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The Arabidopsis UDP-glycosyltransferases UGT79B2 and UGT79B3, contribute to cold, salt and drought stress tolerance via modulating anthocyanin accumulation

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SUMMARY

The plant family 1 UDP-glycosyltransferases (UGTs) are the biggest GT family in plants, which are responsible for transferring sugar moieties onto a variety of small molecules, and control many metabolic processes; however, their physiological significance in planta is largely unknown. Here, we revealed that two Arabidopsis glycosyltransferase genes, *UGT79B2* and *UGT79B3*, could be strongly induced by various abiotic stresses, including cold, salt and drought stresses. Overexpression of *UGT79B2/B3* significantly enhanced plant tolerance to low temperatures as well as drought and salt stresses, whereas the *ugt79b2/b3* double mutants generated by RNAi (RNA interference) and CRISPR-Cas9 strategies were more susceptible to adverse conditions. Interestingly, the expression of *UGT79B2* and *UGT79B3* is directly controlled by CBF1 (CRT/DRE-binding factor 1, also named DREB1B) in response to low temperatures. Furthermore, we identified the enzyme activities of UGT79B2/B3 in adding UDP-rhamnose to cyanidin and cyanidin 3-O-glucoside. Ectopic expression of *UGT79B2/B3* significantly increased the anthocyanin accumulation, and enhanced the antioxidant activity in coping with abiotic stresses, whereas the *ugt79b2/b3* double mutants showed reduced anthocyanin levels. When overexpressing *UGT79B2/B3* in *tt18* (*transparent testa 18*), a mutant that cannot synthesize anthocyanins, both genes fail to improve plant adaptation to stress. Taken together, we demonstrate that *UGT79B2* and *UGT79B3*, identified as anthocyanin rhamnosyltransferases, are regulated by CBF1 and confer abiotic stress tolerance via modulating anthocyanin accumulation.

Keywords: UGT79B2/B3, glycosyltransferase, anthocyanin, CBF1, cold stress, stress tolerance, Arabidopsis.

INTRODUCTION

Low temperature, drought and high salinity are common stresses that adversely affect plant growth and crop production. Harsh environmental factors lead to the overproduction of reactive oxygen species (ROS), which are highly reactive and toxic, and affect many cellular functions by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation (LPO) (Gill and Tuteja, 2010). It is well documented that the induction of the cellular antioxidant machinery for scavenging ROS is important for protecting against various stresses (Ning *et al.*, 2010; You *et al.*, 2014; Hazman *et al.*, 2015), which includes two defense systems: the enzymatic antioxidants and non-enzymatic antioxidants (Gill and Tuteja, 2010). Major enzymatic antioxidants include superoxide dismutase (SOD), catalases (CATs), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), etc. (Gill and Tuteja, 2010), and overexpression of these genes tend to significantly enhance plant tolerance to various abiotic stresses through ROS scavenging (Matsumura *et al.*, 2002; Badawi *et al.*, 2004; Eltayeb *et al.*, 2007; Kim *et al.*, 2008). Non-enzymatic antioxidants are ascorbate, glutathione (GSH), carotenoids, tocopherols and flavonoids (Mittler *et al.*, 2004; Gill and Tuteja, 2010). Flavonoids are among the most bioactive plant secondary metabolites, which even outperform the well-known antioxidants such as ascorbate and α-tocopherols (Hernandez *et al.*, 2009). They serve as ROS scavengers by locating and neutralizing radicals by virtue

of the number and arrangement of their hydroxyl groups attached to the ring structures, and thus are important for plants under adverse environmental conditions (Winkel-Shirley, 2002).

Anthocyanins, a main class of flavonoids, play multiple roles in higher plants. They provide a variety of red, blue and purple colors for flowers, fruits as well as other plant parts, acting as insect and animal attractants (Harborne and Williams, 2000; Winkel-Shirley, 2001). They also serve as important molecules in vegetative tissues to absorb UV and high light irradiation, antioxidants that scavenge ROS and agents acting against microbes in defense responses (Koes *et al.*, 2005; Lepiniec *et al.*, 2006; Pourcel *et al.*, 2007; Mouradov and Spangenberg, 2014). In Arabidopsis, anthocyanin biosynthesis is well characterized at the genetic, enzymatic and product levels. It is primarily controlled by regulatory factors such as MYB, bHLH and WD40 proteins (Borevitz *et al.*, 2000; Gonzalez *et al.*, 2008; Pesch *et al.*, 2015), as well as a series of enzymes such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-sugar:flavonoid-3-O-glucosyltransferase (UFGT) (Winkel-Shirley, 2001). Many genes involved have been identified by screening mutants that show a *transparent testa* (*tt*) phenotype on the seed coat, owing to defects in various steps of the flavonoid biosynthesis pathway (Koornneef, 1990; Shirley *et al.*, 1992). For instance, *TT2* (encoding MYB123), *TT8* (encoding bHLH42) and *TTG1* (*TRANSPARENT TESTA GLABRA 1,* encoding a WD40 protein), act in concert in the regulation of proanthocyanidin accumulation in developing seeds (Nesi *et al.*, 2001). *TT18* encodes an ANTHOCYANIDIN SYNTHASE (ANS) and is involved in proanthocyanin biosynthesis. Loss of *ANS* in *tt18* resulted in a deficiency in anthocyanins and pale-yellow seeds (Abrahams *et al.*, 2002). Glycosylation, catalyzed by UDP-glycosylatransferase (UGTs), is the last step in anthocyanin biosynthesis, and leads to diverse anthocyanin molecules in Arabidopsis (Shi and Xie, 2014). All anthocyanins identified in Arabidopsis contain at least one sugar group. The hydroxyl groups at C3 and C5 positions of cyanidin were found to be most frequently modified by glycosylation (Tohge *et al.*, 2005; Pourcel *et al.*, 2010). Until now, several UGTs have been identified as involved in the anthocyanin biosynthesis pathways. The Arabidopsis UGT78D2 and UGT75C1 have been reported to glycosylate the hydroxyl group at C3 and C5 to form cyanidin 3/5-O-glucosides, respectively (Tohge *et al.*, 2005). And these molecules could be further glycosylated at other positions by accepting different sugar donors. Yonekura-Sakakibara *et al.* (2012) reported that *UGT79B1,* encoding an anthocyanin 3-O-glucoside: 2″-O-xylosyltransferase, could convert cyanidin 3-O-glucoside to cyanidin 3-O-xylosyl(1–>2)glucoside.

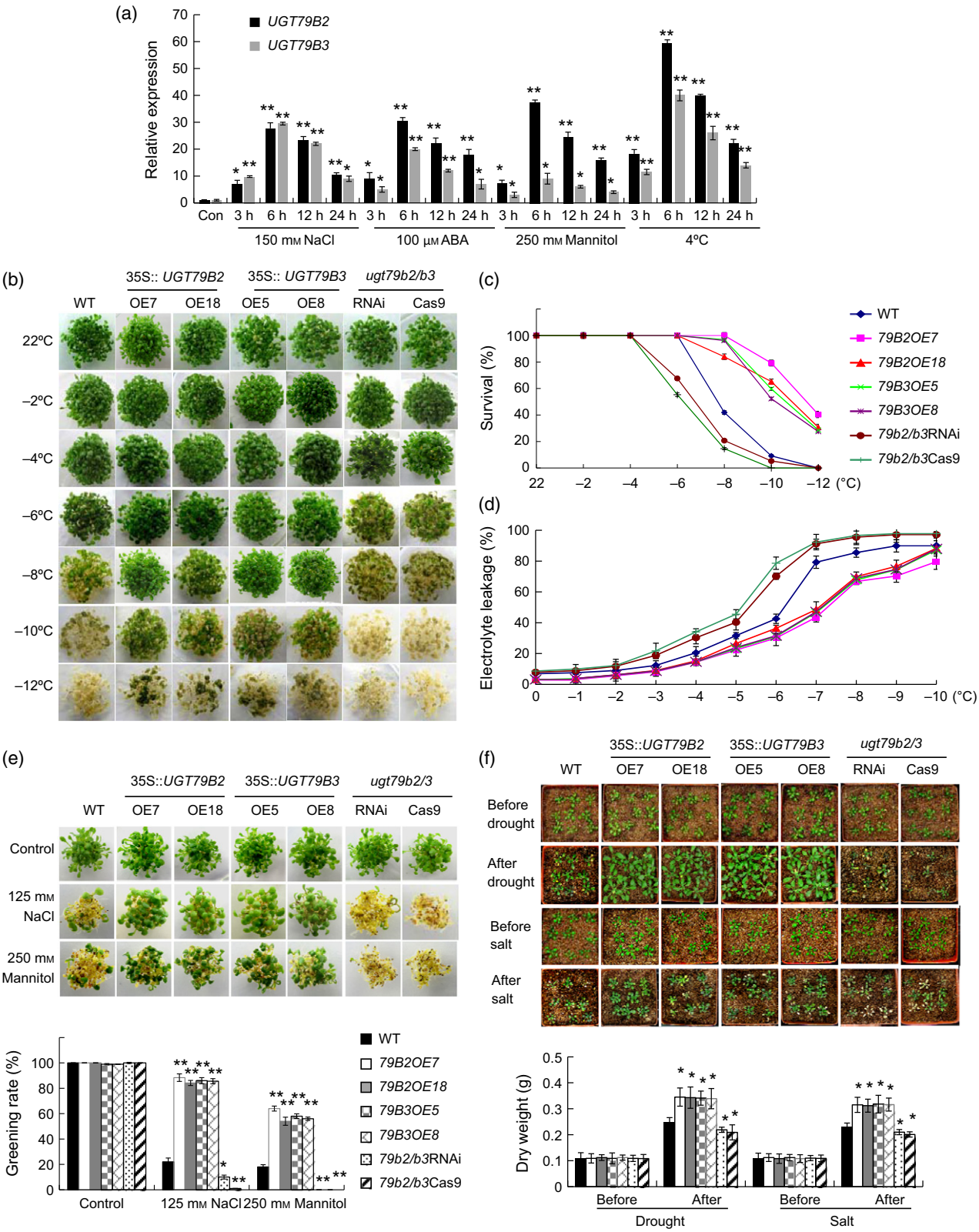
In our study, two Arabidopsis glycosyltransferases, UGT79B2 and UGT79B3, were identified to modify anthocyanins via adding UDP-rhamnose to cyanidin and 3-O-glucoside-cyanidin. Ectopic expression of *UGT79B2/B3* in Arabidopsis leads to the overproduction of anthocyanins, which enhanced ROS scavenging and contributed to abiotic stress tolerance; however, the *ugt79b2/b3* double mutants exhibited reduced anthocyanin accumulation and sensitivity to stress. *UGT79B2* and *UGT79B3* are also found to be regulated by CBF1 in response to cold stress. Based on our study, we demonstrate that UGT79B2 and UGT79B3 play crucial roles in modulating anthocyanin biosynthesis and abiotic stress tolerance.

RESULTS

UGT79B2/B3 is strongly induced by cold and other environmental stimuli

Microarray data deposited on the platform Genevestigator (Zimmermann *et al.*, 2004;) showed that *UGT79B2* and *UGT79B3* were strongly responsive to a variety of environmental stimuli, and especially to low temperatures. To validate this, 2-week-old wild-type Arabidopsis seedlings were exposed to time-course treatment under NaCl, ABA, mannitol and cold (4°C) conditions, respectively, and the *UGT79B2/B3* expression was determined by quantitative real-time PCR (qRT-PCR). Consistent with the online data, *UGT79B2/B3* expression levels were significantly induced under these treatments (Figure 1a). Intriguingly, both *UGT79B2* and *UGT79B3* were upregulated more by cold than by other stress conditions.

Figure 1. Roles of *UGT79B2/B3* in abiotic stresses tolerance. (a) Induction of *UGT79B2/B3* by ABA and abiotic stresses assessed by qRT-PCR. Two-week-old Arabidopsis wild-type (WT) seedlings growing on agar liquid medium under long-day (LD) conditions were treated with a hydroponic solution of 150 mM NaCl, 100 μM ABA and 250 mM mannitol, in cold (4°C), for 3, 6, 12 and 24 h, respectively. *UGT79B2/B3* mRNA levels were assessed by qRT-PCR. Expression in the non-treated sample (Con) was set to 1.0. Asterisks indicate significant differences relative to control conditions (Student's *t*-test: $*P < 0.05$; $**P < 0.01$). (b–d) Tolerance of *UGT79B2/B3* overexpression and mutant plants against low temperatures: 12-day-old plants were subjected to a series of low temperatures below zero (b), and then their tolerance was assessed in terms of survival rate (c) and electrolyte leakage (d). (e) Seedling greening of *UGT79B2/B3* overexpression and *ugt79b2/b3* mutant lines against NaCl and mannitol. Seeds were germinated and continued to grow on MS medium supplemented with NaCl and mannitol, respectively. After 2 weeks, seedling greening (upper) was observed and rates (lower) were calculated. Asterisks indicate significant differences relative to the WT (Student's *t*-test: $*P < 0.05$; $**P < 0.01$). (f) Tolerance of plants to salt and drought stresses in soil (for treatment details, see Experimental Procedures), and the dry weight (lower) of 10 plants were evaluated. At least three biological replicates were performed in this experiment. Asterisks indicate significant differences relative to WT (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).



Generation of UGT79B2/B3 overexpression and loss-of-function lines

To investigate further, we generated 35S::*UGT79B2/B3* overexpression (*UGT79B2/B3OE*) plants and selected two independent homozygous lines with high expression levels (>20-fold) for each gene, *UGT79B2OE7, OE18*, *UGT79-B3OE5* and *OE8*, for further analysis (Figure S1a).

Furthermore, to generate loss-of-function lines for *UGT79B2/B3*, RNAi and CRISPR/Cas 9 strategies were employed. Using an identical sequence present in both *UGT79B2* and *UGT79B3* cDNA, a double-stranded hairpin RNA was designed and transgenic plants were generated. qRT-PCR revealed that the mRNA levels of both *UGT79B2* and *UGT79B3* were significantly knocked down in RNAi lines L1 and L2 (Figure S1b). Here, we used L1 for further analysis, designated as *ugt79b2/b3RNAi*. In addition, with a guide RNA targeting the 5′ coding region of both *UGT79B2* and *UGT79B3* cDNA, the CRISPR/Cas 9 system was constructed and then introduced into Arabidopsis. After sequencing more than 100 transgenic plants, we obtained line 99, designated as *ugt79b2/b3 Cas9*, in which both *UGT79B2* and *UGT79B3* were disrupted by the insertion of a base A at the same target site (Figure S1c). Both *UGT79B2* and *UGT79B3* mRNA levels were not affected in line 99 (Figure S1d).

UGT79B2/B3 confers plant adaptation to low temperatures and other abiotic stresses

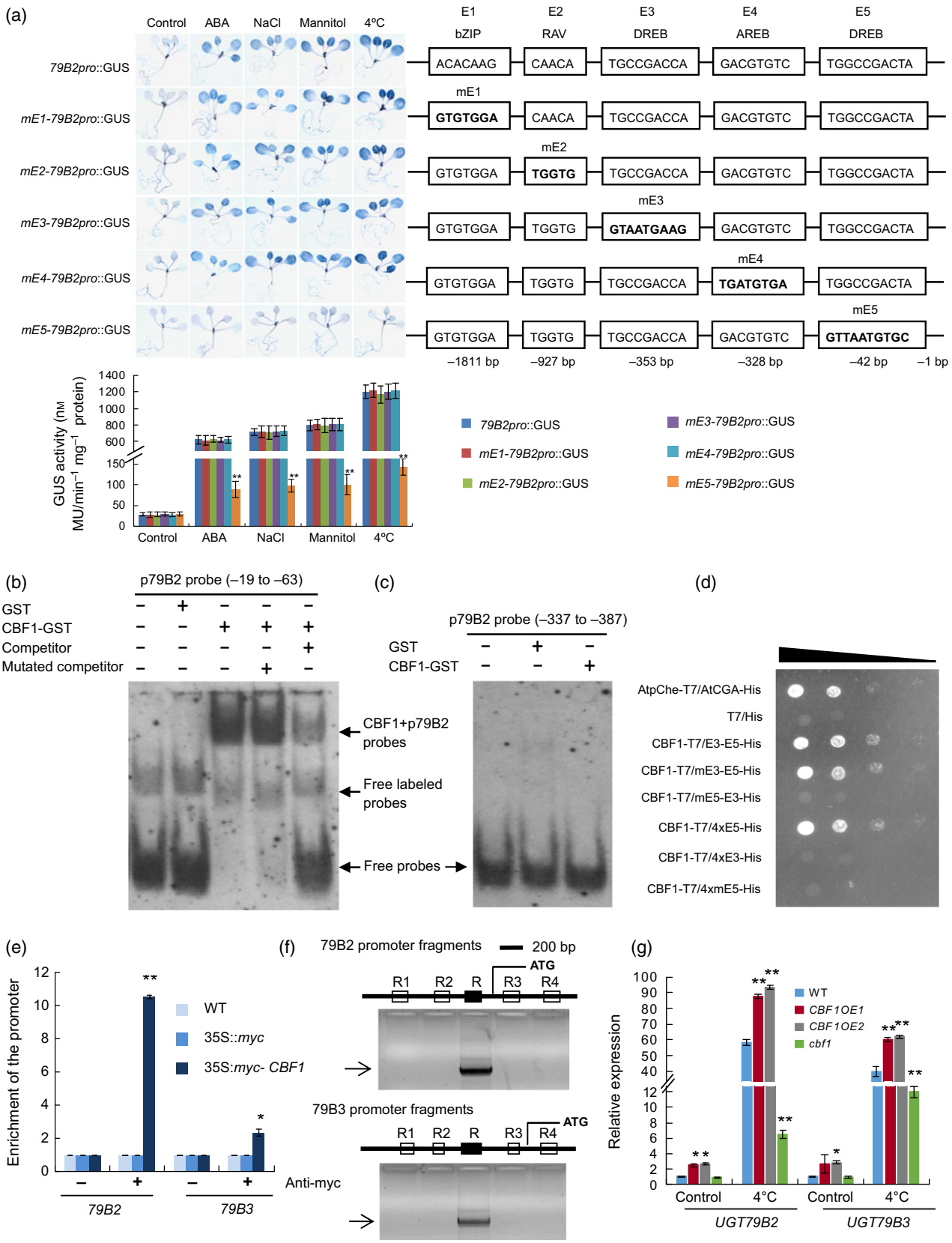
As *UGT79B2/B3* is profoundly induced by cold stress, we first evaluated their roles in adapting to low temperatures. The 12-day-old wild type (WT), *UGT79B2/B3OE*, and the *ugt79b2/b3* RNAi, Cas9 lines were exposed to a series of freezing temperatures after cold acclimation and their survival rates were compared. All seven lines showed 100% survival at 22°C (control), −2°C and −4°C. When the temperature fell lower the *ugt79b2/b3*RNAi and Cas9 lines began to turn yellow first, at −6°C, and then the WT began to turn yellow at −8°C, and the four overexpression lines exhibited the best performances. At −12°C, the *ugt79b2/b3* lines and the WT completely turned white, and the four *UGT79B2/B3OE* lines remained with survival rates of at least 25% (Figure 1b,c). Here, we also observed less ion leakage in the four *UGT79B2/B3OE* plants and more ion loss in the *ugt79b2/b3RNAi* and *Cas9*, lines compared with WT plants (Figure 1d). From both visual assessment and electrolyte leakage assays, cold stress adaptation is greatly improved in *UGT79B2/B3OE* plants, and is obviously reduced when they were functionally deficient.

Additionally, when the *UGT79B2/B3OE* and the mutant lines were exposed to reduced water states, such as salt, drought and osmotic stress, we also observed better performance in the *UGT79B2/B3OE* lines for plant growth (Figure 1e,f) and root elongation (Figure S2), whereas sensitivity was observed in the *ugt79b2/b3* mutant lines. Other overexpression lines showing medium *UGT79B2/B3* transcript levels also showed enhanced tolerance to cold, salt and osmotic stresses, to varying degrees (Figure S3).

UGT79B2 and UGT79B3 are directly regulated by CBF1 in response to cold stress

Next we want to find out why *UGT79B2/B3* could be responsive to various adverse conditions, and how the stress signal is transmitted to *UGT79B2/B3.* Thus, we scanned the upstream regions with the online tool PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/), and found that both *UGT79B2* and *UGT79B3* promoters bear a few stress-regulated *cis*-elements supposed to be bound by different transcription factors. To analyze in detail how *UGT79B2* is regulated by abiotic stresses, we introduced mutations to five stress-related elements present in the *UGT79B2* promoter region, as shown in Figure 2(a), including one bZIP binding element at −1811 bp, one RAV binding element at −927 bp, one AREB binding site at −328 bp and two DREB binding sites, or namely DRE elements: a remote one at −353 bp and a proximal one at −42 bp. Transgenic plants carrying the GUS reporter gene driven by normal or mutated promoters were generated. To investigate the regulatory role of these *cis*-elements, the six transgenic plants were subjected to different stimuli, and GUS assays were performed. We observed that GUS expression is strongly induced by NaCl, mannitol and ABA, and is cold-driven by the normal *UGT79B2* promoter (Figure 2a), which is consistent with the qRT-PCR results (Figure 1a). When bZIP, RAV, AREB and the remote DRE (−353 bp) elements were mutated, GUS expression driven by the mutated promoters is still not affected; however, once the proximal DRE at

Figure 2. Regulation of *UGT79B2/B3* expression by CBF1 transcription factor in response to cold stress. (a) GUS staining (upper) and activity assays (lower) of the transgenic plants driven by *UGT79B2* promoters with mutated elements in response to abiotic stresses. E1–E5 represent five elements present in the *UGT79B2* promoter. mE1–mE5 represent mutated elements. GUS activities are represented by three independent replicates. **Significant difference at $P < 0.01$ relative to GUS activities driven by normal *UGT79B2* promoter. (b, c) Interaction of CBF1 with the DRE element as shown by electrophoretic mobility shift assay (EMSA). Two probes (from −19 to −63 bp) (b) and (from −337 to −387 bp) (c) containing the DRE element within the UGT79B2 promoter were synthesized and employed for this assay. (d) Interaction of CBF1 transcription factors with the DRE elements evaluated by yeast one-hybrid assays: AtpChe-T7/AtCGA-His, positive control; T7/His, negative control. (e) Enrichment of *UGT79B2* and *UGT79B3* promoter fragments performed by ChIP quantitative PCR. Before ChIP, all these plants were cold-treated (4°C) for 12 h, and ChIP was performed using an anti-myc antibody in wild-type (WT) plants, and 35S::*myc* and 35S::*myc-CBF*1 transgenic plants. Asterisks indicate significant differences relative to the WT (Student's *t*-test: *$P < 0.05$; **$P < 0.01$). (f) Other promoter regions (R1–R4) surrounding CBF1-binding sites (R) present in *UGT79B2* and *UGT79B3* promoters were amplified by RT-PCR with specific primers. Arrows indicate fragments bound by CBF1. (g) Two-week-old WT, *CBF1* overexpression and *cbf1* knock-out plants were exposed to cold stress, and then expression levels of *UGT79B2/B3* were assessed by qRT-PCR. Asterisks indicate significant differences relative to the WT (Student's *t*-test: *$P < 0.05$; **$P < 0.01$).



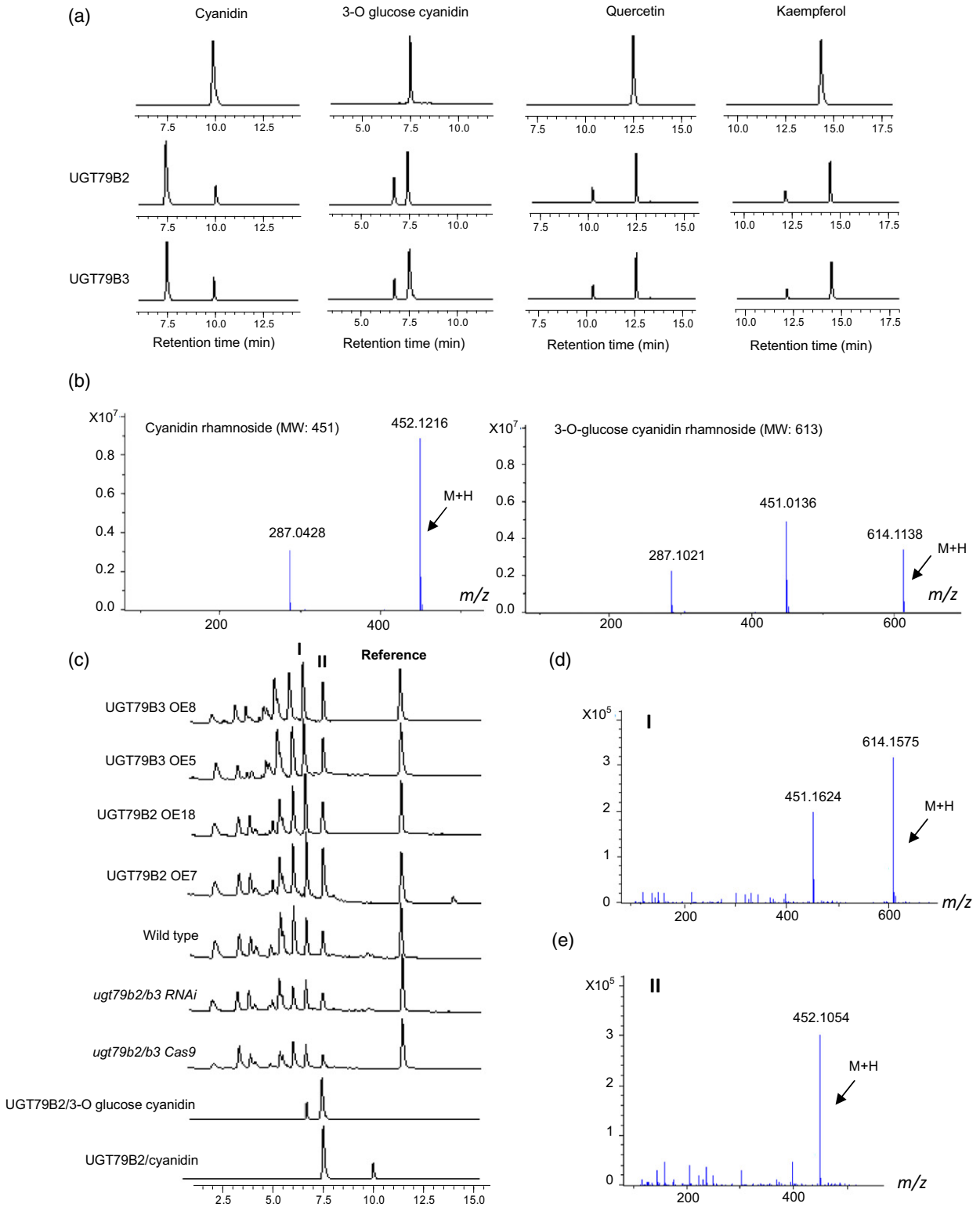


Figure 3. UGT79B2/B3 catalyzing activities towards cyanidin and 3-O-glucose cyanidin. (a) HPLC analysis of reaction products from cyanidin, 3-O-glucose cyanidin, quercetin and kaempferol. (b) LC-MS confirmation of reaction products from cyanidin and 3-O-glucose cyanidin. (c) HPLC analysis of endogenous anthocyanin levels in *UGT79B2/B3OE* and mutant lines. Reaction products from cyanidin and 3-O-glucose cyanidin catalyzed by UGT79B2 were used as controls. (d, e) LC-MS confirmation of endogenous anthocyanins I and II in the plants of (c): I, 3-O-glucose cyanidin rhamnoside; II, cyanidin rhamnoside. (f, g) HPLC analysis of the reaction products from cyanidin, 3-O-glucose cyanidin catalyzed by protein extracts from different plants (f), and the enzyme activity of each line evaluated by conversion rates (g). Asterisks indicate significant differences relative to the wild type (WT) (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).

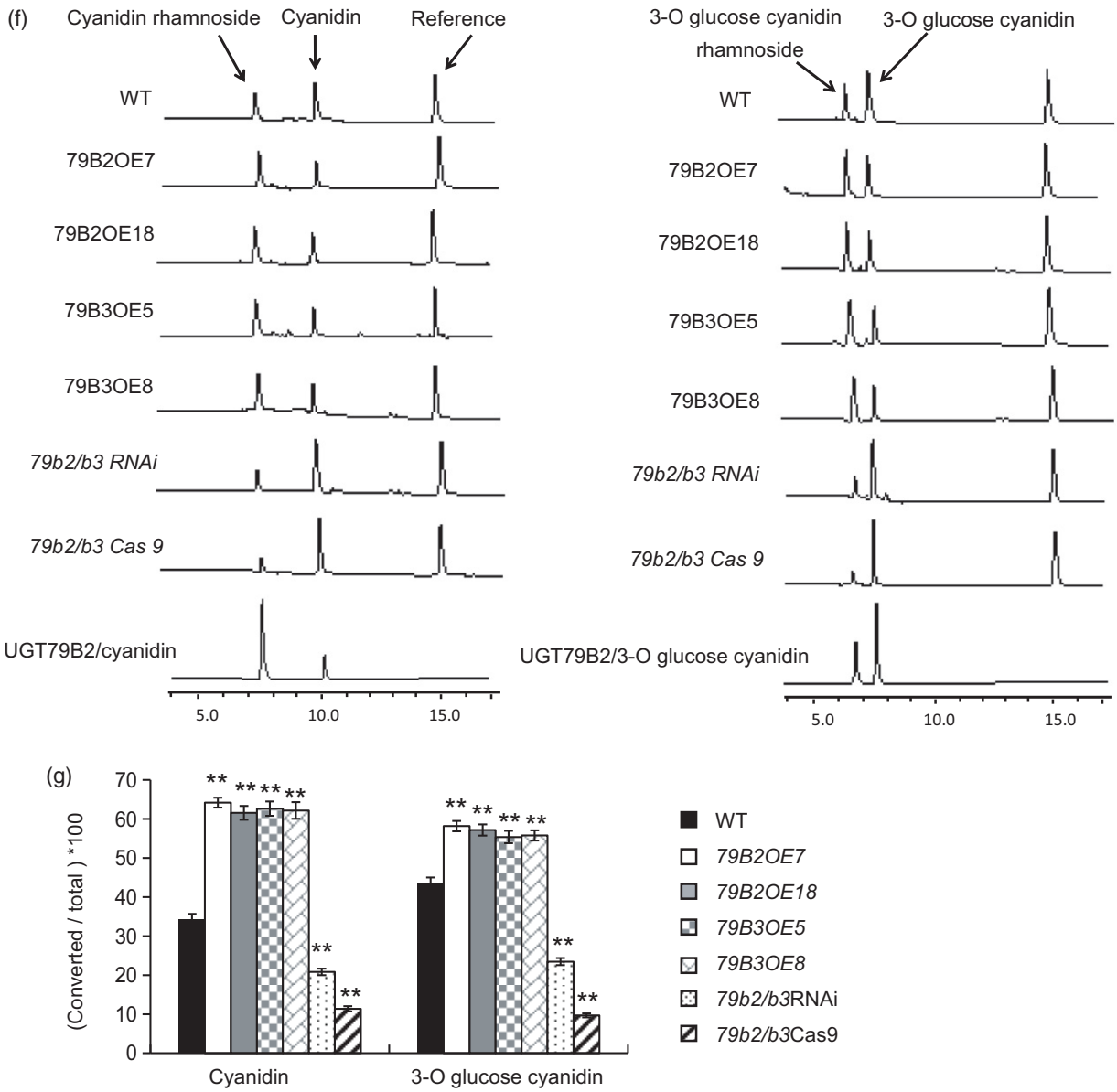


Figure 3. Continued

−42 bp was mutated, GUS staining upon exposure to ABA, NaCl, mannitol and cold was hardly seen, and GUS activity was significantly decreased too (Figure 2a), indicating that the DRE element at −42 bp is essential for the activity of the *UGT79B2* promoter in response to abiotic stresses.

We then selected several typical DREB transcription factors involved in stress regulation, including CBF1/DREB1B, CBF3/DREB1A, DREB2A and DREB2B, and then performed electrophoretic mobility shift assays (EMSAs) with the DRE element at −42 bp. A radioactively labeled DNA probe containing 40–50 bp of the *UGT79B2* promoter surrounding DRE was synthesized and incubated with the purified GST-tagged DREB proteins. We did not see any specific shifts of CBF3/DREB1A, DREB2A and DREB2B with the DRE-surrounding probe at −42 bp (Figure S4); however, specific binding of CBF1 with the DRE-surrounding probe at −42 bp was clearly observed, which could be matched by the competitor (Figure 2b). We also detected binding of the remote DRE fragment at −353 bp with CBF1, but this failed to produce retardation complexes (Figure 2c).

To further confirm these findings, CBF1 binding with DRE elements or mutated DRE elements of the *UGT79B2* promoter was also evaluated by yeast one-hybrid assays.

We observed that the interaction could occur between CBF1 and the promoter fragment ranging from E3 (−353 bp) to E5 (−42 bp), and that an interaction could still occur for the mutated E3 but not for the mutated E5. An interaction could also occur between CBF1 and four tandem-arrayed E5 fragments, but could not occur with four tandem-arrayed E3 fragments, nor with four copies of mutated E5 (Figure 2d). These findings indicate that the interaction of CBF1 with the DRE motif near the transcription starting site is required for *UGT79B2* in response to abiotic stress.

Furthermore, to confirm whether CBF1 directly binds to *UGT79B2* and *UGT79B3* promoters *in planta*, we performed chromatin immunoprecipitation (ChIP) assays. The myc-tagged CBF1 was overexpressed, driven by the CaMV *35S* promoter in Arabidopsis. Both untreated and cold-treated (4°C for 12 h) transgenic plants were subjected to ChIP assay. After precipitation, we detected by qRT-PCR that the DRE-containing region within *UGT79B2* and *UGT79B3* promoters was about 10.0-fold and 2.5-fold enriched with an anti-myc antibody, respectively, in the cold-treated materials (Figure 2e), and about 8.5-fold and 2.0-fold enriched in the untreated plants (Figure S5); however, we did not see any obvious enrichment in wild-type and 35S::*myc* transgenic Arabidopsis samples with or without anti-myc (Figure 2e). Additionally, to rule out any unspecific binding of CBF1 with other sites, we also amplified several genomic regions (R1–R4) flanking the CBF1 binding sites (R) in *UGT79B2* and *UGT79B3* promoters by RT-PCR, and no bands were detected other than R (Figure 2f).

In addition, the *in vivo* role of CBF1 in controlling *UGT79B2/B3* expression was also evaluated using *CBF1* overexpression and knock-out lines. qRT-PCR revealed that *UGT79B2* and *UGT79B3* were more strongly induced by low temperature upon *CBF1* overexpression, whereas they were hardly induced in the *cbf1* mutant (Figure 2g). These data provide reliable evidence that the Arabidopsis *UGT79B2/B3* could be directly regulated by the CBF1 transcription factor in response to cold stress.

CBF3 shares similar functions with CBF1 in regulating the cold response (Gilmour *et al.*, 2004; Novillo *et al.*, 2007). Thus, we also detected *UGT79B2* and *UGT79B3* levels in the *cbf3* mutant, and found that they are not affected by the functional loss of CBF3 (Figure S6).

UGT79B2 and UGT79B3 transfer UDP-rhamnose to cyanidin and cyanidin 3-O-glucoside

To determine why two UGTs could affect plant resistance to abiotic stresses so remarkably, it is necessary to characterize the enzyme activities of proteins UGT79B2 and UGT79B3. A decade ago UGT79B2 and UGT79B3 were predicted to be involved in modifying anthocyanin; however, until now no study had ever verified this hypothesis (Lazar and Goodman, 2006). Thus, to determine the substrate(s) of UGT79B2/B3, we purified the recombinant GST-tagged UGT79B2/B3 protein, and performed the substrate screening towards a broad scope of metabolites in the phenylpropanoid metabolic pathway, including cinnamic acid, ferulic acid, caffeic acid, etc., from the early steps of the phenylpropanoid pathway, and procyanidin, cyanidin, cyanidin 3-O-glucoside, quercetin and kaempferol, from the flavonoid biosynthesis pathway, as well as several compounds in the lignin biosynthesis pathway, such as coniferyl aldehyde and coniferyl alcohol, etc. (Table S1). HPLC analysis indicated that UGT79B2/B3 could only catalyze four compounds – cyanidin, cyanidin 3-O-glucoside, quercetin and kaempferol – converting them into corresponding rhamnosides (Figure 3a). In comparison, they have strong activity towards cyanidin, which is a twofold more powerful catalyst than cyanidin3-O-glucoside; however, both proteins only have very low activities towards quercetin and kaempferol, and have no activity towards others (Table S1). The sugar donor specificity of UGT79B2/B3 was examined with UDP-xylose, UDP-glucose, UDP-arabinose, UDP-rhamnose, UDP-galactose and UDP-glucuronicacid as donors and with cyanidin as the acceptor. No UGT activity was detected for UDP-sugars other than UDP-rhamnose, indicating that UGT79B2/B3 is highly specific to UDP-rhamnose (Table S2). Moreover, the reaction products of UGT79B2/B3 in catalyzing cyanidin and cyanidin 3-O-glucoside were analyzed by LC-MS, and the corresponding glycosides with a rhamnose addition were identified (Figure 3b).

Furthermore, to investigate the endogenous product catalyzed by UGT79B2/B3, the total anthocyanins were extracted from the *UGT79B2/B3OE* and mutant lines, and analyzed by HPLC (Figure 3c). Then LC-MS confirmed the production of cyanidin rhamnoside and 3-O-glucose cyanidin rhamnoside (Figure 3d,e). It is observed that the peaks represent two products that were obviously elevated in *UGT79B2/B3OE* lines, compared with the WT, and were reduced in the *ugt79b2/b3* RNAi and Cas9 lines (Figure 3c). Additionally, we also extracted the total protein from different plants and performed enzyme assays towards cyanidin and 3-O-glucose cyanidin, and the enzyme activities were evaluated by conversion rates (Figure 3f,g). Consistent with above findings, the conversion of the substrates was greatly enhanced upon *UGT79B2/B3* overexpression, whereas it was impeded in *ugt79b2/b3* mutants (Figure 3f,g).

UGT79B2 and UGT79B3 modulate anthocyanin metabolism

To investigate a possible role of *UGT79B2/B3* in anthocyanin metabolism, WT, four *UGT79B2/B3OE* lines and the *ugt79b2/b3* knock-out lines were germinated on MS medium and subjected to constant light for 5 days to enable

anthocyanins to accumulate. It is observed that the four *UGT79B2/B3OE* lines exhibited a deeper purple color at the hypocotyls, whereas the *ugt79b2/b3*RNAi and *CRISPR/Cas9* mutant lines showed lighter color (Figure 4a). When grown on 12% sucrose-containing media, we observed darker leaves in the 17-day-old *UGT79B2/B3OE* plants as a result of greater anthocyanin accumulation (Figure 4a). To make an explicit evaluation, we extracted the total anthocyanins from the plants at different developmental stages, ranging from dry seeds (day 0) to 30-day-old plants. As shown in Figure 4(b) the plants accumulated most anthocyanins on days 5–7, and thereafter the anthocyanin content declined. No differences in anthocyanin levels were observed in dry seeds (day 0) and young seedlings within 3 days. By comparison with the WT, clear discrepancies were seen in 5-day-old plants and older: more anthocyanins accumulated in *UGT79B2/B3OE* plants, and much less anthocyanins accumulated in *ugt79b2/b3* mutants (Figure 4b). Moreover, when 2-week-old plants were exposed to NaCl, mannitol and a series of low temperatures, anthocyanin accumulation against stresses was consistently increased in *UGT79B2/B3OE* lines (Figures 4c and S7) and decreased in the *ugt79b2/b3* lines, compared with that of the WT (Figure 4c).

In addition, to explore the mechanism leading to altered anthocyanin levels, we further investigated whether *UGT79B2/B3* expression resulted in expressional fluctuation of other anthocyanin biosynthesis genes. The expression of five genes involved in different steps of anthocyanin biosynthesis were selected, including *CHS, CHI, F3H, DFR* and *ANS* (Figure 4d). Interestingly, the transcription of all the genes was obviously enhanced in *UGT79B2/B3OE* lines and declined in *ugt79b2/b3* mutants in response to various environmental factors.

From the above results, we have proved that *UGT79B2/B3* was directly regulated by CBF1 in response to cold stress. To see whether the anthocyanin biosynthesis was affected by CBF1, we detected anthocyanin levels in *CBF1* overexpression and mutant plants, and observed that the anthocyanin contents were obviously increased upon CBF1 overexpression, and decreased in the *cbf1* mutants below 4°C (Figure 5a). We also observed that the expression of anthocyanin biosynthesis genes was upregulated in the *CBF1* overexpression plants and downregulated in the *cbf1* mutant, to different degrees (Figure 5b).

In vascular plants, the phenylpropanoid pathway is responsible for the biosynthesis of a variety of metabolites through three major branches: the anthocyanin, flavonol and lignin biosynthesis pathways. Here, we also want to know whether the biosynthesis of some other metabolites in the phenylpropanoid metabolic pathway is perturbed as a result of altered anthocyanin accumulation. Thus, we analyzed the contents of several typical compounds in different plants, including quercetin and kaempferol in the flavonol biosynthesis pathway, and coniferin, sinapinic alcohol glucoside, caffeic acid glucoside and ferulic acid glucoside in the lignin biosynthesis pathway, and we didn't see any change in their accumulation upon altered anthocyanin accumulation (Figure S8).

UGT79B2 and UGT79B3 contribute to abiotic stress tolerance through modulating anthocyanin biosynthesis

From the above results, we confirmed that *UGT79B2/B3* is involved in resisting abiotic stresses as well as modulating anthocyanin metabolism in plants; however, it remains a question whether the contribution of *UGT79B2/B3* to stress tolerance depends on anthocyanin biosynthesis. To address this question, *UGT79B2* and *UGT79B3* were overexpressed in the *tt18* mutant background, in which anthocyanin biosynthesis was completely blocked by disrupting the *ANS* gene (Figure 6a; Yin *et al.*, 2014). *UGT79B2/B3/tt18* transgenic lines with high *UGT79B2/B3* expressional abundances (Figure S9) were selected for stress assays under low temperature, salt and mannitol conditions. The *tt18* mutant was observed to exhibit lower survival rates upon exposure to these stresses (Figure 6b,c), and shorter root length (Figure S10a) under salt and mannitol conditions. Intriguingly, instead of enhancing stress tolerance, overexpression of *UGT79B2/B3* in the *tt18* background showed similar sensitivity as *tt18* to these treatments (Figures 6b,c and S9a), indicating that the contribution of *UGT79B2/B3* to abiotic stress tolerance relys on anthocyanin biosynthesis pathways.

In exploring substrates for UGT79B2 and UGT79B3, we also detected very low activity of both UGTs towards two flavonols, quercetin and kaempferol. The biosynthesis of both anthocyanins and flavonols goes through the same pathway at the outset, and finally divides into two paths, catalyzed by different enzymes from dihydrokaempferol (Figure 6a; Yin *et al.*, 2014). To see whether quercetin biosynthesis is required for stress adaptation of UGT79B2/B3, we also performed overexpression of both genes in the *tt7* mutant (Figure S9), in which quercetin metabolism is blocked by disrupting *TT7*, a gene encoding CYP75B1. Similarly, when subjecting the *tt7* mutant to cold, salt and mannitol treatments, it was more sensitive in comparison with WT (Figures 6b,c and S10b); however, overexpression of *UGT79B2/B3* in *tt7* greatly improved the plant adaptation to these stresses (Figures 6b, c and S10b), suggesting that quercetin biosynthesis is possibly not required for abiotic stress tolerance conferred by *UGT79B2/B3*.

UGT79B2/B3 overexpression enhanced ROS scavenging and upregulated stress-related marker genes in response to abiotic stresses

Anthocyanins are among the most bioactive plant secondary metabolites that serve as well-known antioxidants. Thus, we determined whether ROS levels were altered

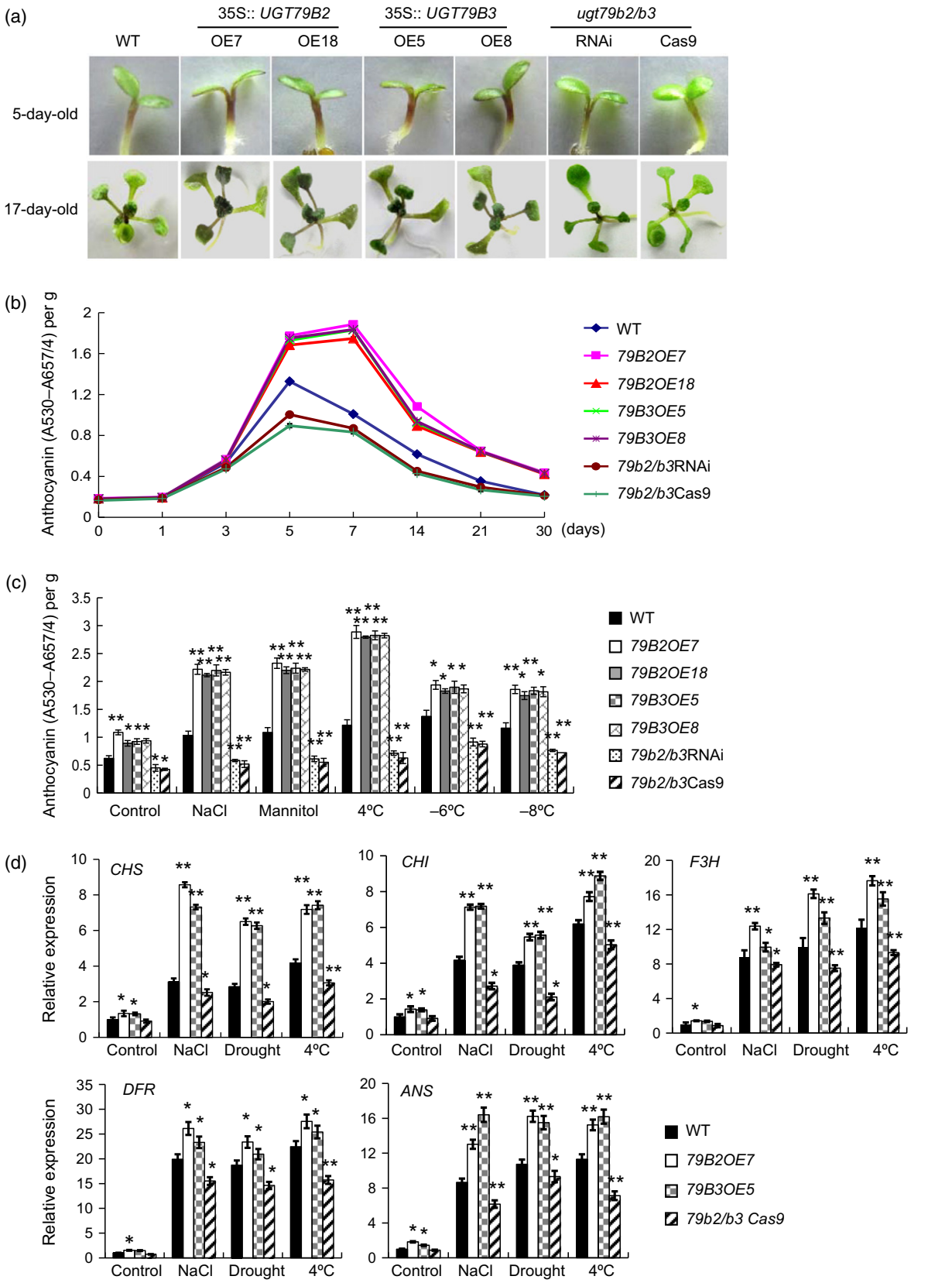


Figure 4. Anthocyanin accumulation was affected by altered *UGT79B2/B3* expression. (a) Anthocyanin accumulation visualized by the purple coloration of wild-type (WT), *UGT79B2/B3OE* and *ugt79b2/b3Cas9* mutant plants (for cultivation conditions, see Experimental Procedures). (b) Anthocyanin accumulation at different developmental stages of the seven different lines. (c) Anthocyanin accumulation under abiotic stress: 2-week-old seedlings were exposed to 150 mM NaCl, 250 mM mannitol and 4°C conditions for 12 h, respectively, and anthocyanin levels were extracted and evaluated by spectrometry. For all extractions and evaluations, at least three replicates were performed. Asterisks indicate significant differences relative to the WT (Student's *t*-test: $*P < 0.05$; $**P < 0.01$). (d) Expression of genes involved in the anthocyanin biosynthesis pathway upon altered *UGT79B2/B3* expression: 2-week-old seedlings were subjected to cold (4°C) and 150 mM NaCl for 12 h, dried in the air for 1 h for drought treatment, respectively, and then gene expressions were analyzed by qRT-PCR. Expression in the non-treated WT sample was set to 1.0. Asterisks indicate significant differences relative to control conditions (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).

upon gain or loss of *UGT79B2/B3* activities. The WT, *UGT79B2/B3OE* and knock-out plants were exposed to 4°C, NaCl and mannitol of different concentrations for 12 h, respectively, and then diaminobenzidine (DAB) and nitrobluetetrazolium (NBT) staining was performed for detecting hydrogen peroxide (H_2O_2) and superoxide, respectively (Figures 7a,b, S11 and S12). The *UGT79B2/B3OE* lines exhibited lighter staining than that of the WT, whereas the *ugt79b2/b3* mutant showed deeper and broader staining. Additionally total anthocyanins were extracted and subjected to FRAP assays, conducted to further test the antioxidant activity of the transgenic lines. The results showed that the *UGT79B2/B3OE* lines have significantly higher antioxidant capacities than those of the WT, whereas the *ugt79b2/b3* mutants exhibited the lowest capacities (Figure 7c), suggesting that UGT79B2/B3 is involved in ROS scavenging through accumulating anthocyanins, and thus enhances tolerance to abiotic stresses. Additionally, the contents of other low molecular weight antioxidants, such as ascorbate and glutathione, were also determined and they showed no differences among these plants, demonstrating that these antioxidants have no contribution to the distinct antioxidant capacity of these plants (Figure S13).

We also detected the expression of several stress-related marker genes in *UGT79B2/B3OE* and the mutant lines, including *CBF1*, *DREB2B*, *RD29A*, *COR47*, and *KIN1*, etc. We did not see any differential expression of them under normal conditions, whereas all these genes were upregulated in *UGT79B2/B3OE* lines and downregulated in the mutants upon exposure to abiotic stresses (Figure S14).

DISCUSSION

UGT79B2 and UGT79B3 are involved in cold stress response under the regulation of CBF1

In this study we revealed the pleiotropic roles of UGT79B2/B3 in improving plant tolerance to a series of adverse environmental factors, including drought, salinity, low temperatures, etc. (Figure 1). In particular they are identified to be involved in cold/freezing tolerance under the regulation of CBF1 (Figure 2), which is distinct from other reported stress-related UGTs, such as UGT74E2, UGT76C2 and UGT71C5 (Tognetti *et al.*, 2010; Li *et al.*, 2015; Liu *et al.*, 2015).

CBF1 encodes a transcriptional activator that plays important roles in cold acclimation (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998). Upon exposure to cold stress, *CBF1* would be transiently induced and control the expression of its downstream target genes, such as *RD29A*, *KIN1*, *COR15A* and *COR47*, *etc.* (Novillo *et al.*, 2004). In our study, using EMSA (Figure 2b), yeast one-hybrid assays (Figure 2d), as well as ChIP assay *in planta* (Figure 2e), *UGT79B2* and *UGTUGT79B3* were also identified to be target genes of CBF1. In some studies, the Arabidopsis CBF1 and CBF3 have been reported to play the same roles in the cold response, in that they show identical expression patterns and control the same subset of COR genes (Gilmour *et al.*, 2004; Novillo *et al.*, 2007); however, in our work we found that the expression of *UGT79B2* and *UGT79B3* is only controlled by CBF1 rather than by CBF3 (Figures 2 and S6). In an earlier study, Fowler and Thomashow (2002) profiled the transcriptomes of warm-grown transgenic Arabidopsis plants that constitutively expressed *CBF1*, *CBF2* and *CBF3*, and observed differences in the set of genes that were upregulated by these transcription factors. Additionally, Novillo *et al.* (2007) found that CBF1 cannot compliment the *CBF3* RNAi line, and vice versa. Thus, together with our findings, it is suggested that CBF1 and CBF3 may not be completely functionally redundant in regulating the cold stress response, and more working patterns of these two factors remain to be investigated.

Apart from cold stress, *UGT79B2* and *UGTUGT79B3* were also responsive to other abiotic stresses, including salinity, drought and osmotic stress, etc. (Figure 1), indicating that these UGTs are likely to be regulated by other transcription factors as well. By introducing mutations to stress-related elements present in the *UGT79B2* promoter, a DRE-motif near the transcription starting site is identified to be crucial for a variety of stress responses; however, after performing EMSA with several DREB factors, we only observed CBF1 binding with the element (Figures 2b and S4). In Arabidopsis, DREB proteins are encoded by at least 56 genes (Sakuma *et al.*, 2002; Agarwal *et al.*, 2006). Thus, we cannot exclude the possibility that other potential DREB factors might have the chance to bind with this crucial DRE element and regulate the response of *UGT79B2* to other environmental factors.

The contribution of UGT79B2/B3 to abiotic stress tolerance is closely associated with anthocyanin accumulation

It is challenging to seek substrates for UGTs. After a lot of screening, we defined the role of UGT79B2/B3 in

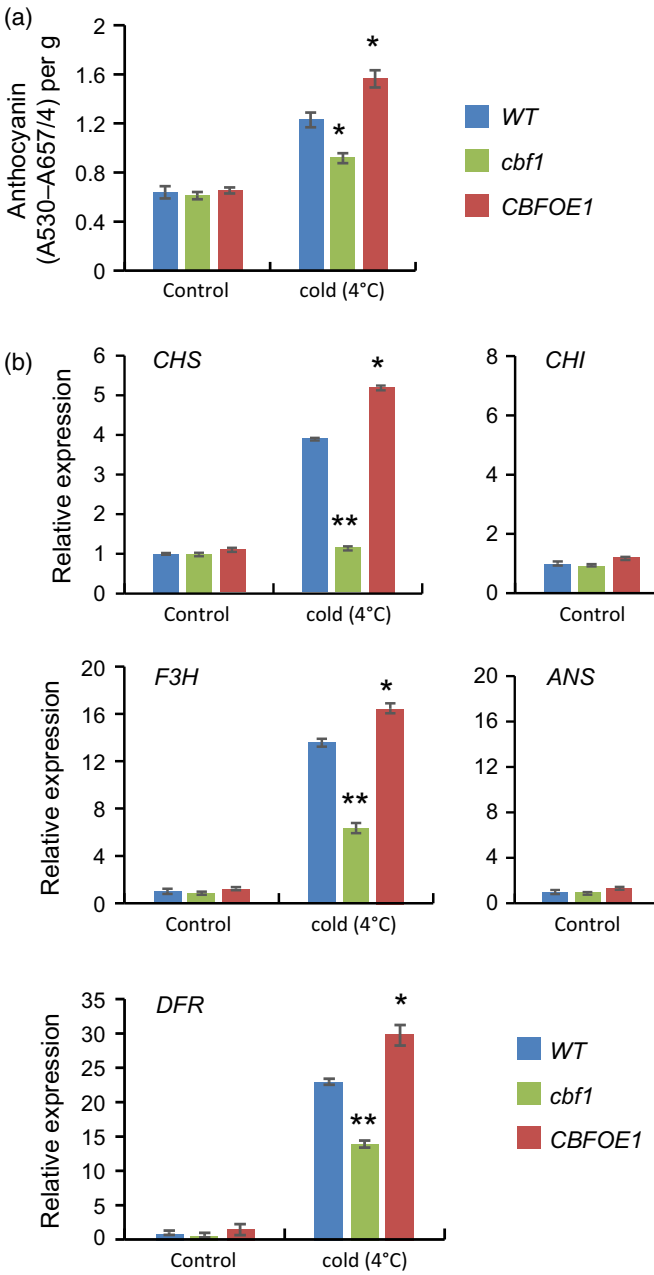


Figure 5. Anthocyanin accumulation was affected by altered *CBF1* expression under cold stress: 2-week-old seedlings growing on MS medium were exposed to 4°C for 12 h. Anthocyanin accumulation (a) and expression of genes involved in the anthocyanin biosynthesis pathway (b) in *CBF1* overexpression and *cbf1* mutant plants were evaluated under normal and cold conditions. For all extractions and evaluations, at least three replicates were performed. Asterisks indicate significant differences relative to the wild type (WT) (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).

modifying anthocyanins (Figure 3; Tables S1 and S2), which also allowed us to explore the contribution of UGT79B2/B3 to stress tolerance. Anthocyanins act as effective antioxidants by neutralizing radicals with their hydroxyl groups. It is documented that anthocyanin accumulation could be triggered by multiple environmental factors, including osmotic stress, extreme temperature, excessive light, salinity and drought stress (Dixon and Paiva, 1995; Steyn *et al.*, 2009; Van Oosten *et al.*, 2013; Ahmed *et al.*, 2014, 2015; Lotkowska *et al.*, 2015; Tian *et al.*, 2015b). Thus, manipulating the expression of anthocyanin biosynthesis genes is believed to alter the accumulation of anthocyanins and to affect the plant response to stresses. Co-expression of flavonoid-specific transcription factors *MYB12* and *PAP1* in Arabidopsis resulted in the overaccumulation of anthocyanins in the plants, and thus brings enhanced tolerance to drought and oxidative stresses (Nakabayashi *et al.*, 2014). Wang *et al.* (2013) also reported that RNA interference of a *DFR* gene in purple sweet potato led to declined anthocyanin accumulation, lower antioxidant activity and higher H_2O_2 levels, which thus reduced the plant tolerance to cold and oxidative stress (Wang *et al.*, 2013). Glycosylation or other modifications of anthocyanins are the last steps in the

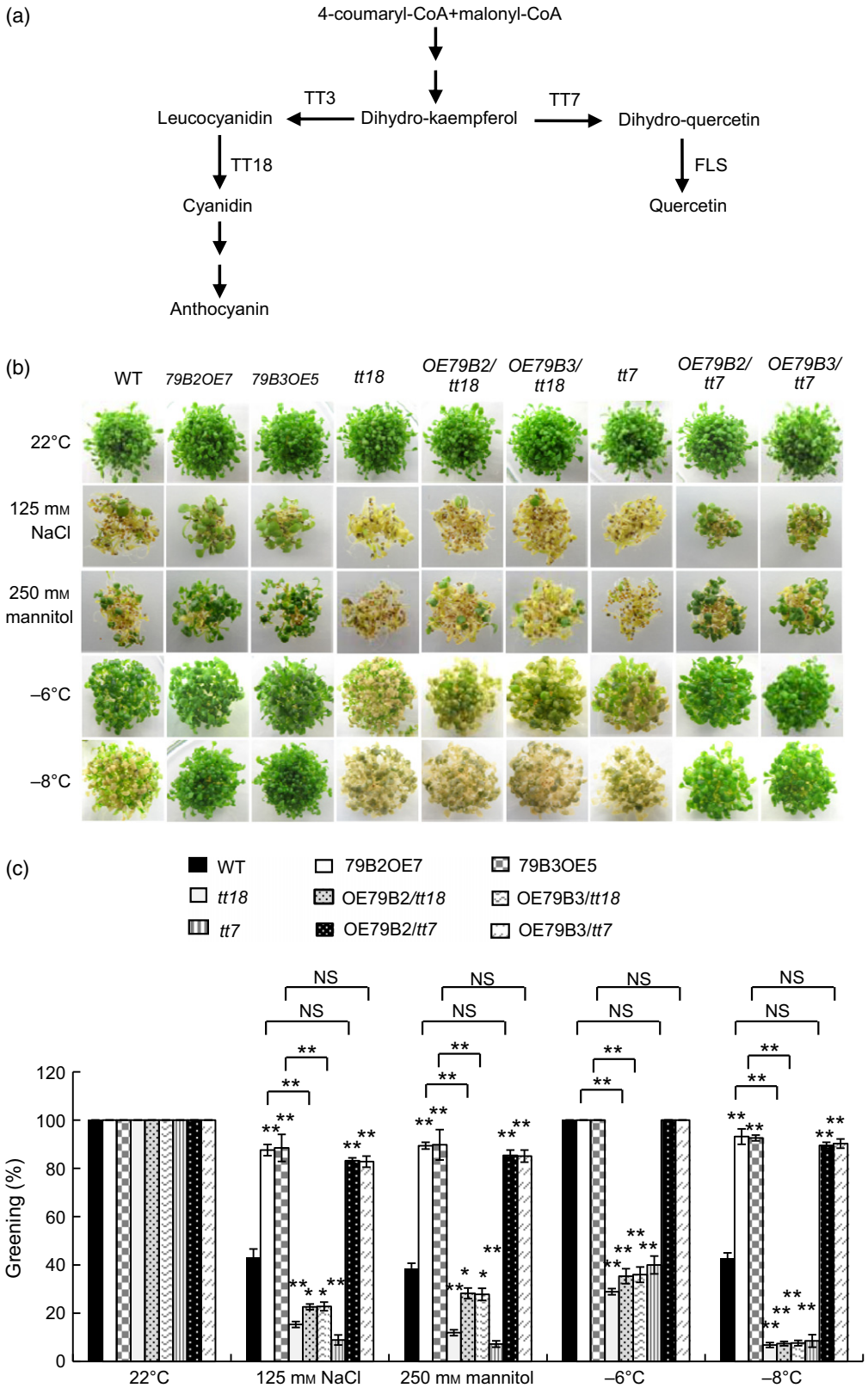


Figure 6. Role of *UGT79B2/B3* against stress in *tt18* and *tt7* mutants. (a) Metabolic pathways involving *TT18* and *TT7*. (b, c) Seedling greening of *OE79B2/B3/tt18* (b) and *OE79B2/B3/tt7* (c) plants when subjected to low temperatures, 250 mM mannitol and 125 mM NaCl, respectively. Asterisks indicate significant differences relative to the wild type (WT), or between the indicated lines (Student's *t*-test: $*P < 0.05$; $**P < 0.01$). NS, not significant.

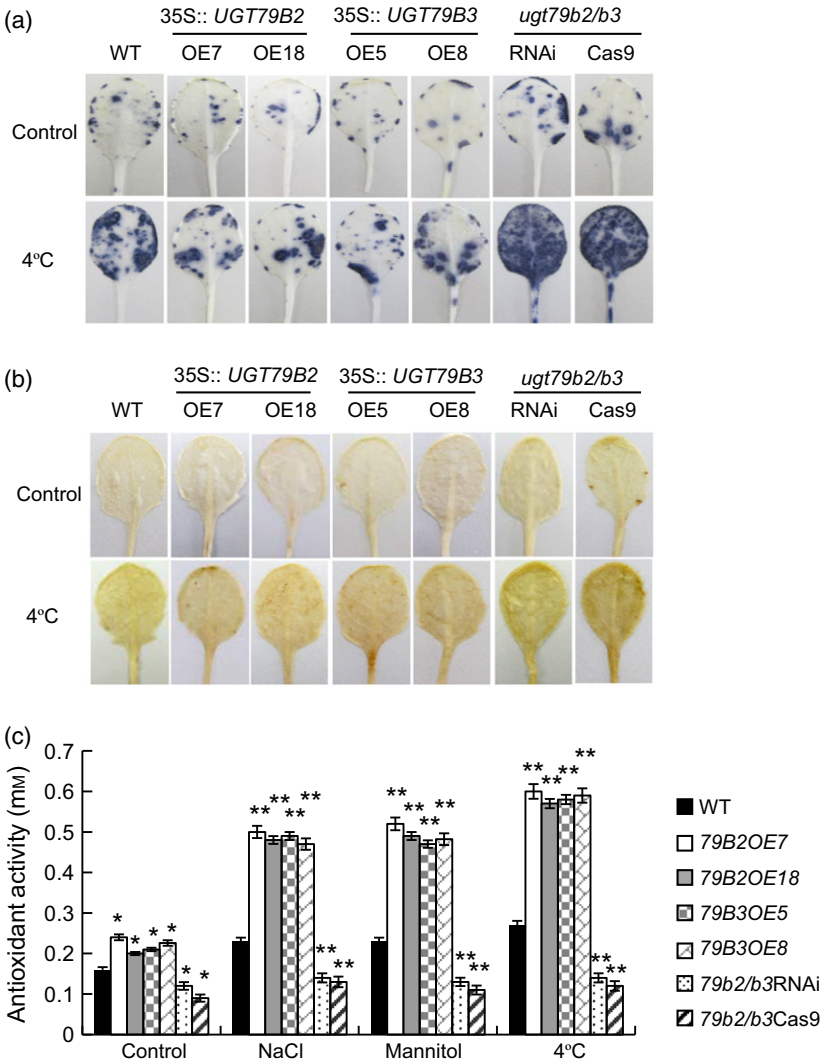


Figure 7. Antioxidant activity conferred by *UGT79B2/B3* overexpression: 4-week-old plants growing in soil were subjected to 4°C, 150 mM NaCl and 250 mM mannitol treatment for 12 h. Plants exposed to 4°C were subjected to NBT (a) and DAB staining (b), respectively. (c) The antioxidant activities of the stress-treated plants were determined. Asterisks indicate significant differences relative to the WT (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).

anthocyanin biosynthesis pathway (Yonekura-Sakakibara *et al.*, 2008). Here, we observed that overexpression of *UGT79B2/B3* in Arabidopsis significantly increased anthocyanin accumulation and enhanced ROS scavenging (Figure 7). In contrast, the *ugt79b2/b3* double mutant accumulated less anthocyanins and more ROS (Figure 7). In support of our hypothesis, excessive expression of *UGT79B2* or *UGT79B3* in *tt18*, which lacks anthocyanins, failed to improve plant adaptation to stress (Figure 6). These observations clearly indicate that the contribution of *UGT79B2/B3* to abiotic stress tolerance is closely associated with anthocyanin accumulation and antioxidant activities *in planta*.

Glycosylation of anthocyanins promotes anthocyanin biosynthesis via feedback activation of the enzyme genes

In our study, we observed that *UGT79B2/B3* overexpression greatly upregulated the expression of critical enzyme genes involved in anthocyanin biosynthesis, including *CHS, CHI, F3H, DFR* and *ANS*, especially under abiotic stress conditions, whereas the knock-out/knock-down of *UGT79B2/B3* obviously decreased their transcription. These findings demonstrate that glycosylation of anthocyanins accelerated the biosynthesis of the anthocyanin, and is closely correlated with the upregulation of the enzyme genes; however, it is important to know why the altered glycosylation of anthocyanins leads to alterations in the transcripts of anthocyanin biosynthesis genes. The anthocyanin metabolic branch belongs to the phenylpropanoid metabolic pathway, which is tightly controlled in a sequential and coordinated manner. Also, it is easy to be disturbed by any altered events as a result of feedback or feedforward regulation. For instance, DFR is involved in catalyzing dihydroquercetin into leucocyanidin in the anthocyanin biosynthesis pathway (Yonekura-Sakakibara *et al.*, 2008). It is reported that overexpression of *McDFR* in *Crabapple* leads to the upregulation of *F3′H*, *ANS* and *UFGT* genes, which greatly accerlerates the biosynthesis of

anthocyanins (Tian *et al.*, 2015a). Glycosylation or other modifications of metabolites in the flavonoids pathway tend to be the final steps in their biosynthesis (Yonekura-Sakakibara *et al.*, 2008). Consistent with our observation, altered glycosylation of flavonols is also found to alter the transcript of enzyme genes involved in their biosynthesis. UGT78D1 and UGT78D2 are responsible for quercetin and kaempferol glycosylation in Arabidopsis (Jones *et al.*,2003; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2008). Yin *et al.* (2012) reported that the knock-out of both genes in the *ugt78d1 ugt78d2* double mutant significantly decreased the expression of many upstream enzymes involved in flavonol biosynthesis, including *C4H*, *CHS*, *F3′H* and *FLS*, etc., which repressed the biosynthesis of flavonols; however, other branches of the phenylpropanoid pathway, such as anthocyanin and lignin biosynthesis, were not or were only marginally affected. This point also agrees with our observation: we did not see any change in the accumulation of flavonols, glycosides of monolignols and phenolic acid compounds upon altered anthocyanin accumulation (Figure S8). Taken together, it is believed that as the plant cells continuously consume aglycones upon constitutive expression of *UGT79B2/B3*, they require more substrates, which in turn stimulates the expression of the upstream enzyme genes and accelerated the biosynthesis of the anthocyanins.

CBF1 is linked to anthocyanin metabolism via UGT79B2/B3 in regulating cold stress adaptation

The cold stress response starts from the perception of the stress signal, governed by transcription factors, followed by the expression of cold-regulated (COR) genes and finally brings about the extensive reprogramming of metabolism, thereby imparting stress tolerance (Kaplan *et al.*, 2004; Zhu *et al.*, 2007; Eremina *et al.*, 2016). Metabolic profiling revealed that cold acclimation increases 75% of the 434 metabolites detected in Arabidopsis plants (Cook *et al.*, 2004). The protective functions of metabolites such as sucrose, trehalose, fructan, maltose, galactinol, proline and glycine betaine are well documented (Chen and Murata, 2002; Stitt and Hurry, 2002; Kaplan and Guy, 2005); however, how the biosynthesis of these metabolites is regulated by cold stress is poorly known.

In our study, we identified that CBF1 could directly control the expression of *UGT79B2* and *UGT79B3* (Figure 2), two glycosyltransferases involved in modulating anthocyanin metabolism (Figure 3), and thus links CBF1 to anthocyanin metabolic pathways in improving cold stress tolerance. Supporting this point, we also found that anthocyanin accumulation and transcription of critical anthocyanin biosynthesis genes could be affected by altered *CBF1* expression (Figure 5). Our findings might represent an integrated mechanism for the plants in coping with cold stress. Together with former studies, it is inferred that anthocyanin accumulation plays important roles in protecting plants against cold stress, and is likely to be regulated by stress-related transcription factor-directed biosynthesis/modification pathways when confronting cold stress.

Moreover, we also observed the upregualtion of stress-related marker genes in *UGT79B2/B3OE* plants and the downregulation of these genes in the *ugt79b2/b3* mutant under stress conditions (Figure S14), but not under normal conditions. In particular, *CBF1* and its target genes like *RD29A*, *COR47*, and *KIN1* are remarkably upregulated upon *UGT79B2/B3OE* in response to cold stress. It is deduced that when coping with low temperatures, anthocyanins are quickly produced and soon spent to clear ROS. As they are consumed, the CBF1-directed anthocyanin biosynthesis is actually enhanced in response to stress as a result of feedback regulation, which in turn activates the transcription of these stress marker genes.

Collectively, a working model was proposed based on our study (Figure 8). When the plants were confronted with cold and other abiotic stresses, such as drought and salinity, CBF1 and other transcription factors could be quickly induced and activate the expression of *UGT79B2/B3* by binding with their promoters, followed by glycosylation of cyanidin and cyanidin 3-O-glucoside, and the overproduction of anthocyanins. Anthocyanins play positive roles in scavenging ROS, which thereby results in enhanced tolerance to cold and other abiotic stresses. It must be noted that this model does not rule out additional substrates of *UGT79B2/B3* and the existence of other mechanisms involved in regulating *UGT79B2/B3* in response to stresses.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was used in this study. The mutant lines *cbf1* (GK-828H07), *cbf3* (SAIL_244_D02), *tt7* (GK_349F05) and *tt18* (SALK_028793) were obtained from the European Arabidopsis Stock Centre (uNASC). All the plants were grown on MS medium or soil under long days (LD, 16 h of light/8 h of dark, 100 μmol m^{-2} s^{-1}) at 22°C.

Plasmid construction and plant transformation

To generate vectors for prokaryotic expression, the coding regions of *UGT79B2* and *UGTUGT79B3* were cloned into the PGEX-2T vector fused with a glutathione-S-transferase (GST) tag. To generate the 35S::*UGT79B2/UGT79B3* construct, the coding region of *UGT79B2* and *UGT79B3* were cloned into the pBI121 binary vector driven by the CaMV *35S* promoter. To generate the RNAi construct for both *UGT79B2* and *UGT79B*3, a 427-bp homologous fragment contained in both *UGT79B2* and *UGT79B3* cDNA was amplified by PCR using the forward primer 5′-TCTAGAGGCGCGCCGAACTCTGATGTCATTGCGATAAGG-3′ and the reverse primer 5′-GGATCCATTTAAATCTGACAATCACTCAGCAAAGACTC-3′. The reversed and forward fragments were both inserted into pFGC5941 with the *Asc*I and *Swa*I sites (underlined), and with the *Bam*HI and *Xba*I sites (highlighted). For generating *UGT79B2/UGT79B3* pro::GUS fusions, the 1600-bp upstream sequence of *UGT79B2* and the

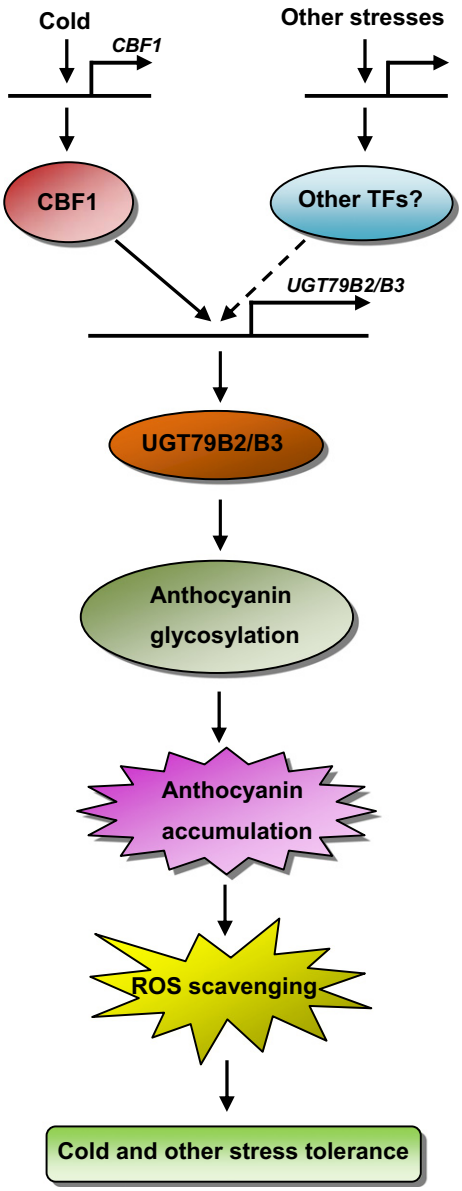


Figure 8. Working model of UGT79B2/B3 involved in abiotic stress response. When plants were confronted with cold and other abiotic stresses, such as drought and salinity, CBF1 and other transcription factors could be induced quickly and activated the expression of *UGT79B2/B3* by binding with their promoters, followed by the glycosylation of anthocyanins, which resulted in the overproduction of anthocyanins. Anthocyanins have positive roles in scavenging reactive oxygen species, which thereby results in enhanced tolerance to cold and other abiotic stresses. Solid lines indicate confirmed pathways in this study or previous studies. Dotted lines indicate predicted or possible pathways.

750-bp full-length intergenic region upstream of *UGT79B3* were cloned into the pBI121 binary vector by replacing the CaMV *35S* promoter. The genome editing of *UGT79B2* and *UGT79B3* based on the CRISPR/Cas9 strategy was carried out by the Hangzhou Biogle Co. (http://www.biogle.cn/). All the constructs were transformed into Arabidopsis Col-0 by Agrobacterium-mediated floral dip (Clough, 2005). The primers used for plasmid construction are listed in Table S3.

Stress assays

We performed cold/freezing treatment according to previous studies (Xin and Browse, 1998; Verslues *et al.*, 2006). Briefly, 12-day-old Arabidopsis seedlings growing on MS medium were incubated for at least 16 h at −1°C, sprinkling the plates with finely crushed ice, and then decreasing the temperature of the freezing chamber by 1°C per hour, with the Petri dishes removed at the desired temperatures. After removal from the freezing chamber, the plates were incubated at 4°C for 12 h in darkness, followed by incubating the Petri dishes under the original growth conditions for 2 days to score the survival of seedlings. The determination of electrolyte leakage was performed according to the protocol described by Lee and Zhu (2010).

For root growth assays, sterilized seeds were sown on MS medium, and when the seedlings grow to 1 cm in length after 3 days they were transferred to MS medium supplemented with different concentrations of NaCl and mannitol. After growing for 2 weeks under stress conditions, the root growth was measured. For drought stress treatment in soil, 1-week-old seedlings growing on MS medium were transplanted into soil and grown for one further week with normal irrigation, and then the 2-week-old plants were photographed. After that, for drought treatment, the plants were left without watering for another 3 weeks, followed by re-irrigation. After recovery for 3 days, the performances of the plants were photographed. For NaCl treatment, 1-week-old seedlings growing on MS medium were transplanted into soil and grown for one further week with normal irrigation, and then the 2-week-old plants were irrigated with 200 mM NaCl solution instead of water for three further weeks. Then the performances of the plants were photographed.

Protein–promoter interaction evaluated by EMSA, yeast one-hybrid and ChIP assays

For performing the EMSA, the coding region of DREB proteins was introduced into the pGEX vector with a GST tag. The DREB-GST recombinant proteins were expressed in *Escherichia coli* XL1-Blue and purified with Glutathione Sepharose™ 4B (GE Healthcare, http://www.gehealthcare.com/en/global_gateway). EMSA was performed with the EMSA Kit (Roche, http://www.roche.com). The promoter probes of *UGT79B2* containing the TGCCGACCA element were synthesized and labeled with digoxin at the 3′ end. The signal was detected by chemiluminescence and exposed to the X-ray films. The probes used in the EMSA assay were listed in Table S3.

To perform yeast one-hybrid asssay, four copies of the core element of the CBF1 binding region were tandem-arrayed and cloned into pHIS2.1 vector. The coding region of CBF1 was cloned into pGADT7. All constructs were transformed into yeast strain AH107. Yeast was grown in SD-Ura-Leu-His medium and then spotted on SD-Ura-Leu-His medium in the presence or absence of 45 mM 3-aminotriazole (Sigma-Aldrich, http://www.sigmaaldrich.com), with different dilutions. The plates were incubated for 3 days at 28°C. Reporter gene activation was checked by cell growth.

For the ChIP assay, samples (1.0–1.3 g) from 3-week-old cold-treated or untreated 35S::myc-CBF1 transgenic Arabidopsis were collected. ChIP was performed using the Pierce™ Agarose ChIP Kit (ThermoFisher Scientific, http://www.thermofisher.com) according to the user's guide. DNA enrichment was analyzed by qRT-PCR. The -fold enrichment of a specific DNA fragment was normalized to that of UBQ10. The primer sets employed to amplify the *UGT79B2* promoter regions for yeast one-hybrid and ChIP assays are listed in Table S3.

Production of recombinant UGT79B2/B3 protein and glycosyltransferase assays

The UDP-sugars for performing enzyme assays were obtained from Qingdao Sugar Biotech Co., Ltd (http://www.qingmeibio.com). Part of the UDP-rhamnose was provided by Dr Min Yang (University College London). The compounds cinnamic acid, ferulic acid, caffeic acid, sinapic acid, coniferin, coniferyl aldehyde, coniferyl alcohol, naringenin, sinapinic alcohol, sinapinic aldehyde, quercetin and kaempferol were obtained from Meryer (Shanghai) Chemical Technology Co., Ltd (http://www.meryer.com). The procyanidin, cyanidin and cyanidin 3-O-glucoside were obtained from Carbosynth (http://www.carbosynth.com).

The recombinant UGT79B2/B3 proteins were expressed in *E. coli* and purified with Glutathione Sepharose^{TM} 4B (GE Healthcare). The glycosyltransferase activity assay was carried out with the conditions described by Hou *et al.* (2004). The products were analyzed by HPLC on a Shimadzu HPLC system (http://www.shimadzu.com) using a 5-μM C18 column (150 × 4.6 mm; Zorbax; Agilent, http://www.agilent.com). A linear gradient with increasing acetonitrile (solvent A) against double-distilled H_2O (solvent B) at a flow rate of 1 ml/min over 35 min was used. Both solutions contained 0.1% trifluoroacetic acid. HPLC conditions were as follows: 0 min, 10% A; 20 min, 75% A; 22 min, 10% A; 35 min, stop. Photodiode array (PDA) was used for the detection of UV-visible absorption in the range of 200–600 nm.

The products were further confirmed by the LC-MS system (Shimadzu, http://www.shimadzu.com). The methods and mobile phases were similar to the HPLC conditions, except that 0.1% acetic acid was used instead of 0.1% trifluoroacetic acid. The mass spectrometer operated in a positive electrospray ionization mode with 50 eV and a probe voltage of 5.0 kV. The dry heater was set to 180°C. The data acquisition and analysis were performed with XCALIBUR 2.0.6.

Determination of anthocyanin accumulation, flavonols, monolignol glycosides and phenolic acid contents

For observing anthocyanin accumulation in 5-day-old plants, seeds were germinated on MS medium and subjected to constant light for 5 days, and the purple coloration at the hypocotyls was photographed. For observing the anthocyanin accumulation of 17-day-old plants, plants were cultured on MS medium containing 3% sucrose in a growth chamber at 22°C, with a 16-h light/8-h dark cycle for 14 days, and then transferred to MS medium containing 12% sucrose for 3 days with constant light, based on a previous study (Yonekura-Sakakibara *et al.*, 2012), and then the purple coloration in the leaves was observed and photographed. Anthocyanin contents were evaluated according to Ronchi *et al.* (1997), and were determined spectrophotometrically at a wavelength of 350 or 530 nm.

Using 2-week-old seedlings as material, flavonols were extracted according to Burbulis *et al.* (1996) and determined spectrophotometrically at 270 nm. Coniferin and sinapinic alcohol glucoside were extracted and their levels were determined according to Lin *et al.* (2016). Phenolic acids were extracted and analyzed with small modifications according to Li *et al.* (2015).

Quantitative RT-PCR

For quantitative real-time PCR, total RNAs were extracted with the Trizol reagent (Takara, now Clontech, http://www.clontech.com). Reverse transcription reactions were performed with the PrimeScript RT reagent kit with gDNA Eraser (Takara). Real-time PCR reactions were performed with a Bio-Rad real-time thermal cycling system using SYBR-Green. All reactions were performed using at least three replicates. Expression levels were normalized with the reference gene *EF1-α*. Primer information for the qRT-PCR assay is included in Table S3.

GUS assays, diaminobenzidine (DAB) and nitrobluetetrazolium (NBT) staining

GUS staining of the seedlings was preformed according to a previous report (Beeckman and Engler, 1994). The determination of GUS activities was performed as described previously (Jefferson *et al.*, 1987). For visualization of H_2O_2 and superoxide generation as a result of abiotic stress, 4-week-old plants growing in soil were treated with mannitol and NaCl. DAB staining was carried out as described by Du *et al.* (2008). The NBT staining was carried out as described by Wang *et al.* (2011).

Determination of total antioxidant capacity, ascorbate and glutathione content

Two-week-old Arabidopsis plants were exposed to 150 mM NaCl, 250 mM mannitol and 4°C conditions for 12 h. Anthocyanins were extracted and the total antioxidant capacity in different plants was determined using ferric reducing ability of plasma (FRAP) methods with a FRAP reagent kit (Beyotime, http://www.beyotime.com) according to the protocol of an earlier study, with slight modifications (Benzie and Strain, 1996). Ascorbate was extracted and determined as previously described. GSSG and GSH content were determined using commercially available kits (S0052; Beyotime), according to the user's manual.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Generation of *UGT79B2/B3OE* plants and double mutants.

Figure S2. Primary root growth of the WT, *UGT79B2/B3OE* lines, and the mutant lines under NaCl and mannitol.

Figure S3. Tolerance of other *UGT79B2/B3OE* lines against cold, salt and osmotic stresses.

Figure S4. DREB1A/CBF3, DREB2A and DREB2B cannot control the expression of *UGT79B2/B3*.

Figure S5. ChIP-quantitative PCR was performed using an anti-myc antibody in untreated wild-type plants, and in 35S::*myc* and 35S::*myc-CBF*1 transgenic plants.

Figure S6. Expression of *UGT79B2/B3* in the *cbf3* mutant exhibited no differences with that in the WT in control and cold conditions.

Figure S7. Anthocyanin accumulation in different *UGT79B2/B3OE* lines.

Figure S8. Levels of some components in the flavonol and lignin biosynthesis pathway in the WT, and in *UGT79B2/B3OE* and mutant lines, determined by HPLC and LC-MS.

Figure S9. Relative mRNA levels of *UGT79B2* and *UGT79B3* in *tt7* and *tt18* mutants, and *OE79B2/B3/tt7* and *OE79B2/B3/tt18* transgenic plants, assessed by qRT-PCR.

Figure S10. Root length of *OE79B2/B3 tt18* (a) and *OE79B2/B3 tt7* (b) plants when subjected to 125 mM NaCl and 250 mM mannitol, respectively.
Figure S11. Hydrogen peroxide (H_2O_2) and superoxide detection in *UGT79B2/B3OE* lines.
Figure S12. Hydrogen peroxide (H_2O_2) and superoxide detection in *ugt79b2/b3* double mutant lines.
Figure S13. Ascorbate and glutathione contents showed no differences in WT, *UGT79B2/B3OE* and *ugt79b2/b3* mutant lines in response to stresses.
Figure S14. Expression of stress-related marker genes in WT, *UGT79B2/B3OE* and *ugt79b2/b3* mutant lines in response to stresses.
Table S1. Specific activity of UGT79B2/B3 in response to different substrates.
Table S2. Relative activity of UGT79B2/B3 in response to different sugar donors.
Table S3. Primers used in this study.

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