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ORIGINAL RESEARCH

COP1 mutation causes low leaf temperature under various abiotic stresses in *Arabidopsis thaliana*

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Abstract

Stomata are microscopic pores on epidermal cells of leaves and stems that regulate water loss and gas exchange between the plant and its environment. Constitutive photomorphogenic 1 (COP1) is an E3 ubiquitin ligase that is involved in plant growth and development and multiple abiotic stress responses by regulating the stability of various target proteins. However, little is known about how COP1 controls stomatal aperture and leaf temperature under various environmental conditions. Here, we show that COP1 participates in leaf temperature and stomatal closure regulation under normal and stress conditions in Arabidopsis. Leaf temperature of *cop1* mutants was relatively lower than that of wild type (WT) under drought, salt, and heat stress and after abscisic acid (ABA), $CaCl_2$, and H_2O_2 treatments. However, leaf temperature was generally higher in both WT and *cop1* mutants after abiotic stress and chemical treatment than that of untreated WT and *cop1* mutants. Stomatal aperture was wider in *cop1* mutants than that in WT under all conditions tested, although the extent of stomatal closure varied between WT and *cop1* mutants. Under dark conditions, leaf temperature was also lower in *cop1* mutants than that in WT. Expression of the genes encoding ABA receptors, ABA biosynthesis proteins, positive regulators of stomatal closure and heat tolerance, and ABA-responsive proteins was lower in *cop1* mutants that that in WT. In addition, expression of respiration-related genes was lower in *cop1* mutants that that in WT. Taken together, the data provide evidence that mutations in *COP1* lead to wider stomatal aperture and higher leaf temperature under normal and stress conditions, indicating that leaf temperature is highly correlated with stomatal aperture.

KEYWORDS
abiotic stress, Arabidopsis, COP1, leaf temperature, stomatal aperture

1 | INTRODUCTION

Plants are sessile organisms. As such, they are unable to avoid adverse environmental conditions in the ways that animals do. Plants adapt to their environment by reprogramming various growth and developmental pathways that enable them to avoid or tolerate a multitude of biotic and abiotic stresses. The trade-off between growth and the ability to withstand stress is a central tenet in plants (Chapin, 1980; Grime, 1977).

Drought is one environmental stress that determines plant survival. One strategy that plants use to minimize water loss during drought is to modulate the opening and closing of microscopic pores in epidermal cells of their above-ground organs called stomata. The size of stomatal pores is regulated by turgor pressure-driven changes in the surrounding guard cells. Changes in guard cell turgor pressure are enabled by potassium ion and sugar accumulation through the action of proton pumps in the plasma membrane and/or modifications in metabolic activity (Poffenroth et al., 1992; Ritte et al., 1999; Schroeder, 1988). Under sufficient water and adequate light conditions, stomatal pores of plants are fully open, allowing them to take up CO_2 and release O_2 (Gotow et al., 1988; Kirschbaum & Pearcy, 1988; Outlaw et al., 1979). On the other hand, plants close their stomatal pores under adverse environments, such as dry air, drought, and dim light, and therefore save their resources for the activation of resistance and survival pathways. In most cases, stomata are closed even if the plant is exposed to only one harmful condition because survival is prioritized over growth. Thus far, several fine-tuning mechanisms are known to be part of the adaptive changes in stomata when a plant is faced with stress (Lawson & Matthews, 2020). The modulation of stomatal pores is considered as the only physical way by which plants rapidly adjust to changing environmental circumstances (Chen et al., 2012; Khanna et al., 2014; Mao et al., 2005; Wang et al., 2010).

Constitutive photomorphogenic 1 (COP1) is an E3 ubiquitin ligase, which contains RING-finger, coiled-coil, and WD40 domains (Deng et al., 1992). It is involved in the signal transduction pathways of many types of stresses by regulating the stability of various proteins in plant and animal cells (Yi & Deng, 2005). In plants, COP1 ubiquitinates photomorphogenic factors, making them targets for degradation by proteasomes. Previous studies identified several COP1 substrates in plants that are involved in photomorphogenesis, circadian rhythms, seedling development, and flowering time. For example, the expression levels or stability of the photoreceptors, phytochrome A (PHYA), phyB, cryptochrome 1 (CRY1), and CRY2, the transcription factors, elongated hypocotyl 5 (HY5), long after far-red light 1 (LAF1), long hypocotyl in far-red 1 (HFR1), constans (CO), early flowering 3 (ELF3), and phytochrome interacting factor 3-like 1 (PIL1), and the nuclear protein, gigantea (GI), were negatively regulated by the E3 ubiquitin ligase activity of COP1 (Jang et al., 2005, 2008, 2010; Luo et al., 2014; Osterlund et al., 2000; Seo et al., 2003, 2004; Wang et al., 2001; Yang et al., 2001; Yu et al., 2008). COP1 is also involved in defense against virus attack, root development, hormone signaling, and miRNA biogenesis (Chico et al., 2014; Cho et al., 2014; Dyachok et al., 2011; Jeong et al., 2010; Luo et al., 2010). Recent studies show that COP1 functions as an E3 ubiquitin ligase for the E3 Small Ubiquitin-related MOdifier (SUMO) ligase, AtSIZ1, and that COP1 is stabilized by AtSIZ1 (Kim et al., 2016, 2017; Lin et al., 2016). Thus, down-regulation of COP1 activity leads to AtSIZ1 accumulation, resulting in the SUMO conjugation of target proteins under various abiotic stress conditions (Kim et al., 2016).

Here, we provide evidence for a role of COP1 in modulating stomatal closure and leaf temperature in the model plant *Arabidopsis thaliana*. Under adequate light conditions, leaf temperature and stomatal aperture of *cop1* mutants were higher and wider, respectively, than those of wild type (WT) under various stress conditions. In darkness, leaf temperature of *cop1* mutants was slightly lower than that of WT, which indicated that wider stomatal aperture of *cop1* mutants caused lower leaf temperature.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The *A. thaliana* Columbia-0 (Col-0) ecotype (WT), *cop1-4*, and *cop1-6* (McNellis et al., 1994) were used in this study. For plants grown on Murashige and Skoog (MS)-supplemented plates, seeds were surface-sterilized in 70% ethanol three times. Seeds were sown on agar plates containing 1/2 MS medium, 1% sucrose, and 0.8% agar (pH 5.8) and stratified at 4°C for 2 days in the dark. After stratification, agar plates containing the seeds were exposed to light at 22°C with 16 h light/8 h dark or 12 h light/12 h dark photoperiods in growth chambers. For plants grown in soil, seeds were directly sown on sterilized soil under light at 22°C with 16 h light/8 h dark or 12 h light/12 h dark photoperiods in growth chambers.

Plants were transferred to cold and heat stress conditions at Zeitgeber time 6 (ZT6) and incubated for 30 min. Plants were also treated with drought stress at ZT3 and incubated for 8 h. To perform NaCl, abscisic acid (ABA), $CaCl_2$, and H_2O_2 treatments, plants were irrigated with the corresponding solutions at ZT6, and plant responses were examined after 24 h. To analyze gene expression by qRT-PCR, plants were collected at ZT6.

2.2 | Cold, heat, and drought treatment

To investigate the effect of cold and heat stress on the leaf temperature and stomatal aperture of *cop1* mutants, seeds of WT, *cop1-4*, and *cop1-6* plants were sown on soil and grown at 22°C for 15 days. The plants were then transferred to 12°C or 30°C growth chambers. After exposing plants to cold and heat for 30 min, they were photographed and used for leaf temperature and stomatal aperture measurements. To assess the effect of drought stress on the leaf temperature and stomatal aperture of *cop1* mutants, seeds of WT, *cop1-4*, and *cop1-6* plants were sown on soil and grown at 22°C for

25 days. Plants were photographed after 8 days of water deficit and used for leaf temperature measurements.

2.3 | Treatment of NaCl, ABA, $CaCl_2$, and H_2O_2

WT, *cop1–4*, and *cop1–6* plants grown at 22°C for 25 days were used to investigate the effect of ABA and NaCl on leaf temperature and stomatal aperture. The plants were watered with 50 μM ABA or 150 mM NaCl solution. Plants were photographed, and leaf temperature and stomatal aperture were measured after 12 or 24 h. To test the effects of calcium ion or hydrogen peroxide on the stomata aperture of *cop1* mutants, WT and *cop1–4* plants were grown on MS media for 25 days and then treated with 5 mM $CaCl_2$ or 1 mM H_2O_2. Stomatal aperture was measured after 24 h.

2.4 | Thermal imaging analysis

WT, *cop1–4*, and *cop1–6* plants from seed directly sown on the soil were used to measure leaf temperature. Thermal images were acquired in the growth chamber using an FLIR T420 infrared camera (FLIR system, Danderyd, Sweden). Photographs were taken from equivalent pixel areas for each genotype. Temperature images were acquired through the FLIR Tools software, and images were analyzed using ImageJ (NIH) with the ThermImageJ plug-in (Tattersall, 2019). For the experiment of dark treatment, 7-day-old WT and *cop1–4* seedlings were transferred to soil for subsequent growth under 12 h light/12 h dark photoperiod at 22°C/19°C in a growth chamber. Twenty-five-day-old plants were used for leaf temperature shift analysis under the dark treatment. Thermal images were acquired at intervals of 24 h starting from the initial light condition (0 h) to different times of dark treatment (24, 48, and 72 h) using the same camera, and the image processing was performed as above.

2.5 | Quantification of stomatal apertures

Stomatal apertures were measured as follows: Epidermal peels were stripped from the leaves of 25-day-old WT, *cop1–4*, and *cop1–6* plants in a growth chamber under the treatment conditions described above. Isolated epidermal peels were mounted on glass slides and observed using a Nikon Eclipse i80 microscope (Nikon). Images were analyzed using ImageJ (NIH). Experiments were repeated three times with two leaves per plant from four to six plants.

2.6 | Measurement of water loss rate

The fresh weights (FWs) of the leaves detached from 21-day-old WT, *cop1–4*, and *cop1–6* plants were obtained every 30 min for 20 h. The amount of water loss was calculated as the difference between the initial FW and desiccated weight divided by the initial FW.

2.7 | Quantitative real-time RT-PCR analysis

Fourteen-day-old WT and *cop1–4* plants grown on agar plates containing MS medium were transferred to liquid MS media with or without 50 μM ABA for 6 h. Total RNA was extracted from the plants with Plant RNA Purification Kit (Qiagen), quantified, and divided into equal amounts. First-strand complementary DNA (cDNA) was synthesized from 2 μg total RNA using a ReverTraAce™ qPCR RT Master Mix with genomic DNA Remover kit (TOYOBO). An equal volume of cDNA was amplified by real-time qRT-PCR (LC480, Roche), according to the manufacturer's protocol. Gene-specific primers and template cDNA were combined with 10 μl of KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems), and the reactions were performed under the following thermal conditions: 95°C for 3 min, 40 cycles of 95°C for 3 sec, and 60°C for 20 sec. The CT values obtained for target genes were normalized to the CT value for *Actin2*. PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi).

2.8 | Western blot analysis

Five-day-old dark-grown WT and *cop1–4* seedlings were transferred to liquid MS media with or without 10 μM ABA under green safety light. After 6 h, total proteins were extracted from the samples and separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The levels of COP1 protein were detected by western blot analysis with an anti-COP1 antibody.

3 | RESULTS

3.1 | *cop1* mutants have lower leaf temperatures under normal growth conditions

Recent studies show that the rate of water loss is faster in *cop1–4* mutant leaves than that in WT, and the stomatal aperture of *cop1–4* mutant leaves is wider than that of WT under normal growth (Chen et al., 2021; Mao et al., 2005; Moazzam-Jazi et al., 2018) and different light conditions (Chen et al., 2021; Mao et al., 2005). These results led us to hypothesize that whole plant body temperature is lower in *cop1* mutants than that in WT. This hypothesis was tested by examining leaf temperature of WT and two *cop1* alleles (*cop1–4* and *cop1–6*) under normal growth conditions. Results showed that *cop1–4* and *cop1–6* mutants had lower leaf temperatures than those of WT (Figure S1a,c), which supports a previous study that showed lower *cop1–4* temperatures than those of WT (Chen et al., 2021).

We next evaluated stomatal aperture of *cop1–4* and *cop1–6* mutants. Results showed that stomatal aperture of *cop1–4* and *cop1–6* mutants was wider than that of WT (Figure S1b,d), which was similar to previously published results (Chen et al., 2021).

3.2 | The effect of salinity and drought stress on the leaf temperature of *cop1* mutants

The importance of *COP1* in plant drought responses is related to its roles in stomatal physiology and development (Mao et al., 2005). Therefore, we examined the effects of NaCl and dehydration on *cop1–4* and *cop1–6* leaf temperature. Results showed that the leaf temperature of *cop1–4* and *cop1–6* mutants was lower than that of WT after 12 h of salt stress (Figure 1a,e).

WT, *cop1–4*, and *cop1–6* plants were subjected to drought treatment by withholding water for 8 days. Results showed that the leaf temperature of *cop1–4* and *cop1–6* mutants was also lower than that of WT (Figure 1b,e), which supported data from a previous study showing that *cop1–4* mutants had lower temperatures than those of WT under drought (Chen et al., 2021).

3.3 | The effect of cold and heat stresses on the leaf temperature of *cop1* mutants

Increases in leaf temperature cause stomatal opening (Kostaki et al., 2020; Urban et al., 2017). By contrast, decreases in leaf temperature induce stomatal closure (Honor et al., 1995; Wilkinson et al., 2001). Thus, the effects of heat or cold stress on the leaf temperature of *cop1–4* and *cop1–6* mutants were evaluated. WT, *cop1–4*, and *cop1–6* plants were subjected to cold stress (12°C) and heat stress (30°C) for 30 min each. Results showed that the leaf temperature was lower in *cop1–4* and *cop1–6* mutants than that in WT under both cold and heat stresses (Figure 1c–e).

The leaf temperature of *cop1* mutants was lower than that of the WT under both stress and normal conditions. We therefore measured stomatal aperture under cold, heat, and salt stress conditions. Stomatal aperture was significantly reduced in WT under cold, heat, and salt stress conditions than that in non-stressed conditions (Figure 2a–d). On the other hand, stomatal aperture of *cop1–4* and *cop1–6* mutants was very slightly reduced under stress conditions than that of the non-stressed controls (Figure 2a–d).

3.4 | Stomata of *cop1* mutants are less sensitive to ABA

ABA signaling in guard cells is important for regulating basal stomatal aperture size and rapid stomatal responses to environmental stimuli

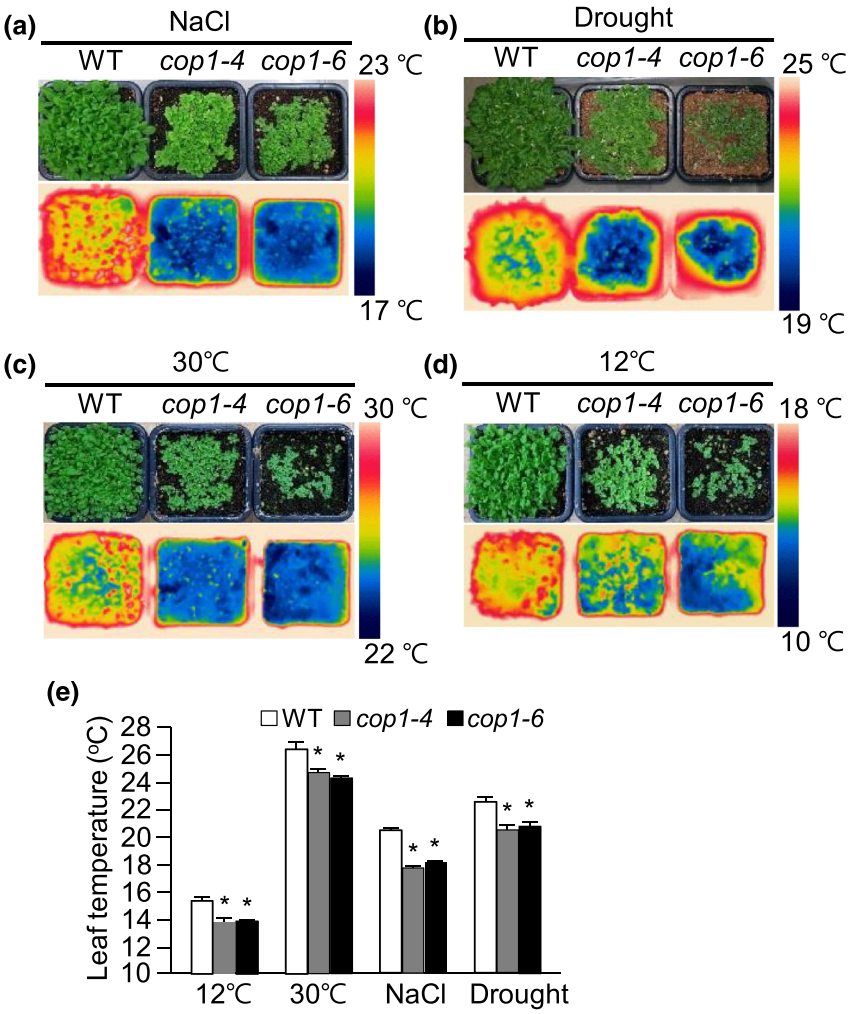


FIGURE 1 *cop1* mutants maintain lower leaf temperature under abiotic stress. (a–d) Representative false-colored infrared images of wild type (WT), *cop1–4*, and *cop1–6* plants under 150 mM NaCl, dehydration, cold (12°C), and heat (30°C) conditions. In cold and heat stress treatments, 15-day-old WT, *cop1–4*, and *cop1–6* plants were exposed to low temperature and high temperature, respectively, for 30 min. In the drought treatment, watering was withheld from 25-day-old WT, *cop1–4*, and *cop1–6* plants, and then their leaf temperature was measured. In the NaCl treatment, 25-day-old WT, *cop1–4*, and *cop1–6* plants were watered with 150 mM NaCl for 12 h. (e) Quantitative analysis of leaf temperature of the genotypes shown in "a–d." Data are mean values ± SD ($n = 3$ pots for each condition). Unshaded bar = WT; gray bar = *cop1–4*; black bar = *cop1–6*. Measurements of the same square pixels from each pot were obtained. Asterisks indicate significant differences between WT and *cop1* (*$p < .05$; Student's *t*-test).

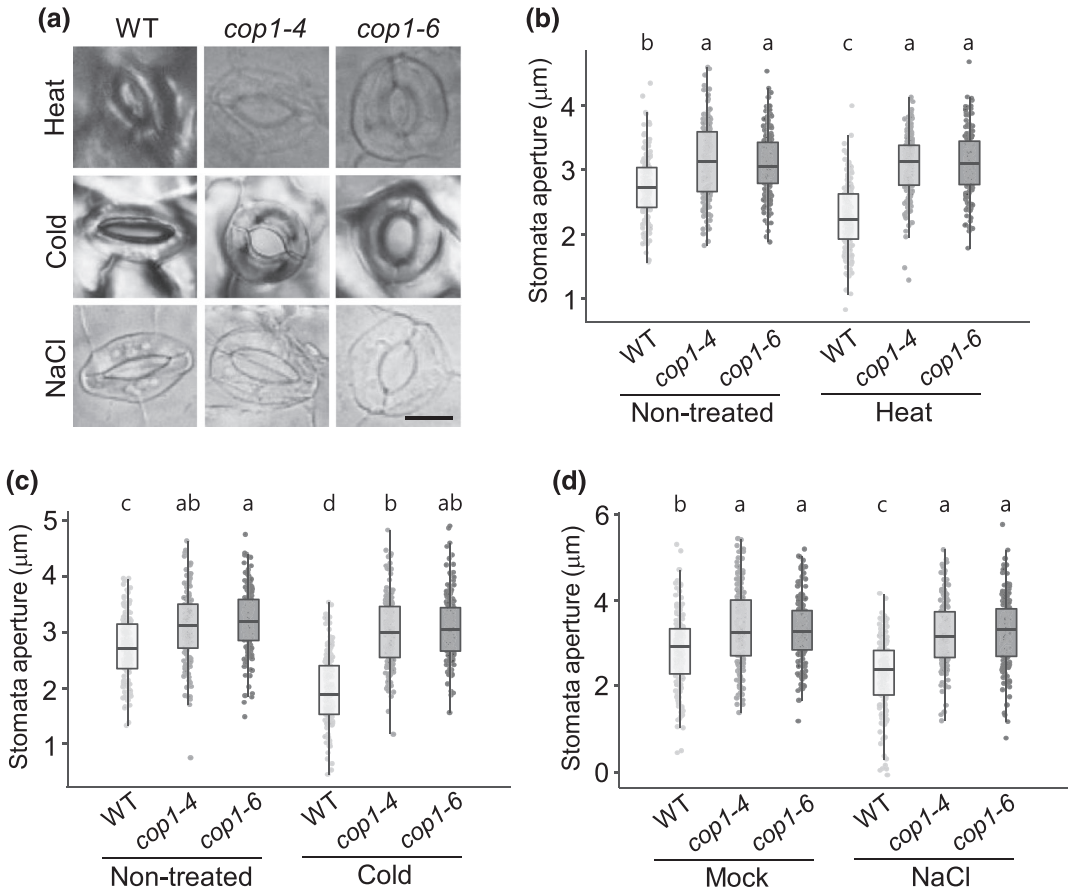
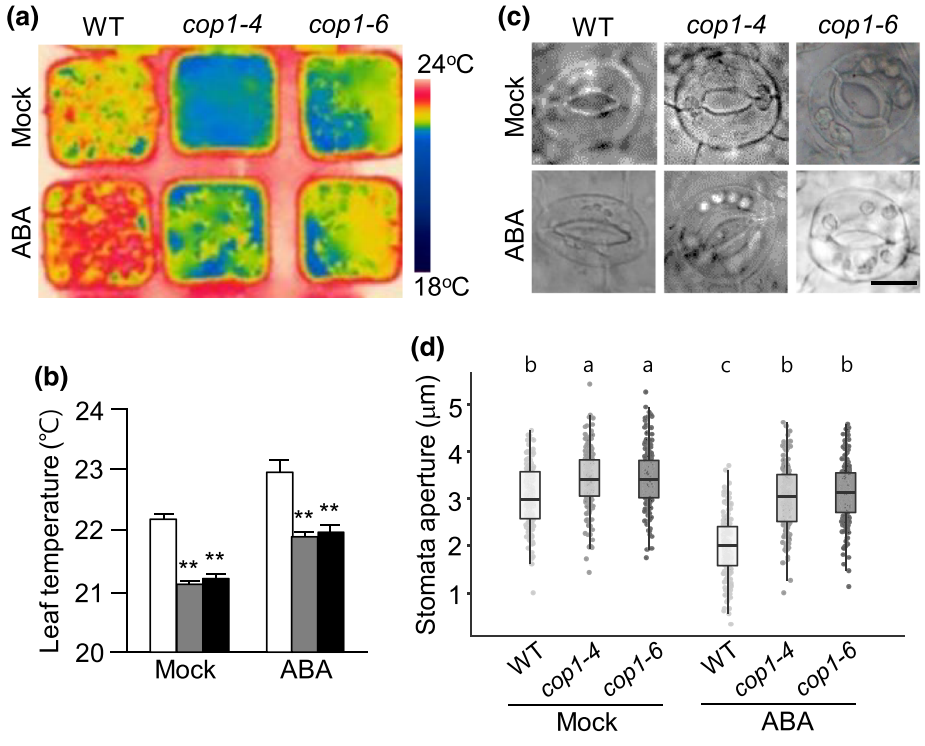


FIGURE 2 Stomatal closure is impaired in *cop1* under abiotic stress. (a) Representative images of wild type (WT), *cop1–4*, and *cop1–6* stomata from leaf epidermal peels. Scale bar, 10 μm. (b–d) Aperture of stomata ($n = 150$) in WT, *cop1–4*, and *cop1–6* plants under heat (b), cold (c), and NaCl stress (d). Stomatal aperture was measured in the same plants as shown in (a). The median, minimum, and maximum values are shown. Different letters indicate statistically significant differences ($p < .05$; one-way ANOVA and Tukey's test).

FIGURE 3 *cop1* mutants maintain low leaf temperatures after abscisic acid (ABA) treatment. (a) Representative false-colored infrared images of wild type (WT), *cop1–4*, and *cop1–6* plants after treatment with 50 μM ABA for 24 h. (b) Quantitative analysis of leaf temperature of the genotypes shown in "a." Data are mean values ± SD ($n = 3$ pots for each condition). Unshaded bar = WT; gray bar = *cop1–4*; black bar = *cop1–6*. Measurements of the same square pixels from each pot were obtained. (c) Representative images of WT, *cop1–4*, and *cop1–6* stomata after ABA treatment. Bar, 10 μm. (d) Stomatal aperture ($n = 150$ stomata) of WT, *cop1–4*, and *cop1–6* plants shown in (a). The median, minimum, and maximum values are shown. Different letters indicate statistically significant differences ($p < .05$; one-way ANOVA and Tukey's test).



(Merilo et al., 2015). Moreover, a previous study shows that ABA-induced stomatal closure is impaired in *cop1-4* and *cop1-6* mutants compared with WT under light and dark conditions (Chen et al., 2021). Thus, we investigated leaf temperature and stomatal aperture of the *cop1-4* and *cop1-6* mutants after ABA treatment. As expected, leaf temperature was lower in *cop1-4* and *cop1-6* mutants than that in WT after ABA treatment. However, leaf temperature was higher in WT and *cop1* mutants than that in untreated samples (Figure 3a,b).

Stomatal aperture of *cop1-4* and *cop1-6* mutants after ABA treatment was narrower than that of mock-treated samples (Figure 3c,d). However, the stomatal aperture of *cop1-4* and *cop1-6* mutants was still wider than that of WT after ABA treatment (Figure 3c,d). These data indicate that *cop1* mutants and WT exhibit significant differences in their stomatal responses to ABA.

3.5 | The effects of ROS and calcium on leaf temperature of *cop1* mutants

Reactive oxygen species (ROS) integrates with ABA signaling pathways to drive stomatal closure (Suhita et al., 2004; Zhang et al., 2001). Therefore, we measured stomatal aperture after hydrogen peroxide (H_2O_2) treatment. Results showed that stomatal aperture of *cop1-4* mutants was slightly narrower after H_2O_2 treatment than that of non-treated samples. Similar to ABA-treated samples, stomatal aperture in WT treated with H_2O_2 was significantly reduced compared with that of non-treated samples (Figure 4a,b). Leaf temperature after H_2O_2 treatment was increased in WT and *cop1-4* plants compared with that of untreated samples (Figure 4c,d).

ABA elevates cytosolic free Ca^{2+} concentration (Gilroy et al., 1991; McAinsh et al., 1990), and cytosolic Ca^{2+} signals are important for stomatal closure (Gobert et al., 2007; Latz et al., 2013; Wang et al., 2015). Therefore, we measured stomatal aperture after $CaCl_2$ treatment. Results showed that the stomatal aperture of *cop1-4* mutants was slightly smaller than that of untreated samples in the $CaCl_2$ treatment. Like in ABA-treated samples, stomatal aperture was significantly reduced in $CaCl_2$-treated WT compared with non-treated samples (Figure 4a,b). Leaf temperature was slightly increased in $CaCl_2$-treated WT and *cop1-4* plants compared with non-treated samples (Figure 4c,d).

3.6 | The effect of ABA on ABA-responsive gene expression in *cop1* mutants

Stomatal aperture affects the rate of water loss. Because the stomatal aperture of *cop1-4* and *cop1-6* mutants was wider than that of WT

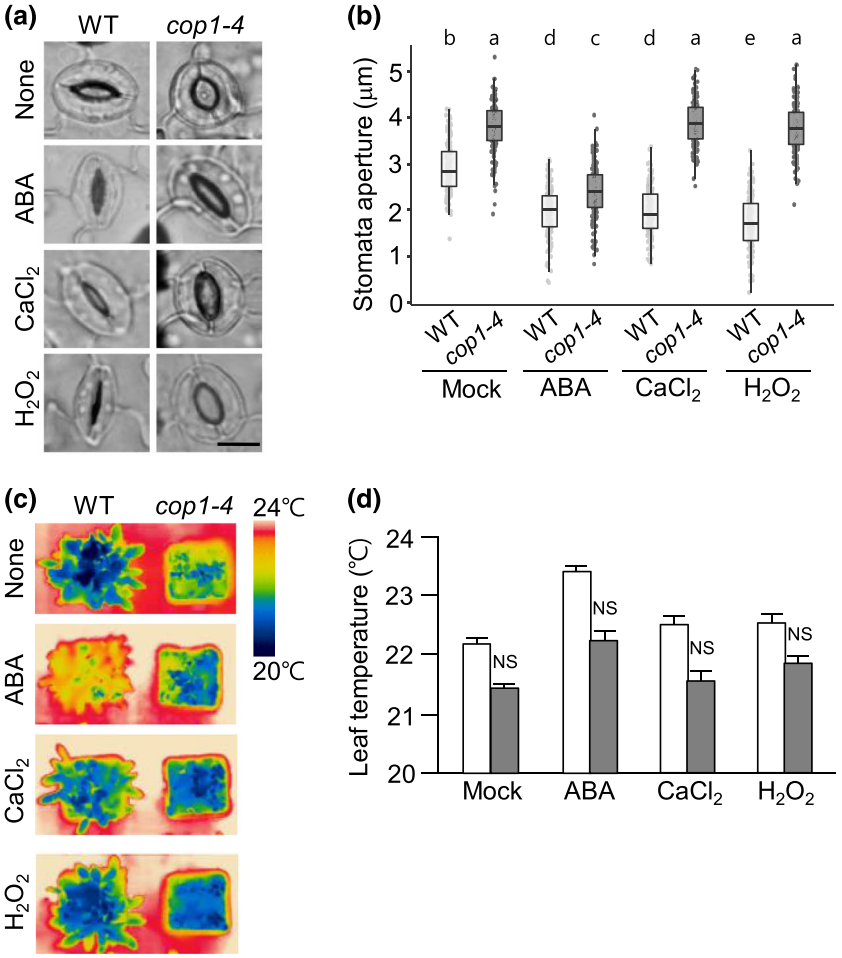


FIGURE 4 The effect of hydrogen peroxide on stomatal closure in *cop1* mutants. (a) Representative images of wild type (WT), *cop1-4*, and *cop1-6* stomata after 50 μM abscisic acid (ABA), 5 mM $CaCl_2$, and 1 mM H_2O_2 treatment. Bar, 10 μm. (b) Stomatal aperture ($n = 150$ stomata) of WT and *cop1-4* plants shown in (a). The median, minimum, and maximum values are shown. Different letters indicate statistically significant differences ($p < .05$; one-way ANOVA and Tukey's test). (c) Representative false-colored infrared images of WT and *cop1-4* after 50 μM ABA, 5 mM $CaCl_2$, and 1 mM H_2O_2 treatments. (d) Quantitative analysis of leaf temperature of the genotypes shown in "a." Data are mean values ± SD ($n = 3$ pots for each condition). Unshaded bar = WT; gray bar = *cop1-4*. Measurements of the same square pixels from each pot were obtained. NS, not significant

under normal and various stress conditions, we measured the rate of water loss in *cop1* mutants. Results showed that water loss of *cop1–4* and *cop1–6* mutants was faster than that of WT (Figure S2). Results presented here support a previous study showing that *cop1–4* mutants lost water at a higher rate than that of WT (Chen et al., 2021).

The wider stomatal aperture and lower leaf temperature of *cop1* mutants after ABA treatment than those of WT led us to hypothesize that *cop1* mutants are less sensitive to ABA. This hypothesis was tested by examining the expression of ABA-responsive genes in *cop1* mutants after ABA treatment. Results showed that the expression of the ABA-responsive genes, *RESPONSIVE TO DEHYDRATION22* (*RD22*), *DESICCATION-RESPONSIVE29* (*RD29*), and *RESPONSIVE TO ABA18* (*RAB18*), was induced by ABA treatment in *cop1–4* mutants, although their levels were approximately twofold lower than those in WT (Figure 5a). These results indicate that ABA signaling is down-regulated in *cop1* mutants and also suggest that the expression and activity of COP1 are affected by ABA. We therefore examined the effect of ABA on the *COP1* gene expression and COP1 protein levels. Results showed that COP1 transcript and COP1 protein levels were not affected by ABA treatment (Figure 5b,c).

3.7 | Leaf temperature of dark-grown *cop1* mutants

All the data thus far showed that *cop1–4* and *cop1–6* mutants maintained a lower body temperature than that of WT under normal and various abiotic stress conditions. Because results were obtained from the plants grown under light conditions, we asked if leaf temperature of *cop1–4* mutants was also lower in the dark. Results showed that leaf temperature was still lower in *cop1–4* mutants than that in WT even under dark conditions (Figure 6a,b).

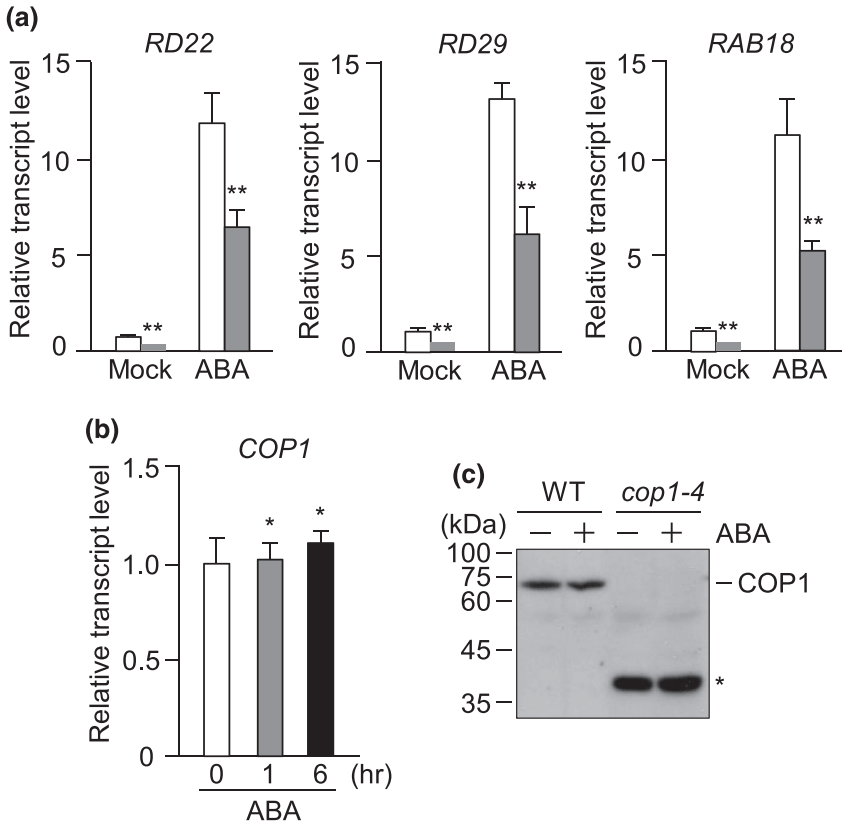


FIGURE 5 Effect of abscisic acid (ABA) on the expression of *COP1* and ABA-responsive genes in *cop1* mutants. (a) Expression levels of *RD22*, *RD29*, and *RAB18* genes were examined by qRT-PCR using the leaves of 14-day-old wild type (WT) and *cop1–4* mutants treated with 50 μM ABA for 6 h. *Actin2* was used as an internal standard. The relative fold expression of non-treated WT was considered as 1. Asterisks indicate statistically significant differences in transcript levels (**$p < .01$; Student's *t*-test) between WT and *cop1–4* mutants. (b) Dark-grown WT seedlings were treated with 10 μM ABA at the indicated time points. Total RNA was extracted from the samples and *COP1* transcript levels were examined by qRT-PCR. Unshaded bar = WT; gray bar = *cop1–4*. Asterisks indicate statistically significant differences in transcript levels (**$p < .1$; Student's *t*-test) between mock-treated and ABA-treated plants. (c) Western blot analysis of constitutive photomorphogenic 1 (COP1) in WT and *cop1–4* seedlings. Total proteins were extracted from dark-grown WT and *cop1–4* seedlings treated with 10 μM ABA for 6 h and separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The levels of COP1 protein were detected by western blot analysis with an anti-COP1 antibody. Asterisk indicates WD40 domain-truncated COP1 protein in *cop1–4* mutants.

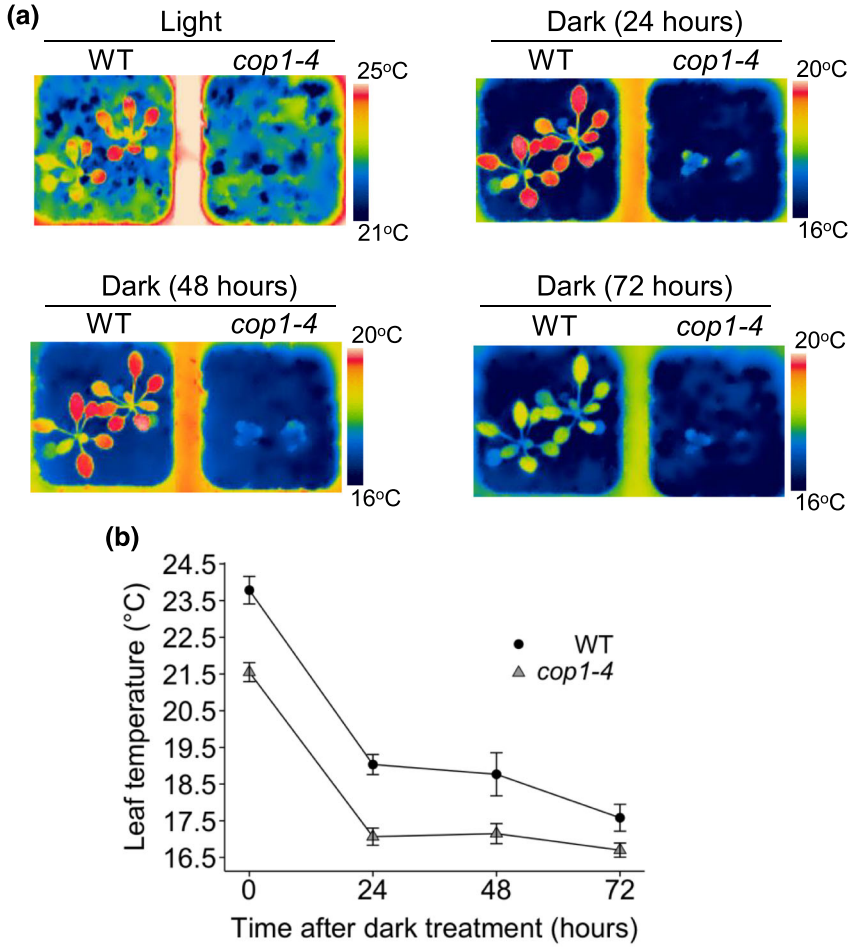


FIGURE 6 Leaf temperature of *cop1* under dark conditions. (a) Representative false-colored infrared images of wild type (WT) and *cop1–4* plants in the light and dark. Light-grown WT and *cop1–4* plants were transferred to dark condition and then photographed at the indicated time points. (b) Quantitative analysis of leaf temperatures of the genotypes shown in "a." Data are mean values ± SD ($n = 3$ pots for each condition).

3.8 | Expression of stomatal-, heat stress-, and respiration-related genes in *cop1* mutants

The wider stomatal aperture and lower leaf temperature of *cop1* mutants than those of WT under various abiotic stresses led us to further examine the expression of the genes related to ABA signaling, stomatal aperture regulation, and heat stress. Results revealed that expression of the genes encoding the ABA receptors, pyrabactin resistance 1 (PYR1) and PYR1-like 1 (PYL1), negative regulators of ABA signaling, ABA-insensitive 1 (ABI1) and ABI2, and ABA biosynthesis-related proteins, ABA deficient 2 (ABA2) and ABA3, was down-regulated in *cop1–4* mutants relative to that in WT (Figure 7).

Similarly, genes encoding positive regulators of stomatal closure (open stomata 1 [OST1] and slow anion channel-associated 1 [SLAC1]), heat tolerance (heat shock protein 101 [HSP101] and HSP70b), and cold tolerance (Cold-Regulated 78 [COR78] and COR15A) were down-regulated in *cop1–4* mutants relative to the WT (Figure 7).

We also examined the relative expression level of respiration-related genes encoding pyruvate kinase 1 (PKP1) and nicotinamide adenine dinucleotide phosphate (NADP) malate dehydrogenase (NADP-MDH) because leaf temperature was low in *cop1* mutant compared with WT in darkness (Figure 6a,b). Results showed that the transcript levels of *PKP1* and *NADP-MDH* were lower in *cop1–4* mutants than those of WT under light and dark conditions (Figure 8).

4 | DISCUSSION

Previous studies report that COP1 plays a crucial role in light-induced stomatal development through cryptochromes and phytochromes (Kang et al., 2009). Furthermore, COP1 controls the opening of stomatal pores during CRY1-, PHOT1-, and PHOT2-mediated blue light signaling. In addition, COP1, together with *PIFs* (phytochrome interacting factors), is involved in PHYB-mediated stomatal opening (Wang et al., 2010). Therefore, we asked if COP1 functions in stomatal aperture-dependent changes in leaf temperature under abiotic stress.

A previous study shows that *cop1–4* and *cop1–6* stomatal apertures are wider than those of WT under normal growth conditions (Chen et al., 2021). In the current study, leaf temperature and stomatal aperture were measured under low and high temperatures and after ABA, $CaCl_2$, H_2O_2, salinity, and drought treatment. Stomata play important roles in salt stress tolerance (Chen & Gallie, 2004; Rahnama et al., 2010; Rajendran et al., 2009). Under salinity conditions, Na^+ and Cl^- induce an increase in leaf tissue ABA concentrations (Geilfus et al., 2018). In addition, ABA sensitivity in response to salinity or

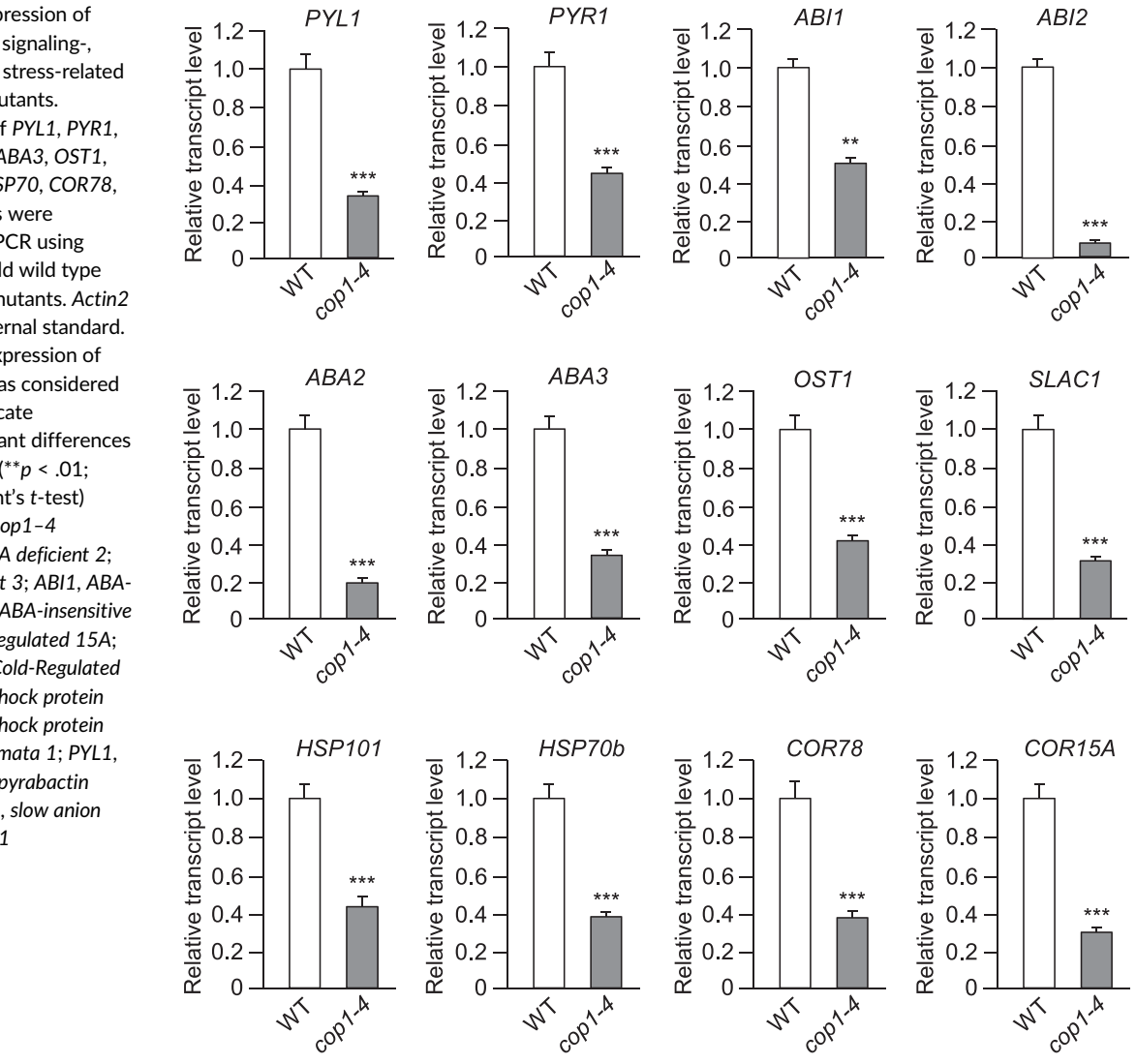


FIGURE 7 Expression of abscisic acid (ABA) signaling-, stomata-, and heat stress-related genes in *cop1–4* mutants. Expression levels of *PYL1*, *PYR1*, *ABI1*, *ABI2*, *ABA2*, *ABA3*, *OST1*, *SLAC1*, *HSP101*, *HSP70*, *COR78*, and *COR15A* genes were examined by qRT-PCR using leaves of 14-day-old wild type (WT) and *cop1–4* mutants. *Actin2* was used as an internal standard. The relative fold expression of non-treated WT was considered as 1. Asterisks indicate statistically significant differences in transcript levels (**$p < .01$; ***$p < .001$; Student's *t*-test) between WT and *cop1–4* mutants. *ABA2*, *ABA deficient 2*; *ABA3*, *ABA deficient 3*; *ABI1*, *ABA-insensitive 1*; *ABI2*, *ABA-insensitive 2*; *COR15A*, *Cold-Regulated 15A*; *COR78(RDA29A)*, *Cold-Regulated 78*; *HSP101*, *heat shock protein 101*; *HSP70*, *heat shock protein 70*; *OST1*, *open stomata 1*; *PYL1*, *PYR1-like 1*; *PYR1*, *pyrabactin resistance 1*; *SLAC1*, *slow anion channel-associated 1*

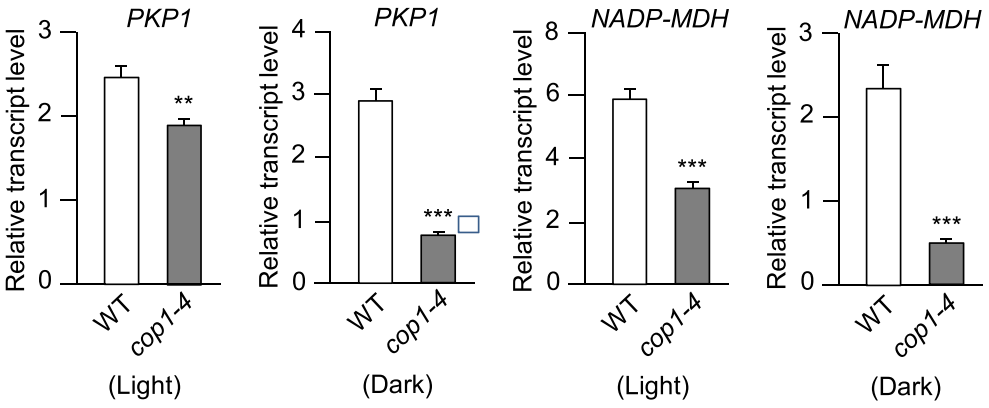


FIGURE 8 Expression of respiration-related genes in *cop1–4* mutants. Expression levels of *PKP1* and *NADP-MDH* genes were examined by qRT-PCR using leaves of 14-day-old wild type (WT) and *cop1–4* mutants. *Actin2* was used as an internal standard. The relative fold expression of non-treated WT was considered as 1. Asterisks indicate statistically significant differences in transcript levels (**$p < .01$; ***$p < .001$; Student's *t*-test) between WT and *cop1–4* mutants. *NADP-MDH*, *nicotinamide adenine dinucleotide phosphate malate dehydrogenase*; *PKP1*, *pyruvate kinase 1*

water deficit affects stomatal movement (Liu et al., 2015; Wilkinson & Davies, 2002). These studies indicate that high salt and ABA induce stomatal closure. In this study, *cop1–4* and *cop1–6* stomatal aperture was narrowed under ABA treatment but was not significantly changed after NaCl treatment (Figures 2d and 3d). However, stomatal apertures were still wider in *cop1–4* and *cop1–6* mutants than those in WT under both conditions (Figures 2d and 3d). Stomatal opening and closing are directly correlated with leaf temperature (Honor et al., 1995; Kostaki et al., 2020; Urban et al., 2017; Wilkinson et al., 2001). Our study showed that leaf temperature was lower in *cop1–4* and *cop1–6* mutants than that in WT under dehydration conditions or after ABA and NaCl treatment (Figures 1e and 2b). Recently, Chen et al. (2021) also showed that leaf temperature of *cop1–4* mutants is lower than that of WT. Taken together, the data suggest that the lower leaf temperature of *cop1* mutants is caused by wider stomatal apertures.

Open stomata cause high transpiration and quickly cool the leaves (Collatz et al., 1991; Jarvis & McNaughton, 1986). We therefore examined *cop1* stomatal apertures and leaf temperatures under low and high temperature conditions. The stomatal apertures of *cop1–4* and *cop1–6* mutants were very slightly reduced after cold and heat treatment, whereas those in WT were significantly narrowed (Figure 2b,c). As expected, leaf temperatures were lower in *cop1–4* and *cop1–6* mutants than those in WT under both conditions, although leaf temperatures of WT and *cop1* plants decreased under cold and increased under heat, respectively (Figure 1e). Again, these results suggest that lower *cop1* leaf temperature than that of WT is caused by wider stomatal aperture.

Salt stress results in a dramatic increase in ABA biosynthesis and H_2O_2 accumulation (Hedrich & Shabala, 2018). In addition, ABA induces H_2O_2 production and increases cytosol Ca^{2+} concentration in guard cells (Arve et al., 2014; Gilroy et al., 1991; McAinsh et al., 1990). Leaf temperature of *cop1* mutants increased after exogenous H_2O_2 or Ca^{2+} treatment, although both chemicals had very little effects on stomatal closing in *cop1* mutants (Figure 4c,d). These observations suggest that stomatal closing of *cop1* mutants is also regulated by H_2O_2 and Ca^{2+}.

Wide stomatal apertures result in high stomatal conductance, which leads to rapid water loss in plants because of increased rates of transpiration (Collatz et al., 1991; Wilkinson & Davies, 2002). Wider stomatal apertures of *cop1–4* and *cop1–6* than those of WT under normal and various abiotic stress conditions suggest that water loss through stomatal pores changes in *cop1* mutants. Indeed, water loss was faster in *cop1–4* and *cop1–6* mutants than that in WT (Figure S2). A recent study also showed higher water loss in *cop1–4* mutants than that in WT (Chen et al., 2021), supporting observations made here that stomatal aperture affects water loss.

Because ABA plays a central role in the regulation of stomatal movements under various stress conditions, the effect of COP1 on the expression of ABA-responsive genes was examined. Transcript levels of *RD22*, *RD29*, and *RAB18* genes were low in *cop1* mutants (Figure 5a). However, expression of these ABA-responsive genes was significantly induced in *cop1* mutants after ABA treatment, although their transcript levels were lower than those in WT (Figure 5a). The increase in transcript levels of ABA-responsive genes in *cop1* mutants was proportional to that in WT. This observation suggests that ABA signaling still occurs in *cop1* mutants but is down-regulated. The expression of the genes related to stomatal closing, ABA perception, and heat and cold tolerance was also down-regulated in *cop1* mutants compared with the WT (Figure 7). These data suggest that expression of genes related to ABA- and abiotic stress-dependent stomatal closure is down-regulated by loss of COP1 function, which implies that *cop1* mutants are desensitized to exogenous ABA treatment, resulting in wider stomatal opening in *cop1* mutants than in the WT. Furthermore, *cop1* mutants are relatively less sensitive to drought and salt stresses than to the ABA treatment, which suggests that the down-regulation of ABA biosynthesis genes drives additional desensitization in *cop1* mutants (Figure 9). In other words, stomatal closing under salt and drought stresses is caused by the accumulation of endogenous ABA; however, the amount of accumulated ABA might be insufficient to cause a statistically significant change in stomatal aperture in *cop1* mutants (Figure 9). However, the difference in leaf temperature between WT and *cop1* mutants was maintained under salt and drought stresses, which suggests that transpiration rates are also reduced in *cop1* mutants under stress conditions. Moreover, these data also indicate that COP1 acts as an upstream positive regulator of ABA signaling. Because ABA signaling was impaired in *cop1* mutants, we examined whether COP1 expression and protein levels were regulated by ABA. Results showed that *COP1* transcript and COP1 protein levels did not change after the ABA treatment (Figure 5b,c). These results suggest that *COP1* gene expression and its protein activity are not directly regulated by ABA; however, COP1 is required for downstream ABA signaling.

Stomatal opening is dependent on the coordinated response of plants to red and blue light (Shimazaki et al., 2007). However, mechanisms underlying dark-induced stomatal closure remain unclear. There are several studies related to dark-induced stomatal closing. For example, WT, blue light receptor mutants (*cry1* and *cry2*), and *cry1cry2* double mutants had completely closed stomata in the dark (Mao et al., 2005). ABA signaling also plays a role in dark-induced stomatal closure as determined by mutant analysis. For instance, the ABA receptor mutants, *q1124* and *s112458*, ABA biosynthesis mutant, *nced3/5*, ABA activation mutants, *bg1* and *bg2*, and ABA degradation mutants, *cyp707a1* and *cyp707a3*, had closed stomata in the dark (Pridgeon & Hetherington, 2021). However, stomatal closure was delayed in *q1124*, *s112458*, *nced3/5*, and *bg1*, but not in *bg2*, *cyp707a1*, and *cyp707a3* (Pridgeon & Hetherington, 2021). Previous reports and results presented here showed that the leaf temperature of *cop1–4* and *cop1–6* mutants was always lower than that of WT under all treatments in the light. Noticeably, leaf temperature of *cop1* mutants was still lower than that of WT under dark conditions (Figure 6b). Recently, Chen et al. (2021) reported that the stomata of *cop1–4* and *cop1–6* mutants closed under dark conditions, although stomata aperture was approximately twofold wider in *cop1* mutants than that in WT. The data indicate that leaf temperature of *cop1* mutants is regulated by stomatal aperture. The results from gene

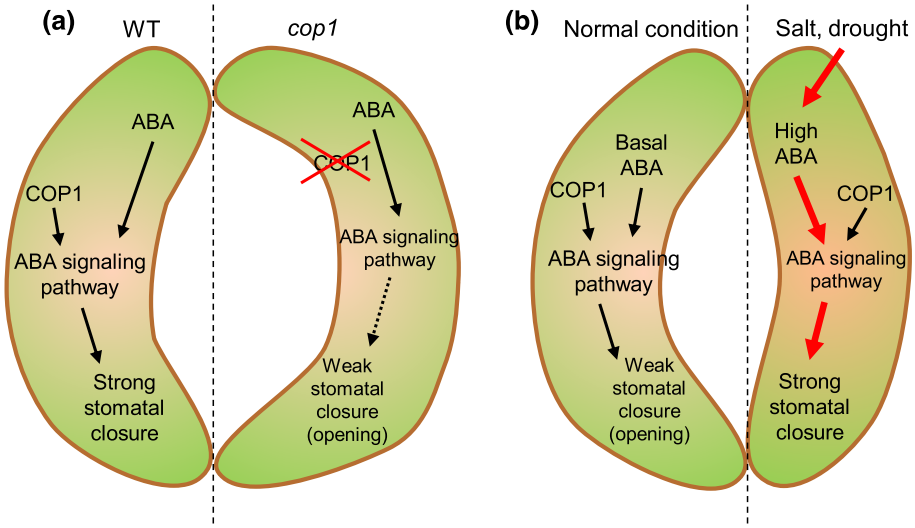


FIGURE 9 Model depicting that constitutive photomorphogenic 1 (COP1) regulates abiotic stress tolerance by inducing stomatal closure via abscisic acid (ABA) signaling. (a) ABA induces stomatal closure in the presence of COP1, because COP1 plays a positive role in the ABA signaling pathway. In the absence of COP1, however, the ABA signaling pathway is down-regulated, which causes less stomatal closure. (b) Under normal conditions, plants synthesize a basal level of ABA, which is insufficient to induce stomatal closure. However, under salt or drought stress conditions, plants synthesize high levels of ABA, which induces stomatal closure. Wider stomatal aperture in *cop1* mutants compared with the wild type (WT) under both normal and abiotic stress conditions indicates that COP1 regulates stomatal closure under all conditions.

expression and stomata aperture analyses showed that ABA signaling-dependent stomatal regulation still occurs in *cop1* mutants. However, this process is down-regulated in *cop1* mutants. Therefore, it is possible that ABA signaling is a component of leaf temperature regulation in *cop1* mutants.

In conclusion, our data showed that under light, stomatal aperture was wider in *cop1* mutants than that in WT and that leaf temperature was lower in *cop1* mutants than that in WT under various stress conditions. *cop1* mutants closed their stomata more in the dark than in light, although stomatal aperture of *cop1* mutant was still wider than that of WT. Thereby, leaf temperature of *cop1* mutants is also lower than that of WT under dark treatment. Taken together, the data presented here and those from other studies reveal that under all growth conditions, the lower leaf temperature of *cop1* mutants than that of WT is affected by wider stomatal aperture.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Joo Yong Kim, Seung Ju Lee, Wang Ki Min, and Seoyeon Cha performed the experiments for research. Hak Soo Seo designed the research. Hak Soo Seo wrote the manuscript. Joo Yong Kim, Seung Ju Lee, Wang Ki Min, Seoyeon Cha, Jong Tae Song, and Hak Soo Seo evaluated the results and the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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