UDP-Glucosyltransferase71C5, a Major Glucosyltransferase, Mediates Abscisic Acid Homeostasis in Arabidopsis[1][OPEN]

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Abscisic acid (ABA) plays a key role in plant growth and development. The effect of ABA in plants mainly depends on its concentration, which is determined by a balance between biosynthesis and catabolism of ABA. In this study, we characterize a unique UDP-glucosyltransferase (UGT), UGT71C5, which plays an important role in ABA homeostasis by glucosylating ABA to abscisic acid-glucose ester (GE) in Arabidopsis (*Arabidopsis thaliana*). Biochemical analyses show that UGT71C5 glucosylates ABA in vitro and in vivo. Mutation of *UGT71C5* and down-expression of *UGT71C5* in Arabidopsis cause delay in seed germination and enhanced drought tolerance. In contrast, overexpression of *UGT71C5* accelerates seed germination and reduces drought tolerance. Determination of the content of ABA and ABA-GE in Arabidopsis revealed that mutation in *UGT71C5* and down-expression of *UGT71C5* resulted in increased level of ABA and reduced level of ABA-GE, whereas overexpression of *UGT71C5* resulted in reduced level of ABA and increased level of ABA-GE. Furthermore, altered levels of ABA in plants lead to changes in transcript abundance of ABA-responsive genes, correlating with the concentration of ABA regulated by UGT71C5 in Arabidopsis. Our work shows that UGT71C5 plays a major role in ABA glucosylation for ABA homeostasis.

Abscisic acid (ABA) is a sesquiterpene hormone that plays an important role in regulating plant growth and development, including seed maturation and dormancy, seed germination, and growth of roots, as well as mediating adaptations to environmental stresses, such as cold, drought, and salinity (Leung and Giraudat, 1998; Himmelbach et al., 1998, 2003). The regulatory effects of ABA mainly depend on its concentration in plant tissue, which is determined by the balance between biosynthesis and catabolism (Nambara and Marion-Poll, 2005). Under drought stress, the concentration of ABA increases up to 100 times in plant tissues to promote stomatal closure and avoid excessive water loss (Raschke, 1987; Zeevaart and Yang, 2005). When the drought condition ceases, the high concentration of ABA rapidly returns to the normal level (Harris et al., 1988; Zeevaart and Creelman, 1988). Genetic screens and analyses of mutants compromised in growth, dormancy, and stomatal control have identified a number of genes involved in ABA biosynthesis and catabolism. Mutations in these genes have profound effects on the development of plants (Zeevaart, 1999; Finkelstein and Rock, 2002; Schwartz et al., 2003; Nambara and Marion-Poll, 2005; Zaharia et al., 2005), which improved our understanding on ABA's biology.

ABA is mainly produced by the de novo biosynthesis pathway through the oxidative cleavage of carotenoids (Milborrow and Lee, 1998; Hirai et al., 2000; Kasahara et al., 2004). In this pathway, zeaxanthin epoxidase catalyzes the formation of all transviolaxthin from zeaxanthin (Marin et al., 1996). Nine cis-epoxycarotenoid dioxygenase (NCED) cleaves carotenoids (Schwartz et al., 2003; Nambara and Marion-Poll, 2005). ABA2 (short-chain alcohol dehydrogenase) converts xanthoxin derived from cleavage of carotenoids into abscisic aldehyde, which is finally oxidized into ABA by abscisic aldehyde oxidase (Seo et al., 2000; González-Guzmán et al., 2004). Recent studies showed that, in addition to the

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de novo ABA biosynthesis (Qin and Zeevaart, 1999; Seo and Koshiba, 2002), the β-glucosidase (BG) homolog Arabidopsis (*Arabidopsis thaliana*) β-glucosidase1/2 (AtBG1/2) generates ABA from abscisic acid-Glc ester (GE) in endoplasmic reticulum and vacuole, respectively (Lee et al., 2006; Xu et al., 2012).

ABA catabolism is mediated through oxidation and conjugation. Oxidation produces 7′-, 8′-, and 9′-hydroxy ABA (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005). The oxidation in the 8′-carbon position of ABA occurs predominantly and is catalyzed by cytochrome P450 707A family (CYP707As), encoding 8′-hydroxylases, to form the unstable intermediate, 8′-hydroxy ABA, which subsequently cyclizes spontaneously to form phaseic acid (Kushiro et al., 2004; Saito et al., 2004). The main conjugation form for ABA is glucosylation, which produces ABA-GE, a storage form and an inactive end product of ABA metabolism (Koshimizu et al., 1966; Zeevaart, 1999; Sauter et al., 2002). ABA glucosylation is a reversible process that is catalyzed by glucosidases and hydrolyases. This process plays a key role in controlling the levels of ABA in plant tissues. AtBG1/2 hydrolyzes ABA-GE to release free ABA when plants suffer stresses (Lee et al., 2006; Xu et al., 2012). Glucosyltransferases, referred to as UDP-glucosyltransferases (UGTs), glucosylate ABA to ABA-GE (Lim et al., 2005). In plants, UGTs consist of a superfamily with 56 families and are generally localized in the cytosol and involved in the biosynthesis of plant natural products, such as flavonoids, phenylpropanoids, terpenoids, and steroids, and the regulation of plant hormones (Ross and O'Neill, 2001; Lim et al., 2005; Bowles et al., 2006; von Saint Paul et al., 2011). UGTs catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules (Pflugmacher and Sandermann, 1998). UGT71B6 is a glucosytransferase that recognizes the naturally occurring (+)-ABA enantiomer, but a mutant of *UGT71B6* (*ugt71b6*) did not exhibit any apparent ABA excessive phenotype (Priest et al., 2005), suggesting that this transferase does not play a major role in ABA catabolism. However, a hypersensitivity to exogenous ABA and high-salt stress during germination in Arabidopsis were seen when *UGT71B6*, *UGT71B7*, and *UGT71B8* were cosuppressed. This observation indicates that glucosylation of ABA plays a critical role in ABA homeostasis (Dong et al., 2014). Nevertheless, the major ABA glucosyltransferase contributing to glucosylation of ABA to ABA-GE remains to be identified.

In this study, we characterized a unique UGT, UGT71C5, in ABA metabolism of Arabidopsis. UGT71C5 has the capacity to glucosylate ABA to ABA-GE in vitro and in vivo. Down-regulation of *UGT71C5* in Arabidopsis results in an increased level of ABA, which correlates with a reduced level of ABA-GE. The transgenic plants with down-regulated *UGT71C5* exhibit an enhanced resistance to drought stress, consistent with the ABA-excessive phenotype in plants. In contrast, up-regulation of *UGT71C5* resulted in a reduced level of ABA and an increased level of ABA-GE. The transgenic plants with up-regulated *UGT71C5* exhibit a reduced resistance to drought stress, consistent with the ABA-deficient phenotype. Altered levels of ABA lead to changes in expressions of ABA-responsive genes in plants. Hence, our work shows that UGT71C5 plays a major role in ABA glucosylation in ABA homeostasis.

RESULTS

ABA-Excessive Phenotypes of *ugt71c5* Mutant and Cytosolic Localization of UGT71C5

The super family of UGTs consists of 56 families in plants, and 26 of them are found in Arabidopsis (Bowles et al., 2006). Previously reported UGTs UGT71B6, UGT75B1, UGT84B1, and UGT84B2, which displayed in vitro glucosylation activity toward ABA, belong to the UGT subfamilies of the family 1 in Arabidopsis (Lim et al., 2005). The *UGT71C5* gene (At1g07240.1; GenBank accession no. NM_100598), coding for 480-amino acid residues (approximately 52 kD), also belongs to the same subfamily in Arabidopsis and shares 57% amino acid identity with UGT71B6 (Supplemental Figs. S1 and S2). These UGTs contain a highly conserved motif in their C-terminal sequences involved in binding to the sugar donor, named as UDPGT (Osmani et al., 2009; http://www.expasy.ch/prosite/; Supplemental Fig. S3).

Analysis of the expression pattern of *UGT71C5* revealed that *UGT71C5* was widely expressed in plant tissues, such as roots, leaves, stems, siliques, and flowers (Supplemental Fig. S4). Phenotypic examination of *ugt71c5* (Arabidopsis Biological Resource Center [ABRC], CS25151) and *ugt71b6* (ABRC, SALK_024685C) mutants showed that the *ugt71c5* mutant was more resistance to drought stress than the *ugt71b6* mutant and wild-type plants after plants suffered dehydration for 18 d (Fig. 1A; Supplemental Fig. S5). Using the software PROSITE motif analysis (http://www.expasy.ch/prosite/), UGT71C5 contains a UGTPG motif at its C terminus that is similar to cytoplasm localized plant secondary product glycosyltransferase (PSPG) motif (Osmani et al., 2009). Hence, it is possible that UGT71C5 localizes to the cytoplasm. To determine the subcellular localization of UGT71C5, we constructed a recombinant UGT71C5 tagged at the C terminus with GFP and expressed it transiently in Arabidopsis protoplasts. The GFP-tagged UGT71C5 was found to distribute throughout the protoplast (Fig. 1B), similar to the cytosolic localization of the other reported UGTs (Ross and O'Neill, 2001; Lim et al., 2005). This observation suggests that UGT71C5 is a cytosolic UGT.

UGT71C5 Catalyzes ABA Glucosylation in Vitro

To show that UGT71C5 is a glucosyltransferase, we tested its transferase activity in vitro. His-tagged UGT71C5 was expressed in *Escherichia coli* and purified by affinity chromatography (Supplemental Fig. S6A); the enzymatic activity was analyzed using reverse-phase HPLC with the

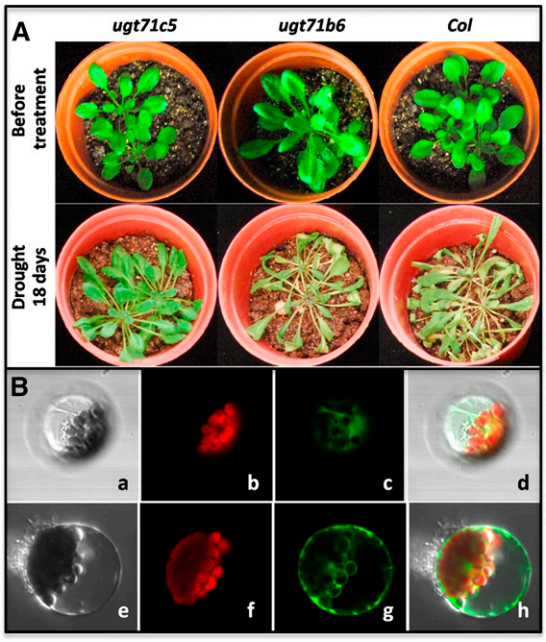

Figure 1. Mutant *ugt71c5* exhibits drought-resistant phenotype, and UGT71C5 localizes in cytoplasm. A, Mutant *ugt71c5* exhibits drought-resistant phenotype. The plants were incubated at 22°C for 3 weeks and treated with dehydration. Upper, Before treatment. Lower, Eighteen days after dehydration. *ugt71c5* and *ugt71b6* are mutants, and Col-0 is the wild type. B, Subcellular localization of UGT71C5:GFP fusion (a–d) and GFP (e–h) in Arabidopsis protoplasts. Cells were analyzed by fluorescence microscopy and photographed after 16 h of incubation for 3 weeks at 22°C after polyethylene glycol-mediated transient expression. a and e, Bright-field fluorescence. b and f, Autofluorescence. c and g, Green fluorescence of UGT71C5:GFP. d and h, Merged.

substrates of ABA and UDP-Glc as the substrates. The product of ABA glucosylation, ABA-GE, was identified by its retention time at 10.5 min, whereas ABA-GE was not detected in the reaction mixture in the absence of ABA (Fig. 2A). When purified recombinant UGT71C5 was added to the reaction mixture, ABA-GE was detected (Fig. 2B). However, ABA-GE was not observed in the reaction mixture containing denatured UGT71C5 (Fig. 2B). The rate of ABA-GE production was both time and dose dependent (Supplemental Fig. S7). The reaction V_{max} was 2.43 nkat mg^{-1}, the K_m was 0.13 mM, and the catalytic number (K_{cat})/K_m was 0.91 mM^{-1} s^{-1}. The low K_m for ABA is consistent with the proposed role of UGT71C5 in ABA glucosylation.

UGT71C5 Exhibits Activity of ABA Glucosyltransferase in Vivo

To determine the function of UGT71C5 in ABA catabolism in Arabidopsis, we generated *UGT71C5* transgenic Arabidopsis lines in sense and antisense orientations. In addition, *UGT71C5* and *UGT71B6 Agrobacterium* spp. transferred DNA (T-DNA) insert mutants *ugt71c5* and *ugt71b6* were used for analysis and comparison (Supplemental Fig. S5).

Quantitative real-time (qRT) -PCR analyses revealed that the transcript levels of the *UGT71C5* up-regulated lines (OEs) were 60-fold higher than those in wild-type plants, whereas the transcripts of *UGT71C5* in down-regulated lines (DNs) were less than one-half of those in the wild type (Fig. 3A). Western-blot analysis using the specific UGT71C5 antibody (Supplemental Figs. S6 and S8) revealed that the expressional levels of *UGT71C5* in *UGT71C5* overexpression lines were 3.7-, 4.9-, and 4.7-fold in OE-11, OE-41, and OE-51, respectively, compared with 1 in wild-type plants (Fig. 3B). In contrast, the levels of UGT71C5 in *UGT71C5* down-expression plants were very weak in DN-73 and undetectable in DN-11 and DN-52 (Fig. 3B).

Enzymatic assay indicated that UGT71C5 glucosylated ABA to ABA-GE in vitro (Fig. 2). To study the action of UGT71C5 in vivo, soluble proteins were

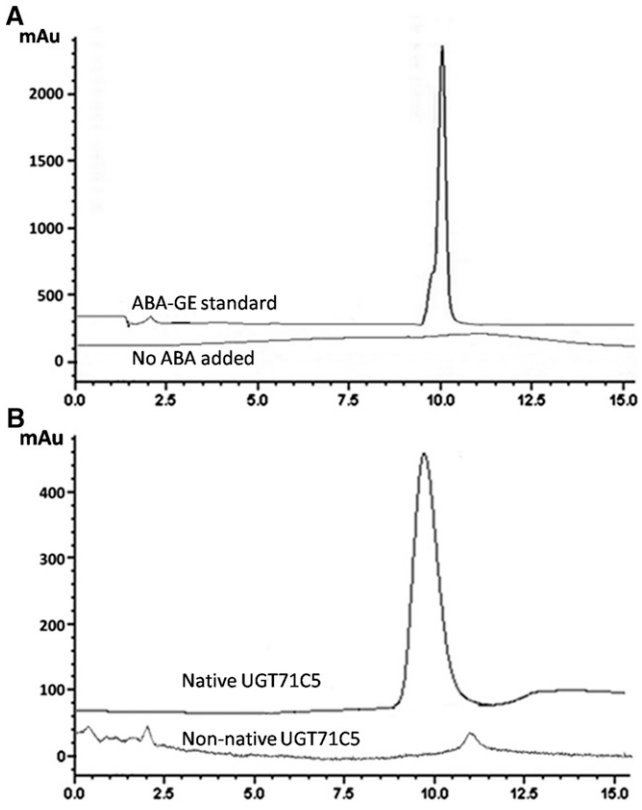

Figure 2. UGT71C5 catalyzes the glucosylation of ABA. A, HPLC analysis of ABA-GE standard substance. The upper trace shows the elution profile of ABA-GE. The lower trace shows the mixture of reaction without ABA. B, UGT71C5 catalyzes ABA to ABA-GE in vitro. The reaction includes UDP-Glc, ABA, and recombinant UGT71C5 extract from *E. coli*. The upper trace shows the reaction with native UGT71C5, and the lower trace shows the reaction with thermodenatured (nonnative) UGT71C5.

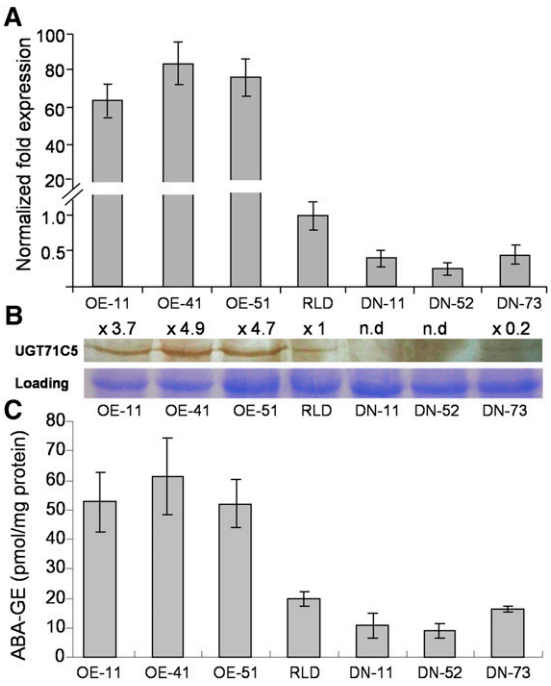

Figure 3. Characterization of *UGT71C5* transgenic lines. A and B, UGT71C5-expressing levels were analyzed by using the qRT-PCR (A) and western-blot (B) techniques. The expression of *UGT71C5* was normalized by the transcripts of *Actin2*. Equal amounts of protein (15 μg) were loaded in each lane, and Coomassie Blue staining was shown as a loading control. C, UGT71C5 exhibits UGT activity in plants. The glucosylation reaction mixture includes UDP-Glc, ABA, and plant protein extracts (1 μg). Data represent the mean of three replicates (±SD). OE-11, OE-41, and OE-51 represent three independent *UGT71C5* overexpressing lines. DN-11, DN-52, and DN-73 represent three independent *UGT71C5* down-expressing lines. RLD indicates the wild type.

extracted from 4-week-old rosette leaves of *UGT71C5*-OEs and *UGT71C5*-DNs transgenic lines and wild-type Reschiev (RLD) plants and used for ABA glucosylation analysis (Fig. 3C). The extracted proteins were incubated with substrates ABA and UDP-Glc for 3 h. The accumulation of ABA-GE indicated that the levels of ABA-GE were 51.9, 62, and 52.6 pmol mg^{-1} fresh weight in the *UGT71C5* up-regulated lines OE-11, OE-41, and OE-51, respectively (Fig. 3C). In comparison, the level of ABA-GE was 22 pmol mg^{-1} fresh weight in the wild type, and the levels were 10.7, 8.7, and 16.2 pmol mg^{-1} fresh weight in *UGT71C5* down-regulated lines DN-11, DN-52, and DN-73, respectively (Fig. 3C). The accumulations of ABA-GE in *UGT71C5* transgenic lines were consistent with the expressional levels of the *UGT71C5* transgene (Fig. 3, B and C). A high level of ABA-GE was detected in the line OE-41 (nearly 62 pmol mg^{-1} fresh weight), which displayed a high expressional level of UGT71C5. These results showed a correlation between the expression levels of UGT71C5 and enzymatic activities of glucosylating ABA in vivo.

Altered Expression of *UGT71C5* Results in Altered ABA-Related Phenotype in Arabidopsis

Our in vitro and in vivo investigations confirmed the enzymatic activity of UGT71C5 in glucosylating ABA to ABA-GE, an inactive form of ABA (Zeevaart, 1999). Therefore, altered expression of UGT71C5 may result in altered levels of ABA and ABA-GE in plants, leading to ABA-deficient or -accumulative phenotype. To test this notion, we assayed germination of seeds and water loss of leaves in *UGT71C5* up- or down-expression lines and the *ugt71c5* mutant plants.

Upon incubation of seeds in Murashige and Skoog (MS) medium for 32 h without vernalization, the germination rates for *UGT71C5* up-regulated transgenic lines OE-11, OE-41, and OE-51 were 65.5%, 67%, and 46%, respectively, whereas wild-type control (RLD) was 30% (Fig. 4A). However, in *UGT71C5* down-regulated transgenic lines DN-11, DN-52, and DN-73, the germination rates were 7%, 23%, and 19%, respectively (Fig. 4A). After 40 h, the germination rates for UGT71C5-OEs were more than 90%, whereas the rates for the wild type were 80%. For UGT71C5-DNs transgenic lines, the rates were less than 70% (Fig. 4A). Hence, the difference between UGT71C5-OEs and UGT71C5-DNs was very clear in MS medium, consistent with the altered levels of ABA and ABA-GE in *UGT71C5* transgenic lines. The ABA-sensitive phenotype was also obvious for the *ugt71c5* mutant, which had a low germination rate of 27% compared with more than 82% in wild-type Columbia-0 (Col-0) Arabidopsis (Fig. 4B).

To further study the impact of exogenous ABA on *UGT71C5* transgenic plants, various concentrations of ABA were added to MS medium. Three days after planting, the impact of exogenous ABA on *UGT71C5* transgenic plants in germination was studied (Fig. 4, C and D). In the presence of lower concentrations of exogenous ABA (0.1–1 μM), *UGT71C5* up-regulated plants displayed ABA-insensitive phenotype with higher germination rates, whereas *UGT71C5* down-regulated plants exhibited more sensitive phenotype with lower germination rates compared with wild-type plants (Fig. 4C). However, in the presence of higher concentrations of exogenous ABA (5 and 10 μM), the differences in seed germination among *UGT71C5* up- and down-expression lines and wild-type plants disappeared, with almost the same germination rate (12%; Fig. 4C). This phenomenon was also observed in the *ugt71c5* mutant and wild-type plants (Fig. 4D). Therefore, our results suggest that down-regulation and knockout of *UGT71C5* result in the delay of seed germination because of excessive ABA in plants. Accordingly, overexpressing *UGT71C5* results in ABA insensitive in seed germination, and exogenous ABA compensates for the deficiency of endogenous ABA glucosylated by overdose of UGT71C5 (Fig. 4C).

UGT71C5 Plays a Negative Role in Drought Resistance

In plants, ABA has an important role in plants' response to drought stress (Zeevaart, 1983; Zhu, 2002).

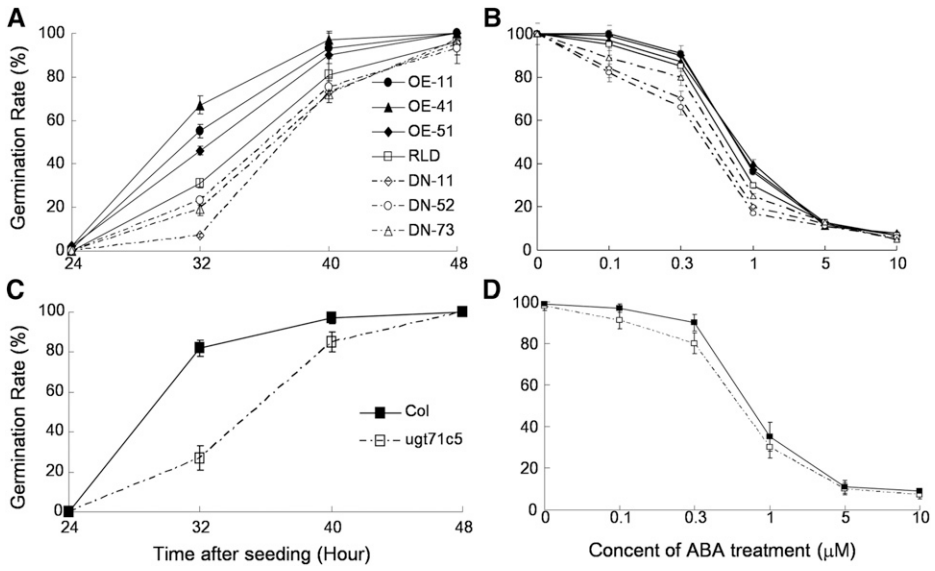

Figure 4. Germination analyses of *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, and wild-type plants. A and B, Plants were planted on MS medium. C and D, Plants were planted on MS medium containing ABA. Data represent the mean of three replicates (±SD). OE-11, OE-41, and OE-51 are three independent *UGT71C5* overexpression lines; DN-11, DN-52, and DN-73 are three *UGT71C5* independent down-expression lines. *ugt71c5* is the mutant, and RLD and Col-0 are wild-type Arabidopsis plants.

To study the function of UGT71C5 in ABA-related drought response, stomatal aperture, water loss, and drought stress response were analyzed using *UGT71C5* transgenic plants (OE and DN plants) and *ugt71c5* and *ugt71b6* mutants. Epidermal peels of 4-week-old leaves were used to analyze stomatal apertures (width and length) in guard cells. The stomatal apertures of *UGT71C5* up-regulated plants OE-11, OE-41, and OE-51 were 0.28, 0.34, and 0.31, respectively, compared with 0.2 in the wild type (RLD; Fig. 5A). However, the stomatal apertures of *UGT71C5* down-regulated plants DN-11, DN-52, and DN-73 were about 0.12 (Fig. 5A). These results were confirmed by microscopy examination (Fig. 5B). The stomatal aperture in the mutant *ugt71c5* was 0.13, which was smaller than that in the mutant *ugt71b6* (0.18) and wild-type (0.21) plants (Fig. 5A). Stomatal closure is a way to reduce water loss for plants to adjust to dehydration (Mansfield, 1976). Consequently, water loss rate and drought tolerance were further analyzed. Leaves with the same age (4 weeks old) and similar size as Arabidopsis were detached and kept in the same environment. After 40 min, the water loss of UGT71C5 up-regulated plants reached 35% of fresh weight, whereas that in *UGT71C5* down-regulated plants and wild-type plants was less than 20% (Fig. 5C). The water loss was 26% in the *ugt71c5* mutant compared with 32% in the *ugt71b6* mutant and 35% in wild-type plants (Fig. 5D).

Altered water loss leads to altered sensitivity to drought stress in plants (Zhu, 2002). When 3-week-old seedlings were withheld water for 12 d, *UGT71C5* down-regulated plants exhibited significantly higher drought tolerance, and *UGT71C5* up-regulated plants exhibited lower drought tolerance (Fig. 5E). After rewatering, *UGT71C5* down-regulated plants survived and completely recovered, whereas wild-type plants survived but with severe damages (Fig. 5E). However, *UGT71C5* up-regulated plants did not survive at all (Fig. 5E). In addition, phenotypic analysis showed that the *ugt71c5* mutant displayed much better drought resistance than the *ugt71b6* mutant and wild-type plants after 18 d of drought treatment (Fig. 1A). Therefore, our results indicate that UGT71C5 plays an important role in ABA-mediated drought resistance.

Deregulation of *UGT71C5* Leads to Dynamic Change in Endogenous ABA/ABA-GE Concentration in Arabidopsis

Phenotypic observation suggests that up- or down-expressing *UGT71C5* results in decreased or increased levels of ABA and accordingly, changes in the level of ABA-GE in Arabidopsis. To confirm the levels of endogenous ABA and ABA-GE regulation by UGT71C5, liquid chromatography (LC) tandem mass spectrometry was used to quantify the levels of ABA and ABA-GE (Supplemental Figs. S9 and S10).

In turgid rosettes, our investigation showed that the levels of ABA were 418, 522, and 368 pmol g^{-1} dry weight in the *UGT71C5* down-expressing lines DN-11, DN-52, and DN-73, respectively, compared with 302 pmol g^{-1} dry weight in the wild type (Fig. 6A). In addition, the level of ABA reached 586 pmol g^{-1} dry weight in the *ugt71c5* mutant compared with 335 pmol g^{-1} dry weight in the *ugt71b6* mutant and 324 pmol g^{-1} dry weight in the wild type (Fig. 6A). In contrast, the levels of ABA were 134, 219, and 147 pmol g^{-1} dry weight in the *UGT71C5* up-regulated lines OE-11, OE-41, and OE-51, respectively (Fig. 6A). However, the levels of ABA-GE in the *UGT71C5* down-regulated lines DN-11, DN-52, and DN-73 were 60, 84, and 55 pmol g^{-1} dry weight, respectively, compared with 95 pmol g^{-1} dry weight in the wild type (Fig. 6A). However, the levels of ABA-GE in the *UGT71C5* up-regulated lines OE-11, OE-41, and OE-51 were 200, 233, and 100 pmol g^{-1} dry weight, respectively (Fig. 6A). In addition, the levels of ABA-GE were 20 pmol g^{-1}

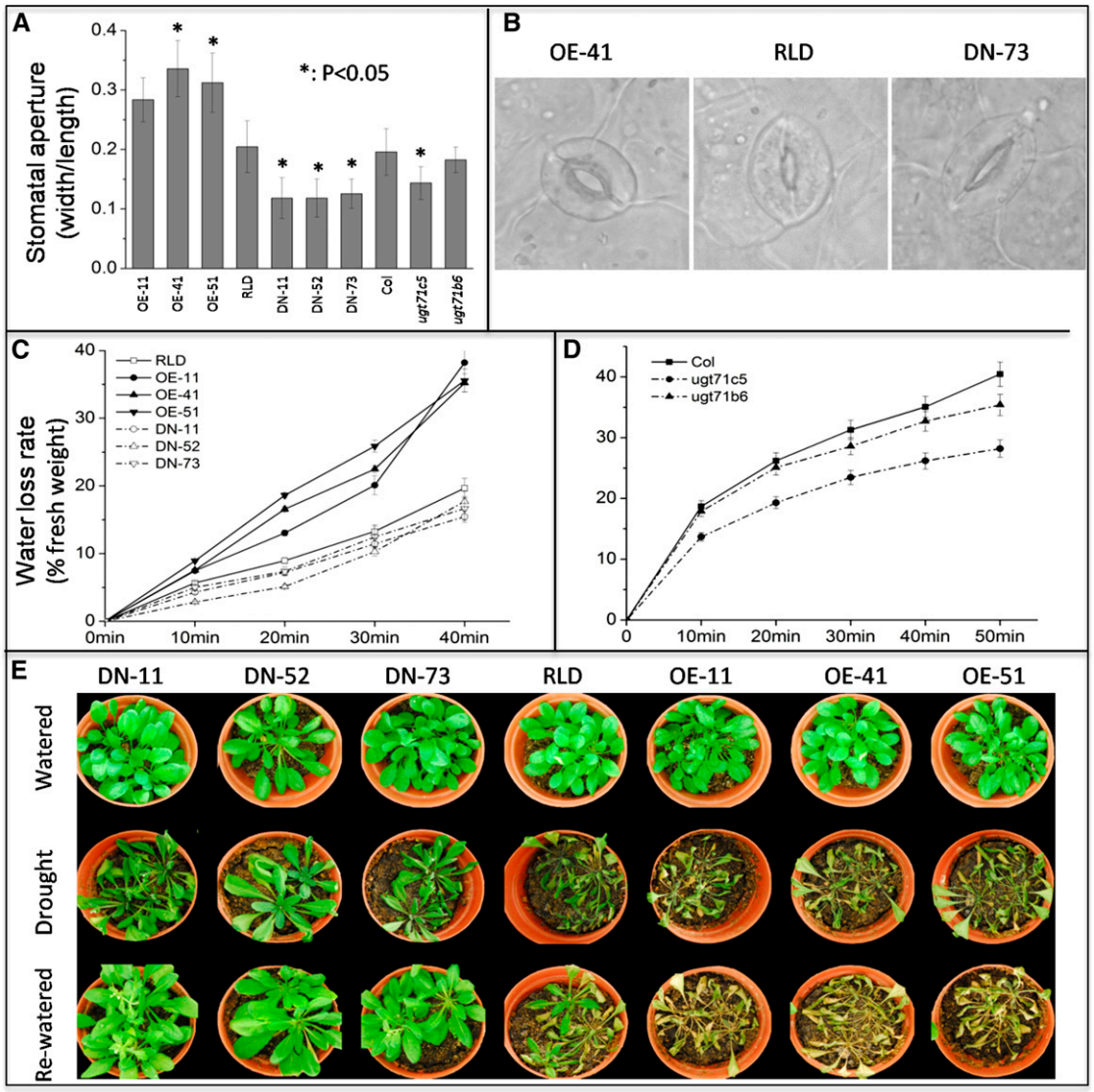

Figure 5. *UGT71C5* down-expression leads to increased drought tolerance. A, Stomatal aperture analyses of *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, and wild-type plants. At least 100 guard cells from each sample were used for measurement. *P* value is below 0.05. B, Stomatal openings of *UGT71C5* overexpression, *UGT71C5* down-expression, and wild-type plants. C and D, Water loss analyses of *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, and wild-type plants. Data were derived from four independent experiments (±SD). E, Phenotypes of *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, and wild-type plants after drought treatment. OE-11, OE-41, and OE-51 are three independent *UGT71C5* overexpression lines; DN-11, DN-52, and DN-73 are three independent *UGT71C5* down-expression lines. *ugt71c5* and *ugt71b6* are mutants, and RLD and Col-0 are wild-type Arabidopsis plants.

dry weight in the *ugt71c5* and *ugt71b6* mutants compared 40 pmol g^{-1} dry weight in wild-type plants (Fig. 6A).

The dynamic state of ABA concentration in plants is well known (Nambara and Marion-Poll, 2005). Drought stress leads to increase in ABA concentration that rapidly reduces to normal level after reirrigation (Harris and Outlaw, 1991). To analyze the levels of ABA and ABA-GE under stress conditions, free ABA and ABA-GE contents in wilted leaves of Arabidopsis were measured after rosette leaves were detached for 3 h. The results revealed that the levels of ABA in *UGT71C5* down-regulated plants reached 4,500 to 5,600 pmol g^{-1} dry weight and that the levels of ABA in *UGT71C5* up-regulated plants were only 2,500 to 2,800 pmol g^{-1} dry weight compared with 3,800 pmol g^{-1} dry weight in the wild type (Fig. 6B). In contrast, the levels of ABA-GE reached 660, 533, and 687 pmol g^{-1} dry weight in *UGT71C5* up-expressing lines OE-11, OE-41, and OE-51, respectively, compared with 206 pmol g^{-1} dry weight in wild-type plants (Fig. 6B). However, the levels of ABA-GE were only 64, 188, and 73 pmol g^{-1} dry weight in

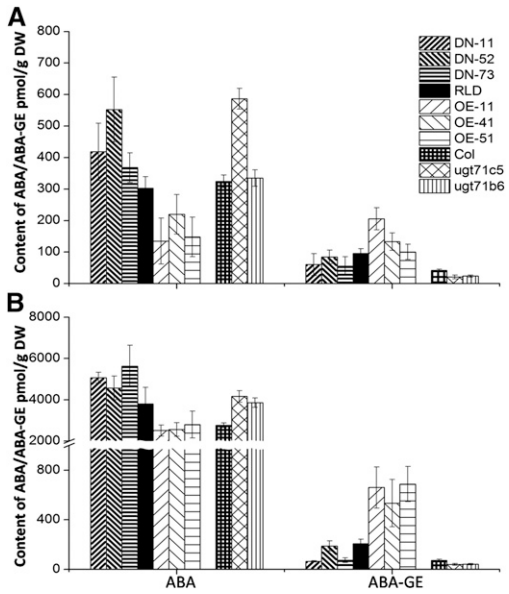

Figure 6. Profiles of endogenous ABA and ABA-GE in *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, *ugt71b6*, and wild-type Arabidopsis plants. Analysis of endogenous ABA and ABA-GE by using the HPLC-mass spectrometry method. Four-week-old rosette leaves were directly ground (A) or detached leaves were left at room temperature for 3 h (B) before profiling. Data are based on three independent replicates (±SD). OE-11, OE-41, and OE-51 are three independent *UGT71C5* overexpression lines; DN-11, DN-52, and DN-73 are three independent *UGT71C5* down-expression lines. *ugt71c5* and *ugt71b6* are mutants, and RLD and Col-0 are wild-type Arabidopsis plants. DW, Dry weight.

UGT71C5 down-expressing lines DN-11, DN-52, and DN-73, respectively (Fig. 6B). In addition, the levels of ABA-GE in *ugt71c5* and *ugt71b6* mutants were 40 pmol g^{-1} dry weight compared with 70 pmol g^{-1} dry weight in wild-type plants (Fig. 6B). Hence, our results confirmed the function of UGT71C5 in glucosylation of ABA in plants.

The Expression of ABA-Responsive Genes Correlates with the Expression Level of *UGT71C5*

Stress conditions lead to the increase of endogenous ABA concentration that induces the expression of ABA-regulated genes (Tuteja, 2007). Our results show that UGT71C5 glucosylates ABA to ABA-GE, and therefore, alteration of *UGT71C5* expression would likely lead to ABA-excessive or -deficient phenotypes. To determine the effect of UGT71C5 in the expression of ABA-responsive genes, qRT-PCR was used to quantify the transcript levels of ABA-responsive genes. Under normal growth conditions, the transcript levels of ABA-responsive genes *CYP707A2*, *RESPONSIVE TO DESSICATION 29A* (*RD29A*), *RD29B*, *RAB GTPASE HOMOLOG B18* (*Rab18*), and *ABA INSENSITIVE5* (*ABI5*) were apparently up-regulated in the *UGT71C5* down-expressing lines and down-regulated in the *UGT71C5* overexpressing lines compared with the wild type (Fig. 7). Higher transcript levels of these genes were found in the *ugt71c5* mutant, and they were higher than those in *ugt71b6* and the wild type (Fig. 7), further confirming the crucial function of UGT71C5 in the glucosylation of ABA in plants. In addition, there was no significant difference in the expression of *NCED3* (Fig. 7). After seedlings were treated with 10 μM ABA for 10 h, however, there were no differences in the transcript levels of ABA-responsive genes among all plants (Fig. 7), suggesting that the altered transcription of the ABA-response genes in the transgenic lines was caused by changes in ABA levels. The expression levels of ABA-responsive genes inversely correlated with the expression level of *UGT71C5*, indicating that UGT71C5 influences the ABA signal network by regulating ABA hemostasis in planta.

DISCUSSION

ABA-mediated responses to various stresses are regulated by the local concentration of ABA that is related to the ABA homeostasis by hydrolyzation and conjugation (Nambara and Marion-Poll, 2005; Millar et al., 2006; Seo et al., 2006). Up to now, hydrolyzation of ABA has been well studied (Cutler and Krochko, 1999; Kushiro et al., 2004). The conjugation of ABA with Glc was investigated at the biochemical level and thought to lead to changes in ABA bioactivity (Boyer and Zeevaart, 1982; Bray and Zeevaart, 1985; Bowles et al., 2006). However, whether the process has a critical role in ABA homeostasis under physiological conditions remains unclear. The conjugation cycle of ABA includes glucosylation of ABA to ABA-GE by glucosyltransferases (UGTs) and hydroxylation of ABA-GE to ABA by BGs. Recent reports reveal that ABA-GE is stored in vacuole and readily transformed to ABA by BGs (AtBG1/2) under stressed conditions (Nambara and Marion-Poll, 2005; Lee et al., 2006; Xu et al., 2012).

It is well known that the ABA concentration increases up to 100-fold when plants suffer drought stress, and it then rapidly reduces to normal level when stressful conditions disappear (Zeevaart and Creelman, 1988; Harris and Outlaw, 1991; Zeevaart and Yang, 2005). It was believed that de novo biosynthesis under normal and abiotic stress conditions contributed to the increased levels of ABA (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). However, recent studies show that mutation in *AtBG*s (*atbg1/2*) leads to ABA-deficient phenotypes, suggesting that hydrolysis of ABA-GE is also an important way to increase the level of ABA (Lee et al., 2006; Xu et al., 2012). However, the UGTs seem to glucosylate ABA, and these UGTs include UGT71B6, UGT73B1, UGT73B3, UGT75B1, UGT75B2, UGT84B1, and UGT84B2, which belong to different groups of family 1 in the superfamily of glucosyltransferases (Ross and O'Neill, 2001; Lim, et al. 2005; http://www.cazy.org/).

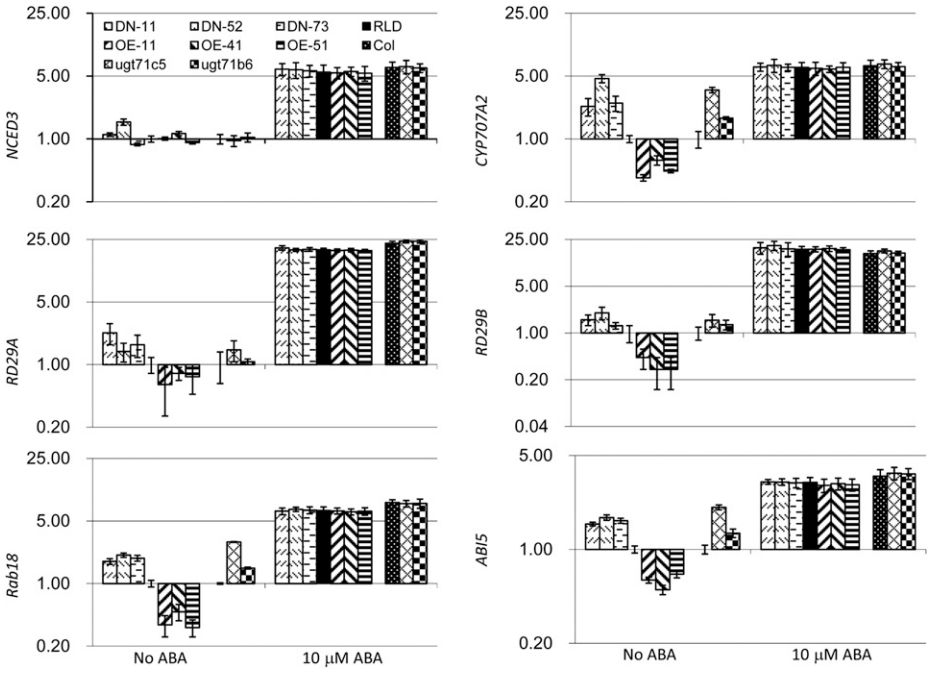

Figure 7. qRT-PCR analyses of ABA-responsive genes in *UGT71C5* overexpression, *UGT71C5* down-expression, *ugt71c5*, *ugt71b6*, and wild-type Arabidopsis plants. The transcript abundances of ABA-responsive genes (*NCED3*, *CYP707A2*, *RD29A*, *RD29B*, *ABI5*, and *Rab18*) were analyzed by using qRT-PCR. Mean values were from three independent technical replicates (±SD) and normalized using *Actin2* transcript. OE-11, OE-41, and OE-51 are three independent *UGT71C5* overexpression lines; DN-11, DN-52, and DN-73 are three independent *UGT71C5* down-expression lines. *ugt71c5* and *ugt71b6* are mutants, and RLD and Col-0 are wild-type Arabidopsis plants.

However, obvious studies did not reveal ABA-related phenotypes involving UGTs (Priest et al., 2006). In this study, we characterized a unique ABA glucosyltransferase, UGT71C5, and showed for the first time, to our knowledge, that ABA glucosylation plays a critical role in controlling ABA concentration in response to stress conditions.

Previously reported UGTs catalyze ABA glucosylation in vitro, but there is no in vivo evidence for their roles in ABA-related phenotype (Priest et al., 2006), suggesting that they are not the major ABA glucosyltransferases. For example, UGT75B1 has been shown to transfer UDP-Glc from Suc synthase to the callose synthase for the synthesis of callose at the site of cell plate (Zhou et al., 2010). UGT84B1 has indole-3-acetate β-glucosyltransferase activity in addition to its ABA glucosyltransferase activity (Grubb et al., 2004). UGT71B6 is a glucosytransferase that recognizes the naturally occurring (+)-ABA enantiomer (Priest et al., 2005). Mutation of *UGT71B6* (*ugt71b6*) in Arabidopsis has no obvious ABA-excessive phenotype, whereas cosuppressions of *UGT71B6* and its two homologs *UGT71B7* and *UGT71B8* displayed an ABA-excessive phenotype (Priest et al., 2005; Dong et al., 2014). These findings indicate that *UGT71B6* is not a major glucosytransferase involved in ABA homeostasis. Compared with other UGTs, UGT71C5 shares high sequence identity with UGT71B6. Importantly, *ugt71c5* mutants exhibit apparently ABA-excessive phenotypes (Fig. 1A; Supplemental Figs. S1 and S2), suggesting that UGT71C5 is a key enzyme in ABA glucosylation in plants. Cytoplasmic location of UGT71C5 in plant cells (Fig. 1B) is consistent with cytoplasmic distribution of ABA. Therefore, there is a possibility that UGT71C5 glucosylates ABA to ABA-GE in cytoplasm to regulate the level of free ABA. Using biochemical and molecular approaches, we confirmed the possibility that Arabidopsis UGT71C5 glucosylates ABA to ABA-GE in vitro and in vivo (Figs. 2 and 3B). Most importantly, deregulation of UGT71C5 leads to dynamic changes in endogenous ABA/ABA-GE concentration (Fig. 6). The content of endogenous ABA and ABA-GE profiled by LC-mass spectrometry in this study confirms the hypothesis that UGT71C5 plays a major role in ABA glucosylation. Down-regulation of *UGT71C5* in plants results in an increased ABA level and a reduced ABA-GE level, whereas up-regulation of *UGT71C5* results in a reduced ABA level and an increased ABA-GE level in turgid or wilted rosette leaves of Arabidopsis (Fig. 6), indicating a major role for UGT71C5 in converting ABA to ABA-GE in plants. Mutation in *UGT71C5* (*ugt71c5*) also results in increased levels of ABA and reduced levels of ABA-GE (Fig. 6), consistent with the observations from down-expressing *UGT71C5* transgenic plants. In contrast, a defect in *UGT71B6* (*ugt71b6*) results in accumulation of ABA in wilted rosettes but not in turgid rosettes (Fig. 6), suggesting that it may function in stressed conditions in addition to UGT71C5. Hence, our investigation suggests that UGT71C5 has more influence on ABA homeostasis than UGT71B6.

Altered ABA homeostasis will lead to altered expression of ABA-responsive genes and plant response to water-deficit stress (Nambara and Marion-Poll, 2005; Tuteja, 2007). In this study, we showed that down-regulation and knockout of *UGT71C5* in Arabidopsis

resulted in increased expressions of ABA-responsive genes, such as *CYP707A2*, *RD29A*, *RD29B*, *Rab18*, and *ABI5*, whereas overexpression of *UGT71C5* resulted in reduced expressions of these genes (Fig. 7). However, a defect in *UGT71B6* (*ugt71b6*) has less influence on the expression of ABA-responsive genes compared with that in *UGT71C5* (*ugt71c5*) in Arabidopsis (Fig. 7). When exogenous ABA was added, the difference in gene expression in *UGT71C5*-deregulated transgenic plants as well as *ugt71c5* and *ugt71b6* mutants disappeared (Fig. 7), indicating that the transcriptional difference of ABA-responsive genes is caused by altered level of endogenous ABA glucosylated by UGT71C5 in plants. Hence, our studies establish the relationship between the function of UGT71C5 and ABA-mediated physiological processes. UGT71B6 has the ability to glucosylate ABA in vivo and raises ABA-GE content; however, UGT71B6 is not able to change endogenous ABA content (Priest et al., 2006). Our investigation also showed that endogenous ABA content in the *ugt71b6* mutant did not change in turgid rosette leaves (Fig. 6A). Therefore, UGT71C5 is a major contributor to ABA signaling network through changing ABA-related gene expression.

Physiological analysis also showed the UGT71C5 functions as a major ABA glucosyltransferase in planta. Overexpressing *UGT71C5* in Arabidopsis results in earlier germination of seeds than in wild-type plants, and the difference is narrowed when exogenous ABA is applied (Fig. 4). Under drought conditions, overexpressing *UGT71C5* in Arabidopsis results in wider openings in stomatal aperture and higher water loss rates, which are typical ABA-deficient phenotypes (Fig. 5). In contrast, down-expressing or knocking out *UGT71C5* leads to ABA-excessive phenotypes, including reduced stomatal aperture and water loss rates (Fig. 5). Compared with *UGT71B6*, a mutation in *UGT71C5* (*ugt71c5*) results in lower stomatal aperture and lower water loss rate than a mutation in *UGT71B6* (*ugt71b6*) in Arabidopsis (Fig. 5). Altered stomatal aperture and water loss disrupt normal response to drought (Mansfield, 1976). Down-expressing and knocking out *UGT71C5* in Arabidopsis lead to enhanced resistance to drought stress compared with overexpressing *UGT71C5* and mutation in *UGT71B6* (*ugt71b6*; Figs. 1A and 5E), further showing that UGT71C5 is a major ABA glucosyltransferase in the mediation of ABA homeostasis.

In plants, the effect of ABA is determined by its concentration. When plants encounter adverse environments, the level of ABA raises to trigger ABA signaling networks to initiate stress responses (Nambara and Marion-Poll, 2005). Production of ABA from de novo biosynthetic pathways and one-step process from ABA-GE hydrolyzed by AtBG1/2 contribute to rapid increase in ABA content necessary for plants to meet physiological needs (Lee et al., 2006; Xu et al., 2012). After the adverse environmental condition disappears, ABA concentration reduces to the normal level (Nambara and Marion-Poll, 2005). However, previous studies leave behind a gap in the conjugation cycle of ABA, although there were some reports that some UGTs might function in the ABA glucosylation (Lim et al., 2003; Priest et al., 2006; Dong et al., 2014). In this study, we provide convincing evidence that UGT71C5 contributes to the reduced level of ABA under normal growth condition by glucosylation of ABA to its inactive storage form, ABA-GE. In the conjugation cycle of ABA to ABA-GE, ABA concentration rises through hydrolyzation of ABA-GE by AtBG1/2 localized in the endoplasmic reticulum and vacuole when plants encounter adverse environment. After abiotic stresses disappear, the high level of ABA is decreased mainly through glucosylation of ABA by UGT71C5 localized in cytoplasm (Fig. 8). Hence, our investigation highlights the importance of UGT71C5 in the conjugation cycle of ABA in ABA homeostasis in plants.

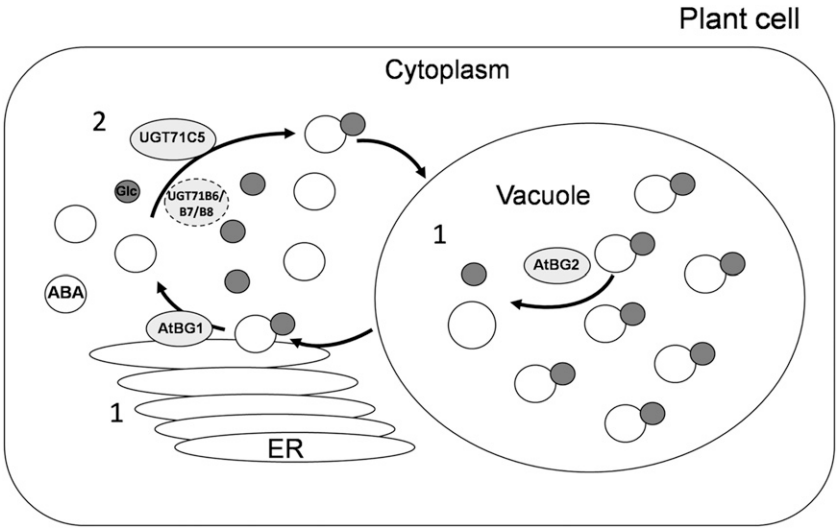

Figure 8. Proposed model on ABA and ABA-GE conversion by UGT71C5 and AtBG1/2 in plant cells. 1, When plants encounter drought stress, ABA concentration rises through ABA-GE hydrolyzation by endoplasmic reticulum (ER)- and vacuole-localized AtBG1/2, respectively. 2, When drought stress disappears, ABA concentration is decreased through ABA glucosylation by cytosolically localized UGT71C5. UGT71B6/B7/B8 may also function in the glucosylation of ABA under drought stress condition.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) accessions RLD and Col-0 are used in this work. *ugt71c5* (CS25151) and *ugt71b6* (SALK_024685c) mutants (Col-0) used in this work were obtained from ABRC (http://abrc.osu.edu/).

For nonsterile culture, seeds were grown in soil (50% vermiculite and 50% nutrient soil) in greenhouses at 22°C under 60% humidity with 16-h-light (250 μmol m^{-2} s^{-1})/8-h-dark cycles. For sterile culture, surface-sterilized seeds were sown on MS medium (Murashige and Skoog, 1962) containing 3% (w/v) Suc and 0.8% (w/v) agar, pH 5.8.

Chemicals

The majority of the chemicals were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/). ABA-GE was purchased from OlChemIm Ltd.

Identification and Confirmation of *ugt71c5* and *ugt71b6* Mutants

To isolate the homozygous *ugt71c5* and *ugt71b6* mutants, genomic DNAs were extracted from rosette leaves and subjected to PCR analysis using gene-specific primers (Supplemental Table S1). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Homozygous mutant was propagated and used for the subsequent experiments.

For semiquantitative and qRT-PCR analyses, total RNA was extracted from 4-week-old Arabidopsis plants in solid MS medium using RNAiso Reagent from TAKARA (http://www.takara.com) according to the manufacturer's instructions. Complementary DNAs (cDNAs) were reverse transcribed from total RNA with the PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA) and quantified with a cycler apparatus (Bio-Rad) with the qPCR Core Kit for SYBR Green (Tiangen). Primer pairs of *ugt71c5* and *ugt71b6* are described in Supplemental Table S1.

Plasmid Construction and Transformation of *UGT71C5*

cDNA of *UGT71C5* was amplified using primers harboring *Bam*HI and *Sma*I sites (Supplemental Table S1), and subsequently cloned into vector pET-32a (Novagen) through *Bam*HI and *Ecl*136II and *pSK-35S::GFP* (Arai et al., 2001) through *Bam*HI and *Sma*I sites, yielding *pET32a-6XHis:UGT71C5* that was transformed to *Escherichia coli* strains BL21 for expression. *pBI221-35S::UGT71C5:GFP* for transient expression in Arabidopsis protoplast was according to a previous report (Yang et al., 2006).

For stable expression of *UGT71C5* in Arabidopsis, transgenic UGT71C5-OE and -DN Arabidopsis lines were generated by transformation with *pCAMBIA2301-35S::UGT71C5* and *pCAMBIA2301-35S::anti-UGT71C5*, which were constructed by replacing GUS of pCAMBIA2301 with the blunted *UGT71C5* cDNA fragment (Supplemental Table S1) in the sense orientation and the antisense orientation into the blunted *Sma*I-*Ecl*136II sites of pCAMBIA2301 (Hajdukiewicz et al., 1994). Transformation of Arabidopsis (RLD) was done according to the floral dip method (Clough and Bent, 1998).

Purification and Glucosylation Assay of UGT71C5

The purification of *pET32a-6xHis:UGT71C5* in *E. coli* (BL21) was done according to the instructions from Invitrogen (http://www.invitrogen.com/). For assays with plant-expressed *UGT71C5*, crude protein extracts from plants were made as described before (Priest et al., 2006). A final volume of 100 μL, including 50 μL of crude extract (30 μg of total protein), 1 mM (±)-ABA, and 5 mM UDP-Glc, was incubated at 30°C for 3 h and stopped by the addition of 10 μL of trichloroacetic acid (240 mg mL^{-1}). ABA and ABA-GE contents in the reaction were profiled by HPLC.

For kinetic assay containing 1 μg of UGT71C5, protein was incubated at 30°C with ABA in 100 μL of reaction mixture containing 5 mM $MgCl_2$ and 10 mM dithiothreitol. Reactions were stopped by adding 10 μL of 240 mg mL^{-1} trichloroacetic acid, and ABA and ABA-GE contents in the reaction mixture were profiled by HPLC using a 5-μm C18 Reverse-Phase HPLC Column (LC-6AD; Shimadzu). A linear gradient with increased concentration of methanol against 0.1 M acetic acid (pH 3.5; triethylamine) from 10% to 80% (v/v) in 30 min was used, and the eluate was monitored at 270 nm. The content of ABA-GE was calculated from four time points and used to determine the kinetic parameters by Michaelis-Menton equation.

Antibody Generation and Western Blotting of Transgenic Plants of *UGT71C5*

Anti-UGT71C5 polyclonal antibody was generated by immunizing rabbits with purified bacterially expressed recombinant UGT71C5 (Supplemental Fig. S6B). Horseradish peroxidase-tagged anti-UGT71C5 antibody was made by Rong Hai Company. Crude proteins of plants were used for western blotting according to standard protocol (Sambrook and Russell, 2001).

Analyses of Expression Pattern of UGT71C5

Total RNA and protein extracts were extracted from Arabidopsis plants (2-week-old root and leaf and 45-d-old leaf, seed, stem, legume, and flower, respectively). For qRT-PCR analysis, cDNAs were reverse transcribed from total RNA with the PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA) and quantified with a cycler apparatus (Bio-Rad) with the qPCR Core Kit for SYBR Green (Tiangen). Primer pairs of *UGT71C5* for qRT-PCR are described in Supplemental Table S1. Extracted proteins were used for western blotting using horseradish peroxidase-tagged anti-UGT71C5 antibody according to the standard protocol (Sambrook and Russell, 2001).

Physiological Analysis of Plants

Seeds of the same age were surface sterilized with $HgCl_2$ and plated on MS medium containing 0.8% (w/v) agar with different concentrations of (±)-ABA. The percentage of germination was scored when cotyledons emerged from the seed coat. Water loss rate was measured as described by Himmelbach et al. (2002). Stomatal aperture was measured according to a previous report (Christmann et al., 2007). The stomatal apertures were pictured and measured with an inverted fluorescence microscope (DMI6000B; Leica).

Transcriptional Analysis of ABA-Responsive Genes

Four-week-old Arabidopsis plants in solid MS medium were incubated in liquid one-half-strength MS with or without 10 μM (±)-ABA for 10 h. Total RNA was extracted from plant tissues using RNAiso Reagent from TAKARA according to the manufacturer's instructions.

For qRT-PCR analysis, cDNAs of ABA-responsive genes were reverse transcribed from total RNA with the PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA) and quantified with a cycler apparatus (Bio-Rad) with the qPCR Core Kit for SYBR Green (Tiangen). Primer pairs of ABA-responsive genes were described in Supplemental Table S1.

Sample Preparation and Mass Spectrometric Analysis of ABA and ABA-GE

Samples of turgid and wilted rosette leaves before solid-phase extraction were treated as described before (Priest et al., 2006); 1 mL of pretreated sample with 20 μL of internal standard (IS) solution (chloromycetin, 14.426 ng mL^{-1}) was loaded to Sepax-UCT C18 Solid-Phase Extraction Cartridges (Sepax). Samples were eluted with 2 mL of buffer containing methanol:distilled, deionized water:acetic acid (80:19:1, v/v/v). The elutant was dried at 40°C under a gentle stream of nitrogen. The residue was reconstituted by the addition of 200 μL of methanol:water:acetic acid (45:54:1, v/v/v); 10-μL aliquots of supernatant were analyzed by LC-mass spectrometry (Agilent).

The mass spectrometer was operated in the negative ion mode using electrospray ionization source. A high electrospray ionization voltage of 4.0 kV was applied to the sprayer. The turbo gas temperature was 450°C. The auxiliary gas flow was 8 L min^{-1}. The settings of nebulizer gas, curtain gas, and collision gas flows were 8, 8, and 6, respectively. All of the gas used in this experiment was high-purity nitrogen. Collision energy was set at 15 eV for ABA and ABA-GE and 25 eV for IS. The detection was made by monitoring the most intensive precursor to fragment transitions at mass-to-charge ratio (*m/z*) 263.1 to 153.0 for ABA, *m/z* 425.1 to 263.0 for ABA-GE, and *m/z* 321.0 to 152.0 for chloramphenicol (IS). The dwell time was 200 ms for analyses.

Sequences of genes mentioned in this article can be found in The Arabidopsis Information Resource (http://www.arabidopsis.org) as the following accession numbers: *UGT71C5* (At1g07240), *UGT71B6* (At3g21780), *UGT71B7* (At3g21790), *UGT71B8* (At3G21800), *UICYP707A2* (At2g29090), *NCED3* (At3g14440), *RD29A* (At5g52310), *RD29B* (At5g52300), *RAB18* (At1g43890), *ABI5* (At2g36270), and *Actin2* (At3g18780).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequence alignment of UGT homologs.

Supplemental Figure S2. Phylogenetic analysis of the UGT homologs.

Supplemental Figure S3. Amino acid sequence alignment of UGT71C5 with its homologs.

Supplemental Figure S4. Expression pattern of UGT71C5 in Arabidopsis.

Supplemental Figure S5. Identification of *ugt71c5* and *ugt71b6* mutants.

Supplemental Figure S6. Purification of UGT71C5 in *E. coli* and titer determination of UGT71C5 antibody.

Supplemental Figure S7. Kinetic assay of UGT71C5.

Supplemental Figure S8. Analysis of the specificity of UGT71C5 antibody.

Supplemental Figure S9. Fragmentation pattern of ABA standard.

Supplemental Figure S10. Fragmentation pattern of ABA-GE standard.

Supplemental Table S1. Primers used in this study.

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