# Characterization of the R2R3-MYB Transcription Factor *CsMYB113* Regulates Anthocyanin Biosynthesis in Tea Plants (*Camellia Sinensis*)

**Liuyuan Shui**
Huazhong Agriculture University
**Meilin Yan**
Huazhong Agriculture University
**Hui Li**
Huazhong Agriculture University
**Pu Wang**
Huazhong Agriculture University
**Hua Zhao**
Huazhong Agriculture University
**Mingle Wang**
Huazhong Agriculture University
**Yu Wang**
Huazhong Agriculture University
**Dejiang Ni**
Huazhong Agriculture University
**Fei Guo** ( guofei@mail.hzau.edu.cn )
Huazhong Agricultural University: Huazhong Agriculture University https://orcid.org/0000-0001-6204-9553

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# Abstract

Tea plant(*Camellia sinensis*) has very long history of cultivation and abundant germplasm resources in China. Purple bud is a characteristic variety, which has attracted the attention of breeding researchers because it accumulated a large number of anthocyanins naturally. In many species, R2R3-MYBtranscription factors (TFs)wereprovedto be involved in the regulation of anthocyanin biosynthesis.Research on anthocyanin metabolism has been relatively clear in some species, but that needs to be further elucidated in tea plants. In this research, anR2R3-MYB transcriptionfactor *CsMYB113* relate to the anthocyanin accumulation regulation was identified from tea plants. Spatial and temporal expressionanalysis revealed differential expression of *CsMYB113*among different tissues and organs, with highest expression occurringin the roots.Subcellular localization assays showed that CsMYB113 localizedin the nucleus.Ectopic expression of CsMYB113increased pigmentation and anthocyanin contentsby the up-regulationof theexpression levelsof genes in anthocyanin biosynthesis pathwayamongdifferent tissues of *Arabidopsis*.Moreover, transient overexpressionof *35S::CsMYB113*in tea plant increased the anthocyanin contents in the leaves.Our results indicated that *CsMYB113* play important role in the anthocyaninbiosynthesis regulation in tea plants. It will also provide useful candidate gene for the modification of anthocyanin metabolism by genetic engineeringin plants.


# Introduction

Anthocyanin, which classified to the sub-class of flavonoids, is one of the most important metabolites existing in horticultural crops (Sun et al. 2016). Anthocyanin has been proved to participate in plant multiple biological and physiological processes including pigmentation, pollen transmission, seed dispersal, UV radiation protection, cold temperatures resistance, drought stress response, and pathogen defense (Karageorgou and Manetas 2006; Liu et al. 2018a; Stuurman et al. 2004; Castellarin et al. 2007; Christie et al. 1994). Moreover, anthocyanin is also exhibit biological activities in humans, such as anticancer, antioxidant, and cardiovascular diseases protection (He and Giusti 2010; Clifford et al. 2015). Due to these benefits, the high anthocyanin content (purple pigmentation) has become one of important trait for the breeders in tea plant (Maritim et al. 2021).

Almost all the pathway genes relate to anthocyanin metabolism were identified to date, and these genes are showing higher similar between many species including tea (Xi et al. 2019; Matsui et al. 2008; Wei et al. 2019). The pathway is catalyzed stepwise by a series of biosynthetic enzymes, such as cinnamate 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3', 5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanin O-methyltransferase gene (AOMT), UDP flavonoid glucosyl transferase (UFGT), anthocyanidin-3-glucoside rhamnosyltransferase (3RT) and methyltransferase (MT) (Jaakola 2013; Perez-Diaz et al. 2016). It’s generally demonstrated that the structural genes directly involved in anthocyanin metabolism are activated by numerous regulators, comprising MYB, basic helix-loop-helix (bHLH), and WD-repeat proteins (WDR) (Xu et al. 2015; Peng et al. 2019; Qi et al. 2020; Deng et al. 2021). These transcription factors (TFs) could independently or combine with cofactors to function as

regulators in anthocyanin metabolism (Baudry et al. 2004; Quattrocchio et al. 2006). In most species, the MYB TFs superfamily is known as the one of largest families. Based on the number of MYB domain repeats, the MYB family can be divided into four classes, including single repeat (1R-MYB), two repeats (R2R3-MYB), three repeats (3R-MYB), and four repeats (4R-MYB) (Dubos et al. 2010). So far, a lot of R2R3-MYB TFs related to the anthocyanin biosynthesis regulation have been identified in many plants, including AtTT2 in *Arabidopsis* (Baudry et al. 2004), MdMYB10 in apple (Espley et al. 2007), IbMYB1a in Sweet potato (Chu et al. 2013), SmMYB1 in eggplant (Docimo et al. 2016), FvMYB10 in strawberry (Zhang et al. 2017) PpMYB15 in peach (Cao et al. 2019), and PpMYB140 in pear (Ni et al. 2021).

Tea (*Camellia sinensis*) as one of the oldest (since 3000 BC) commercial crop and most popular nonalcoholic beverage is widely cultivated in over 50 countries and regions (Fang et al. 2012; Mondal et al. 2004). The popularity of tea is not only attributed to its specific aroma and taste, but also owing to the health benefits for human body. These medicinal properties derived from the various secondary metabolites in tea plants, such as catechins, anthocyanins, and theanine (Shi et al. 2011). However, anthocyanins were trace amount detected in most of the tea varieties (He et al. 2018). In recent years, purple foliage has attracted a lot attention by the tea plant breeding programmers. Many purple strains have been reported in different tea growing countries, such as the Chinese cultivar ‘Ziyan’ and ‘Zijuan’, the Japanese cultivar ‘Sunrouge’ (Hsu et al. 2012; Kerio et al. 2012; Kilel et al. 2013; Jiang et al. 2013). With the great efforts of many researchers, some R2R3-MYB TFs related to the anthocyanin pathway regulation have been identified from tea plants. The R2R3-MYB TFs CsAN1 could combine with *CsGL3* and *CsTTG1* and activate the expression of genes involve in anthocyanin biosynthesis (Sun et al. 2016). In ectopic transgenic tobacco plant leaves, *CHS* and *3GT* were activated by the *CsMYB6A* which result in the significantly increment of anthocyanins (He et al. 2018). In transgenic tobacco lines, CsMYB5a and CsMYB5e were reported to play important role in the regulation of anthocyanins and proanthocyanidins (Jiang et al. 2018). The PAP1-like MYB gene was proposed as a key regulator in controlling anthocyanins metabolism (He et al. 2018; Wei et al. 2016). CsMYB75 promoted the biosynthesis of catechins and anthocyanins by up-regulated the expression of *CsGSTF1* in transgenic tobacco (Wei et al. 2019). Recently, a collection of 122 *R2R3-MYB* TFs have been identified in the chromosome level genome from *Camellia sinensis* (Chen et al. 2021). Therefore, R2R3-MYB TFs related to anthocyanin metabolism regulation still need to be explored fatherly in tea plants.

In this research, the biological function of the MYB TF *CsMYB113* which related to anthocyanin metabolism was studied. The phylogenetic and localization study indicated *CsMYB113* belonging to R2R3-MYB TF family. Ectopic expression of *CsMYB113* in *Arabidopsis* led to significantly increased pigmentation and production of anthocyanins in roots, seeds stems, and leaves. The real-time quantitative PCR (qRT-PCR) analyses revealed *CsMYB113* activate the expression of anthocyanin-related structural genes in 35S: *CsMYB113Arabidopsis* transgenic plants. Moreover, the transient expression assays were carried out in leaves of tea plant for functional verification. This study advances our knowledge relate to the anthocyanin metabolism regulation for tea plant.

# Materials And Methods

## 2.1 Plant materials

Five-year-old tea plants cultivars [*Camellia sinensis* (L.) O. Kuntze cv. '*Fudingdabai*', '*YingShuang*' and '*Wuniuzao*'] were planted in the Tea Germplasm Resources Nursery, Huazhong Agricultural University (HAZU, Wuhan, China). *Nicotiana benthamiana* was used for subcellular localization analysis and *Arabidopsis thaliana* ecotype Columbia was used for genes overexpression experiments. These two plant materials were planted in growth chamber with a 16h light/8h dark photoperiod under illumination of 10000lux. The growth temperature was set to 22/19 °C (light/dark). All the collected samples were rapidly snap-frozen in liquid nitrogen, and then transferred to -80 °C for the further processing.

## 2.2 Total RNA extraction and cDNA synthesis

The RN09-EASYspin plant RNA kit (Aidlab, Beijing, China) was used to isolate total RNA for all the sampled materials. 1% agarose gel electrophoresis was employed to assess the quality for all the extracted RNA. RNA concentrations and integrity were checked by the Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, USA). The single strand cDNA was synthesized from 1 μg of each total RNA using the TRUEscript RT Kit with gDNA Eraser (Aidlab, Beijing, China).

## 2.3 Isolation of CsMYB113 gene and the sequence analysis

The predicted nucleic acid sequences of *CsMYB113* genes have been screened out from *C. sinensis* genome database and our transcriptomic data (Guo et al. 2017; Wei et al. 2018). The open reading frame (ORF) of *CsMYB113* was cloned by using 2×Ultra-Pfu Master Mix (Dye Plus) (Aidlab, Beijing, China) with gene specific primers (forward primer: 5'-ATGGAAGGTGTTCCTTTAGGAG-3'; reverse primer: 5'-TCAAAGATCCCAAAGGTCCAT-3'). PCR products was ligated into pTOPO-Blunt Simple vector (Aidlab, Beijing, China) and checked through sequencing (TSINGKE, Beijing, China). ProtComp 9.0 of softberry (http://linux1.softberry.com) was used to predict the subcellular localization signal of CsMYB113. ProtParam tool (http://web.expasy.org/protparam/) was used to calculate the theoretical molecular weight and isoelectronic point (pI). The ScanProsite (Expasy; SIB Swiss Institute of Bioinformatics, Switzerland) was used to analyze conserved motifs of CsMYB113 proteins. DNAMAN version 6.0 was utilized to multiple sequence alignment analysis.

Based on the Neighbor-joining (NJ) method with 1000 bootstrap replications, molecular Evolutionary Genetics Analysis (MEGA) version 7.0 was used to construct phylogenetic tree. The sequences of R2R3-MYB family from ten plants including tea were downloaded from the BLAST of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). These above gene accession IDs are as follows: CsAN1 (KU745295), AcMYB110a (AHY00342), MdMYB1 (ADQ27443.1), MdMYB3 (AEX08668), MdMYB10 (ACQ45201.1), MdMYB110a (BAM84362.1), AtMYB90/PAP2 (75338996), AtMYB75/PAP1 (75333682), AtMYB123/TT2 (27151707), AtMYB113 (Q9FNV9), AtMYB114(Q9FNV8.1), VvMYBA1 (BAD18977), PcMYB10.6 (AKV89252.1), PcMYB10.1 (AKV89247.1), MrMYB1 (ADG21957), MrMYB2 (ADG21958),

TaMYB14 (AFJ53059), FaMYB11 (AFL02461), VvMYBPA1 (NP_001268160), VvMYBPA2 (ACK56131), ZmMYBP (NP_001278607), CsMYB5a (ATC41981.1), CsMYB5e (ATC41985.1), and CsMYB6A (AQW35194.1).

### 2.4 Gene expression analysis

The gene expression was detected by the real-time quantitative PCR (qRT-PCR) with the Applied Biosystems StepOne Plus™ Real Time PCR System (ABI, Foster City, USA). According to the manufacturer's instructions of 2×SYBR Green qPCR Mix (Aidlab, Beijing, China), the amplification program consisted of one cycle at 94 °C for 3 min, followed by forty cycles at 94 °C for 10 s, 60 °C for 34 s, then finally 60 °C for 1 h. The expression level of genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), Cs*GAPDH* (*Camellia sinensis*) and *AtACTIN2* (*Arabidopsis*) were employed as the internal reference genes to monitor gene expression. Each biological sample was examined with three technical replicates. The gene-specific oligonucleotide primers utilized for qRT-PCR were shown in Supplementary Table S1.

### 2.5 Subcellular Localization analysis

To determine the subcellular localization, The ORF of *CsMYB113* (without the stop codon) was sub-cloned into the BamHI and XbaI site of pCAMBIA2300-GFP vector (Wang et al. 2014) to fuse with GFP by using One Step Cloning Kit (Vazyme, Nanjing, China). Transient expression in tobacco (*Nicotiana benthamiana*) leaves was performed as described previously (Sparkes et al. 2006). With the empty vector as a control*, Agrobacterium tumefaciens* (*GV3101*) harboring *35S::CsMYB113-GFP* and nucleus co-localization marker CBLn-RFP (red) were infiltrated into 4 weeks old tobacco epidermal cells together. The GFP fluorescence in transformed cells was detected under a confocal microscope (Leica, SP8) within 36-48 h. All transient expression experiments were repeated independently more than three times.

### 2.6 Transformation of *Arabidopsis* with *CsMYB113*

The plant expression vectors were constructed by using the Gateway Cloning System (Invitrogen, New York, USA). The completed ORF of *CsMYB113* was cloned into pDONR221 by BP reaction, and then inserted into a pH2GW7 vector by LR reaction. The expression vector (*35S::CsMYB113-pH2GW7*) was transferred into *Agrobacterium tumefaciens* (strain GV3101). The *Arabidopsis* transgene lines were subsequently conducted using the floral-dip method described previously (Clough and Bent 1998). Transgenic plants were choosing according to their resistance of hygromycin (Hyg). Putative transgenic *Arabidopsis* plants were selected on the Murashige and Skoog (MS) solid medium adding Hyg (50 mg/L). The positive transgenic plants were further checked by genomic PCR and qRT-PCR analysis. The homozygous $T_3$ transgene lines were collected for anthocyanin content and qRT-PCR analysis

### 2.7 Transient overexpression of *CsMYB113* in the leaves of tea plant

Transient expression assays in the leaves of tea plant were conducted as reported by Mo et al. (Mo et al. 2015), with some modification. *Agrobacterium tumefaciens* (strain GV3101) harboring empty vector and *35S::CsMYB113-GFP* were cultured overnight in 50ml LB liquid medium with rifampicin (50 mg/L) and spectinomycin (100 mg/L), respectively. After centrifuged, re-suspended and incubated, the suspensions of *Agrobacterium tumefaciens* harboring empty vector and *35S::CsMYB113-GFP* were injected into the different sides of the same leaf using a 1 ml plastic syringe, respectively. Ten days after infiltration, different leaves were collected for the GFP fluorescence observation (Leica DMi8) and anthocyanin biosynthesis analysis.

### 2.8 Anthocyanin extraction and measurement

Anthocyanin in leaves of tea plant was extracted as described previously (Sun et al. 2016). After extracted, the absorbance level of supernatant was detected in two absorbencies (535nm and 650nm), and the contents of anthocyanin were described as $(A_{535}–A_{650})\ g^{-1}$ fresh weight (FW). Each sample was calculated from three repeats. Anthocyanin in different tissues of *A. thaliana* was measured according to (Chen et al. 2018). The supernatant was measured in two absorbencies (530nm and 657nm), and the contents of anthocyanin were described as $A_{530}–0.25\ A_{657}$. Each sample was calculated from three repeats.

### 2.9 Statistical analysis

All data were expressed as a mean value from three technical replicates with error bars indicating ±SD. The one-way ANOVA analysis of variance was used for identification of significant difference. The results were considered statistically significant and indicated with asterisks when $P<0.01$. Different letters indicate significant differences when $\alpha= 0.05$ by using the multiple comparisons.

## Result

### 3.1 Cloning and sequence analysis of *CsMYB113*

The *CsMYB113* gene was cloned from the tea plant cultivar '*Fudingdaba*' by RT-PCR. The complete ORF sequence is 726 bp (Fig. 1B) which encoding for a polypeptide of 241 amino acids. The isoelectric point (pI) and molecular weight (MW) were 9.88 and 27.96 kDa, respectively. The gene sequence BLASTx against the *Arabidopsis* showed the highest similar with *AtMYB113*, and then this gene was named as *CsMYB113*. Sequence analysis via the ScanProsite program revealed that the CsMYB113 protein contained two myb-type HTH (helix-turn-helix) DNA-binding domain profiles at N-terminus. Furthermore, the amino acid sequences similar with CsMYB113 were selected in other species, and multiple sequence alignment was performed. The result suggested that the R2 and R3 domains were highly conserved in these species. Moreover, 13th, 33rd, and 53rd positions of the R2 domain encoded the same tryptophan residues, which were conducive to keep the stability of the 'helix-turn-helix' configuration of the MYB protein domain (Fig. 1C and 2B). Phylogenetic analysis indicated CsMYB113 was divided into subgroup 6 (S6) and similar with MYBs involved in anthocyanin biosynthesis regulation, suggesting that CsMYB113

may play important role in the anthocyanin metabolism regulation. CsMYB113 shared the highest amino acid sequence identity with tea plant CsAN1 (62%) and kiwifruit AcMYB110 (57.5%), respectively.

### 3.2 Relative gene expression analysis of *CsMYB113* and the subcellular localization of its protein

To investigate the expression patterns of *CsMYB113* gene in various tissues, qRT-PCR analysis was conducted to evaluate mRNA expression levels separated from ten tissues of '*Fudingdabai*'. As shown in Fig. 3A, *CsMYB113* transcripts accumulated in all analysed tissues. Overall, the transcript levels of *CsMYB113* were the highest in root, and decreased in the first leaf (FL). Second leaf (SL) showed the lowest expression level, which had no significant difference with other tissues, including seed, bud, third leaf (TL), mature leaf (ML), old leaf (OL), stem, and flower.

To determine the subcellular localization of CsMYB113, the full-length ORF was fused in pCAMBIA2300 vector with the GFP reporter gene driven by the 35S CaMV promoter. Then the construct was transformed into Agrobacterium and then infiltrated into tobacco epidermal cells. The results showed that GFP fluorescence in control (pCAMBIA2300-GFP) was ubiquitous distribution throughout the cell, while the GFP signal of CsMYB113-GFP overlaps with the nucleus co-localization fluorescence signal (as shown in pseudo colours green and red, respectively) (Fig. 3B). It is clearly indicated that CsMYB113 is exclusively localized in the nucleus of plant cell, which is similar with the CsAN1 (Sun et al. 2016). These results indicated that CsMYB113 is a transcription activator.

### 3.3 Overexpression of *CsMYB113* increased the anthocyanin contents in transgenic *Arabidopsis*

To determine the potential role of CsMYB113 in the regulation of anthocyanin biosynthetic pathway, the *35S::CsMYB113* construct was employed to ectopically activated CsMYB113 expression into *Arabidopsis* (Col-0). There was visible phenotypic difference between Col-0 (wide type) plants and the transgenic lines after growing in a 16/8-h (light/dark) photoperiod under illumination of 10000lux in growth chamber. The overexpression of CsMYB113 resulted in the accumulation of anthocyanins in hypocotyl, veins, stems, seeds and roots (Fig. 4A-F). And the anthocyanin contents in four tissues was significantly increased in transgenic *Arabidopsis* compared with wild-type (Fig. 4G and H).

Further research showed that in transgenic lines, the anthocyanin contents in leaf, stem, root, and seed increased by 6.7- , 41.7- , 29.0- , and 4.5- fold compared with the wild-type (average of the three lines), respectively. It's indicated that the relative anthocyanin contents had significant differences in four tissues (Fig. 5A). In order to verify whether the phenomenon is caused by the differential expression of CsMYB113, we detected the expression levels of *CsMYB113* gene in four tissues of wild-type (WT) and three overexpressing homozygous lines. The results showed that the successive decreasing order of the expression levels was tender stem, root, leaf, seed (average of the three lines), which had the same trend as the increase folds of anthocyanin contents (Fig. 5A and B). Therefore, we supposed that the differential expression of the *CsMYB113* gene in four tissues lead to the differences in the anthocyanin contents. At the same time, combined with the results of CsMYB113 is root-specific expression in *C.*

*sinensis*, which could further prove that the expression of *CsMYB113* gene has obvious organizational differences.

### 3.4 Overexpression of *CsMYB113* increased the expression levels of anthocyanin biosynthetic genes

R2R3-MYB TFs play important roles in activating structural genes involved in the anthocyanin biosynthesis. To further study the regulation of *CsMYB113* gene, the expression levels of eight structural genes (*AtPAL*、*AtCHS*、*AtCHI*、*AtF3H*、*AtF3'H*、*AtDFR*、*AtLDOX*、*AtUF3G*) were detected between four tissues of wild-type and transgenic lines, respectively. In general, compared with the wild-type, overexpressing of *CsMYB113* could strongly increase the expression levels of *AtCHI*、*AtF3H*、*AtDFR*、*AtLDOX* and, *AtUF3G* (Fig. 6). In four tissues, the expression level of *F3H* gene was the most significant up-regulated by 5.5-fold in leaves (Fig. 6A), whereas the expression levels of *CHI*, *F3H*, *UF3G* in stems were increased by 13- , 39-, and 114 fold, respectively (Fig. 6B). *F3H*, *DFR* and *UF3G* genes in roots were up-regulated by 42- , 20- , and 40 fold, respectively (Fig. 6C). The expression of each gene in seeds is less than 4- fold (Fig. 6D). These results indicate that CsMYB113 can promote the expression levels of anthocyanin biosynthetic genes, thereby regulating the synthesis and accumulation of anthocyanin. However, the regulation profile has a certain difference, as *CsMYB113* gene mainly up-regulated different structural genes in the four tissues.

### 3.5 Transient expression of GFP protein in leaves of tea plants

With the development of research, transient transformation system has been established in many plants. It has the characteristics of high efficiency, short cycle and fast realization of gene function verification, and has been widely used in herbs (tobacco, tomato, *Arabidopsis*, rice) and woody plants (citrus, poplar). In order to investigate whether this system was suitable for the leaves of tea plant, we constructed a transient expression vector *pK7WG2D* with GFP protein as a reporter. The suspensions bring CsMYB113-GFP plasmid was injected into the leaves of three tea cultivars types ('*Fudingdabai*', '*YingShuang*' and, '*Wuniuzao*'). After infiltration, we detected the GFP fluorescence 10 days later using the inverted microscope. In the non-transformed control, we did not observed any GFP fluorescent signal in the leaves (Fig. 7A2-C2). However, fluorescent signals were obviously detected in the infiltrated parts of the tea plant leaves (Fig. 7A4-C4). These results indicated that the transient expression system could be applied in leaves of tea plant.

### 3.6 Transient Overexpression of *CsMYB113* Stimulated anthocyanin accumulations in the leaves of tea plant

As we found the transient expression system can be applied in leaves of tea plants. In order to further analysed the function of *CsMYB113* gene by using the expression system. We determine the content of anthocyanin and the expression level of *CsMYB113* gene in leaves (Figure 8). Leaves transformed with empty vector *pK7WG2D* (a) and target gene *CsMYB113* (b) were collected respectively. Control was set as the non-transformed leaves (ck). The results showed that the whole leaves grew well and were only slightly damaged near the injection hole. Moreover, phenotypic differences were observed among

different treatments. Transformed with target gene *CsMYB113* (b) appeared slight purple spots in leaves of '*Fudingdabai*' and '*Wuniuzao*' (Fig 8A-C). The anthocyanin contents were significantly increased in leaves transformed with CsMYB113 gene (Fig 8D). There was an almost 2-fold increase in leaves of three cultivars ($p<0.01$).

Meanwhile, qRT-PCR analysis showed that there was a significantly increased in the expression level of *CsMYB113* gene in transformed leaves (Fig 8E). Compare with the non-transformed leaves (ck), the expression level is increase almost 4.5 times in '*Fudingdabai*', and almost 2 times in '*Wuniuzao*' and '*Yingshuang*'. The expression effect in '*Fudingdabai*' was better than that in the other two varieties. These results above further evidence that the *CsMYB113* gene could transient expression in leaves of tea plant and the existence of *CsMYB113* could accelerate the synthesis of anthocyanin in tea leaves to a certain extent.

# Discussion

As a large subclass of MYB family, the R2R3-MYB transcription factor genes play important roles in anthocyanin metabolism regulation. In this research, an R2R3-MYB TF named *CsMYB113* was successfully cloned from tea plant leaves. According to domain organization and phylogenetic analysis, CsMYB113 belongs to the S6 subgroup, which is important for the anthocyanin metabolism regulation (Liu et al. 2015). The protein sequence of CsMYB113 showed highest similarity with the CsAN1 protein (62%) from *Camellia sinensis*. In the 'Zijuan' tea, the activation CsAN1 has been proved specifically unregulated anthocyanin biosynthesis genes to cause abnormal anthocyanin accumulation (Sun et al. 2016). A subcellular localization study showed CsMYB113 is located in the nucleus (Fig. 3B), indicated that CsMYB113 may act as a transcription factor. Taken as a whole, it indicate that CsMYB113 may functions as transcription activating factor involved in the anthocyanin metabolism regulation in tea plants.

Further ectopic transgenic studies were implemented by overexpression of CsMYB113 in *Arabidopsis* plants. As compared to the wide-type of *Arabidopsis*, the overexpressed lines were obviously turned to purple in the $T_3$ homozygous plants, which was in accordance with obviously increased anthocyanin contents (Figure 4). This finding is consistent with the exogenous gene expression patterns seen in *Arabidopsis* of other R2R3-MYB anthocyanin activator genes, such as MdMYB1 from apple (Takos et al. 2006), PUPRLE from cauliflower (Chiu et al. 2010), PyMYB10 from pear (Feng et al. 2010), EsMYBA1 from Herba epimedii (Huang et al. 2013a), MrMYB1 from Chinese bayberry (Huang et al. 2013b), BrMYB2 from Chinese cabbage (He et al. 2020), and FhPAP1 from *Freesia hybrid* (Li et al. 2020).

The R2R3-MYB TFs, which regulating the synthesis of anthocyanin, are playing different roles in the various tissues of plants. In corn, the *C1* gene regulates the biosynthesis of anthocyanin in aleurone (Cone et al. 1986). In *GMYB10* overexpression transgenic tobacco plants (*Nicotiana tabacum*), the leaves, stems, and reproductive tissues turned to purple while no significantly anthocyanin accumulation in petal (Elomaa et al. 2003). Compared with the wild type, the *MdMYBA* overexpressing transgenic tobacco

plants showed obviously increased anthocyanin in the reproductive tissues (Ban et al. 2007). In the *MdMYB3* overexpression tobacco lines, the significantly increased anthocyanin pigmentation was observed in various tissues (Vimolmangkang et al. 2013). When ectopic expressed *PyMYB10* in *Arabidopsis*, the immature seeds accumulated significantly increase anthocyanin content (Feng et al. 2010). In tea plants, the R2R3-MYB TFs showed abundant expression patterns, such as *CsMYB4a* expression was significantly higher in mature leaves, *CsMYB42* is specifically expressed in pollen tubes, *CsMYB47* and *CsMYB17* have the highest expression levels in leaves and buds (Li et al. 2017b; Wang et al. 2019; Chen et al. 2021). In this research, the contents of anthocyanin were determined in various tissues of transgenic Arabidopsis. It is worth noting that there were obvious differences in the level of accumulation of the anthocyanin in the different tissues (leaves, stem, roots, and seeds). The increments of anthocyanin in stem and roots were much higher than that in leaves and seeds (Fig. 4). The leave veins were obviously turned to purple while the mesophyll cells showed green color. We conclude that the *CsMYB113* may play different roles in regulating the synthesis of anthocyanin among various tissues.

It is well known that MYB can promote gene expression levels of anthocyanin biosynthesis to active the anthocyanin accumulation. In transgenic cauliflower plants, up-regulation of *Purple* (*Pr*) gene specifically activated three genes involved in anthocyanin biosynthesis which encoding *F3'H*, *DFR*, and *LDOX* (Chiu et al. 2010). In *35S::LfMYB113* transgenic *Nicotiana tabacum* plants, the expression levels of anthocyanin biosynthetic pathway genes were significantly increased including *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, and *UFGT* (Wen and Chu 2017). When ectopic expressed *GhMYB1a* in tobacco, the expressions levels of *CHS* and *F3H* were significantly up-regulated than other genes related to the anthocyanin biosynthesis (Zhong et al. 2020). In the present study, the genes (*AtPAL*、*AtCHS*、*AtCHI*、*AtF3H*、*AtF3'H*、*AtDFR*、*AtLDOX*、*AtUF3G*) related to anthocyanin biosynthesis were significantly increased in transgenic *Arabidopsis* (stem, root, and seed) overexpressing *CsMYB113*. The relative expression levels increased in stem and root was much higher than that in the seed. There only five genes (*CHI*、*F3H*、*DFR*、*LDOX*、*UF3G*) were significantly increased in the leaves of transgenic *Arabidopsis* lines. These changes are highly consistent with the anthocyanin contents in the different tissues of transgenic lines. MYB TFs have been proved to regulate anthocyanin metabolism by combing with bHLH TFs in many plants (Gonzalez et al. 2008; Liu et al. 2018b; Li et al. 2017a). We conclude that CsMYB113 can integrate with tissue-specific bHLH to increase the transcription levels of anthocyanin biosynthesis, lead to the anthocyanin content increment in different tissues.

In tea plants, the application of stable genetic transformation was limited by the problems of low transformation efficiency and difficulty in vitro regeneration (Mondal et al. 2004). Transient transformation system has many advantages compare to stable transformation, such as short period (the expression levels of genes could be analysis less than 12h after transformation), high efficiency, easy to operation, and wide range of acceptor materials. Therefore, it has been frequently used for the gene function study in strawberry (Hoffmann et al. 2006), grape (Urso et al. 2013), orange (Jia and Wang 2014), persimmon (Mo et al. 2015) and other woody plants. In order to identify the possible function of *CsMYB113* gene in tea plants, the homologous transient expression system was applied in this study. We screen out three cultivars ('*Fudingdabai*', '*YingShuang*' and '*Wuniuzao*'), which were more suitable for

transform. Transient transfection of tea plant leaves with the *CsMYB113* overexpression caused the abnormal anthocyanin increment (Fig. 8), which is consistent with the result in *Arabidopsis*. It proved that *CsMYB113* play a vital role in the anthocyanin regulation in tea plants.

# Conclusions

In the research, a R2R3-MYB TF CsMYB113 related to the regulation of anthocyanin biosynthesis was evaluated from tea plants. CsMYB113 was proved to localize in nucleus. Compared with wild type, some tissues (leaves, stems, roots and, seeds) were observed increased anthocyanin pigmentation inconsistent with the higher anthocyanin content in the *CsMYB113* overexpression *Arabidopsis* plants. The ectopic expressed *CsMYB113* in different tissues of transgenic *Arabidopsis* showed that the expression levels of genes related to anthocyanin biosynthesis were significantly enhanced. A distinguished anthocyanin content increment was detected in tea plant transient overexpression leaves. These results indicated that *CsMYB113* plays a vital role in the regulation of anthocyanin metabolism.

# Declarations

### Contributions

LS and FG designed the experiments. LS, MY and HL performed the experiments. LS and FG wrote the manuscript. PW, HZ, MW, YW and DN revised the manuscript. All authors provided helpful discussions and approved its final version.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


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# Figures

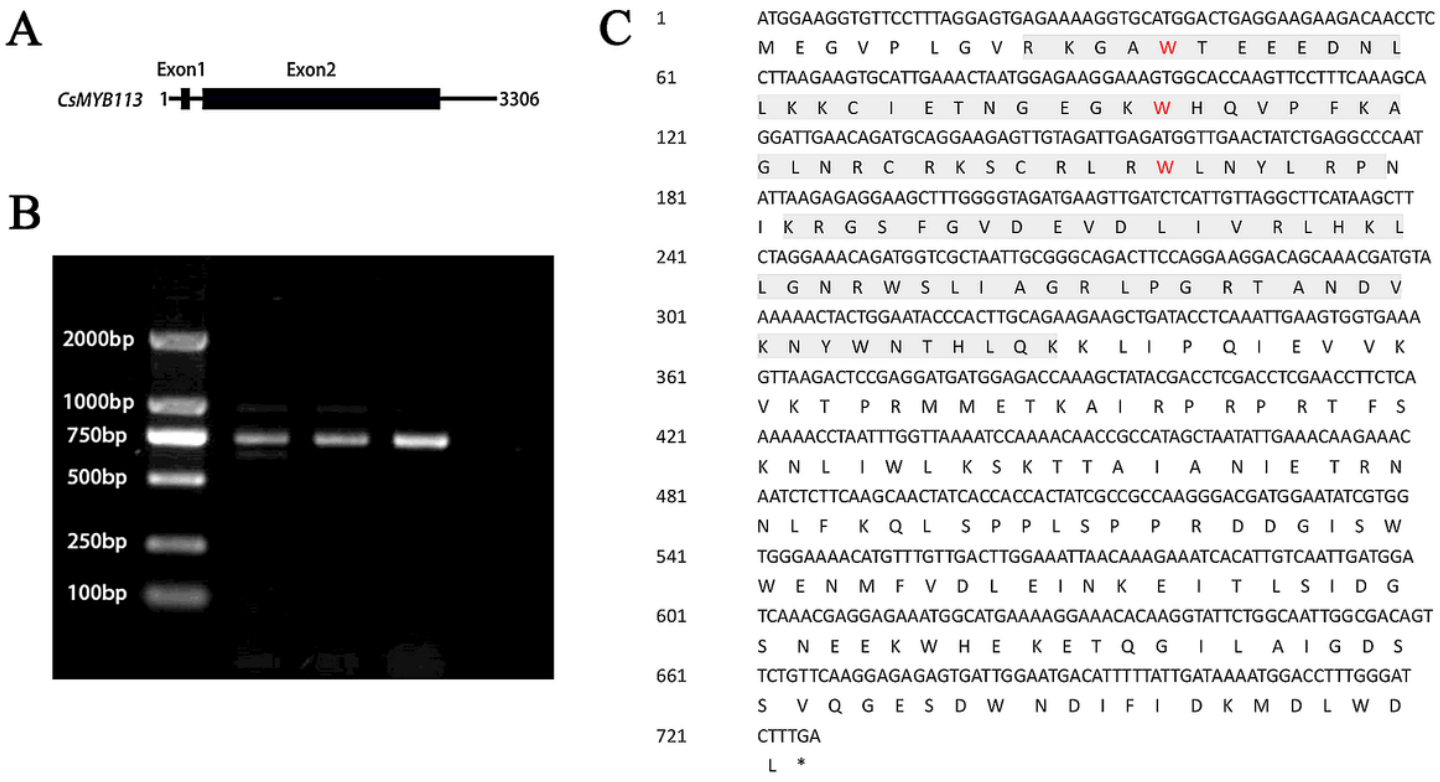
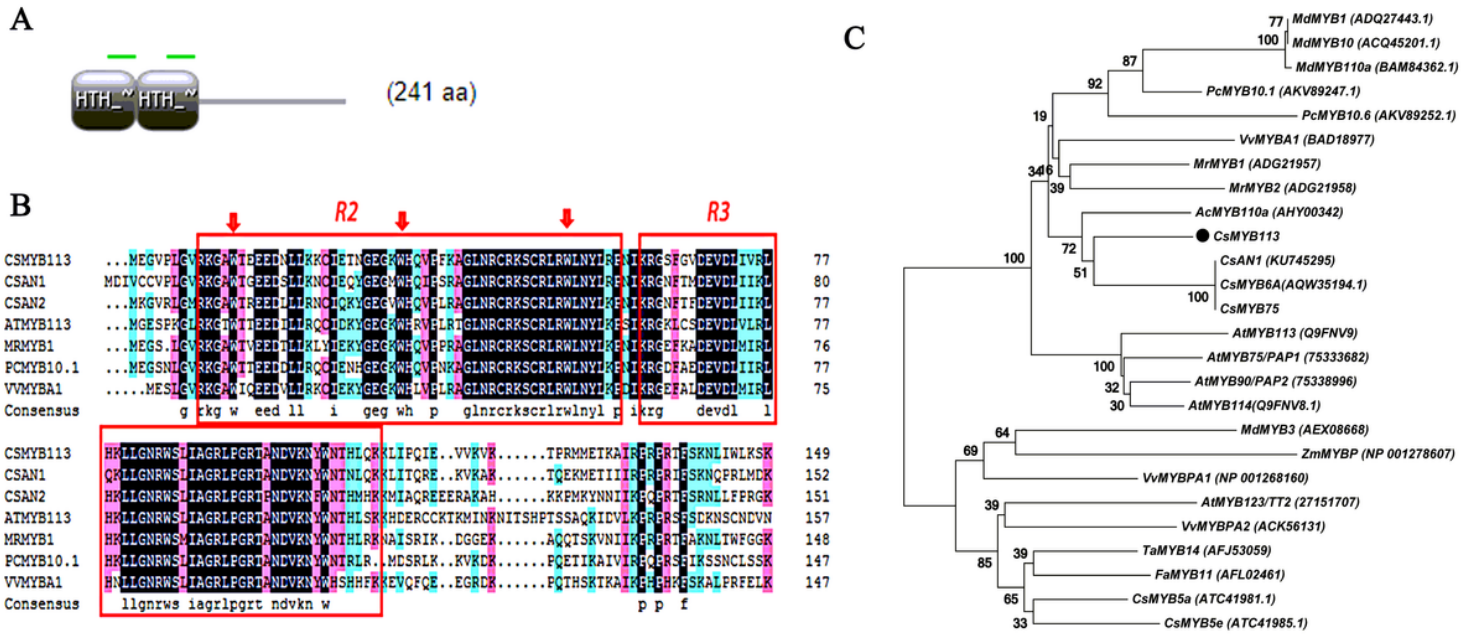


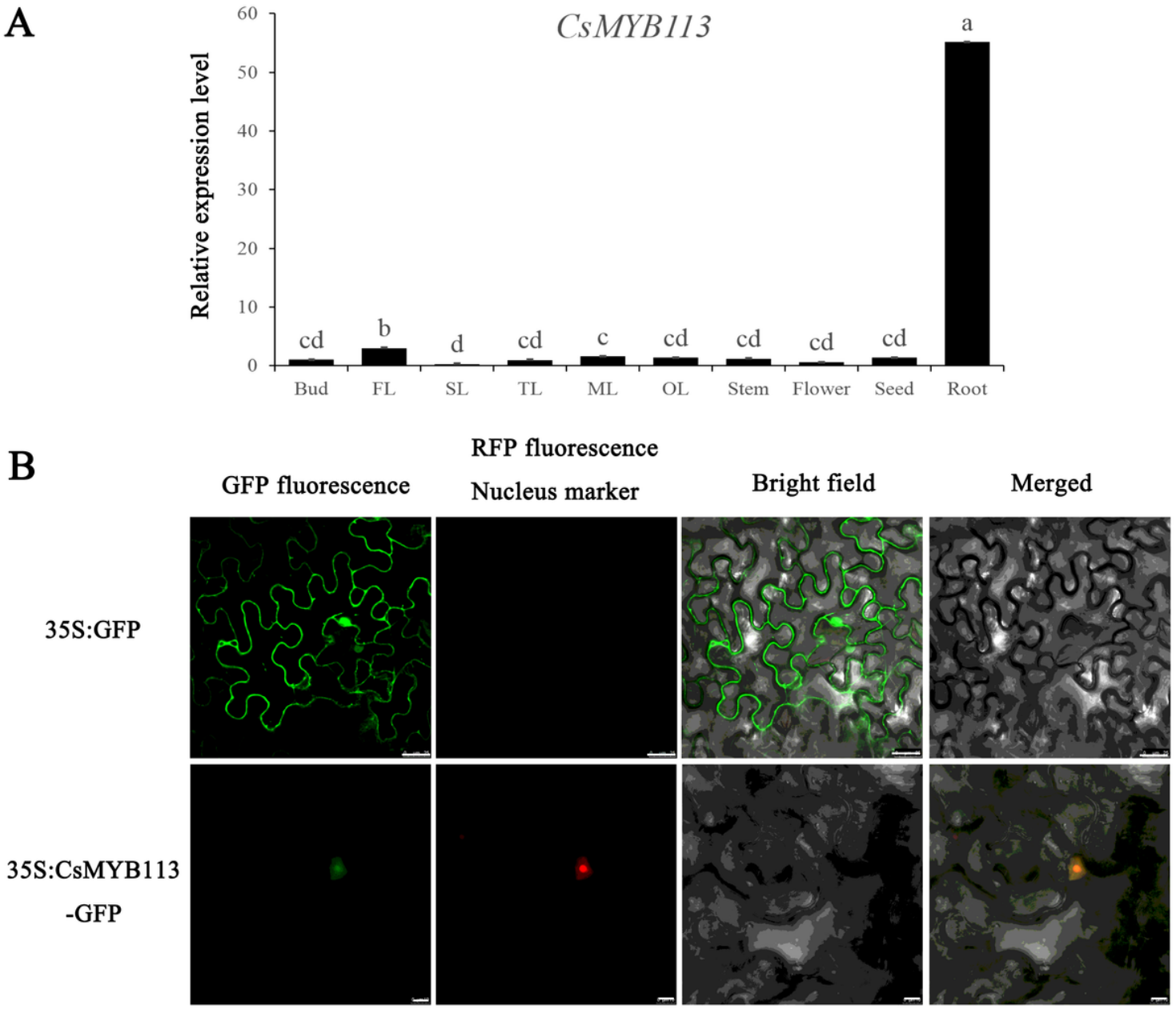

**Figure 1**

Identification and cloning of CsMYB113 gene from tea palnts. A, The predicted full-length of CsMYB113 gene; B, Gel map of amplification products; C, Nucleotides and amino acids sequences of CsMYB113.



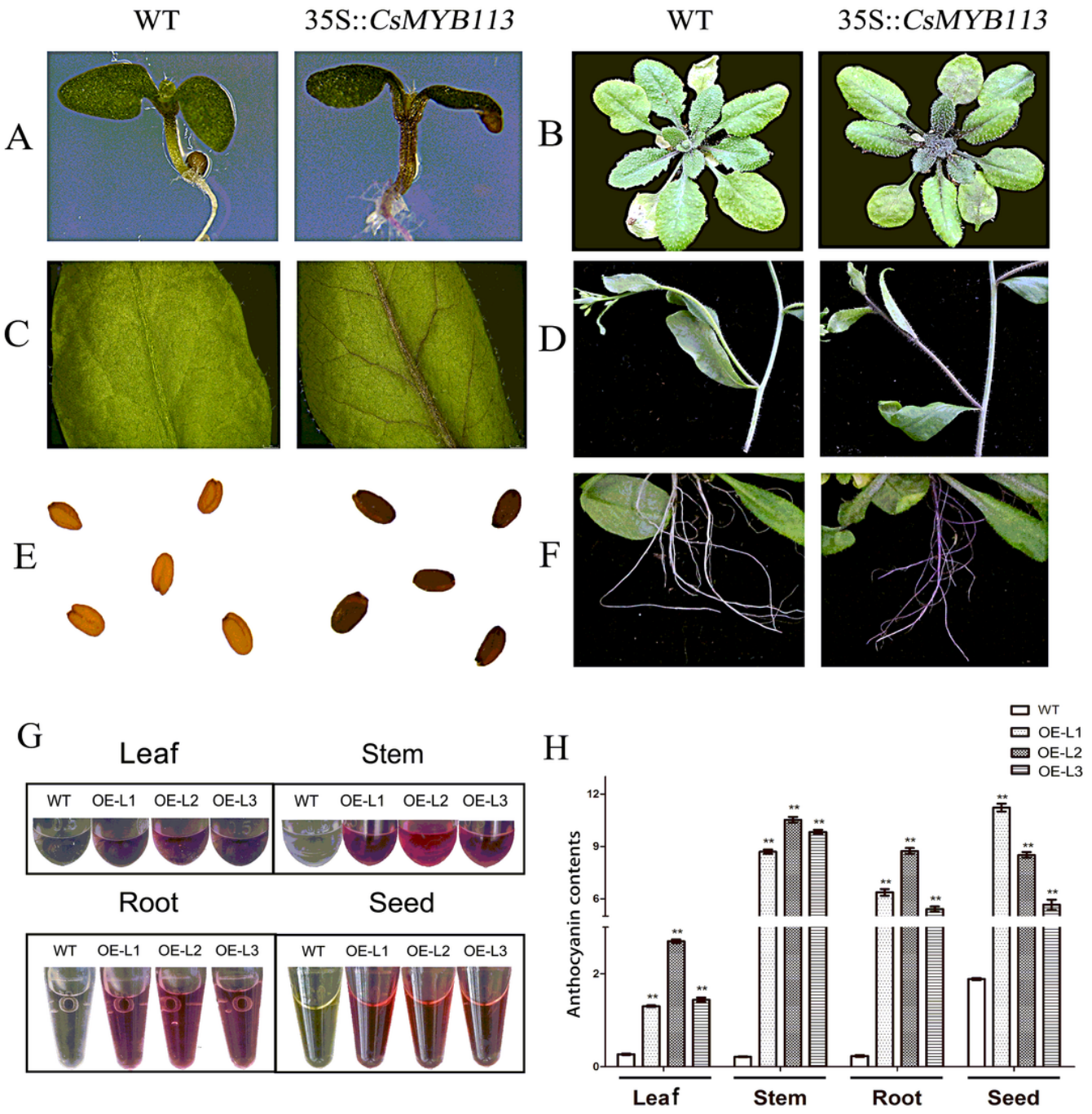

## Figure 2

Conserved motif and phylogenetic analysis of amino acid sequences of CsMYB113 and anthocyanin-associated R2R3-MYB transcription factors in other species. A, Conserved motifs analysis of CsMYB113; B, Multiple sequence alignment of CsMYB113 and reported anthocyanin associated R2R3-MYB transcription factors in other species. C, Phylogenetic tree of CsMYB113 and reported anthocyanin associated R2R3-MYB transcription factors in tea and other plants.



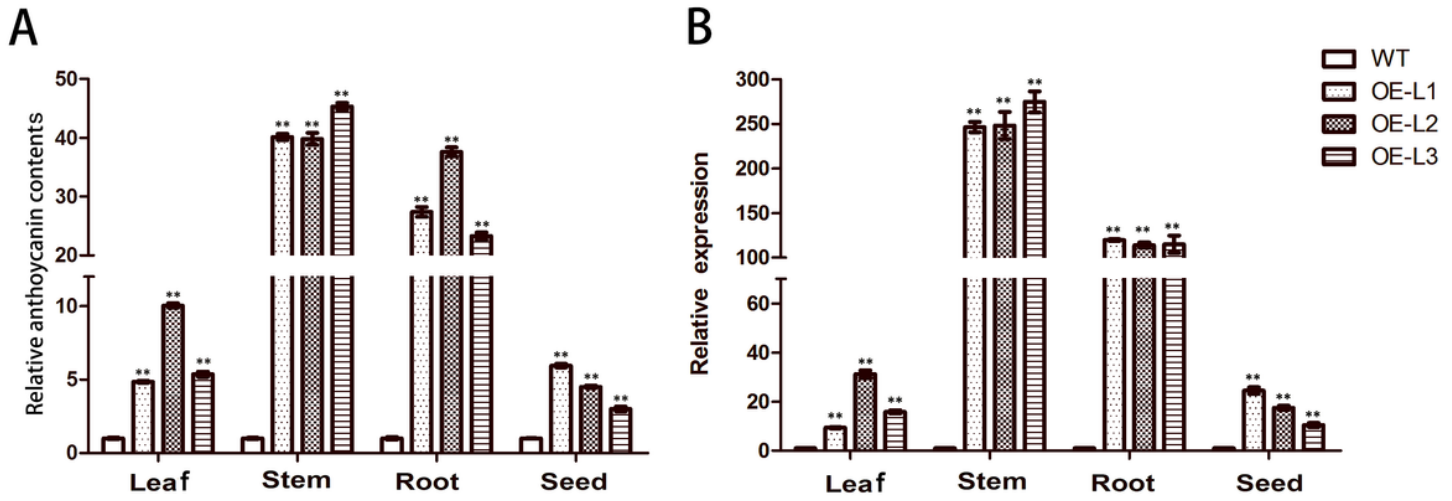

## Figure 3

Expression profile analysis of CsMYB113. A, The expression profile of CsMYB113 genes in different tissues and organs of ‘Fuding dabai’. Data (error bars) are means (±SD) obtained from three technical replicates. Different letters indicate significant differences (α=0.05). B, Subcellular localization analysis of CsMYB113 in tobacco epidermal cells. Scale bar is 25μm in the first row, 10μm in the second row.



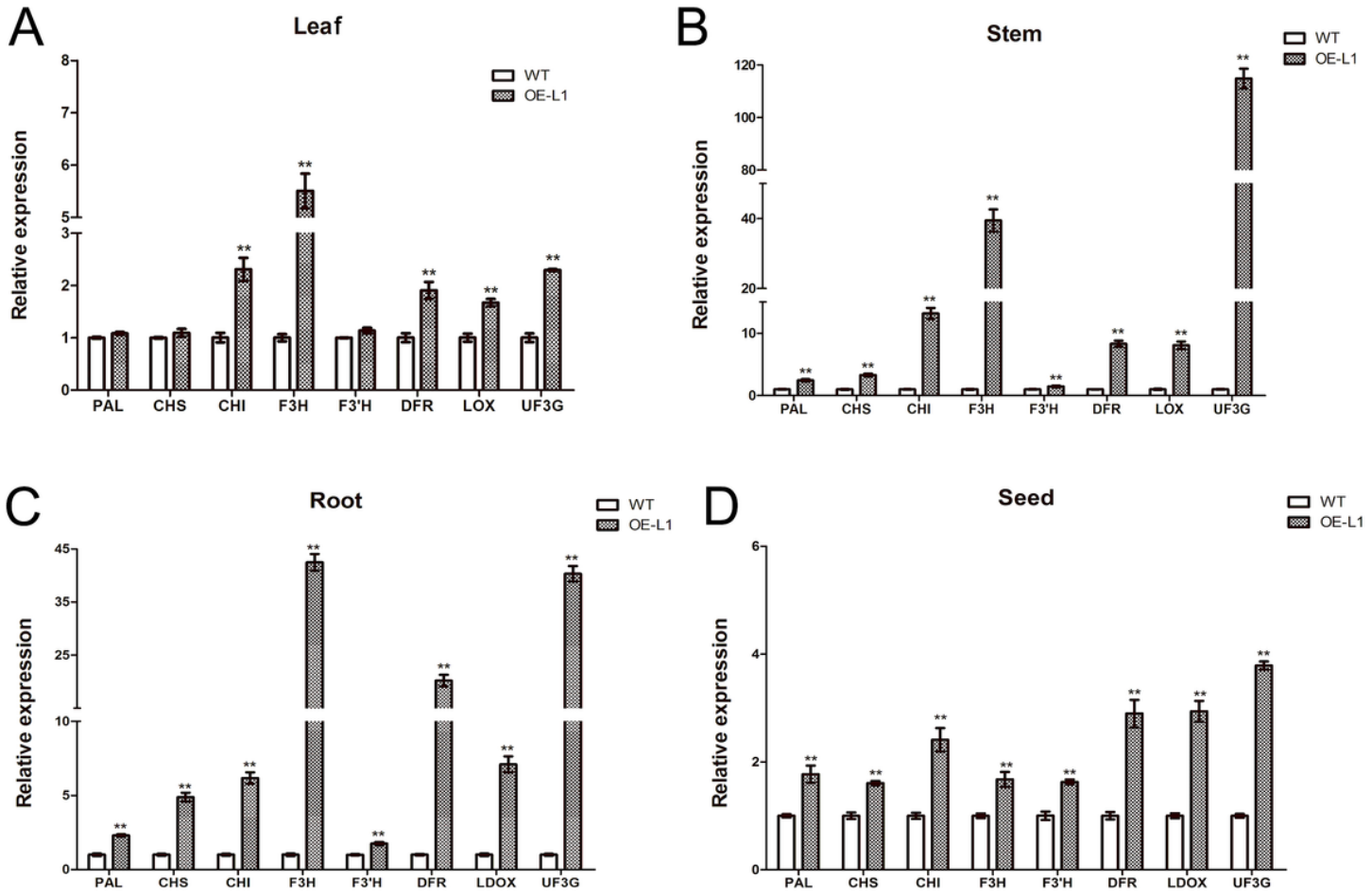

## Figure 4

Phenotypes and anthocyanin contents of transgenic lines and wild type in Arabidopsis. A, Phenotypes at hypocotyls of Arabidopsis seedlings. B, Phenotypes of growing period. C, Phenotypes of veins in the adult plants. D, Phenotypes of stems in the adult plants. E, Phenotypes of seeds. F, Phenotypes of roots. G, Colors of tube during extracting anthocyanins from different tissues. H, Anthocyanins contents in different tissues of Arabidopsis. Data (error bars) are means (±SD) obtained from three technical replicates, asterisks indicate significant differences (P<0.01).



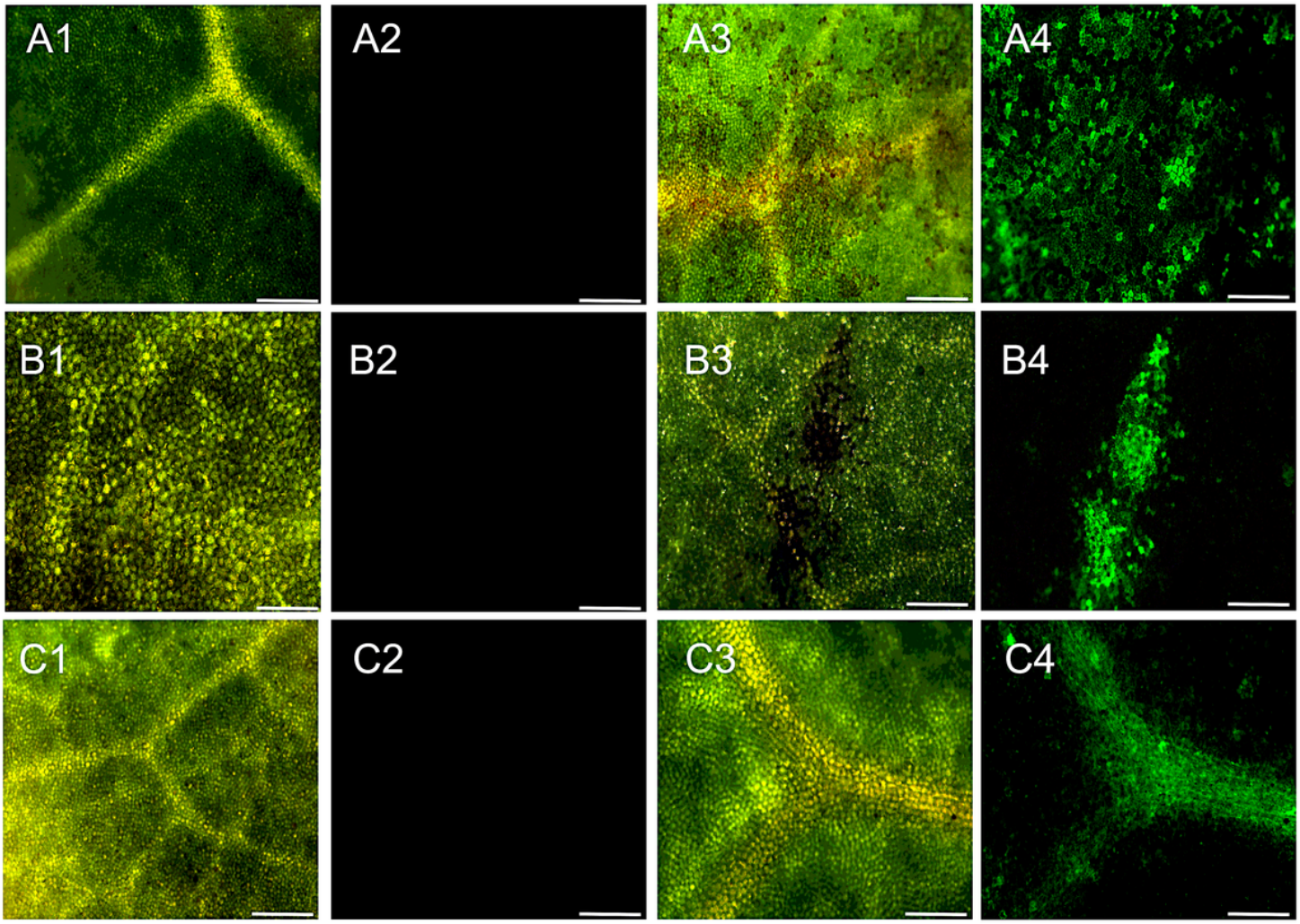

## Figure 5

Relative anthocyanin contents and expression level of CsMYB113 in different tissues of Arabidopsis. A, Increase of anthocyanin contents in different tissues of Arabidopsis. B, Relative expression of CsMYB113 in different tissues of Arabidopsis. Data (error bars) are means (±SD) obtained from three technical replicates. With wild type as control, asterisks indicate significant differences (P<0.01).



## Figure 6

Expression of structural genes of anthocyanin synthesis in different tissues of Arabidopsis. A, Leaf. B, Stem. C, Root. D, Seed. Data (error bars) are means (±SD) obtained from three technical replicates. With wild type as control, asterisks indicate significant differences (P<0.01)




## Figure 7

Transient expression of GFP in leaves of C. sinensis. A-C, Transient expression of GFP in ‘FD’(A), ‘YS’(B) and ‘WNZ’(C). 1, Non-transformed leaves in bright field. 2, GFP fluorescence in non-transformed leaves. 3, Transformed leaves of 35S::CsMYB113-GFP in bright field. 4, GFP fluorescence in transformed leaves of 35S::CsMYB113-GFP. Scale bars= 250 μm. ‘Fudingdabai’: FD, ‘YingShuang’: YS, ‘Wuniuzao’: WNZ.

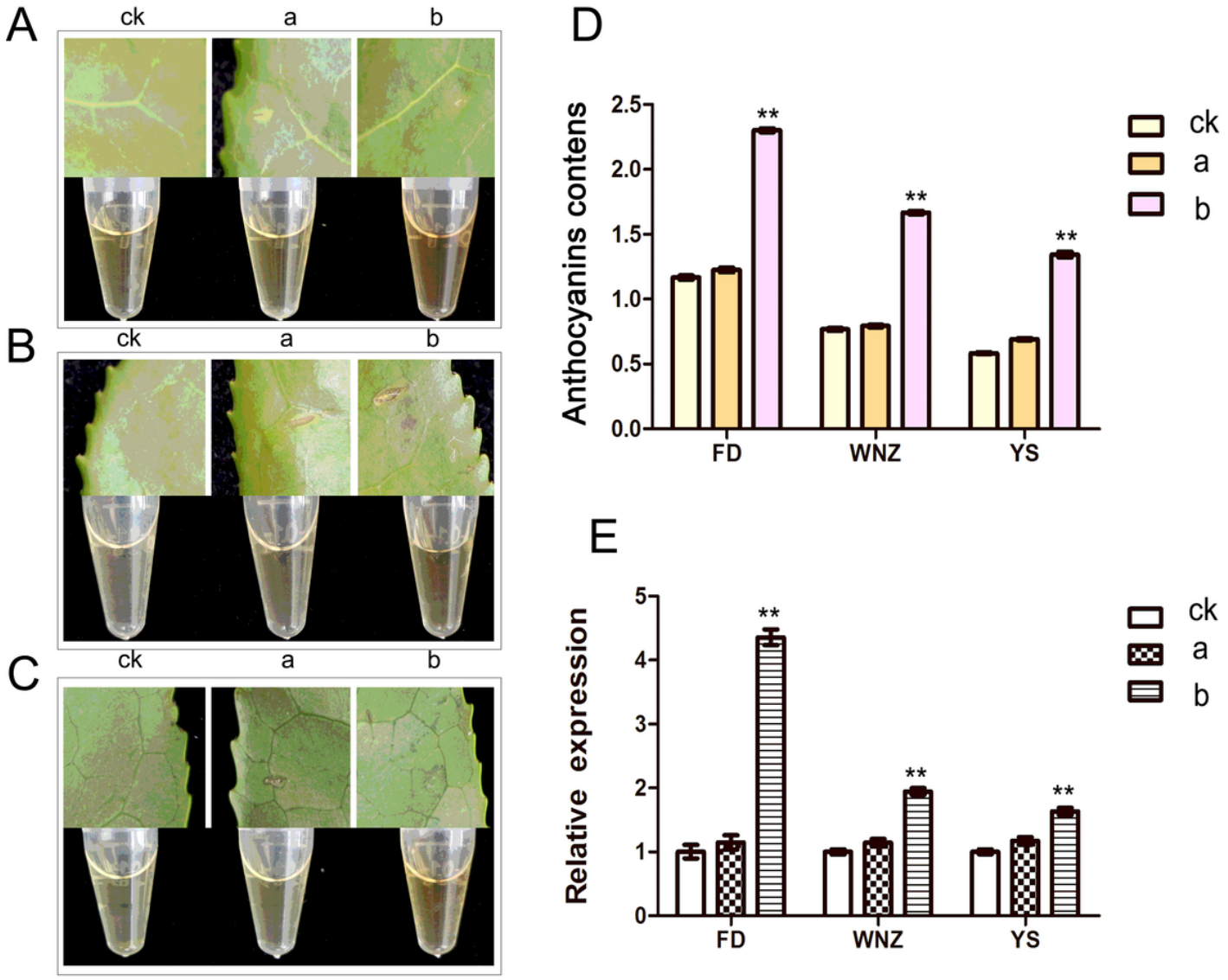



**Figure 8**

A-C: Phenotypes and different colors during extracting anthocyanins with different treatments of 'FD', 'YS' and 'WNZ'. D: Anthocyanin contents of "FD", "WNZ" and "YS" with different treatments; E: Relative expression of CsMYB113 of "FD", "WNZ" and "YS" with different treatments. ck: Non-transformed leaves; b: Transformed leaves with pK7WG2D; c: Transformed leaves with 35S::CsMYB113-GFP. Data (error bars) are means (±SD) obtained from three technical replicates. With ck as control, asterisks indicate significant differences ($P<0.01$). 'Fudingdabai': FD, 'YingShuang': YS, 'Wuniuzao': WNZ.

# Supplementary Files

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