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The *transparent testa4* Mutation Prevents Flavonoid Synthesis and Alters Auxin Transport and the Response of Arabidopsis Roots to Gravity and Light [W]

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We examined whether flavonoids act as endogenous auxin transport regulators during gravity vector and light intensity changes in *Arabidopsis thaliana* roots. Flavonoid deficient *transparent testa4* [*tt4*(2YY6)] seedlings had elevated root basipetal auxin transport compared with the wild type, consistent with the absence of a negative auxin transport regulator. The *tt4*(2YY6) roots had delayed gravitropism that was chemically complemented with a flavonoid intermediate. Flavonoid accumulation was found in wild-type columella cells, the site of gravity perception, and in epidermal and cortical cells, the site of differential growth, but flavonoid accumulation was absent in *tt4*(2YY6) roots. Flavonoid accumulation was higher in gravity-stimulated root tips as compared with vertical controls, with maximum differences coinciding with the timing of gravitropic bending, and was located in epidermal cells. Exogenous indole-3-acetic acid (IAA) also elevated flavonoid accumulation, suggesting that flavonoid changes in response to gravity might be partly as a result of changing IAA distribution. Acropetal IAA transport was also elevated in roots of *tt4*(2YY6). Flavonoid synthesis was repressed in the dark, as were differences in root acropetal transport in *tt4*(2YY6). These results are consistent with light- and gravity-induced flavonoid stimulation that alters auxin transport in roots and dependent physiological processes, including gravitropic bending and root development.

INTRODUCTION

The abundant natural auxin, indole-3-acetic acid (IAA), is required for many important plant functions, including embryo development, growth, and tropic responses. Models for auxin movement suggest that polar auxin transport occurs from cell to cell in many tissues, but the control of transport is not well understood (reviewed in Muday and DeLong, 2001; Friml, 2003). In shoots, auxin moves basipetally from the apex of the plant toward the roots through the inflorescence or hypocotyl, but auxin movement is bidirectional in roots. Acropetal auxin transport, from the root/shoot junction (RSJ) toward the root tip, occurs in the central cylinder along the entire root, whereas from the root tip toward the RSJ, transport occurs in the outer cell layers in the first 7 mm and is termed basipetal (reviewed in Muday and DeLong, 2001).

Differential tissue growth in response to lateral auxin transport across gravitropically or phototropically stimulated organs was postulated in the Cholodny-Went theory (reviewed in Trewavas, 1992). Lateral auxin gradients were detected in shoot tissues by measuring free IAA gradients across gravity stimulated coleoptiles and pulvini tissues (Philippar et al., 1999; Long et al., 2002). In small roots like *Arabidopsis thaliana*, direct IAA measurements across gravity-stimulated roots are not possible. Rather, IAA gradients in gravity-stimulated roots were observed indirectly by visualizing IAA-induced gene expression driving either β-glucuronidase (GUS; Rashotte et al., 2001) or green fluorescent protein reporters (Ottenschläger et al., 2003); yet, it is still unclear how lateral auxin movement is initiated in response to gravitropic or phototropic stimulation (Muday, 2001; Blancaflor and Masson, 2003).

Several proteins function in the control of auxin movement in Arabidopsis. The AUX1 protein is believed to mediate auxin influx into cells (Marchant et al., 1999). Auxin efflux from cells is more complicated, probably involving a protein complex. In Arabidopsis, several PIN proteins, in concert with a regulatory auxin transport inhibitor binding protein, are believed to control cellular auxin efflux and may perform specialized auxin transport regulation in specific tissue locations (reviewed in Friml and Palme, 2002; Muday and Murphy, 2002). In addition, multidrug resistance-like proteins may also participate in auxin transport (Noh et al., 2001) or in the formation of the auxin transport protein complex (Noh et al., 2003). Recent experiments demonstrated gravity-stimulated lateral relocation of PIN3 in roots, suggesting this protein may participate in lateral auxin transport (Friml et al., 2002).

Auxin transport may be regulated during gravity response by small endogenous molecules that change the activity of auxin transport proteins. Flavonoids have been suggested to regulate auxin transport because previous experiments demonstrated that flavonoids displaced binding of synthetic auxin transport

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inhibitors in vitro, with quercetin and kaempferol having the greatest activity (Jacobs and Rubery, 1988). Additionally, in vivo evidence for the regulation of auxin transport by flavonoids was demonstrated by altered auxin transport in Arabidopsis plants with mutations in genes encoding flavonoid biosynthetic enzymes (Murphy et al., 2000; Brown et al., 2001). In these studies, auxin transport was elevated in young seedlings and inflorescences of plants with the *transparent testa4* (*tt4*) mutation, consistent with the absence of an endogenous negative auxin transport regulator. Flavonoid synthesis (Figure 1) is tightly tied to environmental stimuli (Winkel-Shirley, 2002), suggesting that flavonoid synthesis may be regulated under conditions when auxin transport is modulated. For example, when Arabidopsis seedlings are grown in the dark, flavonoid biosynthesis is off, but in the light there is a dramatic induction in flavonoid synthesis, mediated by induced transcription of the genes encoding flavonoid biosynthetic enzymes (Pelletier and Shirley, 1996; Winkel-Shirley, 2002). Flavonoids are quickly and abundantly produced when plants are wounded or during pathogen attacks (Winkel-Shirley, 2002). Therefore, induction of flavonoids in response to stimuli that may require changes in auxin transport may facilitate the action of specific flavonoids in modulating auxin transport.

The goal of these experiments was to determine whether gravity and light lead to changes in auxin transport by modulating flavonoid levels in Arabidopsis roots. Although the Arabidopsis flavonoid-deficient mutant *tt4*(2YY6) has elevated auxin transport in shoots of young seedlings and in mature inflorescence tissues (Murphy et al., 2000; Brown et al., 2001), basipetal auxin transport in roots was not previously directly measured. In this study, the ability of wild-type and *tt4*(2YY6) roots to basipetally transport auxin and to respond to gravity was compared. Flavonoid production was restored in *tt4*(2YY6) by feeding the flavonoid precursor, naringenin (Shirley et al., 1995; Murphy et al., 2000; Brown et al., 2001), to chemically complement the *tt4* mutation and examine the effect on gravity response. Additionally, to determine if changes in auxin transport were directly tied to light-dependent flavonoid synthesis, acropetal auxin transport was examined in dark-grown Columbia (Col) and *tt4*(2YY6), with and without light exposure sufficient to induce flavonoid synthesis. Together, these experiments provide insight into the role of flavonoids in regulation of auxin transport and dependent physiological processes in Arabidopsis roots.

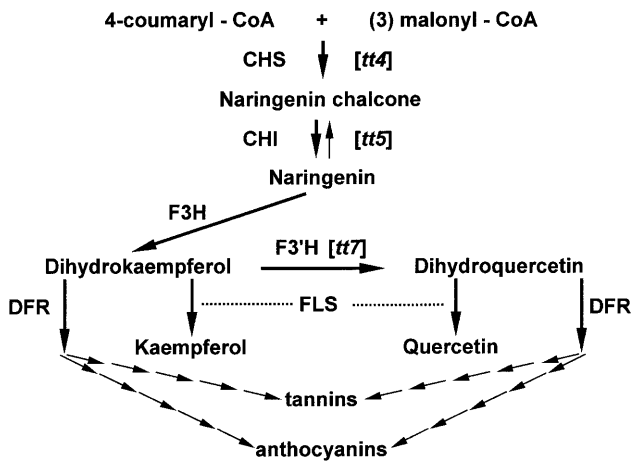


Figure 1. The Phenylpropanoid/Flavonoid Biosynthesis Pathway.

The *tt4*(2YY6) null mutant has a lesion in the chalcone synthase gene (CHS), resulting in no flavonoid production. When naringenin is fed in the growth medium, flavonoid synthesis is restored in the *tt4*(2YY6) mutant.

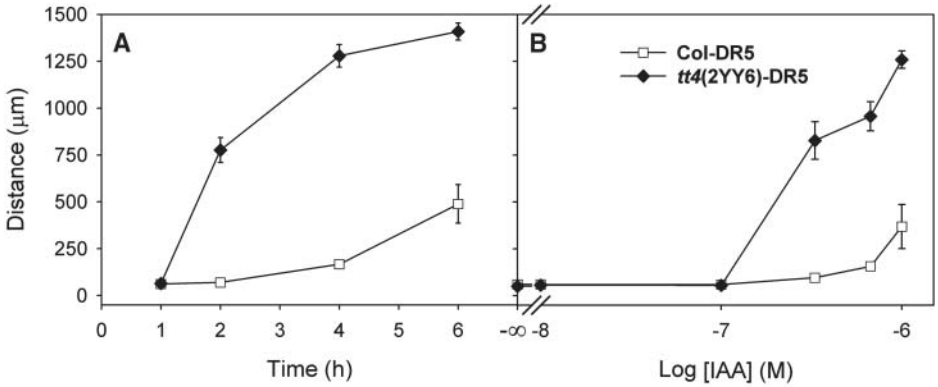
RESULTS

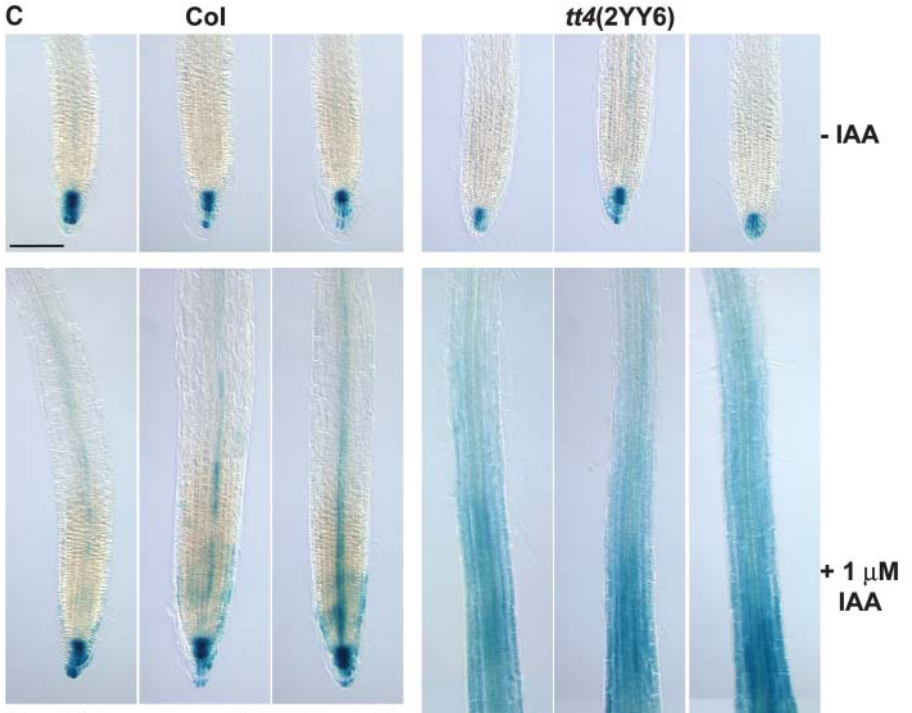
Basipetal Auxin Transport Is Elevated in *tt4*(2YY6) Roots

Previous work suggested that basipetal auxin movement provided the auxin necessary for gravity responses in Arabidopsis roots (Rashotte et al., 2000). Col and *tt4*(2YY6) seedlings were analyzed for root basipetal auxin transport using two methods. First, we quantified the movement of auxin-induced gene expression back from the root tip when auxin was locally applied to the root tip. Col and *tt4*(2YY6) seedlings transformed with the auxin-induced DR5-GUS reporter were treated with and without 1 μM exogenous IAA applied from an agar cylinder at the root tip for 1 to 6 h, and GUS expression was visualized histochemically after a 1 h incubation with substrate. To verify that this assay was reporting basipetal auxin transport differences between Col and *tt4*(2YY6), rather than differences in responsiveness to exogenous IAA, the kinetics of the basipetal movement of IAA-induced GUS expression were examined. The length of the DR5-GUS staining zone was measured as a function of time after application of IAA at the root tip in multiple plants. Figure 2A shows that the distance of DR5-GUS expression from the root tip is greatly increased in *tt4*(2YY6) relative to Col. Furthermore, this assay does not measure acropetal movement of IAA because IAA cylinders applied at 5 and 10 mm from the root tip showed no staining in either *tt4*(2YY6) or Col seedlings (data not shown).

It is possible that the difference in DR5-GUS expression in Col and *tt4*(2YY6) reflects differences in IAA sensitivity. To explore this possibility, the effects of a range of IAA concentrations were compared in Figure 2B. DR5-GUS expression was induced at similar concentrations of IAA, consistent with a similar sensitivity to IAA in both lines. The threshold IAA concentration of 0.33 μM was needed for DR5-GUS expression to extend beyond the root tip in both Col and *tt4*(2YY6). The distance of the IAA-induced gene expression signal was statistically greater in both Col and *tt4*(2YY6) at this concentration compared with 0, 0.01, or 0.1 μM IAA ($P \leq 0.025$, as judged by Student's *t* test). At all IAA concentrations, *tt4*(2YY6) exhibited greater distances of IAA-induced gene expression with no consistent differences in DR5-GUS expression intensity.

The basipetal movement of the IAA-induced gene expression signal is shown in Figure 2C. In the absence of applied IAA, Col and *tt4*(2YY6) seedlings have GUS expression localized to within ~60 μm from the root tip, with a strong expression in the columella cells. In Col treated with IAA at the root tip, GUS expression increased in epidermal tissues further back from the root tip but was patchy, whereas expression remained in the columella and spread into the proximal lateral root cap cells. By





D Light-grown *tt4*(2YY6) seedlings have elevated [^{3}H]-IAA root basipetal auxin transport.

Tissue Source	IAA Transport (pmol)[a]
Col	5.05 ± 0.24
tt4(2YY6)	6.16 ± 0.29[b]

[a] The results are pooled from 4 experiments of 10 seedlings per treatment; the average and SE are reported.
[b] Significantly different as judged by Student's *t*-Test -- $p = 0.005$.

Figure 2. Light-Grown *tt4*(2YY6) Roots Have Elevated Basipetal Auxin Transport.

An agar cylinder containing IAA was applied at the root tip, and the transgenic Col DR5-GUS and *tt4*(2YY6) DR5-GUS seedlings were incubated in the dark for 1 to 6 h. GUS staining was viewed with DIC optics.

(A) One μM IAA was applied to the root tips, and GUS expression was measured as the distance of blue staining from the root tip at several times after application.

(B) The zone of DR5-GUS expression varied with auxin concentration in the agar cylinder.

contrast, *tt4*(2YY6) seedlings showed a considerable increase in the region of IAA-induced GUS expression, and expression diminished in columella cells consistent with elevated basipetal IAA movement from the root tip. The region of auxin-induced GUS expression as measured from the root tip averaged 1300 ± 160 μm in *tt4*(2YY6) versus 174 ± 49 μm in Col (highly significant; $P = 6.7 \times 10^{-12}$; Student's *t* test). The vascular staining that occasionally occurred in Col seedlings was not quantified because this localization is not in the tissues associated with basipetal auxin transport.

Because the DR5-GUS reporter is an indirect measure of auxin movement, IAA transport in the root tips was quantified more directly. We quantified [^{3}H]IAA basipetal movement as described previously (Rashotte et al., 2000), and the data are presented in Figure 2D. Again, significantly more basipetal auxin transport occurred in *tt4*(2YY6) when compared with Col in the region from 1 to 6 mm from the root tip under similar conditions to the DR5-GUS reporter assay. To demonstrate that the IAA has moved away from the site of application, it is essential to remove the root tip in contact with the radioactive agar in this IAA assay. The smallest segment that can be reproducibly removed from the root tip is 1 mm, yet much of the interesting difference between Col and *tt4*(2YY6) roots is in this region that must be discarded. Therefore, it is not surprising that the magnitude of the difference between Col and *tt4*(2YY6) is less using this assay.

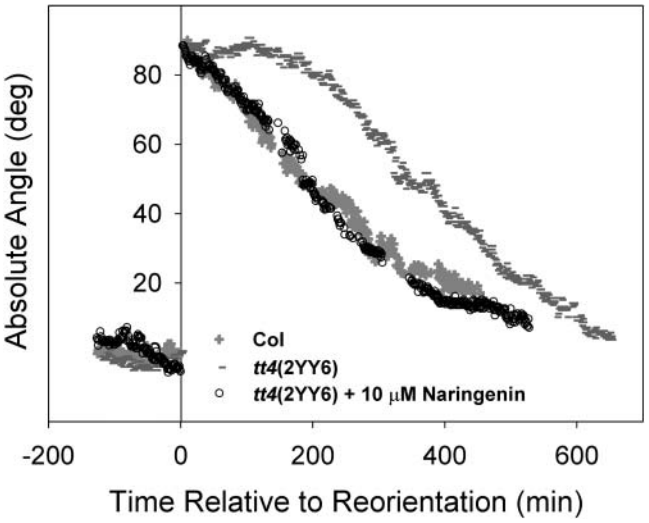


Figure 3. The Gravitropic Response in *tt4*(2YY6) Roots Is Significantly Delayed, but the Delay Is Reversed by Naringenin.

Representative runs from Multi-ADAPT compare characteristic root tip responses of 5 to 6 d light-grown Col, *tt4*(2YY6), and *tt4*(2YY6) plus 10 μM naringenin to gravity stimulation. The seedlings were placed in the dark and grown vertically for ~2 h before gravity stimulation by turning the Petri dish 90°.

tt4(2YY6) Roots Exhibited a Delayed Gravity Response and Naringenin Restored Bending to Wild-Type Levels

To determine whether there were gravitropic response differences between Col and *tt4*(2YY6), we measured root tip responses to a 90° reorientation relative to the gravity vector. Individual root tips were followed using the computer program Multi-ADAPT (Ishikawa and Evans, 1997; Mullen et al., 1998). This software program simultaneously measures the root tip angle and elongation on both sides of the root, providing high temporal and spatial resolution to the analysis of root gravitropic bending. Representative experiments comparing 5- to 6-d *tt4*(2YY6) and Col gravity responses are shown in Figure 3. To quantify the effect on bending, the lag in bend initiation was defined as the time when the root tip absolute angle exceeded and remained beyond 80°. To calculate the difference in timing of bend initiation and in the bend rate, multiple roots were analyzed, and a summary of the experiments is shown in Table 1. Light-grown *tt4*(2YY6) roots exhibited a significant lag early in the gravitropic response compared with light-grown Col ($P = 0.0039$; Student's *t* test). The roots of *tt4*(2YY6) consistently required nearly twice as long to initiate bending, but once *tt4*(2YY6) roots began the gravity response, they bent with a rate of curvature equal to Col.

It was reported previously that flavonoid biosynthesis is restorable in *tt4*(2YY6) by providing naringenin, a flavonoid intermediate that is synthesized after the missing chalcone synthase reaction (Figure 1), as judged by anthocyanin accumulation in seedling cotyledons (Shirley et al., 1995) and diphenylboric acid 2-amino-ethyl ester (DPBA) fluorescence at the RSJ (Murphy et al., 2000). DPBA becomes fluorescent upon binding of flavonoids in vivo. Naringenin concentrations between 1 μM to 1 mM were tested to determine which concentration restored flavonoid synthesis to wild-type levels as judged by DPBA fluorescence. Naringenin at 10 μM restored flavonoid levels to near wild type as discussed later. To examine whether the defect in flavonoid biosynthesis in *tt4*(2YY6) was directly tied to gravity defects, we tested whether growth of *tt4*(2YY6) roots on media containing 10 μM naringenin restored a normal gravity response. Gravitropic curvature in multiple seedlings was determined by Multi-ADAPT, and growth on naringenin restored gravitropic bending to wild-type levels, as indicated in Figure 3 and in the data summary shown in Table 1.

Because the Multi-ADAPT program records the elongation rate of the opposite sides of the root (Ishikawa and Evans, 1997), we analyzed these rates to determine why gravitropic bending in *tt4*(2YY6) is delayed. Although considerable

Figure 2. (continued).

(C) DIC images of roots treated with 1 μM IAA for 5 h. Measurements of auxin-induced GUS expression from the root tip are: −IAA, Col = 65 ± 7.7 μm, *tt4*(2YY6) = 7.9 μm; +IAA, Col = 49 μm, *tt4*(2YY6) = 160 μm. Bar = 100 μm.
(D) Comparison of [^{3}H]IAA basipetal transport in the region 1 to 6 mm from the root tip in Col and *tt4*(2YY6).

Table 1. Root Gravitropic Bending Initiation in *tt4*(2YY6) Seedlings Lags Significantly

Measurement[a]	Col	*tt4*(2YY6)	*tt4*(2YY6) + 10 μM Naringenin
Bending lag (min)	91.1 ± 12.2	153.1 ± 12.2[b]	85.3 ± 8.2
Rate of curvature (degree min^{-1})	0.19 ± 0.020	0.17 ± 0.020	0.21 ± 0.028
Vertical elongation rate (μm min^{-1})			
Right/upper	6.1 ± 0.8	5.4 ± 0.8	6.7 ± 1.1
Left/lower	5.9 ± 0.6	5.9 ± 0.8	6.3 ± 0.7
0 to 30 min after turning elongation rate (μm min^{-1})			
Upper	8.7 ± 0.7[c]	5.6 ± 0.7[b]	7.0 ± 0.9
Lower	8.0 ± 0.8	5.8 ± 1.4	6.3 ± 1.4
30 min to bend initiation elongation rate (μm min^{-1})			
Upper	7.0 ± 1.2	6.1 ± 0.6	10.4 ± 1.7
Lower	7.3 ± 0.9	5.6 ± 0.6	6.0 ± 0.6[d]
Bending elongation rate (μm min^{-1})			
Upper	7.7 ± 0.5	8.9 ± 0.9[c]	7.7 ± 0.7
Lower	6.7 ± 0.4	6.3 ± 0.8	6.5 ± 0.4

[a] The average and SE of roots analyzed by Multi-ADAPT computer software with six, eight, and nine individual 5- to 6-d Col, *tt4*(2YY6), and *tt4*(2YY6) plus 10 μM naringenin seedlings, respectively. Elongation rates were measured in 50-μm segments from the root tip.

[b] Indicates significant differences as compared to Col with $P \leq 0.05$.

[c] Indicates significant difference as compared to the initial elongation rate with $P < 0.05$.

[d] Indicates significant difference between the elongation rates on the upper and lower root with $P < 0.05$.

Student's *t* tests were used for comparisons of *tt4*(2YY6) to Col, or to initial elongation rates, or between upper and lower root elongation rates. In the absence of a footnote, no significant differences exist in these characteristics.

variation existed between individual roots, elongation rates of vertically grown seedlings were not significantly different between Col, *tt4*(2YY6), and *tt4*(2YY6) treated with naringenin (Table 1). These measurements demonstrated that the delay in *tt4*(2YY6) gravitropism is not as a result of a decrease in elongation in *tt4*(2YY6) roots, consistent with a previous report indicating that *tt4*(2YY6) roots have greater overall growth than wild type (Brown et al., 2001). When the elongation rates on either side of the root tip were examined during the initial 30 min after turning the root, elongation accelerated significantly in Col seedlings relative to the vertical control ($P = 0.03$; Student's *t* test), and this acceleration was absent in *tt4*(2YY6) in either the first or second 30-min period after gravity stimulation but occurred after bending had initiated. This acceleration was delayed slightly in *tt4*(2YY6) plus naringenin until 30 min had passed, and then root elongation accelerated. After root bending had initiated in Col, *tt4*(2YY6), or *tt4*(2YY6) plus naringenin, asymmetric root elongation was detected with higher rates on the upper side, resulting in comparable gravitropic curvature. These data are consistent with differential growth in roots occurring as a result of reduced levels of auxin on the upper side of the root, leading to elevated root elongation on that side, and resulting in a downward curvature of the root. Furthermore, the difference in growth immediately after gravity stimulation in *tt4*(2YY6) relative to Col and naringenin-supplemented *tt4*(2YY6) suggests that flavonoids are necessary for the normal growth promotion that occurs in this phase in the wild type. This growth promotion may be because of inhibition of polar basipetal IAA transport from the root tip back and is consistent with elevated growth in response to the resulting decrease in IAA in elongating tissues.

The acceleration of root growth after gravitropic reorientation differs from a previous report also using Multi-ADAPT software, in which Arabidopsis roots exhibited decreased growth after gravity stimulation (Ishikawa and Evans, 1997). There are numerous differences in the ways that the roots were grown in these two analyses, making it difficult to identify the reason for this difference. It is clear that under our growth conditions, we did not detect any growth inhibition, but we did see growth promotion upon reorientation.

Because only the early stages of root gravitropic bending are reduced in *tt4*(2YY6), it was logical to examine whether gravity perception was altered. Direct measurement of the minimum gravity stimuli needed to initiate bending is difficult because sufficient growth to measure curvature occurs much later than the short gravitropic stimulus. The approach used by many investigators to assess gravity perception is to gravity stimulate roots for short periods and then rotate the seedlings on a clinostat for several hours. The clinostat randomizes the gravity vector by rotating the samples continuously at 1*g*, allowing additional gravity-independent elongation, to assess the response to the initial gravity stimulus. The angle of gravitropic curvature change was measured after clinorotation and plotted using the linear-log model (Larsen, 1957) and the hyperbolic method (Perbal et al., 2002) to estimate the minimum time likely to induce bending and determine gravisensitivity, respectively. These graphs are included as supplemental data online. Presentation time, which is the minimum gravity stimulation needed to get a response, was estimated by linear-log analysis and was similar for *tt4*(2YY6) and Col, at 1.1 and 1.2 min, respectively. Our presentation time results were similar to previously published times of 1.2 min (Blancaflor et al.,

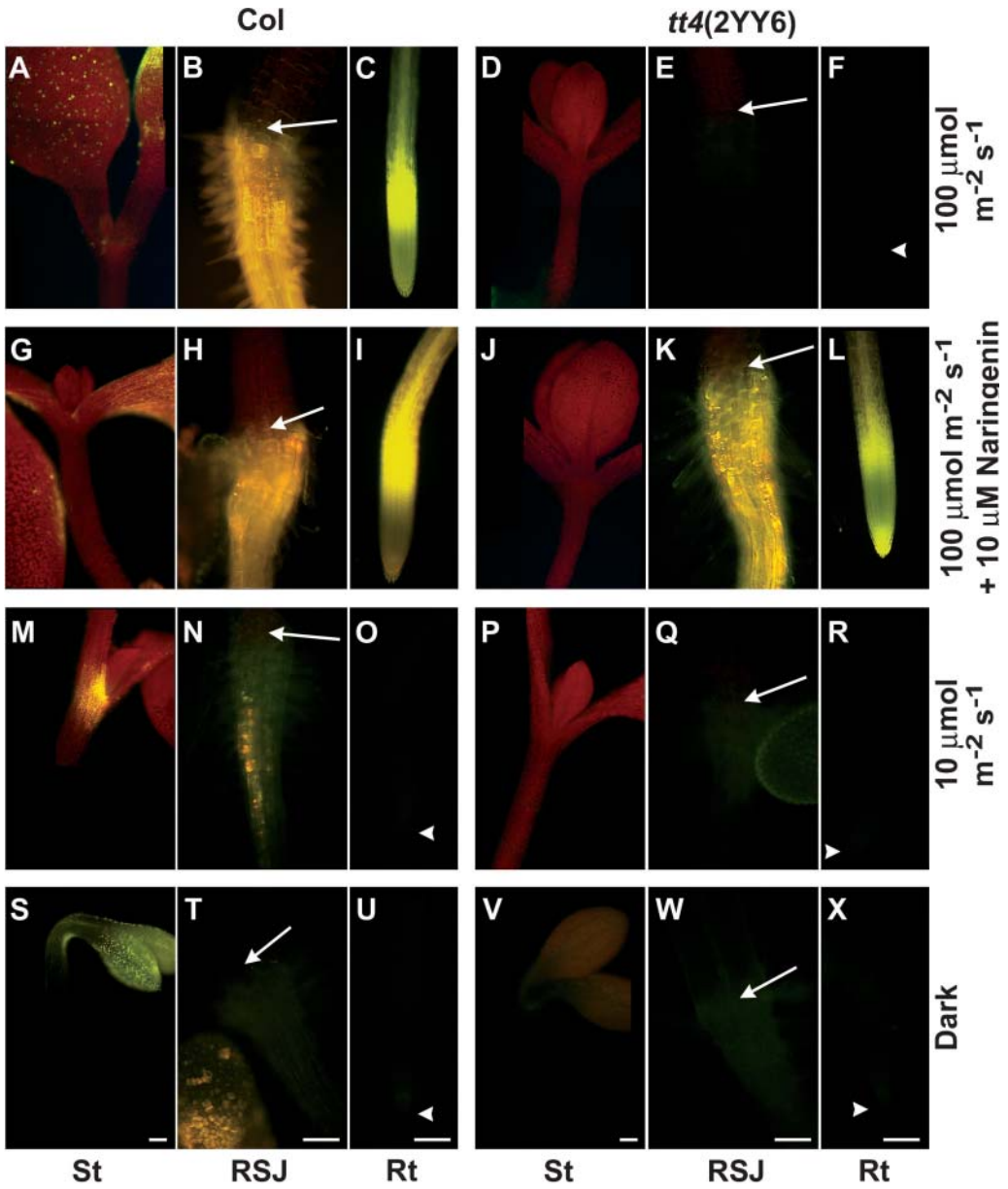


Figure 4. Flavonoids Accumulate in Light-Grown Col.

Flavonoid accumulation in 5-d Col seedlings grown under 100 μmol m^{-2} s^{-1} light (**[A]** to **[C]**), 100 μmol m^{-2} s^{-1} light plus 10 μM naringenin (**[G]** to **[I]**), 10 μmol m^{-2} s^{-1} light (**[M]** to **[O]**), or in the dark (**[S]** to **[U]**). Photographs of 5-d *tt4*(2YY6) seedlings grown under 100 μmol m^{-2} s^{-1} (**[D]** to **[F]**), 100 μmol m^{-2} s^{-1} + 10 μM naringenin (**[J]** to **[L]**), 10 μmol m^{-2} s^{-1} light (**[P]** to **[R]**), or in the dark (**[V]** to **[X]**). Stem (St) montage photographs were assembled to include the cotyledonary node. Exposure times were 0.33 s for St and RSJ images and 0.6 s for root tip images. Arrows indicate the location of the RSJ, and arrowheads the location of nonflavonoid accumulating root tips. The scale bar is 100 μm.

1998) and 1.6 min (Vitha et al., 2000) for light-grown Col. The hyperbolic analysis also suggested that *tt4*(2YY6) was more sensitive to gravity than Col, with gravity sensitivity values of 4.25 and 2.05° g^{-1} min^{-1}, respectively. Therefore, both methods suggest that *tt4*(2YY6) seedlings are not impaired in gravity perception but are perhaps more sensitive than the wild type to a short gravity stimulus.

Flavonoids Accumulate in Tissues that Transport Auxin in Col

DPBA provides a method to observe flavonoid accumulation in plant tissues. DPBA fluoresces gold (emission maxima [E_{max}] = 543 nm) when bound to quercetin and yellow-green (E_{max} = 520 nm) when bound to kaempferol, whereas chlorophyll

autofluorescence is red (E_{max} = 665 to 685 nm; Peer et al., 2001). Col seedlings grown in 100 μmol m^{-2} s^{-1} light accumulated flavonoids in three locations when visualized with epifluorescent microscopy: the shoot apex and cotyledons, the RSJ, and along the root (Figures 4A to 4C). The brightest staining began ~350 to 375 μm from the root tip (Figure 4C). This region of intense fluorescence corresponds to the distal elongation zone in Arabidopsis, which is the site of gravitropic bending (Mullen et al., 1998). Flavonoids were absent in similarly grown *tt4*(2YY6) seedlings (Figures 4D to 4F), although light greenish fluorescence because of sinapate esters was observed as reported previously (Murphy et al., 2000). The absence of DPBA fluorescence in *tt4*(2YY6) is consistent with the lesion in the chalcone synthase gene and indicates that DPBA fluorescence is specific for products of the flavonoid pathway. Growing *tt4*(2YY6) seedlings on medium containing 10 μM naringenin (Figures 4J to 4L) consistently restored flavonoid accumulation in the same tissue locations as in Col grown in the absence (Figures 4A to 4C) or presence of naringenin (Figures 4G to 4I). This dose of naringenin did not affect root elongation or gravity response of wild-type seedlings, although significantly higher naringenin concentrations did affect these two growth processes (Brown et al., 2001). Subtle differences in flavonoid accumulation were detectable after feeding naringenin as compared with untreated Col roots. Naringenin-fed Col roots (Figure 4I) had more gold fluorescence compared with Col without naringenin (Figure 4C), and *tt4*(2YY6) root tips had higher levels of flavonoid accumulation (cf. Figures 4C and 4L, as judged by brighter fluorescence at equal exposure times).

The greater resolution of confocal laser scanning microscopy (CLSM) was used to examine the tissue and cellular localization of flavonoid accumulation (Figure 5). A Col root was examined at low magnification, and the presence of flavonoids in cells along the root was demonstrated with lower concentration at the root tip than in the elongation zone (Figure 5A), paralleling the epifluorescence images in Figure 4. When the root tip was examined at higher magnification, flavonoids were detected in the cells of the root cap (Figures 5B and 5C), including the columella cells, which are the site of gravity perception (Blancaflor et al., 1998), and in the epidermal cells, which are the site of differential gravitropic growth. Naringenin-fed *tt4*(2YY6) seedlings also had flavonoid accumulation in tissues similar to Col seedlings (Figures 5D and 5E). The very faint background fluorescence present after staining *tt4*(2YY6) roots with DPBA is because of sinapate esters, as shown in Figures 5F and 5G (Murphy et al., 2000). Clearly, there is no flavonoid fluorescence present, even though the camera gain was increased to its maximum.

CLSM also facilitated examining the cellular localization of flavonoid accumulation. The DPBA fluorescence occurred in the cytoplasm in both Col and naringenin-fed *tt4*(2YY6) root tips (Figure 5). The amyloplasts are visible as unstained organelles in the center of the columella cells in both Col and *tt4*(2YY6) fed with naringenin. The rest of the cytoplasm of these cells is filled

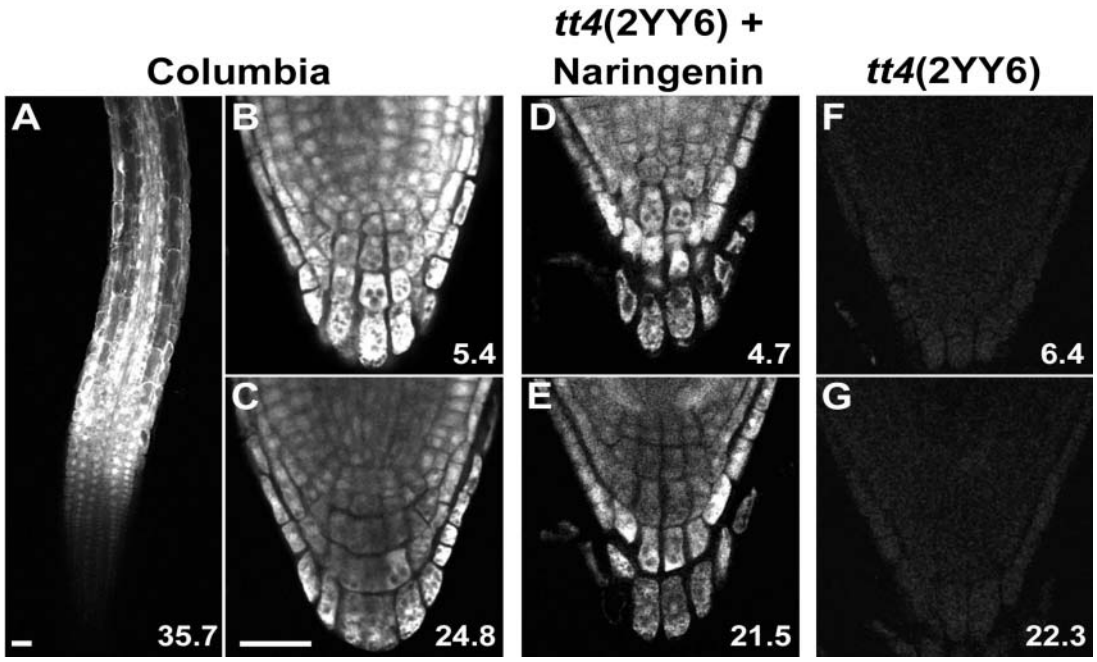


Figure 5. Flavonoids Localize to Columella and Epidermal Tissues in Light-Grown Col and Naringenin-Fed *tt4*(2YY6) Root Tips.

CLSM 10× objective optical section micrograph of DPBA fluorescence in Col **(A)**. Individual optical sections of root tips with a 40× objective are shown for Columbia (**[B]** and **[C]**); *tt4*(2YY6) fed naringenin (**[D]** and **[E]**); and in root tips of *tt4*(2YY6) (**[F]** and **[G]**). Amyloplasts are apparent in **(B)** and **(D)** as dark structures in the center of the cytoplasm of the central columella cells. The approximate depths of individual confocal optical slices (in μm) are indicated in the lower right corner of all panels. Bars = 25 μm.

with flavonoids with slightly greater fluorescence intensity in naringenin-fed *tt4*(2YY6) roots compared with Col. The cytoplasmic localization of flavonoid accumulation is consistent with cytoplasmic orientation of high affinity membrane associated naphthylphthalamic acid (NPA) binding activity from zucchini (*Cucurbita pepo*), which may be the site of flavonoid action (Dixon et al., 1996). DPBA fluorescence was also detectable in the nuclei of Col and naringenin-fed *tt4*(2YY6), although some naringenin-fed *tt4*(2YY6) samples lacked nuclear staining. Accumulation of flavonoids in the nuclei of root tip and elongation zone cells has also been observed by others in the Landsberg *erecta* ecotype (D. Saslowsky and B. Winkel, personal communication). The physiological significance of these differences is unknown, and the significance of nuclear flavonoid accumulation is not clear. What is clear is that the naringenin treatment restored flavonoid synthesis to the cytoplasm of columella cells, which are the site of gravity perception, and to cells of the elongation zone, where differential growth in response to gravity stimulation occurs.

Flavonoid Accumulation Increased in Gravity-Stimulated Col Root Tips

To determine if flavonoid accumulation changed in response to gravity stimulation, DPBA fluorescence was quantified in roots at various time points after gravitropic reorientation using epifluorescence microscopy (Figure 6A). The response was measured by comparing DPBA fluorescence of the apical 40 μm of the root tip, containing the root tip and extending back no farther than the columella cells in gravity-stimulated versus vertically grown roots. Relative DPBA fluorescence as a function of time after reorientation was determined by dividing the average maximum fluorescence intensity after gravity stimulation by the average maximum intensity of the vertical controls. Similar trends are apparent when DPBA fluorescence is reported directly, but the absolute values vary more between individual experiments. The DPBA fluorescence in the root columella cells began increasing at 1 to 1.5 h after gravity stimulation. The fluorescence peaked at 1.5 to 2.5 h after gravity stimulation with a nearly twofold increase in DPBA fluorescence as compared with the vertically grown controls. This peak of gravity-induced flavonoid accumulation corresponded precisely with the time of the maximum difference in the gravity response between *tt4*(2YY6) and Col (Figure 3; Table 1). By contrast, no changes in the bright DPBA fluorescence was observed in the distal elongation zone, either in distance from the root tip or in intensity (data not shown).

Representative DPBA-stained root tips were examined by confocal microscopy in both vertical controls and roots gravity stimulated for 2.25 h, as shown in Figures 6B to 6E. To prevent phototropic responses, light-grown seedlings were placed in the dark in either a vertical or horizontal orientation, as in the above quantitative experiments. In the vertical controls, this dark treatment alters the flavonoid accumulation pattern, resulting in less fluorescence in the columella cells. Gravity stimulation increases the DPBA fluorescence in the root tip, with subtle increases in the columella and more dramatic increases in the outer cell layers (cf. Figures 6B and 6D; cf. Figures 5C and 5E in the outer cell layers). Consistent with the quantification of multiple samples described above, intensity averaged 1.5 times higher in the gravity-stimulated roots when an optical section focused on the epidermal cells was compared with the vertical control ($n = 3$). When optical sections through the root columella cells are quantified, the fluorescence is 1.9 times greater in the outer cells layers in the representative gravity stimulated root tip compared with the vertical control shown in Figures 6E and 6C.

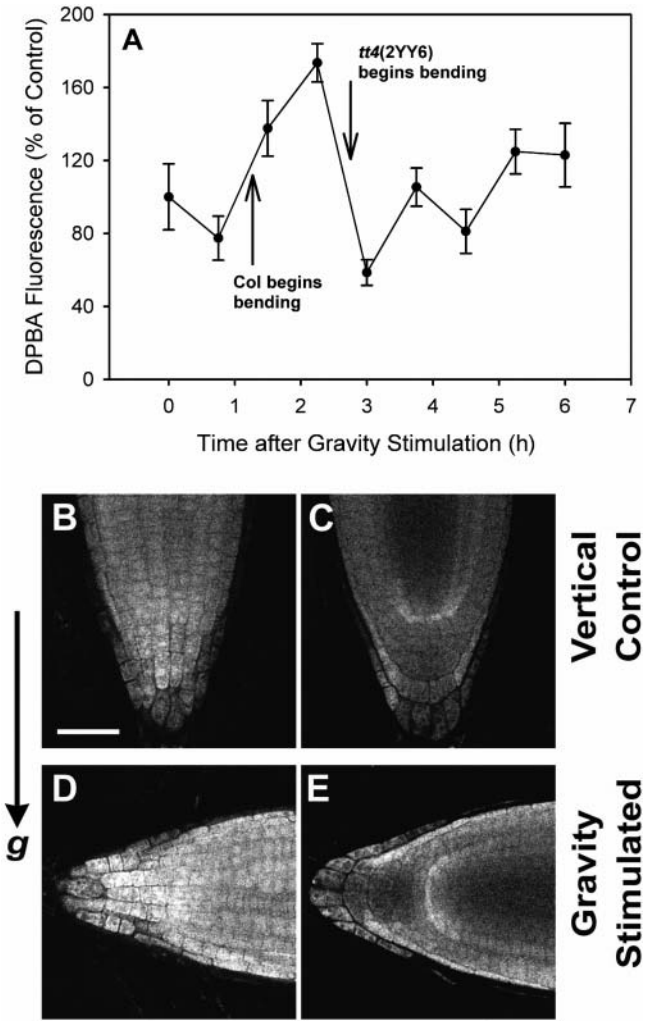


Figure 6. Flavonoid Accumulation Spikes during Gravitropic Stimulation in Col.

(A) The relative DPBA fluorescence determined by dividing average maximum flavonoid accumulation across the 40 μm from the root tip of vertically grown controls versus gravity-stimulated roots measured over time is reported. The results from a representative experiment with 10 seedlings in each experimental category, which was repeated four times with similar results, are shown. Arrows indicate times of gravity-stimulated root bending in Col and *tt4*(2YY6). Error bars are added percent error from the control and the treatment averages.

(B) to **(E)** Col root tips of 2.25 h vertical controls (**[B]** and **[C]**) and gravity stimulated roots (**[D]** and **[E]**). The optical slices in **(C)** and **(E)** are ~15 μm below the root-tip surface that is shown in **(B)** and **(D)**. Scale bar = 40 μm.

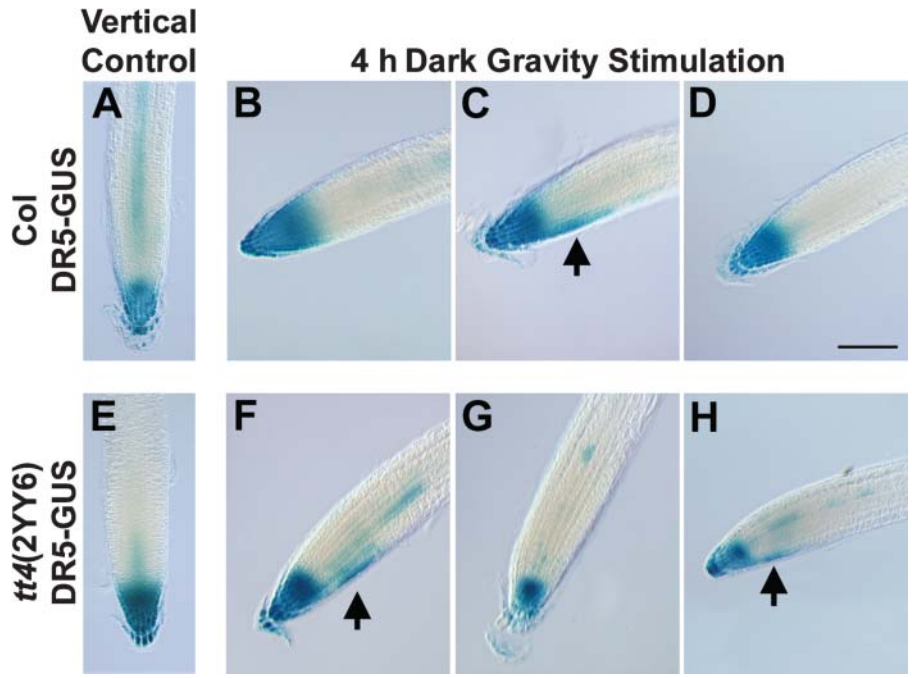


Figure 7. An Auxin-Induced DR5-GUS Expression Gradient Occurs in Gravity-Stimulated *tt4*(2YY6) and Col Roots.

Col (**[A]** to **[D]**) and *tt4*(2YY6) seedlings (**[E]** to **[H]**) were grown vertically in the dark as controls (**[A]** and **[E]**) or gravity stimulated for 4 h in the dark (**[B]** to **[D]** and **[F]** to **[H]**). Arrows on the DIC images indicate the location of the gradient obtained after GUS staining for 3 h. Bar = 100 μm.

Surface plots of DPBA fluorescence intensity generated using Image Pro Plus software are included as supplemental data online and more dramatically illustrate the epidermal flavonoid accumulation in response to gravity stimulation. This elevation of flavonoids in response to gravity in the epidermal cells is consistent with a role for flavonoids in regulating polar auxin transport rather than gravity perception in the columella cells.

Auxin-induced GUS Expression Gradients Were Similar in *tt4*(2YY6) and Col

After prolonged gravity stimulation Arabidopsis root tips transformed with the DR5-GUS reporter showed an auxin-induced GUS expression gradient, which presumably reflects lateral auxin redistribution during gravitropism (Rashotte et al., 2001). We tested for this gradient in gravity stimulated *tt4*(2YY6) and Col roots. The gradient was not observed until 4 to 6 h after gravity stimulation, late in the gravitropic cascade of events (Figure 7). This asymmetry in GUS expression was not found in all roots; in Col it was detectable in >25% of the roots at 4 h after gravity reorientation. This gradient was found at similar times and in similar positions in *tt4*(2YY6) roots and occurred with greater frequency, being found in >50% of the roots 4 and 6 h after gravity stimulation. The DR5-GUS gradient was detectable only after gravitropic stimulation and was never observed in vertically grown Col or *tt4*(2YY6) seedlings. The detection of this gradient in some but not all gravity stimulated roots is consistent with previous observations that the formation of the gradient is transient and more tightly linked to the angle of curvature than the duration of gravitropic stimulation (Rashotte et al., 2001). The timing of the detection of the DR5-GUS expression gradient corresponds to when the gravity response in *tt4*(2YY6) is no longer delayed. Therefore, the formation of the gradient of DR5-GUS expression in *tt4*(2YY6) with even greater frequency is consistent with normal gravity response at this later time after reorientation relative to gravity.

Changing Light Conditions Modulated Flavonoid Synthesis Levels and Root Acropetal Auxin Transport

White light initiates the transcription of genes that encode the flavonoid biosynthesis enzymes (Christie and Jenkins, 1996; Pelletier and Shirley, 1996; Winkel-Shirley, 2002). We examined the effects of light on flavonoid accumulation patterns and correlated the resulting effects with changes in auxin transport. As discussed previously, flavonoids accumulated in Col seedlings grown under 100 μmol m^{-2} s^{-1} white light (Figures 4A to 4C). In etiolated Col seedlings, flavonoid fluorescence was absent except for punctate fluorescence from quercetin and kaempferol in the cotyledons (Figures 4S to 4U). Etiolated *tt4*(2YY6) seedlings exhibited no DPBA fluorescence in any tissue (Figures 4V to 4X). Moving etiolated Col seedlings to the light for increasing time periods (4, 8, 16, 24, and 48 h) gradually increased the amount of flavonoid fluorescence, which after 48 h reached or exceeded the level of fluorescence in Col seedlings

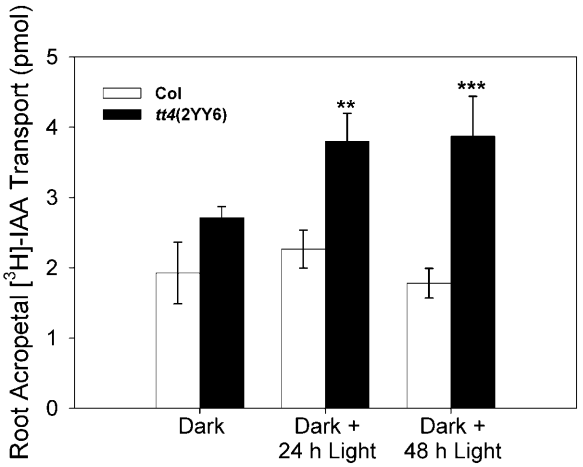


Figure 8. Etiolated Col Seedlings Have Similar Root Acropetal Auxin Transport Compared with *tt4*(2YY6).

Graphs are representative data from one experiment. The experiment was repeated four times with similar results. Seedlings were incubated with 100 nM [^{3}H]IAA applied at the RSJ for 18 h. The apical 5 mm of the roots were excised and measured for radioactive disintegrations. The bars are SE, and statistical analysis was performed using the Student's *t* test. Asterisks indicate the level of significance between Col and *tt4*(2YY6): **, $P = 0.0052$; and ***, $P = 0.00054$. The probability of Col and *tt4*(2YY6) being different in the etiolated state is 0.13.

grown under continuous 100 μmol m^{-2} s^{-1} light levels (data not shown).

Seedlings of etiolated Col were transferred to light to induce flavonoid synthesis and compared with similarly treated *tt4*(2YY6) seedlings. Root acropetal IAA transport was analyzed in etiolated roots and roots returned to the light for 24 and 48 h (Figure 8). In etiolated seedlings, the levels of acropetal IAA transport were similar in Col and *tt4*(2YY6) seedlings ($P = 0.13$; Student's *t* test), consistent with the absence of flavonoids in both treatments. Upon transfer to light, the differences between Col and *tt4*(2YY6) increased with the duration of light treatment, leading to a twofold elevation in transport in the *tt4* mutant ($P < 0.0001$). Surprisingly, the levels of transport remained relatively constant in Col but increased in *tt4*(2YY6) with increasing light. This result is consistent with light increasing auxin production, as reported previously (Bhalerao et al., 2002). This elevated IAA synthesis presumably led to the elevated IAA transport in *tt4*(2YY6), whereas in Col, the increased IAA levels were balanced with increased flavonoid synthesis, which kept transport at similar levels. The difference in acropetal transport of etiolated seedlings treated with light is consistent with elevated transport of auxin from the shoot into the root in light-grown Arabidopsis seedlings, which was measured using a different assay approach (Brown et al., 2001).

IAA-Induced Flavonoid Accumulation

These results are consistent with a role for flavonoids in modulation of auxin transport during gravitropism and with gravity-inducing flavonoid synthesis. A more complex possibility is that the altered auxin distribution in response to gravity stimulation induced local flavonoid synthesis. To test this possibility, we performed experiments to follow changes in flavonoid-induced DPBA fluorescence by adding IAA or NPA to the growth media (Figure 9). The relative DPBA fluorescence was measured in the apical 40 μm of the root tip as described above, by comparing maximum intensity changes in untreated plants compared with those treated with IAA or NPA. After 4 h, flavonoid accumulation increased in seedlings grown on IAA, reaching a fivefold elevation by 24 h. The kinetics of this induction is considerably slower than the gravity-induced flavonoid induction. At the early time points after transfer, IAA caused a decrease in flavonoid accumulation. This difference was significant between the absolute numbers at 0.5 h and 1 h, although normalizing the data to the control masks this significance as the errors add. Therefore, IAA induced flavonoid synthesis, but this effect occurred relatively late after exogenous IAA exposure.

The transfer of seedlings to media containing NPA at levels sufficient to block IAA transport did not increase flavonoid accumulation, although at early times after exposure to NPA, DPBA fluorescence decreased, in parallel to the effect of IAA. The most direct test of whether the flavonoids accumulating in response to gravitropic stimulation were the cause or the result of IAA transport changes was to gravity stimulate seedlings in the presence of NPA, which prevents auxin redistribution and gravitropic bending. The presence of NPA prevented gravity-induced flavonoid accumulation in three separate treatments (data not shown), suggesting that local changes in IAA at the root

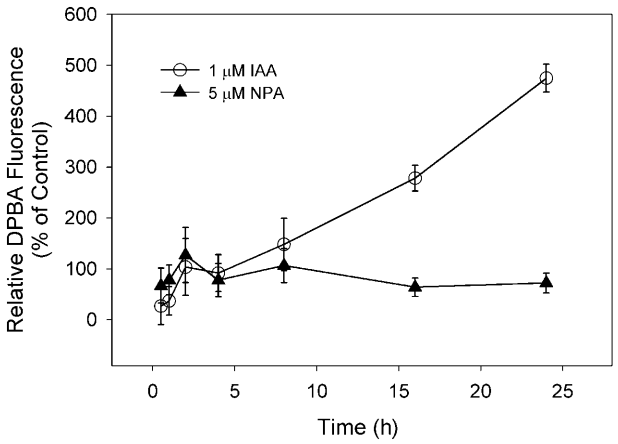


Figure 9. IAA Elevates Flavonoid Accumulation in Col Root Tips.

Seedlings were transplanted to control agar or treatment plates (1 μM IAA or 5 μM NPA) and incubated for the indicated times. DPBA staining was performed, and relative DPBA fluorescence was determined by dividing the average fluorescence intensity of the treatment by the average fluorescence intensity of the control. A representative experiment is shown with 10 seedlings in each experimental category averaged and plotted at the various times. The experiment was repeated three times with similar results. Error bars were obtained by adding percent errors between the control and treatment averages.

tip may play a role in the gravity-induced flavonoid synthesis. These results are consistent with gravity stimulation altering flavonoid levels and with flavonoids altering auxin transport at the root tip, with a complex interplay between these two events.

DISCUSSION

The major goals of these experiments were to determine if the physiological responses of Arabidopsis roots to gravity and light may be mediated through regulation of auxin transport by alterations in flavonoid synthesis. As the Arabidopsis *tt4*(2YY6) null mutant makes no flavonoids, this mutant is invaluable for resolving whether flavonoids regulate auxin transport and whether this regulation has a physiological role in modulating plant growth and development. Jacobs and Rubery (1988) first suggested an in vitro role for endogenous flavonoid action in auxin transport. In vivo studies of Arabidopsis mutants with defects in flavonoid synthesis indicated that auxin transport was elevated in Arabidopsis inflorescences and seedlings, providing further evidence that flavonoids have a role in polar auxin transport (Murphy et al., 2000; Brown et al., 2001). Although changes in inflorescence morphology of mature plants differed in parallel to changes in flavonoid content and auxin transport in *tt4* and wild-type plants, a direct relationship between growth and transport changes in inflorescences was not demonstrated (Brown et al., 2001). Arabidopsis roots provided an ideal system to examine the relationship between auxin transport, flavonoids, and dependent physiological processes and to directly test the hypothesis that environmental conditions regulate flavonoid accumulation and thereby modulate auxin transport.

Consistent with the absence of a negative auxin transport regulator, roots of Arabidopsis seedlings with the *tt4*(2YY6) mutation have elevated auxin transport. Because basipetal auxin transport is necessary for gravity responses in the root tip (Rashotte et al., 2000), we examined basipetal auxin transport in parallel to gravitropic bending. Elevated basipetal auxin transport is apparent in *tt4*(2YY6) compared with Col, both by movement of radiolabeled IAA and by basipetal movement of the IAA-induced gene expression using the DR5-driven GUS reporter. The apparent difference in transport in the *tt4*(2YY6) mutant is much greater in the latter assay because of the increased spatial resolution. The DR5-GUS reporter suggested that IAA moves basipetally at a greater rate and distance from the root tip back in *tt4*(2YY6) than Col. Although this reporter is an indirect measure of auxin movement, examination of direct movement of radiolabeled IAA confirmed the elevated basipetal IAA transport in *tt4*(2YY6) seedlings.

Along with the elevated basipetal IAA movement in the roots of *tt4*(2YY6), there was a significant alteration in gravity response in these roots. Col roots passed the gravitropic bending threshold 90 min after reorientation, whereas *tt4*(2YY6) root-gravitropic bending was not apparent until 150 min after reorientation. Yet, once *tt4*(2YY6) roots began to bend, the rate of bending was similar. Thus, flavonoid action must occur early in gravity responses because later responses are similar to the wild type. Furthermore, to verify that the gravity defect is directly tied to the absence of flavonoids in *tt4*(2YY6) roots, flavonoid synthesis was restored in these roots by growth on media containing the flavonoid precursor, naringenin, at doses that restored flavonoid synthesis to wild-type levels as judged by DPBA staining. Chemical complementation of the flavonoid synthesis mutation ameliorated the lag in *tt4*(2YY6) root gravity responses. Despite the early differences in *tt4*(2YY6) root gravity responses, gravity perception appears unhampered in this mutant. Examination of the presentation time and the gravity sensitivity, two estimates of the minimum time of gravity stimulation that will induce gravitropic bending, were obtained from two graphical methods (Larsen, 1957; Perbal et al., 2002). Both methods indicated that the *tt4*(2YY6) mutant is not impaired in gravity perception. Together, these data indicate that the absence of flavonoids in the *tt4*(2YY6) mutant is sufficient to delay gravity response but not to alter gravity perception or early events in signal transduction. Rather, the timing and localization of flavonoid accumulation is consistent with a role in regulation of auxin movement and the resulting differential growth.

Because gravity perception and initial gravitropic signaling occur in the root columella cells (Blancaflor et al., 1998), we asked whether flavonoids accumulate in these cells and how flavonoid accumulation is changed in response to gravity stimulation. The accumulation of flavonoids was examined in both wild-type and *tt4*(2YY6) seedlings using the molecule DPBA, which fluoresces in response to binding flavonoids. The dye specificity was demonstrated by the absence of the gold and yellow fluorescence in *tt4*(2YY6) plants, in contrast with the wild-type plants. Flavonoids accumulated in the cotyledons and shoot apex, at the RSJ, and at the root tip. Confocal microscopy identified DPBA fluorescence within root columella cells. Furthermore, *tt4*(2YY6) seedlings grown on agar supplemented with low doses of naringenin produced flavonoids at levels and with localization similar to Col, consistent with the restoration of gravity response described above.

An important goal of this study was to examine the connection between gravity stimulation and the flavonoid regulation of auxin transport. We asked whether gravity stimulation altered accumulating flavonoid levels. A spike in flavonoid accumulation occurred between 2 and 2.5 h after gravitropic stimulation. This increase coincided precisely with the time the maximum difference in gravity response occurred between Col and *tt4*(2YY6). CLSM was used to examine these root tips to determine where DPBA fluorescence increased in these roots and to ask if there were gravity-induced asymmetries in DPBA fluorescence. DPBA fluorescence increased more strongly in the outer layer of root cells than in the columella. This is consistent with a role of flavonoids in regulation of auxin transport and differential growth of epidermal cells rather than gravity perception or lateral auxin movement initiated in the columella. Additionally, DPBA gradients were not detected in response to gravity stimulation. Our model is that flavonoids function to reduce auxin transport from the tip back on both sides, and these findings are consistent with that model.

Because *tt4*(2YY6) has elevated basipetal transport and a reduced gravity response, it was important to examine lateral auxin transport at the root tip in response to gravity stimulation. The DR5-GUS reporter develops asymmetries at the root tip of gravity-stimulated plants (Rashotte et al., 2000), and this

expression was examined in *tt4*(2YY6). The *tt4*(2YY6) root tips exhibited asymmetric DR5-GUS expression with similar timing to Col; gradients were observed at 4 to 6 h after gravity stimulation. Indeed, the detection frequency of asymmetric expression was greater in *tt4*(2YY6). Because the gravity defect in *tt4*(2YY6) roots occurs much earlier than the time point at which DR5-GUS asymmetries become evident, the formation of a gradient late in the process is not surprising. This result indicates that the role of flavonoids is in early events in gravity-directed IAA transport, rather than the late events indicated by this reporter.

Our initial model was that flavonoid synthesis modulates auxin movement, but it was also important to determine if the altered IAA transport during gravity response induced flavonoid synthesis. To test this possibility, we grew seedlings on media containing IAA or NPA. Within 4 h after transfer to IAA containing media, an increase in DPBA fluorescence was apparent, and the DPBA fluorescence continued to increase over 24 h. This response is considerably slower than the increase in flavonoids in response to gravity stimulation. By contrast, flavonoid levels were relatively stable in vertical seedlings grown on NPA. Even more significantly, NPA minimized the gravity induced increases in flavonoid synthesis. Flavonoids facilitated gravitropic bending, regulated basipetal IAA transport, and were induced by gravity stimulation and by changes in IAA distribution. These data are consistent with flavonoid and auxin distribution changes being connected by a feedback loop.

To test whether light is another signal that modulates flavonoid levels, we transferred etiolated seedlings to the light for different times and measured root acropetal auxin transport. The induction of gene expression of flavonoid enzymes has been demonstrated previously (Pelletier and Shirley, 1996), and the elevated DPBA fluorescence in response to light (Figure 4) is clear. Etiolated Col and *tt4*(2YY6) had similar auxin transport in the upper root, consistent with the absence of flavonoids in both plants. As the plants were exposed to light and Col initiated flavonoid synthesis, auxin transport in *tt4*(2YY6) became significantly different from Col. Surprisingly, the levels of transport remained relatively constant in Col but increased in *tt4*(2YY6) with increasing light. This result is consistent with light increasing auxin production as reported previously (Bhalerao et al., 2002). Light-induced elevations in IAA synthesis presumably lead to the elevated IAA transport in *tt4*(2YY6), whereas in Col the increased IAA levels were balanced with increased flavonoid synthesis, which kept transport at similar levels.

The physiological significance of the modulation of acropetal auxin transport by flavonoids may be to control lateral root development. Control of lateral root initiation and elongation by acropetal auxin transport from the root into the shoot has been suggested by a number of experiments (Reed et al., 1998; Bhalerao et al., 2002; Casimiro et al., 2003), although this point was initially debated (Casimiro et al., 2001). Consistent with increased acropetal auxin movement in *tt4*(2YY6) are elevated numbers of lateral roots (Brown et al., 2001). An important question remained: Is flavonoid synthesis induced in roots under natural conditions, and are these flavonoids likely to modulate auxin movement into the roots? Col seedlings were grown into agar in a magenta box covered with a charcoal layer and with the agar wrapped in foil to mimic normal light conditions (i.e., aerial tissues in the light and roots in the dark). These seedlings also accumulated flavonoids in the roots (data not shown). Additionally, application of naringenin in an agar cylinder at the RSJ in *tt4*(2YY6) resulted in flavonoid accumulation at the root tip and vice versa (data not shown). This compound relocation is good indication that flavonoids are necessary and used in locations remote from synthesis sites in Arabidopsis seedlings. Furthermore, the profound induction of flavonoid synthesis in response to light and gravity and other environmental signals make these molecules reasonable candidates for communication of environmental changes from the shoots to the roots.

One surprising finding is that *tt4*(2YY6) roots have elevated basipetal auxin transport, which is required for gravitropic bending (Rashotte et al., 2000), yet have reduced gravitropic bending. This is consistent with the phenotype of the *rcn1* mutant, which also has elevated root basipetal auxin transport and a reduced gravity response (Rashotte et al., 2001). In *rcn1*, the gravity response kinetics are much slower at both initiation of bending and curvature. Furthermore, in *rcn1* the formation of asymmetric DR5-GUS expression was delayed along with gravitropic curvature. Excess basipetal auxin flow was suggested to prevent either the formation or perception of the auxin gradient in *rcn1* roots. Consistent with this hypothesis, treatment of roots with low NPA doses restored both auxin transport and gravity response to wild-type levels. The results with *rcn1* and *tt4*(2YY6) are consistent with elevated basipetal IAA interfering with formation of an IAA gradient.

The detailed analysis of elongation rates of gravity-stimulated roots suggests a mechanism for connecting flavonoid synthesis in root tips to regulation of gravitropic bending. In the wild type but not in *tt4*(2YY6), there is a uniform growth promotion after gravity stimulation that is consistent with a reduction of auxin transport into the elongation zone because IAA reduced root elongation. The growth increase and auxin transport decrease precede asymmetric auxin movement and differential growth. Our hypothesis is that flavonoid accumulation in the epidermal and/or cortical cells after gravity stimulation uniformly reduces basipetal transport; thus, roots can reestablish asymmetric IAA transport leading to formation of a lateral IAA gradient through lateral redistribution of IAA transport proteins (Friml et al., 2002).

Several possible mechanisms by which flavonoids modulate auxin transport are possible. There is clear evidence that acropetal auxin transport in the root, as well as basipetal auxin transport in the inflorescence, hypocotyl, and root, are all elevated in the absence of flavonoids. The level of regulation might be directly at the level of binding of flavonoids to auxin transport proteins and inhibition of their activity, as suggested by the in vitro inhibition of NPA binding by flavonoids (Jacobs and Rubery, 1988). Alternatively, flavonoids may regulate the level of phosphorylation of auxin transport proteins (Bernasconi, 1996; Garbers et al., 1996; DeLong et al., 2002). Another possible mechanism is that flavonoids alter either the amount of synthesis or localization of auxin transport proteins, perhaps by phosphorylation of transcription factors that control the synthesis of these auxin carriers (DeLong et al., 2002). The presence of flavonoid accumulation in the nucleus is consistent with this possibility.

Flavonoid synthesis may also influence the expression and/or localization of auxin transport proteins either directly or by altering auxin levels in plants. The member of the *PIN* gene family that is linked to basipetal transport in roots is *AGR1/AtPIN2/EIR1/WAV6* (Boonsirichai et al., 2002), and lateral transport is linked to *AtPIN3* (Friml and Palme, 2002; Friml, 2003). The levels of *PIN2* gene expression have been examined and are significantly elevated in *tt4* compared with the wild type, whereas naringenin decreased the gene expression levels to wild type (W. Peer and A. Murphy, personal communication). The altered flavonoid accumulation may directly affect auxin transport protein synthesis and localization or indirectly affect the process by altering IAA levels. This elevated expression of *PIN2* in *tt4* (W. Peer and A. Murphy, personal communication) is completely consistent with the elevated basipetal auxin transport that is reported here.

Plants respond to gravity and light with a complex cascade of events. The results reported here are consistent with a role for endogenous flavonoids in the changes in basipetal transport of auxin required for gravity responses. Our findings are consistent with flavonoids being required to reduce basipetal IAA transport uniformly in preparation for asymmetric IAA transport needed to establish an IAA gradient across the root tip. The presence of multiple auxin transport proteins in specific root tissues suggests that these proteins may be differentially regulated by flavonoids, either at the level of IAA transport protein activity, expression, and/or localization. The data reported here indicate that when flavonoid compounds are lacking, Arabidopsis roots can still respond to gravity, but they require more time and perhaps use redundant mechanisms to accomplish the same result. Further experimentation will explore the mechanisms for increased flavonoid accumulation in the root tip after gravity stimulation and flavonoid alteration of auxin transport.

METHODS

Chemicals

Naringenin (4′,5,7-trihydroxyflavanone) was purchased from Indofine Chemical (Belle Med, NJ). NPA was purchased from Chem Service (West Chester, PA). [5-^{3}H]IAA was purchased from Amersham (Buckinghamshire, UK; specific activity, 25 Ci mmol^{-1}) or American Radiolabeled Chemicals (St. Louis, MO; specific activity, 20 Ci mmol^{-1}). ScintiVerse scintillation fluid and Triton X-100 were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide salt was purchased from Research Products International (Mt. Prospect, IL). All other chemicals were acquired from Sigma (St. Louis, MO).

Plant Growth Conditions

Col, *tt4*(2YY6) in the Col background, DR5-GUS transgenic line in Col, and *tt4*(2YY6) DR5-GUS seeds [DR5-GUS was crossed into *tt4*(2YY6)] were sterilized by incubation for 1 min in 95% ethanol, then 5 min in freshly prepared 20% (v/v) bleach plus 0.01% (v/v) Triton X-100, and then washed at least five times with sterile water. The sterilized seeds were sown on control plates: 0.8% (w/v) Type M agar (A-4800; Sigma), MS nutrients (macrosalts and microsalts; MSP0501; Caisson Labs, Rexbury, ID), MS vitamins, 1.5% (w/v) sucrose, and 0.05% (w/v) Mes, with pH adjusted to 6.0 with 1 N KOH before autoclaving. Plated seeds were stratified at 4°C in dark conditions 2 to 14 d to induce even germination. The unsealed plates were placed vertically in racks, and the seedlings were grown under various light conditions: total darkness, low light (10 μmol m^{-2} s^{-1}), and standard light (100 μmol m^{-2} s^{-1}) depending on experimental requirements. All assays were performed on 5- to 6-d seedlings measured from the time of sowing, unless otherwise indicated. Seedlings were maintained at room temperature (23°C). All etiolated seedling manipulations were under a dim green safelight (fluorescent lights covered with green Plexiglas; ACR number 2092; fluence rate = ~1 μmol m^{-2} s^{-1}) if required. Naringenin-feeding experiments were performed globally with 10 μM in the medium.

Gravity Stimulation

Multi-ADAPT experiments were performed by transplanting seedlings to control plates, covering the root with molten agar cooled to 35°C, and analyzed on the computer program (Mullen et al., 1998). Image analysis began within ~5 min after agar set. Initial growth was vertical for ~2 h before reorienting the root 90°, and the bending root was followed for an additional 6 to 8 h. The seedling was illuminated with an infrared light–emitting diode and the image produced was captured with a CCD camera and analyzed with Multi-ADAPT software (Mullen et al., 1998) every 60 s. We defined the end of the gravity-induced lag as occurring when the root tip remained past 80°, with 90° equaling horizontal. Root elongation rates were followed in 50-μm segments beginning at the root tip.

Gravity perception (Larsen, 1957) was determined following the protocol of Vitha et al. (2000), and gravity sensitivity was analyzed following Perbal et al. (2002). Seedlings were transplanted to control agar, allowed to acclimatize for at least 1 h, photographed, and then gravity stimulated in the dark for 5, 10, 20, 30, and 40 min. The seedlings were then rotated on a clinostat (rotation = 1 rpm) in the light for 2 h before root tip bending was measured on digital photographs using Adobe Photoshop measurement tools (San Jose, CA).

Auxin Transport Assays

Auxin transport was monitored indirectly using auxin-induced gene expression of β-glucuronide in transgenic Col DR5-GUS (Ulmasov et al., 1997) and *tt4*(2YY6) DR5-GUS (see below). DR5-GUS staining was used to estimate basipetal auxin transport by applying 1 μM or other concentrations of IAA in agar cylinders to the root tips for 5 h or the indicated length of time for the kinetics assay. The GUS staining procedure was performed for 1 h. All seedlings were cleared with 95% (v/v) ethanol overnight and then lactic acid syrup (60%, v/v) overnight, washed with sodium phosphate buffer, mounted in 50% (v/v) glycerol, covered with a cover slip, and viewed with a 10× Zeiss Differential Interference Contrast (DIC) objective on a Zeiss Axioplan microscope (Jena, Germany). Digital photographs were captured with a color-chilled 3CCD camera (model C5810; Hamamatsu, Bridgewater, NJ) and exported into Photoshop as TIFF files. Distance of the region of the root exhibiting auxin-induced GUS expression was measured with the Photoshop measuring tools.

Measurement of radioactive basipetal auxin transport was performed using the method from Rashotte et al. (2000). Basipetal auxin transport assays were set up on control plates with the root tips aligned. A 1-mm thick 100 nM [^{3}H]IAA agar cylinder was applied just touching the root tips, and the roots were incubated for 5 h before excising the 1-mm section touching the agar cylinder and analyzing the radioactivity in the adjacent 5-mm root section. The agar cylinder was prevented from slipping by securing it with metal staples pushed into the agar.

Root acropetal auxin transport assays were assayed after an 18-h incubation period. Etiolated seedlings were transplanted to control plates

on the third day after sowing. The seedlings were subjected to continued darkness, 24 h light, or 48 h light before the assay 5 d after sowing. On the day of the assay, seedlings were transplanted to freshly prepared agar plates with and without NPA (10 μM) with RSJs aligned at least 1 h before applying radioactive agar cylinders to allow for acclimatization. A 100 nM [^{3}H]IAA agar cylinder was applied just below the aligned RSJs, and the seedlings were incubated in the dark in the inverted position to prevent [^{3}H]IAA from collecting along the root. After 18 h the apical 5 mm of the root was excised, placed in 2.5 mL of scintillation liquid in 3-mL scintillation vials, and radioactivity was measured for 2 min on a Beckman scintillation counter (model LS 6500; Fullerton, CA).

Flavonoid Fluorescence Staining and Quantification

Flavonoid compound locations were visualized in vivo by the fluorescence of flavonoid-conjugated DPBA to the compounds after excitation with blue light (Sheahan and Rechnitz, 1992; Murphy et al., 2000; Peer et al., 2001). Plants were grown on agar at the indicated light levels before staining. Assays to determine IAA and NPA influences on DPBA fluorescence accumulation were performed by transplanting 5-d seedlings to control agar, 1 μM IAA, or 5 μM NPA plates and incubating for the indicated times before staining with DPBA and measuring intensities as described below.

Fluorescent staining of whole seedlings was performed according to Murphy et al. (2000), except for the following changes. Staining times were for 5 min using saturated (0.25%, w/v) DPBA and 0.005% (v/v) Triton X-100. Seedlings were then washed for 5 min with 100 mM sodium phosphate buffer (plus 0.005% [v/v] Triton X-100, pH 7.0). Seedlings were mounted on slides in 50% (v/v) glycerol. Fluorescence was achieved by excitation with FITC filters (450 to 490 nm, suppression long pass 515 nm) on a Zeiss Axioplan fluorescence microscope and 10× or 20× Zeiss FLUOR objective. Digital images were captured with a color-chilled 3CCD camera (model C5810; Hamamatsu) and exported into Photoshop as TIFF files. CLSM was performed on a Ziess 510 CLSM. Excitation was with the 488-nm line of an Ar^+ laser with a 505-nm long-pass filter set.

Quantification of fluorescent intensities was performed with Image Pro Plus software (version 4.5.1.29; Media Cybernetics, Carlsbad, CA). Roots were gravity stimulated in the dark and DPBA stained at the desired time points. Grayscale or color images of the root tip below the bright fluorescent zone were collected with a digital camera (model C4742-95; Hamamatsu) on the epifluorescent microscope and exported into Image Pro Plus software as mentioned elsewhere. A freehand line profile was drawn from the root tip through the center of the root, and the software intensity profile was exported to Microsoft Excel (Redmond, WA). The root tip cells were analyzed for intensity and statistically analyzed by comparative statistics by determining the maximum intensity in the first 40 μm of the root tip. Every effort was made to keep exposure times equal. If exposure times were adjusted to prevent saturating DPBA fluorescence, the resulting intensities were normalized to the longest exposure preventing saturation (2000 ms; i.e., the intensity at 500 ms would be multiplied by 4 to correct for normalized intensity with a 2-s exposure). An average of 10 roots were analyzed at each time point and compared with vertically oriented, dark-grown controls.

GUS Staining

GUS staining was performed with Columbia DR5-GUS (Ulmasov et al., 1997) and *tt4*(2YY6) DR5-GUS seedlings according to Rashotte et al. (2001). Seedlings were gravity stimulated in the dark for the required time and then washed with 100 mM sodium phosphate buffer, pH 7.0. Seedlings were then covered with 2 mM X-glucuronide staining solution (Craig, 1992) in situ on the Petri plate, incubated for 3 h at 37°C, and then washed with sodium phosphate buffer, pH 7.0, cleared with 95% ethanol overnight, and then incubated in 60% lactic acid syrup overnight for further clearing. Seedlings were mounted on slides in 50% glycerol and observed using DIC settings on the Zeiss epifluorescent microscope.

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Corrections

Jason M. Ward, Carie A. Cufr, Megan A. Denzel, and Michael M. Neff. (2005). The Dof Transcription Factor OBP3 Modulates Phytochrome and Cryptochrome Signaling in Arabidopsis. Plant Cell **17,** 475–485.

An incorrect column heading was published in Table 1. The incorrect heading read Cotyledon Epidermal Cell Area μM^2 (SE). The correct column heading is Cotyledon Epidermal Cell Area $mm^2 \times 10^{-4}$ (SE).

The authors of the articles listed below discovered an error in a spreadsheet developed by their laboratory. This error resulted in an incorrect conversion of radioactive counts to the appropriate molar units. The absence of a 1000-fold difference led them to report their auxin transport values as pmol instead of reporting them correctly as fmol. The following articles were affected by this conversion error.

Aaron M. Rashotte, Alison DeLong, and Gloria K. Muday. (2001). Genetic and Chemical Reductions in Protein Phosphatase Activity Alter Auxin Transport, Gravity Response, and Lateral Root Growth. Plant Cell **13,** 1683–1697.

The units of radioactive indole-3-acetic acid transport were mislabeled in Figures 3, 4A, and 4B and in Tables 4 and 5, as were the units of radiolabeled benzoic acid in Table 2. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data, since all experiments had internal controls.

Charles S. Buer and Gloria K. Muday. (2004). The *transparent testa4* Mutation Prevents Flavonoid Synthesis and Alters Auxin Transport and the Response of Arabidopsis Roots to Gravity and Light. Plant Cell **16,** 1191–1205.

The units of radioactive indole-3-acetic acid transport were mislabeled in Figure 8. Both control and experimental values should have been reported as fmol rather than pmol. This change in units does not alter the interpretation of the data, since all experiments had internal controls.

The *transparent testa4* Mutation Prevents Flavonoid Synthesis and Alters Auxin Transport and the Response of Arabidopsis Roots to Gravity and Light

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