The maize secondary metabolism glycosyltransferase UFGT2 modifies flavonols and contributes to plant acclimation to abiotic stresses

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- **Background and Aims** Nowadays, the plant family 1 glycosyltransferases (UGTs) are attracting more and more attention since members of this family can improve the properties of secondary metabolites and have significantly enriched the chemical species in plants. Over the past decade, most studies on UGTs have been conducted in *Arabidopsis thaliana* and they were proved to play diverse roles during the plant life cycle. The *Zea mays* (maize) GT1 family comprises a large number of UDP-glycosyltransferase (UGT) members. However, their enzyme activities and the biological functions are rarely revealed. In this study, a maize flavonol glycosyltransferase, UFGT2, is identified and its biological role is characterized in detail.
- **Methods** The UFGT2 enzyme activity, the flavonol and glycoside levels *in planta* were examined by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS). The functions of UFGT2 in modifying flavonols, mediating flavonol accumulation and improving stress tolerance were analysed using two *ufgt2* mutants and transgenic arabidopsis plants.
- **Key Results** By *in vitro* enzyme assay, the maize UFGT2 was found to show strong activity towards two flavonols: kaempferol and quercetin. Two *ufgt2* knockout mutants, Mu689 and Mu943, exhibited obvious sensitivity to salt and drought stresses. The endogenous quercetin and kaempferol glycosides, as well as the total flavonol levels were found to be substantially decreased in the two *ufgt2* mutants, with declined H₂O₂-scavenging capacity. In contrast, ectopic expression of *UFGT2* in arabidopsis led to increased flavonol contents and enhanced oxidative tolerance. Moreover, expression of typical stress-related genes in arabidopsis and maize were affected in *UFGT2* overexpression plants or knockout mutants in response to abiotic stresses. *UFGT2* was also transferred into the arabidopsis *ugt78d2* mutant and it was found to recover the deficient flavonol glycoside pattern in the *ugt78d2* mutant, which confirmed its catalysing activity *in planta*.
- **Conclusion** It is demonstrated in our study that a maize glycosyltransferase, UFGT2, involved in modifying flavonols, contributes to improving plant tolerance to abiotic stresses.

Key words: Abiotic stress, flavonol, glycosyltransferase, kaempferol, maize, quercetin, UFGT2.

INTRODUCTION

Flavonoids, including flavonols and anthocyanins, are a group of specialized secondary metabolites in the plant kingdom, and are reported to have diverse roles, such as providing flower colouring (Mol *et al.*, 1998), affording UV protection (Li *et al.*, 1993), mediating auxin transport (Peer and Murphy, 2007), improving plant defence to biotic/abiotic stresses (Treutter D, 2005) and exerting health-promoting effects (Williams and Grayer, 2004).

Flavonoid biosynthesis derives from the phenylalanine metabolic pathway and has been well characterized in *Arabidopsis thaliana*. Many critical enzymes were identified in earlier studies by screening a series of *transparent testa* (*tt*) mutants, such as *TT3*, *TT4*, *TT5*, *TT6* and *TT7* encoding dihydroflavonol 4-reductase (DFR), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H) and flavanone 3'-hydroxylase (F3'H), respectively. (Koornneef, 1990). Loss of function of these genes severely affects the biosynthesis of

flavonoids, and causes defects in architectural phenotypes (Peer *et al.*, 2001; Buer and Djordjevic, 2009).

Nowadays, flavonoids are attracting more and more attention in regard to their contribution to plant–environment interactions. Earlier studies had reported many cases of flavonoids responsive to UV light. For example, the *A. thaliana* mutant deficient in flavonoid accumulation showed severe sensitivity to UV radiation (Lois and Buchanan, 1994). UV-B irradiation could clearly increase the levels of flavonoids in *Populus trichocarpa* leaves, especially of kaempferol and quercetin (Warren *et al.*, 2003). In recent years, flavonoids were also found to be overaccumulated in response to a wide range of other abiotic stresses, such as cold, heat, salinity, drought, heavy metals, nutrient limitation or air pollution (Nakabayashi *et al.*, 2015). Hernandez *et al.* (2009) suggested a spatio-temporal correlation between the flavonoid accumulation and oxidative stress. Consistently, many studies also revealed that

the higher flavonoid accumulation tends to parallel the upregulation of related biosynthesis genes in response to abiotic stresses. It is reported that the *Oryza sativa* flavonoid pathway genes, *OsDFR* and *OsANS*, were induced by dehydration, high salt and abscisic acid (ABA; Ithal and Reddy, 2004). Ma et al. (2014) found that the transcripts of key flavonoid biosynthesis genes in wheat, including *CHS*, *CHI*, *F3H*, *FLS* (flavonol synthase), *DFR* and *ANS* (anthocyanidin synthase), were rapidly increased under drought stress. Moreover, the upstream regulator MYB112 was reported to control the formation of anthocyanins in response to salinity and high light stresses (Lotkowska et al., 2015). It is also documented that altering the expression of flavonoid biosynthesis genes could affect the plant tolerance to stresses. For instances, an earlier study documented that the arabidopsis flavonoid mutants *tt4*, *tt5* and *tt6* that were deficient in flavonoids were hypersensitive to UV-B irradiation (Li et al., 1993). Meng et al. (2015) reported that overexpression of a tomato F3H-like gene substantially improved the plant tolerance to chilling stress.

Glycosylation is a prominent modification reaction and is often the last step in the biosynthesis of flavonoids (Vogt and Jones, 2000). Flavonoids exist mostly as their glycosides (Winkel-Shirley, 2001) which make them more stable and soluble, and thus facilitate their transportation and accumulation in plant cells (Bowles et al., 2006). Glycosylation of small molecules in plants is catalysed by the family 1 glycosyltransferases, also named UDP-glycosyltransferases (UGTs), which transfer sugar moieties from activated sugar donors to specific acceptor molecules (Bowles et al., 2005). To date, at least eight UGTs have been identified as modifying flavonoids in arabidopsis (Li et al., 2015). Although these UGTs catalyse reactions of the same type of molecules, they differ in their protein sequences, expression patterns, substrates, sugar donors and biological functions. For instance, arabidopsis UGT78D2 catalyses the transfer of UDP-glucose onto cyanidin, kaemferol and quercetin (Yin et al., 2012). However, its two highly homologous enzymes, UGT78D1 and UGT78D3, sharing 71.7 and 75.7 % sequence identities with UGT78D2, respectively, are specific for flavonol aglycones only, with no activity towards cyanidin (Yonekura-Sakakibara et al., 2008). More interestingly, UGT78D1 specifically uses UDP-rhamnose as sugar donor (Yin et al., 2014), while UGT78D3 only accepts UDP-arabinose, rather than UDP-glucose (Yonekura-Sakakibara et al., 2008). Additionally, UGT89C1, which acts downstream of UGT78D1 and UGT78D2, is involved in the secondary glycosylation of the flavonols, and specifically adds UDP-rhamnose onto kaempferol/quercetin-3-glycoside (Yonekura-Sakakibara et al., 2007). Thus, glycosylation of flavonoids by different UGTs leads to specific effects and might represent a delicate tuning to plant growth and development.

Over the past decade, most studies on the biological roles of UGTs have been conducted in arabidopsis, and many family 1 members have been identified to play important roles in diverse pathways. According to our previous study, the *Zea mays* glycosyltransferase family 1 comprises 147 UGT members (Li et al., 2014). However, very few members have been functionally characterized to date. Two decades ago, the first maize glycosyltransferase was identified by analysing the maize mutant of the *bronze* locus (*Bz*). *BRONZE* was identified to be the structural gene coding for the enzyme UDP glucose:flavonoid

glucosyltransferase (UFGT) involved in catalysing the 3-*O*-glucosylation of flavonols or anthocyanidins (Larson and Coe, 1977; Dooner and Nelson, 1977). *BRONZE* is specifically expressed in the endosperm, and is required for anthocyanin pigmentation in the aleurone layer of the seed (Dooner and Nelson, 1977). Disruption of the *Bz* locus by transposons would change the seed colour from purple to bronze (Dooner and Nelson, 1977).

In this study, we characterized a new maize UGT gene, *UFGT2*, by using two maize Mu mutants in which the same gene is disrupted. We found that *UFGT2* could catalyse the glucosylation of two flavonols: kaemferol and quercetin. We also found that loss of *UFGT2* in maize decreased the contents of total flavonols, as well as kaemferol and quercetin glycosides, in both normal and stress conditions. In addition, the two maize *ufgt2* mutants were very sensitive to abiotic stresses such as salinity and drought, while overexpression of *UFGT2* in arabidopsis increased the flavonol accumulation and noticeably improved the plant tolerance to salt and drought stresses. Our findings provide new insights not only in understanding the function of maize *UFGT2*, but also in its potential application in maize breeding for both stress tolerance and health benefits.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of Mu689 and Mu943 mutant alleles were obtained from the UniformMu population, which was constructed by introgression of mutator activity into the W22 genetic background (McCarty et al., 2005). *Arabidopsis thaliana* ecotype Columbia-0 was used in this study. The mutant line *ugt78d2* (SALK_049338) was obtained from the European Arabidopsis Stock Centre (uNASC). Maize plants were grown in soil in a growth chamber under a 16 h light/8 h dark photoperiod at 28 °C. All the arabidopsis plants were grown on Murashige and Skoog (MS) medium or soil under a 16 h light/8 h dark photoperiod at 22 °C.

Plasmid construction and plant transformation

To generate vectors for prokaryotic expression, the coding region of *UFGT2* was cloned into the PGEX-2T vector fused with a glutathione *S*-transferase (GST) tag. To generate the 35S::*UFGT2* construct, the coding region of *UFGT2* was cloned into the pBI121 binary vector driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. All the constructs were transformed into arabidopsis Col-0 by *Agrobacterium*-mediated floral dip. The primers used for plasmid construction are listed in Supplementary Data Table S1.

Stress assays

For measuring the maize seed germination and root growth, the seeds were first surface-sterilized with 70 % ethanol for 5 min, then with 3 % sodium hypochlorite for 10 min, and then rinsed in distilled water. The sterilized seeds were germinated,

and continued growing on filter paper moistened with 25 mM NaCl and 7.5 % polyethylene glycol (PEG) in Petri dishes. After 1 week, the germination rates were calculated and the root lengths were measured. For salt stress treatment, the maize seeds were germinated in normal watered soil, and, when the seedling shoots grew up to about 3 cm long, they were transplanted into soil and watered with 200 mM NaCl for another 3 weeks; the plants were then photographed. For drought stress treatment in soil, the maize seeds were germinated in well-irrigated soil and the seedlings continued growing without watering until they were 3 weeks old; the plants were then photographed.

For the arabidopsis root growth assays, sterilized seeds were sown on MS medium, and when the roots grew up to 1 cm long after 3 d, they were transferred to MS medium supplemented with different concentrations of NaCl and mannitol. After growing for 2 weeks in stress conditions, the root growth was measured. For calculation of the greening rates, seeds were germinated and continued growing on MS medium supplemented with either NaCl or mannitol. After 2 weeks, the numbers of greening seedlings were calculated. For drought stress treatment in soil, 1-week-old seedlings growing on MS medium were transplanted into soil and grown for a further 2 weeks with normal irrigation, and then were photographed. After that, the plants were left without watering for another 3 weeks, followed by re-irrigation. After recovery for 3 d, the plants were again photographed. For NaCl treatment, 1-week-old seedlings growing on MS medium were transplanted into soil and grown for a further 2 weeks with normal irrigation, following which the plants were irrigated with NaCl solution instead of water for three more weeks; the plants were then photographed.

Production of recombinant UFGT2 protein and glycosyltransferase assays

The compounds cinnamic acid, ferulic acid, caffeic acid, sinapic acid, coniferyl aldehyde, coniferyl alcohol, sinapinic alcohol, sinapinic aldehyde, quercetin and kaempferol were bought from Meryer (Shanghai) Chemical Technology Co., Ltd. The procyanidin and cyanidin were obtained from Carbosynth (Shanghai). The plant hormones indole acetic acid (IAA), indole butyric acid (IBA), 6-benzylaminopurine (6-BA), *trans*-zeatin, *cis*-zeatin and ABA were obtained from Sigma-Aldrich (Shanghai, China).

The recombinant UFGT2 protein was expressed in *Escherichia coli* and purified with glutathione–Sepharese™ 4B (GE Healthcare). The glycosyltransferase activity assay was performed following the conditions described by Hou *et al.* (2004). The products were analysed by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system (Japan) using a 5 µm C18 column (150 × 4.6 mm; Agilent Zorbax). A linear gradient with increasing acetonitrile (solvent A) against double-distilled H₂O (solvent B) at a flow rate of 1 mL min⁻¹ 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. The results are presented at 270 nm.

For liquid chromatography–mass spectrometry (LC-MS) analysis (Shimadzu), the methods and mobile phases were similar to 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 software (version 2.0.6).

Determination of flavonol content in maize and arabidopsis

To determine the flavonol content in maize upon exposure to stresses, the imbibed maize seeds were grown in soil in a growth chamber under normal watering conditions until the three-leaf stage. The plants were then treated in Hoagland solution with 300 mM NaCl or 20 % PEG for 2 d. For arabidopsis treatment, the seeds were germinated and kept growing in MS medium until 14 d old, and then exposed to 200 mM NaCl or 16 % PEG for 12 h.

To analyse flavonol content, flavonols were hydrolysed to aglycone forms according to the method previously described (Burbulis *et al.*, 1996). Briefly, flavonols from maize and arabidopsis tissues were extracted in 80 % ethanol at 4 °C for 24 h, followed by adding 1 mol L⁻¹ HCl into the samples which were then kept at 70 °C for 40 min. Then the centrifuged extracts were subjected to HPLC analysis.

Flavonol glycosides from maize and arabidopsis tissues were extracted in 80 % ethanol at 4 °C for 24 h, and then the centrifuged extracts were subjected to HPLC analysis.

Diaminobenzidine staining and determination of H₂O₂

Diaminobenzidine (DAB) staining was carried out as described by Du *et al.* (2008). Briefly, maize leaves were incubated in DAB solution for 16 h, and then immersed in 95 % ethanol, followed by boiling in a water bath to remove the chlorophyll, and then photographs were taken. The content of H₂O₂ in the plants was determined by the Catalase Assay Kit (S0051, Beyotime, Shanghai, China) according to the user's manual.

FIG. 1. Identification of Mu943 and Mu689 mutants. (A) Genomic gene structure of *GRMZM2G117878* and the insertion sites of Mu943 and Mu689 mutants. (B) RT-PCR analysis of the mRNA levels of *GRMZM2G117878* in Mu689 and Mu943 mutants.

Quantitative real-time-PCR

For quantitative real-time PCR (qRT-PCR), total RNAs were extracted with Trizol reagent (Takara). Reverse transcription reactions were performed with the PrimeScript RT reagent kit with gDNA Eraser (Takara). Real-time PCRs were performed with a Bio-Rad real-time thermal cycling

system using SYBR-Green. All reactions were done at least three times. The arabidopsis gene expression levels were normalized with the reference gene *EF1- α* . The maize gene expression levels were normalized with *Ubiquitin*. Primer information for the qRT-PCR assay is included in [Supplementary Data Table S1](#).

FIG. 2. Sensitivity of Mu689 and Mu943 lines to salt and drought stresses in soil. (A) Germination rates of the WT, Mu689 and Mu943 lines. (B) Germination and post-germination growth of the WT, Mu689 and Mu943 upon exposure to 25 mM NaCl and 7.5 % PEG conditions. (C) After 7 d growth under 25 mM NaCl and 7.5 % PEG conditions, the shoot and root lengths were calculated. (D) Seedling growth of the WT, Mu689 and Mu943 upon exposure to 200 mM NaCl and drought conditions. (E) Water loss of leaves detached from WT, Mu689 and Mu943 plants upon exposure to drought stress. At least three biological replicates were performed for these experiments. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to control conditions (Student's *t*-test, *P < 0.05, **P < 0.01).

RESULTS

Characterization of the maize Mu689 and Mu943 mutants in stress response

In order to explore the roles of the maize family 1 glycosyltransferases, we obtained several UGT mutants from the UniformMu population in a uniform W22 inbred background (McCarty *et al.*, 2005). After self-pollination and obtaining the homozygous lines, we subjected the mutants to normal growing conditions as well as environmental stress conditions to observe whether they have morphological changes or responses to adverse conditions. We observed that two mutants, Mu689 and Mu943, exhibited obvious sensitivity to abiotic stresses. The two mutants were found to carry Mu insertion sites in the exon region of the same gene, *GRMZM2G117878* (chr7:24211056–24212727), which was identified as a UDP-glycosyltransferase in our previous study (Li *et al.*, 2014). However, its enzyme activity has not been identified yet. *GRMZM2G117878* is disrupted in the first exon in Mu943, and in the second exon in Mu689 (Fig. 1A). RT-PCR detection indicates that *GRMZM2G117878* is knocked out in both alleles (Fig. 1B). When the seeds of Mu689 and Mu943 were germinated and left to grow on NaCl and PEG, they showed lower germination rates, and shorter shoots and roots compared with the wild type (WT; Fig. 2A–C). Two-week-old maize plants growing in soil were exposed to salt and drought stresses, and the mutants also exhibited weaker growth and were more wilted than the WT (Fig. 2D). Consistently, the two mutants also showed faster water loss in the leaves than the WT (Fig. 2E). During the whole life cycle of Mu689 and Mu943 homozygous lines, they do not show any morphological changes in comparison with

WT W22 separated from the same corn cob, indicating that normal development is not affected by the mutated gene.

In addition, the expression patterns of *GRMZM2G117878* were also determined in different tissues and under abiotic stresses. It was found to be expressed in all tissues, but with relatively higher expression in seedlings and pericarp, and lower expression in silk and tassel (Fig. 3A). It could also be induced by salt, drought and oxidative stresses (Fig. 3B).

GRMZM2G117878 encodes a UDP-glucose:flavonol glycosyltransferase

To know why loss of function of *GRMZM2G117878* results in the plant sensitivity to salt and drought stresses, it is essential to explore its substrate(s) and the related biochemical pathway. Thus, we purified the recombinant GST-tagged *GRMZM2G117878* protein, and screened a series of compounds that we had obtained, including plant hormones such as IAA, IBA, 6-BA, *trans*-zeatin, *cis*-zeatin, ABA and metabolites in the phenylpropanoid pathway such as cinnamic acid, ferulic acid, caffeic acid, quercetin, kaempferol and cyanidin, as well as several compounds in the lignin biosynthesis pathway such as coniferyl aldehyde and coniferyl alcohol (Supplementary Data Table S2). After HPLC analysis and LC-MS confirmation, we found that this UGT had high activity in transferring UDP-glucose onto two flavonols: quercetin and kaempferol (Fig. 4A, B), while it had no or very weak activities towards other compounds (Supplementary Data Table S2). To investigate the biological function of this UGT, we analysed the levels of quercetin and kaempferol glycosides from WT, Mu689 and Mu943 plants. Consistent with the *in vitro* activity, the levels of quercetin and kaempferol glycosides in the two mutants were decreased compared with those of the WT (Fig. 4C). Besides BRONZE, which catalyses the 3-*O*-glucosylation of flavonols or anthocyanidins (Dooner and Nelson, 1977), a new UGT is identified to be involved in glycosylating flavonols, and thus named *UFGT2*.

*The accumulation of total flavonols is decreased in the maize *ufgt2* mutants*

Glycosylation is a critical step in flavonol biosynthesis that could significantly affect the transport, accumulation and secondary modification of the flavonols (Wink, 1997; Frangne *et al.*, 2002). Since *UFGT2* catalyses glucosylation of quercetin and kaempferol, we thus wanted to look at whether the accumulation of the total flavonols is affected in the two maize mutants. We extracted the total flavonols from the dry seeds and 14-day-old seedlings of the WT, Mu943 and Mu689, and then determined their levels (as indicated by quercetin and kaempferol aglycone). As shown in Fig. 5A, the quercetin and kaempferol levels notably decreased in the two mutants in both seeds and seedlings. Furthermore, we also determined the flavonol accumulation under abiotic stresses. Upon exposure to NaCl and PEG conditions, accumulation of flavonols was increased in both the WT and the maize mutants (Fig. 5B), which is agreement with previous studies (Nakabayashi *et al.*, 2015). However, compared with the WT, the two mutants accumulated fewer flavonols (Fig. 5B). To

FIG. 3. Expression pattern of *UFGT2*. (A) *UFGT2* expression in different maize tissues. (B) *UFGT2* expression in response to abiotic stresses. For treatment, the three-leaf stage maize seedlings were subjected to NaCl, PEG and H₂O₂ for different times. At least three biological replicates were performed for these experiments. Error bars represent the s.d. from three biological replicates.

FIG. 4. UFGT2 catalysing activity towards quercetin and kaempferol analysed by HPLC and LC-MS. (A) Catalysing products of purified UFGT2 enzyme towards quercetin and kaempferol analysed *in vitro* by HPLC. Arrows indicate the quercetin/kaempferol glycoside. (B) LC-MS confirmation of the reaction products from quercetin and kaempferol. (C) HPLC analysis of endogenous quercetin and kaempferol glycoside in the maize wild type, Mu689 and Mu943 plants. 1 and 2 indicate the kaempferol and quercetin glycoside, respectively.

confirm these findings further, we also assessed the flavonol contents with spectrophotometry at 270 nm, and observed the same results (Fig. 5C). Additionally, the expression of the

main genes involved in maize flavonol biosynthesis, *ZmFLS1* and *ZmCHI*, was analysed and found to be decreased in the Mu689 and Mu943 mutants under both control and stressed

conditions (Fig. 5D), which agreed with the decreased flavonol levels.

Flavonols have pronounced activity in scavenging reactive oxygen species (ROS) (Agati *et al.*, 2012; Mouradov and

Spangenberg, 2014). To see whether H_2O_2 accumulation was affected in *ufgt2* mutants, WT, Mu943 and Mu689 plants were subjected to NaCl, PEG and H_2O_2 treatments, and H_2O_2 levels were evaluated by DAB staining, and were also determined

FIG. 5. Altered flavonol levels in WT, Mu689 and Mu943 plants. (A) Accumulation of quercetin and kaempferol in the three-leaf stage maize seedlings and dry seeds. Scopletin served as a reference when extracting flavonols. (B) Accumulation of quercetin and kaempferol in three-leaf stage maize plants upon exposure to NaCl and PEG. (C) Quantitative evaluation of flavonol contents determined by spectrophotometry at a wavelength of 270 nm. (D) Altered expression of flavonol biosynthesis genes in the maize *ufgt2* mutants. For treatment, the three-leaf stage WT, Mu689 and Mu943 plants were subjected to 200 mM NaCl and 20 % PEG treatment for 12 h. Expression in the non-treated WT sample was set to 1.0. For all extractions and evaluations, at least three biological replicates were performed. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to control conditions (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

quantitatively. From Fig. 6, we could see that the two mutants showed deeper and more widespread staining (Fig. 6A) as well as higher H_2O_2 levels (Fig. 6B) than the WT.

Overexpression of UFGT2 in arabidopsis increased flavonol accumulation and enhanced the plant stress tolerance

To investigate further the role of *UFGT2* in stress resistance, transgenic arabidopsis plants expressing the maize *UFGT2* open reading frame (ORF) under the control of the CaMV 35S promoter were generated, and the homozygous lines were obtained. We selected three overexpression lines, 35S::*UFGT2* OE1, OE2 and OE3, for further analysis. Of these, OE2 and OE3 showed very high *UFGT2* expression levels, and OE1 had relatively low *UFGT2* levels (Fig. 7A). When WT, OE1, OE2 and OE3 plants were subjected to NaCl and mannitol treatments, and their growing in response to stress conditions was compared, it was observed that OE2 and OE3 showed notably higher germination rates (Fig. 7B), more established seedlings (Fig. 7C, D) and longer root length than the WT (Supplementary Data Fig. S1). On the other hand, OE1 plants exhibited similar growth to the WT (Fig. 7B–D; Supplementary Data Fig. S1). The plants growing in soil were also exposed to drought and salt stresses, and the overexpression lines OE2 and OE3 were observed to survive better (Fig. 7E).

To determine whether the plant stress tolerance is correlated with flavonol accumulation, we evaluated the total flavonols (as indicated by quercetin and kaempferol) in the WT and 35S::*UFGT2* overexpression lines. HPLC analysis revealed that under non-stressed conditions, more flavonols were accumulated in OE2 and OE3 than in the WT. When the four plants were subjected to NaCl and PEG treatments, the flavonol levels were noticeably induced, and more were accumulated in OE2 and OE3 plants than in the WT (Fig. 8A). Quantitative determination showed that the flavonols accumulated in OE2 and OE3 increased >50 % under abiotic stress conditions (Fig. 8B). Consistently, we also found that several flavonol biosynthesis genes including *CHS*, *CHI* and *FLS1* showed much higher expression in the OE2 and OE3 plants than in WT plants under stress conditions (Fig. 8C).

Moreover, we then assessed the growth of WT and *UFGT2* overexpression plants under oxidative stress. As shown in Fig. 9, the *UFGT2* overexpression lines also perform better than WT plants under H_2O_2 conditions (Fig. 9A, B). To compare the ROS-scavenging activity of the plants, H_2O_2 accumulation was also determined after they were exposed to NaCl, PEG and H_2O_2 treatments. DAB staining and quantitative assay of H_2O_2 indicate that less H_2O_2 is accumulated in OE2 and OE3 plants than in the WT (Fig. 9C, D).

FIG. 6. H_2O_2 production in Mu689 and Mu943 when exposed to abiotic stresses. (A) Leaves of three-leaf stage maize seedlings were subjected to NaCl, PEG and H_2O_2 treatment for 3 d, and then H_2O_2 accumulation was visualized by DAB staining. (B) Quantitative determination of H_2O_2 levels in the plants. At least three biological replicates were performed for these experiments. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to control conditions (Student's *t*-test, *P < 0.05, **P < 0.01).

Altered expression of *UFGT2* affected the expression of stress-related genes

To investigate the potential regulating pathways involving *UFGT2* in enhancing stress tolerance, we next monitored the transcript levels of the stress-related marker genes to see whether they can be affected by altered expression of *UFGT2*. Six maize genes were detected, namely *DREB1.8*, *DREB1.9*, *DREB2.7*, *DREB2.8*, *RAB17* and *LEA4*. It was observed that all these genes were downregulated in the maize mutants upon exposure to salt and/or drought stresses (Fig. 10A). Furthermore, the expression of five arabidopsis stress-related genes was also evaluated in the 35S::*UFGT2* transgenic plants,

i.e. *KIN1*, *RD22*, *COR47*, *RD29A* and *RD29B*, and these genes were noticeably upregulated upon *UFGT2* overexpression in response to abiotic stresses. The results imply that these genes might be regulated by some secondary metabolites via a certain feedback pathway, which might partly contribute to the plant stress tolerance.

Maize *UFGT2* recovered the flavonol deficiency in the arabidopsis *ugt78d2* mutant

In arabidopsis, *UGT78D2* mainly contributes to catalyse the glucosylation of quercetin and keampferol. Loss of its function

FIG. 7. Overexpression of *UFGT2* in arabidopsis enhanced the plant's tolerance to abiotic stresses. (A) Relative expression of *UFGT2* in the 35S::*UFGT2* overexpression (OE) plants. (B) Germination rates of the WT and overexpression lines after growing on MS medium supplemented with NaCl or mannitol for 7 d. (C and D) Seedling greening of the WT and overexpression lines upon exposure to 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 the rates (lower) were calculated. (E) Tolerance of the plants to salt and drought stresses in soil (for treatment details, see the Materials and Methods). At least three biological replicates were performed for these experiments. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to the WT (Student's *t*-test, *P < 0.05, **P < 0.01).

FIG. 8. Overaccumulation of flavonols in the 35S::UFGT2 overexpression plants under salt and drought stresses. (A) HPLC analysis of quercetin and kaempferol accumulation in 14-day-old WT and 35S::UFGT2 seedlings upon exposure to 150 mM NaCl and 16 % PEG for 12 h. (B) Quantitative evaluation of flavonol contents determined by spectrophotometry at a wavelength of 270 nm. (C) Altered expression of flavonol biosynthesis genes in 35S::UFGT2 overexpression plants. Expression in the non-treated WT sample was set to 1.0. For all extractions and evaluations, at least three biological replicates were performed. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to the WT (Student's *t*-test, *P < 0.05, **P < 0.01).

results in an altered flavonol glycoside pattern. From the *in vitro* enzyme assays, it is found that the maize UFGT2 also shows high activity towards quercetin and kaempferol, which resembles the arabidopsis UGT78D2. Therefore, to investigate further the role of UFGT2 *in planta*, we transferred the gene into the *ugt78d2* mutant driven by the CaMV 35S promoter to see whether it can complement arabidopsis *ugt78d2* function. We selected two lines, *com1/ugt78d2* and *com2/ugt78d2*, in which UFGT2 was highly expressed, for further analysis

(Supplementary Data Fig. S2), and then we analysed the flavonol glycoside patterns of the WT, *ugt78d2*, *com1/ugt78d2* and *com2/ugt78d2*. In comparison with the WT, knockout of UGT78D2 led to a changed flavonol profile and some peaks were lost/decreased in the *ugt78d2* mutant. However, after expressing UFGT2 in *ugt78d2*, an altered flavonol glycoside pattern was recovered (Fig. 11A). For instance, peaks 1 and 2, representing kaempferol glycoside and quercetin glycoside, respectively, noticeably declined in the *ugt78d2* mutant

FIG. 9. Accumulation of H_2O_2 in 35S::*UFGT2* overexpression plants when exposed to abiotic stresses. (A) Seedling greening of the WT and overexpression lines upon exposure to H_2O_2 treatments. Seeds were germinated and continued to grow on MS medium supplemented with H_2O_2 . After 2 weeks, seedling greening was observed and the rates (B) were calculated. (C) Four-week-old plants growing in soil were subjected to 200 mM NaCl, 16 % PEG and 200 μM H_2O_2 treatment for 12 h, respectively, and then H_2O_2 accumulation was visualized by DAB staining. (D) Quantitative evaluation of H_2O_2 levels in WT and OE plants under stress conditions. For seedling growth under H_2O_2 treatments, DAB staining, H_2O_2 extraction and detection, at least three biological replicates were performed. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to the WT (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

and were recovered in the *com1* and *com2/ugt78d2* plants (Fig. 11B). These findings indicate that *UFGT2* does similar work to *UGT78D2* in arabidopsis, and provide solid evidence for elucidating the *in planta* role of *UFGT2* in flavonol glucosylation.

DISCUSSION

Flavonoids continue to hold the interest of scientists because of their structural diversity, and biological and ecological significance. To date, >10 000 flavonoids have been discovered in the plant kingdom owing to various modifications during flavonoid biosynthesis (Agati *et al.*, 2012), suggesting that a large number of UGTs would be required for modifying various flavonoid molecules. Although some flavonoid-related UGTs have been identified in arabidopsis, more of them remain to be explored to understand flavonoid modification as well as their biological roles, especially in some other plant species.

In this research, a new maize flavonol glycosyltransferase, *UFGT2*, is characterized, which catalyses the glucosylation of keampferol and quercetin. We revealed that the function *UFGT2* resembled that of the arabidopsis *UGT78D2* both *in vitro* and

in planta, and both *ugt2* and *ugt78d2* mutants exhibited altered flavonol glycoside patterns (Fig. 11). However, the total flavonol content in arabidopsis was not affected in the *ugt78d2* single mutant but was substantially decreased in the *ugt78d1 ugt78d2* double mutant. (Yin *et al.*, 2012). Interestingly, the *ugt2* single mutant noticeably affected flavonol accumulation (Figs 5C and 8B), demonstrating that *UFGT2* shares rare or no redundancy with other UGT members, and possibly plays a specific and primary role in the flavonol biosynthesis pathway in maize. Additionally, the *ugt78d2* mutant presents a visible dwarf and branching phenotype, because knockout of *UGT78D2* results in accumulation of kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside, which inhibits auxin transport (Yin *et al.*, 2014). In contrast, the *ugt2* mutants do not show any developmental phenotype. These findings imply that some signalling pathways are fine-tuned by modification of secondary metabolites and vary in different plant species.

We observed that alteration of *UFGT2* expression substantially influenced the transcript levels of the flavonol biosynthesis genes in maize, such as *CHS*, *CHI* and *FLS* (Figs 5D and 8C), which affected the accumulation of total flavonols. Similar effects were also observed in *ugt78d1 ugt78d2* double mutant,

FIG. 10. Expression of stress-related genes is affected by altered *UFGT2* expression under abiotic stresses in maize mutants (A) and the Arabidopsis 35S::*UFGT2* overexpression plants (B). For maize treatment, three-leaf stage WT, Mu689 and Mu943 were subjected to 200 mM NaCl and 20 % PEG treatment for 12 h. For Arabidopsis treatment, 14-day-old WT and overexpression plants were subjected to 150 mM NaCl and 16 % PEG for 12 h, respectively. Expression in the non-treated WT sample was set to 1.0. For plant treatments and qRT-PCR detection, at least three biological replicates were performed. Error bars represent the s.d. from three biological replicates. Asterisks indicate a significant difference relative to the WT (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

CHS, *F3'H*, *FLS1* and *DFR* were significantly lowered in comparison with the WT counterpart (Yin *et al.*, 2012). Moreover, Castellarin *et al.* (2007) suggested that >80 % of the increase

in anthocyanin accumulation may be correlated with mRNA accumulation of major anthocyanin biosynthetic genes, such as *UFGT*, *CHS* and *F3H*. According to the literature, there appear

FIG. 11. UFGT2 complemented the flavonol deficiency of the arabidopsis *ugt78d2* mutant. (A) Flavonol profiles in the WT, *ugt78d2* mutant and the two 35S::*UFGT2* complementation plants. (B) LC-MS confirmation of kaempferol glycoside (peak 1) and quercetin glycoside (peak 2).

to be tight links between the biosynthesis, transport and cellular deposition of the flavonoids (Zhao and Dixon, 2010). It is suggested that vacuolar accumulation of flavonoids is a prerequisite for their biosynthesis (Zhao and Dixon, 2010). The glycosylated forms of flavonoids are found to be the chemical requirement for vacuolar uptake, and most conjugated flavonoids, such as anthocyanin and flavonol glycosides, predominantly reside in the vacuole (Marinova *et al.*, 2007; Peel *et al.*, 2009). Accordingly, Peel *et al.* (2009) found that ectopic expression of *LAP1* in *Medicago*, a MYB transcription factor gene involved in modulating anthocyanin biosynthesis, greatly upregulated the expression of an anthocyanin glycosyltransferase gene, *UGT78G1*, as well as several potential flavonoid transporter proteins. From this point of view, the consistency in expression of core flavonol biosynthesis genes with that

of *UFGT2* might suggest that glucosylation of flavonol by UFGT2 promoted flavonol transport and distribution, which in turn enhanced the metabolic overflow and stimulated flavonol biosynthesis.

Most flavonoids outperform well-described antioxidants, such as ascorbate (vitamin C) and α -tocopherol (vitamin E), owing to their strong capacity to donate electrons or hydrogen atoms (Rice-Evans *et al.*, 1997; Hernandez *et al.*, 2009). In plants, flavonoids mainly exist as glycosides (Winkel-Shirley, 2001). The most reactive hydroxyl groups (7-OH in flavones or 3-OH in flavonols) in flavonoids are generally glycosylated, which protects them from auto-oxidation (Agati and Tattini, 2010), and also facilitates their transport and storage (Wink, 1997; Frangne *et al.*, 2002). It has been hypothesized that changes in cellular redox homeostasis activate the biosynthesis

of flavonoids, particularly flavonol metabolism (Taylor *et al.*, 2005). A few studies also suggest that the activity of MYB transcription factors controlling flavonol biosynthesis is regulated by cellular redox conditions (Heine *et al.*, 2004; Dubos *et al.*, 2010). It is reported that some of the flavonol glycosides are efficient antioxidants during biotic or abiotic stresses. Hectors *et al.* (2014) highlighted the roles of the kaempferol and quercetin glycosides during UV acclimation in Arabidopsis, and found that the concentration of these compounds increased as a result of UV stress. It is suggested that accumulation of flavonol glycosides constitutes a reserve of flavonols which could be easily mobilized at any given time and especially under stress conditions. Thus, controlling the glycosylation of flavonoids was potentially important for the plant acclimation to abiotic stresses. Recently, we found that two flavonoid UGTs, UGT79B2 and UGT79B3, could improve the plant tolerance to abiotic stresses via modulating the accumulation of anthocyanins and eliminating ROS (Li *et al.*, 2017). In our study, we also observed notable induction of *UFGT2* by abiotic stresses, especially by oxidative stress (Fig. 3), and *UFGT2* overexpression decreased the H₂O₂ production in plants (Figs 6 and 9). Taken together, these findings suggest a close link between the cellular redox potential and the control of flavonol accumulation via flavonol glycosylation.

Maize is one of the most widely grown crops in the world, used for food, feed and biofuel. Owing to its value in agriculture and industry, it is promising and indeed urgently required to generate improved maize cultivars. With the development and application of comprehensive metabolomic analysis, more and more studies have focused on deciphering the function and contribution of the small compounds derived from primary or secondary metabolites in plants. Recently, Wen *et al.* (2018) conducted an integrated study on the metabolic networks by a genome-wide association study (GWAS) and uncovered key genes involved in primary metabolism in maize. For example, one of the identified genes, *ZmADT*, is linked to the phenylalanine biosynthesis pathway and provides building modules to secondary metabolites. This research potentially provides important information for breeding nutritionally improved maize via manipulating key genes involved in metabolic pathways, such as *ZmADT*. With the aggravation of environmental deterioration and the demand of people for healthy food, crops with both environmental adaptation properties and rich in nutrient compounds are worth exploring. Flavonols are the most abundant flavonoids in foods and could exert health-promoting effects (Kumar and Pandey, 2013). It will be beneficial to people if we are able to cultivate flavonol-rich crops. In this study, we found that the maize *UFGT2* could modulate flavonol accumulation as well as improve the plant tolerance to abiotic stresses, leading to better understanding of the flavonoid biosynthesis at both the metabolic and gene level in maize. Also, *UFGT2* might be considered as a candidate gene for improving the grain quality and crop traits in stress tolerance in the future.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: root length of the WT and the overexpression lines was measured after growing

for 14 d on MS medium supplemented with NaCl or mannitol. Figure S2: *UFGT2* expression levels in the two 35S::*UFGT2* complementation lines in the *ugt78d2* background evaluated by RT-PCR. Table S1: primers used in this study. Table S2: relative activity of *UFGT2* with different substrates.

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