Developmental Biology 325 (2009) 412–421

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation

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ARTICLE INFO

Article history:
Received for publication 1 August 2008
Revised 9 September 2008
Accepted 1 October 2008
Available online 1 November 2008

Keywords:
TTG1
MYB5
TT2
Seed
Testa
Cell fate

ABSTRACT

A suite of epidermal characters in *Arabidopsis* is under the transcriptional control of a combinatorial complex containing WD repeat, bHLH and MYB proteins. Many genetic, molecular and biochemical means have been employed to identify and characterize a complete minimal set of complex members required for the trichome initiation, root hair spacing, anthocyanin production and seed coat tannin production pathways. In addition, the WD and bHLH proteins required for outer seed coat differentiation have been identified. However, until now the MYB complex member(s) required for this last WD–bHLH–MYB complex-dependent character have remained elusive. Here we identify two MYBs, *AtMYB5* and *TT2*, as partially redundant in regulating this outer seed coat developmental process with *MYB5* having the major role. *MYB5* and *TT2* are shown to be expressed in this outer seed coat domain. We also show that *MYB5* has weak pleiotropic control over trichome development and tannin production and is also expressed in the appropriate places for these functions. *TT8* and the downstream *GL2* and *TTG2* regulators of seed coat development are found to be downregulated in the MYB mutants. Although the TTG1-dependent R2R3 MYBs are considered to be highly pathway specific, identification of MYBs responsible for outer seed coat development allowed for the elucidation of previously undetected novel developmental pleiotropy among these elements.

Introduction

Upon fertilization the integument cells of the ovule differentiate to form the seed coat. The seed coat is a protective structure for the embryo that also contributes to seed dormancy, dispersal and germination. During seed coat development in *Arabidopsis*, the inner cell layer (the endothelium) of the 3 cell layered inner integument accumulate proanthocyanidin (PA) compounds in the vacuole which subsequently oxidize, giving *Arabidopsis* seeds their characteristic brown color (Lepiniec et al., 2006). The outer most cell layer (the epidermis) of the 2 cell layered outer integument undergoes an extensive differentiation process resulting in the deposition of copious amounts of pectinaceous mucilage laterally into the apoplastic space forcing the cytoplasm into a column at the center. The walls of the cytoplasmic columns as well as the radial walls are highly reinforced by secondary thickening, ultimately giving the surface of the mature seed coat its characteristic appearance of raised columellae amidst reticulations of radial walls after dessication (Western et al., 2000; Windsor et al., 2000; Haughn and Chaudhury, 2005). Upon imbibition, mature seeds are enveloped by a layer of extruded hydrophilic mucilage. Analysis of mucilage from wild-type and mutant seeds defective for various aspects of pectin biosynthesis have increasingly revealed the complex structural and compositional nature of the extruded mucilage capsule (Penfield et al., 2001; Usadel et al., 2004; Western et al., 2004; Oka et al., 2007; Macquet et al., 2007a,b; Dean et al., 2007).

Molecular genetic studies revolving around the *transparent testa glabra1* (*ttg1*) mutant of *Arabidopsis* have resulted in a model for the regulation of several developmental processes including seed coat development (Koornneef, 1981; Walker et al., 1999; Zhang et al., 2003; Schiefelbein 2003; Bernhardt et al., 2005; Haughn and Chaudhury, 2005; Lepiniec et al., 2006; Gonzalez et al., 2008). Other aspects of epidermal development impaired in *ttg1* mutants are trichome initiation and development, root hair patterning and anthocyanin production in the plant body. In all cases TTG1 works in a complex with a particular combination of MYB class and bHLH class transcription factors to specify a particular epidermal cell fate. For example, the Glabra1 (GL1) MYB factor along with the Glabra3 (GL3) and Enhancer of Glabra3 (EGL3) bHLH factors are required for trichome initiation. Proper root hair patterning requires the MYB factor Werewolf (WER) together with GL3 and EGL3. PAP1, PAP2, MYB113 and MYB114 MYBs with Transparent Testa8 (TT8), GL3 and EGL3 bHLH factors control the synthesis of anthocyanin pigments while the Transparent Testa2 (TT2) MYB and TT8 bHLH specify the production of proanthocyanidin (PA) pigments in the endothelial layer of the developing seed coat. Lastly, EGL3 and TT8 control outer seed coat differentiation consistent with the expression of both these genes in the developing testa epidermis (Baudry et al., 2006). However, the MYB or MYBs associated with the TTG1 complex that control outer seed coat differentiation have not been previously identified.

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doi:10.1016/j.ydbio.2008.10.005

Downstream direct targets of TTG1/MYB/bHLH transcriptional complexes include other transcription factors such as the Glabra2 (GL2) homeodomain protein and the Transparent Testa Glabra2 (TTG2) WRKY protein (Rerie et al., 1994; Di Cristina et al., 1996; Johnson et al., 2002; Morohashi et al., 2007; Zhao et al., 2008). Both *GL2* and *TTG2* have positive roles in trichome outgrowth and branching and in seed coat mucilage production. *GL2* also regulates root hair patterning while *TTG2* additionally functions in seed coat PA biosynthesis.

All eight of the TTG1 complex-associated MYBs mentioned above cluster into a monophyletic clade (Stracke et al., 2001) with one other MYB, *AtMYB5*, that has no genetically defined function. However, a role in TTG1-dependent developmental pathways is suggested by previous expression work (Li et al., 1996) and 2-hybrid analysis that demonstrates physical interactions between MYB5 and the TTG1-dependent bHLH regulators (Zimmermann et al., 2004). Here we identify *MYB5* as having a major role in controlling the transition of the outer seed coat cell from a non-distinct ovule surface cell to a highly differentiated state, and we show that *MYB5* is expressed in this outer cell layer. In addition, *MYB5* is partially functionally redundant with the *TT2* MYB element in this process. We also show that *GL2*, *TTG2* and *TT8* are downregulated in the developing seed coats of *myb5* and *myb5 tt2* mutants as well as in *egl3 tt8* mutant testas. We also found that *MYB5* is expressed in the inner layer of the inner testa where PA pigments are produced. The double *myb5 tt2* mutant shows a slight visible decrease in pigment production indicating that *MYB5* is partially redundant with *TT2* in this process also. In addition the *myb5* mutant displays a quantitative reduction in trichome branching and overexpression of *MYB5* in the *gl1* mutant restores trichome production. Moreover, *myb5 myb23* double mutants show a lack of stem trichomes in contrast to either single mutant. Thus the identification and characterization of *MYB5* not only fills an important last gap in the transcription factor complexes that regulate mucilage production but also makes possible the novel observation of pleiotropic control of epidermal cell fate pathways by TTG1-dependent R2R3 MYBs of *Arabidopsis*. A revised regulatory model for the positive regulation of TTG1-dependent cell fate pathways is proposed illustrating this previously unobserved mechanism of R2R3 MYB control of these epidermal characters.

Materials and methods

Arabidopsis accessions

The following insertion mutant lines in Col-0 background were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003): SALK 030942 (*myb5-1*), SALK 018613 (*myb23-1*, Kirik et al. 2005), Salk 005260 (*tt2*), SALK 019114 (*egl3*), SALK 030966 (*tt8*). The gene trap line GT11646 (*myb5-2*) was obtained from the Cold Spring Harbor Genetrap Consortium (genetrap.cshl.org). *gl1-1* and *ttg1-1* mutants have been previously described (Koornneef, 1981; Oppenheimer et al., 1991; Walker et al. 1999). The *ProGL2*:*GUS* line was kindly provided by Dr. David Marks. The *ProGL2*:*GUS myb5-1* line was obtained by crossing the *ProGL2*:*GUS* line to *myb5-1* and screening the F2 for GUS positive plants that yielded *myb5* mutant seed. The *ProGL2*:*GUS* line was crossed to the *egl3 tt8* double insertion mutant and a *ProGL2*:*GUS egl3 tt8* line was obtained by screening the F2 for GUS positive plants that yielded seed mutant for both testa color and mucilage production. To obtain the *ProGL2*:*GUS ttg1-1* line *ttg1-1* was crossed to the *ProGL2*:*GUS* line and the F2 screened for bald plants showing residual GUS staining in the few trichomes *ttg1-1* mutants produce.

Plasmid construction

pMYB5PG — an approximately 2.2 kb fragment upstream of the *MYB5* start codon was amplified from Col genomic DNA using the primers below and recombined into pDONR222 (Invitrogen) to produce pGWMYB5P. pGWMYB5P was then used to recombine the *MYB5* promoter fragment into pKGWFS7 GUS vector (Karimi et al. 2002).

forward: 5′attB1-gtcagcaagcttcacaagacc-3′
reverse: 5′attB2-ctccgccgtcttcaacaaagc-3′

p35MYB5 — the *MYB5* Col genomic locus from start to stop codons was amplified using the primers below and recombined into pDONR222 (Invitrogen) to produce pGWMYB5. *MYB5* was then recombined from pGWMYB5 into pK7WG2 (Karimi et al. 2002).

forward: 5′attB1-atgatgtcatgtggtgggaag-3′
reverse: 5′attB2-ctagtcatgtcctaagctag-3′

pTT2PG — an approximately 2.2 kb fragment upstream of the *TT2* start codon was amplified from Col genomic DNA using the primers below and recombined into pDONR222 (Invitrogen) to produce pGWTT2P. pGWTT2P was then used to recombine the *TT2* promoter fragment into pKGWFS7 GUS vector (Karimi et al. 2002).

forward: 5′attB1-tgttgagtcaacagacacgtg-3′
reverse: 5′attB2-ctcacttttctctctcttgtgg-3′

Ruthenium red staining of dry seed

Dry seeds were placed in a 0.01% (w/v) aqueous solution of Ruthenium red until any extruded mucilage was visibly stained. Staining solution was then replaced with water before capturing images.

Sectioning of developing siliques for histochemical detection of GUS activity

Developing siliques 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 1 min to half an hour depending on the reporter line.

SEM of dry seed and light microscopy of developing seed thin sections

Treatment of dry seed and developing seed for Scanning Electron Microscopy (SEM) and light microscopy of plastic-embedded thin sections was performed as described in Windsor et al. (2000).

Gene expression analysis by quantitative-PCR

Total RNA was prepared from developing siliques using a Qiagen RNeasy plant mini kit. 1 μg of total RNA was used to produce first-strand cDNA in 20 μl reverse transcription reactions using a Super-Script II RT kit (Invitrogen). 24 μl PCR reactions were prepared using 1 μl cDNA reaction as template with 12 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). 7 reactions were performed per target in parallel with 5 actin control reactions in at least 2 independent experiments with consistent results. The results of a representative experiment are presented. The following primers were used for target and control gene amplification:

MYB5 forward: 5′ggaggaattacgtcggacgag 3′
MYB5 reverse: 5′tcaatgaccacctgttgccg 3′
TTG2 forward: 5′ttgatgatccaagcagaagcaa 3′
TTG2 reverse: 5′ctgattccactgagccttgcg 3′

TT8 forward: 5′gtcctcaacaacgggtcttgg 3′
TT8 reverse: 5′ttcctcgtctttattgcaccg 3′
Actin forward: 5′tccattcttgcttccctcag 3′
Actin reverse: 5′atcattactcggccttggaga 3′

Results

MYB5 expression in trichomes and developing seed coats

Previous work demonstrated *MYB5* expression in trichomes and developing seeds (Li et al., 1996). To verify expression in trichomes and particularly in integument layers of developing seed coats, a *ProMYB5*:*GUS* construct was created and transformed into Col wild-type *Arabidopsis* line. Emerging first true leaves of young *ProMYB5*:*GUS* seedlings showed uniform expression of the reporter gene (Fig. 1A). As the leaves expanded expression was restricted to trichomes and leaf margins (Fig. 1B). In seedlings with first true leaves fully expanded and with emerging third and fourth leaves, expression was observed primarily in trichomes (Fig. 1C).

Seeds over a broad range of development from *ProMYB5*:*GUS* lines showed expression of the reporter gene (Figs. 1D–I). This was consistent with high levels of *MYB5* expression reported by the AtGenExpress database (Schmid et al., 2005) in early seed stages with mid-globular embryos through late seed stages with green cotyledon embryos. Whole developing seeds dissected out of siliques showed uniform expression and this expression seemed to be in more than one layer of the seed coat (Figs. 1D,E). Interestingly, completely dry seed from reporter lines stained GUS-positive in the epidermal layer, highlighting the surface morphology of the mature seed coat and suggesting expression at very late stages of seed development (Fig. 1F). This pattern was not observed in stained Col wild-type dry seed (Fig. 1G) nor in stained *ProGL3*:*GUS* dry seed (data not shown), consistent with previous demonstrations that *GL3* plays no role in seed coat development (Zhang et al., 2003). Thick cross sections of developing siliques from reporter lines were GUS stained to determine which cell layers of developing seed coats express *MYB5*. Seed coats from seeds with a development range of embryos from the heart to the early upturned-U stages showed expression in the epidermal and endothelial layers where mucilage and PAs are produced respectively (Fig. 1H). Expression was faint but still evident in the epidermal layer at later stages of testa development (Fig. 1I). Taken together, *MYB5*'s expression pattern suggests roles for *MYB5* in trichome development and in the development of the epidermal and endothelial testa cell layers.

MYB5 overexpression results in ectopic trichome production and alters seed coat epidermal development

To better determine its functional capabilities, the *MYB5* genomic locus from start to stop codons was placed under the control of the CaMV 35S promoter. Overexpression in Col wild-type seedlings often resulted in the ectopic production of a few trichomes on cotyledons and hypocotyls (Figs. 2A,B). Similar to *GL1* overexpression, first true leaves of *MYB5* overexpressors showed suppression of trichome formation on the leaf blade (Larkin et al., 1994; Fig. 2D). However, in contrast to *GL1* overexpression, overexpressing *MYB5* resulted in the production of many trichomes in clusters on the margin of leaves (Fig. 2D). *MYB5* was able to rescue trichome production when over-expressed in the bald *gl1* mutant (Fig. 2F).

When placed in water, wild-type seeds extrude mucilage from the epidermal layer of the seed coat. Ruthenium red dye in the water reveals the extruded mucilage as a uniform layer surrounding the seed (Fig. 2G). Seeds from *35S:MYB5* Col lines showed an abnormal ruthenium red staining pattern (Fig. 2H), suggesting an alteration in seed coat development caused by *MYB5* overexpression.

myb5 T-DNA mutant shows reduced trichome branching

A *myb5* T-DNA insertion mutant in the Col0 accession (Salk 030942) was acquired from the Arabidopsis Biological Resource Center (Alonso et al., 2003) and analyzed for phenotypic differences from wild-type. *MYB5* expression was undetectable in *myb5* mutant by quantitative-PCR (Q-PCR) experiments, indicating that *myb5* is a knock-out mutant (Fig. 3A). Although *MYB5* is well-expressed in trichomes on first true leaves no obvious reduction in trichome numbers on first true leaves was detected in *myb5* seedlings compared to Col (data not shown). However, about a 40% drop in the 4-branched trichome class was observed on *myb5* first leaves compared to Col wild-type (Table 1).

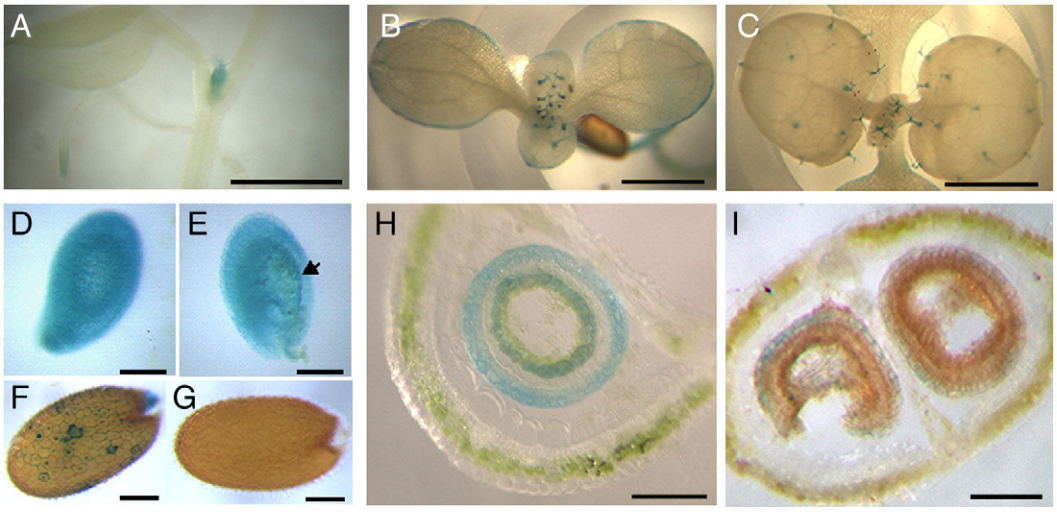

Fig. 1. *ProMYB5*:*GUS* expression pattern in Col wild-type. (A) Seedling with emerging first true leaves. (B) Seedling with more developed, expanding first true leaves than in (A). (C) Seedling with fully expanded first true leaves and emerging third and fourth leaves. (D) Seed dissected from a developing silique. (E) Seed dissected from a developing silique. Arrow indicates part of the endothelial layer of the seed coat outlined by GUS staining. (F) Dry seed showing residual expression in the epidermal layer outlining the seed surface morphology and Col wild-type control (G). (H) Thick cross section of a developing seed at approximately the late torpedo stage. (I) Thick cross section of seeds during late development still showing some expression in the seed coat epidermis. Scale bars: A=500 μm, B and C=1 mm, D to I=100 μm.

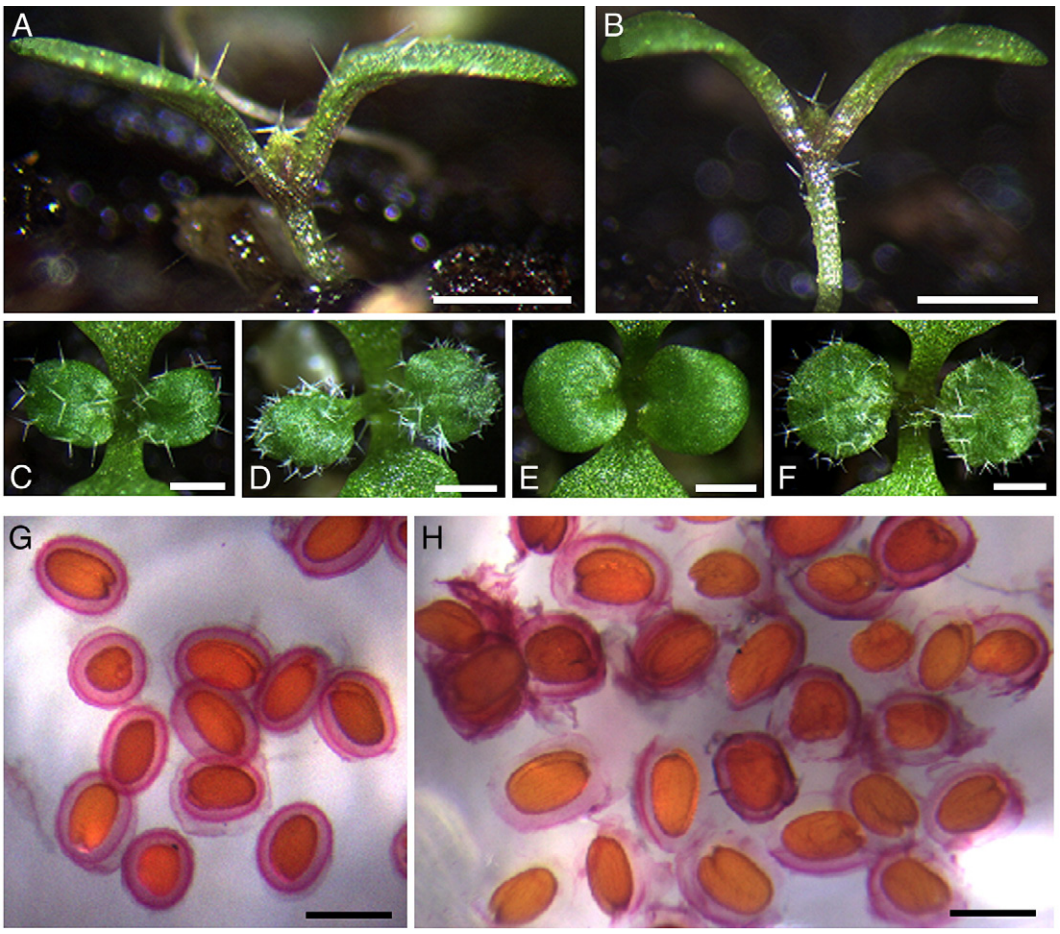

Fig. 2. *MYB5* overexpression. (A) and (B) Col seedlings overexpressing *MYB5* showing ectopic trichome production on cotyledons and hypocotyls. (C) Col wild-type seedling. (D) Col seedling overexpressing *MYB5* with excess trichome production on the periphery of first true leaves but reduced production on the blade compared to (C). (E) *gl1* seedling. (F) *gl1* seedling overexpressing *MYB5*. (G) Ruthenium red staining of extruded mucilage from Col seed. (H) Ruthenium red staining of extruded mucilage from *35S:MYB5* seed. Scale bars: A to H = 500 μm.

It has been shown previously that *myb23* mutants also show no reduction in trichome numbers but do show strong reductions in trichome branching (Kirik et al., 2005). A *myb5 myb23* double mutant line was created to determine if a synergistic trichome phenotype would result from a simultaneous loss of *MYB5* and *MYB23* gene function. Consistent with the results of Kirik et al. (2005) we found that *myb23* mutants are not reduced in total trichome numbers (data not shown) but display a complete loss of 4-branched trichomes and a gain of many 2-branched trichomes on first true leaves not observed in Col (Table 1). *myb5 myb23* double mutants also show no reduction in trichome numbers on first true leaves but do show the same branching defect as *myb23* single mutant (Table 1). However, aborted or under-developed trichomes were observed on the stem of the primary inflorescence of double mutants, in contrast to either single mutant (Fig. 3B), consistent with previously reported expression of *MYB5* and *MYB23* in stems or stem trichomes (Li et al., 1996; Kirik et al., 2001). It was also shown that a mutation in *MYB23* enhances the glabrous phenotype of *gl1* (Kirik et al., 2005). No such effect was observed in *myb5 gl1* double mutants (data not shown). Overall these data demonstrate a relatively minor role for *MYB5* in trichome development.

myb5 T-DNA mutant shows abnormal seed coat development

myb5 seed show an abnormal patchy ruthenium red staining pattern suggestive of reduced seed coat mucilage production during testa development (Fig. 4G). During the course of this work a second *MYB5* mutant line (myb5-2) was obtained from Cold Spring Harbor Gene Trap Consortium that also produces seeds mutant for mucilage extrusion (data not shown). The work presented here was performed with the *myb5* Salk allele. When observed using scanning electron microscopy, *myb5* epidermal seed coat cells show collapsed or partially formed columellae with thin or indistinct radial walls (against the more obvious radial walls from the subepidermal layer immediately beneath) (Fig. 4H); this is in contrast to the surface morphology of Col seed coats showing epidermal cells with raised, reinforced columellae surrounded by prominent reinforced radial walls (Fig. 4B). Thin cross sections of Col wild-type seeds approximately in the late upturned-U stage show the developing wild-type epidermal testa cell morphology seen in Fig. 4C; copious mucilage production at this stage results in large expanded cells while forcing the cytoplasm into a column at the center of the cell (as indicated by the starch granules concentrated in the cytoplasmic column at the center of epidermal cells in Fig. 4C). In contrast, sections of *myb5* seeds at similar developmental stages show flattened epidermal cells compared to the larger expanded wild-type cells indicating decreased mucilage production in mutant cells (Fig. 4I). However, wild-type-like columellae are seldom but occasionally seen in these sections (cell at far right in Fig. 4I with columella of starch granules), consistent with Fig. 4H showing *myb5* testa epidermal cells with missing or partially formed columellae.

Overall this phenotype is consistent with testa mutant phenotypes associated with disruption of TTG1-dependent complexes as observed in *egl3 tt8* double bHLH mutant seed coats (Figs. 4D–F). SEM of the surface of *egl3 tt8* seeds also display epidermal cells with partially

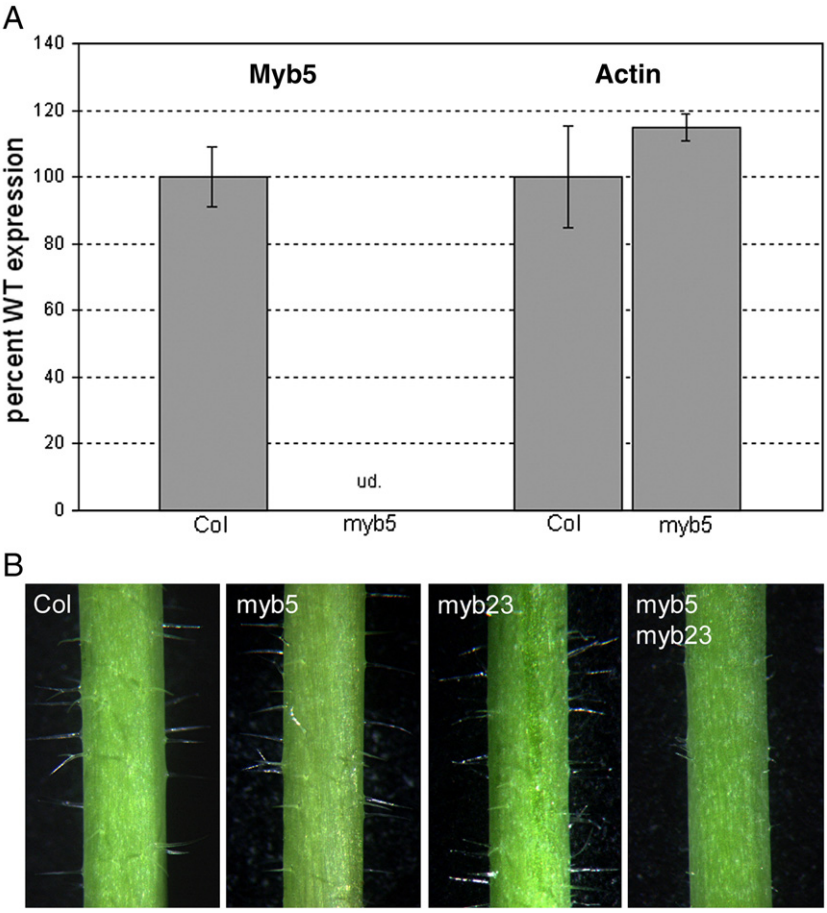

Fig. 3. Characterization of *myb5* mutant. (A) Q-PCR analysis of *MYB5* and actin control expression in Col wild-type and *myb5* mutant reported as percentage of wild-type expression. *MYB5* expression was undetectable (ud.) in *myb5* mutant. Error bars indicate range of expression. (B) Comparisons of trichomes on the stem of primary inflorescences of Col and MYB mutants. Scale bars: B = 1 mm.

formed or collapsed columellae and thin, underdeveloped radial walls while showing the reinforced radial walls from the subepidermal layer (Fig. 4E). Consistent with this is the developing *egl3 tt8* epidermal seed coat layer showing mostly flattened cells producing less mucilage than wild-type cells as observed in thin sections (Fig. 4F). Together these observations indicate a failure of the *myb5* seed coat epidermis to differentiate properly resulting in decreased mucilage production and abnormal cell morphology.

myb5 tt2 double mutant shows a more severe testa developmental defect than either single mutant

Although *myb5* mutant seed coat development is obviously impaired, the *myb5* phenotype suggests the production of some mucilage and in general partial development of the epidermal layer of the testa (Figs. 4G–I). To test the possibility that other TTG1-dependent MYBs may redundantly control testa development a *myb5 tt2* double mutant was created. *TT2* was selected as a likely candidate as it is also active during seed development specifying the production of PA pigments in the endothelial layer of the testa, hence the lighter seed color phenotypes of *tt2* mutants (Nesi et al., 2001). Although *tt2* seed coats appear essentially wild-type for epidermal cell development (Figs. 5B,C), *TT2* expression has previously been demonstrated throughout silique development, peaking during the early globular to torpedo stages and persisting weakly through the mature embryo stage (Nesi et al., 2001). More specifically, concentrated expression of *TT2* has been demonstrated in endothelial cells at the chalazal and micropylar regions of developing seeds bearing a 2.0 kb *TT2* promoter fragment fused to the *uidA* reporter gene, with weaker expression observed in the developing seed body (Debeaujon et al., 2003). Additionally, wild-type seeds of *ProTT2:GUS* lines analyzed here showed expression (albeit weaker than *MYB5*) in the outer most cell layer of developing seed coats (Figs. 5F,G), consistent with a possible role in epidermal testa cell fate determination. Specifically, *TT2* expression in the developing seed coat epidermis appeared variable with most seed sections showing patchy GUS staining in the epidermal layer. Some seed sections showed very little expression (Fig. 5F) and some more uniform expression (Fig. 5G). Whole developing seed dissected from *ProTT2:GUS* lines showed expression primarily at the basal portion of the seed concentrated at the chalazal end with some seed showing weak but uniform expression throughout the seed body, consistent with the results of Debeaujon et al. (2003) (Fig. 5D). GUS stained cross sections through

Table 1
Trichome branching in wild-type and mutant lines

Genotype	Branching phenotype				Total
	1	2	3	4	
Col	0	0.2	76.8	23	422
myb5	0	0.2	85.9	13.9	418
myb23	0.2	15.4	84.4	0	423
myb5 myb23	0.8	16.9	82.3	0	237

Trichomes on first true leaves were counted and the branch phenotype (1, spike; 2, 2 branches, etc.) is reported as the percentage of trichomes with the indicated phenotype. Total is the total number of trichomes counted on first true leaves of each line.

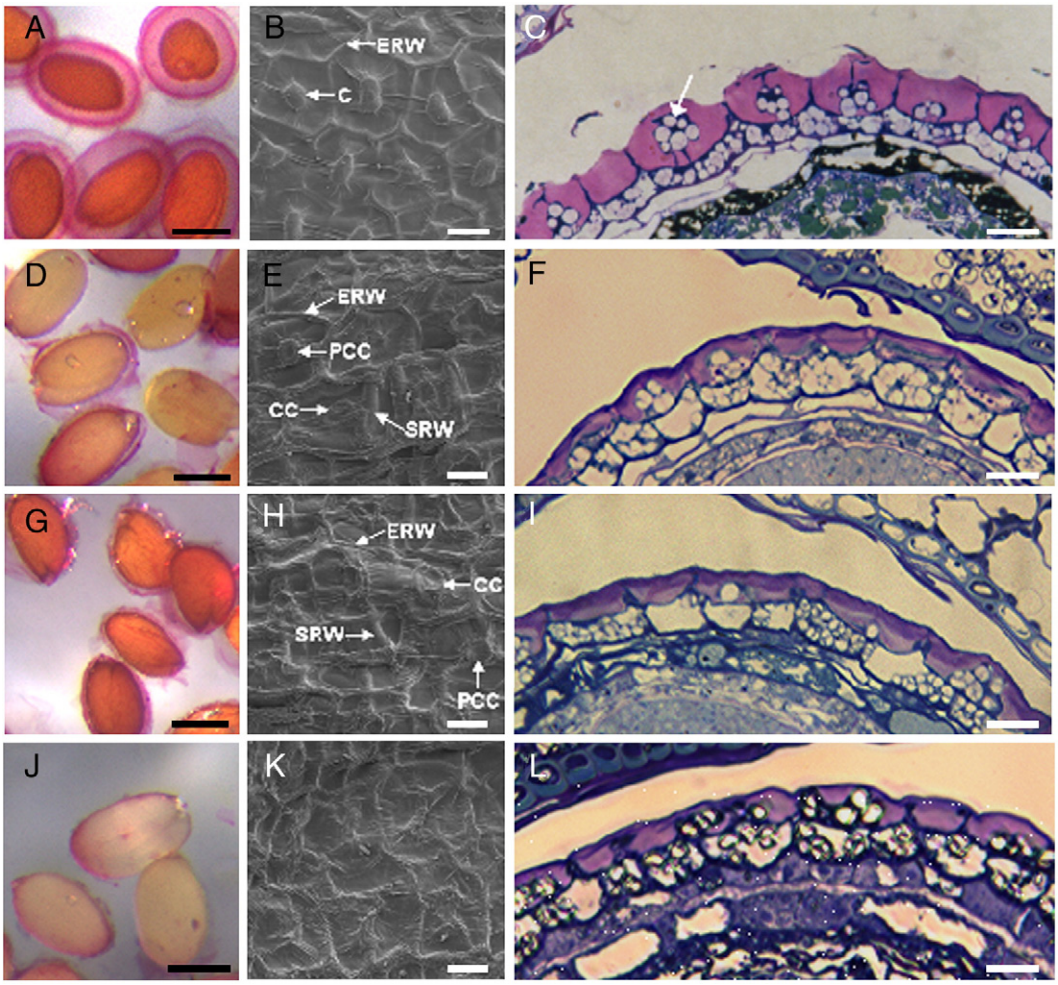

Fig. 4. Phenotypic characterization of MYB and bHLH mutant seed coats. (A) Ruthenium red staining of extruded mucilage from Col seed. (B) SEM of Col dry seed surface. (C) Toluidine blue stained thin cross section of a Col seed at approximately the late upturned-U stage. (D) Ruthenium red staining of extruded mucilage from *egl3 tt8* seed. (E) SEM of *egl3 tt8* dry seed surface. (F) Toluidine blue stained thin cross section of a *egl3 tt8* seed at approximately the late upturned-U stage. (G) Ruthenium red staining of extruded mucilage from *myb5* seed. (H) SEM of *myb5* dry seed surface. (I) Toluidine blue stained thin cross section of a *myb5* seed at approximately the late upturned-U stage. (J) Ruthenium red staining of extruded mucilage from *myb5 tt2* seed. (K) SEM of *myb5 tt2* dry seed surface. (L) Toluidine blue stained thin cross section of a *myb5 tt2* seed at approximately the late upturned-U stage. Arrows in B, E and H point to examples of columella types or indicate radial walls: C, normal columella; PCC, partially collapsed columella; CC, collapsed columella; ERW, epidermal radial wall; SRW, subepidermal radial wall. Arrow in C indicates a cytoplasmic column in a developing seed coat epidermal cell. Scale bars: A, D, G and J=250 μm, B, C, E, F, H, I, K and L=15 μm.

the basal end of developing seed also demonstrated concentrated expression at the chalazal end in cells of the inner-most testa layer (Fig. 5E), consistent with *TT2*'s previously reported expression in the seed coat endothelium and its well-established role in seed coat endothelium development (Nesi et al., 2001; Debeaujon et al., 2003; Lepiniec et al., 2006).

Indeed, *myb5 tt2* mutant testas are more stunted for seed coat epidermal development as indicated by an almost complete lack of extruded mucilage from most seed when ruthenium red stained (Fig. 4J). Also, most *myb5 tt2* seeds viewed by SEM show no obvious columella formation or reinforced epidermal radial walls (Fig. 4K). When 100 testa epidermal cells across 5 different seeds per line viewed by SEM were randomly sampled for the presence of obvious columellae formation (wild-type-like or partial), 32% of the *myb5* cells contained columellae or partially formed columellae in contrast to 4% of the *myb5 tt2* double mutant cells (data not shown). However, light microscopy of toluidine blue-stained thin cross sections of developing *myb5 tt2* seed did not reveal any obvious defects beyond the *myb5* single mutant phenotype (Figs. 4I,L).

Instead of the bright yellow seed *tt2-1* and *tt2-3* mutants show the *tt2* insertion mutant used in this study show a buff-colored seed not unlike those of *tt2-2* mutant previously described (Nesi et al., 2001). Interestingly, *myb5 tt2* double insertion mutant seeds appeared slightly yet noticeably lighter in color than *tt2* seed (Fig. 5A). This suggests a role for *MYB5* in the development of the endothelial cell layer of the testa and is consistent with *MYB5* expression in this layer (Fig. 1H).

GL2, TTG2, and TT8 expression is reduced in MYB and bHLH mutant developing seed

GL2 and *TTG2* are well-established regulators of epidermal cell fate downstream of TTG1–bHLH–MYB transcriptional complexes (Rerie et al., 1994; Di Cristina et al., 1996; Johnson et al., 2002; Morohashi et al., 2007; Zhao et al., 2008). The epidermal layer of both *gl2* and *ttg2* mutant seed coats fail to properly differentiate while the endothelium of *ttg2* mutant testas is additionally defective for PA production. The *TT8* bHLH regulates PA biosynthesis in the inner-most testa layer (Nesi et al., 2000) and controls seed coat epidermal cell fate redundantly with the *EGL3* bHLH (Zhang et al., 2003). Interestingly, *TT8* has been shown to be a direct target of TTG1-dependent transcriptional complexes (Baudry et al., 2006; Gonzalez et al.,

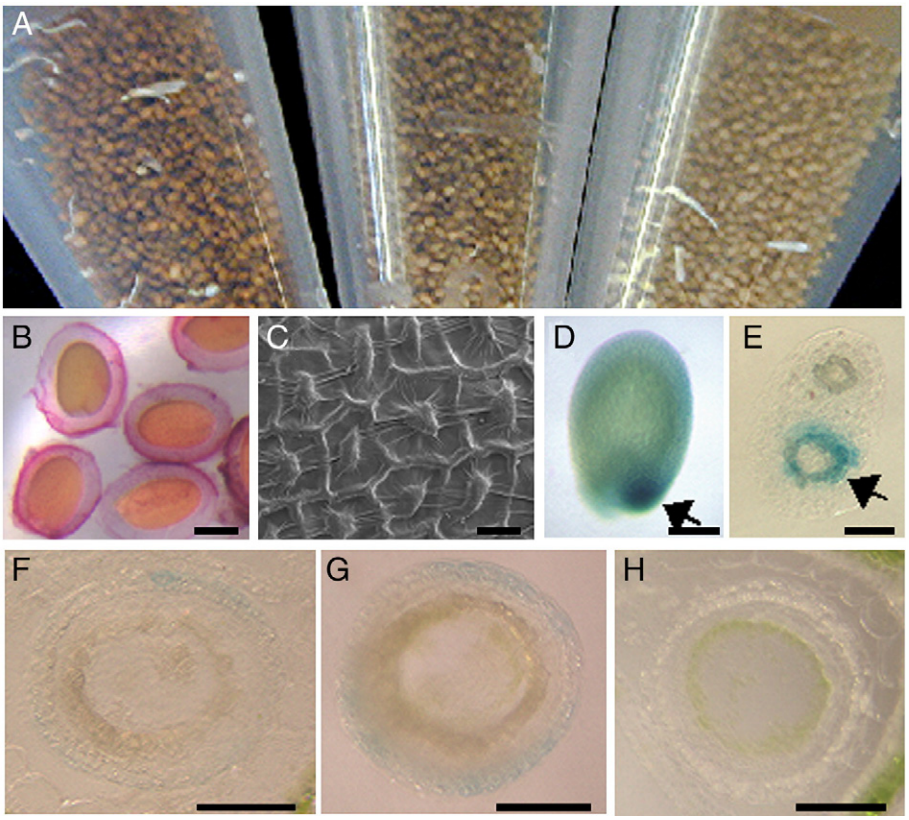

Fig. 5. Characterization of *tt2* single and *myb5 tt2* double mutant seed coats and *ProTT2*:*GUS* expression in developing wild-type seed coats. (A) Comparison of Col, *tt2* and *myb5 tt2* seed color. (B) Ruthenium red staining of extruded mucilage from *tt2* seed. (C) SEM of *tt2* dry seed surface. (D) *ProTT2*:*GUS* expression in a seed dissected from a developing silique. Arrow indicates the chalazal end. (E) Thick cross section through the basal/chalazal end (indicated by arrow) of a developing Col *ProTT2*:*GUS* seed at approximately the late torpedo stage. (F) and (G) Thick cross sections through the center of developing Col *ProTT2*:*GUS* seeds at approximately the late torpedo stage. (H) Thick cross section through a developing Col *ProGL3*:*GUS* as a negative control for (F) and (G). Scale bars: B = 250 μm, C = 15 μm, D, F, G and H = 100 μm, E = 50 μm.

2008). Thus *GL2*, *TTG2* and *TT8* expression profiles were determined in various MYB and bHLH regulatory mutants and compared to wild-type profiles in the context of developing seed coats.

It is well-documented that GL2 is strongly expressed in the embryo at most stages of development and this expression is dependent on TTG1-dependent transcriptional complexes (Lin and Schiefelbein, 2001; Costa and Dolan 2003). Q-PCR analysis of GL2 in developing seeds or siliques would measure mRNA levels reflecting both embryo and seed coats. Consequently, we compared GL2 expression in Col vs. regulatory mutant seed coats specifically by histochemical staining of cross sections of seeds of *ProGL2*:*GUS* lines. Reporter expression was clearly observed (within 1 min of staining) in the epidermal layer of developing seed coats of *ProGL2*:*GUS* Col wild-type seeds (Fig. 6A). However, expression was greatly reduced in this same layer of *ProGL2*:*GUS myb5* developing seeds (Fig. 6B). The epidermal layer of developing seed coats also showed a lack of reporter expression in *egl3 tt8* double mutant and *ttg1* mutant lines bearing the *ProGL2*:*GUS* transgene (Figs. 6C,D), consistent with their known roles in testa development. *ProGL2*:*GUS* expression was rarely observed in the inner-most testa layer of developing wild-type seeds and only faintly after long periods of staining (data not shown).

TTG2 expression has been previously reported to be strong during seed coat development but weak in developing embryos (Johnson et al., 2002). Using a Q-PCR approach, *TTG2* expression in wild-type developing siliques (with seeds at approximately the torpedo to early upturned-U stages) was compared to expression in developing siliques of MYB and bHLH regulatory mutants. *TTG2* expression is reduced in developing siliques of *myb5* and *tt2* single mutants (Fig 6E). Larger reductions in expression were observed in *myb5 tt2* and *egl3 tt8* double mutants (Fig. 6E).

TT8 is broadly expressed during seed coat development in both the endothelial and epidermal layers but is expressed in developing embryos only at late stages beginning at the curled cotyledon stage (Baudry et al., 2006). To determine if *TT8* is under the control of MYB regulators in the context of seed coat epidermal development, *TT8* expression in developing siliques (with seeds at approximately the torpedo to early upturned-U stages) of Col vs. MYB mutant plants was determined using a Q-PCR approach. Although slightly reduced in *myb5* and *tt2* single mutants, developing siliques from *myb5 tt2* double mutants showed a strong reduction in *TT8* expression (Fig. 6F), demonstrating both *MYB5* and *TT2* are positive regulators of *TT8* expression during seed coat development. Based on the expression patterns of *MYB5* and *TT2*, we expect that *TT8* is regulated in both layers by these factors. However, we can't unequivocally tell whether this regulation is in the epidermis or the endothelium or both without further analysis.

Discussion

A pivotal clue to the common regulatory mechanism underlying the developmental pathways defective in the *ttg1* mutant was the discovery that the *R* bHLH anthocyanin regulator from maize completely rescues *ttg1* plants (Lloyd et al., 1992; Galway et al., 1994). In the years following, a number of *Arabidopsis* bHLH and Myb transcriptional regulators were identified that in various combinations play a role in specifying the developmental pathways defined by the *ttg1* mutant. Thus studies centering on *TTG1* have proved invaluable in elucidating a complex transcriptional network important not only for the regulation of flavonoid metabolism but also for cell fate determination, embryo patterning and organ identity (Zhang et al.,

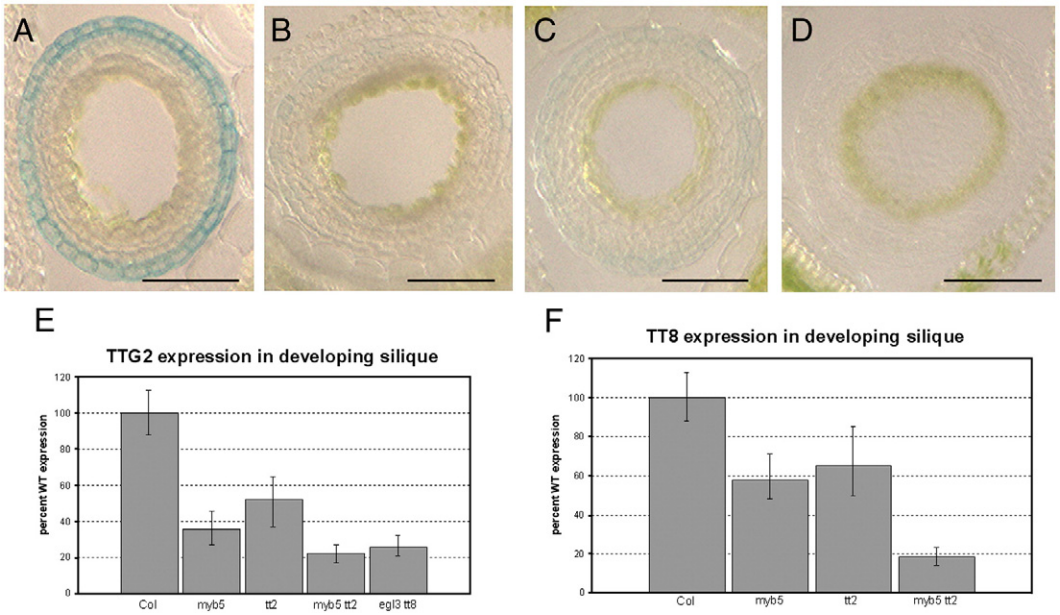

Fig. 6. *GL2*, *TTG2* and *TT8* expression analysis in testa development regulatory mutants. Thick cross sections of developing *ProGL2*:*GUS* seed at approximately the late torpedo stage in the following genetic lines: (A) Col, (B) *myb5*, (C) *egl3 tt8*, (D) *ttg1*. (E) Q-PCR analysis of *TTG2* expression in developing siliques reported as percentage of wild-type expression. Error bars indicate range of expression. (F) Q-PCR analysis of *TT8* expression in developing siliques reported as percentage of wild-type expression. Error bars indicate range of expression. Scale bars: A to D = 100 μm.

2003; Schiefelbein, 2003; Bernhardt et al., 2005; Haughn and Chaudhury, 2005; Lepiniec et al., 2006; Gonzalez et al., 2008) (Fig. 7A). Until now all TTG1-dependent MYBs and bHLHs have been assigned to one or more of the epidermal pathways save one; no MYB associated with a TTG1–complex had been identified governing the fate of the *Arabidopsis* seed coat outer epidermis. Although *MYB61* has been shown to regulate the differentiation of the mucilage-secreting cells of the testa epidermis (Penfield et al., 2001) it seemingly does so via an alternate pathway and MYB61 does not group with the MYB subfamily containing the TTG1-dependent MYB factors (Stracke et al., 2001; Haughn and Chaudhury, 2005). Here we identify the missing MYB as *MYB5*, representing the last regulator to be identified and characterized within this highly studied regulatory framework of plant development. *MYB5* is necessary for the differentiation of the highly specialized mucilage-secreting cells of the seed coat epidermis (Figs. 7A,B). *MYB5* also plays minor roles in trichome development and the differentiation of the PA producing endothelial layer of the *Arabidopsis* seed coat (Figs. 7A,B).

One observation to emerge from studies of TTG1-dependent transcription factors is the specialized nature of the MYBs in contrast to the pleiotropic functions of the bHLHs. For instance, *GL1* and *MYB23* are not only dedicated to the trichome pathway but specialize in particular aspects of trichome development (Kirik et al., 2005). *WER*, *TT2* and the *PAP* MYBs exclusively function in root hair patterning, PA biosynthesis and anthocyanin biosynthesis, respectively (Lee and Schiefelbein, 1999; Nesi et al., 2001; Teng et al., 2005; Gonzalez et al., 2008). On the other hand all the bHLHs function redundantly in more than one developmental pathway: *GL3* regulates trichome initiation, root hair patterning and flavonoid pigment biosynthesis; *EGL3* regulates trichome development, root hair patterning, flavonoid pigment biosynthesis and differentiation of the seed coat epidermis; *TT8* functions in flavonoid pigment biosynthesis and differentiation

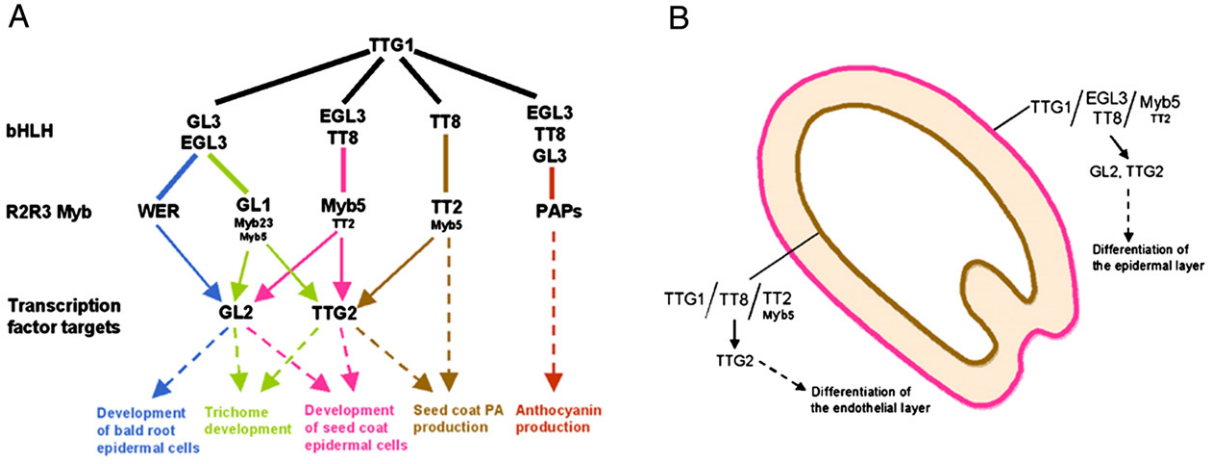

Fig. 7. Models for the regulation of TTG1-dependent developmental pathways. (A) A regulatory network for the positive control of TTG1-dependent epidermal cell fates. Solid lines indicate interactions between members of a complex. Solid arrows indicate direct regulation of *GL2* or *TTG2* targets. Dashed arrows indicate a multi-step differentiation pathway. Colored lines and arrows indicate specific regulator combinations and the pathway controlled. Text size in the case of the MYBs indicates their relative contributions to cell fate regulation. (B) A regulatory model for the differentiation of the seed coat outer and inner layers specified by specific TTG1-dependent transcriptional complexes. Text size in the case of MYB5 and TT2 MYBs indicates their relative contributions to the development of the outer and inner testa layers.

of the seed coat epidermis (Payne et al., 2000; Nesi et al., 2000; Zhang et al., 2003). However, this study reveals at least some pleiotropy at the level of the TTG1-dependent MYBs. *MYB5* primarily regulates the differentiation of the testa epidermis but also plays minor roles in trichome development and PA biosynthesis consistent with its expression pattern. *TT2* is a well-established regulator of PA biosynthesis in the endothelial layer of the testa (Nesi et al., 2001) but it now appears to have a minor role in the development of the testa epidermis as well. Such pleiotropy among the TTG1-dependent MYBs has not been previously observed. Interestingly, previous phylogenetic analysis of the *Arabidopsis* MYB gene family shows that *MYB5* is just excluded from a monophyletic group containing all other TTG1-dependent MYBs (pigment regulators and cell fate/patterning regulators), possibly explaining *MYB5*'s pleiotropic nature and broader expression pattern in contrast to the other MYBs (Stracke et al., 2001). Functional redundancy between *TT2* and another MYB factor particularly for the regulation of *TT8* in PA-accumulating cells has been recently suggested (Baudry et al., 2006). Although a dominant negative approach suggested *PAP1* as a possible regulator of PA biosynthesis in the seed coat (Matsui et al., 2004), recent studies have concluded this to be unlikely (Gonzalez et al., 2008). A more likely candidate for redundancy with *TT2* for the differentiation of PA-accumulating cells of the *Arabidopsis* seed coat is *MYB5*. Developing seeds of *ProMYB5*:*GUS* plants show expression in the endothelial layer of the seed coat. The expression of two key PA biosynthesis regulators, *TT8* and *TTG2*, is reduced in *myb5* developing siliques with more substantial reductions observed in the developing siliques of *myb5 tt2* double mutant plants. Also, while *myb5* seeds are wild-type in color, seeds of *myb5 tt2* double mutant plants appear slightly lighter in shade compared to seeds of the *tt2* insertion mutant used to create the double mutant. Together these observations suggest that MYB5 plays a minor role in the differentiation of the endothelial layer of the testa.

Functional redundancy between *Myb5* and *TT2* extends beyond the differentiation of the seed coat endothelium to include the development of the mucilage-secreting cells of the testa epidermis. While both ruthenium red staining and SEM of mature *tt2* seeds essentially indicate wild-type testa epidermal development, seeds of *myb5 tt2* double mutant are obviously more defective than those of *myb5* single mutant for mucilage secretion/extrusion and testa epidermal cell morphology. Although toluidine blue-stained thin cross sections of *myb5 tt2* developing seeds suggest mucilage production comparable to *myb5* single mutant seeds, it is possible the more severe double mutant phenotype observed in Figs. 4J and K may be due to phenomena besides just the abundance of mucilage (Macquet et al., 2007b; Dean et al., 2007). Consistent with a role for TT2 in the seed coat epidermis is its expression in the developing outer epidermal layer and its contributions to expression of the *TT8* and *TTG2* regulators of seed coat mucilage production. Thus a new (however minor) role for *TT2* is identified in this study as a regulator of seed coat epidermal development redundant with *MYB5*.

Also noteworthy from studies of TTG1-dependent developmental pathways is the exception that the differentiation of the PA-producing cells of the testa endothelium requires only one bHLH, TT8. This is in contrast to the other TTG1 cell fate pathways that are redundantly controlled by two or more bHLHs. No other TTG1-dependent bHLH thus far appears to be expressed in the developing seed coat endothelial layer besides *TT8* (Baudry et al. 2006). This may contribute to the mechanism for the fine-tuned expression of targets such as *BAN* and *GL2* (Debeaujon et al., 2003; this study) and ultimately cell fates in specific layers of the seed coat that otherwise express a very similar set of MYB and bHLH regulators. Transcriptional complexes containing EGL3, which are expressed in the outer seed coat layer but not the inner layer, may result in *GL2* expression restricted to the developing testa epidermis while the lack of EGL3 in the testa endothelium results in other combinations of regulators preferentially activating a different set of targets necessary for the production of PA pigments.

Analyses of *TTG1* and TTG1-dependent transcription factors have uncovered a complex regulatory system controlling various developmental fates in *Arabidopsis*. Among the last processes to be fully characterized at this level is the development of the testa with the identification of *MYB5* and *TT2* as partially redundant regulators of both seed coat epidermal and endothelial differentiation presented here. Recently, studies focusing on mutants defective for pectin biosynthetic enzymes have revealed the complex structural and compositional nature of the mucilage produced in the epidermis of the *Arabidopsis* seed coat (Oka et al., 2007; Macquet et al., 2007a,b; Dean et al., 2007). An emerging seed coat mucilage biosynthetic pathway should enable interesting future studies defining possible pathway regulatory mechanisms by members of TTG1-dependent transcriptional complexes and a clear understanding of mechanisms regulating seed coat development.

Acknowledgments

We thank Greg Hatlestad for insightful comments on the manuscript and other members of the Lloyd lab and the UT Austin Plant Molecular Biology community for helpful discussions. This work was supported by the National Science Foundation (IBN-0344200).

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