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Zinc Finger Protein 1 (ZFP1) Is Involved in Trichome Initiation in *Arabidopsis thaliana*

Aidong Zhang [1,2], Yihua Liu [1], Chunyan Yu [1], Linli Huang [1], Minjie Wu [1], Junyu Wu [1] and Yinbo Gan [1,*]

[1] Zhejiang Key Lab of Crop Germplasm, Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, China; 21116035@zju.edu.cn (A.Z.); 11616003@zju.edu.cn (Y.L.); 11116043@zju.edu.cn (C.Y.); 11316034@zju.edu.cn (L.H.); minjiewu@zju.edu.cn (M.W.); 11516030@zju.edu.cn (J.W.)

[2] Shanghai Key Laboratory of Protected Horticultural Technology, Horticultural Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai 201403, China

* Correspondence: ygan@zju.edu.cn

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Abstract: *Arabidopsis* trichome is specialized structure that develops from epidermal cells, and is an excellent model system for studying various aspects of plant cell development and cell differentiation. Our previous studies have shown that C2H2 zinc finger protein family genes, including *GIS*, *GIS2*, *GIS3*, *ZFP5*, *ZFP6* and *ZFP8*, play an important role in controlling trichome initiation in *Arabidopsis*. Here, our novel results showed a C2H2 zinc finger protein, *ZFP1*, which also plays an important role in trichome initiation in *Arabidopsis*. *ZFP1* over-expression lines display significantly increased trichome number on cauline leaves, lateral branches and main stems in comparison with wild type plants. *ZFP1* RNAi lines and loss-of-function mutants showed the opposite phenotype. Furthermore, our study also found that *ZFP1* mediates the regulation of trichome initiation by cytokinin signaling. The molecular and genetic analyses reveal that *ZFP1* acts upstream of key trichome initiation factors, *GL3* and *TRY*.

Keywords: C2H2 protein; *zinc finger protein 1*; cytokinin; trichome; *Arabidopsis thaliana*

1. Introduction

Trichome, covering on the surface of plants' most aerial parts, could protect plants from many kinds of adverse environments [1–5]. The distribution of trichomes on abaxial and adaxial surface of rosette leaves can also be a phase marker for the vegetative growth phase. The first few rosette leaves only produce trichomes on the adaxial side. The later leaves produce trichomes on both the adaxial side and abaxial side. Based on this feature, a vegetative growth phase can be subdivided into a juvenile phase during which trichomes are only produced on the adaxial side, and an adult phase with trichomes on both sides [6].

Classic molecular and genetic analyses have identified the main regulators in *Arabidopsis*. They are divided into positive regulators whose mutants cover less trichomes, and negative regulators whose mutants display more or clustered trichomes [7]. In *Arabidopsis*, positive regulators mainly belong to three transcription factor groups, including the WD40 repeat protein [8], the R2R3 MYBs [9–11] and the basic helix-loop-helix (bHLH) factors [12,13]. They form a trimetric activator complex, which is called the MYB-bHLH-WD40 complex, and then combine with the promoter of *GLABRA2* (*GL2*) to regulate the trichome initiation [14,15]. A group of single-repeat R3 MYBs, such as CAPRICE (CPC), TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC 1(ETC1), ETC2, ETC3, TRICHOMELESS1(TCL1) and TCL2, act as the negative regulators [16–23]. They compete with R2R3 MYBs to bind to bHLH factors and form a repressor complex [15,24–28]. It is known that trichome development is a very complicated network regulation system. At present, two theoretical models were proposed: the activator–inhibitor model and activator–depletion model. According to activator–inhibitor model, the activators can activate their own inhibitors and up-regulate the expression of them [29,30]. This characteristic leads to the increase of both activators and inhibitors. The inhibitors can neutralize the activators' increase of neighbor cells. However, because of the missing evidence about activators' self-activation, this model is still in doubt. According to the activator–depletion model, TTG1 can move among young tissues and accumulate in cells. TTG1 binds to GL3 so that its movement will enhance the level of GL3. The cells with more GL3 and TTG1 will form trichomes [31].

Phytohormones play an important roles in the biological process of plant [32]. As has been reported, cytokinin stimulates the initiation of trichome in *Arabidopsis* [33]. With the external application of cytokinin, the trichome number in all rosette will significantly increase, but the length of these trichomes will be shorter. Cytokinin can also influence the endoreduplication of trichomes. The DNA content of plant, which is treated by cytokinin, is less than a plant that is grown under normal conditions [33].

microRNA (miRNA) consists of small endogenous, non-coding RNAs of only 20–22 nt in plants, animals and protozoa. Previous research has demonstrated that miRNA regulates trichome development by targeting *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) [34]. SPLs negatively regulate trichome initiation on stem and floral organ in *Arabidopsis*. SPL family has 17 members, and 10 of them are target genes of miRNA156 [35]. The expression level of miRNA156 is highest in young plants, and gradually decreases with the increase of plant age. In contrast, the expression level of *SPLs* is low in young plants, and increase with the increase of plant age. This expression mode is consistent with the phenotype of trichome in stem and floral organ. SPL9 decreases trichome initiation by targeting promoters of *TCL1* and *TRY* [34]. UPL3/KAK mutation encoding HECT domain E3 ligase leads to an increase of trichome branches number and DNA content in nucleus. GL3 may be the target gene of UPL3 [36–38]. ETC2, an m^6A reader, controls trichome branching by affecting mRNA stability in *Arabidopsis* [39].

C2H2 type zinc finger protein, which plays multiple functions, is one of the largest transcription factor families in *Arabidopsis* [40]. As shown in our previous research, *GLABROUS INFLORESCENCE STEMS* (*GIS*), *GIS2*, *GIS3*, *ZINC FINGER PROTEIN 5* (*ZFP5*), *ZFP6* and *ZFP8*, which all encode C2H2 zinc finger proteins, are involved in trichome initiation and root hair development, which display transcriptional hierarchy [5,41–47]. *GIS3* integrates gibberellin (GA) and cytokinin signaling and GIS3 directly targets downstream genes, *GIS* and *GIS2*, to regulate trichome production [46]. *ZFP6* also responds to gibberellin and cytokinin signaling and functions upstream of *ZFP5* [45]. *ZFP5* is involved in gibberellin signal transduction and acts upstream of *ZFP8* [43,44]. All of them play key roles upstream of the trichome initiation complex MYB-bHLH-WD40. Hair (H), which encodes a C2H2 zinc finger protein, regulates multicellular trichome formation in tomato [48]. Recently, Han et al. found that AtZP1, which belongs to C2H2-type zinc finger protein family, negatively regulates root hair initiation and elongation. *AtZP1* acts downstream of *GL2* and regulates root hair initiation by targeting *RHD6*, *RSL4* and *RSL2* transcription [49]. Here, we cloned a C2H2 zinc finger protein *ZFP1* (*At1g80730*), which is a homologous gene of *ZFP5.* Previous researchers have found that *ZFP1* is a unique gene in the *Arabidopsis* genome [50] and is expressed at high levels in the shoot apex of seedling grown under a grow light [51]. It may participate in shoot development and acts downstream of photomorphogenic activation [51]. In our research, we found that *ZFP1* also plays a role in controlling trichome initiation in *Arabidopsis*.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Ecotype Columbia (Col-0), the wild type *Arabidopsis* (*Arabidopsis thaliana*), was used as a control of experiments in this research. Plants were grown in a growth chamber with 20 °C to 22 °C, 95 to

118 µmol m^{-2} sec^{-1}, 16 h light/8 h dark and 70% to 80% relative humidity. *Arabidopsis* seeds were sterilized with 5% (*V/V*) NaClO for 8 min and washed five times with sterile distilled water in laminar flow cabinet. The sterile seeds were transferred on Murashige and Skoog (MS) mediums and incubated at 4 °C for 3 days in darkness. Then, the MS mediums were placed in a growth chamber with growth conditions as described above. Trichome numbers were counted as previously described [41,42].

2.2. Isolation of *zfp1* Loss-of-Function Mutant

zfp1 seeds with T-DNA insertion in the *ZFP1* promoter region were ordered from Nottingham *Arabidopsis* Stock Centre (NASC; catalogue number N669417). Homozygous mutants were selected with kanamycin (50 mg L^{-1}) by testing their progenies' resistance. The presence of T-DNA insertion was confirmed by PCR with gene-specific primers (Table 1).

Table 1. Primers used for construct cloning, mutant identification and quantitative real-time PCR.

Code	Sequence	Use	Reference
ZFP1-FP	5′-GCCGTCGACCAATTCTTTTACTTTCATTCTCTTT-3′	*ZFP1* cloning	
ZFP1-RP	5′-AATGCGGCCGCAGTACAACTTGATCATTAGTGAAAC-3′		
ZFP1-Ri-LP	5′-GTCGACCCTCCTTGCCTTCTTCA-3′	RNAi	
ZFP1-Ri-RP	5′-GCGGCCGCCTATGGTCGGTTTCTGGT-3′		
zfp1-LP	5′-TTTATGAGCGTTTTGGTGACC-3′	mutant specific primers	
zfp1-RP	5′-TCAATGGTGCATATATGATTTGG-3′		
LBb1.3	5′-ATTTTGCCGATTTCGGAAC-3′		
UBQ10-RT-LP	5′-GGTTCGTACCTTTGTCCAAGCA-3′	qRT-PCR	[40]
UBQ10-RT-RP	5′-CCTTCGTTAAACCAAGCTCAGTATC-3′		
ZFP1-RT-LP	5′-GCGGTCACCAAAACGCTCATA-3′	qRT-PCR	
ZFP1-RT-RP	5′-GAATCTGCTGACTGAACCGTGG-3′		
GL3-RT-LP	5′-GAATCTGCTGACTGAACCGTGG-3′	qRT-PCR	[40]
GL3-RT-RP	5′-CAAGAACGTTGTCGATGTGATAATC-3′		
TRY-RT-LP	5′-TTGTCGGTGATAGGTGGGATTT-3′	qRT-PCR	[2]
TRY-RT-RP	5′-ACGGTGAGGCTTGGTATGTTTG-3′		
ARR5-RT-LP	5′-TTGCGTCCCGAGATGTTAGAT-3′	qRT-PCR	[40]
ARR5-RT-RP	5′-TGAGTAACCGCTCGATGAACTTC-3′		

2.3. Construct Cloning

For overexpression and RNAi constructs, the fragments were amplified by PCR from cDNA of Col-0 using PrimeSTAR HS DNA polymerase (TaKaRa Bio Inc., Kusatsu, Japan) and inserted into a pENTR-1A vector (Invitrogen, Calsbad, CA, USA). Then, the fragments recombined into destination vectors with Gateway LR reaction (Invitrogen). The destination vectors were obtained from VIB (Flanders Interuniversity Institute for Biotechnology) as described before [43,45]. The constructs were finally transferred into *Agrobacterium tumefaciens* Stain GV3101 and transformed *Arabidopsis* by floral-dip method as described by Clough and Bent (1998) [52]. The primers used for cloning are list in Table 1.

2.4. Cytokinin Treatment and Gene Expression Analyses

For external cytokinin treatment, 6-benzylaminopurine (6BA, Sigma-Aldrich Corp., St. Louis, MO, USA) was used in this study as we described before [42,43,45]. Plants were sprayed with 100 µmol L^{-1} 6BA and took samples after 2 h for RNA extraction when their inflorescence shoots reached 2 to 3 cm. For trichome phenotype analyses, plants were grown on soil until three to four leaves had emerged and sprayed twice a week with different concentrations of 6BA or mock solution until plants were ready for analyses as described previously [42,45].

2.5. RNA Extraction and Real-Time PCR

Total RNA was isolated with Trizol reagent (Invitrogen). Two µg of total RNA was reversely transcribed to cDNA with M-MLV transcriptase (Promega, Madison, WI, USA) as described in their instruction. The cDNA was diluted four times for real-time PCR. Real-time PCR was performed with SYBR Green PCR mix (Takara). The results of subtraction, which consisted of subtracting the Ct value of *UBQ10* (*At4g05320*) from the Ct value of the target genes, are represented by sign ΔCt. The relative expression of the target genes was obtained by calculating $2^{-\Delta Ct}$ as previously described [46]. The primers used in this study are listed in Table 1.

2.6. Bioinformatic Analyses

The coding sequence (CDS) and protein sequences were acquired from Tair (https://www.arabidopsis.org/). ClustalW (https://www.genome.jp/tools-bin/clustalw) was used for CDS and protein sequences alignment. Jalview software [53] was used for the alignments edit. The protein domain was predicted by SMART (https://smart.embl-heidelberg.de/).

2.7. Statistical Analyses

All data displaying in figures were analyzed by means of ANOVA. SPSS software, version 14.0 (SPSS Inc., Chicago, IL, USA), was used for the significance analysis. Student's t-test was calculated at either 5% (*, $p < 0.05$) or 1% (**, $p < 0.01$) probability to evaluate the difference as described before [54].

3. Results

3.1. Loss of Function of ZFP1 Affects Trichome Initiation

As our previous results showed that *ZFP5* plays a key role in regulating trichome initiation in *Arabidopsis*, we are very curious whether its homologous gene *ZFP1* plays a similar role in trichome development. *ZFP1*, with 687 bp open reading frames encoding 228 amino acids, showed high homologous with other C2H2 zinc finger protein genes (Figure S1) [49]. We ordered *zfp1* mutant (catalog No. N669417) from the Nottingham *Arabidopsis* Stock Centre (NASC). *zfp1* carries a T-DNA insertion in the *ZFP1* promoter region and was verified by genomic PCR. We also constructed an RNAi vector, which silenced the expression of *ZFP1*. We transferred this vector to Col-0 plants (wild type, WT) and selected *ZFP1-Ri-6* line for phenotypic observation (Figure 1). To assess the function of *ZFP1* in regulating trichome initiation, we counted the trichome numbers of WT, *zfp1* and *ZFP1-Ri-6* on their branches, cauline leaves, stem, and sepals. As shown in Figure 2A–C and Figure S2, the mutant and RNAi lines had significantly fewer trichomes than that of WT. These results indicate that *ZFP1* may involve in trichome initiation in *Arabidopsis*.

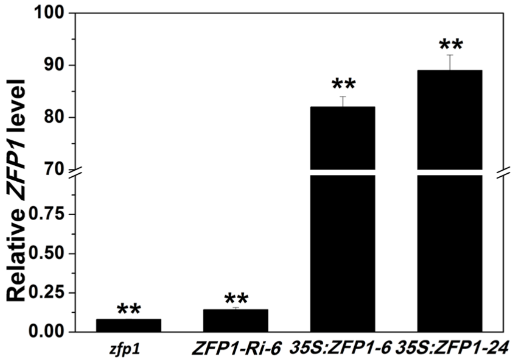

Figure 1. The relative expression level of *ZFP1* in *zfp1*, *ZFP1-Ri-6* and *35S:ZFP1* lines. The values of *UBQ10* were used as control to normalize the expression of target genes. Error bars indicate standard error. ** $p < 0.01$.

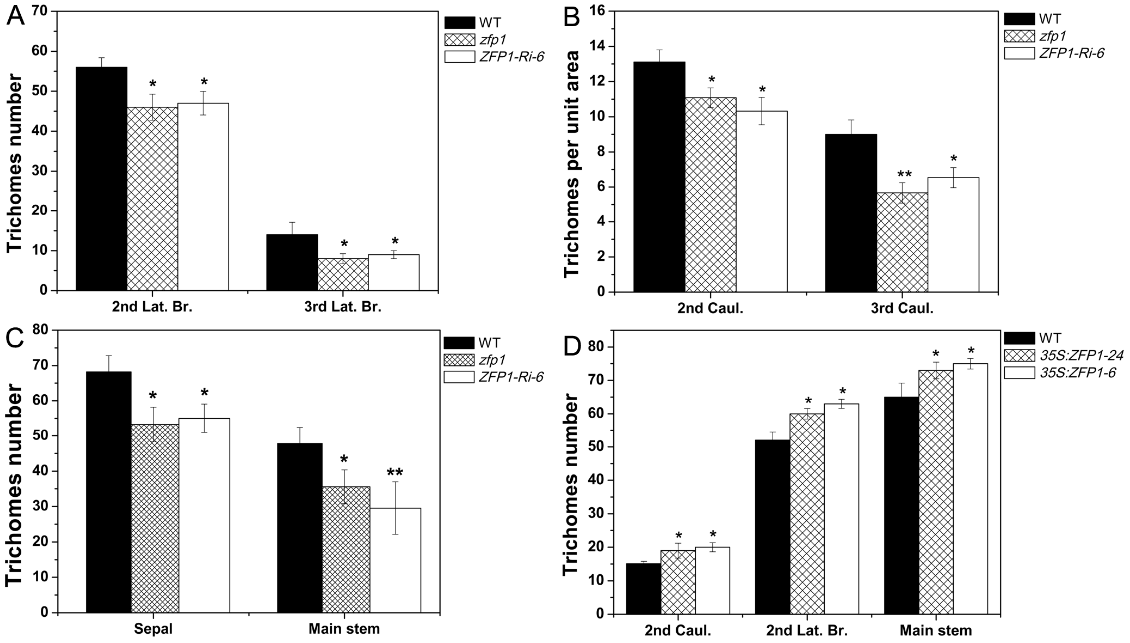

Figure 2. Trichome number on different organs in wild type, *zfp1* mutant, *ZFP1-Ri-6* and *35S:ZFP1* lines. Trichomes number on the second and third branches (**A**), the second and third cauline leaves (**B**), sepals and main stems, (**C**) in wild type, *zfp1* mutant and *ZFP1-Ri-6* line. (**D**) Trichomes number in wild type and *35S:ZFP1* lines. Trichomes number was the average number of counting at least 20 plants. Error bars indicate the standard error. * $p < 0.05$; and ** $p < 0.01$.

3.2. Overexpression of ZFP1 Stimulates Trichome Initiation

To further confirm our hypothesis, we created *35S:ZFP1* transgenic lines and chose two representative overexpression lines (*35S:ZFP1-6* and *35S:ZFP1-24*) (Figure 1). Both lines displayed significantly higher trichomes density on second branches, second cauline leaves and stems than WT plants (Figure 2D). These results further confirm that *ZFP1* positively controls trichome initiation in *Arabidopsis*.

3.3. ZFP1 Is Required for Cytokinin Signal to Control Trichome Initiation during Inflorescence Development

Our previous study showed that C2H2 ZINC FINGER PROTEIN could regulate trichome initiation through cytokinin signaling [42,45]. To investigate whether *ZFP1* plays a similar role in regulating trichome initiation through cytokinin signaling, WT plants were sprayed with 6BA and then sampled after 2 h to test the expression level of *ZFP1*. As shown in Figure 3, the strong induction of a primary cytokinin response gene *ARR5* after 6BA treatment indicates that the effectiveness of cytokinin application signaling. The relative expression of *ZFP1* was also significantly up-regulated by 6BA treatment.

To further confirm the role of cytokinin, *zfp1* mutant and wild type plants were treated with 10 μM and 100 μM 6BA. Then, we counted the trichome numbers. As shown in Figure 4, the trichome numbers increase in *zfp1* plants with 6BA application, which shows a less sensitive phenotype than that of wild type plants on the second branches, second cauline leaves, and third cauline leaves. Taken together, these data indicate that *ZFP1* is required for the cytokinin signal in controlling the trichome initiation in *Arabidopsis*.

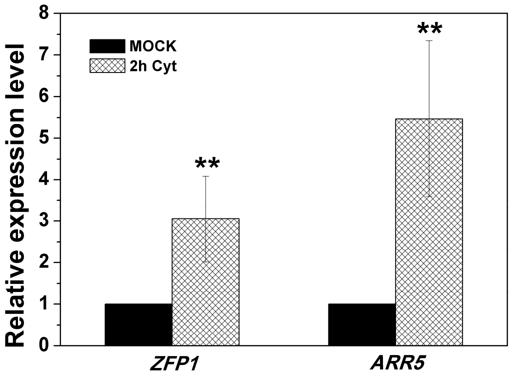

Figure 3. *ZFP1* expression level in wild type when treated with 100 µM 6BA. The values were normalized against the levels of *UBQ10* as a control. Error bars indicate standard error. ** $p < 0.01$.

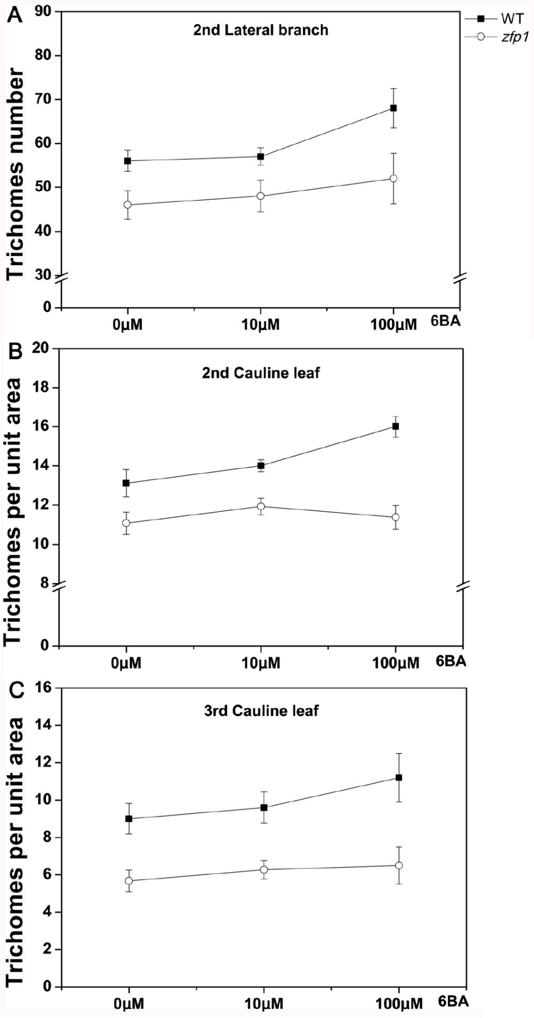

Figure 4. Wild type and zfp1 mutant. Response to 6BA treatment. Trichomes number on the second lateral branches (**A**), second (**B**) and third cauline leaves (**C**) in wild type and zfp1 mutant when treated with different concentrations of 6BA. The trichomes number was the average number of at least 20 plants. Error bars indicate the standard error.

3.4. ZFP1 Acts Upstream of Trichome Initiation Complex

Trichome initiation is mainly regulated by MYB-bHLH-WD40 complex. To verify if *ZFP1* regulates trichome initiation by this pathway, we analyzed the relative expression level of *GL3* and *TRY*.

Our results showed that the expression level of *GL3* in mutant and RNAi line was significantly decreased in comparison to the wild type. The expression level of negative regulator *TRY* was increased in mutant and RNAi line compared with wild type. In contrast, in the *ZFP1* overexpression line, the expression level of *GL3* was up-regulated and *TRY* was down-regulated compared to that of wild type (Figure 5). These results suggest indicate that *ZFP1* acts upstream of the trichome initiation complex.

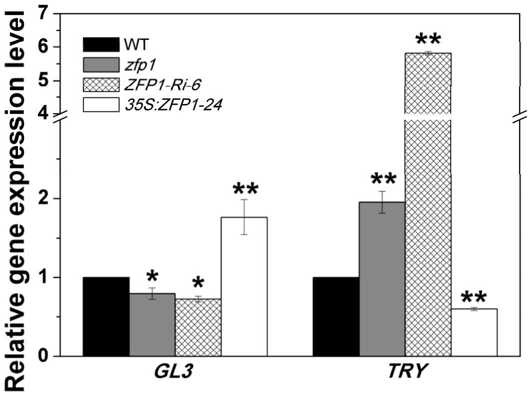

Figure 5. Relative expression level of *GL3* and *TRY* in wild type, *zfp1* mutant and *ZFP1* overexpression line. The values were normalized against the levels of *UBQ10* as a control. Error bars indicate the standard error. * $p < 0.05$; and ** $p < 0.01$.

To further confirm this result, we over-expressed *ZFP1* gene in *gl3* mutant and detected if glabrous phenotype of *gl3* could be restored by over-expression of *ZFP1*. As shown in Figure 6, *35S:ZFP1:gl3* did not rescue *gl3* mutant's phenotype. These data further support the hypothesis that *ZFP1* acts upstream of trichome initiation complex.

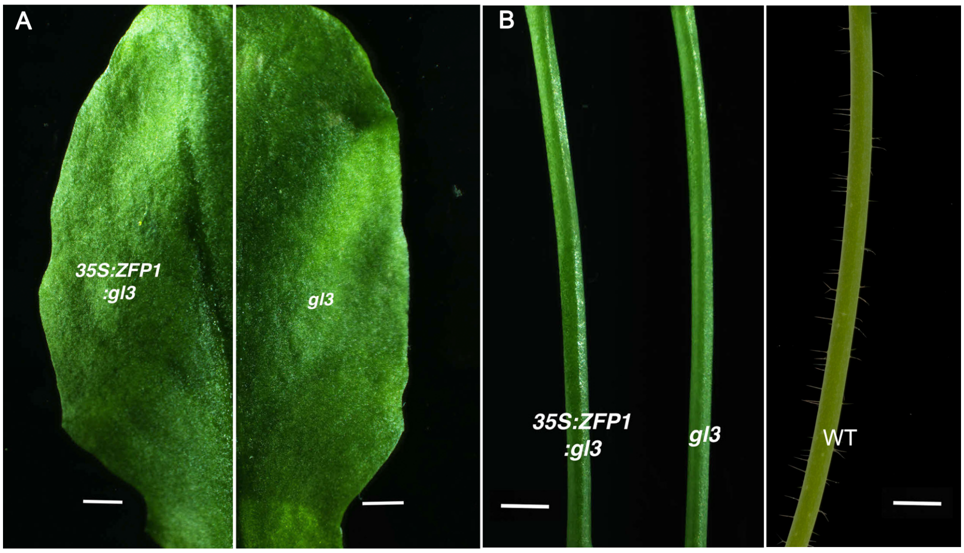

Figure 6. Genetic interaction between *ZFP1* and *GL3* gene. (**A**) Trichome initiation on the rosette leaf of *35S:ZFP1:gl3* and *gl3*. (**B**) Trichome initiation on stem of *35S:ZFP1:gl3*, *gl3*, and wild type. Bars = 2 mm.

3.5. The Expression Patterns of *ZFP1*

Sequence analysis displayed that *ZFP1* contains a C2H2 conserved domain, which is found in all C2H2 zinc finger protein family (Figure S1). To analyze the expression pattern of *ZFP1*, we checked its expression level in different organs. Quantitative RT-PCR data showed that *ZFP1* was mainly expressed in flower, main stem, silique and root but was expressed at lower levels in other organs

(Figure 7). These results were similar with *ZFP1* expression profiles extracted from *Arabidopsis* databases (eFP Brower, http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi) (Figure S3).

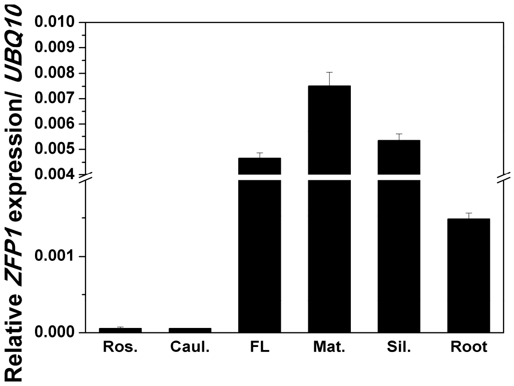

Figure 7. Analysis of *ZFP1* expression in different tissues of wild type. Ros., rosette leaf; Caul., cauline leaf; FL, flower; Mat., main stem; Sil., silique; Root, Root. The values were normalized against the levels of *UBQ10* as a control.

4. Discussion

Our previous results have shown that C2H2 type zinc finger protein family members, *GIS*, *GIS2* and *ZFP8*, could control the trichome development in *Arabidopsis*, but their responses to hormonal signals are diverse [41,42,55]. *GIS* plays a main role in controlling the initiation on branches and *GIS2* regulates the number of trichomes on flowers, while *ZFP8* regulates trichomes development on cauline leaves. As shown in Figure 2, *ZFP1* also plays a predominant role in branches' trichome initiation.

Cytokinin plays a positive role in trichome initiation in *Arabidopsis*. The expression level of *GL1*, *GL3*, *EGL3* and *MYB23* is significantly induced after cytokinin application. In this study, 6BA significantly induced the expression of *ZFP1* and *zfp1* mutant plants were shown to be less sensitive to cytokinin than that of wild type (Figures 3 and 4). At the same time, *ZFP1* acts upstream of trichome initiation complex gene *GL3* that is induced by cytokinin, which means *ZFP1* may respond to cytokinin signaling by *GL3*. These results suggest that exogenous cytokinin induces the expression of *ZFP1*, which increases the expression of *GL3* and eventually promotes trichome initiation in *Arabidopsis* (Figure 8). It is well-known that gibberellin participates in many growth and development processes, including seed germination, hypocotyl elongation, flowering and trichome development. GA biosynthesis mutant *ga1-3* displays glabrous on leaves, and phenotypes are recovered by applying GA [56]. With the treatment of GA, the expression level of *GL1*, *MYB23*, *GL3* and *EGL2* is significantly increased, while the expression of *TRY*, *ETC1* and *ETC2* is significantly decreased [33]. In addition, *ZFP5* responds to gibberellin and acts upstream of *GIS*, *GIS2*, *ZFP8* and the trichome initiation complex [38]. Our previous study also demonstrated that *ZFP6* and *GIS3* could control trichome development by integrating gibberellin and cytokinin signal [45,46]. However, whether *ZFP1* plays any role in control trichome development through GA signal is still unknown and needs to be further investigated.

Trichomes are specialized epidermal cells, protecting plants from abiotic and biotic stresses [43]. Our previous studies have found that *ZINC FINGER PROTEIN 3* (*ZFP3*) participates in the responses of salt stress and osmotic stress by influencing the expression of stress-related genes and changing the proline accumulation and chlorophyll content in *Arabidopsis* [55]. Constitutive expression of *ZFP3* enhances the tolerance of *Arabidopsis* to salt and osmatic stress. *zfp3* loss-of-function mutants were shown to be more sensitive to salt and osmotic stress. With regards to *ZFP3*'s homologous gene, whether *ZFP1* is also involved in abiotic stresses needs to be explored in the future.

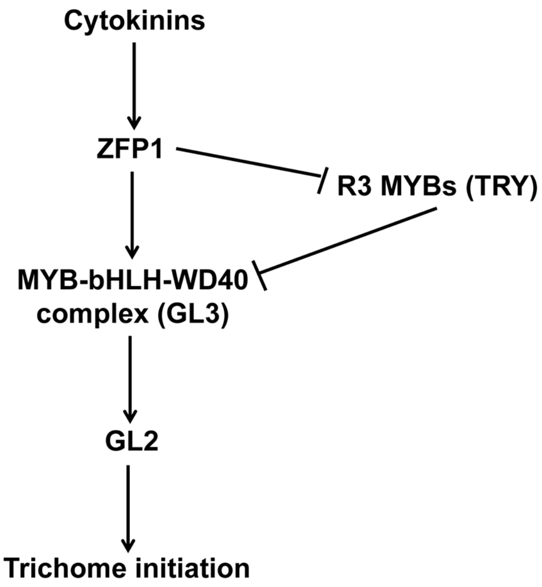

Figure 8. Model of the *ZFP1* in regulating trichome initiation in response to cytokinin in *Arabidopsis*.

5. Conclusions

Our results suggest that *ZFP1* plays a positive role in regulating trichome initiation by acting upstream of key trichome initiation factors, GL3 and TRY, in *Arabidopsis* through the cytokinin pathway.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/12/645/s1, Figure S1: Alignment of CDS sequences (A) and protein sequences (B) of *ZFP1, ZFP2, ZFP3, ZFP4, ZFP5,* and *ZFP7* in *Arabidopsis*, Figure S2: Phenotype of *zfp1* mutant, WT, and *35S:ZFP1*. (A) Phenotype of *zfp1* mutant, WT, and *35S:ZFP1* on sepal. (B) Phenotype of *zfp1* mutant, WT, and *35S:ZFP1* on stem, Figure S3: Expression profiles of *ZFP1* in database of public microarray data.

Author Contributions: Methodology, A.Z. and Y.G.; validation, A.Z., C.Y. and Y.L.; formal analysis, L.H. and M.W.; resources, Y.G.; data curation, A.Z. and J.W.; writing—original draft preparation, A.Z. and Y.G.; funding acquisition, Y.G. All authors have read and agreed to the published version of the manuscript.

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