

Update on *transparent testa* mutants from *Arabidopsis thaliana*: characterisation of new alleles from an isogenic collection

Ingo Appelhagen · Katharina Thiedig ·
Niclas Nordholt · Nina Schmidt · Gunnar Huep ·
Martin Sagasser · Bernd Weisshaar

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Abstract

Main conclusion We present a comprehensive overview on flavonoid-related phenotypes of *A. thaliana tt* and *tds* mutants, provide tools for their characterisation, increase the number of available alleles and demonstrate that *tds3* is allelic to *tt12* and *tds5* to *aha10*.

Flavonoid biosynthesis is one of the best-studied secondary metabolite pathways in plants. In the model system *Arabidopsis thaliana* it leads to the synthesis of three phenolic compound classes: flavonol glycosides, anthocyanins and proanthocyanidins (PAs). PAs appear brown in their

oxidised polymeric forms, and most *A. thaliana* mutants impaired in flavonoid accumulation were identified through screens for lack of this seed coat pigmentation. These mutants are referred to as *transparent testa* (*tt*) or *tannin-deficient seed* (*tds*). More than 20 mutants of these types have been published, probably representing most of the genes relevant for PA accumulation in *A. thaliana*. However, data about the genes involved in PA deposition or oxidation are still rather scarce. Also, for some of the known mutants it is unclear if they represent additional loci or if they are allelic to known genes. For the present study, we have performed a systematic phenotypic characterisation of almost all available *tt* and *tds* mutants and built a collection of mutants in the genetic background of the accession Columbia to minimise effects arising from ecotype variation. We have identified a novel *tt6* allele from a forward genetic screen and demonstrated that *tds3* is allelic to *tt12* and *tds5* to *aha10*.

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I. Appelhagen · K. Thiedig · N. Nordholt · N. Schmidt · G. Huep ·
M. Sagasser · B. Weisshaar (✉)
Department of Biology, Bielefeld University,
Universitätsstrasse 27, 33615 Bielefeld, Germany
e-mail: bernd.weisshaar@uni-bielefeld.de

I. Appelhagen
e-mail: appelha@cebitec.uni-bielefeld.de

K. Thiedig
e-mail: kthiedig@cebitec.uni-bielefeld.de

N. Nordholt
e-mail: nnordhol@cebitec.uni-bielefeld.de

N. Schmidt
e-mail: nschmidt@cebitec.uni-bielefeld.de

G. Huep
e-mail: ghuep@cebitec.uni-bielefeld.de

M. Sagasser
e-mail: sagasser@cebitec.uni-bielefeld.de

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Proanthocyanidins · T-DNA · Vanillin assay

Abbreviations

AGI	Arabidopsis Genome Initiative
Col	Columbia
DMACA	<i>p</i> -dimethylaminocinnamaldehyde
DPBA	Diphenylboric acid 2-aminoethyl ester
EGL3	ENHANCER OF GLABRA 3
MBW	MYB-bHLH-WD40 repeat protein
NFZ	Norflurazon
PA(s)	Proanthocyanidin(s)
PAP1	Production of Anthocyanin Pigment 1
PFG	Production of Flavonol Glycosides
<i>tds</i>	<i>tannin-deficient seed</i>
TLC	Thin-layer chromatography

tt *transparent testa*
 Ws Wassilewskija

Introduction

Among the dark brown coloured seeds of *Arabidopsis thaliana*, yellow seeded mutants are easily discovered. Until now, mutations in 18 genes have been characterised on the molecular level, which result in reduced, altered or absent seed coat pigmentation. The oldest term for this class of mutants is *transparent testa* (*tt*), which explains the morphological basis for the observed phenotype: impaired pigmentation makes the seed coat transparent and reveals the yellow colour of the underlying cotyledons (Bürger 1971; Koornneef 1981, 1990). Another term for the same class of mutants is *tannin-deficient seed* (*tds*; Abrahams et al. 2002) which alludes more to the chemical nature of the defect: lack of the dark brown pigmentation of wild-type seeds, which is due to the accumulation of condensed tannins or proanthocyanidins (PAs) in the seed coat. Defects in seed coat development or in the cells' ability to produce, modify or transport PAs can be detected by the naked eye as well as various staining techniques (as shown e.g. in Debeaujon et al. 2000). Acidic vanillin or *p*-dimethylaminocinnamaldehyde (DMACA) was used in screens to identify mutants with more subtle changes in pigmentation (Abrahams et al. 2002). Identification of PA mutants in *A. thaliana* is facilitated by the fact that all known enzymes of the central flavonoid pathway are encoded by single copy genes, which is not the case in the general phenylpropanoid pathway as well as for the genes encoding flavonol synthases (FLSs) or glycosyltransferases required for the formation of flavonol glycosides and anthocyanins.

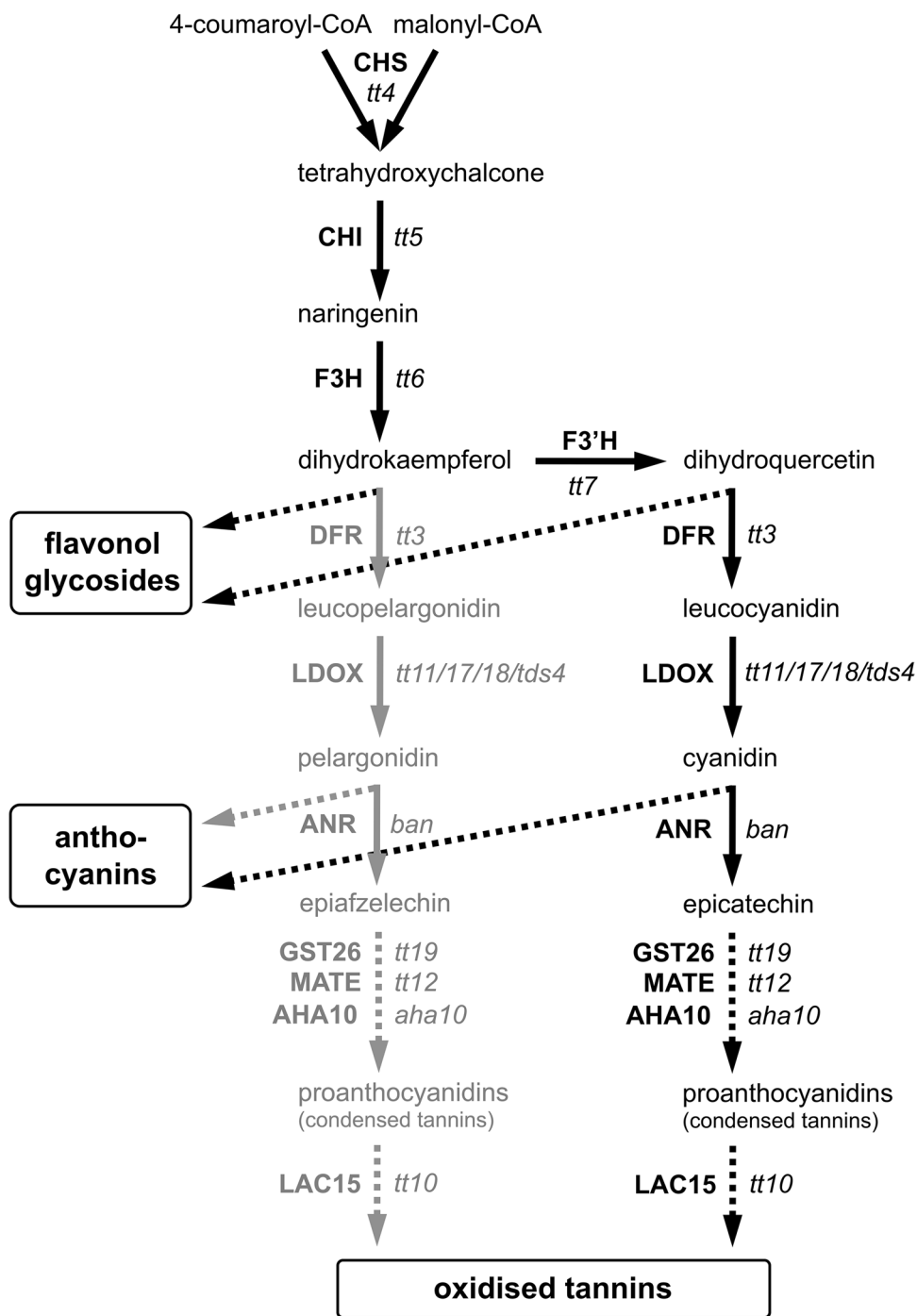
Many of the genes and enzymatic functions encoded by the *tt* loci are known (Fig. 1) and the enzymatic steps of the pathway in *A. thaliana* were comprehensively described in a recent review by Saito et al. (2013). However, open questions exist with regard to developmental control of pigment formation, the molecular function of some regulatory factors and genes involved in deposition and oxidation of the metabolites. Control of flavonoid biosynthesis mainly occurs at the level of transcription (Weisshaar and Jenkins 1998). Flavonol formation is regulated by the R2R3-MYB proteins PFG1 to 3 (Stracke et al. 2007, 2010), whereas expression of enzyme-coding genes required for anthocyanin and PA biosynthesis is controlled by a regulatory network involving the combined action of MYB, bHLH and WD40 repeat proteins (MBW). A ternary complex of MYB75/PAP1, BHLH042/TT8 or BHLH002/EGL3 and WD40/TTG1 is crucial for anthocyanin accumulation in young *A. thaliana* seedlings (Gonzalez et al. 2008; Appelhagen et al. 2011a) and a MYB123/TT2, TT8 and TTG1

complex is the major regulator of PA metabolism in seeds (Baudry et al. 2004). Recently, three additional MBW complexes were shown to contribute to the expression of PA biosynthesis genes in specialised tissues of the seed coat (Xu et al. 2013a). The molecular functions of WIP1/TT1, WRKY44/TTG2 and AGL32/TT16 are less clear. During seed development, pigment accumulation occurs in the innermost cell layer of the seed coat, the endothelium. In wild-type seeds, pigmentation is also found in two specialised areas at the base of the seed termed chalaza and micropyle (Debeaujon et al. 2003; Lepiniec et al. 2006; Fig. 2a, b). *TT16* encodes the MADS box protein Agamous-like 32 and influences PA biosynthesis by determining the identity of the endothelial layer within the ovule (Nesi et al. 2002). Mutants of *TT1* show a phenotype similar to *tt16* with PA accumulation in chalaza and micropyle but reduced and irregular PA spots in the endothelium (Sagasser et al. 2002). The WIP-type zinc finger protein encoded by *TT1* was found to interact with MBW MYB factors like PAP1 and TT2 (Appelhagen et al. 2011b) and TT1 and TT16 were recently shown to be involved in the regulation of *TT8* expression together with TTG2 (Xu et al. 2013b). *TTG2* encodes WRKY44, a factor involved in integument cell elongation, seed mucilage production as well as trichome formation in vegetative parts of the plant, whose expression is influenced by MBW complexes (Johnson et al. 2002; Garcia et al. 2005; Ishida et al. 2007).

In addition to the unresolved questions with regard to the exact roles of TT1, TT16 and TTG2 in the differential accumulation of PAs in different seed coat tissues during development, it is mainly the steps of transport and polymerisation which occur after the biosynthesis of epicatechin that remain to be elucidated (Fig. 1; Zhao et al. 2010). *TT19* for example encodes a glutathione *S*-transferase (Kitamura et al. 2004) but neither glutathionated cyanidin or epicatechin nor ABC transporter mutants with effects on anthocyanin or PA accumulation were detected in *A. thaliana* (Klein et al. 2006). Consequently, other roles of TT19 in PA monomer binding for protection and transport have been proposed (Mueller et al. 2000; Kitamura et al. 2010). In line with the hypotheses of these authors, Sun et al. (2012) found that TT19 can physically interact with and increase water solubility of cyanidin and cyanidin-3-*O*-glucoside and that it can localise to the tonoplast. Francisco et al. (2013) recently showed for the anthocyanin transporter ABCC1 from grape berries that it depends on and co-transporters glutathione, rather than anthocyanin-GSH conjugates.

A different class of transporters involved in PA accumulation is represented by *TT12*, which encodes a multidrug and toxic extrusion protein (MATE; Debeaujon et al. 2001). TT12 was found to transport epicatechin 3'-*O*-glucoside in yeast microsome assays (Zhao and Dixon 2009) but evidence

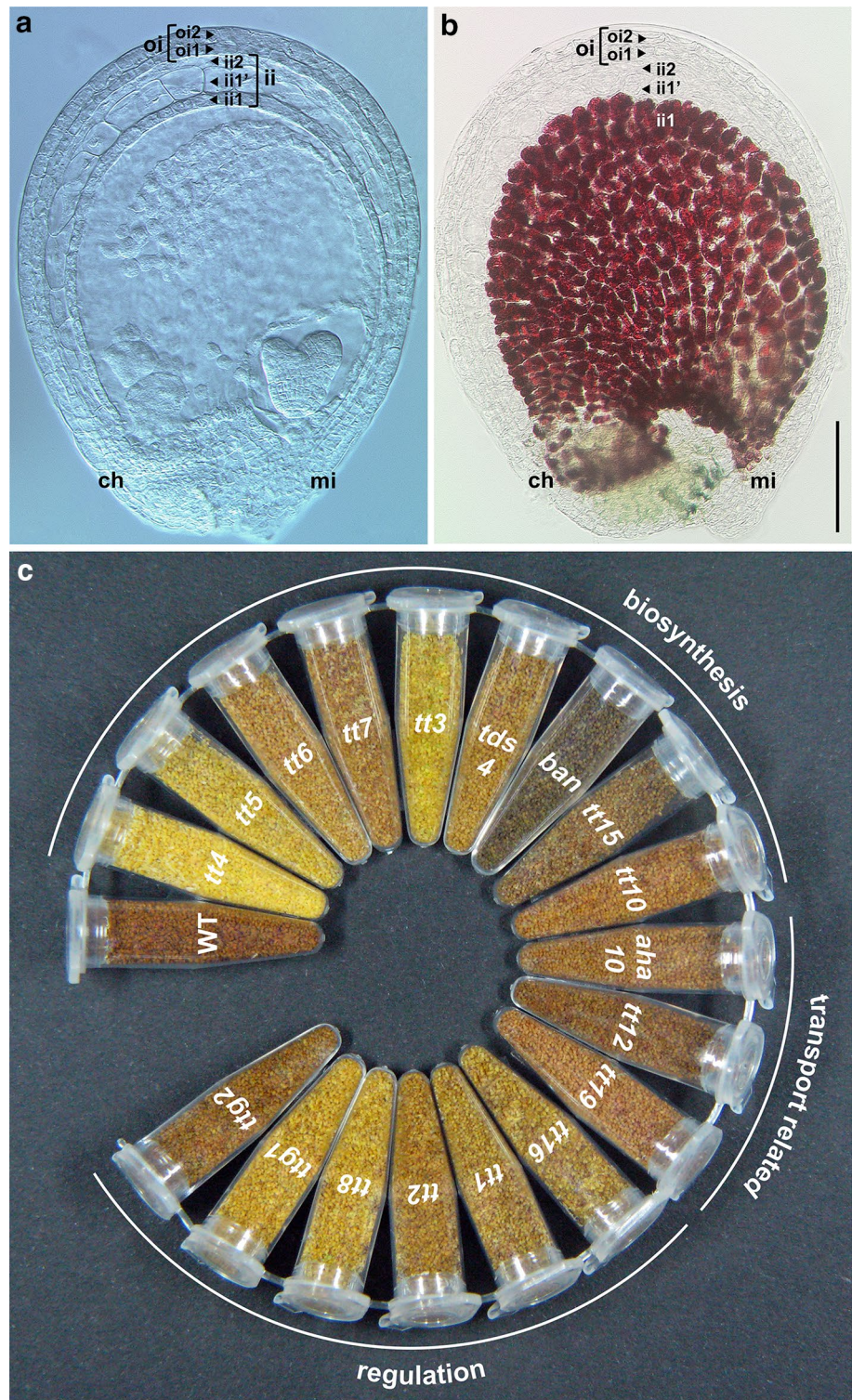
Fig. 1 Flavonoid biosynthesis in *Arabidopsis thaliana*. *Black arrows* illustrate the pathway in wild-type plants, alternative routes found in *f3'h/tt7* mutants are given in *grey*. *Dashed arrows* indicate multiple steps. For full names of gene symbols and enzymes see Table 1



for an epicatechin-specific glycosyltransferase, as detected in *Medicago* (Pang et al. 2008, 2013), or a glycosidase that would be required to cleave off sugar before condensation, is still missing (Routaboul et al. 2012). Consistent with TT12-mediated proton gradient-driven PA precursor transport into the vacuole, effects on seed colour were observed in mutants affected in vacuole acidification, which is the proposed function of the P-type ATPase AHA10 (Baxter et al. 2005) and its putative homologue PH5 from *Petunia hybrida* (Verweij

et al. 2008). Membrane-related defects were also proposed to explain the *tt* phenotype found in the *tt15* mutant, which is defective in a UDP-glucose:sterol-glucosyltransferase (DeBolt et al. 2009). This suggestion is remarkable in the light of the very recent findings by Brillouet et al. (2013), who proposed a chloroplast-derived organelle, named tanosome, for the production of condensed tannins, whose formation would require extensive alterations in the properties of various membranes. So far, the only known enzyme

Fig. 2 *transparent testa* mutants from *Arabidopsis thaliana*. **a** Structure of the seed coat. Cleared wild-type seed at early heart stage viewed with Nomarski optics. **b** Detection of PAs by vanillin/HCl treatment in a Col-0 wild-type seed of the same age as shown in **a**. *Red colour* indicates presence of PAs in the seed coat endothelium (ii1), in chalazal cells and micropylar ii2 cells. **c** Mature seeds of all characterised *transparent testa* (*tt*) mutants. Mutants are classified according to their defects in PA biosynthesis, transport and regulation. Same lines as analysed in Fig. 3. For details on presented alleles and abbreviations see Table 1. *oi* outer integument, *ii* inner integument, *ch* chalaza, *mi* micropyle. Bar 100 μ m



acting after biosynthesis of epicatechin is TT10, a laccase with polyphenol oxidase activity (Pourcel et al. 2005). Other enzymes, potentially required for condensation and oxidation, remain to be identified.

Isolation of novel and more detailed analysis of existing but uncharacterised *tt* or *tds* mutants may help to find

answers to the unresolved questions mentioned above. Some of the reported *tt* mutants have not been characterised in such detail as to clarify if they represent new loci or novel alleles of known *tt* genes. Thus, we have performed a systematic phenotypic characterisation of available *tt* and *tds* mutants. Previous comparisons of *tt* mutants

indicated significant variation in flavonoid content between *Arabidopsis* accessions (Routaboul et al. 2006, 2012). To minimise phenotypic differences between mutants arising from ecotype variation and thus increase comparability of the results, we built a mutant collection in the genetic background of the accession Columbia (Col-0; Fig. 2c). A similar approach was recently taken by Bowerman et al. (2012), albeit with a less comprehensive set of mutants. We present an isogenic mutant set covering 17 distinct *tt* loci, including 14 new alleles, which were isolated from public T-DNA transformant collections. The increased number of available alleles can be applied to analyse allelic series of mutants for a given locus. Together with the description of our isogenic *tt* mutant collection we provide a set of tools to efficiently characterise novel seed colour mutants that are identified in screens by simple and standardised phenotypic tests. We demonstrated the power and applicability of our approach by presenting a novel *tt6* mutant identified in a forward genetic screen and by analysing *tds1*, 2, 3, 5 and 6 which revealed that *tds3* is allelic to *tt12* and that *tds5* is allelic to *aha10*.

Materials and methods

tt collection setup and naming of new alleles

To set up a collection of *tt* mutants in Col-0 genetic background, the SimpleSearch database for GABI-Kat T-DNA insertion lines (<http://www.gabi-kat.de/simplesearch>; Rosso et al. 2003) was screened for FST hits in all described *tt* loci. The SALK T-DNA collection (Alonso et al. 2003) was used, if no suitable GABI-Kat allele was available. The genetic background of lines from both, SALK and GABI-Kat, is Col-0 (Bolle et al. 2013). Selected T2 lines were provided by GABI-Kat or T3 SALK lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). Exact insertion positions for the left border were determined in T2 plants (T3 for SALK lines) according to the confirmation process applied to all GABI-Kat lines. A detailed description for confirmation and genotyping of lines from both collections is given in Bolle et al. (2013). Additional information on GABI-Kat lines, including estimated T-DNA copy numbers, can be found in the SimpleSearch database (Kleinboelting et al. 2012). Homozygous T2 plants were identified by PCR genotyping and subsequently used for flavonoid analysis. Information on oligonucleotides used in this study is given in Supplementary Table S1. Newly identified alleles were numbered to the best of our knowledge in continuation of the existing nomenclature. Table 1 provides an overview of the essential characterisation for all lines representing the Col-0 collection as presented in Figs. 2 and 3. Table 1 also contains

additional alleles identified in the course of this study that are not included in the figures as they showed identical phenotypes, and the table gives evidence for the proposed numbering. The entire mutant set is available from NASC (the set number for the collection is N2105571).

tt15-1 sequence analysis

The *tt15-1* mutant in Col-2 background was originally isolated by Focks et al. (1999; NASC stock N799994). To identify alterations between *tt15-1* and Col-2 sequences, PCR products covering the whole coding sequence of At1g43620 including introns plus 1 kb of upstream sequence were generated on genomic DNA of both genotypes and subjected to sequencing. Oligonucleotides I219/009N were used to amplify and sequence PCR products containing the *tt15-1* mutation.

tds allelism tests

All *tds* lines analysed were kindly provided by Sharon Abrahams and Anthony Ashton. The *tds1*, *tds2* and *tds3-1* mutants were isolated from the Feldmann collection in Wasilewskija (Ws) background, *tds5* and *tds6* are from the Weigel activation tagging collection in Col-7 background (Abrahams et al. 2002). Allelism tests were done by fertilisation of *tds* mutants with pollen of *aha10-6* or *tt12-2* plants. Successful crosses were confirmed in F1 by testing for the heterozygous presence of the T-DNA inherited from the pollen donor. Complementation of the seed coat colour phenotype was analysed in F2 seeds gained from F1 plants whose testa is genetically F1. Both T-DNA border positions were determined in *tds5* by PCR as described for GABI-Kat alleles with the oligonucleotide pairs I388/036N for right border and 8409/I309 for left border. T-DNA border positions in *tds3-1* were addressed using several different oligonucleotides specific for At3g59030 in combination with F-LB102 as left border or F-RB as right border primer, which were successfully used in previous studies to identify T-DNA insertions, without any specific PCR result. PCR reactions also failed on *tds3-1* DNA with all oligonucleotide pairs spanning intron1 and the second half of exon1 of At3g59030. Oligonucleotide pair 045N/I263 was subsequently used to specifically detect the *tds3-1* allele in comparison to *tt12-2*.

tt6-5 analyses

The *tt6-5* mutant was identified as yellow seeded amongst T2 segregants of GABI-Kat line 352E12. Co-segregation of the yellow seed phenotype with the T-DNA insertion of GK-352E12 in At5g65620 was analysed by PCR and inspection of the colour of mature seeds of T2 plants. Candidate

Table 1 The Columbia *transparent testa* mutant collection

Gene product, locus, mutant name	Allele	Collection	Mutation type and insertion site	Genotyping		First characterised in
				Mutant	WT	
Chalcone synthase (CHS) At5g13930 <i>tt4</i>	<i>tt4-15</i>	GK-545D04	T-DNA 5'-UTR	PH39 8409	PH39 I247	This work
	<i>tt4-11</i>	SALK_020583	T-DNA exon 2	I273 LBb1.3	I273 I274	Buer et al. (2006)
Chalcone isomerase (CHI) At3g55120 <i>tt5</i>	<i>tt5-2</i>	GK-176H03	T-DNA exon 4	MR28 8409	MR28 I249	Rosso et al. (2003)
Flavanone 3-hydroxylase (F3H) At3g51240 <i>tt6</i>	<i>tt6-2</i>	GK-292E08	T-DNA 5'-UTR	MM70 8409	MM70 I250	Rosso et al. (2003)
	<i>tt6-5</i>	Isolated from GK-352E12	1 bp deletion exon 2	NP17 RS850	NP17 RS850	This work
Flavonoid 3' hydroxylase (F3'H) At5g07990 <i>tt7</i>	<i>tt7-6</i>	GK-349F05	T-DNA exon 4	ZZ76 8409	ZZ76 I253	Rosso et al. (2003)
	<i>tt7-7</i>	GK-629C11	T-DNA exon 1	04pd 8409	04pd I289	This work
Dihydroflavonol reductase (DFR) At5g42800 <i>tt3</i>	<i>tt3-1*</i>	NASC stock N84	Deletion	B023 B040		Shirley et al. (1992)
Leucoanthocyanidin dioxygenase (LDOX) At4g22880 <i>tt11, tt17, tt18, tds4</i>	<i>tds4-2</i>	SALK_028793	T-DNA exon 2	B078 LBb1.3	B078 B079	Abrahams et al. (2003)
	<i>tds4-4</i>	SALK_073183	T-DNA exon 2	B078 LBb1.3	B078 B079	
Anthocyanidin reductase (ANR) At1g61720 <i>ban, ast</i>	<i>ban-5</i>	SALK_040250	T-DNA intron 1	I276 LBb1.3	I276 I277	Bowerman et al. (2012)
UGT80B1 At1g43620 <i>tt15</i>	<i>tt15-3</i>	GK-337G08	T-DNA exon 13	09kt 8409	09kt I257	This work
	<i>tt15-4</i>	GK-556B06	T-DNA exon 13	09kt 8409	09kt I257	This work
	<i>tt15-5</i>	GK-016H07	T-DNA exon 14	71cs 8409	71cs I258	This work
Autoinhibited H⁺-ATPase 10 (AHA10) At1g17260 <i>aha10</i>	<i>aha10-6</i>	GK-170A07	T-DNA exon 2	I366 8409	I365 I368	This work
MATE family protein TT12 At3g59030 <i>tt12</i>	<i>tt12-2</i>	GK-797D03	T-DNA exon 2	MZ76 8409	MZ76 I263	Kitamura et al. (2010)
Glutathione S-transferase 26 (GST26) At5g17220 <i>tt14, tt19</i>	<i>tt19-8</i>	SALK_105779	T-DNA intron 2	I278 LBb1.3	I278 I279	Wangwattana et al. (2008)
Laccase-like 15 (LAC15) At5g48100 <i>tt10</i>	<i>tt10-7</i>	GK-146E10	T-DNA 5'-UTR	KS53 8409	KS53 I265	This work
	<i>tt10-8</i>	GK-026E11	T-DNA intron 2	01pd 8409	01pd H447	This work
Agamous-like 32 (AGL32) At5g23260 <i>tt16</i>	<i>tt16-4</i>	GK-685G11	T-DNA intron 1	25pj 8409	25pj I266	This work
	<i>tt16-5</i>	GK-962A05	T-DNA 5'-UTR	50lx 8409	50lx I267	This work

Table 1 continued

Gene product, locus, mutant name	Allele	Collection	Mutation type and insertion site	Genotyping		First characterised in
				Mutant	WT	
WIP1 zinc finger At1g34790 <i>tt1</i>	<i>tt1-3</i>	SALK_026171	T-DNA intron 1	S121 LBb1.3	S121 S142	Appelhagen et al. (2010)
MYB123 At5g35550 <i>tt2</i>	<i>tt2-5</i>	SALK_005260	T-DNA exon 3	I280 LBb1.3	I280 I281	Gonzalez et al. (2009)
BHLH042 At4g09820 <i>tt8</i>	<i>tt8-6</i>	GK-241D05	T-DNA intron 2	BW10 8409	BW10 I269	Rosso et al. (2003)
Transparent testa glabra 1 (TTG1) At5g24520 <i>ttg1</i>	<i>ttg1-21</i>	GK-580A05	T-DNA exon 1	ZZ85 8409	ZZ85 I270	This work
	<i>ttg1-22</i>	GK-286A06	T-DNA exon 1	I290 8409	I290 I291	This work
WRKY44 At2g37260 <i>ttg2</i>	<i>ttg2-5</i>	GK-409B01	T-DNA exon 4	07pl 8409	07pl I272	This work

All lines listed are in Col-0 background. In addition, *tt3-1* (in Landsberg *erecta* background, marked by an asterisk) is included to represent all known genes required for seed pigmentation. Mutants shown in Figs. 2 and 3 are given in bold. Additional mutants with identical phenotypes are included for completeness. GABI-Kat line *tt7-6*/GK-349F05 is listed as *tt7-3* in Bowerman et al. (2012). The mutant was renamed because *tt7-3* was already described as an allele from the Weigel collection identified by Abrahams et al. (2002). SALK_040250 was renamed to *ban-5* because *ban-4* was already identified as *ast* (Kitamura et al. 2004). Lines of the GABI-Kat collection are marked with the prefix GK. *ban*, *banyuls*; *ast*, *anthocyanin spotted testa*; *tt*, *transparent testa*; *ttg*, *transparent testa glabra*; *tds*, *tannin-deficient seed*

genes for allelism tests were determined by comparison of flavonoid phenotypes to known *tt* mutants. Allelism to *tt6* was tested by crossing *tt6-5* with *tt6-2*, as described for the *tds* mutants. The *TT6* locus was sequenced in *tt6-5* with a similar approach as described for *tt15-1*. Oligonucleotides NP17/RS850 were subsequently used to amplify and sequence PCR products containing the *tt6-5* deletion.

Histochemical assays and microscopy

A Leica DM5500 microscope equipped with phase contrast and automated differential interference contrast (DIC) condenser was used for bright field images and Leica filter cube A (340–380 nm excitation filter, 425 nm long-pass suppression filter) for epifluorescence applications. Pictures were taken with Diskus digital imaging software (Technisches Buero Hilgers, Koenigswinter, Germany).

Flavonol glycosides in 5-day-old seedlings were stained using diphenylboric acid 2-aminoethyl ester (DPBA, 0.25 % (w/v), 20 % (v/v) ethanol, 0.01 % (v/v) Triton X-100) and visualised by UV light according to Stracke et al. (2007). Seedlings were grown on filter paper soaked with 3 ppm of the bleaching herbicide norflurazon (NFZ, Supelco PS-1044) to avoid unwanted background fluorescence from chlorophyll.

Anthocyanin accumulation was monitored in 5-day-old seedlings grown on NFZ as described above, but supplemented with 4 % sucrose to induce anthocyanin

accumulation. NFZ bleaching was used to improve visibility of the purple anthocyanins. Control plants grown on half strength MS media containing 4 % sucrose without NFZ showed comparable anthocyanin pigmentation.

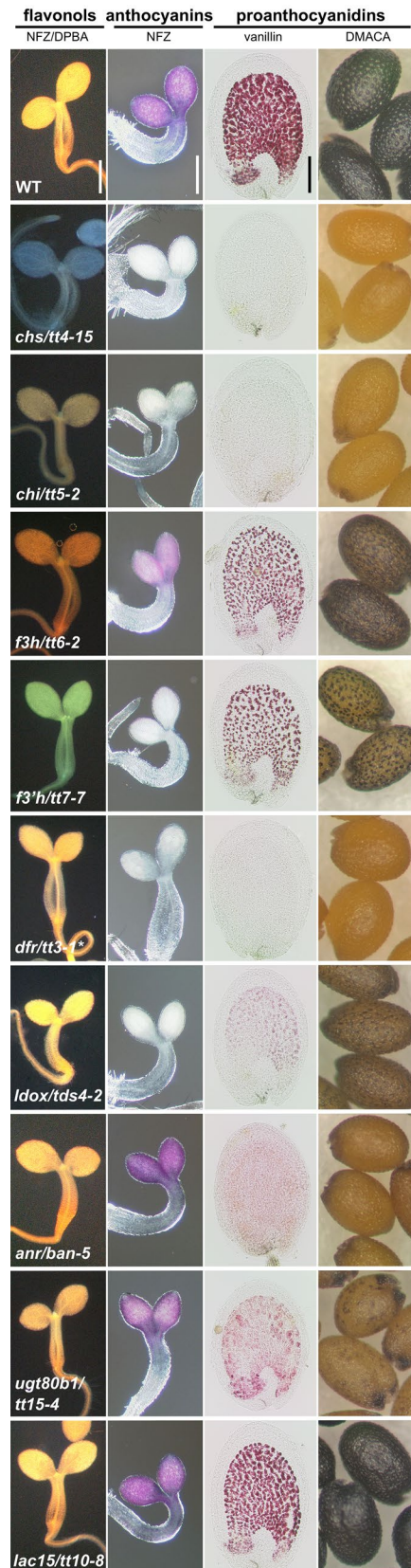
PA accumulation was addressed using acidic vanillin in developing seeds and DMACA for mature seeds as described previously (Debeaujon et al. 2000; Abrahams et al. 2002). Vanillin/HCl treatment leads to a red adduct; the DMACA reaction causes a blue staining in immature seeds and leads to a black appearance of mature seeds. Both dyes are aromatic aldehydes that are specific to a narrow range of flavanols and dihydrochalcones (Sarkar and Howarth 1976). As the latter are missing in *A. thaliana*, the assays are indicative of flavan-3,4-diols (leucoanthocyanidins), flavan-3-ols (like epicatechin) as well as polymeric PAs, in this work collectively named vanillin stainable PAs or PA intermediates.

Results and discussion

Effects of *tt* mutations on the accumulation of the three classes of flavonoids in *A. thaliana* have been presented previously (e.g. in Shirley et al. 1995; Debeaujon et al. 2000; Bharti and Khurana 2003; Bowerman et al. 2012). However, growth conditions, analytical methods and ecotype backgrounds of the respective partial sets of mutants analysed are not comparable between these

Fig. 3 Flavonol glycoside, anthocyanin and proanthocyanidin accumulation in *transparent testa* mutants. *Left column* DPBA-stained seedling visualised under UV light. *Orange* quercetin derivatives, *green* kaempferol derivatives, *blue* sinapates. *Second column* norflurazon-bleached seedlings grown on sucrose for induction of anthocyanin biosynthesis. *Third column* vanillin/HCl-treated immature seeds as presented in Fig. 2. *Fourth column* DMACA-stained mature seed. *Dark brown/black colour* indicates presence of PAs. For untreated mature seeds of all lines see Fig. 2. **a** Col-0 wild type and mutants of structural flavonoid biosynthesis genes. The presented *dfr* mutant carries the *tt3-1* allele in Landsberg *erecta* background (marked with *asterisk*). All other mutants are in Col-0 background. **b** Mutants in genes related to flavonoid transport. **c** Mutants of regulatory loci. The shown *myb123/tt2* mutant (marked with *two asterisks*) carries the only available *tt2* allele in Col background and does not represent the *myb123/tt2* NULL mutant phenotype (see Fig. S2). *Bars* 0.5 mm for seedlings, 100 μ m for vanillin/HCl- and DMACA-treated seeds

a structural mutants



b transport related mutants



c regulatory mutants



studies. Significant variation in secondary metabolite contents between *A. thaliana* accessions has been described for example by Teng et al. (2005) for anthocyanins or by Routaboul et al. (2012) for seed flavonols and PAs. To provide a homogeneous basis for further comparative studies, we set out to create a complete set of mutants in the Col-0 accession (see Table 1 for AGI codes, protein names and abbreviations) and performed systematic analyses on seeds and plants grown under uniform conditions. Figures 2 and 3 summarise the results of the phenotypic characterisation of *tt* and *tds* mutants with respect to their seed colours as well as flavonol, anthocyanin and PA contents.

Flavonol accumulation in *tt* mutant seedlings

Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester, DPBA) is widely used to visualise flavonols, either as a spray reagent on TLC plates or for localisation *in planta*. Reaction with DPBA confers green fluorescence to kaempferol glycosides whereas quercetin glycosides appear orange after treatment (Sheahan and Rechnitz 1992). Both compounds accumulate in light grown *A. thaliana* wild-type seedlings, resulting in a combined fluorescence of saturated orange (Peer et al. 2001; Fig. 3). In our collection of mutants, which originated from screens for altered seed colour, only few lines showed alterations of flavonol staining in seedlings. Most prominent was the blue fluorescence of *chs/tt4-15* seedlings, which is caused by sinapic acid derivatives (Sheahan and Rechnitz 1993). The characteristic colour is visible due to the complete lack of flavonols in this mutant that is defective in the first enzyme of flavonoid biosynthesis, chalcone synthase (Feinbaum and Ausubel 1988; Koornneef 1990). Seedlings of *chi/tt5-2* had a dull yellow appearance under UV after DPBA treatment, which indicates the presence of some flavonols that was already noted by Shirley et al. (1995), Peer et al. (2001) or Routaboul et al. (2006). As CHI catalyses the formation of naringenin chalcone from tetra-hydroxychalcone, its presence in *chi/tt5* may be due to spontaneous isomerisation (Jez et al. 2000; Jez and Noel 2002). Interestingly the amounts of this pathway intermediate seem not to be sufficient for the formation of visible anthocyanins or PAs stainable by vanillin. Seedlings of *f3h/tt6-2* gave a characteristic red fluorescence after treatment with DPBA, which was clearly distinct from the orange observed in wild type. Owens et al. (2008) proposed that in the absence of F3H activity, F3H may convert naringenin to eriodictyol in *f3h/tt6* plants. Eriodictyol can be reduced by DFR and lead to the formation of 3-desoxyflavonoids, which are normally not found in *A. thaliana*, but could cause the red fluorescence observed in *f3h/tt6-2* (Sheahan et al. 1998; Owens et al. 2008). Green fluorescence was found in *f3'h/tt7-7* seedlings after DPBA treatment indicating the presence of kaempferol derivatives

only. This finding is in agreement with the fact that lack of the cytochrome-P450-dependent monooxygenase F3H in the mutant precludes biosynthesis of quercetin glycosides (Fig. 1; Koornneef et al. 1982; Schoenbohm et al. 2000). All other mutants analysed did not show visible differences to wild type with respect to flavonol accumulation, as they are either seed coat specific or affected in steps of flavonoid biosynthesis specifically leading to anthocyanins or PAs. However, when characterising novel yellow seeded mutants, DPBA staining can be used to efficiently determine if they are affected in one of the general steps of flavonoid backbone formation from CHS to F3H.

Anthocyanin accumulation in *tt* mutant seedlings

Mutants in general steps of flavonoid backbone formation are also impaired in anthocyanin accumulation. Whereas seedlings of *chs/tt4-15* and *chi/tt5-2* completely lacked anthocyanins, they were visible in *f3h/tt6-2*. Reduced but detectable anthocyanins were described previously for *tt6-1* (Shirley et al. 1995; Albert et al. 1997) and are discussed in a later section. No pigmentation was visible in *f3'h/tt7-7* seedlings under the growth conditions used. However, it is known that *tt7* is capable of producing anthocyanins as derivatives of pelargonidin instead of cyanidin, as expected (Fig. 1; Koornneef et al. 1982). In addition to the four genotypes mentioned above, the mutants *tt3-1*, *tds4-2*, *tt19-8*, *tt8-6* and *ttg1-22* showed alterations in anthocyanin contents. The *TT3* locus encodes dihydroflavonol 4-reductase (DFR; Shirley et al. 1992), which represents a branchpoint in the flavonoid pathway, separating flavonol biosynthesis from anthocyanin- and PA-specific steps. The latter compounds are therefore not present in knock out plants (Fig. 3; Shirley et al. 1995; Routaboul et al. 2006). Unfortunately, no complete null allele for *dfr/tt3* is available in the accession Col-0. All Col-0 alleles isolated so far carry T-DNA insertions in the *DFR* promoter and either do not show a mutant phenotype (GK-212F02, SALK_099848; Bowerman et al. 2012) or are not null (GK-295C10; Fig. S1). For this reason the classical Landsberg allele *tt3-1* (Koornneef 1990) is shown in Fig. 3 and Fig. S1 to illustrate the phenotype of a full knock out.

Several mutants exist for leucoanthocyanidin dioxygenase (LDOX), also known as anthocyanidin synthase (ANS), and the glutathione *S*-transferase GST26, encoded by the *TT19* locus (Kitamura et al. 2004). Although both genes are essential for anthocyanin accumulation in plants (Fig. 3), mutant alleles were mainly identified in seed colour screens (e.g. *ldox* as *tds4*, *tt11*, *tt17* and *tt18*; Appelhagen et al. 2011a and references therein). The *TTG1* and *TT8* loci encode regulatory factors. WD40/TTG1 is an essential part of MBW complexes regulating, among other processes, anthocyanin accumulation in vegetative parts of the plant as well as

PA accumulation in the seed (Koornneef 1981; Walker et al. 1999; Baudry et al. 2004). A lack of TTG1 therefore results in seedlings without anthocyanins. BHLH042/TT8 also acts in MBW complexes and is absolutely required for PA accumulation in seeds as complex partner of TTG1 and MYB123/TT2. However, in anthocyanin regulatory complexes with TTG1 and MYB75/PAP1, TT8 can be replaced by the partly redundant factor BHLH002/EGL3, provided that it is expressed. This explains the reduced but detectable anthocyanin accumulation observed in seedlings of *bhlh042/tt8-6* plants (Appelhaagen et al. 2011a). All remaining mutants in the collection did not show alterations in flavonol and anthocyanin accumulation in vegetative parts of the plants and can thus be classified as affected in seed-specific genes for PA biosynthesis.

Seed coat pigmentation patterns of *tt* mutants

Differences in PA contents among mature *tt* and *tds* seeds or in comparison to wild type are visible to the naked eye (Fig. 2). However, careful investigation of pigment accumulation during seed development has proven useful to unravel the mechanisms involved. Mutants for anthocyanidin reductase (ANR), the first step specific for PA biosynthesis, for example do not produce seeds with a *tt* phenotype but are even darker than wild type (Fig. 2). In this case, inspection of immature mutant seeds revealed accumulation of anthocyanins in seed coats, which do not occur in this tissue in wild type. This phenotype indicated a redirection in the flux through the flavonoid biosynthetic pathway from PAs to anthocyanins, which was later found to be blocked at ANR in the *ban* mutant (Fig. 1; Devic et al. 1999; Xie et al. 2003). Detailed analysis of seed pigmentation was also key to understand the *tt10* mutation, which results in light brown seeds directly after harvest that darken strongly during storage. As brown seed colour requires oxidative polymerisation of PAs, delayed browning in *tt10* seeds could be explained once the mutant was found to be defective in *LAC15*, encoding an enzyme with polyphenol oxidase activity (Pourcel et al. 2005). These two examples illustrate the importance of a detailed analysis of seed pigmentation patterns over time. For this reason we applied different staining techniques to young and mature seeds, respectively, to characterise our collection. Acidic vanillin was used to visualise accumulation of epicatechin and soluble PAs in seeds carrying embryos of late globular to early heart stage. This developmental stage was chosen, because all relevant cell layers are fully developed until then but no oxidised PAs are visible yet. During maturation a large proportion of PAs gets insoluble by polymerisation, oxidation and reactions with polysaccharides, proteins and other phenolics (Routaboul et al. 2006). Differences in pigmentation among genotypes in mature seeds were visualised using acidic DMACA.

Using this approach, *chs/tt4-15*, *chi/tt5-2* and *dfr/tt3-1* were found to be completely devoid of PAs as expected (Fig. 3). The ectopic anthocyanin accumulation in *anr/ban-5* seed coats described above resulted in a diffuse red staining after vanillin treatment and in light brown seeds after DMACA (Fig. 3), each of which confirmed the absence of PAs in this mutant. Further mutants that were easily identified by DMACA staining include *tt1-3* and *tt16-5*. Both resulted in characteristic stainable chalaza and micropyle together with irregular spots in the endothelium. These two mutants are defective in seed coat-specific regulators (Nesi et al. 2002; Sagasser et al. 2002). Vanillin stainable spots in the endothelium have been observed in several mutant alleles of *wip1/tt1* and therefore do not indicate leakiness of the mutant but are part of the phenotype (Appelhaagen et al. 2011b). The situation is different in the *myb123/tt2* allele presented here, which is the only one available in the genetic background of the ecotype Columbia. The spottiness in vanillin as well as DMACA staining observed in *tt2-5* (Fig. 3b and S2 c, d) is not seen in the classical allele *tt2-1*, which is in Landsberg *erecta* background (Fig. S2 c, d). Sequence characterisation of *tt2-5* revealed a T-DNA insertion close to the end of exon 3 of *MYB123/TT2*. This insertion late in the ORF results in a premature stop codon and leads to reduced transcript levels (Fig. S2b). However, the allele is clearly not NULL (Fig. S2c).

Another mutant with spotty vanillin and DMACA signals was *tt15-4*. The *TT15* locus has been shown to encode UGT80B1, a UDP-glucose:sterol-glucosyltransferase (DeBolt et al. 2009). For our study we also analysed the *tt15-1* allele originally described in Focks et al. (1999) on DNA sequence level. This allele is in Col-2 background and we therefore confirmed by sequencing that genomic DNAs of Col-2 and Col-0 are identical from ATG to stop of *UGT80B1*. In *tt15-1*, we identified a point mutation in the third exon of *UGT80B1* that leads to a premature stop codon most likely resulting in a complete loss of function (Fig. 4d). In accordance with the initial characterisation by Focks et al. (1999), no obvious phenotypic differences to wild type were observed in the mutant with respect to flavonols and anthocyanins. PA accumulation in seed coats, however, was altered in all relevant tissues and resulted in irregular vanillin staining (Fig. 4a, e). DeBolt et al. (2009) describe a reduced content of (acetyl)sterol-glycosides in the mutant resulting in additional pleiotropic effects besides altered seed pigmentation. As sterol-glycosides are compounds of lipid membranes it is tempting to speculate that lack of these may lead to the observed mislocalisation of PAs in *tt15* via effects on membrane properties resulting in altered transport (Pourcel et al. 2007). The recent work by Viotti et al. (2013) can be seen in line with this hypothesis as the authors propose that provacuole formation occurs in a subdomain of the smooth ER that differs in

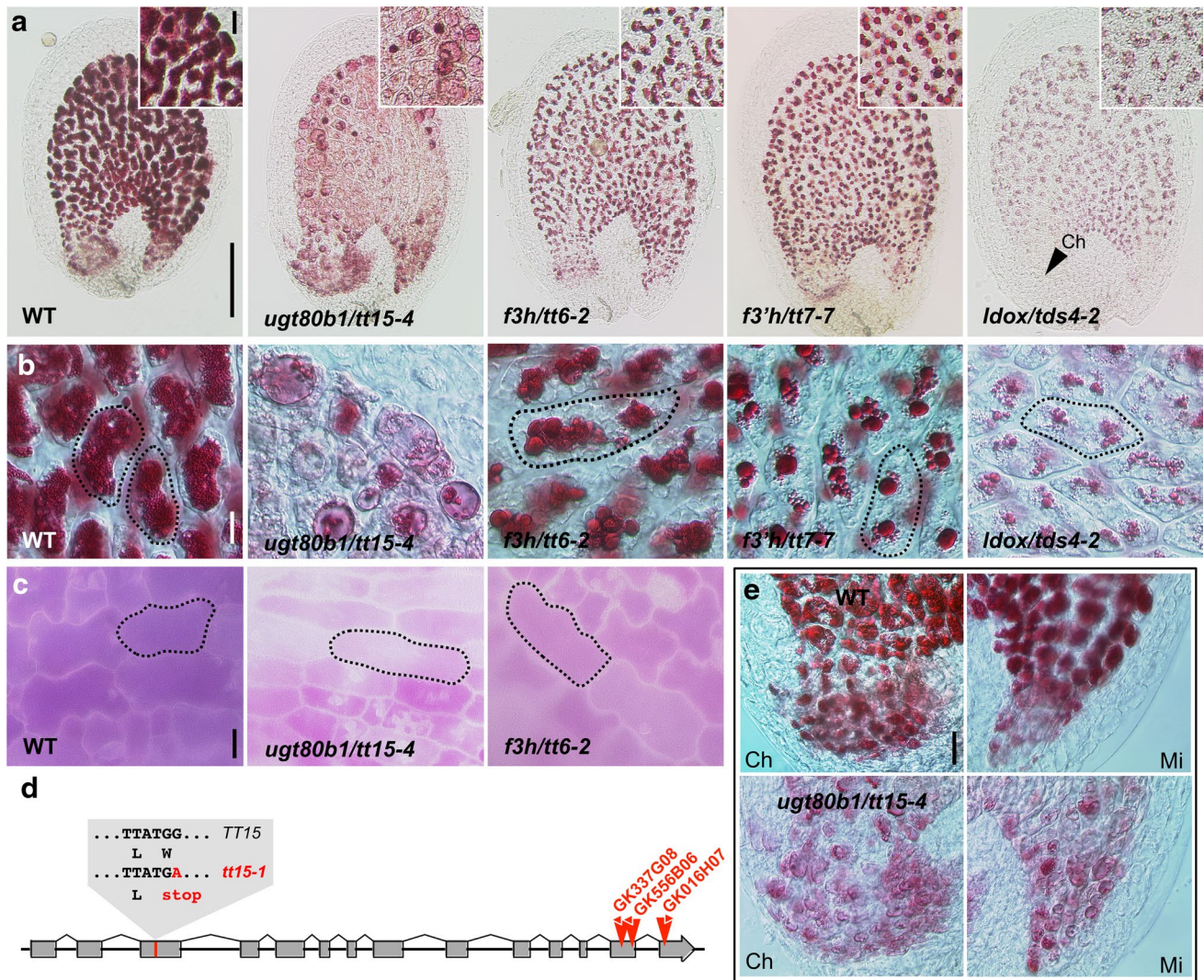


Fig. 4 Proanthocyanidin accumulation in NULL mutants of structural flavonoid biosynthesis genes. **a** Vanillin/HCl-treated immature seeds as shown in Fig. 2. Genotypes as indicated. **b** Endothelial cells of the same lines as shown in **a** with higher magnification. Single cells are outlined with dotted lines. Only wild-type cells show large central vacuoles stained by vanillin/HCl. **c** Anthocyanin accumulation in norflurazon-bleached seedlings. Cells show anthocyanin-filled purple central vacuoles and do not reflect the aberrant PA accumulation pattern in testa cells. **d** Gene model of *tt15* alleles characterised

in this study. Exons are shown as open boxes. T-DNA insertions are marked as red triangles. Left border positions are marked with small triangles. A point mutation in *tt15-1* (red dash) causes a premature stop codon in exon 3. **e** Vanillin/HCl-treated seeds as shown in **a** with focus on chalaza and micropyle. The base of *tt15* seeds appears dark (compare with DMACA picture in Fig. 3) but also shows aberrant pigmentation in the vanillin assay. Bars 100 μm for whole seed and 20 μm for close-up pictures in **a**; 10 μm in **b** and **c**; 20 μm in **e**

lipid composition. If such ER subdomains exist and if they are altered in *tt15* remains to be shown.

Defects in biogenesis of the central vacuole have also been described for all *tt* mutants involved in PA transport processes including *tt19*, *tt12* and *aha10* (Baxter et al. 2005; Kitamura et al. 2010). Our vanillin and DMACA tests also revealed aberrant staining patterns for *f3h/tt6-2*, *f3'h/tt7-7* and *ldox/tds4-2* (Fig. 3). In developing *f3h/tt6-2* seeds, vanillin treatment yielded red staining in all cell types that contain PAs in wild type. However, staining was reduced compared to wild type and did not fill the

cells entirely as if the central vacuoles were fragmented or not fully developed (Fig. 4a, b). Vacuole defects were not observed in hypocotyl cells accumulating anthocyanins (Fig. 4c) but in the PA-containing cells of the endothelium as well as in chalaza and micropyle and resulted in a dark speckled appearance of mature *f3h/tt6-2* seed coats after DMACA treatment. Flavanone 3-hydroxylase (F3H) belongs to the class of 2-oxoglutarate-dependent dioxygenases. The classical *tt6-1* allele encodes a protein that lacks a carboxy-terminal fragment containing conserved amino acid positions essential for Fe(II) binding (Owens

et al. 2008). Protein variants carrying mutations in these positions exhibited no F3H activity in vitro (Lukacin and Britsch 1997), suggesting that flavonoids observed in *tt6-1* should not result from an F3H protein with residual activity. Instead it was proposed that other 2-oxoglutarate-dependent dioxygenases could also have F3H function (Owens et al. 2008). Such multifunctionality of dioxygenases was discussed for other steps in flavonoid biosynthesis (Martens et al. 2010) and has been shown by Stracke et al. (2009) for FLS1 and LDOX. The pigments detected in *tt6* seeds may therefore result from activities of these co-expressed enzymes, whereas pigments in *ldox/tds4-2* seeds (Figs. 3, 4) may be due to FLS1 or F3H.

Epicatechin is the main building block for PA polymerisation in wild-type seeds (Xie et al. 2003). PAs extracted from *f3h/tt7* seeds yield pelargonidin instead of cyanidin after hydrolysis, indicating pigmentation by polymers based on epiafzelechin (Abrahams et al. 2002; Routaboul et al. 2006). Total PA content in *tt7* seeds is reduced when compared to wild type, potentially due to reduced affinity of one or several of the PA-specific enzymes for intermediates that lack 3'-hydroxylation (Abrahams et al. 2002; Routaboul et al. 2006). DMACA staining of *f3h/tt7-7* resulted in seeds with a dotted appearance as already shown by (Abrahams et al. 2002; Fig. 3). Vanillin staining revealed a clearer and finer fragmentation of the vacuoles as observed for *f3h/tt6-2* in cells of endothelium, chalaza and micropyle (Fig. 4). Such structures have not been described for these mutant genotypes before and may indicate a similar mechanism leading to their formation. Small vesicular structures were also found in the endothelium, but not the chalaza, of *ldox/tds4-2* (Fig. 4). Using membrane- and vacuole-specific dyes it was shown that these are rather compartments surrounded by membranes (Abrahams et al. 2003) than subvacuolar inclusions as observed in anthocyanin-containing vacuoles of cotyledons (Pourcel et al. 2010). In *gst26/tt19* mutants, a TT12-GFP fusion protein localising to the tonoplast was found to co-localise with typical vanillin stainable structures (Kitamura et al. 2010). Although LDOX and TT19 act in both, anthocyanin as well as PA biosynthesis, vacuole defects are only observed in PA accumulating but not in anthocyanin-containing tissues (Fig. 4). It was therefore proposed that PA intermediates themselves could be involved as signals in vacuole biogenesis or maintenance (Abrahams et al. 2003). Why accumulation of the same intermediates that leads to defective vacuoles in *ldox/tds4* seed coats does not alter vacuole formation in vegetative tissues, however, needs to be clarified.

Efficient phenotypic characterisation allows directed analysis of *tt* and *tds* mutants

Having established the described methods for flavonoid-specific staining as well as the *tt* mutant collection

characterised with these as a reference framework, we applied our approach to analyse a mutant line that was discovered in the GABI-Kat population based on its phenotype. In the course of a forward genetic screen, yellow seeds were found in the progeny of GABI-Kat line 352E12. Genotyping by PCR and evaluation of the colour of mature seeds of T2 plants revealed no co-segregation of the yellow seed phenotype with the T-DNA insertion of GK-352E12 in At5g65620. Detailed analysis of flavonoid accumulation in the mutant and comparison to the known *tt* mutants led us to the assumption that *F3H/TT6* was the most likely gene to be affected in the mutant. Test crosses to *tt6-2* yielded F1 plants producing yellow seeds (Fig. S3), indicating that *F3H/TT6* was defective in both parents. Sequencing various PCR products from GK-352E12 finally revealed a deletion of one bp at position 709 on genomic level relative to the ATG start codon. The resulting frame shift and subsequent premature stop codon change and truncate the sequence in the region encoding the iron-dependent oxygenase domain (InterPro: IPR005123, containing Pfam motif 03171) including the conserved Fe²⁺ coordinating residue His275 and thus very likely lead to a complete NULL. In continuation of the existing nomenclature, the novel allele was named *tt6-5*.

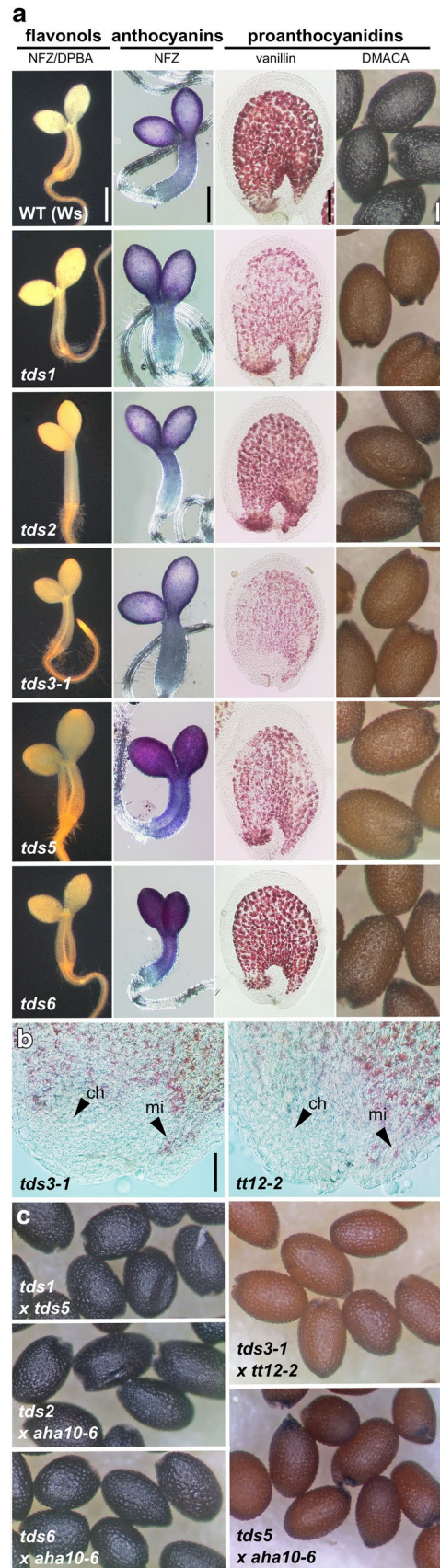
Encouraged by this result we also applied the described strategy to the set of *tds* mutants for which no molecular functions were known so far. The *tds* mutants 1, 2, 3, 5 and 6 showed no prominent alterations in flavonol and anthocyanin contents, when compared to the corresponding wild types (Ws for *tds1*, 2 and 3 and Col-7 for *tds5* and 6; Fig. 5a). Treatment with vanillin/HCl, however, revealed a weak and irregular red colouration of endothelium and micropylar cells in young *tds3* seeds with no signals in the chalazal area. As this phenotype was most comparable to *tt12-2* (Fig. 5b; Debeaujon et al. 2001), we performed testcrosses of *tds3-1* to *tt12-2* and analysed mature seeds from F1 plants. No complementation was observed based on the results from DMACA staining (Fig. 5c), which indicated that *tds3-1* was allelic to *tt12*. Using several combinations of T-DNA and gene-specific primers, we were not able to identify a T-DNA insertion at the *tt12* locus At3g59030 in *tds3-1* by PCR. Thus, we tried to determine the DNA sequence of the entire genomic region. Several primer combinations that were successfully used in wild type to amplify a region containing the 3'-end of the first exon together with the first intron failed on DNA isolated from *tds3-1*. Given the fact that the T-DNA insertion in *tt12-1* was located in the same region at the beginning of intron 1 (Debeaujon et al. 2001) and that *tt12-1* as well as *tds3-1* originated from the Feldmann collection, it is tempting to speculate that both mutants in fact may contain the same allele. In the remaining *tds* mutants 1, 2, 5 and 6 we observed a combination of reduced vanillin stainable PAs

Fig. 5 Phenotypes of *tannin-deficient seed (tds)* mutants and allelism tests. **a** Flavonol glycoside, anthocyanin and PA accumulation as presented in Fig. 2. The background of *tds5* and 6 is Col-7, which is identical to Col-0 (see Fig. 3). **b** Bases of *tds3-1* and *tt12-2* seeds in higher magnification. Chalazal cells of both mutants show no vanillin signals. **c** DMACA-stained mature F2 seeds (with testa from F1 mother plant) of crosses as indicated. Seeds of *tds3-1* × *tt12-2* and *tds5* × *aha10-6* crosses show PA deficiency. *ch* chalaza, *mi* micropyle. Bars 0.5 mm for seedlings and 100 μm for vanillin/HCl- and DMACA-treated seeds in **a**; 50 μm in **b**

in endothelium resulting in lighter colour after DMACA staining together with a clearly pigmented chalaza that resembled the phenotype of *aha10-6* (Figs. 3, 5a). We performed systematic test crosses and observed no complementation in *tds5* × *aha10-6* progeny (Fig. 5c). Using PCR with gene- and T-DNA-specific primers and subsequent sequencing of the PCR products we found that *tds5* contains a T-DNA insertion in intron 19 of *AHA10*. Our identification of *tds5* as novel *aha10* mutant increases the number of available alleles to address open questions with regard to AHA10 function in flavonoid transport.

Conclusions and outlook

The availability of a comprehensive set of mutants in nearly all aspects of flavonoid biosynthesis makes *A. thaliana* an invaluable tool for genetic and biochemical elucidation as well as for biotechnological manipulation of this important pathway in plants. As a basis for further investigations, we have built and systematically characterised a collection of *tt* mutants in the genetic background of the accession Col-0. Our findings in general confirmed characterisations of other alleles of the respective *tt* or *tds* mutants. In addition, for several mutants we observed features that were not described previously. The combination of visible phenotypic differences with staining techniques as presented here yields a nearly unique combination of features for every *tt* mutant known so far. Placing a newly identified mutant with such a phenotype into this grid allows efficient mutant classification, tentative identification of candidate genes for sequencing and reduces the number of required crosses in allelism tests. We have demonstrated the power of this approach by identifying the novel *f3h* allele *tt6-5*, which represents the first mutant that resulted from a forward genetic screen in the GABI-Kat collection. Additional T-DNA insertion lines isolated based on altered seed colour are currently being characterised. Furthermore, we showed that *tds3* is allelic to *tt12* and that *tds5* is allelic to *aha10*. Since another *aha10* allele may be represented by *tt13* (own results and mentioned in Saito et al. 2013) the list of



published seed colour mutants to be characterised could be reduced to *tt9*, which was described by Koornneef (1990) and mapped by Shirley et al. (1995), as well as the *tds* mutants 1, 2 and 6. We hope that the collection presented here will be a valuable resource to address open questions in flavonoid biosynthesis, transport, polymerisation and oxidation e.g. by functional analyses which require crosses with other ecotypes or characterisation of allelic series of mutants.

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Conflict of interest The authors declare that they have no conflict of interest.

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