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# ZMFLS1 PROMOTES OXIDATIVE STRESS RESISTANCE BY REGULATING FLAVONOL BIOSYNTHESIS IN TRANSGENIC *ARABIDOPSIS THALIANA*

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## ABSTRACT

Many biological and abiotic stresses can lead to excessive reactive oxygen species accumulation and oxidative damage in plants. The purpose of the present study was to elucidate the function of maize flavonol synthase 1 (ZmFLS1) in alleviating oxidative stress. The coding sequence of *ZmFLS1* was cloned from maize B73 complementary DNA and was introduced into *Arabidopsis thaliana*. Two *ZmFLS1* overexpression lines, OE-6 and OE-8, showed a significant increase in the accumulation of flavonoids, especially flavonols. The antioxidant capacity test showed that overexpression of *ZmFLS1* resulted in a higher oxygen radical scavenging rate. Consistent with those, transgenic plants with *ZmFLS1* overexpression showed enhanced oxidative stress tolerance and lower oxidative damage. In conclusion, ZmFLS1 promoted plant oxidative stress tolerance through regulating flavonoid biosynthesis and accumulation, especially flavonols, and *ZmFLS1* has the potential in mitigating oxidative damage caused by environmental stress in plants.

**Key words**: ZmFLS1; Flavonols; Oxidative stress; MDA (Malondialdehyde)
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## INTRODUCTION

Biological and abiotic stresses promote the accumulation of ROS (reactive oxygen species) in plant cells (Li *et al*. 2008; Gill and Tuteja 2010; Zhang *et al*. 2014; Qi *et al*. 2018; Li *et al*. 2019). ROS accumulation to appropriate levels effectively transduces stress signals and causes defense responses. However, excessive ROS can oxidize proteins, lipids, nucleic acids, and other macromolecular substances, altering the balance between production and scavenging of oxygen free radical in plants and causing irreversible oxidative damage (Choudhury *et al*. 2017; Mittler 2017). The antioxidant systems in plants, including non-enzymatic and enzymatic systems have crucial protective roles in alleviating stress induced by ROS accumulation and injury (Miller *et al*. 2010; Suzuki *et al*. 2011; Prasad *et al*. 2016; Choudhury *et al*. 2017; Chapman *et al*. 2019).

Flavonols, important secondary metabolites derived from the plant phenylpropanoid pathway, accumulate in various tissues of plants in the form of glycosides and are widely involved in plant growth and plant responses to various stresses (Gill and Tutej, 2010; Nakabayashi *et al*. 2014a; Nguyen *et al*. 2016; Xu *et al*. 2018; Muhlemann *et al*. 2018). Muhlemann *et al*. (2018) showed that flavonols control the growth and integrity of pollen tubes under high-temperature stress by regulating ROS dynamic balance. Wang *et al*. (2016) reported that the Arabidopsis flavonol biosynthesis regulator MYB12 regulates plant abiotic stress tolerance via the accumulation of flavonoids. In Arabidopsis, flavonol accumulation promoted drought tolerance in transgenic Arabidopsis (Nakabayashi *et al*. 2014b). The function of flavonols in plant stress provides a good way for us to improve plant growth and stress tolerance by regulating its biosynthesis.

Flavonols are formed from l-phenylalanine, the initiator of the phenylpropanoid secondary metabolic pathway, which is catalyzed by various key enzymes including phenylalanine ammonialyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid synthase (FLS) and flavanone carboxylase (F3H) (Lepiniec *et al*. 2006; Stracke *et al*. 2007). Among those key enzymes, FLS catalyzes the desaturation of dihydroflavonol to form flavonols which is the last step of the flavonol biosynthesis (Owens *et al*. 2008; Li *et al*. 2013; Nguyen *et al*. 2016). *FLS* genes have been cloned from various plants, and FLS is relatively conservative in structure and function. In *Arabidopsis thaliana*, six AtFLSes have been identified and AtFLSl confers the highest catalytic activity (Owens *et al*. 2008). Nguyen *et al*. (2016) reported that transgenic Arabidopsis with elevated expression of *AtFLS1* showed higher flavonol accumulation and less anthocyanin content, while *Atfls1* mutants showed the opposite phenotype. Li *et al*. (2013) reported that the different expressions of *FtFLS1* and *FtFLS2* in *Tartary buckwheat* led to differential flavonol accumulation, highlighting the different roles of FtFLS1 and FtFLS2 in buckwheat growth and environmental

adaptation. Also, the expression and function of *FLS* genes are affected by plant growth/developmental stage, tissue type, differential environment, etc. suggesting their differential roles in flavonol biosynthesis.

*ZmFLS1* was identified in maize (*Zea mays* L.) B73 genome, and was induced by ultraviolet radiation (Falcone Ferreyra *et al*. 2012). However, the role of ZmFLS1 in catalyzing flavonol biosynthesis and improving plant abiotic stress tolerance has not been confirmed. In this study, we generated two *ZmFLS1* overexpression *Arabidopsis thaliana* lines (OE-6 and OE-8) to investigate the functions of *ZmFLS1* in flavonol synthesis and oxidative stress resistance. Transgenic Arabidopsis with *ZmFLS1* overexpression showed significant promotion in flavonol accumulation and oxidative stress resistance. The functional confirmation of *ZmFLS1* provides an important theoretical foundation for improving flavonoid synthesis and alleviating ROS accumulation and injury in plants by using *ZmFLS1* under stress conditions.

## MATERIALS AND METHODS

***Plant materials and growth conditions:*** In this study, *Arabidopsis thaliana* wild type (WT, Columbia-0) was used for studying the function of ZmFLS1. For germination and growth, 75% ethanol and 0.1% $HgCl_2$ were used to disinfect seeds, then the disinfected seeds were rinsed 3-5 times in sterile water and sown on MS (Murashige and Skoog) plates (0.6% agar) with or without $H_2O_2$ (Hydrogen peroxide) and quercetin. After vernalization for 2-4 d at 4℃, the plates were placed in a growth chamber (20 ± 1℃, light/dark cycle: 16 h light/8 h dark) (Li *et al*. 2019; Hu *et al*. 2019). Germination rate statistics, phenotype observation, and photography were performed during the growth process. When different phenotypes appeared, samples were collected to determine antioxidant capacity and MDA content.

***Oxidative stress treatment:*** Oxidative stress treatment was performed as the method described by Miao *et al*. (2006). During seed germination and seedling growth, 5.0 mM of $H_2O_2$ was added into MS medium for oxidative stress tolerance detection. $H_2O_2$ directly oxidizes plant tissues, causing oxidative damage. Seeds (WT and transgenic lines) were seeded on MS with or without 5 mM $H_2O_2$ and cultured in a growth chamber to observe phenotypes after germination.

***Recombinant vector construction and transformation*:** Recombinant vector construction and transformation were performed according to the methods of Li *et al*. (2019). The CDS (coding sequence) of *ZmFLS1* was amplified using the maize B73 cDNA (complementary DNA) as the template with the specific primers (35s::*GFP-ZmFLS1* F: 5'-gatcctctagaATGGGGGGCGAGACGCACCTGAGCGTGCAGG-3'; 35s::*GFP-ZmFLS1* R: 5'-tatttaaatgtcgaccccgggTTACATGGGGAGCTTGTTGATCTTGCAGTG-3'). The CDS of encoding GFP (Green fluorescent protein) was obtained by PCR (polymerase chain reaction) amplification with specific primers (35s::*GFP* F: 5'-aagcttctgcaggggcccgggATGGGATCCACCATGGTGAGC-3'; 35s::*GFP* R: 5'-cgtctcgccccccatTCTAGAGGATCCGTTCAAGTCTTCT-3') using the template of plasmid pCM3300M-GFP, a maize transgenic vector with GFP CDS. The CDSes of *GFP* and *ZmFLS1* were inserted into a vector (pCAMBIA1300) using a kit (ClonExpressTM II/MultiS One Step Cloning Kit, C112-01/C113-01) (Vazyme Biotech, Nanjing, CN) to create the 35S::*GFP-ZmFLS1* recombinant vector. After sequencing and confirmation, the recombinant vector was introduced into Agrobacterium *tumefaciens* (GV3101). Transgenic seedlings overexpressing *ZmFLS1* were produced by Agrobacterium-mediated floral dipping (Zhang *et al*. 2006; Li *et al*. 2019). Potentially positive transformed seedlings were screened on MS culture medium with hygromycin (25 mg/L) and transplanted into vermiculite and nutrient soil mixture (Nutrient soil: vermiculite=1:1) and cultured in a growth room. Total RNA was isolated and reverse-transcribed with a reverse transcription Kit (M1701, Promega). The actin gene in Arabidopsis (actin specific primers: F 5'-GCACCCTGTTCTTCTTACCGA-3'and R 5'-AGTAAGGTCACGTCCAGCAAGG -3') was used as the internal standard. The expression level of *ZmFLS1* in transgenic seedlings was detected with specific primers (*ZmFLS1* F: 5'-GACGGTGAACAAGGAGAAGAC-3', ZmFLS1 R: 5'-GGCCATGCATGCGACTGGAAT-3') and performed on a Real-Time PCR System (Roche 480II, Roche Diagnostics Corporation, Germany). The relative expression of *ZmFLS1* was calculated according to the method described by Li *et al*. (2019).

***Determination of total flavonoid content:*** Total flavonoids content was determined according to the method described by Jia *et al*. (1999). Samples (transgenic seedlings and the corresponding control) were fully ground and flavonoids were extracted with 1% (V/V) HCl-methanol, then centrifuged for 5 min at 8,000-10,000 rpm. The supernatant was mixed with an equal volume of chloroform, fully mixed, centrifuged, and the obtained supernatant was collected. The specific absorption values of reaction liquid (657 nm, 530 nm, and 340 nm) were measured with spectrophotometric quantification.

***Flavonol staining of whole seedlings:*** Flavonol staining of whole seedlings was performed with the method described by Stracke *et al*. (2007) and An *et al*. (2016). *Arabidopsis thaliana* seeds were sown on filter paper soaked with norflurazon (3 ppm, Supelco; PS-1044)

which blocks carotenoid biosynthesis, and were grown for five days under at 20 ± 1℃, and a 16 h/8 h (light/dark) photoperiod. Then, seedlings were stained with freshly prepared DPBA (diphenylboric acid 2-aminoethyl, D9754, Sigma-Aldrich) dye solution (50 mM KCl, 10 mM MES, 0.1 mM $CaCl_2$, , 0.01% Triton X-100, 2.25 mg mL-1 DPBA, pH 6.2) in the dark at 25-30℃ for at least 2 h. After staining, fresh MES- KCl buffer was used to remove excess DPBA dye. DPBA fluorescence of whole seedlings was visualized on an inverted microscope (Olympus IX73) using an excitation 340-380 nm wavelength and 425 nm long-pass splitter.

***Determination of oxygen free radical scavenging ability*:** The antioxidant capacity was determined by assessing the scavenging capacity of plant seedling extracts on oxygen free radicals. The oxygen free radical scavenging ability of seedling extracts was measured with the methods described by Li *et al*. (2019). The extracts and pyrogallol solution (6 mM) (1:1) were mixed for 4 min and terminated by concentrated hydrochloric acid. The absorbance (320 nm) of the reaction solution was measured to calculate the removal capacity of seedling extracts exposed to $O^{2-}$. An equal volume of seedling extracts and various reagents (including $H_2O_2$ (8.8 mM), salicylic acid-ethanol (9 mM) and ferrous sulfate (9 mM)) were fully mixed, and incubated at 37℃ for 30 min. The special absorbance (512 nm) of the reaction mixture was measured to calculate the removal capacity of seedling extracts to -OH (hydroxyl radicals). The seedling extracts and DPPH (2, 2-Diphenyl-1-picrylhydrazyl) solution (1:1) were mixed and incubated at 25-30℃ for 30 min. The special absorbance (517 nm) was measured to calculate the scavenging ability of seedling extracts to DPPH radical.

***Determination of MDA content:*** MDA content was measured according to the methods described by Quan *et al*. (2004). Samples (0.1 g) were ground and MDA was extracted with 10 mL trichloroacetic acid (10%, v/v). The mixture was centrifuged for 10 minutes (4,000 rpm), then an equal volume of thiobarbituric acid (0.67%) was added to the supernatant, mixed and were reacted in a boiling water bath for 15 minutes. After rapid cooling, the specific absorption values of reaction liquid (532 nm, 600 nm, and 450 nm) were measured with spectrophotometric quantification. MDA content was calculated by the formula [$6.45^{*}$(A532-A600)-$0.56^{*}$A450].

***Statistical analysis:*** All statistics are means (±SD) of at least three independent replications. SPSS 13.0 (Chicago, IL, USA) was used to perform statistical analyses. Significant and extremely significant differences were determined based on $P \leq 0.05$ and $P \leq 0.01$, which represented by "*" and "**", respectively.

## RESULTS

***Generation of transgenic Arabidopsis lines overexpressing ZmFLS1:*** The recombinant vectors (pCAMBIA1300-*GFP* and pCAMBIA1300 -*GFP*-*ZmFLS1*) (Fig. 1A) were introduced into WT (Col-0) by Agrobacterium-mediated transformation. Transformed plants ($T_0$ generation) were screened on MS medium containing hygromycin and at least fifteen transgenic seedlings were identified from our screen. Two *ZmFLS1* overexpression dominant lines (#6, #8) with high *ZmFLS1* expression were selected for subsequent analysis (Fig. 1B).

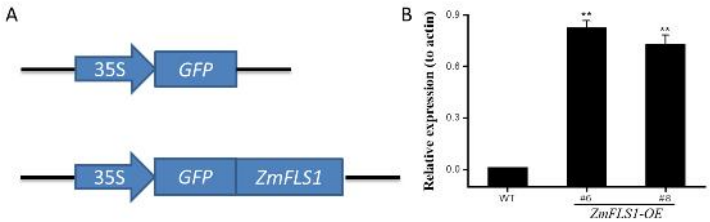


**Fig.1. Schematic structure of the 35s::*GFP-ZmFLS1*and molecular analysis of *ZmFLS*1-expressing lines. (A) Schematic diagram of binary plasmid pCAMBIA1300-*GFP* and pCAMBIA1300-*ZmFLS1-GFP*; (B) Relative expression of *ZmFLS1* in transgenic plants was detected by qRT-PCR. n=3, Error bars are standard deviations. $^{**}P < 0.01$, $^{*}P < 0.05$**

***ZmFLS1 overexpression improves accumulation of flavonols*:** Falcone Ferreyra *et al*. (2012) reported that ZmFLS1 functions as a maize flavonol synthase which is considered to be the key enzyme catalyzing the last step of flavonol biosynthesis. Total flavonoid and anthocyanin (an important class of flavonoids) contents were measured to detect the effects of ZmFLS1 on flavonol synthesis in transgenic Arabidopsis. Total flavonoid content was significantly higher in transgenic seedlings than that in WT (Fig. 2A), while there was no significant difference in anthocyanin content between transgenic plants and the corresponding control (Fig. 2B), indicating the specificity of ZmFLS1 in catalyzing flavonol biosynthesis. The flavonol accumulation in five-day-old Arabidopsis seedlings was determined using flavonol-specific DPBA staining. Consistent with total flavonoid

content, transgenic Arabidopsis seedlings (#6 and #8) showed more orange fluorescence and the relative fluorescence intensity of DPBA in transgenic seedlings was significantly higher than that of the corresponding control, indicating a higher accumulation of flavonols in transgenic seedlings (Fig. 2C, D). These results suggest that the overexpression of *ZmFLS1* in *Arabidopsis thaliana* promotes the synthesis and accumulation of flavonoids, especially flavonols.

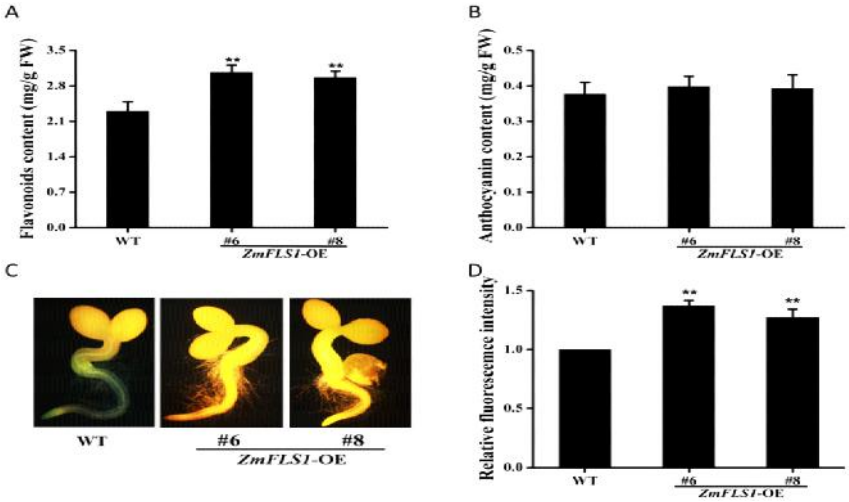


**Fig.2. Comparison of total flavonoid and flavonol accumulation under normal conditions.**

**(A) Three independent experiments were carried out, and the average flavonoid content was obtained; (B) Three independent experiments were carried out, and the average anthocyanin content was obtained; (C) *Arabidopsis thaliana* seeds were infiltrated for 4 days with 16 h light per day on filter paper soaked with 3 ppm. of the bleaching reagent norflurazon (Supelco; PS-1044). The bleached seedlings were stained to saturation for at least 2 h in a freshly prepared solution of 0.25% (w/v) DPBA, 0.00375% (v/v) Triton X-100. Photographs of representative seedlings are shown; (D) The DPBA staining intensity of each sample was quantified by relative gray value which compared with the corresponding control. n=5, Error bars are standard deviations. $^{**}P<0.01$; $^{*}P<0.05$**

***Antioxidant capacity is promoted on transgenic Arabidopsis thaliana*:** As non-enzymatic antioxidants, the promotion of flavonol accumulation likely promotes higher antioxidant capacity. The scavenging capacity of plant extracts to oxygen free radical between transgenic plants and the corresponding control were compared to investigate the effects of flavonol accumulation caused by ZmFLS1. The scavenging capacity of *ZmFLS1*-overexpressing seedlings extracts to $O^{2-}$ was higher than that of WT seedlings extracts (Fig. 3A). The scavenging activity of seedling extracts to DPPH was also higher in transgenic plants and WT, although both transgenic plants and WT showed a downward trend under oxidative stress conditions (Fig. 3B). These results are consistent with flavonol accumulation.

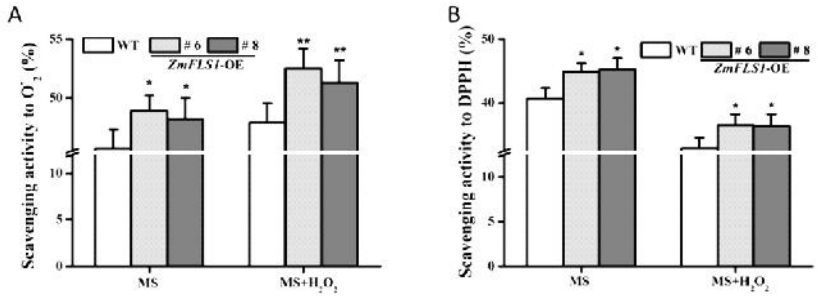


**Fig.3. Comparison of oxygen free radicals scavenging capacities under normal and $H_2O_2$ stress conditions. (A) Scavenging capacity of seedling extracts to $O^{2-}$ under normal and $H_2O_2$ stress conditions. (B) Scavenging capacity of seedling extracts to DPPH under normal and $H_2O_2$ stress conditions. 20-day-old seedlings grown on MS or MS with 5.0 mM $H_2O_2$ were selected to compare the scavenging ability to oxygen free radical ($O^{2-}$ and DPPH) of seedlings extracts. Three independent experiments were carried out, and the average scavenging rate was obtained. Error bars are standard deviations. $^{**}P<0.01$; $^{*}P<0.05$**

***Seed germination and seedling growth under oxidative stress:*** As non-enzymatic antioxidants, flavonols contribute to ROS scavenging and oxidative stress tolerance in plants (Li *et al*. 2019). To detect differences in oxidative stress tolerance between transgenic plants and WT, Arabidopsis seeds were plated on solid MS medium containing 5.0 mM $H_2O_2$ or 5.0 mM $H_2O_2$ and 10 µm quercetin (a type of flavonol) to detect the effect of flavonol accumulation caused by ZmFLS1 on plant growth. As illustrated in Figure 4A, B, there was no obvious difference during germination and seedling growth between *ZmFLS1* overexpression lines and WT on MS or MS with $H_2O_2$ and quercetin. And transgenic lines displayed relatively faster germination and higher tolerance to $H_2O_2$. As a membrane oxidation product, MDA content indicates the degree of damage of plants under oxidative stress. The MDA content of transgenic plants and WT growing on MS with or without $H_2O_2$ or $H_2O_2$ and quercetin was determined. As shown in figure 4C, the accumulation level of MDA was relatively lower in transgenic plants on MS with 5 mM $H_2O_2$ than that of WT, indicating decreased oxidative injury. No significant difference in MDA content was observed between transgenic plants and the corresponding plants on MS or MS with $H_2O_2$ and quercetin. These results suggest that ZmFLS1 regulates the biosynthesis and accumulation of flavonols in *Arabidopsis thaliana*, thereby affecting plant tolerance to oxidative stress.

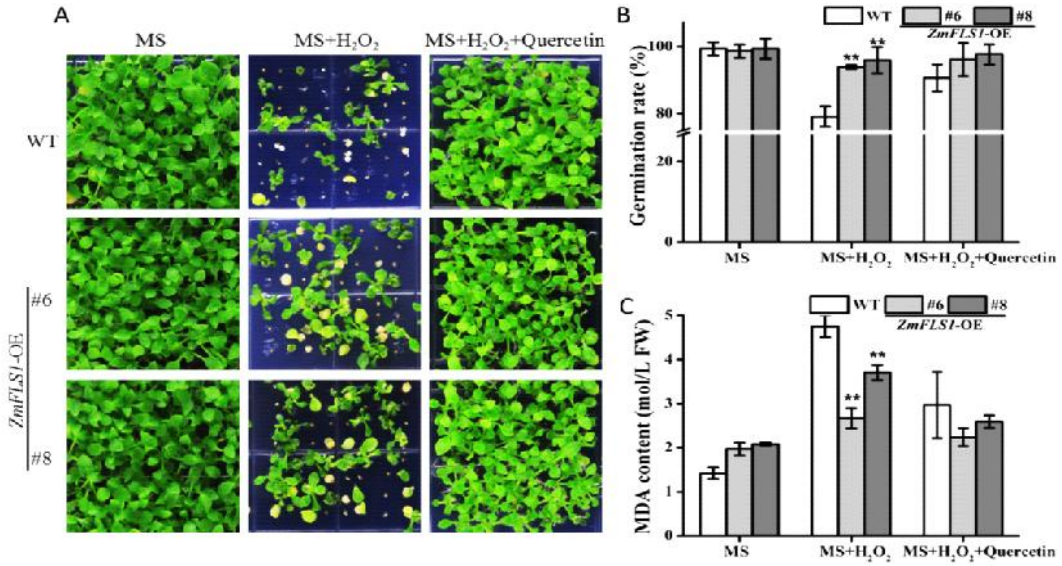


**Fig.4. Seed germination and seedling growth of WT and *ZmFLS1* overexpression lines seedlings under $H_2O_2$ stress. (A) Germination of Arabidopsis seeds grown on MS plates, MS with 5.0 mM $H_2O_2$ and MS with 5.0 mM $H_2O_2$ and 10 µm quercetin for 18 days. Four independent experiments were carried out, and the representative picture was shown, (B) Germination rates of Arabidopsis seeds grown on plates sowing after 4 days; (C) Three independent experiments were carried out, and the average MDA content was obtained. Error bars are standard deviations. $^{**}P<0.01$; $^{*}P<0.05$.**

## DISCUSSION

Flavonoids, such as flavonols, are widely found in many plant tissues, and play significant roles in plant response to various environmental conditions as well as growth and development. The biosynthesis and accumulation of flavonols are regulated by some key enzymes (including CHS, CHI, and FLS), and affect plant stress response and tolerance. Candidate flavonol synthase genes have been identified in many plants, and it is of great significance to study their roles in plant flavonol biosynthesis and stress response. *ZmFLS1* is considered to be an important flavonol synthase gene in maize, but its function in flavonol biosynthesis and plant stress tolerance has not been fully elucidated. *Arabidopsis thaliana* provides a model to investigate the flavonol biosynthesis pathway and perform functional confirmation of candidate genes, providing a useful reference for us to understand gene function. Overexpression of the maize flavonol synthase gene *ZmFLS1* in *Arabidopsis thaliana* was confirmed to promote the accumulation of flavonols in the transgenic plants (Fig. 2), indicating an important role of ZmFLS1 in flavonol biosynthesis.

As non-enzymatic antioxidants, flavonols quench ROS and prevent damage caused by biological and abiotic stress (Gill and Tuteja, 2010; Nakabayashi *et al*. 2014a; Nguyen *et al*. 2016; Baba *et al*., 2017; Maietti *et al*., 2017). Chapman *et al*. (2019) showed that specialized metabolites, including flavonoids are involved in modulating ROS homeostasis during stress

responses. Li *et al*. (2019) showed that flavonoid (especially flavonol) accumulation regulated by MYB111 impairs salt-induced ROS accumulation and damage. Chen *et al* (2019) reported that flavonoid accumulation was negatively regulated by NtMYB4 and thus weakened the ROS-scavenging ability in transgenic tobacco with overexpressing *NtMYB4*. Therefore, we investigated whether the promoted flavonol accumulation induced by ZmFLS1 also promotes plant antioxidant capacity and the scavenging ability of seedling extracts to oxygen free radical. Extracts of *ZmFLS1*- overexpressing seedlings demonstrated an improved scavenging capacity to $O^{2-}$, and DPPH under normal and oxidative stress (Fig. 3), which is consistent with their higher flavonoid/flavonol content.

Under normal conditions, there was no notable difference between transgenic plants with elevated *ZmFLS1* expression and WT during seed germination and seedling growth, indicating that the promoted *ZmFLS1* expression did not cause visible negative effects on plant germination and growth, despite the potential toxicity of flavonoids/flavonols. Under oxidative stress caused by hydrogen peroxide, seed germination and seedling growth were significantly improved in *ZmFLS*-overexpressing transgenic plants (Fig. 4), which is consistent with flavonol/flavonoid content and antioxidant capacity. As a kind of flavonols, quercetin effectively alleviates plant sensitivity to oxidative stress (Fig. 4), indicating that it is flavonols that cause the difference in oxidative stress resistance between transgenic plants and the corresponding control (WT). To some extent, MDA content is a sign of stress injury in plants, and the higher MDA content in transgenic plants under normal growth conditions might be caused by the potential toxicity of flavonols, although it does not cause significant changes to germination and growth, so the fine regulation of plant flavonol synthesis is particularly important.

In conclusion, the correlation between flavonol content, antioxidant capacity, and oxidative stress tolerance indicate that ZmFLS1 improves oxidative stress resistance in transgenic plants by promoting flavonol biosynthesis. The functional characterization of ZmFLS1 in plant abiotic stress provides a theoretical basis for improving plant oxidative stress tolerance through genetic modification.

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**Author's Contributions:** BZ Li, RN Fan designed and guided the research; H Li, YT Fan, and H Zhang performed the experiments and analyzed the data; BZ Li wrote the article; BZ Li, X Zhao, YT Fan, and RN Fan contributed to manuscript editing.

**Competing interests:** The authors have declared that no competing interests exist.

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