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TTG2 controls the developmental regulation of seed coat tannins in *Arabidopsis* by regulating vacuolar transport steps in the proanthocyanidin pathway

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ABSTRACT

The brown color of *Arabidopsis* seeds is caused by the deposition of proanthocyanidins (PAs or condensed tannins) in their inner testa layer. A transcription factor complex consisting of TT2, TT8 and TTG1 controls expression of PA biosynthetic genes, just as similar TTG1-dependent complexes have been shown to control flavonoid pigment pathway gene expression in general. However, PA synthesis is controlled by at least one other gene. *TTG2* mutants lack the pigmentation found in wild-type seeds, but produce other flavonoid compounds, such as anthocyanins in the shoot, suggesting that TTG2 regulates genes in the PA biosynthetic branch of the flavonoid pathway. We analyzed the expression of PA biosynthetic genes within the developing seeds of *ttg2-1* and wild-type plants for potential TTG2 regulatory targets. We found that expression of *TT12*, encoding a MATE type transporter, is dependent on TTG2 and that TTG2 can bind to the upstream regulatory region of *TT12* suggesting that TTG2 directly regulates *TT12*. Ectopic expression of *TT12* in *ttg2-1* plants partially restores seed coat pigmentation. Moreover, we show that TTG2 regulation of *TT12* is dependent on TTG1 and that TTG1 and TTG2 physically interact. The observation that TTG1 interacts with TTG2, a WRKY type transcription factor, proposes the existence of a novel TTG1-containing complex, and an addendum to the existing paradigm of flavonoid pathway regulation.

1. Introduction

Arabidopsis seed coats derive their brown color from the accumulation of proanthocyanidins (PAs), a class of flavonoid chemicals (polymerized flavan-3-ols, or condensed tannins) that protect against a variety of biotic and abiotic stresses and help maintain seed dormancy and viability (Debeaujon, et al., 2003). PAs start out as colorless epicatechin compounds until they are transported to the vacuole where they are polymerized and oxidized as the seed desiccates. Plants with defects in the PA biosynthetic pathway are called *transparent testa* (*tt*) mutants. Many of the genes involved in the synthesis, transport or regulation of PAs have been described (Lepiniec et al., 2006; Appelhagen et al., 2014). In *Arabidopsis*, PAs are only produced in a narrowly defined cell layer in the endothelium of the seed and PA production is strictly coordinated with testa differentiation. Yet gaps remain in our understanding of how this important secondary metabolic pathway is exquisitely developmentally regulated.

Genetic and molecular studies of the anthocyanin flavonoid pathway first elucidated the highly conserved regulatory mechanism involving MYB, basic Helix-Loop-Helix (bHLH) and WD-repeat (WDR) proteins. The emergent regulatory model indicates that a transcriptional complex, consisting of bHLH proteins that physically interact with both MYB and WDR proteins (the MBW complex), controls flavonoid pigment pathway target gene expression (Kubasek et al., 1992; Quattrocchio et al., 1993; Winkel-Shirley et al., 1995; Gonzalez et al., 2008). Thus, the regulation of the flavonoid pigment pathway by the MBW complex is well

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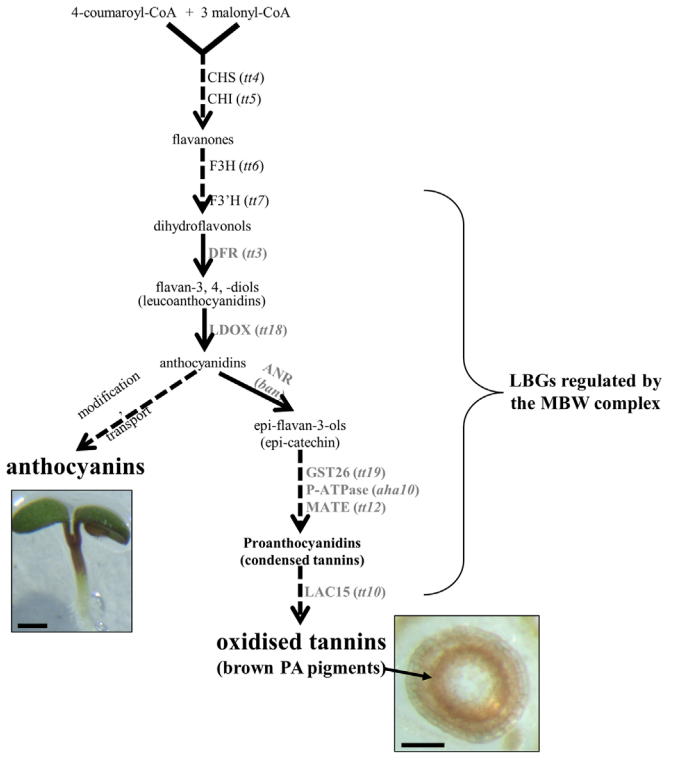

Fig. 1. The flavonoid biosynthetic pathway. The bracket indicates the late biosynthetic genes directly regulated by the MYB-bHLH-TTG1 complex. Dashed lines indicate multiple steps. Steps in gray bold text were tested for regulation by TTG2 in this study. Depicted is a seedling expressing purplish red anthocyanins in the hypocotyl and parts of the cotyledons early in *Arabidopsis* development. Also depicted is a thick cross section through a developing seed showing brownish PA pigments expressed as a diffuse band due to the collapse of the inner testa layers during seed development and the thickness of the section. Scale Bars: 1 mm (seedling), 0.1 mm (seed section).

characterized; in all species studied to date, MBW transcription factor complexes directly regulate a later acting subset of flavonoid biosynthetic genes (or LBGs), with the subset of regulated genes varying between species. Also, the composition of the complex with respect to the MYB and bHLH members can distinguish between anthocyanin structural gene targets (Schwinn et al., 2006; Gonzalez et al., 2008, Xu et al., 2014). However, studies in *Arabidopsis* demonstrate that the MBW complex also regulates developmental pathways of the epidermis, including trichome differentiation, root hair patterning and testa development, by varying MYB and bHLH protein combinations in the complex (Lee and Schielfelbein, 1999; Bernhardt et al., 2003; Zhang et al., 2003; Haughn and Chaudhury, 2005; Gonzalez et al., 2009).

The same MBW regulatory model holds true for the flavonoid pigment pathway leading to PAs in the *Arabidopsis* seed coat. Here, Transparent Testa2 (TT2) and Transparent Testa8 (TT8) are the specific MYB and bHLH transcription factors that, with the TTG1 WD-repeat protein, form the MBW complex regulating LBGs beginning with F3'H, encoded by *TT7* (Gonzalez et al., 2008; Fig. 1). This TT2-TT8-TTG1 complex is functionally distinct from the MBW complex regulating the production of anthocyanins in the plant body; thus by activating PA specific genes, with *Banyuls* (*BAN*) representing the key first PA pathway step, the TT2-TT8-TTG1 complex specifies the production of PA pigments instead of anthocyanins.

In *Arabidopsis,* TRANSPARENT TESTA GLABRA2 (TTG2) is a regulator of several TTG1-dependent epidermal developmental pathways, including developmentally regulated PA biosynthesis in the inner testa layer during seed development (Johnson et al., 2002). TTG2 encodes a WRKY class transcription factor that is directly regulated by MBW complexes (Ishida et al., 2007; Zhao et al., 2008). These transcription factors contain a DNA binding domain of about 60 amino acids containing a conserved WRKY sequence and a novel zinc finger motif (Eulgem et al., 2000; Ulker and Somssich, 2004; Yamasaki et al., 2005). WRKY proteins show high affinity for the W box, an element (C/T)TGAC(T/C) found in the targets of WRKY transcription factors. As a family, WRKY transcription factors in plants primarily regulate stress responses. TTG2 is exceptional in that it regulates developmental processes, including not only PA production, but trichome development in the shoot epidermis and the differentiation of the mucilage-producing cells of the outer seed coat layer (Johnson et al., 2002; Pesch et al., 2014).

In all other species studied thus far the MBW transcription factor complex is sufficient for direct regulation of LBGs of the flavonoid pathway. In *Arabidopsis*, the involvement of TTG2 in PA pigment production represents a novel use of an intermediate transcription factor by the MBW regulatory complex-at least this mode of regulation has not been described for other plants. However, anthocyanin pigment production is unaffected in *ttg2* mutants (Johnson et al., 2002; Fig. 2A), suggesting TTG2 developmentally controls a gene or genes in the PA branch of the flavonoid pathway. Interestingly, the expression of *BAN* (the key first enzymatic step of the PA branch) is not changed in developing seed of *ttg2* mutants as compared to wild-type, but again is directly regulated by an MBW complex (Baudry et al., 2004). Also, it has been shown that *TT8* expression in the endothelium strictly depends on TTG2 but only at the early globular embryo stage of development with *TT8* expression restored in PA-accumulating cells from the heart stage onward (Xu et al., 2013). Otherwise, nothing is known molecularly about how TTG2 might regulate the

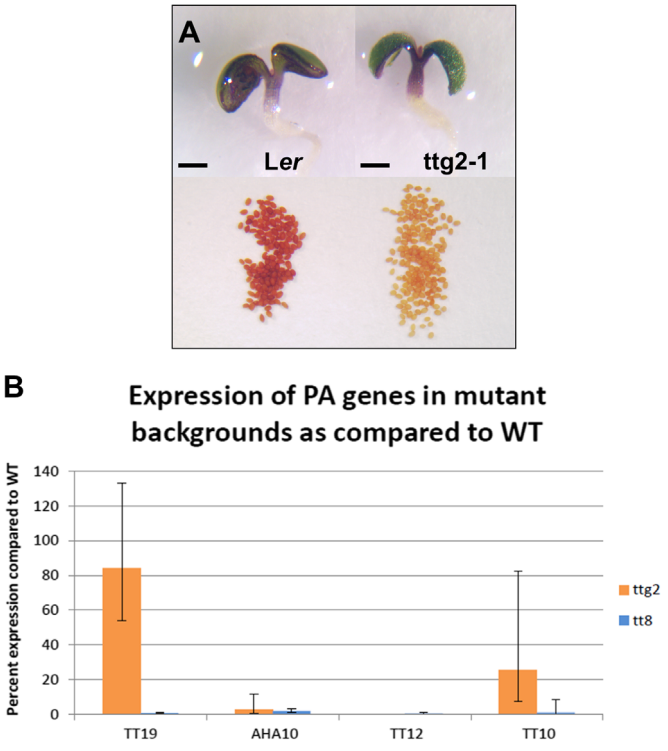

Fig. 2. Flavonoid pigment phenotypes of *ttg2-1* and analysis of flavonoid gene expression in young developing silique. (A) *Ler* and *ttg2-1* five-day-old seedlings (top) and dry seed (bottom). (B) Q-PCR analysis of flavonoid LBG expression in *Ler*, *ttg2-1*, and *tt8-1* young developing silique reported as percentage of wild-type expression. Scale bars: 0.2 mm (seedlings).

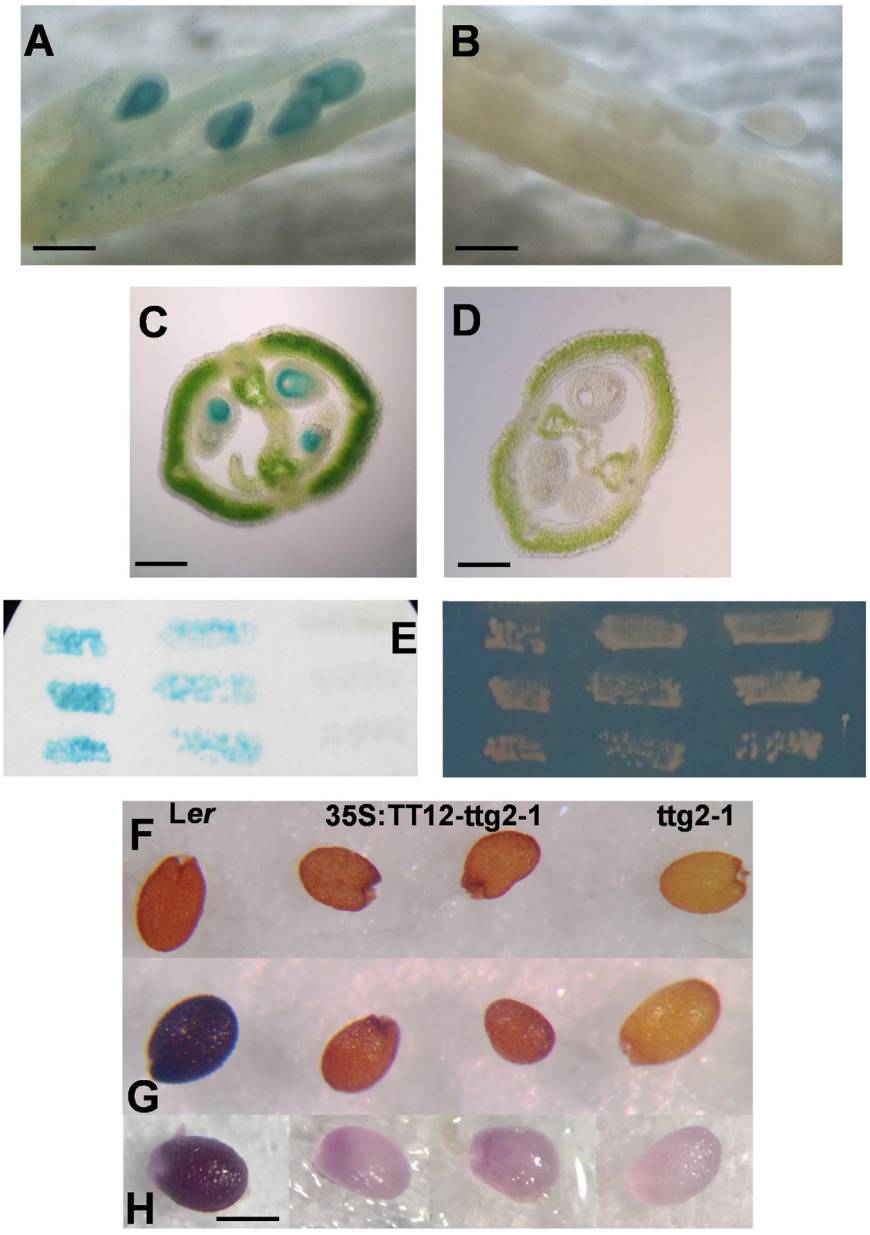

Fig. 3. Characterization of the *TT12* promoter and *TT12* overexpression in *ttg2-1*. *TT12pro:GUS* expression in (A) *Ler* developing whole seed, (B) *ttg2-1* developing whole seed, (C) thick cross section of *Ler* developing silique with young seed, and (D) thick cross section of *ttg2-1* developing silique with young seed. (E) Yeast-one-hybrid analysis assaying for the ability of TTG2 to bind to a 1 kb *TT12* promoter fragment fused to the βgal reporter and integrated into the yeast genome. The first column shows three independently transformed *TT12pro:βgal* yeast reporter lines with a 160aa N-terminally truncated TTG2 protein fused to the Gal4 AD. The second column shows three independently transformed *TT12pro:βgal* yeast reporter lines with full-length TTG2 protein fused to the Gal4 AD. The third column shows three independently transformed *TT12pro:βgal* yeast reporter lines with the Gal4 AD empty vector control. The panel on the right shows yeast patches from which cells were lifted and assays performed. (F) Seed coat color phenotypes of *Ler*, two independent transgenic lines of *ttg2-1* overexpressing *TT12*, and *ttg2-1* without the transgene. (G) DMACA stained seed form *Ler*, two independent transgenic lines of *ttg2-1* overexpressing *TT12*, and *ttg2-1* without the transgene. (H) DMACA stained developing seed at the heart stage from *Ler*, two independent transgenic lines of *ttg2-1* overexpressing *TT12*, and *ttg2-1* without the transgene. Scale bars: A, B =0.5 mm; C, D, F, H =0.2 mm.

flavonoid pathway. This represents an unresolved discrepancy regarding the canonical MBW paradigm for flavonoid pigment pathway regulation. Thus a significant gap remains in our overall knowledge of this important biological phenomenon and in our understanding of the regulatory mechanisms of *Arabidopsis* seed coat development.

Here we begin to reconcile this discrepancy by showing that during seed coat development, TTG2 controls the production of PAs by direct regulation of the *Transparent Testa12* (*TT12*) gene, which encodes a MATE type vacuolar transporter of glycosylated epicatechin PA precursor. In addition, we demonstrate that the lack of *TT12* expression is mainly the cause of the seed coat color phenotype in *ttg2-1* plants. We also show that TTG2 control of *TT12* is dependent on TTG1, and that TTG2 and TTG1 physically interact via a 118 amino acid region of TTG2 located between the WRKY domains. This data suggests that in certain instances TTG1 may form alternative complexes with transcription factors other than MYBs and bHLHs to control gene expression, as also recently suggested by Pesch et al. 2014, which represents a shift from our current model of flavonoid gene regulation.

2. Results

2.1. TT12 expression is dependent on TTG2 function

Seeds of *ttg2-1* mutants are yellow rather than brown, due to an undefined disruption in the flavonoid biosynthetic pathway (Johnson et al., 2002; Fig. 2B). Since *ttg2* mutants are not deficient in anthocyanin production (Appelhagen et al., 2014; Fig. 2A) potential regulatory targets are likely restricted to genes specific to the formation of PAs. Therefore we focused our examination on the expression of genes specific to the production of PAs - *BAN*, *TT10, TT12*, *TT13* (recently shown to encode AHA10, a tonoplast localized P_{3A}-ATPase, Appelhagen et al., 2015), and to genes common to both PAs and anthocyanins, *TT3* and *TT19,* in the *ttg2-1* mutant and its wild-type background in order to identify potential TTG2 regulatory targets. Quantitative PCR (Q-PCR) analysis comparing gene expression in Landsberg *erecta* (L*er*) wild-type and *ttg2-1* developing silique showed that *BAN*, *TT3* and *TT19* transcript levels were unaffected by loss of TTG2 function (data not shown). *TT10*, *TT13* and *TT12* transcript levels are all reduced in the absence of TTG2; *TT10* retains about 25% of its wild-type levels, while *TT13* expression was reduced to less than 3% of wild-type and *TT12* expression was almost totally repressed (Fig. 2B).

We also examined the expression of these genes in the *tt8-1* mutant. TT8 is the bHLH component in the MBW complex that regulates PA production in the *Arabidopsis* seed coat. The expression of all genes tested in the *tt8-1* mutant was no more than 2% of their level in wild-type (Fig. 2B) in agreement with previous studies (Nesi et al., 2000; Lepiniec et al., 2006; Xu et al., 2014). We chose to focus our investigations on TT12 because its expression levels in both the tt8-1 and ttg2-1 backgrounds are considerably reduced (Fig. 2B). Thus *TT12* represents a strong candidate target gene for further elucidating how TTG2 regulates PA pigment production during seed coat development.

To further investigate the relationship between TTG2 and *TT12*, we created a reporter construct consisting of 1587 bp upstream of the *TT12* start codon fused to uidA (*GUS*) reporter gene (*TT12pro:GUS*). L*er* plants expressing this transgene showed *GUS* expression in the inner testa layer of developing seed coats but *ttg2-1* mutants containing the transgene lacked *GUS* expression entirely (Fig. 3A-D), consistent with the Q-PCR data reported above.

2.2. TTG2 physically interacts with TT12 upstream regulatory region

The 5′ cis-regulatory region of *TT12* contains several (T)(T)TGAC (C/T) W-box motifs which match the canonical binding sites for WRKY type transcription factors (Maeo et al., 2001; Ulker and Somssich, 2004; Yamasaki et al., 2005). To investigate the possibility that TTG2 directly regulates *TT12*, we employed a yeast-one-hybrid strategy. A fragment of DNA consisting of 943 base pairs of sequence upstream of the *TT12* start codon was fused to a LacZ reporter gene (*TT12pro:βgal*) and this construct was co-transformed into yeast with either a construct encoding the full-length TTG2 protein or one that truncates 160 amino acids from the N-terminus of the protein but leaves the WRKY domains intact. Indeed, the full length TTG2 protein shows LacZ expression, while the empty vector control shows no LacZ expression. The truncated TTG2 construct containing both WRKY domains shows increased LacZ expression over the full length protein (Fig. 3E). This indicates that TTG2 can bind directly to the *TT12* upstream regulatory region.

2.3. Ectopic TT12 expression partially rescues the ttg2-1 seed coat color phenotype

As mentioned previously, *TT12* expression is virtually undetectable in developing seed coats of the *ttg2-1* mutant. To investigate the restoration of *TT12* expression on the *ttg2-1* seed coat color, we made a construct expressing *TT12* under the control of a cauliflower mosaic virus 35S promoter (*35S:TT12*), which provides strong, ectopic expression of the *TT12* gene. When this transgene was introduced into *ttg2-1,* it partially restored the seed coat color phenotype to a near wild-type appearance (Fig. 3F). This result suggests that the lack of coloration in the seed coat of *ttg2-1* mutants is caused primarily by the lack of TT12 function.

To detect more subtle changes in pigmentation, we used DMACA reagent to stain PAs and epicatechins in seeds (Treutter, 1989; Abrahams et al., 2002). L*er* wild-type seeds were darkly stained by DMACA while *ttg2-1* seeds showed no staining as previously reported (Fig. 3G; Appelhagen et al., 2014). Seeds from *ttg2-1* overexpressing *TT12* were also DMACA negative, though they appeared slightly darker than seeds from the *ttg2-1* mutant. This slightly darker appearance is probably the result of the increased brown pigment in *35S:TT12 ttg2-1* seeds rather than some small amount of DMACA staining. To better evaluate DMACA staining in the absence of PA pigment, young developing seeds at the heart stage were DMACA stained for the presence of PA pigment precursors. In this case, there appears to be slightly more PA pigment precursors in *35S:TT12 ttg2-1* developing seeds compared to *ttg2-1* developing seeds (Fig. 3H). Taken together, these results indicate that the rescue of the *ttg2* seed coat phenotype by ectopic *TT12* expression is not complete (Fig. 3F, G and H).

2.4. TTG2 requires TTG1 in order to regulate TT12

TTG2 is a known direct target of the MBW complex (Ishida et al., 2007), so the reduction in *TT12* expression seen in MBW mutants (Xu et al., 2014) might be explained by a loss of *TTG2* expression. We therefore might expect to see a return of *TT12* expression if *TTG2* were ectopically expressed in a genetic background in which the function of the MBW PA complex is impaired. To investigate this hypothesis we transformed *ttg1-1* plants with a construct that ectopically expresses *TTG2* using the cauliflower mosaic virus 35S promoter (*35S:TTG2*) and compared *TT12* expression between these transgenic plants and their given mutant background. Previous analysis of *TT12* promoter activity via a GUS reporter fusion in *ttg1* developing seed showed no detectable expression (Xu et al., 2014) and we similarly observe a complete absence of expression in the inner testa layer of *TT12pro:GUS* in *ttg1-1* developing seed (Fig. 4A compare to Fig. 3A and C). We therefore analyzed GUS expression in *35 S:TTG2 ttg1-1* plants transformed with the *TT12pro:GUS* construct for any detectable expression above the essentially absent signal in *ttg1-1*. These plants also lacked detectable GUS expression in the inner testa layer, similar to *TT12pro:GUS ttg1-1* plants (Fig. 4B and C); thus the *35 S:TTG2* construct failed to rescue *TT12* expression in the *ttg1-1* background.

2.5. TTG2 physically interacts with TTG1

Despite lacking any recognizable DNA binding domain or nuclear localization signal, TTG1 does enter the nucleus (Walker et al., 1999; Bouyer et al., 2008) and has been shown to interact with other MBW transcriptional complex members. Since our data shows that TTG1 is required for TTG2 to activate *TT12 in vivo*, we tested whether TTG2 and various MBW complex members physically interact using the yeast-two-hybrid system. We constructed Gal4 AD and BD fusions with TTG1, TT8 and TT2 and tested each for interaction with TTG2 AD and BD fusions. As previously reported in the literature (Baudry et al., 2004; Zimmermann et al., 2004), we observed positive LacZ assays when testing for known interactions between TT8 and TT2 and between TT8 and TTG1, but negative results for TT2 and TTG1 (data not shown). LacZ assays

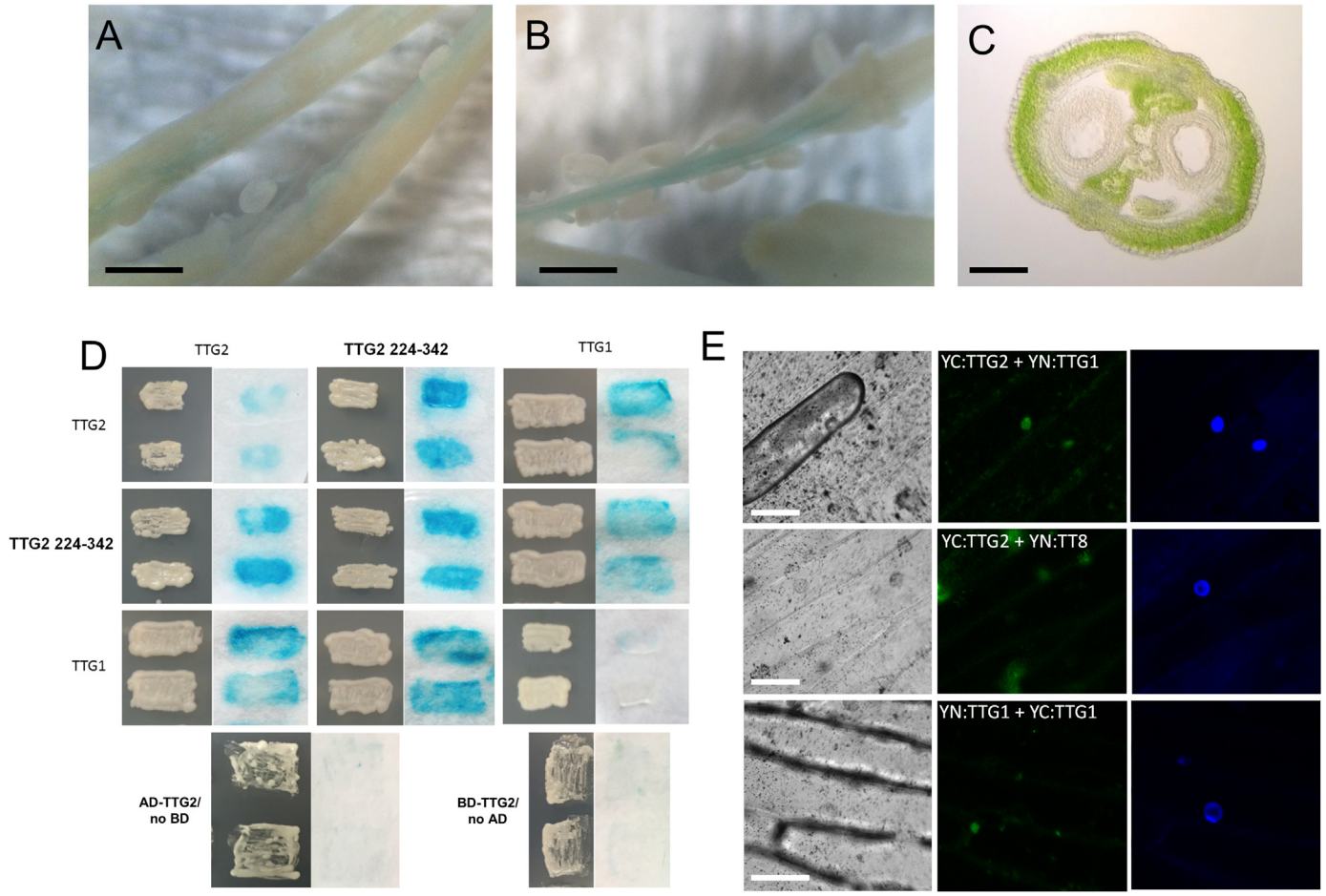

Fig. 4. Characterization of *TT12pro:GUS* expression in *ttg1-1* overexpressing *TTG2* and yeast-two-hybrid analysis of TTG1 and TTG2 proteins. (A) Developing seed of *ttg1-1 TT12pro:GUS* reporter line. (B) Developing seed of *ttg1-1 TT12pro:GUS* reporter line overexpressing *TTG2*. (C) Thick cross section of developing silique with seed of *ttg1-1 TT12pro:GUS* reporter line overexpressing *TTG2*. (D) Yeast-two-hybrid analysis of TTG1 and TTG2 proteins. Two independent βgal assays are shown per interaction test, including reciprocal tests. (E) BiFC analysis of protein interactions in microprojectile bombarded onion cells. TTG2, TTG1 and TT8 were fused at their N-termini to the N-terminal (YN) or C-terminal (YC) half of eYFP. YN and YC fusion combinations were tested for recovery of fluorescence as indicated in the center panels. Left panels are the corresponding bright field images. Right panels are DAPI-stained images revealing the location of nuclei. Scale bars: A, B =0.5 mm, C =0.2 mm, E =0.1 mm.

revealed no interactions between TTG2 and TT8, nor between TTG2 and TT2 (data not shown). However, we did observe positive results in LacZ assays when testing for interaction between TTG2 and TTG1 (Fig. 4D). Also, we observed that TTG2 can interact with itself. These results are consistent with Pesch et al. 2014 which recently showed TTG2 self-interaction and TTG2 interaction with TTG1. Moreover, we localized the domain by which TTG2 both homodimerizes and interacts with TTG1 to a 118 amino acid fragment of TTG2 that lies between the two WRKY domains (TTG2 224-342 in Fig. 4).

We also used bimolecular fluorescence complementation (BiFC) in microprojectile bombarded onion cells to further investigate protein-protein interactions indicated by yeast-two-hybrid. Reconstituted fluorescence in the nucleus was detected when constructs encoding YC:TTG2 and YN:TTG1 were co-expressed in onion cells (Fig. 4E). Although we found that images of microprojectile-bombarded cells with YFP constructs were often accompanied by some degree of YFP signal noise (as seen in Fig. 4E), the use of DAPI stain to reveal nuclei clearly shows co-localization of YFP and DAPI signals in the case of co-bombarded YC:TTG2 and YN:TTG1. However, such YFP and DAPI fluorescence overlap was not detected in the nuclei of cells co-bombarded with YC:TTG2 and YN:TT8 constructs, nor in cells co-bombarded with YN:TTG1 and YC:TTG1 constructs (Fig. 4E). In addition, we measured total corrected YFP fluorescence of nuclei indicated by DAPI staining in Fig. 4E. The two nuclei depicted YC:TTG2-YN:TTG1 top center panel of Fig. 4E have a total corrected fluorescence of 25,467 (YFP fluorescent nucleus on the left hand side) and 14,112 (YFP fluorescent nucleus on the right hand side). In contrast, total corrected YFP fluorescence of nuclei in the YC:TTG2-YN: TT8 and YC:TTG1-YN:TTG1 panels were 345 and 4323, respectively. These BiFC results corroborate the findings of the yeast-two-hybrid studies described above and suggest that TTG2 and TTG1 physically interact in a transcriptional complex in the nucleus of plant cells.

3. Discussion

Flavonoid compounds in *Arabidopsis*, such as anthocyanins, have a range of environmental regulatory components influencing their synthesis. Until very recently, PA synthesis was thought to be strictly produced in the inner testa layer (endothelium, chalaza and micropyle) as part of the stereotypical regime of seed coat development (Lepiniec et al., 2006), with expression of the LBGs coordinately controlled in PA-accumulating cells of the inner testa layer during this window of seed development. However, it now appears that low temperature treatment (16 °C) of *Arabidopsis* plants before flowering results in increased in seed dormancy correlated with significantly increased *TT2* expression (but not *TT8* expression), increased expression of *DFR*, *LDOX* and *BAN* and

subsequently increased seed coat tannin production. High temperature treatment (22 °C) results in the opposite set of observations and constitutes a response mediated by the florigen *Flowering Locus T* (*FT*) in fruit tissues (Chen et al., 2014). Thus, this high temperature response mediated by FT seems to be finely transduced through the repression of MADS transcription factors that positively regulate *TT2* and subsequently PA gene expression and tannin biosynthesis. Unlike with anthocyanins whose production is influenced by a broad host of biotic and abiotic cues, this influence of temperature upon the PA pathway appears to be the only known example of an environmental response in this case. Curiously, this temperature response is not observed in all *Arabidopsis* wild-type accessions. Hence, the expression of PA biosynthetic genes, the transcriptional complexes that controls them and ultimately the deposition of PA pigments are primarily controlled developmentally and do not seem to be heavily influenced by a broad range of environmental cues during testa development. The *Arabidopsis* seed coat therefore represents a unique and interesting genetic model for the study of the developmental regulation of a plant secondary metabolic pathway. However, it will be interesting to learn what, if any, role TTG2 regulatory mechanisms of PA synthesis play in this temperature response.

Besides the biosynthetic genes and the MBW regulators, another class of genes necessary for the deposition of PAs is involved in the transport of monomeric PA precursors into the vacuole (Lepiniec et al., 2006). Although in *Arabidopsis* no glucosyltransferase has been identified that glycosylates epicatechin, the flavan-3-ol unit of polymerized PAs, this is nonetheless an important step as suggested by evidence in other systems. For example, expression of the glucosyltransferase, UGT72L1, from *Medicago* was highly induced by *TT2* overexpression and subsequently shown to catalyze the formation of epicatechin-3′-O-glucoside (E3′OG) (Pang et al., 2008; Pang et al., 2013). E3′OG in turn is the preferred substrate of MATE1 and TT12, both MATE-type transporters from *Medicago* and *Arabidopsis,* respectively. These transporters are expressed in PA-accumulating cells, are localized to the tonoplast, and facilitate vacuolar uptake of E3′OG. Plants deficient for this transport step show tannin deficiencies as a result (Debeaujon et al., 2001; Marinova et al., 2007; Zhao and Dixon, 2009). Additionally in *Arabidopsis*, *TT13/AHA10*, encoding a putative P-type ATPase proton pump, is also required for PA accumulation (Baxter et al., 2005; Appelhagen et al., 2015). Plants mutant for *TT13/AHA10* show a strong reduction in extractable PAs. More recently, both *TT12* and *TT13/AHA10* have been shown to be direct targets of the MBW PA pathway regulatory complex and are thus classified as LBGs (Xu et al., 2014). Although classified as a plasma membrane ATPase based on sequence analysis, TT13/AHA10 was recently shown to localize to the tonoplast, and thus has been hypothesized to establish the electrochemical H+gradient across the vacuolar membrane that is then harnessed by TT12 to transport in E3′OG (Baxter et al., 2005; Marinova et al., 2007; Appelhagen et al., 2015). Therefore, AHA10 and TT12, along with a UGT step not yet identified in *Arabidopsis*, represent a transport pathway for the accumulation of PA monomer units into the vacuole of cells of the inner testa layer.

3.1. Mechanisms of TTG2 Regulation of PA Production: Similarities and Differences of Flavonoid Pathway Control by the MBW complex and TTG2

A recent comprehensive analysis of the flavonoid transcriptional regulatory network (Xu et al., 2014) demonstrated the sophisticated and robust nature of flavonoid structural gene regulation by MBW complexes. The MBW regulatory model seems necessary and sufficient for the direct control of PA pathway genes from *DFR* down to some of the final steps such as transport of PA monomers by TT12 and TT13/AHA10. However, the observation that *ttg2* mutants show PA deficiencies in the seed and that the *TTG2* gene itself is a transcription factor and direct target of MBW complexes (Johnson et al., 2002; Ishida et al., 2007; Zhao et al., 2008) suggested to us that this otherwise well understood flavonoid pathway regulatory model was worthy of further investigation. In this study we demonstrate that TTG2 control of *TT12* expression requires TTG1 (Fig. 4A-C), the WD-repeat protein in the *Arabidopsis* MBW complex that controls flavonoid expression and also *TTG2* itself (Ishida et al., 2007; Morohashi and Grotewold, 2009). Furthermore, our yeast-two-hybrid and *in planta* BiFC data indicate that TTG2 physically interacts with TTG1 (Fig. 4D and E), but not TT8 or TT2 (Fig. 4E, data not shown).

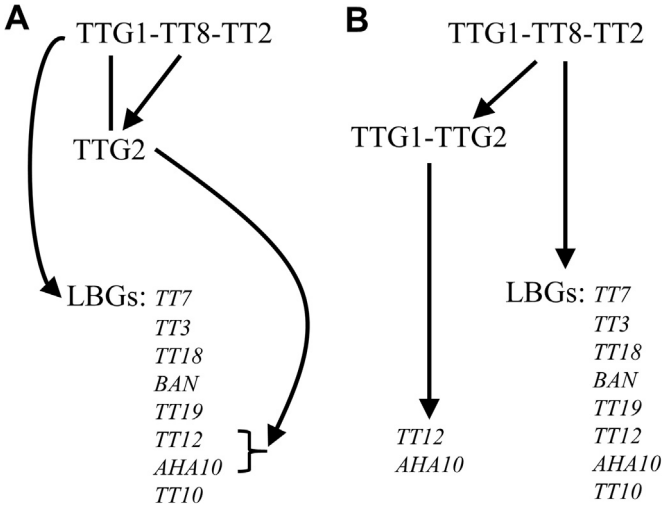

Fig. 5. Possible TTG2 transcriptional regulatory models. In (A) the PA MBW complex controls the expression of the LBGs and *TTG2*. TTG2 then possibly enters the MBW complex via physical interaction with TTG1 and narrows the range of LBG targets to *TT12* and *AHA10*. In (B) the PA MBW complex regulates the LBGs and *TTG2* but TTG2 and TTG1 physically interact to form a separate transcriptional complex that narrowly targets the transport genes *TT12* and *AHA10.* Black lines represent protein-protein interactions. Black arrows represent control of gene expression.

Consistent with previously published observations about the regulation of PA pathway genes by the MBW complex, we observed *TT3*, *BAN*, *TT19*, *TT12*, *TT13/AHA10* and *TT10* to be under the control of TT8 (Debeaujon et al., 2003; Baudry et al., 2004; Xu et al., 2014). In contrast, only *TT12* and *TT13/AHA10* require TTG2 for essentially all of their expression (Figs. 2B and 3A-D), while *TT10* expression is only partially dependent on TTG2. In addition, *TT12* is still weakly expressed in the inner testa layer of *tt8* and *tt2* developing seed, while it is undetectable in *ttg1* and *ttg2* inner testa layers (Figs. 3A-D and 4A-C; Xu et al., 2014). Taken together, these observations of qualitative and quantitative differential flavonoid pathway regulation by the PA MBW complex compared to TTG2 highlight a novel regulatory mechanism for the production of PA pigment by the TTG2 transcription factor.

3.2. A Novel TTG1/TTG2-based Transcriptional Complex for the Regulation of PA Pathway Genes

Our yeast-two-hybrid and genetic data indicate that TTG2 may at some point enter into the MBW complex, via interaction with TTG1, and narrow its target range (Fig. 5A). Alternatively, TTG2 and TTG1 may form a transcriptional complex that regulates a distinct subset of PA genes that is independent of the MYB and bHLH proteins (Fig. 5B). Data presented here and from Xu et al. (2014) which indicate that *TT12* is still weakly expressed in the inner testa layers of *tt8* and *tt2,* but apparently not at all in *ttg1* or *ttg2* inner

testa layers (Xu et al., 2014; Figs. 3A-D and 4A-C), suggest that a scenario in which TTG2 enters a full MBW complex is less likely. Also, if TTG2 were a member of the MBW complex regulating the PA pathway, we would reasonably expect some of the other LBGs regulated by TT8 and TT2 to be down-regulated in *ttg2-1* mutants (i.e., a more common set of shared targets regulated by TT8, TT2 and TTG2), but this has not been shown (Fig. 2; Baudry et al., 2004). Therefore, it is possible that TTG1 and TTG2 form the basis of novel transcriptional complex with a narrowly defined set of targets in the PA pathway. This is not only consistent with the pigment pathway gene expression studies described above, but also consistent with the dependency of TTG2 upon TTG1 for control of *TT12,* and with the yeast-two-hybrid and BiFC results showing interaction of TTG2 with TTG1 but not with TT8 (Fig. 4).

However, it has been recently shown in the context of trichome development that TTG2 physically interacts with TTG1 to strongly and directly enhance *Triptychon* expression, but not the expression of other R3MYB genes that negatively regulate the trichome pathway (Pesch et al., 2014). In addition, no direct physical interaction was shown between TTG2 and a host of other trichome and root hair patterning genes, but TTG2 did interact indirectly with GL3 via a bridge with TTG1. This would suggest a regulatory model as presented in Fig. 5A in which TTG2 does enter the MBW complex to reinforce the expression of a small subset of MBW flavonoid target genes. Thus, interesting parallels can be drawn between TTG2 regulatory mechanisms in the trichome and flavonoid pathways such as the dependence upon TTG1 for activation of TTG2 direct targets (*TRY* and *TT12*) and that TTG2 is required for the expression of only a small subset of genes targeted by the MBW complex, both in the trichome and flavonoid pathways.

Regardless of the exact composition of a complex containing TTG2, the mechanisms of TTG2 regulation described here (namely, the narrow targeting of transport genes) represents a new layer of complexity to PA pigment pathway developmental regulation not otherwise predicted or accounted for by the MBW model (Fig. 5).

Besides TTG2-containing complexes discussed here, there exists additional suggestions of alternate complexes forming in order to regulate flavonoid biosynthesis. Appelhagen et al., (2011) found that TT1, a WIP-type zinc finger protein, could bind to the PAP1 and TT2 MYB proteins involved in anthocyanin and PA regulation respectively. While TTG1 is an integral part of several MBW transcriptional complexes, it does not contain any recognizable DNA binding domains (Walker et al., 1999). However, TTG1 has been shown to interact with GL2 EXPRESSION MODULATOR (GEM), which helps maintain histone methylation (Caro et al., 2007), suggesting that TTG1 may play a role in making DNA accessible for transcription factors. This may explain why TTG2 requires TTG1 for proper control of *TT12*.

Additionally, we localized the interaction domain on TTG2 to a 118 amino acid region that lies between the two WRKY domains. We discovered in our yeast-two-hybrid analysis that TTG2 can homodimerize via this same 118 amino acid region. Although it has been speculated in the literature that WRKY proteins may dimerize via a WRKY domain (Eulgem et al., 2000), here we show TTG2 dimerization via an unpredicted protein-protein interaction domain localized between the WRKY domains.

3.3. TTG2 regulates TT12 and TT13/AHA10

The regulation of the LBGs by MBW complexes has been well studied, but the *ttg2* mutant seed coat phenotype suggested a regulatory sub-routine that is just being uncovered. The work presented here corroborates previous findings that PA biosynthesic genes (including *TT12* and *TT13/AHA10*) depend on the TT2-TT8-TTG1 complex for normal expression (Fig. 2B; Debeaujon et al., 2003; Baudry et al., 2004; Xu et al., 2014; Appelhagen et al., 2015). However, our Q-PCR data show that *TT12* and *TT13/AHA10* are also dependent on TTG2 for expression, while other LBGs are unaffected by the absence of TTG2. Our analysis does show a reduction in *TT10* transcript levels, but this reduction is minor in comparison to the almost total ablation of expression seen for *TT12*. This could be significant, but we think that the reduction we see in *TT10* expression is more likely the result of a feedback mechanism since *TT10* encodes a laccase-like protein that is thought to operate downstream of TT12 and TT13/AHA10 mediated transport of PA precursors into the tonoplast (Fig. 1; Pourcel et al., 2005). However, it is interesting that TTG2 exerts weak control over the expression of epidermal patterning genes such as *CPC* and *GL2,* besides the strong direct control of *TRY* expression (Pesch et al., 2014). This is yet another possible parallel between TTG2 regulation of the trichome and flavonoid pathways in which TTG2 shows both strong and weak control of particular pathway genes.

Based on the extreme *TT12* message reduction observed in *ttg2-1* mutant compared to wild-type plants, we investigated the regulatory relationship between TTG2 and *TT12*. The *TT12 promoter-GUS* construct confirmed that TT12 expression was virtually abolished in a *ttg2* mutant (Fig. 4A-D). Furthermore, we found that ectopic expression of *TT12* in a *ttg2-1* mutant partially restored much of the brown color to the normally yellowish seed coat (Fig. 3F). Finally, we showed that TTG2 can bind to the 5′ cis regulatory region of the *TT12* gene in a yeast-one-hybrid assay (Fig. 3E). This is consistent with the presence of two canonical W-box WRKY binding elements and three other sequence elements that are very similar to this motif. Together, these data provide strong evidence that TTG2 directly regulates *TT12*.

However, this is not the entire story. Expression of the *35S:TT12* construct in a *ttg2* mutant did not fully restore wild-type levels of seed tannin formation, as made clear by the lack of DMACA staining in these seeds (Fig. 3G and H). The function of another target (or targets) of TTG2 must still be needed to restore proper PA production. *TT13/AHA10* is a likely candidate. In this work we show that *TT13/AHA10* expression in *ttg2* mutants is only about 2% of that seen in wild-type (Fig. 2B) which indicates that this gene is regulated by TTG2, either directly or indirectly. Also, the seeds of *tt13/aha10* mutants have more brown pigmentation than *ttg2* mutants, but they lack DMACA staining, similar to what we see in the *35 S:TT12 ttg2* plants (Baxter et al., 2005; Appelhagen et al., 2014; Appelhagen et al., 2015; Fig. 3F and G). There are two (C/T) TGAC(T/C) motifs within 600 base pairs of the *TT13/AHA10* start codon, so it seems possible that a WRKY type transcription factor may regulate this gene. We might also expect that ectopic expression of *TT13/AHA10* and *TT12* together will fully restore the seed coat phenotype to *ttg2* mutants; but whether it does or does not, the overall conclusion remains well-supported by the findings presented here and elsewhere in the literature (Debeaujon et al., 2003; Baudry et al., 2004; Xu et al., 2014) that TTG2 regulation of the flavonoid pathway represents a novel regulatory mechanism for the production of PA pigment in the seed coat.

3.4. TTG2 as a Possible Regulator of the PA Transport Sub-Pathway

It is possible that the lack of TT13/AHA10 and TT12 function is not the sole basis of the *ttg2* seed coat pigment phenotype. Because both TT12 and TT13/AHA10 are involved in the transport of PA precursors into the vacuole, it is tempting to speculate that TTG2 may regulate yet other features of transport mechanisms late in the PA pathway. In *Medicago* the gene *UGT72L1* encodes a glucosyltransferase responsible for the glycosylation of epicatechin to E3′OG, but intensive genetic screens and phylogenetic analyses have yet to reveal an obvious ortholog of this gene in *Arabidopsis* (Lepiniec et al., 2006; Pang et al., 2008; Zhao and Dixon, 2009;

Pang et al., 2013). When *UGT72L1* promoter activity was assessed in *Arabidopsis*, its expression overlapped well with that reported for *TTG2,* not just in developing seed but in leaf trichomes as well as other vegetative tissues including hypocotyls and leaf primordia, suggesting that a similar gene could be a regulatory target of TTG2 (Johnson et al., 2002; Pang et al., 2013). It would be interesting to determine if this profile of the *Medicago UGT72L1* promoter activity in *Arabidopsis* depends on TTG2. If so, it would imply that TTG2 regulation of the PA pathway extends to a possible UGT step. Regardless of the outcome of this search, it is obvious that there is more to learn about the regulation and development of this offshoot of the flavonoid pathway.

4. Materials and methods

4.1. Arabidopsis accessions

The mutant lines *ttg1-1* and *ttg2-1* are in the Landsberg *erecta* (L*er*) ecotype and have been previously described (Koornneef, 1981; Walker et al., 1999; Johnson et al., 2002). The *tt8-1* mutant is in the Enkheim-2 (En-2) ecotype and has been previously described (Nesi et al., 2000). Plants were grown in soil at 21 °C in continuous white light, or seedlings were grown on germination medium containing 3% sucrose as described (Zhang et al., 2003).

4.2. Plasmid Construction

p35S:TTG2-the *TTG2* Col0 genomic locus from start to stop codons was amplified using the primers below and recombined into pDONR222 (Invitrogen) to produce pETTG2. *TTG2* was then recombined from pETTG2 into pB7WG2 (Karimi et al., 2002). Gateway recombination sequences were included on all appropriate primers but are not shown.

TTG2fwd: 5′attB1-ATGGAGGTGAATGATGGTGAAA-3′.

TTG2rev: 5′attB2-TCAAATTGTTTGCTTAGAAAGTTGTG-3′.

pTT12pro:GUS- an approximately 1.5 kb fragment upstream of the *TT12* start codon was amplified from Col0 genomic DNA using the primers below and recombined into pDONR222 (Invitrogen) to produce pETT12pro. pETT12pro was then used to recombine the *TT12* regulatory fragment into pKGWFS7 GUS vector (Karimi et al., 2002).

TT12pro3fwd: 5′attB1-AGGTATAAGAGAAGAAGGTC-3′.

TT12pro3rev: 5′attB2-GGTCCGTTTATTAGTTCCTC-3′.

p35S:TT12-the *TT12* Col0 genomic locus from start to stop codons was amplified using the primers below and recombined into pDONR222 (Invitrogen) to produce pETT12. *TT12* was then recombined from pETT12 into pB7WG2 (Karimi et al., 2002).

TT12fwd: 5′attB1-ATGAGCTCCACAGAGACATACG-3′.

TT12rev: 5′attB2-TTAAACACCTGCGTTAGCCATC-3′.

pADTTG2-the *TTG2* coding sequence without introns was amplified from an *Arabidopsis* Col0 silique cDNA prep using the primers TTG2fwd and TTG2rev (sequence listed above) and cloned into pDONR222 (Invitrogen) to produce pETTG2cds. *TTG2* was then recombined from pETTG2cds into pACT, the yeast-two-hybrid GAL4 activation domain vector.

pBDTTG2-*TTG2* coding sequence from pETTG2cds was recombined into pGBT9, the yeast-two-hybrid GAL4 DNA binding domain vector.

pADTTG2-224-342-354 base pairs of *TTG2*, encoding amino acids 224–342 between the two WRKY sequences, was amplified from a Col0 silique cDNA prep using the *TTG2* primers listed below and cloned into pDONR222 (Invitrogen) to produce pETTG2-224-342cds. This *TTG2* fragment was then recombined from pETTG2cds into pACT.

TTG2-224-342fwd: 5′attB1-TCTTGTCCTCTTCCCGGCGTG-3′.

TTG2F6rev: 5′attB2-TGAGCCTTGCGATACTCCTGC-3′.

pBDTTG2-224-342-the 354 base pair fragment of *TTG2* coding sequence in pETTG2-224-342 was recombined into pGBT9.

pADTTG1-the *TTG1* coding sequence was amplified from a Col0 genomic DNA using the primers below and cloned into pDONR222 (Invitrogen) to produce pETTG1. *TTG1* was then recombined from pETTG1 into pACT.

TTG1fwd: 5′attB1-ATGGATAATTCAGCTCCAGATTCG-3′.

TTG1rev: 5′attB2-TCAAACTCTAAGGAGCTGCATTTTG-3′.

pBDTTG1-*TTG1* coding sequence from pETTG1 was recombined into pGBT9.

pTT12proY1H-an approximately 1 kb fragment upstream of the *TT12* start codon was amplified from Col0 genomic DNA using the primers below and recombined into p4P1R DONR vector (Deplancke et al., 2004) to produce pE4TT12pro. pE4TT12pro was then used to recombine the *TT12* regulatory fragment into pMW3 LacZ reporter vector (Deplancke et al., 2004).

TT12pro2fwd: 5′attB4-GGGCCAATCCGACCTAATAG-3′.

TT12pro2rev: 5′attB1R-GGTCCGTTTATTAGTTCCTC-3′.

pYC-TTG2-the *TTG2* coding sequence from start to stop codons was amplified form Col-0 silique first strand cDNA using the primers below and recombined into pDONR222 (Invitrogen) to produce pETTG2CDS. *TTG2* was then recombined from pETTG2CDS into pSAT5A-DEST-C(175-END)EYFP-N1 (ABRC; https://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm), resulting in the C-terminal portion of YFP fused to the N-terminus of TTG2.

TTG2fwd: 5′attB1-ATGGAGGTGAATGATGGTGAAA-3′.

TTG2rev: 5′attB2-TCAAATTGTTTGCTTAGAAAGTTGTG-3′.

pYN-TTG1-*TTG1* coding sequence from pETTG1 was recombined into PSAT4A-DEST-N(1-174)EYFP-N1 (ABRC; https://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm), resulting in the N-terminal portion of YFP fused to the N-terminus of TTG1.

pYC-TTG1-*TTG1* coding sequence from pETTG1 was recombined into pSAT5A-DEST-C(175-END)EYFP-N1 (ABRC; https://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm), resulting in the C-terminal portion of YFP fused to the N-terminus of TTG1.

pYN-TT8-the *TT8* coding sequence from start to stop codons was amplified form L*er* silique first strand cDNA using the primers below and recombined into pDONR222 (Invitrogen) to produce pETT8CDS. *TT8* was then recombined from pETT8CDS into PSAT4A-DEST-N(1-174)EYFP-N1 (ABRC; https://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm), resulting in the N-terminal portion of YFP fused to the N-terminus of TT8.

TT8fwd: 5′attB1-ATGGATGAATCAAGTATTATTCCG-3′.

TT8rev: 5′attB2-CTATAGATTAGTATCATGTATTAT-3′.

4.3. Sectioning of developing siliques for histochemical detection of GUS activity

Young developing siliques (2–5 days after flowering (daf) or during the globular stage) from promoter:GUS lines were embedded in 5% low melt agarose. 30–40 μm cross sections of developing siliques were cut using a Vibratome Series 1000 Plus tissue sectioning system (Ted Pella, Inc.). Sections were placed in a drop of staining reagent containing 1 mg/ml X-Gluc substrate and 1 mM potassium ferricyanide/potassium ferrocyanide. Staining was observable within 10–30 min or not at all depending on the reporter line.

4.4. Yeast-two-hybrid and yeast-one-hybrid analysis by LacZ staining lift assays

Integration of the pTT12proY1H plasmid into the yeast strain YM4271 was performed as described (Deplancke et al., 2004) to create the TT12 promoter:LacZ reporter bait line for yeast-one-hybrid analysis. Yeast-two-hybrid analysis was performed in the Y190 yeast line. All LacZ assays and analysis were performed as previously described (Payne et al., 2000; Zhang et al., 2003).

4.5. Gene expression analysis by quantitative-PCR

Total RNA was prepared from young developing siliques, about 2–5 daf or during the globular stage see above, using a Qiagen RNeasy plant mini kit. 2 µg of total RNA was used to produce first-strand cDNA in 20 µl reverse transcription reactions using a Super-Script III RT kit (Invitrogen). 10 µl PCR reactions were prepared using 1 µl cDNA reaction as template with 5 µl 2X SuperPower Syber Q-PCR mixture (ABI) and run on a spectrofluorometric thermal cycler (ABI 7900HT). Target primers were used at 200 nM final concentration and 200 nM actin primers were used in separate control reactions. The comparative cycle threshold method was used to analyze Q-PCR results (User Bulletin 2, ABI PRISM Sequence Detection System). Three biological replicates were performed for each experiment with each replicate containing four reactions per target (including the actin control target) with consistent results. The combined results from the three replicate experiments are presented. The following primers were used for target and control gene amplification:

TT3 fwd: 5′-CGTTTGAAGGTGTTGATGAGAA-3′.
TT3 rev: 5′-TGAAGTTAACCCCATGTCCG-3′.
BAN fwd: 5′-GAAGAGGGCTTGTCGATTCCG-3′.
BAN rev: 5′-TACATCTCATTGATCCCCATATTCG-3′.
TT19 fwd: 5′-CTATGGCTGATTTGACGCACA-3′.
TT19 rev: 5′-TCTTCCAAGACGGTCTATCCG-3′.
AHA10 fwd: 5′-ATCTCTCAGAGAAGTCGTTCCG-3′.
AHA10 rev: 5′-CTAGAAGCCTGGCGATTTCTGC-3′.
TT12 fwd: 5′-CTTGGAGTTGCTGGGATCTGGT-3′.
TT12 rev: 5′-TCTCTTGATTCCAGTTGCCG-3′.
TT10 fwd: 5′-CAGATTCGTAGCTGATAATCCCG-3′.
TT10 rev: 5′-CCCACGTTTGATGTCTATCCAAG-3′.
ACT fwd: 5′-TCCATTCTTGCTTCCCTCAG-3′.
ACT rev: 5′-ATCATTACTCGGCCTTGGAGA-3′.

4.6. Microprojectile bombardment of BiFC constructs

Micro-projectile bombardment was performed as previously described in Zhao et al. (2008) except that the tissue bombarded was a layer of white onion cells. ImageJ (https://imagej.nih.gov/ij/) was used to quantify YFP fluorescence in nuclei of micro-projectile bombarded cells. Area of the selected nuclei, integrated density and mean fluorescence of background areas were measured and used to calculate corrected total nuclear fluorescence by subtracting the integrated density from the product of the area of selected nuclei and the mean fluorescence of background readings.

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