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# TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*

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**Summary**

**Genetic analyses have demonstrated that together with TTG1, a WD-repeat (WDR) protein, TT2 (MYB), and TT8 (bHLH) are necessary for the correct expression of *BANYULS* (*BAN*). This gene codes for the core enzyme of proanthocyanidin biosynthesis in *Arabidopsis thaliana* seed coat. The interplays of TT2, TT8, and their closest MYB/bHLH relatives, with TTG1 and the *BAN* promoter have been investigated using a combination of genetic and molecular approaches, both in yeast and *in planta*. The results obtained using glucocorticoid receptor fusion proteins *in planta* strongly suggest that TT2, TT8, and TTG1 can directly activate *BAN* expression. Experiments using yeast two- and three-hybrid clearly demonstrated that TT2, TT8, and TTG1 can form a stable ternary complex. Furthermore, although TT2 and TT8 were able to bind to the *BAN* promoter when simultaneously expressed in yeast, the activity of the complex correlated with the level of *TTG1* expression in *A. thaliana* protoplasts. In addition, transient expression experiments revealed that TTG1 acts mainly through the bHLH partner (i.e. TT8 or related proteins) and that TT2 cannot be replaced by any other related *A. thaliana* MYB proteins to activate *BAN*. Finally and consistent with these results, the ectopic expression of *TT2* was sufficient to trigger *BAN* activation in vegetative parts, but only where *TTG1* was expressed. Taken together, these results indicate that TT2, TT8, and TTG1 can form a ternary complex directly regulating *BAN* expression *in planta*.**

**Keywords: bHLH, flavonoid, MYB, proanthocyanidin, transcription factor, TTG1.**

## Introduction

Flavonoid compounds, mainly anthocyanins, flavonols, and proanthocyanidins (PAs), are major plant secondary metabolites that fulfill a multitude of functions during plant development (Winkel-Shirley, 2001). In *Arabidopsis thaliana*, PAs specifically accumulate in the inner integument and in the pigmented chalazal strand of the seed coat. The presence of these polymerized flavonoids with antioxidant properties contributes to a protection barrier for the embryo and has been shown to enhance seed coat-imposed dormancy and seed longevity (Debeaujon *et al.*, 2000; Winkel-Shirley, 1998). The first enzymatic step committed to PA biosynthesis is catalyzed by an anthocyanidin reductase encoded by the *BANYULS* gene (*BAN*; Xie *et al.*, 2003). *BAN* expression is strictly limited to PA-accumulating cells during seed coat development (Debeaujon *et al.*, 2003). This specific expression pattern appears to be mainly conferred by TT2, an R2R3-MYB transcription factor (TF) encoded by the *TRANSPARENT TESTA2* gene (Debeaujon *et al.*, 2003; Nesi *et al.*, 2001). Nevertheless, two additional regulatory genes were shown to participate in the control of *BAN* expression (Nesi *et al.*, 2000), namely *TRANSPARENT TESTA8* (*TT8*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*). These genes encode, respectively, a basic helix-loop-helix TF (bHLH; Nesi *et al.*, 2000) and a WD-repeat protein (WDR; Walker *et al.*, 1999) that are also involved in the regulation of anthocyanin accumulation in vegetative tissues. Two other regulators of

the PA pathway in seeds have been recently identified, a zinc-finger protein (TT1, Sagasser *et al.*, 2002) and a MADS-box TF (TT16, Nesi *et al.*, 2002). These TFs are thought to act upstream of TT2, probably through the regulation of seed coat development (Debeaujon *et al.*, 2003; Nesi *et al.*, 2002; Sagasser *et al.*, 2002). How the putative direct regulators of PA metabolism, TT2, TT8, and TTG1, act together in activating *BAN* expression remains unknown.

The formation of TF complexes is known to be a fundamental process in fine-tuning gene activity in eukaryotes. In plants, the cooperation between R2R3-MYB and bHLH proteins was shown to be crucial for the expression of flavonoid biosynthesis genes in *Zea mays* (Goff *et al.*, 1992), for trichome development (Payne *et al.*, 2000), and ABA-regulated gene expression (Abe *et al.*, 2003) in *A. thaliana*. For instance, although the MYB factor P alone is able to activate the expression of the genes of the phlobaphene pathway in *Z. mays* (Grotewold *et al.*, 1994), other MYBs from the C1/PL family require a direct interaction with bHLHs of the R/B family to induce anthocyanin biosynthesis (Goff *et al.*, 1992). Interestingly, a few amino acid changes in the MYB domain were sufficient to confer similar functional properties to P (i.e. interaction with R and activation of usual targets of the C1/R complex; Grotewold *et al.*, 2000). This work has revealed the importance of combinatorial interactions between the MYB and the bHLH factors controlling the flavonoid pathway, to specifically regulate the expression of target genes. Moreover, these interactions may be involved, at least partially, in some of the functional specificity displayed by members of these two major plant TF gene families (Heim *et al.*, 2003; Riechmann *et al.*, 2000; Stracke *et al.*, 2001).

In plants for which flavonoid metabolism was genetically investigated (mainly *Antirrhinum majus*, *Petunia hybrida*, *Perilla frutescens*, and *A. thaliana*), MYBs and bHLHs homologous to the *Z. mays* C1 and R proteins were identified as regulatory factors (for review see Irani *et al.*, 2003). The ectopic expression of C1, R, and DELILA in heterologous plant systems revealed the conservation of the combinatorial interactions between MYBs and bHLHs from different species for anthocyanin regulation (Bradley *et al.*, 1998; Lloyd *et al.*, 1992; Mooney *et al.*, 1995). However, these experiments also highlighted important functional differences (Mooney *et al.*, 1995) and suggested the existence of additional regulatory factors.

One class of these regulators consisted of the remarkably conserved WDR proteins, AN11 in *P. hybrida* (de Vetten *et al.*, 1997), TTG1 in *A. thaliana*, PFWD in *P. frutescens* (Sompornpailin *et al.*, 2002), and PAC1 in *Z. mays* (Carey *et al.*, 2004). In *A. thaliana*, TTG1 not only regulates flavonoid metabolism, but is also involved in trichome organogenesis, root hair spacing, and biosynthesis of seed coat mucilage (Koornneef, 1981). These cell differentiation processes occurring in epidermal tissues have another regulator in common, namely TTG2, a WRKY-type protein (Johnson *et al.*, 2002). Interestingly, some MYB and/or bHLH proteins have also been found to be involved in the regulation of all these pathways, suggesting the existence of a conserved regulatory mechanism (Borevitz *et al.*, 2000; Kirik *et al.*, 2001; Lee and Schiefelbein, 1999; Oppenheimer *et al.*, 1991; Payne *et al.*, 2000; Penfield *et al.*, 2001; Zhang *et al.*, 2003) (Table 1). As the ectopic expression of R complements the defects observed in a *ttg1* mutant, it was proposed that TTG1 could regulate the pathways in which *A. thaliana* R homologues are involved (Lloyd *et al.*, 1992).

**Table 1** R2R3-MYB and bHLH factors of the TTG1-dependent pathways

| | Root-hair spacing | Trichome initiation | Flavonoid metabolism | Mucilage biosynthesis |
|---|---|---|---|---|
| MYB | WER | GL1, AtMYB23 | TT2, PAP1/2 | MYB61 |
| bHLH | GL3, EGL3 | GL3, EGL3 | TT8, EGL3 | TT8, EGL3 |
| WDR | TTG1 | TTG1 | TTG1 | TTG1 |

The WDR domain is found in proteins implicated in various eukaryotic cellular functions that involve multiple simultaneous or consecutive protein–protein interactions (Smith *et al.*, 1999). Consistent with this property, TTG1 has been shown to interact in the yeast two-hybrid system with two bHLH factors (GLABRA3/GL3 and ENHANCER OF GLABRA3/EGL3; Payne *et al.*, 2000; Zhang *et al.*, 2003). A similar interaction between a WDR protein and a TF has already been described in plants, as for instance, between the bZIP protein HY5 and the COP1 WDR domain (Ang *et al.*, 1998; Torii *et al.*, 1998). However, analysis of the TTG1 sequence reveals no other known specific domain than the WDR and its precise regulatory function remains poorly understood (Payne *et al.*, 2000; Walker *et al.*, 1999).

Here, we report a set of molecular and genetic experiments performed to elucidate the nature and specificity of the interactions between TT2, TT8, and TTG1 proteins, and with the *BAN* promoter. The results are consistent with the formation of a TT2–TT8–TTG1 ternary complex that directly regulates *BAN* expression *in planta*. A model is proposed that presents the role of each member of this protein complex in the precise determination of *BAN* expression pattern in the *A. thaliana* seed coat.

## Results

### *TT2, TT8, and TTG1 directly activate* BAN *expression in* A. thaliana *siliques*

Previous work has shown that *BAN* expression is greatly reduced or null in the *tt2*, *tt8*, and *ttg1* mutants, indicating that *BAN* is either a primary or a secondary target of TT2, TT8, and/or TTG1 (Debeaujon *et al.*, 2003; Nesi *et al.*, 2000).

To distinguish between these possibilities, we introduced the *35S:TT2-GR*, *35S:TT8-GR*, and *35S:TTG1-GR* constructs into the corresponding mutant backgrounds (*tt2-1*, *tt8-1*, and *ttg1-13*, respectively). Thus, transgenic plants that ectopically express fusion proteins with the glucocorticoid receptor (GR) domain were generated. The fusion with GR enables the control of the subcellular localization of the chimeric proteins through binding to a cytoplasmic complex preventing their action. Dexamethasone (DEX) disrupts the complex allowing release and translocation into the nucleus of a pool of active protein. A DEX treatment in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, still induces the transcription of direct target genes, whereas the transcription of further downstream genes is inhibited. This method has already contributed to the detection of proximal targets of several TFs, including the *P. hybrida* anthocyanin regulator AN1 (Spelt *et al.*, 2000).

Transgenic *ttg1-13* mutants transformed with the *35S:TTG1-GR* construct were obtained and all presented the typical *ttg1* phenotype. However, 10 days after a single DEX application on seedlings, young emerging leaves started to develop trichomes (Figure 1b). The repetition of DEX treatment on young siliques resulted in the production of brown seeds similar to the wild-type instead of fully yellow seeds characteristic of *ttg1* (Figure 1c–e). Furthermore, a treatment of 2-day-old germinating seedlings restored anthocyanin production in the hypocotyl and at the cotyledon margin (not shown), demonstrating that the fusion of TTG1 with GR is functional.

The activation of *BAN* expression was determined by quantitative RT-PCR on total mRNA of young developing siliques. In wild-type siliques, *BAN* is expressed at 40% of *EF* expression (the *EF1α A4* gene is used as an internal control), whereas no transcript is detected in *tt2-1*, *tt8-1*, or *ttg1-13* in the same conditions (Figure 2a). The activation of TTG1-GR in siliques by incubation in a 10 μM DEX solution for 6 h (see Experimental procedures) resulted in a highly significant and reproducible expression of *BAN* (two independent transgenic plants were tested and a representative result obtained for one transgenic plant is shown in Figure 2b). When a CHX treatment (100 μM) preceded DEX application and continued for 6 h, *BAN* was still activated to a similar


**Figure 1.** Effect of DEX treatment on *ttg1* plants transformed with *35S:TTG1-GR* construct.
(a) Three-week-old untransformed *ttg1* seedling treated at day 10 by a single application of a 10 μM DEX solution on leaves. DEX application has no effect on the *ttg1* glabrous phenotype.
(b) Three-week-old *ttg1* seedling transformed with *35S:TTG1-GR* construct and treated at day 10 by a single application of a 10 μM DEX solution on leaves. Young emerging leaves present regular-shaped trichomes (arrowheads).
(c) *Transparent testa* seeds of an untreated *ttg1* plant transformed with *35S:TTG1-GR* construct.
(d) Seeds of a *ttg1* plant transformed with *35S:TTG1-GR* construct, after DEX treatment by application of a 10 μM solution on the whole plant during silique development. Some *transparent testa* seeds were expected according to the presence of fully mature siliques before the start of DEX treatments.
(e) Wild-type seeds.
Bars = 1 cm (a, b); 200 μm (c–e).

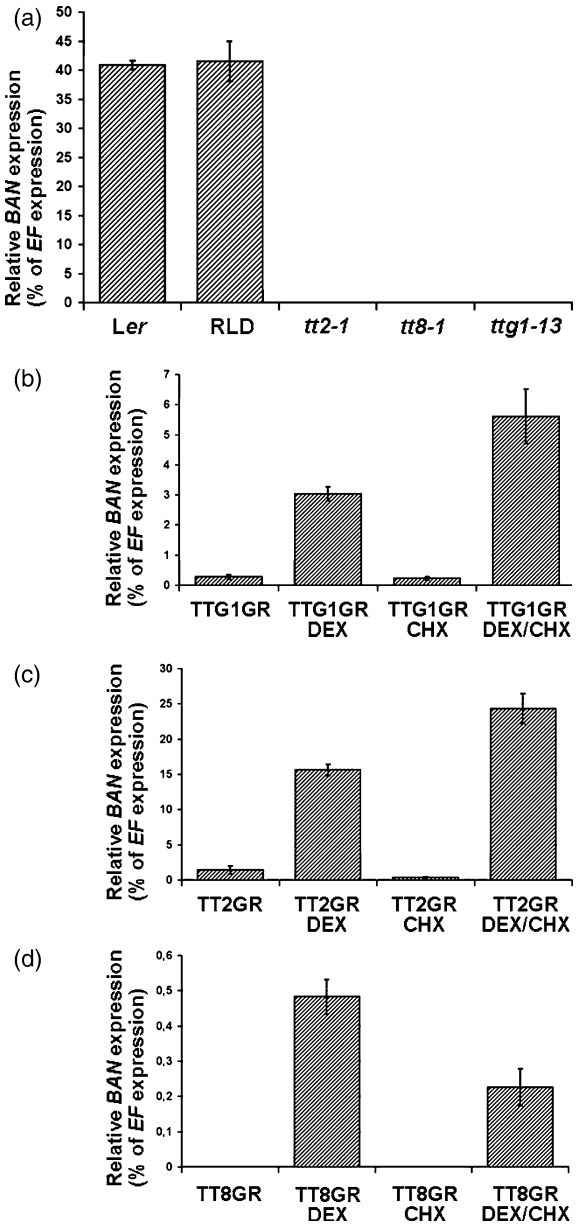



**Figure 2.** Direct activation of *BAN* expression by TTG1-GR, TT2-GR, and TT8-GR fusion proteins in *A. thaliana* siliques.

The level of *BAN* mRNA was measured by quantitative RT-PCR in young developing siliques (see Experimental procedures). It is presented as a percentage of the constitutive expression level of the gene *EF1α A4* (*EF*; mean ± standard deviation of three quantifications on one cDNA sample).

(a) Comparison of *BAN* expression level in the siliques of L*er* and RLD ecotypes, and of the *tt2-1* (L*er*), *tt8-1* (Enkheim), and *ttg1-13* (RLD) mutants.

(b–d) *BAN* expression level in the siliques of transgenic plants expressing TTG1-GR (b), TT2-GR (c), or TT8-GR (d) in the corresponding mutant background. In each case, the *BAN* expression level was quantified after 6 h incubation of siliques in four conditions: mock, DEX, CHX, or both DEX and CHX treatment. This shows the results of a reproducible induction obtained on one transgenic plant. This induction is representative of the results obtained with at least two independent transgenic plants for each construct. It is important to notice that the difference in the level of *BAN* activation between the conditions DEX and DEX/CHX is not significant with regard to the repetitions made on different transgenic plants and should be attributed only to the variation of the biological material present in each sample.

level (Figure 2b), suggesting that it is a primary target of TTG1. In line with this, *BAN* expression was investigated in plants transformed with the *35S:TT2-GR* or *35S:TT8-GR* constructs. Similarly, DEX treatment resulted in a significant induction of *BAN* in siliques after 6 h of induction (Figure 2c,d). Reproducible inductions were obtained in the presence of CHX, providing evidence that *BAN* is also a primary target of TT2 and TT8. However, despite a significant *BAN* activation, TT8-GR was probably not fully functional (unlike TT2-GR and TTG1-GR), because the level of *BAN* activation remained weak after a 6-h induction (< 1% EF for all the transgenic plants tested). In addition, the *tt8* phenotype was not complemented after a DEX treatment of siliques or seedlings (not shown).

### *TT2, TT8, and TTG1 interact to form a ternary complex*

Possible interactions between TT2, TT8, and TTG1 were investigated by two-hybrid experiments. A total number of 16 yeast clones containing different combinations of GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD) fusion proteins (Figure 3a) were tested for the expression of the three reporter genes namely *ADE2*, *HIS3*, and *LacZ*. First, plating the clones on a medium lacking adenine and histidine, and supplemented with 3-aminotriazole (AT) to a final concentration of 15 mM revealed the expression of the two auxotrophic markers (*ADE2* and *HIS3*) in the clones no. 8, 11, 12, 13, 14, and 16 (Figure 3a). This result suggested the occurrence of relevant interactions in these clones, because the growth of all the control clones was inhibited in these conditions (clones no. 1-7). The expression of the third reporter gene *LacZ* was investigated by quantitative assays using *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The β-galactosidase activities confirmed the interaction results obtained for *ADE2* and *HIS3* (Table 2). Taken together, these results demonstrated significant interactions between TT8 and TTG1 (clone no. 14 and 16), TT2/TT8 (clone no. 11 and 13), and TT2/TTG1 (clone no. 12). A high *LacZ* activation was detected in clones no. 14 and 16 with 3.9 ± 0.3 and 19.9 ± 2.6 UβGal, suggesting a high-affinity interaction between TT8 and TTG1. The interaction between TT2 and TTG1 was demonstrated only in one direction (no. 12), a phenomenon that is often encountered in two-hybrid experiments (Burbulis and Winkel-Shirley, 1999; Estojak *et al.*, 1995). TT2 was shown to homodimerize, as the clone no. 8 was positive for the three reporter genes. On the contrary, TT8 or TTG1 homodimer formation was not clearly supported in these yeast experiments.

In order to investigate the ability of TT2, TT8, and TTG1 to form a complex of three proteins, three-hybrid experiments were performed. In combinatorial interactions, the addition of the third protein is expected to enhance the stability of the chimeric GAL4 and to increase the activation of the reporter genes. Clones no. 11–16 previously described were trans-

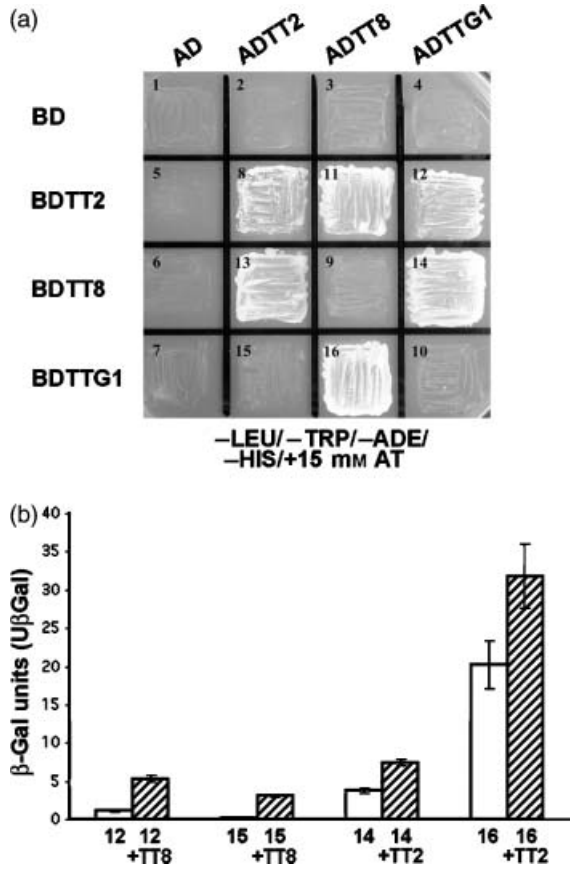



**Figure 3.** Cooperative interaction of TT2, TT8, and TTG1 in yeast two-hybrid experiments.

(a) The yeast strain was co-transformed with the indicated combinations of TT2, TT8, and TTG1 fused to AD or BD. The interactions tested concerned the control clones (1–7), the homodimer formation (8–10), and the heterodimer interactions (11–16). Activation of two of the reporter genes, *ADE2* and *HIS3*, was assayed by plating the clones on the appropriate selective medium.

(b) β-Galactosidase activity was monitored in clones no. 12, 14, 15, and 16 co-transformed with the third partner expressed in the pTFT1 vector. Comparison between β-galactosidase activity in these new clones and the former ones is presented. The β-galactosidase activity is shown in β-Gal units. Results are mean ± standard deviation of four independent yeast clones.

ADE, adenine; AT, 3-aminotriazole; HIS, histidine; LEU, leucine; TRP, tryptophan.

formed with a plasmid allowing the expression of the third protein. *LacZ* activation was monitored in the new clones and compared with the former ones (Figure 3b). A significant increase in β-galactosidase activity was detected in clones no. 12 and 15, both transformed with TT8. As no interaction between AD-TTG1 and BD-TT2 was detected in two-hybrid for clone no. 15, this additional result suggested that TT8 is able to form a bridge between TT2 and TTG1. Accordingly, *LacZ* activation increased in clones no. 14 and 16 transformed with TT2, indicating that the addition of TT2 can strengthen the interaction between TT8 and TTG1. These results were consistent with the two-hybrid results and demonstrate that TT8 is able to interact simultaneously with TT2 and TTG1. Moreover, they suggest a cooperative binding of TT2, TT8, and TTG1 to form a complex of three proteins *in vivo*.

### *TT2, TT8, and TTG1 bind simultaneously to the* BAN *promoter*

Analysis of the interactions between TT2, TT8, and TTG1 with the *BAN* promoter was performed with one-hybrid experiments. A yeast strain that presented the *HIS3* reporter gene under the control of a functional *BAN* promoter (see Experimental procedures; Debeaujon *et al.*, 2003) was constructed and transformed with different combinations of plasmids allowing the expression of TT2, TT8, and/or TTG1 (Figure 4).

A single transformation of the strain with TT2, TT8, or TTG1 fused to AD gave no positive interaction results, suggesting that none of the three proteins was able to activate transcription alone (Figure 4a). However, the expression of both TT2 and TT8 resulted in specific growth of the strain on media lacking histidine, except when TT8 was expressed in fusion with BD (Figure 4b–d). These results demonstrate that the presence of both TT2 and TT8 is necessary for the binding to the *BAN* promoter in yeast. The direct interaction between TT2 and TT8, revealed in two-hybrid experiments, suggests that they might act as a complex. The BD-TT8 fusion probably does not have the right conformation to allow the activation of the *BAN* promoter by any of the TT2/BD-TT8 complexes.

**Table 2** Quantification of the third reporter gene *LacZ* confirming the yeast two-hybrid interaction results

| | | | | | Interaction results | |
|---|---|---|---|---|---|---|
| Yeast clones | AD construct | BD construct | β-Galactosidase activity (UβGal) | Control clones (UβGal) | *LacZ* | *ADE2/HIS3* |
| 8 | AD-TT2 | BD-TT2 | 1.123 ± 0.121 | 2: 0.08/5: 0.641 | + | + |
| 11 | AD-TT8 | BD-TT2 | 1.291 ± 0.206 | 3: 0.024/ 5: 0.641 | + | + |
| 13 | AD-TT2 | BD-TT8 | 0.96 ± 0.199 | 2: 0.08/ 6: 0.005 | + | + |
| 12 | AD-TTG1 | BD-TT2 | 1.1 ± 0.053 | 4: 0.051/5: 0.641 | + | + |
| 15 | AD-TT2 | BD-TTG1 | 0.167 ± 0.021 | 2: 0.08/ 7: 0.02 | – | – |
| 14 | AD-TTG1 | BD-TT8 | 3.9 ± 0.3 | 4: 0.051/6: 0.005 | ++ | + |
| 16 | AD-TT8 | BD-TTG1 | 19.9 ± 2.6 | 3: 0.024/ 7: 0.02 | +++ | + |

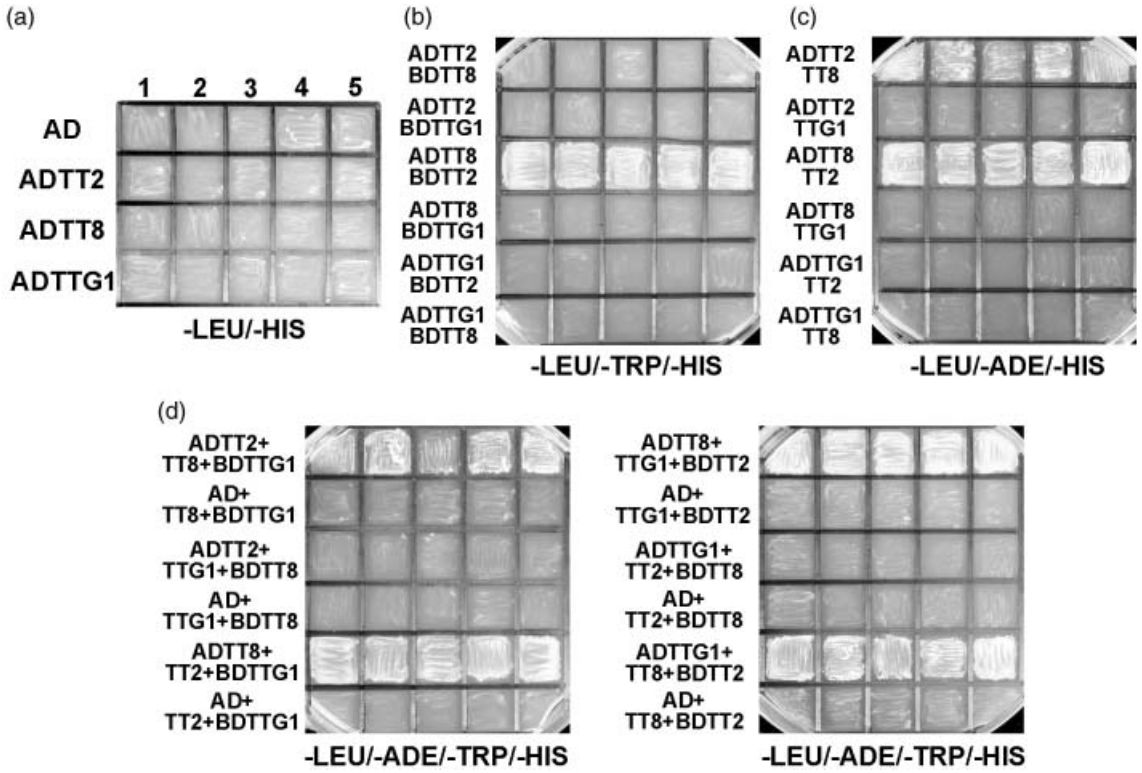



**Figure 4.** Cooperative interaction of TT2, TT8, and TTG1 with the *BAN* promoter in yeast one-hybrid experiments.
Yeast clones are grown on appropriate media to maintain the expression of the vectors and to test for the activation of the *HIS3* reporter gene. Results obtained for five independent colonies are presented.
(a) Single transformants.
(b, c) Double combinations.
(d) Triple combinations.
ADE, adenine; HIS, histidine; LEU, leucine; TRP, tryptophan.

Noteworthy, TTG1 appears not to be required for the recognition of the *BAN* promoter by the TT2/TT8 complex in yeast. Similarly, TTG1 is not likely to influence the stability of TT2/TT8 interaction with the *BAN* promoter in yeast because no variation in the *HIS3* expression level occurred when TTG1 was added in the positive clones of Figure 4b,c (data not shown). However, the positive result obtained for the clone expressing AD-TTG1, TT8, and BD-TT2 is very important (Figure 4d). This is because in this clone, the AD necessary for the activation of the reporter gene is fused to TTG1, demonstrating that TTG1 participates in the complex binding to the *BAN* promoter. Taken together, these results indicate that the ternary complex made of TT2, TT8, and TTG1 can directly bind to the *BAN* promoter.

### *TTG1 controls* BAN *activation through TT8*

Transient expression experiments were conducted in protoplasts derived from cultured *A. thaliana* cells. The effect of the ectopic expression of various combinations of MYBs and bHLHs factors on *BAN* activation was determined by quantifying the GUS activity generated from a *BAN:uidA* reporter construct containing the functional *BAN* promoter. In this system, *TTG1* is expressed as revealed by RT-PCR experiments, while no expression of *TT2*, *TT8*, or the other effectors tested was detectable (data not shown).

Transient transformation of the protoplasts with *35S:TT2* or *35S:TT8* did not induce any activation of the reporter gene. However, co-transformation with these two constructs resulted in a significant GUS activity, confirming that both TT2 and TT8 are necessary to activate *BAN* in plant cells (Figure 5a). As TTG1 has been shown to be involved in the direct induction of *BAN* expression and interacts with TT2 and TT8 (this study), we decided to test its effect on TT2/TT8 activity in protoplasts. Remarkably, *TTG1* overexpression strongly increased the *BAN* activation initially conferred by *35S:TT2* and *35S:TT8* (Figure 5a). In addition, the effect of a reduction in *TTG1* expression was tested by RNA interference (RNAi, see Experimental procedures). Co-transformation with *35S:TT2*, *35S:TT8*, and the *TTG1RNAi* construct resulted in a significant twofold decrease in GUS activity when compared with the result obtained without RNAi. Thus, the activation of *BAN* by TT2/TT8 correlated with the expression level of *TTG1*, indicating that TTG1 has a

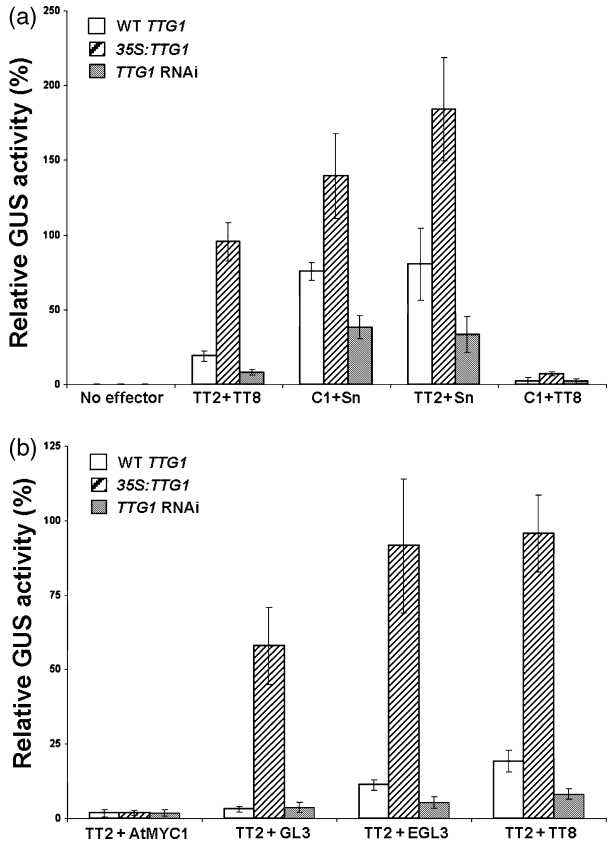



**Figure 5.** Effect of MYB/bHLH combinations on *BAN* expression depending on the *TTG1* expression level in *A. thaliana* protoplasts.
Relative GUS activity is presented in percentage of the GUS activity generated by a *35S:uidA* construct. The effects of different MYB/bHLH combinations were tested with a wild-type *TTG1* expression level, *TTG1* overexpression, or *TTG1* RNAi. Results are mean ± absolute deviation of eight independent transfection experiments.
(a) Comparison of the activity of TT2/TT8 and the maize regulators C1/Sn.
(b) Comparison of the activity of the TT2/bHLHs combinations.

quantitative effect on the efficiency of the TT2/TT8 complex in protoplasts.

The *Z. mays* flavonoid regulators C1 (MYB) and Sn (bHLH) were also tested in this system. Surprisingly, the combination of *35S:C1* and *35S:Sn* constructs strongly activated the *BAN:uidA* reporter gene four times more efficiently than TT2/TT8 (Figure 5a). Therefore, cross-expressions were investigated to determine whether this difference concerned the MYB/bHLH complex as a whole or more specifically, the expression or the function of one component of the complex. In these experiments, TT2/Sn gave an induction similar to C1/Sn (Figure 5a), demonstrating that MYB partners are not responsible for the quantitative difference observed between the *Z. mays* and *A. thaliana* TFs. In addition, TT2/TT8 was more sensitive to *TTG1* overexpression (fivefold induction) than C1/Sn or TT2/Sn (twofold induction), suggesting that TTG1 mainly acts through TT8. On the contrary, the C1/TT8 combination was not able to activate *BAN* significantly. Taken together, these results demonstrate that TTG1 has a quantitative effect on *BAN* expression in plant protoplasts, at least through TT8, the bHLH component of the TT2/TT8 complex. Moreover, TT8 and Sn presented major functional differences, in their ability to interact with C1 and in their requirement for TTG1 to induce high levels of *BAN* expression in *A. thaliana* protoplasts.

### BAN *activation is specifically conferred by TT2 in* A. thaliana

In order to assess the specificity of each component of the TT2/TT8 complex for the activation of the *BAN* promoter, combinations of TT2-related MYB and TT8-related bHLH factors were investigated in protoplasts. The *A. thaliana* MYB and bHLH proteins presenting the highest structural similarity with TT2 (e.g. PAP1, PAP2, WER, GL1, AtMYB23, and AtMYB111 – subgroups 5, 6, 7, and 15; Stracke *et al.*, 2001) or TT8 (e.g. TT8, GL3, EGL3, and AtMYC1 – subgroup IIIf; Heim *et al.*, 2003) have been tested.

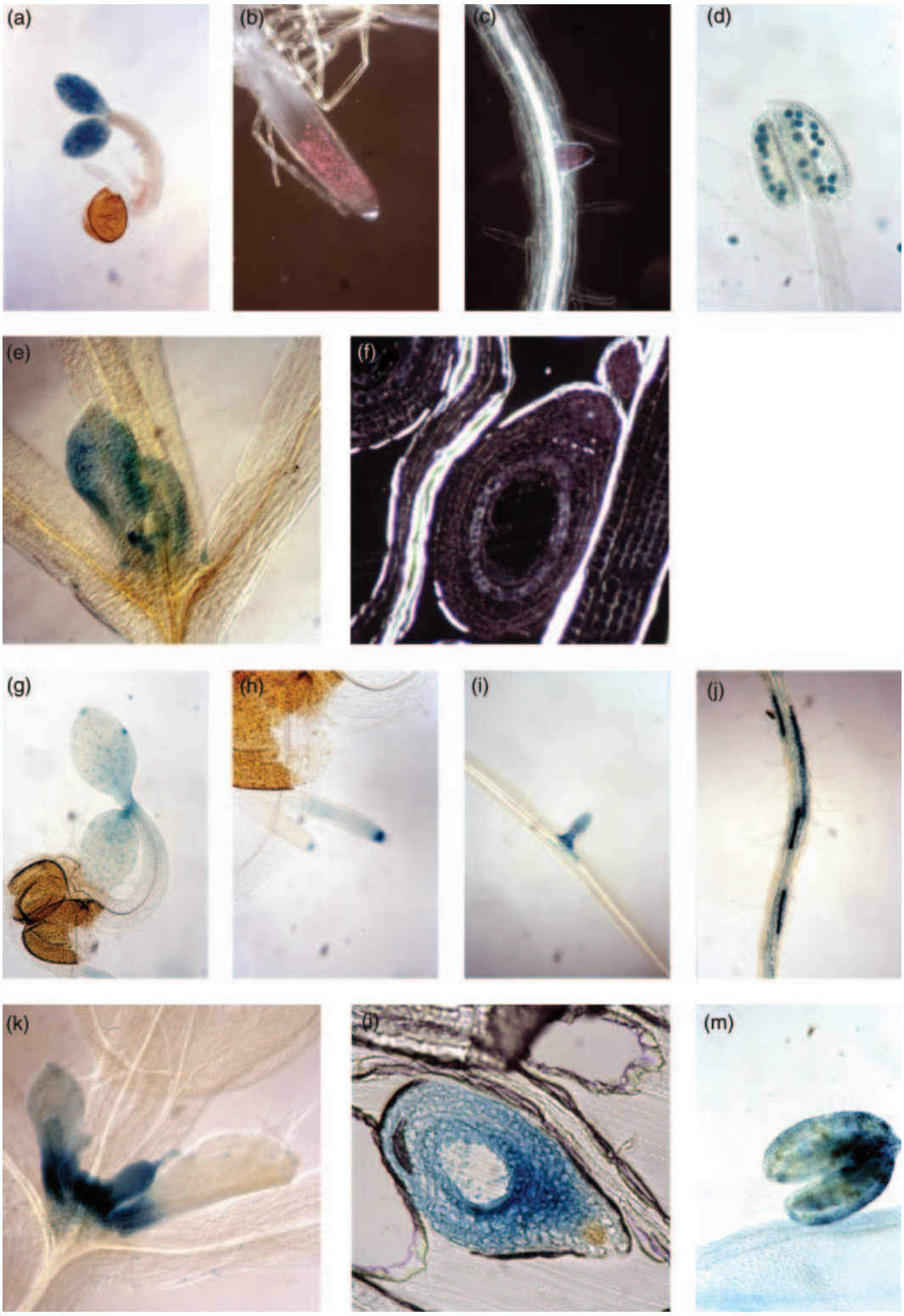
The combinations between *35S:TT2* and *35S:EGL3* or *35S:GL3* caused a significant induction of the *BAN:uidA* reporter construct depending on *TTG1* expression (Figure 5b). The level of induction produced by TT2/EGL3 was similar to that conferred by TT2/TT8, whereas TT2/GL3 induction was lower. These results may indicate differences of the TT2/bHLH complexes in their affinity for the *BAN* promoter or, as already observed with TT8 and Sn, they could indicate variations in TTG1 requirement among these *A. thaliana* bHLH homologues. For instance, *TTG1* overexpression produced an eightfold induction of TT2/EGL3 similar to the results obtained with TT2/TT8 (fivefold) whereas an 18-fold induction is obtained for TT2/GL3. However, the last member of the subgroup IIIf, AtMYC1, did not confer any activation of the reporter, highlighting the specificity of the response observed for TT8, EGL3, and GL3.

None of the binary combinations between the closest *A. thaliana* TT2-related MYB and any of the bHLHs resulted in a significant activation of the *BAN* promoter, even when TTG1 was overexpressed (data not shown). These results demonstrate that TT8 can be replaced by closely related bHLHs and suggest that TT2 is specifically required for *BAN* activation in *A. thaliana*. Overall, TT2/TT8 was the most effective combination of *A. thaliana* regulators to activate *BAN* in protoplasts.

### *TTG1 controls the ectopic expression of* BAN in planta

Nesi *et al.* (2001) demonstrated that the ectopic expression of *TT2*, generated by a *70S:TT2* construct, is able to induce the expression of *BAN* in *A. thaliana* roots. As already noticed, the GUS activity generated *in planta* from a *BAN:uidA* construct in a *70S:TT2* background does not match exactly with the pattern of activity of the *70S*

promoter driving *TT2* expression (Figure 6; Debeaujon *et al.*, 2003). In these plants, *BAN* expression is restricted to particular domains probably depending on the requirement for additional regulatory elements. TT8 is not likely to be a limiting factor as previous analyses by RT-PCR (Nesi *et al.*, 2000) and studies of the activity of *TT8* promoter *in planta* with a *TT8:uidA* construct (A. Baudry and L. Lepiniec, unpublished results) demonstrated that *TT8* is expressed in



vegetative tissues. Furthermore, it could be replaced *in planta* by some homologous bHLHs (this study) and ectopically induced in a *70S:TT2* background (Nesi *et al.*, 2001).

In order to check for a potential involvement of TTG1 in *BAN* ectopic expression, we investigated the activity of the *TTG1* promoter with the introduction of a *TTG1:uidA* construct *in planta* (Figure 6). The activity of this promoter corresponds to the domains where ectopic *BAN* activation was detected in *70S:TT2* × *BAN:uidA* plants. In both types of transgenic plants, GUS activity was detected in the cotyledons and in the root tip of germinating seedlings (Figure 6a,b,g,h), as well as in the secondary root tip and in the emerging leaves of 10-day-old seedlings (Figure 6c,e,i,k). In the seed coat, the *BAN* promoter was activated in the five layers of the integument at the globular stage in *70S:TT2* × *BAN:uidA* plants (Figure 6l), whereas it is normally restricted to PA-accumulating cells in the wild type. This expression correlated with the activity of the *TTG1* promoter at the same developmental stage (Figure 6f). Despite a strong activity of the *TTG1* promoter in wild-type pollen (Figure 6d), no GUS activity was detected in the pollen of *70S:TT2* × *BAN:uidA* plants. However, this defect can be attributed to a lack of activity of the *70S* promoter in *A. thaliana* pollen (Figure 6m). Taken together, these results indicate that the activity of the *BAN* promoter in a *70S:TT2* background correlates with the activity of the *TTG1* promoter.

## Discussion

### *TT2, TT8, and TTG1 can interact simultaneously*

TFs of the bHLH family are known to be involved in protein–protein interactions, inducing specific DNA binding and activation of target genes. In plants, some bHLHs form complexes with R2R3-MYB TFs (Goff *et al.*, 1992), or with closely related bHLHs (Fairchild *et al.*, 2000). In this study, we demonstrate that TT8, a bHLH factor regulating flavonoid metabolism in *A. thaliana*, directly interacts with two other regulatory proteins: TT2 (MYB factor) and TTG1 (WDR protein). Moreover, the ability of TT8 to simultaneously interact with TT2 and TTG1 and to form a bridge between these two proteins was demonstrated in three-hybrid experiments. Nevertheless, these proteins may not be arranged in a linear array because an interaction was also detected between TT2 and TTG1 in two-hybrid analysis. This latter interaction was detected only for a specific orientation of the fusion proteins, suggesting a weaker interaction or an interaction that is more sensitive to the structure of the chimeric proteins. Furthermore, co-expression of TT2 with TT8 and TTG1 significantly increased *LacZ* activation. This effect suggests that TT2 has a synergistic influence on TT8/TTG1 interaction, most likely through a bridging between TT8 and TTG1 as indicated by the two-hybrid experiments. These results are all consistent with the existence of a ternary complex composed of TT2, TT8, and TTG1.

Although to our knowledge, no interaction between an MYB and TTG1 has been demonstrated to date in two-hybrid experiments, a direct interaction might occur between such factors. A thorough study of the genetic interactions between the allelic series of *gl1* and *ttg1* provided early evidence that TTG1 and GL1 can associate (Larkin *et al.*, 1999; Schnittger *et al.*, 1999). More particularly, allele-specific interactions were demonstrated between a weak allele of GL1, named *gl1-EM2*, and a strong allele of *TTG1*, *ttg1-1* (Schnittger *et al.*, 1999). This last mutant expresses a truncated version of the TTG1 protein that is potentially not able to interact with GL3, the bHLH partner (Payne *et al.*, 2000), suggesting the existence of a direct interaction between GL1 and TTG1. Further characterization of the *gl1-EM2* mutation and a confirmation of the interaction results by other means will be necessary to address this question.

Several lines of evidence suggest that the ternary protein complex composed of TT2, TT8, and TTG1 might bind to the *BAN* promoter in plant cells. TT2, TT8, and TTG1 are all expressed in the PA-accumulating cells of the seed coat and the ectopic expression of the three proteins in the same tissues is sufficient to activate *BAN* (Debeaujon *et al.*, 2003; this study). Furthermore, the analysis of TT2 and TT8

**Figure 6.** Correlation between the activity of the *TTG1* promoter in the wild-type and the activity of the *BAN* promoter in a *70S:TT2* background.
Histochemical localization of the GUS activity generated by *TTG1:uidA* in the wild-type (a–f) is compared with the activity of the *BAN* promoter in a *70S:TT2* background (g–l), and with the activity of the *35S* promoter in the wild-type (m). Representative pictures of the results obtained with independent transformants are shown.
(a) Two-day-old germinating seedling (×5).
(b) Root of a 2-day-old germinating seedling (dark field, ×20).
(c) Secondary root (dark field, ×20).
(d) Stamen (×20).
(e) Emerging leaves of a 10-day-old seedling (×10).
(f) Section of a seed at the globular stage (dark field, ×20).
(g) Three-day-old germinating seedling (×5).
(h) Root of a 3-day-old germinating seedling (×10).
(i) Secondary root (×10).
(j) Primary root (×10).
(k) Emerging leaves of a 10-day-old seedling (×10).
(l) Section of a seed at the globular stage (×20).
(m) Stamen (×20).

sequences reveals the presence of putative nuclear localization signals (NLS), and GFP fusion experiments have shown *in planta* that TT2 and TT8 are localized in the nucleus (Nesi *et al.*, 2001; A. Baudry and L. Lepiniec, unpublished results). TTG1 subcellular localization is more controversial as no typical NLS or DNA-binding domain was detected in its sequence and the *P. hybrida* homologue AN11 was predominantly localized in the cytoplasm (de Vetten *et al.*, 1997; Walker *et al.*, 1999). However, recent results on another homologue from *Perilla frutescens* (PFWD) reveal that this WDR could be transferred from the cytoplasm to the nucleus of onion epidermal cells, when co-expressed with the bHLH protein MYC-RP (Sompornpailin *et al.*, 2002). Similarly, TTG1 could be transferred into the nucleus, after its interaction with TT8 and/or TT2 in the cytoplasm. Interestingly, preliminary experiments conducted in *A. thaliana* protoplasts confirmed that a functional GFP–TTG1 fusion protein could enter into the nuclear compartment (M. Heim, M. Hulskamp, and B. Weisshaar, unpublished results), consistent with the formation of a ternary complex composed of TT2, TT8, and TTG1 in the nucleus of PA-accumulating cells.

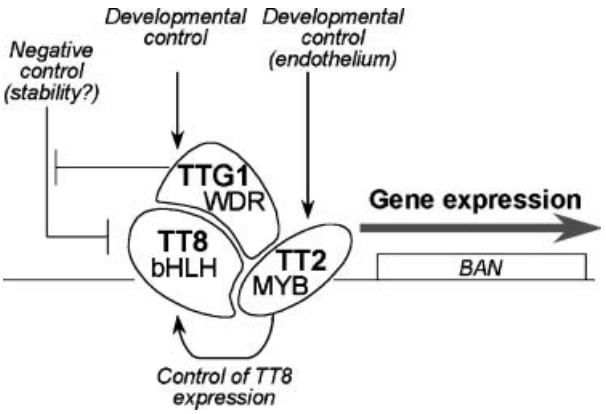



**Figure 7.** Model for the structure and regulation of the transcriptional activating complex composed by TT2, TT8, and TTG1.
TT2, TT8, and TTG1 form a transcriptional complex capable of directly activating the expression of *BAN*. TT2 is responsible for the specific recognition of the promoter, in combination with TT8. TTG1 regulates the activity of these proteins *in planta*, potentially through TT8 stability. The specificity and the level of *BAN* expression might result from a balance between the developmental control of *TT2*, *TTG1*, and *TT8* expression and a putative post-translational negative regulator. The possible formation of homodimers is not taken into account.

### *Specific functions of TT2 and TT8 in mediating direct activation of the* BAN *promoter*

A set of molecular and genetic experiments carried out in yeast and *in planta* provided evidence that the complex formed by TT2 and TT8 directly activates *BAN* expression. Using DEX-inducible fusion proteins, we have shown *in planta* that *BAN* belongs to the proximal targets of TT2 and TT8. In yeast one-hybrid experiments both TT2 and TT8 were necessary and sufficient to bind to the *BAN* promoter. The cooperation between TT2 and TT8 was confirmed in *A. thaliana* protoplasts. All these results are consistent with the model described for MYB/bHLH interaction in the regulation of anthocyanin biosynthesis in *Z. mays* (Goff *et al.*, 1992; Grotewold *et al.*, 2000). However, in *A. thaliana*, the coexistence of anthocyanin and PA biosynthesis and the structural similarity between the regulators of these two pathways raised the question of the molecular mechanisms leading to their specific activation. Several reports highlighted some functional homologies between the MYBs and the bHLHs regulated by TTG1 (Table 1). For instance, the ectopic expression of the *Z. mays* R protein in *ttg1* complements the various defects in regulatory pathways involving different bHLHs in *A. thaliana* (Lloyd *et al.*, 1992; Payne *et al.*, 2000; Zhang *et al.*, 2003). In addition, WER and GL1 were demonstrated to be functionally equivalent, their involvement in root-hair patterning or trichome organogenesis depending only on their specific expression pattern (Lee and Schiefelbein, 2001). In the present study, by testing several MYB/bHLH combinations, we were able to unravel functional specificities among TT2, TT8, and the MYBs/bHLHs involved in TTG1-dependent regulatory pathways.

First, TT2 was the only *A. thaliana* MYB able to activate *BAN* expression in combination with TT8, EGL3, GL3, or Sn in *A. thaliana* protoplasts. This result indicates that TT2 has unique structural properties such as the formation of homodimers revealed by the two-hybrid experiments. TT2 probably presents a specific DNA-binding activity, different from that of PAP1/PAP2, its closest *A. thaliana* homologues regulating anthocyanin metabolism (Borevitz *et al.*, 2000). Interestingly, C1 which was the only other MYB able to activate *BAN* expression, is the closest TT2 homologue among the MYB known to date (Nesi *et al.*, 2001; Stracke *et al.*, 2001). These results provide molecular evidence that in the TT2/TT8 complex, the MYB protein conveys the specific recognition of the target DNA (Figure 7).

Second, our results indicate that TT8 is not the only *A. thaliana* bHLH able to activate *BAN* as EGL3 and GL3, in combination with TT2 and TTG1, also activated *BAN* when overexpressed in *A. thaliana* protoplasts. Potentially, TT8 might participate in DNA binding, via a mechanism conserved among the three TTG1-dependent bHLHs (Figure 7). *BAN* promoter was weakly activated in the chalazal area of *tt8* developing seeds, suggesting that TT8 is not the only bHLH able to induce *BAN* expression *in planta* (Debeaujon *et al.*, 2003). EGL3 and/or GL3 are likely to be responsible for this residual *BAN* expression. Despite the important functional similarity among TT8, EGL3, and GL3, some of the results also suggest a difference in their ability to activate *BAN in planta*. Indeed, when expressed under the same promoter in combination with TT2, TT8 gave a significantly higher level of induction than EGL3 and much higher than GL3. These results are consistent with the recent genetic

data reported by Zhang *et al.* (2003) demonstrating that the ectopic expression of EGL3 complements *tt8* mutation, unlike the ectopic expression of GL3. However, if this difference is the consequence of a different affinity of the TT2/bHLH complexes for the sequence of the *BAN* promoter, a variation in TT2/bHLH complexes accumulation, or in TTG1 requirement remains to be investigated. To conclude on the fully functional equivalence between TT8 and EGL3, it would be necessary to test whether the expression of EGL3 under the control of *TT8* promoter complements *tt8* defects.

### *Hypotheses on the regulation of TT2/TT8 activity by TTG1*

This study reveals new insights into the regulatory function of TTG1 in PA biosynthesis (Figure 7). It controls directly *BAN* expression *in planta* and forms a ternary protein complex with the TFs TT2 and TT8. Furthermore, *BAN* activation by the TT2/TT8 complex correlates with the level of TTG1 expression in *A. thaliana* protoplasts. This result is consistent with the report of Tsuchiya *et al.* (2004) supporting a quantitative effect of TTG1 *in planta*, in the regulation of abnormal anthocyanin accumulation in the *fus3* embryo. However, TTG1 does not seem to be necessary for the specific recognition of the target DNA, even if it can participate in the TF complex directly binding to the *BAN* promoter. Indeed, the ectopic expression of TT2 and TT8 was sufficient to bind to the *BAN* promoter in yeast and to activate *BAN* in *A. thaliana* protoplasts, in the absence of TTG1. Furthermore, comparisons between TT2/TT8 and C1/Sn combinations indicated that TTG1 regulates *BAN* expression mainly by affecting TT8 function. Interestingly, this property appears to be conserved for the TTG1-dependent bHLHs (e.g. TT8, EGL3, and GL3), but not for Sn (or to a significantly lower extent). Indeed, the TT2/Sn combination is able to strongly activate *BAN* in protoplasts when TTG1 is silenced. This result is consistent with the report of Lloyd *et al.* (1992) and enables the explanation of the phenotypic complementation of *ttg1* by ectopic expression of R.

TTG1 ability to co-activate the TT2/TT8 complex was also supported *in planta*, by investigating the ectopic expression of *BAN* induced by a *70S:TT2* construct. This expression was restricted to the domains in which the *TTG1* promoter is active. However, this comparison also raised some questions about the mode of action of TTG1. During seed coat and root development, the *TTG1* promoter was activated early and transiently (Figure 6b,c,f). Although the activity of the *BAN* promoter in *70S:TT2* plants correlated with the activity of the *TTG1* promoter at early developmental stages in these tissues, it remained strongly and specifically activated in PA-accumulating cells (Debeaujon *et al.*, 2003) and in atrichoblasts (Figure 6j) after *TTG1* expression stopped. This result may reflect a long turnover of TTG1 depending on specific cell types. Alternatively, in the presence of TT2, TTG1 could be necessary to initiate *BAN* activation via the induction of an auto-activated loop able to maintain a constant level of *BAN* expression in PA-accumulating cells. Consistent with this hypothesis, TT2 positively influences *TT8* expression (Nesi *et al.*, 2001) and the investigation of both the TT8 and TTG1 effect on *TT8* expression is underway.

Nevertheless, the importance of TTG1 regulation remains intriguing. Many TFs controlling plant secondary metabolism are regulated by external stimuli (Vom Endt *et al.*, 2002). Accordingly, TTG1 might participate in the modulation of the level of flavonoid accumulation in response to external signals. In *A. thaliana*, anthocyanin accumulation at the distal edges of the cotyledons and in the epidermal layer of the hypocotyl is regulated by TT8 and TTG1 (Kubasek *et al.*, 1998). This regulation is under developmental control, but can be modulated by specific light conditions. In *Z. mays*, the light-induced accumulation of anthocyanins has been shown to be the result of the increase of *C1*, *PL*, or *Sn* expression (Piazza *et al.*, 2002). In addition to a similar transcriptional control, a post-translational regulation of TT8 by TTG1 might enhance light-induced accumulation of flavonoid compounds in *A. thaliana* seedlings. Similarly, in *Brassica carinata*, PA accumulation is quantitatively influenced by temperature (Marles *et al.*, 2003).

Payne *et al.* (2000) proposed that TTG1 acts through a transient stabilization of a bHLH partner. Our results of a quantitative effect of TTG1 on TT8 activity are consistent with this hypothesis. The direct interaction of TTG1 with TT8 might prevent TT8 degradation. Besides, the relative instability of the TT8 protein might partially explain the weak *BAN* activation conferred by DEX induction of TT8-GR *in planta*, preventing the accumulation of the fusion protein in the cytoplasm. Interestingly, recent data support the existence of negative regulators affecting TTG1-dependent pathways (Figure 7). For instance, the *icx1* mutant isolated by a mutagenesis of *CHS:uidA* plants, shows a complex phenotype altered in many epidermal pathways, including an upregulation of anthocyanin biosynthesis enzymes (Wade *et al.*, 2003). *UPL3/KAKTUS* is another negative regulator described recently and it encodes an HECT ubiquitin-protein ligase that represses excess branching and endoreplication of trichomes (Downes *et al.*, 2003; El Refy *et al.*, 2003). It is thought to act through the negative regulation of GL3. Similarly, control through rapid turnover of TT8 in tissues lacking TTG1 might be important to restrict more efficiently the expression of *BAN* in the pigmented cell layer (Debeaujon *et al.*, 2003).

## Experimental procedures

### *Yeast two- and three-hybrid assays*

Two-hybrid analyses were performed in the strain PJ69-4a containing *ADE2*, *HIS3*, and *LacZ* as reporter genes (James *et al.*, 1996).

**Table 3** Sequence of the primers used in this study

| Primer | Sequence[a] (5′⇒3′) |
|---|---|
| TTG1-*Bsa*I | GCACCACGGTCTCCCATGGATAATTCAGCTCC |
| TTG1-*Sal*I | GAAACTGTCGACTCAAACTCTAAGGAGCTG |
| BAN1H1 | CTCTTGTGAATTCTTGGTAGATG |
| BAN1H2 | GTAAGAGTCTGTCTAGAGATTGTAC |
| TT2B1 | *att*B1-ATGGGAAAGAGAGCAACTAC |
| TT2B2 | *att*B2-CACAAGTGAAGTCTCGGAGCC |
| TT8B1 | *att*B1-ATGGATGAATCAAGTATTATTC |
| TT8B2 | *att*B2-CTAGATTAGTATCATGTATTATG |
| TTG1B1 | *att*B1-ATGGATAATTCAGCTCCAGA |
| TTG1B2 | *att*B2-CAACTCTAAGGAGCTGCATTT |
| pTTG1-5′ | *att*B1-AACTCTCTTATGAACCGCACC |
| pTTG1-3′ | *att*B2-AGTGAGAGGAGAGTTTTGAGGT |
| BAN5 | ACATTTGCTGTGCTTACAACACAAGT |
| BAN6 | CGAAAGCCTTCATTGATAAGTTTTTGCG |
| EF1F | CTGGAGGTTTTGAGGCTGGTAT |
| EF1R | CCAAGGGTGAAAGCAAGAAGA |
| S181 | GGTCCATGGTTGTACTTTTGAAATTA |
| S182 | AGAGGATCCAACGTCGTCGTCAGC |
| L55 | CACCATCTGAATATACAGAAGCCC |
| L56 | GATCTGGATAACGAATCTGG |
| MJ191 | *att*B2-CTATAGATTAGTATCATGTATTATGACTTGGTGG |
| MJ192 | *att*B1-CCATGGATGAATCAAGTATTATTCCGGCAG |
| MJ193 | *att*B2-TTAACATATCCATGCAACCCTTTGAAGTGCC |
| MJ194 | *att*B1-CCATGTCTTTGACAATGGCTGATGGTGTAGAA |
| MJ195 | *att*B2-TTAAAACAAATCACCAATGACTCTTCTCAGC |
| MJ196 | *att*B1-CCATGGCAACCGGAGAAAACAGAACGGTG |
| MJ197 | *att*B2-TCAACAGATCCATGCAACCCTTTGAAGTGC |
| MJ198 | *att*B1-CCATGGCTACCGGACAAAACAGAACAACTG |
| RS351 | *att*B1-CCATGGGAAAGAGAGCAACTAC |
| RS353 | *att*B2-TCAACAAGTGAAGTCTCGGAGCC |

[a]*att*B1 and *att*B2 refer to the corresponding Gateway™ recombination sequences.

Quantitative β-galactosidase assays and three-hybrid analyses were performed in the strain Y190 (Harper *et al.*, 1993). The *TT2*, *TT8*, and *TTG1* full-length cDNAs were cloned in pAS2ΔΔ and pACTIIst vectors (Fromont-Racine *et al.*, 1997) to be expressed in yeast as fusions with BD or AD. To this end, *TT2* and *TT8* cDNAs were excised from the pLBR19-TT2 and pLBR19-TT8 vectors (Nesi *et al.*, 2000, 2001) by *Nco*I and *Sal*I digestion. *TTG1* cDNA was amplified by PCR with the primers TTG1-*Bsa*I and TTG1-*Sal*I (Table 3), and the product was digested by *Bsa*I and *Sal*I. The resulting *TT2*, *TT8*, and *TTG1* inserts, presenting *Nco*I and *Sal*I/*Xho*I-compatible cohesive ends, were cloned in pAS2ΔΔ and pACTIIst digested by *Nco*I/*Sal*I or *Nco*I/*Xho*I, respectively. In order to express three proteins in the same strain, an additional expression vector was used: pTFT1 (Egea-Cortines *et al.*, 1999). *TT2*, *TT8*, and *TTG1* cDNAs were excised from pAS2ΔΔ by an *Msc*I/*Sal*I digestion and cloned in pTFT1 digested by *Eco*RI, blunt-ended by the mung bean nuclease, and then digested by *Sal*I. The constructs were sequenced to ensure that no mutation was introduced by PCR and that the cDNAs were in frame with AD, BD, or the SV40 NLS in the pTFT1 constructs.

pAS2ΔΔ, pACTIIst, and pTFT1 present different selective genetic markers to be maintained in yeast (*TRP1*, *LEU2*, and *ADE2*, respectively). Yeast was co-transformed using the AcLi/SSDNA/PEG method (Gietz and Woods, 2002) and transformants were selected on appropriate media. 3-AT was added in media lacking histidine, and several concentrations were tested, from 5 to 50 mM. β-galactosidase activity (UβGal) was measured on liquid cultures using ONPG as a substrate, as described in the Matchmaker-yeast protocol handbook (Clontech, Palo Alto, CA, USA). The results presented are averages from four independent clones and the standard deviation observed is acceptable for that kind of test according to Serebriiskii and Golemis (2000).

### *Yeast one-hybrid assay*

The reporter plasmid was constructed by inserting a 236-bp fragment of the *BAN* promoter (−193 to +43 relatively to *BAN* transcription start site) into the pHISi vector (Alexandre *et al.*, 1993). This fragment was amplified by PCR with the primers BAN1H1 and BAN1H2 (Table 3), digested by *Eco*RI and *Xba*I and inserted into pHISi between the *Eco*RI and *Xba*I sites. This plasmid was then digested with *Nco*I and integrated into the yeast strain YM4271 (Liu *et al.*, 1993), at the *URA3* locus. The resulting yeast strain, selected on medium lacking uracil, contained the *HIS3* reporter gene under the control of the whole regulatory sequences of the *BAN* promoter. It was co-transformed with the pACTIIst, pAS2ΔΔ, and pTFT1 vectors expressing *TT2*, *TT8*, or *TTG1* and assayed for *HIS3* expression on media lacking histidine.

### *Construction of* 35S:TT-GR *and* TTG1:uidA *transgenes and plant transformation*

*TT2*, *TT8*, and *TTG1* cDNA were introduced into the binary vector pR1R2ΔGR, to enable *in planta* expression of in-frame fusions with the amino acids 512-795 of the GR protein, under the control of the *35S* promoter. The pR1R2ΔGR vector was constructed by introduction of a Gateway™ rfB cassette (Invitrogen, Carlsbad, CA, USA) into pBI-ΔGR (Lloyd *et al.*, 1994), linearized by *Xba*I and blunt-ended with the Klenow fragment. The cDNA were amplified without the stop codon by the TT2B1/TT2B2, TT8B1/TT8B2, or TTG1B1/TTG1B2 primer sets (Table 3). The amplification products were transferred to the pDONR207 entry vector (Invitrogen) by a BP recombination reaction, sequenced, and subsequently transferred to pR1R2ΔGR by a LR recombination reaction.

The *TTG1* promoter construct used in this study corresponds to region −1496 to +59 bp relative to the *TTG1* transcription start site and was amplified from Wassilewskija (WS) genomic DNA with the pTTG1-5′/pTTG1-3′ primer set (Table 3). The PCR product was introduced by a BP recombination reaction into pDONR207, sequenced, and transferred to the binary vector pBI101GUS (F. Divol, J.C. Palauqui, and B. Dubreucq, to be published elsewhere) by an LR recombination reaction, to obtain a transcriptional fusion between the *TTG1* promoter and *uidA* reporter gene.

The resulting binary vectors were electroporated into *Agrobacterium tumefasciens* C58C1pMP90 strain (Koncz and Schell, 1986) and used for agroinfiltration of inflorescences (Bechtold *et al.*, 1993) of the corresponding mutants (*tt2-1*, *tt8-1*, or *ttg1-13*) for *35S:TT-GR* constructs or WS for *TTG1:uidA*. Kanamycin-resistant transformants were selected on Murashige and Skoog medium and then transferred to the soil for further characterization.

### *DEX induction experiments and RNA analysis*

DEX (Sigma-Aldrich, Steinheim, Germany) induction was performed *in planta* by application of a 10 μM DEX and 0.015% Silwet L77 solution on leaves or siliques (Wagner and Sablowski, 2002). DEX induction for the monitoring of *BAN* expression in siliques was performed in 24-well plates. For each condition, four siliques (2-

5 days after pollination) were taken on *35S:TT-GR* transgenics, opened, and incubated in 100 mM phosphate buffer, pH 7.2, 0.1% triton X-100, 10 mM $Na_2$-EDTA, and 100 μM CHX when necessary (Spelt *et al.*, 2000; CHX Ready made; Sigma-Aldrich). Vacuum was applied for 30 min to ensure effective penetration of CHX. Thereafter, DEX was added to a 10 μM final concentration, and penetration was facilitated by a second round of 30 min vacuum. After 3 h on an orbital shaker, the incubation buffer was replaced by a freshly prepared buffer (10 μM DEX and/or 100 μM CHX), and incubation was carried on for an additional 3 h (Wagner and Sablowski, 2002).

Total RNA was extracted from siliques using the Genelute total RNA miniprep kit (Sigma-Aldrich), according to the manufacturer's recommendations. The extracts were treated with 30 units of RNAse-free DNAse I (Qiagen, Hilden, Germany) and eluted with RNAse-free water. Reverse transcription and real time RT-PCR using SYBR Green (Roche, Penzberg, Germany) and a Roche light cycler to detect *BAN* expression level were performed as described in Baud *et al.* (2003). A specific primer set (BAN5/BAN6, Table 3) was designed to amplify a 153-bp fragment on *BAN* cDNA. The results of *BAN* expression in siliques were standardized to the constitutive expression level of the gene *EF1αA4* (*EF*) determined with EF1F/EF1R primer set (Table 3; Baud *et al.*, 2003).

### *Histochemical detection of GUS activity*

GUS staining was performed as described in the report of Debeaujon *et al.* (2003) in the presence of 0.5 or 3 mM potassium ferricyanide/potassium ferrocyanide for *TTG1:uidA* plants or *70S:TT2* × *BAN:uidA* plants, respectively. Resin embedding and sections of GUS-stained developing seeds were realized as explained in Baud *et al.* (2003).

### *Co-transfection of* A. thaliana *protoplasts*

The At7 cell culture, protoplast isolation, co-transfection, and determination of standardized GUS activity were carried out as described by Hartmann *et al.* (1998), except that 30 μg of plasmid DNA was used for the PEG-mediated DNA transfer into protoplasts: 10 μg of the *BAN:uidA* reporter construct; 0.5 μg of each effector construct; if added, 10 μg of the *TTG1RNAi* construct; 5 μg of the standardization plasmid pBT8-*UBI:LUCm3*, expressing the luciferase transformation control; and an inactive luciferase expression vector (pBTΔLUC) to complete the amount of 30 μg of DNA. In this work, eight different experiments were taken into account to get a statistical overview. The error bars display the average of the absolute deviation.

For *BAN:uidA* reporter construct, a 1496-bp fragment of the *BAN* promoter (−1453 to +43 relative to the *BAN* transcription site) was amplified with the primers S181 containing an *Nco*I-restriction site and S182 containing a *Bam*HI-restriction site (Table 3), and cloned into pBT8-*35S:uidA* digested by *Bam*HI and *Nco*I, replacing the *35S* promoter cassette. A second reporter construct was made by transferring the 236 bp fragment described above in the one-hybrid experiment into pBT8-*35S:uidA* and confirmed the co-transfection results obtained with the 1496 bp reporter (data not shown). According to Debeaujon *et al.* (2003), these two constructs contain the whole regulatory sequences of the *BAN* promoter.

The *TTG1RNAi* construct used for transient *TTG1* silencing was constructed using the Gateway™ destination vector pJawohl8-RNAi. A 236-bp fragment spanning the 5′UTR from position -200 up into the first exon to position +36 of *TTG1* was amplified using primers L55 and L56 (Table 3). The PCR product was purified and cloned via directional TOPO cloning into the entry vector pENTR/D-TOPO (Invitrogen). After sequencing, the fragment was transferred into pJawohl8-RNAi via an LR recombination reaction. For *TTG1* overexpression, *TTG1* cDNA was excised from the two-hybrid vector pAS2ΔΔ-TTG1 by digestion with *Msc*I and *Sal*I, and inserted into pLBR19 digested by *Hin*dIII, blunt-ended by the Klenow fragment and then digested by *Sal*I.

The other effectors used in this study were constructed using the pBTdest vector, except the expression constructs for the *Z. mays* regulators *C1* and *Sn* which have been described previously (Hartmann *et al.*, 1998). The pBTdest was constructed by digesting pBT8-*35S* with *Xho*I and *Sma*I, filling in the overhanging ends with the Klenow fragment, and inserting a Gateway™ rfB cassette (Invitrogen). Full-length cDNAs were amplified using primer sets containing the *att*B1 and *att*B2 recombination sequences (*GL3*: MJ197/MJ198; *EGL3*: MJ193/MJ196; *AtMYC1*: MJ194/MJ195; *TT8:* MJ191/MJ192; *TT2*: RS351/RS353; Table 3). The amplification products were recombined into pDONR201 (Invitrogen), sequenced, and then transferred into pBTdest via a LR recombination reaction.

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The accession numbers for the sequences mentioned in this article are as follows: *AtMYB23*: At5g40330; *AtMYB111*: At5g49330; *AtMYC1*: AtbHLH012/At4g00480; *BAN*: At1g61720; *EGL3*: AtbHLH002/At1g63650; *GL1*: AtMYB0/At3g27920; *GL3*: AtbHLH001/At5g41315; *MYB61*: AtMYB61/At1g09540; *PAP1*: AtMYB75/At1g56650; *PAP2*: AtMYB90/At1g66390; pBTdest vector: AJ551314; pJawohl8-RNAi vector: AF408413; *TT2*: AtMYB123/At5g35550; *TT8*: AtbHLH042/At4g09820; *TTG1*: At5g24520; and *WER*: AtMYB66/At5g14750.