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Research article

Characterization of *Arabidopsis thaliana FLAVONOL SYNTHASE 1* (*FLS1*) -overexpression plants in response to abiotic stress

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ABSTRACT

Flavonoids are an important group of secondary metabolites that are involved in plant growth and contribute to human health. Many studies have focused on the biosynthesis pathway, biochemical characters, and biological functions of flavonoids. In this report, we showed that overexpression of *FLS1* (*FLS1-OX*) not only altered seed coat color (resulting in a light brown color), but also affected flavonoid accumulation. Whereas *fls1-3* mutants accumulated higher anthocyanin levels, *FLS1-OX* seedlings had lower levels than those of the wild-type. Besides, shoot tissues of *FLS1-OX* plants exhibited lower flavonol levels than those of the wild-type. However, growth performance and abiotic stress tolerance of *FLS1-OX*, *fls1-3*, and wild-type plants were not significantly different. Taken together, *FLS1* can be manipulated (i.e., silenced or overexpressed) to redirect the flavonoid biosynthetic pathway toward anthocyanin production without negative effects on plant growth and development.

1. Introduction

Flavonoids are an important group of plant secondary metabolites involved in plant signaling pathways that mediate responses to ambient environmental challenges. In plants, flavonoids can be subdivided into several classes including anthocyanins, flavonols, flavonones, etc. Anthocyanins function in UV protection, defense, and pollinator attraction, whereas flavonols help to regulate auxin polar transport (Santelia et al., 2008; Pollastri and Tattini, 2011). Many lines of evidence indicate that both biotic and abiotic stresses can stimulate flavonoid accumulation in *Arabidopsis* (Winkel-Shirley, 2002; Fini et al., 2011). Furthermore, flavonoids are known to be natural antioxidants in cells (Howard et al., 2002). Accordingly, these metabolites are thought to play positive roles in plant abiotic stress tolerance (Di Ferdinando et al., 2012). However, *Arabidopsis tt4* mutants, which are devoid of flavonoid accumulation, do not differ significantly from wild-type plants in survival rate in response to drought stress conditions (Nakabayashi et al., 2014).

Many studies on flavonoids have elucidated their biosynthesis pathways, biochemical characteristics, and biological functions in plant development as well as human health. The basic chemical structure of flavonoids consists of three rings (C6–C3–C6) that associate with hydroxyl (OH) groups and degrees of oxidation, acylation, methylation, and polymerization in various configurations, resulting in many kinds of compounds (Kumar and Pandey, 2013). Anthocyanins generally dissolve easily in water and transmit various colors (blue, purple, or red) depending on pH and chelation of metal ions, whereas flavonols (including kaempferol and quercetin) have a yellow color in crystal form and are marginally soluble or even insoluble in water. Most plant flavonoids occur as glycosides that are generated by replacing the OH group

Abbreviations used: ABA, Abscisic acid; DAB, 3,3′-Diaminobenzidine; DPBA, Diphenylboric acid 2-aminoethylester; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; GFP, Green fluorescent protein; GUS, β-glucuronidase; MS, Murashige and Skoog; ROS, Reactive oxygen species; RT-PCR, Reverse transcription polymerase chain reaction.

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with sugar, and these glycosides are preferentially absorbed by humans (Romagnolo and Selmin, 2012). Numerous in vivo and in vitro studies examining the biological functions of flavonoids in humans and other animals have been conducted (Wang and Stoner, 2008; Romagnolo and Selmin, 2012; Lin et al., 2015). These compounds have positive effects with respect to anti-oxidation, anti-cell proliferation, apoptosis stimulation, anti-inflammation, anti-angiogenesis, anti-invasiveness, and induction of cell differentiation (Wang and Stoner, 2008; Romagnolo and Selmin, 2012). In addition, in vivo studies have demonstrated positive actions of flavonoids in the prevention of cancers of the colon, skin, and lung (Wang and Stoner, 2008).

Anthocyanins and flavonols are synthesized via the phenylpropanoid metabolic pathway, in which each compound is produced from dihydroflavonol via two different steps catalyzed by dihydroflavonol 4-reductase (DFR) (EC 1.1.1.219) or FLS (EC 1.14.11.23), respectively. The *Arabidopsis thaliana* genome contains six *FLS* genes referred to as *FLS1* to *FLS6* (Owens et al., 2008). *FLS1* is functionally the most important and has been studied more extensively than the others (Owens et al., 2008). In this study, to further examine whether we can use genetic manipulation to redirect the flavonoid biosynthetic pathway and increase production of specific flavonoids such as quercetin or kaempferol, we characterized transgenic plants that ectopically express the *FLS1* gene. Briefly, shoot tissues of *FLS1-OX* plants exhibited lower kaempferol levels that those of the wild-type and, interestingly, *FLS1-OX* plants had reduced seed coat pigmentation. While it is conceivable that this reduced seed coat pigmentation increases sensitivity to various abiotic stresses, no differences between *fls1-3*, *FLS1-OX*, and wild-type seedlings were detected in response to salt and drought stresses.

2. Materials and methods

2.1. Plant materials and culture conditions

Seeds of *A. thaliana* wild-type (Col-0), *fls1-3* mutant (Kuhn et al., 2011), *DR5:GUS,* and *DR5:GFP* were surface sterilized and stored at 4 °C for 3 d before seeding on half-strength Murashige and Skoog (1/2× MS) medium. The 1/2× MS medium was supplemented with 2% (w/v) sucrose and 1.2% (w/v) phytoagar and the pH was adjusted to 5.8 before autoclaving. Additional compounds, including ABA and mannitol, were sterilized by filtration and added to the warm autoclaved medium. Growth chamber conditions were set as follows: temperature, 23 ± 1 °C; light intensity, 50–55 μmol photons m^{-2} s^{-1}; light cycle, 16:8 h (light:dark); relative humidity, ~70%.

2.2. DNA construction and transgenic plant generation

The full-length *FLS1* cDNA (*AT5G08640*) was isolated by a reverse transcription polymerase chain reaction (RT-PCR) using specific primers (Supplementary Table S1). The purified PCR product was then cloned into the *pEarleyGate 203* vector (Earley et al., 2006) and transformed into wild-type plants (Col-0) using the *Agrobacterium tumefaciens* (strain GV3101) floral dip method (Clough and Bent, 1998). Approximately 1 kb of the *FLS1* promoter, containing the *core promoter element* (*TATA-box*), *common cis-acting element* (*CAAT-box*), and *MYB binding site* (*MBS*) (Supplementary Fig. S1), was cloned into the *pMDC162* vector to generate the *FLS1pro:GUS* construct. This construct was then transformed into wild-type plants (Col-0) as described above.

2.3. qRT-PCR

Seedlings exhibiting normal growth were used for RNA extraction and first-strand cDNA preparation. Quantitative RT-PCR reactions were performed using specific primers (Supplementary Tables S1).

2.4. GFP visualization in root tips

Five-day-old *DR5:GFP* (Benkova et al., 2003) seedlings were transferred to experimental conditions for 24 h and visualized under a confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss, Oberkochen, Germany) for green fluorescent protein (GFP).

2.5. Histochemical GUS assay

GUS assays were conducted following the protocol of Jefferson et al. (1987) with some modifications. Seedlings were incubated in GUS solution at 37 °C for 6–12 h, after which chlorophyll was removed by soaking the seedlings in 100% ethanol at 70 °C. Pictures were taken using a digital microscope (Leica EZ4D).

2.6. Anthocyanin quantitative assay

To measure anthocyanin content, seedlings were homogenized in liquid nitrogen. The powdered samples were used for anthocyanin extraction and measurement as described in Mancinelli et al. (1988).

2.7. Flavonoid staining in seedlings

Seedlings were grown and stained in diphenylboric acid 2-aminoethylester (DPBA) solution as previously described (Lewis et al., 2011). Pictures were taken under a confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss) for GFP (kaempferol) and yellow fluorescent protein (YFP) (quercetin).

2.8. High-performance liquid chromatography

Thirty-five-day-old shoot parts of wild-type (Col-0) and *FLS1-OX #5* plants were used for flavonols measurement by high-performance liquid chromatography (HPLC) (Ultimate 3000 HPLC system, Dionex, Sunnyvale, CA, USA). The extracts were analyzed on an YMC-Triart C18 + column (4.6 mm × 250 mm, 5 μm particle size) (YMC, Kyoto, Japan) with a mobile phase solution of 100% methanol (MeOH) and 0.1% formic acid. The flow rate was 0.6 mL/min and injection volume was 10 μL. The temperature conditions were 4.5 °C for the autosampler and 30 °C for the column oven. The UV detector was set at 360 nm. Quercetin and kaempferol were used as the standards.

2.9. Preparation of plant extracts and UPLC-Q-Tof MS analysis

Approximately 200 mg each of fresh *A. thaliana* wild-type (Col-0) and *FLS1-OX #5* samples were chopped into small slices and subjected to ultrasonic extraction in 10 mL MeOH for 30 min. After extraction, the samples were filtered through a 0.2-μm membrane filter. Samples were then analyzed using an Acquity ultra-performance liquid chromatography system (Waters, Millford, MA, USA) coupled to a Micromass Q-Tof Micro mass spectrometer (Waters, Manchester, UK). An Acquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm) was used to perform the chromatographic separation. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The starting eluent was 10% B. The proportion of B was increased linearly to 13% from 0 to 3 min, held constant at 13% until 12 min, increased linearly to 100% from 12 to 17 min, held constant at 100% until 19.5 min, returned to the initial composition (10% B) at

19.5 min, and was then held constant for 2.5 min to re-equilibrate the column. The flow rate was 0.3 mL/min and the sample injection volume was 2 μL. The column and samples manager were maintained at 35 °C and 15 °C, respectively. The mass spectrometer was operated in negative ion mode and the optimized MS conditions were as follows: capillary voltage of 2800 V, cone voltage of 70 V, source temperature of 100 °C, desolvation temperature of 300 °C, cone gas flow rate of 20 L/h, and desolvation gas flow rate of 800 L/h. To ensure mass accuracy and reproducibility, leucine-enkephalin was used as the reference lock-mass compound at a concentration of 500 pg/μL and a flow rate of 3 μL/min. Identification of the flavonol glycosides was performed based on their elution time and mass spectrometric fragmentation pattern in comparison with data reported by Besseau et al. (2007). Quantitation of flavonol glycosides was performed by analyzing three technical replicates of each extract and measuring the absorbance at 345 nm, and kaempferol and quercetin were used as standards for quantification of kaempferol glycosides and quercetin glycosides, respectively.

2.10. 3,3′-Diaminobenzidine (DAB) staining

DAB staining was carried out following the protocol of Joseph (2009) with some modifications. Seedlings were incubated in DAB solution for 3 h and chlorophyll was removed by soaking the seedlings in 100% ethanol at room temperature. Pictures were taken using a digital microscope (Leica EZ4D).

2.11. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay to test radical scavenging activity

The radical scavenging activity of seedling extracts was estimated according to the methods of Tohge et al. (2005). The seedlings were extracted in 70% v/v acetone in water and the dried extracts were diluted in ethanol (100%) to various concentrations. The absorbance of the reacted mixtures of extracts and DPPH solutions was measured at 517 nm.

2.12. Statistical analyses

Statistical analyses were carried out by Duncan's tests at a 95% confidence level.

3. Results and discussion

3.1. FLS1 expression in response to ABA

ABA stimulates the expression of genes involved in the anthocyanin biosynthesis pathway in grape (Ban et al., 2003; Gagne et al., 2011). As shown in Fig. 1, ABA increased the expression of *CHALCONE SYNTHASE* (*CHS*), *DFR*, and a stress marker gene *COLD-REGULATED 15A* (*COR15A*) in 7-day-old wild-type seedlings, even at a low concentration (0.1 μM), whereas the *FLS1* expression seemed to be insensitive to ABA treatments (Fig. 1). On the other hand, flavonol accumulation in root tips was increased in response to ABA (0.1 μM) (Supplementary Fig. S2). Moreover, drought stress (induced by mannitol treatments at 200 and 300 mM) also increased flavonol levels (Supplementary Fig. S2). Based on these results, we speculate that ABA can induce the expression of *CHS* encoding for a key enzyme in flavonoid biosynthesis pathway and this can result in abundant presence of flavonol precursors leading to higher flavonol levels. Flavonol accumulation may be very sensitive to changes in the ambient environment and may regulate subsequent events including root development; this response enables plants to grow in unfavorable conditions.

Flavonols are known to be natural auxin transport inhibitors, and increased accumulation of these compounds can affect auxin flow from the shoot to the root of a plant (Santelia et al., 2008; Pollastri and Tattini, 2011). We used two types of transgenic plants harboring *DR5*, an artificial promoter containing auxin response cis-element which is responsive to auxin accumulated in plants to drive the expression of *GUS* and *GFP* genes (*DR5:GUS* and *DR5:GFP*, respectively) (Benkova et al., 2003). In previous study, Guo et al. (2012) used the *DR5:GUS* seedlings (12-day-old) and found that ABA can decrease *GUS* expression in whole seedlings including in root caps. In current study, we tested the response of *DR5:GUS* and *DR5:GFP* seedlings (7-day-old) to ABA treatments (ranging from 0.1 to 10 μM) using GUS staining or direct observations under a confocal microscope. As shown in Fig. 2, GUS staining as well as GFP signals in the root tips indicated that low concentrations of ABA (0.1 and 0.5 μM) significantly decreased auxin response in comparison to those in higher ABA concentrations (1 and 10 μM) (Fig. 2). Mannitol treatment also increased flavonol accumulation, leading to reduced auxin response in root tips, especially for the 200 mM mannitol (Fig. 2 and S2). In a previous study, we showed that low concentrations of ABA (0.1 μM) promote lateral root growth (Nguyen et al., 2013). This effect may be related to flavonoid accumulation because *TRANSPARENT TESTA GLABRA 1* knock-out (*ttg1*) mutant, which lacks the TTG1 transcription factor that functions in regulation of expression of genes involved in flavonoid biosynthesis, did not exhibit a similar response to that of wild-type seedlings (Nguyen et al., 2013). In this study, the relationship between ABA and root growth was further investigated. From these results, it is understood that ABA can partially regulate auxin response via the control of flavonol accumulation. We can thus speculate that the increase in flavonols in response to ABA treatment may decrease the auxin response and result in alterations of auxin signaling in roots. This alteration may then trigger the formation of lateral root primordia.

3.2. The FLS1 promoter exhibits strong activity in the bud and flower

To examine the *FLS1* expression pattern, *FLS1* transcript levels were quantified in various tissues. As shown in Fig. 3a, *FLS1* expression was very high in the flower and bud in five-week-old plants. To confirm the causal role of the *FLS1* promoter in this expression pattern, we generated transgenic plants in which the approximately 1-kb region upstream of the *FLS1* gene was fused to the *GUS* reporter gene. As shown in Fig. 3b, the *FLS1* promoter had strong activity in the bud and flower. These results were consistent with previous studies examining *FLS1* expression and flavonol accumulation in various plant tissues (Peer et al., 2001; Owens et al., 2008). High expression of *FLS1* is positively correlated with strong flavonol accumulation in flowers, buds, and the junction of roots and shoots (Peer et al., 2001). As shown in Fig. 3b, *FLS1* was highly expressed in the root (Fig. 3b), a result that is consistent with a previous study reporting that flavonol accumulation is higher in the root than in the shoot tissues (Kuhn et al., 2011). Furthermore, when seedlings were grown in dark conditions, *FLS1* expression was not only very high in the roots but also in the hypocotyls, relative to its expression in the same tissues of light-grown seedlings (Fig. 3b). In the flavonoid biosynthesis pathway, there are two branches directed to anthocyanins and flavonols. Whereas the MYB/bHLH/WD40 (MBW) complex regulates the expression of *DFR*, *LDOX* (*LEUCOANTHOCYANIDIN DIOXYGENASE*) and *UF3GT* (*FLAVONOID 3-O-GLUCOSYLTRANSFERASE*) and requires the presence of light, *FLS* expression is regulated by MYB12 (Mehrtens et al., 2005; Stracke et al., 2007) which is responsive to ethylene or auxin (Lewis et al., 2011). Moreover, the expression of *MYB12* is regulated by the ELONGATED HYPOCOTYL 5 (HY5) transcription factor under visible

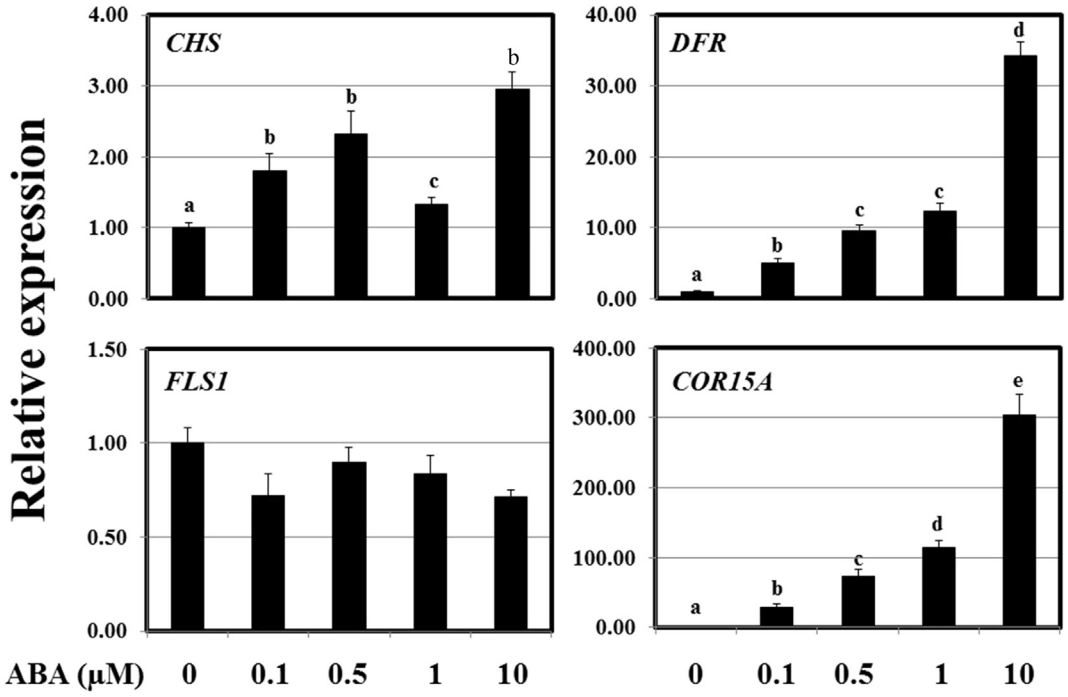


Fig. 1. The transcript level of *FLS1* in response to ABA. Seven-day-old wild-type seedlings were transferred to 1/2× MS medium supplemented with the indicated concentrations of ABA for 12 h and were then used for RNA extraction and qRT-PCR. *ACTIN2* was used as a control. Specific primer pairs are shown in Supplementary Table S1. qRT-PCR was repeated for statistical analysis (n = 3 biological replicates). The letters reflect significant differences ($p < 0.05$).

light conditions (Stracke et al., 2010). On the other hand, flavonols interfere with auxin polar transport, resulting in the control of root development (Santelia et al., 2008; Grunewald et al., 2012). As such, the light-dependent nature of *FLS1* expression in the hypocotyl suggests that the regulation of flavonol biosynthesis may be involved in the process of photomorphogenesis.

3.3. Decreased seed coat pigmentation in FLS1-OX plants

To redirect the biosynthesis pathway toward production of flavonols, *FLS1* was overexpressed in wild-type plants (Col-0 background). After selection for homozygous plants, we confirmed the expression level of *FLS1* in these transgenic plants by qRT-PCR (Fig. 4a). In addition, we measured flavonol and anthocyanin accumulation in *FLS1-OX* (#5 and #6) as well as *fls1-3* mutant and wild-type seedlings. Consistent with the observation of high expression of *FLS1* in the *FLS1-OX* (#5 and #6) seedlings, the flavonol contents in root tips of the overexpression lines were slightly higher than in the wild-type (Fig. 4b). In contrast to our expectation, however, flavonol levels in the shoots were much lower in *FLS1-OX* #5 than in wild-type plants (Fig. 4c and d). These observations were similar to the results obtained by Kuhn et al. (2011), where the authors also observed slightly reduced flavonol contents in *Arabidopsis* lines that overexpressed the *FLS1* gene (Kuhn et al., 2011). In a previous study, Kuhn et al. (2011) reproposed a model reported by Winkel (2004) that was considered "metabolic channeling" for metabolome formation in flavonoid biosynthesis; this model showed that there is competition between FLS1 and other enzymes involved in anthocyanin biosynthesis and that *FLS1* may not function as a limiting factor in flavonol accumulation. This may help to explain why the overexpression of a single gene (*FLS1*) does not successfully increase flavonol accumulation.

Interestingly, the quantitative data in Fig. 4d showed that there was a difference in the ratio of kaempferol and quercetin (kaempferol:quercetin) between wild-type and the *FLS1-OX* #5 plants. The kaempferol level was higher than that of quercetin in wild-type plants (kaempferol > quercetin), whereas the quercetin level was higher than that of kaempferol in *FLS1-OX* #5 plants (kaempferol < quercetin). In a previous study, Wisman et al. (1998) found that quercetin levels were unchanged in the *fls1* mutant and thus proposed that another related enzyme with higher affinity for dihydroquercetin than for dihydrokaempferol may be present (Wisman et al., 1998). This result supports our observation of similar quercetin levels in wild-type and *FLS1-OX* #5 plants. These results imply that biosynthesis of flavonoids is regulated in a highly complicated manner, and thus requires further studies. In previous studies, flavonols were shown to be a natural inhibitors of auxin polar transport (Santelia et al., 2008; Pollastri and Tattini, 2011). Moreover, the phytohormone auxin can induce flavonol accumulation (Lewis et al., 2011). From these reports, it is likely that overexpression of the *FLS1* gene caused negative feedback regulation resulting in the greater reduction of flavonols in *FLS1-OX* plants than in wild-type plants. Our results clearly indicate that we may not expect over-accumulation of flavonols due to simple overexpression of the *FLS1* gene in plants.

Owing to the low flavonol content in *fls1-3* mutants, anthocyanin accumulation was apparently increased in these mutant seedlings (Fig. 4e). As shown in Fig. 4e, the anthocyanin level of *fls1-3* mutants was about two-fold higher than that of wild-type seedlings and was significantly lower in *FLS1-OX* transgenic plants than in wild-type seedlings. These results were consistent with those of a previous study (Kuhn et al., 2011). Moreover, the *FLS1-OX* transgenic plants not only showed reduced endogenous anthocyanin content but also exhibited a change in seed coat color from brown (as observed in wild-type) to light brown (Fig. 5). This alteration of seed coat color is similar to observations of *TRANSPARENT TESTA 2* knock-out (*tt2*) or *ttg1* mutants, which have a bright yellow seed color due to defect in proanthocyanidins accumulation (Nesi et al., 2001; Baudry et al., 2004).

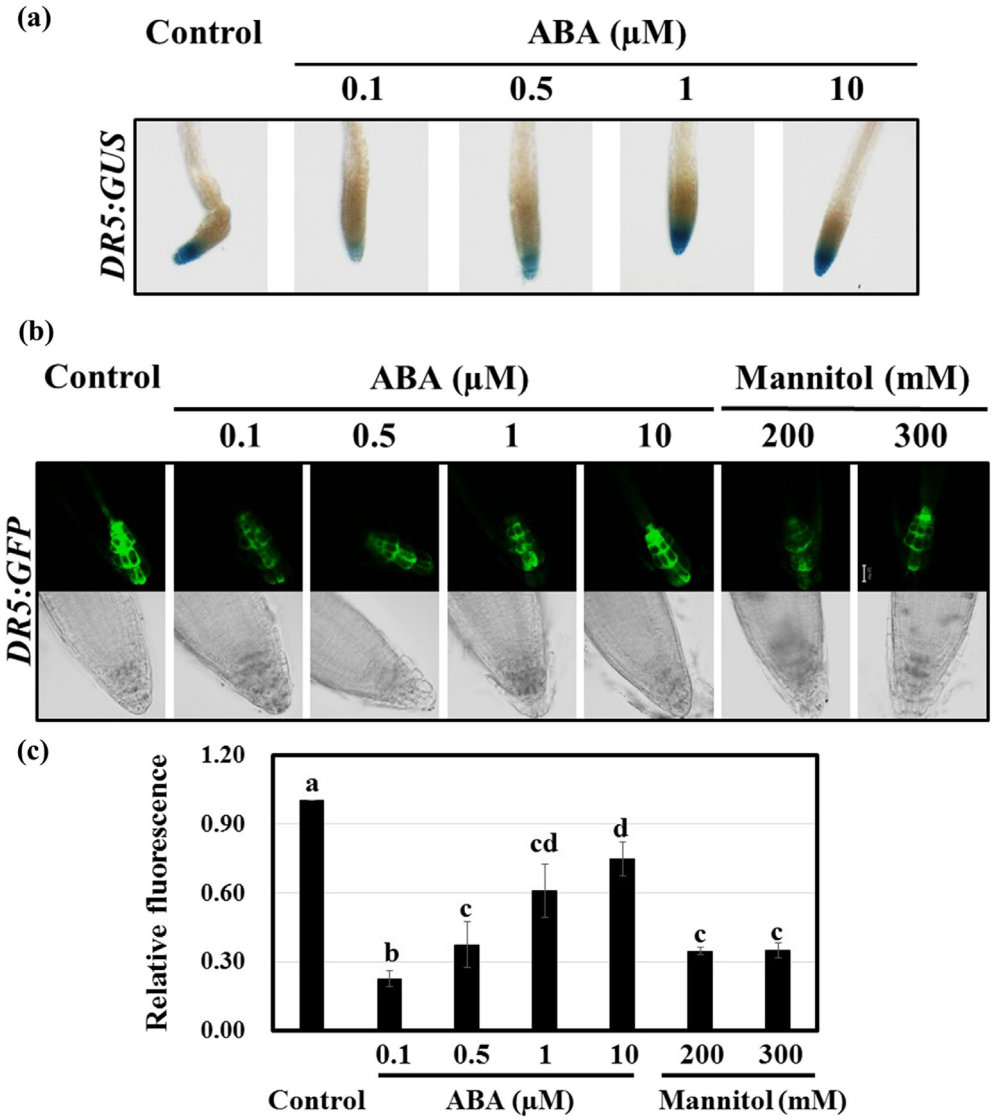


Fig. 2. The effects of ABA on auxin accumulation in root tips. (a and b) Seven-day-old *DR5:GUS* and *DR5:GFP* seedlings were transferred to 1/2× MS medium supplemented with the indicated concentrations of ABA and mannitol for 12 h prior to GUS staining or GFP observation using a confocal microscope. (c) The relative fluorescence was calculated by relative the GFP fluorescence of samples in response to ABA or mannitol treatments to those of control samples. The experiment was repeated for statistical analysis (n = 3 biological replicates). The letters reflect significant differences ($p < 0.05$).

It is very important to understand, in detail, the regulation mechanism of the flavonoid biosynthesis pathway so that we can easily control and produce particular flavonoids for agricultural, culinary, and medicinal applications. Flavonols have been reported to have positive effects in the neutralization of free radicals, which themselves increase the likelihood of cancer (Romagnolo and Selmin, 2012; Ahn et al., 2014). In a previous study, it was shown that the overexpression of *MYB12* can enhance flavonol accumulation in comparison to that of wild-type plants (Mehrtens et al., 2005). In this study, we aimed to increase the flavonol content in *Arabidopsis* by the overexpression of *FLS1* gene, which encodes for a key enzyme in flavonol biosynthesis. However, the results showed that *FLS1-OX* lines reduced levels of both anthocyanin and flavonol in plants. Moreover, *FLS1-OX* plants exhibited reduced pigmentation in seed coats. The overexpression of *MYB12*, which encodes for a transcription factor, resulted in the enhanced accumulation of flavonols, because it functions as a master activator of several structural genes (such as *CHS*, *F3H*, and *FLS*) in flavonol biosynthesis pathway (Mehrtens et al., 2005; Stracke et al., 2007). However, our results imply that the simple overexpression of the *FLS1* gene cannot result in the enhanced accumulation of flavonols. On the other hand, as *FLS1* encodes for an enzyme, translational or post-translational regulations also have the potential to influence FLS1 function. We are currently examining the post-transcriptional regulation of *FLS1* expression.

3.4. Abiotic stress responses of FLS1-OX and fls1-3 seedlings

Previous results have shown that *PRODUCTION OF ANTHOCYANIN PIGMENT 1-Dominant* (*pap1-D*) plants, which have higher endogenous anthocyanin levels, have stronger tolerance to abiotic stress than wild-type plants (Nakabayashi et al., 2014). In this study, reactive oxygen species (ROS) accumulation was observed in wild-type and *fls1-3* seedlings in response to abiotic stress conditions (Fig. 6a). After a 6 h treatment with salt stress (NaCl, 200 mM), ROS levels in wild-type and *fls1-3* seedlings were not significantly

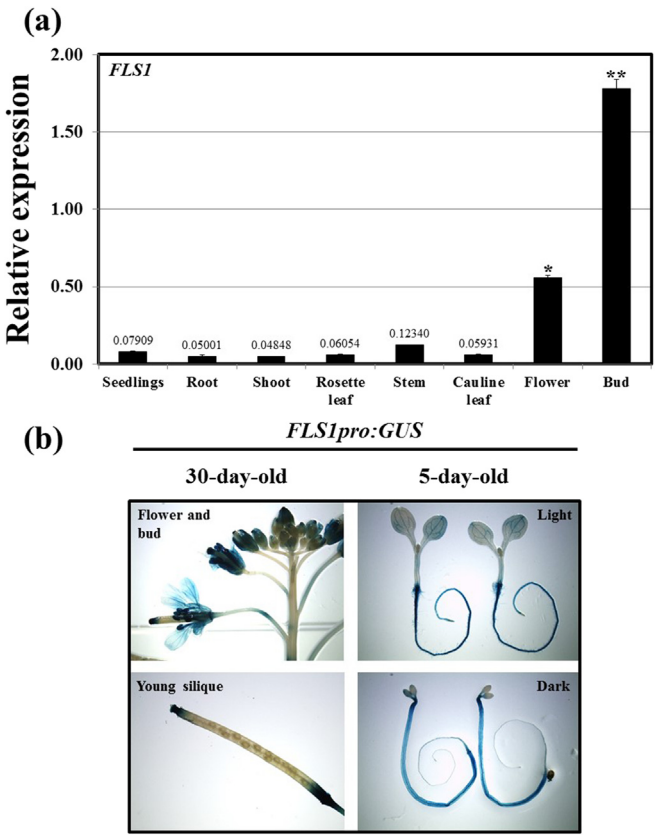


Fig. 3. *FLS1* expression pattern. (a) The RNA samples for qRT-PCR were extracted from 7-day-old seedlings (whole seedlings, root, and shoot) and 35-day-old plants (rosette leaf, stem, cauline leaf, flower, and bud). *ACTIN2* was used as a control. The specific primer pairs are shown in Supplementary Table S1. qRT-PCR was biologically repeated for statistical analysis (n = 3). The stars reflect significant differences ($p < 0.05$). (b) The 30-day-old *FLS1pro:GUS* plants were used for GUS staining and indicated the *GUS* expression in flower and bud, young silique, and 5-day-old seedlings of *FLS1pro:GUS* were grown on 1/2× MS medium in normal light or dark conditions.

different (Fig. 6a). In addition, as shown in Fig. 6b, wild-type and *fls1-3* plants showed similar phenotypes when grown in normal conditions. Following this, the responses of *FLS1-OX* and *fls1-3* seedlings to abiotic stress conditions were tested (Fig. 7). After transferring seedlings to the indicated media, primary root elongation was measured and the results are shown in Fig. 7. While all stress conditions (i.e., ABA, drought, and salt) reduced primary root length, this decrease was similar for wild-type and genetically modified plants (*FLS1-OX* and *fls1-3*) (Fig. 7). Based on these observations, genetic manipulation of the *FLS1* gene can promote biosynthesis of preferred metabolites without harmful effects related to growth performance and stress tolerance in *FLS1-OX* and *fls1-3* plants.

Previously, Nakabayashi et al. (2014) reported that although *MYB12-OX* plants accumulate higher levels of flavonols, their survival rate is similar to that of wild-type plants after drought treatment. However, *pap1-D* and double overexpression lines generated by crossing *pap1-D* and *MYB12-OX* plants demonstrate high tolerance to drought stress (Nakabayashi et al., 2014). Our results also indicate that a minor alteration in flavonoid accumulation associated with *FLS1* overexpression does not affect stress responses or growth performance in comparison to that of wild-type plants (Figs. 4, 5 and 7). The overexpression of *TaFLS1* (*FLS1* from *Triticum aestivum*) in *A. thaliana* could result in greater primary root elongation in response to salt stress than observed in plants that harbor only empty vectors (Wang et al., 2014). The difference in root elongation between *TaFLS1-OX* and *FLS1-OX* plants in response to salt stress indicates that the various *FLS1* genes are responsible for diverse functions. Recently, a study by Vu et al. (2015) found that the overexpression of *Brassica napus FLS* (*BnFLS-OX*) can increase flavonol accumulation in *Arabidopsis*. In addition, there are six *FLS* genes in *Arabidopsis* (Owens et al., 2008) implies that each FLS can play a unique role in flavonol biosynthesis, although these functions have yet to be clarified. As such, further studies are required to elucidate the individual and combined functions of the various flavonoids with respect to plant stress tolerance.

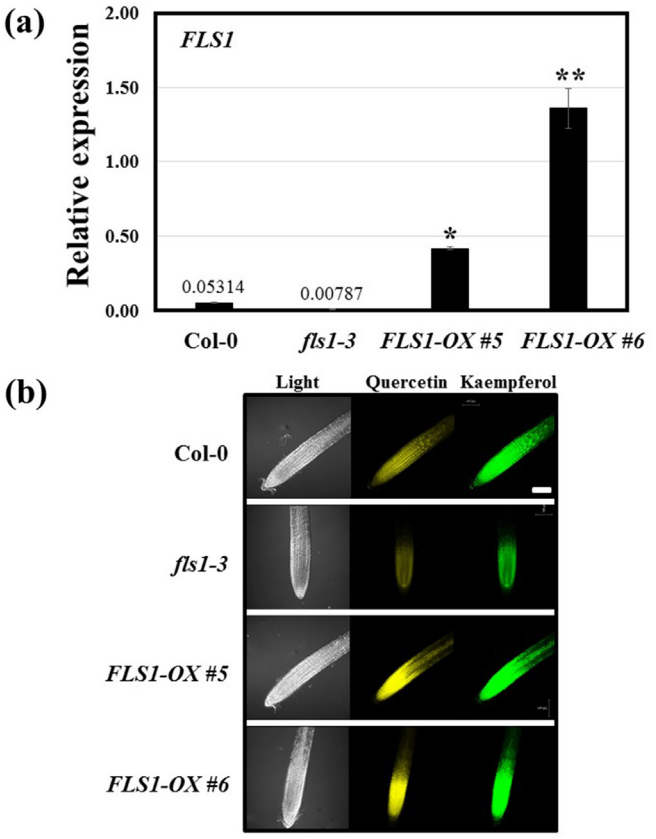
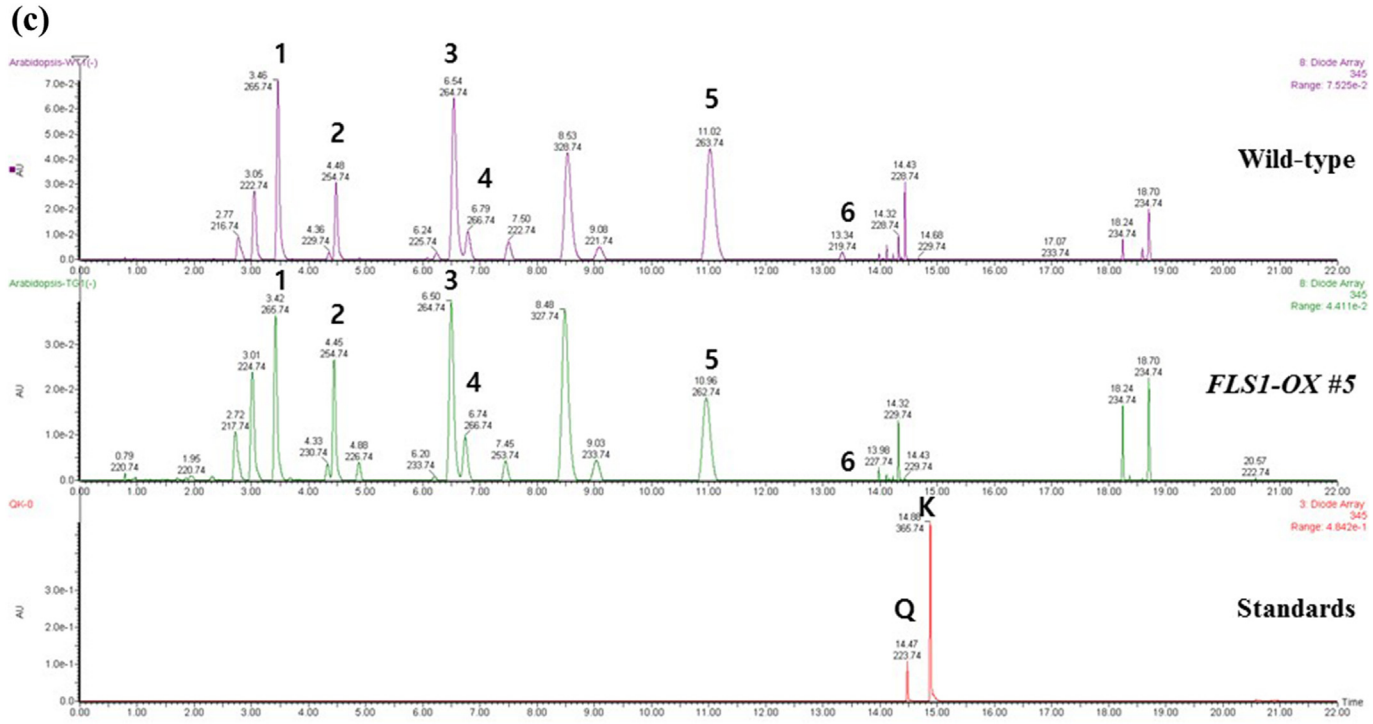


Fig. 4. Flavonol and anthocyanin accumulation in *FLS1-OX* and *fls1-3* seedlings. (a) RNA samples for qRT-PCR were extracted from 5-day-old wild-type (Col-0), *fls1-3*, and *FLS1-OX* (#5 & #6) seedlings. *ACTIN2* was used as a control. Specific primer pairs are shown in Supplementary Table S1. qRT-PCR was repeated for statistical analysis (n = 3 biological replicates). Stars indicate significant differences ($p < 0.05$). (b) Five-day-old wild-type (Col-0), *fls1-3*, and *FLS1-OX* (#5 & #6) seedlings were used for DPBA staining to observe flavonol accumulation in root tips. White bar = 100 μm. (c) Thirty-five-day-old shoot of wild-type (Col-0) and *FLS1-OX* #5 plants were used for quantitative assays (UPLC-Q-Tof MS analysis) to show the flavonols glycosides content. **(1)** Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1->2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside; **(2)** Quercetin 3-*O*-β-D-glucopyranosyl 7-*O*-α-L-rhamnopyranoside; **(3)** Kaempferol 3-*O*-β-D-glucopyranoside 7-*O*-α-L-rhamnopyranoside; **(4)** Quercetin 3,7-di-*O*-α-L-rhamnopyranoside; **(5)** Kaempferol 3,7-di-*O*-α-L-rhamnopyranoside; **(6)** Quercetin 7-*O*-α-L-rhamnopyranoside and Quercetin 3-*O*-α-L-rhamnopyranoside. (d) Thirty-five-day-old shoot of wild-type (Col-0) and *FLS1-OX* #5 plants were used for quantitative assay (HPLC) to show flavonol contents. Sugar residues were removed from flavonols before measurement. The experiment was repeated for statistical analysis (n = 3 biological replicates). The letters reflect significant differences ($p < 0.05$) of kaempferol or quercetin levels between Col-0 and *FLS1-OX* #5. (e) Five-day-old wild-type (Col-0), *fls1-3*, and *FLS1-OX* (#5 & #6) seedlings were used for anthocyanin measurements. The relative anthocyanin level was calculated by relative the anthocyanin levels of *fls1-3* and *FLS1-OX* (#5 & #6) to those of wild-type (Col-0) seedlings. The experiment was repeated for statistical analysis (n = 3 biological replicates). The letters reflect significant differences ($p < 0.05$).



	Wild-type			*FLS1-OX #5*		
						n=3
	Average (%)	SD	RSD (%)	Average (%)	SD	RSD (%)
1 (K)	0.117	0.000	0.215	0.057	0.001	1.181
2 (Q)	0.046	0.001	1.224	0.038	0.001	1.483
3 (K)	0.156	0.002	1.425	0.093	0.001	1.419
4 (Q)	0.023	0.000	0.576	0.020	0.000	0.824
5 (K)	0.204	0.001	0.527	0.085	0.001	1.600
6 (Q)	0.007	0.000	0.000	0.000	0.000	0.000

K = Kaempferol glycosides Q = Quercetin glycosides

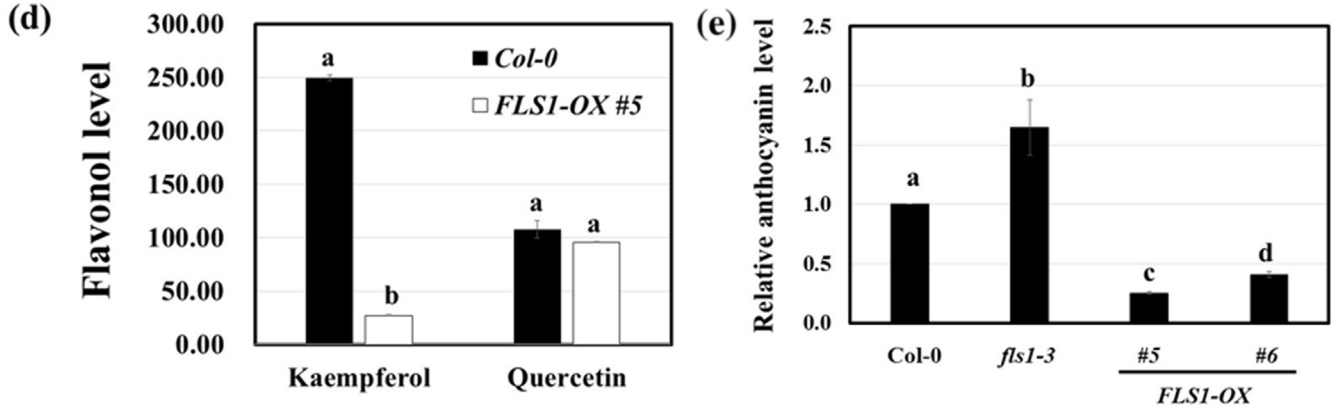


Fig. 4. (*continued*)

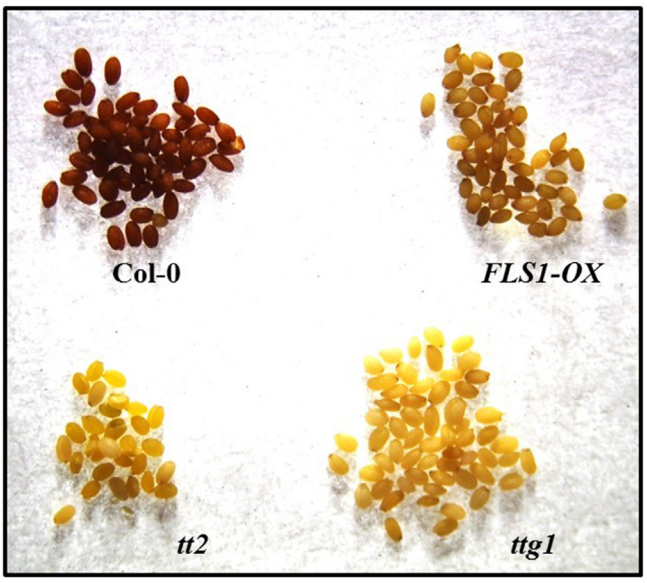


Fig. 5. Alteration of seed color in *FLS1-OX* plants. The seeds of wild-type (Col-0), *FLS1-OX*, *ttg1*, and *tt2* plants were observed and images were taken using a digital microscope (Leica EZ4D).

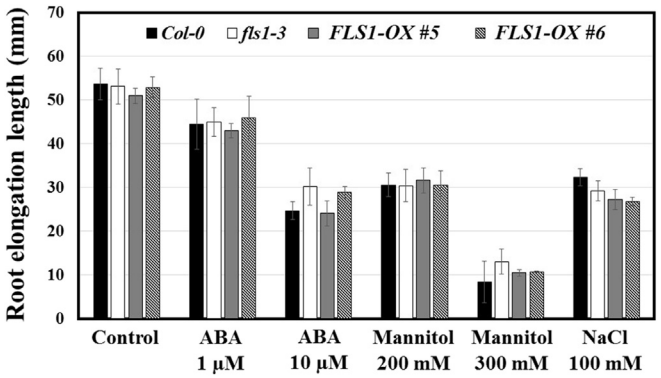


Fig. 7. The primary root length of *FLS1-OX* and *fls1-3* seedlings in response to abiotic stresses. Five-day-old wild-type (Col-0), *fls1-3*, and *FLS1-OX* (#5 & #6) seedlings were transferred to stress-inducing media (indicated conditions) and the elongated fragments of main roots (mm) were measured after 7 d of growth in these conditions (n = 30).

3.5. Radical scavenging activity of fls1-3 seedling extracts

While it has been reported that soluble anthocyanins had very little effect on radical scavenging activity, the increased level of proanthocyanidins in seeds of *pap1-D* mutant can enhance this radical scavenging activity (Tohge et al., 2005). Here, we compared the radical scavenging activities from the wild-type, *fls1-3,* and *pap1-D* seedlings (Fig. 8). The levels of anthocyanins were analyzed and shown in Fig. 8a. Anthocyanin levels were relatively higher in *fls1-3* mutant and *pap1-D* seedlings (by 2 and 5 times, respectively) than in wild-type (Col-0) seedlings (Fig. 8a). However, the radical

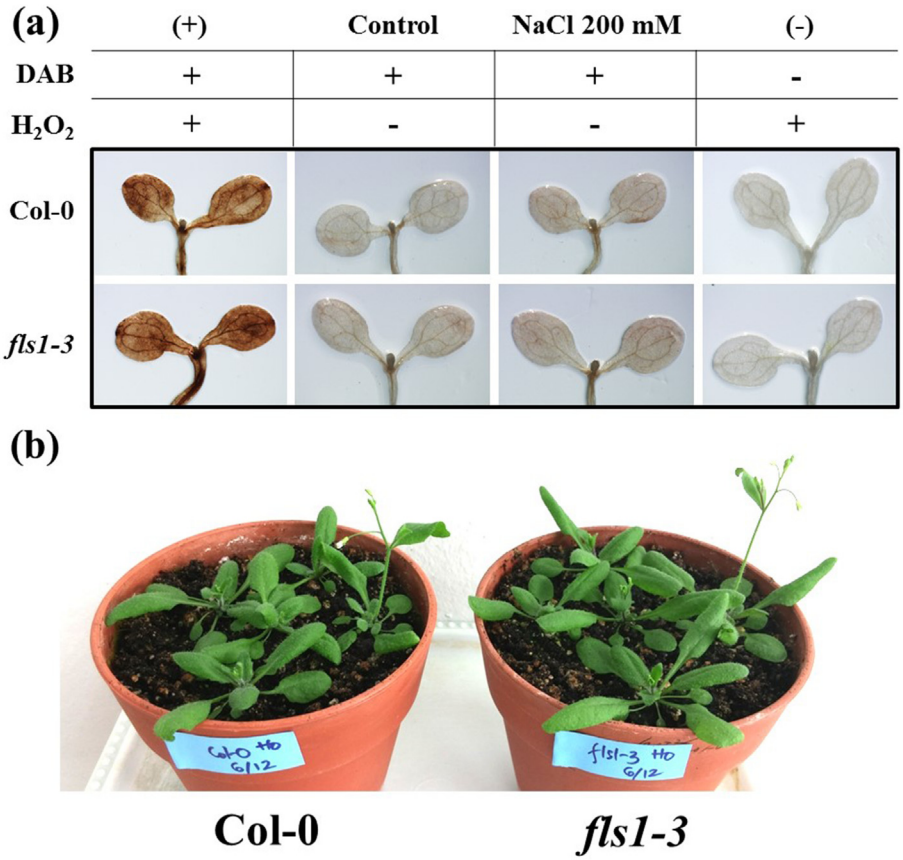


Fig. 6. ROS accumulation and growth performance of wild-type and *fls1-3* plants. (a) Seven-day-old wild-type (Col-0) and *fls1-3* seedlings were grown on 1/2× MS medium and used for the indicated treatments for 6 h. They were then stained with DAB solution to visualize ROS accumulation. In the negative control (−), the seedlings were stained with solution without DAB addition, whereas in the positive control (+), H_2O_2 (30%) was supplied. (b) 20-day-old wild-type and *fls1-3* plants growing in normal conditions.

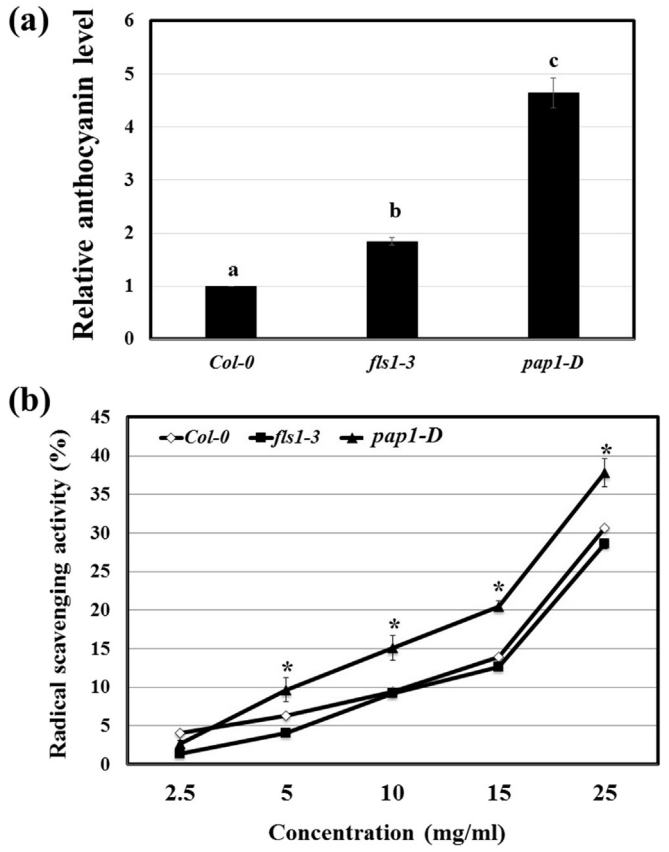


Fig. 8. Radical scavenging activity of *fls1-3* seedling extracts. (a) Five-day-old wild-type (Col-0), *fls1-3*, and *pap1-D* seedlings were used for anthocyanin measurements. The relative anthocyanin level was calculated by comparing anthocyanin levels of *fls1-3* and *pap1-D* to those of wild-type (Col-0) seedlings. The experiment was repeated for statistical analysis (n = 3 biological replicates). The letters reflect significant differences ($p < 0.05$). (b) Five-day-old wild-type (Col-0), *fls1-3*, and *pap1-D* seedlings were grown on 1/2× MS medium and used for DPPH assay.

scavenging activity of extracts of *fls1-3* seedlings was similar to that of wild-type seedlings, which was significantly lower than that of *pap1-D* seedlings (Fig. 8b). Based on these results, we thought that the marginally increased anthocyanin levels in *fls1-3* seedlings may not cause increased radical scavenging activity, as observed in *pap1-D* plants which themselves accumulate very high levels of anthocyanins and other flavonoids (Borevitz et al., 2000; Nakabayashi et al., 2014). In *pap1-D* plants, the overexpression of *PAP1*, which encodes a transcription factor that regulates the expression of many genes involved in the flavonoid biosynthesis pathway, results in the production of various flavonoids that have various levels of radical scavenging activity (Borevitz et al., 2000; Tohge et al., 2005; Solfanelli et al., 2006; Nakabayashi et al., 2014).

3.6. The expression of genes involved in flavonoid biosynthesis in FLS1-OX and fls1-3 seedlings

The expression levels of genes involved in the flavonoid biosynthesis pathway were analyzed in *FLS1-OX* and *fls1-3* seedlings. As shown in Fig. 9, expression levels of *PAP1*, *CHI*, and *UF3GT* were not significantly different between *FLS1-OX*, *fls1-3*, and wild-type seedlings. However, *MYBL2* expression was higher in *fls1-3* than in *FLS1-OX* and wild-type seedlings (Fig. 9). *DFR* expression was lower in *FLS1-OX* plants than in *fls1-3* mutant and wild-type seedlings (Fig. 9). The flavonoid biosynthesis pathway is precisely regulated by many factors in plant systems (Winkel-Shirley, 2002; Fini et al., 2011). In our study, we used the knock-out mutant *fls1-3* and transgenic plants that overexpressed *FLS1*, which encodes an enzyme involved in flavonoid biosynthesis; as such, the expression levels of other genes in the pathway were not expected to be affected. Nevertheless, the expression of *MYBL2* was higher in *fls1-3* mutants and *FLS1-OX* plants relative to wild-type plants (Fig. 9). We propose that there is a regulatory feedback mechanism in the flavonoid biosynthesis pathway, and suggest that further studies are needed to examine this prediction.

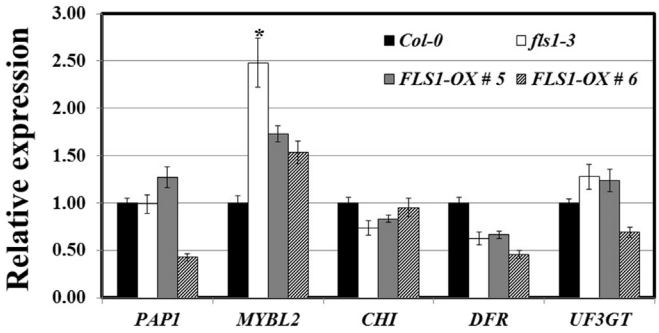


Fig. 9. Transcript levels of genes involved in flavonoid biosynthesis in *FLS1-OX* and *fls1-3* seedlings. RNA samples for qRT-PCR were extracted from 5-day-old seedlings of wild-type (Col-0), *fls1-3* and *FLS1-OX* (#5 & #6). *ACTIN2* was used as a control. Specific primer pairs are shown in Supplementary Table S1. qRT-PCR was repeated for statistical analysis (n = 3 biological replicates). Stars indicate significant differences ($p < 0.05$).

Author contribution statement

HL, SWH, DL and HNN designed the experiments. HNN, JHK, JK, CYJ, and WL performed the experiments. HNN and HL wrote and revised the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.03.010.

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