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Weird fingers: Functional analysis of WIP domain proteins

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ABSTRACT

WIP proteins form a plant specific subfamily of C2H2 zinc finger (ZF) proteins. In this study, we functionally characterized the WIP domain, which consists of four ZF motifs, and discuss molecular functions for WIP proteins. Mutations in each of the ZFs lead to loss of function of the TT1/WIP1 protein in *Arabiopsis thaliana*. SV40 type nuclear localisation signals were detected in two of the ZFs and functionally characterized using GFP fusions as well as new mutant alleles identified by TILLING. Promoter swap experiments showed that selected WIP proteins are partially able to take over TT1 function. Activity of the *AtBAN* promoter, a potential TT1 target, could be increased by the addition of TT1 to the TT2–TT8–TTG1 regulatory complex.

1. Introduction

Zinc fingers (ZFs) are among the most widespread motifs in eukaryotic proteins. They are classified according to the number and order of cysteine and histidine residues, which coordinate zinc ions to maintain characteristic tertiary protein structures. Most prominent are C2H2 ZFs consisting of a ββα-structure. In many zinc finger proteins (ZFPs) of this class, helix positions-1, 3 and 6 have been identified to contact DNA in a sequence specific manner [1]. C2H2 ZF motifs were also shown to bind RNA and to mediate protein interactions [2,3]. In accordance with these different molecular functions, ZFPs were found to be involved in diverse biological processes including chromatin modification, transcriptional regulation and ubiquitin dependent protein degradation [4].

In animals, C2H2 ZFs are typically arranged in tandem with a conserved spacing of seven amino acids between adjacent fingers to attach to neighbouring target DNA triplets [1]. In plants, only about 20% of C2H2 ZFs are arranged in tandem, whereas the majority of the ZFPs contains one single or several dispersed ZF motifs [5]. The A1 family of plant ZFPs, belongs to the minority of proteins with tandemly arranged ZFs and is characterised by four ZF motifs [5]. Subgroup A1d of this family has six members in *Arabidopsis thaliana*, all of which have their ZFs in a highly conserved C-terminal region, called WIP domain, according to its initial three amino acids (Fig. 1) [6]. ZF1 and 3 in this domain fit well to the consensus of the "classical" C2H2 type represented by patterns like Pfam PF00096 or Prosite PS00028, whereas ZF2 and 4 contain an uncommon spacing of the zinc coordinating residues. The four WIP ZFs display motifs known to be relevant for DNA binding, including conserved primary DNA recognition positions [1], but neither DNA binding nor other molecular functions have been shown so far for any WIP and any other A1 C2H2 ZFP [5].

TT1/WIP1 is specifically expressed in endothelial cells during seed coat development and loss of function causes a *transparent testa* (*tt*) phenotype [6]. *WIP2* was found to play a role in the development of the transmitting tract, which is important for pollen tube growth, and is therefore also referred to as *NO TRANSMITTING TRACT* (*NTT*) [7]. Petricka et al. have reported that mutations in *WIP6* lead to alterations in leaf vein patterning and thus renamed the gene to *DEFECTIVELY ORGANIZED TRIBUTARIES5* (*DOT5*) [8]. Recently, *CmWIP1* was shown to control carpel abortion and the development of unisexual male flowers in melon [9].

In this report we give the first experimental evidence that the predicted zinc coordinating residues of all ZFs in the WIP domain are crucial for its function and show that nuclear localisation

Abbreviations: ZF, zinc finger; ZFP, zinc finger protein; TT, transparent testa; PA, proanthocyanidin; NLS, nuclear localisation signal

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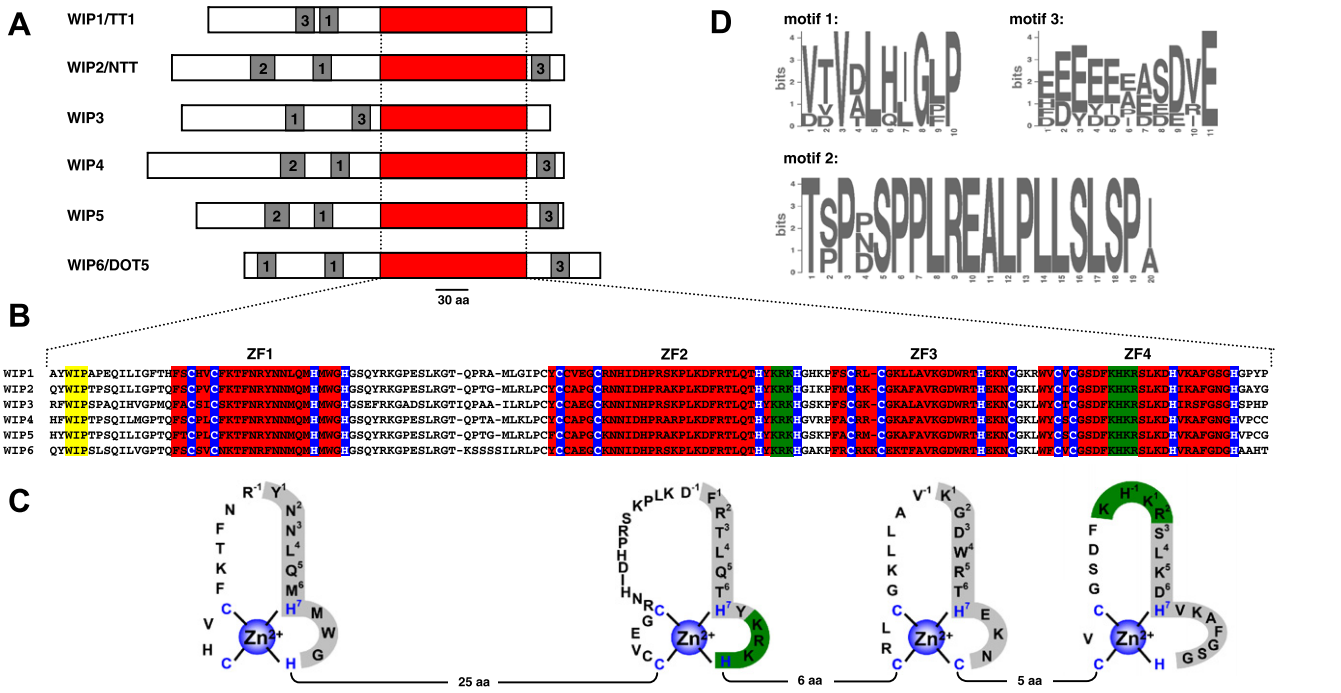

Fig. 1. Predicted motifs in the *A. thaliana* WIP protein family. (A) Schematic representation of domain architecture including results of MEME analysis [21]. (B) WIP domain amino acid alignment (sequences derived from the Col-0 reference genome). The initial three conserved amino acids are eponymous for the protein family (yellow). ZF motifs are marked in red. Zinc coordinating residues are emphasised in blue. NLSs are highlighted in green. (C) Proposed structure of ZFs in TT1 according to predictions in [5]. Amino acids are numbered relative to the first putative helical residue. The α-helical region is shaded in grey. Spacing between zinc coordinating residues of adjacent fingers is given in amino acids (aa). (D) Sequence logos of conserved motifs detected by MEME analysis.

signals (NLSs) are part of the atypically extended C2H2 ZF structures. Furthermore we present promoter activation studies, which indicate that WIPs might be involved in protein interactions rather than in or in addition to nucleic acid binding.

2. Materials and methods

2.1. Plant material

The *tt1-1* allele was characterised previously [6]. The *tt1-3* mutant (SALK_026171; [10]) is in Columbia-0 background and carries a T-DNA insertion in the intron of *TT1* (left boarder at position +1054 from the start ATG; GenBank accession BZ662681). The *tt1-6* and *tt1-7* alleles are derived from an EMS mutagenised *A. thaliana* population generated in the GABI-TILL project. Primers G086 (5′-TCTTGAATGAAACATTACTATGCTCGGTCA-3′) and G087 (5′-TGCATGGTCAATTTTGTATTGATCTGTTTTT-3′) were used for TILLING. Mutations were confirmed by sequencing.

2.2. Plasmid constructs and plant transformation

All plasmids and the oligonucleotides used for their generation are listed in Supplementary Table 1. *TT1f* mutant- and *WIP* promoter swap-constructs were assembled in the binary vector $pPro_{TT1}$-TT1. This vector is a derivative of pANGUS [11] in which the TATA-GUS fragment was replaced by the 1.1 kb *TT1* promoter-*TT1* cDNA cassette of $pPro_{TT1}$-TT1::GFP described previously [6]. Mutations in *TT1f1* to *TT1f4* as specified in Supplementary Table 1 were introduced by site directed mutagenesis. *WIP* cDNAs were amplified by PCR, cloned into pBT8-35S-LUCm3 [12] replacing the LUC ORF and transferred to $pPro_{TT1}$-TT1 as either *Nco*I/*Sac*I or *Nco*I/*Kpn*I fragments replacing *TT1*. All constructs were transformed into *Agrobacterium tumefaciens* GV3101 pMP90RK and transformed into *tt1-1* by floral dipping. WIP-GFP fusions were expressed under 2x *35S CaMV* promoter control in pAVA393 as described in [6]. Plasmids used for transient promoter activation studies are based on pBT8-35S-LUCm3 and pBT10-GUS [12]. The TT2, TT8 and TTG1 effector constructs were described before [13].

2.3. PA analysis

Vanillin staining of immature seeds was carried out as described in [14]. For PA quantification a protocol developed for rapeseed [15] was adapted for arabidopsis. Mature seeds (10 mg) were ground in 0.3 mL acidic methanol and PA polymers were subsequently hydrolysed at 95 °C for 20 min into coloured anthocyanidins by adding 1.2 mL butanol-HCl and 40 μL ferric reagent. After boiling, absorption of the clear supernatant was measured at 550 nm and cyanidin content was calculated using a calibration curve prepared with cyanidin chloride (Sigma–Aldrich).

2.4. Protoplast transfection, GFP localisation and BAN promoter activation assay

Reporter and effector constructs, At7 protoplast isolation and transfection experiments were carried out as described previously [12,13]. BY2 cells were treated as described in [16]. After 20 h incubation in the dark, GFP fluorescence was analysed with a Leica DM5500 microscopy system.

3. Results and discussion

3.1. Zinc finger structure

Formation of zinc fingers in WIP domains was inferred from high sequence similarity of ZF1 and 3 to known ZFPs like TFIIIA [6]. ZF2 and 4 however have only been found in WIP domain proteins so far and differ from the established C2H2 consensus by a

predicted extended hairpin structure (ZF2) or an enlarged helix (ZF4; Fig. 1B and C). To test the functionality of the ZFs in the WIP domain, we generated variants of WIP1/TT1 carrying amino acid substitutions in at least one of the predicted zinc coordinating histidine or cysteine residues of each single ZF (TT1f1 to TT1f4) and in combination for the uncommon ZFs 2 and 4 (TT1df). In *A. thaliana*, loss of TT1 function results in yellow seeds due to a lack of proanthocyanidins (PAs) that is specific to the innermost cell layer of the seed coat (endothelium) but does not affect chalaza and micropyle at the seed base ([6,17] Fig. 2B). This mutant phenotype can be complemented by expression of *TT1* under the control of a 1.1 kb *TT1* promoter fragment [6] (Fig. 2B and C). Complementation of the *tt1-1* mutant therefore represented a visible and quantifiable system to investigate the functionality of the TT1 variants. At least 20 independent transgenic lines for each construct were analysed by semiquantitative RT-PCR to ensure expression levels of the respective *TT1* variant comparable to wildtype (not shown). PA precursors in immature seeds of the produced transgenics were visualized by vanillin staining [18] and the degree of browning of mature seeds by condensed and oxidised PAs was visually

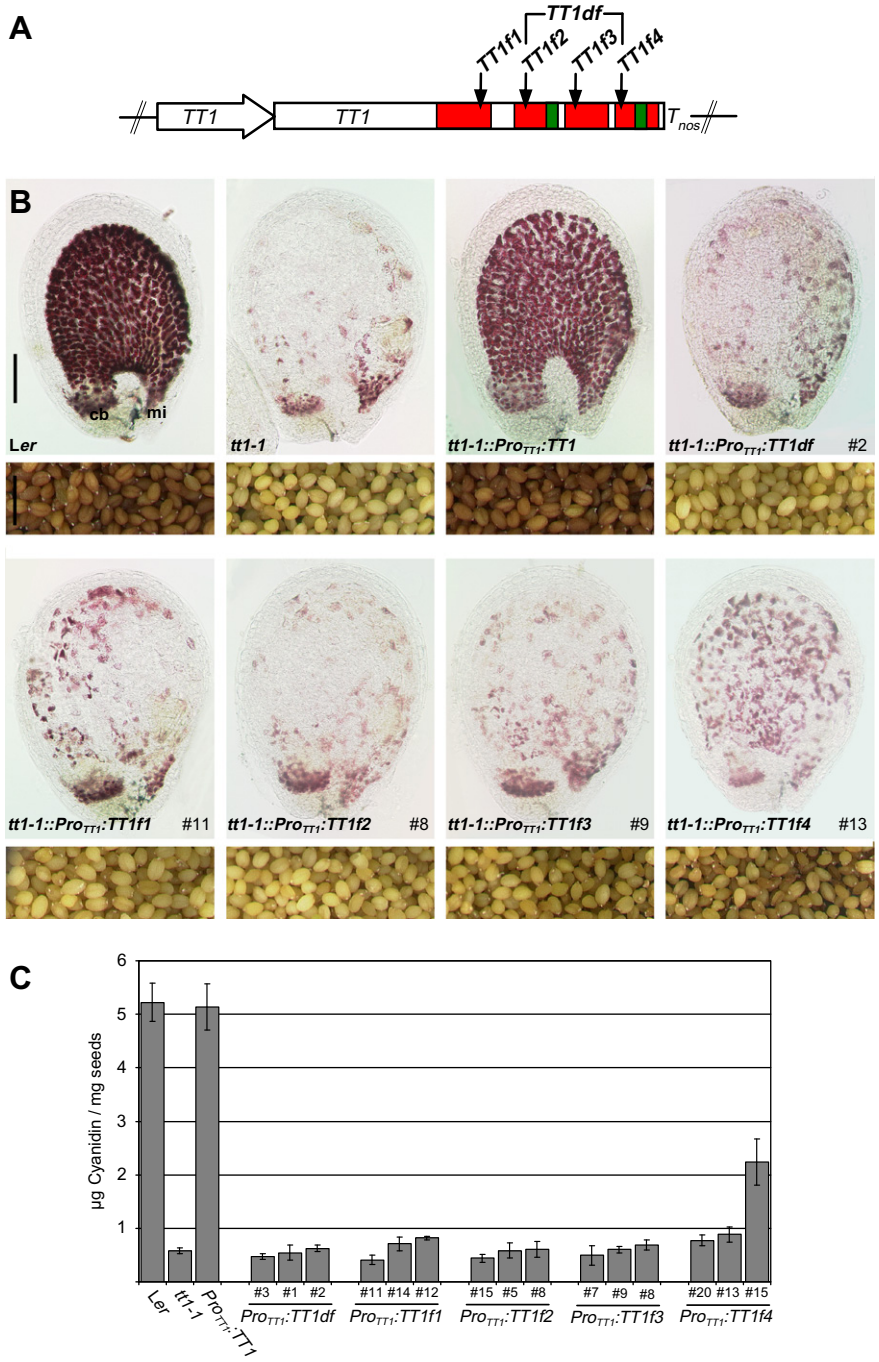

Fig. 2. Complementation of *tt1-1* with *TT1* variants. (A) Schematic representation of the constructs containing substitutions for predicted zinc coordinating residues. Arrow, *TT1* promoter; open box, *TT1* coding sequence; red, ZF motifs; green, NLSs; T_{nos}, *A. tumefaciens nos* terminator. (B) Upper panels: immature T2 seeds of representative lines expressing the Pro_{TT1}*:TT1f* transgenes stained with vanillin (scale bar = 100 µm). Red colour indicates presence of PA precursors. Lower panels: Mature seeds of the lines shown above, untreated (scale bar = 1 mm). Genotypes as indicated. Complementation of *tt1-1* with wildtype *TT1* demonstrates the functionality of the 1.1 kb *TT1* promoter fragment used. cb, Chalazal bulb; mi, micropyle. (C) PA quantification in mature T3 seeds of the genotypes shown in (B). Hashes indicate identity of three independent lines.

inspected and quantified photometrically after hydrolysis into coloured anthocyanidins (Fig. 2C). Compared to *tt1-1*, pigmentation was slightly increased in the transgenic lines (especially for the *TT1f4* construct). However, none of the tested TT1 variants was able to completely restore PA production in the mutant.

This indicates that the cysteine and histidine residues mutated in the TT1 variants are essential for function and makes it likely that the respective sequences form the predicted ZFs. The fact that the disruption of each individual ZF leads to a dysfunctional WIP protein suggests that every single motif is crucial and that all four motifs have to act in concert to fulfil WIP function.

3.2. Nuclear localisation signals

We have shown previously that a TT1-GFP fusion protein is exclusively localized to the nucleus in transiently transfected *A. thaliana* protoplasts [6]. For this study we applied the same approach to the complete set of WIP proteins using arabidopsis At7 as well as tobacco BY2 cells and observed nuclear localisation in all cases (Fig. 3A). WIP proteins range in size from 34 to 47 kDa, which is close to the diffusion barrier of the nuclear pore complex [19]. All WIPs contain two monopartite basic sequences with similarity to SV40 NLS [20] located within ZF2 and 4. For functional characterization, the predicted NLS residues were replaced by alanine residues in TT1 and the respective GFP fusion proteins were analysed for their subcellular localisation. Since varying distribution phenotypes were observed in the At7 protoplast system, the results were evaluated statistically (Fig. 3B). Introducing mutations in only one of the two putative signals (TT1m1- or TT1m2-GFP, respectively) resulted in reduced frequencies of nuclear localisation. The most prominent reduction was achieved with a TT1 version lacking both NLSs (TT1dm-GFP).

In planta confirmation of the NLSs studies was possible with two new *tt1* alleles identified by TILLING. Both alleles, named *tt1-6* and *tt1-7*, are point mutations (*tt1-6*: G > A at position +1289 from the start ATG; *tt1-7*: C > T at +1294), which lead to $R^{222} > H^{222}$ or $H^{224} > Y^{224}$ substitutions in the first NLS of TT1, respectively. In homozygous condition, both mutations decrease pigmentation and confer yellow to pale brown seed coats (Fig. 3C and D). Vanillin staining of seeds of the two new mutants showed clearly reduced PA accumulation in endothelial cells when compared to wildtype, although the reduction is not as strong as in *tt1-1* (containing the classical allele in genotype Landsberg *erecta*; Fig. 2B) and *tt1-3*, which has the same genetic background as the TILLING alleles (Columbia-0). Backcrosses of *tt1-6* and *tt1-7* with *tt1-1* did not result in phenotypic complementation in the F1, confirming that in both lines the *tt1* mutation causes the phenotype.

As the point mutations in both alleles affect the first NLS, mislocalisation of TT1 represented a possible explanation for the observed phenotypes. To address this question we tested the subcellular distribution of GFP fusions of these TT1 variants in the At7 protoplast system (Fig. 3B). Both variants lost their exclusively nuclear localisation, consistent with the alanine substitution approach described above. The $R^{222} > H^{222}$ substitution from *tt1-6* resulted in a reduction of nuclear localisation comparable to TT1m1-GFP, illustrating that the replacement of a single arginine residue by another basic amino acid is sufficient to influence the functionality of the NLS.

The first NLS comprises one of the zinc coordinating histidine residues of ZF2. This makes it likely that the alanine substitution approach as well as the *tt1-7* mutation, which affects this histidine, not only destroy the NLS but also ZF2 itself. The strongly reduced pigmentation of *tt1-7* seeds is therefore in line with the results of the ZF mutagenesis described above. In the case of *tt1-6* however the point mutation lies in a region where no effect on ZF formation is expected. Hence, we assume that the observed strong mislocalisation of the fusion protein is primarily a result of the NLS mutation and is not caused by disruption of the ZF. Reduced pigmentation of *tt1-6* seeds in turn illustrates the importance of correct nuclear localisation for TT1 function. Taken together, these findings indicate that both NLSs identified are necessary for correct subcellular localisation of TT1, and that TT1 probably contains additional signals that are involved in nuclear import. As the described NLSs are completely conserved in all WIP proteins, it can be assumed that these sequences are required for nuclear import of the other WIPs as well.

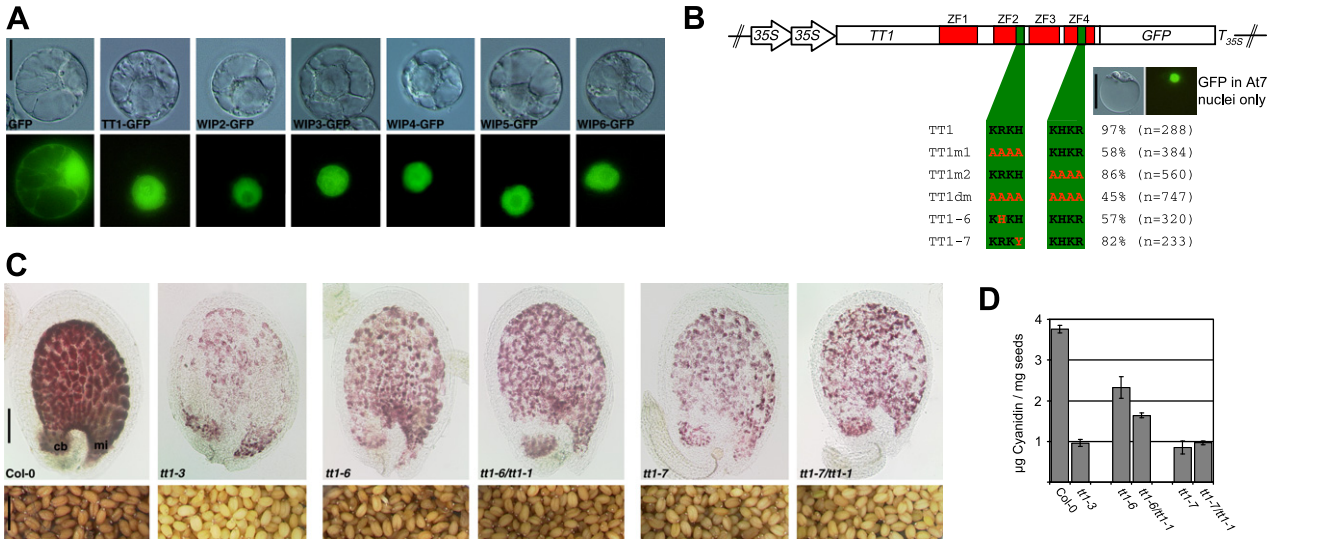

Fig. 3. Nuclear localisation of WIP proteins. (A) Localisation of constitutively expressed WIP-GFP fusion proteins in transiently transfected BY2 protoplasts. Upper panels: bright field image using Nomarski optics. Lower panels: GFP fluorescence. Scale bar = 20 µm. (B) Schematic representation of the constructs containing mutations in predicted NLSs and localisation frequencies of the resulting NLS mutated TT1-GFP proteins in At7 protoplasts. Arrows, *CaMV35S* promoter; open box, translational fusion of *TT1* and *GFPm5* coding sequence; red, ZF motifs; green, NLSs; T_{35S}, *CaMV 35S* terminator. Total number of evaluated fluorescent cells (*n*) and percentage of cells showing exclusively nuclear localisation of GFP fluorescence. Scale bar = 20 µm. (C) Phenotypes of the TILLING alleles *tt1-6* and *tt1-7* and heterozygous plants resulting from backcrosses to *tt1-1*. Vanillin staining of immature seeds and untreated mature seeds of the indicated genotypes as in Fig. 2. (D) PA quantification in mature seeds of the genotypes shown in (C).

3.3. Promoter swap experiments

ZFPs often contain domains in addition to ZF motifs that provide functional specificity. Among WIP proteins amino acid sequence conservation is largely restricted to the ZF domain. Using MEME analysis [21], conserved signatures were identified outside of the WIP domain (Fig. 1D). WIP proteins 2, 4 and 5 are characterized by the presence of motif 2, which is equivalent to the one described in [9]. All six WIPs share motif 1 and contain stretches rich in glutamate, which could be parts of acidic activation domains (motif 3). To test this hypothesis, TT1 and a TT1 fragment containing theses residues were fused to the GAL4-DNA binding domain and co-transfected with a GAL4 responsive *GUS* reporter into arabidopsis protoplasts. However, no significant activation was observed (data not shown).

To elucidate the importance of the less conserved parts of the proteins, WIP2, 3, 4 and 6 were tested for their ability to fulfil TT1 function by expressing the corresponding coding sequences in the *tt1-1* mutant under control of the *TT1* promoter. Instead of WIP5, which is highly similar to WIP4 (see Fig. 1A), we included a truncated *TT1* variant in the experiment, which only codes for the WIP domain (*WIP-D*). For each construct several transgenic lines were investigated for their ability to replace TT1 in seed coat pigmentation and representative pictures are shown in Fig. 4A. The isolated WIP domain of TT1 was not able to induce pigmentation. The expression of all tested full length *WIPs*, however, led to enhanced PA production compared to the background of the transgenics, although wild-type levels were not reached. Hence, it seems that the tested WIP family members are at least partially able to restore TT1 function.

Failed complementation by WIP-D shows that the conserved ZF domain alone is not sufficient to mediate TT1 function and suggests that functions encoded by the less conserved parts of the WIPs are required in addition. Motif 2 was already shown to be necessary for proper function of *Cm*WIP1 in melon [9]. Motif 1 is present in all six WIPs and therefore represents another promising candidate for further investigation. In summary, we conclude that all WIPs can act in similar ways and may only differ in their spatial and temporal expression patterns and their targets.

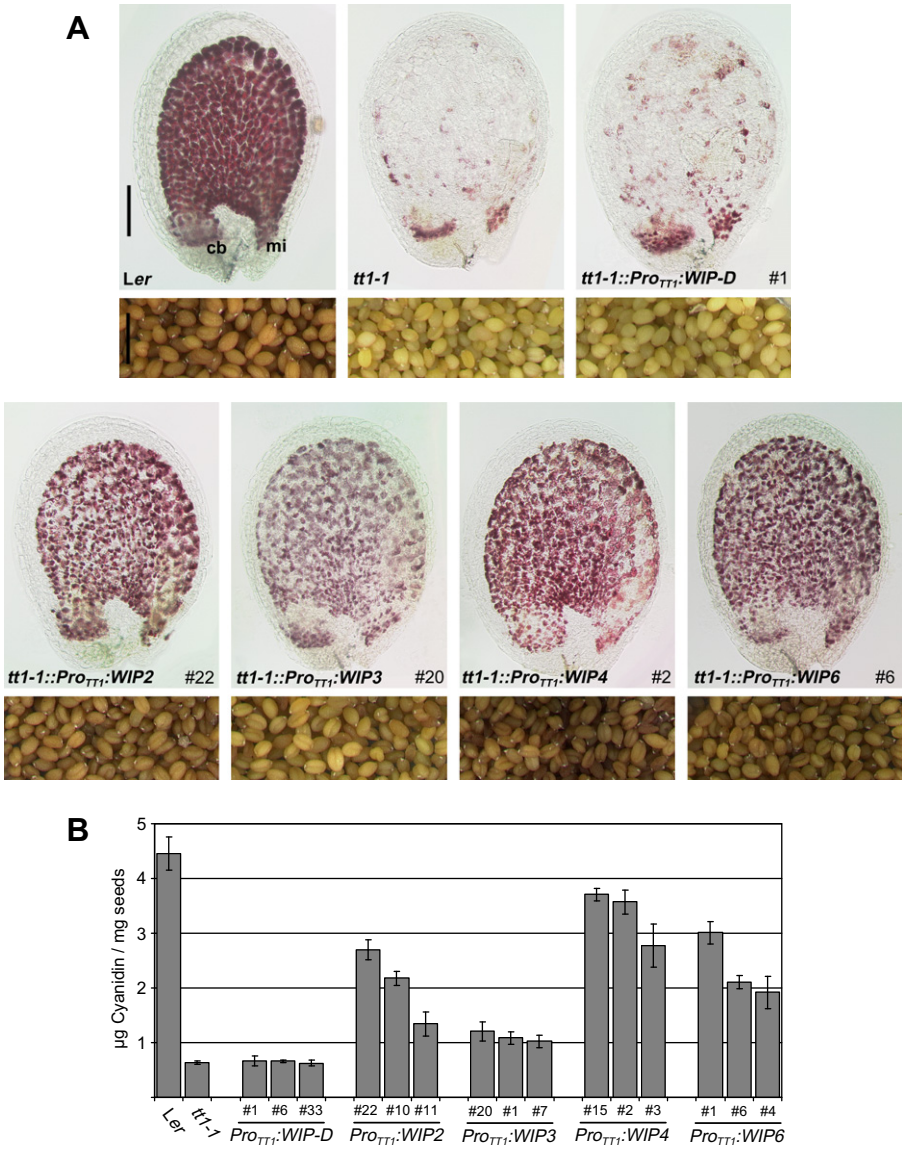

Fig. 4. *WIP* promoter swap experiments. (A) Full length coding sequences of the indicated *WIPs* and of a truncated *TT1* coding for the WIP domain only (*WIP-D*) were expressed under the control of the *TT1* promoter in *tt1-1* background. Vanillin staining of immature seeds and untreated mature seeds of the indicated genotypes as in Fig. 2. (B) PA quantification of mature T3 seeds of genotypes shown in (A). Hashes indicate identity of three independent lines.

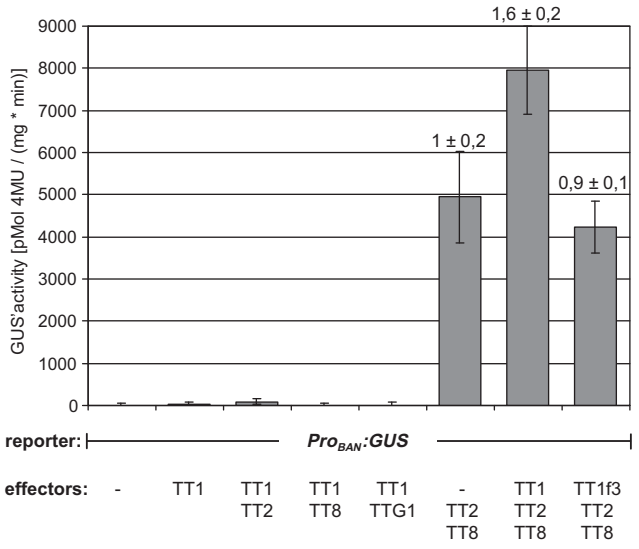

Fig. 5. TT1 effects on *BAN* promoter activation. Co-transfection experiments with At7 protoplasts were performed to analyse the activation of a Pro_{BAN}*:GUS* reporter by different effectors. Normalised GUS activity is given. Values above the error bars indicate fold induction relative to values obtained in co-transfections with TT2 and TT8. Results are derived from six independent transfection experiments.

3.4. Effects on BAN promoter activation

The presence of ZF motifs and the observed nuclear localisation indicates that WIP proteins may act as transcriptional regulators. However, DNA binding of WIP proteins has not been demonstrated so far. A potential target gene for TT1 in *A. thaliana* is *BANYULS (BAN)* which encodes anthocyanidin reductase, a key enzyme of the PA pathway [22], and reduced expression of *BAN* in *tt1-1* had been shown already [6]. To test if TT1 is able to activate the *BAN* promoter, co-transfection experiments were performed in arabidopsis protoplasts using Pro_{BAN}*:GUS* as a reporter. TT1 alone was not able to activate the *BAN* promoter in this assay (Fig. 5) and activation was also not observed when TT1 was used in fusion to the VP16 activation domain (data not shown). This indicates that TT1 does not bind directly to *cis* regulatory elements in the *BAN* promoter.

BAN expression is known to be synergistically controlled by a ternary complex consisting of the MYB protein TT2, the BHLH protein TT8 and the WD40 protein TTG1 [13]. TTG1 is endogenously expressed in At7 cells, which allows activation of the *BAN* promoter by combined co-transfection of TT2 and TT8 (Fig. 5). Co-transfection experiments using TT1 in combination with either TT2 or TT8 did not yield *BAN* promoter activity. However, when TT1 was applied in combination with TT2 and TT8, *BAN* promoter activity was increased about 1.6-fold over the effect of the TT2 and TT8 control. This induction is low but reproducible and was not observed when TT1f3 was used as an effector confirming that it requires correct formation of at least this ZF.

Based on these results we propose that TT1 may be involved in the formation of the TT2–TT8–TTG1 protein complex via protein–protein interactions rather then binding DNA directly. Several C2H2 ZFPs are known to act in a similar manner [3]. One example is the FOG-type ZF protein U-shaped from drosophila, which binds to GATA-1. Together they form a promoter associated complex controlling gene expression [23].

The fact that according to the "DNA recognition code" [1,5] only 5 of the 12 potential base determinant helix positions have high specificity to particular DNA bases, may also indicate an involvement of TT1 in protein interactions rather then DNA binding. However, it remains to be elucidated, if such proposed protein–protein interactions are mediated by the ZF motifs or protein parts outside of the WIP domain and, if they are the common functional principle for all WIP proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.007.

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