TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in *Arabidopsis thaliana*

Antoine Baudry†, Michel Caboche and Loïc Lepiniec*
Seed Biology Laboratory, UMR 204 INRA/INAPG, Jean-Pierre Bourgin Institute, Route de Saint-Cyr, 78026 Versailles Cedex, France

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*For correspondence (fax +33 1 30 83 30 99; e-mail lepiniec@versailles.inra.fr).
†Present address: Steve Kay Laboratory, Department of Biochemistry, Institute of Childhood and Neglected Diseases, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Summary

The control of *TT8* expression was investigated in this study, and it was demonstrated that it constitutes a major regulatory step in the specific activation of the expression of flavonoid structural genes. First, the GUS activity generated *in planta* from a *TT8::uidA* construct revealed cell-specific activation of the *TT8* promoter consistent with the known involvement of the TT8 bHLH factor in proanthocyanidin, anthocyanin and mucilage biosynthesis. Moreover, the activity of this reporter construct was strongly affected in *ttg1*, *TT2* overexpressers (OE), and *PAP1-OE*, suggesting interplay between *TT2*, *PAP1*, *TTG1* and the activation of the *TT8* promoter *in planta*. To further investigate the mechanisms involved, we used 35S::*TT2-GR* and 35S::*TTG1-GR* transgenic plants (expressing fusion proteins with the glucocorticoid receptor), as well as one-hybrid experiments, to determine the direct effect of these factors on *TT8* expression. Interestingly, *in vivo* binding of TT2 and PAP1 to the *TT8* promoter was dependent on the simultaneous expression of TT8 or the homologous bHLH factors GL3 and EGL3. Consistent with these results, the activity of the *TT8::uidA* reporter was strongly affected in the seed endothelium of a *tt8* mutant. Similarly, a strong decrease in the level of *TT8* mRNA was detected in the siliques of a *gl3*·*egl3* mutant and in plants that express a dominant negative form of the *PAP1* protein, suggesting that *TT8* expression is controlled by different combinations of MYB and bHLH factors *in planta*. The importance of this positive feedback mechanism in the strong and specific induction of proanthocyanidin biosynthesis in the seed coat of *Arabidopsis thaliana* is discussed.

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

Introduction

The combinatorial control of structural gene expression is a central feature of the specific accumulation of flavonoid compounds in plants. The interplay between transcription factors (TFs) of the R2R3-MYB and basic helix-loop-helix (bHLH) families was first described in *Zea mays* with the interaction of C1 (MYB) and R (bHLH) for the activation of anthocyanin biosynthesis (Goff *et al.*, 1992). Extensive studies of flavonoid regulation in different plant species have demonstrated the conservation of MYB/bHLH interactions in the control of flavonoid biosynthesis (Quattrocchio *et al.*, 2006). Furthermore, an additional class of regulatory factors involved in this process has been isolated, and is characterized by a highly conserved sequence composed of five WD repeats (WDR; Carey *et al.*, 2004; de Vetten *et al.*, 1997; Walker *et al.*, 1999).

In *Arabidopsis thaliana*, proanthocyanidins (PA, oligomers of the flavan-3-ol 2,3-cis-(-)-epicatechin) accumulate in the inner integument and in the pigmented chalazal strand of the seed coat (Debeaujon *et al.*, 2003). This specific pattern is conferred by the collective action of *TT2* (MYB), *TT8* (bHLH) and *TTG1* (WDR) in regulation of the expression of the PA structural genes (Lepiniec *et al.*, 2006). One
important target that controls the first enzymatic step specifically committed to PA biosynthesis is the anthocyanidin reductase encoded by BANYULS (BAN; Xie et al., 2003). In a previous study, we have shown that a ternary complex composed of TT2, TT8 and TTG1 is able to bind the BAN promoter to activate transcription of the BAN gene in planta (Baudry et al., 2004). Interestingly, similar MYB/bHLH/WD40 complexes in A. thaliana are implicated in the regulation of trichomes and root-hair organogenesis, and of mucilage biosynthesis in the seed coat, suggesting that a common regulatory module has been retained in plants for the control of several epidermal cell differentiation pathways (Broun, 2005).

R2R3-MYB and bHLH represent two major families of TF in plants, with 125 and 133 members in the A. thaliana genome (Heim et al., 2003; Stracke et al., 2001), respectively. The subgroup IIIf of the bHLH family contains the closest homologues of TT8, namely GL3 and EGL3 (Heim et al., 2003). These bHLHs are involved in regulation of the TTG1-dependent pathways of A. thaliana (i.e. trichome and root-hair production, mucilage, anthocyanin and PA biosynthesis) in a partially redundant manner (Zhang et al., 2003). For instance, TT8, GL3 and EGL3 control anthocyanin accumulation in leaves; mucilage biosynthesis in the outer integument of the seed coat is induced by TT8 and EGL3; and TT8 is necessary for PA accumulation in the endothelium (Nesi et al., 2000; Zhang et al., 2003). Essentially, the activation of such diverse pathways is the consequence of interactions between these bHLH and more specific MYB proteins (Lepiniec et al., 2006). Thus, the implication of TT8 in anthocyanin and PA metabolism is the result of interactions with the homologous MYB proteins PAP1/PAP2 and TT2, respectively (Baudry et al., 2004; Zimmermann et al., 2004).

bHLH factors might also be involved, to some extent, in the specific recognition of the target DNA. In Z. mays, anthocyanin accumulation is controlled by the C1/R complex, whereas P (MYB) can activate phlobaphene biosynthesis alone. Comparison of C1 and P protein sequences allowed amino acids in the MYB domain to be identified that, once mutated in P, were sufficient to confer the interaction with R (Grotewold et al., 2000). In addition to its ability to activate transcription of the usual targets of P, the resulting mutant protein was able to activate specific genes of anthocyanin biosynthesis, a new property conferred by the interaction with R. These results suggested that, through their interaction with bHLH, MYB regulators of the flavonoid pathway display new DNA-binding specificities.

Similarly, the WDR protein is likely to have a direct influence on the activity of the MYB/bHLH complex. Interaction results suggest that TTG1 is involved in a ternary complex with MYB and bHLH factors controlling PA biosynthesis, trichome organogenesis, and root-hair patterning (Baudry et al., 2004; Bernhardt et al., 2003; Payne et al., 2000). Moreover, three-hybrid experiments in yeast have demonstrated that TTG1 is able to interact simultaneously with TT2 and TT8 (Baudry et al., 2004). This interaction increased the activity of the TT2/TT8 complex in the induction of BAN transcription in planta, but did not seem to modify the specificity of the complex for DNA. This implies that TTG1 could affect the functionality of the bHLH partner, and might participate in the activation, modification or stabilization of this TF (Baudry et al., 2004; Payne et al., 2000).

Essential data are still lacking concerning the transcriptional regulation of the regulators of flavonoid biosynthesis, which might be the primary step in the control of the specific accumulation of flavonoid compounds in plants. Based on the clear correlation between the BAN expression pattern and the activity of the TT2 promoter in PA-accumulating cells (Debeaujon et al., 2003), it has been suggested that the control of TT2 expression is the key determinant of PA biosynthesis in the A. thaliana seed coat. In contrast, the TTG1 promoter is activated transiently in the whole seed coat at the globular stage of embryo development (Baudry et al., 2004). Detailed analysis of TT8 expression remains to be carried out. Preliminary results suggested a possible role of its MYB partners TT2 and PAP1. Indeed, overexpression of these genes in A. thaliana induced the ectopic accumulation of TT8 mRNA (Nesi et al., 2001; Tohge et al., 2005). Similarly, the accumulation of AN1 mRNA, encoding a bHLH factor homologous to TT8, is activated by AN2 and AN4, the MYB factors controlling anthocyanin production in Petunia hybrida flowers (Spelt et al., 2000). Although no such relationships have been found in Z. mays (Carey et al., 2004), these results suggest that, at least in dicotyledonous species, the control of bHLH gene expression by other flavonoid regulators is an important feature in the regulation of flavonoid accumulation.

In this study, the precise pattern of TT8 expression was investigated in planta by characterization of the GUS activity generated from a transcriptional fusion between the TT8 promoter and the uidA reporter gene. The results are consistent with previous data obtained by semi-quantitative RT-PCR analysis (Nesi et al., 2000), and demonstrated that TT8 regulation occurs at the transcriptional level. In addition, the cell-specific pattern is also consistent with the diverse functions of TT8 in seeds (PA and mucilage biosynthesis) and in vegetative parts (anthocyanin biosynthesis). Possible alterations to the activity of this TT8::uidA construct were investigated in various genetic backgrounds, namely tt2, ttg1, TT2 overexpressers (OE) and PAP1-OE. The results indicate that TT8 expression is regulated by TT2, PAP1 and TTG1 at the transcriptional level. Using 35S::TT2-GR and 35S::TTG1-GR transgenic plants (expressing fusion proteins with the glucocorticoid receptor, GR), as well as one-hybrid experiments, we have demonstrated the direct effect of these factors on TT8 expression. Moreover, the binding of TT2 and PAP1 to the TT8 promoter in yeast was dependent...
on the co-expression of TT8 or the homologous bHLH factors GL3 and EGL3. Consistent with these results, the activity of the TT8::uidA reporter was strongly affected in the seed endothelium of a tt8 mutant. Similarly, a strong decrease in the level of TT8 mRNA was detected in the gi3 × egl3 double mutant and in plants that express a dominant negative form of the PAP1 protein, suggesting that TT8 expression is controlled by several combinations of MYB and bHLH factors in planta. The functional importance of this positive feedback in the strong and specific activation of flavonoid biosynthesis is discussed.

Results

Characterization of the activity of the TT8 promoter in A. thaliana

A transcriptional fusion between a 1517 bp fragment of the TT8 promoter and the uidA reporter gene (encoding GUS) was introduced into wild-type (WT) A. thaliana plants to investigate the mechanisms that control TT8 expression in planta. Histochemical analyses were performed with several independent transformants and revealed an activation of the reporter construct in developing seeds and in seedlings, consistent with the previous results of semi-quantitative RT-PCR experiments performed to investigate TT8 expression (Nesi et al., 2000). These results demonstrate the importance of transcriptional regulation for TT8 expression. Additionally, the results revealed that the activity of the TT8 promoter is restricted to specific cell types (Figure 1).

In seeds, a strong GUS activity was detected in the micropylar area, the endothelial layer and the chalazal pigment strand of the seed coat (corresponding respectively to regions 1, 2 and 3 defined in Debeaujon et al., 2003). This activity was the highest at the globular and heart stages of embryo development (Figure 1a–c), and it fits well the peak of BAN expression and the accumulation of PA in these cells. However, some activity remained in the endothelium at the curled cotyledon and mature stages of embryo development when the expression of BAN and other regulators of PA biosynthesis (TT2 and TTG1) is no longer detected (Figure 1d; Baudry et al., 2004; Debeaujon et al., 2003). At the heart stage, the outermost cell layer of the seed coat, where mucilage accumulates, started to show strong GUS activity that persisted until later stages of embryo development (Figure 1b–d). Finally, a significant GUS activity was detected in the embryonic hypocotyl, at late stages of embryo development (curled cotyledon and mature embryo stages; Figure 1d,e).

A strong GUS activity was also detected in seedlings, 2 days after germination, in the upper part of the hypocotyl and at the cotyledon margin (Figure 1m). This activity persisted in 3-day-old germinating seedlings, with an extension to the upper half of the hypocotyl (Figure 1o).

This specific pattern perfectly matches that of anthocyanin accumulation at the same stages of development (Figure 1l). In 10-day-old germinating seedlings, the GUS activity was restricted to young emerging leaves (Figure 1n) and was similar to the pattern of GUS activity generated from a TTG1::uidA construct (Baudry et al., 2004). No GUS activity was detected in the roots of TT8::uidA plants.

The activity of the TT8 promoter is induced by TT2 or PAP1 overexpression and is altered in the ttg1 mutant

Two independent transgenic plants carrying the TT8::uidA reporter construct were selected as representative of the different transformants (n = 16) and crossed with a set of A. thaliana mutants to investigate TT8 regulation. First, this construct was introduced into TT2-OE and PAP1-OE (pap1-D; Borevitz et al., 2000; Nesi et al., 2001). In these plants, previous analyses have shown an ectopic accumulation of TT8 mRNA in roots (in TT2-OE; Nesi et al., 2001) or a significant increase of TT8 expression in seedlings (in PAP1-OE; Tohge et al., 2005), suggesting that TT2 and PAP1 can induce TT8 expression in planta. These results were confirmed in our experiments, as a GUS activity and therefore activity of the TT8 promoter were induced in the roots of TT2-OE × TT8::uidA plants. In addition, the GUS activity was not only restricted to cotyledon margins, as observed in WT seedlings, but also activated inside cotyledons, showing a patchy pattern due to spots of GUS activity in the guard cells of the stomata (Figure 1p). As expected, this ectopic activation of the TT8 promoter in cotyledons correlated with that observed for the activity of a BAN::uidA construct in TT2-OE (Baudry et al., 2004). The same results were obtained with the PAP1-OE × TT8::uidA plants, in both roots and cotyledons, at the same developmental stages (Figure 1q). These results demonstrate that TT2 or PAP1 overexpression is sufficient to induce the ectopic transcription of TT8.

The activity of the TT8::uidA construct was then investigated in tt2 and ttg1 mutant backgrounds. As TT2 induced TT8 expression when ectopically expressed in planta, one would expect a reduction in the activity of TT8::uidA in the tt2 mutant if this regulatory mechanism were required for TT8 expression in seeds. However, we observed no quantitative or qualitative differences in the activity of TT8::uidA in tt2 seeds, compared with WT. A possible explanation is that TT8 might be redundantly regulated by different MYB factors in PA-accumulating cells.

Conversely, a strong decrease in the GUS activity generated from the TT8::uidA construct was revealed in both seeds and seedlings of the ttg1 mutant, but in a cell-type-dependent manner. For instance, in seedlings, some activity remained at the tip of the cotyledons and at the top of the hypocotyl, but not at the cotyledon margins (Figure 1r). Similarly, in developing seeds, a significant but weak GUS
activity was still detectable in the chalazal pigment strand of the seed coat (region 3) and in the embryonic hypocotyl (Figure 1f–h). In contrast, no GUS activity could be detected in the inner (regions 1 and 2) or outer integument of the seed coat, indicating that TTG1 is necessary for activation of the TT8 promoter in these cells.

Figure 1. Activity of the TT8 promoter in Arabidopsis thaliana seeds and seedlings of different genetic backgrounds. The histochemical localization of the GUS activity generated from a TT8::uidA construct introduced into WT (a–e, m–o), ttg1-1 (f–h, r), tt8-1 (i–k), TT2-OE (p) and PAP1-OE (q) is presented and compared with the pattern of anthocyanin accumulation in WT germinating seedlings (l). For GUS experiments, representative pictures of the results obtained with several independent transformants are shown.

(a, f, i) Sections of seeds at the globular stage of embryo development (f, late globular stage; i, early globular stage).
(b, g, j) Sections of seeds at the heart stage.
(c) Transversal section of a seed at the torpedo stage.
(d) Section of a seed at the curled cotyledon stage.
(e) Dissected mature embryo.
(h, k) Sections of seeds at the mature embryo stage.
(l) Two-day-old germinating seedling grown on norflurazon.
(m) Two-day-old germinating seedling.
(n) Emerging leaves of a 10-day-old seedling.
(o–r) Three-day-old seedlings.

The bar corresponds to 90 μm (a–c), 110 μm (d, e, n), 105 μm (f, g, i, j), 140 μm (h, k), and 380 μm (l, m, o–r). c, chalaza; en, endothelium; m, micropyle; ml, mucilage layer; WT, wild-type.
TT2 and TTG1 directly activate TT8 expression in A. thaliana siliques

To gain further insights into the mechanisms by which TT2 and TTG1 control TT8 expression, we used transgenic plants expressing TT2-GR and TTG1-GR fusion proteins (as previously characterized by Baudry et al., 2004). In these plants, each fusion protein is constitutively expressed, but inactive without the addition of dexamethasone (DEX). After induction, the chimeric TF can activate its primary and secondary targets. Simultaneous treatment with cycloheximide (CHX) blocks de novo protein production and thus the activation of secondary targets, allowing the identification of the primary targets that are still transcribed. In a previous study, this approach demonstrated that BAN transcription is directly controlled by TT2, TT8 and TTG1 in A. thaliana seeds (Baudry et al., 2004). Similar experiments were performed in this study to determine whether TT8 expression is directly controlled by TT2 or TTG1 (Figure 2).

The optimal conditions defined by Ohgishi et al. (2001) were used to ensure a significant transcriptional induction by DEX and the complete block of de novo protein synthesis in the presence of CHX (see Experimental procedures). The efficiency of DEX and CHX treatments was confirmed using plants carrying the GVG construct (35S::GAL4-VP16-GR) and a luciferase reporter gene (LUC) inducible by GVG (Ohgishi et al., 2001). As shown in Figure 2(a), after incubation of 35S::GVG x GAL4UAS::LUC seedlings with DEX for 6 h, activation of LUC production was clearly detectable. However, in the presence of both DEX and CHX, LUC activity was no longer detected even though LUC transcription was not affected, indicating that LUC production was inhibited and that CHX-mediated inhibition of protein production was efficient.

TT8 expression was investigated in the siliques of the 35S::TT2-GR and 35S::TTG1-GR transgenic plants. These constructs introduced into the tt2-1 (Ler ecotype) and ttg1-13 (RLD ecotype) mutants can restore PA synthesis after DEX treatment, demonstrating that the fusion proteins are fully functional (Baudry et al., 2004). A primer set was designed for quantitative RT-PCR in order to specifically measure the amount of TT8 transcripts (Figure 2b–d). Consistent with the results described for the activity of the TT8 promoter in tt2

Figure 2. Direct activation of TT8 expression by TTG1-GR and TT2-GR.
(a) The efficiency of in planta DEX and CHX treatments was tested on transgenic plants carrying both the GVG construct (35S::GAL4-VP16-GR) and GVG-inducible LUC reporter construct (GAL4UAS::LUC; Ohgishi et al., 2001). The results obtained after incubation of seedlings over 6 h under four different conditions (mock, DEX, CHX and DEX/CHX) are presented. The LUC luminescence from plants was imaged using a high-sensitivity camera system. The transcription of the LUC reporter gene in the presence of DEX or DEX/CHX was confirmed by a semi-quantitative RT-PCR analysis on seedlings; the constitutive expression level of the EF1αA4 gene (EF) was used as a control.

(b–d) TT8 expression was measured by quantitative RT-PCR in young developing siliques (2–4 days after pollination). The results are presented as a percentage of the constitutive expression level of the EF gene (mean ± SD of three quantifications on one representative cDNA sample). The differences between the means are all statistically significant at P < 0.05. (b) Comparison of TT8 expression level in the siliques of Ler and RLD ecotypes, and of the tt2-1 (Ler) and ttg1-13 (RLD) mutants. (c, d) Comparison of TT8 expression level in the siliques of transgenic plants expressing TTG1-GR (c) or TT2-GR (d) in the ttg1-13 and tt2-1 mutants, respectively. In each case, the TT8 expression level was quantified after 6 h of incubation of the siliques under four conditions: mock, DEX, CHX, or both DEX and CHX treatment. The results of a reproducible induction obtained using one transgenic plant are shown. This induction is representative of the results obtained with two independent transgenic plants for each construct.
and ttg1, TT8 expression was only affected in the ttg1 background (Figure 2b). Mild but significant and reproducible induction was obtained with two independent transgenic plants for each construct. In both cases, this induction of TT8 expression by DEX was conserved in the presence of CHX, demonstrating that TT8 is a direct target of TT2 and TTG1 in planta.

Homologous MYB and bHLH bind simultaneously to the TT8 promoter

Possible direct interactions between TT2, TT8, TTG1 and the TT8 promoter were investigated in yeast one-hybrid experiments. A transcriptional fusion between the TT8 promoter (identical to the promoter fragment used for expression analyses in planta) and the HIS3 reporter gene was introduced into yeast by homologous recombination. This reporter strain was then transformed with different combinations of plasmids allowing the expression of TT2, TT8 and/or TTG1 in yeast (Figure 3). As previously observed in the study of TT2, TT8 and TTG1 interactions with the BAN promoter, only simultaneous expression of AD-TT8 and BD-TT2 allowed growth of the reporter strain on medium lacking histidine (Figure 3a,b), thus revealing a direct interaction of these proteins with the TT8 promoter. As a direct interaction has been demonstrated between TT2 and TT8 (Baudry et al., 2004), these proteins probably bind to the TT8 promoter as a complex. Interestingly, these results suggest that TT8 might be involved in the control of its own transcription.

Additional combinations of MYB and bHLH factors were tested in yeast for interaction with the TT8 promoter. Previous results indicated the occurrence of direct interactions between TT2, PAP1 and TT8, GL3 and EGL3 (Baudry et al., 2004; Zimmermann et al., 2004), and different combinations of these factors were tested in yeast. Positive interaction results were obtained when BD-PAP1 was transformed in combination with AD-TT8, AD-EGL3 and AD-GL3, indicating that these MYB/bHLH complexes can bind directly to the TT8 promoter (Figure 3c). In contrast, AD-EGL3/BD-TT2 and AD-GL3/BD-TT2 combinations were not able to activate the reporter gene. TT2 is known to interact with these bHLH factors in vivo; these negative results might therefore be attributed to a poor affinity of the TT2/EGL3 and TT2/GL3 complexes for the TT8 promoter. Taken together, these results suggest that a broad set of MYB/bHLH complexes potentially participates in activation of the TT8 promoter in planta.

TT8 controls its own transcription in planta

To confirm that TT8 controls its own transcription in planta, the activity of the TT8::uidA construct was investigated in the tt8 mutant. For this purpose, two independent lines (the same TT8::uidA transgenic lines mentioned above for crosses with TT2-OE, PAP1-OE and ttg mutants) were crossed with the tt8-1 mutant. In this background, alterations to the WT pattern of GUS activity were observed (Figure 1i–k), indicating that the activity of the TT8 promoter is controlled by TT8, but only in specific cell types. First, the GUS activity was strongly reduced in cells corresponding to PA-accumulating cells in the WT (Figure 1i,j), although it was still detectable in the chalazal pigment strand (region 3), as already observed in the ttg1 background (Figure 1f,g). In addition, no GUS activity could be detected in the embryonic hypocotyl at late stages of embryo development (Figure 1k). Otherwise, a WT pattern of GUS activity was revealed in the

Figure 3. Cooperative interaction of MYB (TT2, PAP1) and bHLH (TT8, EGL3 and GL3) factors with the BAN and TT8 promoters in yeast one-hybrid experiments. Yeast clones were grown on appropriate media to maintain the expression vectors and to test for activation of the HIS3 reporter gene. Results obtained for five independent colonies are presented.
(a) TT2, TT8 and TTG1 interactions with the BAN promoter.
(b) TT2, TT8 and TTG1 interactions with the TT8 promoter.
(c) PAP1, EGL3 and GL3 interactions with the TT8 promoter.
AD, GAL4 activation domain; BD, GAL4 DNA-binding domain.

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TT8 expression is controlled by homologous MYB and bHLH factors

One attractive hypothesis to explain why the activity of the TT8 promoter is not affected in the tt2 mutant or in some specific cell types in the tt8 mutant is that, in these mutants, other MYB or bHLH factors act redundantly with TT2 or TT8 to control TT8 expression. For instance, it has been shown that in the outer integument of the seed coat, TT8 and EGL3 redundantly control mucilage biosynthesis (Zhang et al., 2003). Therefore, it is tempting to speculate that EGL3 might also be involved in TT8 regulation in these cells (Figure 1k).

To test this hypothesis, we measured the level of TT8 expression in the siliques of gl3 x egl3, tt8 x egl3 and tt8 x gl3 x egl3 mutants at the heart stage of embryo development (Figure 4; Zhang et al., 2003). It is important to note that the tt8-1 mutant allele used is caused by a point mutation that could affect mRNA stability (Nesi et al., 2000). Thus, even if a clear decrease (50%) in the level of TT8 expression was detected in the tt8-1 mutant compared with WT, it is not possible to distinguish between two possibilities: a decrease due to the absence of a functional TT8 protein (a result that would be consistent with the auto-activated loop mechanism) or a lower stability of the mutated TT8 transcript. However, in both WT and tt8-1 backgrounds, the introduction of the egl3 mutation clearly affected TT8 expression (a decrease corresponding to 30% of the WT level in both cases). By contrast, the gl3 mutation had almost no effect (comparing the levels of TT8 expression in tt8 x egl3 and tt8 x gl3 x egl3). These results confirm that EGL3 is involved in the regulation of TT8 expression in siliques. As the EGL3 promoter is activated in the outer integument of the seed coat, as revealed by the GUS activity generated in planta from an EGL3::uidA construct (Figure 5a; Zhang et al., 2003), EGL3 is a good candidate for TT8 regulation in the seed coat. It might be responsible for the remaining activation of the TT8 promoter described in the outer integument of tt8 seeds (Figure 1k). In addition, the activity of the GL3 promoter clearly correlates with that of the TT8 promoter in the cotyledons of germinating seedlings (Figure 5f,g,h), suggesting that GL3 could potentially control TT8 expression in these cells.

The possible redundancy between different MYB factors for TT8 regulation in the seed coat was investigated by monitoring the level of TT8 expression in the siliques of 35S::PAP1-SRDX plants (Hiratsu et al., 2003). In these plants, a fusion protein between PAP1 and a transcriptional repression domain of 12 amino acids (EAR motif) is overexpressed, leading to the dominant repression of PAP1 target genes and the inhibition of flavonoid biosynthesis in seeds and seedlings (Hiratsu et al., 2003). Interestingly, in the siliques of 35S::PAP1-SRDX plants, several flavonoid structural genes are downregulated, including BAN (Figure 4b; Matsui et al., 2004). In our experiments, a significant decrease in TT8 expression at the heart stage of embryo development (Figure 4a) was also detected. As PAP1 can bind to the TT8 promoter in vivo, this result is consistent with the direct inhibition of MYB-mediated TT8 activation in siliques. In addition, as observed in tt8 x gl3 x egl3 and in ttg1 mutants, some TT8 expression still remains in the 35S::PAP1-SRDX plants analysed (20–30%), suggesting that other factors potentially participate in TT8 regulation, independently of MYB–bHLH–TTG1 complexes.

Discussion

TT8 expression correlates with PA, anthocyanin and mucilage production

The activity of the TT8 promoter was investigated in planta by analysis of the GUS activity generated from a TT8::uidA reporter construct. Our results are fully consistent with the previous characterization of TT8 function (Nesi et al., 2000; Zhang et al., 2003), suggesting that TT8 activity might be related to the activity of its promoter. Indeed, in this report, we have shown that the TT8 promoter is induced in cells accumulating flavonoid compounds (i.e. PA and anthocyanins). In seeds, TT8 expression starts in the endothelial layer of the seed coat and is maximal at the globular and heart stages of embryo development, consistent with its role in the direct regulation of BAN transcription (Baudry et al., 2004;
Debeaujon et al., 2003). However, unlike TT2 and TTG1, TT8 expression persisted in the endothelium until late stages of development (mature embryo). In young seedlings, TT8 expression was detected in the upper part of the hypocotyl and at cotyledon margins, corresponding to the sites of anthocyanin production after germination. Accordingly, TT8 has been shown to be involved in the regulation of anthocyanin accumulation in seedlings, in a redundant manner with the bHLH factors encoded by GL3 and EGL3 (Zhang et al., 2003). Moreover, the activity of the TT8 promoter correlated with the peak of expression of flavonoid structural genes in seedlings, 3 days after germination (Kubasek et al., 1998).

Another metabolic pathway redundantly regulated by TT8 and EGL3 is mucilage biosynthesis in the outermost cell layer of the seed coat (Zhang et al., 2003). These pectic polysaccharides are formed during the torpedo and curled cotyledon stages of embryo development, concomitant with an important cell differentiation, which leads to the formation of the columellae (Haughn and Chaudhury, 2005). Expression of TT8 was clearly observed in the outermost cell layer of the seed coat at these stages, and this probably corresponds to the activation of mucilage biosynthesis by TT8 and an unknown MYB partner.

The detection of TT8 promoter activity in the embryonic hypocotyl during late stages of embryogenesis was rather unexpected. Interestingly, the GL3 and EGL3 promoters are also activated in the radicle and in the embryonic hypocotyl at the same stages (Figure 5c,e). Previous results have demonstrated that, during this period of embryo development, root and hypocotyl epidermal patterning are pre-established to ensure the correct differentiation of epidermal cells after seed germination (Lin and Schiefelbein, 2001). TT8 might therefore be implicated in this pre-patterning of the hypocotyl during late embryogenesis. Similarly, the strong activation of the GL3 promoter at cotyledon margins of the mature embryo can be correlated to the post-germination pattern of anthocyanin accumulation in seedlings (compare Figures 1l and 5f).

**Specific combinations of MYB and bHLH factors induce TT8 expression in planta**

The introduction of the TT8::uidA construct in TT2-OE and PAP1-OE demonstrated the ability of these two flavonoid regulators of the MYB family of TFs to activate TT8 transcription in planta. Moreover, the results obtained with inducible TT2-GR fusion proteins indicate that TT2 controls TT8 transcription without intermediate protein synthesis. This activation might be conferred by a direct binding of the MYBs to the TT8 promoter as demonstrated in the yeast one-hybrid experiments. However, the lack of modification of the activity of the TT8 promoter in the tt2 mutant may be due to functional redundancy between several MYB factors for TT8 regulation in the seed coat. A dominant negative approach with one of the homologous MYBs was used to investigate this possibility. As expected, a large reduction in the amount

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**Figure 5.** EGL3 and GL3 are possible substitutes for TT8 in the seed coat and in germinating seedlings. The pattern of EGL3 (a–d) and GL3 (e–h) promoter activities in WT A. thaliana is presented.
(a, e) Mature embryo stages.
(b, f) Dissected mature embryos.
(c, g) Two-day-old germinating seedlings.
(d, h) Three-day-old germinating seedlings.
Bar corresponds to 50 µm (a), 115 µm (b, e, f) and 380 µm (c, d, g, h).

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of TT8 mRNA was detected in the siliques of 35S::PAP1-SRDX plants, consistent with inhibition of MYB-mediated TT8 activation and with previous results demonstrating BAN inhibition (Matsui et al., 2004). The identity of the other MYB factors potentially participating in the regulation of TT8 in PA-accumulating cells remains unknown. Interestingly, the study of flavonoid biosynthesis in petunia has shown that the activation of anthocyanin structural genes is intimately linked to other cell differentiation processes (Koes et al., 2005). For instance, in the same cells, AN1 (bHLH factor) can interact with AN2 and PH4 (MYB factors) to activate anthocyanin biosynthesis and acidification of the vacuole, respectively. Similarly, TT8 might form part of different MYB/bHLH complexes controlling different aspects of the differentiation of PA-accumulating cells. This hypothesis could also explain why, in these cells, the activation of the TT8 promoter does not match exactly with that of the TT2 promoter, and persists even at later stages of embryo development.

The results obtained in yeast suggest that at least TT2 and PAP1 can bind to the TT8 promoter, but only when a bHLH partner is present, as already observed for BAN regulation (Baudry et al., 2004). However, we have identified in these experiments some functional differences between TT8, GL3 and EGL3. Indeed, TT2 was able to bind to the TT8 promoter only when it was simultaneously expressed with TT8 but not with GL3 or EGL3, suggesting that TT2/GL3 and TT2/EGL3 complexes could not activate TT8 expression in planta. Another difference highlighted in our experiments concerns the self-regulation of these bHLH factors. Our results support the participation of TT2 and PAP1 in a self-activated, positive loop controlling TT8 expression, and suggest that GL3 and EGL3 are potentially implicated in similar mechanisms in the seed coat and in cotyledons. However, recent genetic analyses suggest that, in the root, a closely related MYB factor (WEREWOLF, WER) has an opposite effect on the activity of GL3 and EGL3 promoters, and could inhibit the expression of these genes at the transcriptional level (Bernhardt et al., 2005). Further investigation will be necessary in order to conclude whether this can be related to functional differences between WER and TT2/PAP1 in planta. Interestingly, these differences would explain why, in a tt8 mutant, TT2 overexpression was not able to induce BAN in roots (Nesi et al., 2001).

**TTG1 controls TT8 expression in planta**

TTG1 appears to be another important factor controlling TT8 expression in planta. TTG1 is known to form ternary complexes with MYB and bHLH factors (Baudry et al., 2004; Payne et al., 2000) and could potentially take part in the MYB–bHLH complexes interacting with the TT8 promoter, as previously demonstrated for BAN regulation (Baudry et al., 2004). It is important to emphasize that TTG1 is also involved in the regulation of TT8 activity through an unknown post-translational mechanism, probably via a direct interaction with the TT8 protein (Baudry et al., 2004). The effect of TTG1 on TT8 activity might also be mediated by this mechanism regulating the activity of the bHLH factors (TT8, GL3 and EGL3), before or simultaneously with bHLH binding to the TT8 promoter.

The activity of the TT8 promoter is severely affected in ttg1 and tt8, thus reflecting the importance of the self-activated loop for TT8 regulation, especially in the seed coat. However, the residual activation of the TT8 promoter in the chalaza of these mutants is intriguing and indicates that neither TTG1 nor TT8 are necessary for the maintenance of TT8 expression in these cells. The detection of a low but significant amount of TT8 mRNA in the siliques of tt8 × gl3 × egl3 or 35S::PAP1-SRDX plants suggests that TT8 activation in the chalaza might be independent of regulation by the MYB/bHLH/TTG1 complexes and thus conferred by a supplementary, unknown activator (Figure 6).

**Functional importance of the feedback regulation of TT8 expression**

Our results show that activation of the TT8 promoter participates in the regulation of flavonoid metabolism in *A. thaliana*. As soon as TT8 transcription is activated in specific cells (for instance in the chalaza or in the upper part of the seed coat), the residual activation of the TT8 promoter is severely affected in ttg1 and tt8, thus reflecting the importance of the self-activated loop for TT8 regulation, especially in the seed coat. However, the residual activation of the TT8 promoter in the chalaza of these mutants is intriguing and indicates that neither TTG1 nor TT8 are necessary for the maintenance of TT8 expression in these cells. The detection of a low but significant amount of TT8 mRNA in the siliques of tt8 × gl3 × egl3 or 35S::PAP1-SRDX plants suggests that TT8 activation in the chalaza might be independent of regulation by the MYB/bHLH/TTG1 complexes and thus conferred by a supplementary, unknown activator (Figure 6).
of the hypocotyl), a self-activated loop controlling TT8 expression is induced by TT2, PAP1 and TTG1 (Figure 6). Concomitant with TT8 regulation, the MYB/TT8/TTG1 complexes could induce the transcription of PA or anthocyanin structural genes (Baudry et al., 2004; Zimmermann et al., 2004). This self-activated feedback loop is initially controlled by MYB factors and TTG1, allowing the synchronization of TT8 expression with the expression of its partners. Subsequently, the auto-activation would confer a strong and very specific induction of the structural genes in the endothelium or at cotyledon margins. It will be interesting to investigate whether such mechanisms are also involved in the regulation of other bHLH.

The factor allowing the initial TT8 activation in the seed coat remains to be identified. However, we cannot exclude the possibility that TT2 or other MYB factors can bind to the TT8 promoter in the absence of TT8 but with a lower efficiency and could be also responsible for initiating TT8 expression. A weak binding to the TT8 promoter might be undetectable in yeast because of the necessity to bypass a threshold level conferring sufficient activation of the reporter gene and allowing yeast growth on selective medium. TT2 might thus be able to activate the loop in every cell expressing TTG1. This last hypothesis is consistent with our results demonstrating the ectopic activation of TT8 in roots and in cotyledons of TT2-OE.

This feedback regulation of TT8 expression raises additional questions concerning the control of the specific pattern of PA accumulation in the innermost layer of the A. thaliana seed coat. The TT2 expression pattern is thought to be the main determinant of this specificity (Lepiniec et al., 2006; this study). However, many eukaryotic TFs, especially to be the main determinant of this specificity (Lepiniec et al., 2006; this study). However, many eukaryotic TFs, especially those controlling anthocyanin biosynthesis, can bind to the TT8 promoter (Kanno et al., 2002). In addition, a recent study has revealed that the three layers of the inner integument have been described previously (Nesi et al., 2000). Production of the 35S::PAP1-SRDX plants (Col-0 ecotype) was performed by M. Ohme-Takagi and colleagues (AIST, Tsukuba, Japan) as described in Hiratsu et al. (2003); 35S::PAP1-SRDX1 and 35S::PAP1-SRDX2 correspond to two independent transformants. The 35S::GVG × GAL4AD::LUC (Col-0 ecotype) plants were kindly provided by Aoyama and colleagues (Kyoto University, Japan) and are described in Ohgishi et al. (2001). The ttg1-1 (Ler ecotype), ttg1-1 (Ler ecotype) and ttg1-13 (RLD ecotype) plants have been described previously (Nesi et al., 2000; Walker et al., 1999). All experiments were performed with both ttg1-1 and ttg1-13 and identical results were obtained with these two reference alleles of the ttg1 mutation. In vitro and greenhouse culture conditions were as detailed by Nesi et al. (2000). The carotenoid biosynthesis inhibitor, norflurazon (Sandoz, Basel, Switzerland) was used to induce photobleaching of WT germinating seedlings, highlighting the normal pattern of anthocyanin accumulation. For this purpose, seeds were germinated on filter paper imbibed with a 100 µM norflurazon solution in distilled water.

**Construction of the TT8::uidA transgene and plant transformation**

The TT8 promoter construct used in this study corresponds to region ~1501 to +17 bp relative to the TT8 transcription start site and was amplified from the bacterial artificial chromosome F17A8 (Col-0 ecotype) containing the complete genomic sequence of the TT8 gene (Nesi et al., 2000), with the pTT8-5/pTT8-3′ primer set (Table 1). The PCR product was introduced by a BP recombination reaction (Gateway, Invitrogen, Paisley, UK) into pDONR207, sequenced, and transferred into the binary vector pBi101GUS (F. Divol, J.C. Palaqui, and B. Dubreucq, unpublished data) by an LR recombination reaction, to obtain a transcriptional fusion.

**Experimental procedures**

**Plant materials and growth conditions**

The tt8-1 allele used in this study originated from the Kranz and Robbelen Arabidopsis Information Service collection. Both tt8-1 and the corresponding Enkheim-2 WT ecotype were provided by the Nottingham Arabidopsis Stock Center (UK; stock center seed numbers N111 and N138 respectively). The same tt8-1 allele was used for crosses with gl3 and egl3 mutants (both in Ler ecotype) by Zhang et al. (2003), to obtain the tt8 × gl3 and tt8 × gl3 × egl3 plants. In this allele, a point mutation is responsible for the phenotype, a G to A change at position +1874 of the TT8 gene, causing non-splicing of the last intron. A longer transcript is produced, encoding a non-functional protein with 28 additional amino acids (Nesi et al., 2000). Production of the 35S::PAP1-SRDX plants (Col-0 ecotype) was performed by M. Ohme-Takagi and colleagues (AIST, Tsukuba, Japan) as described in Hiratsu et al. (2003); 35S::PAP1-SRDX1 and 35S::PAP1-SRDX2 correspond to two independent transformants. The 35S::GVG × GAL4AD::LUC (Col-0 ecotype) plants were kindly provided by Aoyama and colleagues (Kyoto University, Japan) and are described in Ohgishi et al. (2001). The ttg1-1 (Ler ecotype), ttg1-1 (Ler ecotype) and ttg1-13 (RLD ecotype) plants have been described previously (Nesi et al., 2000; Walker et al., 1999). All experiments were performed with both ttg1-1 and ttg1-13 and identical results were obtained with these two reference alleles of the ttg1 mutation. In vitro and greenhouse culture conditions were as detailed by Nesi et al. (2000). The carotenoid biosynthesis inhibitor, norflurazon (Sandoz, Basel, Switzerland) was used to induce photobleaching of WT germinating seedlings, highlighting the normal pattern of anthocyanin accumulation. For this purpose, seeds were germinated on filter paper imbibed with a 100 µM norflurazon solution in distilled water.

**Table 1** Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence* (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1αA4UP</td>
<td>ATGCCGAGGACTGCCTTCTCAT</td>
</tr>
<tr>
<td>EF1αA4RP</td>
<td>TTGGCCGCAACCTTATGCTGATCA</td>
</tr>
<tr>
<td>EF1-F</td>
<td>CTGGAGGGTTTGGACTGTTAT</td>
</tr>
<tr>
<td>EF1-R</td>
<td>CCAAGGGTGAAAGCAGAAG</td>
</tr>
<tr>
<td>EGL3-Ncol</td>
<td>CATGCCATGGAAACGAGAACCACAG</td>
</tr>
<tr>
<td>EGL3-SaM</td>
<td>ACAGGTGACCTTAACATATCCATGCAACC</td>
</tr>
<tr>
<td>GL3-Ncol</td>
<td>CATGCCATGGCGATGACGAACAG</td>
</tr>
<tr>
<td>GL3-SaM</td>
<td>ACAGGTGACCTTCAGACATCCATGCAACC</td>
</tr>
<tr>
<td>LUC1218</td>
<td>AGGCGCCAAGGCTTGA</td>
</tr>
<tr>
<td>LUC1439</td>
<td>AACAGCGCCGCACCCAG</td>
</tr>
<tr>
<td>PAP1-Ncol</td>
<td>CATGCCATGGACCGCTCTGTCGACAGAG</td>
</tr>
<tr>
<td>PAP1-SaM</td>
<td>ACAGGTGACCTTCAGACATCCATGCAACC</td>
</tr>
<tr>
<td>pTT8-5′</td>
<td>atbF1-TTACCAATTATTTTCTACA</td>
</tr>
<tr>
<td>pTT8-3′</td>
<td>atbF2-GTCTCTCTTATCAAAAT</td>
</tr>
<tr>
<td>TT8-1</td>
<td>CGGCCGTTCACTTAGCTGACT</td>
</tr>
<tr>
<td>TT8-2</td>
<td>CAGCCTTCTCGCCG</td>
</tr>
<tr>
<td>TT8-1H1</td>
<td>CCCATTAGAATTCTACATATTGTG</td>
</tr>
<tr>
<td>TT8-1H2</td>
<td>GCTCTCTCTGAGAATCATTAAC</td>
</tr>
</tbody>
</table>

*atbF1 and atbF2 refer to the corresponding Gateway™ recombination sequences.
between the TT8 promoter and the uidA reporter gene (encoding GUS). The resulting binary vector was electroporated into Agrobacterium tumefaciens C58C1mpMP90 strain (Koncz and Schell, 1986) and used for agroinfiltration of inflorescences (Bechtold et al., 1993) of WT *Arabidopsis thaliana* plants of the Wassilewskija ecotype. Kanamycin-resistant transformants were selected on MS medium and then transferred to soil for further characterization.

**Histochemical detection of GUS activity**

GUS staining was performed as described by Debeaujon et al. (2003) in the presence of 3 mM potassium ferricyanide/potassium ferrocyanide, except for TT8::uidA x ttg1 plants for which the concentration was decreased to 0.5 mM. Resin embedding and sectioning of GUS-stained developing seeds were carried out as described by Baudry et al. (2004).

**DEX induction experiments and RNA analysis**

DEX induction of TT8 expression in siliques was performed on transgenic plants containing the 35S::TT2-GR and 35S::TTG1-GR constructs as described by Baudry et al. (2004). For each treatment, four siliques (2–5 days after pollination) were taken from 35S::TT7-GR transgenics, opened, and incubated in 24-well plates in 100 mM phosphate buffer, pH 7.2, 0.1% Triton X-100, 10 mM Na2-EDTA, and 100 μM CHX when required (Spelt et al., 2000; CHX Ready made, Sigma-Aldrich, Steinheim, Germany). A vacuum was applied for 30 min to ensure effective infiltration of CHX. Then DEX was added to a final concentration of 10 μM, and vacuum applied for a further 30 min. After 3 h on an orbital shaker, incubation buffer was replaced by a freshly prepared buffer (10 μM DEX and/or 100 μM CHX), and incubation continued for 3 h (Baudry et al., 2004; Wagner and Sablowski, 2002).

Total RNA were extracted from siliques using the Genelute total RNA miniprep kit (Sigma-Aldrich), according to the manufacturer’s recommendations. Extracts were treated with 30 units of RNase- lase-free DNase I (Qiagen, Hilden, Germany) and eluted with RNase-free water. Reverse transcription and real-time RT-PCR were carried out using SYBR green (Roche, Penzberg, Germany) and a Roche light cyclor to detect TT8 expression level, as described by Baudry et al., 2004. A specific primer set (TT8-1H1 and TT8-1H2, Table 1) determined a 149 bp fragment on TT8 cDNA. The specificity of the amplification was confirmed by sequencing the reaction product. The results of TT8 expression in siliques were standardized to the constitutive expression level of the gene EF1α4 (EF) determined with the EF1-F/EF1-R primer set (Table 1; Baudry et al., 2004).

**Monitoring of LUC expression**

LUC production in 35S::GVG × GAL4UAS::LUC seedlings was assayed using a CCD camera (ISIS 4, Photonic Science Ltd, Robertsbridge, UK) and the Photolite image acquisition software (Photonic Science Ltd., Robertsbridge, UK). 5 min after spraying plantlets with a solution containing 5 mM luciferin (Analytical Bioluminescence Laboratory, San Diego, CA, USA) and 0.01% Triton X-100. Images were captured for 2–5 min using a threshold value of 30 and a map value of 250. Total RNA was extracted from plantlets using the Genelute total RNA miniprep kit (Sigma-Aldrich) to confirm the transcription of the LUC gene. RT-PCR analyses were performed as described by Nesi et al. (2000), using 30 cycles of amplification with the LUC1218/LUC1439 and EF1α4AUP/EF1α4ARP primer sets, specific to LUC and EF cDNA, respectively.

**Yeast one-hybrid assay**

The reporter plasmid was constructed with the pHISi vector (Baudry et al., 2004). A 1481-bp fragment of the TT8 promoter was amplified by PCR using the TT8::uidA construct as a template and the primers TT8-1H1 and TT8-1H2 (Table 1). The fragment was then digested by EcoRI and XbaI and cloned into pHiSi between the EcoRI and XbaI sites. This plasmid was digested with Apal and integrated into the yeast strain YM4271 at the URA3 locus. The resulting yeast strain, selected on medium lacking uracil, contained the HIS3 reporter gene under the control of the complete regulatory sequence of the TT8 promoter. The effectors (TT2, TT8 or TTG1) were expressed with the pACTII, pAS2ΔA, and pTFT1 vectors in fusion with the GAL4 activation domain (AD), the GAL4 DNA-binding domain (BD) or the SV40 nuclear localization sequence, respectively, as described in Baudry et al. (2004). To express PAP1, GL3 and EGL3 in yeast, the PAP1, GL3 and EGL3 full-length cDNAs were amplified with the appropriate primer sets (Table 1) to introduce Ncol and SalI cleavage sites at the 5’ and 3’ ends, respectively, and the products were digested by Ncol and SalI. The resulting GL3 and EGL3 inserts were cloned into pACTII digested by Ncol/Xhol, and the PAP1 insert was introduced into pAS2ΔA digested by Ncol/SalI. The TT8::HIS3 strain was co-transformed using the AclisSSDNA/PEG method (Gietz and Woods, 2002), and transformants presenting single, double or triple combinations of vectors were selected on appropriate media.

**Acknowledgements**

We thank Alan Lloyd and Toni Gonzalez for seeds of gl2 x egl3, tt8 x egl3, tt8 x gl3 x egl3 double and triple mutants, GL3::uidA and EGL3::uidA reporter lines; Takashi Aoyama for the 35S::GVG x GAL4UAS::LUC transgenic plants; Masaru Ohme-Takagi for the gift of the 35S::PAP1-SRDX lines; and Fanchon Divol, Bertrand Dubreucq and Jean-Christophe Palaqui for the pH101GUS plasmid. We also would like to thank François Parcy and Pascal Genschik for helpful comments during this work, Alessandro Alboresi and Hoai-Nam Truong for their help with the luciferase imaging, and Christian Dubos and Helen North for critical reading of the manuscript. This work was supported by GABI-Ge´no-plante project NO2001-00045, including a postdoctoral fellowship to A.B.

**References**


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Debeaujon, I., Nesi, N., Caboche, M. and Lepiniec, L. (2003) The accession numbers for the sequences mentioned in this article are as follows: BAN: At1g61720; EF1αA4: At5g60390; EGL3: AtbHLH002/At1g63650; GL3: AtbHLH001/At5g41315; PAP1: AtMYB75/At1g56650; TT2: AtMYB123/At5g35550; TT7: AtbHLH042/At4g09820; TTG1: At5g24520.


The accession numbers for the sequences mentioned in this article are as follows: BAN: At1g61720; EF1αA4: At5g60390; EGL3: AtbHLH002/At1g63650; GL3: AtbHLH001/At5g41315; PAP1: AtMYB75/At1g56650; TT2: AtMYB123/At5g35550; TT7: AtbHLH042/At4g09820; TTG1: At5g24520.