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FvMYB44, a Strawberry *R2R3-MYB* Transcription Factor, Improved Salt and Cold Stress Tolerance in Transgenic *Arabidopsis*

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Abstract: MYB (v-MYB avian myeloblastosis viral oncogene homolog) transcription factor (TF) family has numerous members with complex and diverse functions, which perform an integral role in regulating the plant's response to adversity. This study used cloning to obtain a novel MYB TF gene from the diploid strawberry *Fragaria vesca*, which was given the designation *FvMYB44*. Subcellular localization results showed that the protein of FvMYB44 was a nuclear localization protein. The resistance of *Arabidopsis thaliana* to salt and low temperature stresses was greatly enhanced by the overexpression of *FvMYB44*. When subjected to salt and temperature stress, transgenic plants showed higher proline and chlorophyll concentrations and higher superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities than wild-type (WT) and unloaded line (UL) of *A. thaliana*. In contrast, WT and UL lines had higher malondialdehyde (MDA) content and reactive oxygen species ROS (O_2^- and H_2O_2) content. These findings suggest that *FvMYB44* may perform a role in controlling the response of *A. thaliana* to cold and salt stress.

Keywords: *Fragaria vesca*; *FvMYB44*; salt stress; cold stress; *Arabidopsis thaliana*

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1. Introduction

In the process of growth, plants will inevitably encounter environmental stress, which will affect plant growth and development. More seriously, the damage caused is irreversible [1]. In particular, these abiotic stresses, such as soil and water stress, low temperature, high temperature, high salt, and nutrient deficiencies, have caused a decline in the yield and quality of horticultural crops year after year, and these problems need to be addressed urgently in many countries [2–4]. A growing body of research suggests that plants can receive external signals that are transmitted to cells through some pathway, resulting in adaptive mechanisms [5,6]. As molecular biology technology has developed recently, it has been found to be effective to use genome editing to increase the ability of plants for environmental adaptation. Transcription factors and their regulatory roles are among them, and they are crucial to the study of plants.

In the plant genome, MYB transcription factors as one of the largest transcription factor families are responding plants to their surroundings [7]. The majority of MYB proteins function as TFs. MYB TF is a class of eukaryotic TF made up of 51 or 52 amino acid (aa) residues and 1 or more conservative domains characterized by 1 to 4 MYB repeats. The DNA recognition helix that results from these aa residue pairs can make immediate contact with the DNA [8]. The MYB domain is a 51–52 amino acid peptide containing a series of

highly conserved amino acid residues and spacer sequences, MYB-TF is categorized into 4 categories: 1R-MYB (MYB related), 2R-MYB (R2R3-MYB), 3R-MYB (R1R2R3-MYB), and 4R-MYB (R1R2R2R2R1/2-MYB) [9–11].

MYB TF was first found in the Zea mays [12], so far, have been shown in many kinds of plants in a variety of MYB gene families [13,14]. Moreover, lots of research have proved that when plants are disturbed by the outside world, MYB TF can respond to enhance the ability of plants to cope with these adverse conditions. According to Bian et al. [15], overexpression of GmMYB811 resulted in high levels of seed germination and seedling emergence despite high salt and dehydration conditions, and increased resistance of soybean seedlings to salt and drought stress. It was demonstrated that FvMYB24 functioned as a positive regulator of strawberry salt tolerance via binding to the SOS1 promoter [16]. MdMYB124 and MdMYB88 can be positively involved in regulating the expression of cold endurance and cold related genes under cold stress in apples and *A. Thaliana* through C-Repeat Binding Factor-dependent and C-Repeat Binding Factor-independent pathways [17]. Previous articles explain a lot of the functions of MYB transcription factor, the regulation of accumulation of volatile substances, anthocyanins, and flavonols in strawberry fruit mediated by MYB TF was studied most [18–20], but few studies have been conducted on the association of MYBs genes with cold tolerance and salt tolerance in strawberry.

It is important to consider the role of functional gene expression regulatory networks mediated by TFs in plant response paths to various stressors. In such signal networks, the response of plants to MYB TFs is crucial. MYB can modulate the expression of certain genes response with stress, thereby enhancing plant adaptation to adversity [21–23]. The expression of defense-respond genes XsPOD, XsNCED1, XsCAT1, XsP5PCS, XsSOD, XsABI3, XsABI5, and XsABF2Xs was discovered to be directly or indirectly controlled by XsMYB30 to improve the yellowhorn's tolerance to drought and salt. [24]. Expression of the *Arabidopsis AtMYB44* gene confers drought/salt-stress tolerance in transgenic soybean [25]. This increased the tolerance of soybean to salt and drought, enabling it to survive and grow tenaciously in challenging conditions. Moreover, MbMYBC1 can promote the expression of cold response genes such as *DREB1A*, *COR15a*, *ERD10B* and *COR47* under cold stress [26].

This work describes the isolation and cloning of a novel MYB gene from *F. vesca* and the identification of the functions of chill and salt tolerance in *Arabidopsis thaliana*. Finally, all this information indicated that *FvMYB44* was identified as an important candidate gene for improving chill and salt stress tolerance of *F. vesca*. This result gives researchers a theoretical basis to investigate how *FvMYB44* contributes to salt and cold tolerance in strawberries. In addition, it can serve as a theoretical basis for additional study on the molecular biology of MYB transcription factor genes in the emergence of resistance in *F. vesca*.

2. Materials and Methods

2.1. Materials, Growing Situation, and Treatment for Plants

The seeds of *F. vesca* came from Northeast Agriculture University Harbin China, and the seedlings were cultured on Murashige and Skoog (MS) medium, including indole-3-butyric acid (IBA) (Adamas, 133-32-4, Shanghai, China) and 6-benzylaminopurine (6BA) (Adamas, 1214-39-7, Shanghai, China), or in matrix with a 2:1 ratio of soil to vermiculite. With a relative humidity of 70% and a temperature of 22 °C, all plants were cultured in plant growth chamber (Lichen RGX-600F, Shanghai, China), setting photoperiod to 16 h light and 8 h dark [27]. The fully mature leaves fully developed young leaves, freshly emerged roots, and freshly emerged stems were chosen to measure the relative mRNA levels. Therefore, 30 good growth condition seedlings were selected and broken up into 6 groups, each group including 5 seedlings. The first group was the control group and the material was cultured in a tissue culture chamber (22 °C), while the second and third groups were cultured in a low temperature environment at 4 °C and a high temperature environment at 37 °C, respectively [28]. Another 3 groups received 15% PEG6000 (Adamas, 25322-68-3, Shanghai, China), 200 mm NaCl (Adamas, 7647-14-5, Shanghai, China), and

100 µm ABA (Adamas, 14375-45-2, Shanghai, China) treatments, respectively, to replicate dryness, salinity, and ABA disposal. After 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h of stress disposal, the leaves, stems, and roots of the seedlings were quickly submerged in liquid nitrogen and maintained at −80 °C to avoid any interference with the RNA extraction procedure that would follow [29].

2.2. Isolation and Cloning of FvMYB44

OminiPlant RNA Kit (Conway Collection, Beijing, China) was applied to extract total RNA from roots, stems, and laminas (both immature and mature leaves), and RNase-Free DNase I was used to purify the RNA. From a Takara First-strand cDNA Synthesis SuperMixture kits, Oligo dT was used for cDNA synthesis, reversed transcription synthesis of cDNA first strand (Takara, 6210A, Tokyo, Japan). A pair of primers that are specific to genes (*FvMYB44-F* and *FvMYB44-R*; Table S1) was designed. The target region was amplified by PCR using the cDNA as a template, and the ASY-T1 vector (TransGen Biotech, CT01-01, Beijing, China) was then ligated to the PCR product in order to detect positive ones and get the sequence [30].

2.3. Subcellular Localization of FvMYB44

The transient expression vector of *FvMYB44* was made by inserting *FvMYB44* fragment into *SalI* and *BamHI* based on the enzymatic cut site on vector pSAT6-GFP-N1 (*FvMYB44-slF* and *FvMYB44-slR*; Table S1) [31]. The *FvMYB44*-containing combination plasmid was injected into tobacco outer epidermal cells using the *Agrobacterium tumefaciens* injection technique, while the empty 35S:GFP plasmid served as a control. The confocal microscopy was used to identify the position of the FvMYB44-GFP combination protein (LSM 900, Zeiss, Oberkochen, Germany).

2.4. Sequence Analysis and Structure Prediction of FvMYB44

Multiple alignment of the FvMYB44 and other MYB transcription factor sequences from various species was operated using DNAMAN 5.2 (https://www.lynnon.com/support.html (accessed on 1 September 2021)) (LynnonBiosoft, San Ramon, CA, USA), and phylogenetic trees were created using the neighbor-joining method using MEGA7 (http://www.megasoftware.net (accessed on 5 September 2021)) [32]. Based on the ExPASy website (https://web.expasy.org/protparam/ (accessed on 11 September 2021)), the primary protein structure of FvMYB44 was analyzed. The domain and tertiary protein structures of FvMYB44 were predicted using the SMART website (http://smart.embl-heidelberg.de/ (accessed on 11 September 2021)) and SWISS-MODEL website (https://swissmodel.expasy.org/ (accessed on 11 September 2021)), respectively [33].

2.5. Expression Analysis of FvMYB44

Plants were treated with abiotic stress and the expression levels of *FvMYB44* in different tissues were tested by qPCR. qRT-PCR primers of *FvMYB44*-qF and *FvMYB44*-qR were designed based on the conservative series of *FvMYB44* (Table S1). The PCR reaction system was arranged with ddH_2O 9 µL, 2xMix 12.5 µL, 1 µL primer, and 1.5 µL cDNA. The primers should be diluted with 10 times their own volume of ddH_2O according to the Oligo instruction manual. Reaction procedure: 94 °C for 30 s; 95 °C 5 s, 54 °C 40 s, 72 °C 30 s; 35 cycles were performed at 72 °C for 10 min, and the PCR products were stored at 4 °C [33]. The internal reference gene was the Actin gene of *F. vesca*, and primers *FvActin*-F, *FvActin*-R were showed in Table S1. For more than three samples, the statistical analysis of the expression levels of pertinent mRNA in stems, immature leaves, roots, and mature leaves using one-way ANOVA. Using the $2^{-\Delta\Delta Ct}$ technique, in young leaves and roots the expression levels of the target gene were examined [34].

2.6. Stress Treatment and Determination of Related Physiological Indexes in A. thaliana

The 5′ and 3′ ends of the *FvMYB44* cDNA were attached with *SalI* and *BamHI* digestion sites by PCR using the *FvMYB44*-F and *FvMYB44*-R primers. Then, to create the pCAMBIA2300-*FvMYB44* overexpression vector, the target fragment of *FvMYB44* was joined to *SalI* and *BamHI* of the PCAMBIA2300 vector. Using the inflorescence-mediated method, GV3101-mediated transfer of *Agrobacterium rhizogenes* into the Columbia ecotype *A. thaliana*. In order to screen transgenic lines, MS medium, including 50 mg/L kanamycin, was applied. The transgenic lines were finally identified by qRT-PCR method analysis, and WT (wild type), and UL (unloaded line with empty vector) were set as the control. The T3 transgenic lines were used for further analysis. To statistically assess the relative expression level of *FvMYB44* in transgenic *A. thaliana*, one-way ANOVA were used.

A. thaliana cultured in one-half MS medium with T3 transgenic lines (L1, L3, and L4) and WT, UL. After the plants have grown for 10 days, the seedlings were transplanted into fertile nutrient pots with soil and vermiculite after having their cotyledons revealed (soil:vermiculite = 2:1). In each pot, four seedlings were placed. All *A. thaliana* were split into 2 groups of 20 plants each based on the various stress treatments. One group was treated with −8 °C cold incubation in a chamber for 14 h and later put back to normal condition for recover culture for 7 days. Another was cultivated with water for 3 days after being irrigated with 200 mm NaCl for the first 7 days. After these treatments, the phenotypic characteristics of the plants need to be observed and, at the same time, the survival rate of each strain needs to be calculated. After stress treatment, WT, UL, and transgenic lines samples were further determined for physiological and biochemical indexes. The absorbance of chlorophyll solutions was determined for all lines according to the method of Sartory and Grobbelaar [35], and calculations were made to determine the amount of chlorophyll using the formula published by Wellburn [36]. The activities detection of CAT, POD, SOD, O_2^-, and H_2O_2 were assessed in Zhang's study [37]. Using MDA Analysis Kit (TBA method), the MDA content was determined [38]. The proline content in the sample was extracted and determined as Qu [39].

2.7. Statistical Analysis

The one-way variance was examined using the SPSS 21.0 program (IBM, Chicago, IL, USA). The mean of three replicate trials served as the basis for all data, the standard deviation (SD) was computed. Significant statistical differences were denoted by the notations * $p \leq 0.05$, ** $p \leq 0.01$.

3. Results

3.1. Cloning and Bioinformatic Analysis of FvMYB44

From the results of sequence analysis, the total length of *FvMYB44* is 561 bp (Figure S1). The protein encoded by ExPASy-ProtParam had a theoretical isoelectric point of 8.86 and a theoretical molecular weight of 19.991 kDa by the *FvMYB44* gene. Ser, Gly, Ala, and Leu residues made up 10.8%, 10.2%, 9.1%, and 8.6% of the 186 amino acids that made up the protein FvMYB44, respectively. Furthermore, the FvMYB44 protein was hydrophilic, and hydrophilic coefficient was −0.541.

Sequence comparison of MYB proteins from 10 other varieties with the FvMYB44 protein revealed a conserved DNA-binding structural domain unique to the MYB transcription factor family (Figure 1a). Phylogenetic tree analysis revealed that FvMYB44 and *Rosa chinensis* RcMYB73 (XP_024164121.2) had the highest homology, indicating that they were the most similar in genetic evolution (Figure 1b). Through analyzing the protein secondary structure of FvMYB44, the results showed that it included 46.35% α-helix, 13.31% β-coil, 5.11% extended strand, and 39.45% random coil (Figure 2a). The sequence of FvMYB44 contained a conserved SANT domain at 10-59, 62-110 aa, as Figure 2b showed. According to the whole results, FvMYB44 belongs to the R2R3-MYB family. The investigation discovered that the FvMYB44 protein has an HTH region (Figure 2c), and the findings were cleverly congruent with the secondary structure of the protein predicted in the earlier

paper. In addition, the SWISS-MODEL website can predict the tertiary structure of the FvMYB44 protein.

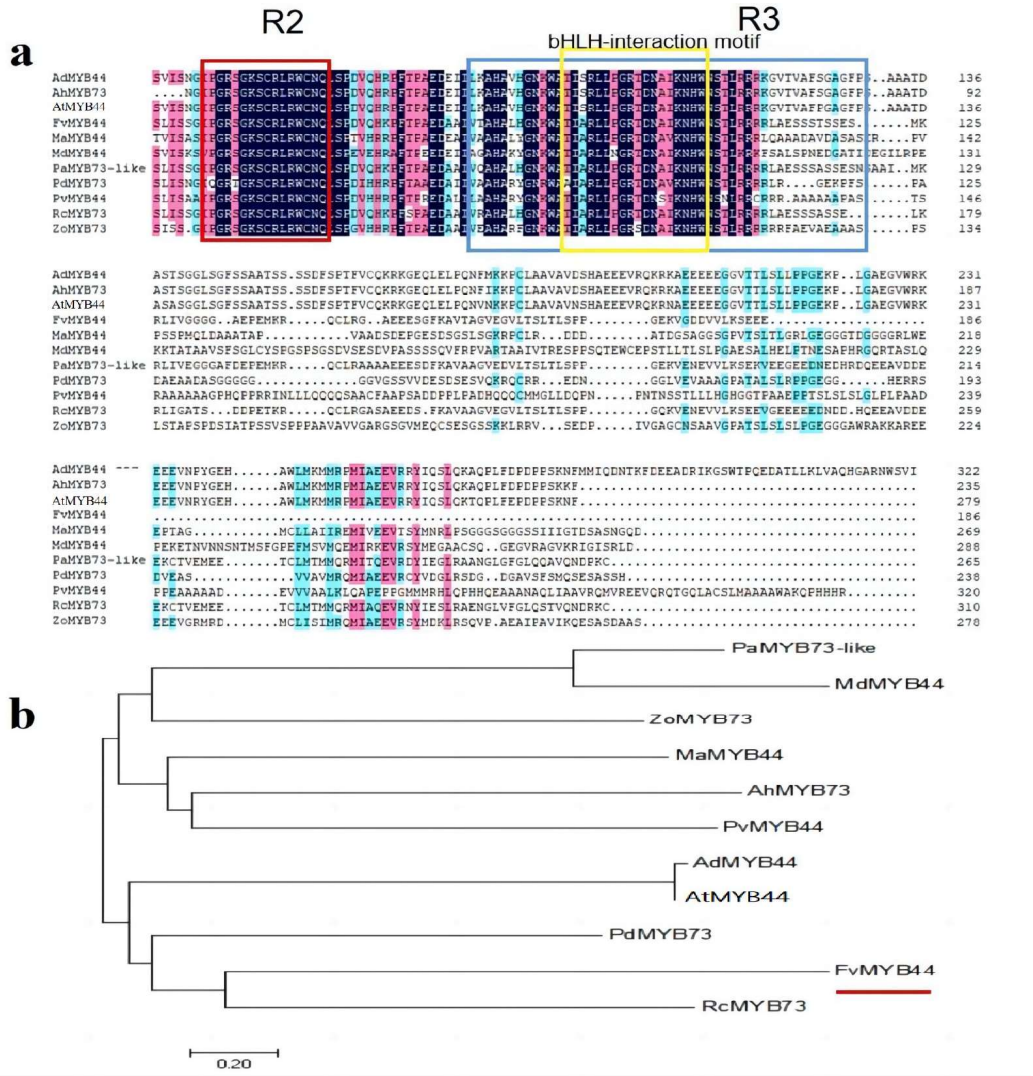


Figure 1. Contrast and evolutionary relationship between FvMYB44 and MYB transcription factors in different species. (**a**) Homology comparison among FvMYB44 protein and MYB protein in other plants. Red and blue boxes mean the conserved regions of the MYB amino acid sequence. (**b**) MYB protein phylogenetic tree analysis in FvMYB44 and other plants, where FvMYB44 was underlined in red. The accession numbers are as follows: RcMYB73 (*Rosa chinensis*, XP_024164121.2), PaMYB73-like (*Potentilla anserina*, XP_050387456.1), PdMYB73-like (*Phoenix dactylifera*, XP_008805112.1), ZoMYB73 (*Zingiber officinale*, XP_042414516.1), AtMYB44 (*Arabidopsis thaliana*, NP_201531), MaMYB44 (*Musa acuminata*, XP_009400144.1), AhMYB44 (*Arachis hypogaea*, XP_025609138.1), AdMYB44 (*Arachis duranensis*, XP_015931281.1), MdMYB44 (*Malus domestica*, NP_001315871.1), and PvMYB44 (*Panicum virgatum*, XP_039803171.1).

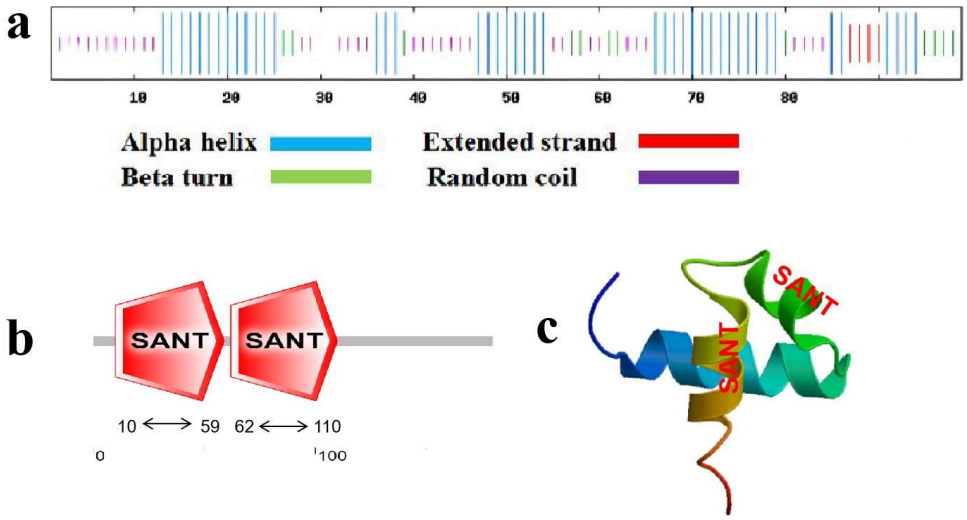


Figure 2. Predicted FvMYB44 protein structure and domains. (**a**) prediction of protein secondary structure; (**b**) prediction of protein domains; (**c**) prediction of tertiary structure.

3.2. FvMYB44 Was Localizated onto Nucleus

Using *A. tumefaciens* injection method, 35S::FvMYB44-GFP fusion plasmid was transferred into tobacco epidermal cells. In Figure 3, the results of confocal microscope observation were shown out. The fluorescence of the 35S:GFP plasmid was used as a contrast and has a wide distribution in the cell structure (Figure 3a). However, the fluorescent signal of 35S:FvMYB44-GFP was only visible in the nucleus (Figure 3d). Therefore, it can be tentatively confirmed that the FvMYB44 protein functions in the nucleus.

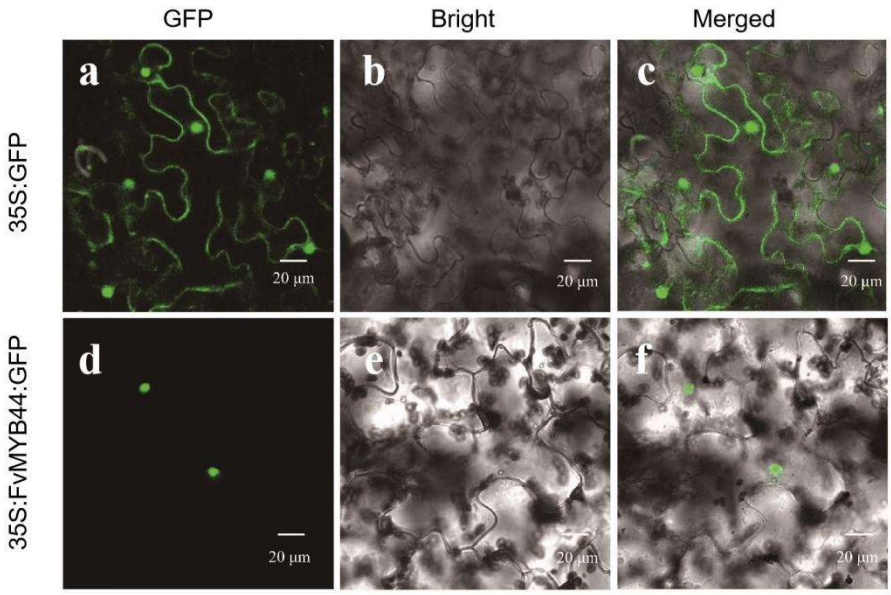


Figure 3. Subcellular localization of FvMYB44 in tobacco leaf epidermal cells. The 35S:GFP and 35S:FvMYB44 plasmids were injected into the cells by *Agrobacterium tumefaciens* injection method. (**a**,**d**) GFP fluorescence, (**b**,**e**) Bright-field, (**c**,**f**) Merged. Bar = 50 μm.

3.3. Expression Level Analysis of FvMYB44 in F. vesca Seedlings

Figure 4 shows the RT-qPCR results of *FvMYB44* in the organs of *F. vesca*, including new leaves, old leaves, roots, and stems. The expression level of *FvMYB44* was nearly the same in roots and young leaves, while the expression level was significantly higher than stems and mature leaves. In young leaves, the peak expression time of *FvMYB44* was 8 h,

4 h, 8 h, 6 h, and 2 h with the five different stresses of salt, cold, dehydration, ABA, and heat, respectively, with an overall trend of increasing, then decreasing expression. Gene expression in roots followed a similar pattern, reaching its peak at 4 h, 2 h, 4 h, 6 h, and 6 h, respectively. Moreover, compared to other treatments, salt stress and low temperature both enhanced the degree of *FvMYB44* expression in these two organs (Figure 4b,c). These results demonstrated that *FvMYB44* was more sensitive to salt and cold than other stressors.

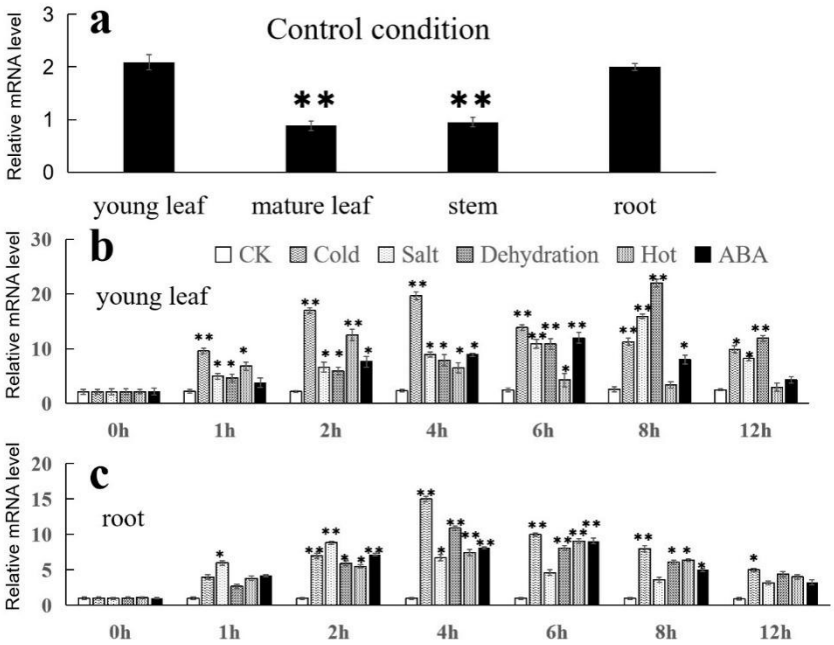


Figure 4. Quantitative RT-PCR analysis of the *FvMYB44* expression pattern in *F. vesca*. (**a**) *FvMYB44* expression in various tissues under non-stress conditions. (**b**,**c**) Expression of *FvMYB44* in new leaves and roots over time in the presence of control and dehydration (15% PEG6000), heat (37 °C), cold (4 °C), salt (200 mM NaCl), and abscisic acid (100 M ABA). The standard deviation is indicated by error bars. The error bars with asterisks above them show that the treatment and control groups differ significantly from one another. (* $p \leq 0.05$, ** $p \leq 0.01$).

3.4. Increased Salt Tolerance in A. thaliana Due to Overexpression of FvMYB44

To further understand the function of *FvMYB44* in these two conditions, transgenic *Arabidopsis* were cultivated in low-temperature, high-salt conditions. Upon addition of *FvMYB44*-specific primers, it was discovered that only L1, L2, L3, L4, and L5 were identified as transgenic plants, with L1, L3, and L4 having higher expression of *FvMYB44*, as shown in Figure 5a. WT and UL were employed as control lines among them.

We irrigated all the healthy plants (WT, UL, L1, L3, and L4) with 200 mM NaCl. The phenotypes of each plant were evaluated one week after treatment. The WT and UL lines had extremely chlorotic leaves, small plants, and obvious signs of shrinking and withering. However, for transgenic lines, only a small portion of the plants exhibited marginal yellowing. The WT and UL lines were unable to restart normal growth after 3 days of irrigation with water, while the transgenic lines were able to do so to some extent. Although the transgenic *Arabidopsis* was more affected by drought, it was able to survive normally after resuming growth and the leaves started to gradually recover within two weeks. The WT and UL lines were unable to restart normal development after three days of irrigation with water, whereas the transgenic strain was able to do so to some extent. WT, UL, L1, L3, and L4 had corresponding survival rates of 22%, 26%, 76%, 79%, and 75%, respectively. According to (Figure 5c), transgenic *Arabidopsis* survived under salt stress significantly better when *FvMYB44* was overexpressed.

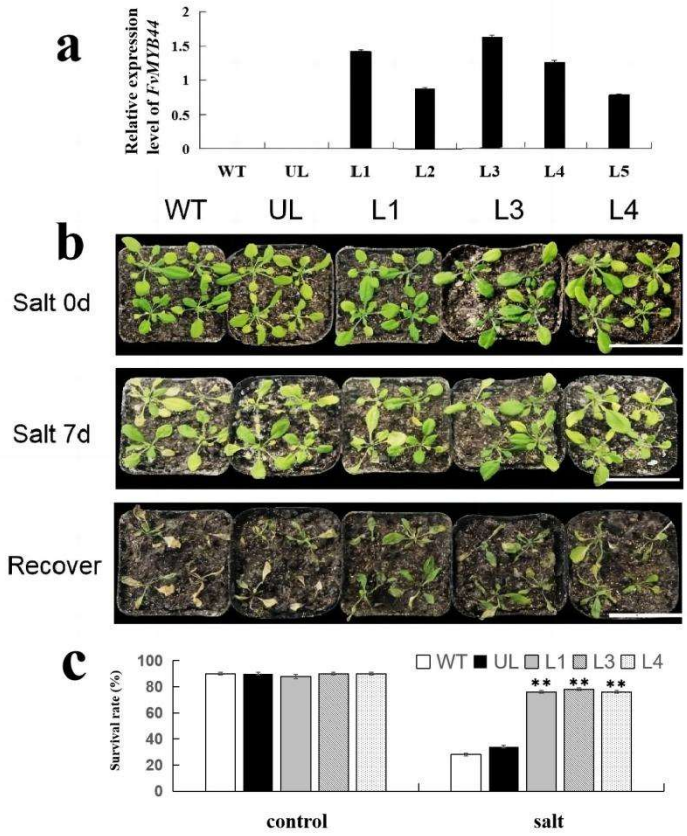


Figure 5. Transgenic *A. thaliana* lines that overexpress *FvMYB44* grow when exposed to salt stress. In WT, UL, and 5 *FvMYB44*-overexpression lines (L1, L2, L3, L4, and L5), the relative expression level of *FvMYB44* was shown in (**a**). (**b**) WT, UL, and transgenic lines (L1, L3, and L4) phenotypes cultivated in a control environment, salt treatment (irrigation with 200 mM NaCl for 7 days), and recovery from salt treatment (irrigation with water for 3 days) Bar = 5 cm. (**c**) WT, UL, and transformed lines (L1, L3, and L4) survival rates. Three replicate experiments were conducted. The standard deviation is indicated by error bars. Significant differences between the WT and UL, transformed lines are denoted by asterisks (** $p \leq 0.01$).

Figure 6 showed there were no appreciable differences among all plants in the physiological and biochemical indicators tested under control circumstances. However, the *FvMYB44* transgenic *Arabidopsis* showed higher CAT, SOD, and POD activities, chlorophyll and proline levels than WT and UL lines after 7 days of growth under high salt treatment. In the transgenic lines, the MDA content was lower than in the WT and UL lines. After salt stress treatment, the chlorophyll content of L1, L3, and L4 decreased by 0.59-, 0.56-, and 0.64-fold, respectively, compared to the WT lines under control conditions. Their MDA contents increased by 0.33-, 0.33-, and 0. 38-fold, respectively. The increase in proline content of the transgenic lines was significant and could be seen to be 3.41, 3.4, and 4.8 times higher compared to the control treatment. SOD activity increased approximately 2.5-fold and POD activity increased 1.15-, 1.43-, and 1.37-fold, respectively, in all transgenic *Arabidopsis*, but CAT activity increased less, only 1.21-, 1.02-, and 1.01-fold, respectively. After salt treatment, the O_2^-/H_2O_2 concentration increased to some extent in all lines. There appeared to be a significant difference in O_2^- content between WT and FvMYB114 transgenic *Arabidopsis*. Compared to the control, there was a twofold increase in WT and UL lines, with 1.32-, 1.28-, and 1.42-fold increases in O_2^- content in L1, L3, and L4, respectively. Both WT and UL lines also showed an average 2-fold increase in hydrogen peroxide (H_2O_2) content (2.31- and 2.22-fold), but FvMYB114 transgenic *Arabidopsis* (L1, L3, and L4) showed 1.55-, 1.56-, and 1.59-fold increases in hydrogen peroxide content, respectively. These results suggest that overexpression of *FvMYB44* under salt stress not only enhances the ability of

plants to scavenge reactive oxygen species, but also reduces membrane lipid peroxidation in transgenic *Arabidopsis* and protects biofilms, in addition to improving the survival of plants under salt stress.

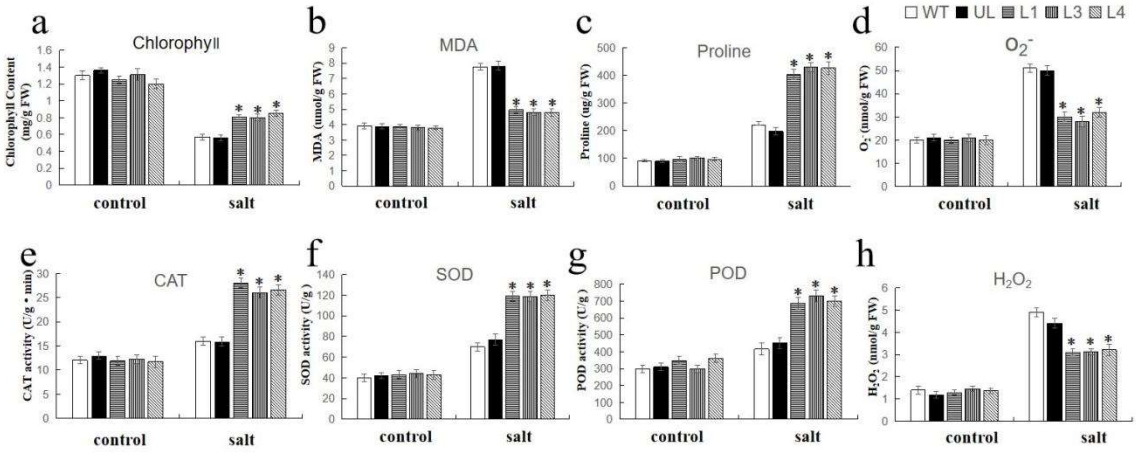


Figure 6. The physiological indicators of salt treatment on the WT, UL transgenic *A. thaliana* lines (L1, L3, and L4) overexpress *FvMYB44* (200 mm NaCl treatment for 7 days). Chlorophyll (**a**), MDA (**b**), proline (**c**), O_2^- (**d**), and (**h**) hydrogen peroxide (H_2O_2) content, and (**e**) CAT, (**f**) SOD, and (**g**) POD activity. The transgenic lines (L1, L3, and L4), the UL, and the WT are distinguished from each other by asterisks above each error bar. (* $p \leq 0.05$). The index level of WT was used as the study control group.

3.5. Overexpression of FvMYB44 Increases Cold Tolerance in A. thaliana

As can be seen from Figure 7, when *A. thaliana* (WT, UL, L1, L3, L4) were grown under normal conditions, they all had similar leaf phenotypes and showed little difference from each other. However, after the temperature was lowered to −8 °C, the phenotypes of these plants were significantly different after 14 h due to the altered environment. The WT and UL lines showed a reduction in leaf size and number, but the L1, L3, and L4 plants were only slightly injured. After a week of recovery growth, when the plants returned to normal temperature, it was found that the low temperature produced irreversible damage to the WT and UL lines, with browning of their leaf color and significant reduction in leaf diameter, and their survival rates were only 23% and 25%, respectively. The transgenic lines L1, L3, and L4 had greater survival rates (76%, 80%, and 79%, respectively).

According to the findings in Figure 8, the concentrations of MDA, proline, chlorophyll content, O_2^-, and H_2O_2, and the activities of SOD, POD, and CAT were almost equal in each strain under the control conditions (22 °C). However, after cold treatment, the chlorophyll content decreased in all lines, but L1, L3, and L4 lines decreased less. Compared to WT and UL, transgenic *A. thaliana* showed noticeably higher POD, SOD, CAT, chlorophyll, and proline activity, but reduced MDA concentrations. In WT and UL, MDA content was greater. After the application of cold stress in this study, the chlorophyll content of the *FvMYB44*-OE lines decreased significantly by 0.56-, 0.63-, and 0.59-fold compared to the WT strain under control conditions. In contrast, the MDA content of the transgenic strain increased 0.35-, 0.37-, and 0.32-fold, with the most significant increase in proline content, which increased 4.61-, 4.1-, and 4.7-fold, respectively. The CAT activity of *FvMYB44* overexpressing *Arabidopsis* increased 1.11-, 1.23-, and 0.98-fold, SOD activity increased 2.5-, 2.3-, and 2.3-fold, and POD activity increased 1.37-, 1.38-, and 1.41-fold. O_2^- expression levels in WT and FvMYB114 transgenic *Arabidopsis* were considerably different, according to these results. O_2^- levels rose 2.17, 2.11, 1.32, 1.34, and 1.39 times higher in WT, UL, L1, L3, and L4, respectively, than in the control, but H_2O_2 levels rose 2.31, 2.56, 1.46, 1.58, and 1.53 times higher, respectively. In other words, the high-level expression of *FvMYB44* contributes to a higher capacity for plants to withstand cold temperatures.

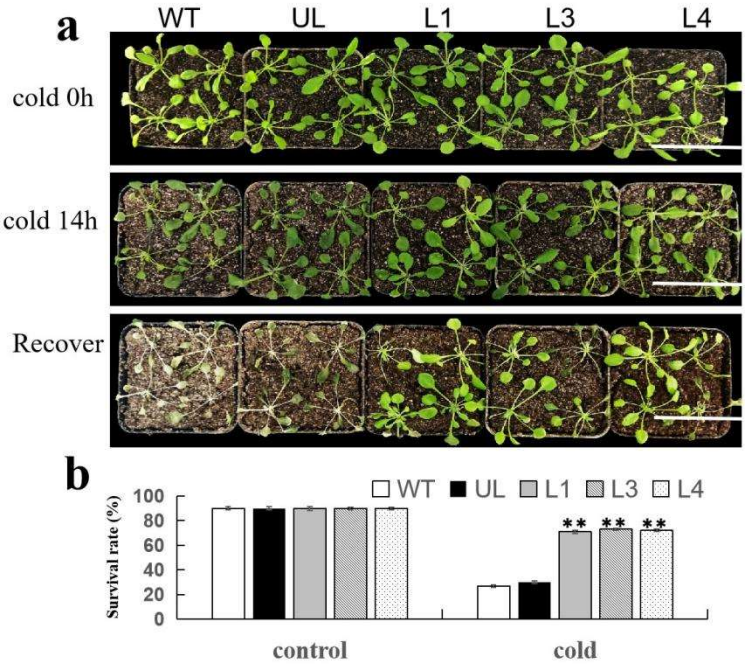


Figure 7. Transgenic *A. thaliana* that is overexpressing *FvMYB44* grows when kept at low temperatures. (**a**) Phenotypes of WT, UL and *FvMYB44* overexpression lines (L1, L3, and L4) under control conditions (22 °C), after exposure to low temperature (−8 °C) and one week after resumption of growth. Bar = 5 cm. (**b**) The percentage of WT, UL, and transgenic lines that survived in a control condition and after being exposed to cold. Three replicate experiments were conducted. The standard deviation is indicated by error bars. Significant differences between the WT and UL, transformed lines are denoted by asterisks (** $p \leq 0.01$).

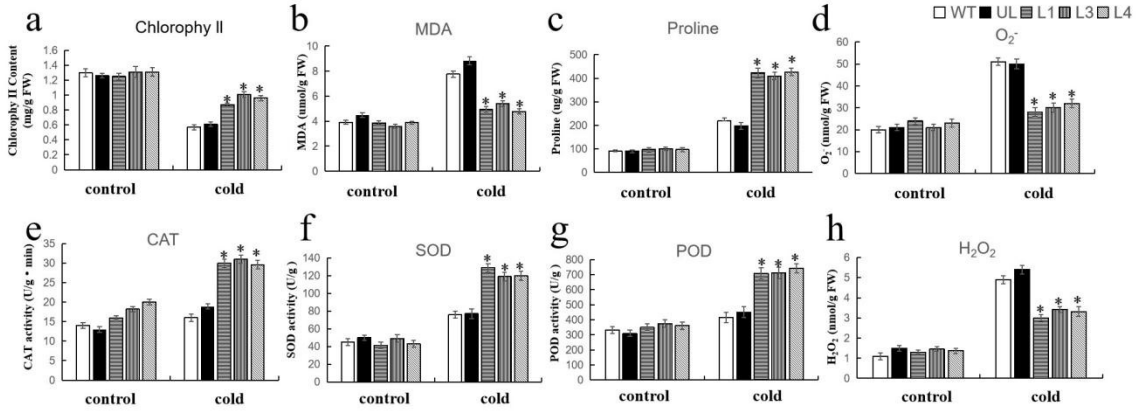


Figure 8. WT, UL transgenic *A. thaliana* lines (L1, L3, and L4) overexpressing *FvMYB44* under cold treatment (−8 °C for 14 h) have the following physiological indicators. Chlorophyll (**a**), MDA (**b**), proline (**c**), O_2^- (**d**), and (**h**) hydrogen peroxide (H_2O_2) content, and (**e**) CAT, (**f**) SOD, and (**g**) POD activity. The transgenic lines (L1, L3, and L4), the UL, and the WT are distinguished from each other by asterisks above each error bar. (* $p \leq 0.05$). The index level of WT was used as the study control group.

4. Discussion

In recent years, environmental changes have brought about many irreversible changes in plant growth and development. As sessile organisms, plants have gradually evolved a variety of complex defense mechanisms in the face of external environmental changes [40,41]. MYB can participate in the regulation of metabolic response processes and hormone signaling, assisting plants to respond to abiotic stresses in various ways to maintain healthy

plant growth and development, and is closely related to plant yield [42,43]. Understanding the process by which MYB transcription factors in plants adapt to complex environments is crucial for crop development and breeding.

WT forest strawberries were used as experimental materials in this study. The experimental materials were pre-cultured in MS medium supplemented with auxin and cytokinin in order to promote seedling growth and improve seedling resistance [41]. Utilizing homologous cloning, the *FvMYB44* gene sequence was retrieved by cloning after the proper primers were produced in DNAMAN software. Sequence analysis results showed that the 561 bp nucleotide sequence of *FvMYB44* encoded 186 aa (Figure 1). A unique SANT-MYB DNA binding conserved domain was identified in *FvMYB44*. This demonstrated that it belongs to the R2R3-MYB gene (Figure 2). Subcellular localization in tobacco leaf cells by *Agrobacterium* injection revealed that FvMYB44 was localized in the nucleus (Figure 3). A previous study found that FvMYB44 and MdMYB are homologous [44], so MdMYB44 and other several specific genes were selected for homology analysis (Figure 1). According to the results of the phylogenetic tree, FvMYB44 and RcMYB73, which belong to the *Rosaceae* family, are most closely related genetically [45], while *AtMYB44* has been shown to perform an important role in plant resistance to abiotic stresses [25], which is close to the findings of FvMYB44, implying that FvMYB44 may also respond to abiotic stresses. Gene expression patterns become an integral part of studying the role of genes. For this reason, to test our conjecture, we analyzed the expression level of *FvMYB44* gene in different periods and in different tissue sites in strawberry. Gene expression is time-specific and spatially specific. Figure 4 demonstrated how this trait was reflected in the expression of *FvMYB44* in *F. vesca*. In Figure 4, the expression levels of *FvMYB44* were in the order of new leaves, roots, stems, and old leaves from highest to lowest, respectively. The higher expression level of new leaves could be that immature leaves are more sensitive to external stimuli [41]. Different stresses might trigger the expression of *FvMYB44* in roots and immature leaves, and its amount of expression altered over time. This experiment suggests that *FvMYB44* may be more susceptible to the effects of low temperature and salt stress, which points the way for further research. The WT strawberry without stress treatment was used as standard, and the relative mRNA level of WT did not changed, though it changed a lot under abiotic stress. It has been shown that the decrease in agricultural productivity in recent years is closely related to temperature, salt stress, and drought stress, the abiotic stress affect the plant growth and development directly, and ABA as hormones transduction signal also promoted plant response [46–48]. MYB TF has been found to be essential for plant responses to adversity. In this case, each line (WT, UL and *FvMYB44*-OE *Arabidopsis*) was placed in a high salt and low temperature environment for a sustained period of incubation (Figures 5 and 7). Transgenic lines developed better than WT and UL after a period of cultivation, even though all *A. thaliana* would be slightly harmed. This supported the findings of other investigations and suggested that increasing the expression of *FvMYB44* could enhance *A. thaliana*'s resilience to extremes of cold and salinity.

The morphological characteristics, physiological and biochemical changes that occur in plants under stress are their response to environmental changes [49]. When plants are confronted with adversity, they rapidly produce large amounts of ROS, which upsets the intracellular redox balance and causes serious cell damage [50]. Under difficult growth conditions, SOD, POD, and CAT are important defensive enzymes that remove ROS from cells to lessen cellular damage. Thus, their behavior can be utilized to gauge the extent of plant damage in unfavorable conditions [51]. MDA, one of the end products of lipid peroxidation in plant cell membranes, can combine with proteins and nucleic acids to make them inactive, thus further damaging biological membranes and intracellular substances [52]. Stress can affect chlorophyll content, hence, the use of chlorophyll content to represent the level of resistance of plants [53]. As a component of plant proteins, proline is free in the plant body, not only as a cytoplasmic regulatory substance, but also to stabilize the structure of biomolecules, regulate the redox potential of cells, and improve plant resistance. [54,55]. In this study, CAT, SOD, and POD activities rose after salt and cold

treatments in all lines, although these activities increased more noticeably in the *FvMYB44* transgenic *Arabidopsis* (Figures 6 and 8). Although chlorophyll contents dropped following treatment in all lines, the contents of transgenic lines (L1, L3, and L4) decreased more slowly than those of UL and WT plants, with increases in MDA, O_2^-, and H_2O_2 contents being less pronounced in transgenic *Arabidopsis* than in UL and WT. Compared to the UL and WT, transgenic *Arabidopsis* had a higher proline content increase. These findings showed that low temperature and high salt stress can trigger the response mechanism in *FvMYB44*, and that *FvMYB44* overexpression significantly improved plant stress resistance.

5. Conclusions

In the present study, *FvMYB44* from *F. vesca* was isolated, cloned, and characterized. Young leaves and roots have a higher expression of *FvMYB44*, which is tissue specific. A functional analysis showed that overexpression of *FvMYB44* is closely related to plant stress responsiveness. The current findings suggest that *FvMYB44* can increase plants' tolerance to low temperatures and salt stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041051/s1, Figure S1:The total length of *FvMYB44*; Table S1: List of primers used in this study.

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