ALA-Induced Flavonols Accumulation in Guard Cells Is Involved in Scavenging H_2O_2 and Inhibiting Stomatal Closure in *Arabidopsis* Cotyledons

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5-aminolevulinic acid (ALA), a new plant growth regulator, can inhibit stomatal closure by reducing H_2O_2 accumulation in guard cells. Flavonols are a main kind of flavonoids and have been proposed as H_2O_2 scavengers in guard cells. 5-aminolevulinic acid can significantly improve flavonoids accumulation in plants. However, whether ALA increases flavonols content in guard cells and the role of flavonols in ALA-regulated stomatal movement remains unclear. In this study, we first demonstrated that ALA pretreatment inhibited ABA-induced stomatal closure by reducing H_2O_2 accumulation in guard cells of *Arabidopsis* seedlings. This result confirms the inhibitory effect of ALA on stomatal closure and the important role of decreased H_2O_2 accumulation in this process. We also found that ALA significantly improved flavonols accumulation in guard cells using a flavonol-specific dye. Furthermore, using exogenous quercetin and kaempferol, two major components of flavonols in *Arabidopsis* leaves, we showed that flavonols accumulation inhibited ABA-induced stomatal movement by suppressing H_2O_2 in guard cells. Finally, we showed that the inhibitory effect of ALA on ABA-induced stomatal closure was largely impaired in flavonoid-deficient *transparent testa4* (*tt4*) mutant. In addition, exogenous flavonols recovered stomatal responses of *tt4* to the wild-type levels. Taken together, we conclude that ALA-induced flavonol accumulation in guard cells is partially involved in the inhibitory effect of ALA on ABA-induced H_2O_2 accumulation and stomatal closure. Our data provide direct evidence that ALA can regulate stomatal movement by improving flavonols accumulation, revealing new insights into guard cell signaling.

Keywords: abscisic acid (ABA), 5-aminolevulinic acid (ALA), flavonol, hydrogen peroxide, stomatal opening

INTRODUCTION

Stomatal movements regulate plant gas exchange with the environment, and thus are critical for plant growth and development (Wang et al., 2014). Numerous environmental or hormone factors can trigger stomatal movement (Kim et al., 2010). Perhaps because the difficulty in balancing CO_2 uptake and transpirational water loss, to date, most studies related to stomatal movements have focused on regulation of stomatal closure, while fewer studies have been made to enhance stomatal

opening to increase plant photosynthesis. However, enhancing stomatal aperture to improve the uptake of CO_2 by terrestrial plants is an increasingly important problem due to global climate changes (Wang et al., 2014). 5-Aminolevulinic acid (ALA), a natural plant growth regulator, is known to improve plant photosynthesis under both normal (Hotta et al., 1997) and stressful conditions (Hotta et al., 1998; Nishihara et al., 2003; Wang et al., 2004; Liu et al., 2011; Zhang et al., 2012; Ali et al., 2013b). Recent investigations showed that ALA did not affect stomata development, but significantly inhibited stomatal closing (An et al., 2016a), indicating increasing stomatal aperture is an important mechanism of ALA-mediated improvement of photosynthesis. Most importantly, ALA improved plant drought tolerance simultaneously (An et al., 2016a), further suggesting its great application potential in agriculture and forestry.

Among the regulators of stomatal movement, abscisic acid (ABA) is the most important hormone in provoking stomatal closing (Dodd, 2003; Tanaka et al., 2005). The molecular and cellular mechanisms involved in stomatal movements have been particularly well characterized in the context of ABA-induced stomatal closure (Li et al., 2006). Hydrogen peroxide (H_2O_2) is an important signaling molecule in plants and has been proved as an essential signal in ABA-induced stomatal closure (Pei et al., 2000; Rhee, 2006). We recently demonstrated that ALA treatment inhibited ABA-induced stomatal closure by reducing H_2O_2 accumulation in guard cells (An et al., 2016a). These results revealed the interaction between ALA and ABA in regulating stomatal movement and indicated a crucial function of H_2O_2 in this interaction. However, how ALA reduces ABA-induced H_2O_2 accumulation is largely unknown.

Hydrogen peroxide content in guard cells is tightly regulated by the generating and scavenging systems. Hydrogen peroxide in plant cells can be synthesized via several routes (Neill et al., 2002), and more than one source of H_2O_2 have also been proposed for guard cells. The enzymatic sources of H_2O_2 that have been revealed in guard cells include NADPH oxidase (Kwak et al., 2003; Desikan et al., 2006), peroxidases (He et al., 2011), copper amine oxidase (An et al., 2008), and polyamine oxidase (Hou et al., 2013). Among these, plasma membrane NADPH oxidase has been identified as a main source of ABA-induced H_2O_2 production in guard cells of *Arabidopsis* (Kwak et al., 2003). Comparatively, the scavenging mechanism of H_2O_2 in guard cells which suppresses stomatal closure has received little attention. Although it has been well documented that reactive oxygen species (ROS) in plant cells can be rapidly detoxified by various cellular enzymatic and small molecule antioxidants (Mittler et al., 2004), direct evidence on how H_2O_2 is scavenged in guard cells during stomatal opening is still lacking. Miao et al. (2006) showed that glutathione peroxidase 3 (AtGPX3) functioned as a ROS scavenger in ABA signaling. Munemasa et al. (2013) found that depletion of glutathione contributed to a higher level of ABA-induced H_2O_2 accumulation, indicating glutathione is also a H_2O_2 scavenger in ABA signaling. Our previous study suggested that ALA reduced H_2O_2 in guard cells mainly through accelerating its elimination (An et al., 2016a). However, until now, little is known about how ALA scavenges H_2O_2 in guard cells.

Many plant secondary metabolites act as antioxidants and can affect ROS concentrations (Chobot and Hadacek, 2011). Flavonoids are an important group of plant secondary metabolites that perform as antioxidants (Nakabayashi et al., 2014; Nguyen et al., 2016). Flavonols are among the most abundant flavonoids in plants (Winkel-Shirley, 2002; Martens et al., 2010). The flavonol branch pathway has remained intact for millions of years, and is almost exclusively involved in the responses of plants to a wide range of environmental stimuli (Pollastri and Tattini, 2011). Flavonols may act as defense molecules, signaling molecules, antioxidants, auxin transport inhibitors, and developmental regulators (Agati and Tattini, 2010; Pandey et al., 2015; Kuhn et al., 2016). Although flavonols have been well-documented for their antioxidant capacity *in vitro* (Yamasaki et al., 1997; Nakabayashi et al., 2014), their antioxidant capacity *in planta* is still a matter of controversy. In *Arabidopsis*, the main flavonoids in leaves are normally flavonols, including quercetin and kaempferol (Pelletier et al., 1999), and they accumulate mainly in the vacuoles of epidermal cells (Martens et al., 2010). Recently, Watkins et al. (2014) further demonstrated that in *Arabidopsis* flavonols accumulated specifically in guard cells and acted as a ROS scavenger in guard cells. 5-aminolevulinic acid can significantly improve flavonoids accumulation in fruits (Xie et al., 2013; Chen et al., 2015), leaves (Xu et al., 2011) and roots (Xu et al., 2010). However, no information is available on how ALA affects flavonols content in plants. We hypothesized that ALA may accelerate H_2O_2 removal by improving flavonols accumulation in guard cells and hence inhibit ABA-induced stomatal closure.

5-aminolevulinic acid pretreatment showed similar promotive effect on plant photosynthesis to concurrently applied ALA. However, whether ALA pretreatment also function through regulating stomatal movement remains unclear. Therefore, in this study, first, we investigated the effect of ALA pretreatment on stomatal movement and found that ALA pretreatment also inhibited ABA-induced stomatal closure by reducing H_2O_2 accumulation in guard cells. Then, using this experimental system and a flavonol-specific dye, we examined the effect of ALA on flavonols accumulation in guard cells and the influence of flavonols accumulation on stomatal movement. Furthermore, the role of flavonols accumulation in ALA-induced stomatal movement was investigated through a comparison of wild-type *Arabidopsis* plants and *transparent testa4* (*tt4*), the *Arabidopsis* chalcone synthase (CHS) mutant which is flavonoid-deficient. Our data provide direct evidence for ALA-mediated improvement of flavonols accumulation and demonstrate its positive role in ALA-induced stomatal movement, revealing new insights into guard cell signaling.

MATERIALS AND METHODS

Plant Materials and Growth Conditions
Arabidopsis (*Arabidopsis thaliana*) of wild-type (Col-0, WT), ALA-over-producing transgenic lines (*YHem1*) (Zhang et al., 2010) and CHS *tt4* mutant that derived from Col-0 background were used in this study. Seeds were surface sterilized with

bleaching power (5%, w/v) for 20 min, washed with sterilized water three times, then germinated and grown on Murashige and Skoog (MS) medium. Seedlings were cultured in a growth chamber at 25°C under a Photosynthetic Photo Flux Density (PPFD) of 150 μmol$\cdot$m$^{-2}\cdot$s^{-1} in 12 h light/12 h dark cycles.

Pretreatment with ALA, Exogenous Flavonol, and Treatment of ABA and H$_2$O$_2$

Two-week-old seedlings were used for experiments. Seedlings of WT, *YHem1,* and *tt4* with uniform height and size were carefully taken out of MS medium, washed off the medium, immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) and incubated under light conditions (PPFD 240 μmol m^{-2} s^{-1}) for 2 h to open the stomata. These seedlings were then pretreated with ALA or exogenous flavonol before being exposed to ABA or H$_2$O$_2$. For ALA pretreatment, *YHem1* seedlings were immersed in opening buffer for another 4 h, and seedlings of WT or *tt4* were immersed in the opening buffer supplemented with 0.5 mg L^{-1} ALA for a further 4 h under light conditions. For exogenous flavonols pretreatment, seedlings with pre-opened stomata were immersed in the opening buffer supplemented with quercetin (0–100 μM) or kaempferol (0–100 μM) for a further 1 h under light conditions (PPFD 240 μmol m^{-2} s^{-1}). For ABA or H$_2$O$_2$ treatment, pre-treated seedlings were immersed in the opening buffer supplemented with 10 μM ABA (Sigma-Aldrich, MO, USA) or 200 μM H$_2$O$_2$. Then, 10–12 seedlings of each treatment were randomly selected at 30-min intervals for 2 h and the cotyledons were picked with tweezers and used for stomatal bioassay and measurement of endogenous H$_2$O$_2$ and flavonol content. To avoid any potential rhythmic effects on stomatal aperture, experiments were always started at 8:00 am of the day.

Stomatal Bioassay

Because the obtainment of suitable rosette leaves needs more than 4 weeks, to expedite the experimental process, the cotyledons from two-week-old seedlings were used for experiments in this study. Stomatal bioassay was performed directly on the cotyledons because cotyledons are thin and pale green, allowing us to easily observe stomatal movements without peeling off the epidermal tissues (Amodeo et al., 1996; Jiang et al., 2014; Shang et al., 2016). Stomatal apertures were observed by a light microscope (Nikon TE100, 400×), using a fitted camera (MShot Digital Imaging System), and measured with Adobe Photoshop 6.0 (Adobe systems, CA, USA). In each treatment, 30 randomly selected apertures were scored at each time point and experiments were repeated three times. The data presented are means of 90 measurements ± standard errors (SE).

Measurement of Endogenous H$_2$O$_2$ and Flavonol Content Using Confocal Laser-Scanning Microscopy

Endogenous H$_2$O$_2$ was measured with the fluorescent indicator H$_2$DCF-DA as described by An et al. (2016a). Seedlings after each treatment were placed into Tris-KCl buffer (10 mM Tris and 50 mM KCl, pH 6.5) containing H$_2$DCF-DA (Sigma-Aldrich,

USA) at 50 μM for 30 min, in the dark at 25°C. Endogenous flavonol was measured with the fluorescent indicator dye diphenylboric acid 2-aminoethyl ester (DPBA) as described by Watkins et al. (2014). After each treatment, seedlings were placed into Mes-KCl buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) containing 0.01% (v/v) Triton X-100 and DPBA (Sigma-Aldrich, USA) at 2.52 mg mL^{-1} for 30 min, in the dark at 25°C. After staining, excess H$_2$DCF-DA and DPBA dye were removed with fresh Tris-KCl buffer and Mes-KCl buffer, respectively, in the dark. The cotyledons were picked with tweezers and their fluorescence was observed using a laser scanning confocal microscope (Leica TCS SP2, LSCM), with the following settings: ex = 488 nm, em = 525 ± 15 nm, power 50%, zoom 16, mild scanning, frame 512 × 512. Chlorophyll signal was collected between 650 to 700 nm. The DCF or DPBA fluorescence intensities were measured by placing a region of interest (ROI) around the guard cell. The intensity values within each ROI were recorded and averaged. In the determination of H$_2$O$_2$ and flavonol, at least 3 biological replicates were performed and 5 images were taken for each biological replicate.

Statistical Analysis

All data were taken from at least three independent experiments. Statistical analysis was performed using SPSS statistical computer package (version 16.0 SPSS Inc. Chicago. IL). Data was compared with the control or among treatments by analysis of variance (ANOVA) to discriminate significant differences at $P<0.05$ or $P<0.01$ followed by least significant difference tests (LSD).

RESULTS

ALA Pretreatment Inhibits ABA-Induced H$_2$O$_2$ Accumulation and Stomatal Closure

Previously, we have demonstrated that ALA inhibits ABA-induced stomatal closure via down-regulation of H$_2$O$_2$ accumulation when ALA is applied together with ABA (An et al., 2016a). To further determine whether ALA pretreatment has the same effect, we compared stomatal responses in cotyledons of two-week-old *Arabidopsis* to ABA with or without ALA pretreatment for 4 h. ABA significantly reduced stomatal aperture of wild-type (WT) plants after 30 min and decreased it by almost 50% within 2 h (**Figures 1A,B**). When ABA was applied to *YHem1* (ALA-overproducing transgenic plants) or ALA-pretreated WT plants, ABA-induced stomatal closing was significantly suppressed. The time course for stomatal movement illustrated that the inhibition of ALA on ABA-induced stomatal closure was initiated after ABA application and lasted for at least 2 h (**Figure 1B**). These results indicate that ALA pretreatment inhibits ABA-induced stomatal closure.

To determine whether ALA pretreatment inhibits ABA-induced stomatal closure also by reducing ROS accumulation, we estimated the H$_2$O$_2$ content in guard cells using a fluorescent dye, H$_2$DCF-DA. We found that ABA treatment for 1 h significantly increased H$_2$O$_2$ accumulation in guard cells of

FIGURE 1 | ALA pretreatment inhibits ABA-induced stomatal closure by reducing H$_2$O$_2$ accumulation in guard cells. (A,B) ALA pretreatment inhibits ABA-induced stomatal aperture. Two-week-old wild-type *Arabidopsis* pretreated with exogenous 0.5 mg L^{-1} ALA under light (240 μmol m^{-2} s^{-1}) for 4 h and *YHem1*-transgenic *Arabidopsis* incubated under the same light condition for 4 h were immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) alone or containing 10 μM ABA for 2 h under light (240 μmol m^{-2} s^{-1}). The cotyledons were picked with tweezers at 30-min intervals for 2 h for the record of guard cell images and determination of stomatal apertures. **(A)** Images of guard cell after 1 h of different treatments. Scale bar: 10 μm. **(B)** Time courses of stomatal responses in WT and *YHem1* to different treatments. Values are the means of 90 measurements ± SE from three independent experiments. Different letters on the same time point indicate significant differences at *P* = 0.05 level. **(C,D)** ALA pretreatment reduces ABA-induced H$_2$O$_2$ accumulation in guard cells. The cotyledons of the above treated seedlings were picked with tweezers at 1 h of treatment and loaded with 50 μM H$_2$DCF-DA for 30 min in darkness at 25°C. Fluorescence of the cotyledons **(C)** was observed using a confocal microscope. For each treatment, DCF fluorescence is shown in green and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 μm. **(D)** DCF intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. Different letters indicate significant differences at *P* = 0.05 level.

WT plants to more than two-fold of control plants. Without ABA application, the *YHem1* transgenic plants and ALA-pretreated WT plants showed the same level of DCF fluorescence intensity with non-pretreated WT plants (**Figures 1C,D**). When ABA was applied to *YHem1* or ALA-pretreated WT plants, ABA-induced H$_2$O$_2$ accumulation was suppressed by about 35%. These results indicate that, similar to the effect of ALA treatment, ALA pretreatment also reduces ABA-induced H$_2$O$_2$ accumulation and then inhibits stomatal closure.
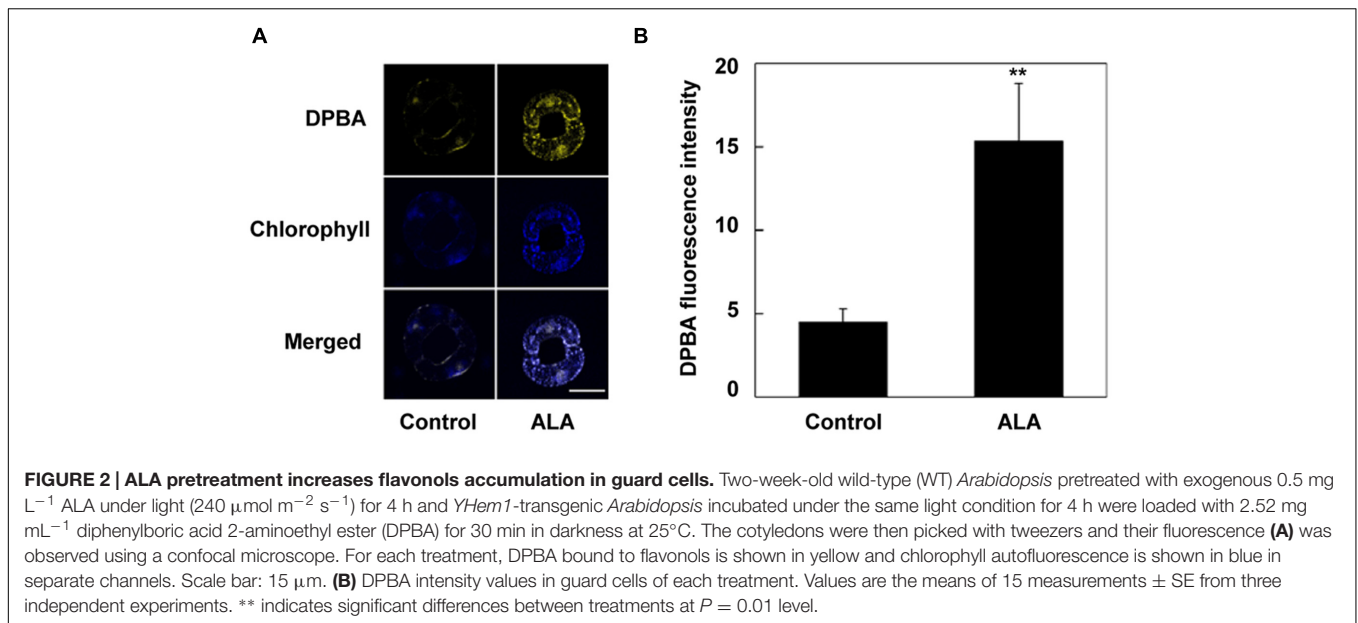
ALA Pretreatment Increases Flavonols Accumulation in Guard Cell

A role of flavonols in suppressing H$_2$O$_2$ accumulation and stomatal closure has been suggested (Watkins et al., 2014). To determine whether flavonols accumulation contributes to ALA-mediated reduction of H$_2$O$_2$ content, we first investigated the effect of ALA on flavonols content in guard cells using a flavonol-specific fluorescent dye, DPBA.

We found that ALA pretreatment for 4 h significantly increased flavonols accumulation (**Figure 2**). The DPBA fluorescence intensity in guard cells of ALA-pretreated plants was about 2.44-fold higher than that in control plants.

Flavonols Inhibit ABA-Induced Stomatal Closure

To determine whether flavonols accumulation in guard cells can regulate stomatal movement, we investigated the effects of exogenous quercetin and kaempferol, two major components of flavonol in *Arabidopsis* leaves, on ABA-induced stomatal closure. First, to choose the appropriate concentration of exogenous flavonol, we detected the flavonols content in guard cells after treatment with quercetin or kaempferol of different concentrations for 1 h. Results showed that the concentrations equal to or higher than 10 μM of quercetin and kaempferol significantly increased endogenous flavonols content (**Figures 3A,B**). Since flavonols content in guard cells of plants

FIGURE 2 | ALA pretreatment increases flavonols accumulation in guard cells. Two-week-old wild-type (WT) *Arabidopsis* pretreated with exogenous 0.5 mg L^{-1} ALA under light (240 μmol m^{-2} s^{-1}) for 4 h and *YHem1*-transgenic *Arabidopsis* incubated under the same light condition for 4 h were loaded with 2.52 mg mL^{-1} diphenylboric acid 2-aminoethyl ester (DPBA) for 30 min in darkness at 25°C. The cotyledons were then picked with tweezers and their fluorescence **(A)** was observed using a confocal microscope. For each treatment, DPBA bound to flavonols is shown in yellow and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 μm. **(B)** DPBA intensity values in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. ** indicates significant differences between treatments at $P = 0.01$ level.

treated with 10 μM quercetin and kaempferol was similar to that in ALA-pretreated plants (**Figures 2** and **3A,B**), we choose 10 μM quercetin and kaempferol for the following experiments.

Stomatal bioassays showed that quercetin or kaempferol alone did not influence stomatal aperture (**Figures 3C,D**). When applied together with ABA, both of them significantly suppressed ABA-induced stomatal closure, compared to the effect of ABA alone. After being treated for 2 h, stomatal aperture of plants treated with ABA and quercetin or kaempferol was about 50% higher than that of plants treated with ABA alone (**Figure 3D**). These results demonstrated that flavonols accumulation in guard cells inhibited ABA-induced stomatal closure, indicating that flavonols might play an important role in the inhibition of ABA-induced stomatal closure by ALA.

Flavonols decrease H$_2$O$_2$ Accumulation in Guard Cells and Inhibit H$_2$O$_2$-Induced Stomatal Closure
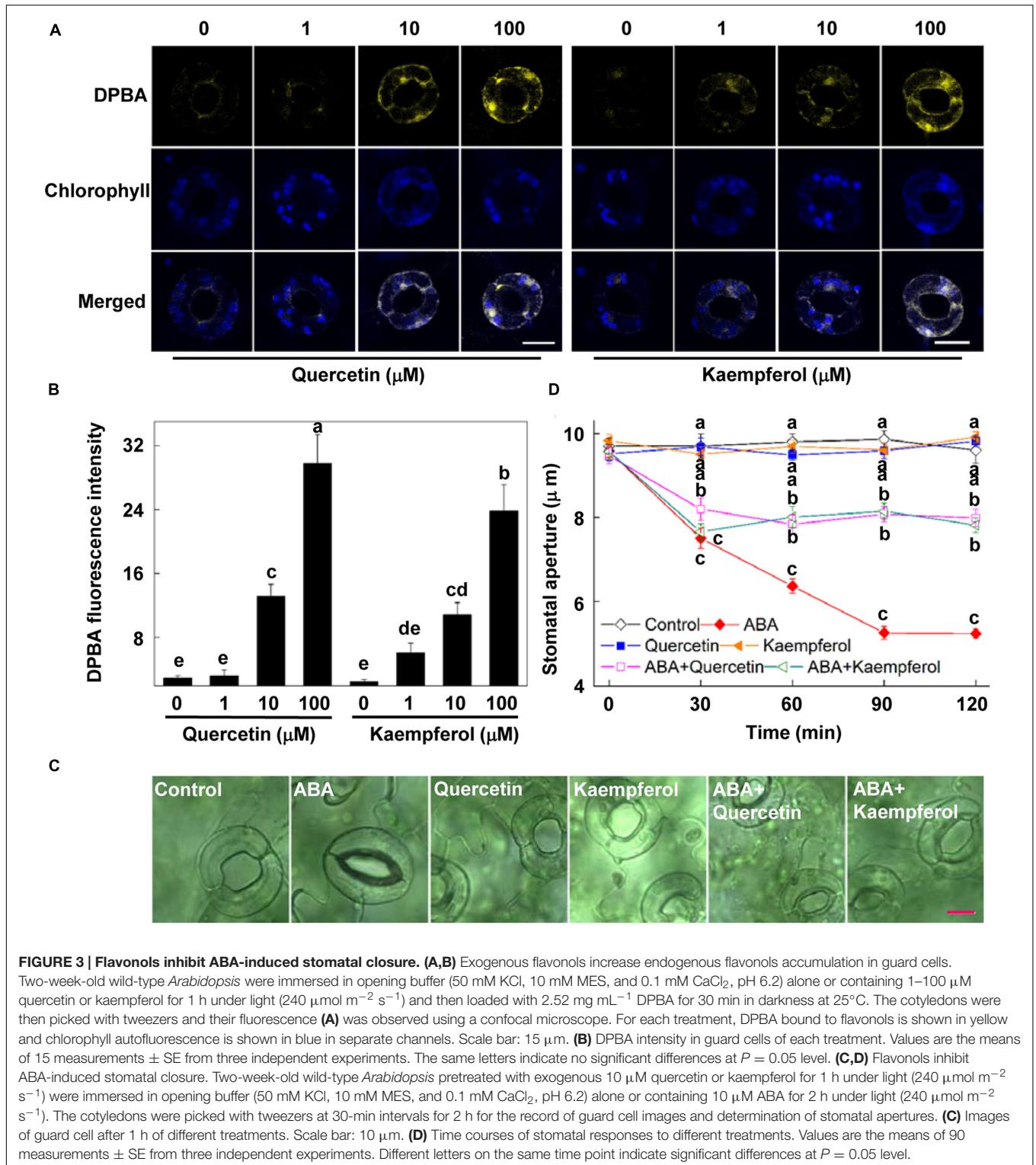
To determine whether flavonol accumulation inhibits ABA-induced stomatal closure by reducing H$_2$O$_2$ content in guard cells, we investigated the effects of exogenous flavonols on H$_2$O$_2$ content in guard cells. We found that both quercetin and kaempferol significantly decreased ABA-induced H$_2$O$_2$ accumulation in guard cells (**Figures 4A,B**), consistent with their effects on flavonols accumulation. These results indicate that flavonols accumulation reduces H$_2$O$_2$ content and consequently suppresses ABA-induced stomatal closure.

To confirm the effect of flavonols accumulation on H$_2$O$_2$ content and its role in stomatal closure, we evaluated whether flavonols inhibited H$_2$O$_2$-induced stomatal closure. Results showed that both quercetin and kaempferol significantly suppressed H$_2$O$_2$-induced stomatal closure (**Figures 4C,D**). After being treated for 2 h, stomatal aperture of plants treated with H$_2$O$_2$ and quercetin or kaempferol was about

30% higher than that of plants treated with H$_2$O$_2$ alone (**Figure 4D**). These results confirmed that flavonols accumulation could scavenge H$_2$O$_2$ in guard cells and inhibit stomatal closure.

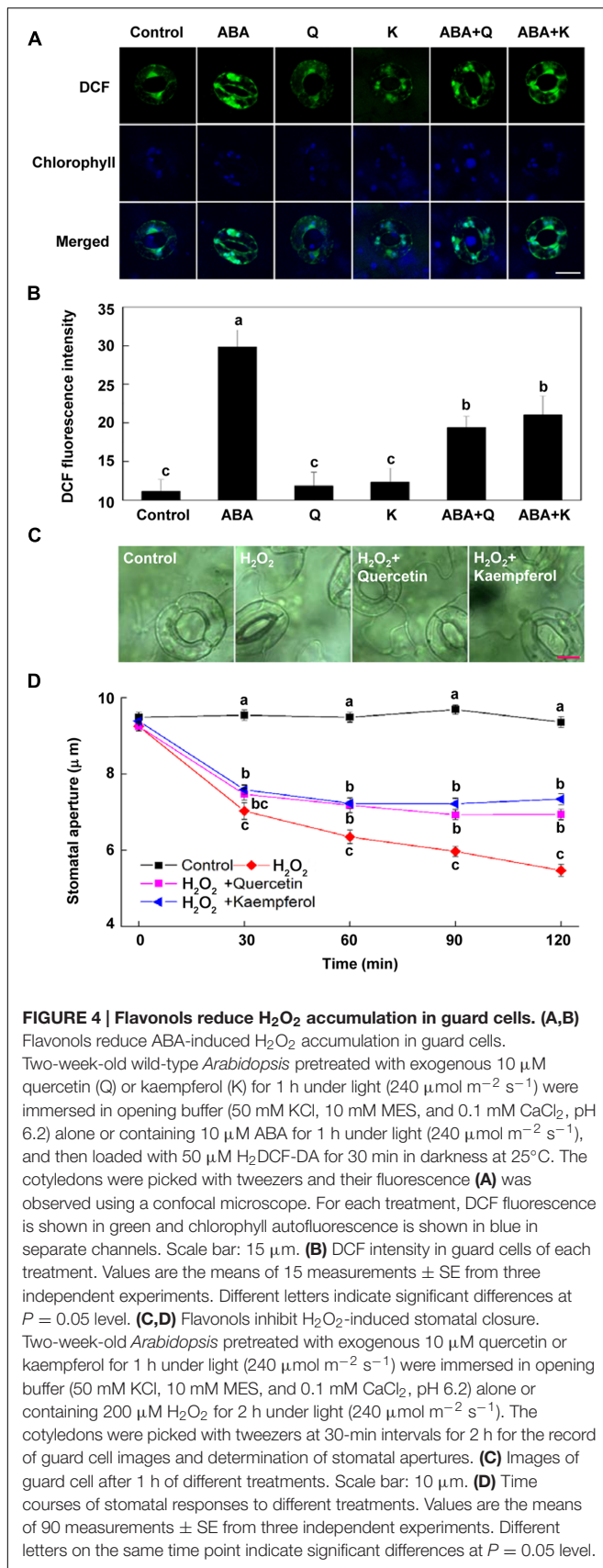## Flavonols Accumulation Involves in ALA-Regulated Stomatal Movement

The above results indicate that ALA-induced flavonols accumulation mediates ALA's inhibitory effects on H$_2$O$_2$ accumulation and stomatal closure. To verify the role of flavonols accumulation, we investigated the effect of ALA on ABA-induced stomatal closure using the *transparent testa 4* (*tt4*) *Arabidopsis* mutant that makes no flavonoids (Nakabayashi et al., 2014). **Figures 5A,B** showed that flavonols did not accumulate in the *tt4* mutant, even with the application of exogenous ALA. The *tt4* mutant showed similar stomatal aperture to WT plants under control conditions (**Figures 5C,D**). ABA significantly reduced stomatal aperture of WT and *tt4*. However, stomatal response of *tt4* to ABA was significantly faster and greater than that of WT. Stomatal aperture of *tt4* was 31.97% and 17.61% lower than that of WT after 30 min and 120 min of ABA treatment, respectively. When ABA was applied together with ALA, ABA-induced stomatal closure was significantly inhibited by ALA in either WT or *tt4*. However, compared with WT plants, *tt4* showed significantly greater ABA-induced stomatal closure response when ABA and ALA were applied together. Stomatal aperture of *tt4* was about 30% lower than that of WT at each time point under ABA+ALA treatment. Measurement of H$_2$O$_2$ content showed that, compared with their corresponding control, ABA significantly increased H$_2$O$_2$ content in the guard cells of both WT and *tt4*, and the H$_2$O$_2$ accumulation in *tt4* was greater than that in WT (**Figures 5E,F**). When ABA was applied together with ALA, ABA-induced H$_2$O$_2$ accumulation was significantly decreased. Furthermore, compared with WT, *tt4* showed remarkably higher

FIGURE 3 | Flavonols inhibit ABA-induced stomatal closure. (A,B) Exogenous flavonols increase endogenous flavonols accumulation in guard cells. Two-week-old wild-type *Arabidopsis* were immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl₂, pH 6.2) alone or containing 1–100 μM quercetin or kaempferol for 1 h under light (240 μmol m^{-2} s^{-1}) and then loaded with 2.52 mg mL^{-1} DPBA for 30 min in darkness at 25°C. The cotyledons were then picked with tweezers and their fluorescence **(A)** was observed using a confocal microscope. For each treatment, DPBA bound to flavonols is shown in yellow and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 μm. **(B)** DPBA intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. The same letters indicate no significant differences at $P = 0.05$ level. **(C,D)** Flavonols inhibit ABA-induced stomatal closure. Two-week-old wild-type *Arabidopsis* pretreated with exogenous 10 μM quercetin or kaempferol for 1 h under light (240 μmol m^{-2} s^{-1}) were immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl₂, pH 6.2) alone or containing 10 μM ABA for 2 h under light (240 μmol m^{-2} s^{-1}). The cotyledons were picked with tweezers at 30-min intervals for 2 h for the record of guard cell images and determination of stomatal apertures. **(C)** Images of guard cell after 1 h of different treatments. Scale bar: 10 μm. **(D)** Time courses of stomatal responses to different treatments. Values are the means of 90 measurements ± SE from three independent experiments. Different letters on the same time point indicate significant differences at $P = 0.05$ level.

H_2O_2 content when ABA and ALA were applied together. These results indicate that ALA inhibits ABA-induced stomatal closure in both WT and *tt4* by decreasing ROS accumulation in guard cells, but this inhibitory effect of ALA is impaired in *tt4* mutant, compared to WT. Our data verified that flavonols accumulation played an important role in ALA-regulated stomatal movement.

To further confirm the role of flavonols accumulation, we detected whether exogenous flavonols could restore stomatal responses of *tt4* mutant to ABA and ALA. Results

FIGURE 4 | Flavonols reduce H_2O_2 accumulation in guard cells. (A,B)
Flavonols reduce ABA-induced H_2O_2 accumulation in guard cells.
Two-week-old wild-type *Arabidopsis* pretreated with exogenous 10 μM
quercetin (Q) or kaempferol (K) for 1 h under light (240 μmol m^{-2} s^{-1}) were
immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH
6.2) alone or containing 10 μM ABA for 1 h under light (240 μmol m^{-2} s^{-1}),
and then loaded with 50 μM H$_2$DCF-DA for 30 min in darkness at 25°C. The
cotyledons were picked with tweezers and their fluorescence **(A)** was
observed using a confocal microscope. For each treatment, DCF fluorescence
is shown in green and chlorophyll autofluorescence is shown in blue in
separate channels. Scale bar: 15 μm. **(B)** DCF intensity in guard cells of each
treatment. Values are the means of 15 measurements ± SE from three
independent experiments. Different letters indicate significant differences at
$P = 0.05$ level. **(C,D)** Flavonols inhibit H_2O_2-induced stomatal closure.
Two-week-old *Arabidopsis* pretreated with exogenous 10 μM quercetin or
kaempferol for 1 h under light (240 μmol m^{-2} s^{-1}) were immersed in opening
buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) alone or
containing 200 μM H$_2$O$_2$ for 2 h under light (240 μmol m^{-2} s^{-1}). The
cotyledons were picked with tweezers at 30-min intervals for 2 h for the record
of guard cell images and determination of stomatal apertures. **(C)** Images of
guard cell after 1 h of different treatments. Scale bar: 10 μm. **(D)** Time
courses of stomatal responses to different treatments. Values are the means
of 90 measurements ± SE from three independent experiments. Different
letters on the same time point indicate significant differences at $P = 0.05$ level.

showed that 10 μM quercetin or kaempferol increased the
endogenous flavonols content in *tt4* guard cells to the WT
level (**Figures 6A,B**). Although ABA-induced stomatal closure
response was greater in *tt4* mutant than in control plants,
exogenous flavonols induced stomatal responses of *tt4* recoverd
to the WT level (**Figures 5D** and **6C**). After exposure to ABA for
2 h, the stomatal aperture of *tt4* was 23.26% lower than that of
WT, however, it recovered to about 105% of that of WT when
exogenous flavonols were applied concurrently (**Figures 5D** and
6C). We further demonstrated that exogenous flavonols reduced
H_2O_2 accumulation in *tt4* to nearly WT levels (**Figures 4A,B**
and **7**). These results confirmed the important role of flavonols
in scavenging H_2O_2 and then inhibiting stomatal closure.

DISCUSSION

It has been well-documented that ALA, a new plant growth
regulator, significantly improves plant photosynthesis and
growth (Akram and Ashraf, 2013). However, its underlying
mechanisms remain largely unknown. Stomatal aperture is
a major limiting factor in photosynthesis and plant growth
(Lawson et al., 2014; Wang et al., 2014). Our recent study
showed that ALA significantly inhibited stomatal closing (An
et al., 2016a), indicating ALA-induced stomatal opening is
an important reason for the improvement of photosynthesis.
Here, we demonstrated that ALA pretreatment also significantly
inhibited ABA-induced stomatal closing. These results are
consistent with previous reports that both ALA treatment (Hotta
et al., 1997; Nishihara et al., 2003; Tian et al., 2014) and
pretreatment (Liu et al., 2011; Zhang et al., 2012; An et al.,
2016b) universally improved plant photosynthesis. Although
stomatal opening will inevitably increase transpirational water
loss, which might decrease plant drought tolerance, several
studies have shown that ALA largely improve drought tolerance
of various plant species (Li et al., 2011; Liu et al., 2011, 2013;
Kosar et al., 2015). Our previous study provides direct evidence
that ALA inhibits stomatal closing but significantly improves
plant drought tolerance simultaneously (An et al., 2016a).
Increasing stomatal aperture indeed accelerates transpirational
water loss, but transpiration simultaneously enhances water and
nutrient uptake into plants, which is vital to plant growth.
It has been reported that ALA significantly increased uptake
of several nutrients, such as phosphate (Yao et al., 2006; Ali
et al., 2014), nitrogen (Wei et al., 2012; Ali et al., 2014),
sulfur (Ali et al., 2014), potassium calcium, magnesium, iron,
copper, and zinc (Naeem et al., 2010; Zhang et al., 2015). 5-
aminolevulinic acid also widely improves plant root activity and
root growth (Kosar et al., 2015; An et al., 2016a,b), which is
important for water and nutrient uptake and hence for plant
growth and stress resistance (Sharp et al., 2003; Schachtman
and Goodger, 2008). Therefore, the higher plant photosynthesis
and the stronger root water/nutrient uptake induced by ALA
are important mechanisms underlying ALA-improved drought
tolerance. Although the exact mechanisms behind the paradox
between enhanced drought tolerance and promoted stomatal
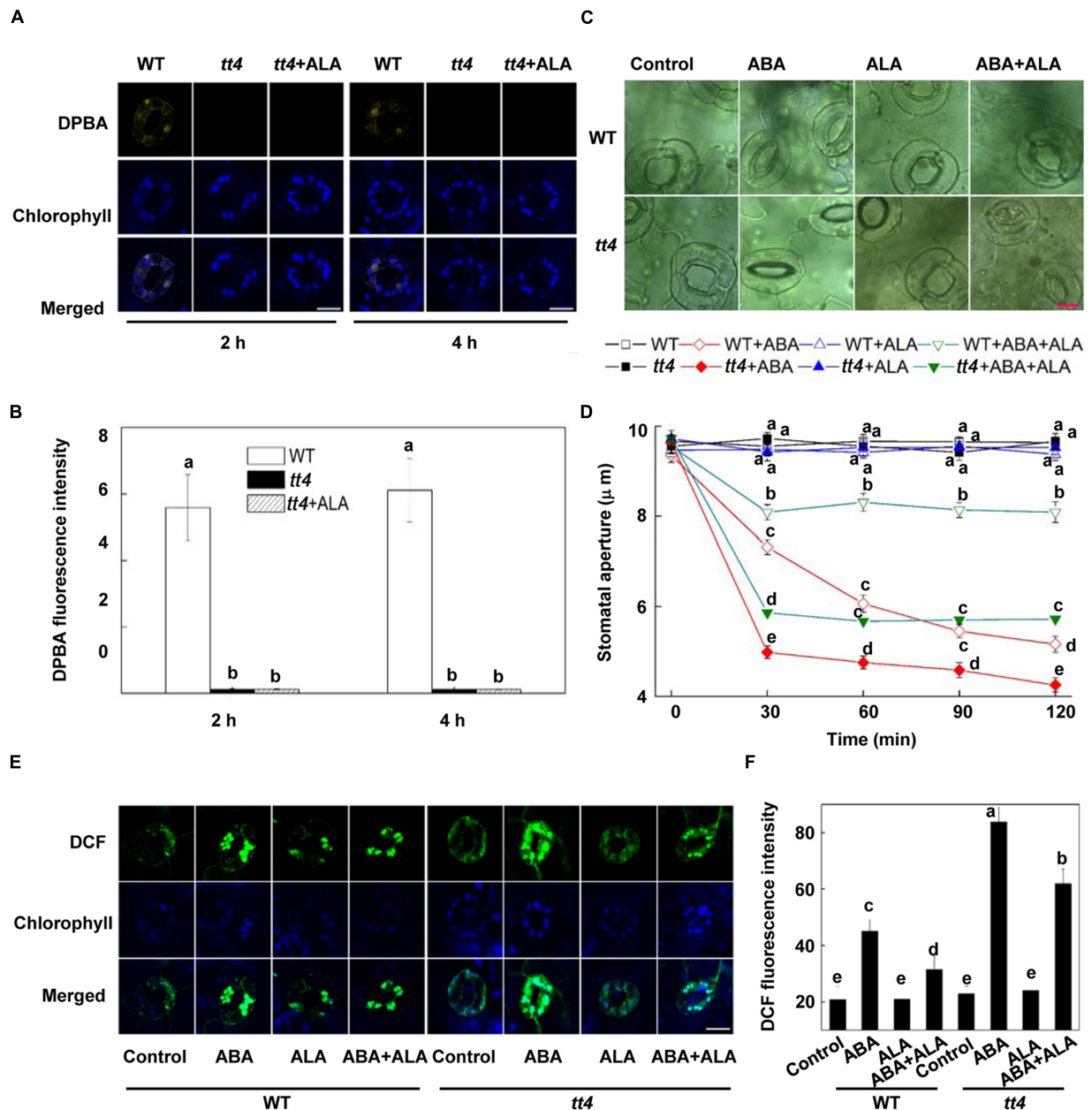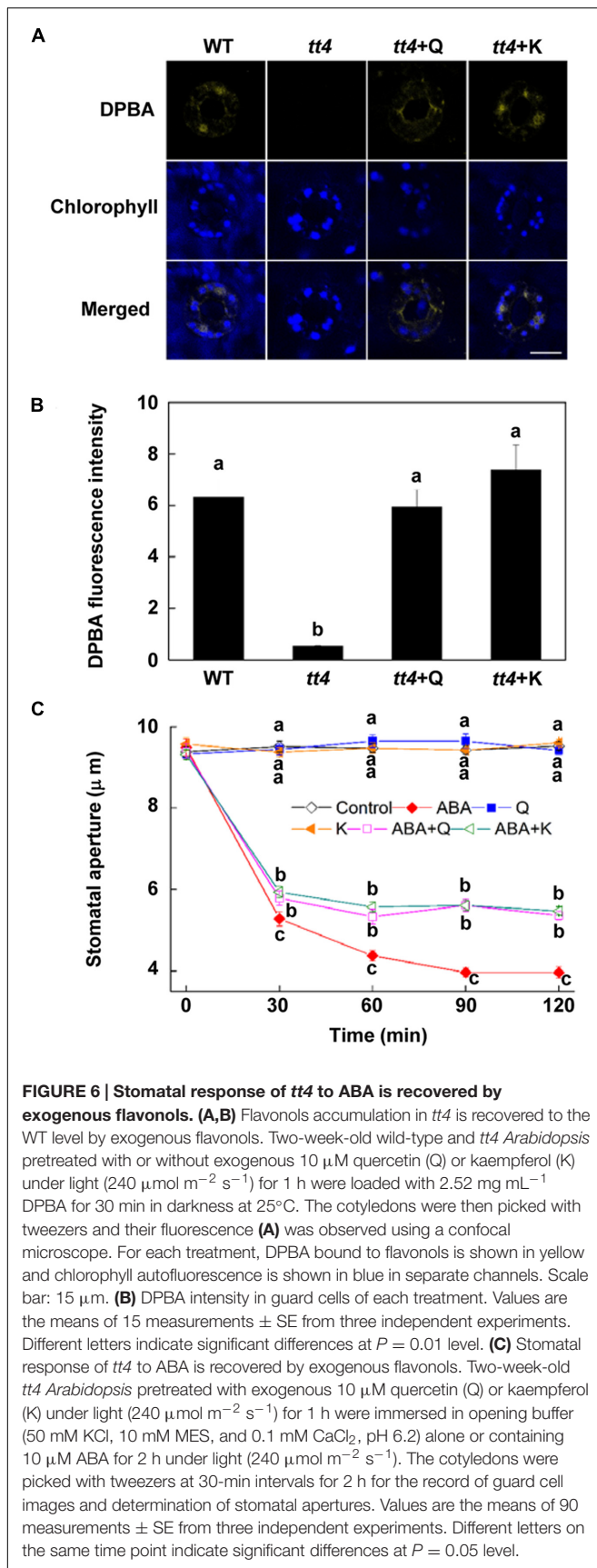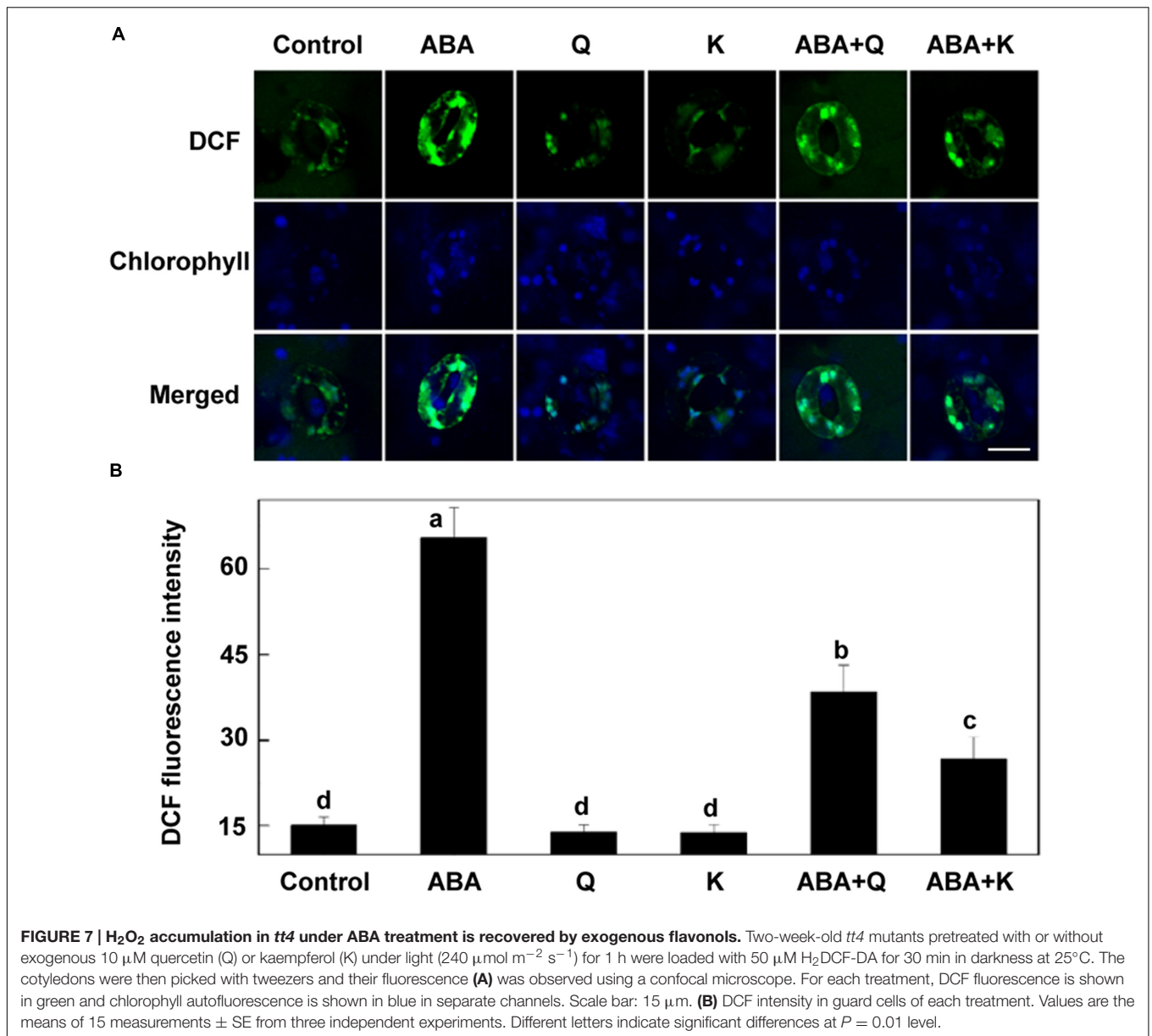opening still remain elusive, the obtained results suggest the great

FIGURE 5 | The inhibitory effect of ALA on ABA-induced stomatal closure is impaired in *tt4* mutant. (A,B) Flavonols accumulation is absent in *tt4* mutant pretreated with or without ALA. Two-week-old wild-type and *tt4 Arabidopsis* pretreated with exogenous 0.5 mg L^{-1} ALA under light (240 µmol m^{-2} s^{-1}) for 2 h or 4 h were loaded with 2.52 mg mL^{-1} DPBA for 30 min in darkness at 25°C. The cotyledons were then picked with tweezers and their fluorescence **(A)** was observed using a confocal microscope. For each treatment, DPBA bound to flavonols is shown in yellow and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 µm. **(B)** DPBA intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. Different letters indicate significant differences at *P* = 0.01 level. **(C,D)** The inhibition of ABA-induced stomatal closure by ALA is impaired in *tt4* mutant. Two-week-old wild-type and *tt4 Arabidopsis* pretreated with exogenous 0.5 mg L^{-1} ALA under light (240 µmol m^{-2} s^{-1}) for 4 h were immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) alone or containing 10 µM ABA for 2 h under light (240 µmol m^{-2} s^{-1}).The cotyledons were picked with tweezers at 30-min intervals for 2 h for the record of guard cell images and determination of stomatal apertures. **(C)** Images of guard cell after 1 h of different treatments. Scale bar: 10 µm. **(D)** Time courses of stomatal responses in WT and *tt4* to different treatments. Values are the means of 90 measurements ± SE from three independent experiments. Different letters on the same time point indicate significant differences at *P* = 0.05 level. **(E,F)** The inhibition of ABA-induced H$_2$O$_2$ accumulation in guard cells by ALA is impaired in *tt4* mutant. The cotyledons of the seedlings treated as shown in Figures C and D were picked at 1 h of treatment and loaded with 50 µM H$_2$DCF-DA for 30 min in darkness at 25°C. Fluorescence of the cotyledons **(E)** was observed using a confocal microscope. For each treatment, DCF fluorescence is shown in green and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 µm. **(F)** DCF intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. Different letters indicate significant differences at *P* = 0.05 level.

FIGURE 6 | Stomatal response of *tt4* to ABA is recovered by exogenous flavonols. (A,B) Flavonols accumulation in *tt4* is recovered to the WT level by exogenous flavonols. Two-week-old wild-type and *tt4 Arabidopsis* pretreated with or without exogenous 10 μM quercetin (Q) or kaempferol (K) under light (240 μmol m^{-2} s^{-1}) for 1 h were loaded with 2.52 mg mL^{-1} DPBA for 30 min in darkness at 25°C. The cotyledons were then picked with tweezers and their fluorescence **(A)** was observed using a confocal microscope. For each treatment, DPBA bound to flavonols is shown in yellow and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 μm. **(B)** DPBA intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. Different letters indicate significant differences at $P = 0.01$ level. **(C)** Stomatal response of *tt4* to ABA is recovered by exogenous flavonols. Two-week-old *tt4 Arabidopsis* pretreated with exogenous 10 μM quercetin (Q) or kaempferol (K) under light (240 μmol m^{-2} s^{-1}) for 1 h were immersed in opening buffer (50 mM KCl, 10 mM MES, and 0.1 mM CaCl$_2$, pH 6.2) alone or containing 10 μM ABA for 2 h under light (240 μmol m^{-2} s^{-1}). The cotyledons were picked with tweezers at 30-min intervals for 2 h for the record of guard cell images and determination of stomatal apertures. Values are the means of 90 measurements ± SE from three independent experiments. Different letters on the same time point indicate significant differences at $P = 0.05$ level.

application potential of ALA in agriculture and forestry for a sustainable low-carbon society.

5-aminolevulinic acid not only increases plant drought tolerance but also can improve plant tolerance to various other abiotic stresses, such as salt (Nishihara et al., 2003), cold (Hotta et al., 1998), heat (Zhang et al., 2012), low light (Wang et al., 2004), and heavy metal stresses (Ali et al., 2013a; Tian et al., 2014). A common mechanism behind ALA's function is the improvement of antioxidant capacity and the reduction of ROS accumulation. Guard cells represent a powerful single-cell model system for understanding signal transduction mechanisms in plants (Munemasa et al., 2015). Our recent research showed that ALA significantly reduced H$_2$O$_2$ content in guard cells and consequently inhibited stomatal closure (An et al., 2016a). Here, we further showed that ALA pretreatment also significantly reduced H$_2$O$_2$ accumulation in guard cells and inhibited stomatal closure. Together, these results indicate that ALA-mediated H$_2$O$_2$ decreasing can last for a long time. In guard cells, it contributes to ALA-induced stomatal opening, and in plant organs, such as leaves and roots, it helps decreasing abiotic stress-induced oxidative damage in plant tissues.

Hydrogen peroxide is an important second messenger in ABA signaling in guard cells (Pei et al., 2000). Its content in guard cells is tightly regulated by the production and scavenging systems. ABA-induced H$_2$O$_2$ accumulation is dependent on H$_2$O$_2$ synthesis in *Arabidopsis*, and the NADPH oxidase subunits, RESPIRATORY BURST OXIDASE HOMOLOG D and F (*Atr*bohD and *Atr*bohF, respectively) are required for the production of H$_2$O$_2$ during ABA-induced stomatal closure in guard cells (Kwak et al., 2003). However, recent research indicates that ALA-caused decrease of H$_2$O$_2$ in guard cells is not primarily through the inhibition of H$_2$O$_2$ synthesis but mainly through accelerating its elimination (An et al., 2016a). How ALA removes H$_2$O$_2$ in guard cells remains unclear. In this work, we provide evidence that ALA-induced flavonol accumulation plays an important role as a H$_2$O$_2$ scavenger in ALA-mediated stomatal movement in *Arabidopsis*. The following results support this conclusion: (i) ALA stimulated flavonols accumulation in guard cells; (ii) exogenous quercetin and kaempferol, two main kinds of flavonols in *Arabidopsis*, resulted in flavonols accumulation in guard cells and inhibited ABA- and H$_2$O$_2$-induced stomatal closure by decreasing H$_2$O$_2$ in guard cells; (iii) the inhibition of ABA-induced H$_2$O$_2$ accumulation and stomatal closure by ALA were impaired in *tt4* mutant which is flavonol deficient; (iv) exogenous quercetin and kaempferol restored the stomatal responses of *tt4* mutant to ABA. Watkins et al. (2014) first reported the signaling role of flavonol accumulation in ethylene-induced stomatal movement. Our data confirm the role of flavonol as a H$_2$O$_2$ scavenger in guard cell signaling. Although flavonols have been proposed as natural antioxidants (Nakabayashi et al., 2014; Nguyen et al., 2016), its antioxidant capacity *in planta* has long been debated. Our data together with report of Watkins et al. (2014) provide direct evidence that flavonols can scavenge H$_2$O$_2$ in guard cells, confirming its antioxidant capacity *in planta* and revealing new components in guard cell signaling. However, in the present study, ALA's effects on H$_2$O$_2$ accumulation and stomatal closure were not

FIGURE 7 | H$_2$O$_2$ accumulation in *tt4* under ABA treatment is recovered by exogenous flavonols. Two-week-old *tt4* mutants pretreated with or without exogenous 10 μM quercetin (Q) or kaempferol (K) under light (240 μmol m^{-2} s^{-1}) for 1 h were loaded with 50 μM H$_2$DCF-DA for 30 min in darkness at 25°C. The cotyledons were then picked with tweezers and their fluorescence **(A)** was observed using a confocal microscope. For each treatment, DCF fluorescence is shown in green and chlorophyll autofluorescence is shown in blue in separate channels. Scale bar: 15 μm. **(B)** DCF intensity in guard cells of each treatment. Values are the means of 15 measurements ± SE from three independent experiments. Different letters indicate significant differences at *P* = 0.01 level.

totally inhibited, but just partially impaired in *tt4* mutant. These results indicate that the inhibitory effect of ALA pretreatment on ABA-induced stomatal closure is not completely dependent on flavonols accumulation, and further studies are needed to reveal other factors which also involve in this process.

Flavonols are ubiquitous phenolic plant secondary metabolites (Thompson et al., 2010). Previous studies in the past decades have indicated that ALA can stimulate phenylpropanoid metabolism and improve flavonoid accumulation in plants (Xu et al., 2010, 2011; Xie et al., 2013; Chen et al., 2015). However, no specific information is available on the effect of ALA on flavonols accumulation in plants. In the present study, using a flavonol-specific dye, we proved that ALA significantly improved flavonols accumulation in guard cells in *Arabidopsis*, providing direct evidence of ALA-stimulated flavonol accumulation in plants.

Flavonols are involved in various physiological processes in plants including growth, development, and defense response to various stresses (Agati and Tattini, 2010; Pandey et al., 2015; Kuhn et al., 2016). Nakabayashi et al. (2014) suggest that flavonoid over-accumulation can enhance tolerance to multiple stresses. As mentioned earlier, improving antioxidant capacity was a common mechanism behind ALA-conferred plant tolerance to various stresses. Here, we demonstrated that ALA-induced flavonols accumulation decreases H$_2$O$_2$ content in guard cells. Therefore, flavonols accumulation may play important roles in ALA-induced improvement of plant antioxidant capacity. Besides their roles in plant biology, flavonols have been reported to provide several health benefits to mankind owing to their antioxidant and anti-inflammatory properties (Lee et al., 2015). Moreover, consumption of flavonol-rich food is helpful in

lowering the risk of certain diseases (Lea, 2015; Pandey et al., 2015). For example, quercetin and kaempferol have synergistic actions in inhibiting cancer cell proliferation (Arima et al., 2002). Taken together, flavonols not only play important roles *in planta* but also have a close connection with human health. How to improve the flavonols content in plant has become a hot research topic in the field of biology (Jia et al., 2015). In this work, we prove that ALA stimulates flavonols accumulation in guard cells, indicating that application of ALA would be a potential method to improve flavonols in leaves of plants. Further studies will be carried out to elucidate the mechanisms how ALA improves the flavonols content in plants, which is largely unknown.

In summary, the data presented here demonstrate that ALA significantly enhances flavonols content in guard cells, and the flavonols accumulation contributes to the inhibitory effect of ALA on ABA-induced stomatal closure by suppressing H_2O_2 accumulation in guard cells. In flavonol-deficient *tt4* mutant, the inhibitory effect of ALA on ABA-induced H_2O_2 accumulation and stomatal closure was not completely suppressed, indicating that ALA-induced stomatal movement is not totally dependent on flavonols accumulation and there should be other pathways also involved in this physiological process. Our results provide new insights into the signal transduction pathway of ALA-induced stomatal movement, and add new potential method to improve plant flavonol content which shows multiple beneficial effects on plant itself and our humankind. The molecular mechanism how ALA regulates flavonol synthesis in plants is worthy of further study.

AUTHOR CONTRIBUTIONS

YA and LW conceived and designed research. YA, XF, LL, and LX carried out all the experiments. YA and XF analyzed the data. YA and LW wrote the manuscript. All authors read and approved the manuscript.

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REFERENCES

Agati, G., and Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection. *New Phytol.* 186, 786–793. doi: 10.1111/j.1469-8137.2010.03269.x

Akram, N. A., and Ashraf, M. (2013). Regulation in plant stress tolerance by a potential plant growth regulator, 5-aminolevulinic acid. *J. Plant Growth Regul.* 32, 663–679. doi: 10.1007/s00344-013-9325-9

Ali, B., Huang, C. R., Qi, Z. Y., Ali, S., Daud, M. K., Geng, X. X., et al. (2013a). 5-Aminolevulinic acid ameliorates cadmium-induced morphological, biochemical, and ultrastructural changes in seedlings of oilseed rape. *Environ. Sci. Pollut. Res. Int.* 20, 7256–7267. doi: 10.1007/s11356-013-1735-5

Ali, B., Wang, B., Ali, S., Ghani, M. A., Hayat, M. T., Yang, C., et al. (2013b). 5-Aminolevulinic acid ameliorates the growth, photosynthetic gas exchange capacity, and ultrastructural changes under cadmium stress in *Brassica napus* L. *J. Plant Growth Regul.* 32, 604–614. doi: 10.1007/s00344-013-9328-6

Ali, B., Xu, X., Gill, R. A., Yang, S., Ali, S., Tahir, M., et al. (2014). Promotive role of 5-aminolevulinic acid on mineral nutrients and antioxidative defense system under lead toxicity in *Brassica napus*. *Ind. Crop. Prod.* 52, 617–626. doi: 10.1016/j.indcrop.2013.11.033

Amodeo, G., Talbott, L. D., and Zeiger, E. (1996). Use of potassium and sucrose by onion guard cells during a daily cycle of osmoregulation. *Plant Cell Physiol.* 37, 575–579. doi: 10.1093/oxfordjournals.pcp.a028983

An, Y. Y., Liu, L. B., Chen, L. H., and Wang, L. J. (2016a). ALA inhibits ABA-induced stomatal closure via reducing H_2O_2 and Ca^{2+} levels in guard cells. *Front. Plant Sci* 7:482. doi: 10.3389/fpls.2016.00482

An, Y. Y., Qi, L., and Wang, L. J. (2016b). ALA pretreatment improves waterlogging tolerance of fig plants. *PLoS ONE* 11:e0147202. doi: 10.1371/journal.pone.0147202

An, Z. F., Jing, W., Liu, Y. L., and Zhang, W. H. (2008). Hydrogen peroxide generated by copper amine oxidase is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *J. Exp. Bot.* 59, 815–825. doi: 10.1093/Jxb/Erm370

Arima, H., Ashida, H., and Danno, G. (2002). Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. *Biosci. Biotech. Bioch.* 66, 1009–1014. doi: 10.1271/bbb.66.1009

Chen, L., Guo, Y. R., Bai, G., Sun, J. J., and Li, Y. (2015). Effect of 5-aminolevulinic acid and genistein on accumulation of polyphenol and anthocyanin in 'Qinyang' apples. *J. Anim. Plant Sci.* 25, 68–79.

Chobot, V., and Hadacek, F. (2011). Exploration of pro-oxidant and antioxidant activities of the flavonoid myricetin. *Redox Rep.* 16, 242–247. doi: 10.1179/1351000211Y.0000000015

Desikan, R., Last, K., Harrett-Williams, R., Tagliavia, C., Harter, K., Hooley, R., et al. (2006). Ethylene-induced stomatal closure in *Arabidopsis* occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J.* 47, 907–916. doi: 10.1111/j.1365-313X.2006.02842.x

Dodd, I. C. (2003). Hormonal interactions and stomatal responses. *J. Plant Growth Regul.* 22, 32–46. doi: 10.1007/s00344-003-0023-x

He, J. M., Yue, X. Z., Wang, R. B., and Zhang, Y. (2011). Ethylene mediates UV-B-induced stomatal closure via peroxidase-dependent hydrogen peroxide synthesis in *Vicia faba* L. *J. Exp. Bot.* 62, 2657–2666. doi: 10.1093/Jxb/Erq431

Hotta, Y., Tanaka, T., Bingshan, L., Takeuchi, Y., and Konnai, M. (1998). Improvement of cold resistance in rice seedlings by 5-aminolevulinic acid. *J. Pestic. Sci.* 23, 29–33. doi: 10.1584/jpestics.23.29

Hotta, Y., Tanaka, T., Takaoka, H., Takeuchi, Y., and Konnai, M. (1997). Promotive effects of 5-aminolevulinic acid on the yield of several crops. *Plant growth Regul.* 22, 109–114. doi: 10.1023/A: 1005883930727

Hou, Z. H., Liu, G. H., Hou, L. X., Wang, L. X., and Liu, X. (2013). Regulatory function of polyamine oxidase-generated hydrogen peroxide in ethylene-induced stomatal closure in *Arabidopsis thaliana*. *J. Integr. Agr.* 12, 251–262. doi: 10.1016/S2095-3119(13)60224-5

Jia, H. F., Wang, J. A., Yang, Y. F., Liu, G. S., Bao, Y., and Cui, H. (2015). Changes in flavonol content and transcript levels of genes in the flavonoid pathway in tobacco under phosphorus deficiency. *Plant Growth Regul.* 76, 225–231. doi: 10.1007/s10725-014-9990-0

Jiang, Y., Wu, K., Lin, F., Qu, Y. N., Liu, X. X., and Zhang, Q. (2014). Phosphatidic acid integrates calcium signaling and microtubule dynamics into regulating ABA-induced stomatal closure in *Arabidopsis*. *Planta* 239, 565–575. doi: 10.1007/s00425-013-1999-5

Kim, T. H., Bohmer, M., Hu, H., Nishimura, N., and Schroeder, J. I. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. *Annu. Rev. Plant Biol.* 61, 561–591. doi: 10.1146/annurev-arplant-042809-112226

Kosar, F., Akram, N. A., and Ashraf, M. (2015). Exogenously-applied 5-aminolevulinic acid modulates some key physiological characteristics and antioxidative defense system in spring wheat (*Triticum aestivum* L.) seedlings under water stress. *S. Afr. J. Bot.* 96, 71–77. doi: 10.1016/j.sajb.2014.10.015

Kuhn, B. M., Errafi, S., Bucher, R., Dobrev, P., Geisler, M., Bigler, L., et al. (2016). 7-Rhamnosylated flavonols modulate homeostasis of the plant hormone auxin and affect plant development. *J. Biol. Chem.* 291, 5385–5395. doi: 10.1074/jbc.M115.701565

Kwak, J. M., Mori, I. C., Pei, Z. M., Leonhardt, N., Torres, M. A., Dangl, J. L., et al. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J.* 22, 2623–2633. doi: 10.1093/Emboj/Cdg277

Lawson, T., Simkin, A. J., Kelly, G., and Granot, D. (2014). Mesophyll photosynthesis and guard cell metabolism impacts on stomatal behaviour. *New Phytol.* 203, 1064–1081. doi: 10.1111/Nph.12945

Lea, M. A. (2015). Flavonol regulation in tumor cells. *J. Cell. Biochem.* 116, 1190–1194. doi: 10.1002/jcb.25098

Lee, N. K., Jeong, J. H., Oh, J., Kim, Y., Ha, Y. S., and Jeong, Y. S. (2015). Conversion of flavonols kaempferol and quercetin in mulberry (*Morus Alba* L.) leaf using plant-fermenting *Lactobacillus plantarum*. *J. Food Biochem.* 39, 765–770. doi: 10.1111/jfbc.12176

Li, D. M., Zhang, J., Sun, W. J., Li, Q., Dai, A. H., and Bai, J. G. (2011). 5-Aminolevulinic acid pretreatment mitigates drought stress of cucumber leaves through altering antioxidant enzyme activity. *Sci. Hortic.* 130, 820–828. doi: 10.1016/j.scienta.2011.09.010

Li, S., Assmann, S. M., and Albert, R. (2006). Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. *PLoS Biol.* 4:e312. doi: 10.1371/journal.pbio.0040312

Liu, D., Pei, Z., Naeem, M., Ming, D., Liu, H., Khan, F., et al. (2011). 5-Aminolevulinic acid activates antioxidative defence system and seedling growth in *Brassica napus* L. under water-deficit stress. *J. Agron. Crop Sci.* 197, 284–295. doi: 10.1111/j.1439-037X.2011.00465.x

Liu, D., Wu, L. T., Naeem, M. S., Liu, H. B., Deng, X. Q., Xu, L., et al. (2013). 5-Aminolevulinic acid enhances photosynthetic gas exchange, chlorophyll fluorescence and antioxidant system in oilseed rape under drought stress. *Acta Physiol. Plant.* 35, 2747–2759. doi: 10.1007/s11738-013-1307-9

Martens, S., Preuss, A., and Matern, U. (2010). Multifunctional flavonoid dioxygenases: flavonol and anthocyanin biosynthesis in *Arabidopsis thaliana* L. *Phytochemistry* 71, 1040–1049. doi: 10.1016/j.phytochem.2010.04.016

Miao, Y. C., Lv, D., Wang, P. C., Wang, X. C., Chen, J., Miao, C., et al. (2006). An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell* 18, 2749–2766. doi: 10.1105/tpc.106.044230

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498. doi: 10.1016/j.tplants.2004.08.009

Munemasa, S., Hauser, F., Park, J., Waadt, R., Brandt, B., and Schroeder, J. I. (2015). Mechanisms of abscisic acid-mediated control of stomatal aperture. *Curr. Opin. Plant Biol.* 28, 154–162. doi: 10.1016/j.pbi.2015.10.010

Munemasa, S., Muroyama, D., Nagahashi, H., Nakamura, Y., Mori, I. C., and Murata, Y. (2013). Regulation of reactive oxygen species-mediated abscisic acid signaling in guard cells and drought tolerance by glutathione. *Front. Plant Sci.* 4:472. doi: 10.3389/fpls.2013.00472

Naeem, M., Jin, Z., Wan, G., Liu, D., Liu, H., Yoneyama, K., et al. (2010). 5-Aminolevulinic acid improves photosynthetic gas exchange capacity and ion uptake under salinity stress in oilseed rape (*Brassica napus* L.). *Plant Soil* 332, 405–415. doi: 10.1007/s11104-010-0306-5

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., et al. (2014). Enhancement of oxidative and drought tolerance in *Arabidopsis* by overaccumulation of antioxidant flavonoids. *Plant J.* 77, 367–379. doi: 10.1111/tpj.12388

Neill, S., Desikan, R., and Hancock, J. (2002). Hydrogen peroxide signalling. *Curr. Opin. Plant Biol.* 5, 388–395. doi: 10.1016/S1369-5266(02)00282-0

Nguyen, N. H., Kim, J. H., Kwon, J., Jeong, C. Y., Lee, W., Lee, D., et al. (2016). Characterization of *Arabidopsis thaliana* FLAVONOL SYNTHASE 1 (FLS1)-overexpression plants in response to abiotic stress. *Plant Physiol. Biochem.* 103, 133–142. doi: 10.1016/j.plaphy.2016.03.010

Nishihara, E., Kondo, K., Parvez, M. M., Takahashi, K., Watanabe, K., and Tanaka, K. (2003). Role of 5-aminolevulinic acid (ALA) on active oxygen-scavenging system in NaCl-treated spinach (*Spinacia oleracea*). *J. plant physiol.* 160, 1085–1091. doi: 10.1078/0176-1617-00991

Pandey, A., Misra, P., and Trivedi, P. K. (2015). Constitutive expression of *Arabidopsis* MYB transcription factor, AtMYB11, in tobacco modulates flavonoid biosynthesis in favor of flavonol accumulation. *Plant Cell Rep.* 34, 1515–1528. doi: 10.1007/s00299-015-1803-z

Pei, Z. M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G. J., et al. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406, 731–734. doi: 10.1038/35021067

Pelletier, M. K., Burbulis, I. E., and Winkel-Shirley, B. (1999). Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. *Plant Mol. Biol.* 40, 45–54. doi: 10.1023/A:1026414301100

Pollastri, S., and Tattini, M. (2011). Flavonols: old compounds for old roles. *Ann. Bot.* 108, 1225–1233. doi: 10.1093/aob/mcr234

Rhee, S. G. (2006). H_2O_2, a necessary evil for cell signaling. *Science* 312, 1882–1883. doi: 10.1126/science.1130481

Schachtman, D. P., and Goodger, J. Q. D. (2008). Chemical root to shoot signaling under drought. *Trends Plant Sci.* 13, 281–287. doi: 10.1016/j.tplants.2008.04.003

Shang, Y., Dai, C., Lee, M. M., Kwak, J. M., and Nam, K. H. (2016). BRI1-associated receptor kinase 1 regulates guard cell ABA signaling mediated by open stomata 1 in *Arabidopsis*. *Mol. Plant* 9, 447–460. doi: 10.1016/j.molp.2015.12.014

Sharp, R. E., Bohnert, H. J., Springer, G. K., Davis, G. L., Schachtman, D. P., Wu, Y. J., et al. (2003). Root growth maintenance during water deficits: physiology to functional genomics. *J. Exp. Bot.* 54, 20–20. doi: 10.1093/jxb/erh276

Tanaka, Y., Sano, T., Tamaoki, M., Nakajima, N., Kondo, N., and Hasezawa, S. (2005). Ethylene inhibits abscisic acid-induced stomatal closure in *Arabidopsis*. *Plant Physiol* 138, 2337–2343. doi: 10.1104/pp.105.063503

Thompson, E. P., Wilkins, C., Demidchik, V., Davies, J. M., and Glover, B. J. (2010). An *Arabidopsis* flavonoid transporter is required for anther dehiscence and pollen development. *J. Exp. Bot.* 61, 439–451. doi: 10.1093/jxb/erp312

Tian, X., Ali, B., Qin, Y. B., Malik, Z., Gill, R. A., Ali, S., et al. (2014). Alleviation of lead toxicity by 5-aminolevulinic acid is related to elevated growth, photosynthesis, and suppressed ultrastructural damages in oilseed rape. *Biomed. Res. Int.* 2014, 1–11. doi: 10.1155/2014/530642

Wang, L. J., Jiang, W. B., and Huang, B. J. (2004). Promotion of 5-aminolevulinic acid on photosynthesis of melon (*Cucumis melo*) seedlings under low light and chilling stress conditions. *Physiol. Plant.* 121, 258–264. doi: 10.1111/j.0031-9317.2004.00319.x

Wang, Y., Noguchi, K., Ono, N., Inoue, S., Terashima, I., and Kinoshita, T. (2014). Overexpression of plasma membrane H^+-ATPase in guard cells promotes light-induced stomatal opening and enhances plant growth. *Proc. Nat. Acad. Sci. U.S.A.* 111, 533–538. doi: 10.1073/pnas.1305438111

Watkins, J. M., Hechler, P. J., and Muday, G. K. (2014). Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. *Plant Physiol.* 164, 1707–1717. doi: 10.1104/pp.113.233528

Wei, Z. Y., Zhang, Z. P., Lee, M. R., Sun, Y. P., and Wang, L. J. (2012). Effect of 5-aminolevulinic acid on leaf senescence and nitrogen metabolism of pakchoi under different nitrate levels. *J. Plant Nutr.* 35, 49–63. doi: 10.1080/01904167.2012.631666

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. *Curr. Opin. Plant Biol.* 5, 218–223. doi: 10.1016/S1369-5266(02)00256-X

Xie, L., Wang, Z. H., Cheng, X. H., Gao, J. J., Zhang, Z. P., and Wang, L. J. (2013). 5-Aminolevulinic acid promotes anthocyanin accumulation in Fuji apples. *Plant Growth Regul.* 69, 295–303. doi: 10.1007/s10725-012-9772-5

Xu, F., Cheng, S. Y., Zhu, J., Zhang, W. W., and Wang, Y. (2011). Effects of 5-aminolevulinic acid on chlorophyll, photosynthesis, soluble sugar and

flavonoids of ginkgo biloba. *Not. Bot. Hortic. Agrobo.* 39, 41–47. doi: 10.15835/nbha3915880

Xu, F., Zhu, J., Cheng, S. Y., Zhang, W. W., and Wang, Y. (2010). Effect of 5-aminolevulinic acid on photosynthesis, yield, nutrition and medicinal values of kudzu (*Pueraria phaseoloides*). *Trop. Grasslands* 44, 260–265.

Yamasaki, H., Sakihama, Y., and Ikehara, N. (1997). Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H2O2. *Plant Physiol.* 115, 1405–1412. doi: 10.1104/pp.115.4.1405

Yao, S. M., Wang, W. J., and Chen, G. X. (2006). Effects of 5-aminolevulinic acid on phosphate absorption and distribution in rice using 32P tracer method. *Plant Nutr. Fert. Sci.* 12, 70–75.

Zhang, J., Li, D. M., Gao, Y., Yu, B., Xia, C. X., and Bai, J. G. (2012). Pretreatment with 5-aminolevulinic acid mitigates heat stress of cucumber leaves. *Biol. Plant.* 56, 780–784. doi: 10.1007/s10535-012-0136-9

Zhang, L. Y., Feng, X. X., Gao, J. J., An, Y. Y., Tian, F., Li, J., et al. (2015). Effects of rhizosphere-applied 5-aminolevulinic acid (ALA) solutions on leaf physiological characteristics and fruit quality of apples. *Jiangsu J. Agr. Sci.* 31, 158–165.

Zhang, Z. P., Yao, Q. H., and Wang, L. J. (2010). Expression of yeast Hem1 gene controlled by *Arabidopsis* HemA1 promoter improves salt tolerance in *Arabidopsis* plants. *BMB Rep.* 43, 330–336. doi: 10.5483/Bmbrep.2010.43.5.330