Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Identification of lipids and lipid-binding proteins in phloem exudates from *Arabidopsis thaliana*

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Abstract

The phloem plays a crucial role in assimilate and nutrient transport, pathogen response, and plant growth and development. Yet, few species have yielded pure phloem exudate and, if proteins need to be analysed, those species may not have sequenced genomes, making identification difficult. The enrichment of *Arabidopsis thaliana* phloem exudate in amounts large enough to allow for metabolite and protein analysis is described. Using this method, it was possible to identify 65 proteins present in the *Arabidopsis* phloem exudate. The majority of these proteins could be grouped by response to pathogens, stress, or hormones, carbon metabolism, protein interaction, modification, and turnover, and transcription factors. It was also possible to detect 11 proteins that play a role in lipid/fatty acid metabolism (aspartic protease, putative 3-β-hydroxysteroid dehydrogenase, UDP-sulphoquinovose synthase/SQD1, lipase, PIG-P-like protein: phosphatidylinositol-*N*-acetylglucosaminyltransferase), storage (glycine-rich protein), binding (annexin, lipid-associated family protein, GRP17/oleosin), and/or signalling (annexin, putative lipase, PIG-P-like protein). Along with putative lipid-binding proteins, several lipids and fatty acids could be identified. Only a few examples exist of lipids (jasmonic acid, oxylipins) or lipid-binding proteins (DIR1, acyl-CoA-binding protein) in the phloem. Finding hydrophobic compounds in an aqueous environment is not without precedence in biological systems: human blood contains a variety of lipids, many of which play a significant role in human health. In blood, lipids are transported while bound to proteins. The present findings of lipids and lipid-binding proteins in phloem exudates suggest that a similar long-distance lipid signalling exists in plants and may play an important role in plant growth and development.

Key words: Fatty acids, lipid-binding proteins, lipids, long-distance signalling, long-distance transport, phloem, phloem exudates, proteome.

Introduction

Plants have developed two long-distance transport systems: the xylem for water and mineral distribution from the roots to the remainder of the plant; and the phloem, which was originally thought to be responsible simply for the transport of assimilates. The latter has evolved to optimize longitudinal flow in the sieve elements by removing any obstacles in the form of organelles and by increasing the porosity of the cell walls at the longitudinal ends (van Bel and Knoblauch, 2000). While it is possible that sieve elements may still contain nuclei, vacuoles, and organelles during their early development, those cell components disintegrate, leaving the sieve elements with only the plasma membrane and a thin cytoplasm containing endoplasmic reticulum, phloem-specific plastids, and a few, often dilated, mitochondria (Shah and Jacob, 1969; Cronshaw, 1981; Behnke and Schulz, 1983; Behnke, 1991; van Bel and Knoblauch, 2000). No functional protein synthesis machinery is thought to exist. As a result, sieve tube proteins are assumed to be synthesized in the companion cells and transported into the sieve elements via plasmodesmata (Raven, 1991; Hayashi *et al.*, 2000). However, recent findings by Lin *et al.* (2009) suggest that at least some components of the protein synthesis machinery are present in pumpkin phloem sap, raising the possibility that limited translation may occur.

The view of the phloem function has evolved from that of simple assimilate transport to a trafficking system for pathogen response and developmental regulators (Citovsky and Zambryski, 2000; Ding *et al.*, 2003; Wu *et al.*, 2003; Haywood *et al.*, 2005). The phloem is essential for the transport of mineral nutrients, plant viruses, virus-induced silencing, defence and resistance against pathogen infection, and signalling of environmental conditions (Lucas *et al.*, 1995; Ryabov *et al.*, 1999; Burton *et al.*, 2000; Seo *et al.*, 2001; Yoo *et al.*, 2004; Suárez-López, 2005; Kehr, 2006; Lough and Lucas, 2006). It contains a multitude of compounds: small molecules (Chen *et al.*, 2001; Corbesier *et al.*, 2003), peptides and proteins (Fisher *et al.*, 1992; Sakuth *et al.*, 1993; Schobert *et al.*, 1995; Kühn *et al.*, 1997; Marentes and Grusak, 1998; Kehr *et al.*, 1999; Xoconostle-Cazares *et al.*, 1999; Haebel and Kehr, 2001; Hoffmann-Benning *et al.*, 2002; Giavalisco *et al.*, 2006; Lin *et al.*, 2009), and nucleic acids (Kühn *et al.*, 1997; Ruiz-Medrano *et al.*, 1999; Yoo *et al.*, 2004; Haywood *et al.*, 2005). Moreover, interactions between several proteins or RNA and proteins to facilitate or regulate phloem transport appear more and more likely (Xoconostle-Cazares *et al.*, 1999; Aoki *et al.*, 2002, 2005; Yoo *et al.*, 2004; Lee *et al.*, 2005; Kehr and Butz, 2008; Ham *et al.*, 2009). The contents of the phloem are now known to be so complex that phloem transport has been called the 'information superhighway' of plants (Jorgensen *et al.*, 1998). It is important for the signalling of environmental conditions as well as in the pathogen response of plants.

Many plant viruses have hijacked the phloem transport mechanisms to spread throughout the infected plant (Waigmann *et al.*, 2004; Gopinath and Kao, 2007). On the other hand, plants are able to show a systemic response to infection, which makes long-distance communication within the plant essential (Durrant and Dong, 2004; Truman *et al.*, 2007). Plant hormones such as systemin and jasmonic acid (JA), which elicit a pathogen response, and several pathogenesis-related proteins have been shown to be transported in the phloem in response to pathogen infection (Schillmiller and Howe, 2005; Truman *et al.*, 2007; Thorpe *et al.*, 2007; summarized in Lough and Lucas, 2006).

Similarly, the floral stimulus is formed in the leaves in response to an inductive photoperiod and translocated through the phloem to the apical meristem (Lang, 1965; Zeevaart, 1976). It was shown that the interaction between *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) in the plant is essential for the induction of flowering (An *et al.*, 2004; Ayre and Turgeon 2004) and that the FT protein in *Arabidopsis* and cucurbits and its analogue Hd3a in rice (Kojima *et al.*, 2002; Corbesier *et al.*, 2007; Lin *et al.*, 2007; Tamaki *et al.*, 2007) are the long-distance signal for flowering.

The difficulty in working with plant phloem lies in its capacity to seal itself upon wounding. Only in few plant species is it possible to obtain reasonable amounts of phloem exudate through cuts of the petiole (cucurbits; Balachandran *et al.*, 1997; Haebel and Kehr, 2001), shallow cuts or punctures in the stem or petiole (lupine; Marentes and Grusak, 1998; Hoffmann-Benning *et al.*, 2002; *Brassica*

napus; Giavalisco *et al.*, 2006), or by using aphids (Fisher *et al.*, 1992; Doering-Saad *et al.*, 2002; Walz *et al.*, 2002; Barnes *et al.*, 2004). The first two of the approaches mentioned above are not feasible due to the fragile nature of *Arabidopsis* inflorescence stems and petioles. Aphids introduce several proteins into the phloem with their saliva (Kehr, 2006; Will and van Bel, 2006). This leads to a limited transcriptional reprogramming (Thompson and Goggin, 2006) and the reduction in normal callose deposition and P-protein accumulation, and has the potential to change the phloem composition. For this reason, it was decided to use a method employing EDTA, which had been successfully used in other plants (King and Zeevaart, 1974; Hoffmann-Benning *et al.*, 2002). Phloem sap obtained in this way can be used to analyse proteins, small molecules, lipids, and RNAs (Guelette *et al.*, 2007; Deeken *et al.*, 2008).

With the analytical methodology becoming increasingly more sensitive, the complexity of the phloem exudates becomes even more apparent. The next logical step in the study of the function of phloem macromolecules is not only the establishment of comprehensive databases of vascular genes and phloem-mobile transcripts, proteins, and metabolites, but to understand the functions of the phloem macromolecules. This will help to better understand previously unknown long-distance transport and signalling pathways. The present study represents a further step towards the characterization of phloem-mobile proteins and metabolites. A successful attempt to collect *Arabidopsis* phloem exudate in large enough amounts to allow for the analysis of metabolites, proteins, and, to a lesser extent, RNA, is described. While the role of RNA, viruses, and many proteins is extensively studied, little is known about fatty acids and lipids and their transport throughout the phloem (Madey *et al.*, 2002; Guelette *et al.*, 2007; Behmer *et al.*, 2010). The present findings suggest that lipids and lipid-binding proteins are indeed present in the phloem sap. Since the phloem is an aqueous environment, lipids are not necessarily expected. Yet, other aqueous biological systems such as human blood contain a variety of lipids. Typically these are bound to proteins. Some lipid–protein complexes are transported to other tissues for storage, modification, or degradation; others transport essential vitamins or play a role in signalling (Blaner, 1989; Glatz *et al.*, 1995; Charbonneau *et al.*, 2009). In several cases, these lipid–protein complexes serve as messengers and affect transcription factor activity (Tontonoz *et al.*, 1994; Nagy and Szanto, 2005). Here it is hypothesized that plant phloem lipids and lipid-binding proteins have regulatory and transport functions in plants similar to those in animals and that they may play an important role in stress-related and developmental signalling beyond what is already known about hormones such as JA.

Materials and methods

Growth of plant material

Arabidopsis thaliana var. Columbia seedlings were surface sterilized in 20% bleach/1% Triton X-100 for 20 min, washed several times

with water, and plated on a Petri dish containing half-strength Murashige and Skoog Basal medium (Sigma-Aldrich). They were incubated for 2 d in darkness in the refrigerator followed by a 5 d incubation at 22 °C. Seedlings were planted in soil and grown at 22 °C during the day and at 18 °C during the 8 h night (LD).

Harvest of phloem exudate

Phloem exudate harvest was started 2–3 h after the onset of light. *Arabidopsis* rosette leaves from 6-week-old plants were cut and re-cut in 20 mM K_2-EDTA, pH 7.0, placed in groups of 10 in a microtube containing 1.2 ml of the same solution, and incubated for 1 h in a dark and humid atmosphere. After 1 h, the solution was discarded. The petioles were washed thoroughly with water to remove all residual EDTA and placed in a new microtube containing 1.2 ml of deionized water. Exudates were collected for 6–8 h in a dark, humid atmosphere, immediately frozen in liquid nitrogen, lyophilized, and stored at –80 °C. For preparation of phloem RNA, 100 U of RNase inhibitor (Roche) was added per ml of deionized water into which the phloem exudate was collected.

Analysis of proteins in the phloem exudates

Phloem exudate was analysed using an Ultrafast Microprotein Analyzer system with a peptide microtrap and a C_{18} reverse-phase column (Michrom BioResources, Auburn, CA, USA). Samples were resuspended in 100 μl of deionized water and loaded onto the peptide microtrap. The gradient went from 5% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) in water to 65% ACN/0.1% TFA in water in 42 min. The flow rate was 500 μl min^{-1}. Fractions of interest were further analysed using matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) or Edman sequencing. Edman sequencing was performed by the MSU- Research Technology Support Facility.

Alternatively, phloem exudates from 20 leaves per replicate (three replicates in total) were subjected to 15% SDS–PAGE. Protein bands were excised and digested with trypsin according to Shevchenko *et al.* (1996). The digested peptides were extracted into 60% ACN/1% TFA and the appearance of tryptic fragments was monitored using MALDI-TOF-MS. For liquid chromatography–tandem mass spectrometry (LC-MS/MS), samples were dried and resuspended into 2% ACN/0.1% formic acid. LC-MS was performed on a Capillary LC system (Waters Corp., Milford, MA, USA) coupled to an LCQ DECA ion trap mass spectrometer (Thermofinnigan, San Jose, CA, USA) equipped with a nanospray ionization source. The sample was trapped onto a Peptide Cap Trap (Michrom BioResources) and flushed onto a 5 cm×75 μm ID picofrit column packed with 5 μm ProteoPep C18 material (New Objective, Woburn, MA, USA). Peptides were eluted with a gradient of 2–95% ACN in 0.1% formic acid at a flow rate of 200 nl min^{-1} for 60 min. Peptides/proteins were identified using the programs SEQUEST, MASCOT, or gpm.org. Carbamidomethyl cysteine was set as the fixed modification and oxidation of methionine was allowed. Up to two missed tryptic sites were permitted. Peptide tolerance was set to 2.5 Da, and MS/MS tolerance was set to 0.8 Da. Positive identifications were confirmed by individually comparing MS/MS spectra. Positive identification required at least two unique peptides per proteins counting only peptides with significant scores (95% confidence per peptide; >2.5 for SEQUEST). Gupta and Pevzner (2009) have suggested that inclusion of single-hit proteins does not necessarily increase the false discovery rate. Four additional proteins with a single peptide identification were included due to the fact that their mass corresponded to the predicted size from the gel and they were seen in at least two independent preparations. One protein was submitted to Edman sequencing (Ed in Supplementary Table S1 available at *JXB* online) at the Michigan State University Research Technology Support Facility. Database searches using individual tryptic fragments were performed using the BLAST searches at

NCBI (http://www.ncbi.nlm.nih.gov/blast). Predicted function/localization of proteins is based on the NCBI, GO (http://www.e-bi.ac.uk/QuickGO/), and/or Bar (http://bar.utoronto.ca/welcome.htm) databases as well as the cited literature.

Analysis of metabolites in the phloem exudates (modified from Roessner et al., 2000)

Phloem exudate from 20 *Arabidopsis* leaves was resuspended, pooled, and phase partitioned with chloroform:methanol (1:1). Both phases were dried individually and derivatized using methoxyamine in pyridine and BSTFA [*N,O*-bis-(trimethyl silyltrifluoroacetamide)] with 1% trimethylchlorosilane. The metabolites were then separated by gas chromatography–mass spectrometry (GC-MS) using a JOEL XMS-AX505H system with a DB17/MS column (J&W Scientific, 30 m×0.32 mm ID×0.25 μm film; temperature program: 70-5, 5/min-320-5; data in Fig. 1) or an Agilent 5890N GC system coupled to a 5973 MSD using a DB 5MS column (J&W Scientific, 30 m×0.25 mm ID×0.25 μm film; temperature program: 50-2, 10/min-320-5; data in Supplementary Fig. S3 at *JXB* online). Identification was carried out using the NIST library. Only compounds with a score >80 that passed additional individual comparison were included in the list. Data sets from four independent preparations were compared. Metabolites included in Table 1 had to be present in at least three of those preparations and absent in the controls. Two sets of controls were used for the phloem metabolite analysis: one 'blank' sample applying the complete GC-MS preparation and analysis to 1.2 ml of deionized water (only reagent peaks were visible; not shown) and one minus-EDTA sample where leaves/petioles had never been submerged in EDTA but collection occurred directly into deionized water (see Fig. 1). Reagent peaks were visible only in the first
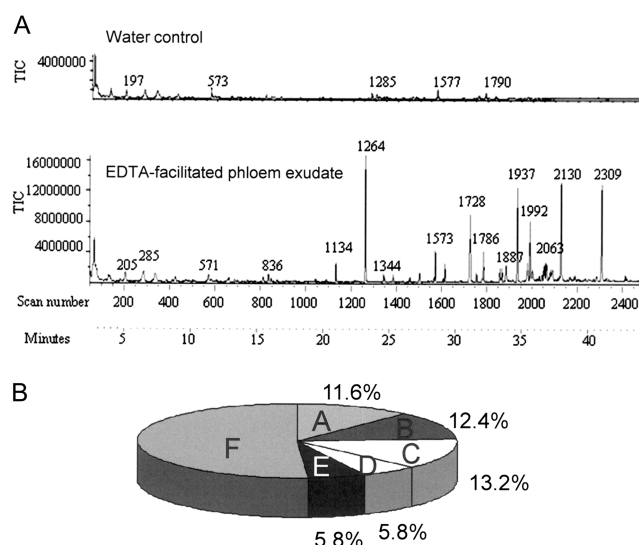

Fig. 1. (A) Gas chromatogram showing the separation of metabolites from the chloroform phase of *Arabidopsis thaliana* phloem exudates. The bottom panel displays metabolites present in the exudates of petioles incubated first in EDTA to prevent the sealing of the phloem. The top panel demonstrates the absence of any major metabolite peaks if the petioles were incubated in water (water control). The water control was fitted to the same scale as the EDTA extract. Both panels are a typical representative of three biological replicates. (B) Distribution of metabolites detected in *Arabidopsis* phloem exudate. They were grouped into amino acids (A), sugars and sugar derivatives (B), fatty acids/-esters (C), organic acids and phosphates (D), others (E), and unknowns (F).

Table 1. Metabolites identified in *Arabidopsis thaliana* phloem exudate

Lipids marked with an asterisk are related to those found by Madey *et al*. (2002). Numbers correspond to peak # in Supplementary Figure S3 at *JXB* online.

Amino acids	Sugar/-alcohols phosphates	Fatty acids/-esters	Organic acids	Others
Alanine	Ribose	Nonanoic acid*	Propanoic acid (1)	Uridine
Valine	Fructose	Dodecanoic acid*	Ethyl-phosphoryc acid	Bis-ethyl-ethane amine
Leucine	Glucose	Octadecanoic acid butyl ester	Butanoic acid	Phytosterol-like cpd (13)
Glycine	Galactitol	Tetradecanoic acid*	Malic acid (4)	Urea
Isoleucine	Galactonic acid	Pentadecanoic acid	2-Methoxy-pentanedioic acid	Cinnamic acid
Serine	*Myo*-inositol (9)	Octadecanedioic acid	Acetic acid	Dehydroabietic acid (12)
Threonine	5 Hexoses (7)	Palmitelaidic acid	Maleic acid (3)	Cyclopentanapth azepinone
Aspartic acid	2-O-Glycerol-galactopyranoside	Palmitic acid (8)	Succinic acid	Phosphate
Glutamine	*Myo*-inositol-P	Octadecanoic acid methyl ester	Propanedicarboxylic acid (6)	
Proline (5)	Sucrose	11-*trans* octadecanoic acid		
Phenylalanine	Melibiose (14)	Octadecanoic acid (11)		
Asparagine		Eicosanoic acid		
Tyrosine		9,12,2-OH Octadecanoic acid methyl ester		
		Docosanoic acid methyl ester*		
		Oleic acid (10)		

10 min of each GC-MS. To confirm the presence, identity, and relative ratios of sucrose:glucose and sucrose:fructose, standard solutions of these sugars were derivatized both individually and as a 1:1:1 mixture, and analysed using GC-MS as described above. Analysis of the peak areas from the chromatogram of the mixture confirmed the maintenance of the 1:1:1 ratio between those sugars and thus no impact of BSTFA (not shown).

Analysis of phloem exudate for the presence of mRNA

RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), digested with DNase for 30 min at 37 °C, and repurified. RNA concentration and quality were determined with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). The 260/280 ratios of the mRNA were in the range of 1.68–2.16. A 200 ng aliquot of mRNA was reverse transcribed using the Omniscript RT Kit (Qiagen). Semi-quantitative RT-PCR to ensure phloem sap purity was performed monitoring 18S (F, TCAACTTTCGATGGTAGGATAGTG; R, CCGTGTCAG-GATTGGGTAATTT; product size, 144 bp), Rubisco small subunit (SSU; F, CATCCCCGGCTCAGGCC; R, AAGGAATC-CACTTGTTGCGG; product size, 230 bp), Rubisco large subunit (LSU; F, GCTACCACATCGAGCCCG; R, CGGCACAAAA-TAAGAAACGG; product size, 372 bp), pathogen and circadian controlled (PCC1; At3g22231; F, TCCTCACTCCTCAGC-TCCTC; R, GTTTGGGCAACGACTTCTGT; product size, 363 bp), and ubiquitin-conjugating enzyme (UBC9; At4g27960; F, TGGCTTCGAAAAGGATCTTG; R, TCGATATGGTGAG-TGCAGGA; product size, 290 bp). The annealing temperature for all reactions was 53 °C/40 cycles using a PCR master mix (Promega; *Taq* DNA polymerase). The identity of the PCR products was confirmed based on the expected product size (see figures in parentheses above) and sequencing of representative bands: PCR products were cloned into pGEM T-Easy (Promega), transformed into *Escherichia coli* strain DH5α using heat shock treatment, and inserts sequenced following a plasmid preparation (Wizard *plus* SV Miniprep; Promega).

Analysis of lipids in phloem exudates

Phloem exudates were phase partitioned against chloroform:methanol (1:1, v/v), concentrated under N₂, and submitted to either thin-layer chromatography (TLC; acetone:methanol:water; 90:30:7) or LC-MS. For the latter, the hydrophobic components of the phloem exudates were separated using a LC-20AD Ultrafast liquid chromatograph (Shimadzu, Kyoto, Japan) on a C18 column (5 cm×2.1 mm×2.7 μm; Supelco, Bellefon, PA, USA) with a gradient from 30% solvent A (10 mM ammonium acetate) to 100% solvent B (100% isopropanol/10 mM ammonium acetate), and identified using electrospray ionization (ESI)-MS (3200 QTRAP; AB/MDS). Initial identification of lipids was based on their *m/z* plus the presence of at least two predicted MS/MS fragments using the LipidView software by AB/MDS. To improve lipid identification, subsequent lipid analysis was performed using a Waters LCT Premier mass spectrometer (LC-TOF-MS) with multiplexed CID (collision-induced dissociation, at 20, 35, 50, 65, and 80 V) and a gradient from 10% A (10 mM ammonium formate) to 99% B (acetonitrile:isopropanol; 1:2) on the column described above using both positive and negative ion mode. Data are representatives of three biological replicates.

Results and Discussion

Extraction of phloem exudate without cellular cross-contamination

Collection and analysis of the phloem sap are complicated by the fact that, in most plants, the phloem seals itself upon wounding, and no exudates are secreted. To prevent this, *Arabidopsis* petioles were incubated for 1 h in EDTA. This initial exudate was discarded. The petioles were washed, followed by collection of the exudate into water using the method previously described for *Perilla* (King and Zeevaart 1974; Hoffmann-Benning *et al.*, 2002) and modified for *Arabidopsis* (Maeda *et al.*, 2006; Guelette *et al.*, 2007; Deeken *et al.*, 2008; see Supplementary Fig. S1 at *JXB* online). These authors had thoroughly tested the effect of EDTA on the phloem exudation in *Perilla crispa*. They had shown that exudation was maximal during the first 4 h and continued at a slightly reduced rate after that. They also determined that EDTA stimulates the exudation of assimilates and leads to less callose accumulation in the sieve pores. The conclusion was that EDTA chelates Ca²⁺ ions,

which would otherwise participate in reactions that seal the phloem (King and Zeevaart, 1974). Indeed, it was shown later that, as a result of plasma membrane leakage, injury, or turgor disruption, P-protein crystalloids present in legume sieve tubes disperse and block the sieve pores. This process is facilitated by Ca^{2+} and inhibited by EDTA (Knoblauch *et al.*, 2001). At the same time, no effect of EDTA on the cell ultrastructure was observed, indicating that it can be safely used for phloem exudate collection. A similar method used to analyse glucosinolates and tocopherols, respectively, in the phloem also showed no adverse effect on phloem loading and transport (Chen *et al.*, 2001; Maeda *et al.*, 2006). To summarize, phloem exudate collection methods using a range of EDTA concentrations from 10 mM to 20 mM have been published and have shown no adverse effects. To reduce interference of EDTA with chromatography and gel electrophoresis, the plants were moved into water after 1 h and only the later part of the exudate was used (Supplementary Fig. S1 at *JXB* online; Hoffmann-Benning *et al.*, 2002). Thus, rather than exudation into EDTA, phloem is exudated into water (EDTA-facilitated exudation).

Collection of exudates for different lengths of time showed the same protein pattern after 1, 5, and 9 h of exudation. Particularly during the first 5 h the density of all protein bands increases with the increase in exudation time, confirming that as the collection time increases, more protein is accumulated and that exudation has not ceased during longer collection times. Except for a band at ~50 kDa, the density of bands relative to each other appears the same and no new bands appear, suggesting that cell damage is unlikely and that collection for 5–9 h is optimal (Supplementary Fig. S2 at *JXB* online). To account for proteins or metabolites that would leak from cut cells, the same number of leaves was incubated for the first hour in water instead of EDTA and then the exudate was collected into water. Exudation into water without prior EDTA treatment does not yield metabolites or proteins detectable by GC-MS (Fig. 1) or high-performance liquid chromatography (HPLC; Fig. 2). On the other hand, collection into water after a short incubation with EDTA leads to the detection of a large number of proteins and metabolites (Figs 1–7).

Extraction of mRNA from leaves, petioles, and phloem exudate followed by RT-PCR shows the presence of Rubisco small and large subunit mRNA in leaves and petioles but not in the phloem exudate (Fig. 3). On the other hand, mRNAs for UBC9 (At4g27960) and PCC1 (At3g22231) were found to be present in the phloem exudates (Fig. 3). Both had previously been described in phloem exudates (Deeken *et al.*, 2008). The absence of Rubisco mRNAs indicates that compounds detected and identified are indeed largely derived from the phloem sap and are not due to intracellular contamination leaked through the cut surface or membranes. The amounts of fructose and glucose relative to sucrose were determined using GC-MS after exudation for 1 h. Identification of fructose, sucrose, and glucose was based on the retention time of individual standards as well as a mixture, and on identification through the NIST library. It revealed
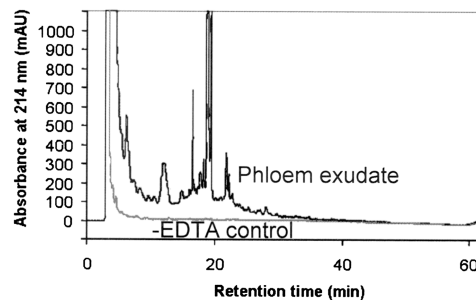

Fig. 2. HPLC chromatogram of *Arabidopsis* phloem exudates extracted into water after previous incubation with EDTA (black) or without EDTA (water control; grey).

Fig. 3. Presence of mRNAs for 18S, Rubisco small and large subunit (RbcS and RbcL), ubiquitin-conjugating enzyme (UBC), and PCC1 (pathogen and circadian controlled) in samples from leaf (L), petiole (P), and phloem exudates (Ph) as determined by RT-PCR. The picture shows a representative of three independent biological replicates.

that sucrose was the predominant sugar/metabolite in the exudates, with a peak area ratio of 1:7.6 and 1:8 for glucose:sucrose and fructose:sucrose, respectively (Supplementary Fig. S3 at *JXB* online). This matches the findings of Deeken *et al.* (2008) whose ratios of glucose to sucrose (96 nmol g FW^{-1}) in phloem exudates were between 1:4 and 1:8. Peaks eluting in the first 10 min were identified as reagent peaks and are marked with an asterisk. True metabolite peaks are indicated with a number and cross-referenced in Table 1.

Identification of metabolites in *Arabidopsis* phloem exudate

Metabolites were analysed using total phloem exudates as well as separated hydrophobic and hydrophilic fractions.

GC-MS revealed the presence of at least 121 distinct metabolite spectra in the phloem exudates. Of these, ~50% gave rise to spectra of known identity (Table 1; Fig. 1A, B: hydrophobic fraction). Eleven percent of the metabolite spectra belonged to amino acids, 12% to sugars and sugar derivatives, 13% to fatty acids and their derivatives, and 6% to organic acids and phosphates. Amino acids and sugars (monosaccharides and disaccharides) are assimilates expected to be present in the phloem. As expected, sucrose was the most abundant metabolite present (Supplementary Fig. S3 at *JXB* online).

The presence of *myo*-inositol and *myo*-inositol phosphate in the phloem has interesting implications. *Myo*-inositol phosphate is largely known as a precursor of phytic acid, a storage compound for phosphate in seeds (Hegeman and Grabau, 2001). It also plays a central role in plant metabolism, signal transduction, and cell wall biosynthesis (Loewus and Murthy, 2000). Since both molecules can be readily converted into each other by highly regulated enzymes (*myo*-inositol kinase; *myo*-inositol phosphatase), *myo*-inositol phosphate may also be a source for *myo*-inositol. This, in turn, is rate limiting for the synthesis of phosphatidylinositol bisphosphate and can thus either directly or indirectly play a role in signalling in the phloem (Ananieva and Gillaspy, 2009; Munnik and Vermeer, 2010).

It was possible to detect several fatty acids and their derivatives (Table 1). The presence of lipids/fatty acids in the phloem has been reported before, when small amounts of lipids and large amounts of free fatty acids were identified in the phloem of canola (Madey *et al.*, 2002). Recently, Behmer *et al.* (2010) suggested the presence of phytosteroids and cholesterol in the phloem sap. Like in Madey *et al.* (2002), fatty acids were detected in this study. Several of these were short and medium length and medium length, saturated fatty acids (non-anoic acid, dodecanoic acid, tetradecanoic acid, and pentadecanoic acid), including odd-chain length fatty acids (propanoic acid, nonanoic acid, and pentadecanoic acid). Propanoic acid and butanoic acid are grouped with organic acids, although they are considered to be short-chain fatty acids (Oxford Dictonary of Chemistry; www.cyberlipid.org). Saturated, even-numbered fatty acids make up 10–40% of the total fatty acids in most naturally occurring lipids, with C_{14}–C_{18} fatty acids being the most abundant (http://lipidlibrary.aocs.org). Shorter chain and odd-numbered fatty acids are not typically present in plant membranes and, thus, appear to be specific to the plant phloem. Yet their function remains unknown. They could simply be transported in the phloem; however, a role in pathogen response or signalling has been discussed in the literature (Sanz *et al.*, 1998; Hamberg *et al.*, 1999).

The presence of fatty acids raises the possibility of more complex lipids in the phloem sap. TLC confirmed the presence of a small amount of lipids in the phloem exudate (Fig. 4). Five of the lipids have retention times different from those present in leaves. This suggests that they are not contaminants but may instead perform separate functions in the phloem. LC-MS confirmed the occurrence of lipids and other hydrophobic compounds in exudates (Fig. 5A).
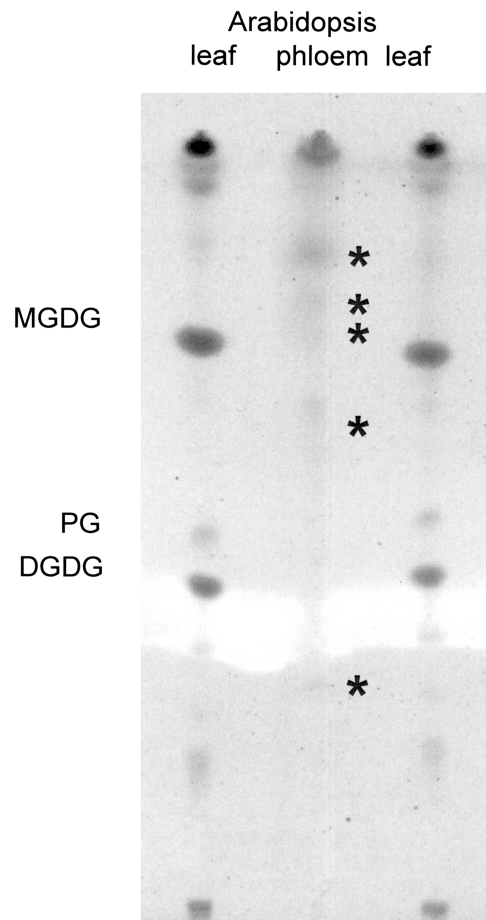

Fig. 4. Thin-layer chromatogram comparing lipids from *Arabidopsis* leaves and phloem exudates. The asterisks indicate lipids specific for phloem exudates. PG, phosphatidylglycerol; MGDG, monogalactosyldiacyglycerol; DGDG, digalactosyldiacyl-glycerol.

Mass over charge-based characterization of ions in conjunction with detection of at least two predicted MS/MS fragments using lipid profiler as well as manual interpretation (Fig. 5B) suggests the presence of phosphatidic acid (PA), lysophosphatidic acid (LPA), phosphatidylglycerol (PG), phosphtidylinositol (PI) and its phosphates (PIPs), di- and triacylglycerols (TAGs), and phosphatidylcholine (PC). An example is given for the lipid at the retention time of 16.1 min [PC 34:3 (16:0, 18:3); Fig. 5B]. PA and PIPs are known second messengers in plants (Wang, 2004). However, little is known about their long-distance transport. These findings raise the possibility that they could also play a role in long-distance signalling.

Identification of proteins present in *Arabidopsis* phloem exudate

In the past, several labs have isolated phloem exudates and separated the proteins therein. Some of these experiments were to follow-up specific proteins or look at specific functions (Schobert *et al.*, 1995; Kühn *et al.*, 1997; Balachandran *et al.*, 1997; Xoconostle-Cazares *et al.*, 1999;

Hoffmann-Benning *et al.*, 2002; Lee *et al.*, 2005); others attempted to obtain a more comprehensive protein profile (Fisher *et al.*, 1992; Sakuth *et al.*, 1993; Marentes and Grusak 1998; Kehr *et al.*, 1999; Haebel and Kehr, 2001; Giavalisco *et al.*, 2006). Over 1000 proteins have been detected in those exudates (Lough and Lucas, 2006; Lin *et al.*, 2009). Many of those, however, have just been characterized as spots on a gel with no clear identification (Kehr, 2006). In addition, many of those spots on the gel correspond to identical proteins with modifications such as

Fig. 5. (A) Lipid profile of phloem exudates of *Arabidopsis* (chloroform phase) using LC-MS/positive ion mode. Mass spectra of the lipid at a retention time of 16.1 min were generated at aperture 1 voltages of 35, 50, and 80 V in both positive (B) and negative (C) ion mode to show lipid fragmentation. PC, phosphatidylcholine; PA, phosphatidic acid; TAG, triacyl glycerol; *, detergent; N/D, not determined due to multiple peaks in the spectra. Chromatograms are representatives of three biological replicates for each sample.

phosphorylation and glycosylation (Nakamura *et al.*, 1995; Taoka *et al.*, 2007). A previous very comprehensive proteomics study of phloem proteins was done in a close relative of *Arabidopsis*, *Brassica napus*, and yielded 101 distinct genes out of 140 gel spots (Giavalisco *et al.*, 2006). This shows that large numbers of phloem proteins can be identified if the species has its genome sequenced or is closely related to one that does. The fact that many of the protein spots identified from their two-dimensional gels correspond to identical proteins (22% based on identical accession numbers) suggests that a large number of post-translational modifications are present in phloem proteins.

Using a combination of HPLC and Edman sequencing or SDS–PAGE (Fig. 6) followed by in-gel digests and LC-MS/MS, it was possible to distinguish 65 proteins present in *Arabidopsis* phloem exudate (Supplementary Table S1 at *JXB* online; Figs 6, 7). Many (23%) of those appear to be identical to the proteins described by Giavalisco *et al.* (2006) for *Brassica* (indicated on Supplementary Table S1). The identified proteins could be grouped into the following categories based on their predicted or known function according to Fig. 7.

Carbohydrate metabolism: This group included 14 proteins, most of which had been found in the phloem of other plant species (Walz *et al.*, 2002; Giavalisco *et al.*, 2006). It was discussed that all enzymes of the glycolytic pathway are present in the phloem but are repressed due to the low oxygen levels (Geigenberger, 2003). Fructose-2,6-bisphosphatase and triosephosphate isomerase have now been added to this list.

Stress, pathogen, or hormone response proteins (16): Several disease-resistance proteins (RPP1-WsB and RPP8-like protein), components of the my
rosinase system (myrosinase and myrosinase-binding protein), as well as several other pathogen response proteins
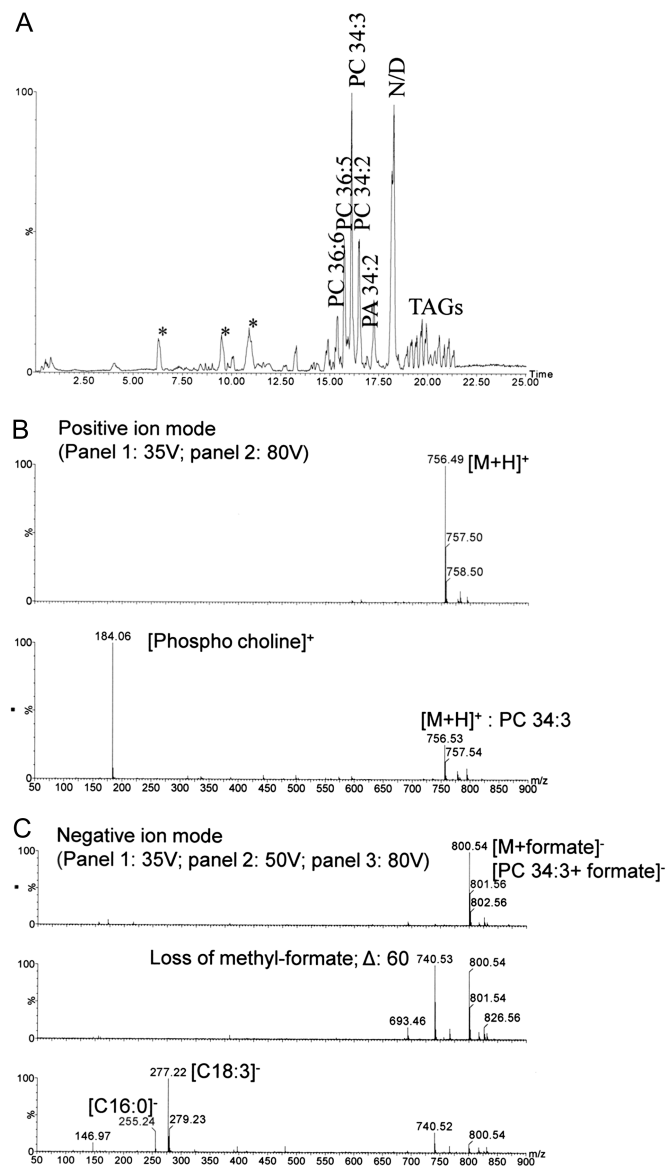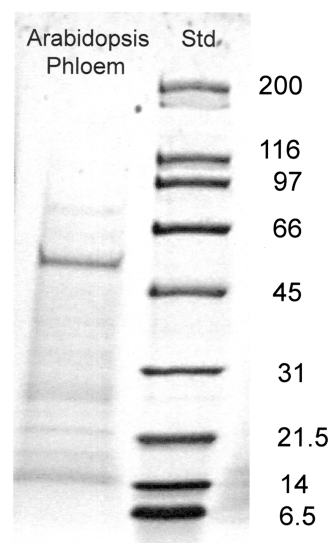
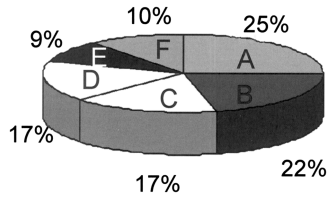**Fig. 6.** SDS–PAGE of proteins from *Arabidopsis* phloem exudate.

Fig. 7. Distribution of proteins detected in *Arabidopsis* phloem exudate. Proteins were grouped by function into pathogen/stress response (A), carbohydrate metabolism (B), protein binding/modification/metabolism (C), lipid/fatty acid binding/metabolism/signalling/storage (D), DNA/RNA/nucleotide binding (E), and others/unknown function (F).

(a thaumatin-like protein, major latex protein, and Bet v1 allergen-like protein) were detected. All had been described previously in the phloem (Giavalisco *et al.*, 2006; Lin *et al.*, 2009; Dafoe *et al.*, 2010). The Bet v1 family protein belongs to the group of PR-10 pathogenesis-related proteins. Experiments have shown that it has the chemical ability to bind fatty acids and cytokinins (Mogensen *et al.*, 2002) as well as brassinosteroids, and may play a role in plant growth and development (Markovic-Housley *et al.*, 2003), but probably not in brassinosteroid trafficking (Symons and Reid, 2004). Some proteins in this group have been shown to be induced by auxin (actin 7/2, Zn-binding dehydrogenase, and glutathione *S*-transferase), abscisic acid (uncharacterized phloem protein UPP3), JA (myrosinase-binding protein), or to play a role during oxidative (superoxide dismutase CSD 1 and 2; Walz *et al.*, 2002) or anaerobic stress (alanine aminotransferase; Good and Muench, 1992), or in cold, drought, and salt tolerance (glyoxylase I, monodehydroascorbate reductase, and carbonic anhydrase). The function and presence in the phloem of most of these proteins has been described by Giavalisco *et al.* (2006) and Lin *et al.* (2009). This is the first description of the putative Zn-binding dehydrogenase, alanine aminotransferase, and glyoxylase 1 in the phloem.

Nucleotide-, DNA-, or RNA binding proteins (six): These include a possible transcription factor (unidentified phloem protein UPP6) as well as RNA-binding proteins (GRP7 and UPP5). In addition, several of the stress response proteins listed above contain nucleotide-binding sites (RPP8, ethylene-responsive RNA-helicase, and 14-3-3 like proteins—two subunits). Most could be interesting in light of the fact that mRNA appears to be transported through the phloem and acts as a signalling molecule, a process that is facilitated by RNA-binding proteins (Heintzen *et al.*, 1997; Xoconostle-Cazares *et al.*, 1999; Yoo *et al.*, 2004; Aoki *et al.*, 2005; Deeken *et al.*, 2008; Ham *et al.*, 2009; Lin *et al.*, 2009).

'Plastidal' proteins (11): As in Giavalisco *et al.* (2006) in *B. napus*, a small number of proteins which have previously been described as associated with chloroplasts or mitochondria were found. In general, most of the proteins present in phloem exudates are assumed to be synthesized in the companion cell and transported into the sieve elements (Fisher *et al.*, 1992; Sakuth *et al.*, 1993; Dannenhofer *et al.*, 1997; Ruiz-Medrano *et al.*, 2001) though a new discussion about protein translation in phloem sap has been initiated (Lin *et al.*, 2009). Eleven proteins were found that are known to be located predominantly in the chloroplast. Among these are the two Rubisco subunits which have been previously described in phloem exudates (Marentes and Grusak, 1998; Giavalisco *et al.*, 2006; Lin *et al.*, 2009; Rodriguez-Medina *et al.*, 2011). Since the 'plastidal' proteins detected are mostly nuclear encoded, it is possible that they also have been actively transported into the sieve element from companion cells through plasmodesmata and serve a related function here. This transport has been shown to be a highly regulated process needing the presence of chaperones (Aoki *et al.*, 2002, 2005) or kinases (Lee *et al.*, 2005). A series of green fluorescent protein (GFP) transport and other experiments has shown that the size exclusion limit (SEL) for proteins trafficking through plasmodesmata companion cells to the sieve element can be up to 67 kDa, though some labs report a SEL of only 25 kDa (Oparka *et al.*, 1999; Fisher and Cash-Clark, 2000; Kim *et al.*, 2002; Stadler *et al.*, 2005; Martens *et al.*, 2006). This would be sufficient to explain the presence of the vast majority of proteins (plastidal and others) detected in the phloem. Another explanation for their presence lies in the fact that during development, sieve elements have gradually lost their nucleus and most of their organelles (van Bel, 2003; Lough and Lucas, 2006). However, angiosperm sieve elements still contain P- and S-type plastids and few dilated mitochondria, though the functionality of the latter is still debated (van Bel, 2003). The plastidal proteins detected here could be components of those organelles.

Protein modification, folding, turnover, and stability, and protein–protein interaction (11 proteins): The presence and the role of components of the translational apparatus, the ubiquitin–proteasome pathway, as well as that of kinases, proteases, and chaperones had been described previously and will not be discussed here (Aoki *et al.*, 2005; Nakamura *et al.*, 1995; Schobert *et al.*, 1995; Giavalisco *et al.*, 2006; Lin *et al.*, 2009). One protein, annotated as a phosphoglycerate mutase/putative acid phosphatase-like protein, is interesting in the context of phosphate homeostasis or signalling. It contains a histidine phosphatase domain which is found in histidine acid phosphatases and phytases (*myo*-inositol hexakisphosphate phosphohydrolases). Proteins with this domain function in metabolism, signalling, and regulation (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=209303). Though not (yet) annotated as such, one possible function, rather than cleaving a phosphate of a protein, could be to liberate the phosphate from the *myo*-inositol backbone (Mullaney and Ullah, 1998). It could thus play a major role not just in phosphate metabolism, but also in regulating the relative ratios of *myo*-inositol phosphate and *myo*-inositol available for osmoprotection and signalling. This is of particular

significance since its potential substrates have also been detected in the phloem exudate (see above).

14-3-3 proteins are small conserved proteins, which bind an array of regulatory proteins such as kinases, phosphatases, ATPases, and transmembrane receptors. They play an important role in regulatory processes and signal transduction. In plants they activate transcription by binding to activators in the nucleus (Ferl, 1996; Fu *et al.*, 2000; Emi *et al.*, 2001). They have been shown to act as intracellular receptors for rice Hd3a and, thus, could play a role in flowering (Taoka *et al.*, 2011). Interestingly, free Raf kinases appear to be bound to 14-3-3 proteins in animal systems; however, once they bind to liposomes/membranes, the 14-3-3 protein dissociates (Hekman *et al.*, 2006). This suggests that 14-3-3 proteins may modulate binding of proteins to lipids.

Lipid/fatty acid-binding proteins: Eleven proteins with a role in lipid/fatty acid metabolism (aspartic protease, 3-β-hydroxy dehydrogenase, SQD1, and class 3 lipase) and storage (glycine-rich protein), binding (annexin, lipid-associated family protein, and GRP17), and signalling (annexin, putative GDSL lipase, MLP/Bet vI-like protein, and PIG-P; Table 2) were detected. Lipids and fatty acids are not typically expected in the phloem exudates. As the phloem exudate is an aqueous environment, it is not too unexpected that the lipids described above may be bound to proteins. Annexin and a lipid transfer protein (LTP) had previously been reported in the phloem exudate. LTP/DIR1 was shown to play a role in systemic acquired resistance (Maldonado *et al.*, 2002; Mitton *et al.*, 2009). However, no connection was made to phloem lipids. Annexin, one of the lipid-binding proteins, can be cytoplasmic but has also been shown to bind phospholipids/membranes and is involved in Ca^{2+} signalling as well as callose formation (Andrawis *et al.*, 1993; Mortimer *et al.*, 2008). Under stress conditions, *Medicago* annexin AnxMs2 localizes in the nucleolus even though it contains no typical nuclear localization signal. This suggests that it plays a role in intracellular membrane trafficking and signal transduction (Kovács *et al.*,

Table 2. Putative lipid-binding proteins identified from phloem exudates

Protein identification	Accession number	Mol. wt (kDa)
Lipase class 3 family protein	At4g16820	58
Aspartic protease	At4g04460	56
GRP17 (oleosin)	At5g07530	53
Putative PIG-P	At2g39435	50
Sqd1	At4g33030	44
Putative lipase/hydrolase	At1g29660	40
Annexin	At1g35720	36
Putative 3-β-hydroxysteroid dehydratase/isomerase	At2g37660	35
Lipid-associated family protein	At4g39730	20
Bet vI allergen	At1g23130	18
GRP7	At2g21660	17

1998).GRP17 has previously been identified in *Arabidopsis* pollen (Mayfield *et al.*, 2001). It contains an oleosin domain. Seed-localized oleosin prevent lipid aggregation and control oil-body size (Mayfield and Preuss, 2000). It is conceivable that it plays a similar role in the phloem sap.

Two proteins that are listed above as pathogen response proteins are the major latex protein-like protein and the Bet vI allergen-like protein. They belong to the Bet vI-like superfamily, which contains proteins with low sequence similarity but a common hydrophobic fold. Members of this superfamily include ceramide transfer proteins, phosphatidylcholine transfer proteins, phosphatidyinositol transfer proteins, acetyl-CoA hydrolases, and HD-Zip transcription factors from plants (Radauer *et al.*, 2008). The discovery of representatives of the proteins in phloem exudates suggests that they may play a role not just in transmembrane lipid transport but also in long-distance lipid transport and signalling.

Three other proteins could have a role in long-distance lipid signalling: two putative lipases and a putative PIG-P protein. Lipases have been implicated in salicylic acid signalling (Feys *et al.*, 2001) and wound response (Guan and Nothnagel, 2004). The PIG-P protein is subunit P of a phosphatidylinositol *N*-acetylglucosaminyltransferase. The enzyme catalyses the transfer of *N*-acetylglucosamine to phosphatidylinositol in the first step of glycosylphosphatidylinositol (GPI) anchor synthesis. GPI anchors are glycolipids which anchor proteins to membranes (Watanabe *et al.*, 2000). Interestingly, *myo*-inositol, which is not only necessary for the synthesis of phosphatidyl bisphosphate (PIP_2), a signalling compound, but can also be a part of the GPI anchor, was also found. In animals, GPI anchors may directly interact with receptor proteins and signal neural and brain development (Peles *et al.*, 1997; Ferrando-Miguel *et al.*, 2004). Only few GPI-anchored proteins have been found in plants. One group, the arabinogalactan proteins (AGPs), have been shown to contain a classical C-terminal GPI anchor signal sequence (Schultz *et al.*, 1998): their synthesis is thought to occur in the endoplasmic reticulum followed by secretion into the apoplast. There the AGP could be cleaved by phospholipases and act as signal molecules. It is possible that GPI-anchored proteins participate in cell-to-cell or even long-distance interaction. Similarly, inositol-3-phosphate and PIP_2 are important second messengers in animals as well as in plants (Ananieva and Gillaspy; 2009; Munnik and Vermeer, 2010); in plants, PA and LPA also appear to be relevant (Katagiri *et al.*, 2005; Munnik and Testerink, 2009). PA has been proposed to play a role in ABA-, wound-, and pathogen-related signalling (Katagiri *et al.*, 2005; Testerink and Munnik, 2005). Representatives of all three lipid groups (PIP, PA, and LPA) were identified in the samples and confirmed by MS/MS.

Throughout this study of phloem exudates, several fatty acids and lipids, some of which are known factors in intracellular signaling (PA and PIP) have been detected. In addition, proteins with the predicted, or in some cases known, ability to bind and transport these lipids and again

a role in intracellular signalling were found (i.e. annexin; Andrawis *et al.*, 1993). Together, this suggests that these lipids may not only be involved in intracellular signalling but may also play an important role in long-distance lipid signalling and that the lipid-binding proteins function as 'chaperones' which move lipids into and/or throughout the phloem. Therefore, lipids and lipid-binding proteins appear to have a larger and more complex function in systemic and developmental signalling throughout the plant than previously thought—a novel concept that could open up an entirely new field of study.

Supplementary data

Suppplementary data are available at *JXB* online.

Figure S1. Flow chart of phloem extraction and experimental set-up.

Figure S2. Time course of the appearance of proteins collected into water.

Figure S3. GC-MS profile of total (non-phase-partitioned) phloem exudates after 1 h of exudation into water (after EDTA treatment).

Table S1. Proteins identified in *Arabidopsis thaliana* phloem exudate.

Acknowledgements

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