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RESEARCH PAPER

# Architectural phenotypes in the *transparent testa* mutants of *Arabidopsis thaliana*

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## Abstract

**Flavonoids are low molecular weight secondary plant metabolites with a myriad of functions. As flavonoids affect auxin transport (an important growth-controlling hormone) and are biologically active in eukaryotes, flavonoid mutants were expected to have undescribed architectural phenotypes. The *Arabidopsis thaliana transparent testa* (*tt*) mutants are compromised in the enzymatic steps or transcriptional regulators affecting flavonoid synthesis. *tt* mutant seedlings were grown on hard-slanted agar (a stress condition), under varying light conditions, and in soil to examine the resulting growth patterns. These *tt* mutants revealed a wide variety of architectural phenotypes in root and aerial tissues. Mutants with increased inflorescences, siliques, and lateral root density or reduced stature are traits that could affect plant yield or performance under certain environmental conditions. The regulatory genes affected in architectural traits may provide useful molecular targets for examination in other plants.**

**Key words:** Aglycone, *Arabidopsis*, auxin, ethylene, flavonoid, plant architecture.

## Introduction

Flavonoids are secondary metabolites found throughout the plant kingdom. They have diverse roles in plants and animals. In plants, flavonoids are allelopathic (Bais *et al.*, 2004), modulate reactive oxygen levels (reviewed in Taylor and Grotewold, 2005), inhibit auxin transport (reviewed in Peer and Murphy, 2007), act in defence (Treutter, 2005), provide flower colouring (Mol *et al.*, 1998), are required for pollen viability in some species (Coe *et al.*, 1981; Mo *et al.*, 1992; Taylor and Jorgenson, 1992), signal to symbiotic organisms (Redmond *et al.*, 1986; Djordjevic *et al.*, 1987; Wasson *et al.*, 2006), and afford UV protection (Li *et al.*, 1993). Flavonoids may exert their effects indirectly, for example, through the modulation of plant hormones or reactive oxygen species, although direct effects are possible. The evidence for direct affects comes from studies with pollen fertility. Adding the aglycone kaempferol to infertile petunia pollen devoid of flavonoids rapidly restores fertility (Mo *et al.*, 1992) and affects gene transcription (Guyon *et al.*, 2000). Flavonoids also have other roles in plants. They modulate the extent of somatic embryogenesis (Imin *et al.*, 2007) and accumulate in the progenitor cells of root organs in legumes (Morris and Djordjevic, 2006; Mathesius *et al.*, 1998, 2000). It has recently been shown that *Arabidopsis* seedlings are capable of selectively taking up exogenously applied aglycones, and that the synthesized flavonoid compounds are capable of long distance cell-to-cell movement (Buer *et al.*, 2007, 2008).

Flavonoids are also active in animal systems where they have antioxidant properties (Kandaswami and Middleton, 1994), modulate angiogenesis (Fotsis *et al.*, 1993), interact with kinases (O'Prey *et al.*, 2003), cause apoptosis (Kuntz *et al.*, 1999), and are oestrogenic (Miksicek, 1993). These findings show that flavonoids are bioactive, and the molecular targets include multi-drug resistance transporters (now ABCB transporters; Verrier *et al.*, 2008) in plants and animals (Taylor and Grotewold, 2005).

In *Arabidopsis*, the flavonoid pathway is well characterized at the genetic, enzymatic, and product levels, and the genes involved are sequenced (Fig. 1). Previous studies explored the irregularities that flavonoid-pathway

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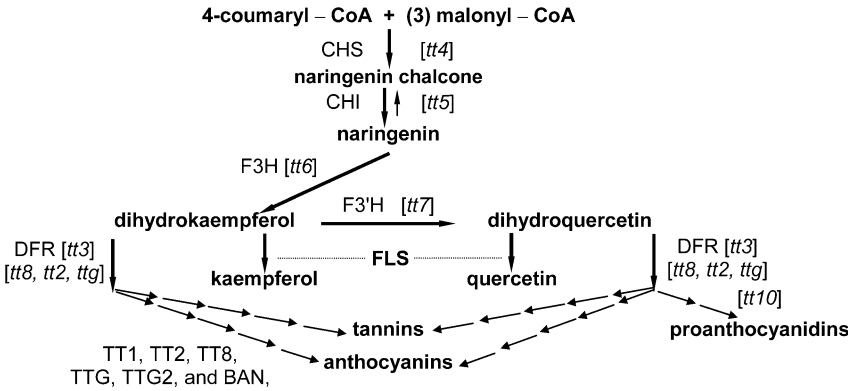


**Fig. 1.** The flavonoid pathway in *Arabidopsis*. Shown are the locations of the *transparent testa* mutations included in these experiments (in brackets). Mutations *tt1*, *tt2*, *tt8*, and *ttg* are in regulatory genes involved in several points of the pathway. The gene products affected by these regulatory mutations are *TT2* which is a R2R3 repeat MYB transcription factor; *TT8* which is a bHLH transcription factor; and *TTG* which encodes a WD40 repeat gene. These proteins operate as a complex to induce BANYLUS expression to form anthocyanins as downstream products of the pathway. *TT1* is a WIP family zinc finger transcription factor and *TTG2* is a WRKY type transcription factor that acts downstream of *TTG1*. The mutated genes and the affected products are: *TT3* (DFR: dihydroflavonol reductase); *TT4* (CHS: chalcone synthase), *TT5* (CHI: chalcone isomerase), *TT6* (F3H: flavonol 3-hydroxylase), *TT7* (F3′H: flavonol 3′-hydroxylase), and *TT10* an enzyme for biflavonol conversion and oxidizing procyanidins to proanthocyanidins in the seed testa. The figure is adapted from Buer *et al.* (2007), (www.plantphysiol.org), Copyright American Society of Plant Biologists.

mutations cause, especially in relation to seed colour and viability (Debeaujon *et al.*, 2000). All the mutants used in this study have defective proanthocyanidin accumulation in the seed that generates the *transparent testa* (*tt*) phenotype (Koornneef, 1990). A few architectural phenotypes of flavonoid-pathway mutants are known. These include a delay in the gravity response in *tt4*, a mutant that cannot make flavonoids (Buer and Muday, 2004). The *transparent testa glabra* (*ttg*) mutants lack trichomes (Larkin *et al.*, 1994) and have root hair differences (Galway *et al.*, 1994). Recently, cell shape irregularities were found in *rol1* mutants (Ringli *et al.*, 2008), although this mutant has not been used in this study. Brown *et al.* (2001) concluded that the flavonoid-induced branching phenotype found in the *tt4*(2YY6) mutant of *Arabidopsis* was due to the lack of flavonoids, but subsequent experimentation showed that this was due to a second mutation, *max4* (Bennett *et al.*, 2006). To our knowledge, no systematic assessment of architectural phenotypes in the *transparent testa* mutants has been conducted.

Flavonoid accumulation in plants can be visualized using diphenyl boric acid 2-amino ethyl ester (DPBA), a flavonoid-specific dye (Peer *et al.*, 2001; Buer and Muday, 2004; Buer *et al.*, 2007). In *Arabidopsis*, DPBA forms a highly fluorescent complex with quercetin and kaempferol generating golden and green fluorescence, respectively (Buer *et al.*, 2007; Fig. 2). Other flavonoid intermediates and background phenolics produce greatly reduced fluorescence with DPBA compared to quercetin and kaempferol (Buer *et al.*, 2007).

Due to the biological activity of flavonoids in plants and animals, we reasoned that there should be more morphological phenotypes other than seed colour and the few previously described architectural phenotypes in the *tt* mutants. Advantage was taken of the many mutants available in the flavonoid pathway in *Arabidopsis* in the Landsberg *erecta* ecotype. These included mutants with genetic lesions affecting enzymatic gene products and mutants in several regulatory genes (Table 1; Fig. 1). Most genes in the flavonoid pathway are single copy (Winkel-Shirley, 2001), simplifying experimentation. A wide range of new shoot and root morphological phenotypes for the *transparent testa* mutants is reported here, and it is shown that environmental conditions influence the observed phenotypes.

## Materials and methods

### Chemicals

All chemicals are from Sigma unless otherwise noted. Dihydrokaempferol was obtained from TransMIT, Marburg, Germany.

### Plant growth conditions

All seeds are from the Arabidopsis Biotechnology Resource Centre (Ohio State University). Seeds were stored in airtight glass vials after drying at 28 °C and exhibited no loss in germination for months. Seedlings of Landsberg *erecta* (L*er*) and the *transparent testa* mutants were grown in a single line ~1 cm apart in unwrapped 150 mm Petri dishes. For each experiment, there were two plates of each mutant with 18 seedlings per plate. The plates were placed at a 45° angle in continuous light in an incubator at 22–23 °C. The MS medium with Gamborg's B5 vitamins (Sigma M0404) was hardened with Type 'M' agar (Sigma; at 1.5%) without sucrose unless otherwise stated. The different day lengths

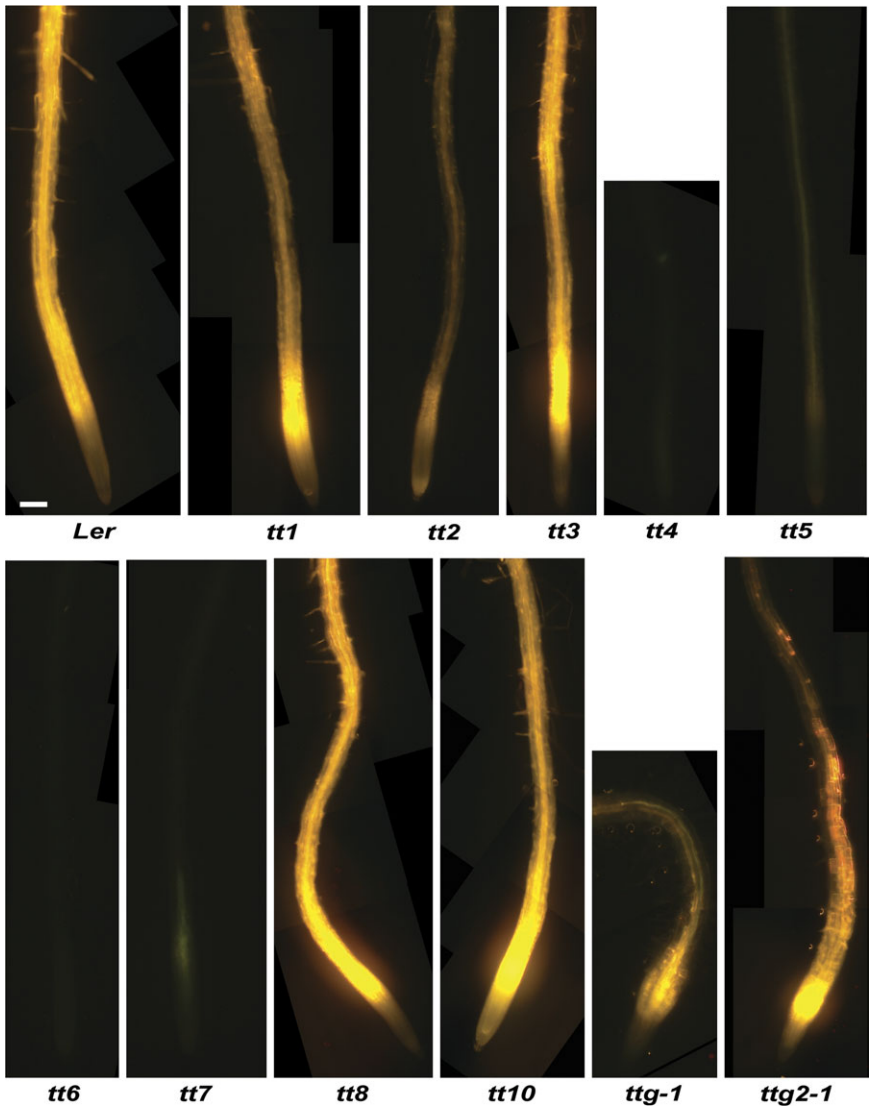


**Fig. 2.** DPBA-flavonoid fluorescence in roots of *transparent testa* mutants varies according to the aglycone that accumulates in the organ. Most seedlings accumulate quercetin that fluoresces a bright golden yellow complexed with DPBA. The mutants *tt4* and *tt6* have very dim fluorescence from background sinapate esters and naringenin, respectively. Naringenin-DPBA fluoresces over 400 times less strongly than quercetin- or kaempferol-DPBA complexes (Buer *et al.*, 2007). The *tt7* mutant shows a dim fluorescence from dihydroquercetin-DPBA and *tt5* shows fluorescence from the spontaneous reaction forming naringenin and subsequent downstream products. Several mutants have obvious DPBA fluorescence in the root hairs. Seedlings were analysed for fluorescence 5 d following germination. Seedlings were grown without sucrose in the medium. The bar=100 μm.

other than continuous light were long days (16 h day) and short days (8 h day). All seedlings were analysed at 5 d following germination unless otherwise stated.

To test if high $CO_2$ atmospheres or ethylene caused root 'nodules', seedlings were grown in 5000 ppm $CO_2$ obtained by supplementation in a growth cabinet and high ethylene atmospheres by wrapping plates with Nescofilm.

The soil medium for pot growth was the Research School of Biological Sciences, Controlled Environment Facilities, *Arabidopsis* mix. Pots were 8×8 cm. Feeding naringenin to *tt4* seedlings occurred in pots and the seedlings were watered with 10 μM naringenin three times a week, beginning at the basal leaf stage and continuing until pollen formation.

The long-term root growths were cleared by placing root tissue in 100% ethanol overnight, and then an overnight incubation in lactic acid (88%).

### *DPBA fluorescence*

DPBA fluorescence analysis was as previously described by Buer *et al.* (2007). All experiments were performed on 5-d-old seedlings grown without sucrose unless otherwise noted.

### *Measurements*

Root hair density was determined by counting the root hairs in the focal plane on one side of the root and dividing this number by the length of the root across the counted

**Table 1.** The *transparent testa* mutants from *Arabidopsis* used in these experiments

All seeds were obtained from the Ohio Biotechnology Resource Center, University of Ohio. Abbreviations: TF, transcription factor; EMS, ethylmethane sulphonate; unk, unknown.

| Line | Enzyme lesion | Locus[a] | Mutagen | Reference |
|---|---|---|---|---|
| L*er* | Wild type | – | – | – |
| *tt1-1* | WIP zinc finger protein | At1G34790 | X-rays | Sagasser *et al.*, 2002 |
| *tt2-1* | MYB domain protein 123 | At5G35550 | X-rays | Nesi *et al.*, 2001 |
| *tt3-1* | Dihydroflavonol 4-reductase | At5G42800 | X-rays | Shirley *et al.*, 1995 |
| *tt4-1* | Chalcone synthase | At5G13930 | EMS | Koornneef, 1990 |
| *tt5-1* | Chalcone isomerase | At3G55120 | fast neutrons | Shirley *et al.*, 1995 |
| *tt6-1* | Flavonone 3-hydroxylase | At3G51240 | EMS | Pelletier and Shirley, 1996 |
| *tt7-1* | Flavonoid 3′-hydroxylase | At5G07990 | EMS | Schoenbohm *et al.*, 2000 |
| *tt8-1* | bHLH domain protein | At4G09820 | unk | Nesi *et al.*, 2000 |
| *tt10-1* | Laccase-like protein | At5G48100 | EMS | Pourcel *et al.*, 2005 |
| *ttg-1* | WD40 repeat protein | At5G24520 | EMS | Walker *et al.*, 1999 |
| *ttg2-1* | WRKY TF | At2G37260 | unk | Ishida *et al.*, 2007 |

[a] Locus locations are from TAIR.

distance. All measurements were made above the root hair initiation site. Lateral root density was determined by counting the number of lateral roots on both sides of the root and dividing by the root length across the measurement. All measurements were made using ImageJ software freely available from NIH on 600 dpi TIFF images generated on a Canon flatbed scanner. Root skew was measured by the angle scribed from the root/shoot junction to the root tip as measured by ImageJ. At least 25 roots were averaged from three separate experiments. Vertically down has been defined as –90 degrees.

### *Statistical analysis*

All statistical comparisons are with the wild type unless indicated otherwise. Statistically significant differences were determined with Student's *t*–test assuming equal or unequal variances determined by *F*–test.

## Results

### *DPBA-flavonoid fluorescence differs between* transparent testa *mutants*

DPBA was used to compare flavonoid accumulation in all the *transparent testa* mutants (Fig. 2). For the mutants affected in the enzymatic steps of flavonoid synthesis, the results follow that of the known biochemical pathway (Fig. 1). The wild-type roots are golden coloured due to DPBA-quercetin fluorescence. By contrast, *tt4* and *tt6* show very dim fluorescence due to background sinapate esters and naringenin accumulation, respectively. Mutations downstream of quercetin production showed the expected fluorescence of quercetin-DPBA conjugation. The chalcone isomerase mutant, *tt5*, showed dim golden fluorescence. This is most likely due to the previously described low-level spontaneous conversion of naringenin chalcone to naringenin (Mol *et al.*, 1985; Cisak and Mielczarek, 1992) and the subsequent formation of quercetin. The examination of several mutants affected in regulatory genes (*tt8*, *ttg*, and *ttg2*) showed the strong golden fluorescence of quercetin. However, *tt2* (a mutant with a perturbation in a regulatory MYB transcription factor; Table 1) had reduced golden fluorescence compared to the wild type, indicating that this mutation affects flavonoid accumulation in tissues in addition to the testa.

### *Root hair morphology differences among the* transparent testa *mutants*

As *ttg* has root hair differences compared to the wild type (Galway *et al.*, 1994), other *transparent testa* mutants were examined for alterations to root hairs at 5 d after germination. The measurements included: the distance from the root tip to the root-hair initiation zone, root hair density, root hair length, and root hair DPBA fluorescence (Table 2). Several mutants differed from the wild type. The *ttg* mutant was similar to the previously reported literature (Galway *et al.*, 1994), except that the distance from the root tip to the root hair initiation zone was greater under our conditions. Considerable variation occurred among the other mutants in relation to the distance from the root tip to the root hair initiation zone, as well as root hair density, and the presence or absence of flavonoid compounds in the root hairs themselves. The *tt2* and *tt7* mutants are nearly devoid of root hairs and *tt6* and *ttg* had very long root hairs compared to the wild type. By contrast, *tt2* and *ttg2* had significantly shorter root hairs. The *tt7*, *ttg*, and *tt2* mutants were affected in most or all root hair parameters examined, whereas, the *tt1* mutant was unaffected. The *ttg* and *ttg2* mutants lack flavonoid fluorescence in the root hairs. This is due to the absence of flavonoid fluorescence in the epidermal cell layer of these mutants.

### *Root morphology differences in the* transparent testa *mutants*

As sucrose can have hormone-like properties (Smeekens, 2000), the *transparent testa* mutants were analysed for

**Table 2.** Root hair phenotypes exist between the *transparent testa* mutants

All seedlings grew on MS media without sucrose, 1.5% agar, and plates at 45° with continuous light and analysed after 5 d following germination. All statistical comparisons are with the wild type. Statistically significant differences were determined with Student's *t*–test assuming equal or unequal variances determined by *F*–test.

| Line | Distance to root hair (μm) | Root hair length (μm) | Root hair density (no. $\mu m^{-1}$) | Root hair DPBA fluorescence |
|---|---|---|---|---|
| L*er* | 853.3±52.9 | 175.5±7.1 | 0.013±0.001 | + |
| *tt1-1* | 1014.0±51.0 | 186.1±9.3 | 0.013±0.0003 | + |
| *tt2-1* | **1097.9±78.2**[a] | **87.6±10.2**[b] | **0.0093±0.0008**[b] | + |
| *tt3-1* | **1072.8±44.9**[a] | 143.6±20.6 | 0.011±0.0007 | + |
| *tt4-1* | **1135.9±79.5**[a] | 159.0±12.6 | 0.013±0.001 | — |
| *tt5-1* | **1299.2±74.6**[a] | 156.2±18.8 | 0.011±0.0007 | — |
| *tt6-1* | **1153.2±92.4**[a] | **415.4±53.9**[b] | 0.012±0.0005 | — |
| *tt7-1* | **1382.1±108.8**[a] | **297.1±16.7**[a] | **0.0085±0.0005**[b] | — |
| *tt8-1* | **1273.0±126.9**[a] | **111.9±9.9**[b] | 0.012±0.001 | + |
| *tt10-1* | 919.7±39.5 | 117.6±11.4 | **0.015±0.001**[a] | + |
| *ttg-1* | **1599.1±69.5**[b] | **331.2±21.2**[b] | **0.027±0.004**[a] | — |
| *ttg2-1* | 804.0±158.5 | **104.1±16.0**[a] | 0.015±0.001 | — |

[a] $P < 0.05$.
[b] $P < 0.001$.

any root architecture phenotypes using slanted hard-agar plates without sucrose and continuous light. Several root phenotypes emerged (Fig. 3; Table 3). The mutants *tt1*, *tt3*, *tt4*, *tt8*, and *tt10* were altered in the skewing angle of root elongation across the agar surface. The *tt4* and *tt10* mutants had looping roots, but the looping occurred later in growth in the *tt10* seedlings. Several mutants had more (*tt6*) or fewer (*tt4* and *tt8*) lateral roots compared to the wild type, which was measured as lateral root density (the number of emerged lateral roots $mm^{-1}$ root length). The *tt4* mutant produced significantly fewer lateral roots than the wild type under the conditions used here. This result differed from those previously reported using MS salts, 1.5% sucrose, and a vertical orientation (Brown *et al.*, 2001). However, when lateral root density of *tt4* was analysed using the previously reported conditions (with sucrose), the results were congruent (Table 4; Brown *et al.*, 2001).

### transparent testa *mutants show phenotypes in the aerial tissues*

The mutants were examined for aerial phenotypes including inflorescence height, the number of inflorescences, and silique density. After germinating seeds on 9 cm plates, seedlings were transplanted to pots, and grown under long-day conditions. Table 5 summarizes the resulting phenotypes and representative images of the inflorescences are in Fig. 5. Interestingly, *tt8*, *tt10*, and *ttg* had significantly increased inflorescence production compared to the wild type. The *tt8* and *ttg2* mutants showed increased inflorescence height and *ttg* showed decreased inflorescence height and increased silique density. The *tt4* mutant did not show increased aerial branching compared to the wild type, which is consistent with the published results (Bennett *et al.*, 2006).

### *The effect of exogenous addition of flavonoids on the* tt6 *long hypocotyl and* tt4 *looping phenotypes*

Growing seedlings on slanted hard-agar plates revealed two additional aerial differences. These included significantly longer hypocotyls on the *tt6* mutant seedlings and longer petioles on the *tt8* mutant seedlings (Fig. 4). The longer hypocotyls of *tt6* plants were also apparent when plants were grown on vertical plates and in the presence of sucrose.

An attempt was made to reverse the long hypocotyl phenotype in *tt6* by adding aglycones downstream of the lesion to the seedlings via the medium. The *tt6* hypocotyls grew longer with dihydrokaempferol addition, and the lateral root density increased with the addition of dihydrokaempferol or dihydroquercetin (Table 6). Adding kaempferol or quercetin did not affect hypocotyl length.

The *tt4* mutant showed a root looping phenotype in the no sucrose, slanted-agar assay. Adding naringenin to the medium of the *tt4* mutant reversed the looping phenotype with increasing concentration (Table 7); however, the phenotype did not completely rescue the phenotype. Growing *tt4* seedlings in long day conditions (16 h light) reduced the looping phenotype, and short days (8 h light) abolished it completely. The looping phenotype was also sensitive to normal experimental manipulations. Moving the plates to make daily measurements, for example, abolished root looping. Thus, for all the slanted-plate experiments reported here, the plates remained stationary until scanned.

### *Aberrant outgrowths form on* tt4 *and wild-type roots after prolonged periods in agar supplemented with sucrose*

The growth of *tt4* seedlings in agar in the presence and absence of naringenin over long periods resulted in aberrant

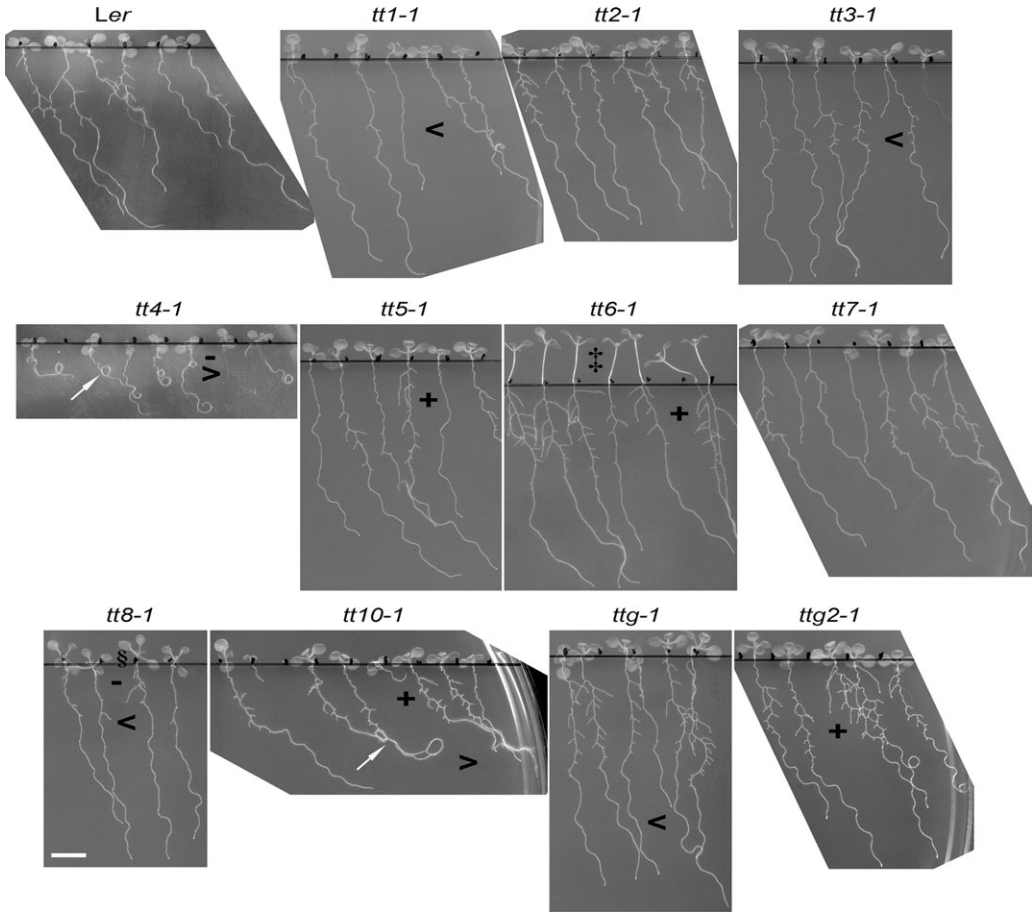


**Fig. 3.** Root phenotypes of the various *transparent testa* mutants. Growing seedlings on 1.5% slanted agar induced several different phenotypes in the seedlings. All seedlings grew without sucrose in the medium under continuous light. The scans were performed at 9 d following germination. Interesting phenotypes were looped roots in *tt4* and *tt10* (arrow), increased (+) or decreased (–) lateral roots compared to wild type, greater root (>) or lesser (<) skewing, elongated hypocotyls in *tt6* (‡), and elongated petioles in *tt8* (§). The scale bar=8 mm.

**Table 3.** Summary of phenotype differences in transparent testa mutants

Growth conditions were MS salts, no sucrose, continuous light, on 1.5% agar, and plates tilted at 45°. Seedlings were scanned 9 d following germination. n.m., not measured

| Mutation | Hypocotyl length (mm) | Petiole length (mm) | Skew (°)[a] | Looping roots (%) | Lateral roots (no.) | Lateral root density (no. mm$^{-1}$ root length) |
|---|---|---|---|---|---|---|
| L. *erecta* | 1.6±0.07 | 1.2±0.04 | –65.0±2.5 | 0 | 9.5±0.7 | 0.17±0.01 |
| *tt1-1* | n.m. | n.m. | **–71.5±1.5**[b] | – | 8.6±0.8 | 0.18±0.02 |
| *tt2-1* | n.m. | n.m. | –68.4±1.1 | – | 8.9±0.5 | **0.24±0.01**[c] |
| *tt3-1* | n.m. | n.m. | **–79.7±0.9**[c] | – | 8.3±0.5 | 0.17±0.009 |
| *tt4-1* | n.m. | n.m. | **–53.1±2.5**[b] | **75.0±8.3** | **1.4±0.3**[cd] | **0.05±0.01**[cd] |
| *tt5-1* | n.m. | n.m. | –68.9±1.6 | – | 11.5±0.7 | **0.22±0.01**[b] |
| *tt6-1* | **7.8±0.2**[c] | n.m. | –69.0±1.6 | – | **11.9±0.8**[b] | **0.25±0.01**[c] |
| *tt7-1* | n.m. | n.m. | –62.4±1.5 | – | 10.3±0.8 | 0.20±0.02 |
| *tt8-1* | n.m. | **1.5±0.05**[c] | **–77.4±1.2**[c] | – | **5.5±0.6**[c] | **0.13±0.01**[b] |
| *tt10-1* | n.m. | n.m. | **–42.7±1.3**[c] | **38.9±5.6** | 9.1±0.7 | **0.24±0.02**[b] |
| *ttg-1* | n.m. | n.m. | **–76.8±1.4**[c] | – | 10.2±1.0 | 0.20±0.02 |
| *ttg2-1* | n.m. | n.m. | –62.2±1.0 | – | 11.0±0.7 | **0.23±0.01**[c] |

[a] The angle the roots grow across the plate. The view is from the bottom of the plate, through the agar. Vertically down is –90°.
[b] $P$ <0.05 Results of Student's $t$–test for equal or unequal variance determined by $F$–test. All comparisons are with Landsberg *erecta*, the wild type.
[c] $P$ <0.001.
[d] These data are contrary to previous literature, but the growth conditions are entirely different. This is generally tested with at least 1.5% sucrose in the media. See Table 4.

**Table 4.** Growth conditions affect lateral rooting in *tt4*

Growth conditions: MS salts, 1.5% sucrose, and vertical orientation. Lateral root density was scored 9 d following germination.

| Ecotype/mutation | Lateral root density (No. mm$^{-1}$) |
|---|---|
| L. *erecta* | 0.43±0.022 |
| *tt4-1* | **0.57±0.015**[a] |
| Col | 0.43±0.010 |
| *tt4-2* | **0.53±0.013**[a] |

[a] Statistical comparisons are between wild type and mutant within the same ecotype. Comparisons were analysed with *F*–test for variance and two-tailed Student's *t*–test depending on results of *F*–test. $P < 0.001$; $n=20$.

**Table 5.** Summary of the aerial phenotype differences in selected *transparent testa* mutants

Growth conditions: pots under long days and sampling was after 9 weeks, following senescence.

| Mutation | Inflorescence height (mm) | 1° Inflorescences (no.) | Silique density (no. mm$^{-1}$) |
|---|---|---|---|
| L. *erecta* | 281.4±10.7 | 1.5±0.1 | 0.17±0.01 |
| *tt3-1* | 268.8±11.9 | 1.3±0.1 | **0.24±0.009**[b] |
| *tt4-1* | 246.7±24.0 | 1.3±0.2 | 0.26±0.02 |
| *tt5-1* | 266.7±10.9 | 1.3±0.3 | 0.27±0.01 |
| *tt8-1* | **348.0±15.3**[b] | **4.5±0.3**[b] | 0.25±0.01 |
| *tt10-1* | 263.1±13.2 | **3.5±0.4**[b] | 0.30±0.02 |
| *ttg-1* | **226.4±6.6**[b] | **3.5±0.3**[b] | **0.37±0.03**[a] |
| *ttg2-1* | **335.0±23.6**[a] | 2.3±0.5 | 0.31±0.03 |

[a] $P < 0.05$, results of Student's *t*–test for equal or unequal variance determined by *F*–test. All comparisons are with Landsberg *erecta*, the wild type. $n \geqslant 20$.
[b] $P < 0.001$, results of Student's *t*–test for equal or unequal variance determined by *F*–test. All comparisons are with Landsberg *erecta*, the wild type. $n \geqslant 20$.

root growths that visually resembled root nodules (Fig. 5). These root outgrowths required at least 40 d to appear. Adding naringenin to the medium retarded their formation. However, after 90 d, the growths appeared on the wild-type roots as well. These root outgrowths formed in addition to normal appearing lateral roots and probably represent aborted lateral roots or, perhaps, determinate lateral roots. Observation of the cleared root outgrowths viewed under higher magnification indicated that they initiate from the pericycle (Fig. 5E). The possibility was checked that these root growths were the product of elevated ethylene or $CO_2$ due to inadequate aeration of the medium. These root outgrowths did not form under high carbon dioxide or ethylene atmospheres (data not shown).

### *Root application of naringenin restores pollen fluorescence in* tt4

It was demonstrated previously that certain flavonoids are capable of root-to-shoot movement in *Arabidopsis* (Buer *et al.*, 2007, 2008). It was determined if naringenin addition to the agar medium could restore the wild-type seed colour to *tt4* mutants. Naringenin addition did not complement the seed colour. Work by Hsieh and Huang (2007) indicated that flavonoids accumulate in Brassicaceae pollen. It was ascertained if feeding naringenin to *tt4* would restore pollen fluorescence. Figure 6 shows that flavonoid fluorescence is restored to *tt4* pollen by feeding 10 μM naringenin to seedlings growing in soil under long-day conditions. These results are consistent with reciprocal grafts where wild-type cross-feeding occurs in wild-type/*tt4* combinations (Buer *et al.*, 2008).

## Discussion

### *New architectural phenotypes in* transparent testa *mutants*

Although extensively studied, the *transparent testa* mutants have little information available on specific architectural phenotypes resulting from flavonoid level perturbations. This study examined the regulatory and enzymatic mutants affected in the flavonoid pathway from the Landsberg *erecta* ecotype and showed new phenotypes in these mutants. Mutations in the various genes of the pathway led to differential fluorescence in the mutants caused by alterations in flavonoid accumulation. The type and intensity of the DPBA-flavonoid fluorescence observed was due to the presence or absence of flavonoids or the intermediates that accumulate because of pathway alterations (Peer *et al.*, 2001). The mutants *tt3*, *tt8*, *ttg*, and *tt10* are blocked from producing anthocyanins and accumulated more quercetin than the wild type except for *tt2*. Since TT2 occurs in the same part of the flavonoid pathway, one would expect the accumulation of quercetin to be equal in these mutants. The *TT2* gene expresses strongly and transiently in immature seed and works synergistically with *TT8*, *TTG1*, and *BANYLUS* to synthesize the downstream products of the flavonoid pathway (Nesi *et al.*, 2001). Although, Nesi *et al.* (2001) did not detect *TT2* transcript in seedlings, leaves, or stems at 4 d, or in roots on 10 d, these results clearly show that, relative to the control, flavonoid levels are affected in the roots of the *tt2*, *tt8*, and *ttg1* mutants at 5 d. It is possible that Nesi *et al.* (2001) missed *TT2* expression in roots under their time constraints. It is also possible that *TT2* expresses at very low levels or in a few select cells.

Several new architectural defects were discovered in the *tt* mutants compared to the wild type. These differences included the root looping phenotypes in *tt4* and *tt10*, increased lateral root density in *tt5*, *tt6*, and *tt10*, decreased lateral root density in *tt4* if grown on hard slanted agar, and the long hypocotyl phenotype in *tt6*. The regulatory mutant, *tt8*, had a long petiole phenotype. Differential effects in root hair length or density or the distance from the root tip to the root hair initiation zone were found in all mutants except *tt1*, with *tt2*, *tt7,* and *ttg1* affected in all three measures. The *tt8, tt10,* and *ttg* mutants also had

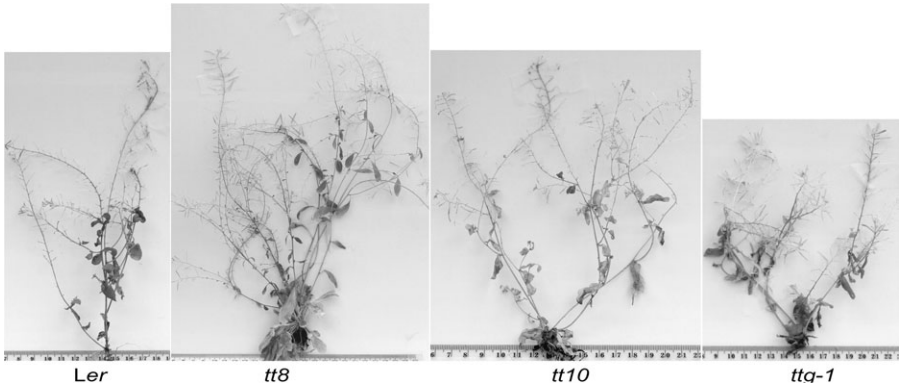


**Fig. 4.** The aerial phenotypes in some *transparent testa* mutants have multiple inflorescences. The multiple inflorescences of *tt8*, *tt10*, and *ttg-1* are compared to the wild type and clearly have more inflorescences. The photographs were taken 9 weeks following transplanting to pots. The scale is provided by a ruler shown at the bottom of each panel with major gradations in cm.

**Table 6.** The long hypocotyl phenotype and lateral root density in *tt6* increases with the addition of DHK

Seedlings were grown on hard-slanted agar without sucrose and continuous light. Statistical comparisons are between wild type and mutant unless stated otherwise. Comparisons were analysed with *F*–test for variance and two-tailed Student's *t*–test depending on the results of the *F*–test. Data were pooled from two independent experiments, $n \geqslant 20$.

| Ecotype/mutant | MS | DMSO | N | DHK | DHQ | K | Q |
|---|---|---|---|---|---|---|---|
| Hypocotyl length (mm) | | | | | | | |
| L*er* | 1.9±0.1 | 2.0±0.1 | 2.0±0.1 | 1.9±0.1 | 1.7±0.1 | 1.7±0.1 | 1.9±0.1 |
| *tt6* | 8.5±0.2[a] | 8.4±0.1[a] | 8.6±0.2[a] | 9.2±0.2[ab] | 8.6±0.2[a] | 8.8± .2[a] | 8.7±0.2[a] |
| Lateral root density (no. $mm^{-1}$) | | | | | | | |
| L*er* | 0.17±0.01 | n.t.[c] | n.t. | 0.23±0.02 | 0.24±0.01 | n.t. | n.t. |
| *tt6* | 0.25±0.01 | n.t. | n.t. | 0.29±0.02[c] | 0.35±0.01[b] | n.t. | n.t. |

[a] $P < 0.001$
[b] Comparison between *tt6* on MS and DHK, $P < 0.01$. The other data points were not different from *tt6* on MS.
[c] Not tested.

increased inflorescence production compared with the wild type and there were differences in silique density, inflorescence height, and number in *ttg*. The absence of flavonoid fluorescence in *ttg* and *ttg2* root hairs is because flavonoid fluorescence is absent from the root epidermal cell layer (Fig. 2). Specific localization of flavonoid fluorescence was noted previously in *tt4* (Buer *et al.*, 2007), so such an occurrence in other *transparent testa* mutants is possible.

The distance from the root tip to when root hairs began elongating in *ttg* was considerably greater in our experiments than reported by Galway *et al.* (1994). This may be an ethylene-induced difference; indeed the plates were wrapped with Parafilm (Schiefelbein and Somerville, 1990), and ethylene accumulation occurs under these circumstances (Buer *et al.*, 2003), while our experiments were in unwrapped plates. The root hair density in the *ttg* mutant in our experiments was about double the density compared to the wild type, similar to previous work (Galway *et al.*, 1994).

### *Flavonoid interactions with auxin, ethylene, and touch perception*

One possible explanation for these phenotypes is the effect that certain flavonoids (especially quercetin and kaempferol) exert on auxin levels in the plant through modulation of auxin transport (Jacobs and Rubery, 1988). Auxin is thought to regulate lateral root formation (reviewed in Fukaki *et al.*, 2007) and indeed, Dubrovsky *et al.* (2008) show that localized auxin levels are the critical determinant in setting up stem cell niches for lateral root formation. A connection with root looping and slanting across the agar surface and auxin fluxes is also apparent (Lucas *et al.*, 2008), and the formation of lateral roots at the apex of root waves depends on auxin (De Smet *et al.*, 2007). Root hair formation is also related to auxin transport and ethylene levels (reviewed in Bibikova and Gilroy, 2003), and previous reports describe root hair phenotypes in flavonoid mutants (Galway *et al.*, 1994; Bharti and Khurana, 2003; Mo *et al.*, 1992).

As discussed above, growing seedlings on hard-slanted agar caused several phenotypes, and there are several postulates as to why these differences occur. It is suggested that the regular waving, looping, and skewing is a combination of thigmotropism and a positive response to gravitropism (Okada and Shimura, 1990) or root–gel interactions (Thompson and Holbrook, 2004). Ethylene is an influencing factor (Buer *et al.*, 2003), as well as nutrient conditions in the medium including sucrose (Buer *et al.*, 2000). A new mutant in *Arabidopsis* with resistance to auxin also has

**Table 7.** The looping phenotype in *tt4* is reversed by adding naringenin to the medium under continuous light

Combined results from five experiments of two replicates of nine seedlings within each experiment. The seedlings grew on hard-slanted agar without sucrose and were stationary until scanned.

| Treatment | L*er* (% looping roots) | *tt4-1* (% looping roots) |
|---|---|---|
| MS control | 0.43±0.03 | **97.7±2.1**[a] |
| DMSO control | 0.38±0.03 | **89.8±8.3**[a] |
| N ($5\times10^{-7}$ M) | 0.81±0.03 | **74.3±7.7**[a] |
| N ($5\times10^{-6}$ M) | 0.22±0.01 | **55.6±2.9**[a] |
| N ($5\times10^{-5}$ M) | 0.31±0.01 | **9.6±2.9**[a] |

[a] Comparisons are between wild type and mutant within each category. Statistical analysis is from two-tailed Student's *t*–test assuming unequal variance according to *F*–test of variance, $P < 0.0001$.

perturbed root phenotypes (Fortunati *et al.*, 2008). Complicating matters are experiments that show an interaction between ethylene and flavonoids (Buer *et al.*, 2006). Many mutants have aberrant root phenotypes, but it is still unclear how these growth patterns are manifested (Oliva and Dunand, 2007), but many of them occur in auxin transport mutants. The lack of auxin transport inhibition during the oscillations from positive to negative gravitropic responses possibly caused the root looping phenotype in *tt4*. Adding naringenin to the medium nearly reversed the looping phenotype from *tt4* suggesting a direct role for flavonoids in this phenotype. Ringli *et al.* (2008) also noted auxin-dependent and independent effects in plant growth and cell shape in flavonoid glycoside mutants. A recent report implicated Multidrug Resistance-Like4 (MDR4) (now ABCB4; Verrier *et al.*, 2008) regulation by flavonols during gravitropic responses showing that *ABCB4* and *TT4* are epistatic (Lewis *et al.*, 2007).

Daylength and the manual movement of plates affected the presence or absence of the root-looping phenotype in *tt4*. Because of this, the plates remained stationary during these experiments. It was noted that *tt4* roots of pot-grown plants do not display a root-looping phenotype. Therefore, researchers should consider the use of more realistic growth conditions if the architectural phenotypes shown in this study are translated to other plant systems.

### *Analysis of flavonoid pathway mutants: fertile ground to improve plant yield or tailor plant architecture?*

Some phenotypes are worth further investigation for the possibility of increasing yields or adapting plants to local soil conditions. For example, multiple inflorescences occurred in *tt8*, *tt10,* and *ttg*. Other mutants showed possible desirable yield-enhancing phenotypes such as shorter stature (*ttg*) and significantly increased silique density (*tt3* and *ttg*). Other mutants showed increased lateral root density or root hair density, traits beneficial for growth in poor soil conditions (Lynch, 2007). The flavonoid regulatory mutants (*tt1*, *tt2*, *tt8*, and *ttg*) are difficult to interpret as they operate in concert at several points in the pathway and effects of the mutations are probably pleiotropic.

There may be some possible problems applying these phenotypes to agronomy. Many of the early flavonoid pathway mutants are UV sensitive (Li *et al.*, 1993), which could be problematic for field-grown plants. However, the susceptibility to UV is compensated for by higher sinapate ester production in *tt* mutants (Sheahan, 1996). The *tt8* and *ttg* regulatory mutants accumulate more quercetin than the wild type, and although UV sensitivity was not tested, it is probably not an issue because of this accumulation. Seed storage problems have been reported for the *tt* mutants (Debeaujon *et al.*, 2000), which would be an issue related to agronomic uses.

The mutations responsible for these plant architecture phenotypes with the potential to affect yield occur in transcription factors that are the potential targets of the second generation of molecular biology (Busov *et al.*, 2008; Century *et al.*, 2008). Major advances in agronomy are necessary to increase yields to feed the projected nine billion-world population that will require food by 2050 (Cohen, 2003).

### *Low-level quercetin production in* tt5

The individual mutants in the enzymatic steps within the flavonoid pathway are relatively straightforward, as they accumulate intermediates consistent with the corresponding lesions in the pathway. Since the mutated genes occur as single copies, they are expected to be solely responsible for the results found, unless flavonoid perturbations have pleiotropic effects or the mutations are leaky. The only mutant that gave a less definitive result was *tt5* (chalcone isomerase) where there was evidence of trace amounts of golden fluorescence (indicative of quercetin formation). Previous experimentation with this *tt5* allele showed naringenin chalcone accumulation at the root–shoot junction plus an unidentified peak with a chalcone and flavanone skeleton with a retention time equal to quercetin (Peer *et al.*, 2001), and they speculated on a spontaneous isomerization of naringenin chalcone to form naringenin. Shirley *et al.* (1995) and Pelletier *et al.* (1999) showed kaempferol in seeds and flowers by thin-layer chromatography and HPLC, respectively, also supporting the hypothesis that the *tt5* mutation does not lead to a complete metabolic block at chalcone isomerase. There is experimental evidence of the spontaneous isomerization of naringenin chalcone to naringenin, which occurs at physiological pH (Mol *et al.*, 1985; Cisak and Mielczarek, 1992). This spontaneous isomerization is consistent with the low levels of golden fluorescence seen in experiments with *tt5*.

### *The long hypocotyl phenotype of* tt6

The inability to reverse the *tt6* long hypocotyl phenotype by adding dihydrokaempferol may indicate that naringenin accumulation caused the extension of the tissue. Supplementation with dihydrokaempferol would restore downstream flavonoid synthesis past the block in the pathway;

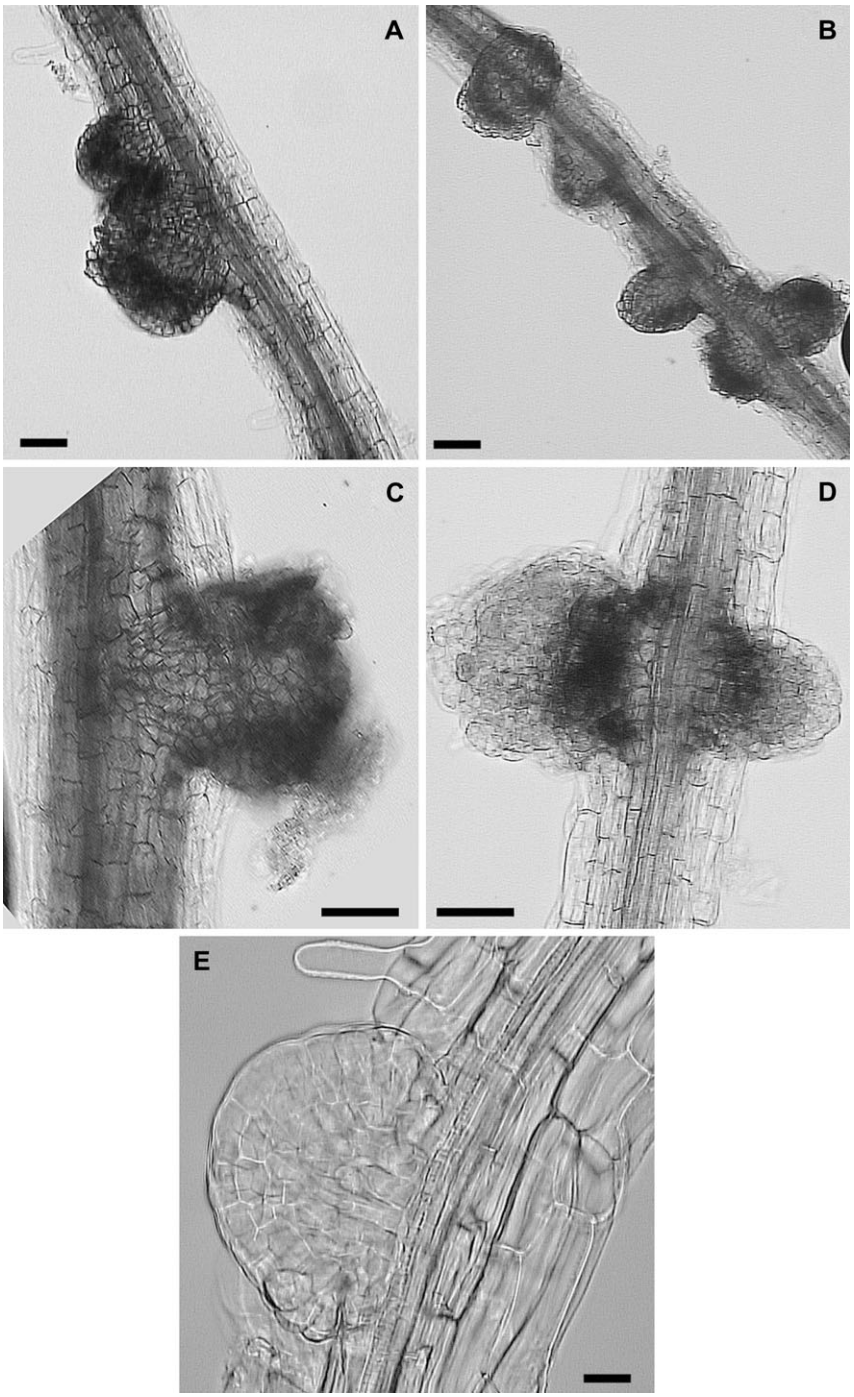


**Fig. 5.** Aberrant root outgrowths on *tt4* roots at 40 d. (A–D) Aberrant growths that resemble root nodules are shown. Typically, each plant produced 100s of root outgrowths averaging 1 per mm root length, and often the structures were clustered in groups (B). A cleared root outgrowth is shown in (E). Cleared roots indicated that initiation is from the pericycle. Scale bars: (A)–(D) 50 μm; (E) 20 μm.

however, it would not alleviate the accumulation of naringenin due to the mutation. Indeed, adding dihydrokaempferol to *tt6* seedlings increased the hypocotyl length. There are indications the mutant may be leaky (Peer *et al.*, 2001; Wiseman *et al.*, 1998), but we did not see any downstream flavonoid products in our DPBA staining experiments. This phenotype requires further investigation.

The long-hypocotyl phenotype in *tt6* is similar to that of the *hy5* mutant in *Arabidopsis* (Lee *et al.*, 2007), which lacks flavonoids in the root and has increased auxin transport (Sibout *et al.*, 2006). HY5 is a transcriptional regulator that binds to the chalcone synthase promoter (Lee *et al.*, 2007).

### *Phenotypes of* tt4 *mutants*

Interestingly, day length was important for the looping phenotype seen in *tt4* roots. The root looping decreased with decreasing day length and disappeared entirely when

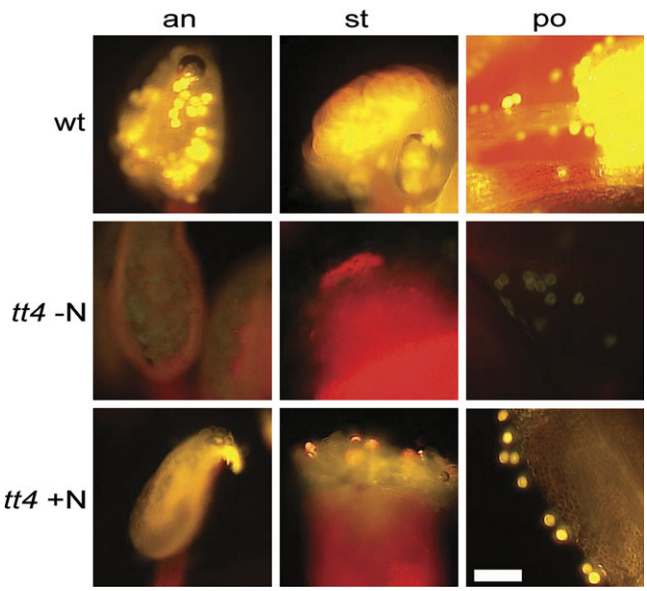


**Fig. 6.** Feeding naringenin to *tt4* restores flavonoid fluorescence to pollen. *tt4* seedlings were watered three times weekly with 10 μM naringenin. Following pollen formation, flower tissue was analysed for flavonoid fluorescence using DPBA. The wild-type fluorescence is similar to that reported by Peer *et al.* (2001). Abbreviations: an, anther; st, stigma; po, pollen. Bar=100 μm.

seedlings were grown under 8 h light regimes. Although light regulates flavonoid-producing enzymes, it is difficult to decipher how day length interacts with flavonoid levels and root gravi- and thigmotropism.

Recent experiments by Hsieh and Huang (2007) probably explain the inability to revert *tt4* seed colour back to the wild type by adding exogenous naringenin to plants. They showed that flavonoids in the Brassicaceae end up in the pollen surface rather than the seed testa. The testa tissue derives from ovular tissue, and thus is maternal in origin (Debeaujon *et al.*, 2000). Adding exogenous naringenin to *tt4* seedlings from the basal leaf stage until pollen formation resulted in the restoration of flavonoid fluorescence in the pollen coat at a slightly lower level than the wild type under our conditions. This supports the long-distance movement of flavonoids in *tt4* plants shown previously (Buer *et al.*, 2007, 2008).

The root outgrowths during long-term seed colour complementation experiments are remotely similar to aberrant lateral roots created by drought in Brassicaceae (Vartanian *et al.*, 1994). We believe the outgrowths are aborted lateral roots, as they arise from the pericycle. The seedlings also have normal lateral roots intermingled with these outgrowths (data not shown) and why some lateral roots would abort and not others is not understood.

## Conclusion

Perturbation in flavonoid levels resulting from mutations directly or indirectly regulating the flavonoid pathway caused a wide range of morphological features of *Arabidopsis* plants. Overall, flavonoid effects on auxin movement are a unifying theme that may explain many of the phenotypes observed in this study. However, it is unclear whether flavonoids act directly as regulatory agents or indirectly through auxin accumulation or movement, or both (Peer and Murphy, 2007). The recent work by Santelia *et al.* (2008) supports modulation through PIN localization. The direct effect of flavonoids on plant growth and architecture is the focus of our further experiments within the *transparent testa* mutants.

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