Localizacion of Flavonoids in the Yellow Lupin Seedlings and Their UV-B-absorbing Potential

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Quantification of the flavonoids in yellow lupin (Lupinus luteus; Leguminosae) seedlings revealed that a flavone glucoside, 7-O-β-(2-O-β-rhamnosyl)glucosyl-4’5,7-trihydroxyflavone (apigenine 7-O-β-neohesperidoside), is rich in the epicotyl and cotyledon. In hypocotyls and roots, 8-C-β-glucosyl-4’5,7-trihydroxyisoflavone (genistein 8-C-β-glucoside) was a predominant flavonoid constituent. The roles of the localized flavonoids are briefly discussed relating to defense against biotic and abiotic external stresses.

Introduction

Numerous isoflavones are known to occur in several lupins (Fukui et al., 1973; Harborne et al., 1976; Ingham et al., 1983; Hashidoko et al., 1986; Tahara et al., 1990; Shibuya et al., 1991). In addition, other classes of flavonoids, 3-O-methylflavonols and prenylated dihydroflavones, were found in Lupinus luteus (Tahara et al., 1987 and 1994). As a part of flavonoid dynamism of lupins, we have determined the localization of flavonoid constituents in the seedlings of L. luteus cv. Topaz grown under different light conditions (Katagiri et al., 2001).

Constituents 1 and 2 isolated and identified by spectroscopic methods (MS, UV, and 1H and 13C NMR analyses) have already been reported for L. luteus. The former C-glucoside (Zapesochnaya and Laman, 1977) was the predominant flavonoid in root and hypocotyls, while the latter flavone glucoside localized in the aerial part has been estimated by mass spectroscopic techniques (GC-MS and MS/MS) (Franski et al., 1999). In the present study, 2D NMR techniques were applied to unambiguously assign 13C NMR signals, because there are some discrepancies in the carbon assignment of 2 from different sources (Rao and Rao, 1982; Stein and Zinsmeister, 1990).

Results and Discussion

HPLC analyses of alkaline hydrolyzed MeOH extracts of the separated organs (root, cotyledon, epicotyl, and hypocotyls) from 7-day-old lupin seedlings grown under a 16 h light/8 h dark regime revealed the presence of two major flavonoids, 1 and 2 (Fig. 1), which are differently localized in the organs (Katagiri et al., 2000 and 2001). Apigenin 7-O-β-neohesperidoside (2) was localized and accumulated in the aerial parts, particularly in younger epicotyls (Table 1).

Extracts were kept at 40°C for 2 h in 0.4 N aqueous ammonia to hydrolyze acylated flavonoid glycosides, because HPLC performance of malonylated glycosides was not efficient under the current HPLC conditions. After this alkaline treatment of MeOH extracts from roots and hypocotyls, the peak of genistein 7-O-β-glucoside (3, tR = 12.3 min) increased significantly. This result suggested that a major part of genistein 7-O-β-glucoside (3) detected is originally present as an ester form of 3, probably malonylated at 6"-position (Shibuya et al., 1991; Katagiri et al., 2000). In contrast, neither genistein 8-C-β-glucoside (1) nor apigenin 7-O-β-neohesperidoside (2) showed any increase of peak intensity after the alkaline treatment.

It is well known that flavonoid complexes in green leaves are resistant agents against ultraviolet-B (UV-B, 280–315 nm radiation) (Harborne and Williams, 2000; Ryan et al., 2001). Therefore, the remarkable accumulation of apigenin 7-O-β-neohesperidoside (2) in epicotyls and cotyledons of the seedlings seems most reasonable. A relative UV-B absorption value of a methanol extract from each organ (v/fr.-w) was high in the order of epicotyls,
A significant amount of apigenin 7-O-β-neohesperidoside (2) was also detected in cotyledons of etiolated 4-day-old seedlings (Table III). Under irradiation with continuous light for 48 h, the content of flavone glycosides and isoflavone glucosides was not increased in the intact cotyledons of etiolated seedlings, but enhanced in the cotyledons excised from the stem. This result suggested that in the intact cotyledon flavonoid glycosides biosynthesized under a 24 h-light regime are transported rapidly into other organs. Incidentally, genistein 8-C-β-glucoside (1) in the excised and intact cotyledons had approx. the same concentration, so that compound 1 likely plays a topical role in an early stage of the seedling. In yellow lupin seedlings, prenylated isoflavones, luteone and wightone, are known to be the major constitutive and inducible antifungal substances when the tissues had been stressed (Harborne et al., 1976; Ingham et al., 1983), and these antifungal isoflavones were also present in excised cotyledons.

Biosynthetic pathways of flavonoids have been well studied (Heller and Forkmann, 1994), and flavones and isoflavones branch off from the common intermediate dihydroflavone, naringenin (or liquiritigenin). However, the regulation mechanism of biosynthesis and transportation of flavonoid in plant tissues has little been studied. The roots of yellow lupin grown up in sunlight accumulated prenylated naringenins and their derivatives together with large amounts of isoflavones (Tahara et al., 1994). However, no naringenin or any of its derivative (glycosides, complex flavanones...
Table I. Contents of the flavonoids in yellow lupin.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age (day)</th>
<th>4</th>
<th>7</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicotyl</td>
<td>0.03</td>
<td>0.14</td>
<td>0.22</td>
<td>15.1</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>0.38</td>
<td>0.50</td>
<td>0.30</td>
<td>1.8</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>0.32</td>
<td>0.38</td>
<td>0.37</td>
<td>1.5</td>
</tr>
<tr>
<td>Root</td>
<td>0.14</td>
<td>0.17</td>
<td>0.20</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table II. UV-B protection potential of yellow lupin tissues from greenish and etiolated seedlings.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fresh wt.*</th>
<th>UV-B absorbing ratio**</th>
<th>Fresh wt.* UV-B abs. Ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicotyl</td>
<td>1.12</td>
<td>3.67</td>
<td>2.98</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>5.69</td>
<td>4.08</td>
<td>1.00**</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>2.34</td>
<td>2.03</td>
<td>0.39</td>
</tr>
<tr>
<td>Root</td>
<td>2.15</td>
<td>3.05</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* 10 seedlings.  
** Integrated OD value from 280 to 315 nm for the methanol extract corresponding to 100 mg of fresh lupin tissues was measured. The integrated OD value of the cotyledon described above was determined as 1.00, relative UV-B absorbing ratio of the other tissues or every tissue from seedlings of 15 days-grown under 16 h-light and of 7 days-grown in the dark were as shown here.  
*** _; not determined.

and so on) was detected in our analyses. Instead of dihydroflavone derivatives, large amounts of a flavone glycoside, apigenin 7-O-β-neohesperidoside (2) were found, particularly in the cotyledons and epicotyls. Thus, yellow lupin seedlings that show topical accumulation of different types of flavonoids in each organ are likely a good plant material to study the regulation system of flavonoid biosynthesis, including switching on and off key pathways.

**Experimental**

*Plant materials*  
The seeds of *Lupinus luteus* cv. Topaz were soaked in running tap water for 48 h and germinated at 23 °C in 16 h-light (10 klux)/8 h-dark or 24 h in the dark in a vermiculite layer in plastic boxes for certain periods. The harvested seedlings were divided into roots, hypocotyls, cotyledons and epicotyls, and these organs were subjected to
qualitative and quantitative analyses of flavonoids and UV-B (280–315 nm) absorption scanning.

Isolation of glycosyl flavonoids.

After being chopped, yellow lupin seedlings (ca. 50 g) were extracted twice, at first with MeOH (200 ml) and then with 80% MeOH (200 ml × 2). The combined extracts were filtrated, concentrated to remove MeOH and diluted four times with water. The diluted solution applied to a porous polystyrene-gel column (30 ml bed volume) pre-equilibrated with water, and the column was washed subsequently with 16%, 32%, 54% and 100% MeOH. Two flavonoid glycosides eluted with 54% MeOH were isolated by prep. TLC in 100% MeOH. Two flavonoid glycosides eluted subsequently with 16%, 32%, 54% and 100% MeOH. Two flavonoid glycosides eluted with 54% MeOH were isolated by prep. TLC in CHCl₃-Me₂CO-MeOH-water (= 6:3:4:1, v/v, Rf 0.60 for CHCl₃-Me₂CO-MeOH-water (= 6:3:4:1, v/v, Rf 0.60 for 1 and 0.49 for 2). Analytical and preparative TLC were performed as described in our earlier papers (Hashidoko et al., 1986; Tahara et al., 1990).

Isolated flavonoid glycosides applied to spectroscopic analyses were identified as genistein 8-C-β-glucoside (1) and apigenin 7-O-β-neohesperidoside (2) by comparison with literature data (the former, Zapesochnyaya and Laman, 1977; and the latter, Rao and Rao, 1982; Stein and Zinsmeister, 1990). Position of the rhamnosylation on the β-glucosyl moiety of 2 was unambiguously assigned at C”-2 with following process: in the HMBC (in DMSO-d₆) as shown below, an anomeric carbon of C-1” (100.3) indicated a cross peak with a proton signal assignable as H-2” at ca. 3.50. Because signals of methine protons also overlapped in this region, we carried out a peracetylation of compound 2 in acetic anhydride/pyridine and measured its HH-COSY spectrum (in CD₃OD). Signals of the methine protons at hydroxylated carbons on the sugar moieties showed a downfield shift due to acetylation of the hydroxyl groups, so that the proton signal of rhamnosylated methine carbon remained upfield together with two signals assignable to H-5” and H-5”. In the HH-COSY proton signal at δ 3.96 was assignable as H-2” on the peracetylated derivative of 2, due to its vicinal coupling (by J = 7.6 Hz) with signal (δ 5.25) of an 1”-anomeric proton at the glucosyl moiety.

2-D NMR analyses of apigenin 7-O-β-neohesperidoside (2)

1H and 13C NMR data in DMSO-d₆ were assigned by 2D NMR experiments (HMBC and HMQC) by using a Bruker AMX500 (1H: 500 MHz and 13C:125 MHz).

13C NMR: carbon(s), δ ppm, (class: p, primary; s, secondary; t, tertiary; and q, quaternary), (HMBC correlation from shown proton(s)): C-2, 164.0 (q) (→ H-3, and H-2’ and 6’); C-3, 103.0 (t); C-4, 181.7 (q) (→ H-3), C-5, 160.9 (q) (→ OH-5 and H-6); C-6, 99.2 (t) (→ OH-5 and H-8); C-7, 162.3 (q) (→ H-1”, H-6 and H-8); C-8, 94.4 (t) (→ H-6); C-9, 156.8 (q) (→ H-8); C-10, 105.3 (q) (→ OH-5, H-6 and H-8); C-11, 120.7 (q) (→ H-3, and H-3’ and 5’); C-12, 184.8 (t) (H-6’ and H-2’); C-13, 151.9 (t) (→ H-5’ and H-3’); C-14, 163.1 (q) (→ H-2’ and 6’); C-15, 97.7 (t) (→ H-2’); C-16, 76.2 (t) (→ H-1”); C-17, 77.1 or 76.9 (t); C-18, 69.6 (t); C-19, 76.9 or 77.1 (t); C-20, 60.4 (s); C-1”, 100.3 (t) (→ H-2”); C-2”, 70.4 (t) (→ H-1” and H-4”); C-3”, 70.5 (t); C-4”, 71.8 (t) (→ H-6”); C-5”, 68.3 (t) (→ H-4” and H-1”); C-6”, 18.1 (p) (→ H-4”).

Table III. Effect of light irradiation on flavonoid contents in excised and intact cotyledons from yellow lupin seedlings etiolated for 4 days.

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Excised*</th>
<th>Intact**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
<td>Dark</td>
</tr>
<tr>
<td>Isoflavones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein 8-C-β-glucoside</td>
<td>0.27</td>
<td>0.31</td>
</tr>
<tr>
<td>2’-Hydroxyisoflavin 7-O-β-glucoside</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Genistein 7-O-β-glucoside</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>2’-Hydroxyisoflavin</td>
<td>0.36</td>
<td>0.09</td>
</tr>
<tr>
<td>Luteone</td>
<td>0.01</td>
<td>0.25</td>
</tr>
<tr>
<td>Wighteone</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td>Flavone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin 7-O-β-neohesperidoside</td>
<td>0.70</td>
<td>0.67</td>
</tr>
<tr>
<td>Total identified flavonoids</td>
<td>1.50</td>
<td>1.50</td>
</tr>
</tbody>
</table>

* Cotyledons separated from the seedling stem.
** Intact cotyledons on the seedling stem.
\(^1\)H NMR: proton(s), \(\delta\) ppm, coupling pattern(s, singlet; d, doublet; and t, triplet), coupling constant(s) Hz: H-3, 6.852, s; OH-5, 12.957, s; H-6, 6.354, d, \(J = 2.0\) Hz; H-2', 7.924, d-like, \(J = 8.8\) Hz; H-3' and 5', 6.927, d-like, \(J = 8.8\) Hz; H-1", 5.215, d, \(J = 7.3\) Hz; H-2", ca. 3.50, t-like, ca. 7 Hz; H-3"/H11032 and 6"/H11032, 7.924, d-like, \(J = 8.8\) Hz; H-3"/H11032, H-4"/H11032 and H-5"/H11032, overlapped around 3.2–3.5 ppm; H2"–6", ca. 3.5 and 3.8; H-1", 5.114, s-like; H-2"", H-3"", H-4"", around 3.7, 3.3, 3.21; H-5"", ca. 3.74; H3–6", 1.186, d, \(J = 6.2\) Hz.

**Quantification of yellow lupin flavonoids**

Small amounts of each organ (ca. 2.5 g) were sliced and put into 10 ml of MeOH including 2-phenylchromone as the internal standard and homogenized with a Polytron at 4 °C. The homogenate was centrifuged at 3,000 \(\times\) g for 15 min at 4 °C. The resulting supernatant was cleaned up with a disposable column cartridge Bond Elut (C18, 200 mg/3 ml tube) and then analyzed with HPLC according to our previous methods for measuring isoflavone constituents of white lupin (Katagiri et al., 2000). Under the same HPLC condition, two glycosylflavonoids genistein 8-C-\(\beta\)-glucoside (1) and apigenin 7-\(\beta\)-neohesperidoside (2), which are not observed in MeOH extract from white lupin, were separately eluated at Rt 11.3 and 13.5 min, together with genistein 7-\(\beta\)-glucoside (3), respectively (Fig. 2). To determine the concentration of these compounds in each organ, relative peak areas toward that of the internal standard 2-phenylchromone were recorded at 263 nm. The factors to calibrate for quantification were 2.40 and 0.89 for genistein 8-C-\(\beta\)-glucoside (1) and apigenin 7-\(\beta\)-neohesperidoside (2), respectively. The calibration curves were linear in a range between 0.41 and 8.25 nmol under the present conditions. The factors for other compounds are shown by Katagiri et al. (2000). In order to hydrolyze acylated flavonoid glycosides in MeOH extracts [mainly genistein 7-\(\beta\)-(6"-malonyl)glucoside], each extract was kept at 40 °C for 2 h in 0.4 M aqueous ammonia and the resulting hydrolysate was analyzed as usual (Fig. 2).

**UV scanning analysis.**

Each organ was sliced and put into MeOH (4 ml per 1 g-f.w.) and homogenized by a Polytron at 4 °C. The first homogenate was centrifuged at 3,000 \(\times\) g for 15 min at 4 °C. The precipitates were re-extracted twice with 80% MeOH. The supernatants thus obtained by centrifugation were combined. A portion of the combined extract equivalent to 0.1 g of fresh plant material was adjusted to 25 ml with 80% MeOH. The diluted extract was subjected to a Hitachi U-3210 spectrophotometer using 10 mm of quartz cuvettes and absorbance of UV-B was automatically integrated in a range of 280 to 315 nm.

**Acknowledgment**

We thank Mr K. Watanabe of our graduate school, for FI-MS analysis.


