Article

A TCP Transcription Factor in *Malus halliana, MhTCP4*, Positively Regulates Anthocyanins Biosynthesis

Jiaxin Meng †, Jiao Yin †, Han Wang and Houhua Li *

Abstract: Anthocyanins belong to a group of flavonoids, which are the most important flower pigments. Clarifying the potential anthocyanins biosynthesis molecular mechanisms could facilitate artificial manipulation of flower pigmentation in plants. In this paper, we screened a differentially expressed gene, MhTCP4, from the transcriptome data of *Malus halliana* petals at different development stages and explored its role in anthocyanins biosynthesis. The transcriptome data and qRT-PCR analysis showed that the expression level of MhTCP4 gradually decreased from the flower color fades. Tissue specific expression analysis showed MhTCP4 was expressed in the petal, leaf, and fruit of *M. halliana*, and was highly expressed in the scarlet petal. Overexpression of MhTCP4 promoted anthocyanins accumulation and increased pigments in infected parts of *M. ‘Snowdrift’* and *M. ‘Fuji’* fruit peels. In contrast, when endogenous MhTCP4 was silenced, the anthocyanins accumulation was inhibited and pigments decreased in the infected peels. The qRT-PCR analysis revealed that overexpression or silence of MhTCP4 caused expression changes of a series of structural genes included in anthocyanins biosynthesis pathway. The yeast two-hybrid assays indicated that MhTCP4 did not directly interact with MhMYB10. Furthermore, the yeast one-hybrid assays indicated that MhTCP4 did not directly bind to the promoter of MhMYB10, but that of the anthocyanins biosynthesis genes, MhCHI and MhF3′H. Dual luciferase assays further confirmed that MhTCP4 can strongly activate the promoters of MhCHI and MhF3′H in tobacco. Overall, the results suggest that MhTCP4 positively regulates anthocyanins biosynthesis by directly activated MhCHI and MhF3′H in *M. halliana* flowers.

Keywords: anthocyanins; MhTCP4; flower color; *Malus halliana*

1. Introduction

*Malus halliana* is a species of garden plants with high ornamental value. Flower color is an important ornamental value of plants. With the flower opening, color of *M. halliana* petals gradually fade. The process of flower fade can be divided into three stages, including small bud stage (S1), initial-flowering stage (S2) and late-flowering stage (S3). The color of petals at S1 is scarlet, S2 is pink and S3 is light pink to white. Our previous study has substantiated that the concentration of anthocyanins, cyanidin-3-O-galactoside, in the flower color fade [1]. At present, the pathway of anthocyanins biosynthesis has been basically identified, and the molecular regulatory network of that is also gradually being improved in *Malus* spp. The structural genes include phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonoid 3′-hydroxylase (*F3′H*), dihydroflavonol 4-reductase (*DFR*), anthocyanin synthase (*ANS*), and UDP-glucose: flavonoid 3-O-glucosyltransferase (*UGFT*), advance biochemical reactions in anthocyanin biosynthesis [2,3]. In addition, the protein complex MYB-Basic-Helix-Loop-Helix (bHLH)-WDR (MBW) has been known as the core regulator in anthocyanins biosynthesis [4,5]. In *Malus*, the three alleles, MmMYB1, MmMYB10, and MmMYBA, were widely reported to...
play positive roles in anthocyanins biosynthesis under nature or stress [6–8]. Moreover, the other transcription factor (TF) families were also reported as anthocyanins biosynthesis regulators in recent years. For example, PyWRKY26 and PybHLH3 co-targeted the promoter of PyMYB114 to enhance anthocyanins biosynthesis and transport in red-skinned pears [9]; and MdNAC52 positively regulates anthocyanins biosynthesis by interacting with MdMYB9 and MdMYB11 promoters [10].

In the recent years, increasing evidence suggests that TCPs play important roles in anthocyanins biosynthesis in plants [11]. TCP (TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL FACTORS) is a large TF family in higher plants. The TCP proteins play important roles in plant growth and development regulation, plant stress response, and multiple signal transduction pathways [12–14]. The typical feature of the TCP family is that it contains a bHLH conserved domain consisting of a longer basic region, two helix and a loop [15]. TCP family is divided into Class I and Class II according to its structural domain characteristics. The differences between them are as follows: one is that Class II has four more amino acids than the TCP domain of Class I, and the other is that the protein of Class II contains a specific R domain rich in 18–20 arginine and glutamate-cysteine-glutamate structure (ECE) [16,17]. Class II can further be divided into two clades, CYC/TB1 clade and CIN clade [15]. Most CYC/TB1-like proteins have R domain, like AtTCP1, AmCYC, and OsTB1, whereas most CIN-like proteins have lost R domain, like AtTCP3, AtTCP4, OsPCF5, and AmCIN [15,18–20]. Previous studies have shown that both Class I and Class II TCP proteins are involved in the regulation of anthocyanins biosynthesis. In A. thaliana, Class I transcription factor, AtTCP15, has been proved to regulate anthocyanins accumulation under high light [21]. And BrTCP15, a Class I TCP, inhibits anthocyanin biosynthesis pathway under low light treatments in Brassicarapa [22,23]. Class II TCP AtTCP3 interacting with R2R3-MYB proteins, promotes flavonoid biosynthesis [11]. There is also Class II TCP in Camellia sinensis that named CsTCP3 promoting the anthocyanins accumulation by interacting with CsTT8 and modulating the transactivation activity of the promoters of CsANS1 and CsANR1 [24].

In Malus, only one Class I TCP gene, MdTCP46, was found to interact with MdMYB1, and enhance the binding activity of MdMYB1 to its target anthocyanins biosynthesis gene promoters at high light intensity [25]. Thus, the identification and function analysis of Class II TCP related to anthocyanins biosynthesis regulation still need explored. In this study, a Class II TCP, named MhTCP4, was isolated from M. halliana petals. Transient over-expression and silencing assays revealed that MhTCP4 positively regulates anthocyanins biosynthesis in flower pigmentation. This study provides insights into the transcriptional regulatory mechanisms of TCP family, which increases understanding of the mechanism of pigmentation changes in flowers and therefore will contribute to breed plants with desirable color traits.

2. Results

2.1. Bioinformatics Analysis of TCP Transcription Factor Family in Malus halliana

In petals of M. halliana RNA-Seq data, 21 candidate TCPs were screened (Table S1). The expression heat map of FPKM from RNA-seq was shown in Figure 1A. Notably, the expression of MhTCP4 gradually decreased during flower development, with FPKM values ranging from 203.55 to 517.54, which were higher than that of other MhTCPs (Table S1; Figure 1A). In addition, the expression of MhTCP4 was significantly positively correlated with the anthocyanins content and the correlation coefficient was as high as 0.94 (Figure 1B). The anthocyanins content used in correlation heat map was cyanidin 3-galactoside content in M. halliana petals detected by HPLC-DAD, as reported in our previous study [1]. These results proved that MhTCP4 could play an important role in anthocyanins biosynthesis.
Figure 1. Expression and correlation analysis of MhTCPs in *Malus halliana*. (A) Heat map of the expression of MhTCPs in the petals at three stages. S1, small bud stage; S2, initial-flowering stage; and S3, late-flowering stage. MhTCP4 is marked by the black dot. (B) Heat map representing the correlation between MhTCPs expression and the anthocyanin content in the different development stages of petals. Yellow dot represents a positive correlation, and the blue dot represents a negative correlation. MhTCP4 is marked by the black dot.

Phylogenetic analysis was performed on the 21 candidate MhTCPs and 22 *A. thaliana* AtTCPs from the TAIR. The 21 MhTCPs were divided into 2 classes. Based on differences in their TCP domains, MhTCP2, MhTCP4, MhTCP5, MhTCP10-1, MhTCP10-2, and MhTCP13 were subdivided into CIN clade of Class II, and only MhTCP18 was subdivided into CYC/TB1 clade of Class II, meanwhile, other MhTCPs were divided into Class I (Figure 2A). Through MEME motif analysis, motif 1 and motif 2 were detected in all of 21 TCP protein sequences, which were the basic regions of TCP family (Figure 2B). Compared with class II proteins, the class I proteins were detected four-amino acid deletion in the basic domain (Figure 2C). To determine the chromosomal distribution of the candidate TCP genes, we mapped them into the *Malus × domestica* HFTH1 Whole Genome version 1.0 by the MapChart software. The results of chromosomal distribution of the candidate TCPs showed that 21 TCP genes were randomly distributed among 14 chromosomes, in which, Chr06 had three members; Chr10, Chr13, Chr16, and Chr17 contained two genes; and Chr01, Chr02, Chr03, Chr07, Chr10, Chr11, Chr12, Chr14 and Chr15 only contained one gene, respectively (Figure 2D).
Figure 2. Cont.
2.2. Sequence and Phylogenetic Analysis of MhTCP4

In order to explore the role of MhTCP4 in anthocyanins biosynthesis, the coding sequence of MhTCP4 from the petals of *M. halliana* in S1 stage was isolated. Sequence analysis showed that MhTCP4 gene encodes 546 amino acids. Secondary structure analysis of MhTCP4 protein showed that the conserved domain is mainly maintained by a long basic region, two α-helix and a β-turn containing 5 amino acids (Figure 3A). Then 19 orthologous proteins of MhTCP4 were identified by Blastp, including 11 TCPs in *A. thaliana*, 5 TCPs in rice, 2 TCPs in *Anthirrinum majus* and 1 TCP in maize. Subsequently, phylogenetic analysis of the 21 proteins showed that MhTCP4 was most closely related to OsPCF5, AtTCP3, AtTCP4, and AtTCP10 (Figure 3B). The amino acid sequence alignment of 20 homologous genes, including MhTCP4, shows that the TCP conserved region of these genes is a bHLH...
domain composed of about 60 amino acid residues (Figure 3C). Referring to the report of Jessica et al. [16], further amino acid sequence analysis confirmed that MhTCP4, AtTCP3, AtTCP4, AtTCP10, and OsPCF5 all belong to CIN clade (Figure 3D). These results suggested that MhTCP4 may have similar functions of these genes in CIN clade.

Figure 3. Cont.
were Blast, based on UniProtKB/Swiss-Prot database. The species, gene names, and GeneBank accession numbers are as follows: *Oryza sativa* Indica Group (OsTB1, Q8LN68.1), *Oryza sativa* Indica Group (OsPCF6, A2XMN1.1), *Oryza sativa* Indica Group (OsPCF5, A2WM14.1), *Zea mays* (ZmTEOSINTE BRANCHED 1, Q93WI2.2), *Antirrhinum majus* (AmDICHOTOMA, Q9SNW8.1), *Arabidopsis thaliana* (AtTCP1, Q9FYG7.1), *Arabidopsis thaliana* (AtTCP2, Q93V43.1), *Arabidopsis thaliana* (AtTCP3, Q9MAH8.1), *Arabidopsis thaliana* (AtTCP4, Q8LPR5.1), *Arabidopsis thaliana* (AtTCP5, Q9FME3.1), *Arabidopsis thaliana* (AtTCP12, A0AQW4.1), *Arabidopsis thaliana* (AtTCP13, Q9S7W5.1), *Arabidopsis thaliana* (AtTCP17, Q9LEZ9.1), *Arabidopsis thaliana* (AtTCP18, A1YKT1.1), *Arabidopsis thaliana* (AtTCP24, Q9C758.1), *Oryza sativa* Indica Group (OsPCF5, A2WM14.1), *Oryza sativa* Indica Group (OsPCF7, Q8LT05.2), *Oryza sativa* Indica Group (OsPCF6, A2XMN1.1), *Oryza sativa* Indica Group (OsPCF8, Q2QM59.1), *Oryza sativa* Indica Group (OsTB1, Q8LN68.1), *Zea mays* (ZmTEOSINTE BRANCHED 1, Q93WI2.2), *Antirrhinum majus* (AmDICHOTOMA, Q9SNW8.1), *Antirrhinum majus* (AmCYCLOIDEA, O49250.1).

2.3. Analysis of Tissue Specific Expression of MhTCP4 in *Malus halliana*

The expression pattern of MhTCP4 in different tissues of *M. halliana* was detected via qRT-PCR. The results showed that MhTCP4 gene was expressed in leaf, fruit, and petal, but the expression level in petal (S2) was significantly higher than that in fruit or leaf (Figure 4A). In addition, the qRT-PCR results showed that the expression of MhTCP4 gene gradually reduced from S1 to S3 stage in the petal of *M. halliana* (Figure 4B).
Figure 4. MhTCP4 tissue specific expression analysis and spatio-temporal differential expression analysis in petals of Malus halliana. (A) The tissue-specific expression of MhTCP4 was analyzed. The left y-axis denotes the RNA relative expression obtained by qRT-PCR. (B) Spatio-temporal differential expression of MhTCP4 in the petals of Malus halliana. S1, S2 and S3 represent the three different stages of the petals of Malus halliana. Error bars represent the SEs of three biological replicates and three technical replicates. * p < 0.05, ** p < 0.01 using the t-test.

2.4. Overexpression of MhTCP4 Promoted Anthocyanins Biosynthesis in Malus spp.

The obvious red pigmentation was observed in fruit peels of M. ‘Snowdrift’ and M. ‘Fuji’ infected by the bacterial liquid containing recombinant plasmid, respectively. In contrast, no significant phenotypic changes were observed in the peels infected by the empty vector (Figure 5A). Subsequently, the expression level of MhTCP4 in the experimental group and the control group was detected by qRT-PCR. The results showed that the expression level of MhTCP4 was up-regulated in the experimental group (Figure 5B). Besides, the anthocyanins from the peels of the infected site were extracted and detected by HPLC. The results showed that an anthocyanin, cyanidin 3-galactoside, was detected in fruit peels of M. ‘Snowdrift’ and M. ‘Fuji’ (Figure 5C). Compared with the control group, the content of cyanidin 3-galactoside in the fruit peels of M. ‘Snowdrift’ and M. ‘Fuji’ overexpressed MhTCP4 was increased by 1.2-fold and 2.5-fold, respectively (Figure 5C). From this point of view, the overexpression of MhTCP4 significantly promote the anthocyanins biosynthesis. To further clarify the effects of MhTCP4 overexpression on the molecular regulation pathway of anthocyanins biosynthesis, qRT-PCR analysis was performed on 7 structural genes including MhPAL, MhCHS, MhCHI, MhF3’H, MhDFR, MhANS, and MhUFGT. Compared
with the control group, the expression of six structural genes included MhPAL, MhCHI, MhF3’H, MhDFR, MhANS, and MhUFGT, except that of MhCHS showed an up-regulation trend, which are consistent with that of MhTCP4 (Figure 5D). The results indicated that the overexpression of MhTCP4 caused the up-regulation of the expression of anthocyanins biosynthesis pathway genes. Overall, these results suggested that the MhTCP4 gene overexpression promotes anthocyanins biosynthesis.

Figure 5. Cont.
Figure 5. Transient overexpression of MhTCP4 in fruit peels of Malus ‘Snowdrift’ and Malus ‘Fuji’. (A) Fruit peel coloration around injection sites. MhTCP4-pBI121 were used for overexpression with the pBI121 vector. Empty vectors were the control. (B) The cyanidin 3-galactoside content of transgenic and control fruit peels. (C) The expression levels of MhTCP4 in MhTCP4-overexpression and control fruit peels. (D) The expression levels of anthocyanins biosynthesis genes in MhTCP4-overexpression and control fruit peels. Error bars represent the SEs of three biological replicates and three technical replicates. * p < 0.05, ** p < 0.01 using the t-test.

2.5. MhTCP4 Silencing Inhibits the Anthocyanins Biosynthesis in Malus spp.

Compared with the control group, the red pigmentation accumulation was not observed in fruit peels of M. ‘Snowdrift’ where MhTCP4 was silenced. In fruit peels of M. ‘Fuji’, a large amount of red pigmentation was accumulated after infection in control group, but the infected parts of ‘peels infected with MhTCP4-TRV2 were remained yellow (Figure 6A). In addition, obvious red pigmentation was observed on the non-infected parts (Figure 6A). Then we performed qRT-PCR detection on the peels of the infected sites in the experimental group and the control group, and the results showed that MhTCP4 gene in the experimental group was significantly down-regulated both in the infected parts in M. ‘Snowdrift’ and M. ‘Fuji’ (Figure 6B). The HPLC results showed that the content of cyanidin 3-galactoside in the infected parts of M. ‘Snowdrift’ peels and M. ‘Fuji’ peels in the experimental group was about 0.8-fold and 0.2-fold of that in the control group, respectively (Figure 6C). And it is speculated that endogenous silencing of MhTCP4 inhibits the anthocyanins biosynthesis in peels. In MhTCP4 silencing peels, the expression of structure genes showed a down-regulation trend, which was consistent with that of MhTCP4 (Figure 6D). Overall, these results suggested that the MhTCP4 gene endogenous silencing inhibits anthocyanin biosynthesis.
Figure 6. Cont.
2.6. MhTCP4 Not Interacts with MhMYB10

Our previous study substantiated MhMYB10 (HF36879) positively regulate anthocyanins biosynthesis in M. halliana [1]. It would be interesting to analyze whether MhTCP4 is capable of interacting with MhMYB10. As shown in Figure 7, all of these yeast co-transformants could grow normally on SD/-Trp-Leu. However, only the positive control (pGBK7-T5 + pGADT7-T) yeast strain grew normally and turned blue on SD/-Trp-Leu-His-Ade (supplemented with 40 mg mL\(^{-1}\) X-α-gal), whereas pGBK7-MhTCP4 + pGADT7-MhMYB10 and the negative control did not grow in the same medium (Figure 7). These results indicated MhTCP4 did not interact with MhMYB10.
were co-expressed with MhTCP4-AD (Figure 8A). Meanwhile, the negative control and other
values were used as positive control.

2.7. MhTCP4 Binds with and Actives Promoter of the Anthocyanins Biosynthesis Genes, MhCHI
and MhF3’H

To further determine the functions of MhTCP4 during anthocyanins biosynthesis,
its potential role in binding to the promoter sequences of target genes was examined.
In酵母双杂交实验, only proMhCHI-pHIS2 and proMhF3’H-pHIS2 could grow
on TDO (SD/-His/-Leu/-Trp) plate with 100 mM 3-AT when the fused pHIS2 vectors
were co-expressed with MhTCP4-AD (Figure 8A). Meanwhile, the negative control
and other combinations did not grow (Figure 8A). These results suggested that MhTCP4
could not bind to the promoters of the transcription factor, MhMYB10, but that of anthocyanins
biosynthesis genes, MhCHI and MhF3’H.

Figure 7. Yeast two-hybrid between MhTCP4 and MhMYB10. The CDS of MdTCP4 and MhMYB10
were fused to the pGBKT7 and pGADT7 vectors, respectively. The pGBKT7-53 + pGADT7-T vectors
were used as positive control.

Figure 8. Effect of MhTCP4 on the promoters of anthocyanins-related genes. (A) Y1H analysis
of the binding of MhTCP4 and the promotors related to anthocyanins biosynthesis. DDO, double-
dropout medium (SD/-Leu/-Trp); TDO, triple-dropout medium (SD/-His/-Leu/-Trp). (B) Luciferase
complementation imaging assays showing that MhTCP4 activates the promotors of MhCHI and
MhF3’H. (C) The luminescence intensity of promotors of MhCHI and MhF3’H increased by MhTCP4.
Values are means ± SE of three biological replicates. ** p < 0.01 using the t-test.
Then, the dual-luciferase assays were performed to detect whether MhTCP4 could activate the promoters of MhCHI and MhF3’H. As shown in Figure 8B, few LUC luminescence signals were observed from the vectors containing the 62SK and proMhCHI-LUC or 62SK and proMhF3’H-LUC. However, the vectors containing MhTCP4-62SK and proMhCHI-LUC or MhTCP4-62SK and proMhF3’H-LUC vectors produced more stronger LUC luminescence signals (Figure 8B). Compared with the empty vector, the overexpression of MhTCP4 significantly increased the luminescence signals (Figure 8B). Compared with the empty vector, MhTCP4 increased the MhCHI and MhF3’H promoter activities by 9.76-fold and 11.86-fold, respectively (Figure 8C). The results were consistent with luminescence signal phenotype. Collectively, these results proved that MhTCP4 activates MhCHI and MhF3’H expression by directly binding to their promoters.

3. Discussion

It is a common phenomenon that the flower color often gradually fades during development, which are due to the decreased of anthocyanins biosynthesis [26–28]. In this study, 21 candidate TCPs were screened from transcriptome data in M. halliana petals at S1, S2 and S3 stages. Among them, the expression of MhTCP4 was significantly positively correlated with anthocyanins content of flower development. Through sequence and phylogenetic analysis of MhTCP4, 19 orthologous proteins of MhTCP4 were identified. These orthologous proteins are reported to play important roles in flavonoid biosynthesis, leaf morphogenesis, hypocotyl elongation, petal growth and development, leaf development, cell proliferation, and photoperiod flowering regulation [12,18,20,22,29–31]. Among them, we found its amino acid sequence and motifs were more similar with AtTCP3, which belong to CIN clades of TCP Class II and reported as flavonoid biosynthesis promotor [11]. On account of the functional conservation of TCPs, we speculated that MhTCP4 may positively regulate anthocyanins biosynthesis as AtTCP3. In order to confirm the function of MhTCP4, the transient overexpression and silencing were performed in fruit peels of M. spp. The MhTCP4 overexpression in fruit peels of M. ‘Snowdrift’ and M. ‘Fuji’ induced cyanidin 3-galactoside accumulation and promoted the expression of structural genes related to anthocyanins biosynthesis. By contrast, anthocyanins content and structural gene expression decreased significantly when MhTCP4 were silenced in peels. Therefore, these results suggest that MhTCP4 is a functional TCP TF, which promoting anthocyanins biosynthesis.

Except AtTCP3, we have looked at another gene, OsPCF5, which also phylogenetically close to MhTCP4. In the previous studies, OsPCF5 and its homologous genes, including OsPCF8 and OsPCF6, have been reported as important regulators response to low temperature stress [14,32]. Another homologous gene, OsPCF7, is an important regulatory gene for rice growth and development, and plays multiple roles in rice plant architecture [33]. These studies on the functions of OsPCF5 and its homologous genes also provide new insights for MhTCP4 to regulate other functions besides anthocyanin biosynthesis. For instance, in terms of flower development or response to stress in M. halliana, whether MhTCP4 has a function similar to OsPCF5 remains to be explored in the future.

The previous study has reported AtTCP3 interacts with R2R3-MYB proteins including PAP1 and PAP2 in Arabidopsis [33]. TCP family genes encode proteins sharing the TCP domain, a 59-amino acid bHLH motif [15,26], which allows DNA binding and protein-protein interactions [27,28]. However, in this study, the yeast two-hybrid results showed MhTCP4 did not interact with MhMYB10 by the repeated tests. Similar result occurs in MdTCP3, which is also belonged to TCP Class II in Malus. MdTCP3 could not physically interact with MdMYB1, an allele of MYB10 with similar protein motifs [25]. Although the conserved TCP domain provides the possibility to interactions, other parts of the proteins with highly divergent, fast evolving sequences outside the TCP domain of different species are, essential for their functional specificity, thus species differences in function may exist [15]. In addition, combined with yeast one-hybrid assay results, the results suggested that MhTCP4 protein could not interact with MhMYB10 protein or directly bind to the promoter of MhMYB10 to regulate the anthocyanins biosynthesis.
Meanwhile, we confirmed that MhTCP4 directly binds to the promoters of MhCHI and Mhf3′H, while not binds to MhCHS, MhDFR, MhANS, MhUFGT through yeast one-hybrid assays. In Malus, MdTCP46, a Class I TCP gene, is found to enhance the binding activity of MdMYB1 to the promoters of its target gene including MdDFR and MdUFGT [25]. In addition, previous studies reported MdMYB10 activates the promoter of MdDFR and MdMYBA bounds specifically to MdANS promoter region [4,6,7]. These results showed that, compared with MYB10 and its alleles, MhTCP4 regulates different anthocyanins biosynthesis genes. This is further evidence that the functional role of MhTCP4 is completely independent of MhMYB10. Thus, we extrapolate that, in M. halliana flowers, MhTCP4 and MhMYB10 concurrently regulate anthocyanins biosynthesis, but the MhTCP4 and MhMYB10 regulates different structural genes, respectively.

We noticed that the expression of PAL and CHS in M. ‘Snowdrift’ and M. ‘Fuji’ with transient silence MhTCP4 did not orderly change following the expression of MhTCP4. The same situation was reported in previous studies. For example, on the promotion of strawberry anthocyanin biosynthesis by FvTCP9, FvTCP9 interference led to up-regulation of PAL expression [34]. We speculated that there is a negative feedback regulatory mechanism between anthocyanins content and the upstream structural genes like PAL and CHS, since MhTCP4 could not directly regulate the expression of PAL and CHS [35]. The UV-B induction was performed during the transient expression assays in fruit peels [36]. Anthocyanins, key compounds for stress resistance, could not be formed in the peels due to the MhTCP4 silencing [37–39]. The absence of anthocyanins caused negative feedback regulation of upstream structural genes, causing an increase in PAL and CHS expression. In addition, when overexpressing MhTCP4, the CHS gene expression was inconsistent in the peels of the two specie fruits. This might be due to the sampling time deviation of these two species, resulting in different transient expression levels of CHS. Especially in the case that MhTCP4 cannot directly activate CHS, there is a degree of uncontrollable expression of the CHS gene. The regulatory network of the anthocyanin biosynthesis pathway is very complex, and further studies are still needed.

In this study, we functionally characterized the MhTCP4 in M. halliana and proved that MhTCP4 positively regulates anthocyanins biosynthesis. For the first time, we found that MhTCP4 directly activates the promoters of MhCHI and Mhf3′H without interacting with MhMYB10. The activation of MhCHI and Mhf3′H expressions gradually reduces due to the decreased expression of MhTCP4, then the anthocyanins synthetic substrate content is decreases and flower fades. Overall, our findings provide new sight on the mechanisms of anthocyanins biosynthesis and will facilitate artificial manipulation of flower pigmentation in ornamental plants.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

M. halliana petals, M. ‘Snowdrift’ fruits and M. ‘Fuji’ fruits were used as research materials, M. halliana and M. ‘Snowdrift’ all grew in Northwest A&F University, Shaanxi, Yangling, China (34°20′ N, 108°24′ E), M. ‘Fuji’ fruits were collected from “Chunhua Tiandi” Orchard in Chunhua County, Shaanxi Province.

Petals of M. halliana were collected from three different periods (S1, S2, S3). Due to the fruits of M. halliana are not able to turn red, the fruits of M. ‘Snowdrift’ and M. ‘Fuji’ were used to transient expression experiment. M. ‘Snowdrift’ fruits were bagged for 20 days, and M. ‘Fuji’ fruits were bagged for 35 days after falling flower until used, respectively. The petals, leaves and peels were immediately frozen in liquid nitrogen, then they were stored at −80 °C for further experiments.

4.2. RNA Sequencing Data Analysis

Library construction, RNA sequencing (RNA-Seq), RNA assembly, and DEG analysis of Malus halliana petals at S1, S2, and S3 stages, each with three biological replicates, were performed and reported in our previous study [1].
4.3. Bioinformatics Analysis of TCP Gene Family in Malus halliana

Compared with Genome Database for Rosaceae (GDR, https://www.rosaceae.org/) (accessed on 16 May 2019), the candidate TCP genes were screened from M. halliana transcriptome database. The heat map of gene expression based on FPKM was constructed by local software TBtools [40]. The FPKM values of the MhTCP TF family genes were taken in the transcriptome, and the total anthocyanins contents of the three development stages (S1, S2, S3) in M. halliana were taken, and the local software TBtools was used to draw the correlation analysis heatmap [40].

The TCP protein sequences of M. halliana and A. thaliana were alignment. The alignment results were imported into MEGA 7, and the neighbor joining (NJ) method was used to construct a phylogenetic tree [41]. The parameter settings were as follows: bootstrap method 1000; P-distance model; partial deletion; cutoff 50. Then, the online software Evolvview (www.evolgenius.info/evolview) (accessed on 20 May 2019) was used to beautify the phylogenetic tree [42]. Online software MEME (https://meme-suite.org/meme/) (accessed on 18 June 2019) and local software TBtools was used to identify and visualize the conserved domain motif [40,43]. To determine the chromosomal distribution of the candidate TCP genes, we mapped them into the Malus × domestica HFTH1 Whole Genome version 1.0 by the MapChart software [44].

The obtained MhTCP4 gene sequence was analyzed, and the CDS (coding sequence) was translated by DNAMAN software. The translated amino acid sequence was analyzed by SWISS-MODEL online software (https://swissmodel.expasy.org/) (accessed on 15 December 2019) to analyze the three-dimensional protein structure of MhTCP4 conserved domain [45]. To analyze the potential functionality of MhTCP4, the orthologous genes which have been annotated in other species of MhTCP4 gene were analyzed by Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (accessed on 21 July 2020).

4.4. Extraction of Total RNA and Isolation of the MhTCP4 Genes

Total RNA was extracted from M. halliana petals with EZNA Plant RNA Kit (R6827-01, Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s protocols, after which 1000 ng RNA was used as the template to reverse-transcribe to first-strand cDNA with the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). According to the gene login number HF03916, the coding DNA sequence (CDS) of MhTCP4 homologous gene (MdTCP4) was downloaded from GDR, and a pair of upstream and downstream primers was designed using Primer premier 5.0 software (Table S2). Next, PCR products were attached to pMD-19T vector (TaKaRa, Dalian, China).

4.5. Overexpression and Silence Recombinant Plasmid Construction

To overexpress the MhTCP4 gene, a plant binary expression vector pBI121 that containing the CaMV 35S promoter was choosed. Restriction sites were introduced at both ends of upstream and downstream primers to amplifying CDS of MhTCP4 gene, and then inserted between the Sac I and Xma I double restriction sites of pBI121 vector to construct the recombinant plasmid 35S: MhTCP4-pBI121 (Table S2).

In order to specifically silence MhTCP4, the primers were designed to clone a 355 bp sequence in the 3′ untranslated region (3′UTR) of MhTCP4, which was used in the subsequent VIGS experiment (Table S2). The recombinant plasmid was constructed using the modified TRV2 virus vector. After analyzing the restriction sites of 3′UTR-MhTCP4 and TRV2 vector, the 3′UTR-MhTCP4 was inserted between the Sma I and BamH I restriction sites of the TRV2 vector to generate gene silencing recombinant plasmid MhTCP4-TRV2.

4.6. MhTCP4 Overexpression or Silence in Fruit Peels of Malus ‘Snowdrift’ and Malus ‘Fuji’

The constructed recombinant plasmid was transformed into Agrobacterium tumefacienes strain GV3101. For overexpression of MhTCP4 gene, based on the method of Li et al. [46], the method of infection was adjusted appropriately. The fruits of M. ‘Snowdrift’ and M. ‘Fuji’ were injected by 1 mL sterile needle removal syringe. Empty vector, pBI121, was
used as control. All the treated fruits were placed in the dark at 4 °C for 24 h and then cultured in a 17 °C culture chamber under continuous light for 24 h, supplemented with UV-B (254 nm–315 nm, Philips, 40 W), phenotype of infected fruit was observed 4 or 8 days after infection.

VIGS experiment was adapted from the method of Li et al. [46]. The A. tumefacienes containing TRV1 and MhTCP4-TRV2 were mixed in a 1:1 ratio. At the same time, TRV1 and empty TRV2 plasmid were mixed in a 1:1 ratio as the control group. Then the mixed solution was incubated in a shaker at 28 °C for 90 r for 3 h in the dark. The infection treatment and the store conditions of M. ‘Snowdrift’ and M. ‘Fuji’ fruits are the same as that mentioned above.

4.7. qRT-PCR Analysis

Total RNA was extracted from the peels of the infected site and was reverse-transcribed into first-strand cDNA using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China) according to the manufacturer’s instructions. The real-time quantitative PCR using 2 × SYBR real-time PCR mixture kit (BioTeKe, Beijing, China) on the StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). Three biological replicates with three technical replicates were performed for each genetic test, and the transcript abundance was calculated according to the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Supplementary Table S2.

4.8. HPLC Analysis

The extraction of phenolic compounds was described previously [8,47]. The extraction supernatant was absorbed and filtered through a 0.45 µm filter, which was analyzed by HPLC coupled with a diode array detector (DAD) (Shimadzu LC-2030C Liquid Chromatograph, Shimadzu, Kyoto, Japan; Inertsil C-18 column, 5.0 µm particle size, 4.6 mm × 250 mm). HPLC separation was performed using a linear gradient of A (10% formic acid dissolved in water) and B (10% formic acid and 1.36% water in acetonitrile) at 30 °C at a flow rate of 1.0 mL·min$^{-1}$. Phenolic compounds were identified by reference to the UV spectral absorption peak and retention time of known standards, the content of phenolic compounds was calculated using standard calibration curves. Three biological replicates with three technical replicates were measured for each sample.

4.9. Yeast Two-Hybrid Assay

The CDS of MhTCP were inserted into pGBK7 vector between Nde I and BamH I, and MhMYB10 was inserted into pGADT7 vector Nde I and BamH I, respectively. The primers used for the Y2H assays are listed in Supplementary Table S2. The recombinant plasmids were co-transformed into yeast strain Y2H Gold and plated on medium lacking Trp and Leu (SD/-Trp-Leu) at 28 °C. Then the Yeast transformed with the positive control (pGBK7-53 + pGADT7-T), pGBK7-MhTCP + pGADT7-MhMYB10 and the negative control (pGBK7-MhTCP + pGADT7; pGBK7 + pGADT7-MhMYB10) were transferred to medium lacking Trp, Leu, His, and Ade (SD/-Trp-Leu/-His-Ade) with 40 mg·mL$^{-1}$ X-α-Gal for interaction screening.

4.10. Yeast One-Hybrid Assay

The promoter fragments of the anthocyanins biosynthesis related gene including MYB10, CHS, CHI, F3′H, DFR, and ANS, were amplified from genomic DNA extracted from M. halliana petals were inserted into the reporter vector pHIS2 between EcoR I and Sac I. The CDS of MhTCP4 was inserted into the pGADT7 vector between Nde I and BamH I. The primers used for Y1H assay are listed in Supplementary Table S2. The recombinant plasmids were co-transformed into yeast strain Y187 and plated on screening medium lacking Trp and Leu (SD/-Trp-Leu) at 28 °C. Then screened on selective medium (SD/-Leu/-Trp/-His) containing the optimal concentration of 100 mM 3-amino-1,2,4-triazole (3-AT).
4.11. Dual LUC Reporter Assay

To screen for downstream genes of MhTCP4, the ORF sequence of MhTCP4 were inserted into pGreenII 62-SK effector vectors between EcoR I and Kpn I. The promoter fragments from MhCHI and MhF3'H were inserted into the reporter vector pGreenII 0800-LUC between Sal I and BamH I. The empty vector pGreenII 62-SK was used as a negative control effector. Transformation and infiltration were performed as described in Yang et al. [48]. The luciferase signals were observed by an imaging apparatus (Plant View 100, Guangzhou, China). The transcriptional activity was examined by a luciferase detection Kit (Transgen, Beijing, China). The transcriptional abilities were expressed by the LUC/REN ratio.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23169051/s1.

Author Contributions: Conceptualization, H.L.; methodology, H.L.; software, J.Y. and J.M.; validation, J.M., H.W. and J.Y.; formal analysis, J.M. and H.W.; resources, H.L.; writing—original draft preparation, J.M. and J.Y.; writing—review and editing, J.M. and H.L.; visualization, J.M. and J.Y.; supervision, H.L.; funding acquisition, H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Natural Science Foundation of China (32171862), Shaanxi Academy of Forestry Science and Technology innovation plan special (SXLK2020-0201), and Shaanxi Key R&D Plan (2021NY-067).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Han, M.L.; Yin, J.; Zhao, Y.H.; Sun, X.W.; Meng, J.X.; Zhou, J.; Shen, T.; Li, H.H.; Zhang, F. how the color fades from *Malus halliana* flowers: Transcriptome sequencing and DNA methylation analysis. *Front. Plant Sci.* 2020, 11, 576054. [CrossRef] [PubMed]
2. Xie, X.B.; Li, S.; Zhang, R.F.; Zhao, J.; Chen, Y.C.; Zhao, Q.; Yao, Y.X.; You, C.X.; Zhang, X.S.; Hao, Y.J. The bHLH transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant Cell Physiol.* 2020, 61, 284–292. [CrossRef] [PubMed]
3. Liu, Y.; Tikunov, Y.; Schouten, R.E.; Marcelis, L.; Visser, R.; Bovy, A. Anthocyanin biosynthesis and degradation mechanisms in solanaceous vegetables: A review. *Front. Chem.* 2018, 6, 52. [CrossRef]
4. Allan, A.C.; Hellens, R.P.; Laing, W.A. MYB transcription factors that colour our fruit. *Trends Plant Sci.* 2008, 13, 99–102. [CrossRef]
5. Yan, H.; Pei, X.; Zhang, H.; Li, X.; Zhang, X.; Zhao, M.; Chiang, V.L.; Sederoff, R.R.; Zhao, X. MYB-mediated regulation of anthocyanin biosynthesis. *Int. J. Mol. Sci.* 2021; 22, 3103. [CrossRef]
6. Espley, R.V.; Hellens, R.P.; Putterill, J.; Stevenson, D.E.; Kutty-Amma, S.; Allan, A.C. Red colouration in apple fruit is due to the activity of the MYB transcription factor, *MdMYB10*. *Plant J.* 2007, 49, 414–427. [CrossRef]
7. Ban, Y.; Honda, C.; Hatsuyma, Y.; Igarashi, M.; Bessho, H.; Moriguchi, T. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant Cell Physiol.* 2007, 48, 958–970. [CrossRef]
8. Meng, J.X.; Gao, Y.; Han, M.L.; Liu, P.Y.; Yang, C.; Shen, T.; Li, H.H. In vitro anthocyanin induction and metabolite analysis in *Malus spectabilis* leaves under low nitrogen conditions. *Hortic. Plant J.* 2020, 6, 284–292. [CrossRef]
9. Li, C.; Wu, J.; Hu, K.D.; Wei, S.W.; Sun, H.Y.; Hu, L.Y.; Han, Z.; Yao, G.F.; Zhang, H. *PyWRKY26* and *PybHLH3* cotargeted the *PyMYB114* promoter to regulate anthocyanin biosynthesis and transport in red-skinned pears. *Hortic. Res.* 2020, 7, 37. [CrossRef]
10. Sun, Q.; Jiang, S.; Zhang, T.; Xu, H.; Fang, H.; Zhang, J.; Su, M.; Wang, Y.; Zhang, Z.; Wang, N.; et al. Apple NAC transcription factor *MdNAC52* regulates biosynthesis of anthocyanin and proanthocyanidin through *MdMYB9* and *MdMYB11*. *Plant Sci.* 2019, 289, 110286. [CrossRef]
11. Li, S.; Zachgo, S. TCP3 interacts with R2R3-MYB proteins, promotes flavonoid biosynthesis and negatively regulates the auxin response in *Arabidopsis thaliana*. *Plant J.* 2013, 76, 901–913. [CrossRef]
12. Li, C.; Potuschak, T.; Colon-Carmona, A.; Gutierrez, R.A.; Doerner, P. *Arabidopsis* TCP20 links regulation of growth and cell division control pathways. *Proc. Natl. Acad. Sci. USA* 2005, 102, 12978–12983. [CrossRef]
13. Finlayson, S.A. *Arabidopsis* Teosinte Branched1-like 1 regulates axillary bud outgrowth and is homologous to monocot Teosinte Branched1. *Plant Cell Physiol.* 2007, 48, 667–677. [CrossRef]
42. He, Z.L.; Zhang, H.K.; Gao, S.H.; Lercher, M.J.; Chen, W.H.; Hu, S.N. Evolview v2: An online visualization and management tool for customized and annotated phylogenetic trees. *Nucleic Acids Res.* **2016**, *44*, W236–W241. [CrossRef]

43. Bailey, T.L.; Boden, M.; Buske, F.A.; Frith, M.; Grant, C.E.; Clementi, L.; Ren, J.Y.; Li, W.W.; Noble, W.S. MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res.* **2009**, *37*, W202–W208. [CrossRef]

44. Voorrips, R.E. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* **2002**, *93*, 77–78. [CrossRef]

45. Guex, N.; Peitsch, M.C. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* **1997**, *18*, 2714–2723. [CrossRef]

46. Li, Y.Y.; Mao, K.; Zhao, C.; Zhao, X.Y.; Zhang, H.L.; Shu, H.R.; Hao, Y.J. MdCOP1 ubiquitin E3 ligases interact with MdMYB1 to regulate light-induced anthocyanin biosynthesis and red fruit coloration in apple. *Plant Physiol.* **2012**, *160*, 1011–1022. [CrossRef]

47. Meng, J.X.; Wei, J.; Chi, R.F.; Qiao, Y.H.; Zhou, J.; Wang, Y.L.; Wang, H.; Li, H.H. MrMYB44-Like negatively regulates anthocyanin biosynthesis and causes spring leaf color of *Malus* ‘Radiant’ to fade from red to green. *Front. Plant Sci.* **2022**, *13*, 822340. [CrossRef]

48. Yang, S.; Zhang, M.; Xu, L.; Luo, Z.; Zhang, Q. MiR858b inhibits proanthocyanidin accumulation by the repression of *DkMYB19* and *DkMYB20* in persimmon. *Front. Plant Sci.* **2020**, *11*, 576378. [CrossRef]