



Research article

Constitutive expression of *GmF6'H1* from soybean improves salt tolerance in transgenic Arabidopsis

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ABSTRACT

Coumarin plays a pivotal role in plant response to biotic stress, as well as in the mediation of nutrient acquisition. However, its functions in response to abiotic stresses are largely unknown. In this work, a homologous gene, *GmF6'H1*, of *AtF6'H1*, which encodes the enzyme catalyzing the final rate-limiting step in the biosynthesis pathway of coumarin, was isolated from soybean. *GmF6'H1* protein shares very high amino acid identity with *AtF6'H1*, and expression of *GmF6'H1* in *atf6'h1* can successfully restore the decreased coumarin production in the T-DNA insertion mutant. Further study revealed that the expression of *GmF6'H1* in soybean was remarkably induced by salt stress. Constitutive expression of *GmF6'H1* in Arabidopsis, driven by 35S promoter, significantly enhanced the resistance to salt of transgenic Arabidopsis. All these results suggest that *GmF6'H1* can be used as a potential candidate gene for the engineering of plants with improved resistance to both biotic and abiotic stresses.

1. Introduction

With the global wide increase of saline-alkali lands, salt stress has become one of the major environmental factors that limit the normal growth and production of plants (Chinnusamy and Zhu, 2003). To date, a large number of studies showed that the response and resistance of plant to salt stress involve sophisticated physiological and molecular mechanism, on which many genes play a critical role by regulating the osmotic pressure of cytoplasm, the stability of cell membrane, the induction of ROS, and so on (Leshem et al., 2007; Pastori and Foyer, 2002; Zhu, 2002).

Coumarin (anhydride of o-coumaric acid) is a kind of white crystalline lactone naturally synthesized from phenylalanine in some plants (Kai et al. 2006, 2008; Murray et al., 1982). It is produced from the metabolic pathway of phenylalanine (Brown, 1962; Fritig et al., 1970; Kai et al. 2006, 2008). The biosynthetic pathway of coumarin has been established using an Arabidopsis T-DNA mutant of *At3g13610* gene, which encodes a 2-oxoglutarate-dependent dioxygenase (designated as *F6'H1*) catalyzing the biosynthesis of scopoletin via ortho-

hydroxylation of feruloyl CoA. The coumarin nucleus, benzo-2-pyrone, is derived from cinnamic acid (phenylacrylic skeleton), which is produced from a branch of phenylpropanoid pathway. A p-coumaroylshikimate/quinate 3'-hydroxylase (*C3'H*) plays a critical role in the production of coumarin (Kai et al., 2006; Nair et al., 2002; Schoch et al., 2001). Another 2-oxoglutarate-dependent dioxygenase, designated as *F6'H2*, induced by 2,4-D treatment in shoots, is possibly responsible for the biosynthesis of coumarin in the above ground parts (Kai et al., 2008).

Some coumarin compounds have been identified as phytotoxic metabolites, and function in the defense response of plants against biotic stresses (Baillieul et al., 2003; Garcia et al., 1995; Gnonlonfin et al., 2012; Liu et al., 2017; Prats et al., 2007; Shimizu et al. 2000, 2005; Sun et al., 2014). Using gene silencing technology, scopoletin was found to have strong antifungal activity in tobacco (Sun et al., 2014). The multiple functions of coumarin were also embodied as its effects in mediating iron acquisition under Fe deficiency condition (Chen et al., 2017; Clemens and Weber, 2016; Fourcroy et al., 2014; Rodríguez-Celma et al., 2013; Schmid et al., 2014). As a kind of plant growth

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regulator, coumarin regulated plant growth and development (Lupini et al., 2014). In Arabidopsis, it was recently reported that coumarin (scopoletin), as did auxin, stimulated the development of cell structure (Graña et al., 2017), and low temperature dramatically induced the accumulation of coumarin scopolin (Döll et al., 2018). In rice, coumarin treatment significantly increased the resistance to cold stress of seedlings. Further studies indicated that coumarin treatments helped to remain the content of chlorophyll, protect the degradation of protein, and promote the accumulation of free proline under cold stress condition (Döll et al., 2018).

Although the functions of coumarin in plant growth and response to biotic stress have been investigated, very little is known about its effects on abiotic stress in plants. In this work, we report that *GmF6'H1* from soybean (*Glycine max* L. Merr.) has a function in plant response to salt stress. Constitutive expression of *GmF6'H1* in Arabidopsis significantly increased the salt tolerance of transgenic plants.

2. Materials and methods

2.1. Plant materials and growth conditions

GmF6'H1 was cloned from soybean variety *Jidou12*. Arabidopsis *thaliana* Columbia-0 (Col-0) ecotype and *atf6'h1* were used for transformation. The T-DNA insertion mutant *atf6'h1* was obtained from the Arabidopsis Biological Resource Center. Basically, all plants were grown in the greenhouse under cool white fluorescent light with a 16 h light/8 h dark photoperiod at 22–25 °C as described previously (Tang et al., 2010).

2.2. Cloning and expression analyses of *GmF6'H1*

GmF6'H1 (GenBank: KJ463390.1) was isolated from soybean with gene specific primers (*GmF6'H1*-F: 5'-AAATGTCTTCCACTCCCACCAA TCT-3' and *GmF6'H1*-R: 5'-TCATATCATGGCAAATTCAT AG-3'). Amino acid sequence alignment was performed using MEGA 5.1 program. For salt stress treatments, ten-day-old soybean seedlings were subjected to the treatment with different concentrations of NaCl for 24 h, or with 200 mM NaCl for different time periods under hydroponic condition. Plant tissues were collected at the indicated time points for total RNA extraction.

Total RNA was isolated from different tissues of six-week-old soybean plants. After treated with DNase I to remove contaminated genomic DNA, first-strand cDNA was reversely transcribed using PrimeScript™ Reverse Transcriptase (Takara, Cat.No. D2680S). qRT-PCR was performed with SYBR-Green using RealMasterMix (SYBR Green) (TIANGEN, China, FP202). The soybean ubiquitin gene (*GmUbiq*, D28123) was used as the internal control (Xia et al., 1994). Gene-specific primers *GmF6'H1*-qF: 5'-ATGTCTTCCACTCCCACC-3' and *GmF6'H1*-qR: 5'-AACTAGAGGCCAACCTGA C-3' were used for *GmF6'H1*, and *GmUbiq*-F: 5'-TCTGACACCATGACAATGTG-3' and *GmUbiq*-R: 5'-CTTCTGGATGTTGTAGTCAGC-3' were used for *GmUbiq*, respectively.

2.3. Arabidopsis transformation and transgene confirmation

To generate plant transformation construct, the CDS of *GmF6'H1* was cloned into a modified pCAMBIA-2301 vector under the control of CaMv 35S promoter (Jin et al., 2017). The resultant construct was transformed into *Agrobacterium tumefaciens* GV3101 for Arabidopsis (WT and *atf6'h1*) transformation as described previously (Clough and Bent, 2010). Five-day-old WT and *atf6'h1* seedlings grown on Murashige and Skoog (MS) medium (Sigma-Aldrich) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar were transplanted into soil and grown in greenhouse for 4 weeks before transformation was performed. After screening on MS medium with 50 µg/L kanamycin, putative transgenic plants were transferred to soil for propagation. About 10–15

T_2 independent transgenic lines were confirmed by PCR using the genomic DNA as template and RT-PCR with gene specific primers (*GmF6'H1*-qF and *GmF6'H1*-qR for *GmF6'H1*; and *AtACT2*-qF: 5'-ATT ACCCGATGGG CAAGTCA-3' and *AtACT2*-qR: 5'-CACAAAACGAGGGCT GGAACA-3' for *AtActin2*, respectively) (Bustin et al., 2009; Wongsurawat et al., 2010). The Arabidopsis *AtActin2* (AY096381) was used as the internal control (Yan et al., 2017).

2.4. Salt stress assays of transgenic plants

WT, *atf6'h1* and homozygous T_3 transgenic plants were used for all the analyses. For germination assays, seeds were sown side by side to minimize the deviation. At least 100 seeds of each line were used for each experimental treatment, and three biological replicates were performed for statistical analyses. For each comparison, seeds were sown on MS medium with 2% sucrose and 0.8% agar, supplemented with or without 150 mM NaCl for 7 or 10 days. Germination rate was defined as that the cotyledons have obviously expanded and turned green. Different batches of seeds were used in the germination assays. For seed germination rate analyses with coumarin treatments, wild type Arabidopsis seeds were soaked in different concentrations of coumarin (0, 2, 5, 10 ng/mL) for 5 h after sterilization, and transferred onto filter paper soaked with 150 mM NaCl for 7 days. To determine the tolerance of transgenic plants at flower periods, plants at the bolting stage were irrigated with 200 mM NaCl for 14 days. Photographs were taken and the numbers of siliques were counted. For leaf disk assays, fully expanded leaves of three-week-old WT and transgenic plants grown in greenhouse were selected and incubated in 200 mM NaCl under continuous white light for 24 h. Disks floated in distilled water were served as control. Chlorophyll content in the leaves was determined as described by Lichtenthaler (1987).

2.5. Leaf water loss assays

Three-week-old seedlings of individual wild type and transgenic plants were used for water loss analyses. The fresh weights were determined at designated time intervals. The percentage of initial fresh weight at each time point was used to determine the amount of water loss. These measures were taken three times.

2.6. Coumarin content analyses

Roots of three-week-old wild type, *atf6'h1* and transgenic Arabidopsis plants were grounded into fine power in liquid nitrogen, soaked in methanol for 22 h, and centrifuged for 10 min at 15 000 g. The supernatant of each sample was subjected to HPLC analysis for the content analyses of scopoletin and scopolin as described previously (Kai et al., 2006). Methanol containing 4-methylumbelliferone (4MU) was used as an internal standard.

3. Results

3.1. Isolation of the putative *GmF6'H1* gene from soybean

In Arabidopsis, the ortho-hydroxylation of cinnamic acid is a key step in the phenylpropanoid pathway for coumarin synthesis (Brown, 1962; Sun et al., 2015). A feruloyl-CoA 6'-hydroxylase, designated as F6'H1, was firstly testified to catalyze the formation of scopoletin from feruloyl-CoA (Kai et al., 2006). To obtain their homologous gene from soybean, a putative F6'H1, designated as *GmF6'H1* (KJ463390.1), was identified. *GmF6'H1* shares 62% homology with *AtF6'H1* (Fig. 1a). For *GmF6'H1*, two genomic fragments were identified in chromosomes 18 and 8, suggesting that *GmF6'H1* may exist as isoform or a two-copy gene. Phylogenetic tree analysis showed that *GmF6'H1* is more closely related to *MtF6'H1* (XM_013604042.2), compared with other F6'H1 members already reported (Fig. 1b).

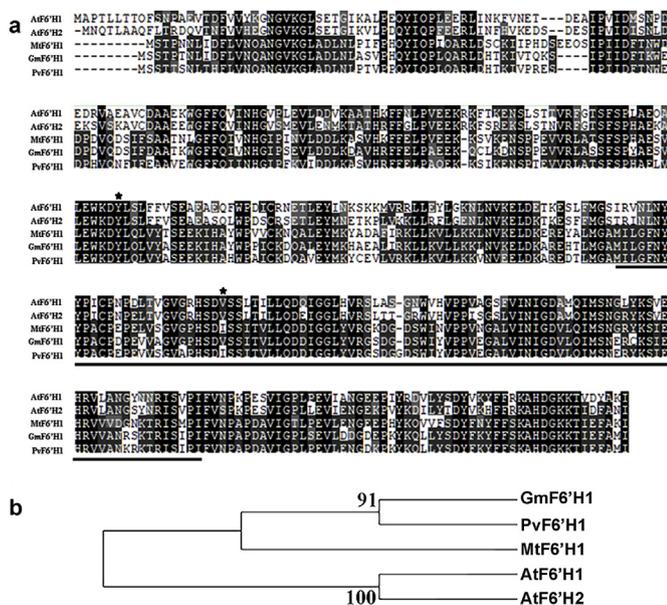


Fig. 1. Amino acid sequence alignment and phylogenetic tree of F6'H1 from different plants. (a) Comparison of F6'H1 proteins from *Arabidopsis thaliana* AtF6'H1 (BT011745.1) and AtF6'H2 (BT012156.1), *Medicago truncatula* MtF6'H1 (XM_013604042.2), *Glycine max* GmF6'H1 (KJ463390.1), and *Phaseolus vulgaris* PvF6'H1 (XM_007146169.1). Residues are highlighted in black and grey according to the level of conservation. Black stars represent two conserved amino acid residues, Tyr151 and Val238. 2OG-Fe(II) motif is underlined in black. (b) Phylogenetic tree of F6'H1 protein in different plants. Phylogram was conducted with MEGA 5.1. The branch lengths were proportional to sequence divergence.

3.2. *GmF6'H1* is induced by salt stress in soybean

As a first step to understand the possible role of *GmF6'H1* in plant response to abiotic stress, we performed qRT-PCR (quantitative real-time reverse transcription-PCR) and investigated its expression pattern in different tissues of six-week-old soybean plants grown under normal condition (Fig. 2a). *GmF6'H1* showed ubiquitous expression in roots, stems, leaves and siliques, with the most abundant expression in roots (Fig. 2b). We also examined the transcript abundance of *GmF6'H1* in ten-day-old soybean seedlings with different salt treatments. qRT-PCR analyses demonstrated that *GmF6'H1* was strongly induced by salt (Fig. 2c and d). When treated with different concentrations of NaCl for 24 h, the highest transcript level was observed with 200 mM NaCl treatment (Fig. 2c). When treated with 200 mM NaCl for different time periods, the highest transcript level was observed after 72 h (Fig. 2d).

3.3. Coumarin promotes seed germination under salt stress condition

To further understand the possible function of coumarin, we investigated the effects of different concentrations of coumarin on seed germination of *Arabidopsis* under both normal and salt stress condition. As shown in Fig. 3, although high concentrations of coumarin (5, 10 ng/mL) inhibited the germination of *Arabidopsis* seeds under both normal and salt stress conditions, low concentration of coumarin (2 ng/mL) only slightly inhibited the germination of *Arabidopsis* seeds under normal condition (Fig. 3a, c), and significantly promoted the germination of *Arabidopsis* seeds under salt stress condition (Fig. 3b, d). After 7 days on MS medium supplemented with 150 mM NaCl, 42.1% of seeds treated with 2 ng/mL coumarin germinated, but only 29.8% of the control seeds (treated with 0 ng/mL coumarin) germinated.

3.4. *GmF6'H1* is the functional homologue of AtF6'H1

To dissect the biological function of GmF6'H1 protein, we constructed transgenic *Arabidopsis* lines heterologously expressing *GmF6'H1* in *atf6'h1* mutant for functional complementation test. Although the *Arabidopsis* mutant of AtF6'H1 (*atf6'h1*) showed normal growth phenotype as did the wild type (Fig. S1a), the expression of AtF6'H1 was knocked out and the production of coumarin was knocked down in the mutant (Figs. S1b and c). First, we confirmed the expression of *GmF6'H1* transgene in *atf6'h1* by RT-PCR analysis (Fig. S1b). Expression of *GmF6'H1* did not caused any phenotypic change in the complementary plants (Fig. S1a), but successfully restored the production of scopletin to the same level as that in the WT plants (Fig. S1c). Then, we performed salt tolerance assessment of WT, *atf6'h1* and complementary plants. Under normal growth condition, seeds of all the three kinds of plant materials germinated well (Fig. 4a, c). However, in the presence of 150 mM NaCl, the germination rate of *atf6'h1* was significantly lower than that of the wild type plants (Fig. 4b, d). Expression of *GmF6'H1* restored the germination rate of to the same level of WT plants (Fig. 4b, d). These data indicate that GmF6'H1 protein can substitute for the corresponding *Arabidopsis* AtF6'H1 component, thereby is a functional orthologue of its *Arabidopsis* counterpart.

3.5. Constitutive expression of *GmF6'H1* increases salt tolerance in transgenic *Arabidopsis*

To further evaluate the potential function of *GmF6'H*, we generated transgenic *Arabidopsis* plants constitutively expressing *GmF6'H1*, under the control of the cauliflower mosaic virus 35S promoter (Fig. S2a). At least 25 independent transgenic lines were successfully obtained, and the integration of transgene into the *Arabidopsis* genome was initially confirmed by PCR analyses (Fig. S2b). Further analyses by RT-PCR verified the expression of *GmF6'H1* in all selected transgenic lines (Fig. S2c). We selected three independent transgenic lines (G1, G2, G6) expressing *GmF6'H1* for subsequent phenotypic analyses.

We first examined the germination rate of WT and three independently generated transgenic lines (G1, G2 and G6) on MS medium supplemented with or without 150 mM NaCl. Under normal germination condition, no significant difference was observed in regarding to the germination rates between wild type and all transgenic lines (Fig. 5a, c). However, under high salt stress condition (150 mM NaCl), the germination rates of transgenic lines were significantly higher than that of the wild type plants. After 7 days on MS medium supplemented with 150 mM NaCl, only 23% of wild type, while more than 50% of transgenic seeds germinated (Fig. 5b, d). These results were consistent with our previous observations that exogenous application of coumarin promoted the seed germination under salt stress condition (Fig. 3b, d).

We further compared the salt tolerance of wild type and different transgenic lines grown in potted soil in greenhouse. Plants at the bolting stage were irrigated with 200 mM NaCl. Constitutive expression of *GmF6'H1* did not change the overall development or plant morphology of transgenic *Arabidopsis* as transgenic lines G1, G2 and G6 all grew well under normal growth condition (Fig. 6a). However, after they were treated with 200 mM NaCl for 2 weeks, obvious differences were observed between wild type and transgenic plants. While wild type plants were severely stunted in the growth and became wilted, transgenic lines appeared to be less impaired by salt stress (Fig. 6b). Although salt stress considerably affected the growth of all the plants in general, transgenic plants showed at a more vigorous status, maintained higher chlorophyll content, and produced more siliques than did the wild type plants (Fig. 6c–f).

Since the synthesis of chlorophyll was inhibited under salt stress, leaf sections were more sensitive to salt stress (Lv et al., 2008; Smethurst et al., 2009). Therefore, we further analyzed the salt tolerance of leaf disks from transgenic plants grown in greenhouse. Leaf disks from three-week-old WT and transgenic plants were exposed to

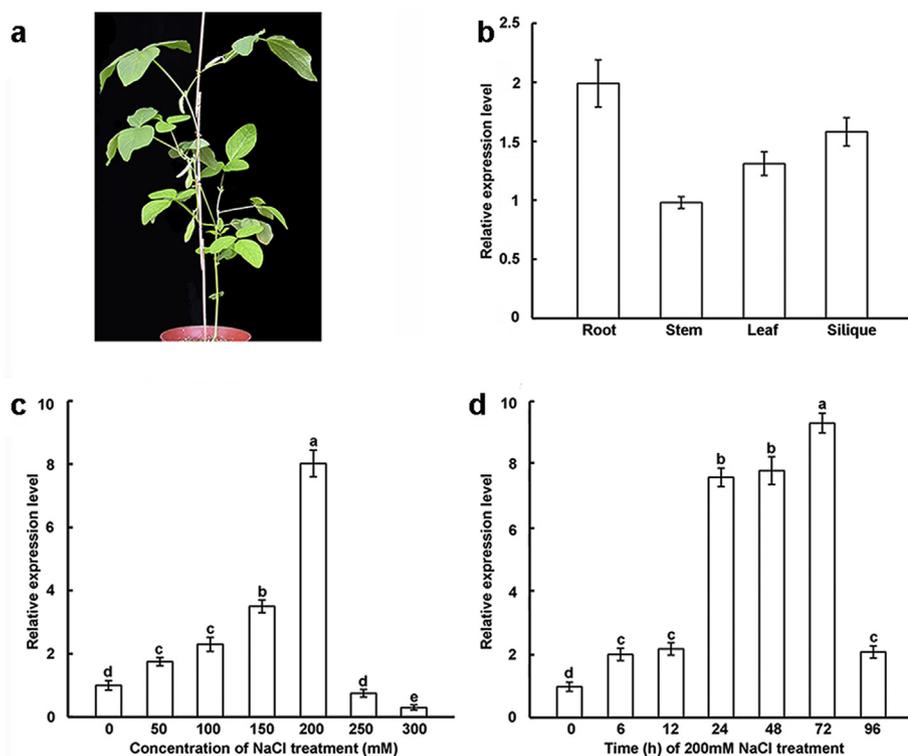


Fig. 2. *GmF6'H1* expression analyses. (a) Phenotype of soybean plant for gene expression pattern analysis. (b) Expression levels of *GmF6'H1* in different plant tissues as indicated. (c) Expression levels of *GmF6'H1* after treated with different concentrations of NaCl for 24 h. (d) Expression levels of *GmF6'H1* treated with 200 mM NaCl for different time periods. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

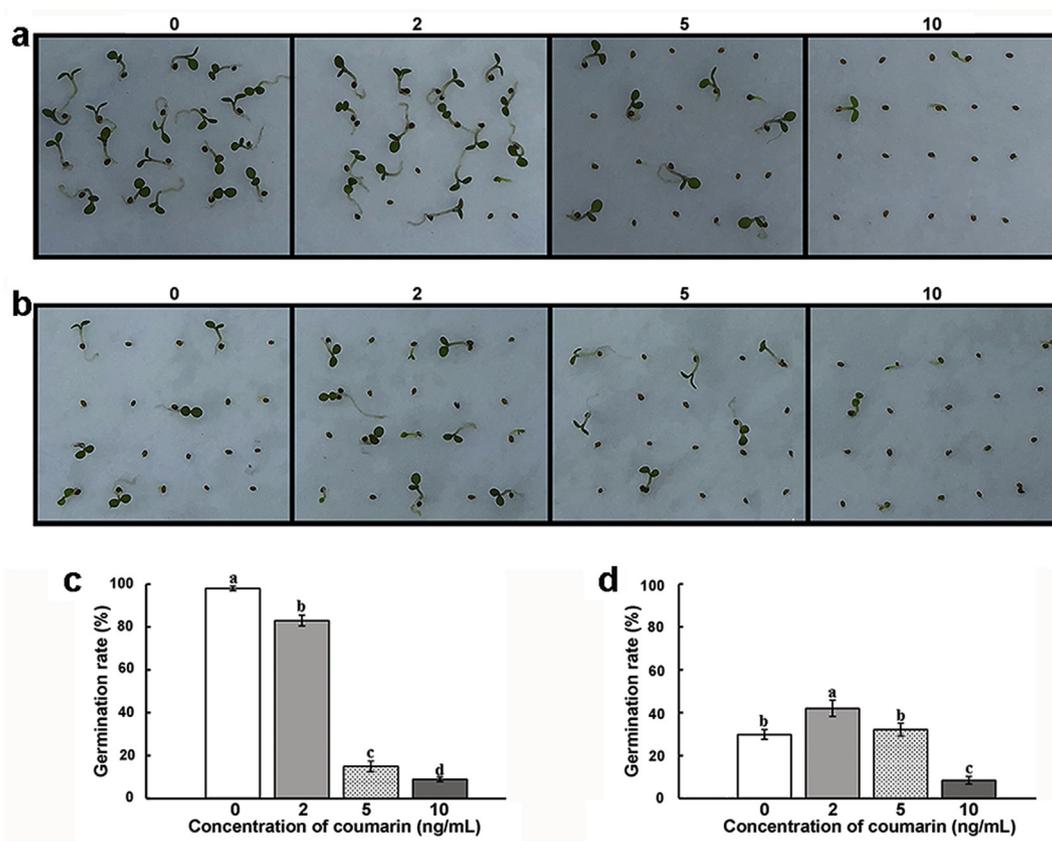


Fig. 3. Exogenous coumarin application promoted seed germination of wild type *Arabidopsis* under salt stress condition. (a) Seed germination after treated with different concentrations of coumarin (0, 2, 5, 10 ng/mL) on filter paper soaked with sterile water for 7 days. (b) Seed germination after treated with different concentrations of coumarin (0, 2, 5, 10 ng/mL) on filter paper soaked with 150 mM NaCl for 7 days. (c, d) Germination rates of seeds in (a) and (b), respectively. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

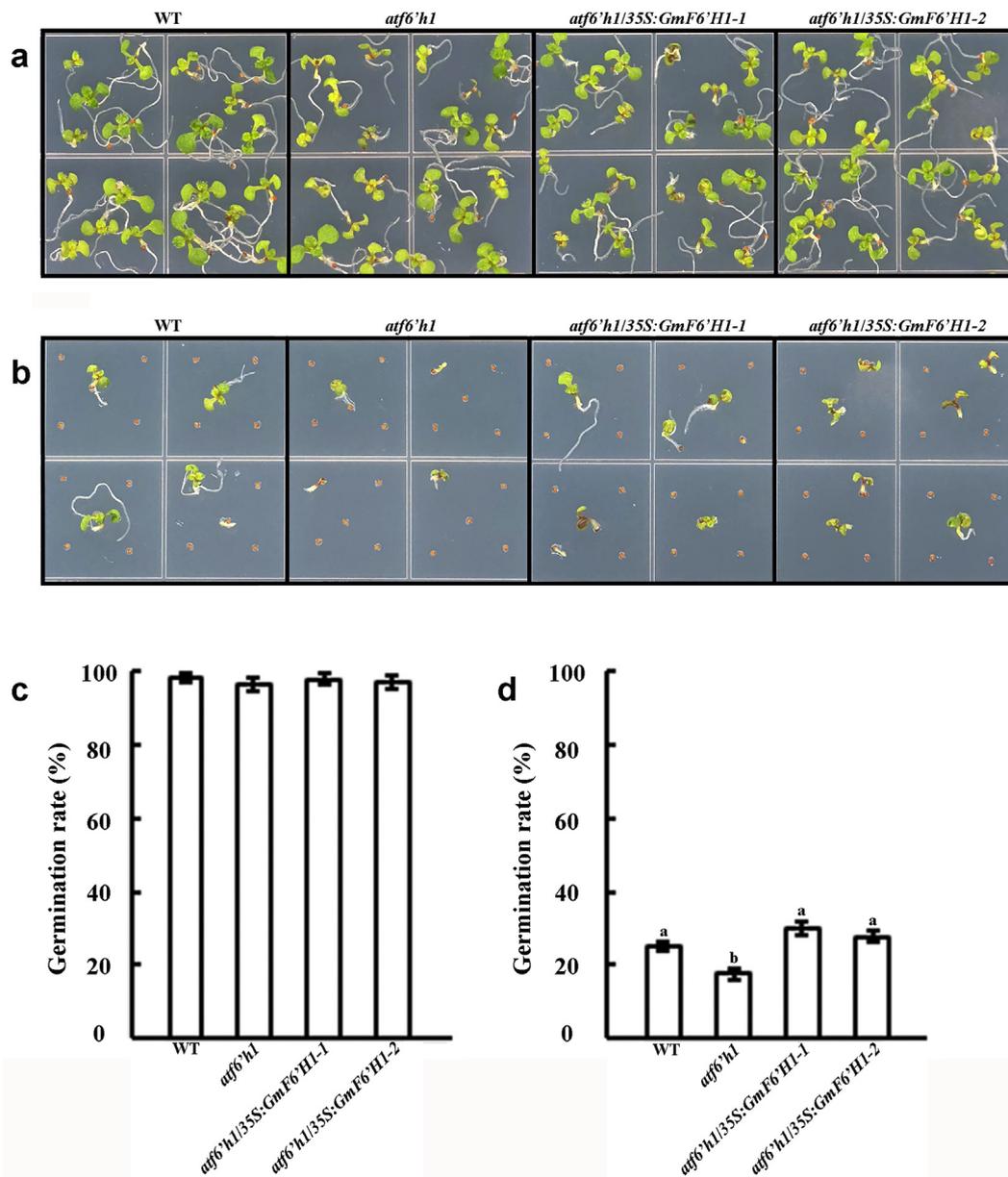


Fig. 4. Expression of *GmF6'H1* in *atf6'h1* restored its germination rate to the same level of wild type plants under salt stress condition. (a) Seeds of wild type, *atf6'h1* and two complementary lines were germinated on MS medium for 10 days. (b) Seeds of wild type, *atf6'h1* and two complementary lines were germinated on MS medium supplemented with 150 mM NaCl for 10 days. (c, d) Germination rates of seeds in (a) and (b), respectively. WT, wild type; *atf6'h1*, know out mutant of *AtF6'H1*; *atf6'h1/35S:GmF6'H1-1* and *atf6'h1/35S:GmF6'H1-2*, two complementary lines. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

distilled water or 200 mM NaCl for 24 h. Under control (no salt) condition, no significant difference was observed between WT and transgenic plant (Fig. 7a). But at high salt (200 mM NaCl) condition, leaf disks from transgenic plants showed more tolerance to the stress (Fig. 7b), and maintained relatively higher content of chlorophyll (Fig. 7c and d).

3.6. *GmF6'H1* promotes coumarin production in transgenic *Arabidopsis*

To confirm the enhanced salt tolerance of transgenic *Arabidopsis* plants were indeed a contribution of *GmF6'H1* gene expression, we examined the endogenous concentration of coumarin in both wild type and transgenic plants. As expected, the content of coumarin in all tested transgenic lines were significantly higher than that in wild type (Fig. 7e).

The enhanced salt tolerance of transgenic plants may also be due to

the reduced water loss by transpiration. Therefore we examined the fresh weight (FW) of three-week-old seedlings of wild type and different transgenic lines over different time intervals as an indicator of transpiration water loss (Ko et al., 2006). The most rapid water loss happened within the first two hours (Fig. 7f). Transgenic plants only lost about 40%, whereas WT plants lost almost 60% of their FW in 2 h (Fig. 7f).

4. Discussion

In *Arabidopsis*, the ortho-hydroxylation of cinnamic acid is a key step in the phenylpropanoid pathway for coumarin synthesis (Brown, 1962; Sun et al., 2015). A feruloyl-CoA 6'-hydroxylase, designated as F6'H1, was firstly testified to catalyze the formation of scopoletin from feruloyl-CoA (Kai et al., 2006). Although sequence alignment analysis revealed that F6'H1 shared higher homology with other plant 2-

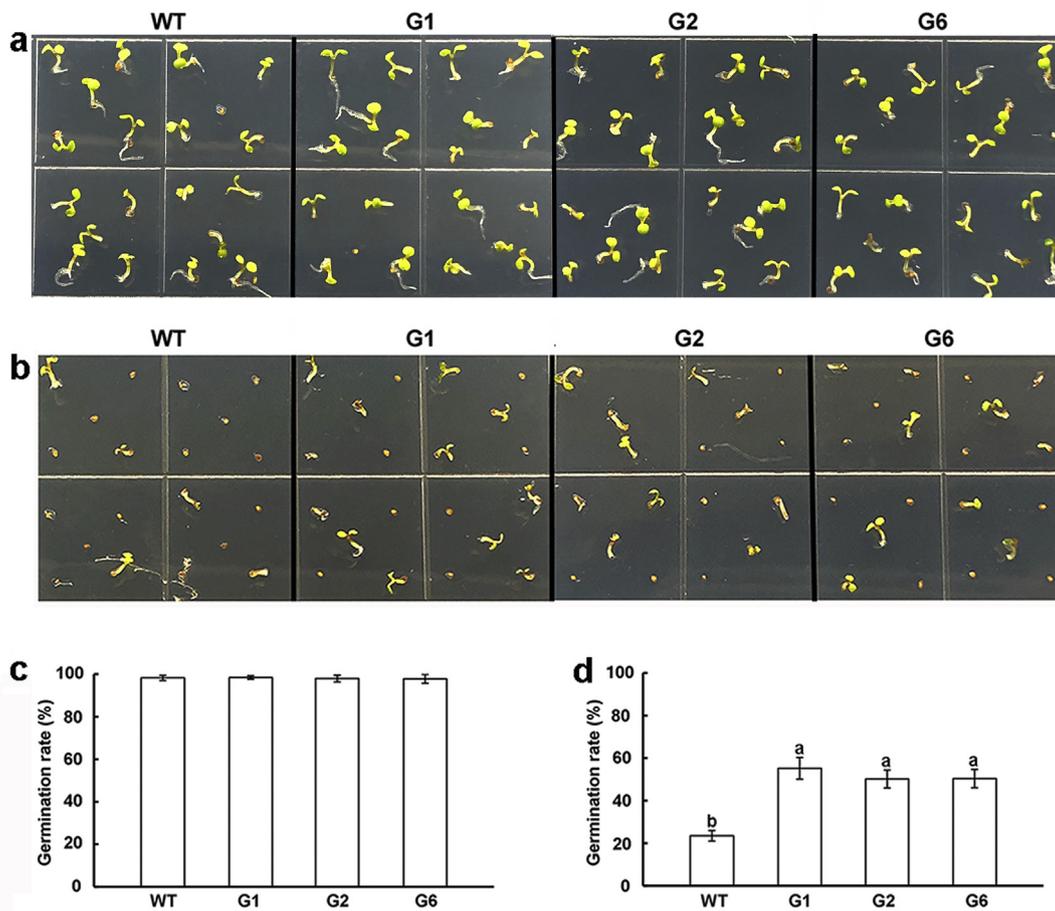


Fig. 5. Expression of *GmF6'H1* increased the germination rates of transgenic plants under salt stress condition. (a) Seeds of wild type and different transgenic lines were germinated on MS medium for 7 days. (b) Seeds of wild type and different transgenic lines were germinated on MS medium supplemented with 150 mM NaCl for 7 days. (c, d) Germination rates of seeds in (a) and (b), respectively. WT, wild type; G1, G2 and G6, different transgenic lines. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

oxoglutarate dependent dioxygenase, F6'H1 family proteins possessed a conserved Fe(II)-binding motif (Sun et al., 2015; Vialart et al., 2012). Crystal structure analysis of AtF6'H1 showed that its substrate specificity was not only dependent on 2-oxoglutarate and Fe(II), Tyr151 and Val238, two conserved amino-acid residues, also played a vital role (Sun et al., 2015). Both AtF6'H1 (At3g13610) and AtF6'H2 (At1g55290) belong to the 2-oxoglutarate-dependent dioxygenase family, and are responsible for the biosynthesis of scopoletin (Kai et al., 2008). The deduced amino acid sequence of *GmF6'H1* also contains the conserved 2OG-Fe(II) motif, which is typical for the oxygenase superfamily, suggesting that it is a specific feruloyl-CoA 6'-hydroxylase (Fig. 1a).

Previously, it was shown that fluorescence compounds identified as coumarins were produced to defend against fungal pathogens (Chong et al., 2002; El Oirdi et al., 2010; Sun et al., 2014). And the key gene for coumarin synthesis, F6'H, was verified (Kai et al. 2006, 2008), and its expression could be induced by pathogenic microorganism attack (Sun et al., 2014). In Arabidopsis, the expression of *AtF6'H2* was induced by 2,4-D in shoots (Kai et al., 2008). In tobacco, the transcription of *Naf6'H1* could be up-regulated by JA signaling activated by fungus, to promote the production of coumarins (Sun et al., 2014). Meanwhile, *AtF6'H1* was also found to be induced by Fe deficiency. The resultant coumarins was helpful to mediate Fe mobilization (Schmid et al., 2014). Other studies also showed cold stress could promote the production of coumarin (Döll et al., 2018). The induced expression of *GmF6'H1* by salt stress indicated that *F6'H* gene could be regulated by both biotic and abiotic stresses (Fig. 2c and d; Zhang and Liu, 2015).

As a growth regulator, coumarin plays a critical role in plant germination and growth (Lupini et al., 2014). We found that high concentration of coumarin (10 ng/mL) inhibited the seed germination of Arabidopsis under both normal and salt stress conditions (Fig. 3a, c). This is consistent with the previous report that coumarin inhibited wheat seed germination only at high concentrations over 200 μ M (Abenavoli et al., 2006). However, low concentration of coumarin (2 ng/mL) significantly promoted the germination of Arabidopsis seeds under salt stress conditions (Fig. 3b, d). The concentration-dependent manner on seed germination illustrates that coumarin may have hormonal characteristics. Since phytohormone can effectively help plants to remove free radicals and reactive oxygen species, our observations indicate that under salt stress condition, low concentration of coumarin can alleviate the detrimental effects of high salt stress on the germination of Arabidopsis seeds, possibly in the same way (Slocum et al., 1984).

Previously, coumarin was found to enable to chelate the Fe(III) under Fe deficient condition (Schmid et al., 2014; Siwinska et al., 2018), and by an indirect way, coumarin was able to modulate the plasma membrane H^+ -ATPase activity to promote nitrate uptake in maize roots (Lupini et al., 2018). The resistance to salt stress was improved in transgenic Arabidopsis plants during both germination and growth (Fig. 5a–d; 6a–f). Consistently, the content of coumarin in all tested transgenic lines were significantly higher than that in wild type (Fig. 7e). Therefore, the increased salt tolerance of transgenic plants may be a result of promoted endogenous production of coumarin, which has helped to increase the uptake and utilization of plant

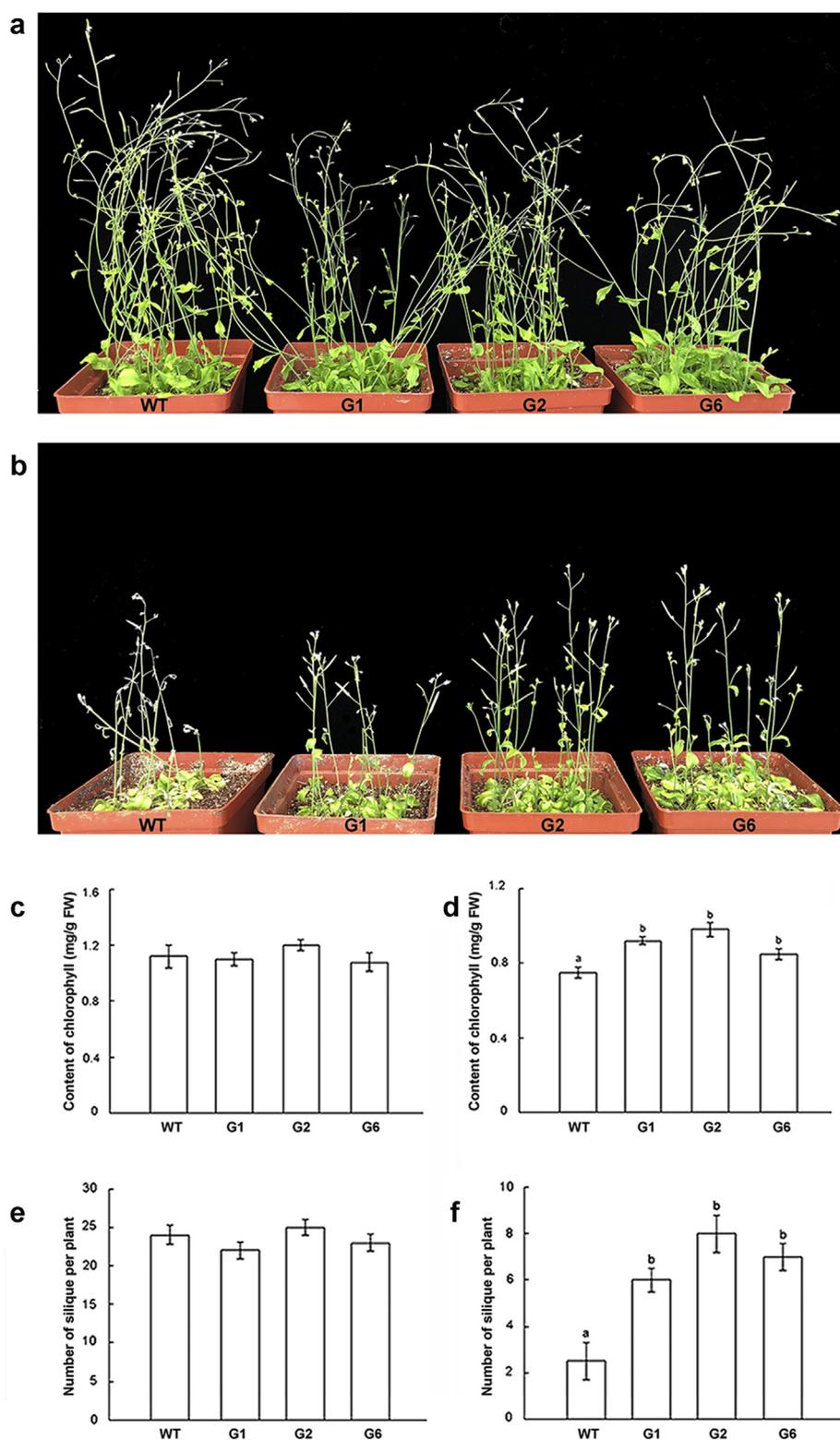


Fig. 6. Salt tolerance assays of transgenic plants. (a) Phenotypes of wild type and different transgenic lines at bolting stage were irrigated with plain water for 14d. (b) Phenotypes of wild type and different transgenic lines at bolting stage were irrigated with 200 mM NaCl for 14d. (c, d) Leaf chlorophyll contents of wild type and transgenic plants in (a) and (b), respectively. (e, f) The numbers of siliques per plant of wild type and transgenic plants in (a) and (b), respectively. WT, wild type; G1, G2 and G6, different transgenic lines. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

nutrients under stress condition. Analysis of water loss also indicated that the enhanced salt tolerance of transgenic plants may also be due to the reduced water loss by transpiration (Fig. 7f). All these results demonstrate that constitutive expression of *GmF6'H1* increased the endogenous level of coumarins and enhanced the salt tolerance in transgenic Arabidopsis, possibly by promoting the uptake of nutrients and regulating the stability of cell membrane.

Studies on the functions of *F6'H1* homologues in other plant species

have revealed that *F6'H1* genes played a prominent role in plant response to biotic stresses (Baillieul et al., 2003; Garcia et al., 1995; Gnonlonfin et al., 2012; Liu et al., 2017; Prats et al., 2007; Shimizu et al. 2000, 2005; Sun et al., 2014) and iron acquisition under Fe deficiency condition (Chen et al., 2017; Clemens and Weber, 2016; Fourcroy et al., 2014; Rodríguez-Celma et al., 2013; Schmid et al., 2014). Our findings demonstrate that *GmF6'H1* is a functional homologue of *AtF6'H1* for the production of coumarin in Arabidopsis, and

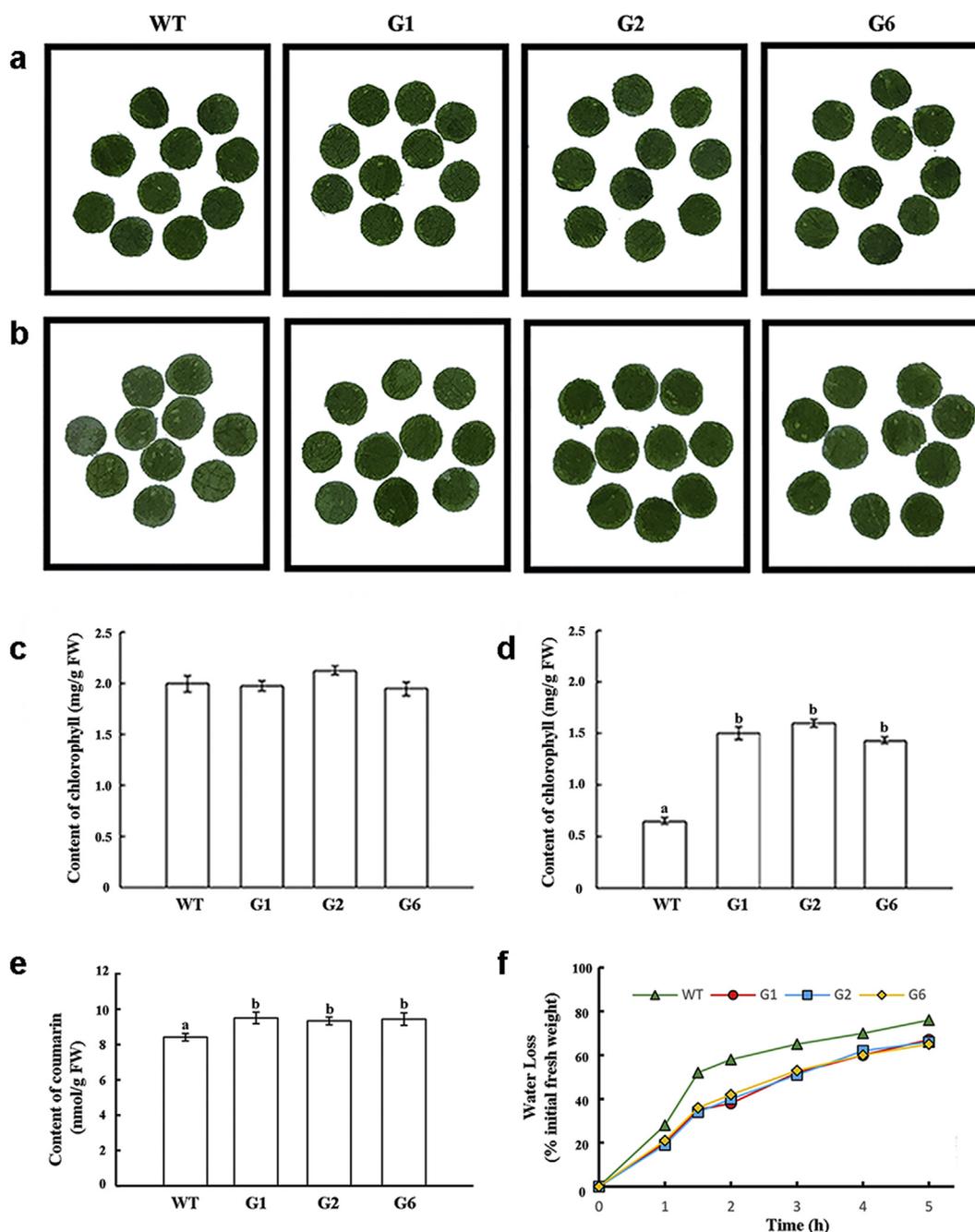


Fig. 7. Salt tolerance, coumarin content and water loss analyses of leaf disks and three-week-old seedlings of wild type (Col-0) and different transgenic lines. (a, b) Phenotype of leaf disks soaked in 0 and 200 mM NaCl for 24 h, respectively. (c, d) Chlorophyll contents of leaf disks in (a) and (b), respectively. (e) Coumarin contents. (f) Water loss tests. WT, wild type; G1, G2 and G6, different transgenic lines. Data are shown as mean \pm SD from three replicates. Significant difference ($P < 0.05$) is based on ANOVA (Tukey's multiple comparison test).

also plays a role in plant response to salt stress. Therefore, *F6'H* family has a broad role in plant response to different stresses. By genetically manipulating its expression, endogenous coumarin production can be increased to promote the salt resistance in plants. As soybean is a model leguminous crop plant, understanding the biological functions of *GmF6'H1* will provide direct evidence for the breeding of crop plants with increased resistance to both biotic and abiotic stresses.

Author contributions

CD and TM carried out gene cloning, Arabidopsis transformation, salt stress analyses, and wrote the manuscript. SS, LG, LH, ZW, YZ, and ML confirmed the expression of transgene. XG examined the content of

coumarin. YS, JL, YY, HZ edited the manuscript. JZ designed the overall study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.06.027>.

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