RESEARCH PAPER

TRANSPARENT TESTA 16 and 15 act through different mechanisms to control proanthocyanidin accumulation in Arabidopsis testa

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Abstract

Flavonoids are secondary metabolites that fulfil a multitude of functions during the plant life cycle. In Arabidopsis proanthocyanidins (PAs) are flavonoids that specifically accumulate in the innermost integuments of the seed testa (i.e. endothelium), as well as in the chalaza and micropyle areas, and play a vital role in protecting the embryo against various biotic and abiotic stresses. PAs accumulation in the endothelium requires the activity of the MADS box transcription factor TRANSPARENT TESTA (TT) 16 (ARABIDOPSIS B-SISTER/AGAMOUS-LIKE 32) and the UDP-glycosyltransferase TT15 (UGT80B1). Interestingly tt16 and tt15 mutants display a very similar flavonoid profiles and patterns of PA accumulation. By using a combination of genetic, molecular, biochemical, and histochemical methods, we showed that both TT16 and TT15 act upstream the PA biosynthetic pathway, but through two distinct genetic routes. We also demonstrated that the activity of TT16 in regulating cell fate determination and PA accumulation in the endothelium is required in the chalaza prior to the globular stage of embryo development. Finally this study provides new insight showing that TT16 and TT15 functions extend beyond PA biosynthesis in the inner integuments of the Arabidopsis seed coat.

Key words: Arabidopsis thaliana, proanthocyanidins, seed coat, seed, tannins, testa, TT15, TT16.

Introduction

Flavonoids are secondary metabolites well known for the coloration of plant tissues that fulfil a multitude of functions during the plant life cycle, from protection against environmental stresses to modulation of plant growth and development (Lepiniec et al., 2006; Grunewald et al., 2012). In addition flavonoids are antioxidant molecules presenting beneficial properties for human health and are well known to impact the organoleptic, nutritive, and processing characteristics of
feeds (Lepiniec et al., 2006). In Arabidopsis seeds, flavonols and proanthocyanidins (PAs; also called condensed tannins) are the main flavonoids (Routaboul et al., 2006, 2012). PAs specifically accumulate in the innermost integuments of the seed testa (i.e. endothelium) as well as in the chalaza and the micropyle areas. They play a crucial role in protecting the embryo against various biotic and abiotic stresses (Winkel-Shirley, 1998) and in modulating seed dormancy, longevity, and dispersion (Debeaujon et al., 2000; Windsor et al., 2000; Bueso et al., 2014). Once oxidized, PAs confer a brown colour to mature seeds (Pourcel et al., 2005) enabling the visual screening of mutants impaired in flavonoid accumulation named transparent testa (tt) (Shirley et al., 1995).

PA biosynthesis is catalysed by a series of enzymes encoded by genes belonging to two main groups, the early biosynthetic genes (EBGs) and the late biosynthetic genes (LBGs) (Xu et al., 2014). The EBGs encode proteins whose activity provides precursors for the whole flavonoid biosynthetic pathway. The LBG group comprises genes encoding proteins involved in PA precursor biosynthesis as well as proteins involved in PA modification and compartmentalization (Appelhagen et al., 2015). In addition to the EBGs and LBGs, TT10/LAC15 encodes a LACCASE-type flavonoid oxidase involved in the oxidative polymerization of PAs (Pourcel et al., 2005), and TT15/UGT80B1 (UDP-GLUCOSE:STEROLGLUCOSYLTRANSFERASE) and TT9/GFS9 encode proteins that are involved in vesicular trafficking controlling PA accumulation in the vacuole (Warnecke et al., 1997; Focks et al., 1999; DeBolt et al., 2009; Ichino et al., 2014). Interestingly, TT15 has also been proposed to be the causative gene underlying a natural variation in PA accumulation occurring between the Col-0 and Cvi-0 Arabidopsis accessions (Routaboul et al., 2012).

Genes involved in the transcriptional control of PA biosynthesis have also been characterized. For instance, specific R2R3-MYB (MYB123/TT2 and MYB5) and R/B-like bHLH (TT8/bHLH42, GL3/bHLH00 and EGL3/bHLH02; subgroup IIIf) transcription factors (TFs) together with TRANSPARENT TESTA GLABRA 1 (TTG1, WD repeat family, suggesting that TT16 may be involved in the transcriptional regulation of PA biosynthesis that belongs to the MADS box family (Shore and Sharrocks, 1995; Nesig et al., 2002). MADS box TF can multimerize and form heterotrimERIC complexes in order to regulate its target genes (de Folter et al., 2006; Immink et al., 2009). In plants, MADS box TFs were found to regulate development of organs such as flowers, ovules, seeds, leaves and roots and play an important role in the establishment of the Arabidopsis seed testa (Riechmann and Meyerowitz, 1997; Smyth, 2000; Ng and Yanofsky, 2001; Nesig et al., 2002; Debeaujon et al., 2003; de Folter et al., 2006; Prasad and Ambrose, 2010; Prasad et al., 2010; Mizzotti et al., 2012, 2014; Ehlers et al., 2016; Ezquer et al., 2016; Figueiredo et al., 2016; Xu et al., 2016). The expression of TT16 orthologous genes is restricted in angiosperm and gymnosperm species to female reproductive organs, mainly the integuments of the ovules (Becker et al., 2002; Nesig et al., 2002; Kaufmann et al., 2005; Tonaco et al., 2006; Deng et al., 2012; Chen et al., 2013; Rhodes et al., 2014). Unlike most of the other regulatory tt mutants, tt16 accumulates PAs in the chalaza and micropyle areas while they are absent from the endothelium (Nesig et al., 2002). It is noteworthy that both the flavonoid profile and the pattern of PA accumulation displayed by the tt16 mutant are similar to those of tt15 suggesting that both genes may act on the same genetic pathway (Nesig et al., 2002; Routaboul et al., 2006, 2012; DeBolt et al., 2009). Interestingly, TT2 ectopic expression restores PA accumulation in tt16 indicating that TT16 acts upstream of the PA biosynthetic pathway, and that the ability of the cells to accumulate PA is not directly dependent on TT16 activity (Nesig et al., 2002). The lack of TT16 activity is also associated with ectopic cell divisions leading to disorganized and irregularly shaped PA-accumulating cells (Nesig et al., 2002). This phenotype is exacerbated in the tt16 stk1agl11 (seed-stick) double mutant (Mizzotti et al., 2012). These results demonstrate that TT16 is involved in the transcriptional control of endothelium development. More recently, TT16 function (together with GORDITA, GOA/AGL63, its closest homologue) was associated with nucellus degeneration following ovule fertilization (Erdmann et al., 2010; Xu et al., 2016). In addition, TT16 together with SHATTERPROOF 1 and 2 (SHP1/AGL1 and SHP2/AGL5, two closely related MADS-box TFs) was shown to be involved in the control of endosperm development and in the coordination of cell divisions in ovule integuments and seed coat development (Ehlers et al., 2016). Several studies have shown that TT16 can interact in vivo with various members of the MADS box protein family, suggesting that TT16 may be involved in the transcriptional control of additional facets of seed coat development (de Folter et al., 2005; Kaufmann et al., 2005; Tonaco et al., 2006; Immink et al., 2009). In canola, another member of the Brassicaceae, beside the exhibition of abnormal endothelium development and decreased PA content, the expression of most genes known to be involved in the PA biosynthetic pathway, as well as several related genes such as TTG2, was significantly reduced in Bnttt16 mutant lines compared with wild-type plants (Deng et al., 2012; Chen et al., 2013).

In order to refine our understanding of the roles and mode of action of TT16 and TT15 during seed development and PA biosynthesis, a combination of genetic, molecular, biochemical, and histochemical methods was used. We demonstrated that TT16 and TT15 act upstream of the PA biosynthetic pathway through two distinct genetic
pathways. We then demonstrated that the activity of TT16 in regulating cell fate and PA accumulation in the endothelium is required prior to the globular stage in the chalaza area. Finally this study showed that TT16 and TT15 activities extend beyond PA biosynthesis in the endothelium, as TT16 most probably regulates the fate of the inner integuments of the testa, whereas TT15 plays a role at the whole plant level.

Materials and methods

All PCRs were carried out using high-fidelity Phusion DNA polymerase, according to the manufacturer’s instructions (Thermo Scientific Finnzymes). PCR products were subsequently sequenced after recombination or cloning into their destination vectors. All the primers used in this study are described in Supplementary Table S1 at JXB online. Expression analyses (qRT-PCR) were performed as described in Dubos et al. (2008).

Plant material

Arabidopsis accession Wassilewskija (WS) was used as wild-type control. The mutant lines tt16-1 (dxt32; Nesni et al., 2002) and tt15-2 (cob16; Routaboul et al., 2012) were obtained from the Versailles Biological Resource Centre (http://publiclines.versailles.inra.fr). The double tt15 tt16 mutant was obtained by crossing the tt15-2 and tt16-1 alleles. Plants expressing β-glucuronidase (GUS) under the control of the TT8 and TT15 promoters are described in Xu et al. (2013, 2014a). All methods and conditions used for plant growth, plant transformation, and selection for transgenic lines were as previously reported by Nesni et al. (2000).

Studied Arabidopsis gene IDs

The Arabidopsis gene IDs were as follows: BANYULS/ANR, At1g61720; CHS/T4,T, At1g53930; TT2/3YBI23, At1g53550; TT8/HHL042, At4g09820; TT15/UG8081, At1g43620; TT16/AGL32/ABS, At5g23260.

Proanthocyanidin staining and measurement

PA staining of 4-day-old seeds was carried out using vanilline reagent as described in Debeaujon et al. (2003). Quantitative PA measurements were carried out on 15 mg of dried seeds according to Routaboul et al. (2012) using methanol–aceton–water–trifiuroacetic acid (30/42/20/0.05, v/v/v/v) to maximize PA extraction. Samples were measured in triplicates in two independent biological repetitions.

Con structs

Fusions of TT16 (pTT16: 1597 bp prior to the ATG) and BAN (pBAN: 236 bp prior to the ATG) promoters to the Gateway™ recombination cassette were carried out as described for the promoter of TT8 (1.5 kb prior to the ATG) in Dubos et al. (2008). Briefly, pTT16 and pBAN were PCR-amplified from genomic DNA (WS) with the pTT16-5’/HindIII/pTT16-3’/XbaI and pBAN-5’/ HindIII/pBAN-3’/XbaI primer pairs, respectively. The obtained DNA fragments were subsequently cloned into the pBIB-Hyg-GTW vector (Dubos et al., 2008) digested with HindIII and XbaI, giving the pBIB-Hyg pTT16:GTW and pBAN:GTW vectors.

TT2 and TT15 coding sequences (CDSs) were PCR-amplified from WS cDNAs using the cTT2/B1/cTT2/B2 and cTT15/B1/cTT15/B2 primer pairs, respectively. Genomic TT16 (gTT16), which corresponds to the DNA sequence between the ATG and stop codons, including both exons and introns, was PCR-amplified from genomic DNA (WS) using the cTT16-gATG-B1/cTT16-gSTOP-B2 primer pair. The same primers were also used to amplify from WS cDNA cTT16/ABS1 (long: 759 bp) and cTT16S/ABS1 (short: 744 bp) CDSs corresponding to the two splice variants of TT16. The obtained DNA fragments were then BP-recombined into the pDONR207 vector (Gateway™).

TT2 and TT15 CDSs were recombined into the pMDC32 vector (Curtis and Grossniklaus, 2003) for overexpression (which contains two copies of the 35S minimal promoter from the cauliflower mosaic virus, p70S).

cTT16/ABS1 and cTT16S/ABS1 CDSs were LR-recombined into the pTT16:GTW vector.

gTT16 was LR-recombined into pTT16:GTW, pTT8:GTW, pBAN:GTW, and pMDC32 vectors.

TT2 promoter (2 kb prior to the ATG) for GUS analysis was cloned into pDONR207 and then LR-recombined into pGW3 (Nakagawa et al., 2007).

For each construct, 6–12 independent transgenic plants were analysed, and representative observations are presented.

Histochemical detection of GUS activity

GUS staining for seeds expressing promoter:uidA gene fusion constructs were performed as described in Berger et al. (2011). For each construct, 6–12 independent transgenic plants were analysed, and representative observations are presented.

Immunofluorescence labelling of cell wall

Four-day-old siliques were collected on ice (extremities were removed, and the remaining part was cut into two pieces) and incubated for 1 h at 4 °C in the fixation buffer (1× PBS (Eurobio), 2% formaldehyde, and 0.1% triton X-100) after vacuum treatment (three times). Samples were then dehydrated using a series of increasing ethanol concentrations in PBS (30%, 50%, 70%, 90%, 100%) at 4 °C (2 h each). Siliques were then stained using toluidine blue (0.01% in absolute ethanol), and transferred into a 2:1 followed by a 1:1 absolute ethanol–water–hexane (2% w/v, 2 h each). Siliques were then incubated twice for 3 h in 100% wax solution at 40 °C before polymerization. Cross sections of 8 μm were finally cut using a Leica RM2165 microtome, and sample ribbons were placed on a drop of sterile water (Versol) on polyethylene slides, and left to dry overnight at 37 °C. Immunolabelling using the JIM4 and JIM8 monoclonal antibodies (PlantProbes, Leeds, UK) was carried out as described in Macquet et al. (2007). Samples were then observed using a confocal microscope (Leica TCS-SP2 AOLS, Leica Microsystems). Spectral bands from 498 to 567 nm were selected in order to specifically detect the Alexa Fluor 488 fluorescence.

Results

TT15 acts upstream of the PA biosynthetic pathway

The two mutants tt16 and tt15 share a very similar pattern of flavonoid accumulation. They both have lower content of the major accumulated flavonols (quercitrin and quercetin-3-rhamnoside) and very little PAs (Routaboul et al., 2006, 2012) when compared with wild-type (WT) seeds. Both tt16 and tt15 mutants accumulate PA mostly in the chalaza and micropyle areas. Expression analysis (quantitative RT-PCR) showed that the tt15 had decreased PA accumulation strongly correlated with a decrease of BAN mRNA levels (and to a lesser extent of CHS, the first EBG of the PA biosynthetic pathway) (Fig. 1A and Table 1), which was similar to what
Table 1. Analysis of mature seed PA content (soluble and insoluble PAs) in wild-type, tt16, tt15, and transgenic lines used for tt16 and tt15 complementation assays

Proanthocyanidin content is expressed as mg cyanidin g⁻¹ seed.

<table>
<thead>
<tr>
<th>Line</th>
<th>Soluble</th>
<th>Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.97 ± 0.02</td>
<td>3.52 ± 0.04</td>
</tr>
<tr>
<td>tt16</td>
<td>0</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>p70S:gTT16 in tt16</td>
<td>12.05 ± 0.06</td>
<td>4.07 ± 0.01</td>
</tr>
<tr>
<td>p70S:TT2 in tt16</td>
<td>0.92 ± 0.10</td>
<td>1.22 ± 0.05</td>
</tr>
<tr>
<td>pTT16:TT16L in tt16</td>
<td>0</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>pTT16:TT16S in tt16</td>
<td>0</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>p70S:TT15 in tt16</td>
<td>0</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>tt15</td>
<td>0</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>p70S:TT15 in tt16</td>
<td>4.34 ± 0.11</td>
<td>2.00 ± 0.01</td>
</tr>
<tr>
<td>p70S:TT2 in tt15</td>
<td>1.56 ± 0.02</td>
<td>1.64 ± 0.01</td>
</tr>
<tr>
<td>p70S:TT16 in tt15</td>
<td>0</td>
<td>0.43 ± 0.03</td>
</tr>
</tbody>
</table>

was previously observed in tt16 developing seeds (Nesi et al., 2002; Debeaujon et al., 2003). To confirm that TT15 acts upstream the PA biosynthetic pathway, like TT16, the TT2 coding sequence (CDS) was overexpressed in tt15 (using the p70S promoter). As a positive control for this complementation assay, overexpression of the TT15 CDS was also carried out using the same promoter. In both cases PA accumulation increased in immature and mature transgenic seeds, indicating that, similarly to TT15, TT16 functions upstream of the PA biosynthetic pathway (Fig. 1B and Table 1).

TT15 and TT16 affect PA accumulation in an independent manner

Departs from the above-described similarities, contrary to tt16, the endothelium cell shape of tt15 does not seem to be affected, suggesting that TT15 does not function upstream of TT16 (Nesi et al., 2002; Debeaujon et al., 2003). Conversely, we found that the activity of TT15 promoter was unaffected in tt16, when compared with WT seeds (Fig. 2A). Rather, the phenotypic defects observed in vegetative tissues upon gTT16 overexpression (i.e. stunted plants with curly leaves, reduced flower size and shrunken silique; Nesi et al., 2002) were accentuated in tt15, suggesting that the two proteins act in different genetic pathways (Fig. 2B and Supplementary Fig. S1). To investigate this hypothesis further, cross-complementations of tt16 and tt15 were carried out. For this purpose the TT16 genomic region (gTT16) and TT15 CDSs were overexpressed (p70S promoter) in tt15 and tt16, respectively (Fig. 2C). Although both constructs were able to complement their respective mutants (see previous paragraph for TT15 CDS; Fig. 1B), no cross-complementation was observed, indicating that the function of each gene does not rely on the function of the other (Fig. 2C and Table 1). It is noteworthy that both gTT16 and TT2 CDSs were able to complement tt16 when overexpressed with the same promoter (p70S; Figs 2C and 4, Supplementary Fig. S2, and Table 1), confirming the functionality of the DNA fragments used and the position of TT2 downstream of TT16 in the PA biosynthetic pathway (Nesi et al., 2002; Debeaujon et al., 2003). These data also confirm that the competency of PA-accumulating cells to synthetized and accumulate PA remain conserved in tt16 mutant seeds. Lastly, we found that the tt15 tt16 double mutant displayed a transparent testa phenotype that was similar to that of tt16 (i.e. no additive effect), confirming that TT15 and TT16 act through distinct genetic routes (Fig. 3).

TT16 activity extends beyond the control of the sole endothelium development

In order to further characterize the different cellular impacts of tt16 and tt15 mutations, immunohistolabelling experiments were carried out. The rationale was that in Arabidopsis seeds the i1 (PA-accumulating cells—endothelium) and the...
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ii1′ (parenchymatous cells) cell layers are derived from the ovular endothelium through periclinal divisions (Debeaujon et al., 2003); the formation of these two cell layers is achieved once the ovule becomes mature. Two markers were used for these experiments, namely the JIM4 and JIM8 monoclonal antibodies that target specific arabinogalactan-proteins present in the cell wall of different cell layers of the testa. Markers of the cell wall have been selected because various studies have demonstrated that cell wall properties can vary between different cell types and in response to developmental signals (Cassab, 1998; Freshour et al., 2003). The JIM4 antibody specifically marked the ii1′ cell layer in WT seed coat (Fig. 4A). Interestingly, no JIM4 labelling was observed in tt16 seeds, in contrast to what was observed in tt15 seeds (Fig. 4A). It is noteworthy that JIM4 labelling was restored in tt16 seeds overexpressing gTT16, but not in tt16 seeds overexpressing TT2 or TT15 CDSs (Debeaujon et al., 2003). JIM8 antibody specifically labelled three cell layers of the testa in WT seeds, namely the ii1 (endothelium, PA-accumulating cells), inner integument 2 (ii2) and outer integument 2 (oi2, mucilage-producing cells) cell layers (Fig. 4B). This labelling pattern was also observed for the tt15 mutant as well as for tt16 lines overexpressing gTT16 (Debeaujon et al., 2003). JIM8 labelling of the ii1, ii2 and oi2 cell layers

![Fig. 2. TT15 and TT16 affect PA accumulation through two independent genetic pathways. (A) Pattern of TT15 promoter activity in wild-type (WT) and tt16 seeds (globular stage) revealed by the detection of GUS activity. (B) Effect of gTT16 overexpression on WT and tt15 vegetative tissues highlighting that growth defects (stunted plants with curly leaves and reduced flower size; Nesi et al., 2002) are enhanced in tt15 mutants when compared with WT plants. (C) Cross-complementation experiments in which gTT16 (the genomic sequence comprising the introns and exons present between the start and stop codons of TT16 as TT16 is expressed under two spliced variants, TT16L/ABS1 and TT16S/ABS2; Nesi et al., 2002) and TT15 CDSs were overexpressed in tt15 and tt16, respectively. gTT16 and TT2 CDSs were overexpressed in tt16 in order to confirm the functionality of the DNA fragments that were used and the downstream position of TT2 when compared with TT16 in the PA biosynthetic pathway, respectively (Debeaujon et al., 2003). Upper panels: 4-day-old (globular stage) seeds treated with vanilline reagent, which specifically stains PAs red (whole mount); lower panels: seed colour.]
PA-accumulating cells upon fertilization (Debeaujon et al., 2003). Interestingly, we found that TT2 expression remains restricted to the micropyle area in the tt16 mutant indicating that TT16 activity is required for the progression of TT2 expression within the PA-accumulating cells (Fig. 5A). TT8 expression in tt16 seeds is also lower in the endothelium (ii1) when compared with WT seeds (Xu et al., 2013).

To investigate if there was a developmental time frame in which TT16 activity was required for proper PA biosynthesis and accumulation in seeds, two different promoters were used to drive the expression of TT16 for tt16 complementation experiments (Fig. 5B). For this purpose the promoters of TT8 (pTT8) and BAN (pBAN) were used, as the expression of the corresponding genes in PA-accumulating cells initiates at distinct developmental stages (Nesi et al., 2000; Debeaujon et al., 2003; Supplementary Fig. S3). Because TT16 is expressed under two alternative mRNA forms, the genomic sequence comprising the introns and exons present between the start and stop codons was used (gTT16). We first successfully complemented the transparent testa (tt) phenotype of tt16 using the TT16 promoter (pTT16, ~1.6 kb prior to the start codon), confirming that the genomic DNA fragment containing the TT16 CDS was functional (Fig. 5B). We observed a similar tt16 complementation when pTT8 was used, but not with pBAN (Fig. 5B).

This observation suggests that there is a developmental frame in which PA-accumulating cells can perceive signals associated with TT16 activity (i.e. prior to the globular stage). In addition, data gathered on pTT16:gTT16:GUS (~1.6 kb) and pTT8:GUS activities (restricted to the chalazal area in WT and tt16, respectively; Xu et al., 2013, 2016) and TT8 expression (initiating from the chalazal area prior to fertilization; Supplementary Fig. S3) demonstrated that the expression of TT16 in the chalazal area prior to the globular stage was sufficient to trigger PA accumulation. In support of this conclusion it was also recently shown that when pTT16 (~1.6 kb) is fused to the uidA reporter gene, no GUS activity is observed in seeds, whereas the expression of TT16 in PA-accumulating cells requires the regulatory sequences that are present up to 3.4 kb prior to the TT16 start codon (Ehlers et al., 2016; Xu et al., 2016).

TT16 genomic sequence spanning the CDS region plays a key role in the control of PA accumulation in seeds

In Arabidopsis TT16 is alternatively spliced into two CDSs that are 759 bp (TT16L/ABSI) and 744 bp (TT16S/ABSI) long (Nesi et al., 2002). In another member of the Brassicaceae, Brassica napus (canola), four homologues of the Arabidopsis TT16L variant (i.e. BnTT16.1 to BnTT16.4) displaying between 75 and 80% identity at the protein level were identified and characterized; each of them complements the tt16 mutation when ectopically expressed using the CaMV 35S promoter (Chen et al., 2013). To determine if the genomic region of TT16 plays a role in regulating TT16 expression, we carried out tt16 complementation assays in which TT16L and TT16S CDSs as well as gTT16 were expressed under the control of...
Control of proanthocyanidin accumulation by TT16 and TT15

Immature 4-day-old (globular stage) seeds of WT, tt16, and tt16 transgenic lines were thus treated with vanilline reagent, which specifically stains PAs red (Fig. 6), and then the PA content was measured in dry seeds (Table 1). PA-accumulating cells (i.e. endothelium, chalaza, and micropyle) of WT seeds were stained red, whereas only the chalaza and micropyle areas were stained in tt16 seeds. This observation correlated with the strong decrease in PA content measured in tt16 dry seeds when compared with WT (Routaboul et al., 2006). Surprisingly, although the expression of gTT16 complements the tt16 mutant, no complementation was observed when TT16L or TT16S CDS was expressed under the control of pTT16. (Figs 5B and 6). These data are fully consistent with previous results showing the importance of TT16 genomic sequence for the correct expression of the gene (Xu et al., 2016).

Discussion

Previous analyses have demonstrated that tt16 and tt15 mutants share a very similar pattern of flavonoid accumulation in the seed coat (Routaboul et al., 2006, 2012). Both mutants have altered accumulation of PA in the endothelium, whereas PAs still accumulate in the chalaza and micropyle areas (Fig. 2). In this study, we showed that TT15, like TT16, acts upstream the PA biosynthetic pathway, but through a distinct genetic route (Fig. 7). Interestingly, we also found that the growth defects triggered by gTT16 overexpression in vegetative tissues were accentuated in tt15, indicating that TT15’s function extends beyond PA accumulation in seeds to whole plant development (Fig. 2B and Supplementary Fig. S1; DeBolt et al., 2009).

TT16 is a key regulator of endothelial cell fate; however it was still unclear if its role within the testa extended beyond the development of this cell layer. In order to explore this we used for immunolabelling experiments two different markers targeting the cell wall of distinct cell layers of the seed testa (Fig. 4 and Supplementary Fig. S2). These markers were the JIM4 (the ii1′ cell layer) and JIM8 (PA-accumulating cells and the ii2 and oi2 cell layers) monoclonal antibodies directed against different arabinogalactan epitopes. Through this approach we confirmed that TT16 is involved in the transcriptional control of cell fate determination of the two most inner integuments (ii1 and ii1′) of the seed testa and that the cells issued from ectopic divisions derived from the ii1 cell layer (Nesi et al., 2002). In support of this finding it was recently shown that TT16 and SHATTERPROOF 1 and 2 (SHP1/AGL1 and SHP2/AGL5) play an antagonistic role in the control of ii1′ cell layer development (Ehlers et al., 2016). The mucilage extrusion defects observed in tt16 or tt16 shp1 shp2 mutants when compared with WT seeds indicate that TT16 function in testa development is broader than primarily thought (Ehlers et al., 2016). The fact that the alteration of TT16 activity impacts cell wall properties of the ii1′ cell layer suggests that TT16 may, like STK, play a role in the control of structural and mechanical properties of the seed testa (Exquer et al., 2016). In contrast, no difference was observed between WT and tt15 seeds confirming the idea that TT16 and TT15 functions are independent. Altogether these data demonstrate that TT15 and TT16 are involved in PA accumulation in the endothelium through different pathways, and that their functions extend beyond this tissue.

With the aim to get new insights into the role that TT16 plays during seed development we investigated if there was...
a developmental time frame in which TT16 activity was required for proper PA biosynthesis and accumulation. Complementation experiments of tt16 were carried out by expressing gTT16 under the control of three different promoters that are active at distinct developmental stages in PA-accumulating cells (i.e. pTT16, pTT8, and pBAN genes). This strategy revealed that TT16 activity is required in the chalaza area prior to the globular stage of embryo development for proper endothelium development and PA accumulation (Fig. 1). Moreover, based on TT16 expression data (Mizzotti et al., 2012; Xu et al., 2016) and the pattern of pTT16, pTT8, and pBAN activity in Arabidopsis seeds (Debeaujon et al., 2003; Xu et al., 2013) it is likely that TT16 activity in the chalaza area prior to fertilization is sufficient to trigger endothelium cell fate determination.

While studying the potential role of TT16 spliced variants (TT16L/ABS I, 759 bp, and TT16S/ABS II, 744 bp) during seed development, we confirmed that a TT16 genomic sequence spanning the CDS region plays a key role in the control of PA accumulation in seeds (Fig. 2). In addition, and unlike what has been found in canola (Brassica napus), we found that TT16L (as well as TT16S) was not sufficient to restore tt16 mutant phenotypes (Chen et al., 2013). Taken together these observations suggested that the intronic regions of TT16 are necessary for the initiation of PA biosynthesis in the endothelium and must thus contain key regulatory sequences. The importance of the intragenic sequences for the control of gene expression has already been reported for other MADS box TFs. This is for example the case for AGAMOUS (AG), a key transcriptional regulator of floral organ specification, for which most of the regulatory elements that control its expression are located in the second intron of the gene (Kaufmann et al., 2010). Whether or not regulatory sequences are located within the introns of TT16 will need further investigation.

Another important question that remains to be addressed is determining the role that TT16S plays during seed development, in particular in the light of the work of Chen et al. (2013) mentioned above. Is the dimerization between TT16L and TT16S previously reported (de Folter et al., 2005) necessary for TT16 activity? This is, for example, the case with STK/AGL11 (SEEDSTICK; Kaufmann et al., 2005), another MADS box TF that is partially redundant with TT16 in regulating endothelium development (Mizzotti et al., 2012; Fig. 7). Close petunia homologues of TT16 (FLORAL BINDING PROTEIN 24; FBP24) and STK (FBP11) were also shown to interact in vivo in yeast and in plant cells (Tonaco et al., 2006).
Taken together these data could suggest that part of TT16’s activity relies on the formation of (dimeric or trimeric) protein complexes involving TT16 and STK.

Nevertheless, with respect to PA accumulation in seeds, TT16 and STK present antagonistic activities. TT16 promotes PA accumulation in the endothelium (Nesi et al., 2002) whereas STK inhibits the expression of genes involved in PA biosynthesis and accumulation, as well as those such as TT8, EGL3 and TT16 encoding regulatory proteins in the inner integument 2 (ii2) (Mizzotti et al., 2014). Interestingly, the presence of STK in the chalaza and micropyle areas in mature ovules does not inhibit the accumulation of PA, whether TT16 is present or not (Debeaujon et al., 2003; Xu et al., 2013; Mizzotti et al., 2014). This observation suggests that an additional regulatory mechanism controls the expression of PA biosynthetic genes in the chalaza and micropyle areas, being dominant over STK inhibition and independent of TT16 induction (Fig. 7). TT16 could also repress the
deposition of STK-dependent repressive marks on the chromatin of genes involved in PA biosynthesis (Mizzotti et al., 2014), or conversely, TT16 may facilitate the deposition of chromatin marks associated with an active chromatin state at these loci.

**TT16** and **GOA/AGL63** (GORDITA) are paralogous genes (Erdmann et al., 2010). To date, GOA function has been associated with the control of fruit size, through the modulation of cell expansion (Prasad and Ambrose, 2010; Prasad et al., 2010). GOA and TT16 have additive roles in seed coat development as revealed by the phenotype of the goa tt16 double mutant whose seeds display phenotypic defects associated with each mutation; long and narrow 01 cells for goa seeds and flat and irregularly shaped ii1 cells that lack PA accumulation for tt16 seeds (Prasad et al., 2010). One of the hypotheses associated with this observation would be that both genes play a similar role in cell fate determination, but in different cell layers because of distinct promoter activities (Prasad and Ambrose, 2010), and through different molecular mechanisms. This later assumption is supported by the fact that GOA possesses a specific protein–protein interaction domain (the ‘deviant’ domain, DD) that results in the absence of shared protein interaction partners with the other ABS proteins (Erdmann et al., 2010). Recently, it was demonstrated that TT16 acts redundantly with GOA to promote nucellus degeneration upon fertilization leading to the formation and correct positioning of the chalazal endosperm (Xu et al., 2016). Nevertheless, the role that GOA plays in the chalaza area remains to be elucidated (Prasad et al., 2010).

PA biosynthesis and accumulation in Arabidopsis seeds is regulated at the transcriptional level by various MYB--bHLH–WDR (MBW) protein complexes (Baudry et al., 2004; Thévenin et al., 2012; Xu et al., 2013, 2014a, b, 2015). Similar MBW complexes have been shown to be involved in cell fate determination of trichomes and root hairs. Cell-to-cell movement of proteins involved in these MBW complexes was found to be central in this process (Bernhardt et al., 2005; Balkunde et al., 2011). PA accumulation in seeds initiates in the chalaza and micropyle areas before spreading throughout the whole endosperm, following the expression pattern of **TT2** and **TT8** (Debeaujon et al., 2003; Xu et al., 2013). In contrast, PA accumulation in tt16 is restricted to the chalaza and micropyle areas, as is the case for TT2 expression (Fig. 1). Similarly, the expression of TT8 in tt16 is unaffected in the chalaza and micropyle areas whereas its spreading throughout the endosperm is delayed (Xu et al., 2013; Supplementary Fig. S1). Because the overexpression of TT2 is sufficient to overcome the transparent testa phenotype of tt16, it could be hypothesized that cell-to-cell communication from the chalaza and micropyle areas toward the endosperm is central for initiating PA biosynthesis in the endosperm. Nevertheless, it cannot be excluded that such a signal could derive from other seed tissues (e.g. ii’ cell layer, endosperm) in response to TT16 activity. In addition a recent study has highlighted the importance of auxin production in the endosperm to initiate the development of the Arabidopsis seed coat, a process involving another MADS box TF, AGL62 (Figueiredo et al., 2016). Indeed, these hypotheses would require additional experiments in order to be validated, and necessitate identifying the signal (e.g. MBW protein members). The identification and characterization of genes that are directly regulated by TT16 in the seed tests will be the next challenge in order to clearly understand how TT16 activity synchronizes cell fate determination, PA accumulation and seed development.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. Growth defects in vegetative tissues (stunted plants with curly leaves and reduced flower size) due to gTT16 over-expression are enhanced in tt15 mutants when compared with wild-type plants.

Fig. S2. Arabidopsis seed immunohistolabelling using monoclonal antibodies targeting specific arabinogalactan proteins present in the cell wall of the testa, in tt16 and tt15 complementation experiments.

Fig. S3. Pattern of TT8 promoter activity in developing wild-type ovules and seeds revealed by the detection of GUS activity.

Table S1. Primers used in this study.

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**References**


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