

## Two novel disaccharides, rutinose and methylrutinose, are involved in carbon metabolism in *Datisca glomerata*

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**Abstract** *Datisca glomerata* forms nitrogen-fixing root nodules in symbiosis with soil actinomycetes from the genus *Frankia*. Analysis of sugars in roots, nodules and leaves of *D. glomerata* revealed the presence of two novel compounds that were identified as  $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-D-glucose (rutinose) and  $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-1-*O*- $\beta$ -D-methylglucose (methylrutinose). Rutinose has been found previously as a/the glycoside part of several flavonoid glycosides, e.g. rutin, also of datiscin, the main flavonoid of *Datisca cannabina*, but had not been reported as free sugar. Time course analyses suggest that both rutinose and methylrutinose might play a role in transient carbon storage in sink organs and, to a lesser extent, in

source leaves. Their concentrations show that they can accumulate in the vacuole. Rutinose, but not methylrutinose, was accepted as a substrate by the tonoplast disaccharide transporter SUT4 from Arabidopsis. In vivo  $^{14}\text{C}$ -labeling and the study of uptake of exogenous sucrose and rutinose from the leaf apoplast showed that neither rutinose nor methylrutinose appreciably participate in phloem translocation of carbon from source to sink organs, despite rutinose being found in the apoplast at significant levels. A model for sugar metabolism in *D. glomerata* is presented.

**Keywords** Apoplastic phloem loading · Carbon storage · Disaccharides · Methylrutinose · Rutinose · Sucrose transporter

### Abbreviations

MS	Mass spectroscopy
DCI	Direct chemical ionization
ESI	Electron spray ionization
NMR	Nuclear magnetic resonance
OAc	Acetyl
SE/CC	Sieve element/companion cell complex
MDH	Malate dehydrogenase
TEM	Transmission electron microscopy

### Introduction

Only a small number of freely occurring disaccharides are known to exist in plants. Sucrose is the most abundant one, trehalose is found in smaller quantities in several plant species and small quantities of gentiobiose have been found in, e.g., tomato fruits (Dumville and Fry 2003), but otherwise free disaccharides are associated with the degradation of complex glycosides, like maltose, which occurs during

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degradation of starch and melibiose, which occurs during degradation of  $\alpha$ -galactosylsucrose storage oligosaccharides (Avigad 1982). A major part of carbon produced during photosynthesis is channelled into the biosynthesis of sucrose that serves as the transport carbohydrate in most plants (Zimmermann and Ziegler 1975; Giaquinta 1983). Sucrose can also be stored at high concentrations in sink organs (Saftner et al. 1983). Some plants transport other carbohydrates in addition to sucrose. These are typically sugar alcohols (sorbitol, mannitol, dulcitol, myoinositol and volemitol) and oligosaccharides based on sucrose, namely the raffinose oligosaccharide series (e.g., raffinose, stachyose and verbascose) or fructans (Zimmermann and Ziegler 1975; Häfliger et al. 1999; Wang and Nobel 1998). Transport carbohydrates enter the phloem at the minor veins, the endings of the leaf vascular network that are in close contact with the mesophyll cells (Esau 1967). The anatomy of phloem companion cells in minor veins indicates the phloem loading mechanism. Numerous branched plasmodesmata connect companion cells with the bundle sheath cells in symplastic phloem loaders, making entire symplastic transfer of sugars from mesophyll into the phloem possible, while in apoplastic phloem loaders these connections are scarce (Gamalei 1984; Turgeon et al. 1993). The companion cells of evolved apoplastic phloem loaders tend to show transfer cell anatomy, i.e. an increase in plasma membrane surface by cell wall ingrowths (Pate and Gunning 1969). Symplastic phloem loading is usually correlated with the presence of high amounts of non-sucrose sugars (members of the raffinose oligosaccharide family or sugar alcohols) in the phloem, while apoplastic phloem loaders translocate almost exclusively sucrose, which is loaded from the apoplast into the phloem via sucrose-proton symporters (Zimmermann and Ziegler 1975; Gamalei 1984; Sauer and Stolz 1994).

Here, we describe the identification of two novel disaccharides,  $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-D-glucose (rutinose) and  $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-1-O- $\beta$ -D-methylglucose (methylrutinose), in a dicotyledonous plant *Datisca glomerata*. *D. glomerata* is an actinorhizal plant, i.e., it can form root nodules that host nitrogen-fixing actinomycetes from the genus *Frankia*. Carbon and nitrogen partitioning have been only superficially studied in actinorhizal species. The Datisceae family is represented by two perennial species, *D. glomerata* that occurs in riparian habitats throughout Baja California, Mexico and CA, USA and *D. cannabina* that is found along streams and rocky hillsides in the eastern Mediterranean region and the Himalayas (Davidson 1973). In the present study, we show that rutinose and methylrutinose are involved in transient carbon storage in *D. glomerata*. We also explore the possibility that these disaccharides are involved in long-distance carbon transport in this plant.

## Materials and methods

### Plant material

*Datisca glomerata* (Presl.) Baill seeds were obtained from plants in Vaca Hills, CA, USA and germinated in a greenhouse on soil (T 25 Frühstorfer Erde; Archut, Lauterbach-Wallenrod, Germany) mixed with one-third volume of sand. After 2–3 months, plantlets were transferred to an aerated hydroponic system containing one-fourth strength Hoagland's solution (Hoagland and Arnon 1938). For nodulation, N-free one-fourth strength Hoagland's solution was used and plants were infected with crushed nodules upon transfer to the hydroponic system in a greenhouse with 16 h light (ca. 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). For time course experiments, plants in hydroponic culture were transferred to a growth chamber with 23°C and a fixed light–dark regimen (8 h dark, 16 h light or 10 h dark, 14 h light, respectively; ca. 350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Plants grown at 16 h light received 5 mM  $\text{KNO}_3$  each week, while plants receiving 14 h light were subjected to N limitation. This was achieved by the omission of replenishment of the  $\text{KNO}_3$  in the growth medium of these plants. After 3 weeks both N-supplied and N-limited plants were sampled for carbohydrate analysis. Samples were taken 30 min before the end of the dark or light phase, respectively. Plants for experiments with in vivo  $^{14}\text{C}$  labeling were grown on pot soil in a greenhouse. Leaves of *Datisca cannabina*, *Coriaria japonica*, *Begonia kellermannii*, *Cercocarpus betuloides*, *Ceanothus* sp., *Colletia cruciata*, *Dryas drummondii* and *Dryas octopetala* were harvested in the local botanical garden.

### Sugar analysis

Plant organs were frozen in liquid nitrogen, stored at  $-80^\circ\text{C}$ , ground in liquid nitrogen and extracted with 5 ml of a chloroform/methanol mixture (1.5/3.5; v/v). After incubation on ice for 30 min, the mixture was extracted twice with 3 ml of double-distilled  $\text{H}_2\text{O}$  each. The aqueous phases were combined and dried completely in a rotary evaporator at 37°C. The dried residues were dissolved in ultra pure  $\text{H}_2\text{O}$  (Millipore, Schwalbach, Germany), filtered through a syringe with a cellulose-nitrate membrane (0.45  $\mu\text{m}$ ; Schleicher and Schuell, Dassel, Germany) and used for HPLC analysis. Sugars were separated over an anion exchange column MA1 (CarboPAC10; Dionex Corp., Sunnyvale, CA, USA) with a pre-column (CarboPAC10 Guard; Dionex Corp.) and eluted with 600 mM NaOH (Malinckrodt Baker BV, Deventer, The Netherlands) using an LC-9A pump from Shimadzu (Kyoto, Japan), with a flow rate of 0.4  $\text{ml min}^{-1}$ . The autosampler (#2157; LKB/Pharmacia, St. Albans, UK) was thermostated at 12°C. Sugars were detected by a thin layer amperometric

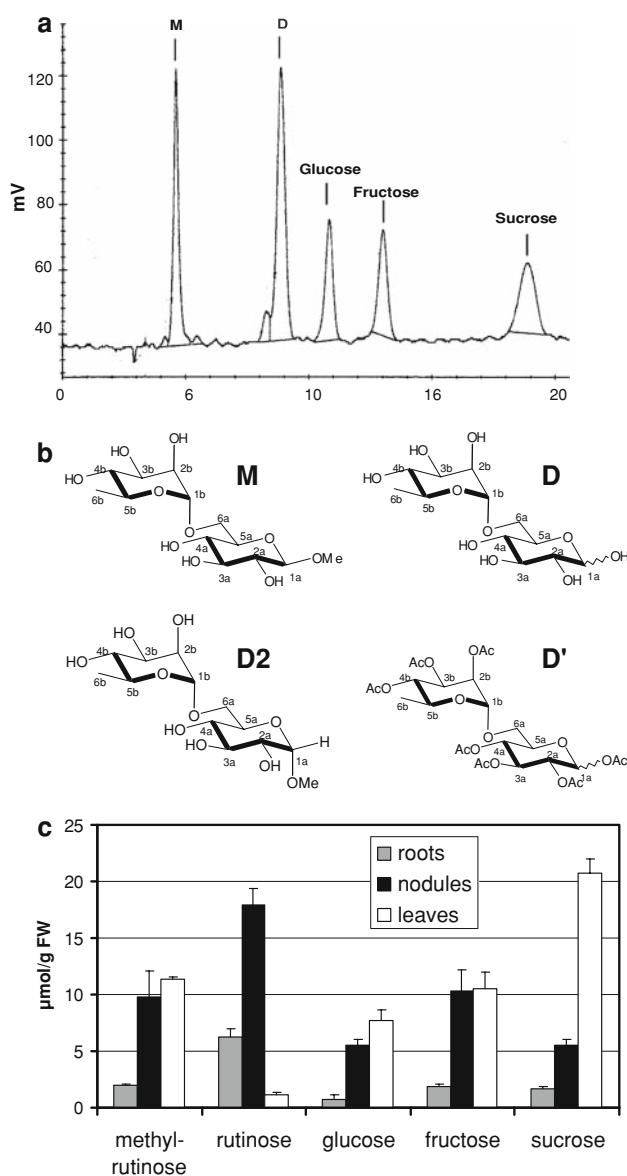
cell (Model 5200, ESA, Chelmsford, MA, USA) with a gold electrode using a pulse amperometric detector (Coulchem II, ESA). Chromatograms were evaluated using the program Peaknet 5.1 (Dionex, Idstein, Germany).

Preparative isolation of two unknown compounds from chloroform/methanol extracts of nodulated *D. glomerata* root systems

The method of Pharr et al. (1987) was used with some modifications. 150 g of *D. glomerata* was ground to a fine powder in liquid nitrogen and extracted in 1.5 l of a chloroform/methanol mixture as described above. 900 ml of double-distilled H<sub>2</sub>O were added and phases were separated by centrifugation for 20 min at 11,600g. The aqueous phase was saved and the organic phase was again extracted with 900 ml H<sub>2</sub>O. The combined aqueous phases were concentrated to 100 ml in a rotary evaporator at 37°C. 20 g of cation exchanger Dowex 50Wx8 (200–400 mesh, in H<sup>+</sup>-Form; Serva, Heidelberg, Germany) were added and stirred for 30 min. Ca. 10 g of Polyclar AT (Serva) was added to the filtered extract and stirred for 30 min before pelleting in a tabletop centrifuge. The supernatant was concentrated to 10 ml in a rotary evaporator at 37°C and applied to an anion exchange column filled with Dowex 1 × 8 (100–200 mesh, OH<sup>-</sup> form; Serva). Elution was done with 0.2 M NaOH at a rate of 2.5 ml/min, and sugar composition of the fractions was determined by HPLC. The fractions containing D and M, respectively, were combined and neutralized using 5 M HCl and concentrated to 10 ml in a rotary evaporator at 37°C. NaCl was separated from the sugars by descendent paper chromatography on Whatman Chr 17 paper (Whatman, Maidstone, UK) in n-propanol/ethyl acetate/H<sub>2</sub>O (7:1:2, by vol.). Sugars were stained on cut off strips in detection solution (2.5 ml of saturated AgNO<sub>3</sub> in 500 ml acetone) and eluted from the chromatogram in double-distilled H<sub>2</sub>O in an ultrasonication bath for 20 min (Sonifier B15, Branson Ultrasonics, Geneva, Switzerland) three successive times. The eluates were combined and concentrated in a rotary evaporator at 37°C. Aliquots were dried in a Speed Vac concentrator and used for analysis.

Determination of the structures of the isolated compounds D and M

After isolation of the compounds **M** and **D** their structure (Fig. 1b) was determined by applying NMR and MS. To find out the connecting points of the monosaccharide units of **M** and **D**, acyl protection of all hydroxy groups was performed with an 1:2 mixture of acetic acid anhydride (Ac<sub>2</sub>O), pyridine and catalytic amounts of 4-(dimethylamino)pyridine (DMAP). After introduction of the acyl



**Fig. 1** Identification and distribution of rutinose and methylrutinose. **a** A sugar HPLC chromatogram of a nodule extract showing two peaks (labeled with “D” and “M”, respectively) that could not be identified via comparison with standards. **b** Chemical structure of rutinose (**D**) and methylrutinose (**M**). The product of the chemical acetylation of **D** (**D'**) showed that the connection between the monosaccharides was 1b (rhamnose) to 6a (glucose). Chemical methylation of rutinose led to a  $\alpha$ -glycosidic bond in the 1a position (**D2**), in contrast to the  $\beta$ -glycosidic bond in the natural substance (**M**). **c** Sugar contents of roots (gray bars), nodules (black bars) and leaves (white bars) of greenhouse-grown *D. glomerata* (150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Average values from three parallel greenhouse-grown series (150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) are shown

protecting groups, the connecting points were identified by applying the NMR correlation spectroscopy methods H,H-COSY and C,H-COSY. After acylation, all secondary hydroxy groups of **M** and **D** at positions 2a/b to 4a/b showed a chemical shift around  $\delta = 5.0$  which is the

expected value for secondary acylated hydroxy groups (Jung et al. 1989). The corresponding 6a protons showed a shift about  $\delta = 3.7$ . These results confirm that the connection between the monosaccharides is 1b-6a.

The  $\alpha$ -glycosidic linkage at 1b of **M** and **D** and the  $\beta$ -glycosidic linkage at 1a of structure **M** were also confirmed by applying H,H-COSY and C,H-COSY spectroscopy after the introduction of the acyl protecting groups. For the proton at position 1b of **M'** and **D'**, we found a Singulett which is typical for an  $\alpha$ -glycosidic linkage. For the proton at 1a of compound **M'** we found a Duplett with a coupling constant of 8.0 Hz which is also the expected value for a  $\beta$ -glycosidic linkage.

The molecular mass of the shown unprotected natural products **M** and **D** were confirmed by applying the electron spray ionization method (ESI) and the direct chemical ionization method (DCI). For **D**, we found 349.5 g/mol which is the molecular weight plus  $\text{Na}^+$  (ESI) and for **M**, we found 358 g/mol which is the molecular weight plus  $\text{NH}_4^+$  (DCI).

Chemical identification of **D** and **M** as rutinose and methylrutinose

$^1\text{H-NMR}$ -Spectra were taken with Varian (Palo Alto, CA, USA) spectrographs XL-200 (200 MHz), VXR-200 (200 MHz), Unity 300 (300 MHz), INOVA A 500 (500 MHz) and UNITY INOVA-600 (600 MHz) and Bruker (Karlsruhe, Germany) spectrograph AMX-300 (300 MHz). Chemical shifts are given in  $\delta$ -scale units. Internal standard was tetramethyl silane (TMS) ( $\delta_{\text{TMS}} = 0.00$  ppm) or chloroform ( $\delta_{\text{CHCl}_3} = 7.24$  ppm). Coupling constants are given in Herz (Hz).  $^{13}\text{C-NMR}$ -spectra were taken with Varian models XL-200 (50 MHz), VXR-200 (50 MHz), Unity 300 (75 MHz), INOVA A 500 (125 MHz) und UNITY INOVA-600 (150 MHz) and Bruker spectrograph AMX-300 (75 MHz). Internal standards were tetramethyl silane ( $\delta_{\text{TMS}} = 0.00$  ppm) or deuteriochloroform ( $\delta_{\text{CDCl}_3} = 77.00$  ppm). Chemical shifts were reported on the  $\delta$  scale relative to  $\text{CDCl}_3$  or TMS as an internal standard. Mass spectra were taken with MAT95 (Finnigan Corp., San Jose, CA, USA). Thin-layer chromatography (TLC) plates SIL G/UV<sub>254</sub> (0.25 mm) were obtained from Machery-Nagel (Düren, Germany). Silica gel for column chromatography was obtained from Machery-Nagel (particle size 40–63  $\mu\text{m}$ ).

*Acetyl O-(2,3,4-tris-O-acetyl- $\alpha$ -L-rhamnopyranosyl)-(1  $\rightarrow$  6)-2,3,4-tris-O-acetyl- $\alpha,\beta$ -D-glucopyranoside (D')*

To a solution of **D** (10.0 mg, 30.7  $\mu\text{mol}$ ) in a 2:1 mixture of pyridine and  $\text{Ac}_2\text{O}$  (5.0 ml), a catalytic amount of DMAP was added, the mixture was stirred for 18 h at room temper-

ature, washed with ice water (25 ml) and extracted with  $\text{CH}_2\text{Cl}_2$  (50 ml). The organic layer was washed successively with 1 N HCl, saturated  $\text{NaHCO}_3$  solution, water and brine, dried over  $\text{MgSO}_4$  and concentrated in vacuum. The residue was purified by column chromatography (pentane/EtOAc 1:10) to give **D'** (2.40 mg, 10.4%):  $R_f = 0.68$  (pentane/EtOAc 1:10).

$^1\text{H-NMR}$  (600 MHz,  $\text{CDCl}_3$ ,  $\alpha$ - :  $\beta$ -anomers, 1:1 mixture)

$\delta = 1.18$  (d,  $J = 6.0$  Hz, 3 H,  $\text{CH}_3$ ), 1.98–2.22 (10  $\times$  s, 21 H, 7  $\times$  OAc), 3.52 (dd,  $J = 11.3$ , 6.5 Hz, 0.5 H, 6a- $\text{H}_a$ ,  $\alpha$ -anomers), 3.56 (dd,  $J = 11.3$ , 6.5 Hz, 0.5 H, 6a- $\text{H}_a$ ,  $\beta$ -anomers), 3.72 (m<sub>c</sub>, 1H, 6a- $\text{H}_b$ ), 3.84 (m<sub>c</sub>, 1.5 H, 5a-H,  $\beta$ -anomers, 5b-H), 4.07 (m<sub>c</sub>, 0.5 H, 5a-H,  $\alpha$ -anomers), 4.68 (2  $\times$  s, 1 H, 1b-H), 5.04 (m<sub>c</sub>, 2 H, 2a-H,  $\alpha$ -anomers, 4a-H,  $\beta$ -anomers, 4b-H), 5.09 (m<sub>c</sub>, 1 H, 2a-H,  $\beta$ -anomers, 4a-H,  $\alpha$ -anomers), 5.20 (m<sub>c</sub>, 2.5 H, 3a-H,  $\beta$ -anomers, 2b-H, 3b-H), 5.43 (t,  $J = 9.4$  Hz, 0.5 H, 3a-H,  $\alpha$ -anomers), 5.64 (d,  $J = 9.4$  Hz, 0.5 H, 1a-H,  $\beta$ -anomers), 6.28 (d,  $J = 4.2$  Hz, 1a-H,  $\alpha$ -anomers).

$^{13}\text{C-NMR}$  (150 MHz,  $\text{CDCl}_3$ ,  $\alpha$ - :  $\beta$ -anomers, 1:1 mixture)

$\delta = 17.2$  (C-6b), 20.8 (7  $\times$  C(O) $\text{CH}_3$ ), 66.3, 66.5, 66.6, 66.8 (C-6a,  $\alpha$ -anomers +  $\beta$ -anomers, C-5b), 68.6, 68.8, 68.9 69.2, 69.4, 69.6, 69.7, 70.2, 70.9, 71.0, 72.7, 73.8 (C-5a,  $\alpha$ -anomers +  $\beta$ -anomers, C-2a,  $\alpha$ -anomers +  $\beta$ -anomers, C-4a,  $\alpha$ -anomers +  $\beta$ -anomers, C2b, C3b, C4b, C-3a,  $\alpha$ -anomers +  $\beta$ -anomers), 88.93 (C-1a,  $\alpha$ -anomers), 91.61 (C-1a,  $\beta$ -anomers), 98.0, 98.3 (C-1b,  $\alpha$ -anomers +  $\beta$ -anomers), 168.8, 169.2, 169.4, 169.6, 169.8, 169.9, 170.0, 170.1, 170.3 (7  $\times$  C(O) $\text{CH}_3$ ).

*$\alpha$ -L-Rhamnopyranoside-(1  $\rightarrow$  6)-glucose (D)*

MS (ESI):  $m/z$  (%) 349.5 (100) [ $\text{M} + \text{Na}$ ] $^+$ ;  $\text{C}_{12}\text{H}_{22}\text{O}_{10}$  (326.13)

*MethylO-(2,3,4-tris-O-acetyl- $\alpha$ -L-rhamnopyranosyl)-(1  $\rightarrow$  6)-2,3,4-tris-O-acetyl- $\beta$ -D-glucopyranoside (M')*

To a solution of **M** (10.0 mg, 29.4  $\mu\text{mol}$ ) in a 2:1 mixture of pyridine and  $\text{Ac}_2\text{O}$  (5.0 ml) a catalytic amount of DMAP was added, the mixture was stirred for 18 h at room temperature, washed with ice water (25 ml) and extracted with  $\text{CH}_2\text{Cl}_2$  (50 ml). The organic layer was washed successively with HCl (1 N), saturated  $\text{NaHCO}_3$  solution, water and brine, dried over  $\text{MgSO}_4$  and concentrated in vacuum. The residue was purified by column chromatography (pentane/EtOAc 1:10) to give **M'** (7.40 mg, 34.7%):  $R_f = 0.72$  (pentane/EtOAc 1:10).



<sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>)

$\delta$  = 1.18 (d,  $J$  = 6.0 Hz, 3 H, CH<sub>3</sub>), 1.89, 1.92, 2.01, 2.12 (4 × s, 6 × OAc), 3.42 (s, 3 H, OMe), 3.62 (m<sub>c</sub>, 3 H, 6a-H<sub>2</sub>, 5a-H), 3.88 (m<sub>c</sub>, 1 H, 5b-H) 4.08 (dq,  $J$  = 6.0 Hz, 9.8 Hz), 4.4 (d,  $J$  = 8.1 Hz, 1 H, 1a-H), 4.78 (s, 1 H, 1b-H), 4.91 (t,  $J$  = 9.8 Hz, 2 H, 2a-H, 4a-H), 5.03 (t,  $J$  = 9.8 Hz, 1 H, 4b-H), 5.18 (t,  $J$  = 9.8 Hz, 1 H, 3a-H), 5.22 (m<sub>c</sub>, 2 H, 2b-H, 3b-H).

<sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>)

$\delta$  = 17.2 (C-5b), 20.57, 20.60, 20.67, 20.77, 20.82 (4 × s, 6 × C(O)CH<sub>3</sub>), 57.00 (OCH<sub>3</sub>), 66.55, 66.90, 68.90, 69.36, 69.53, 70.95, 71.26, 72.78, 73.24 (C-2a, C-3a, C-4a, C-5a, C-6a, C-2b, C-3b, C-4b, C-5b), 98.12 (C-1b), 101.4 (C-1a), 169.4, 169.5, 169.9, 170.0, 170.2 (6 × C(O)CH<sub>3</sub>).

*α*-L-Rhamnopyranoside-(1 → 6)-1-O-β-D-methylglucoside (M)

MS (DCI):  $m/z$  (%) 358 (4 %) [M + NH<sub>4</sub>]<sup>+</sup>, 212 (100) [M-Rhamnose + H+NH<sub>4</sub>]<sup>+</sup>; C<sub>13</sub>H<sub>24</sub>O<sub>10</sub> (340.14).

Methyl O-(*α*-L-rhamnopyranosyl)-(1 → 6)-2-*α*-D-glucopyranoside (D2)

To an HCl saturated MeOH solution, **D** (5.0 mg, 15.3 μmol) was added and stirred at room temperature for 4 h. The solution was concentrated in vacuo and analyzed by mass spectroscopy and TLC.  $R_f$  = 0.27 (EtOAc:MeOH 6:1); MS (ESI)  $m/z$  (%): 339.8 (100) [M-H]<sup>-</sup>, 363.7 (app. 1%) [M + Na]<sup>+</sup>; C<sub>13</sub>H<sub>24</sub>O<sub>10</sub> (340.14).

## Confirmation of the chemical identification

To confirm the saccharide composition of **D** (rutinose) and **M** (1-O-β-methyl-rutinose), acid hydrolysis was performed. 0.5–1 mM of **D** or **M**, respectively, were boiled for 1 h in 50 μl 1 M HCl, lyophilized twice and then dissolved in 50 μl ultra pure H<sub>2</sub>O. HPLC analysis showed that rhamnose and glucose had been formed. To confirm that glucose was located at the reducing end, both disaccharides were digested enzymatically, followed by HPLC analysis of the digestion products. When 1 mM rutinose or methylrutinose was treated in 71 mM sodium acetate, 96 mM EDTA with *α*-glucosidase (buffer pH 6.0; 0.05 U *Saccharomyces cerevisiae* enzyme from ICN, Irvine, CA, USA) or *β*-glucosidase (buffer pH 5.0; 0.05 U almond enzyme from Sigma, St. Louis, MO, USA), respectively, subsequent HPLC analysis did not show any digestion. However, when either disaccharide was treated with naringinase (*α*-rhamnosidase) from *Penicillium decumbens* (0.05 U; Sigma), HPLC

analysis of the products showed that rhamnose and glucose were formed. Hence, it was confirmed that both disaccharides consisted of glucose and rhamnose, with rhamnose at the non-reducing end.

## Chemical synthesis of methylrutinose from rutinose

To exclude that the structure **M** is an artefact due to the extraction conditions, the introduction of a methyl group at position 1a of compound **D** was performed using HCl gas and methanol (Tietze and Eicher 1991). The formed methyl derivative **D2** (Fig. 1b) was afterward compared with the natural product **M** by applying TLC and showed a different  $R_f$  value than compound **M**. Because we have shown above that the 1a position of compound **M** has a *β*-glycosidic linkage, the synthesized methyl derivative has the *α*-linkage based on different  $R_f$  values [ $R_f$  = 0.27 for **D2** compared with 0.21 for **M** (EtOH:MeOH 6:1)]. The structure **D2** was confirmed by applying electron spray ionization (ESI) mass spectroscopy (MS). We found the same molecular weight for **D2** and **M**.

## Different extraction methods

For ethanol extraction, about 300 mg *D. glomerata* material frozen in liquid nitrogen was transferred into a scintillation vial with 2 ml of 10 mM Hepes buffer pH 7.0 in 80% ethanol prewarmed to 80°C and incubated in a shaking water bath at 80°C for 30 min. Then, the liquid was transferred to an evaporation glass and the powder was extracted again with 1 ml buffer in 80% ethanol. For extraction with acetone, *D. glomerata* material frozen and ground in liquid nitrogen was transferred to a pre-cooled plastic tube on ice containing 5 ml of 80% acetone. Sugars were extracted over night at 4°C. For extraction with perchloric acid, *D. glomerata* material was frozen and ground in liquid nitrogen. Then, 1 ml of 10% HClO<sub>4</sub>, 5 mM EGTA was added to the powder and ground further without cooling. After the sample had melted, it was transferred to a centrifuge tube. After centrifugation, the supernatant was neutralized to ca. pH 7 using 5 M KOH, 1 M triethanolamine and incubated on ice for 15 min. The precipitated KClO<sub>4</sub> was removed by centrifugation.

In each case, extracts were dried in a rotary evaporator at 37°C, and the residues were prepared for HPLC analysis as described above.

## Isolation and analysis of apoplastic fluid

Apoplastic fluid was obtained from leaves according to the method of Speer and Kaiser (1991) and Lohaus et al. (2001). Fully expanded leaves were taken from plants in the middle of the light period. Leaves were infiltrated with

2 mM icecold  $\text{CaCl}_2$  in a 50 ml syringe, carefully blotted dry and immediately centrifuged at 160g for 3–9 min. This solution was used instead of distilled  $\text{H}_2\text{O}$  because  $\text{Ca}^{2+}$  effectively seals the sieve elements, thus preventing contamination with phloem sap that occasionally occurs when sieve elements are damaged during infiltration-centrifugation (W. M. Kaiser, University of Würzburg, Germany, personal communication). Using centrifugation, 2–30  $\mu\text{l}$  of the diluted apoplastic fluid could be obtained. The cellular contamination of the apoplastic fluid was tested by measuring malate dehydrogenase (EC 1.1.1.37) activity in the leaf extracts and in the apoplastic fluid samples (Lohaus et al. 1995). Malate dehydrogenase assays were performed in 100 mM MOPS buffer pH 7.5 containing 0.5 mM NADH and 2 mM oxaloacetate in a volume of 1 ml. Reactions were started by the addition of leaf extract (5  $\mu\text{l}$ ) or apoplastic fluid samples (10–20  $\mu\text{l}$ ) and the increase of absorbance was measured at 334 nm. The measured contaminations of apoplastic fluid samples were about 0.2–0.3% of bulk malate dehydrogenase activity in the leaves.

Sugars in the apoplastic fluid samples were analyzed by HPLC. Apoplastic sugar concentrations in the leaves were determined on the basis of the dilution factor  $F = (V_{\text{apoplast}} + V_{\text{gas space}})/V_{\text{apoplast}}$ . The volume of the apoplastic gaseous space was calculated from the difference of the fresh weight of leaves before and after the infiltration. Apoplastic concentrations were calculated under the assumption that the apoplastic water phase of leaves makes up about 10% of the leaf volume (Speer and Kaiser 1991).

#### Transmission electron microscopy

For TEM, small samples of leaves (2–3 mm  $\times$  2–3 mm) were vacuum infiltrated for 1 h in 2.5% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.3 and fixed for 12 h at 4°C. Postfixation took place in 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 1 h at 4°C. After dehydration in a graded acetone series, the samples were embedded in Spurr's resin (Sigma, SPR-LV) and polymerized at 60°C for 12 h in 100  $\mu\text{l}$  PCR vials. Ultrathin sections (60–80 nm in thickness) were cut with a glass knife, mounted on 50-mesh copper grids, contrasted with 4% uranyl acetate and lead citrate for 25 and 10 min, respectively, and observed with a transmission electron microscope H-600 (Hitachi, Tokyo, Japan).

#### Analysis of sucrose transporter expression in *D. glomerata*

RNA was isolated from *D. glomerata* using the Invisorb Spin Plant-RNA Mini Kit (Invitex, Berlin, Germany). First-strand cDNA was synthesized from total RNA isolated from mature leaves of *D. glomerata* using reverse transcriptase from MBI (St. Leon-Rot, Germany). This cDNA

was used as a template for PCR with degenerate primers as described by Knop et al. (2001). A 336-bp fragment of a *D. glomerata* sucrose transporter was amplified and characterized by cloning in pGEM-T Easy (Promega, Madison, WI, USA) and sequencing. Sequencing was carried out using an ABI Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Norwalk, CT, USA). Sequence comparisons were performed using the software package from the Wisconsin genetics computer group (Altschul et al. 1990).

#### Functional analysis of sucrose transporters

$^{14}\text{C}$ -sucrose uptake was analyzed as described by Gahrtz et al. (1994).

#### In vivo $^{14}\text{C}$ labeling

Five plants grown in pot soil at 16 h light/8 h dark regimen at PPFD 140  $\mu\text{mol m}^{-2} \text{s}^{-1}$  were exposed to the air containing 167.98 MBq/l  $^{14}\text{CO}_2$  at total concentration  $^{14}\text{CO}_2 + ^{12}\text{CO}_2$  1%, for 15 min in the middle of the light period. After the labeling, plants were put on the usual growth regimen. Source leaves (third from the top of the branch) were harvested from the plants after 1, 2 and 24 h after the labeling. The leaves were divided into laminae and petioles, fixed in boiling 80% ethanol and further extracted with 80, 60 and 40% ethanol for 30 min each at 60°C. The extracts were combined, and the volume was reduced by evaporation. The recovery of the label in disaccharides was studied by TLC/autoradiography.

#### TLC/autoradiography of extracts

Water:ethanol extracts from laminae and petioles of *D. glomerata* were analyzed chromatographically using TLC sheets covered with silica gel 60 (Merck, Darmstadt, Germany) developed in butanol:acetic acid:water (3:1:1, by vol.) three times in the same direction. In this way, good separation of glucose, fructose and rhamnose from the disaccharides was achieved, but sucrose and rutinose were only partially separated. Therefore, extracts were subjected to hydrolysis by either invertase or naringinase prior to chromatography to eliminate either sucrose or rutinose, respectively. For this purpose, the extracts were dried in a rotary evaporator at 40°C and redissolved in dd  $\text{H}_2\text{O}$ . Then, extracts were divided into three aliquots. Two aliquots were supplemented with 1/20 volume of 1 M Na-acetate buffer pH 4.0 and either invertase from baker's yeast (Sigma) or naringinase ( $\alpha$ -rhamnosidase) from *P. decumbens* (Sigma) to a final concentration of 2 U/ml. Then, the aliquots were incubated at 25°C for 30 min, followed by neutralization by the addition of NaOH, and chromatography as described

above. The chromatograms were exposed to Kodak MIN RS films for 8 days.

#### Study of the uptake of $^{14}\text{C}$ -sucrose from the apoplast in *D. glomerata* leaves

Source leaves were cut from plants, and the petioles were re-cut under water and placed in MES-buffered solutions (20 mM, pH 6.0) containing uniformly labeled  $^{14}\text{C}$ -sucrose (0.01 mM at  $10^{10}$  Bq/mmol) either without further additives or with additionally 20 mM of  $^{12}\text{C}$ -sucrose or 20 mM of  $^{12}\text{C}$ -rutinose, respectively. After 30 min of feeding, the petioles were placed in dd  $\text{H}_2\text{O}$  for the next 30 min. Afterwards, parts of the leaf laminae containing minor veins were excised, dried quickly at  $80^\circ\text{C}$  and exposed to Kodak MIN RS films for 8 days.

#### $\alpha$ -L-Rhamnosidase assays

Plant material was ground in liquid nitrogen and re-suspended in reaction buffer pH 5.5 (either 50 mM Na-succinate or 50 mM Na-succinate, 150 mM NaCl, 1 mM EDTA, 1 mM  $\text{MgCl}_2$ ) or in reaction buffer pH 7.0 (either 50 mM potassium phosphate or 50 mM potassium phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM  $\text{MgCl}_2$ ). Cell debris was spun down and the protein extract was purified over PD-10 desalting columns (Amersham Biosciences). Protein contents were determined according to Bradford (1976). 100  $\mu\text{l}$  of extract was assayed in a volume of 1 ml reaction buffer. As a substrate, either 25  $\mu\text{l}$  of a 40 mM solution of rutin in methanol or 150  $\mu\text{l}$  of 5 mM *p*-nitrophenyl- $\alpha$ -L-rhamnopyranoside in dd  $\text{H}_2\text{O}$  were added and the mixture was incubated at  $25^\circ\text{C}$  for 1 h. In the case of rutin, reactions were stopped by extraction with 200  $\mu\text{l}$   $\text{CHCl}_3$ . 500  $\mu\text{l}$  of the supernatant was dried and resuspended for HPLC analysis. In the case of *p*-nitrophenyl- $\alpha$ -L-rhamnopyranoside, 300  $\mu\text{l}$  of reaction mixture added to 1 ml 2 M  $\text{Na}_2\text{CO}_3$ , and the  $\text{OD}_{405}$  was determined.

## Results

Analysis of sugars from roots, nodules and leaves of *Datisca glomerata* revealed the presence of two novel disaccharides

High-performance liquid chromatography analysis was used to study the composition of soluble carbohydrates in chloroform/methanol extracts from leaves, roots and nodules of *D. glomerata*. The results showed that besides sucrose, glucose and fructose, two unknown carbohydrates were present at considerable concentrations (Fig. 1a). The two unknown compounds, called D and M, were isolated

from nodulated *D. glomerata* root systems, and their structure was determined by mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR) after derivatization of the isolated compounds. They were found to represent rutinose ( $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-D-glucose) and methylrutinose ( $\alpha$ -L-rhamnopyranoside-(1  $\rightarrow$  6)-1-*O*- $\beta$ -D-methylglucoside; Fig. 1b). The amounts of sucrose, glucose, fructose, methylrutinose and rutinose in leaves, roots and nodules of *D. glomerata* are shown in Fig. 1c. It should be noted that when plants were grown under  $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  light intensity, but different light/dark regimens, levels of rutinose and methylrutinose could vary dramatically (up to 60  $\mu\text{mol/g}$  fresh weight rutinose were detected in nodules) while the levels of glucose, fructose and sucrose did not change much (data not shown).

Could rutinose and/or methylrutinose represent artifacts?

Rutinose has been reported as the glycosidic part of flavonoid glycosides (reviewed by Avigad 1982), but to our knowledge had not been found as a free sugar before. *Datisca cannabina*, a close relative of *D. glomerata*, is known to contain a high level of rutinose-containing flavonoid glycosides, mainly datiscin (5,7,2'-trihydroxyflavone-3-*O*-rutinose; Zapesochnaya et al. 1982a). Therefore, the presence of high amounts of free rutinose in *D. glomerata* raised the question whether it was released from datiscin chemically or enzymatically during the extraction of sugars. Chemical hydrolysis of the glycosidic bonds in datiscin or other flavonoid glycosides in the course of the preparation of chloroform/methanol extracts should be excluded. To eliminate this possibility, rutin (quercetin-3-*O*-rutinoside) was subjected to the procedure and HPLC analysis of the resulting extract showed that neither rutinose nor rhamnose had been liberated (data not shown). This left the possibility of enzymatic hydrolysis by flavonol 3-*O*- $\beta$ -heterodisaccharidases as has been reported e.g. for rutinases from the seeds of tatar buckwheat (*Fagopyrum tataricum* Gaertner, Yasuda and Nakagawa 1994; *Fagopyrum esculentum* Moench, Baumgertel et al. 2003). However, large amounts of rutinose and methylrutinose were found in chloroform/methanol-, ethanol-, perchloric acid- and acetone-extracts of *D. glomerata* organs that had been shock-frozen in liquid nitrogen (data not shown), i.e. under conditions where even enzymes that, like rutinases, tolerate 20% DMSO in the reaction medium (Yasuda and Shinoyama 1995) should not be active.

The question remained whether 1- $\beta$ -*O*-methylrutinose was a chemical or enzymatic artefact due to the methylation of rutinose in the presence of methanol. The chemical option could be excluded, since chemical methylation of rutinose yielded only 1- $\alpha$ -*O*-, not 1- $\beta$ -*O*-methylrutinose

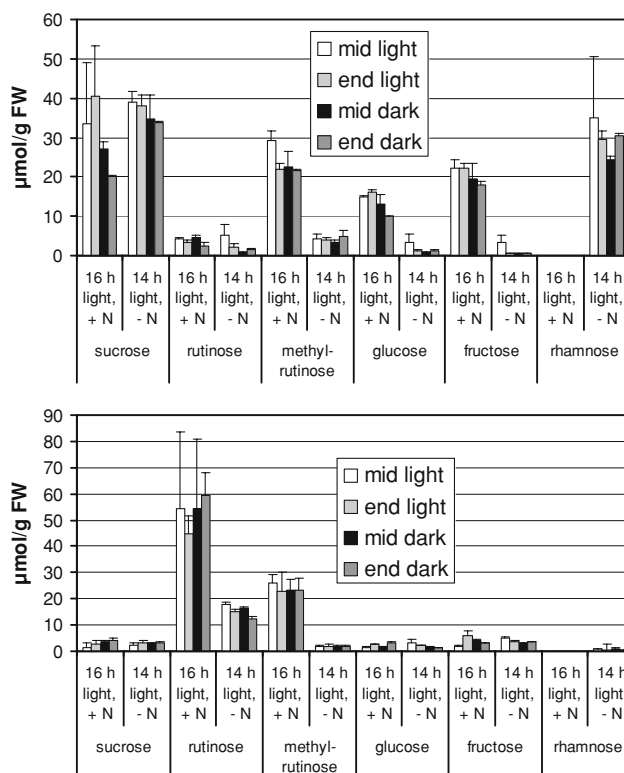
(Fig. 1b, compare M and D2). Enzymatically produced artefacts might occur because the abovementioned flavonol 3-*O*- $\beta$ -heterodisaccharidases are capable of transesterification: they produce  $\beta$ -1-*O*-alkyl disaccharide esters when alcohols are present (Yasuda and Shinoyama 1995). However, 1- $\beta$ -*O*-methylrutinose was also found in extracts prepared using ethanol, acetone or perchloric acid, i.e. in the absence of a potential methyl donor (data not shown). Hence, 1- $\beta$ -*O*-methylrutinose is neither a chemically nor an enzymatically produced artifact.

Are there more plant species that contain free rutinose?

Rutinose was not known to exist as a free sugar in plants before, and Datisceae are not closely related to agriculturally used and therefore well examined plant species. Therefore, the possibility existed that rutinose was present in several plant families whose sugar composition had not yet, or only superficially, been examined. Furthermore, since *Datisca* spp. are actinorhizal plants able to enter a root nodule symbiosis with actinomycetous bacteria from the genus *Frankia*, it was tempting to speculate that rutinose had some function in relation to the symbiosis. Therefore, chloroform–methanol extracts from leaves of several actinorhizal plant species, namely Cucurbitales (*D. cannabina*, *Coriaria japonica* from the Coriariaceae family), Rosales (*Colletia cruciata*, *Cercocarpus betuloides*, *Ceanothus* sp. from the Rhamnaceae family and *Dryas drummondii* and *D. octopetala* from the Rosaceae family), and a closely related non-actinorhizal plant species, *Begonia kellermannii* (Begoniaceae are Cucurbitales and phylogenetically close to Datisceae; Zhang et al. 2006) were examined for the presence of rutinose using HPLC. Rutinose was found only in *D. cannabina* (data not shown).

Role of rutinose and methylrutinose in carbon partitioning in leaves and roots of *D. glomerata*

The diurnal dynamics of sugar contents in leaves and non-nodulated roots of *D. glomerata* were examined (Fig. 2). To better understand the possible role of rutinose and methylrutinose in carbon partitioning, plants grown with and without N limitation were compared. It is well known that N limitation strongly increases the allocation of photoassimilates into roots and promotes root growth (see, e.g. Fichtner et al. 1995 and references therein). Importantly, in both N-supplied and N-limited plants carbon availability exceeded the demands to a nearly similar extent as reflected by similar levels of starch accumulation in leaves of both variants (data not shown). This was achieved by adjusting the light period of both variants (16 h for N-supplied plants vs. 14 h for N-limited plants).



**Fig. 2** Time course of sugar contents ( $\mu\text{mol/g}$  fresh weight) in roots and leaves of *D. glomerata*. Plants were grown in aerated hydroponic culture at 16 h light, 8 h dark or 14 h light, 10 h dark, respectively, and samples (in duplicate) were taken for determination of sugar content in the middle of the light phase, 30 min before the end of the light phase, in the middle of the dark phase and 30 min before the end of the dark phase. The plants grown at 16 h light received 5 mM  $\text{KNO}_3$  each week. The  $\text{KNO}_3$  in the growth medium of the plants grown at 14 h light had not been replenished since 3 weeks at the harvesting date

Although circadian variations of sugar levels in both variants were rather low, strong differences were observed between N-supplied and N-limited plants. Leaves of N-supplied plants accumulated high levels of sucrose, methylrutinose, glucose and fructose, while rutinose levels were lowest and no rhamnose was detected. In leaves of N-limited plants, sucrose content did not change, but the levels of glucose and fructose were dramatically reduced. This indicates that less sucrose was deposited in the vacuoles of these leaves (because leaf hexoses derive from the cleavage of sucrose by vacuolar invertase; Huber 1989), probably due to an increase in sucrose export from the leaves to the roots. Strikingly, high levels of rhamnose were detected in these leaves. As rutinose/methylrutinose is produced from glucose and rhamnose, the accumulation of rhamnose indicates a block of rutinose/methylrutinose synthesis, most likely due to the limited availability of glucose. Accordingly, the levels of methylrutinose strongly decreased; however, the levels of rutinose, being generally low in leaves, were only slightly reduced upon N limitation. In



roots of N-supplied plants, rutinose represented the dominant disaccharide, and methylrutinose levels were about half of those of rutinose. Both rutinose and methylrutinose levels in roots far exceeded the levels of sucrose and hexoses (Fig. 2, see also Fig. 1c). In roots of N-limited plants, the levels of sucrose and hexoses remained low and those of rutinose and methylrutinose decreased dramatically, although they still exceeded sucrose levels. These results suggest that in both leaves and roots, rutinose and methylrutinose are synthesized primarily when carbohydrates are available in excess which is not the case in N-limited plants. In this situation, increased export of carbohydrates from the leaves and intense carbohydrate metabolism in roots to support enhanced root growth are expected to lower the availability of monosaccharides for the synthesis of rutinose and methylrutinose in both leaves and roots. Thus, rutinose and methylrutinose seem to participate in carbon storage in leaves and sink organs under conditions when carbon availability exceeds the demands.

#### *D. glomerata* is an apoplastic phloem loader

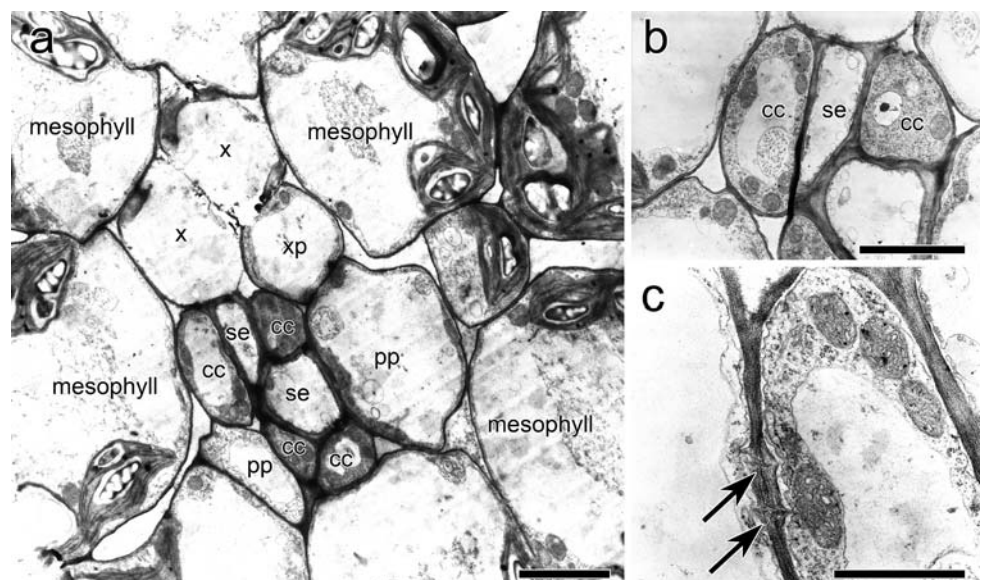
The presence of rutinose and methylrutinose in both leaves and underground organs of *D. glomerata*, and their preferential accumulation in underground organs, raised the question whether rutinose and/or methylrutinose can be synthesized in leaves and transported to the sink organs via the phloem. To analyze the possible role of these disaccharides in long-distance carbon transport, the phloem loading mechanism in *D. glomerata* leaves had first to be understood. For this purpose, transmission electron microscopical (TEM) analysis of the minor vein phloem was performed. The minor veins in *D. glomerata* typically consist of two companion cell/sieve element complexes

(SE/CCs) and a few phloem parenchyma cells that are irregularly positioned within the minor veins (Fig. 3a). Each SE/CC includes one sieve element and a pair of companion cells (Fig. 3a). Sieve elements have no nacreous thickenings (Fig. 3b). The companion cells lack cell wall ingrowths and contain no plasmodesmal fields at the mesophyll interface although they contain a single plasmodesmata (Fig. 3b, c). Altogether, the terminal phloem of *D. glomerata* can be classified as type 2A (primitive apoplastic loader with ordinary companion cells) according to the nomenclature developed by Gamalei (1995).

#### Rutinose is the dominating sugar in the apoplast of *D. glomerata* leaves

The study of minor vein anatomy showed that the phloem loading in *D. glomerata* should occur mainly apoplastically, i.e. rely on substrate-specific sugar transporters in the plasma membrane of companion cells, driven by the proton motive force (Sauer and Stolz 1994). HPLC analysis of sugar levels in the apoplastic fluid of leaves of *D. glomerata* revealed that rutinose was the dominating sugar in this compartment, reaching concentrations between 2.7 and 12 mM (Table 1). No sucrose was found in the apoplast of *D. glomerata* leaves, but glucose and fructose were detected at nearly similar concentrations, suggesting that they were derived from the artifactual hydrolysis of sucrose by apoplastic invertase during the isolation procedure. The presence of invertase activity in the apoplastic fluid was confirmed by adding sucrose to the apoplastic fluid where it was promptly cleaved to glucose and fructose (data not shown). Further confirmation of the presence of sucrose in the apoplast was obtained using 50 mM MES buffer, pH 6.5, instead of 2 mM CaCl<sub>2</sub> for the

**Fig. 3** Electron micrographs of *D. glomerata* minor veins. CC companion cell, SE sieve element, PP phloem parenchyma, X xylem element, XP xylem parenchyma. **a** Overview of a minor vein. **b** Upper sieve element-companion cell complex on an adjacent section. **c** Detail of the left companion cell and adjacent mesophyll cell shows two plasmodesmata (arrows). The size bars denote 5  $\mu\text{m}$  (**a**), 2.5  $\mu\text{m}$  (**b**) and 1  $\mu\text{m}$  (**c**), respectively



**Table 1** Sugar concentrations in leaf apoplastic fluid of *D. glomerata* [mM]

Growth conditions	Methylrutinose	Rutinose	Glucose	Fructose	Sucrose
Soil-grown plants	1.71 ± 0.48	12.62 ± 4.58	8.59 ± 2.23	6.89 ± 2.07	n.d.
Hydroponically grown plants	0.78 ± 0.20	7.53 ± 2.39	4.45 ± 0.36	3.44 ± 0.19	n.d.

Leaves were taken at the middle of the light period from plants grown either on pot soil or in hydroponic culture at 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

Data represent average values from five leaves taken from five different plants per each variant  $\pm$  SD

n.d. not detected

isolation of the apoplastic fluid, yielding sucrose concentrations of  $1.72 \pm 0.78$  mM with glucose and fructose levels between 1 and 5 mM (data not shown). The apoplastic fluid contained only traces of methylrutinose (Table 1). Assuming that most glucose and fructose in the apoplast were derived from the cleavage of sucrose, rutinose still represented ca. 55% of apoplastic sugars in soil-grown plants and ca. 59% in hydroponically grown plants, respectively, while sucrose represented maximally 30 and 27 %, respectively. The rest was made up of hexoses. Thus, the sugar composition in the leaf apoplast was strikingly different from the sugar composition in total leaves where rutinose concentrations were about seven times lower than sucrose concentrations (Figs. 1c, 2 vs. Table 1).

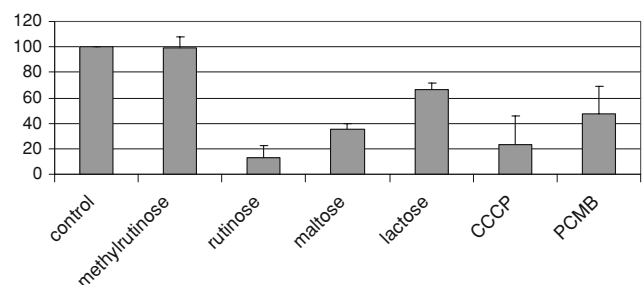
#### Type III sucrose transporters accept rutinose, but not methylrutinose as substrate

The presence of rutinose in the leaf apoplast of *D. glomerata* suggested that rutinose, along with sucrose, may be loaded from the apoplast into the phloem and transported from leaves to the roots and nodules. This would require rutinose to be accepted by disaccharide transporters present on the plasma membrane of SE/CC in the minor vein phloem of *D. glomerata*. The most abundant disaccharide transporters here can be expected to be sucrose/proton symporters. There are three types of sucrose/proton symporters in plants (Aoki et al. 2003; Reinders et al. 2006). Recently, it was found that these transporters have broader substrate specificity than previously thought. Type I transporters can transport phenolic  $\beta$ -glucosides and two  $\alpha$ -glucosides (Chandran et al. 2003; Sivitz et al. 2005, 2007). At least two different type I sucrose transporters accept the non-glucoside biotin as a substrate (Ludwig et al. 2000).

In roots, nodules and leaves of *D. glomerata*, RT-PCR using degenerate oligonucleotide primers based on the conserved amino acid regions of sucrose transporters led to the amplification of a cDNA fragment of type III transporter (SUT4) in all cases (GenBank accession AJ459485; data not shown); no cDNA fragments representing members of other sucrose transporter classes could be amplified. The SUT4 subfamily of sucrose transporters is characterized by

low affinity and high-transport capacity and had been implicated in phloem loading in source leaves, and in phloem unloading in strong sinks (Weise et al. 2000). However, recently AtSUT4, the member of this transporter family from Arabidopsis, and the homolog from barley have been found to localize to the vacuolar membrane, not the plasma membrane (Endler et al. 2006), as was also found to be the case for the homolog from *Lotus japonicus*, LjSUT4 (Reinders et al. 2008).

Rutinose and methylrutinose had never been tested as substrates for sucrose transporters. The ability of rutinose and methylrutinose to inhibit the uptake of  $^{14}\text{C}$ -sucrose by yeast strain DBY2617 expressing the AtSUT4 ORF (Weise et al. 2000) was examined. As shown in Fig. 4, uptake of sucrose by AtSUT4 was inhibited by rutinose, maltose and lactose, with rutinose having the strongest inhibitory effect. Methylrutinose did not affect sucrose uptake (Fig. 4). Hence, we conclude that AtSUT4 can transport rutinose, but not methylrutinose. Substrate specificity of type III disaccharide transporters thus seems to depend on a terminal glucose unit, independent on whether the glucose is connected via the reducing or the non-reducing end.



**Fig. 4** Substrate specificity of AtSUT4 in yeast cells. Uptake of 3 mM  $^{14}\text{C}$ -sucrose by strain SEY2102 (Emr et al. 1983) containing AtSUT4 in pDR196 (Weise et al. 2000) was analyzed in the presence of inhibitors and of competing sugars. The sulfhydryl group inhibitor PCMB and the uncoupler CCCP were used as inhibitors and added to a final concentration of 50  $\mu\text{M}$ . Uptake of 3 mM  $^{14}\text{C}$ -sucrose was also analyzed in the presence of competing non-labeled sugars added at a threefold excess (final concentrations of 9 mM). Each bar represents results from at least three independent experiments. Standard deviations are given

Neither rutinose nor methylrutinose is transported in the phloem of *D. glomerata*

The fact that vacuolar disaccharide transporters accept rutinose as a substrate seemed to strengthen the idea that rutinose might follow the same transport pathways as sucrose. To determine whether rutinose could be taken up from the apoplast into the phloem via sucrose transporters, *D. glomerata* leaves were fed via petioles with buffered <sup>14</sup>C-sucrose solution either without additives or supplied with 20 mM of <sup>12</sup>C-sucrose or 20 mM of <sup>12</sup>C-rutinose, respectively. After 30 min of exposure, followed by a chase period of 30 min, parts of the leaf lamina containing minor veins were cut off and autoradiographs were obtained (Fig. 5). <sup>12</sup>C-sucrose inhibited the accumulation of <sup>14</sup>C-sucrose in the vascular tissue, while <sup>12</sup>C-rutinose did not. Hence, rutinose does not compete with sucrose for sucrose transporters in the plasma membrane of the phloem companion cells.

Analysis of *D. glomerata* minor vein architecture by TEM suggested that some minor capacity for symplastic loading of assimilates did exist. Furthermore, it could not be ruled out that rutinose might enter the phloem from the apoplast via a transporter that did not accept sucrose as a substrate. Therefore, we used in vivo <sup>14</sup>C labeling to investigate the possibility that freshly synthesized (methyl-) rutinose might be loaded from mesophyll cells into the phloem. Plants were exposed to <sup>14</sup>CO<sub>2</sub> for 15 min in the middle of the light period, and the recovery of the label in disaccharides extracted from leaf laminae and leaf petioles was studied 1, 2 and 24 h after labeling using TLC/autoradiography. At all time points, sucrose was strongly labeled but no label could be detected in rutinose or methylrutinose (data not shown). These results indicated that the time for newly assimilated carbon to become incorporated into rutinose is longer than would be expected for a carbohy-

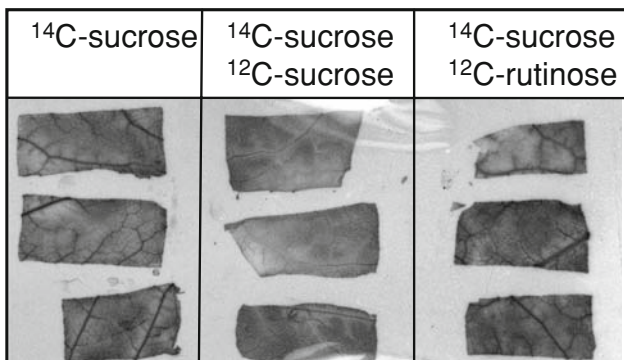
drate participating in the translocation from leaves to sink organs.

### Metabolism of rutinose and methylrutinose

Roots, nodules and leaves of *D. glomerata* contain an activity that demethylates methylrutinose to rutinose, i.e. a β-glucosidase activity, as was observed when extraction of sugars from frozen plant tissue was performed with water (data not shown). Equally, all organs of *D. glomerata* contain an activity that liberates rutinose from rutin (quercetin-3-O-rutinose), i.e. a flavonol 3-O-β-heterodisaccharidase activity as has been found in *Fagopyrum* sp. (Yasuda and Nakagawa 1994; Baumgertel et al. 2003). When rutin, dissolved in methanol, was added to protein extract from roots, nodules or leaves, rutinose and methylrutinose were formed (data not shown) indicating that like the enzyme from *Fagopyrum tataricum* (Yasuda and Nakagawa 1994), the flavonol 3-O-β-heterodisaccharidase from *D. glomerata* can perform transglycosylation of rutinose in the presence of short-chain alcohols.

As was shown during their chemical identification, rutinose and methylrutinose are cleaved into (methyl-)glucose and rhamnose by naringinase, a fungal α-L-rhamnosidase. A plant α-L-rhamnosidase has been characterized in seeds of *Fagopyrum esculentum* (Bourbouze et al. 1976). The presence of α-L-rhamnosidases in roots, nodules and leaves of *D. glomerata* was examined using the synthetic substrate *p*-nitrophenyl-α-L-rhamnopyranoside under the conditions described by Bourbouze et al. (1976) and with modifications. Because the high flavonoid content of *D. glomerata* interferes with the detection of yellow *p*-nitrophenol, protein extracts were purified via Sephadex G25 columns before assays. No *p*-nitrophenyl-α-L-rhamnopyranoside cleaving activity was detected.

There is evidence that not every α-L-rhamnosidase accepts *p*-nitrophenyl-α-L-rhamnopyranoside as a substrate (Yu et al. 2002). Because protein extracts from roots, nodules and leaves liberated rutinose from rutin, rutin was also used as substrate, and reaction products were analyzed by HPLC. Free rutinose was detected in all cases, but never glucose or rhamnose. Similarly, when rutinose was used as a substrate directly, formation of glucose and/or rhamnose could not be detected (data not shown). Hence, no α-L-rhamnosidase activity could be detected in roots, nodules and leaves from *D. glomerata*.



**Fig. 5** Uptake of <sup>14</sup>C-sucrose by *D. glomerata* source leaves. *D. glomerata* leaves were fed buffered <sup>14</sup>C-sucrose solution either without additives, or supplied with 20 mM <sup>12</sup>C-sucrose or 20 mM <sup>12</sup>C-rutinose, respectively. After washing, parts of the laminae containing minor veins were excised, dried and autoradiographed

### Discussion

Two novel disaccharides were identified in this study in *Datisca glomerata*, the reducing sugar rutinose and its non-reducing β-methylated derivative methylrutinose. The

occurrence of rutinose in *Datisca* sp. is not surprising per se, since it represents the glycoside of the most common flavonoid in Datisceae, datiscin. However, the fact that it occurs as a free disaccharide is indeed surprising, since there are few free disaccharides known in plants. Methylrutinose, to our knowledge, has not been described before. Analysis of sugar contents of roots, nodules and leaves showed that both rutinose and methylrutinose were present in sink and source organs, but that sucrose was the dominating disaccharide in source leaves while rutinose was the dominating disaccharide in sink organs.

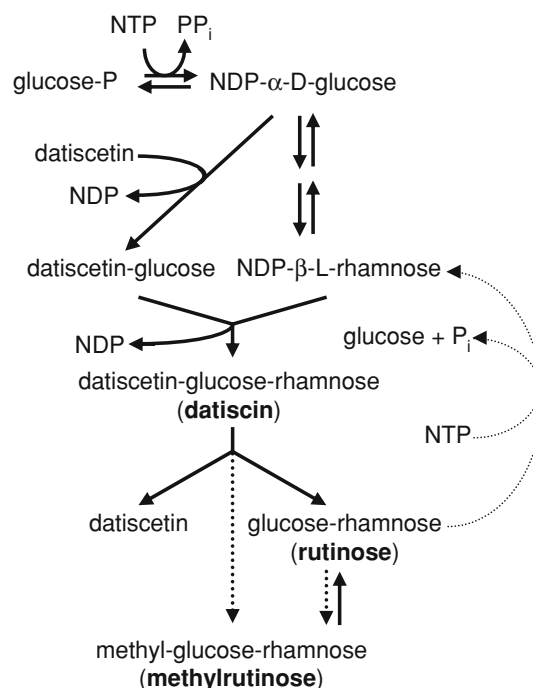
Free rutinose metabolism does not seem widespread in the plant kingdom

Up to now, rutinose was only found as a free sugar in members of the Datisceae, *D. glomerata* and *D. cannabina*. The family Datisceae contains only one genus, *Datisca*, represented by two species. Leaves from several other actinorhizal plant species from different families, Coriariaceae, Rosaceae and Rhamnaceae, did not contain rutinose. The closest relatives of Datisceae are the non-actinorhizal families Tetramelaceae and Octomelaceae, represented by the tropical trees *Tetrameles nudiflora* and *Octomeles sumatrana* (Zhang et al. 2006), which have not been examined. But even a member the next closely related plant family, Begoniaceae, was shown not to contain rutinose as a free sugar. Hence, rutinose metabolism seems to have evolved specifically among the Datisceae, or maybe also among Tetramelaceae and Octomelaceae.

Rutinose and methylrutinose in carbon metabolism of *D. glomerata*

Sink-source relationships are regulated via plant sugar metabolism, whose control is central to plant development. The analysis of N-supplied versus N-limited plants has suggested that rutinose/methylrutinose is not central to *D. glomerata* C metabolism but are formed when sucrose demands have been filled and might serve as an intermediate carbon storage forms in the vacuoles of leaves and sink organs. Hence, the question arises on how two new disaccharides could be integrated in standard sugar metabolism in *D. glomerata*.

An obvious possibility for the biosynthesis of rutinose/methylrutinose involving only well-known enzyme activities would be the sequential glycosylation of datiscin with glucose and rhamnose, followed by a one-step deglycosylation of the resulting datiscin by a flavonol 3-*O*- $\beta$ -heterodisaccharidase to yield rutinose, or, in case of transglycosylation, methylrutinose, as depicted in Fig. 6. Flavonol 3-*O*- $\beta$ -heterodisaccharidases from *Fagopyrum* sp. (Yasuda and Nakagawa 1994; Baumgertel et al. 2003) have been shown to be



**Fig. 6** Hypothetical model for (methyl-) rutinose metabolism in *D. glomerata*. Solid arrows denote reactions shown in *D. glomerata* or in other plants, dashed arrows show hypothetical reactions. NDP- $\beta$ -L-rhamnose is synthesized via NDP- $\alpha$ -D-glucose. Sequential glycosylation of the flavonol datiscin with nucleotide sugars leads first to datiscin-glucose (datiscanin; Zapesochneya et al. 1982b) and then to datiscin-rutinoid (datiscin). A flavonol 3-*O*- $\beta$ -heterodisaccharidase cleaves datiscin into datiscin and rutinose or catalyzes a transglycosylation yielding datiscin and methylrutinose; this reaction can take place in extracts from roots, nodules and leaves. In all those organs, methylrutinose can be demethylated by a  $\beta$ -glucosidase of unknown subcellular localization, yielding rutinose. It is not clear whether rutinose can be methylated to methylrutinose, or whether methylrutinose can only be formed from datiscin by transglycosylation. Since no  $\alpha$ -rhamnosidase activity was detected and since rhamnose never was found to accumulate in sink organs of *D. glomerata* to any significant extent, in contrast with other sugars, rutinose cleavage is postulated to yield NDP- $\beta$ -L-rhamnose

glycosylated proteins with a pH optimum in the acidic range (pH 4.8 for the enzyme from *F. esculentum*; pH 5 for the enzyme from *F. tataricum*), implying that they are active in the vacuole or apoplast. In the presence of alkyl donors, these enzymes catalyze transglycosylations, an activity that would lead directly to the synthesis of methylrutinose from rutin dissolved in methanol; such a flavonol 3-*O*- $\beta$ -heterodisaccharidase activity has been detected in *D. glomerata* in that the addition of rutin (quercetin-rutinose) dissolved in methanol to extracts of roots, nodules or leaves led to the production of free rutinose and methylrutinose (data not shown). The addition of rutinose to protein extracts from roots, nodules or leaves of *D. glomerata* did not lead to the production of methylrutinose, supporting the concept of methylrutinose production via transglycosylation by a flavonol 3-*O*- $\beta$ -heterodisaccharidase.



Most flavonol glycosyltransferases are soluble cytosolic enzymes (Vogt and Jones 2000), but the substrates are likely to be localized in membranes due to their low solubility in water, and membrane-associated glycosyltransferases are known (Miller et al. 1999). Thus, synthesis of datiscin might take place in the plasma membrane or in the tonoplast, and an apoplastic or vacuolar 3-*O*- $\beta$ -heterodisaccharidase would liberate rutinose or catalyze a transglycosylation leading to methylrutinose synthesis, at the other side of the membrane. It seems likely that the 3-*O*- $\beta$ -heterodisaccharidase is a vacuolar enzyme, so the synthesis of datiscin would take place in the tonoplast. At any rate, if methylrutinose would be produced in the apoplast, it would have to be demethylated by an apoplastic  $\beta$ -glucosidase to enter the symplast, since disaccharide transporters do not seem to accept methylrutinose as a substrate (Fig. 4).

Analysis of apoplastic fluid from source leaves revealed that methylrutinose could not be detected in this compartment, while rutinose was abundant (Table 1). Significant amounts of glucose and fructose were also found which are likely to represent the products of sucrose hydrolysis, since apoplastic fluid contained invertase activity (data not shown). Because apoplastic  $\beta$ -glucosidases are documented in plants (Dietz et al. 2000), the lack of methylrutinose might be due to its demethylation to rutinose in the course of the isolation of the apoplastic fluid. However, methylrutinose added to apoplastic fluid was not demethylated (data not shown), indicating either that no such activity exists in the apoplast of *D. glomerata*, or that *D. glomerata* contains an apoplastic  $\beta$ -glucosidase that is, in contrast with the enzyme described by Dietz et al. (2000), insoluble.

No  $\alpha$ -L-rhamnosidase activity could be found in roots, nodules or leaves of *D. glomerata*, either in the soluble or in the insoluble fraction. Since hydrolase activities should be easily detectable, this negative result indicates that the mechanism of rutinose degradation in plants may differ from that in fungi. Recently, it was shown that sucrose synthase has rather broad substrate specificity (Römer et al. 2001) making it a candidate for a rutinose-degrading enzyme, but sucrose synthase did not cleave rutinose (B. Sauerzapfe and L. Elling, personal communication). It is tempting to speculate based on these results that rutinose is degraded by a reversal of the rhamnosyl transferase reaction that is active in the biosynthesis of datiscin. Plant rhamnosyl transferases that transfer a rhamnosyl residue from UDP- or dTDP-rhamnose to a  $\beta$ -glucosylated flavone have already been characterized (Bar-Peled et al. 1991; Kroon et al. 1994). The question is whether the reverse reaction would also work with free rutinose instead of datiscin. In this case, degradation of rutinose would likely take place in the cytosol (or, for datiscin, at the cytosolic face of the tonoplast) and yield glucose and UDP- or dTDP-rham-

nose (see Fig. 6). The metabolization of UDP- or dTDP-rhamnose in plants has not yet been examined.

#### Transport of rutinose and methylrutinose

The high rutinose concentrations that can be reached in plant organs (Fig. 1c) in combination with the fact that rutinose is accepted as a substrate by a type III sucrose transporter from *Arabidopsis* (Fig. 4) indicate that it can be transported across the tonoplast membrane and accumulates in vacuoles. Furthermore, rutinose represents the most abundant sugar in the leaf apoplast (Table 1), indicating that it can be released into the apoplast. Because the analysis of minor vein anatomy in *D. glomerata* leaves that resolves the phloem loading mechanism (Gamalei 1984; Turgeon et al. 1993) had shown that this species should be an apoplastic phloem loader (Fig. 3), our results seemed to imply that rutinose would be able to enter the phloem.

Three types of sucrose transporters are known (Reinders et al. 2008), types I and II locating to the plasma membrane and type III to the vacuolar membrane and their substrate specificities differ. Type I plasma membrane sucrose transporters from *Arabidopsis thaliana* (SUC2 and SUC9) can transport  $\alpha$ - and  $\beta$ -glucosides, even phenolic ones including arbutin, fraxin and esculin (Chandran et al. 2003; Sivitz et al. 2007). The substrate specificity of the vacuolar type III transporters seems to be somewhat higher (Reinders et al. 2008), while type II sucrose transporters have a very narrow substrate specificity (Meyer et al. 2000; Sivitz et al. 2007). To date, only type I sucrose transporters, the group with the lowest substrate specificity, have been implicated in phloem loading (Gottwald et al. 2000; Hackel et al. 2000). Hence, the fact that rutinose was accepted by type III sucrose transporters and by the mesophyll plasma membrane sucrose transporters seemed to imply that it would also be transported into companion cells. However, rutinose does not interfere with the uptake of sucrose from the apoplast into phloem companion cells (Fig. 5). Thus, phloem loading in *D. glomerata* seems to involve sucrose transporter(s) with a higher substrate specificity than those present in vacuolar membranes or in the plasma membranes of mesophyll cells. The results of the *in vivo*  $^{14}\text{C}$  labeling analysis seemed to rule out the possibility that either rutinose or methylrutinose represents a carbon transport form in *D. glomerata*.

Methylrutinose is not accepted as a substrate by sucrose transporters, but can be converted into rutinose by the action of  $\beta$ -glucosidases (see above) which in plants can be present in the apoplast (Dietz et al. 2000), in the ER (Matsushima et al. 2002), plastids (Kristoffersen et al. 2000; Nikus et al. 2003) and vacuoles (Santana et al. 2002), but have not yet been reported in the cytosol. It is an open question whether methylrutinose occurs outside the vacuole;

the simplest assumption would be that methylrutinose is present only in vacuoles where the reducing disaccharide rutinose can be methylated for storage purposes, and is demethylated to rutinose for mobilization.

### Concluding remarks

The unusual sugar metabolism in Datisceae seems to represent a quirk of nature which could evolve without major changes in enzyme and transporter equipment but does not offer any obvious advantages.

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