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Role of *Arabidopsis* RAP2.4 in Regulating Light- and Ethylene-Mediated Developmental Processes and Drought Stress Tolerance

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ABSTRACT Light and the plant hormone ethylene regulate many aspects of plant growth and development in an overlapping and interdependent fashion. Little is known regarding how their signal transduction pathways cross-talk to regulate plant development in a coordinated manner. Here, we report functional characterization of an AP2/DREB-type transcription factor, *Arabidopsis* RAP2.4, in mediating light and ethylene signaling. Expression of the *RAP2.4* gene is down-regulated by light but up-regulated by salt and drought stresses. RAP2.4 protein is constitutively targeted to the nucleus and it can bind to both the ethylene-responsive GCC-box and the dehydration-responsive element (DRE). We show that RAP2.4 protein possesses an intrinsic transcriptional activation activity in yeast cells and that it can activate a reporter gene driven by the DRE *cis*-element in *Arabidopsis* protoplasts. Overexpression of *RAP2.4 or* mutation in *RAP2.4* cause altered expression of representative light-, ethylene-, and drought-responsive genes. Although no salient phenotype was observed with a *rap2.4* loss-of-function mutant, constitutive overexpression of *RAP2.4* results in defects in multiple developmental processes regulated by light and ethylene, including hypocotyl elongation and gravitropism, apical hook formation and cotyledon expansion, flowering time, root elongation, root hair formation, and drought tolerance. Based on these observations, we propose that *RAP2.4* acts at or downstream of a converging point of light and ethylene signaling pathways to coordinately regulate multiple developmental processes and stress responses.

Key words: *Arabidopsis*; RAP2.4; transcription factor; light signaling; ethylene response; drought tolerance.

INTRODUCTION

As sessile organisms, plants have evolved a battery of photoreceptors to sense the various parameters of their ambient light environment and adjust their growth and development accordingly. *Arabidopsis* seedling photomorphogenic development has been adapted as a model system to study the molecular mechanisms regulating light signaling in higher plants. In the dark, *Arabidopsis* seedlings undergo skotomorphogenesis (etiolation) and exhibit long hypocotyls, closed cotyledons and apical hooks, and development of the proplastids into etioplasts. In contrast, light-grown *Arabidopsis* seedlings undergo photomorphogenesis (de-etiolation) and exhibit short hypocotyls, open and expanded cotyledons, and development of the proplastids into green mature chloroplasts (McNellis and Deng, 1995). *Arabidopsis* uses two major classes of photoreceptors to mediate seedling de-etiolation: the blue (B)/UV-A (320–500 nm) absorbing cryptochromes (cry1 and cry2), and the red (R)/far-red (FR) light (600–750 nm) sensing phytochromes (phyA–phyE) (Briggs and Olney, 2001). cry1 plays a major role in response to high intensities of blue light, whereas cry2 is the primary photoreceptor for low-intensity blue light (Lin et al., 1998). Among the phytochromes, phyB to phyE predominantly regulate light responses under continuous R and white light in a conditionally redundant manner, with phyB playing a dominant role (Reed et al., 1994; Franklin et al., 2003), whereas phyA is the primary photoreceptor mediating the high irradiance response to continuous FR light (FRc), including inhibition of hypocotyl elongation, opening of apical hook, expansion of cotyledons, accumulation of anthocyanin, and FRc pre-conditioned blocking of greening (Nagatani et al., 1993; Whitelam et al., 1993).

Molecular and genetic studies in *Arabidopsis* have identified numerous signaling intermediates that are either specific for individual photoreceptors or shared by multiple types of photoreceptors (for reviews, see Neff et al., 2000; Quail, 2002; Wang and Deng, 2004). Notably, many of the identified

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signaling intermediates encode transcription factors or transcriptional regulators (such as HY5, HYH, FHY3, FAR1, LAF1, HFR1, PAT1, PIF1, PIF3, PIF4, COG1 and OBP3) (Oyama et al., 1997; Ni et al., 1998; Hudson et al., 1999; Fairchild et al., 2000; Bolle et al., 2000; Ballesteros et al., 2001; Huq and Quail, 2002; Holm et al., 2002; Wang and Deng, 2002; Park et al., 2003; Huq et al., 2004; Ward et al., 2005a) or proteins involved in the control of the abundance of these transcriptional regulators, including the COP/DET/FUS proteins, the SPA1-4 proteins, and the F-box proteins AFR and EID1 (Dieterle et al., 2001; Harmon and Kay, 2003; Serino and Deng, 2003; Laubinger et al., 2004). In addition, recent transcriptome analyses have suggested that a key feature of light signal transduction mediated by both phytochromes and cryptochromes is regulation of genome expression by transcriptional cascades, which lead to subsequent photomorphogenic development (Ma et al., 2001; Tepperman et al., 2001; Jiao et al., 2003). However, the majority of the identified light-responsive transcription factors have not yet been functionally characterized.

The *Arabidopsis AP2*-family genes have been shown to function as key developmental regulators or important mediators of responses to various environmental stress signals (such as cold, salt, and drought) (for reviews, see Sakuma et al., 2002; Nakano et al., 2006). A role of *AP2*-family genes in regulating light signaling is suggested by the observation that several *Arabidopsis AP2*-family genes, including *RAP2.4* (At1g78080), are among the genes whose transcript levels are significantly and rapidly regulated by light (Tepperman et al., 2001; Ma et al., 2002, 2003; Jiao et al., 2003; Feng et al., 2005). Consistent with this, a recent study reported that overexpression of an AP2-type transcription factor, SOB2/DRN-like, suppresses the long-hypocotyl phenotype of the *phyB-4* mutant (Ward et al., 2005b). Moreover, *Arabidopsis RAP2.4* and its putative orthologs from *Medicago truncatula* (*MtWXP1*), maize (*ZmDBF1*), and cotton (*GhDBP2*) are induced by various environmental stresses, such as cold, abscisic acid (ABA), NaCl and drought treatments (Kizis and Pages, 2002; Cheong et al., 2002; Zhang et al., 2005; Feng et al., 2005; Huang and Liu, 2006), suggesting that *Arabidopsis RAP2.4* and its homologous genes may share a conserved function in mediating responses to light and other environmental stresses. Here, we report molecular and functional characterization of *Arabidopsis RAP2.4*. Our results suggest that *RAP2.4* likely defines a cross-talk point of light and ethylene signal transduction pathways, and plays a multifaceted role in regulating *Arabidopsis* development and drought stress tolerance.

RESULTS

Regulation of *RAP2.4* Expression

Previous microarray studies suggested that accumulation of *Arabidopsis RAP2.4* was down-regulated by white light (Ma et al., 2003), but up-regulated by wounding, salt, and drought stresses (Cheong et al., 2002; Feng et al., 2005). To confirm these observations, we first performed a Northern blot analysis to compare *RAP2.4* expression under different light conditions and in response to hormones/stresses treatments. *Arabidopsis* seedlings were first grown under darkness for 4 d, then transferred to FR, R, and B light for 24 h. As shown in Figure 1A, mRNA accumulation of *RAP2.4* was significantly reduced by exposure to all three light wavelengths, suggesting that all light wavelengths act to repress *RAP2.4* mRNA accumulation. In addition, expression of *RAP2.4* was clearly induced by treatments with NaCl and drought stress, but not obviously affected by treatments with 1-aminocyclopropane-1-carboxylic acid (ACC, an immediate substrate of ethylene biosynthesis), auxin (supplied as IAA) or ABA (Figure 1B). Further, quantification of a *RAP2.4* promoter-driven beta-glucuronidase (*RAP2.4p::GUS*) reporter gene expression using the substrate 4-methylumbelliferyl β-D-glucuronide (4-MUG) confirmed down-regulation of *RAP2.4* expression by various wavelengths of light (Figure 1C). Quantitative real-time RT-PCR analysis also confirmed induction of *RAP2.4* mRNA accumulation by salt and drought stresses, but not by treatment with ACC (Figure 1D).

RAP2.4 Is Localized in the Nucleus

RAP2.4 contains a single AP2 domain (Jofuku et al., 1994; Okamuro et al., 1997) and belongs to the DREB (dehydration-responsive element-binding proteins) subfamily of AP2 proteins (Sakuma et al., 2002). Presumably, RAP2.4 acts as a transcription factor to regulate nuclear gene expression required for light responses. To provide evidence for such a notion, we translationally fused the full-length *RAP2.4* coding region with the green fluorescent protein (GFP) under the control of the strong 35S promoter (*GFP-RAP2.4*), and transiently expressed it in the onion epidermal cells by particle bombardment. As shown in Figure 2A, GFP-RAP2.4 fusion protein was detected exclusively in the nucleus under both darkness and white light conditions, suggesting that RAP2.4 is constitutively nuclear-localized. This result is consistent with a putative role of RAP2.4 acting as a transcription factor.

RAP2.4 Binds to the DRE and GCC-Box Elements and Acts as a Transcriptional Activator

Several AP2 domain-containing proteins have been shown to bind to either the ethylene-responsive GCC-box (core sequence AGCCGCC) or the dehydration responsive element (DRE, core sequence TACCGACAT) (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998), while some others have been shown to bind to both the DRE and GCC-box elements (Hao et al., 2002; Sakuma et al., 2002). To directly test the DNA-binding activity of RAP2.4, we cloned the full-length *RAP2.4* coding region into a GST fusion protein expression vector and GST-RAP2.4 fusion protein was expressed in *E. coli* and purified. Gel electrophoretic mobility shift assay showed that GST-RAP2.4 fusion protein, but not GST by itself, can bind to both the DRE and GCC-box in a dosage-dependent manner (Figure 2B). Excess amounts of unlabeled cold probes can effectively reduce or abolish the binding of

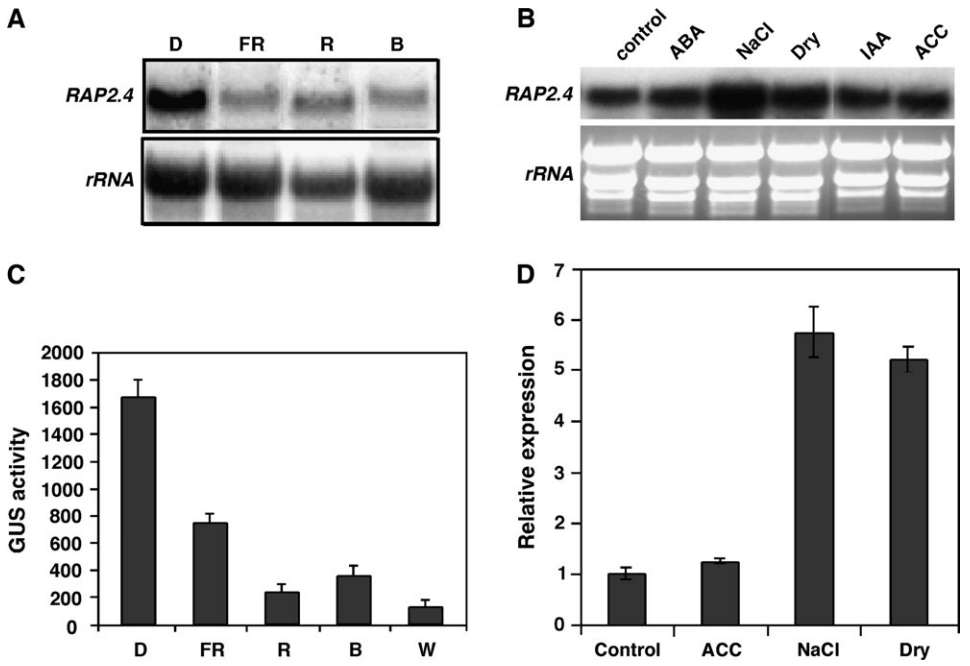

Figure 1. Regulation of *RAP2.4* Expression by Light, Hormones, and Stresses.

(A) Northern blot analysis showing that *RAP2.4* mRNA accumulation is reduced by exposure to FR, R, and B for 24 h. An *18S* rRNA blot is shown below as a loading control. D, darkness; FR, far-red; R, red; B, blue light.

(B) Northern blot analysis showing that *RAP2.4* expression is clearly up-regulated by salt (200 mM NaCl for 6 h) and drought treatment (drying on lab bench for 6 h), but not obviously affected by ACC (100 μM, 12 h), ABA (100 μM, 6 h), or IAA (20 μM, 2 h). 5-day-old seedlings were used for the experiment. A fluorescence image of rRNA is shown below as a loading control.

(C) Quantification of *RAP2.4p::GUS* reporter gene activity using 4-MUG as the substrate. Five-day-old seedlings grown under different light conditions were used for the assay. D, darkness; FR, far-red; R, red; B, blue; W, white light.

(D) Real-time RT-PCR analysis showing induction of *RAP2.4* expression by salt and drought treatments, but not by ACC. For (C) and (D): the means and standard deviations (bars) of triplicate experiments are shown.

GST-RAP2.4 to the labeled DRE or GCC-box element (Figure 2C). Further, base substitutions at the C4, G5, and C7 of DRE element or mutations at the G2 and G5 positions of the GCC-box greatly reduced or eliminated the binding (Figure 2D and 2E). These results suggest that RAP2.4 can bind specifically to both the DRE and GCC-box *cis*-elements.

Several AP2 proteins have been shown to function either as transcriptional activators or repressors (Stockinger et al., 1997; Fujimoto et al., 2000). A conserved ERF (ethylene-responsive element-binding factors)-associated amphiphilic repression (EAR) motif [L/FDLNL/F(x)P] has been found to be essential for the repressive activity of several *Arabidopsis* ERF proteins (Ohta et al., 2001). RAP2.4 lacks such a motif and thus likely acts as a transcriptional activator. To test this, we first fused the *RAP2.4* full-length open reading frame with the LexA DNA-binding domain, and cotransformed it with the p8op-*LacZ* reporter gene into the yeast strain EGY48. The reporter gene plasmid carries the *LacZ* reporter gene under the control of eight copies of LexA operators and the minimal TATA region from the GAL1 promoter (Clontech). LexA-RAP2.4, but not LexA by itself, significantly activated the *LacZ* reporter gene expression in yeast cells (Figure 3A). This result indicates that RAP2.4 is capable of activating gene expression.

Next, we tested the transcriptional activation activity of RAP2.4 in *Arabidopsis* protoplasts using the *4x DRE::GUS* reporter gene in which four tandem copies of DRE element from the *rd29* gene was fused with the 35S minimal promoter to drive the *GUS* gene expression (Kim et al., 2002). The effector plasmid (RAP2.4 driven by double copies of the strong 35S promoter or empty vector control) was cotransformed into *Arabidopsis* leaf protoplasts with the *4x DRE:GUS* reporter gene and a *35S::Luciferase* reference plasmid (Figure 3B). The reference plasmid serves as an internal control for transformation efficiency. As expected, *35S::RAP2.4* significantly activated the *4x DRE::GUS* reporter gene expression in *Arabidopsis* protoplasts (Figure 3C), thus supporting a role for RAP2.4 in transcriptional activation of DRE-containing gene expression in plant cells.

Overexpression of *RAP2.4* Results in Defects in Multiple Seedling De-Etiolation Responses

To characterize the *in vivo* function of *RAP2.4*, we obtained two *Arabidopsis* T-DNA insertion mutant alleles of *RAP2.4* (Salk_020767 and Salk_093377). The T-DNAs are inserted into the single exon of *RAP2.4* in both alleles. The T-DNA insertions were confirmed by PCR and sequencing of the flanking

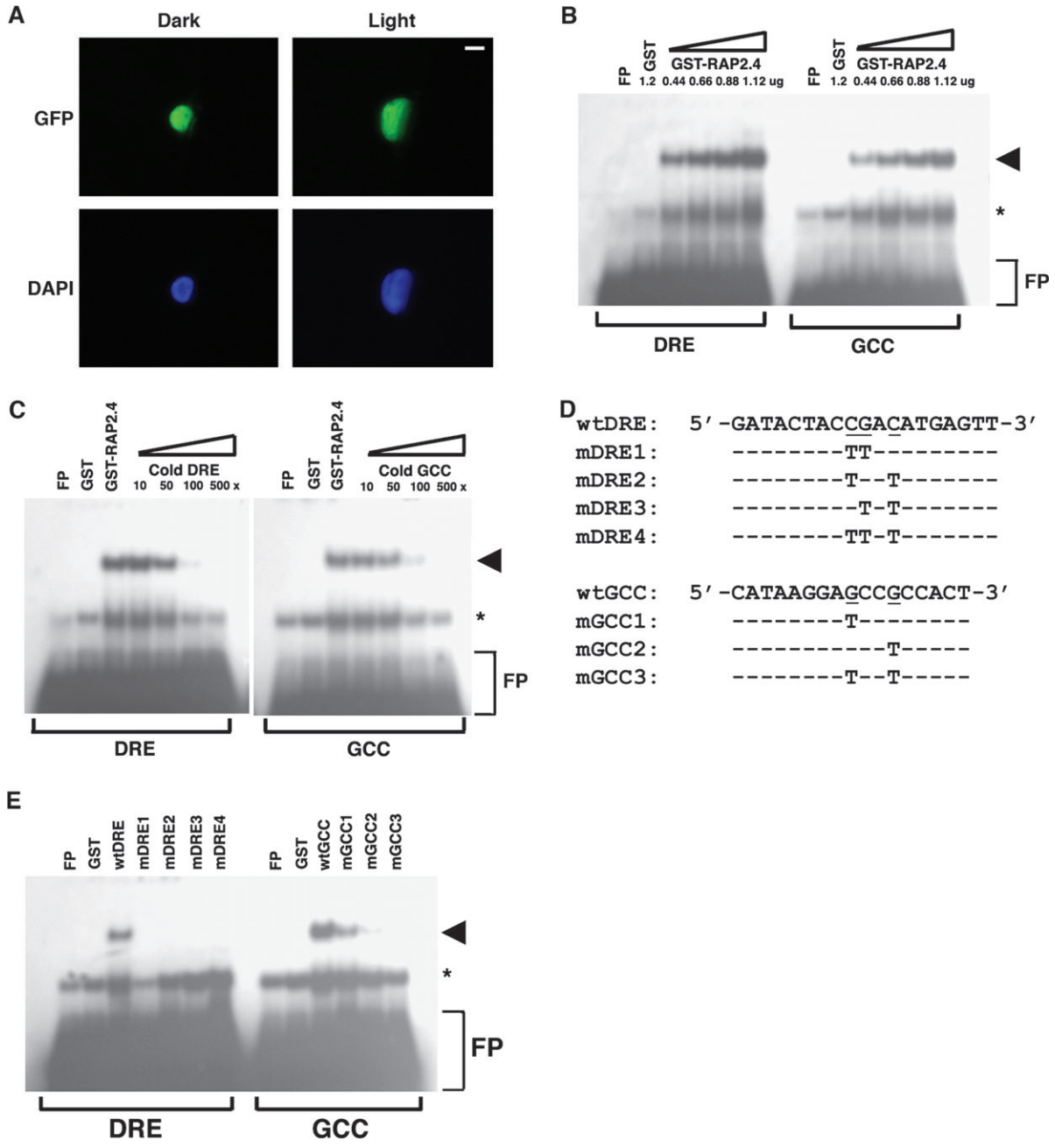

Figure 2. RAP2.4 Localizes to the Nucleus and Binds to the GCC-Box and DRE Elements.

(A) The top panels show that GFP-RAP2.4 fusion protein is targeted into the nucleus of onion epidermal cells under both dark and white light conditions. The bottom panels are 4′, 6-diamidino-2-phenylindole (DAPI) staining images of the corresponding cells showing the positions of the nuclei. Bar = 50 μm.

(B) Dosage-dependent binding of GST-RAP2.4 to the DRE and GCC-box. Two copies of DRE and GCC-box were used as probes in the gel retardation assay. The DNA-protein-binding complexes are indicated with an arrowhead. FP, free probe.

(C) Cold probe competition assay. 10×, 50×, 100×, and 500×excess amounts of cold probe DNA were added to the reaction mixtures to compete with the labeled probes. FP, free probe.

(D) Wild-type and mutant sequences of DRE and GCC-box used in the DNA-binding assay. The C4, G5, and C7 nucleotides of the DRE element and the G2 and G5 nucleotides of the GCC-box are underlined.

(E) Binding assay with wild-type and mutated DRE and GCC sequences as probes. 1.2 μg of GST protein was used for all binding assays. For (B) to (E), 0.44 μg GST-RAP2.4 was used for binding assays with the DRE element, and 0.66 μg GST-RAP2.4 was used for binding assays with the GCC-box. FP, free probe.

The lower band (indicated with a star) above the free probes in Figure 2B, 2C, and 2E likely represents non-specific oligo-forms of the probes.

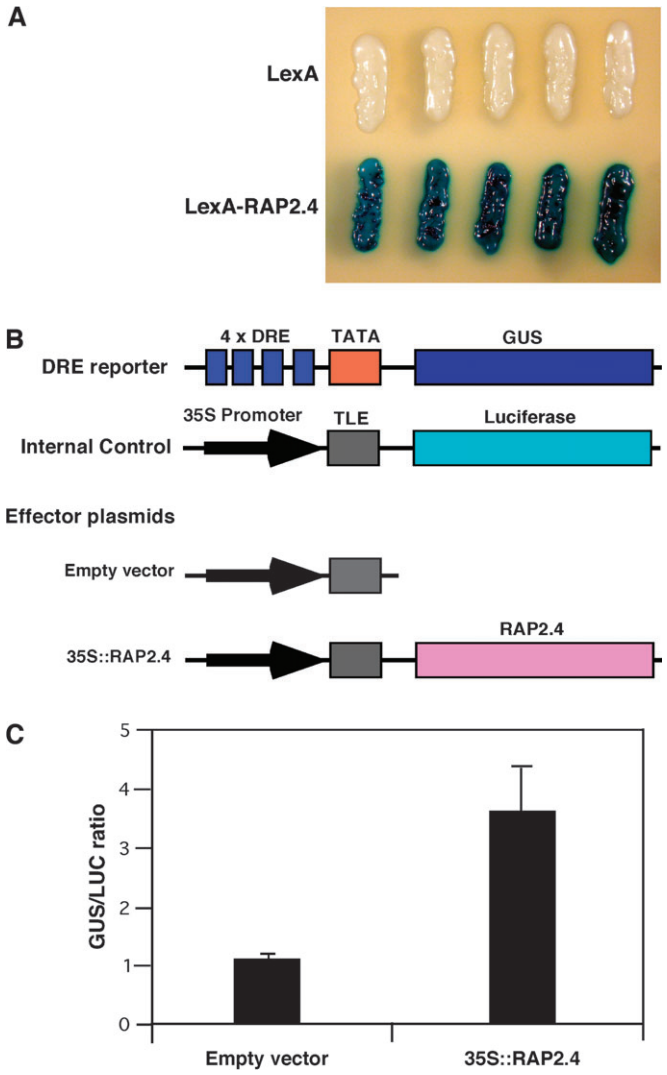

Figure 3. RAP2.4 Activates Reporter Gene Expression in Yeast and *Arabidopsis* Protoplasts.

(A) Plate assay showing that LexA-RAP2.4, but not LexA by itself, activates the *LacZ* reporter gene expression in yeast cells.
(B) Diagram of the various reporter and effector constructs used in the *Arabidopsis* protoplasts transient expression assay.
(C) RAP2.4 activates the *4x DRE::GUS* reporter gene expression in *Arabidopsis* protoplasts. Bars represent standard deviations of triplicate experiments.

sequences (Figure 4A). Northern blot analysis revealed that in the *rap2.4–1* allele (Salk_020767), *RAP2.4* mRNA accumulation was abolished, suggesting that it likely represents a null allele, whereas in the *rap2.4–2* allele (Salk_093377), *RAP2.4* expression levels were not significantly affected, but apparently had a higher molecular weight (Figure 4B). Thus, we used the *rap2.4–1* allele in subsequent functional analysis. To complement loss-of-function mutant analysis, we also generated multiple independent *RAP2.4* overexpression lines (*RAP2.4-ox* plants, driven by the constitutive 35S promoter). Northern blot analysis showed that the transgene is highly expressed in four independent lines (A4, H1, C1, and E5) (Figure 4C). Two of the *RAP2.4-ox* lines, H1 and C1, were randomly selected for subsequent functional analyses.

Under continuous darkness, the *RAP2.4-ox* plants had nearly normal hypocotyl elongation, but slightly reduced apical hook curvature, compared with wild-type and *rap2.4–1* mutant seedlings (Figure 4D; also see Figure 6A). Strikingly, the *RAP2.4-ox* plants developed clearly shorter hypocotyls under a wide range of intensities of continuous FR, R, and B light (Figure 4D and 4E). These observations suggest that *RAP2.4* acts as a positive regulator of light signaling in regulating hypocotyl elongation, and it is shared by both the phytochrome and cryptochrome photoreceptors. Microscopic analysis revealed that the reduction in hypocotyl length is primarily caused by reduced hypocotyl cell length, rather than cell number (data not shown).

Interestingly, the *RAP2.4-ox* seedlings possessed smaller cotyledons when grown under continuous FR, R, and B light conditions, compared with the wild-type and *rap2.4–1* mutant seedlings (Figure 5A and 5B), suggesting that *RAP2.4* plays a negative role in light promotion of cotyledon expansion. Northern blot analysis revealed that the induction of two light-responsive genes involved in photosynthesis—*CAB3* (encoding chlorophyll a/b binding protein, required for chlorophyll accumulation) and *RBCS* (encoding small subunit of ribulose-1, 5-biphosphate carboxylase)—was reduced in the *RAP2.4-ox* plants, but enhanced in the *rap2.4–1* mutant when dark-grown seedlings (4 d old) were transferred into R light for 6 h (Figure 5C). Moreover, phyA and phyB are known to be responsible for the agravitropic growth of hypocotyls under FR and R light, respectively. The hypocotyls of *RAP2.4-ox* seedlings exhibited an enhanced negative gravitropic growth under continuous R and FR light conditions, compared with the wild-type and *rap2.4–1* mutant seedlings (Figure 5D). This observation suggests that *RAP2.4* negatively regulates phytochrome-mediated agravitropic growth of hypocotyls. However, chlorophyll and anthocyanin accumulation was not significantly affected in either the *RAP2.4-ox* or the *rap2.4–1* mutant seedlings under FR, R or B light conditions (data not shown).

To further substantiate a functional role of *RAP2.4* in regulating light signaling in *Arabidopsis*, we generated *phyA/RAP2.4-ox*, *phyB/RAP2.4-ox*, and *cry1/RAP2.4-ox* double homozygous plants by crossing the various photoreceptor mutants with the *RAP2.4-ox* transgenic plants (line H1). The *phyA/RAP2.4-ox* double mutants displayed a phenotype identical to the *phyA* single mutant under FR light (Supplemental Figure 1A), suggesting that the hyper-photomorphogenic phenotype of *RAP2.4-ox* transgene is fully dependent on functional phyA, and thus supporting *RAP2.4* as an authentic signaling molecule in regulating phyA-mediated FR light responses. Similarly, the *phyB/RAP2.4-ox* and *cry1/RAP2.4-ox* seedlings possessed elongated hypocotyls, similar to the *phyB* and *cry1* single mutant, under R and B light conditions, respectively (Supplemental Figure 1B and 1C). These results suggest that *RAP2.4*-mediated inhibition of hypocotyl elongation

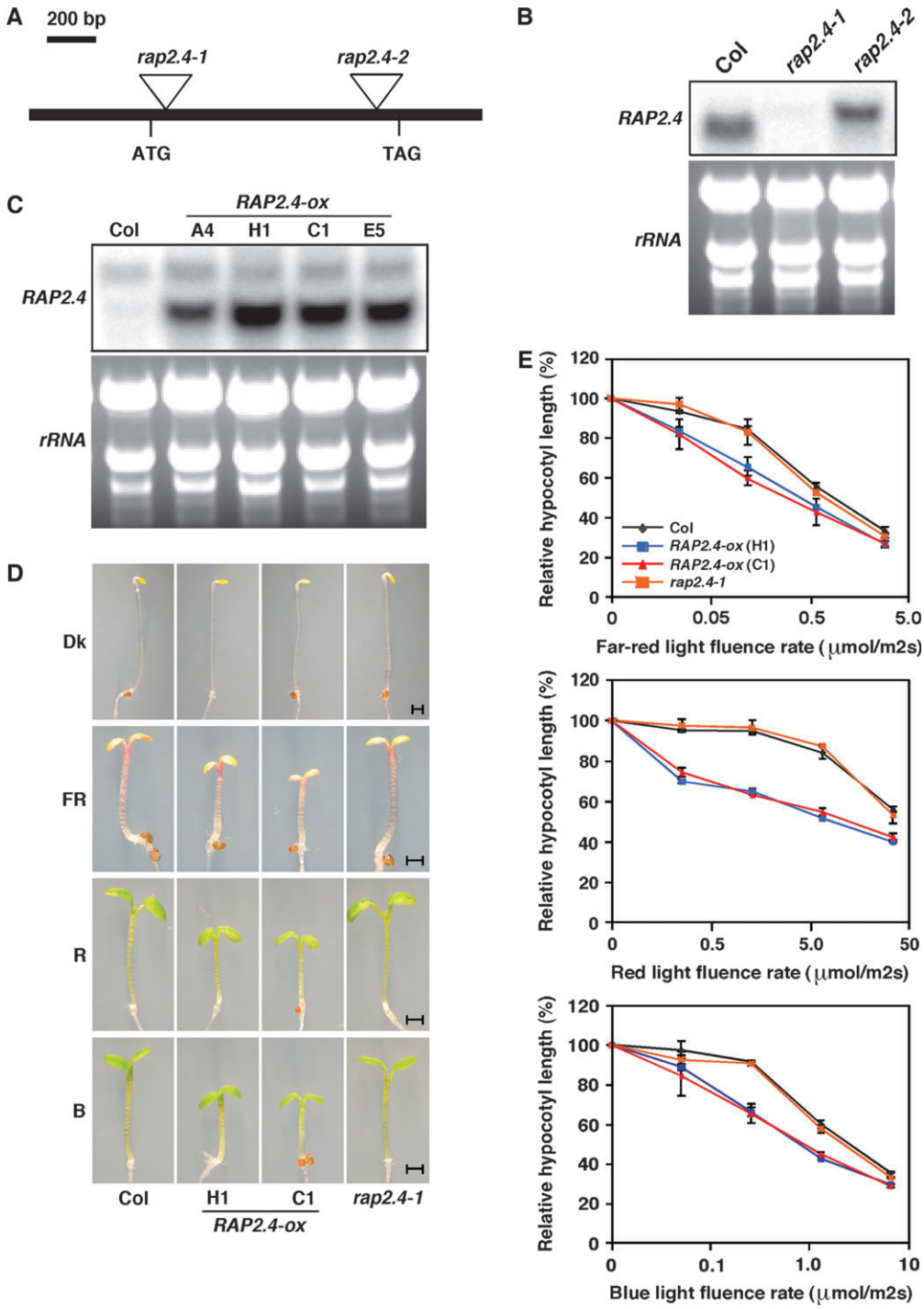

Figure 4. Loss- and Gain-of-Function Mutants of the *RAP2.4* Gene.

(A) Schematic structure of *rap2.4–1*and *rap2.4–2* T-DNA insertion alleles. The black rectangles represent the *RAP2.4* coding region with a single exon. Triangles represent T-DNA insertions.

(B) Northern blot analysis of the *rap2.4–1*and *rap2.4–2* mutants. A fluorescent image of rRNA is shown below as a loading control.

(C) Northern blot analysis of *RAP2.4* mRNA accumulation in wild-type and four independent overexpressing transgenic lines (A4, H1, C1, and E5). A fluorescence image of rRNA is shown below as a loading control.

(D) The *RAP2.4-ox* seedlings show reduced apical hook curvature in darkness. Under continuous FR, R, and B lights, the *RAP2.4-ox* seedlings possess clearly shorter hypocotyls. The *rap2.4–1*mutant seedlings responded normally under all light conditions. The seedlings are 4 d old. Bar = 1 mm.

(E) Fluence-rate responses of 4-day-old *RAP2.4-ox* plants, *rap2.4–1*, and wild-type (Col) seedlings under far-red, red, and blue light conditions. Bars denote standard deviations from 20 seedlings.

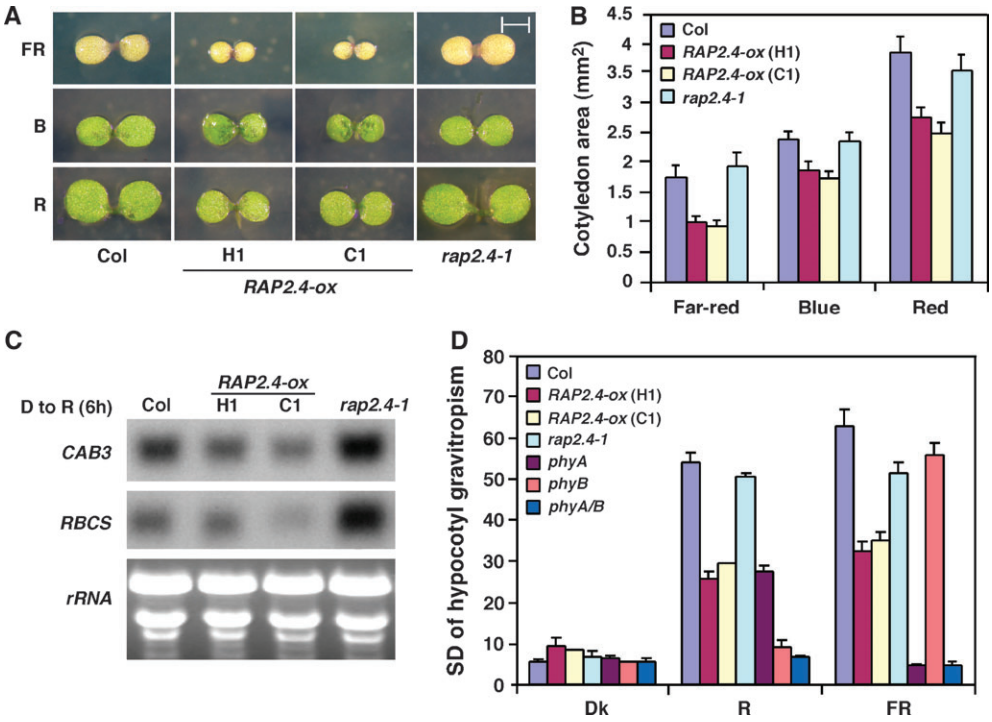

Figure 5. Multiple De-Etiolation Defects of the *RAP2.4-ox* Plants.

(A) *RAP2.4-ox* seedlings possess reduced cotyledons under FR, R, and B light conditions (4-day-old seedlings). Bar = 1 mm. FR, far-red; R, red; B, blue light.

(B) Quantification of cotyledon area of wild-type, *RAP2.4-ox*, and *rap2.4–1*mutant seedlings. Bars represent standard deviations of 30 seedlings.

(C) Altered induction of *CAB3* and *RBCS* in the *RAP2.4-ox* and *rap2.4–1*mutant seedlings in response to red (R) light. A fluorescence image of rRNA of the duplicate gel is shown below to indicate approximately equal loading of RNA. D to R (6 h): Seedlings were grown in darkness for 4 d then transferred to red light for 6 h.

(D) Quantification of the hypocotyl angles of wild-type, *RAP2.4-ox*, *rap2.4–1*, *phyA*, *phyB*, and *phyA/B* double mutant seedlings under continuous darkness (Dk), far-red (FR), or red (R) light conditions. The orientation of hypocotyls angle was measured as the standard deviations (SD) around the vertical 0^0. Values represent the pooled SD for a minimum of 100 seedlings from triplicate experiments. High SDs indicates randomization of hypocotyls.

under R and B light is largely dependent on functional phyB and cry1, respectively. The slightly shorter hypocotyls of *cry1/RAP2.4-ox* seedlings, compared with *cry1* mutant seedlings, indicate that other blue light photoreceptors (such as cry2) may still be functional in suppressing hypocotyls elongation under blue light conditions.

Overexpression of *RAP2.4* Promotes Early Flowering

We also observed that the *RAP2.4-ox* plants displayed a slight but reproducible early flowering phenotype (about 1.5–2 d earlier) under long-day conditions (16 h light/8 h darkness) (Supplemental Figure 2A). Consistent with this, the *RAP2.4-ox* plants possessed fewer rosette leaves than wild-type plants at bolting (Supplemental Figure 2B), suggesting that *RAP2.4* acts to promote flowering under long-day conditions. Under short-day conditions (8 h light/16 h darkness), the *RAP2.4-ox* plants and *rap2.4–1* mutants plants flowered around the same time as the wild-type control plants (data not shown). At mature stage, the *RAP2.4-ox* plants are normal, like wild-type plants, and seeds setting are not affected (data not shown).

Overexpression of *RAP2.4* Causes Defects in Multiple Ethylene-Mediated Responses

The ability of RAP2.4 protein to bind the ethylene-responsive GCC-box prompted us to examine a possible role of *RAP2.4* in mediating ethylene responses. We compared hypocotyl elongation, cotyledon expansion, and gravitropic growth of hypocotyls of dark-grown *RAP2.4-ox* plants with wild-type and *rap2.4–1* mutant seedlings in the presence and absence of exogenously supplied ACC. We found that the apical hooks of dark-grown *RAP2.4-ox* seedlings had reduced curvature either in the absence or presence of exogenous ACC (Figure 6A, Supplemental Figure 3A). Moreover, in the presence of ACC, dark-grown *RAP2.4-ox* seedlings had reduced elongation of hypocotyls that are more agravitropic than wild-type and *rap2.4–1* mutant plants (Figure 6B, Supplemental Figure 3B). Student's *t*-test analysis indicates that the differences in hypocotyl lengths among these genotypes are statistically significant. These observations suggest that *RAP2.4* plays an inhibitory role in ethylene-induced apical hook formation and negative gravitropism of hypocotyl growth, but promotes ethylene inhibition of hypocotyl

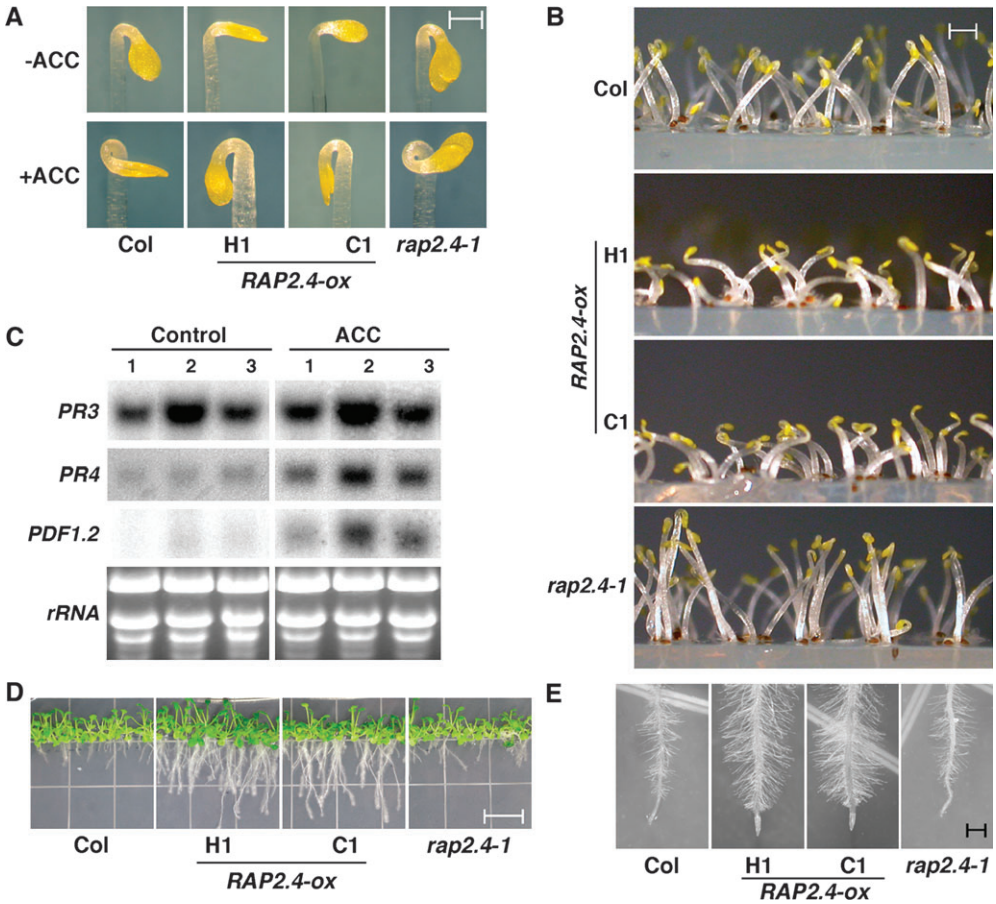

Figure 6. Altered Ethylene Responses of the *RAP2.4-ox* Plants.

(A) Reduced apical hook curvature of dark-grown *RAP2.4-ox* seedlings (4-day-old) in the absence or presence of 10 μM ACC. Bar = 0.5 mm.

(B) Images of 5-day-old seedlings grown on 10 μM ACC supplemented plate. Bar = 2 mm.

(C) Northern blot analysis showing increased expression of *PR3*, *PR4*, and *PDF1.2* in the *RAP2.4-ox* plants (line H1) in the absence or presence of ACC (100 μM for 24 h). Lane 1: Col wild-type; lane 2: *RAP2.4-ox* (H1); lane 3: *rap2.4–1*.

(D) Representative root images of wild-type (Col), *RAP2.4*-ox plants, and *rap2.4–1*mutant seedlings grown on media supplemented with 20 μM of ACC for 10 d. Bar represents 10 mm.

(E) *RAP2.4*-ox plants develop more and longer root hairs than wild-type (Col) and *rap2.4–1*mutant seedlings. The seedlings were grown on media supplemented with 20 μM of ACC for 10 d. Bar represents 10 mm.

elongation in dark-grown seedlings. Further, Northern blot analysis revealed that the expression of a number of ethylene-responsive GCC-box containing genes, including *PR3*, *PR4*, and *PDF1.2* (Solano et al., 1998; Hass et al., 2004), was increased in the *RAP2.4-ox* plants compared with wild-type and *rap2.4–1* mutant plants, in response to prolonged ACC treatment (100 μM ACC for 24 h). Increased expression of *PR3* in the *RAP2.4-ox* plants is also evident in the absence of ACC (Figure 6C). The observed changes in the expression levels of these genes support the *in vivo* relevance for the GCC-box binding activity of RAP2.4.

Ethylene also acts to inhibit root elongation and promote root hair formation and root hair elongation (Masucci and Schiefelbein, 1994; Pitts et al., 1998). Strikingly, when grown on media supplemented with various concentrations of ACC, the *RAP2.4-ox* seedlings developed much longer primary roots (Figure 6D, Supplemental Figure 3C) and produced significantly more root hairs that are much longer in length compared with wild-type and *rap2.4–1* mutant seedlings (Figure 6E). These observations suggest that *RAP2.4* negatively regulates ethylene inhibition of root elongation, but promotes ethylene-mediated root hair formation and root hair elongation.

To further confirm a regulatory role of RAP2.4 in mediating ethylene signaling, we constructed *ein2*/*RAP2.4-ox* double homozygous plants. The *ein2* mutant is insensitive to ethylene inhibition of hypocotyl elongation in etiolated seedlings (Guzman and Ecker, 1990). As shown in Supplemental Figure 4, the short-hypocotyl phenotype of *RAP2.4-ox* was completely suppressed by the *ein2* mutation in the double homozygous plants, suggesting that RAP2.4 likely acts upstream of EIN2 in the ethylene signaling pathway.

Overexpression of *RAP2.4* Confers an Enhanced Tolerance to Drought Stress

The finding that RAP2.4 is capable of binding to the DRE element led us to examine whether drought tolerance might be

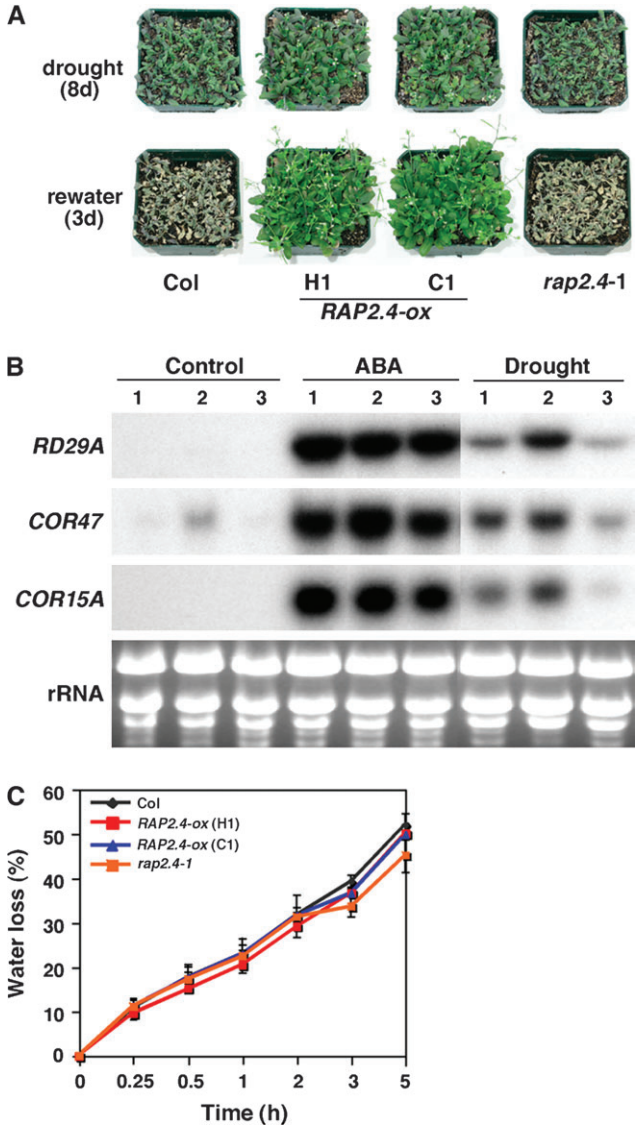

Figure 7. Enhanced Drought Tolerance of the *RAP2.4-ox* Plants.

(A) Enhanced drought tolerance of the *RAP2.4-ox* plants. Watering was withheld for 8 d (upper panel), then re-watered for 3 d (lower panel). This experiment was repeated four times with similar results. Three-week-old plants were used for this experiment.

(B) Northern blot showing that the expression of a number of CRT/DRE-containing genes (*RD29A*, *COR47*, and *COR15A*) is elevated in *RAP2.4-ox* plants, but slightly reduced in the *rap2.4–1* mutant plants, in response to drought stress (drying on lab bench for 6 h), but not to ABA treatment (100 μM for 6 h). Lane 1, Col wild-type; lane 2, *RAP2.4-ox* (H1); lane 3, *rap2.4–1*.

(C) Water loss measurement in the wild-type, *RAP2.4-ox*, and *rap2.4–1* mutant plants. Each data point represents the mean of duplicate measurements (n = 8 each). Error bars represent standard deviations.

affected by *RAP2.4* overexpression and *rap2.4–1* mutation. After withholding water for 8 d, wild-type and *rap2.4–1* mutant plants wilted, but the *RAP2.4-ox* plants remained turgid. After re-water for 3 d, no wild-type or *rap2.4–1* mutant plants recovered, whereas all *RAP2.4-ox* plants recovered well and continued their development to normal flowering and set seeds (Figure 7A). Consistent with the enhanced tolerance to drought stress, we found that the expression levels of a number of genes containing DRE or a similar *cis*-element named the C-repeat (CRT, core sequence TGGCCGAC), including *RD29A*, *COR47*, and *COR15A*, increased in the *RAP2.4-ox* plants, but slightly reduced in the *rap2.4–1* mutant plants in response to drought stress. These genes responded to ABA treatment in a similar fashion in wild-type, *RAP2.4-ox*, and *rap2.4–1* mutant plants (Figure 7B). Thus, it is most likely that *RAP2.4* regulates their expression through the CRT/DRE elements, rather than the ABA-responsive element (ABRE) present in the promoters of these genes. To test whether the enhanced drought tolerance of the *RAP2.4-ox* plants might be due to reduced water loss during leaf transpiration as a result of ABA-induced stomatal closure, rosette leaves of 4-week-old wild-type, *RAP2.4-ox*, and *rap2.4–1* mutant plants were detached from approximately the same locations and kept on the lab bench (abaxial side face up in weighing dishes) and loss of fresh weight was monitored at different time points. No significant differences in water-loss rates were found among these genotypes (Figure 7C). The *RAP2.4-ox* plants and *rap2.4–1* mutant plants also responded normally to ABA inhibition of seed germination and root elongation (data not shown). Together, these results suggest that the enhanced drought tolerance of the *RAP2.4-ox* plants likely resulted from altered drought-specific responsive gene expression through an ABA-independent pathway. Consistent with this notion, the expression of *RAP2.4* is up-regulated by salt and drought treatment, but not significantly affected by ABA (Figure 1B and 1D).

DISCUSSION

RAP2.4 Acts as a Transcriptional Activator Regulating Expression of DRE- and GCC-Box-Containing Genes

Arabidopsis RAP2.4 has a single AP2 domain and was classified into the A-6 group (Sakuma et al., 2002) or group I (Nakano et al., 2006) of the dehydration-responsive element-binding proteins (DREB) subfamily of AP2 family, based on amino acid sequence alignment (Supplemental Figure 5A). The AP2 domain is a novel DNA-binding domain that consists of approximately 60 amino acid residues (Jofuku et al., 1994; Okamuro et al., 1997). The solution structure of the AP2 domain of AtERF1 reveals that this highly conserved domain consists of a three-stranded β-sheet and one α-helix running almost parallel to the β-sheet. It contacts DNA via the conserved Arg and Trp residues in the β-sheet (Allen et al., 1998). Previous studies have shown that the Val14 and Glu19 conserved in the β-sheet of the AP2 domain of DREBs are important for determining the DNA-binding specificity of DREB1A and DREB2A (Sakuma et al., 2002). The AP2 domains of group A-6 (including RAP2.4) and a subgroup of A-5 DREB proteins contain the conserved Val14, but lack the conserved Glu19 (Sakuma et al., 2002) (Supplemental Figure 5B). The DNA-binding specificity of DREB

proteins lacking Glu19 has remained unknown. In this study, we showed that RAP2.4 is targeted into the nucleus independently of the light conditions, and that RAP2.4 can bind specifically to both the ethylene-responsive GCC-box and the dehydration-responsive DRE *cis*-elements. Further, we showed that RAP2.4 activates reporter gene expression in yeast cells and *Arabidopsis* protoplasts. The *in vivo* significance of RAP2.4 DNA-binding to these elements is further supported by the findings that the expression of a number of GCC-box and CRT/DRE element containing genes are up-regulated in the *RAP2.4-ox* plants, but reduced in the *rap2.4–1* mutant plants. Taken together, these observations support that RAP2.4 acts as a *bona fide* transcription factor involved in the regulation of ethylene- and dehydration-responsive gene expression.

Arabidopsis RAP2.4 Likely Defines a Cross-Talk Point of Light- and Ethylene-Signaling Pathways

The *AP2* gene family is one of the largest gene families in *Arabidopsis* (with over 140 members) and members of this gene family have been shown to function as key developmental regulators or important mediators of responses to pathogenic and various environmental stress signals (such as cold, salt, and drought) (Sakuma et al., 2002; Feng et al., 2005; Nakano et al., 2006). Notably, most functional studies on *AP2*-family genes were based on gain-of-function mutations or overexpression analyses (Wilson et al., 1996; Solano et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Ward et al., 2005b; Chen et al., 2007). A few studies employing loss-of-function mutants provided evidence for genetic redundancy among related *AP2*-family genes in regulating ethylene and cytokinin responses (Alonso et al., 2003; Rashotte et al., 2006). The lack of robust morphological phenotype of the *rap2.4–1* loss-of-function mutant is thus most likely due to genetic redundancy of other *RAP2.4*-related genes belonging to the same cluster (Supplemental Figure 5A) of the *Arabidopsis AP2* gene family. Consistent with this, examination of the *Arabidopsis* microarray database at the GENEVESTIGATOR (www.genevestigator.ethz.ch) (Zimmermann et al., 2004) revealed that expression of several members of this group, including At1g36060, At2g22200, At5g65130, and At1g64380, similar to *RAP2.4*, is also down-regulated by light. Moreover, examination of the expression patterns of these *RAP2.4*-related genes at the AtGenExpress Web page (www.weigelworld.org/resources/microarray/AtGenExpress/) generated by Dr Weigel's group (Max Planck Institute for Developmental Biology, Tübingen, Germany) revealed that *RAP2.4*, At1g36060, At2g22190, At1g64380, and At4g39780 share highly overlapping expression patterns with basal expression in green seedlings and abundant expression in leaves, floral organs, roots, and seeds (Schmid et al., 2005). Determination of the specific and overlapping functions of these related genes in regulating light and other stress responses would require a systematic effort and higher-order mutant analysis.

Our results presented in this study support that *RAP2.4* plays a dual role in regulating different photo-responses, based on the molecular and phenotypic defects observed in both loss- and gain-of-function mutants. It seems that RAP2.4 acts as a positive regulator of light inhibition of hypocotyl elongation, but negatively regulates cotyledon expansion, negative gravitropic growth of hypocotyl, and induction of certain light-responsive genes (such as *CAB* and *RBCS*). Similar cases in which some genes involved in light signaling appear to have opposing roles in regulating hypocotyl elongation and cotyledon expansion have been previously reported (Ward et al., 2005a; Khanna et al., 2006). In addition, *RAP2.4* negatively regulates ethylene-mediated inhibition of root elongation, negative gravitropism of hypocotyl growth, and ethylene-induced apical formation, but enhances ethylene-mediated promotion of root hair formation and root hair elongation. These results suggest that RAP2.4 likely defines a cross-talk point of light and ethylene signal transduction pathways.

The gaseous hormone ethylene plays a critical role in the regulation of developmental programs throughout the plant lifecycle, such as seed germination, cell elongation, fertilization, abscission, fruit ripening, and seed dispersal. Ethylene biosynthesis is induced in response to a variety of biotic and abiotic stresses, such as pathogen attack, wounding, high and low temperature, and drought (Wang et al., 2002). Ethylene has therefore been suggested to be a mediator of the stress response. Previous studies have documented antagonistic or overlapping roles of light and ethylene in regulating multiple aspects of plant development. For example, ethylene inhibits hypocotyl elongation and induces exaggerated apical hooks in dark-grown seedlings but promotes the opening of apical hook and hypocotyl elongation in the light (Smalle et al., 1997; Knee et al., 2000). It has also been reported that ethylene acts antagonistically with light in regulating negative gravitropic growth of hypocotyls and stems (Wheeler et al., 1986; Golan et al., 1996). However, the molecular mechanisms mediating the cross-talk between light and ethylene signaling pathways remain largely unknown. It has been suggested that light reduces ethylene production through promoting ACC malonylation or modulating the expression of ACC oxidase (*ACO*) and ACC synthase (*ACS*) genes, and stability/activity of these enzymes (Jiao et al., 1987; Finlayson et al., 1998; Vandenbussche et al., 2003; Foo et al., 2006; Lee et al., 2006). There is also a growing body of evidence suggesting that the signaling pathways of both light and ethylene cross-talk with the signaling pathways of auxin and other phytohormones (Halliday and Fankhauser, 2003; Li et al., 2004; Stepanova et al., 2005; Stepanova and Alonso, 2005). Our results suggest that RAP2.4 may act to mediate the cross-talk between the light and ethylene signaling pathways through regulating the expression of ethylene-responsive GCC-box and dehydration-responsive element (DRE)-containing genes (Supplemental Figure 6). This notion is consistent with a recent report that light is necessary for cold- and drought-induced gene expression mediated by the CRT/DRE element in *Arabidopsis* (Kim et al., 2002). Determination of the downstream target genes of *RAP2.4* and the genetic epistasis relationship between *RAP2.4* and other

known light and ethylene signaling components may help further define the molecular mechanisms underlying the interaction between light and ethylene signaling pathways.

Modulation of Drought Stress Tolerance by *AP2*-Family Transcription Factors May Entail Distinct Mechanisms

Previous studies have implicated several *Arabidopsis AP2*-family genes in drought stress tolerance, including *DREBs*/CBFs (for DRE-binding proteins/C-repeat-binding factors), *AtERF7*, and the *SHN* clade genes (Stockinger et al., 1997; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Nakashima et al., 2000; Haake et al., 2002; Aharoni et al., 2004; Song et al., 2005). However, different mechanisms might be used by these *AP2* family transcription factors to mediate drought stress tolerance. For example, both the *DREB1* and *DREB2* genes are induced by water stress or cold and their transcripts accumulate at high levels shortly after initiation of the stress treatments, but neither of these genes is induced by exogenous ABA, suggesting that their function is independent of this hormone (Liu et al., 1998). In addition, the drought tolerance conferred by overexpression of the *SHN* clade genes (*SHN1*/*WIN1*, *SHN2*, and *SHN3*) in *Arabidopsis* is reported to be associated with increased cuticular wax levels (Aharoni et al., 2004; Broan et al., 2004). On the other hand, AtERF7 was found to act as a transcriptional repressor in ABA responses and affect drought stress tolerance through influencing plant transpirational water loss (Song et al., 2005). Similarly, the enhanced tolerance to osmotic (salt and drought) stresses in transgenic *Arabidopsis* overexpressing ZmDBF1 (a maize AP2 domain-containing protein sharing all the conserved motifs of RAP2.4; Nakano et al., 2006), and GmDREB2 (a soybean AP2 domain protein belonging to the A-5 group of DREB subfamily of AP2/EREBP family) is believed to involve transcriptional regulation in response to ABA (Kizis and Pages, 2002; Saleh et al., 2006; Chen et al., 2007). Our findings that expression of *RAP2.4* is induced by drought and high-salinity stresses but not by ABA treatment favor the notion that the enhanced drought tolerance of the *RAP2.4-ox* plants might be mainly through regulation of ABA-independent water stress-inducible gene expression. Consistent with this notion, transgenic alfalfa (*Medicago sativa*) overexpressing WXP1—a *Medicago truncatula* protein with the closest related protein in *Arabidopsis* being RAP2.4—showed improved drought tolerance and increased cuticular wax accumulation on leaf surface (Zhang et al., 2005). Future studies to determine the mechanisms by which RAP2.4 confers drought stress tolerance may aid in the design of novel strategies for improving drought stress tolerance in crop plants.

METHODS

Plant Materials and Growth Conditions

The T-DNA mutant *rap2.4–1* (Salk_020767), and *rap2.4–2* (Salk_093377) were ordered from ABRC (Ohio State University, Columbus, OH). The T-DNA insertion sites were confirmed by PCR and sequencing. Their ecotype is Columbia (Col). The *phyA-211* (Reed et al., 1994), *phyB-9* (Reed et al., 1993), and *cry1-304* (Mockler et al., 1999) mutants are also derived from the Col background. The *phyAB* double mutant was described in Osterlund et al. (2000). The *ein2* mutant was described in Guzman and Ecker (1990). The *rap2.4–1* mutant has been backcrossed twice with its wild type to remove/reduce potential background mutations. Homozygous lines were selected by Kanamycin antibiotic resistance and verified by PCR genotyping.

The *phyA*/*RAP2.4-ox*, *phyB*/*RAP2.4-ox*, *cry1*/*RAP2.4-ox*, and *ein2*/*RAP2.4-ox* double homozygous plants were derived from genetic crosses of their corresponding single parental mutants (for *RAP2.4-ox*, the transgenic line H1 was used*)*. Putative double mutants were selected in F2 generation and confirmed in F3 generation based on the mutant phenotype and antibiotic selection.

Surface sterilization and cold treatment of the seeds and seedling growth conditions for different light sources were described previously (Yang et al., 2005), unless otherwise indicated. Seedlings were grown on germination agar plates containing 1% sucrose for phenotypic analysis. For hypocotyl length, cotyledon area, and apical hook curvature measurements, seedlings were photographed with a digital camera (Nikon Coolpix 4500, Japan) and the measurement was taken with the NIH Image software.

Plasmid Construction

In order to get cDNA clone of the *RAP2.4* gene, total RNA was extracted from Col wild-type seedlings using Plant RNeasy Mini Kits (Qiagen USA, Valencia, CA). RT-PCR was carried out to amplify the open reading frame of *RAP2.4* as previously described (Lin and Wang, 2004). The primers were RAP2.4-F1 (5′-CTC GAG TCG GTA CCG GAT CCA TGG CAG CTG CTA TGA ATT TG-3′) and RAP2.4-F2 (5′-GAG CTC TCT AGA CTA AGC TAG AAT CGA ATC CC-3′) with suitable restriction sites added for cloning. The PCR fragment was cloned into the pTOPO-Blunt vector (Invitrogen, Carlsbad, CA) to generate pTOPO-RAP2.4 and its sequence was confirmed by sequencing. To generate GFP-RAP2.4 fusion gene, a *BamHI-XbaI* fragment containing *RAP2.4* full-length open reading frame was released from pTOPO-RAP2.4 and cloned into the *Bgl*II-*Xba*I digested pRTL2-mGFP(S65T) vector (von Arnim et al., 1998), giving rise to pRTL2-GFP-RAP2.4.

To generate the *RAP2.4* overexpression construct, the pTOPO-RAP2.4 plasmid was digested by *Xho*I and *Sac*I to release the *RAP2.4* cDNA, and the fragment was inserted into the pF3PZPY122 binary vector (Feng et al., 2003) digested with SalI and SacI, generating pPZPY-RAP2.4, in which the *RAP2.4* cDNA was driven by the 35S constitutive promoter. To generate the *RAP2.4p::GUS* reporter gene construct, a 2.0-kb fragment of *RAP2.4* promoter (including 5′-UTR) was amplified from Col genomic DNA with primers 5′-TGG ATC CTG GGA ATG GGA TCA CTC ATA G-3′ and 5′-TCC ATG GAA TTC ATA GCA GCT GCC ATT TAA AA-3′, and cloned into the pGEM-T Easy vector (Promega, Madison, WI) to result in pGEM-RAP2.4p. The *RAP2.4* promoter fragment was released from pGEM-RAP2.4p by *Bam*HI and *Nco*I

digestion, and inserted into the corresponding sites of the pCAMBIA3301 vector (CAMBIA, www.cambia.org) to replace the CaMV 35S promoter, generating RAP2.4p::GUS. The pPZPY-RAP2.4 and RAP2.4p::GUS constructs were electroporated into the *Agrobacterium* strain GV3101 and then introduced into *Arabidopsis* Col wild-type plants via a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on germination plates containing 200 μg/ml Gentamycin (for pPZPY-RAP2.4) or 20 μg/ml glufosinate-ammonium (for RAP2.4p::GUS). We selected about 40 T1 transgenic lines and allow them to self-produce T2 seeds. T2 plants with single T-DNA insertion were selected for phenotypic analyses. For most experiments, homozygous T3 or T4 transgenic plants were used.

To construct the GST-RAP2.4 expression plasmid, the pTOPO-RAP2.4 plasmid was digested with *Sac*I (then blunted) and *Xho*I to release the *RAP2.4* open reading frame, which was then inserted into *Not*I (then blunted) and *Sal*I-digested pGEX-5X-1 vector (Amersham Biosciences, Sunnyvale, CA) to generate pGST-RAP2.4 plasmid.

To generate the pLexA-RAP2.4 plasmid, the full-length RAP2.4 cDNA was amplified with primers RAP2.4-B (5′-AGGATCCTCATGGCAGCTGCTATGAATTTG-3′) and RAP2.4-NX (5′-TCCATGGTCTAGACTAAGCTAGAATCGAATCCC-3′) using pTOPO-RAP2.4 as the template to modify the cloning sites. The cDNA was then cloned into the pGEM-T Easy vector, resulting in pGEM-RAP2.4. After sequencing confirmation, the pGEM-RAP2.4 was digested with *Bam*HI and *Nco*I to release the RAP2.4 fragment, which was then ligated into the corresponding sites of the pLexA vector (Clontech, Mountain View, CA) to generate in-frame fusion of the LexA DNA-binding domain with RAP2.4.

To generate the RAP2.4 effector construct for the reporter gene assay in *Arabidopsis* protoplasts, pTOPO-RAP2.4 plasmid was digested with *Xho*I (then blunted) and *Xba*I, and then the released RAP2.4 fragment was inserted into *Sma*I and *Xba*I digested pRTL2 vector (von Arnim et al., 1998) to generate 35S::RAP2.4 plasmid.

PCR Genotyping

Genomic DNA was prepared with DNeasy Plant Mini Kits (Qiagen USA, Valencia, CA). PCR primers used for verifying the *rap2.4–1* and *rap2.4–2* mutants were RAP2.4-F1, RAP2.4-F2, and the T-DNA left border primer LB (5′-CGG AAC CAC CAT CAA ACA GG-3′). The PCR method was described previously (Lin and Wang, 2004).

Northern Blotting

Plant total RNA was extracted from seedlings using RNeasy Plant Mini Kits. Northern blotting analysis was performed as previously described (Yang et al., 2005). A full-length cDNA fragment of *RAP2.4* was used for probe labeling. Fragments of *RBCS*, *CAB3*, and 18S rRNA used for labeling were described by Wang and Deng (2002). cDNA fragments of *PDF1.2*, *PR3*, *PR4*, *RD29A*, and *COR47* genes were generated by RT-PCR for probe labeling. The primers used are as follows: 5′-CATCATGGCTAAGTTTGCTTC-3′ and 5′-GGTAGATTTAACATGGGACGTAAC-3′ for *PDF1.2*; 5′-AGAACAGAATCCTGCTTCAG-3′ and 5′-CGTTAACGAAGGATCTTTGG-3′ for *PR3*; 5′-ATGAAGATCAGACTTAGCATAAC-3′ and 5′-AGCTCATTGCCACAGTCGAC-3′ for *PR4*; 5′-AGTGATCAAACAGAGGAACC-3 and 5′-TCTGAAACAGCCGACTCTTC-3′ for *RD29A*; 5′-ATGGCTGAGGAGTACAAGAAC-3′ and 5′-GCATGATAACCTGGAAGCTTC-3′ for *COR47*. The cDNA clone for *COR15A* gene was obtained from ABRC and digested by *Eco*RI and *Sal*I. The signals were quantified with a PhosphoImager (Storm 840, Amersham Biosciences, Sunnyvale, CA). The relative expressions were calculated by normalizing each signal against that of *18S rRNA*.

Real-Time RT-PCR

For real-time RT-PCR analysis, the RNA samples were reverse-transcribed into first-strand cDNA using StrataScrip Reverse Transcriptase (Stratagen, La Jolla, CA). Then, PCR was carried out using gene-specific primers and SYBR Premix ExTaq reagent (Takara Mirus Bio, Madison, WI) with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The primers are 5′-GGT TTC GTC GCC AGA TCT AT-3′ and 5′-AGC GGT GGA CTC TTC AAA CT-3′ for *RAP2.4*, and 5′-TTC CTT GAT GAT GCT TGC TC-3′ and 5′-TTG ACA GCT CTT GGG TGA AG-3′ for *ubiquitin* gene. The *RAP2.4* expression levels were normalized to the *ubiquitin* gene.

Hormone Treatment

For root elongation and lateral root formation assays, seeds were placed on germination plates and grown vertically for 3 d under continuous white light. The seedlings were then transferred to new plates (unsupplemented or supplemented with hormones). The positions of the primary root tips were marked. The seedlings were then grown vertically under continuous white light for additional 10 d and the new root growth was measured with a ruler. The percentage of relative root elongation was calculated based on control plants grown on unsupplemented media. For ethylene triple response assay, seedlings were grown in germination plates containing 10 μM ACC under darkness for either 4 or 5 d. For ethylene-responsive gene expression assay, 5-day-old seedlings were treated with ACC for 24 h before RNA extraction.

Preparation of GST-RAP2.4 Fusion Protein

The pGST-RAP2.4 plasmid was transformed into the bacterial strain BL21(DE3). To prepare bacterial extract, the transformed cells were inoculated in LB and grown overnight. The culture was diluted (1:100) in fresh media and cultured at 37°C until the cell OD_{600} was 0.8. The cells were induced with 0.1 mM IPTG (Isopropyl-1-thio-β-D-galactopyranoside) for 3 h at 25°C and harvested by centrifugation. The cell pellet was resuspended in 1× PBS, 1 mM PMSF, and lysed with French Press. The cell lysate was applied on the Glutathione Sepharose 4B column (GE Healthcare Bio-Sciences Corp, NJ). The column was washed with 1× PBS and bound proteins were eluted with an elution buffer (50 mM Tri-HCl, pH 8.0, 10 mM Glutathione).

Gel Mobility Shift Assay

The DRE and GCC-box sequences used for probe were 5′-TTGATACTACCGACATGAGTTGATACTACCGACATGAGTT-3′ and 5′-TTCATAAGAGCCGCCACTCATAAGAGCCGCCACT-3′, respectively. To prepare probes, oligonucleotide sets were annealed by boiling each of the complementary oligonucleotides for 5 min and slowly cooling to room temperature. The annealed nucleotides were labeled by Klenow fill-in reaction in the presence of [^{32}P]dATP. The binding reactions (20 μl) were performed with the following binding buffer: 25 mM HEPES, pH 7.5, 5% Glycerol, 40 mM KCl, 1 mM DTT, 0.5 mM EDTA. Binding reactions were incubated at RT for 20 min and analyzed by electrophoresis on 6% nondenaturing polyacrylamide gels in 0.5× Tris-Borate-EDTA buffer.

Transcriptional Activation Assay in Yeast

The pLexA-RAP2.4 (or pLexA empty vector) was cotransformed with the p8op-*LacZ* reporter gene (Clontech) into the yeast strain EGY48, and the transformants were selected on amino acid drop-off plates without Histidine (selection marker for pLexA plasmids) and Ura (selection marker for the p8op-*LacZ* plasmid). For measuring β-galactosidase activity, plate assay was performed as previously described (Wang and Deng, 2002).

Transient Gene Expression in Onion Cells

GFP-RAP2.4 was transiently expressed in onion epidermal cells using particle bombardment and examined as described previously (Lin and Wang, 2004).

Protoplast Transient Expression Assay

Isolation of *Arabidopsis* protoplast and the PEG-mediated transfection procedure were performed as described (Kovtun et al., 2000). The *4×DRE::GUS* reporter gene, 35S::RAP2.4 effector plasmid (or empty vector control), and the *35S::LUC* construct (internal control) were cotransformed into protoplasts. After transformation, the protoplasts were incubated in darkness for 16–18 h. The protoplasts were pelleted and resuspended in 100 μl of 1× CCLR buffer (Promega). For β-glucuronidase (GUS) enzymatic assay, 5 μl of the extract was incubated with 50 μl 4-methylumbelliferyl β-D-glucuronide (MUG) assay buffer (50 mM sodium phosphate pH 7.0, 1 mM MUG, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% sarkosyl, 0.1% Triton X-100) at 37°C for 1 h, and the reaction was stopped by adding 945 μl of 0.2 M Na_2CO_3. For luciferase (LUC) activity assay, 5 μl of the extract was mixed with 50 μl of luciferase assay substrate (Promega). The GUS and LUC activities were measured using a Modulus Luminometer/Fluometer (Turner Biosystems, Sunnyvale, CA). The reporter gene expression levels were expressed as GUS/LUC ratios.

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Supplementary Material

The following supplementary material is available for this article online:

Supplemental Figure 1. Double Mutant Analysis.

Quantifications of hypocotyl lengths under the indicated light conditions. FR, far-red; R, red; B, blue.

Supplemental Figure 2. Early Flowering Phenotype of the *RAP2.4-ox* Plants under Long-Day Conditions.

(A) *RAP2.4-ox* plants are early flowering under long-day conditions.

(B) Quantification of 'days to flowering' and 'rosette leave number at bolting'. Bars stand for standard deviations.

Supplemental Figure 3. Altered Ethylene Responses of the *RAP2.4-ox* Plants.

(A) Quantification of apical hook curvature of dark-grown *RAP2.4-ox* seedlings (4 d old) in the absence or presence of 10 μM ACC. Bars stand for standard deviations of 30 seedlings.

(B) Quantification of the relative hypocotyl lengths of 5-day-old seedlings of different genotypes grown on plate supplemented with 10 μM ACC relative to seedlings grown on unsupplemented plate Bars stand for standard deviations of 30 seedlings.

(C) Relative root elongation on various concentrations of ACC. Bars represent standard deviations of 30 seedlings.

Supplemental Figure 4. Genetic Epistatsis Analysis between RAP2.4 and EIN2.

Images of 4-day-old etiolated seedlings grown in the absence (top panels) and presence of 10 μM ACC (lower panels). Bar = 2 mm.

Supplemental Figure 5. A Phylogenetic Tree of *RAP2.4* and Related Genes.

(A) A phylogenetic tree of *RAP2.4* and its related genes based on deduced amino acid sequences. The plot was obtained by the Cluster Method of the MegAlign program (DNAstar, Madison, WI).

(B) Amino acid sequence alignment of the AP2 domains from the *Arabidopsis* RAP2.4 cluster. The *Medicago* WXP1 (Zhang et al., 2005), maize DBF1 (accession No. AAM80486), and cotton GhDBP2 (accession No. AY619718) proteins were also included for comparison. The conserved Val14 residue is indicated by the arrow.

Supplemental Figure 6. A Model Illustrating the Regulation and Function of *RAP2.4*.

RAP2.4 expression is up-regulated by salt, drought, and wounding, but repressed by light. The translated RAP2.4 protein binds to both the ethylene-responsive GCC-box and dehydration-responsive DRE elements to regulate late responsive gene expression, leading to altered light- and ethylene-regulated responses.