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RESEARCH PAPER

Hexoses as phloem transport sugars: the end of a dogma?

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Abstract

According to most textbooks, only non-reducing carbohydrate species such as sucrose, sugar alcohols, and raffinose-family sugars function as phloem translocates. Occasional abundance of reducing sugar species (such as hexoses) in sieve-tube sap has been discarded as an experimental artefact. This study, however, discloses a widespread occurrence of hexoses in the sieve-tube sap. Phloem exudation facilitated by EDTA provided evidence that many of the members of two plant families (Ranunculaceae and Papaveraceae) investigated translocate >80% of carbohydrates in the form of hexoses. Representatives of other families also appear to translocate appreciable amounts of hexoses in the sieve tubes. Promoting effects of EDTA, activities of sucrose-degrading enzymes, and sugar uptake by micro-organisms on hexose contents of phloem exudates were checked. The rate of sucrose degradation is far too low to explain the large proportions of hexoses measured in phloem exudates; nor did other factors tested seem to stimulate the occurrence of hexoses. The validity of the approach is further supported by the virtual absence of hexoses in exudates from species that were known as exclusive sucrose transporters. This study urges a rethink of the existing views on carbohydrate transport species in the phloem stream. Hexose translocation is to be regarded as a normal mode of carbohydrate transfer by the phloem equivalent to that of sucrose, raffinose-family sugars, or sugar alcohols.

Key words: Carbohydrate translocation, family-related transport sugars, hexose, Papaveraceae, phloem transport, Ranunculaceae, sieve tubes.

Introduction

As early as 1968, Arnold concluded that transport sugars in the phloem cannot possibly be hexoses. He suggested that a good translocate should be non-reducing so that it does not interact non-enzymatically with proteins in the sieve tube sap. In his view, sucrose was therefore the ideal translocate to be protected from enzymatic attack until it arrives at its destination (Arnold, 1968). In this way, the flow of carbon could be controlled by one or two key sucrose-hydrolysing enzymes in sink tissues (for further details, see Turgeon, 1995).

Comprehensive tests of the phloem-sap composition including several hundreds of species (Zimmermann and Ziegler, 1975) lent support to Arnold's claims. However, sucrose turned out to be not the only carbohydrate transported. Three major types of organic C-carriers were identified in phloem sap: sucrose, galactosyl-oligosaccharides (or RFO-family oligosaccharides), and sugar alcohols which all have the absence of free ketone or aldehyde groups in common. Occasionally, hexoses were detected in phloem sap (Meyer-Mevius, 1959), but these were dismissed as being artefacts (Ziegler, 1975). Categorical disregard of hexoses found in phloem sap (Richardson and Baker, 1982; Richardson *et al*., 1984; Kuo-Sell, 1989) became the rule corroborated by circumstantial evidence against hexoses as regular components of phloem sap provided by molecular studies (von Schaewen *et al*., 1990; Dickison *et al*., 1991; Sonnewald *et al*., 1991; Heineke *et al*., 1992; Riesmeier *et al*., 1993, 1994).

There exists a dramatic difference in carbohydrate composition between mesophyll and sieve tube exudate of many dicotyledonous species (Schrier, 2001). Hence, the borderline between production and transport compartments in the minor veins seems to play a decisive role in determining the nature of the C-translocate in sieve tubes (van Bel, 2003; Hafke *et al*., 2005). The inability of minor-vein sieve elements to accumulate hexoses is

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presumed to explain the minimal hexose level in phloem sap of apoplasmically phloem-loading species. Transformants in which sucrose carriers involved in phloem loading were knocked out (Riesmeier *et al*., 1993, 1994) showed almost full blockage of phloem loading and strongly reduced growth. The same applies to plant transformants expressing an apoplasmic yeast invertase which cleaves sucrose into hexoses (von Schaewen *et al*., 1990; Dickison *et al*., 1991; Sonnewald *et al*., 1991; Heineke *et al*., 1992).

Several arguments were invoked to explain the occasional presence of hexoses in phloem exudates which conflicts with the putative incapability to hexose loading. The most obvious explanation was that hexoses leaking out from the cut surface contaminate phloem exudates. Another source of hexoses may be sucrose hydrolysis by sucrose-degrading enzymes released from the cut surface (Richardson and Baker, 1982; Groussol *et al*., 1986). Furthermore, EDTA or EGTA used to suppress Ca^{2+}-induced sieve-tube plugging in exudation experiments (King and Zeevaart, 1974) may remove calcium anchors of the plasma membrane. As a result, cells may become leaky and cellular hexoses may flow out during the exudation period (Pate, 1980; Girousse *et al*., 1991). All in all, a massive body of evidence forces us to believe that hexoses are not natural components of sieve tube sap, a view that has been adopted by all leading textbooks.

Yet, there remains room for doubts regarding the absence of hexoses in phloem sap. First of all, the claims regarding the artefactual presence of hexoses induced by EDTA or by sucrose-degradation are poorly documented. Moreover, a few species (for example, among the cucurbits) seem to contain considerable amounts of hexoses in exuding sieve tube sap (Meyer-Mevius, 1959; Richardson and Baker, 1982). Last but not least, hexoses were encountered in exudates of several species (Schrier, 2001; Schrier *et al*., 2000) to such high concentrations that it was difficult to dismiss them as artefacts. The present study demonstrates that representatives of some plant families (Ranunculaceae, Papaveraceae) translocate almost exclusively hexoses, while the phloem sap contains appreciable proportions of hexoses in members of several other families.

Materials and methods

Plant material

Plants were grown under ambient outdoor conditions (a moderate land climate) in the botanic garden of the Justus-Liebig-University, Giessen, Germany. Herbaceous dicotyledonous plant species with relatively large leaves and distinct petioles were selected for experimentation. For some species, the term 'petiole' was not correct, but used for convenience (for example, in *Eranthis hyemalis* the inflorescence stem was employed). Plants under investigation were: *Achillea millefolia*, *Aconitum napellus*, *Anemone nemorosa*, *Centranthus ruber*, *Consolida regalis*, *Corydalis cava*, *Dahlia* hybrid, *Digitalis purpurea*, *Echium vulgare*, *Epilobium angustifolium*, *Eranthis hyemalis*, *Fumaria officinalis*, *Galinsoga parviflora*, *Geum urbanum*, *Lupinus polyphyllus*, *Lythrum salicaria*, *Nigella arvensis*, *Papaver rhoeas*, *Phlox paniculata*, *Phytolacca americana*, *Primula veris*, *Pulsatilla vulgaris*, *Ranunculus ficaria*, *Rudbeckia deamii*, *Saponaria officinalis*, *Scrophularia nodosa*, *Valeriana officinalis*, *Valerianella locusta*, and *Vicia faba*. These species collectively cover the growing season between January and October.

Exudation experiments

Full-grown leaves were cut from the plant close to the petiole base, while the site of cutting was submerged under a 5 mM EDTA (2-Na-ethylenediamine tetraacetic acid) solution to prevent ready plugging of the sieve plates. After ~15 min of EDTA-incubation, the petioles were placed into 2 ml Eppendorf cups with 1 ml exudation medium; the number of leaves (one to five) fitted into one cup was dependent on their size. The respective standard exudation media, freshly prepared before each experiment, contained 1 mM MES [2-(*N*-morpholino)ethanesulphonic acid]/KOH buffer (pH 7 or 5) either without or with 2.5 mM EDTA. Standard exudation experiments lasted 6 h. Exudation media were replaced each hour by 1 ml of fresh medium frozen in liquid nitrogen, and immediately stored in a deep-freezer (–30 °C). Exudation was executed under standardized conditions in a climate chamber (BK 5060EL; Heraeus Instruments, Hanau, Germany) under a light intensity of 3500–5000 lx at plant height (halogen metal vapour radium HRI BT 400W/D Osram lamps; Osram, Munich, Germany) at 20 °C and a relative air humidity of 100%. The composition of exudation samples was determined by high-pressure liquid chromatography (HPLC).

Carbohydrate analysis of exudates

Approximately 0.025 g of Polyclar (Serva, Heidelberg, Germany)—a phenol-binding substance—was added to the frozen 1 ml exudate samples in 2 ml Eppendorf cups. The cups were thawed in boiling water for 10 min, cooled down quickly with ice and centrifuged (Mikro22R, Hettich, Tüttlingen, Germany) at 4 °C for 10 min at 1000 *g*. An aliquot of 0.6 ml was taken from the supernatant for carbohydrate determination by HPLC (HPAE-PAD installed in a DX 500 system; Dionex, Sunnyvale, CA, USA) using a 250 mm length/3 mm in diameter analytical CarboPac^{TM} PA20 column (Dionex) with a 50 mm guard column for isocratic separation (35 mM NaOH). The standard reference solution contained myo-inositol, D(+)-trehalose, D(+)-glucose, D(–)-fructose, D(+)-sucrose, (D+)-melizitose-monohydrate, D(+)-raffinose-pentahydrate, and stachyose-trihydrate, each at a concentration of 10 mg l^{-1} as related to the carbohydrate part of the compounds. The reference sugars were purchased from Fluka, Buchs, Switzerland, except sucrose and raffinose which were from Carl Roth, Karlsruhe, Germany). Sugar content was calculated as mg l^{-1} and carbohydrate fractions expressed as the percentage of the total identified.

Sugar analysis of leaf tissues

The leaves used previously in exudation experiments were ground in liquid nitrogen. Frozen tissue (100–180 mg} was weighed in 2 ml Eppendorf cups, and ~0.025 g of Polyclar and 1 ml of cold water were added. Probes were immediately placed in boiling water (10 min) to stop all enzyme activity, then agitated on a table shaker (KL2; Edmund Bühler, Tübingen, Germany; 420 movements min^{-1}) for 10 min at 4 °C, and centrifuged for 10 min at 10 000 *g* on ice. The supernatant was collected and the pellet was re-suspended in 0.75 ml of distilled water, shaken for 1 h and centrifuged for 10 min on ice. After species-dependent dilution of the aggregated first and second supernatant, sucrose, glucose, and

fructose contents in 40 µl of the supernatant probes were measured enzymatically in a microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA) using a sugar determination kit (Boehringer, Mannheim, Germany; cat. no. 10 716 260 035). The amounts prescribed in the Boehringer manual were adapted to the 40 µl aliquots. Sugar content was measured as mg g^{-1} FW and carbohydrate fractions expressed as the percentage of the total identified.

Activity of sucrose-degrading enzymes in exudation media

Leaves of *Anemone nemorosa*, *Corydalis cava*, *Eranthis hyemalis*, *Fumaria officinalis*, *Primula veris*, *Ranunculus ficaria*, and *Vicia faba* were placed with their cut petiole ends into 10 ml test tubes containing 6 ml 2.5 mM EDTA-solutions buffered by 1 mM MES/KOH at pH 5 or pH 7. After 1 h, the leaves were removed and transferred to a second set of test tubes with 6 ml of identical exudation media. After another hour, the leaves were removed from these tubes. The test tubes (containing the exudates collected during the first hour and second hour separately) were placed at 20 °C under standard light conditions to obtain a time-course of the hexose/sucrose content for 6 h. Therefore, each hour after removal of the leaf, 1 ml was sampled from the medium and frozen in liquid nitrogen. The frozen samples were treated with Polyclar (see Carbohydrate analysis of exudates), thawed, and analysed for carbohydrates by HPLC. Possible alterations in the hexose:sucrose ratios provide information on the breakdown rate by sucrose-degrading enzymes.

Impact of EDTA on exudate composition

Potential leakage of hexoses from EDTA-injured cells was measured using *Fumaria officinalis* and *Pulmonaria officinalis* petioles from which the leaf blades had been removed. The petioles were placed into 1 ml 1 mM MES/KOH solutions (pH 7) with or without 2.5 mM EDTA in 2 ml Eppendorf cups to allow carbohydrate release from the basal cut surface under standard conditions. The apical cut surface was sealed with paraffin to prevent drying. During 6 h, the release samples were replaced each hour and immediately frozen in liquid nitrogen. The carbohydrate composition of the leakage fluid was determined by HPLC.

Potential artefactual stimulation of hexose exudation by EDTA having invaded via the xylem vessels was also checked by comparison of exudation from *Eranthis hyemalis* leaves under standard light conditions or darkness. Exudation took place into 2 ml Eppendorf cups filled with 1 ml 2.5 mM EDTA in 1 mM MES/KOH buffer (pH 7) for 6 h. The exudation samples were replaced each hour and immediately frozen. The carbohydrate composition of the exudation samples was determined by HPLC as mentioned under the heading 'Carbohydrate analysis of exudates'.

Resorption of hexoses and sucrose by the cut petiole surface

Petioles of *Corydalis cava*, *Eranthis hyemalis*, and *Ranunculus ficaria* with or without the leaf blade were placed with the basal cut end into 2 ml Eppendorf tubes containing 1 ml 0.1 or 1 mM ^{14}C(U)-labelled sucrose, ^{14}C(U)-labelled glucose, or ^{14}C(U)-labelled fructose (Amersham Biosciences, UK) solutions buffered by 1 mM MES/KOH at pH 7. Of petioles without leaf blades, the apical cut end was sealed with paraffin to prevent drying. Uptake was carried out under standard light conditions (see Exudation experiments). After 40 min, sugar uptake through the basal cut surface was measured by dispensing two 0.3 ml samples of the uptake media into vials filled with 4.5 ml Aquasafe scintillation fluid (Zinsser Analytic, Maidenhead, UK). In addition, leaf discs (10 mm in diameter, 13–17 mg in weight) and the shredded apical and basal 1 cm of the petioles were destructed in 1 ml of a solubilizer (Irgasolv Tissue Solubilizer; CIBA-Geigy, Basel Switzerland) overnight at 40 °C and mixed with 4.5 ml Aquasafe scintillation fluid. All probes were measured in a scintillation counter (Canberra Packard, Meriden, CT, USA).

Visualization of microbial contamination of the petiole tissue

Microbial contamination sitting on the petioles of *Eranthis hyemalis*, *Corydalis cava*, and *Ranunculus ficaria* was visualized by dipping the excised petiole ends for 3 min in a 1% methylene blue solution. Stained objects on the epidermis were observed under a microscope (Leica DMBL; Leica, Wetzlar, Germany) at a magnification of ×400 and ×630 (objectives Leica NPLAN ×40/0.65, Leica PL APO ×63/1.20 W CORR, respectively).

Results and discussion

Impact of EDTA on phloem exudation

In a first set of experiments, the efficiency of the Ca^{2+}-chelator EDTA as a means of maintaining phloem exudation was investigated. EDTA-concentrations as high as 20 mM have been reported to leave phloem exudation (King and Zeevaart, 1974) and phloem loading (Urquhart and Joy, 1981) unaffected. Since high concentrations may have deleterious effects on plasma membrane structure (Pate, 1980), it is important to search for the lowest effective concentration of EDTA. According to concentration plots, 5.0 mM EDTA is a maximally effective and a relatively harmless concentration (Groussol *et al*., 1986). Therefore, 5.0 mM EDTA has been applied in more recent exudation studies (Girousse *et al*., 1991; Weibull *et al*., 1991; van Bel *et al*., 1994; Olesinski *et al*., 1996; Almon *et al*., 1997). Lower EDTA-concentrations have not been tested thus far (Groussol *et al*., 1986). To assess the lowest effective concentration with the slightest damage, phloem exudation was compared in the presence of 2.5 mM or 5.0 mM EDTA (Table 1). An arbitrary choice of the species investigated (Table 1) shows that, with a few exceptions (e.g. *Podophyllum hexandrum*), the exudation rates were in the same range with a tendency to a better exudation at 5.0 mM EDTA. However, 2.5 mM EDTA solutions were used in the subsequent experiments in order to minimize harmful EDTA effects, since the exudation yield was sufficient for reliable HPLC measurements of sugars (Table 1).

Carbohydrate exudation from intact leaves, measured by placing cut petioles into a medium with and without 2.5 mM EDTA (Fig. 1), was compared for species with major differences in phloem sugar composition and mode of phloem loading (e.g. van Bel *et al*., 1994; Flora and Madore, 1996). It appears that carbohydrate exudation into the control medium (without EDTA) virtually stopped in most species (Fig. 1). In a few species, however, exudation into the EDTA-free medium was not fully inhibited, indicative of some spontaneous phloem bleeding (*Consolida regalis*, *Digitalis purpurea*, *Lythrum salicaria*, and *Nigella arvensis*; Fig. 1).

The sharp decline in carbohydrate exudation into EDTA-free media in many species (Fig. 1) is ascribed to a large amount of sugar leakage from the cut petiole surface to the first exudation sample during the first 15 min incubation period after petiole excision. Thus, only exudation samples collected between 2 h and 6 h after excision actually represent phloem sap composition.

Table 1. *Average exudation rates of sieve-tube carbohydrates (mg l^{-1}) from the petioles of several monocotyledons and dicotyledons arbitrarily selected for presentation*

The samples (the numbers are given in parenthesis after the species name) were collected in the 2nd or 3rd hour of exudation in media containing 2.5 or 5.0 mM EDTA, pH 7.0.

	Exudation treatment	
	2.5 mM	5.0 mM
Aristolochia macrophylla (6)	16.5	26.4
Canna carnea (6)	5.1	2.9
Corydalis cava (5)	4.0	6.0
Fumaria officinalis (4)	5.0	4.8
Helleborus sp. (2)	4.3	0.9
Iris germanica (6)	0.9	7.8
Podophyllum hexandrum (6)	2.3	98.7
Ranunculus ficaria (18)	11.4	10.7
Urtica dioica (6)	7.5	17.6

In the species investigated, appreciable EDTA-induced exudation of carbohydrates was maintained during the entire collection period of 6 h, with the exception of a very low yield for *Valeriana officinalis* (Fig. 1). The exudation profiles were consistently species-dependent with both increasing (14 species) and decreasing (six species) carbohydrate exudation trends observed during a 6 h period (Fig. 1).

Effect of the medium pH on the exudation

Subsequently, the effect of medium pH on the exudation rates was measured. The medium pH may have a major impact given the exceptional pH of the phloem sap. Moreover, the apparent carbohydrate composition of phloem sap has been linked to pH-dependent invertase activity (Groussol *et al.*, 1986) in the exudation medium. In general, exudation profiles and carbohydrate composition were similar at pH 5 and pH 7 (see Figs S1, S2 in the Supplementary data at *JXB* online), but the exudation performance was distinctly higher at pH 7 for all species with the exception of *Consolida regalis* (see Figs S1, S2 in the Supplementary data at *JXB* online) and *Nigella arvensis* (see Fig. 2 and Fig. S2 in the Supplementary data at *JXB* online). Therefore, all subsequent experiments were carried out using an exudation medium buffered at

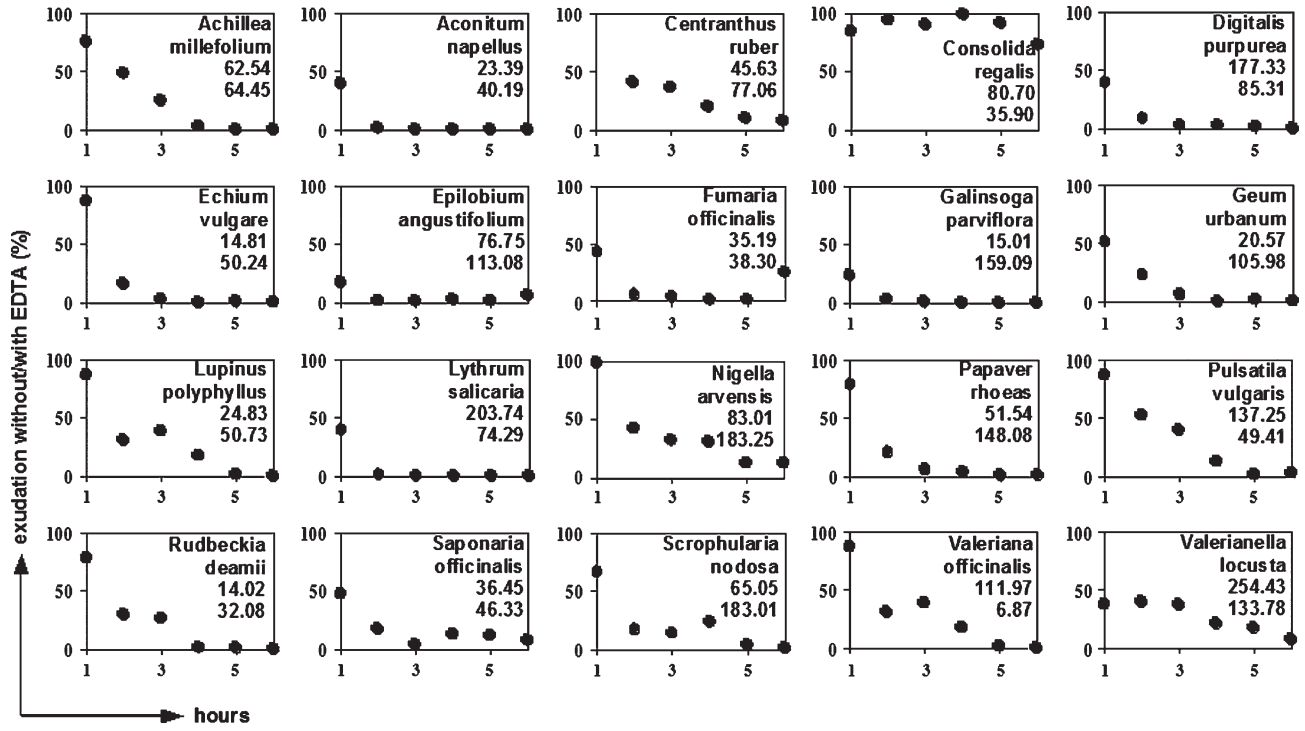

Fig. 1. Effect of 2.5 mM EDTA on carbohydrate exudation from the petioles of excised leaves of 20 herbaceous dicotyledonous species in comparison to the absence of EDTA. The graph represents the percentage of carbohydrate exudation into EDTA-free (pH 7) as compared with the exudation into EDTA-containing pH 7 media (*y*-axis) during 6 h of exudation after excision (*x*-axis). At the right-hand side of each figure, the total amount of carbohydrates (mg l^{-1}) of the first (1st-hour) and last exudation (6th-hour) sample are inserted. At least two replicates were performed for each species.

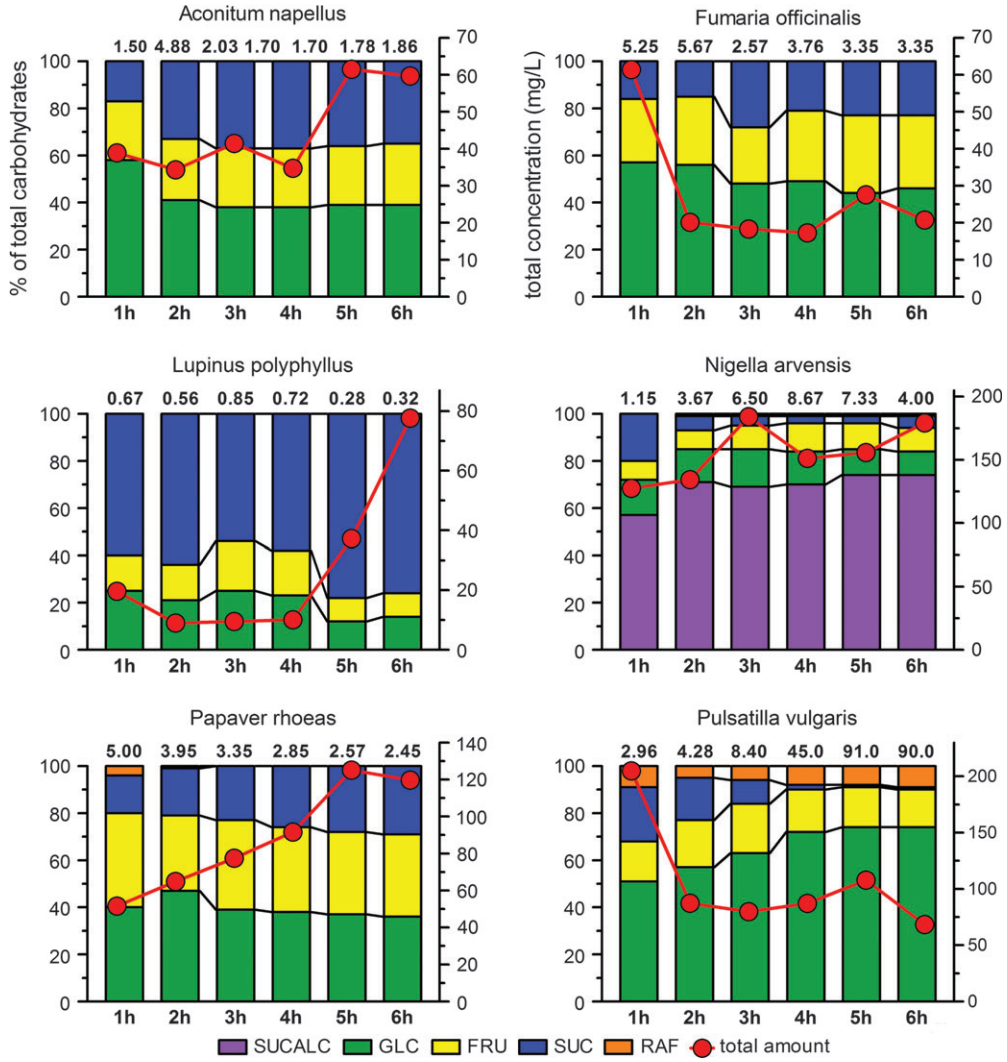

Fig. 2. Time-related carbohydrate exudation profiles from the petioles of excised leaves of six herbaceous dicotyledonous species. The bars represent the relative carbohydrate composition (left *y*-axis, % of total carbohydrate). Samples exuded into media with pH 7.0 and 2.5 mM EDTA were collected every hour. The numbers on top of each bar represent the hexose:sucrose ratios in the respective exudates samples. The identity of sugar alcohols is not absolutely certain in all cases. The red circles represent the total amount of carbohydrates in each single exudate sample (right *y*-axis, total concentration, mg l^{-1}). At least two replicate experiments were performed for each species.

pH 7. Why the exudation performance is better at pH 7 (cf. Groussol *et al*., 1986) remains unclear; the most obvious explanation is a higher degree of Ca^{2+}-chelation at pH 7, possibly due to better accessibility of the EDTA carboxyl groups.

The amounts of carbohydrate exuded are relatively stable from the second hour on, but exhibit certain trends in exudation yield (Fig. 1). Time-dependent species-specific upward or downward exudation tendencies (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online) are mostly identical to those shown before (Fig. 1).

The conclusion that exudates collected after 1 h have an origin different from that of the 2nd- to the 6th-hour samples (Fig. 1) was corroborated by carbohydrate analyses (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online). The divergent sugar composition of the 1st-hour sample in most species seems to confirm that the 2nd- to 6th-hour samples (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online) actually reflect the phloem sap composition.

As expected (cf. Zimmermann and Ziegler, 1975), the carbohydrate composition of exudates was highly species-specific and showed a variety of phloem translocates both at pH 5 and pH 7 (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online). Typical sucrose transporters (*Achillea millefolium*, *Echium vulgare*, Fig. S1 in Supplementary data; *Galinsoga parviflora*, *Lupinus polyphyllus*, Fig. 2 and Fig. S1 in Supplementary data; *Dahlia* hybrid, *Rudbeckia deami*, Table 2), species mainly transporting raffinose-related sugars (*Catharanthus roseus*,

Table 2. *Hexose:sucrose ratios in phloem exudates (first column) and leaf tissues of 24 dicotyledonous herbs*

Exudation from *Fumaria* leaves was carried out in spring and autumn. The ratios were calculated from the amounts of carbohydrate in 2nd- to 6th-hour exudation samples collected from cut leaf petioles (second column). From the same samples, the contribution of hexoses (glucose and fructose) and sucrose to the total carbohydrate content was calculated for each species (third column). The other carbohydrates identified are listed in the fourth column. In addition, hexose:sucrose ratios in ground leaf tissues (leaf extracts) are presented in the fifth column. The sixth column lists the corresponding plant families. Sugar alcohols were not all identified with absolute certainty. The number of experiments is given in parenthesis. RAF: raffinose; SUGALC, sugar alcohols; STA, stachyose.

	Hexose:sucrose ratio in exudates	Contribution of hexoses and sucrose to carbohydrates identified (%)	Other transport carbohydrates identified	Hexose:sucrose ratio in leaves	Family
Late winter/early spring					
Corydalis cava	4.1 (18)	95–98	RAF+STA	1.57 (4)	Papaveraceae
Fumaria officinalis	6.2 (18)	89–92	RAF	0.46 (5)	Papaveraceae
Primula veris	0.84 (12)	80–95	RAF+STA	1.98 (3)	Primulaceae
Anemone nemorosa	10.0 (24)	100		1.71 (8)	Ranunculaceae
Eranthis hyemalis	5.2 (6)	88–92	RAF+STA	1.93 (9)	Ranunculaceae
Ranunculus ficaria	4.2 (6)	100		1.12 (8)	Ranunculaceae
Late summer/early autumn					
Dahlia hybrid	0.13 (10)	100		1.13 (2)	Asteraceae
Galinsoga parviflora	0.71 (10)	100		6.2 (2)	Asteraceae
Rudbeckia deamii	0.32 (5)	100		0.16 (1)	Asteraceae
Echium vulgare	0.42 (15)	100		1.69 (5)	Boraginaceae
Saponaria officinalis	0.88 (15)	100		0.61 (3)	Caryophyllaceae
Lupinus polyphyllus	0.41 (15)	100		0.39 (3)	Fabaceae
Lythrum salicaria	9.5 (15)	40–65	SUGALC+RAF	4.7 (3)	Lythraceae
Fumaria officinalis	5.4 (34)	100		1.85 (8)	Papaveraceae
Papaver rhoeas	4.9 (15)	96–100	RAF	1.49 (3)	Papaveraceae
Digitalis purpurea	6.2 (25)	50–70	SUGALC	2.82 (5)	Plantaginaceae
Phlox paniculata	1.75 (10)	90	RAF	0.60 (2)	Polemoniaceae
Aconitum napellus	4.8 (30)	100		0.77 (6)	Ranunculaceae
Consolida regalis	9.2 (15)	5–10	SUGALC	1.41 (3)	Ranunculaceae
Nigella arvensis	5.7 (15)	15–50	SUGALC	1.63 (3)	Ranunculaceae
Pulsatilla vulgaris	21.0 (29)	100		5.4 (6)	Ranunculaceae
Scrophularia nodosa	0.71 (15)	40–50	RAF+SUGALC	1.15 (3)	Scrophulariaceae
Centranthus ruber	0.94 (10)	100		0.75 (2)	Valerianaceae
Valeriana officinalis	2.33 (10)	100		2.99 (2)	Valerianaceae
Valerianella locusta	1.26 (5)	55	RAF+SUGALC	1.26 (1)	Valerianaceae

Epilobium angustifolium; results not shown), species having preferential phloem transport of sugar alcohols (*Consolida regalis*, *Lythrum salicaria*, Fig. S1; *Nigella arvensis*, Fig. 2), and species having appreciable amounts of hexoses in the phloem sap (e.g. *Aconitum napellus*, *Fumaria officinalis*, *Papaver rhoeas*, *Pulsatilla vulgaris*, Fig. 2) were found.

Taken together, the present observations seem to indicate that EDTA-facilitated exudation is a valid experimental method for collecting pure phloem sap and lend support to the view that exudates collected from the 2nd hour on largely represent the natural composition of phloem sap.

Abundance of hexoses in the phloem sap of several species

The amount and composition of the carbohydrates in the exudates strongly varied between the species (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online) due to differences in phloem sugar concentration and the size of the leaves. For a good comparison of the hexose contribution between samples and between species, the hexose:sucrose ratio [mg l^{-1} (glucose+fructose)/mg l^{-1} sucrose) was calculated for each sample (Fig. 2; Figs S1, S2 in the Supplementary data). In particular, representatives of the Ranunculaceae exhibited an abundance of hexoses in the exudates. The hexose:sucrose ratio in the exudates of *Aconitum napellus* and *Pulsatilla vulgaris* is far over 1.00, which indicates a predominance of hexoses (Fig. 2). Moreover, in two other Ranunculaceae, *Consolida regalis* (Figs S1, S2 in the Supplementary data) and *Nigella arvensis* (Fig. 2; Fig. S2 in the Supplementary data), the contribution of mono- and disaccharides to the entire carbohydrate content is relatively low, but the hexose:sucrose ratios are high.

An extended data set (Table 2) reveals a sheer dominance of hexoses in the phloem sap of most Ranunculaceae. The hexose:sucrose ratios in the exudate of seven representatives of the Ranunculaceae vary between 4.21 (*Ranunculus ficaria*) and 20.95 (*Pulsatilla vulgaris*) (Table 2). In conclusion, the contribution of hexoses to

phloem-sap carbohydrates is >80% in several Ranunculaceae (Fig. 2, Table 2).

Representatives of the Papaveraceae, (Table 2), *Corydalis cava*, *Papaver rhoeas*, and *Fumaria officinalis*, also show hexose:sucrose ratios between 4.07 and 6.16 (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online; Table 2), which is equivalent to a hexose contribution between 80% and 86% as for the non-RFO sugars. Furthermore, other species scattered throughout dicotyledonous families (e.g. *Digitalis purpurea*, 6.19; *Phlox paniculata* 1.75; *Valeriana officinalis*, 2.33; *Valerianella locusta*, 1.26) also exhibit hexose:sucrose ratios which seem to exclude potentially artefactual levels of hexoses in the exudates (Table 2). For *Fumaria officinalis*, the only species examined here that is growing and flowering both in spring and early autumn, the season did not exert an effect on the hexose:sucrose ratio (Table 2).

By contrast, a number of other species showed low hexose:sucrose ratios in the exudate (Table 2) in agreement with the high sucrose concentrations in phloem sap reported for families such as Asteraceae and Fabaceae (Zimmermann and Ziegler, 1975). The results indicate that EDTA-mediated phloem exudation yielded a species-specific variation of sugar composition similar to that obtained with other techniques (Zimmermann and Ziegler, 1975; Flora and Madore, 1996).

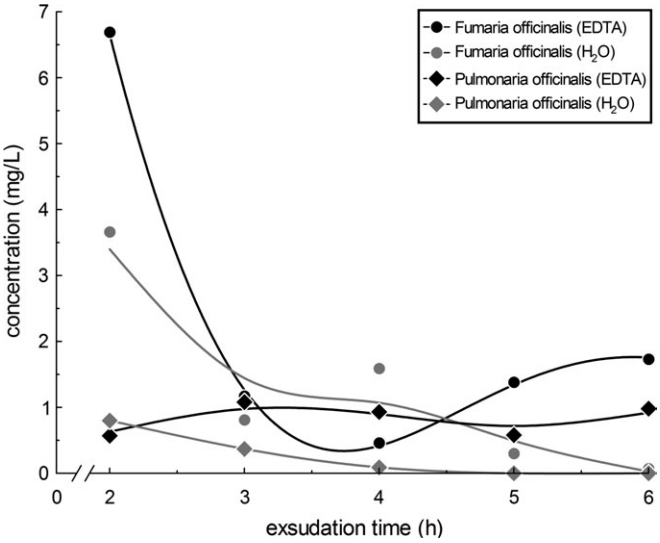

Fig. 3. Effect of EDTA on the amounts of carbohydrates in exudates from blade-less petioles of *Pulmonaria officinalis* and *Fumaria officinalis* into 1 mM MES/KOH (pH 7) solutions between the 2nd and 6th hour after the start of the exudation. Exudate samples collected after 1 h are omitted. Symbols and lines: black, 2.5 mM EDTA; grey, controls without EDTA.

Hexose:sucrose ratios in exudates and leaf blades

The strikingly high amounts of hexoses in the exudates of several species (Fig, 2; Figs S1, S2 in the Supplementary data at *JXB* online, Table 2) did not conform to the general belief that phloem transport of hexoses is virtually non-existent and physiologically undesirable. Therefore, much effort was invested in establishing that exudation samples really reflect phloem sap composition (Figs 3, 4; Tables 2–4).

A strong argument to presume sucrose to be the predominant carbohydrate translocate is the selective accumulation of sucrose by sieve tubes (von Schaewen *et al*., 1990; Dickison *et al*., 1991; Sonnewald *et al*., 1991; Heineke *et al*., 1992; Riesmeier *et al*., 1993, 1994). The apparent incapability to phloem loading of hexoses in the few species studied has led to the opinion that hexoses cannot occur in phloem sap. According to this concept,

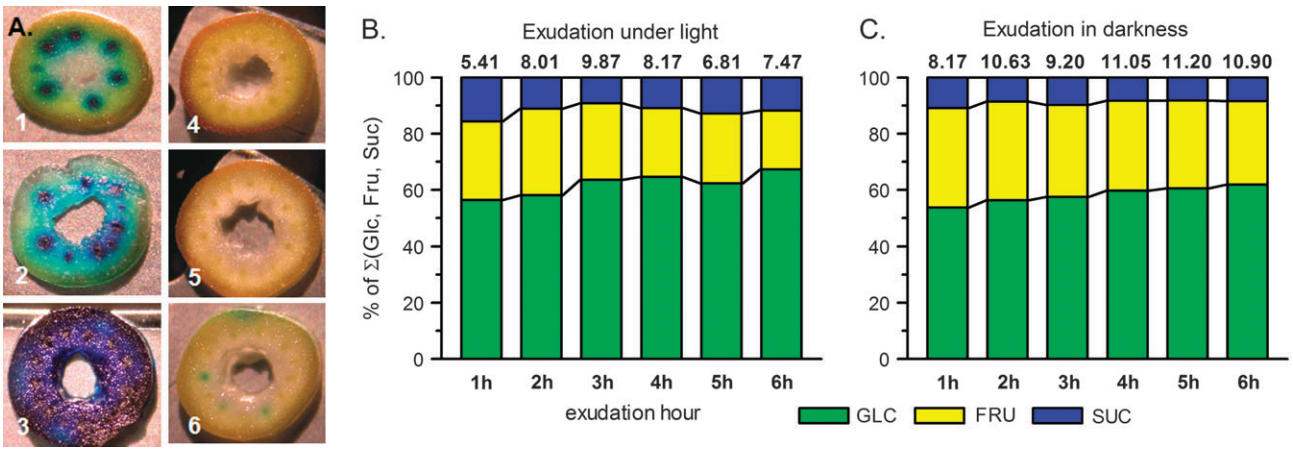

Fig. 4. Effect of EDTA on hexose:sucrose ratios in the exudate of *Eranthis hyemalis*. (A) Cross-sections of petioles of *Eranthis* at about three-quarters (pictures 1, 4), half (pictures 2, 5), and one-quarter (pictures 3, 6) petiole length counted from the blade insertion. The cut ends of the petioles had been placed in a 0.3% methylene blue solution under light (pictures 1–3) and darkness (pictures 4–6) for 6 h. (B, C) The percentage of carbohydrate composition in successive 2.5 mM EDTA-containing samples (pH 7) under light (B) and darkness (C) collected during 6 h of exudation. The numbers on top of each bar are the hexose:sucrose ratios per sample.

Table 3. *Sucrose-to-hexose conversion during exudation expressed as loss of sucrose after 6 h (%)*

Petioles with leaf blades were placed in exudation fluid (2.5 mM EDTA, pH 5 or 7, 1 mM MES/KOH). After a collection period of 1 h (1st-hour exudation samples), the leaves were transferred for another hour to other test tubes with the identical exudation medium (2nd-hour samples). The test tubes then contain a mixture of sugars and potentially sucrose-degrading enzymes released from the cut petiole. This approach allowed '*in vitro*' quantification of sucrose breakdown rates in exudates without the further input of materials from the petioles. Sucrose conversion by sucrose-degrading enzymes was monitored during the subsequent 6 h. Data represent the loss of sucrose after 6 h as the percentage of the sucrose present at the start of the '*in vitro*' experiment both in 1st- and 2nd-hour samples. The number of experiments is given in parenthesis after each species name.

	pH			
	7		5	
	1st hour	2nd hour	1st hour	2nd hour
Anemone nemorosa (4)	12.6	0.8	0.0	9.7
Corydalis cava (3)	22.5	2.8	16.0	17.9
Eranthis hyemalis (1)	13.8	0.0	15.8	0.0
Fumaria officinalis (1)	21.0	9.6	1.9	0.0
Primula veris (1)	15.2	8.7	8.6	17.5
Ranunculus ficaria (2)	10.0	0.5	0.0	2.9
Vicia faba (5)	7.5	2.2	n.d.	n.d.

Table 4. *Uptake rates (in μg h^{-1}) of ^{14}C-labelled (0.1 or 1.0 mM) glucose, fructose, and sucrose by excised blade-less petioles and by excised petioles with leaf blades of* Eranthis hyemalis, Ranunculus ficaria, *and* Corydalis cava

Solutes were taken up via the cut surface during 40 min. The uptake data represent the amounts of sugar accumulated by the petiole with leaf blade or by the petiole only, respectively.

	Petioles with leaf blades		Blade-less petioles	
	Concentration applied (mM)		Concentration applied (mM)	
	1.0	0.1	1.0	0.1
Eranthis hyemalis (μg h^{-1})				
Glucose	27.57	1.65	4.05	0.19
Fructose	19.49	1.70	3.43	0.38
Sucrose	41.13	3.54	7.50	0.77
Ranunculus ficaria (μg h^{-1})				
Glucose	10.65	1.00	3.59	0.27
Fructose	6.81	0.78	2.19	0.24
Sucrose	15.92	1.64	4.00	0.46
Corydalis cava (μg h^{-1})				
Glucose	26.03	2.16	13.52	1.65
Fructose	18.52	2.62	9.41	1.51
Sucrose	37.58	2.62	24.03	2.98

mesophyll hexoses must be converted into sucrose on their way to the sieve element/companion cell complexes in order to get access to the sieve tubes.

Yet, it is doubtful if an apparent incapability of phloem loading of hexoses fully excludes the possibility of hexose accumulation. In *Ricinus*, where sucrose is the sole carbohydrate in phloem bleeding sap under natural conditions, large amounts of hexoses are transported when an excess of hexoses is supplied to the bare cotyledons of seedlings (Kallarackal and Komor, 1989). Thus, several species may have a concealed capacity to hexose loading or have not been tested properly with regard to hexose loading. As a second explanation for the paradox between the incapability to load hexose and an abundant presence of hexose in the phloem sap, companion cells convert sucrose loaded at the vein termini into hexoses along the phloem-loading pathway (Nolte and Koch, 1993). In this frame, it is worth noting that the monosaccharide level of the phloem sap varies with the site of collection (Richardson and Baker, 1982).

In sucrose-transporting species, sucrose accumulation at the vein termini renders the hexose:sucrose ratios to be higher in leaf extracts than in phloem exudates (Schrier, 2001). In reverse, higher hexose:sucrose ratios in exudates than in leaf extracts would lend credibility to the view that hexoses act as a prominent phloem translocate. Therefore, hexose:sucrose ratios were compared between leaf blade extracts and phloem exudates (Table 2).

In the seven species of the Ranunculaceae investigated, the hexose:sucrose ratios in exudates are three to six times higher than those in leaf tissues (Table 2). This holds both for spring species (*Anemone nemorosa*, *Eranthis hyemalis*, *Ranunculus ficaria*) as well as for summer/autumn species (*Aconitum napellus*, *Nigella arvensis*, *Pulsatilla vulgaris*). Similar ratio differences between leaf extract and phloem exudate also emerge in three papaveracean species (*Fumaria officinalis*, *Corydalis cava*, *Papaver rhoeas*) and in a few species such as *Digitalis purpurea*, *Phlox paniculata*, and *Lythrum salicaria* scattered throughout several plant families (Table 2). It should be noted that the few representatives from sucrose-transporting families tested (van Bel *et al*., 1994; Schrier, 2001) show low hexose:sucrose ratios (Table 2). All in all, presumptive hexose-translocating species possess higher hexose:sucrose ratios in exudates than in leaves.

Does the presence of hexoses in phloem exudates result from the activity of sucrose-degrading enzymes?

Occurrence of hexoses in the phloem sap has been discredited in the past by claims that hexoses in sieve-tube exudates are artefacts induced by experimental approaches. These contentions are formulated as heading questions and checked.

One of the potential sources of artefactual hexoses is the activity of sucrose-degrading enzymes released from the petiole cutting surface into the exudate (Richardson and Baker, 1982; Groussol *et al*., 1986; Girousse *et al*., 1991). Surprisingly little research has been done on this point and, hence, there is hardly solid evidence in favour

of sucrose breakdown by invertase or other sucrose-degrading enzymes in the exudates (Groussol *et al*., 1986).

In order to track breakdown of sucrose in exudates, excised leaves were removed from exudation test tubes after 1 h (1st-hour exudates) and transferred to a second set of test tubes from which the leaves were removed again after 1 h (2nd-hour exudates). Breakdown of sucrose in the 1st-hour and 2nd-hour exudates remaining in the test tubes was monitored by taking hourly samples during the following 6 h. The rationale is that time-dependent loss of sucrose quantifies the activity of sucrose-degrading enzymes released from the petiole into the exudation medium. This approach allows a quantitative estimate of the sucrose breakdown in the exudation experiments. Breakdown of innate sucrose by sucrose-degrading enzymes was measured at pH 5 and pH 7 in view of the potential pH-dependence of sucrose-degrading enzymes.

This approach revealed some degree of sucrose degradation in the exudation medium for almost all species, albeit that the rates of sucrose breakdown strongly differed between species and pH conditions (Table 3). The breakdown rate of sucrose in the 1st-hour exudates is higher at pH 7, whereas the opposite seems to hold for 2nd-hour exudates (Table 3). Our provisional interpretation is that the sucrose-degrading activities in the 0–1 h samples and in the 1–2 h samples may be of a dissimilar origin.

What is the rate of sucrose degradation under the present experimental conditions?

In a simplified approach, the hexose:sucrose ratio produced by sucrose hydrolysis (y) is calculated from the percentage of the breakdown of sucrose (x) present in the exudates at the start of the breakdown experiments (Table 3). The formula $y=2x/(100–x)$ takes into account that breakdown of one sucrose molecule results in the production of two hexose molecules and starts from the assumption that the breakdown is an approximately linear process. In none of the species did sucrose breakdown exceed 22.5% of the initial amount of sucrose (*Corydalis cava*; Table 3). Starting from a 100% sucrose exudate, a sucrose breakdown of 22.5% after 6 h would result in a hexose:sucrose ratio of 0.41 in the exudate according to the above formula. However, sucrose is only degraded for just 1 h in exudation experiments (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online), so that the actual breakdown rates must be considerably lower. The maximal breakdown rate of 4% per hour (22.5%/6 = ~4%, *Corydalis cava*; Table 3) is equivalent to hexose:sucrose ratios of about 0.08. As far as is known, the only publication on sucrose degradation in exudates (Groussol *et al*., 1986) reported a sucrose breakdown of 7% in 4 h in alfafa (which is equivalent to 10.5% after 6 h leading to an hexose:sucrose ratio of 0.23). This breakdown rate is in the order of the values found in this study (Table 3) for sucrose-translocating species.

To correct potential background errors in the calculations, an arbitrary 10-fold 'security zone' has been adopted. This implies that phloem-exudate hexose:sucrose ratios over 0.10 (*Corydalis cava*; Table 3) are not taken as evidence for hexose translocation. Instead, hexose:sucrose ratios of 1.0 are taken as the limit value that cannot possibly be attributed to sucrose breakdown. Hence, hexose:sucrose ratios above 1.0 are believed to result from high native hexose concentrations in phloem sap. Collectively, sucrose degradation rates (Table 3) and hexose:sucrose ratios (Table 2; Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online) suggest that Ranunculaceae and Papaveraceae translocate high proportions of hexoses via the phloem. Furthermore, phloem sap in members of several other dicotyledonous families (Table 2) must contain appreciable amounts of hexoses.

Are hexoses in the exudate due to cellular leakage induced by EDTA?

Another critical point in these experiments is the effect of EDTA on plasma membrane functioning. It is conceivable that EDTA either weakens membrane function at the cut surface or creeps into the petiole driven by transpiration during the exudation period and attacks the membranes of cells along the transport pathway. The first may lead to hexose release directly into the exudation medium. The second to unspecific release of carbohydrates into the sieve tubes along the vascular pathway, resulting in an increased hexose level in the exudate.

In order to investigate harmful EDTA effects, carbohydrate release from the cut surface of blade-less petioles into an exudation medium with or without 2.5 mM EDTA was monitored first. Leaf blades were removed to knock-out the influence of transpiration and the supply of carbohydrates from the leaf blade. With the hexose translocator *Fumaria officinalis*, carbohydrate release into the exudation medium was doubled by EDTA (aggregated 11.07 mg l^{-1} versus 6.15 mg l^{-1}; Fig. 3). Presence of EDTA tripled carbohydrate release from the cut surface in the sucrose translocator *Pulmonaria officinalis* (aggregated 3.90 mg l^{-1} versus 1.26 mg l^{-1}). Since carbohydrate release did not increase in the course of EDTA treatment (Fig. 3), EDTA does not seem to exert a deleterious effect on the surface cells. The putative stimulus of carbohydrate release is ascribed to the usual chelation effect of EDTA on sieve tube exudation.

In view of EDTA-translocation in the transpiration stream, potential EDTA-induced leakage from cells along the transport pathway was investigated in intact leaves. Application of 0.3% methylene blue solutions under standard light conditions showed that the exudation medium indeed creeps into the petiole of *Eranthis*

hyemalis despite 100% relative humidity in the exudation chamber (Fig. 4A, pictures 1–3). Methylene blue uptake was much reduced in darkness (Fig.4A, pictures 4–6). Consequently, time-dependent deleterious EDTA effects would be much more visible in light in view of the larger contact surface with EDTA. Therefore, a higher EDTA-induced release of hexose or/and sucrose-degrading enzymes would increase hexose:sucrose ratios in the exudates in the light as compared with darkness. However, hexose:sucrose ratios were similar in exudates collected under light and darkness (Fig. 4B, C). Slightly higher hexose:sucrose ratios in darkness conflict with any positive effect of EDTA on hexose:sucrose ratios (Fig. 4B, C). In conclusion, EDTA-effects on hexose:sucrose ratios seem to be negligible in the exudation experiments (Fig. 2; Figs S1, S2 in the Supplementary data at *JXB* online).

Although EDTA effects were often employed as an argument against the validity of exudation experiments, the burden of proof is weak and mainly founded on circumstantial evidence obtained with other approaches. Collection of phloem sap from cut aphid stylets invariably provided evidence that sucrose is the sole carbohydrate compound of phloem sap in several grasses (Fukumorita and Chino, 1982; Hayashi and Chino, 1990; Ohsima *et al*., 1990), a few legumes (Barlow and Randolph, 1978; Girousse *et al*, 1991), and Scrophulariaceae (Knop *et al*., 2001). In the bleeding sap of *Ricinus communis*, too, sucrose was virtually the only carbohydrate component (Kallarackal and Komor, 1989). Hence, absence of hexoses in exudates acquired without chemical manipulation was taken as conclusive evidence for the general absence of hexoses in phloem sap. As the presence of hexoses in exudates collected using EDTA was interpreted as an artefact, the sole presence of sucrose was even taken as proof for collection of pure phloem sap (Kallarackal and Komor, 1989; Girousse *et al*, 1991).

Yet, documentation with regard to the artefactual character of hexoses is poor and equivocal. Comparative analysis of phloem sap collected by stylets and EDTA mediation showed a considerable shift in amino acid composition in *Medicago sativa* (Girousse *et al*., 1991). This shift was absent in *Hordeum vulgare* and *Avena sativa* (Weibull *et al*., 1991). For alfafa, 10% hexoses and 90% sucrose were detected in EDTA exudates, while sucrose was the only phloem component in stylet exudates (Girousse *et al*., 1991). The difference in exudation time in 5.0 mM EDTA treatments probably explains the discrepancy between the results for the cereals (1.5 h) and for alfalfa (22 h). EDTA has a fair chance of imposing changes on membranes and metabolism during an exudation period of 22 h (Girousse *et al*., 1991). As in the present experiments much shorter EDTA treatments (Weibull *et al*., 1991) may not dramatically alter the original phloem composition.

Are hexoses in the exudate due to selective resorption via the cut surface?

Selective resorption through the cut surface may also influence exudate composition. High hexose contents could result from intense, selective resorption of sucrose as compared with hexoses. This possibility was tested in the following series of experiments. Uptake of 0.1 and 1.0 mM [^{14}C]glucose, [^{14}C]fructose, and [^{14}C]sucrose by blade-less and blade-bearing petioles of *Eranthis hyemalis*, *Corydalis cava*, and *Fumaria officinalis* was compared. Although the distribution profiles were unique for each single species (Table 4), species-specific uptake rates by blade-less petioles did not show significant differences between the sugars when the differences in molecular weight are taken into account (Table 4). The expectedly higher, transpiration-sustained sugar uptake rates by blade-bearing petioles were also similar for each single species (Table 4). Thus, the uptake results contradict prevalent resorption of sucrose by petiole tissues and exclude a causal relationship between selective resorption and high hexose:sucrose ratios.

Are hexoses in the exudate due to microbial infection?

Differential uptake of sugars or cleavage of sucrose by micro-organisms on the petioles may also affect the sugar composition of the exudate. Following application of methylene blue, the epidermal coverage by fungal spores, short fungal hyphae, and bacteria was <3% at the start of exudation and even lower at the end of the exudation period for the petioles of *Erantis hyemalis*, *Consolida regalis*, and *Ranunculus ficaria* according to microscopic screening (results not shown). The low coverage and propagation rates, possibly suppressed by the presence of EDTA, seem to exclude that differential uptake of sugars by micro-organisms affected sugar composition of exudates.

Concluding remarks

There seems to be a number of reasons why the overwhelming presence of hexoses in phloem sap of several species has not been discovered before:

(i) As the principal reason, herbaceous families such as Ranunculaceae, Papaveraceae, and Scrophulariaceae were not or hardly investigated in the comprehensive study of Zimmermann and Ziegler (1975) and several prior studies on carbohydrate composition of phloem sap.

(ii) The bark exudation technique applied in the above-mentioned study mostly only functions with woody species. In general, trees and shrubs do not seem to translocate hexoses, although this view may also need to be revised. In *Sambucus nigra* phloem sap collected by using EDTA, 76% of the carbohydrates

were found in the form of sucrose and 24% as hexoses. The presence of hexoses is unlikely to be an artefact given the fact that hexoses still made up 16% of the carbohydrates in stylet exudates obtained with the aphid *Aphis sambuci* (AJE van Bel, A Biehl, unpublished data).

(iii) Collection of phloem exudates via aphid stylets was limited for the major part to gramineous (wheat, Hayashi and Chino, 1986; rice, Hayashi and Chino, 1990; maize, Ohsima *et al*., 1990; oats and barley, Weibull *et al*., 1991) and leguminous (alfafa, Girousse *et al*., 1991) crops, plant groups reputed for sucrose transport.

(iv) The few direct comparisons between aphid- and EDTA-mediated phloem exudation produced inconclusive results (Girousse *et al*, 1991; Weibull *et al*., 1991), but were interpreted as an argument against the use of EDTA.

(v) Studies conferring the absence of hexose carriers in transgenic plants pertained to solanacean crops (potato, tomato, tobacco) which predominantly translocate sucrose.

(vi) Effects of extracellular invertase in transformants (von Schaewen *et al*., 1990; Dickison *et al*., 1991; Sonnewald *et al*., 1991; Heineke *et al*., 1992) hint at a role of sucrose-degrading enzymes in exudates, a claim hardly verified (Groussol *et al*., 1986).

Hexoses may be accumulated and transported by sieve tubes in many more species than the restricted number reported here. It is important to note that hexose translocation may not be limited to a few families, but may occur dispersed through the angiosperm plant families, preferably in herbs. Hexose translocation should not be regarded as an exception, a coincidence or an artefact, but rather as a mode of carbohydrate transfer by the phloem equivalent to the translocation of sucrose, raffinose-family oligosaccharides, or sugar alcohols.

Supplementary data

The following data are available in the online version of this article:

Fig. S1. Exudation patterns and quantities at pH 7 as an extension of Fig. 2.

Fig. S2. Exudation patterns and quantities at pH 5.

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