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ORIGINAL PAPER

TTG1-mediated flavonols biosynthesis alleviates root growth inhibition in response to ABA

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Abstract

Key message **Our results demonstrate that the flavonoids biosynthetic pathway can be effectively manipulated to confer enhanced plant root growth under water-stress conditions.**

Abstract Abscisic acid (ABA) is one of most important phytohormones. It functions in various processes during the plant lifecycle. Previous studies indicate that ABA has a negative effect on root growth and branching. Auxin is another key plant growth regulator that plays an essential role in plant growth and development. In contrast to ABA, auxin is a positive regulator of root growth and development at low concentrations. This study was performed to help understand whether flavonoids can suppress the effect of ABA on lateral root growth. The recessive *TRANSPARENT TESTA GLABRA 1* (*ttg1*) mutant was characterized on ABA and sucrose treatments. It was determined that auxin mobilization could be altered by modifying flavonoids biosynthesis, which resulted in alterations of root architecture in response to ABA treatment. Moreover, transgenic *TTG1-overexpression* (*TTG1-OX*) seedlings exhibited enhanced root length and lateral root number compared to wild-type seedlings grown under normal or stress conditions. Genetic manipulation of the flavonoids biosynthetic pathway could therefore be employed successfully for the improvement of plant root systems by overcoming the inhibition of ABA and some abiotic stresses.

Keywords TTG1 · ABA · Flavonoids · Anthocyanin · Root growth inhibition · *Arabidopsis thaliana*

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Introduction

Plants are very susceptible to environmental challenges imposed by biotic and abiotic factors such as pathogens, herbivores, low temperatures and drought. To guard against these stresses, plants undergo developmental changes, exhibiting plasticity in the architecture of shoot and root systems in a given environment. For example, plants generate plenty of lateral roots under normal conditions, while lateral root initiation and elongation are limited under drought conditions to maximize the elongation of the primary root to reach water deep in the soil (Malamy 2005; van der Weele et al. 2000; Xiong et al. 2006). Developmental plasticity has evolved over a long period of time and may contribute to the "real-time adaptation" of plants that allows them to acclimate to specific surroundings.

Roots provide an optimal system for studying developmental plasticity, a characteristic feature of plant growth (Malamy 2005). One of the main determinants of root system architecture is lateral root (LR) initiation and

growth. This developmental process is influenced by endogenous and exogenous factors, such as auxin, abscisic acid (ABA), drought stress and osmotica (Fukaki and Tasaka 2009; Deak and Malamy 2005). Auxin, a crucial phytohormone, is produced mainly in the shoot apex and is transported toward the root system via polar auxin transporters (Ljung et al. 2001). Polar flows of auxin control organ development, cell elongation and cell division, resulting in the alteration of shoot and root system architecture in responses such as phototropism, gravitropism and thigmotropism (reviewed in Peer et al. 2011). In contrast, ABA inhibits root growth and functions as a negative regulator in LR emergence (Pilet 1975; Deak and Malamy 2005). Exogenously applied ABA suppresses the emergence of LR primordia from the parent root prior to the activation of the LR meristem (De Smet et al. 2003). ABA-induced LR inhibition reportedly occurs through an auxin-independent pathway (Fukaki and Tasaka 2009). The fact that the application of auxin to medium could not rescue LR inhibition in response to ABA suggests that an ABA-sensitive, auxin-independent checkpoint is involved at the post-emergence stage (De Smet et al. 2003). On the contrary, the *Arabidopsis* recessive mutant *axr2-1/iaa7* is resistant to exogenous ABA whereas the *slr-1/iaa14* mutant is hypersensitive to ABA in root growth inhibition assays, suggesting that Aux/IAA-dependent auxin signaling also affects the plant root response to ABA (Fukaki and Tasaka 2009).

Flavonoids are secondary metabolites that are divided into various subclasses, including flavanones, isoflavonoids, anthocyanins, flavonols, catechins, flavones and proanthocyanidins, on the basis of structure. Although they are non-essential for plant growth and development, flavonoids play species-specific roles in nodulation, fertility, the defense system and UV protection (Peer and Murphy 2007). The coordinated up-regulation of flavonoids biosynthesis-related genes has been well established, and several structural genes for flavonoids biosynthesis have been identified in various plant species (Olsen et al. 1993; Shirley et al. 1995). The *Arabidopsis thaliana* genes *TRANSPARENT TESTA 2* (*TT2*), *TRANSPARENT TESTA 8* (*TT8*) and *TTG1* encode the MYB, bHLH and WD40 transcription factors, respectively; these proteins function together in a transcriptional complex to drive the expression of flavonoids biosynthetic genes such as the *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *LEUCOANTHOCYANIDIN DIOXYGENASE* (*LDOX*) and *ANTHOCYANIDIN REDUCTASE* (*ANR*) (Lepiniec et al. 2006; Dubos et al. 2008; reviewed in Francesca et al. 2006). In the other branch of the flavonoids biosynthetic pathway, plants can produce compounds called flavonols. Flavonols is a class of secondary metabolites that belong to the flavonoids group. Flavonols differ from the other flavonoids classes by the degree of oxidation of the central pyran ring (Skerget et al. 2005). In animal cells, flavonols can inhibit the generation of ROS (reactive oxygen species), which results in the protection of cells from oxidative damage (Pollastri and Tattini 2011). Many lines of evidence indicate that flavonols may control plant growth and development by redirecting the polar auxin transport flow (Pollastri and Tattini 2011; Santelia et al. 2008). These compounds are known to function as regulators of auxin transporters, leading to alterations in shoot and root branching (Peer and Murphy 2007).

Although many lines of evidence suggest that ABA inhibits LR growth through modulation of Aux/IAA-dependent auxin signaling components, the molecular mechanisms underlying their crosstalk remain elusive. It has been recently reported that ABA triggers biosynthesis of phenolic compounds to activate defense mechanisms against UV stress (Berli et al. 2010). To address whether flavonoids play a role in ABA-induced LR inhibition, we studied *ttg1* seedlings, which have impaired flavonoid biosynthesis (Walker et al. 1999), and plants overexpressing *TTG1*. We reasoned that altered levels of flavonoid accumulation might affect LR growth in response to ABA. LR growth following ABA treatment was reduced in *ttg1* seedlings and increased in plants overexpressing *TTG1*. Our results demonstrate that the flavonoid biosynthetic pathway can be effectively manipulated to confer enhanced plant root growth under water-stress conditions.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia and Landsberg), the recessive mutant of *TRANSPARENT TESTA GLABRA 1* (*ttg1*), *CHSpro::GUS* (Oh et al. 2011a) and *DR5::GUS* lines (Oh et al. 2011b), *PAP1-D* (CS3884), *aba3* (CS157), *tt2* (CS83) were employed in these experiments. Seeds were sterilized and stored at 4 °C for 3 days before seeding on half-strength Murashige and Skoog (1/2 × MS) medium containing 2 % (w/v) sucrose (pH 5.7). Plant samples were cultured in a growth chamber under the following conditions: temperature: 23 ± 1 °C; light intensity: 50–55 μmol photons m^{-2} s^{-1}; light/dark cycle: 16 h/8 h; relative humidity: 70 %. Four-day-old seedlings were transferred to 1/2 × MS media containing the indicated supplements.

Construction and generation of transgenic plants

Transgenic *Arabidopsis* plants expressing the full-length cDNA of *TTG1* (*At5g24520*) were generated. Briefly, full-

length *TTG1* cDNA was amplified by PCR and cloned into the *pMDC32* vector, which contains the cauliflower mosaic virus (*CaMV*) 35S promoter. To obtain transgenic plants that overexpress *TTG1*, wild-type plants were transformed with the *Agrobacterium tumefaciens* strain *GV3101* harboring the construct *CaMV35S::TTG1* using the floral dip method (Clough and Bent 1998). Homozygous T_4 transgenic (*TTG1-OX*) seeds were used for all experiments.

RT-PCR

Ten-day-old seedlings of wild-type and *TTG1-OX* (lines #4 and #5) were used for RNA extraction and cDNA preparation. RNA extraction was performed using the Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. PCR reactions using the cDNA described above were carried out with specific primer sets (supporting information Table S1) and repeated using different numbers of cycles (30 and 40 cycles).

Anthocyanin measurement

For visualization of anthocyanins, seeds of mutants were grown for 3 days on 1/2 × MS medium containing 50 µM norflurazon (chlorophyll inhibitor). Seedlings were then observed and photographed using a Leica EZ4D microscope.

To quantify the levels of anthocyanins, 7-day-old wild-type, *TTG1-OX* (lines #4 and #5), *ttg1* and *PAP1-D* seedlings grown on 1/2 × MS medium were used for anthocyanin extraction. Approximately 50 mg of tissue samples were homogenized in liquid nitrogen, and anthocyanin levels were determined as described in Mancinelli et al. (1988) with some modifications. In brief, anthocyanins were extracted overnight at 4 °C in 250 µl of 1 % (v/v) HCl in methanol. A total of 250 µl distilled water and 250 µl chloroform was added to the samples to separate the anthocyanins from the chlorophyll. Samples were then vortexed and centrifuged for 2 min at 3,000×*g* to remove the chlorophyll. The aqueous phase was examined by measuring the optical density at A_{535} and A_{650}. Anthocyanin levels were calculated using the simple formula $(A_{535} - A_{650})$/fresh weight.

Flavonoids staining in seedlings

Seven-day-old seedlings of each mutant and wild-type seedlings were grown and stained as previously described (Sheahan and Rechnitz 1993; Murphy et al. 2000; Lewis et al. 2011) with some modifications. Briefly, seedlings were stained for 7 min using saturated (0.25 %, w/v) DPBA with 0.02 % (v/v) Triton X-100 and were then washed in distilled water for 5 min. Samples were visualized using a Confocal Laser Scanning Microscope (LSM 5 Exciter, Carl-Zeiss) with an Ar-laser (458/488/514 nm) for GFP and a 543 nm HeNe-laser for YFP.

Histochemical GUS assay

GUS assays were performed using GUS solution containing 1 mM X-gluc, 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1 % Triton X-100. Seedlings were incubated in this solution at 37 °C for 6 h and decolorized with 70 % ethanol before observation using a Leica EZ4D microscope (Jefferson et al. 1987).

Statistical analyses

Each experiment was repeated three times using at least ten samples every time. Statistical analysis was done by one-way ANOVA Tukey's test at a 95 % confidence level.

Results

The ttg1 seedlings exhibited retarded root growth in response to ABA treatment

To examine whether the alteration of flavonoids can effect root growth in seedlings in response to ABA treatment, *ttg1* recessive mutant and wild-type 4-day-old seedlings were transferred to 1/2 × MS medium supplemented with the indicated concentrations of ABA and sucrose (Fig. 1). In the normal level of sucrose applied medium (2 % w/v), the number of LRs of wild-type seedlings decreased when the ABA concentration was increased in the medium (Fig. 1b). However, the lowest concentration of ABA (0.1 µM) facilitated lateral root growth in wild-type seedlings. The *ttg1* mutants were much more sensitive to ABA inhibition of LR growth than the wild type at higher concentration of ABA at 4 % sucrose (Fig. 1). We found that the phenotype of the *ttg1* mutant in response to ABA was compensated by the overexpression of the *TTG1* gene. From this result, we hypothesized that decreasing flavonoids biosynthesis can have a negative effect on root development in response to ABA.

Increase of flavonoids biosynthesis resulted in an increase in LR number in response to ABA treatment

Sucrose is a factor that stimulates the accumulation of anthocyanins in plants by altering the expression of genes in the flavonoids biosynthetic pathway (Solfanelli et al.

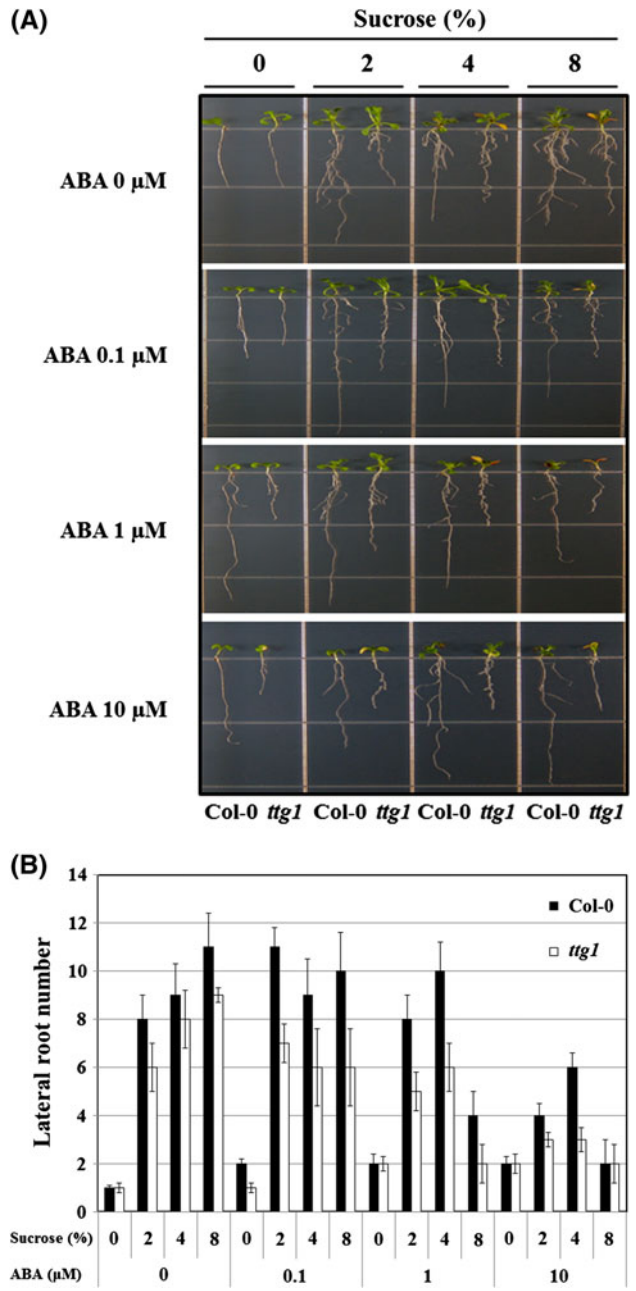

Fig. 1 Growth of *ttg1* and wild-type (Col-0) seedlings in response to ABA and sucrose. **a** Seeds were germinated and grown on normal 1/2 × MS medium, and 4-day-old seedlings were transferred to 1/2 × MS medium supplemented with ABA and sucrose, as indicated in the figure. Pictures were taken on the seventh day after transfer. **b** Numbers of lateral roots (LRs) for *ttg1* and Col-0 seedlings from **a** were plotted. The data shown are from three separate experiments performed at 23 °C ($n = 20$ per experiment)

2006). In this study, we altered the content of sucrose in the medium (from 0 to 8 %) to try to enhance the level of endogenous flavonoids, and we hoped that this alteration would affect the development of the root system upon exposure to ABA (Fig. 1). Indeed, we observed a clear increase in LR number in both wild-type and *ttg1* seedlings when the sucrose content was increased from 2 to 8 % in medium lacking ABA (Fig. 1b). Enhancing the sucrose concentration of the medium can also help overcome ABA-mediated inhibition of root system development. Indeed, in the presence of ABA (1 and 10 µM), higher concentrations of sucrose (2–4 %) also led to an increase in LR number (from ~8 to ~10 and ~4 to ~6) (Fig. 1b). However, a higher concentration of sucrose (8 %) along with ABA (1 and 10 µM) resulted in a decrease in the number of LRs in both the wild-type and *ttg1* seedlings (Fig. 1b).

To support the above results, we attempted to test the expression of *CHS* by growing *CHSpro::GUS* transgenic seedlings and measuring the level of anthocyanins in response to ABA and/or sucrose (Fig. 2). From these results, we realized that an increase in exogenously applied sucrose as positively correlated with the expression of *CHS*, especially when the sucrose content was shifted from 2 to 4 % in the medium. Moreover, the anthocyanins content was also enhanced as long as increased sucrose as well as ABA concentration was used (Fig. 2b). We also determined the level of anthocyanins in the ABA-deficient mutant, *aba3*, in response to sucrose and/or ABA (Fig. 2c). Taken together, we found that an increase in flavonoids biosynthesis resulted in the enhancement of LR number in response to ABA treatment.

Increase of flavonols accumulation in TTG1-OX seedlings

The full-length cDNA of *TTG1* was constructed under the control of the *CAMV35S* promoter and transformed into wild-type *Arabidopsis thaliana*. After three generations of selection, two independent lines (#4 and #5) from the T_4 generation were isolated. Genotype checking confirmed that these lines were homozygous and were genuine overexpression mutants (Fig. 3a). The T_4 seeds from these lines were used in later experiments.

Because *ttg1* was reported to exhibit lower levels of accumulation of anthocyanins than the wild-type, we measured anthocyanins levels in *TTG1-OX* transgenic seedlings. We used the *PAP1-D* mutant as a positive control because it has been previously reported to accumulate high levels of anthocyanins (Borevitz et al. 2000). As shown in Fig. 3b, c, the seedlings that overexpressed *TTG1* were also shown to contain lower anthocyanin levels than the wild-type. This led us to examine the transcript levels of flavonoids biosynthetic genes that were reported to be target genes of TTG1 regulation. In this experiment, the level of *DFR* transcript was slightly lower in *TTG1-OX #4* and *TTG1-OX #5* seedlings than in the wild-type, while the expression of *FLAVONOL SYNTHASE* (*FLS1*) was enhanced in these mutants (Fig. 4a). We consequently determined the level of flavonols accumulation in seedlings

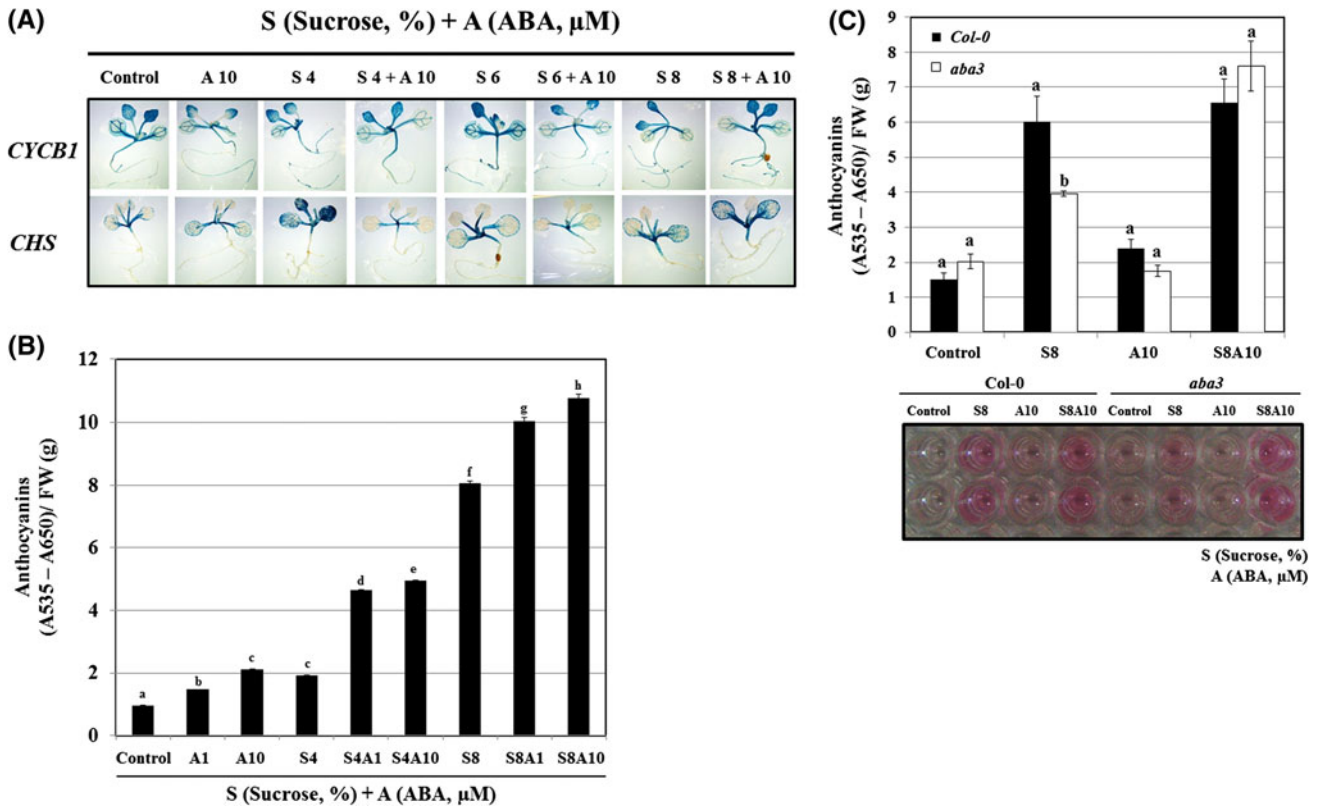

Fig. 2 The effects of sucrose and/or ABA on the expression of *CYCB1pro::GUS*, *CHSpro::GUS* and the accumulation of anthocyanins. **a** *Arabidopsis* T_3 homogenous transgenic seeds harboring *CYCB1pro::GUS* or *CHSpro::GUS* were germinated and grown on 1/2 × MS medium for 10 days and transferred to 1/2 × MS medium supplemented with ABA and sucrose as indicated in the figure. Seedlings were incubated for 6 h before visualization of GUS staining. The seedlings shown were typical of three separate experiments performed at 23 °C ($n = 10$ per experiment). **b** Seven-day-old wild-type (Col-0) seedlings growing on 1/2 × MS medium were treated with the indicated concentrations of sucrose and ABA for 24 h and the level of anthocyanins was measured. A one-way ANOVA Tukey's test ($P < 0.05$) was performed to determine which treatments significantly affected anthocyanin accumulation. *Lower-case letters above the column* indicate significant differences. *Vertical bars* indicate standard error ($n = 3$). **c** Seven-day-old wild-type (Col-0) and *aba3* seedlings growing on 1/2 × MS medium were treated with 8 % sucrose and 10 μM ABA for 24 h and the level of anthocyanins was measured. A one-way ANOVA Tukey's test ($P < 0.05$) was performed to determine which treatments resulted in a significant difference in anthocyanin accumulation between wild-type and mutant seedlings. *Lower-case letters above the column* indicate significant differences. *Vertical bars* indicate standard error ($n = 3$)

of these mutants. The results, shown in Fig. 5, indicate that levels of kaempferol (green color) and quercetin (yellow color) were enhanced in *TTG1-OX* seedlings compared to the wild-type and the other mutants for control. As shown in Fig. 5a, the *tt2* mutant lacking in the proanthocyanidin biosynthesis served as a negative control. In contrast, ABA-deficient mutant *aba3* accumulated slightly more quercetin, which we did not expect (Fig. 5a). We then asked whether ABA and/or sucrose can alter the level of flavonols. As shown in Fig. 5b, ABA and/or sucrose were able to alter the level of quercetin and kaempferol.

To examine whether the wild type *TTG1* gene can complement the altered root phenotype of the *ttg1* mutant, we introduced the *35S::TTG1* construct into *ttg1* plants. The *TTG1/ttg1* seedlings had longer primary roots than *ttg1* seedlings (Fig. S1).

Enhancement of root development of TTG1-OX seedlings in response to ABA and drought treatment

Because *ttg1* seedlings showed retarded root growth on medium supplemented with ABA, we examined the response of *TTG1-OX* seedlings to ABA treatment. As expected, the *TTG1-OX* seedlings exhibited stronger root growth in ABA medium than did the wild-type (Fig. 6a, b). Moreover, we also observed that *TTG1-OX* seedlings produced many more LRs and longer primary roots than did the wild-type upon exposure to drought stress (Fig. 6a, b). We employed the *PAP1-D* mutant as a positive control for increased flavonoids biosynthesis. The *PAP1-D* seedlings were transferred to ABA medium under the same experimental conditions used for *TTG1-OX* seedlings. The results clearly showed that both the *PAP1-D* and *TTG1-OX* seedlings exhibited the same root development response

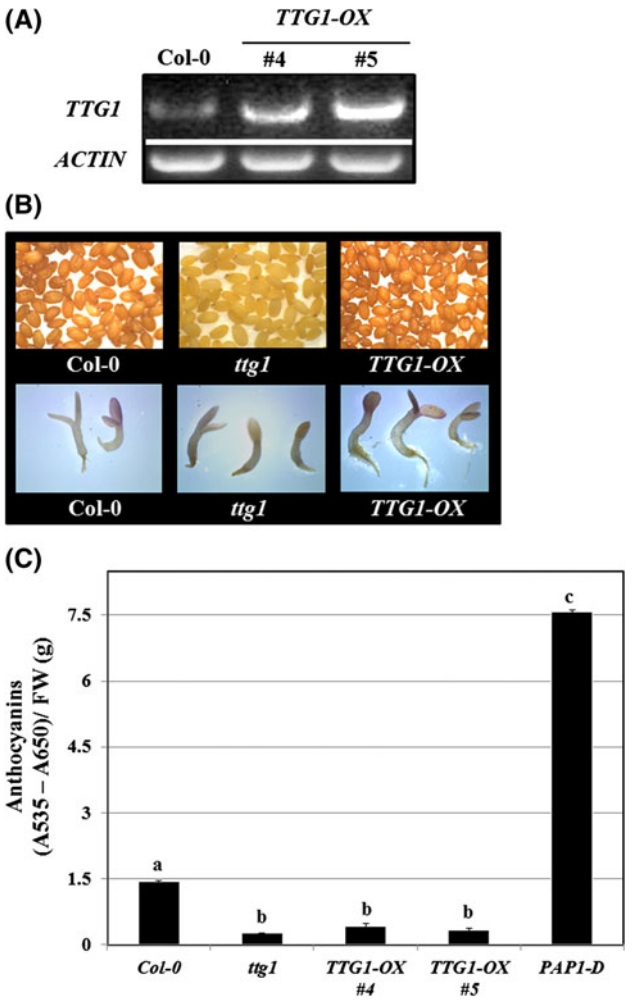

Fig. 3 Reduced level of anthocyanins in *TTG1-OX* seedlings. Full-length *TTG1* cDNA was cloned under the control of the *CaMV 35S* promoter to generate *TTG1-OX* seeds. **a** The overexpression of *TTG1* was examined using *RT-PCR*. Actin is shown as the internal loading control. Ten-day-old seedlings were grown on normal 1/2 × MS medium before total RNA extraction for RT-PCR. **b** Phenotypes of wild-type, *ttg1* and *TTG1-OX* seeds or seedlings. Seed coat color (300 mg seeds of each) and the accumulation of anthocyanins ($n = 50$ per each experiment) are shown. For visualization of anthocyanins, seeds of mutants were grown for 3 days on 1/2 × MS medium containing 50 μM norflurazon (inhibitor of chlorophyll formation). Seedlings then were then observed, and pictures were taken using a Leica EZ4D microscope. *Purple color* indicates anthocyanin accumulation. **c** Ten-day-old seedlings grown on normal 1/2 × MS medium were ground, and anthocyanins were extracted in aqueous solution and subjected to measurement. The seeds or seedlings shown were typical of three separate experiments performed at 23 °C. A one-way ANOVA Tukey's test ($P < 0.05$) was performed to indicate significant differences in anthocyanin accumulation between the wild-type and mutants. *Lower-case letters* (a, b, c) were marked above the column to reflect significant differences. *Vertical bars* indicate standard error ($n = 3$) (color figure online)

upon exposure to ABA (Fig. 6a). The expression levels of some stress-marker genes were therefore examined to confirm whether these mutants indeed exhibited increased levels of stress tolerance. However, as shown in Fig. 6c, the results showed that transcript levels of *COLD-REGULATED 15A* (*Cor15A*) and *KIN1* were quite similar in wild-type and *TTG1-OX* seedlings. This demonstrates that the enhanced root growth of *TTG1-OX* seedlings exposed to stress or ABA treatment were not due to a simple mediation of stress-tolerance signaling pathways.

Differences in root architecture of TTG1-OX seedlings are related to auxin activity

Auxin is the most important phytohormone for root development. The *TTG1-OX* seedlings showed enhanced root architecture not only in response to ABA treatment but also under normal conditions (Fig. 6a, b). The above results also indicate that this enhanced root development did not occur through the enhancement of stress-marker gene expression (Fig. 6c). Additionally, flavonols, which are auxin polar transport inhibitors, showed increased accumulation in *TTG1-OX* seedlings. For these reasons, we hypothesized that the expression of auxin-related genes is altered in *TTG1-OX* seedlings. In fact, the transcript levels of *IAA3* and *IAA17* were lower in transgenic seedlings (Fig. 7). The repression of these genes has been shown to affect root system development and adaptation (Kim et al. 2006; Tian and Reed 1999). Next, we intended to examine the auxin sensitivity upon ABA treatment in combination with sucrose (Fig. 8). To do this, transgenic seedlings containing an auxin marker construct (*DR5::GUS*) were treated with ABA and sucrose (Fig. 8). These results demonstrate that ABA and sucrose have an antagonistic relationship with regard to the auxin sensitivity of plant cells (Fig. 8).

Taken together, we can speculate that genetic modification of the flavonoids biosynthetic pathway somehow affects auxin activation, resulting in changes in root architecture.

Discussion

It is well established that modifications in root system architecture are consequences of a balance between stimulatory and repressive signaling cascades. For example, ABA is proposed to inhibit the formation of LRs from primordia, while auxin accumulation is important for lateral root initiation and elongation (Deak and Malamy 2005). Although root architecture is primarily determined by phytohormones such as auxin and ABA, recent reports suggest that flavonoids exert a critical influence on root architecture as well. According to a previous report, the root architectures of several mutants devoid of certain genes that function in flavonoids biosynthesis are quite

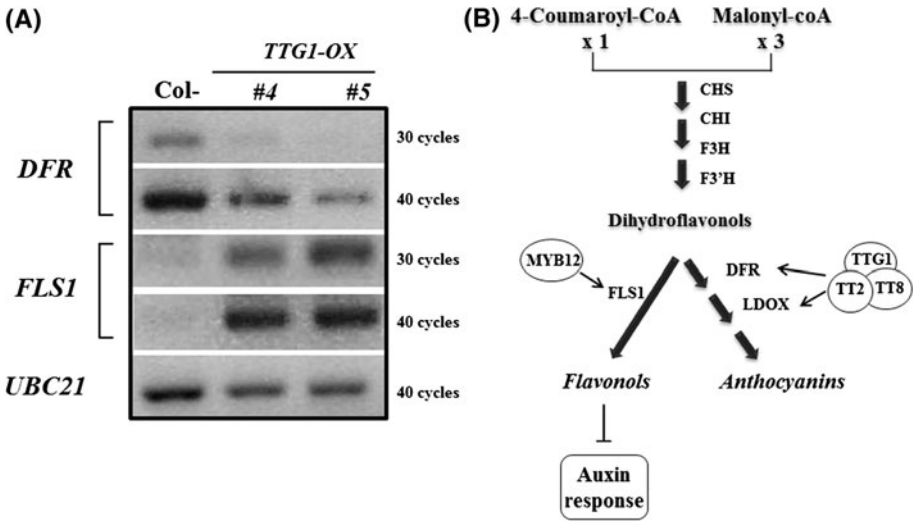

Fig. 4 The transcript levels of the *DFR* and *FLS1* genes in wild-type and *TTG1-OX* seedlings. **a** The transcript levels of flavonoids biosynthetic genes *DFR* and *FLS1* were determined in wild-type and *TTG1-OX #4* and *#5* seedlings on normal 1/2 × MS medium. Several rounds of *RT-PCR* were performed, and *PCR* results from two experiments using different numbers of cycles are shown. *UBIQUITIN-CONJUGATING ENZYME 21* (*UBC21*) was used as the internal loading control. Ten-day-old seedlings were grown on normal 1/2 × MS medium before total RNA extraction for *RT-PCR*. **b** Simplified model of the flavonoids biosynthetic pathway. First, chalcone synthase (CHS) catalyzes the condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA. The later steps in this pathway are catalyzed by a series of enzymes, leading to the production of two main types of final products: anthocyanins and flavonols, i.e., auxin transport inhibitors. (*CHI* chalcone isomerase, *F3H* flavonol 3-hydroxylase, *F3′H* flavonol 3′-hydroxylase, *FLS* flavonol synthase, *DFR* dihydroflavonol-4-reductase, *LDOX* leucoanthocyanidin dioxygenase)

different from those of the wild-type (Buer and Djordjevic 2009). For example, *tt4* and *tt8* seedlings showed fewer LRs when compared to wild-type (Buer and Djordjevic 2009). Moreover, there is an array of convincing evidence that there is a link between flavonoids and auxin polar transport (Murphy et al. 2000; Santelia et al. 2008; Peer and Murphy 2007). By using *tt4* seedlings, Murphy et al. (2000) indicated that roots of *tt4* released more auxin into the medium than did the wild-type (Murphy et al. 2000). Recently, it was proposed that flavonoids affect the redirection of the auxin stream by exerting a specific influence on the location of PIN (PIN-FORMED) (Santelia et al. 2008). Based on these reports, we hypothesize that ABA may modulate the biosynthesis of flavonoids to alter the direction of auxin polar transport in an attempt to suppress LR growth when plants are suffering from drought stress. TTG1 is an important transcription factor involved in flavonoids biosynthesis. Moreover, TTG1 functions in various processes during plant morphogenesis including root hair and trichome formation (Gonzalez et al. 2008; Walker et al. 1999; Zhang et al. 2003); thus, we exploited the *ttg1* mutant to examine its response to ABA with regard to LR growth (Fig. 1). On 0 % sucrose medium, ABA inhibition of LR growth was not observed, while on medium containing higher concentrations of sucrose along with ABA, the *ttg1* mutants differed considerably from the wild-type. Moreover, the number of LRs increased with increasing levels of sucrose (2–4 %), despite the presence of ABA (1 μM). According to Deak and Malamy (2005), more than 4.5 % sucrose in the medium may produce abnormal effects on plant growth due to high osmotic stress. A significant decrease in the number of LRs in the *ttg1* mutant demonstrates that this mutant is highly sensitive to ABA. It appears that the increased biosynthesis of flavonoids resulting from sucrose treatment might lead to decreased sensitivity to ABA in both wild-type and *ttg1* plants (Fig. 1). This reasoning was further supported by our observation of the regulation of the *CHS* gene that we studied using transgenic plants harboring the *GUS* reporter gene under the control of the *CHS* promoter (Fig. 2a). The *CHS* gene is fairly sensitive to sucrose, leading to increased flavonoids biosynthesis (Solfanelli et al. 2006). However, GUS activity was severely suppressed by the presence of ABA plus 4 % sucrose in the medium (Fig. 2a). Notably, the inhibitory effect of ABA on GUS activity was not obvious if the seedlings were incubated in medium containing more than 4 % sucrose (Fig. 2a). Our results are in accordance with the previous studies that showed that sucrose can enhance flavonoids biosynthesis, leading to an alteration of endogenous flavonols accumulation that in turn affects root architecture (Solfanelli et al. 2006; Peer et al. 2011).

The *ttg1* mutant is more sensitive to ABA than wild-type. This led us to test our hypothesis using the transgenic *TTG1-OX* seedlings. At first, we expected to see enhanced anthocyanin biosynthesis in the *TTG1-OX* seedlings, which

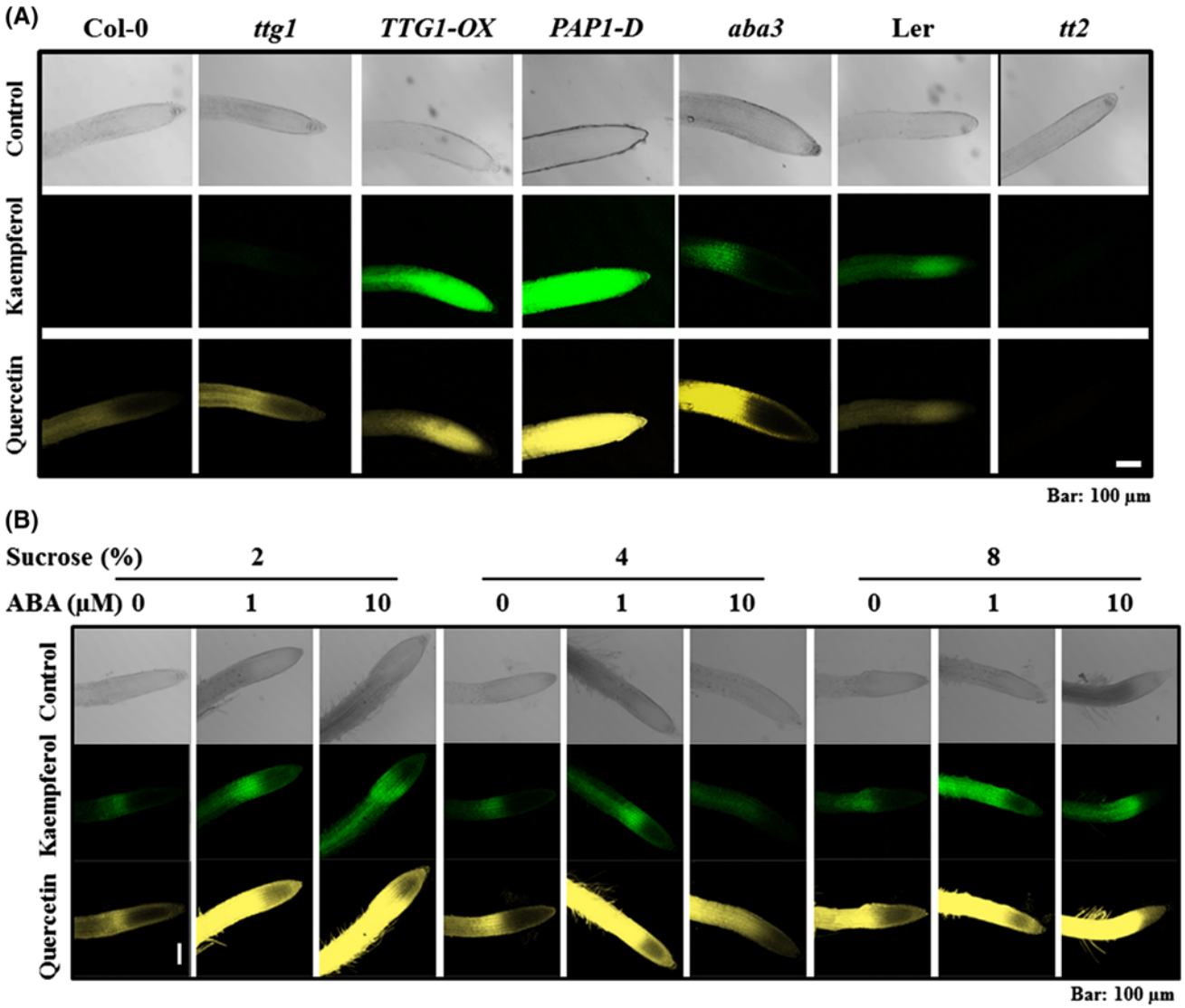

Fig. 5 Kaempferol and quercetin (flavonols) accumulation in wild-type and *TTG1-OX* seedlings. **a** Seven-day-old wild-type (Col-0 and Ler), *TTG1-OX*, *PAP1-D*, *ttg1*, *tt2* and *aba3* seedlings grown on normal 1/2 × MS medium were used for DPBA staining for the visualization of kaempferol and quercetin accumulation (*Bar* 100 µm). The roots shown were typical of three separate experiments performed at 23 °C ($n = 10$ seedlings per experiment). **b** Seven-day-old wild-type (Col-0) seedlings were transferred to 1/2 × MS medium supplemented with indicated concentrations of sucrose and ABA for 24 h and used for DPBA staining for the visualization of kaempferol and quercetin accumulation (*Bar* 100 µm). The roots shown were typical of three separate experiments performed at 23 °C ($n = 10$ seedlings per experiment)

turned out not to be the case (Fig. 3). Even more surprising, the anthocyanin level was diminished in the transgenic seedlings (Fig. 3c). The roots of *ttg1* mutants accumulate more quercetin, which belongs to the flavonols group, than wild-type plants (Buer and Djordjevic 2009). In contrast, the levels of kaempferol and quercetin were reduced in the *tt4* and *tt7* mutant seedlings (Lewis et al. 2011). Indeed, *TTG1-OX* accumulated kaempferol and quercetin rather than anthocyanins (Figs. 3c, 5). Therefore, we hypothesized that the increase in LR number in *TTG1-OX* is a consequence of altered levels of flavonols accumulation (Figs. 4, 5). Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) is a rate-limiting enzyme involved in the biosynthesis of anthocyanins (Martens et al. 2003). Anthocyanins and catechins are no longer synthesized when the *DFR* gene is deactivated in barley and *Arabidopsis*, which indicates that this gene is vital for the production of anthocyanins (Olsen et al. 1993; Shirley et al. 1995). The decreased level of the *DFR* transcript in *TTG1-OX* seedlings in comparison to the level in wild-type seedlings may be responsible for the reduced accumulation of anthocyanins in the transgenic seedlings (Figs. 3c, 4a). Similarly, the *DFR* transcript is reduced in *TT2 overexpression* (*TT2-OX*) seedlings (Nesi et al. 2001). It remains unclear why

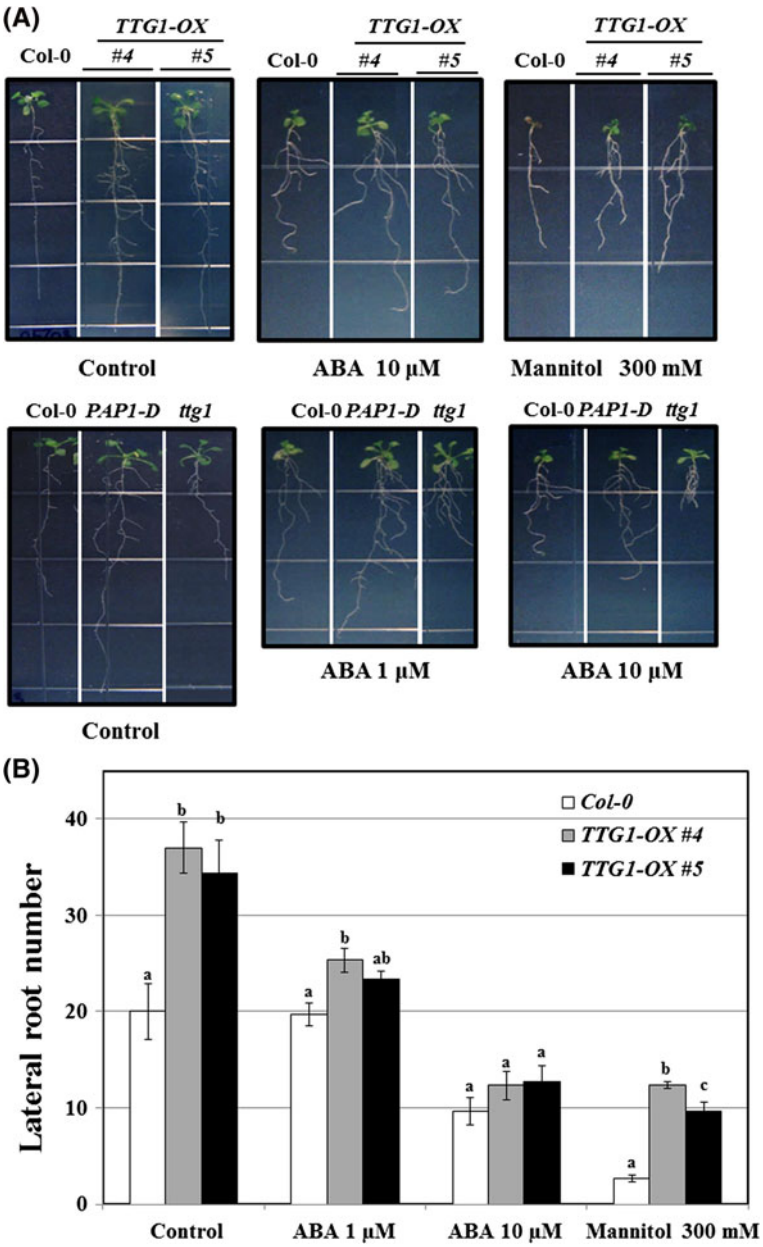

Fig. 6 Growth phenotypes of wild-type, *TTG1-OX*, *PAP1-D* and *ttg1* seedlings grown on 1/2 × MS medium supplemented with 10 µM ABA or 300 mM mannitol. **a** Four-day-old seedlings were transferred to test medium and allowed to grow for 10 days before being photographed. The seedlings shown were typical of three separate experiments performed at 23 °C ($n = 15$ seedlings per experiment). **b** The numbers of LRs in these seedlings were counted under a microscope. The data shown are from three separate experiments performed at 23 °C ($n = 10$ per experiment). One-way ANOVA Tukey's test ($P < 0.05$) was performed to indicate a significant difference in LR number between wild-type and mutants per treatment. *Lower-case letters* (*a*, *b*, *c*) were marked above the column to reflect significant differences. *Vertical bars* indicate standard error ($n = 30$). **c** The transcript levels of the stress-marker genes *Cor15A* and *KIN1* were determined for wild-type and *TTG1-OX #4* and *#5* seedlings on normal 1/2 × MS medium. Several rounds of *RT-PCR* were performed, and *PCR* results from two experiments using different numbers of cycles are shown. *UBC21* was used as the internal loading control. Ten-day-old seedlings were grown on normal 1/2 × MS medium before total RNA extraction for *RT-PCR*

the accumulation of the *DFR* gene transcript is lower in *TTG1-OX* and *TT2-OX* seedlings than in wild-type seedlings. The enhanced level of TTG1 and TT2 protein in *TTG1-OX* and *TT2-OX* seedlings, respectively, may interfere with the structure of the transcriptional complex comprising TTG1, TT2 and TT8. It is plausible that a complex with only two components forms. Whereas the level of the *DFR* transcript was reduced in *TTG1-OX* plants, the level of the *FLS1* transcript was greatly increased. This may explain the enhanced levels of flavonols (kaempferol and quercetin) in *TTG1-OX* seedlings in comparison to levels in wild-type seedlings (Figs. 4a, 5).

The role of ABA in flavonoid accumulation is controversial, because the level of anthocyanins is enhanced by ABA treatment in some plants (Jiang and Joyce 2003; Jeong et al. 2004), and reduced in others (Guruprasad and Laloraya 1980; Ozeki and Komamine 1986). As demonstrated in our study (Fig. 5b), ABA appears to increase the kaempferol and quercetin at 2 % sucrose. However, in the presence of 4 or 8 % sucrose, the accumulation of flavonols in response to ABA shows concentration dependent manner, indicating that the regulation of flavonoid biosynthesis is very complicated. The precise function of ABA in the regulation of flavonoid biosynthesis requires further study.

Abscisic acid is produced at a high level when plants are exposed to abiotic stresses such as water stress (Jiang and Zhang 2002). It seems that the enhanced number of LRs in *TTG1-OX* was not a result of the elicitation of the abiotic

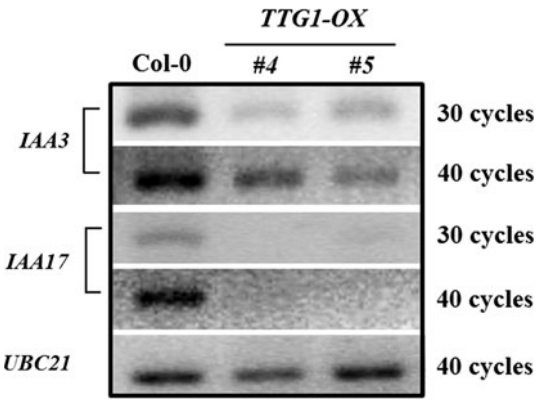

Fig. 7 The transcript levels of the *IAA3* and *IAA17* genes in wild-type and *TTG1-OX* seedlings. Several rounds of *RT-PCR* were performed, and *PCR* results from two experiments using different numbers of cycles are shown. *UBC21* was used as the internal loading control. Ten-day-old seedlings were grown on normal 1/2 × MS medium before total RNA extraction for *RT-PCR*

et al. 2003) led us to examine the auxin sensitivity of wild-type seedlings in response to ABA. We found that the transcript levels of auxin responsive genes such as *IAA3* and *IAA17* were fairly decreased in *TTG1-OX* compared to wild-type (Fig. 7). We also treated transgenic seedlings containing an auxin marker construct (*DR5::GUS*) with ABA and sucrose (Fig. 8). These results demonstrate that ABA and sucrose have an antagonistic relationship in the auxin sensitivity of plant cells (Fig. 8). It is possible that the interference of the auxin transport system that occurred in the *TTG1-OX* seedlings through an alteration of flavonols biosynthesis could change local auxin accumulation, leading to an increase in LR growth in the presence of ABA. Although the molecular mechanism underlying the ability of low concentrations of ABA to facilitate lateral root growth remains unclear, our results demonstrate that ABA regulates the accumulation of anthocyanins and

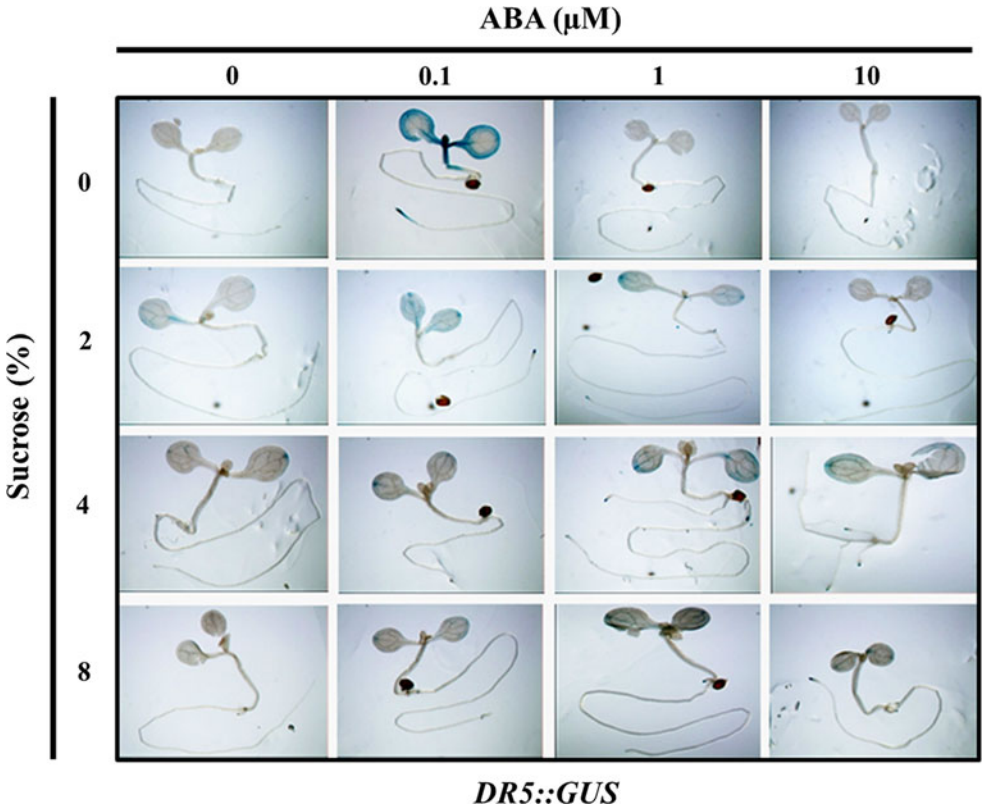

Fig. 8 The effects of sucrose and ABA on the GUS expression in *DR5::GUS* transgenic seedlings. Four-day-old *DR5::GUS* transgenic seedlings grown on normal 1/2 × MS medium were transferred to 1/2 × MS medium supplemented with the indicated concentrations of ABA and sucrose and incubated for 6 h before GUS staining. The seedlings shown were typical seedlings from three separate experiments performed at 23 °C ($n = 10$ per experiment)

stress-signaling pathway, because the transcript levels of stress-responsive marker genes such as the *Cor15A* and the *KIN1* were not altered in *TTG1-OX* under normal conditions (Fig. 6c). A previous report showing that ABA inhibits LR growth at the post-emergence step (De Smet

flavonols, which may alter the proper distribution of auxin in root systems.

Water scarcity is a frequently occurring abiotic stress that has a decisive effect on plant growth and development and that limits crop productivity (Sadras and Milroy 1996;

Ceccarelli and Grando 1996). Taken together, our results offer the biotechnological potential for increasing crop yield in which the genetic manipulation of the transcription factor involved in the flavonoids biosynthetic pathway could confer enhanced plant root growth under water-stress conditions in the field.

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