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Variation in Expression and Protein Localization of the PIN Family of Auxin Efflux Facilitator Proteins in Flavonoid Mutants with Altered Auxin Transport in *Arabidopsis thaliana* [W]

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Aglycone flavonols are thought to modulate auxin transport in *Arabidopsis thaliana* via an as yet undefined mechanism. Biochemical studies suggest that flavonoids interact with regulatory proteins rather than directly with the PIN auxin efflux facilitator proteins. Auxin transport is enhanced in the absence of flavonoids (*transparent testa4* [*tt4*]) and reduced in the presence of excess flavonols (*tt7* and *tt3*). Steady state *PIN* mRNA levels in roots inversely correlate with auxin movement in *tt* mutants. *PIN* gene transcription and protein localization in flavonoid-deficient mutants appear to be modulated by developmental cues and are auxin responsive. Modulation of *PIN* gene expression and protein distribution by localized auxin accumulations occurs in the wild type as well. Flavonoids inhibit auxin transport primarily at the shoot apex and root tip and appear to modulate vesicular cycling of PIN1 at the root tip. In some auxin-accumulating tissues, flavonoid increases and changes in flavonoid speciation are subsequent to auxin accumulation.

INTRODUCTION

The plant hormone auxin is synthesized primarily in the shoot apex and transported in a polar fashion to other parts of the plant, where it acts upon many aspects of plant growth and development. Despite the identification of components of the auxin efflux apparatus, factors that modulate auxin efflux have not been elucidated. Since Jacobs and Rubery (1988) reported that flavonoid compounds displace the auxin efflux inhibitor (AEI) 1-naphthylphthalamic acid (NPA) from zucchini (*Cucurbita pepo*) hypocotyl microsomes, endogenous plant flavonoids have been considered likely natural regulators of cellular auxin efflux and consequent polar auxin transport. Specific sites of flavonoid action were recently suggested by the identification and characterization of *Arabidopsis thaliana* NPA binding protein complexes (Murphy and Taiz, 1999a, 1999b; Murphy et al., 2000, 2002) with characteristics similar to higher and lower affinity sites previously characterized in maize (*Zea mays*) and zucchini (Katekar and Geissler, 1979, 1980; Michalke et al., 1992; Bernasconi et al., 1996). The flavonols quercetin and kaempferol were found to compete with NPA bound to these sites in a biphasic fashion (Murphy et al., 2000) and interact with associated plasma membrane protein complexes (Murphy et al., 2002).

The lower affinity NPA binding complex was found to contain a flavonol-sensitive plasma membrane aminopeptidase, AtAPM1. AtAPM1 is an ortholog of mammalian bifunctional microsomal aminopeptidases that consists of a highly conserved enzymatic domain sensitive to NPA-like phthalimide inhibitors (Komoda et al., 2001) and a protein–protein interaction domain involved in trafficking of membrane transport proteins (Murphy et al., 2002). The higher affinity complex contained AtPGP1, AtPGP2, and AtMDR1/AtPGP19 (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2003), which are orthologs of flavonoid-sensitive human plasma membrane Multidrug Resistance (MDR) p-glycoproteins (Phang et al., 1993; Hooijberg et al., 1997). Mutations in *AtMDR1*, *AtPGP1*, and their maize and sorghum (*Sorghum bicolor*) orthologs (*Br2* and *Dwarf3*, respectively) result in defective auxin transport and growth (Noh et al., 2001; Geisler et al., 2003; Multani et al., 2003).

Molecular genetic and immunohistochemical evidence link the localization of the *pin-formed* (PIN) auxin efflux facilitator family to the polarity of auxin transport. PIN1 is the best-characterized member of the family. Distinct patterns of PIN localization observed in roots and shoots (Geldner et al., 2001, 2003; Benkova et al., 2003; Noh et al., 2003; Blakeslee et al., 2004) suggest that polar auxin transport may be regulated in a tissue-specific manner. Although PIN1 is ubiquitously localized in early embryos, it becomes restricted to precambial tissues and, subsequently, to the vascular tissue (Gälweiler et al., 1998; Steinmann et al., 1999). In mature tissues, PIN1 is localized at the plasma membrane at the basal side of xylem parenchyma cells, as well as in flanking cortical cells of hypocotyls in young seedlings (Noh et al., 2003). PIN2 is localized at the anticlinal and basal sides of root epidermal, cortical, and lateral cap cells

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and at the outer anticlinal side of root cortical cells (Müller et al., 1998; Boonsirichai et al., 2003). PIN3, apparently involved in lateral redistribution of auxin, has been localized to lateral surfaces of shoot endodermal cells and on all sides of the root columella cells in vertically grown roots (Friml et al., 2002b). PIN4, an apparent auxin sink generator, has been localized to the boundaries of the quiescent center and surrounding cells (Friml et al., 2002a). Recent evidence that PIN proteins in *atmdr1* mutant hypocotyls are mislocalized (Noh et al., 2003) suggests that MDR/PGP proteins may mediate flavonoid–PIN interactions because MDR/PGP activity is flavonoid sensitive (Murphy et al., 2002).

The widespread use of flavonoids as kinase inhibitors (Senderowicz, 2000) and evidence that nonflavonoid kinase inhibitors can also displace NPA from microsomal membranes (Bernasconi, 1996) suggest that flavonoids modulate auxin transport at these and other sites by altering protein and/or lipid phosphorylation (Rashotte et al., 2001; DeLong et al., 2002). This interpretation is strongly supported by evidence that the NPA-insensitive *rcn1* mutant of Arabidopsis harbors a lesion in a phosphatase 2A subunit ortholog (Garbers et al., 1996; Rashotte et al., 2001) and the *pinoid* (*pid*) auxin transport mutant has a defect in a putative Ser/Thr kinase C gene (Christensen et al., 2000; Benjamins et al., 2003). Consistent with a proposed role as generalized autocrine effectors (Murphy et al., 2000), flavonols could influence auxin accumulation and transport by modulating multiple phosphorylation events: quercetin is an effective inhibitor of mammalian PI-3, MAPK, PKC, and Akt/PKB kinases, as well as phospholipase A2 and phosphodiesterases (Graziani et al., 1983; Gschwendt et al., 1983; Levy et al., 1984; Muday and Murphy, 2002; Spencer et al., 2003). In addition to modulating transport protein trafficking and function, flavonoids could also potentially influence upstream phosphorylation-dependent signal transduction (Agullo et al., 1997) or nuclear transcription (Ciolino et al., 1999; Xing et al., 2001) mechanisms.

The *transparent testa* (*tt*) mutants of Arabidopsis provide an ideal system for studying flavonoid regulation of auxin transport because they harbor mutations in genes encoding regulatory, enzymatic, and transport components of flavonoid biosynthesis (Koornneef et al., 1982; Shirley et al., 1995; Nesi et al., 2000, 2001, 2002; Debeaujon et al., 2001; Shikazono et al., 2003). Of the mutations in the early enzymes of the biosynthetic pathway (*tt4*, *tt5*, *tt6*, *tt7*, and *tt3*), the chalcone synthase mutant *tt4* is the most widely studied because it produces no flavonoids (Shirley et al., 1995). Higher rates of auxin transport have been found in young *tt4* seedlings, which also exhibit auxin leakage from the root tips; treatment with either the flavonoid biosynthetic intermediate naringenin or NPA restored auxin transport to wild-type levels (Murphy et al., 2000; Brown et al., 2001). No reduction in NPA binding sites was found in microsomes prepared from *tt4* mutants (Brown et al., 2001), suggesting that flavonoids modulate auxin regulatory proteins rather than alter their abundance.

The aglycone flavonols kaempferol and quercetin are the predominant flavonoids in young 4- to 5-d wild-type seedlings (Murphy et al., 2000; Peer et al., 2001). In light-grown seedlings, quercetin accumulates at the shoot apex, in a cone-shaped pattern in the upper root, and at the root tip; kaempferol accumulates at the root tip and in a single ring of cells delineating the root from the shoot; naringenin chalcone accumulates from the boundary of the root distal elongation zone through the proximal elongation zone (Murphy et al., 2000; Peer et al., 2001). Treatment of *tt4* with the flavonoid biosynthetic intermediate naringenin results in accumulation patterns of kaempferol and quercetin similar to the wild type (Murphy et al., 2000). This report focuses on early flavonoid biosynthetic pathway mutants with altered levels of quercetin and kaempferol, that is, *tt4* (no flavonoid production); *tt7*, a flavonone 3′-hydroxylase mutant accumulating excess kaempferol; and *tt3*, a dihydroflavonol reductase mutant accumulating excess kaempferol and quercetin (Peer et al., 2001).

It is the objective of this study to determine whether changes in endogenous flavonol levels alter the expression of *PIN* genes and subsequent subcellular localization of their gene products. Flavonols might regulate the function of the PIN auxin efflux facilitators via three mechanisms. (1) Flavonol displacement of microsomal NPA binding, described above, suggests a direct interaction with a regulator of the PIN protein at the plasma membrane. (2) Alteration of PIN1 asymmetric relocalization at the plasma membrane by AEIs after brefeldin A (BFA)–mediated internalization (Geldner et al., 2001, 2003) suggests that flavonols could modulate the cycling of PIN proteins on and off the membrane. (3) Alterations in mammalian gene transcription induced by treatment with exogenous flavonols (Ciolino et al., 1999; Xing et al., 2001) suggests that flavonoids could also directly or indirectly regulate the abundance of PIN proteins by modulating *PIN* gene transcription or mRNA/protein stability, presumably through phosphorylation events.

We first analyzed auxin transport profiles of *tt* mutants to determine which tissues could be best used for an analysis of flavonoid–PIN interactions. We then investigated the expression and localization of *PIN* genes and their gene products in the same *tt* mutants. Using combinations of *tt* mutants and AEIs, we ascertained what changes reflect altered auxin movement to the tissues examined and what changes may represent direct flavonoid regulation of expression, protein targeting, or protein stability. Finally, we interpreted the results to propose the tissue-specific sites of flavonoid regulation of auxin transport.

RESULTS

Auxin Transport Is Altered in Flavonoid Mutants

To determine what effect altered flavonoid compositions would have on auxin transport in whole tissues, a more refined auxin transport assay (Geisler et al., 2003) than reported previously (Murphy et al., 2000; Brown et al., 2001) was used to compare wild-type and *tt* mutant transport profiles. Unlike previous studies, the flavonol-accumulating *tt3* and *tt7* mutants were also assayed. In all cases, auxin leakage from the root tip was counted as part of the auxin delivered to the root (Murphy et al., 2000). Differences noted in *tt* mutant transport assays were, however, confirmed by gas chromatography–mass spectrometry determination of free auxin as reported previously (Brown et al., 2001).

Although auxin transport from the shoot apex to the root–shoot transition zone (R-S TZ) at 5 h in *tt4* seedlings (which accumulate no flavonoids) was not different from the wild type ($P > 0.05$), significantly less auxin was transported to the R-S TZ in *tt7* and *tt3* seedlings (which accumulate excess kaempferol or kaempferol and quercetin, respectively) compared with the wild type ($P = 0.001$; Figure 1). However, in transport assays conducted over a period of 3.5 h, only *tt4* seedlings exhibited radioactivity in the R-S TZ (data not shown), indicating that accelerated auxin transport occurs in *tt4* hypocotyls. Serial sectioning (2-mm sections) of lower hypocotyls indicated that auxin transport was primarily inhibited in the shoot apex of *tt7* and *tt3* seedlings (data not shown).

By contrast, significantly more auxin from the shoot apex reached the root tip in *tt4*, indicating elevated levels of auxin transport ($P < 0.05$), whereas no difference was observed in auxin transport from the shoot to the root in *tt7* and *tt3* ($P > 0.05$; Figure 1). In contrast with *tt4* (Murphy et al., 2000), auxin leakage from *tt7* and *tt3* root tips was not observed. Auxin transport in *tt* mutants was not different from the wild type in etiolated seedlings that lack flavonoids ($P < 0.05$).

Coapplication of the early flavonoid biosynthetic intermediate naringenin with radiolabeled auxin significantly decreased auxin transport from the shoot apex to the R-S TZ in both *tt4* and the wild type compared with untreated wild type ($P < 0.001$ and $P = 0.03$, respectively; Figure 1). Auxin transport from shoot apex to root was not different in the wild type plus naringenin and *tt4* plus naringenin compared with untreated wild type, restoring wild-type auxin transport to the *tt4* root tip ($P > 0.05$; Figure 1). The resulting auxin transport profiles were not different from *tt7* and *tt3* ($P > 0.05$). These results strongly suggest that auxin transport is modulated by flavonoids at the shoot tip.

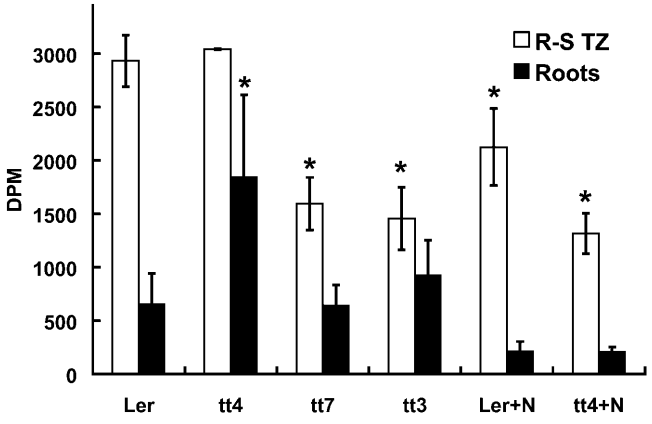

Figure 1. Altered Auxin Transport in 4.5-d Flavonoid Mutant Seedlings.

Auxin transport from shoot apex to R-S TZ and root tips of 4.5-d seedlings. Altogether 20 nM naringenin (N) was coapplied with radiolabeled auxin in L*er* plus N and *tt4* plus N treatments. The mean L*er* wild-type values were 2,933.3 ± 137.6 disintegrations per minute (DPM) at the R-S TZ and 655.8 ± 162.5 DPM at the root tip. Values shown are means ± SD for two independent experiments. The experiment was replicated with identical results. *, Significance was determined by ANOVA followed by Tukey post hoc test; $P < 0.05$ compared with untreated wild type.

The endogenous levels of free indoleacetic acid (IAA) in 5-d *tt4* hypocotyls were not different from the wild type ($P > 0.05$), whereas *tt4* roots had twofold more endogenous free IAA than wild-type roots ($P < 0.01$; Table 1), suggesting that the increased IAA in the *tt4* root is due to elevated auxin transport to the root. By contrast, the endogenous free IAA levels at the apex and the base of inflorescence stems were not different between *tt4* and the wild type (Brown et al., 2001). Auxin conjugate levels were not determined.

Tissue-Specific *PIN* Expression Is Altered in *tt* Mutants

We examined the expression levels of *PIN1*, *PIN2/AGR1/WAV6/EIR1*, and *PIN4/AGR3* in the wild type and *tt* mutants to determine if flavonoids affected *PIN* mRNA levels. *PIN2* expression was not examined in the hypocotyls since Chen et al. (1998) had previously shown that, in light-grown tissue, *AGR1/PIN2* is only expressed in roots.

In *tt4* shoots, *PIN* expression was not significantly different from wild-type shoots (Table 2). In *tt4* roots, *PIN1* mRNA was below the limit of detection, whereas *PIN2* mRNA was 23-fold greater than the wild type ($P < 0.001$); *PIN4* expression was not different from the wild type (Table 2).

In *tt3* shoots, *PIN* expression was not significantly different from wild-type shoots (Table 2). Expression of *PIN1* in *tt3* roots was threefold greater than the wild type ($P < 0.01$); *PIN2* and *PIN4* expression were not different from the wild type (Table 2). Some changes in *PIN4* expression in response to auxin may be undetected because of the small number of cells in which it is expressed.

Naringenin Treatment Restores Wild-Type *PIN* Expression in *tt4*

Previously, we showed that naringenin treatment restored flavonoid accumulation in *tt4* (Murphy et al., 2000) and auxin transport (Murphy et al., 2000; Brown et al., 2001; Figure 1). Therefore, we hypothesized that naringenin treatment may restore *PIN* expression in *tt4*. Naringenin treatment did not alter *PIN* expression in *tt4* shoots (Table 2). *PIN1* expression was not different from wild-type levels in *tt4* plus naringenin roots, whereas it was not detectable in untreated *tt4* roots (Table 2). *PIN2* expression was not different from the wild type in *tt4* plus naringenin roots, whereas they were significantly greater in untreated *tt4* roots (Table 2). Treatment with naringenin did not alter *PIN4* expression in *tt4* roots (Table 2). Hence, naringenin treatment restored *PIN1* and *PIN2* expression to wild-type levels in *tt4* roots.

PIN Expression Responds to Altered Auxin Transport

Increase of Transported Auxin by Application at Shoot Apex

Because alterations of auxin transport appear to induce changes in *PIN* gene expression (Blakeslee et al., 2004) and exogenous application of auxin to wild-type seedlings phenocopied the *tt4* root phenotype (data not shown), we hypothesized that some of the observed changes in *PIN* expression in *tt* mutants might be

Table 1. Endogenous Free Auxin Quantitations in 5-d Seedlings

	Free Auxin	
	ng g^{-1} Fresh Weight	
Line	Shoots	Roots
L*er*	52 ± 19.3	14 ± 7.3
tt4	64 ± 22.1	28 ± 9.1[a]

[a] Significantly different from the wild type; Student's *t* test, $P < 0.01$; $n = 3500$ seedlings per replicate.

induced by altered auxin movement and not direct interactions of flavonoids with auxin transport mechanisms. We therefore investigated the effects of exogenous application of auxin on *PIN* expression. *PIN1* and *PIN2* expression in wild-type plus IAA roots was like that observed in *tt4*: *PIN1* was not detected, and *PIN2* and *PIN4* expression increased (Table 3).

Auxin Transport Inhibitors

As low levels of NPA restored wild-type auxin transport to *tt4* despite no loss of NPA binding sites (Murphy et al., 2000; Brown et al., 2001), we hypothesized that comparison of *PIN* expression in AEI-treated wild type and flavonoid-deficient mutants would help localize regions of direct regulation of auxin efflux by flavonoids. The 2,3,5-triiodobenzoic acid (TIBA) inhibits auxin efflux by directly competing with IAA at the efflux site (Lomax et al., 1995). As such, TIBA treatment reduces the rate of auxin transport and shortens the auxin gradient observed in untreated hypocotyls but would not be expected to compete with flavonoid binding to a regulatory site. On the other hand, NPA would be expected to compete with flavonoid binding to one or more regulatory sites. These experiments provide a useful comparison with TIBA because NPA does not bind the auxin efflux carrier site and is more effective in restricting labeled IAA to the shoot apex (data not shown).

In TIBA-treated wild-type seedlings, shoot and root expression of *PIN1* was increased, *PIN2* expression increased in roots, and *PIN4* expression decreased in shoots (Table 3). The changes in *PIN1* expression in the wild-type plus TIBA shoots and roots were similar to expression patterns observed in the *tt3* mutants, and *PIN2* expression was enhanced similarly to untreated *tt4*, in which auxin that is usually redirected in a basipetal flow from the wild-type root apex leaks out of the root tip (Table 3). When roots of *tt4* seedlings were treated with TIBA, *PIN1* expression reverted to at least untreated wild-type levels (Table 3). These results suggest that altered *PIN* gene expression in *tt* mutants reflects changes in auxin transport from the apices.

The increased *PIN1* expression in NPA-treated wild-type shoots and roots was similar to TIBA-treated seedlings, as was increased *PIN2* expression in roots (Table 3). However, *PIN4* expression responded differently to NPA than to TIBA. Expression of *PIN4* in shoots increased in the wild type plus NPA, whereas it decreased in the wild type plus TIBA (Table 3). When *tt4* was treated with NPA, *PIN1* expression in roots was restored to wild-type levels, and *PIN2* was elevated as in untreated *tt4* roots (Table 3).

Therefore, *PIN* expression is responsive to fluxes in auxin concentration: *PIN1* expression in roots is decreased in conditions of enhanced auxin movement (*tt4* and Landsberg *erecta* [L*er*] plus IAA) and increases when auxin movement is inhibited (*tt3*; treatment with TIBA, NPA, and naringenin). *PIN2* increases when auxin levels are high in the roots. Auxin responsiveness of *PIN4* expression was not conclusive from these experiments. Flavonoids indirectly affect *PIN* expression by modulating auxin transport (*tt4* and *tt3*).

Table 2. Relative Expression ± SD and Graphical Summary of *PIN* Genes in Wild-Type and *tt* 5-d Seedlings

	Relative Transcript Levels[a]					
	PIN1	*PIN2*	*PIN4*	*PIN1*	*PIN2*	*PIN4*
Shoots						
L*er*	1 ± 0.36	NE	1 ± 0.46	—	NE	—
tt4	0.04 ± 1.17	NE	0.03 ± 0.64	—	NE	—
tt4 + N	0.25 ± 1.58	NE	2.8 ± 1.59	—	NE	—
tt3	2.39 ± 1.67	NE	3.28 ± 2.97	—	NE	—
Roots						
L*er*	1 ± 0.36	1 ± 1.19	1 ± 0.26	—	—	—
tt4	Not detected	23.96 ± 0.7[b]	0.28 ± 0.61	↓↓	↑↑	—
tt4 + N	0.01 ± 1.58	2.3 ± 1.25	0.21 ± 1.59	—	—	—
tt3	3.59 ± 0.84[b]	0.65 ± 0.41	2.18 ± 3.39	↑	—	—

[a] Relative values of *PIN1*, *PIN2*, and *PIN4* expression as a percent of the wild type with β-tubulin as the endogenous correction factor were calculated. Relative expression from real-time PCR analyses were normalized to untreated L*er* shoots for shoot data and untreated L*er* roots for root data, respectively. *PIN2* expression in the shoots was not examined (NE) since Chen et al. (1998) showed *PIN2* was not expressed in light-grown hypocotyls. For the treatment study, 10 nM naringenin (N) was used. The experiment was replicated, $n = 3$. ↑↑, >10-fold increase; ↑, twofold to 10-fold increase; ↓, reduction; ↓↓, below detection; and —, not different from the wild type.

[b] Statistically different from the wild type, determined by ANOVA followed by Student-Newman-Keuls post hoc test ($P < 0.05$).

Table 3. Relative Expression ± SD and Graphical Summary of *PIN* Genes in Wild-Type and *tt* 5-d Seedlings in Response to Altered Auxin Transport

	Relative Transcript Levels[a]					
	PIN1	PIN2	PIN4	PIN1	PIN2	PIN4
Shoots						
L*er*	1 ± 0.36	NE	1 ± 0.46	—	NE	—
L*er* + IAA	0.1 ± 2.47	NE	0.15 ± 0.97	—	NE	—
L*er* + TIBA	11.17 ± 0.85[b]	NE	0.01 ± 0.36[b]	↑↑	NE	↓
tt4 + TIBA	0.84 ± 0.82	NE	0.03 ± 2.08	—	NE	—
L*er* + NPA	85.97 ± 0.82[b]	NE	38.6 ± 0.69[b]	↑↑	NE	↑↑
tt4 + NPA	12.88 ± 0.76[b]	NE	0.68 ± 0.8	↑↑	NE	—
Roots						
L*er*	1 ± 0.36	1 ± 1.19	1 ± 0.26	—	—	—
L*er* + IAA	Not detected	5.2 ± 2.1[b]	5.21 ± 1.97[b]	↓↓	↑	↑
L*er* + TIBA	26.65 ± 0.91[b]	225 ± .054[b]	0.16 ± 1.07	↑↑	↑↑	—
tt4 + TIBA	381.7 ± 0.95[b]	0.35 ± 0.80	9.87 ± 1.01[b]	↑↑	—	↑
L*er* + NPA	68 ± 2.6[b]	258 ± 2.46[b]	2.61 ± 0.68	↑↑	↑↑	—
tt4 + NPA	2.34 ± 0.76	6.00 ± 0.96[b]	0.46 ± 0.68	—	↑	—

[a] Relative values of *PIN1*, *PIN2*, and *PIN4* expression as a percent of the wild type with β-tubulin as the endogenous correction factor were calculated. Relative expression from real-time PCR analyses were normalized to untreated L*er* shoots for shoot data and untreated L*er* roots for root data, respectively. *PIN2* expression in the shoots was not examined (NE) since Chen et al. (1998) showed *PIN2* was not expressed in light-grown hypocotyls. For the treatment studies, 1 μM IAA, 10 μM TIBA, or 10 μM NPA was used. The experiment was replicated, $n = 3$. ↑↑, >10-fold increase; ↑, twofold to 10-fold increase; ↓, reduction; ↓↓, below detection; and —, not different from the wild type.

[b] Statistically different from the wild type, determined by ANOVA followed by Student-Newman-Keuls post hoc test ($P < 0.05$).

PIN Localization Responds to Altered Auxin Transport

PIN Localization Is Altered in *tt* Mutants

We examined PIN localization in flavonoid-deficient seedlings using polyclonal antibodies raised against PIN1, PIN2, and PIN4 to determine if the pattern of protein localization was altered. A new anti-PIN4 antibody was prepared (see Methods). Localization of PIN1, PIN2, and PIN4 in wild-type seedlings was consistent with previous reports (Gälweiler et al., 1998; Müller et al., 1998; Steinmann et al., 1999; Friml et al., 2002a; Noh et al., 2003; Blakeslee et al., 2004).

PIN1 signal was not detected in the *pin1* mutant (Figure 2A). In wild-type roots, a strong PIN1 signal was detected at the plasma membrane at the basal side of the vascular cells, and a weak signal was observed in cortical tissues (Figure 2B). In *tt4* root tips, PIN1 signal was delocalized from the plasma membrane in the vascular tissue and adjacent cortical tissues (Figure 2C). PIN1 localization in *tt7* was reduced in the vascular tissue, and a signal was more abundant in the cortical and epidermal cells (Figure 2D). In *tt3*, PIN1 signal was stronger in the vascular tissue compared with the wild type and was also found in the cortex (Figure 2E).

Only a nonspecific epidermal surface PIN2 signal was observed in the *pin2* mutant (Figure 2F); this nonspecific signal was also observed in the wild type and *tt* mutants. PIN2 was localized at the basal sides of root epidermal cells as well as at the basal and outer periclinal sides of cortical cells in the wild type (Figure 2G). PIN2 localization among the *tt* seedlings was similar to the wild type, with basal localization in the epidermis and cortex and lateral localization in the cortical cells (Figures 2H to 2J).

PIN4 signal was not observed in *pin4* mutant roots (Figure 2K). In wild-type root tips PIN4 was localized on the apical side of the quiescent center cells, vascular meristem initials, and their daughter cells; nonpolar localization was observed in the columella initials and columella C1/S1 (Friml et al., 2002a; Figure 2L). PIN4 localization was restricted to the bottom of the quiescent center cells in *tt4* in 60% of the seedlings examined and absent in the remaining 40% (Figure 2M; Supplemental Figures 1A to 1C). In *tt7* and *tt3*, PIN4 signal was observed throughout the root tip, and localization near the membrane was weak (Figures 2N and 2O).

Basal membrane localization of PIN1 in the auxin-sensitive mid-hypocotyl region of *tt4* mutants was slightly disrupted (Supplemental Figures 1D and 1E). No differences in PIN1 localization were observed in the root transition zone among the *tt* mutants (Supplemental Figures 1F to 1I). Although the auxin transport and growth studies described above suggest that the apical region of seedlings is most likely the site of flavonoid regulation of auxin transport, differences between the localization of PIN proteins at the shoot apices of wild-type and *tt* mutant seedlings could not be resolved with direct immunofluorescence techniques (data not shown).

PIN1 Localization Responds to Increase in Transported Auxin

Since application of exogenous auxin to wild-type phenocopied *tt4* root tips (data not shown) and *PIN1* expression in *tt4* (Table 3), we investigated the affects of altering auxin movement on PIN1 localization in the wild type. When a microdroplet of auxin was

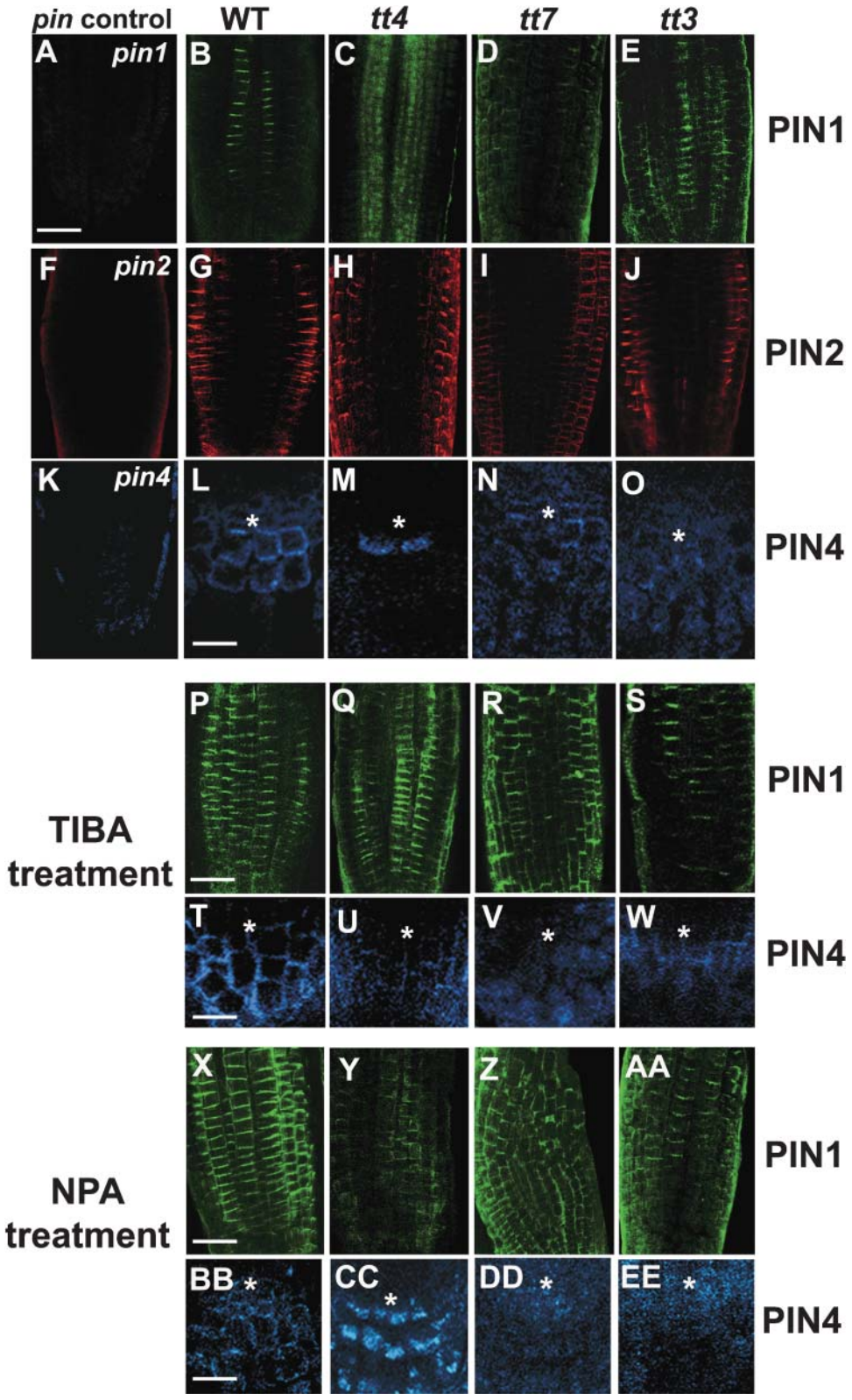

Figure 2. PIN1, PIN2, and PIN4 Immunolocalization in Roots of 5-d Flavonoid Mutant Seedlings.

applied to wild-type shoot apex, an increased asymmetric PIN1 signal was observed in distal elongation zone (Supplemental Figure 1N). However, when a microdroplet of auxin was applied to the root apex, PIN1 signal was delocalized from the plasma membrane in addition to the asymmetric localization (Supplemental Figure 1O).

Treatment with Auxin Efflux Inhibitors Restores PIN Localization in tt4

Because AEIs were able to restore wild-type auxin transport (Murphy et al., 2000; Brown et al., 2001) and *PIN1* expression levels in *tt4*, we reasoned that treatment with AEIs might restore PIN localization. Because the subcellular localization and tissue-specific distribution of PIN1 and PIN4 in *tt* mutants were altered, we visualized both proteins using concentrations and durations of AEI treatments that do not interfere with PIN1 cycling (Geldner et al., 2001).

In addition to the normal vascular PIN1 localization, the distribution of PIN1 localization expanded to the cortical cells in wild-type root tips treated with TIBA or NPA (Figures 2P and 2X); these patterns were also observed in AEI-treated *tt* seedlings (Figures 2Q to 2S, and 2Y to 2AA), thereby restoring wild type–like vascular PIN1 localization to *tt4* (Figure 2Q). PIN1 localization was similar in untreated and AEI-treated *tt7* and *tt3* (Figures 2R and 2S).

PIN4 signal in TIBA-treated wild-type roots tips was stronger in all cells, including the columella and meristem daughter cells, than in the untreated wild type (Figures 2T and 2BB). PIN4 distribution in *tt4* root tips was partially restored after TIBA treatment with localization expanding to the columella and meristem initials and their daughter cells, although the signal was weak (Figure 2U). In TIBA-treated *tt7*, PIN4 localization was observed diffusely throughout the cells of the root tip (Figure 2V). In *tt3*, tissue-specific PIN4 localization was partially restored in TIBA-treated seedlings (Figure 2W). PIN4 signal was delocalized from the sides of the cells in NPA-treated *tt* seedlings (Figures 2CC to 2EE), although NPA partially restored wild-type distribution in *tt4* root tips (Figure 2CC).

Therefore, PIN1 subcellular localization is flavonoid sensitive, and tissue-specific distribution is auxin responsive at the root tip. PIN4 localization is auxin responsive and flavonoid sensitive and distribution is auxin responsive. PIN2 localization and distribution are neither auxin nor flavonoid responsive.

Flavonoid Effects on PIN Localization

Exogenous Flavonoid Treatment Restores PIN Localization in tt4

Treatment of *tt4* with the flavonoid biosynthetic intermediate naringenin (1 nM) resulted in restoration of wild-type PIN1 and partial restoration of wild-type PIN4 localization patterns (Figures 3A and 3D).

Kaempferol and quercetin treatments of *tt4* were used to mimic the endogenous accumulations of these compounds observed in *tt7* and *tt3*. PIN1 and PIN4 localizations in kaempferol-treated *tt4* resembled those in untreated *tt7* (Figures 3B and 3E); quercetin-treated *tt4* resembled untreated *tt3* (Figures 3C and 3F). Taken together, these data suggest that flavonoids act as autocrine effectors by modulating auxin retention in cells in which they are synthesized and adjacent cells, and this, in turn, affects *PIN* expression and localization.

PIN1 Is Retained in BFA Bodies after Flavonol Washout in Flavonoid-Deficient Tissues

Geldner et al. (2001) have suggested that AEIs interfere with PIN1 vesicle trafficking in wild-type root tips, as PIN1 protein remains in perinuclear BFA bodies after BFA washout with TIBA and NPA. Presumably, both AEIs interfere with PIN1 relocalization by interrupting an interaction between one or more NPA binding regulatory proteins and the PIN1 efflux facilitator (see Muday et al., 2003). To date, the root tip is the only region where cycling has been reported.

If NPA mimics endogenous flavonols, then, in an otherwise flavonoid-deficient background, kaempferol and quercetin should interfere with PIN1 relocalization in a fashion similar to TIBA and NPA. In *tt4* root tips treated with BFA, PIN1 signal was observed in BFA bodies (similar to BFA-treated wild type), in addition to the diffuse distribution also observed within the untreated cells (Figure 3G). Following buffer washout in BFA-treated *tt4*, PIN1 signal was diffuse, as in untreated *tt4* (Figure 3H); an additional localization at the plasma membrane in the vascular tissue was also observed. This suggests that BFA treatment itself may have other effects on cellular trafficking in the absence of flavonoids.

In the wild type, after BFA washout with a mixture of kaempferol and quercetin, PIN1 signal was observed at the plasma membrane (Figure 3I); however, in the region of the root tip that

Figure 2. (continued).

Confocal images of PIN immunohistochemical localizations. Asterisks indicate the position of quiescent center cells in PIN4 localization images. PIN2 and PIN4 confocal images have been false-colored; color is not as a result of autofluorescence. Bars in **(A)** to **(K)**, **(P)** to **(S)**, and **(X)** to **(AA)** = 25 μM; **(L)** to **(O)**, **(T)** to **(W)**, and **(BB)** to **(EE)** = 10 μM.

(A) to **(E)** PIN1 localization (green) in *pin1* **(A)**, L*er* **(B)**, *tt4* **(C)**, *tt7* **(D)**, and *tt3* **(E)**.

(F) to **(J)** PIN2 localization (red) in *pin2* **(F)**, L*er* **(G)**, *tt4* **(H)**, *tt7* **(I)**, and *tt3* **(J)**.

(K) to **(O)** PIN4 localization (blue) in *pin4* **(K)**, L*er* **(L)**, *tt4* **(M)**, *tt7* **(N)**, and *tt3* **(O)**.

(P) to **(S)** PIN1 localization in seedlings treated with 10 μM TIBA in L*er* **(P)**, *tt4* **(Q)**, *tt7* **(R)**, and *tt3* **(S)**.

(T) to **(W)** PIN4 localization in seedlings treated with 10 μM TIBA in L*er* **(T)**, *tt4* **(U)**, *tt7* **(V)**, and *tt3* **(W)**.

(X) to **(AA)** PIN1 localization in seedlings treated with 1 μM NPA in L*er* **(X)**, *tt4* **(Y)**, *tt7* **(Z)**, and *tt3* **(AA)**.

(BB) to **(EE)** PIN4 localization in seedlings treated with 1 μM NPA in L*er* **(BB)**, *tt4* **(CC)**, *tt7* **(DD)**, and *tt3* **(EE)**.

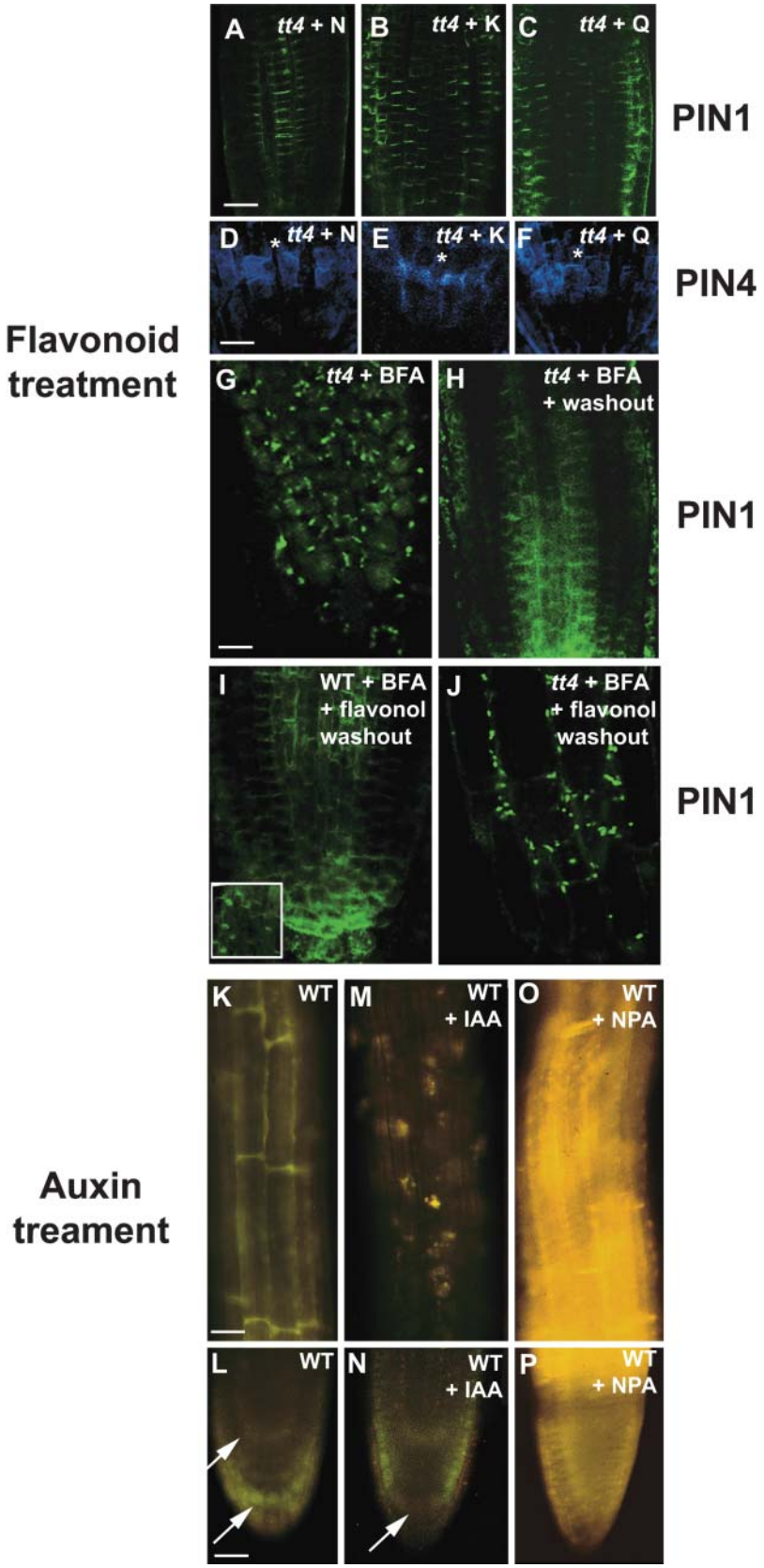

Figure 3. Flavonoid–Auxin Interactions in Roots.

lacks flavonols (Murphy et al., 2000; Peer et al., 2001), PIN1 signal remained in BFA bodies (Figure 3I, inset). Flavonol washout also resulted in an expansion of PIN1 distribution into other cell types, similar to the expanded distribution observed in *tt7* and *tt3*. In *tt4*, PIN1 signal was retained in BFA bodies and did not resume its usual asymmetric localization at the plasma membrane after flavonol washout of BFA (Figure 3J). In addition, the diffuse intracellular signal seen in untreated cells was no longer observed.

This suggests that wild-type cells that already contain flavonols are conditioned to their presence and are insensitive to the effects of exogenous flavonol application in terms of cellular trafficking of PIN1 protein. By contrast, in tissues that do not normally contain flavonols or are not conditioned to the presence of flavonols (tissue-specific regions in wild-type roots and in *tt4*), the flavonols interfere with normal cellular trafficking.

Flavonoid Localization and Speciation Respond to Altered Auxin Movement

Increase of Transported Auxin by Application at Shoot Apex

The proximal elongation zone is immediately above the naringenin chalcone-containing region that brightly fluoresces when visualized with diphenylboric acid 2-aminoethyl ester (DPBA) (Murphy et al., 2000; Peer et al., 2001). Cells in this region and the root tip were examined for alterations in flavonoid accumulation and localization in response to auxin movement. In untreated 5-d wild-type seedlings, kaempferol fluorescence was observed at the plasma membrane in the proximal root elongation zone and in the pericycle, columella initials, and columella and lateral cap cells; kaempferol and quercetin were both observed in the root cap (Figures 3K and 3L; Supplemental Figures 1J to 1M). When exogenous auxin was applied at the shoot apex of 4.5-d seedlings (as for auxin transport assays) and the seedlings were stained with DPBA 5 h later, fluorescent aggregations of quercetin were observed in the proximal elongation zone, C3/S3 cells, and lateral root cap (Figures 3M and 3N); epidermal accumulation of naringenin chalcone was also observed (data not shown).

Auxin Efflux Inhibitors

When NPA was applied in a strip above the R-S TZ to inhibit auxin movement to the root tip (see Methods), no change in flavonoid localization was noted in the elongation zone or root tip (data not shown). However, when NPA was applied globally for 48 h, diffuse kaempferol and quercetin localization was observed in the root tip and quercetin aggregations in the proximal elongation zone, pericycle, and root cap were observed (Figures 3O and 3P).

DISCUSSION

Flavonoid Modulation of Auxin Transport Is Tissue Specific

The auxin transport profiles of *tt* mutant and wild-type seedlings indicate that flavonoids regulate basipetal movement of auxin primarily at sites of synthesis (shoot apex) and redirection (root tip). Consistent with previous results (Murphy et al., 2000), auxin transport from shoot tip to root tip is enhanced in the absence of flavonoids (*tt4* and dark-grown wild type) and is reduced in the presence of excess kaempferol and quercetin (*tt7* and *tt3*). Although no significant difference in auxin transport was observed between wild-type and *tt4* hypocotyls at the 5-h time

Figure 3. (continued).

(A) to **(J)** Confocal images.
(K) to **(P)** Epifluorescence images.
(A) to **(F)** Five-day *tt4* seedlings treated with exogenous flavonoids. PIN1 immunolocalization (green) in *tt4* seedlings treated with 1 nM naringenin (N) in **(A)**, 10 nM kaempferol (K) in **(B)**, and 1 nM quercetin (Q) in **(C)**. PIN4 localization (blue) in *tt4* seedlings treated with 1 nM naringenin (N) in **(D)**, 10 nM kaempferol (K) in **(E)**, and 1 nM quercetin (Q) in **(F)**. Asterisk is above quiescent center cells in PIN4 localization images.
(G) and **(J)** PIN1 localization in 5-d root tips treated with BFA and after flavonol washout.
(G) PIN1 is observed in BFA bodies after BFA treatment in *tt4*.
(H) PIN1 signal is diffuse in addition to polar localization in the vascular tissue after BFA washout in *tt4*.
(I) PIN1 signal is observed at the plasma membrane after BFA washout with flavonols in wild-type root tips. Inset, PIN1 signal remains in BFA bodies in a nonflavonol-containing region of the root tip.
(J) PIN1 remains in BFA bodies after flavonol washout of BFA in *tt4*.
(K) to **(P)** Flavonoid fluorescence in 5-d L*er* seedlings stained with DPBA.
(K) Kaempferol fluorescence is observed at the plasma membrane in the proximal elongation zone of 4.75-d untreated L*er*. This is the region above the brightly fluorescing naringenin chalcone area (Murphy et al., 2000; Peer et al., 2001).
(L) Kaempferol fluorescence is observed in pericycle, columella initials, and columella tiers 1, 2, and 3; kaempferol and quercetin fluorescence is observed in the root cap in 4.75-d untreated L*er*. Arrows point to kaempferol fluorescent columella initials and tier 3/story 3 columella cells.
(M) Quercetin aggregates are observed in the proximal elongation zone of 4.75-d L*er* seedlings treated with a droplet of IAA placed at shoot apex and stained 5 h after IAA application (as for auxin transport assays).
(N) Root tip of 4.75-d L*er* plus IAA droplet placed at shoot apex and stained 5 h after application (as for auxin transport assays). Arrow points to tier 3/story 3 columella (C3/S3) cells with quercetin fluorescence after IAA treatment.
(O) Proximal elongation zone of 5-d L*er* transferred to NPA at 3 d (long-term global NPA treatment).
(P) Root tip of 5-d L*er* transferred to NPA at 3 d (long-term global NPA treatment).
Bars in **(A)** to **(C)** = 25 μM; **(D)** to **(J)** = 10 μM; and **(K)** to **(P)** = 25 μM.

point used in the assays shown in Figure 1, assays conducted for shorter periods or under slightly different conditions (Murphy et al., 2000) indicate that auxin transport in *tt4* hypocotyls is also enhanced compared with the wild type. The morphological changes and cortical shedding observed in *tt4* root tips are also consistent with the observed alterations in auxin transport and further identify the root tip as a site of flavonoid action. However, not all auxin transported to the root tip is available for basipetal redirection, as enhanced auxin leakage has been observed in *tt4* root tips (Murphy et al., 2000), and enhanced retention is evident in *tt7* and *tt3* (Figure 1).

Although aglycone flavonols accumulate at the R-S TZ as well as the root and shoot apices in the wild type (Murphy et al., 2000; Peer et al., 2001), the data presented here suggest that the R-S TZ is not a primary site of flavonoid modulation of auxin transport. However, flavonols may still function in cortical auxin retention, diversion of auxin from the transport stream, and at points of organ initiation.

Flavonoid Modulation of PIN Cycling

Flavonoids appear to directly modulate membrane trafficking of PIN1 in the root tip, where PIN1 cycling has been clearly demonstrated (Geldner et al., 2001, 2003). As *tt4* is a loss-of-function mutant, the resulting lack of flavonoids alters PIN1 cycling. When flavonoid-deficient *tt4* seedling roots are treated with BFA, PIN1 is sequestered in intracellular bodies (BFA bodies) identical to those seen in the wild type (Geldner et al., 2001). However, unlike wild-type root tips, when BFA treatment of *tt4* is followed by flavonol washout, PIN1 remains localized in BFA bodies, just as has been shown with NPA and TIBA washouts (Geldner et al., 2001). As is the case with NPA or TIBA treatments, endogenous or exogenously applied flavonols alone do not induce formation of intracellular bodies containing PIN1. Taken together with the differences in *PIN1* expression exhibited in root tips after TIBA and NPA treatments, these results support models that place aglycone flavonols as a subset of NPA binding sites. The lack of PIN1 retention in BFA bodies in wild-type roots after flavonoid washout suggests that flavonoids inhibit a protein modification or interaction required for PIN1 retention before sequestration in the BFA compartment. An auxin-responsive component on PIN1 trafficking may be active in tissues lacking flavonols as well: PIN1 is delocalized from the plasma membrane in *tt4* roots and in wild-type root tip regions that lack flavonols after exogenous application of auxin to the root apex.

Although AGR1/PIN2 cycling has been demonstrated (Boonsirichai et al., 2003), no evidence of direct flavonoid modulation of PIN2/AGR1 membrane cycling was observed. However, flavonoid modulation of PIN4/AGR3 cycling or functionality cannot be ruled out because the enlarged root tips and additional row of root cap cells observed in 4- to 5-d *pin4* seedlings (Friml et al., 2002a) are also present in *tt4* seedlings. Additionally, PIN4 is delocalized from the sides of the cell in *tt7*, *tt3*, and *tt* mutants treated with flavonols or AEIs. This suggests that both auxin and flavonols modulate PIN4 association with the membrane. PIN4 is thought to function as an auxin sink in root meristem cells and is essential for re-export of shoot-derived auxin to graviresponsive columella cells (Friml et al., 2002a). Consistent with this hypothesis is the absence of PIN4 protein in *tt4* roots. Under conditions of increased auxin transport (e.g., *tt4*), more auxin is delivered to the root tip, and an auxin sink is created by default; hence, PIN4 protein is not required.

Flavonoid modulation of PIN cycling in the shoot apex could not be confirmed. However, the auxin transport studies presented here, recent reports of PIN1 redirection in the shoot apical meristem (Reinhardt et al., 2003), and colocalization of asymmetric flavonoid accumulation (data not shown) with PIN1 delocalization in hypocotyls exposed to unidirectional light (Blakeslee et al., 2004) support the likelihood that flavonoid-sensitive cycling mechanisms similar to those found in root tips function in growing shoot tissues.

We hypothesize that the flavonoid-sensitive plasma membrane aminopeptidase AtAPM1 (Murphy et al., 2002) may mediate flavonoid-PIN1 interactions because specific AtAPM1 inhibitors reduce PIN1 plasma membrane localization (A. Bandyopadhyay and A.S. Murphy, unpublished data). In addition, the full-length form of AtAPM1 containing both trafficking and catalytic domains is enriched in wild-type seedling membranes during periods of maximal aglycone flavonol accumulation (Murphy et al., 2002), whereas the truncated form containing only the catalytic domain predominates in all *tt4* membranes examined.

A role for MDR/PGP proteins that copurify with AtAPM1 (Murphy et al., 2002) in flavonoid-sensitive cycling is also possible because PIN1 localization was found to be disrupted in *mdr/pgp* mutant hypocotyls (Noh et al., 2003). MDR/PGP proteins have been shown to alter cycling mechanisms in yeast (Fritz et al., 1999), and vesicular cycling of flavonoid-sensitive MDR/PGPs themselves has been described in mammals (Kipp et al., 2001; Di Pietro et al., 2002). However, recent mutational and cellular transport studies of AtPGP1 and AtPGP19 (AtMDR1) indicate that, in addition to mediating PIN localization, MDR/PGPs exhibit direct, energy-dependent, NPA- and flavonoid-sensitive auxin transport (J.J. Blakeslee and A.S. Murphy, unpublished data).

Flavonoids May Modulate Phosphorylation of Vesicular Trafficking Proteins

Observed increases in *tt4* cortical shedding and lateral roots are similar to NPA-reversible phenotypes seen with overexpression of *PID*, a gene encoding an ortholog of flavonoid-sensitive mammalian protein kinase C proteins (Benjamins et al., 2001). However, excess flavonol accumulations either do not inhibit PID function or are developmentally distinct from regions of *PID* expression because mutants that overproduce flavonols (*tt3* and *tt7)* lack the severe developmental phenotypes seen in *pid* mutants. However, if models placing RCN1 at an early stage of PIN vesicle trafficking (Muday and Murphy, 2002) are correct, the observed results are consistent with flavonoid inhibition of phosphatase 2A activity.

Altered Auxin Transport in *tt* Mutants Modulates *PIN* Expression and PIN Distribution

Flavonoids appear to modulate PIN cycling, subcellular localization, and transport function. However, changes in *PIN* gene

expression observed in *tt* mutants are apparently indirect effects of altered apical auxin transport because these changes can be mimicked by experimental manipulation of auxin levels in the wild type and *tt* mutants using AEIs or exogenous IAA. Similarly, the observed changes in tissue-level PIN distribution in the root tip appear to reflect altered auxin levels rather than direct flavonoid interactions with transport proteins. For example, in *tt3* and *tt7* root tips, flavonol accumulation and consequent inhibition of basipetal auxin transport to tissues immediately above results in enhanced *PIN1* expression and expanded PIN1 protein distribution without altering subcellular localization.

Distribution and subcellular localization of PIN2/AGR1 were not altered in any of the flavonoid mutants examined, although *PIN2* expression and PIN2 protein abundance (as indicated by increased immunofluorescence signal) increased in the absence of flavonoids. The increase in PIN2 protein in the absence of flavonoids is consistent with the increased amount of auxin available for redistribution. This suggests that *PIN2* gene expression is auxin responsive rather than flavonoid responsive, whereas PIN2 protein distributions and subcellular localization is neither auxin nor flavonoid responsive.

Conclusion

These results indicate that, in Arabidopsis seedlings, flavonoids directly modulate auxin transport at the sites of auxin synthesis (shoot apex) and redirection (root tip). Observed changes of PIN expression and localization in other tissues appear to be secondary effects of altered auxin transport. Flavonoids appear to alter PIN cycling at the root tip but may also modulate direct auxin transport by MDR/PGPs as well.

Each *PIN* gene examined had different auxin and flavonoid sensitivities with regard to expression patterns and protein localization, but in all cases the changes observed are consistent with both *tt* mutant growth phenotypes and auxin transport profiles. For example, PIN1 primarily regulates the delivery of auxin from the shoot tip to root tip (Gälweiler et al., 1998). *PIN1* gene expression is auxin responsive; PIN1 protein localization is both auxin and flavonoid responsive; PIN1 is delocalized from the membrane in the absence of flavonoids; and distribution expands in the presence of excess flavonols. These responses are consistent with proposed PIN1 function, increased auxin transport from shoot apices in *tt4* mutants, and resulting auxin-related phenotypes such as increased primary root growth, increased lateral root formation, and increased numbers of secondary inflorescences (Brown et al., 2001). They are also consistent with reduced auxin transport observed in *tt3* and *tt7*. However, the relatively small impact on root growth and other auxin-related growth phenotypes seen in these mutants (W.A. Peer and A.S. Murphy, unpublished data) indicates that expanded PIN1 protein distribution compensates for flavonoid inhibition of transport to some extent.

PIN2 primarily mediates basipetal auxin transport in root tissues in the root transition zone and below (Müller et al., 1998). *PIN2* expression is auxin responsive, but protein localization is neither auxin responsive nor flavonoid sensitive; wild-type localization patterns are observed in the *tt* mutants examined. This is consistent with a relatively small impact of flavonoid biosynthesis mutations on root gravitropism. *PIN4* expression is not flavonoid responsive, but protein localization is auxin responsive and flavonoid sensitive; PIN4 localization and distribution is reduced in the absence of flavonoids and expands in the presence of excess flavonols. The root phenotypes noted in *tt4* and *pin4* mutants noted above are consistent with these results.

The subtle growth and tropic response phenotypes of *tt* mutants (Peer et al., 2001; Buer and Muday, 2004) and normal gravitropic responses found in dark-grown wild-type seedlings that lack flavonoids (Jensen et al., 1998) suggest that flavonoids are nonessential regulators of auxin transport mechanisms. Although both flavonoids and auxin accumulate in regions of new growth (Ljung et al., 2001; Peer et al., 2001; Marchant et al., 2002), flavonoids also play well-documented roles in defense, free radical protection, and microbial interactions (Fox et al., 2001; Harvaux and Kloppstech, 2001; Ryan et al., 2001). As such, in flavonoid-accumulating regions of apical growth, flowering, root and shoot branching, wounding, and rhizobial nodulation (Vogt et al., 1994; Mathesius et al., 1998; Murphy et al., 2000; Mathesius, 2001; Peer et al., 2001), flavonoids could be regarded as multifunctional autocrine effectors rather than specific regulators. However, specific and localized interactions of flavonoids with auxin transport mechanisms have been identified in the work described here. Mutational, cell biological, and biochemical analysis of PIN, MDR/PGP, and AtAPM1 functions using flavonoids as inhibitors now in progress are expected to clarify how flavonoids modulate these target proteins.

METHODS

Plant Materials

Arabidopsis thaliana L*er*, *tt7* (At5g07990), and *tt3* (At5 g42800) seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH); *tt4* (UV118a; At5g139030) seeds, a null mutant for chalcone synthase, were the kind gift of Brenda Winkel (Virginia Tech, Blacksburg, VA).

Quantitation of Endogenous Free Auxin

Seedlings were grown as described previously (Murphy and Taiz 1995). Five days after planting seedlings were harvested, the cotyledons were excised, and seedlings were cut in half at the R-S TZ. Roots and shoots were collected in lots of 500, and auxin quantifications were performed according to the method of Chen et al. (1988).

Auxin Transport Assays

Auxin transport assays were conducted on intact light-grown seedlings as described previously (Geisler et al., 2003), with the following refinements: Seedlings were grown 4.5-d after germination. Before assay, 10 seedlings were transferred to vertically discontinuous filter paper strips saturated in one-quarter MS and allowed to equilibrate for 2 h. Auxin solutions used to measure transport were made up in 0.25% (w/v) agarose containing 2% (v/v) DMSO and 25 mM Mes, pH 5.2. A 0.1-μL microdroplet containing 500 nM unlabeled IAA and 500 nM [^{3}H]IAA (specific activity 25 Ci/mmol; American Radiochemicals, St. Louis, MO) was placed on the shoot apical tip of seedlings using a modified microliter Hamilton syringe. For coapplication, naringenin was added to the IAA and agarose to a final concentration of 20 nM before agarose solidification. Applications were performed using a Narishige micromanipulator

(Narishige Scientific Instrument Lab, Tokyo, Japan) mounted to a Florod LFA microscope-guided x-y laser cutter (Florod, Gardena, CA). Seedlings were then incubated in the dark for 5 h. After incubation, the hypocotyls and cotyledons were removed. A 2-mm section of filter paper, upon which the R-S TZ was centered, was harvested, along with the 2-mm segment of tissue containing the root-shoot transition zone. Additionally, a 4-mm basal filter paper-strip section containing the final 1 to 2 mm of root tissue as well as any root tip leakage was also harvested. A 0.2-μL treatment with [^{14}C]IAA (specific activity 1 mCi/mm) was used instead of 0.1 μL of [^{3}H]IAA to investigate auxin transport and accumulation in cotyledons and hypocotyls. See http://www.hort.purdue.edu/hort/research/murphy/protframe.htm for complete details.

Real-Time PCR

Seedlings were grown as described by Murphy and Taiz (1995). Addition of <1% (w/v) sucrose to the media had no measurable effect on *PIN* expression. Addition of >1% (w/v) sucrose to the media resulted in reproducible changes in *PIN* expression.

At 5 d, roots and shoots were divided at the transition zone using a double-edged razor blade. For AEI treatments, seedlings were grown on half-strength MS, 1% (w/v) phytagar plates, and transferred to plates with 10 μM of TIBA, 10 μM of NPA, 10 nM naringenin, or 1 μM IAA for 4 h before harvesting.

RNA extraction and PCR conditions were as described by Blakeslee et al. (2004). The reference gene β-*tubulin* was used to quantitate the relative transcript number of each target *PIN* gene in each tissue type; values were calculated and statistically analyzed as described by Livak and Schmittgen (2001). Briefly, to normalize the data, the ΔC_T value, or cycle number where a statistical difference is measured above background, is determined by subtracting the average gene 1 value (*PIN*) from the average gene 2 value (β-*tubulin*). The $\Delta\Delta C_T$ value is calculated by subtracting the ΔC_T calibrator value, an arbitrary constant; therefore, the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. The range for gene 1 relative to gene 2 is determined from the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the standard deviation of the $\Delta\Delta C_T$ value. See Livak and Schmittgen (2001) for details. Statistical analyses were then conducted using a one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls post hoc analysis. The amplification efficiencies of the target genes *PIN1* (At1g73590) (1.95), *PIN2* (At5g57090) (1.9), and *PIN4* (At2g01420) (1.99) were nearly equal to the reference gene β-*tubulin* (At5g12250) (1.96) efficiency. Primer sequences for *PIN1* were 5′-CGT GGA GAG GGA AGA GTT TA-3′ (forward), 5′-AAC ATA GCC ATG CCT AGA CC-3′ (reverse); *PIN2*, 5′-TAT CAA CAC TGC CTA ACA CG-3′ (forward), 5′-GAA GAG ATC ATT GAT GAG GC-3′ (reverse); *PIN4*, 5′-ACA ACG CCG TTA AAT ATG GA-3′ (forward), 5′-AGA CCC CAT TTT ATT CAG CC-3′ (reverse); β-*tubulin*, 5′-TGG GAA CTC TGC TCA TAT CT-3′ (forward), 5′-GAA AGG AAT GAG GTT CAC TG-3′ (reverse).

Antibody Generation and Purification

Anti-PIN1 and anti-PIN2/AGR1 antibodies have been previously described (Gälweiler et al., 1998; Boonsirichai et al., 2003). A polyclonal anti-PIN4/AGR3 antibody was generated from a 157 amino acid PIN4 polypeptide (from 265 to 422) overexpressed from a 571 bp-long PIN4 cDNA fragment in a pBAD/GIII vector (Invitrogen, Carlsbad, CA) in *E. coli* Top10 cells and used to immunize rabbits. Anti-PIN4 antibody was affinity-purified according to standard protocols (Harlow and Lane, 1988).

Immunohistochemical Localizations

All reagents were from Sigma (St. Louis, MO), except where noted. Seedlings were grown on 1% (w/v) phytagar plates and one-quarter MS basal salts, pH 4.85, without sucrose. Addition of <1% (w/v) sucrose to the media had no measurable effect on PIN localization or flavonoid speciation. Addition of >1% (w/v) sucrose to the media resulted in reproducible changes in tissue-specific PIN localization and flavonoid speciation. Altogether 100 seedlings were examined per treatment; results presented are consistent in 80% to 90% of the seedlings, except where noted for PIN4 localization in *tt4*.

Immunohistochemical localizations and microscopy were as described previously (Noh et al., 2003), with the following modifications: Seedlings were digested with 0.5% (w/v) pectolyase Y-23 (Seishin, Tokyo, Japan) and 1% (w/v) BSA and/or biotin/avidin D (v/v; Vector Labs, Burlingame, CA) and permeabilized for 1 h in 10% (v/v) DMSO and 1% (v/v) Nonidet P-40. The antibody dilutions were 1:400 for anti-PIN1 and 1:250 for anti-PIN2 and anti-PIN4. A confocal laser scanning microscope (Eclipse 800; Nikon, Tokyo, Japan) equipped with an argon laser (488 nm; Bio-Rad, Hercules, CA) was used for immunofluorescence imaging. Green HeNe laser (543 nm, 1.4 mW) and a red laser diode (638 nm, 5 mW) were used for autofluorescence detection.

For the inhibitor studies, 5-d seedlings were incubated in 1 μM NPA for 4 h and 10 μM TIBA for 2 h. To test the effect of flavonoid precursors, 2-d *tt* seedlings were transferred to plates coated with 1 nM quercetin, 1 nM naringenin, or 10 nM kaempferol. Five-day *tt4* seedlings were treated with 10 μM BFA for 2 h and then washed with a 1 μM mixture of quercetin and kaempferol for 2 h before immunolocalization analysis as described above. For microdroplet auxin application treatments, seedlings were grown as for auxin transport studies for 4.5 d and placed on a discontinuous filter paper system, in which they were sandwiched between two 2-mm filter paper strips centered on the R-S TZ. For NPA treatment, these filter paper strips were soaked with 10 μM NPA.

Flavonoid identification and localizations with DPBA were as described previously (Murphy et al., 2000; Peer et al., 2001) and were visualized with an epifluorescent microscope equipped with an FITC filter (excitation 450 to 490 nm, suppression LP 515 nm).

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