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Overexpression of a Flavonol Synthase Gene from *Apocynum venetum* Improves the Salinity Stress Tolerance of Transgenic *Arabidopsis thaliana*

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Abstract
This study may enhance our understanding of *AvFLS* function in plants under salt stress and may provide a new tool for the improvement of plant salt tolerance in the field. We isolated and identified *AvFLS* from *Apocynum venetum.* To further characterize the potential role of *AvFLS* in salt tolerance and to explore the relationship between FLS and salt resistance, we generated transgenic *Arabidopsis* lines overexpressing *AvFLS*. Tissue-specific expression analysis and salt-stress experiments identified *AvFLS* as a salt-inducible gene that is highly expressed in leaves of *A. venetum*. Subcellular localization analysis showed that *AvFLS* was located in the cytoplasm, consistent with other plant FLS proteins.The overexpression of *AvFLS* in *Arabidopsis thaliana* significantly improved the salt-stress tolerance of the transgenic plants: under salt stress, transgenic *Arabidopsis* exhibited improved flavonoid accumulation, seed germination rate, plant growth, chlorophyll content, and fresh weight compared to wild-type plants. Comparison of malonaldehyde (MDA), soluble sugar, and proline contents between the transgenic and wild-type plants indicated that the improved salt tolerance associated with *AvFLS* overexpression was due to decreased membrane damage. *AvFLS* overexpression also led to the upregulation of endogenous *Arabidopsis* genes involved in flavonoid biosynthesis. The results of this study demonstrate the potential utility of the *AvFLS* gene for molecular crop breeding, both to increase the contents of valuable flavonoids and to improve crop productivity in saline fields.

Keywords Salt tolerance · FLS · *Apocynum venetum* · Transgenic Arabidopsis · Heterologous expression

1 Introduction

Soil salinity is one of major abiotic stressors of plants, affecting both growth and yield. Plants adapt to changes in habitat by continuously adjusting energy flow and secondary metabolic pathways to maintain growth and development while resisting biotic and abiotic stresses (Goyal et al. 2012; Bita et al., 2019; Thakur et al. 2019; Yadav et al. 2021). Flavonoids, a class of important secondary metabolites, are extensively distributed across the plant kingdom and can be divided into six categories: flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanins (Agati et al. 2012). Flavonoids play important roles in plant development, growth, and stress resistance (Hartmann 2007; Vickers et al. 2009). Flavonols are important component of flavonoids and are critical for the regulation of plant growth and development, as well as the response to biotic and abiotic stresses (Reginato et al. 2014; Flowers and Muscolo 2015; Shah and Smith 2020). For example, flavonols promote the growth of plant roots under drought stress and alleviate the inhibitory effects of abscisic acid on lateral root growth (Nguyen et al. 2013). Similarly, 5-aminoallylic acid induces the accumulation of flavonols in the guard cells of *Arabidopsis thaliana*, and flavonols inhibit ABA-induced stomatal closure by scavenging hydrogen peroxide; this process regulates external gas flow under stress conditions (An et al. 2016). Finally,

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comparisons of the effects of flavonoids among various drought-tolerant tomato varieties demonstrated that, under drought stress, the increase in flavonoid content in drought-tolerant varieties was significantly greater than that in drought-sensitive varieties (Sánchez-Rodríguez et al. 2011).

Flavonoids are synthesized using common key enzymes such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), and dihydroflavonol reductase (DFR). Flavonols and anthocyanins are synthesized via the same pathway before diverging at the point of dihydroflavonol production. At this point, dihydroflavonols are converted to anthocyanins or flavonols by DFR or FLS, respectively (Vu et al. 2015; Park et al. 2019). Flavonol synthase (FLS), a member of the 2-oxoglutarate-dependent dioxygenase (2-ODD) family, is an important rate-limiting enzyme in the plant flavonol metabolic pathway (Muir et al. 2001; Tanaka et al. 2008). FLS not only participates in plant growth and development, but also plays an important role in the plant defense response to biotic and abiotic stresses, such as ultraviolet radiation and pathogen invasion (Xu et al. 2012; Fang et al. 2019; Li et al. 2020; Yu et al. 2020; Dong and Lin 2021). Indeed, FLS activity was first reported in suspension cultures of parsley cells irradiated with ultraviolet (Britsch et al. 1981). Ultraviolet radiation led to a significant increase in plant flavonoid contents, and flavonols, the production of whose is catalyzed by FLS, can protect plants such as corn from ultraviolet damage (Falcone Ferreyra et al. 2010). For example, the *Ginkgo biloba FLS* (*GbFLS*) gene is induced in response to ultraviolet light, sodium chloride (NaCl), exogenous abscisic acid, and salicylic acid (Xu et al. 2012), thereby promoting flavonoid accumulation and improving salt tolerance. Two other 2-ODD genes in the flavonoid anabolic pathway, anthocyanin synthase (*ANS*) and *flavanone 3-hydroxylase* (*F3H*), might be somewhat interchangeable or complementary with *FLS* (Kawai et al. 2014; Guo et al. 2020; Wang et al. 2021a, 2021b, 2021c, 2021d). For example, *Arabidopsis* FLS1 partially catalyzes F3H, while *Arabidopsis* ANS shares some of the functions of FLS (Owens et al. 2008; Stracke, et al. 2009; Martens et al. 2010).

The flavonol content of various plants is strictly regulated by the *FLS* genes (Verhoeyen et al 2002; Ma et al. 2014; Wang et al. 2016; Xu et al. 2020a, b). *FLS* genes have been well-studied in many plants (Koes et al. 1994; Falcone Ferreyra et al. 2012; Fujino et al. 2018; Yonekura-Sakakibara et al. 2019; Liu et al. 2021). The full-length *FLS* gene sequence was first cloned from petunia (Froemel et al. 1985) and has subsequently been identified and cloned from a variety of other plants, including *Petunia hybrida* (Holton et al. 1993), *A. thaliana* (Owens et al. 2008; Preuß et al. 2009; Falcone Ferreyra et al. 2010), *Litchi chinensis* (Liu et al. 2018), *Fagopyrum tataricum* (Li et al. 2012), *Scutellaria baicalensis* (Kim et al. 2014), *Ginkgo biloba* (Xu et al. 2012), *Camellia sinensis* (Jiang et al. 2020), *Acacia confusa* (Toh et al. 2013), *Vaccinium uliginosum* (Zhang et al. 2016a, b), and *Oryza sativa* (Park et al. 2019). *FLS* gene functions and characteristics may differ both among and within species. For example, all five *FLS* genes from *Vitis vinifera* are expressed in the flowers, but only *VvFLS4* and *VvFLS5* are expressed in fruits (Fujita et al., 2003). Similarly, the functional properties of the six *FLS* genes found in *A. thaliana* (*AtFLS1–6*) differ: the AtFLSl protein has very strong catalytic activity, AtFLS3 has very low enzymatic activity, and the remaining AtFLS proteins have no enzymatic activity (Owens et al. 2008). In addition, the transcriptional abundance of *Triticum aestivum FLS* (*TaFLS1*) under salt stress was significantly greater in the salt-tolerant variety SR3 as compared to the salinity-sensitive cultivar Jinan 177 (JN177) (Wang et al. 2014). *TaFLS1* improved the salinity tolerance of *A. thaliana* at the seedling stage but not at the germination stage. (Wang et al. 2016). Finally, the overexpression of *AtFLS1* in *A. thaliana* significantly altered seed coat color and flavonoid accumulation without affecting growth performance or abiotic stress tolerance as compared to the wild type (Nguyen et al. 2016).

Apocynum venetum Linn. (Apocynaceae) is a perennial, halophytic forage plant with high medicinal value and strong stress resistance that grows on saline-alkali lands, sandy wastelands, and floodplains in northern China. *A. venetum* is rich in flavonoids (Chen et al. 2020; Xu et al. 2021; Yang et al. 2021; Abubakar et al. 2022), and salinity stress was shown to significantly increase flavonol content (Xie et al., 2014; Jiang et al. 2020; Xu et al. 2020a, b; Wang et al. 2021a, b, c, d). However, the physiological and biochemical mechanisms underlying the antioxidant protection conferred by flavonoids in *A. venetum* plants adapted to salt stress remain unclear. Previously, we cloned the key enzyme gene in the flavonoid biosynthesis pathway of *A. venetum*, namely *AvFLS* (Guo et al. 2019). To further characterize the potential role of *AvFLS* in salt tolerance and to explore the relationship between FLS and salt resistance, we generated transgenic *Arabidopsis* lines overexpressing *AvFLS*. This study may enhance our understanding of AvFLS function in plants under salt stress and may provide a new tool for the improvement of plant salt tolerance in the field.

2 Materials and methods

2.1 Cloning and analysis of *AvFLS*

2.1.1 Plant materials and salt stress treatments

A. venetum seeds were collected from wild plants in Shaya County, Xinjiang Uygur Autonomous Region, China (40˚92´N, 82˚21´E; 957 m). Seedlings were cultured as

previously described (Guo et al. 2022). To assess the response of the *FLS* gene of *A. venetum* (*AvFLS*) to salt stress, six-week-old plantlets were subjected to one of two treatments: (1) To determine the tissue-specificity of the *AvFLS* gene under control conditions and under salt stress, NaCl (0, 50, or 300 mM) was added to the hydroponic growth solution. After two weeks of salt stress, seedling roots, leaves, and stems were collected, frozen in liquid nitrogen, and stored at − 80 °C. (2) To determine the expression patterns of *AvFLS* under prolonged salt stress, plants were subjeted to 0, 50, 100, 150, 200, or 300 mM NaCl stress treatment for 144 h. Leaves were sampled at 6 and 144 h. At each sampling point, the second pair of unfolded leaves (from the top) were collected, immediately frozen in liquid nitrogen, and stored at − 80°C for RNA extraction.

2.1.2 Gene cloning and bioinformatics analysis

The full-length cDNA of *AvFLS* was downloaded from the GenBank database (accession no. MK391176.1) (Guo et al. 2019). The coding sequence was amplified using the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech) with the gene-specific primers AvFLS-F1, AvFLS-F2, AvFLS-R1, and AvFLS-R2 (Table S1). Sequences from other species homologous to the predicted AvFLS protein sequence were identified and aligned using DNAMAN (Ver. 7.0) (Liu et al. 2018; Guo et al. 2019; Li et al. 2021). Phylogenetic analysis was carried out using the neighbor joining method (bootstrap resampling test with 1000 replicates) in MEGA version 10.0 software (https://megasoftware.net/). *Eustoma russellianum* was used as the outgroup (Wang et al. 2021a, b, c, d; Guo et al. 2022; Wang et al. 2022a, b).

2.1.3 *AvFLS* expression patterns and subcellular localization

To compare *AvFLS* expression patterns among tissues and treatment groups, RNA was first isolated from the collected samples and reverse transcribed to cDNA as previously described (Jeong et al., 2018;Wang et al. 2021a, b, c, d ;Zhang et al., 2021). We performed real-time quantitative PCRs (qRT-PCRs) using the cDNA template and the housekeeping gene Actin as an internal control, as described previously (Zhang et al., 2021). PCRs were performed on an ABI Prism7500 (Applied Biosystems, USA). The gene-specific primers (AvFLS-qRT-F/R) and actin primers (Actin-qRT-F/R) used for PCR are shown in Table S1. Three biological replicates and three technical replicates of each sample were performed. The comparative CT method ($2^{-\Delta\Delta Ct}$) (Wang et al. 2021a, b, c, d;Zhang et al., 2021;Wang et al. 2022a, b) was used to calculate the relative expression of each target gene.

Using the gateway method, the full-length coding sequence of AvFLS was fused to the modified pCAMBIA1302-GFP vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter to generate the 35S::AvFLS-GFP fusion construct. The 35S::AvFLS-GFP and 35S::GFP fusion proteins were introduced into different epidermal cells of *Nicotiana tabacum* using the *Agrobacterium*-mediated transformation method (Zhang et al. 2016a, b). The transformed *N. tabacum* leaves were cultivated for 2–6 days and then examined under a confocal laser scanning microscope (Zeiss Lsm 700, Zeiss, Jena, Germany).

Effects of salt stress on transgenic *Arabidopsis* overexpressing *AvFLS.*

2.1.4 Generation of transgenic Arabidopsis overexpressing *AvFLS*

The *AvFLS* fragment was inserted into the pEarleyGate 100 vector, and the recombinant protein was transformed into wild-type (WT) *A. thaliana* (Columbia-0) using the *Agrobacterium*-mediated floral dip method as previously described (Holton, et al. 1993; Muir,et al. 2001; Li et al. 2021). The specific primers used are listed in Supplementary Table S1. Successfully transformed seeds were selected on MS medium containing 50 mM kanamycin as previously described (Nguyen, et al. 2016; Wang, et al. 2021a, b, c, d). The T3 generation transgenic lines (OE1) with the highest levels of *AvFLS* expression and with 100% resistance to kanamycin were considered homozygous and were thus selected for further analysis.

2.1.5 Salt-stress treatment and effects on biomass

To test the response of *AvFLS*-transgenic *Arabidopsis* to salinity stress, WT and OE1 seeds were seeded in 1/2 MS medium containing 0, 25, 50, 75, or 100 mM NaCl. Once cotyledons appeared, the number of germinated seeds was counted every 24 h for seven days. The seed germination percentage was calculated as the total number of seeds germinated over the seven day period/total number of seed planted × 100% (Ellis et al. 1986).

Four-week-old WT and OE1 seedlings were stressed using 100 mM NaCl. Leaves were collected from the treated plants at 0 h, 6 h, 24 h, 72 h, 120 h, and 168 h after salt treatment for the assessment of biomass, photosynthetic pigments, soluble sugar, proline, malondialdehyde (MDA), and total flavonoids. Each parameter was measured in six replicates. Fresh samples were weighed, oven-dried at 60 ± 65°C for 16 h (Delgado-Pertíñez et al. 2000), and weighed again to obtain seedling dry and fresh weights.

2.1.6 Measurement of physiochemical parameters

Chlorophyll a and chlorophyll b were extracted from leaves (0.1 g) using a mixture of acetone, ethanol and water in a volume ratio of 4.5:4.5:1. A spectrophotometer (Shimadzu, UV-1800, Suzhou, China) was used to measure absorbance at 663 nm and 645 nm. Chlorophyll concentrations were calculated as follows (Gratani 1992):

$$\text{Chlorophylla} = \left(12.21 \times D_{663} - 2.96 \times D_{645}\right) \times V \div (1000 \times W),$$
$$\text{Chlorophyllb} = \left(22.88 \times D_{645} - 4.67 \times D_{663}\right) \times V \div (1000 \times W), \text{and}$$
$$\text{Totalchlorophyll} = \text{chlorophylla} + \text{chlorophyllb},$$

where D_{663} and D_{645} correspond to absorbance at 663 and 645 nm, respectively; v is the extraction volume (ml); and W is the leaf weight (g).

Soluble sugar content was measured using Anthrone colorimetry (Yemm and Willis 1954). We constructed a standard curve reflecting the linear relationship between the mass concentration of glucose (X) and the absorbance value (Y) as follows: $y = 9.095x - 0.0075$ ($R^2 = 0.998$) (Supplementary Fig. 1A). This standard curve was reliable for glucose concentrations of 0–0.1 mg/ml. Proline was extracted and quantified using the method of Bates et al. (1973); malondialdehyde content was measured using a thiobarbituric acid reaction following Heath and Packer (1968); and total flavonoid concentration was determined using the aluminum nitrate method (Park et al. 1997) with rutin as the standard.

2.1.7 Expression patterns of genes associated with stress tolerance

To explore the possible molecular mechanisms underlying the participation of FLS in the salt-stress response, we used qRT-PCR to quantify the transcript levels of five genes encoding enzymes in the flavonoid biosynthesis pathway (*AtCHI*, *AtCHS*, *AtANS*, *AtF3H*, and *AtFLS1*) in transgenic and WT plants under salt stress. Four-week-old WT and OE1 seedlings were exposed to 100 mM NaCl for 0 h, 6 h, 24 h, 72 h, and 120 h. Samples were then collected for RNA extraction and cDNA synthesis. RNA was isolated from the collected samples and reverse transcribed to cDNA as previously described (Wang et al. 2021a, b, c, d; Zhang et al., 2021). qRT-PCRs were performed as described above using gene-specific primers (Supplementary Material Table 1).

2.1.8 Statistical analysis

All data are presented as means ± standard deviation (SD). Significant differences among treatments were identified using one-way ANOVAs in SPSS 20.0, followed by Duncan's multiple range tests. We considered $p < 0.05$ statistically significant.

3 Results

3.1 Analysis of AvFLS in *A. venetum*

3.1.1 Bioinformatic sequence analysis

The full-length cDNA sequence of *AvFLS* had an open reading frame of 1212 bp and was predicted to encode a polypeptide containing 335 amino acids, with a predicted molecular weight of 38.33 kDa and a predicted isoelectric point of 6.11. Multiple sequence alignments showed that the deduced amino acid sequence of AvFLS was highly homologous with known FLS proteins from other plant species (Fig. 1).

A GenBank conserved domain database search and functional analysis revealed that AvFLS possessed putative conserved domains belonging to the 20G-FeII Oxy super family, as well as a highly conserved N-terminal region found in proteins with 2-oxoglutarate/Fe(II)-dependent dioxygenase activity. Importantly, the positions of many of the conserved sequence motifs identified in AvFLS, including the HXD motif for ligating ferrous iron and the RXS motif for binding 2-oxoglutarate (2OG), were similar between the AvFLS sequence and the other FLS sequences (Fig. 1). Phylogenetic analysis recovered AvFLS in a sister relationship with the FLS protein from *Coffea arabica* and within a larger clade comprised of the FLS proteins from *Solanum lycopersicum*, *Nicotiana tabacum, Gentiana triflora*, *Eustoma grandiflorum,* and *Sesamum indicum* (Fig. 2).

3.1.2 Expression patterns and subcellular localization

To explore the possible role played by *AvFLS* in the response of *A. venetum* to salt stress, we quantified its expression patterns under control and salt-stress conditions using qRT-PCR. *AvFLS* was ubiquitously expressed in all tested tissues (Fig. 3). *AvFLS* was significantly upregulated in the leaves as compared to the roots and stems across all treatment groups, and *AvFLS* expression level increased significantly as salinity increased (Fig. 3). These results suggested that AvFLS may be involved in the stress response of *A. venetum*, similar to other known *FLS* genes such as *MdFLS1* in *Malus domestica* (Li et al. 2021) and *BnFLS* in *Brassica napus* (Vu et al. 2015).

As salt stress was prolonged, the expression patterns of *AvFLS* in *A. venetum* leaves changed substantially. After 6 h, relative *AvFLS* gene expression was significantly greater in leaves exposed to 200 and 300 mM NaCl as compared to leaves exposed to 100 mM NaCl or less (Fig. 4). However, after 144 h of salt treatment, the *AvFLS* gene was

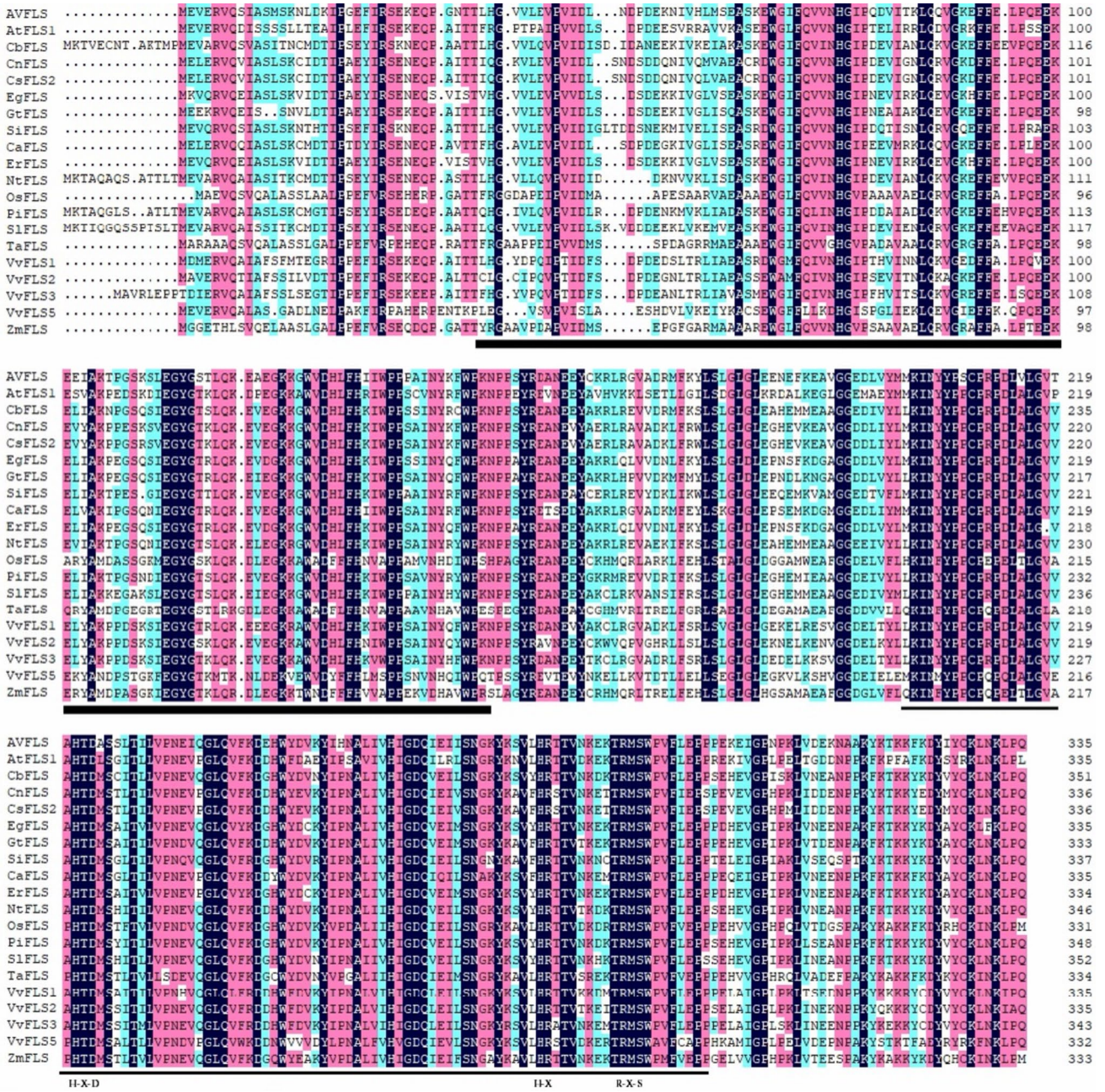

Fig. 1 Relationship between AvFLS and other known FLS proteins from representative plant species. Multiple sequence alignment. Thick black line indicates the highly conserved N-terminal 2-ketoglutarate domain; fine black line indicates the 2OG-Fe (II) oxygenation enzyme domain. The regions marked H–X-D, H–X, and R-X-S correspond to the regions conserved across genes in the 2-ODD superfamily

significantly upregulated in the leaves exposed to 200 and 300 mM NaCl as compared to all other treatment groups (Fig. 4). This suggested that the *AvFLS* gene was induced by salt stress, and that the response fluctuated over time. This may indicate that *AvFLS* plays an important role in the resistance of *A. venetum* to salt stress.

To determine the subcellular location of AvFLS, the recombinant AvFLS-green fluorescent protein (GFP) was transformed into *N. tabacum* mesophyll protoplasts. The pCAMBIA1302GFP vector was used as a control. The control GFP protein was expressed throughout the cell, while the GFP signal of AvFLS was localized in the cytoplasm (Fig. 5). As the AvFLS does not possess a transmembrane-spanning domain (Guo et al. 2019), we concluded that AvFLS was a cytoplasmic protein.

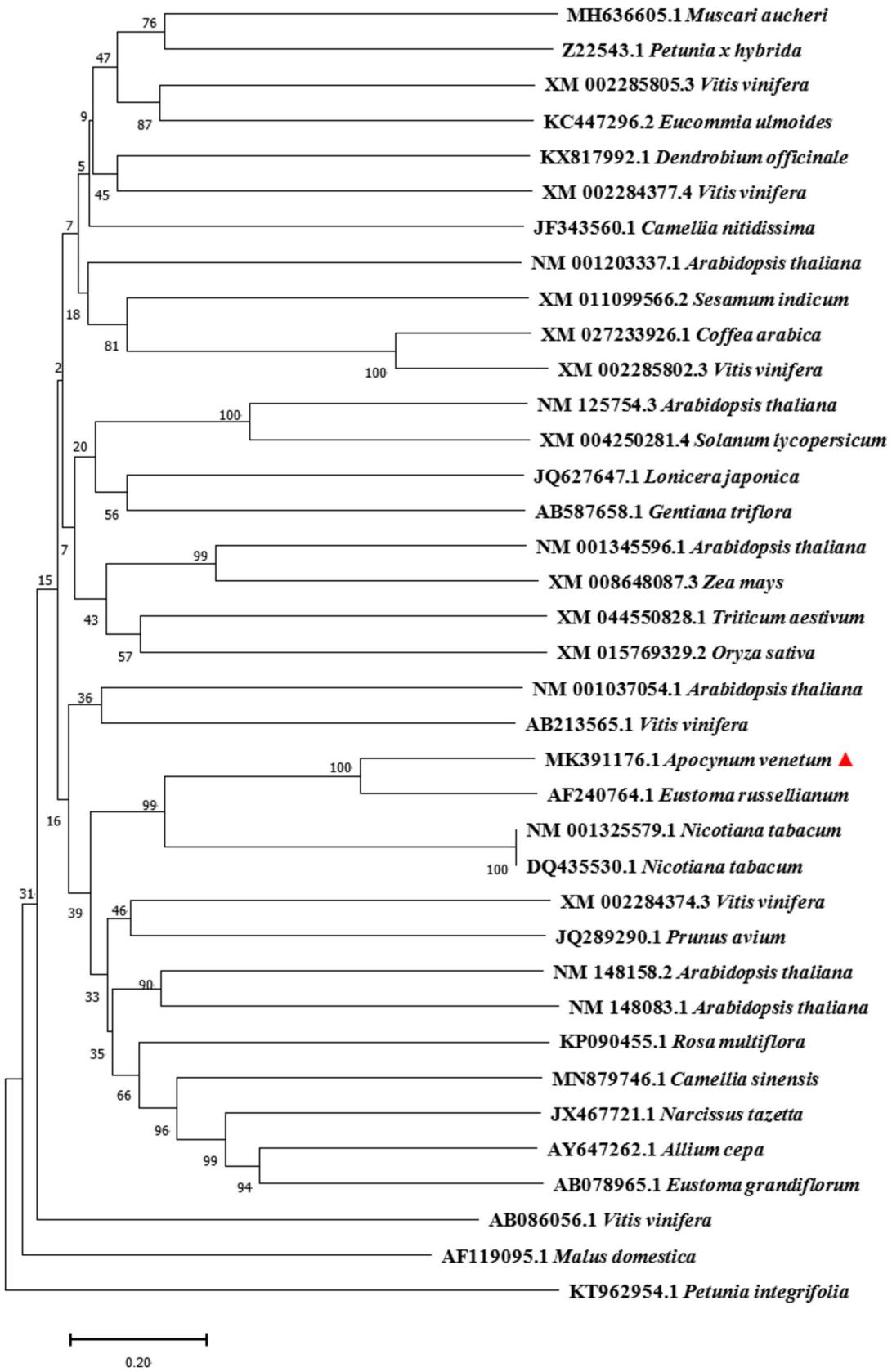

Fig. 2 Phylogenetic tree analysis of *AvFLS* and other 2-ODD superfamily genes

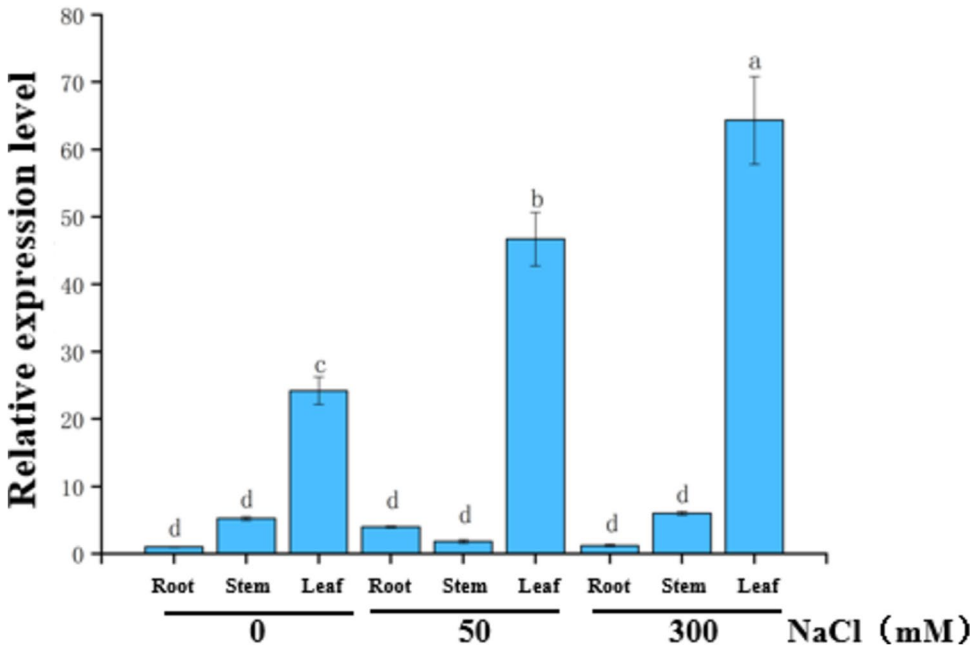

Fig. 3 Gene expression patterns of *AvFLS* in various tissues of *A. venetum* in response to salt stress. The values shown are the means ± SDs of three biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

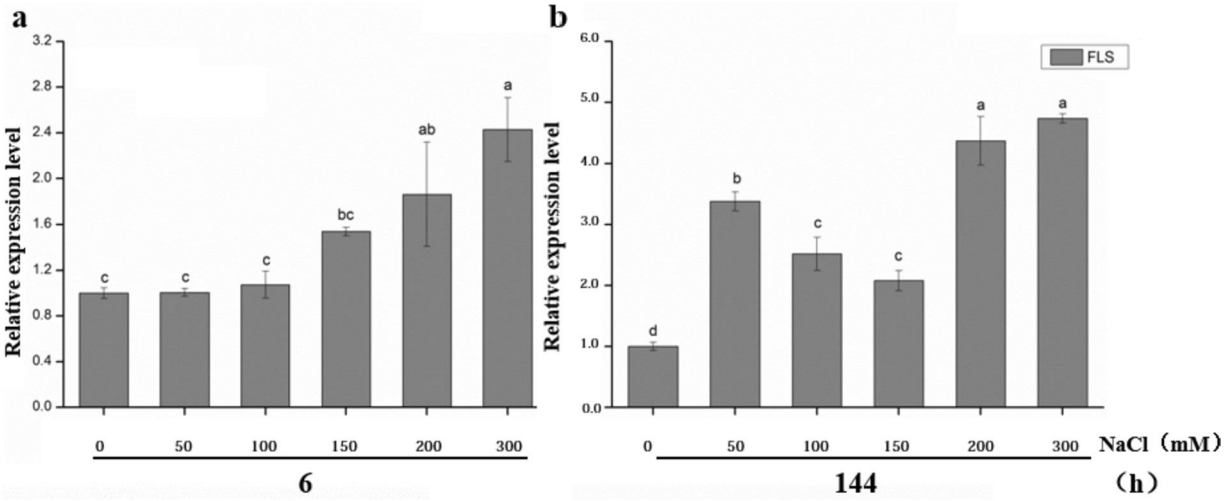

Fig. 4 Relative expression level of the *AvFLS* gene in the leaf tissues of *A. venetum* exposed to various concentrations of NaCl (0–300 mM). (**a**) After 6 h of salt treatment. (**b**) After 144 h of salt treatment. The values shown are the means ± SDs of three biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

3.2 Effects of AvFLS overexpression on the salinity tolerance of *Arabidopsis*

3.2.1 Growth performance was improved in transgenic Arabidopsis under salt stress

Under control conditions (0 mM NaCl), there was no significant difference in seed germination rate between the WT and transgenic plants (Fig. 6a). Exposure to 25–100 mM NaCl for 7 d significantly decreased the germination rate of both the WT and overexpression line OE1, but the germination rate was significantly higher in the transgenic line as compared to the WT across all treatment groups (Fig. 6a).

After growth at control conditions for five weeks, the transgenic *Arabidopsis* were somewhat larger than the WT plants (Fig. 6b). After four-week-old WT and transgenic plants were treated with 100 mM NaCl for one week, the growth of both lines was severely curtailed. However, the growth of the transgenic line was noticeably better than that of the WT line (Fig. 6c).

Under control conditions, the biomass of the transgenic plants was greater than that of the WT plants (both wet and dry

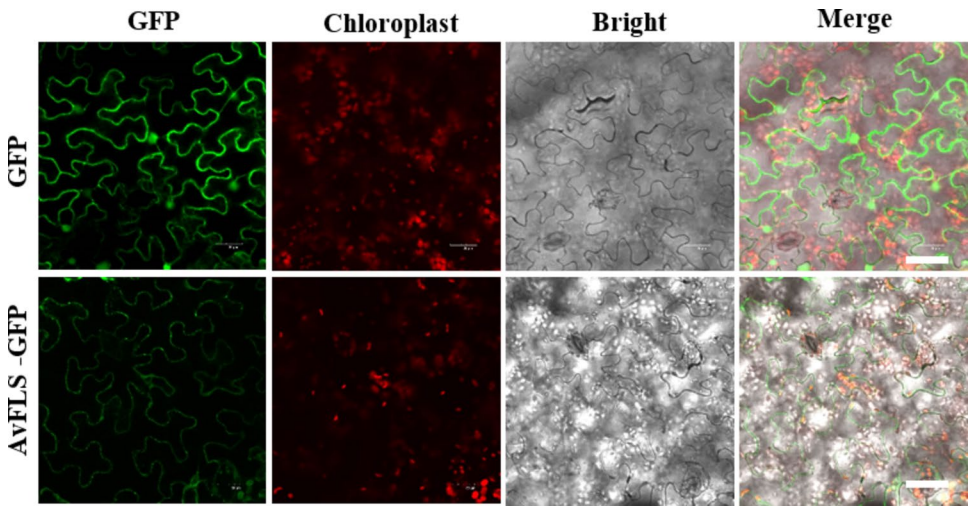

Fig. 5 Subcellular localization of AvFLS. The recombinant 35S::AvFLS-GFP and the pCAMBIA1302-GFP control vector were transiently expressed in *N. tabacum* protoplasts. Scale bars: 50 μm

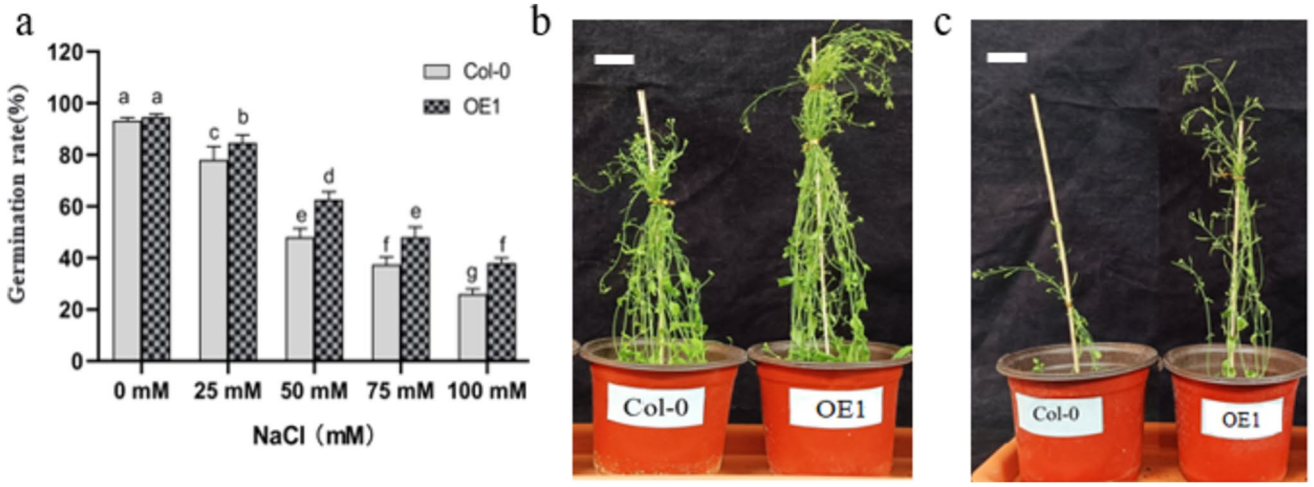

Fig. 6 Growth performance of transgenic *Arabidopsis* overexpressing *AvFLS* as compared to WT *Arabidopsis* under salt stress. **a** *AvFLS* expression in *AvFLS*-over-expressing (OE1) and wild-type (Col-0) *Arabidopsis* and in *Apocynum venetum*. **b** OE1 and Col-0 *Arabidopsis* seed germination in 1/2 MS medium supplemented with NaCl (0, 25, 50, 75 and 100 mM). **c** Five-week-old OE1 and Col-0 *Arabidopsis* seedlings grown under control conditions (without NaCl). **d** Four-week-old OE1 and Col-0 *Arabidopsis* seedlings after treatment with 100 mM NaCl for one week. The values shown are the means ± SDs of six biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$). Scale bars: 5 cm

weights; Fig. 7). Although the biomass of both lines decreased significantly under increased salinity, the biomass of the transgenic line remained significantly higher than that of the WT (Fig. 7): after 7 d of exposure to 100 mM NaCl, the fresh and dry weights of the transgenic plants were 24.95% and 14.08%, respectively, greater than the fresh and dry weights of the WT (Fig. 7).

3.2.2 *AvFLS* overexpression mitigated the negative effects of salinity on the physiochemical parameters of Arabidopsis

Chlorophyll a, chlorophyll b, and total chlorophyll contents generally decreased in both WT and transgenic *Arabidopsis* seedlings as exposure to 100 mM NaCl was prolonged.

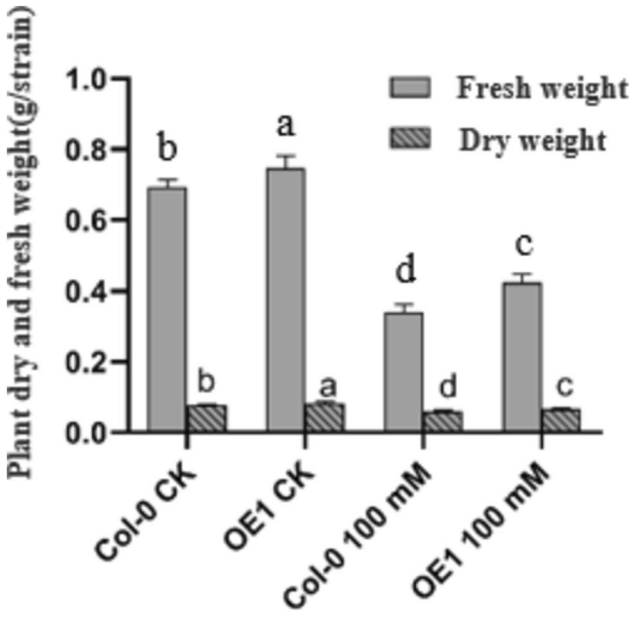

Fig. 7 Dry and fresh weights of transgenic and wild-type *Arabidopsis* under salt stress. The values shown are the means ± SDs of three biological replicates. Error bars represent the standard deviation of six replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

However, chlorophyll a content in the transgenic plants was significantly greater than that in the WT plants at all times points measured ($P < 0.05$): after 0 h, 6 h, 24 h, 72 h, 120 h, and 168 h of exposure to 100 mM NaCl, the chlorophyll a content of the transgenic plants was 39.33%, 7.62%, 28.45%, 59.64%, 32.03%, and 93.08%, respectively, greater than that of the WT plants (Fig. 8A). Chlorophyll b content in the transgenic plants was also significantly greater than that in the WT plants at all times points measured except 6 h ($P < 0.05$): after 0 h, 6 h, 24 h, 72 h, 120 h, and 168 h of exposure to 100 mM NaCl, the chlorophyll b content of the transgenic plants was 19.50%, 6.8%, 27.40%, 77.72%, 109.72%, and 112.94%, respectively, greater than that of the WT plants (Fig. 8B). Similar to the trend in chlorophyll a content, total chlorophyll levels in the transgenic plants were significantly greater than those in the WT plants at all times points measured ($P < 0.05$; Fig. 8C).

Soluble sugar content fluctuated in both transgenic (OE1) and WT *A. thaliana* as the duration of salt stress (100 mM NaCl) was prolonged (Fig. 9a). However, the soluble sugar content of the transgenic line (OE1) was significantly greater than that of the WT at 0 h, 6 h, 24 h, and 120 h (Fig. 9a). At 168 h, the soluble sugar content of the WT was significantly greater than that of the transgenic line, and at 72 h, there was no significant difference in soluble sugar content between the lines (Fig. 9a and Supplementary Material Fig. 10a).

In both transgenic and WT *Arabidopsis* leaves, proline content gradually increased with time under salt stress (100 mM NaCl): after 24 h, 72 h, 120 h, and 168 h of salt stress, proline content in the WT leaves increased 2.29-fold, 6.60-fold, 6.68-fold, and 13.73-fold compared to control conditions (0 h), while proline content in the transgenic leaves increased 6.69-fold, 5.09-fold, 11.44-fold, and 24.30-fold compared to control conditions (Fig. 9b). However, the proline content of the transgenic *A. thaliana* leaves treated with 100 mM NaCl for 72 h was lower than that of the WT, and this difference was maintained until the end of the experiment (Fig. 9b and Supplementary Material Fig. 10b).

Under control conditions (0 h), MDA content in the WT *Arabidopsis* leaves was significantly greater than that in the transgenic leaves and, as salt stress was prolonged, MDA content increased significantly in both WT and transgenic lines (Fig. 9c). However, the MDA content of the transgenic leaves was significantly lower than that of the WT leaves at all time points except 72 h and 120 h (Fig. 9c). The MDA content of the transgenic leaves was 30.7%, 35.3%, 16.3%, and 16.7% lower than that of the WT leaves at 0 h, 6 h, 24 h, and 168 h, respectively. This suggested that that the overexpression of *AvFLS* reduced

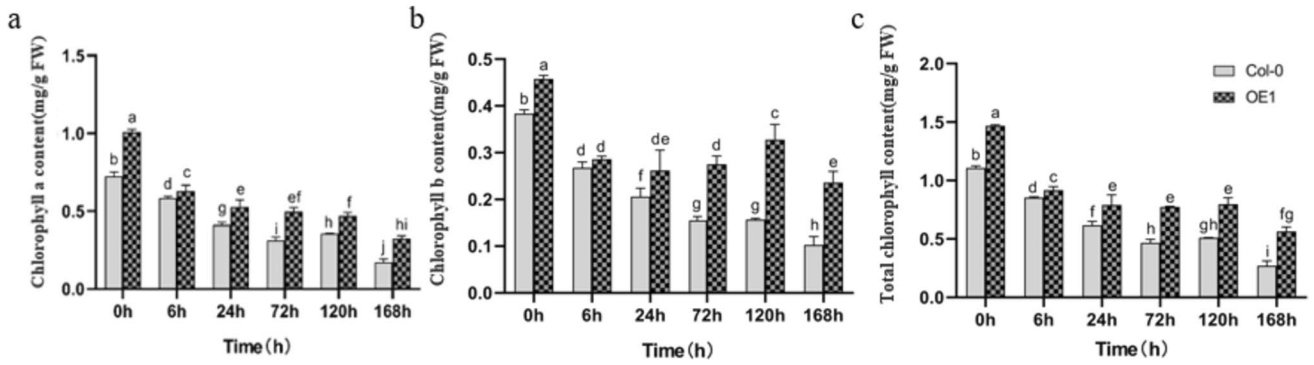

Fig. 8 Chlorophyll content of transgenic and wild-type *Arabidopsis* under salt stress. **a** Chlorophyll a content. **b** Chlorophyll b content. **c** Total chlorophyll content. The values shown are the means ± SDs of six biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

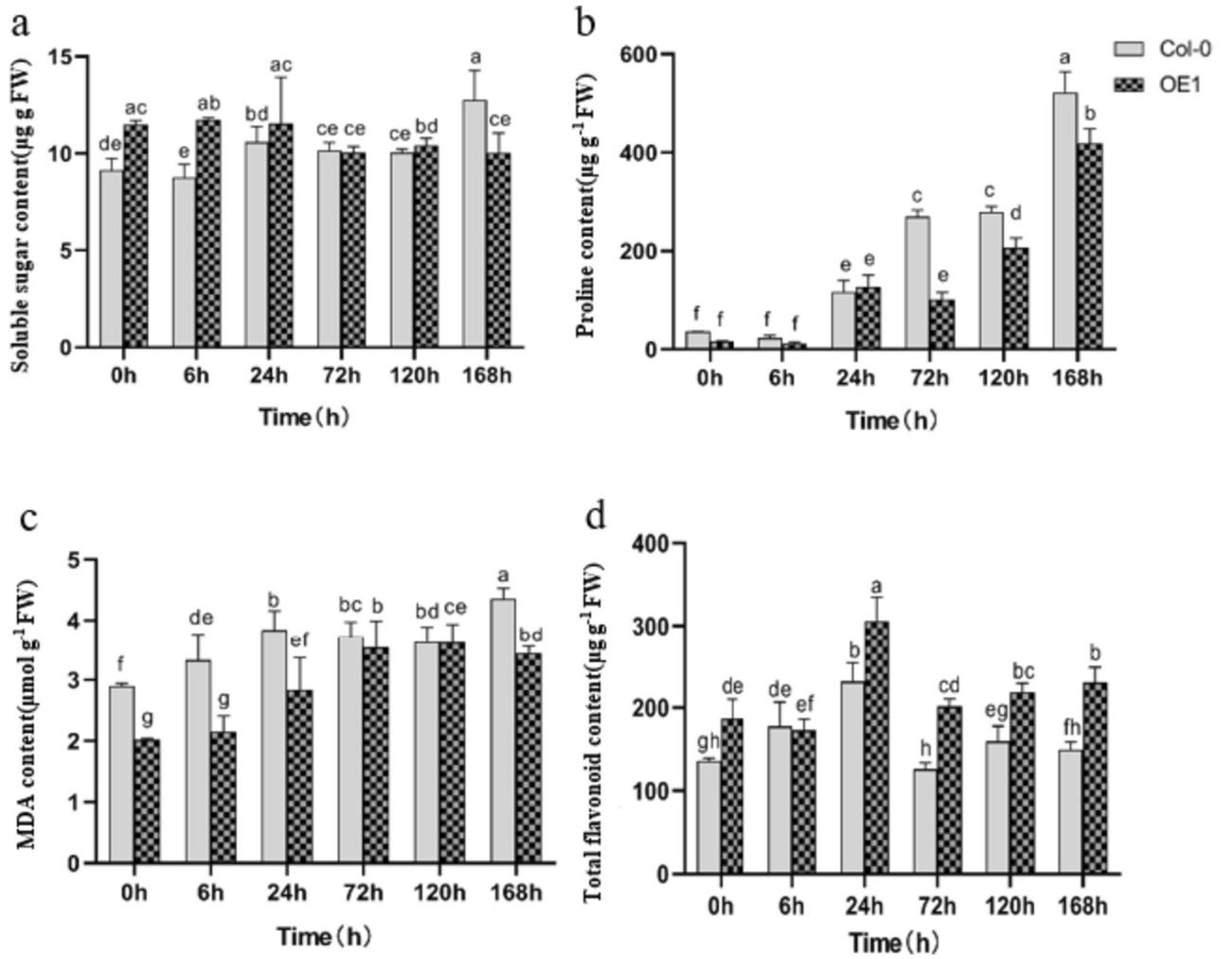

Fig. 9 Physiological indexes in the leaves of transgenic *A. thaliana* overexpressing *AvFLS* (OE1) and wild-type *A. thaliana* (Col-0) under prolonged salt stress (100 mM NaCl). **a** Soluble sugar content. **b** Proline content. **c** MDA content. **d** Total flavonoid content. The values shown are the means ± SDs of six biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

the degree of salt-induced membrane lipid peroxidation in *Arabidopsis*, reducing MDA production and the associated tissue damage.

The standard curve of rutin was $Y = 12.537x + 0.00184$ ($R^2 = 0.9979$), and this standard curve had good linearity for rutin concentrations of 0–0.07 mg/L (Supplementary Material Fig. 10b).

At all time points except 6 h, total flavonoid content was significantly greater in the transgenic leaves than in the WT leaves (Fig. 9d). At 0 h, 24 h, 72 h, 120 h and 168 h, total flavonoid contents in the transgenic leaves were 37.2%, 32.2%, 60.3%, 37.1%, and 54.3%, respectively, greater than total flavonoid contents in the WT leaves (Fig. 9d). This suggested that the overexpression of *AvFLS* might significantly increase total flavonoid content in *A. thaliana*.

3.2.3 *AvFLS* overexpression upregulated the expression of flavonoid pathway genes in Arabidopsis

The heterologous expression of the *FLS* gene affects the synthesis of total flavonoids and anthocyanins in plants, and may also lead to the downregulation or non-expression of endogenous genes (Ohno et al. 2011; Nguyen, et al. 2016; Jeyaraj et al. 2017; Jiang et al. 2020). Under control conditions, the relative expression of the *AtFLS* gene did not differ significantly between the transgenic (OE1) and WT plants (Fig. 10a). However, under salt stress, *AtFLS* was significantly upregulated in the WT plants as compared to the transgenic plants at all time points except 72 h (Fig. 10a). The expression of *AvFLS* peaked in the OE1 plants after 24 h of salt treatment; at the same time point, the expression of *AtFLS* was relatively low (Fig. 10b).

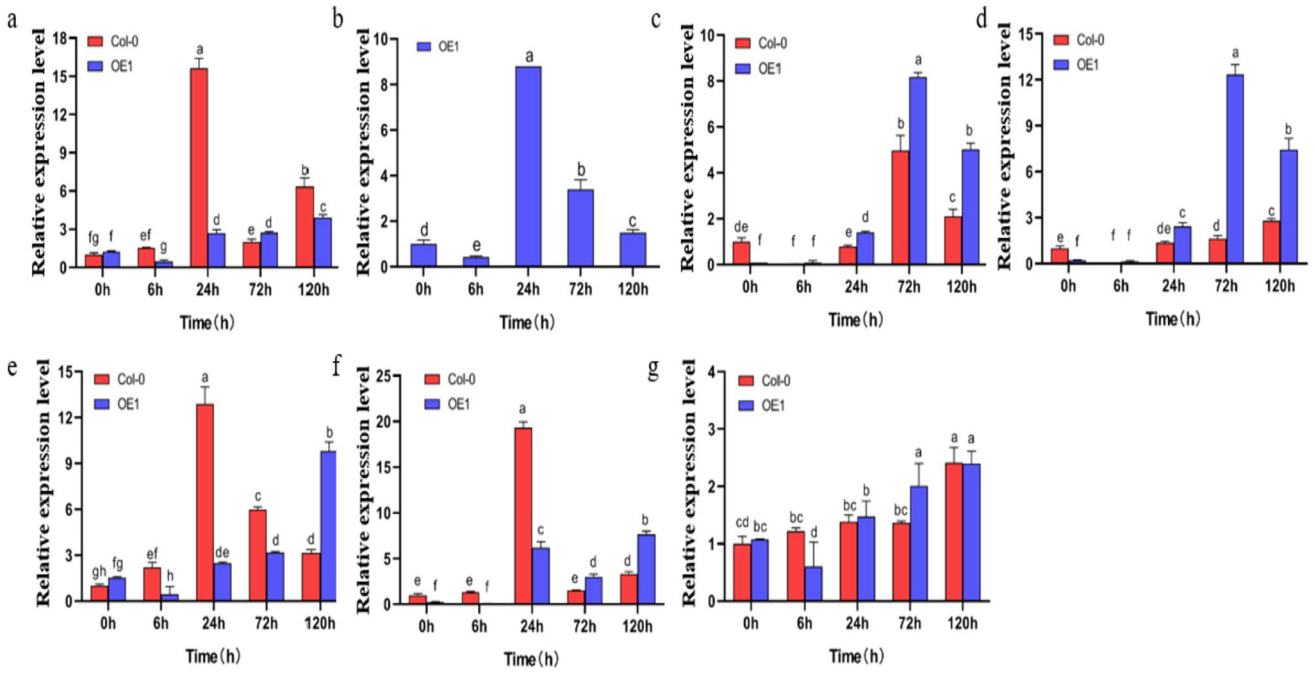

Fig. 10 Expression levels of genes related to flavonoid synthesis pathway under salt stress. **a** Expression of the *Arabidopsis* flavonol synthase gene (*AtFLS*) in Col-0 and OE1 plants. **b** Expression of the *A. venetum* flavonol synthase gene (*AvFLS*) in OE1 plants. **c** Expression of the *Arabidopsis* dihydroflavonol-4-reductase gene (*AtDFR*) in Col-0 and OE1 plants. **d** Expression of the *Arabidopsis* anthocyanin synthase gene (*AtANS*) in Col-0 and OE1 plants. **e** Expression of the *Arabidopsis* flavanone 3-hydroxylase gene (*AtF3H*) in Col-0 and OE1 plants. **f** Expression of the *Arabidopsis* chalcone synthase gene (*AtCHS*) in Col-0 and OE1 plants. **g** Expression of the *Arabidopsis* chalcone isomerase gene (*AtCHI*) in Col-0 and OE1 plants. The values shown are the means ± SDs of three biological replicates. Error bars represent the standard deviation of three replicates. Different letters within a treatment group indicate significant differences based on one-way ANOVAs ($P < 0.05$)

AvFLS overexpression also affects the expression patterns of some genes in the flavonoid synthesis pathway of *A. thaliana*. Two genes in the anthocyanin metabolic pathway, *AtDFR* and *AtANS,* were significantly upregulated in the transgenic line as compared to the WT after 24 h, 72 h, and 120 h of salt stress (Fig. 10c, d). Similarly, the genes encoding flavanone 3-hydroxylase (*AtF3H*; Fig. 10e) and chalcone synthase gene (*AtCHS;* Fig. 10f) were significantly upregulated in the transgenic lines as compared to the WT lines after 120 h of salt stress. The chalcone isomerase gene (*AtCHI*) was significantly upregulated in both the transgenic and the WT lines as salt stress was prolonged; there was no significant difference in expression between the two lines (Fig. 10g).

4 Discussion

Flavonol synthase is a key enzyme in the plant flavonoid metabolic pathway and therefore plays an important role in flavonoid biosynthesis. Plant flavonoids, which are regulated by the flavonoid metabolic pathway, determine flower and seed-coat colors (Lou et al. 2014; Zhang et al. 2018). In addition, increases in flavonoid content can increase plant tolerance of biotic and abiotic stresses (Yuan, et al. 2015; Baskar et al. 2018; Li et al. 2018), because flavonoids can act as antioxidants or pro-oxidants to eliminate or reduce the tissue damage associated with the reactive oxygen species produced as a result of adverse environmental conditions (Lee et al. 2004; Tattini et al. 2004; Eghbaliferiz and Iranshahi 2016). Therefore, further systematic physiological and molecular experiments of FLS gene will be helpful to further reveal the mechanism of *FLS* gene in improving plant stress.

In this study, we successfully isolated and characterized *AvFLS* cDNA from *A. venetum*, demonstrated that *AvFLS* encodes a functional protein, and showed that *AvFLS* was ubiquitously expressed throughout *A. venetum,* including leaves, stems, and roots. *AvFLS* expression levels were higher in the leaves and lower in the stems and roots, these results are consistent with the research results of Wang et al.,(2021a, b, c, d).

To further investigate the flavonol synthase function of the FLS protein from *A. venetum*, the amino acid sequence of AvFLS was compared with predicted FLS sequences from other species using a phylogenetic tree. Phylogenetic analyses recovered *A. venetum* in a close relationship with

predicted FLS proteins from *Capsicum baccatum* (Wang et al. 2022a, b), *Nicotiana tabacum* (Shi et al. 2017), and *Solanum lycopersicum* (Morimoto and Tao 2016; Gao et al. 2018).

At the subcellular level, *AvFLS* appeared to be localized in the cytoplasm and not in the nucleus, consistent with the observation that flavonoids are synthesized and localized in the cytoplasm in *A. thaliana* (Saslowsky et al. 2005; Kuhn et al. 2011) and *Dendrobium officinale* (Yu et al. 2020). However, there are many flavonoid metabolic enzymes that have been localized in the cytoplasm, including CHS, CHI, and FLS (Kuhn et al. 2011).

In response to stress, plants may synthesize osmotic regulators, such as proline, soluble sugars, and betaine to improve stress resistance (Farooq et al. 2009; Hao et al. 2021). Proline accumulation is triggered by environmental stress (Hare and Cress 1997; Maggio et al., 2021) to protect cells from stress-associated damage (Hong et al. 2000; Kavi and Sreenivasulu et al., 2014). We observed that salt stress significantly increased the proline content of the leaves of both WT *Arabidopsis* seedlings and *Arabidopsis* seedlings overexpressing *AvFLS*. After 168 h of salt stress (100 mM NaCl), the proline content in the leaves of the WT was 522.8 μg· g^{-1}, a 13.7-fold increase compared to proline content under control conditions (0 h). Similarly, the proline content in the transgenic leaves was 419.37 μg·g^{-1} after 168 h of salt stress, corresponding to a 24.3-fold increase in proline content compared to control conditions (0 h). Under control conditions and during the early stages of salt stress (0 h, 6 h, and 24 h), soluble sugar content was significantly greater in the transgenic plants than in the WT plants. Thus, *AvFLS* overexpression improved osmoregulation and increased soluble sugar accumulation in *Arabidopsis,* enhancing resistance to salt stress. Similarly, the overexpression of the *FLS* gene of *Chrysanthemum morifolium* (*CmFLS*) improved the stress resistance of tobacco (Wang et al. 2021a, b, c, d).

Photosynthesis provides the energy required for plant growth and development, and is thus critical for increases in plant biomass and economic crop yield (Richards et al., 2006; Long et al. 2006; Zhu et al. 2008; Zhu et al. 2010). Chlorophyll content reflects the efficiency of plant photosynthesis. Therefore, chlorophyll concentration indicates the physiological state of the plant and can be considered a biochemical marker of stress tolerance (Arnon 1949; Rahneshan et al., 2018). Indeed, chlorophyll concentration has been specifically identified as an important physiological indicator of plant salt tolerance (Singh and Gautam 2013; Bernal-Vicente et al. 2018). Under stress conditions, the chlorophyll content of plants with strong stress resistance is higher than the chlorophyll content of plants with poor stress resistance (Billings and Mooney 1968; Quick et al. 1992; Mukami et al. 2019). After 168 h of salt exposure, the contents of both chlorophyll a and chlorophyll b were significantly greater in the transgenic *Arabidopsis* seedlings overexpressing *AvFLS* that in WT plants, indicating that the transgenic plants were more resistant to salt stress. Consistent with this, when the *Euphorbia kansui* flavonol synthase gene (*EkFLS*) was overexpressed in *A. thaliana*, chlorophyll content increased, affecting photosynthesis and promoting stress resistance (Wang et al. 2021a, b, c, d).

Under abiotic stress, plants produce large amounts of peroxides, and the resulting oxidative damage to plant cell membranes produces MDA (Parvanova et al. 2004). Due to their polyphenolic structure, flavonoids act as strong antioxidants in plants, scavenging excess free radicals (Djeridane et al. 2006). Increases in flavonoid content improve the antioxidant capacity of plants, which in turn improves plant stress tolerance (Ashraf 2009; Akula and Ravishanka 2011). Here, MDA contents in the transgenic plants were lower than those of the WT under control and salt-stress conditions, indicating that *AvFLS* overexpression reduced the lipid peroxidation of cell membranes in *Arabidopsis.*

Studies have shown that in some cases, when the overexpressed exogenous gene is highly similar to the endogenous gene, both genes in the transgenic plant are inhibited (i.e., co-suppression; Facchini 2001). For example, the overexpression of the rice (*Oryza sativa*) FLS gene *OsFLS* in tobacco led to the downregulation of the early and late biosynthetic genes in the flavonoid pathway; the endogenous *FLS* gene *NtFLS* was particularly strongly inhibited (Park et al. 2019). Here, the expression levels of *AtFLS* did not differ significantly between the transgenic and WT *A. thaliana* lines under control conditions, but *AtFLS* was significantly downregulated in the transgenic plants as compared to the WT under salt stress. This indicated that overexpression of the *AvFLS* gene moderately suppressed the expression of the endogenous *AtFLS* gene in *Arabidopsis*. However, due to the strong upregulation of the *AvFLS* gene, total flavonoid content was significantly greater in the *AvFLS*-overexpression line under both salt-stress and control conditions. Consistent with this, *AvFLS*-overexpressing transgenic tobacco plants accumulated flavonoids to a greater extent than WT plants, which resulted in a significant positive effect on the recovery of growth under salinity stress (Wang et al. 2021a, b, c, d).

FLS transcript levels affect the contents of various flavonols in plants, and thus participate in the regulation of the plant response to various external stresses (Khare et al. 2018; Sharma et al. 2019; Hou et al. 2020). For example, the contents of flavonols, such as quercetin and kaempferol, increased significantly in Tartary buckwheat seedlings under salt stress, and the *FtFLS2* gene was simultaneously significantly upregulated (Li et al. 2018). Similarly, ultraviolet radiation increased flavonol accumulation and strongly upregulated *F3H* and *FLS* in soybean (Kim et al. 2008). It also has been reported that *EkFLS* overexpression is strongly

correlated with an increase in flavonoid synthesis and therefore in abiotic stress tolerance in *Arabidopsis* (Wang et al. 2021a, b, c, d). In addition, *FtFLS2* was upregulated by SA and NaCl, affecting the flavonol biosynthetic pathway (Li et al. 2013). Similarly, overexpression of *BnFLS* increased flavonol accumulation and exhibited enhanced tolerance to multiple abiotic stressors in the transgenic plants (Vu et al. 2015). Treatment with the flavonoid quercetin not only alleviated the adverse effects of mannitol-induced osmotic stress, but also upregulated *CHI* and *FLS* in *Apocynum* seedlings (Yang et al. 2021). The heterologous expression of the *FLS* gene led to the downregulation or non-expression of endogenous genes in *N. tabacum* (Jiang et al. 2020), while the overexpression of *AvFLS* in *Arabidopsis* downregulated the endogenous *FLS* gene *AtFLS* in our research work. In vivo, *FLS* and *DFR* genes competitively determine the accumulation of flavonols, anthocyanins, and flavan-3-ols in plants (Mahajan et al. 2011; Zhao et al. 2014; Luo et al. 2016; Akita et al. 2018). However, the overexpression of *AvFLS,* the *FLS* gene from the halophyte *A. venetum,* in *Arabidopsis* upregulated not only the genes involved in flavonoid biosynthesis but also the genes involved in anthocyanin synthesis. That is, the expression levels of *AtDFR* and *AtANS* in the transgenic plants after 24 h, 72 h, and 120 h of salt stress were significantly higher than those in wild-type *Arabidopsis* at the same time points. Interestingly, studies have shown that, in *FLS*-overexpressing plants, the early genes in the flavonoid synthesis pathway (*CHS* and *F3H*) were not expressed or were expressed at low levels (Park et al. 2019; Li et al. 2021). Our results showed that the expression levels of *AtCHS* and *AtF3H* in transgenic plants treated with salt for 6 h and 24 h were significantly lower than those of wild-type plants at the same time points. However, these genes were significantly upregulated in the transgenic plants compared to the wild-type plants after 120 h salt treatment. At the same time, we also found that the falvonoid content of transgenic *Arabidopsis* overexpressing *AvFLS* was significantly greater than that of WT *Arabidopsis* after 120 h of salt treatment. The upregulation of these genes indicated that the activity of the flavonoid synthesis pathway increased in response to salt stress in the transgenic overexpression line, corresponding to the observed significant increase in total flavonoid content.Together, these results indicate that *FLS* genes in halophytes and glycophytes may play different functions under salt stress, and it is necessary to continue to study the related mechanisms in subsequent studies.

5 Conclusion

Overexpression of the *AvFLS* gene in transgenic *Arabidopsis* improved plant growth and development under salt stress compared to the WT. In transgenic *Arabidopsis*, the expression level of the endogenous *AtFLS* gene differed significantly from that of wild type. In addition, *AvFLS* expression in transgenic *Arabidopsis* increased with prolonged salt stress. This study not only helped to clarify the molecular mechanisms of salt-tolerance genes, but also provided a theoretical basis for the further mining of *Apocynum* genes, the molecular breeding of plants with improved resistance to salt stress, and the consequent improvement of crop quality.

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Declarations

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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