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Effect of aluminum and fluoride on R2R3-MYB transcription factor characterization and expression in *Camellia sinensis*

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Abstract

Tea plant (*Camellia sinensis*) is a typical plant that accumulates abundant aluminum (Al) and fluoride (F). Al and F play vital roles in the growth and development in tea plant. MYB (myeloblastosis) is one of the largest transcription factor families in plant, which plays an important role in the stress responses. As the largest and multifunctional subfamily of MYB, R2R3-MYB subfamily participates in multiple abiotic stresses in plant. However, there are few studies about R2R3-MYB in tea plant, especially in the accumulation of Al and F during its growth. Here, we identified 43 *CsMYB* genes from the transcriptome database of tea plant and analyzed the relative expression in Al and F treatments. According to the *Arabidopsis thaliana* classification, 43 *CsMYB* genes are divided into 18 subgroups via phylogenetic tree analysis. All 43 *CsMYB* proteins have the typical R2R3-MYB domain by MEME prediction. Moreover, 9 *CsMYB* genes (*CsMYB11*, *CsMYB17*, *CsMYB29*, *CsMYB38* to *CsMYB43*) that related to abiotic stress were selected from 43 *CsMYB* genes for a further study, and the relative expressions showed that they are tissue-specifically expressed. In addition, the results of different concentrations of Al treatments showed that *CsMYB11*, -17, -29, -38 and -41 are stimulated by Al treatment. The results of different concentrations of F treatments showed that the *CsMYB11*, -17, -38, -39, -40, -41, -42 and -43 participate in F accumulation of *C. sinensis* roots. Our research establishes a solid foundation for further investigation into the molecular functions of R2R3-MYB transcription factors in *C. sinensis*.

Additional key words: *Arabidopsis thaliana*, phylogenetic tree, stress response, tea growth and development.

Introduction

Tea plant [*Camellia sinensis* (L.) O. Kuntze] is a leaf-type crop. It is known to be a strong accumulator of aluminum (Mukhopadhyay *et al.* 2012). Under acidic conditions, Al is accumulated in tea plant mainly in leaves, especially in old leaves up to 5.6 g(Al) kg⁻¹(f.m.) and less in young leaves 1 g(Al) kg⁻¹(f.m.) (Wong *et al.* 1998). Tea leaves take up Al during growth and contain up to 30 g(Al) kg⁻¹ (d.m.) (Tolra *et al.* 2011). However, tea plants with high Al content do not exhibit symptoms of toxicity, rather growth stimulation (Hajiboland *et al.* 2013). Previous studies have showed that the tea plant physiological characteristics and metabolites are changed in response to Al (Hajiboland

et al. 2013, Xu *et al.* 2016). Xu *et al.* (2017) also find 755 and 1059 differentially expressed proteins in tea roots and leaves under Al stress, respectively. These results enhance understanding of the stimulatory effects of Al on tea plant. Recent study has revealed that the myeloblastosis (MYB) transcription factor (TF) families are involved in response of tea roots to high Al content (Li *et al.* 2017). Nonetheless, the molecular mechanism of tea plants response to Al is still unclear.

Tea plant is often assumed to be highly tolerant to fluoride (F) as well, as they can accumulate a lot of F without showing symptoms of toxicity (Ruan and Wong

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Abbreviations: DBD - DNA binding domain; MYB - myeloblastosis; RT-qPCR - reverse transcription quantitative polymerase chain reaction; TF - transcription factor.

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2001). Fluorides are accumulated mainly in leaves and increased with age of tea leaves. The highest F content is found in fallen leaves, followed by mature leaves, young leaves, branches, and roots (Fung *et al.* 1999). Meanwhile, high F treatment can influence photosynthesis, antioxidant defense, and cell ultrastructure of tea leaves (Cai *et al.* 2016). Previous reports demonstrated that F is taken up by tea roots through active rather than passive processes (Zhang *et al.* 2013). In addition, the F uptake of tea plant is affected by many environmental factors, such as the pH and other mineral elements Ca^{2+} and Al^{3+} (Ruan *et al.* 2004, Yang *et al.* 2016). Recent studies have also found that many genes of tea plant are involved in the absorption of F (Li *et al.* 2017). However, little is known about the molecular mechanisms of F accumulation in tea plant.

The MYB TF family exists widely in eukaryotes and it is one of the largest and most diverse families of TF in the plant kingdom (Riechmann *et al.* 2000). The distinguishing property of MYB proteins is a highly conserved MYB domain consisting of 1 - 4 imperfect tandem repeats (MYB-repeat) at the N-terminus. The MYB repeat is 50 - 53 amino acids in length and contains three regularly distributed tryptophan (or phenylalanine) residues. Each MYB-repeat encodes three α -helices, with the second and third helices forming a helix-turn-helix structure, which recognizes and binds to the DNA major groove at the specific recognition site C/TAACG/TG (Stracke *et al.* 2001). The MYB family is divided into different types according to the number of MYB-repeats: 4R-MYB, 3R-MYB (R1R2R3-MYB), 2R-MYB (R2R3-MYB), and 1R-MYB (R1/R2 MYB) type (Dubos *et al.* 2010). The 4R-MYB group is the smallest and contains four R1/R2-like repeats, it is rarely known of their functions in plants (Dubos *et al.* 2010). The 3R-MYB group is also small, and its constituents function in cell cycle control (Ito 2005, Haga *et al.* 2007). The 2R-type MYB genes constitute the largest plant subfamily and are thought to have evolved from the same ancestor as 3R-MYB genes (Zhou *et al.* 2015). The R2R3-MYB is one of the largest families of MYB TF, which shared an R2R3

domain with 104 amino acid residues, including five conserved tryptophan residues. More than over 100 R2R3-MYB members have been reported in dicots and monocots (Wilkins *et al.* 2009). Whole genome identification of R2R3-MYB has been carried out in different plant species. For example, 126 R2R3-MYB genes were found in *Arabidopsis* (Dubos *et al.* 2010), 192 in poplar (Wilkins *et al.* 2009), 157 in maize (Du *et al.* 2012a) and 244 in soybean (Du *et al.* 2012b). They have been implicated in multiple biological processes, such as biotic and abiotic stresses (Lippold *et al.* 2009, Segarra *et al.* 2009), determination of cell shape (Noda *et al.* 1994), and regulation of differentiation (Xie *et al.* 2010). For example, *AtMYB59* regulates root development through the control of cell cycle progression at the root tips (Mu *et al.* 2009) and *AtMYB77* regulates lateral root formation by modulating the expression of auxin-inducible genes (Shin *et al.* 2007). *AtMYB68* (subgroup 14) is a root growth specific regulator, impacting overall plant development under unfavourable conditions (*e.g.* high temperature) (Feng *et al.* 2004). *AtMYB96* has been shown to be involved in regulation of lateral root growth in response to drought stress *via* the ABA-auxin signalling network (Seo and Park 2009). Extensive studies in *Arabidopsis* have provided a better understanding of the R2R3-MYB gene family. In tea plant, the R2R3-MYB is found to be related to the flavonoid biosynthesis (Zhao *et al.* 2013). In addition, a recent study has showed that the purple foliage coloration of tea plant is activated by the R2R3-MYB (Sun *et al.* 2016). Whereas little is known about this family in tea plant under abiotic stresses, especially under Al and F stresses.

In this study, we screened and identified 43 *C. sinensis* R2R3-MYB genes, and analyzed the relative expression in different tissues and after Al and F treatments. The results of this study can reveal the structures and functions of the *CsMYB* genes and serve as a basis for future research to elucidate the regulatory mechanisms of R2R3-MYB transcription factor in tea plant.

Materials and methods

Plants and Al and F treatments: The two-year-old cutting seedlings of tea [*Camellia sinensis* (L.) O. Kuntze] cv. Longjingchangye were pre-incubated in control nutrient solution in a greenhouse under a temperature of 23 °C, a 70 % relative humidity, a 14-h photoperiod, and an irradiance of 250 - 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 2 weeks. Then tea plants were grown hydroponically in an incubator with 0, 0.4, and 2 mM Al^{3+} or 0, 8, and 16 mg dm^{-3} F^{-} and growth and the uptake of Al^{3+} and F^{-} were determined. Root samples were collected after 0, 3, 6, 12, and 24 h. In addition, different tissues from conventionally grown tea plant were collected, including roots, stems, first and fourth leaves, buds, flowers, pollen and pollen tubes, and

fruits. All the samples were immediately frozen in liquid nitrogen and then stored at -80 °C until required.

Phylogenetic tree construction and sequence analysis of the *CsMYB* family: We searched for all the putative MYB proteins that retrieved from *C. sinensis* transcriptome data (Pan *et al.* 2016) and unpublished transcriptome data (NCBI accession: SRP149468). Using *BLAST* search of amino acid sequences, we detected whether the *CsMYBs* contain highly conserved DNA binding domain (DBD) or not. Then we determined the sequence similarity of the *CsMYBs* with *Arabidopsis MYBs* by NCBI. After excluding the *CsMYBs* that lack

DBD, we finally identified the 43 *CsMYBs* which can be used for further analysis. The MYB family of *Arabidopsis* was downloaded from the plant TF database (<http://planttfdb.cbi.pku.edu.cn/>). The amino acid sequences of MYB proteins from tea and *Arabidopsis* were aligned. Subsequently, we constructed an unrooted phylogenetic tree by using the neighbor-joining method and bootstrap analysis (1 000 replicates) in *MEGA* 6.0

(<http://www.megasoftware.net>). Motif location was analyzed using *MEME* v. 4.10.1 (<http://meme-suite.org/tools/meme>). With the following parameter settings: the distribution of motifs, 0 or 1 per sequence; maximum number of motifs to find 20; minimum width of motif 6; maximum width of motif 53. In addition, only motifs with an *e*-value $\leq 1 \times 10^{-10}$ were retained for further analysis.

Fig. 1. The phylogenetic tree was constructed between tea plant and *Arabidopsis*. The sequences include 43 *CsMYB* proteins and 126 *AtMYB* proteins.

Reverse transcription quantitative PCR analysis: The total RNA was isolated from all samples by using the RNA isolation kit (*Huayueyang*, Beijing, China) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized from samples RNA using the *Prime*

*Script*TM RT reagent kit (*TaKaRa*, Dalian, China) and then diluted 10-fold. The *Csβ-actin* gene was selected as an internal reference to normalize the expression of the selected *CsMYB* genes. The sequences of all primers are listed in Table 1 Suppl. The profile of cycling was based

on *SYBR Premix Ex-Taq* kit® (*TaKaRa*). The RT-qPCR was performed on a *Bio-Rad* (Hercules, USA) *CFX96* platform under conditions: denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 30 s; and then 95 °C for 10 s; a final extension at 65 °C for 5s and then 95 °C for 5 s. Each reaction was performed in a total volume of 20 mm³ containing 0.4 mm³ (10 mM) of each gene-specific primer, 2 mm³ of diluted cDNA as the

template, 7.2 mm³ of ddH₂O, and 10 mm³ of *SYBR Premix Ex Taq* (2×) mixture. Each independent cDNA sample was performed with three independent biological replications. We used the $2^{-\Delta\Delta CT}$ method to calculate the expressions of *CsMYB* genes relative to the *Csβ-actin* gene (Pfaffl 2001). The average of three $2^{-\Delta\Delta CT}$ values obtained from the triplicate experiments showed the final expression.

Fig. 2. The conserved motif of CsMYB proteins. The unrooted phylogenetic tree was constructed using the conserved domain sequences using *MEGA*. Motifs of R2R3-MYB proteins were identified by *MEME*. Each motif is represented by a colored box and numbered.

Results

To identify R2R3-MYB family genes in tea plant, *BLAST* searches were carried out against tea plant genomes, including *C. sinensis* transcriptome data (Pan *et al.* 2016) and *NCBI* data. Among the potential *CsMYB* genes, 43 *CsMYB* genes encoding MYB domain from the transcriptome database of tea plant were selected for further investigation and their corresponding amino acid sequences were confirmed using *NCBI BLAST* program (Table 2 Suppl.). These genes were named *CsMYB01* to *CsMYB43*. In order to confirm the obtained classifications and to further categorize the *CsMYB* family, a phylogenetic tree based on the alignment of the 126 *AtMYB* proteins and 43 *CsMYB* proteins was constructed (Fig. 1). According to the classification of R2R3-MYB family genes in *Arabidopsis*, 43 *CsMYB* proteins in tea plant were further identified as belonging to 18 subgroups. Among the *CsMYB* subgroups, subgroups 7, 9, 13, 16, 17, 20, and 25 have one *CsMYB* gene; subgroups 2, 3, 15, 18, 21, and 24 have two *CsMYB* genes; subgroup 1 has three *CsMYB* genes; subgroup 8 has four *CsMYB* genes; subgroup 4 has five *CsMYB* genes; and subgroups 14 and 22 have six *CsMYB* genes. Given the above classification, the 43 *CsMYB* genes were subjected to further analyses.

To further reveal the diversification of 43 *CsMYB* genes, 20 distinct conserved motifs were identified in

C. sinensis R2R3-MYB proteins using *MEME* (Fig. 2). All of the closely related members in the phylogenetic analysis had common conserved motifs among the R2R3-MYB proteins, which was consistent with those results of the presented phylogenetic analysis. In this study, all of the *CsMYB* genes have the typical R2R3-MYB domain (Fig. 3). In general, the basic regions of the R2 and R3 repeats had 50 basic residues. Highly conserved tryptophan residue was located in the 10, 30, and 50 positions of the R2 repeat, and the 31 and 50 positions of the R3 repeat. Motifs 1, 2, and 3 consist of the integrated R2R3-MYB domain. Motifs 4 and 5 were the shortest with 10 amino acids. The motif 4 was not observed in subgroups 1, 16 - 18, 20 - 22, and 25. Motif 5 was absent in subgroups 1, 21, and 25. Five *CsMYB* proteins belonged to subgroup 4, while only one protein (*CsMYB14*) had no motif 6. Motifs 8 and 9 had the longest sequence of 49 amino acids, followed by motif 19. Motif 9 was only found in two *CsMYB* proteins (*CsMYB30* and *CsMYB31*). Only subgroup 15 has motifs 11 and 12. Motifs 13, 14, and 16 were not observed in subgroup 22. Six *CsMYB* proteins belonged to subgroup 22, while two *CsMYB* proteins (*CsMYB38* and *CsMYB39*) had the same motifs. Motifs 15, 17, and 20 was absent in subgroup 18.

Fig. 3. The logos of R2 (A) and R3 (B) MYB repeats highly conserved across 43 R2R3 MYB proteins in *Camellia sinensis*. Asterisks represent the conserved tryptophan residues (Trp) in the MYB domain.

The RT-qPCR analysis was performed to elucidate the expression patterns of *CsMYB* genes in different tissues of *C. sinensis* (Fig. 4). The *CsMYB* genes showed a diverse expression patterns. For example, *CsMYB11* (subgroup 4) and *CsMYB40* (subgroup 22) displayed the highest expression in flowers and a relatively low expression in fruits. *CsMYB17* (subgroup 13) revealed the highest expression in pollen and a relatively low expression in fruits. *CsMYB29* (subgroup 17) and *CsMYB38* (subgroup

22) are expressed strongly in roots. *CsMYB39* (subgroup 22) showed the highest expression in stems and a relatively low expression in pollen. *CsMYB41* (subgroup 22) and *CsMYB42* (subgroup 22) revealed high expressions in pollen tubes. The expression of *CsMYB43* (subgroup 22) was mostly observed in fruits and pollen tubes, whereas low expression was observed in other organs.

To detect the impact of Al and F on the expression of the nine *CsMYB* genes (*CsMYB11*, *CsMYB17*, *CsMYB29*,

CsMYB38, and *CsMYB40* to *CsMYB43*), tea plants were treated with a range of Al^{3+} and F^- concentration. According to the preliminary experiments, the new roots of *C. sinensis* grew faster under 0.4 mM Al^{3+} than under

0 and 2 mM Al^{3+} treatment. The *CsMYB11* (subgroup 4) was down-regulated under 0.4 and 2 mM Al^{3+} . The expression of *CsMYB17* (subgroup 13) was the highest after 3 and 12 h exposure to 0.4 mM Al^{3+} . *CsMYB17*

Fig. 4. The relative expressions of 9 *CsMYB* genes in different tissues (roots, stems, first leaves, flowers, fourth leaves, pollen, pollen tubes).

Fig. 5. The relative expression of 9 *CsMYB* genes at different Al^{3+} treatments (0, 0.4, and 2 mM) for 0 to 24 h.

(subgroup 13) displayed a significant induction within 3 h at 2 mM Al^{3+} , but the transcript accumulation declined to normal levels after 24 h. Under 0.4 mM Al^{3+} , the gene *CsMYB29* (subgroup 17) was highly expressed within 3 h, but had a low expression later. The *CsMYB38* (subgroup 22) had the highest expression at 12 h under 0 and 0.4 mM Al^{3+} and was efficiently induced also after 6 h under 2 mM Al^{3+} . Furthermore, the expression of *CsMYB38* (subgroup 22) at 2 mM Al^{3+} for 6 h was higher than that at 0 and 0.4 mM Al^{3+} for 12 h. The *CsMYB41* (subgroup 22) was highly expressed at 24 h under 0.4 mM Al^{3+} treatment. Compared with the control groups, *CsMYB39*, *CsMYB40*, *CsMYB42*, *CsMYB43* (subgroup 22) had similar expressions under Al^{3+} treatment and their expressions

decreased from 3 to 24 h (Fig. 5).

Under the F^- treatment (Fig. 6), it is apparently observed that *CsMYB11* (subgroup 4), *CsMYB17* (subgroup 13), *CsMYB40*, *CsMYB42*, and *CsMYB43* (subgroup 22) have similar expressions under 8 mg(F^-) dm $^{-3}$. They all reached the high expressions at 3 h and their expressions further increased at 24 h. The expression of *CsMYB38* (subgroup 22) and *CsMYB41* (subgroup 22) peaked at 6 h under 8 mg dm $^{-3}$ F^- . In addition, compared with the control group, *CsMYB29* (subgroup 17) was observed to be down-regulated at 3 h and then increased at 6 h under 0 mg(F^-) dm $^{-3}$. The *CsMYB17* (subgroup 13) was highly expressed at 0 mg(F^-) dm $^{-3}$ within 3 h, but down-regulated later.

Fig. 6. The relative expression of 9 *CsMYB* genes at different F^- treatments (0, 8, and 16 mg dm $^{-3}$) for 0 to 24 h.

Discussion

In previous studies, the R2R3-MYB TF has been identified in *Arabidopsis* (Dubos *et al.* 2010), in poplar (Wilkins *et al.* 2009), in rice (Katiyar *et al.* 2012), and in some other plants. Although individual R2R3-MYB genes have been identified in tea plant (Zhao *et al.* 2013, Sun *et al.* 2016), systematic analysis of the R2R3-MYB family has not been studied. Thus, we identified and analyzed 43 *CsMYB* genes of this family in tea plant using transcriptome data and found that they all encode proteins with the R2R3-MYB domain. These *CsMYB* genes can be classified into 18 subgroups. According to the previous studies, we screened 9 *CsMYB* candidate genes that are related to abiotic stresses from 43 *CsMYB* genes and used them for a

further study. The relative expressions of these MYB genes in different tissues were analyzed for exploring their functions in *C. sinensis*.

We analyzed tissue-specific expression patterns of *CsMYB* genes (Fig. 4). The *CsMYB29* (subgroup 17) showed the highest expression in roots and Mu *et al.* (2009) showed that *AtMYB59*, also belonging to subgroup 17, regulates root development through the control of cell cycle progression at the root tips. Therefore, we predict that *CsMYB29* might participate in the development of tea roots *via* control the root tip cell cycle progression. *CsMYB38* to *CsMYB43* were clustered in the same subgroup 22 (Fig. 1). According to the previous studies,

AtMYB77 (subgroup 22) regulates lateral root formation by modulating the expression of auxin-inducible genes (Shin *et al.* 2007). In this study, *CsMYB38* (subgroup 22) was also found to be highly expressed in the roots, indicating that *CsMYB38* may participate in the growth of tea roots. In addition, *AtMYB70* and *AtMYB77* (subgroup 22) show up-regulation during stem development (Ko *et al.* 2004). *CsMYB39* showed the highest expression in stems as well. *CsMYB40* (subgroup 22) showed the highest expression in flowers, while *CsMYB41* and *CsMYB42* showed the highest expressions in pollen tubes and the expression of *CsMYB43* was mostly observed in fruits and pollen tubes. The above results indicate that the various members of *CsMYB* (subgroup 22) might function in different tissues of *C. sinensis*.

The roots growth of *C. sinensis* is stimulated by Al^{3+} and F^- (Fung *et al.* 2008, Zhang *et al.* 2013). In the present study, we detected the expression patterns of 9 *CsMYB* genes in *C. sinensis* roots after exposure to Al^{3+} and F^- . Tea plant is known to be an Al^{3+} hyperaccumulator and the growth of roots is promoted at lower Al^{3+} concentrations but inhibited at high concentrations (Mukhopadhyay *et al.* 2012). Comparing to control (0 mM Al^{3+}), *CsMYB11* (subgroup 4), *CsMYB17* (subgroup 13) and *CsMYB29* (subgroup 17) were up-regulated by Al treatment, suggesting that *CsMYB11*, -17 and -29 might influence root growth of *C. sinensis* in response to Al treatment. In addition, functional studies on R2R3-MYB revealed that R2R3-MYB genes in the same subgroup not only possess similar functions but also are co-expressed after stress treatments (Liu *et al.* 2010). For example, the *AtMYB44*, *AtMYB70*, *AtMYB73*, and *AtMYB77* were classified into subgroup 22 and showed similar temporal and spatial expression pattern (Shin *et al.* 2007, Fan *et al.* 2014). Interestingly, *CsMYB38* to *CsMYB43* were grouped into subgroup 22 (Fig. 5). Compared with the control, *CsMYB39*, -40, -42 and -43 were all down-regulated by Al^{3+} treatments. It implies that the expression of *CsMYB39*, -40, -42 and -43 are negatively regulated by the uptake of Al^{3+} . In addition, the *CsMYB38* gene had the highest expression at 2 mM Al^{3+} . It is suggested that the Al^{3+} treatment could efficiently promote the expression of

CsMYB38 in tea roots. *CsMYB41* was highly expressed at 0.4 mM Al^{3+} . The results implied that appropriate concentration of Al^{3+} could improve the expression of *CsMYB41* (subgroup 22) in tea roots.

Some R2R3-MYB family members have been found to be related to the flavonoid biosynthesis and purple foliage coloration of tea plants (Zhao *et al.* 2013, Sun *et al.* 2016). However, the R2R3-MYB family involved in the process of absorption of F of tea plant has not yet been reported. In the present study, the expression of *CsMYB11* (subgroup 4) and *CsMYB17* (subgroup 13) peaked after 3 h at 8 mg(F^-) dm⁻³. It is reasonable to speculate that the 8 mg(F^-) dm⁻³ could efficiently improve the expression of *CsMYB11* and *CsMYB17*. In addition, the functions of some *Arabidopsis* R2R3-MYB members, especially of the subgroup 22, have been well characterized experimentally and phylogenetically analyzed (Jung *et al.* 2008). In the present study, the relatively high expressions of *CsMYB38*, -39, -40, -41, -42, and -43 genes in response to the 8 mg dm⁻³ F treatment suggest that these genes may somehow influence F^- accumulation. In contrast, *CsMYB29* (subgroup 17) gene expression is inhibited during exposure to F^- . These data combined with the *C. sinensis* root growth patterns under different concentrations of F^- lead us to speculate that *CsMYB11*, -17, -38, -39, -40, -41, -42 and -43 are involved in F accumulation of *C. sinensis* roots.

In conclusion, 43 *CsMYB* genes were identified for the first time in *C. sinensis*. We systematically analyzed their bioinformatic characteristics and found they can be classified into 18 subgroups. Furthermore, we select 9 *CsMYB* genes for exploring their expression patterns in different tissue and under Al and F stresses. These genes showed a diverse expression pattern in *C. sinensis* tissues. In addition, the expressions of 9 *CsMYB* genes under Al^{3+} and F^- stress indicate that *CsMYB11*, -17, -38, and -41 might function in the response to Al and F stresses and deserve further research. These findings are useful to expand our understanding of molecular responses to Al and F treatment in tea plant. Taken together, this study would provide a solid foundation for future functional investigation of the R2R3-MYB family in tea plant.

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