Flavonoid Accumulation Patterns of Transparent Testa Mutants of Arabidopsis

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Flavonoids have been implicated in the regulation of auxin movements in Arabidopsis. To understand when and where flavonoids may be acting to control auxin movement, the flavonoid accumulation pattern was examined in young seedlings and mature tissues of wild-type Arabidopsis. Using a variety of biochemical and visualization techniques, flavonoid accumulation in mature plants was localized in cauline leaves, pollen, stigmata, and floral primordia, and in the stems of young, actively growing inflorescences. In young Landsberg erecta seedlings, aglycone flavonols accumulated developmentally in three regions, the cotyledonary node, the hypocotyl-root transition zone, and the root tip. Aglycone flavonols accumulated at the hypocotyl-root transition zone in a developmental and tissue-specific manner with kaempferol in the epidermis and quercetin in the cortex. Quercetin localized subcellularly in the nuclear region, plasma membrane, and endomembrane system, whereas kaempferol localized in the nuclear region and plasma membrane. The flavonoid accumulation pattern was also examined in transparent testa mutants blocked at different steps in the flavonoid biosynthesis pathway. The transparent testa mutants were shown to have precursor accumulation patterns similar to those of end product flavonoids in wild-type Landsberg erecta, suggesting that synthesis and end product accumulation occur in the same cells.

Flavonoids comprise a diverse group of phenolic compounds that serve a variety of ecological and physiological functions in plants (Stafford, 1990; Mathesius et al., 1998a; Debeaujon et al., 2000). A possible role for phenolics in the regulation of auxin retention was first proposed by Stenlid (1976) and Marigo and Boudet (1977). One group of flavonoids, the aglycone flavonols, has been shown to inhibit polar auxin transport, thereby promoting auxin retention. In zucchini hypocotyls, aglycone flavonols, has been shown to inhibit polar auxin transport, thereby promoting auxin retention in clover roots (Mathesius et al., 1998b).

Murphy et al. (2000) recently demonstrated tissue-specific localization of flavonoids in Arabidopsis seedlings. Quercetin, kaempferol, and naringenin chalcone (NC) were shown to be concentrated in three tissues: the upper hypocotyl (UH), the hypocotyl-root transition zone type affecting seed coat color in Arabidopsis results from defects in various steps in the flavonoid biosynthesis pathway (Koornneef et al., 1982; Koornneef, 1990; Shirley et al., 1995; Fig. 1). As such, the tt mutants make it possible to analyze the effects of individual flavonoids on auxin transport and growth characteristics. The tt4 alleles contain mutations in the gene encoding CHS and, as there is only a single gene encoding this enzyme in Arabidopsis, these plants are deficient in flavonoids (Saslowsky et al., 2000). In a recent series of studies, two tt4 alleles were examined and were found to have altered auxin transport in seedlings and inflorescence tissue, as compared with wild-type seedlings (Murphy et al., 2000, Brown et al., 2001). In one of those studies (Murphy et al., 2000), treatment of seedlings with naringenin, a flavonoid precursor, restored flavonoid biosynthesis in the tt4 mutant, resulting in the normal auxin distribution profile and loss of auxin efflux from the mutant root. Thus, there is mounting evidence that auxin retention or transport in vivo is regulated by the accumulation of aglycone flavonoids.

Additional evidence in support of the hypothesis that flavonoids act to regulate auxin transport is that these compounds accumulate in tissues where auxin transport may be regulated. Murphy et al. (2000) recently demonstrated tissue-specific localization of flavonoids in Arabidopsis seedlings. Quercetin, kaempferol, and naringenin chalcone (NC) were shown to be concentrated in three tissues: the upper hypocotyl (UH), the hypocotyl-root transition zone

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Figure 1. Schematic diagram of the flavonoid biosynthetic pathway. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase. Specific tt mutations are indicated. Each enzyme product can potentially undergo glycosylation.

(TZ), and the distal elongation region of the root. These same tissues have been shown to be sites of auxin accumulation in Arabidopsis seedlings, as determined by direct measurement of radiolabeled indole-3-acetic acid transport (Murphy et al., 2000) and by the expression of auxin-responsive reporter genes (Ulmasov et al., 1997; Sabatini et al., 1999). Therefore, flavonoids appear appropriately distributed to regulate auxin movement.

It is probable that only specific flavonoid compounds are going to be active as regulators of auxin movement in plants. To determine which compounds are active, a number of mutants with lesions at different enzymes in the flavonoid biosynthetic pathway were examined. The tt4 mutations in the chalcone synthase (CHS) gene are deficient in flavonoid accumulation, but accumulate an excess of sinapate esters (Saslowsky et al., 2000); the tt5 lesion in the CHI gene, accumulates NC in place of flavonols, but has wild-type levels of sinapate esters; the tt7 mutant in the flavonoid 3'-hydroxylase gene (F3'H) accumulates an excess amount of kaempferol; and a tt3 lesion in the gene encoding DFR, accumulates excess amounts of quercetin and kaempferol, as predicted from the pathway and confirmed experimentally (Koornneef et al., 1982; Shirley et al., 1995; Graham, 1998).

In this paper flavonoid-deficient tt mutants have been used to determine if flavonols are transported from their site of synthesis to their site of accumulation. The flavonoid content in whole seedlings of Arabidopsis and the tt mutants was previously determined by Shirley et al. (1995) and Burbulis et al. (1996) using thin-layer chromatography (TLC), HPLC, and mass spectroscopy (MS), and by Sheahan (1996) and Sheahan and Cheong (1998) by HPLC and diode array analysis. These previous publications did not provide information on where these compounds were accumulating. In a previous study (Murphy et al., 2000), visualization of tissue-specific flavonoid accumulation was limited to 4-d whole wild-type seedlings with the intention of correlating flavonoid localization with regions of auxin accumulation. Here, the accumulation of flavonol precursors and their end products in specific tissue segments and subcellular regions were characterized in wild-type and tt mutant plants by a combination of histochemical fluorescence staining with diphenylboric acid-2-aminoethyl ester (DPBA), two-dimensional TLC, HPLC, fluorometry, UV/vis spectroscopy, and MS. The results from these analyses indicate that the location of flavonoid accumulation is unchanged when the later reactions of the pathway are inhibited.

In this report we have also expanded flavonoid localization studies in seedlings, extended the studies to include mature plants, and supplemented histochemical fluorescence assays with chemical analyses to confirm the identity of flavonoid intermediates. We have applied these studies to a range of tt mutants blocked at various stages in the flavonoid biosynthetic pathway to determine the localization of flavonoid intermediates.

RESULTS

Identification and Quantification of Flavonoid Species in Wild-Type Arabidopsis Tissues

Flavonoids in wild-type and mutant seedlings were identified in three main zones: the cotyledonary node, the hypocotyl/root TZ, and the root tip. After dissection of seedlings, the endogenous flavonoids in the three zones were extracted and identified by two-dimensional TLC, HPLC, fluorometry, UV/vis spectroscopy, and MS by comparison with known standards (hereafter, for the sake of brevity, referred to as “spectral analysis”). Aglycones and substituted flavonoids were detected. For each region the major flavonoid skeletons identified as well as the specific genetic lesion found in each tt mutant are indicated in Table I. In all cases except tt6, the flavonoid intermediate expected to accumulate as a result of the specific biosynthetic defect was detected. Naringenin is the intermediate expected to accumulate in tt6; however, it was never detected in mutant or wild-type seedlings. In addition, the lesion in tt6 is leaky, and therefore, end-product flavonoids were also detected.

To obtain more quantitative information on the flavonoid concentration in these tissue segments, the same three segments were excised and the aglycone flavonols were purified by HPLC and were quantified by UV/vis spectroscopy and mass spectrometry. The amounts of quercetin and kaempferol in the different regions are presented in Table II. In the wild type, the TZ/UR is the major site for accumulation of quercetin and kaempferol followed by the UR. Kaempferol accumulated in the LR, and a small
amount of quercetin was found in the LH (Table II). High sds found in the normalized root tip concentration shows reflected the difficulty in obtaining accurate weights of those tissues.

In HPLC analyses of seedling sections, it was possible to determine the relative abundance of aglycone flavonoids. In Table III, the proportion of total flavonoids that are aglycone flavonols is reported as a function of age and region of the seedling. The cotyledon contained mainly glycosylated flavonoids, with the amounts of aglycone flavonols decreasing as the tissue matured (Table III). In the UH, aglycone flavonols were the predominant flavonoids at 3 d, decreasing over time (Table III). In the LH, aglycone flavonols peaked at 5 d and decreased to one-third the total amount of flavonoids at 7 d (Table III). The same pattern was observed in the TZ/UR, with aglycone flavonols predominating at 5 d and glycosylated flavonoids comprising the majority at 7 d (Table III). In the LR, aglycone flavonols appeared to be the principal form at every time point (Table III).

Quantitation of Flavonoid Species in Tissues of tt Mutants of Arabidopsis

To determine how flavonoid distribution was affected by alterations in the enzymes in the flavonoid biosynthetic pathway, flavonoids were quantified in the tt mutants. Flavonoids were not detected in any section of tt4 seedlings (Table II), as expected by the mutation in the CHS gene, which encodes an enzyme early in the flavonoid pathway. The tt3 seedlings have a mutation in the gene encoding DFR, which is late in the flavonoid biosynthetic pathway, so flavonoids such as quercetin and kaempferol would be expected to accumulate. In tt3 seedlings, the TZ/UR was the primary site of quercetin and kaempferol accumulation. Compared with Landsberg erecta (Ler) seedlings, tt3 contained significantly more quercetin in the UH, LH, and TZ/UR, and tt3 also contained more kaempferol in the LH than wild-type seedlings (Table II). Whereas kaempferol alone was detected in Ler in the LR, kaempferol and quercetin accumulated in tt3 (Table II).

Flavonols were not detected in tt5 (Table II), and a peak consistent with NC, the intermediate expected to accumulate in this mutant, was detected. However, a peak containing a chalcone and flavonone skeleton with approximately the same retention time as quercetin and at a concentration of 5.3 ± 0.4 mg mg fresh weight−1 was observed in the UH in tt5. As NC does not have this retention time, spontaneous isomerization of NC to naringenin may have resulted in the subsequent formation of a flavanone structure in tt5.

The tt7 mutant accumulated significantly more kaempferol in the UH and TZ/UR than did Ler (Table II), which is expected based on the mutation in the gene encoding F3′H. Within the kaempferol peaks, a minor chalcone skeleton (<1%) was detected spectroscopically. Like tt5, tt7 also had peaks with approximately the same retention time as quercetin: 7-methyl-chalcone accumulated in the UH (63.3 ± 3.3 mg mg fresh weight−1) and to a much lesser extent in the LH (5.3 ± 0.7 mg mg fresh weight−1). A similar peak in the TZ/UR region was insufficient for definitive spectroscopic analysis. The accumulation of these other flavonoid compounds is not expected, as this mutant would not be predicted to exhibit altered conversion from flavanones and fla-

Table I. Summary of tt mutations and major flavonoids accumulated and tissue-specific flavonoid localization in 5-d seedlings determined histochemically and confirmed biochemically

<table>
<thead>
<tr>
<th>Line</th>
<th>Enzyme Mutateda</th>
<th>Flavonoid Accumulatedb</th>
<th>Cotyledonal Node</th>
<th>TZ</th>
<th>Root Tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>tt4</td>
<td>CHS (null phenotype)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>tt5</td>
<td>CHI (null)</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>tt6</td>
<td>F3H (leaky)</td>
<td>Naringenin(^d)</td>
<td>NC, kaempferol, quercetin</td>
<td>NC, kaempferol, quercetin</td>
<td>NC, kaempferol</td>
</tr>
<tr>
<td>tt7</td>
<td>F3′H (null phenotype)</td>
<td>Kaempferol</td>
<td>Kaempferol</td>
<td>Kaempferol</td>
<td>Kaempferol</td>
</tr>
<tr>
<td>tt3</td>
<td>DFR (null)</td>
<td>Excess quercetin, kaempferol</td>
<td>Quercetin, kaempferol</td>
<td>Quercetin, kaempferol</td>
<td>Quercetin, kaempferol</td>
</tr>
</tbody>
</table>

\(^a\) Adapted from Koornneef et al. (1982); Shirley et al. (1995); and Wisman et al. (1998). \(^b\) n.d., None detected. \(^c\) NC, Naringenin chalcone. \(^d\) Unidentified orthodihydroxy or orthotrihydroxy flavonoid accumulated (Sheahan et al., 1998).

Table II. HPLC determination of mean flavonol composition ± so of 5-d-old Arabidopsis seedlings divided into sections

<table>
<thead>
<tr>
<th>Line</th>
<th>Quercetin</th>
<th>Kaempferol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mg fresh wt</td>
<td>ng/mg fresh wt</td>
</tr>
<tr>
<td>Ler</td>
<td>26.5 ± 4.2</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>tt3</td>
<td>137 ± 6.2(^*)</td>
<td>15 ± 0.8(^*)</td>
</tr>
<tr>
<td>tt4</td>
<td>0 ± 0.0*</td>
<td>0 ± 0.0*</td>
</tr>
<tr>
<td>tt5</td>
<td>0 ± 0.0*</td>
<td>0 ± 0.0*</td>
</tr>
<tr>
<td>tt7</td>
<td>0 ± 0.0*</td>
<td>0 ± 0.0*</td>
</tr>
</tbody>
</table>

\(^*\) Asterisk indicates P < 0.001 compared with Ler by Student-Neumann-Keuls post hoc.
The results described above indicate that the concentrations of flavonoid intermediates change between wild-type and mutant plants. To examine the distribution of flavonoids in intact seedlings, a DPBA fluorescence time-course study showing the flavonoid accumulation patterns at different stages of development was performed, first in wild-type Ler and later with mutants. Each flavonoid-DPBA conjugate has a unique fluorescent color and a different distribution of flavonoids in intact seedlings, a DPBA fluorescence time-course study showing the flavonoid intermediates change between wild-type and mutant plants. To examine the LR seems to prevent leakage of auxin from the root tip (Murphy et al., 2000).

Time-Course of Flavonoid Staining

The accumulation of flavonoids may be the result of altered feedback mechanisms in the mutants, and the compounds' subsequent availability to other enzymes. In the LR, kaempferol was also detected in tif7 as in Ler. The presence of kaempferol in the LR seems to prevent leakage of auxin from the root tip (Murphy et al., 2000).

<table>
<thead>
<tr>
<th>Percent of Aglycone Flavonols</th>
<th>Cotyledon</th>
<th>UH</th>
<th>LH</th>
<th>TZ/UR</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>22 ± 10</td>
<td>79 ± 14</td>
<td>78 ± 16</td>
<td>86 ± 15</td>
<td>106 ± 32</td>
</tr>
<tr>
<td>5</td>
<td>19 ± 7.9</td>
<td>14 ± 10</td>
<td>96 ± 16</td>
<td>96 ± 5.2</td>
<td>103 ± 20</td>
</tr>
<tr>
<td>7</td>
<td>14 ± 11</td>
<td>10 ± 9.9</td>
<td>36 ± 25</td>
<td>35 ± 15</td>
<td>87 ± 27</td>
</tr>
</tbody>
</table>

Table III. Average ratio of aglycone flavonols ± so to total flavonoids in 3-, 5-, and 7-d-old wild-type seedling sections by HPLC, fluorimetry, UV/vis spectroscopy, and mass spectroscopy (see "Materials and Methods")

They were calculated by dividing the nanograms per milligram of aglycone fresh wt flavonols by the nanograms per milligram fresh wt of the total flavonoids.

The flavonoid staining patterns in each region were restricted to the actively growing tissues: the flowers, immature siliques, and inflorescence stems, cauline and rosette leaves, floral primordia, stigmata, and pollen (Fig. 3A, inset). In contrast, only blue sinapate ester autofluorescence was visible in unstained plants (Fig. 3B). However, in older mature plants that no longer had elongating inflorescence stems, the flavonoid staining was restricted to the actively growing tissues: the flowers, immature siliques, and upper inflorescence stems (Fig. 3C). In Ler, the principal flavonoid observed in the flowers and upper inflorescences was quercetin (gold), and the stigma papillae and pollen were also rich in quercetin (Fig. 3C, inset). The higher concentration of quercetin in these tissues was verified by spectral analysis. This flavonoid staining pattern occurred in all plants that were examined. In HPLC analyses of wild-type inflorescence stem segments, aglycone flavonoids were concentrated in the growing tissues and at the apex, and decreased toward the base, as shown in Figure 4. Flavonoids at the base were in the form of aglycones, glycosylated derivatives.
Figure 2. Time course of flavonoid accumulation in stained Ler seedlings viewed through an FITC filter. Size bars are 0.125 mm for A, B, C, D, E, and F and are 0.100 mm for G, H, and I. A through C, Cotyledonary nodes of 3-, 5-, and 7-d seedlings, respectively. The amount of quercetin (gold fluorescence) accumulation increases over time, and at 7 d quercitrin (glycosylated quercetin, yellow-orange fluorescence) is observed. Arrows point to cotyledonary nodes. A (inset), Autofluorescence of 3-d cotyledonary node; red color is due to chlorophyll. D through F, Hypocotyl/root TZ of 3-, 5-, and 7-d seedlings, respectively. The amounts of quercetin and kaempferol (yellow-green fluorescence) reach a maxima at 5 d, with kaempferol occurring in a ring of cells above the cone-shaped region of quercetin containing cells. Arrows point to transition zones. D (inset), Autofluorescence of 3-d TZ; red autofluorescence is due to chlorophyll and pale green autofluorescence is due to sinapate esters. G through I, Root tips of 3-, 5-, and 7-d seedlings, respectively. NC (yellow fluorescence) is observed in the distal elongation zone and kaempferol in the root cap. Arrows point to distal elongation zone (G–I) and root cap (H and I). The non-staining area is the meristematic region. Root tips lacked sufficient autofluorescence to photograph.
tives, and anthocyanin intermediates (Fig. 4). In segments containing cauline leaves (indicated by asterisks), the leaves and petioles were removed from the stem prior to HPLC analysis, and such cauline leaf nodes contained increased amounts of aglycone flavonoids (Fig. 4). Anthocyanins were most concentrated at the base of the stem, and decreased in concentration toward the apex (Fig. 4).
Flavonoid Staining in tt Mutants

Flavonoids accumulated in the same regions in the tt mutants as in wild-type seedlings. An unstained TZ of a tt3 seedling showing background autofluorescence is presented in Figure 5A. Autofluorescence was encountered in all of the seedlings as a result of the presence of chlorophyll (red fluorescence) and sinapate esters (faint green fluorescence) in the epidermis and cell walls; fah1-2 seedlings were used as a control for sinapate ester autofluorescence. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; data not shown). In all of the tt mutants, chloroplasts were observed in the upper one-third of the root. In tt5 (Fig. 5C), NC (yellow) was observed in the TZ, and in tt6 (Fig. 5D), a mixture of chalcone derivatives and end-product flavonoids were observed. In stained tt4(85) seedlings, only autofluorescence of the chlorophyll and sinapate esters was observed (Fig. 5B). In addition, tt4(85) had the same staining phenotype as the true null tt4 (UV118a; Figure 7A, or complete plasmolysis, as in Figure 7B. Under mild plasmolysis conditions, quercetin (gold) fluorescence was localized in the plasma membrane at the basal ends of root cells, as well as in the nuclear region (Fig. 7A). Under more stringent plasmolysis conditions, fluorescence was observed in the nuclear region, plasma membrane, and endomembrane system (Fig. 7B). In tt7, which only accumulates kaempferol (as shown in Table II),
the yellow-green fluorescence of the DPBA-kampferol complex was localized in the nuclear region under mild plasmolysis conditions, as shown in Figure 7C. In tt5, which accumulates NC (as shown in Table I), fluorescence is observed throughout the cytosol (Fig. 7D). This pattern was observed in >90% of the seedlings observed.

**DISCUSSION**

Although the ability of aglycone flavonoids to inhibit auxin efflux and plasma membrane binding of the transport inhibitor NPA has been established for some time (Jacobs and Rubery, 1988; Bernasconi, 1996), evidence supporting a role for endogenous flavonoids in auxin transport has only recently been demonstrated (Murphy et al., 2000; Brown et al., 2001). Here we present evidence that flavonoids co-localize spatially and temporally with regions of auxin accumulation. Localization of aglycone flavonoids in areas of organ transition and maturation suggests that flavonoids may influence developmental processes through controlling the distribution of auxin in these tissues.

**Flavonoid Accumulation**

Flavonoid accumulation in seedlings is developmentally regulated and parallels the expression of the early genes of flavonoid biosynthesis, CHS, CHI, and flavonone 3-hydroxylase (Pelletier et al., 1999). The flavonoids accumulate in a tissue-specific pattern that corresponds to areas of auxin accumulation (Ulmasov et al., 1997; Sabatini et al., 1999) and PIN gene expression (Galweiler et al., 1998; Muller et al., 1998; Friml et al., 2000). The areas of flavonoid accu-
mulation also define TZ between different organs, including the cotyledonary node transition, root-shoot transition, and transition from the meristematic to elongation zones in roots. The role of this accumulation may be to modulate auxin flow between these different organs. The cotyledonary node TZ between the UH and the epicotyl is based on the vascular tissue differences at the node and that of the subtending hypocotyl (Busse and Evert, 1999). The hypocotyl/root TZ is generally the lower boundary of chloroplast formation and is the uppermost region where suberized Casparian strips form in the root endodermis (A. Murphy, unpublished data). A single ring of epidermal cells at this boundary also produces a profusion of epidermal root hairs. In roots, the distal elongation zone is a TZ between the meristematic region and the central elongation zone, and in Arabidopsis begins about 100 microns from the root tip (Mullen et al., 1998), which is the region of intense flavonoid staining in the root tip. In adult plants, flavonoid accumulation is restricted to actively growing or maturing tissue.

Accumulation of flavonoid derivatives is also developmentally regulated. Our HPLC/MS/spectroscopic analysis of whole and sectioned seedlings confirmed earlier reports (Sheahan and Cheong, 1998; Pelletier et al., 1999) that glycosylated flavonoids comprise a large proportion of the total flavonoids as early as 5 d, but also indicated that they are limited to the cotyledons and the UH until 7 d. Aglycone forms predominate in the hypocotyl/root TZ and root tip, and it is not until 7 d that the majority of flavonoids are glycosylated in all sections. This biochemical analysis corresponds to the in situ DPBA staining patterns of whole seedlings. Since the aglycone forms are hydrophobic and associated with membranes,
their localization in the TZ is consistent with their role in regulating auxin efflux. Both the flavonoid species present and its localization are important in regulation of auxin distribution.

**Flavonoid Accumulation in tt Mutants**

Flavonoid profiles in the mutants are generally altered according to predictions based on the biochemical pathway, with one exception that indicates that one reaction may be more complex than expected. Naringenin is the intermediate expected to accumulate in *tt*6, but was not detected by any of the methods used here. This result may be explained by a report from Sheahan et al. (1998) that an unidentified orthodihydroxy or orthotrihydroxy flavonoid is, in fact, the predominant flavonoid accumulating in *tt*6. This suggests that naringenin is unstable or is quickly converted into another compound. As the flavonoid biosynthetic pathway is under feedback control (Pelletier et al., 1999), and naringenin itself regulates transcription of genes encoding its biosynthetic enzymes (Pelletier et al., 1999). The accumulation of other flavonoid compounds in *tt*5 and *tt*7 may be the result of altered feedback mechanisms in the mutants and the compounds’ subsequent availability to other enzymes.

Developmental differences were noted in the *tt* mutants compared with wild type, and differences in *tt*4 are discussed in the companion paper by Brown and coworkers (2001). It is now possible to begin to investigate the underlying causes of these developmental defects.

**Localization**

The intermediates and final products of the flavonoid pathway accumulate in the same cells and tissues in the *tt* mutants and wild type, respectively. This result supports the hypothesis that flavonoids are synthesized in the same cells in which they accumulate. Quercetin localization in the plasma membrane at the basal ends of cells is consistent with the basally located AtPIN1 (Galweiler et al., 1998) and NPA-binding protein that Jacobs and Gilbert (1983) described, lending further support that flavonoids are endogenous regulators of auxin transport (Jacobs and Rubery, 1988; Murphy et al., 2000; Brown et al., 2001). Nuclear localization of kaempferol and quercetin, perhaps as derivatives, suggests that they may function as antioxidants or transcriptional regulatory factors, as sulfonated forms of flavonoids have been identified in the nucleus (Grandmaison and Ibrahim, 1996). The subcellular localization of these flavonoids in Arabidopsis is consistent with the membrane association of the enzyme complexes of the flavonoid pathway (Shirley, 1999) and with previous studies (Sheahan and Cheong, 1998; Hutangura et al., 1999).

**Flavonoids and NPA Amidase/Aminopeptidase (AP) Activity Colocalize**

The regions of flavonoid staining in Arabidopsis—cotyledonary node, hypocotyl-root TZ, distal elongation zone and root cap, and bases of root primordia along the vascular tissue, stigmata, flowers, and primordia—are identical to the regions stained in biochemical assays of NPA amidase activity, and Tyr-, Trp-, and Pro-AP activity (Murphy and Taiz, 1999a, 1999b). This, along with the observations that Tyr-, Trp-, and Pro-β-naphthylamide conjugates have NPA-like activity, and that flavonoids inhibit NPA-binding and AP activity, suggests a role for flavonoids and APs in stimulating auxin retention in plant tissues (Murphy et al., 2000). However, biochemical evidence suggests that aglycone flavonoids do not directly bind the catalytic site of the plasma membrane APs (Murphy et al., 2000) and, therefore, modulate AP activity indirectly.
CONCLUSIONS

There is increasing evidence that auxin retention, i.e. the inhibition of auxin transport, is as important as auxin transport itself in determining the steady-state distribution of auxin in the plant. Localized regions of auxin retention can lead to auxin accumulation, which, in turn, may trigger developmental events. flavonoids have long been suspected to be positive regulators of auxin retention despite being less effective at inhibiting auxin transport than artificial inhibitors such as NPA (Jacobs and Rubery, 1988; Faulkner and Rubery, 1992). Membrane-bound aglycone flavonoids specifically colocalize with regions of auxin accumulation in Arabidopsis seedlings and mature plants. Since flavonoid precursors also colocalize to these regions, it appears that they act in the same cells in which they are synthesized. This flavonoid localization suggests that aglycone flavonoids function as autocrine effectors that have a role in auxin distribution.

MATERIALS AND METHODS

Reagents and Seed Stocks

All chemicals were from Sigma (St. Louis). Col-0, Ler, tt3-1(84), tt4(85), tt5-1(86), tt6-1(87), and tt7-1(88) seeds in the Ler background, and fah1-2 seeds were obtained from the Arabidopsis Biological Resource Center at Ohio State University, and the null mutant seeds the Arabidopsis Biological Resource Center at Ohio State University, fah1-2 (Saslowsky et al., 2000). Yellow seeds of (UV118a) were generous gifts from Brenda Winkle-Shirley (UV118a) after 5 min of staining. Flavonoid fluorescence of stained seedlings visually increased up to 15 min, then darkened was observed. Flavonoid fluorescence of flowers was viewed with an epifluorescence microscope.
Firmly by comparison with profiles published by Graham (1991), Burbulis et al. (1996), Sheahan et al. (1998), and Pelletier et al. (1999).

Flavonoid glycosides and aglycones were prepared for analysis from seedling sections as described by Burbulis et al. (1996) and were analyzed by HPLC using a C18 column (4.2 x 250 mm, 5 μm, Supelco, Bellefonte, PA) with a CC 410 pump (Perkin Elmer, Norwalk, CT) with UV absorbance monitoring at 254 nm with a UV/vis absorbance monitor (Spectraflow 773, Kratos, Ramsey, NJ) and fluorescence monitoring with excitation at 367 nm and emission at 515, 520, 527, 534, or 543 nm (Waters 870, Waters, Bedford, MA) to confirm identities of individual peaks. Separation was achieved with a gradient of 2% (v/v) methanol in water (pH 3.0 with H3PO4) and acetonitrile at a flow rate of 1 mL min⁻¹ with a highly concave profile (16 min for unhydrolyzed flavonoids and 30 min for aglycone products of acid hydrolysis). The values were integrated and normalized to the injection of naringenin standard (20 μL of 10 ng μL⁻¹ in MeOH). Results shown do not include quantitation of peaks identified spectrophotometrically as sinapate esters, although sinapate contents of seedlings were consistent with those found in the literature (Sheahan, 1996; Ruegger et al., 1999). Peaks were also analyzed spectrofluorimetrically, and matched previously reported data (Chapple et al., 1992; Sheahan, 1996; Sheahan and Cheong, 1998). Peaks were collected and analyzed spectrophotometrically according to Markham (1982) and Harborne et al. (1975). Molecular weights of flavonoid skeletons of individual peaks were identified by electrospray + MS, as previously described (Murphy et al., 1999a).

Flavonoid and Anthocyanin Content of Inflorescence Stems

Inflorescence stems were divided into 2-mm sections. Flavonoids were extracted and analyzed by HPLC as above. Anthocyanins were extracted and analyzed by spectrophotometry (Adamse et al., 1989).

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LITERATURE CITED


Koornneef M (1990) Mutations affecting the testa color in Arabidopsis. Arabidopsis Inf Serv 28: 1–4


Marigo G, Boudet AM (1977) Relations polyphenols croissance: mise en évidence d’un effect inhibiteur des com-
posés phénoliques sur le transport polarisé de l’auxine. Physiol Plant 41: 197–202


Murphy A, Taiz L (1999a) Naphthylphthalamic acid is enzymatically hydrolyzed at the hypocotyl-root transition zone and other tissues in Arabidopsis thaliana seedlings. Plant Physiol Biochem 37: 413–430


Stafford H (1990) Flavonoid Metabolism. CRC Press, Boca Raton, FL

