Exogenous ethanol treatment alleviates oxidative damage of *Arabidopsis thaliana* under conditions of high-light stress

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**Abstract** Abiotic stresses, such as high light and salinity, are major factors that limit crop productivity and sustainability worldwide. Chemical priming is a promising strategy for improving the abiotic stress tolerance of plants. Recently, we discovered that ethanol enhances high-salinity stress tolerance in *Arabidopsis thaliana* and *rice* by detoxifying reactive oxygen species (ROS). However, the effect of ethanol on other abiotic stress responses is unclear. Therefore, we investigated the effect of ethanol on the high-light stress response. Measurement of chlorophyll fluorescence showed that ethanol mitigates photo inhibition under high-light stress. Staining with 3,3′-diaminobenzidine (DAB) showed that the accumulation of hydrogen peroxide (H2O2) was inhibited by ethanol under high-light stress conditions in *A. thaliana*. We found that ethanol increased the gene expressions and enzymatic activities of antioxidative enzymes, including ASCORBATE PEROXIDASE1 (*AtAPX1*), Catalase (*AtCAT1* and *AtCAT2*). Moreover, the expression of flavonoid biosynthetic genes and anthocyanin contents were upregulated by ethanol treatment during exposure to high-light stress. These results imply that ethanol alleviates oxidative damage from high-light stress in *A. thaliana* by suppressing ROS accumulation. Our findings support the hypothesis that ethanol improves tolerance to multiple stresses in field-grown crops.

**Key words:** *Arabidopsis thaliana*, ethanol, high-light stress, reactive oxygen species.

**Introduction**

Light provides a source of energy for photosynthesis and plays a crucial role in various plant developmental processes (Jiao et al. 2007). However, plants often encounter high-light intensity that exceeds their photosynthetic capacity, known as high-light stress. High-light stress causes the accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), superoxide anion (O2•−), and singlet oxygen (¹O2), as a result of the transport of electrons and transfer of excitation energy (Apel and Hirt 2004; Pospíšil 2016). Excess ROS accumulation leads to membrane oxidation and the disruption of photosystems resulting in severe cellular damage (Farnese et al. 2016). Thus, plants have evolved several efficient protective mechanisms to avoid the accumulation of ROS to harmful levels. Superoxide dismutase (SOD) converts O2•− into H2O2, which is subsequently converted into H2O by ascorbate peroxidase (APX) and catalase (CAT). These three enzymes have a major role in scavenging O2•− into H2O (Bowler et al. 1992; Shigeoka et al. 2002; Willekens et al. 1995). In addition, many nonenzymatic molecules, such as ascorbate, glutathione, and flavonoids, including anthocyanins, serve as antioxidants and contribute to the protection against ROS (Foyer and Shigeoka 2011; Pietta 2000). ROS detoxifying enzymes and antioxidants function in protecting the photosynthetic apparatus from oxidative damage (Yoshio-Nishimura 2016).

Recently, chemical priming has gained attention because it has been shown that single compounds often improve multi-stress in various crop species (i.e., nitroprusside) (Sako et al. 2020). Previously, we revealed that the exogenous application of ethanol enhanced salinity stress tolerance in *Arabidopsis thaliana* and rice (Nguyen et al. 2017). Transcriptome analysis showed that ethanol treatment upregulated the gene expressions of ROS-related transcription factors and scavengers. Furthermore, ethanol increased total APX activity and reduced H2O2 accumulation to improve salinity stress tolerance in plants. High-light intensity causes the production of ROS, which triggers oxidative damage.

Abbreviations: ROS, reactive oxygen species; H2O2, hydrogen peroxide; APX, ascorbate peroxidases; DAB, 3,3′-diaminobenzidine.

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to the photosystem, known as photoinhibition. We hypothesized that ethanol treatment can remove ROS produced not only by salinity but also by high-light stress and may enhance tolerance to high-light stress. In this study, we investigated the effect of ethanol on high-light stress in *A. thaliana*. The results indicate that ethanol treatment increases antioxidant enzyme activities and the expression of flavonoid biosynthetic pathway-related genes as well as reduced ROS accumulation under high-light conditions, leading to the amelioration of photodamage.

**Materials and methods**

*Plant materials and growth conditions*

*A. thaliana* (ecotype Columbia-0) seeds were sterilized and stratified for 4 days at 4°C. Seeds were sown in Murashige and Skoog (MS) medium supplemented with 1% sucrose at 22°C under long day conditions (16h/8h light/dark) and a light intensity of 40–60 μmol photons m⁻²s⁻¹. For ethanol treatment, Col-0 seeds were sown in MS medium containing 10 mM (0.059 v/v%) ethanol and grown for 2 weeks under the same light conditions as the control. For high-light stress, 2-week-old *A. thaliana* plants were grown on MS medium containing 10 mM ethanol treated with or without high-light for 24 h were followed by anthocyanin measurement. Anthocyanin content was calculated as (A₅₃₅−2.2A₆₅₀)/g·FW.

**Enzyme assay**

Two-week-old *A. thaliana* plants grown on MS medium containing 10 mM ethanol treated with or without high-light stress for 24 h were analyzed using enzymatic assays. The experiment was performed using three biological replicates. Proteins were extracted from three plants and the APX assay and CAT assay were performed as described previously (Du et al. 2008; Nguyen et al. 2017). The protein content was determined as described previously.

**Chlorophyll fluorescence measurements**

Chlorophyll fluorescence of leaves was measured at room temperature using a Junior-PAM (Walz, Effeltrich, Germany). Before the chlorophyll fluorescence measurements, plants were dark-adapted for 30 min. The results represent the mean values of at least four measurements.

**Results**

**Ethanol mitigates photoinhibition under conditions of high-light stress**

To investigate the effect of ethanol treatment on the plant growth and high-light stress response, we evaluated the growth and photosynthetic efficiency using 2-week-old *A. thaliana* plants grown on medium containing 10 mM ethanol. We confirmed that the plant growth on medium with 10 mM ethanol for two weeks was not significantly different compared with that of non-treated plants (Supplementary Figure S1A, B). We monitored the maximum photochemical efficiency of PSII (Fv/Fm) in leaves from untreated and ethanol-treated plants for 3 days under high-light stress condition. Photosynthetic activity in control plants was decreased with incubation time under high-light stress. In contrast, ethanol-treated plants showed a lower reduction of photosynthetic activity for at least 2 days (Figure 1), which suggests that ethanol treatment alleviates the damage to photosynthesis under high light.

**Ethanol treatment prevents hydrogen peroxide overaccumulation under conditions of high-light stress**

We previously showed that ethanol treatment repressed the accumulation of H₂O₂ under salinity stress conditions (Nguyen et al. 2017). In addition to salinity, other abiotic stress conditions, such as high-light, drought, and cold, are known to trigger ROS production in plants (Farnese et al. 2016; Mullineaux et al. 2018). To investigate whether ethanol detoxifies ROS produced...
by high-light stress, the accumulation of \( \text{H}_2\text{O}_2 \) under high-light stress in ethanol-treated plants was verified by using DAB staining (Figure 2A, B). Leaves of plants treated with high light for 72 h were significantly stained by DAB, which indicates that \( \text{H}_2\text{O}_2 \) was significantly accumulated. On the other hand, DAB staining showed that ethanol treatment resulted in a significantly reduced accumulation of \( \text{H}_2\text{O}_2 \) in plants (Figure 2A, B). These results indicate that ethanol treatment detoxifies \( \text{H}_2\text{O}_2 \) produced by high-light stress.

**Ethanol enhances the detoxification of ROS under conditions of high-light stress**

Ethanol reduced \( \text{H}_2\text{O}_2 \) accumulation under high-light stress conditions. We confirmed whether ethanol activates the transcription of ROS detoxifying enzymes similar to that occurring during salinity stress. Since cytosolic \text{AtAPX1} is required for the protection of chloroplasts against ROS (Davletova et al. 2005) and ethanol treatment upregulates \text{AtAPX1} expression levels during salinity stress, we investigated the expression of \text{AtAPX1} under high-light conditions. Consistent with previous reports (Davletova et al. 2005), \text{AtAPX1} expression was increased by high-light exposure. Furthermore, ethanol treatment induced \text{AtAPX1} expression levels under high-light conditions (Figure 3A). In addition to APX, catalase (CAT) and glutathione peroxidase (GPX) activities are also important to detoxify \( \text{H}_2\text{O}_2 \) (Apel and Hirt 2004). Thus, we evaluated expression levels of \text{AtCAT1}, \text{AtCAT2} and \text{AtGPX7}, which have functions in light response (Gaber et al. 2012; Vanderauwera et al. 2004). \text{AtCAT1} and \text{AtCAT2} expressions were increased in plants treated with ethanol under high-light stress (Figure 3B, C). On the other hand, there was no significant difference in \text{AtGPX7} level in plants treated with or without ethanol, even though the expression was induced by high-light stress in both plants treated with or without ethanol (Supplementary Figure S2). These results suggest that ethanol activates \text{AtAPX1} and \text{AtCAT1,2} in plants. To clarify whether ethanol regulates APX and CAT activities under high-light stress, the enzyme assays were performed using plants treated with high light for 24 h. The results indicated that total APX activity and total CAT activity were higher in ethanol-treated plants compared with untreated controls under conditions of high-light stress (Figure 3D, E). In contrast, no significant differences for APX and CAT activities were observed between ethanol-treated and untreated control plants under non-stress conditions. Our data indicate that ethanol induces the transcription of \text{AtAPX1}, and \text{AtCATs} genes and enhances APX and CAT activities under high-light stress.

**Ethanol upregulates the expression of flavonoid biosynthesis-related genes under conditions of high-light stress**

Flavonoids, such as anthocyanin, are antioxidative compounds and accumulate in plant tissues in response to different types of abiotic stresses, including high light (Dixon and Paiva 1995; Vanderauwera et al.
Anthocyanin was reported to modulate ROS levels under abiotic stress conditions in vivo (Xu et al. 2017). A previous transcriptome analysis showed that both ethanol treatment and salinity stress induced the expression of flavonoid biosynthetic pathway-related genes (Nguyen et al. 2017). We examined the expression of the R2R3-MYB transcription factors AtMYB12 and AtMYB111, in ethanol-treated plants under high-light stress by qRT-PCR. MYB12 and MYB111 are key regulators of the flavonol pathway and are induced by high light. The induction correlates with flavonol biosynthesis (Mehrtens et al. 2005; Stracke et al. 2007). The expression of the AtMYB12 and AtMYB111 genes was highly upregulated in ethanol-treated plants under high-light conditions compared with non-treated plants (Figure 4A, B). Anthocyanin biosynthetic genes such as AtDFR and AtLDOX also increased in ethanol-treated plants under high-light conditions (Figure 4C, D). We confirmed that anthocyanin content was higher in plants treated with ethanol than non-treated plants under high-light stress (Figure 4E). These results suggest that reduced H$_2$O$_2$ accumulation under high-light stress by ethanol treatment may result from increased activity of detoxification enzymes and the biosynthesis of antioxidants.

### Discussion

In the present study, we showed that ethanol treatment enhances transcription and activities of antioxidant enzymes under conditions of high-light stress. In addition, the expression of anthocyanin biosynthesis-related genes and anthocyanin contents were also upregulated by exogenous ethanol treatment. The activation of these antioxidant systems by ethanol was associated with a reduction in H$_2$O$_2$ accumulation, leading to the mitigation of photoinhibition under high-light stress in *A. thaliana*. Photoinhibition is determined by the balance between the rate of photodamage to photosystem II (PSII) and the rate of its repair (Takahashi and Murata 2008). ROS were reported to inhibit the translation of factors required for the repair of PSII (Nishiyama et al. 2011). Our findings show that ethanol activates detoxifying H$_2$O$_2$ under high-light conditions, suggesting that decreased ROS levels following ethanol treatment enhance the repair of PSII and mitigate photodamage.

Ethanol treatment activated the antioxidant system under high-light stress condition not normal conditions. A previous report showed that mild osmotic treatment for 10 days altered histone modification, and several
modified genes showed different transcriptional response not under the normal condition but under the second stress treatment (Sani et al. 2013). Further, some reports suggested that histone modifications, including acetylation, methylation, and phosphorylation were affected by ethanol exposure in utero (Mandal et al. 2017). These results imply that ethanol treatment might affect histone modification for the rapid response to high-light stress, although further analysis is necessary to reveal how ethanol affects epigenetic regulation.

High-light and other abiotic stresses often occur concomitantly, and high light aggravates plant growth under abiotic stress, such as that occurring with salinity (Chen et al. 2017). Light is an inevitable factor during the application of ethanol to crops in the field. A recent report showed that managing the overall levels of ROS is essential for plant survival during multi-stress (Zandalinas et al. 2021). So far, we have shown that ethanol treatment mitigates the accumulation of ROS under high-light and salinity stress. Therefore, ethanol is a promising chemical priming agent for enhancing multi-abiotic stress tolerance of field crops.

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Author contributions
KS conceived the idea for the project and designed the experiments. KS and RN performed the experiments. KS, MT and MS wrote the manuscript.

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Figure 4. Gene expression of flavonoid biosynthesis-related transcription factors is upregulated by ethanol treatment under conditions of high-light stress. (A–D) Relative expression of AtMYB111 (A), AtMYB12 (B), AtDFR (C), and AtLDOX (D) after high-light exposure for 0 and 6 h in the presence or absence of 10 mM ethanol. The expression level of the unstressed plants grown on normal MS medium was set as 1. Three biological repeats were performed. Error bars represent the mean ± SE. Statistical significance was determined by ANOVA followed by post-hoc Tukey’s test. Means that differed significantly (p < 0.05) are indicated by different letters.

(E) Anthocyanin contents of the seedlings treated with high-light stress for 0 and 24 h. Three biological repeats were performed. Error bars represent the mean ± SE. Statistical significance was determined by ANOVA followed by post-hoc Tukey’s test. Means that differed significantly (p < 0.05) are indicated by different letters.
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