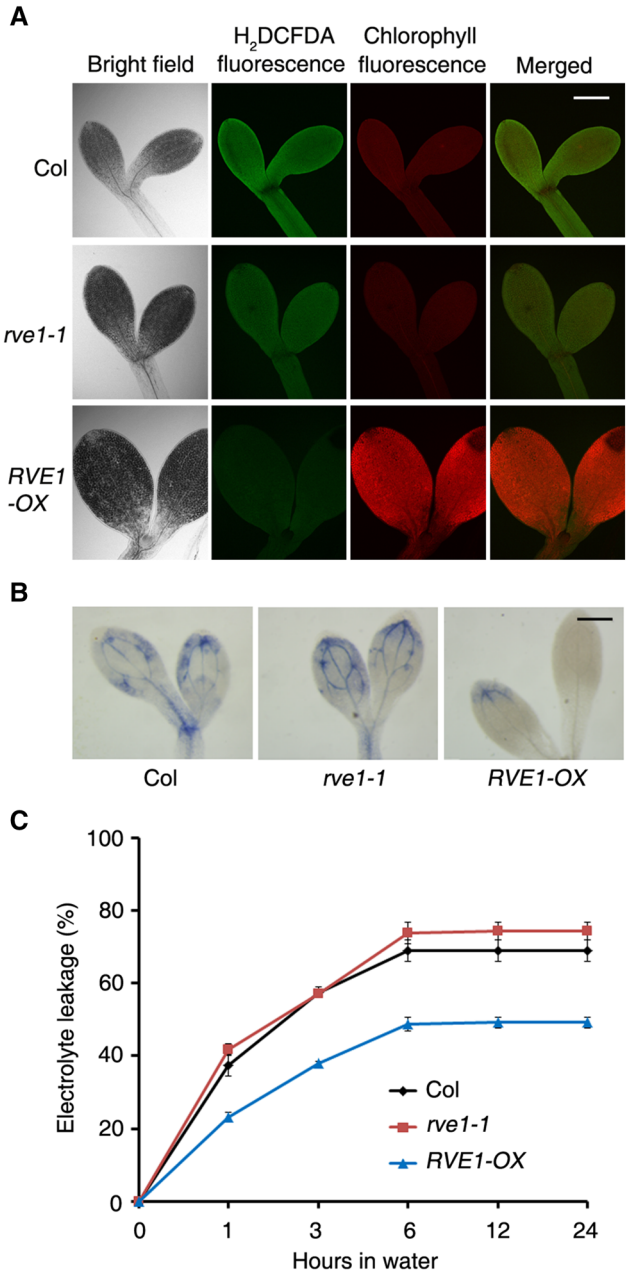

Fig. 5 RVE1 regulates ROS production and cell death. **a** Cellular ROS levels in the cotyledons of wild-type (Col), *rve1*, and *RVE1-OE* seedlings. H_2DCFDA fluorescence (*green*) indicates ROS and chlorophyll auto-fluorescence is shown in *red*. **b** Trypan blue staining of cotyledons of wild-type (Col), *rve1*, and *RVE1-OE* seedlings. For **a** and **b**, 6-day-old etiolated seedlings were exposed to white light (150 μmol m^{-2} s^{-1}) for 2 d. *Bars* denote 200 μm. **c** Six-day-old etiolated seedlings were exposed to light (150 μmol m^{-2} s^{-1}) for 12 h and immersed in water, and electrolyte leakage was measured periodically. Data are Mean ± SD, $n = 3$

RVE1 regulates ROS-responsive gene expression

We previously identified a number of ROS-regulated genes that were directly bound by light signaling transcription factors (Chen et al. 2013). ZAT10 (ZAT ZINC FINGER PROTEIN10) plays a prominent role in ROS signaling and *NDB2* (encoding NAD(P)H dehydrogenase) is an oxidative stress-induced gene, while SIB1 (SIGMA FACTOR BINDING PROTEIN1) and ERF4 (ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR4) respond to singlet oxygen generation (Miller et al. 2008; Laloi et al. 2007; Ho et al. 2008). To examine whether RVE1 modulates the expression of these ROS-regulated genes, 6-day-old etiolated seedlings were illuminated with white light for 1 or 3 h. As shown in Fig. 6, the transcript levels of *ZAT10*, *SIB1*, *ERF4*, and *NBD2* were gradually increased after light exposure. After 3 h of illumination, the expression levels of these genes were much lower in *RVE1-OE* seedlings than in those of the wild type. However, after 1 or 3 h of light treatment, the expression of *ZAT10*, *ERF4*, and *NBD2* was drastically increased in the *rve1* mutant compared with the wild type (Fig. 6). These data indicate that RVE1 represses ROS-responsive gene expression during de-etiolation.

Discussion

The central clock proteins, CCA1 and LHY, have been extensively studied and ensure accurate timekeeping of the circadian clock during all seasons (Pruneda-Paz and Kay 2010; Zhang and Kay 2010). RVE1 controls daily rhythms of auxin production by integrating the circadian and auxin signaling pathways (Rawat et al. 2009). Two RVE1 homologous, RVE2/CIRCADIAN 1 (CIR1) and RVE7/EARLY-PHYTOCHROME-RESPONSIVE 1 (EPR1), seem to act primarily as clock outputs via unknown mechanisms (Zhang et al. 2007; Kuno et al. 2003). In addition to regulating clock rhythms, members of the RVE family are involved in other plant responses. For instance,

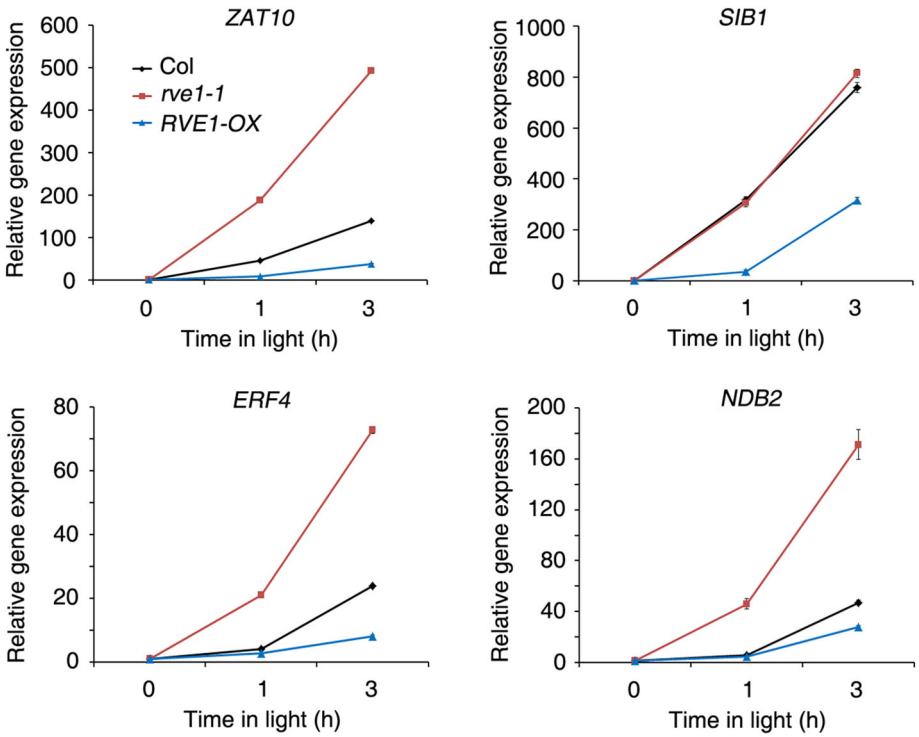

Fig. 6 RVE1 regulates ROS-responsive gene expression. qRT-PCR showing the relative expression of various ROS-responsive genes, including *ZAT10*, *SIB1*, *ERF4*, and *NDB2*. Six-day-old etiolated seedlings were kept in darkness or transferred to white light (150 μmol m^{-2} s^{-1}) for 1 and 3 h. Relative expression levels were normalized to that of *UBQ1*. Data are Mean ± SD of three technical replicates. The experiments were repeated twice with similar results

constitutively expressed *CIR1* displayed delayed flowering, longer hypocotyls, and reduced seed germination (Zhang et al. 2007). Loss of *RVE8* also caused early flowering and elongated hypocotyls (Rawat et al. 2011).

Our study provides evidence that RVE1 regulates chlorophyll biosynthesis and seedling greening through, at least in part, modulating *PORA* expression. First, yeast one-hybrid, EMSA, and ChIP assays showed that RVE1 physically binds to the promoter of *PORA* through the EE-box cis-element (Fig. 1). Second, using qRT-PCR analysis, we showed that RVE1 promotes *PORA* expression (Fig. 2). Third, overexpression of *RVE1* resulted in higher greening rate, less ROS accumulation, and cell death, and reduced ROS-responsive gene expression than the wild type (Figs. 4, 5, 6). In addition, we observed that the greening rate and the expression of some ROS-regulated genes were altered by *RVE1* mutation. There might be functional redundancy of *RVE1* with other homologous proteins. Future studies using higher order mutants involving other *RVE* genes are required to address this possibility.

CCA1 and PSEUDO-RESPONSE REGULATORs (PPRs) have been reported to mediate the seedling greening response via unidentified mechanisms (Kato et al. 2007). Overexpression of *CCA1* promotes the greening of dark-grown seedlings, whereas constitutive expression of *PPR1* has the opposite effect (Kato et al. 2007). CCA1 might act partly by modulating the level of *PORA*, since CCA1 is also able to bind directly to the *PORA* promoter and regulate its expression (Ni et al. 2008).

POR enzymes play critical roles in chlorophyll biosynthesis and affect plant greening during the dark-to-light transition (Runge et al. 1996; Sperling et al. 1997). These enzymes can be regulated at the post-translational level. A chloroplast membrane-localized protein CHAPERONE-LIKE PROTEIN OF POR1 interacts with POR and stabilizes its isoforms (Lee et al. 2013). Previous studies showed that the transcript level of *PORA* is directly modulated by EIN3 and DELLA proteins (Zhong et al. 2009; Cheminant et al. 2011). Our study identified RVE1 as a novel direct regulator of *PORA* expression. RVE1, EIN3, and DELLAs may act independently or coordinately to maintain proper *PORA* levels in response to exogenous signals (such as light) and endogenous cues (such as ethylene and gibberellic acid). It has been shown that *PORA* and *PORB* were similarly regulated by light and rhythm (Matsumoto et al. 2004). However, the expression of *PORB* was reduced by both *RVE1* mutation and overexpression. Other factors are likely involved in regulating *PORB* transcript level.

Besides directly regulating *PORA* expression, RVE1 also negatively modulates the expression of several chlorophyll biosynthesis genes through an unknown mechanism that reduces Pchlide accumulation in the cotyledons of dark-grown seedlings. Upon illumination, excess Pchlide may generate ROS or free radicals, resulting in photo-oxidization in cells (Reinbothe et al. 1996, Buhr et al. 2008). Therefore, the increased greening rate for *RVE1-OX* seedlings is caused by both the high expression of *PORA* and the reduced levels of Pchlide in darkness,

which reduce the amount of photobleaching that occurs after illumination. Consistent with this notion, the cotyledons of *RVE1-OX* seedlings barely accumulated ROS or exhibited cell death and had low levels of ROS-responsive genes expression after light exposure (Fig. 5). Thus, RVE1 likely acts as a crucial mediator that promotes chlorophyll biosynthesis and chloroplast development and maximizes the ability of plants to transition from heterotrophic to autotrophic growth.

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