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Adaptation of aphid stylectomy for analyses of proteins and mRNAs in barley phloem sap

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Abstract

Sieve tubes are transport conduits not only for photo-assimilates but also for macromolecules and other compounds that are involved in sieve tube maintenance and systemic signalling. In order to gain sufficient amounts of pure phloem exudates from barley plants for analyses of the protein and mRNA composition, a previously described stylectomy set-up was optimized. Aphids were placed in sealed cages, which, immediately after microcauterization of the stylets, were flooded with water-saturated silicon oil. The exuding phloem sap was collected with a capillary connected to a pump. Using up to 30 plants and 600 aphids (*Rhopalosiphum padi*) in parallel, an average of 10 µl of phloem sap could be obtained within 6 h of sampling. In first analyses of the macromolecular content, eight so far unknown phloem mRNAs were identified by cDNA-amplified fragment length polymorphism. Transcripts in barley phloem exudates are related to metabolism, signalling, and pathogen defence, for example coding for a protein kinase and a pathogen- and insect-responsive WIR1A (wheat-induced resistance 1A)-like protein. Further, one-dimensional gel electrophoresis and subsequent partial sequencing by mass spectrometry led to the identification of seven major proteins with putative functions in stress responses and transport of mRNAs, proteins, and sugars. Two of the discovered proteins probably represent isoforms of a new phloem-mobile sucrose transporter. Notably, two-dimensional electrophoresis confirmed that there are >250 phloem proteins awaiting identification in future studies.

Key words: Aphid, barley, cDNA-AFLP, mRNA, phloem, protein, *Rhopalosiphum padi*, signalling, stylectomy, two-dimensional gel electrophoresis.

Introduction

The past few years have brought new insights into functions of the phloem in addition to the transport of assimilates from source leaves to sink organs. Meanwhile, systemic signalling is an issue of increasing importance. For instance, phloem translocation of auxin was shown to be essential for regulation of polar growth (Kramer and Bennett, 2006), while salicylic acid and jasmonic acid were detected in sieve tube exudates and suggested to be involved in systemic acquired pathogen resistance (Durrant and Dong, 2004) and the systemic wound reaction (Schilmiller and Howe, 2005).

Besides such small and highly mobile substances, macromolecules present in sieve tubes are being increasingly studied. The major reason is that mature sieve elements (SEs) lose their capability for transcription and translation during differentiation, and therefore any macromolecule in the phloem transport fluid is likely to be imported from the adjacent companion cells (CCs) and constitutes a potential signalling compound. Phloem-mobile

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mRNAs were shown to be involved in the regulation of meristem differentiation and leaf development (Lough and Lucas, 2006; Kehr and Buhtz, 2008). By the use of grafting approaches, it was, for example, demonstrated that mRNAs of genes that were mutated in a *KNOTTED*-like transcription factor or the gene *GIBBERRELIC ACID INSENSITIVE* were systemically transported from mutant stocks via the phloem into scions, where they caused the mutant phenotype in differentiating young leaves (Kim *et al.*, 2001; Haywood *et al.*, 2005).

That proteins can also transfer information via the phloem was recently demonstrated in several published studies describing the phloem transport of the protein FLOWERING LOCUS T that leads to the induction of flowering in different plant species (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Tamaki *et al.*, 2007; Aki *et al.*, 2008). Moreover, some proteins function in distal signalling as carriers for messenger molecules such as RNAs, proteins, and lipid-derived compounds (van Bel and Gaupels, 2004; Kehr, 2006). On the other hand, proteins also maintain efficiency and longevity of sieve tubes, by protecting SEs from biotic and abiotic stresses. For example, an antioxidant defence system and a set of protease inhibitors were detected in phloem exudates, and other proteins are involved in sugar metabolism (Hayashi *et al.*, 2000; Walz *et al.*, 2002, 2004; Giavalisco *et al.*, 2006).

There is meanwhile clear evidence that phloem macromolecules fulfil an important role in whole-plant integration of developmental processes and stress responses, and this underlines the need for a deeper knowledge about the macromolecular composition of the phloem transport fluid. Using the model plants *Ricinus communis*, *Brassica napus*, and various cucurbits, which allow easy sampling by incisions in stems and petioles, >100 proteins and hundreds of mRNAs were identified (Hayashi *et al.*, 2000; Barnes *et al.*, 2004; Walz *et al.*, 2004; Doering-Saad *et al.*, 2006; Giavalisco *et al.*, 2006; Kehr, 2006; Omid *et al.*, 2007; Ruiz-Medrano *et al.*, 2007). Vilaine *et al.* (2003) analysed the mRNA content of isolated celery (*Apium graveolens*) phloem strands by cDNA macroarray and construction of a cDNA library with subsequent sequencing of clones. However, with this approach it was not possible to distinguish between immobile CC- and mobile sieve tube-derived transcripts. Bearing in mind that the total number of both proteins and mRNAs is >1500 in pumpkin sieve tubes, as estimated by Lough and Lucas (2006), efficient sampling and screening techniques are urgently required.

To date, most comprehensive approaches are underway in dicots and only little progress was achieved in deciphering the macromolecular phloem composition of the economically highly important monocot species. In rice, 111 proteins could be identified (Ishiwatari *et al.*, 1995; Fukuda *et al.*, 2004*a*, *b*; Suzui *et al.*, 2006; Aki

et al., 2008), whereas no phloem proteins from other monocot species are as yet known. In addition, six mRNAs were found in barley and rice coding for thioredoxin h, actin, oryzacystatin-1, sucrose transporter 1, proton ATPase, and aquaporin (Sasaki *et al.*, 1998; Doering-Saad *et al.*, 2002).

While sieve tube exudates from incisions were shown to be contaminated by cell contents of injured tissues surrounding the phloem (Ruiz-Medrano *et al.*, 1999), stylectomy is regarded to yield pure phloem sap (Fisher *et al.*, 1992; Sasaki *et al.*, 1998; Doering-Saad *et al.*, 2002). In this report, the application of a high-throughput stylectomy set-up for identification of authentic barley phloem mRNAs and proteins is described.

Materials and methods

Plant material and aphids

Hordeum vulgare L. cv. Ingrid plants were grown in pots placed in controlled-environment chambers illuminated by artificial light sources (Philips SON-T Agro 400, HRI-BT 400, Radium Lampenwerk, Wipperfürth, Germany; light intensity 180 μmol m^{-2} s^{-1}) under a 16 h day–21 °C/8 h night–18 °C regime at a relative humidity of 70%. The plants were used for phloem sap collection 8–12 d after germination. *Rhopalosiphum padi* aphids were cultivated on 2- to 4-week-old barley plants enclosed in perspex boxes (~50 cm×50 cm×60 cm) covered with a gauze cloth, under continuous light (60 μmol m^{-2} s^{-1}, HRI-BT 400, Radium Lampenwerk, Wipperfürth, Germany), at room temperature and a relative humidity of 60%.

Aphid stylectomy

Between five and 10 aphids were placed into cages manufactured from propylene tubes, two of which were fixed onto the upper surface of each first barley leaf with a solvent-free glue (Fig. 1A). After the aphids had settled overnight, stylets were cut with a microcautery device (Fig. 1B, D) (HF-microcautery unit CF-50, Syntech, Hilversum, The Netherlands) according to Fisher and Frame (1984). Immediately after cutting off the stylets, cages were flooded with water-saturated silicon oil DC 200 (Sigma-Aldrich, St Louis, MO, USA) to prevent contamination or evaporation of the exudates (Fig. 1C), and leaves were transferred to a second platform. Every 30 min phloem exudates were collected using a borosilicate microcapillary with a tip diameter of 0.1 mm and backloaded with oil (Fig. 1C, E). The capillary was connected to a pump, and pressure could be fine-tuned with a valve. Exudates were collected for 6 h while keeping the pooled samples on ice, and the total volume was estimated with a microlitre-scaled capillary.

RNase test of phloem samples

RNA integrity/degradation was analysed by detecting the rRNA peaks of a barley leaf total RNA sample on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) following the Agilent RNA 6000 Nano assay reagent kit guide using the Eukaryote Total RNA Nano assay. A 200 ng aliquot of the RNA sample was incubated with serial dilutions of RNase A (20 mg ml^{-1}; Sigma), a barley total leaf extract, or with phloem sap samples in a 10 μl reaction volume for 30 min at 25 °C. A 1 μl aliquot of each of these reactions was separated on the Agilent 6000 chip.
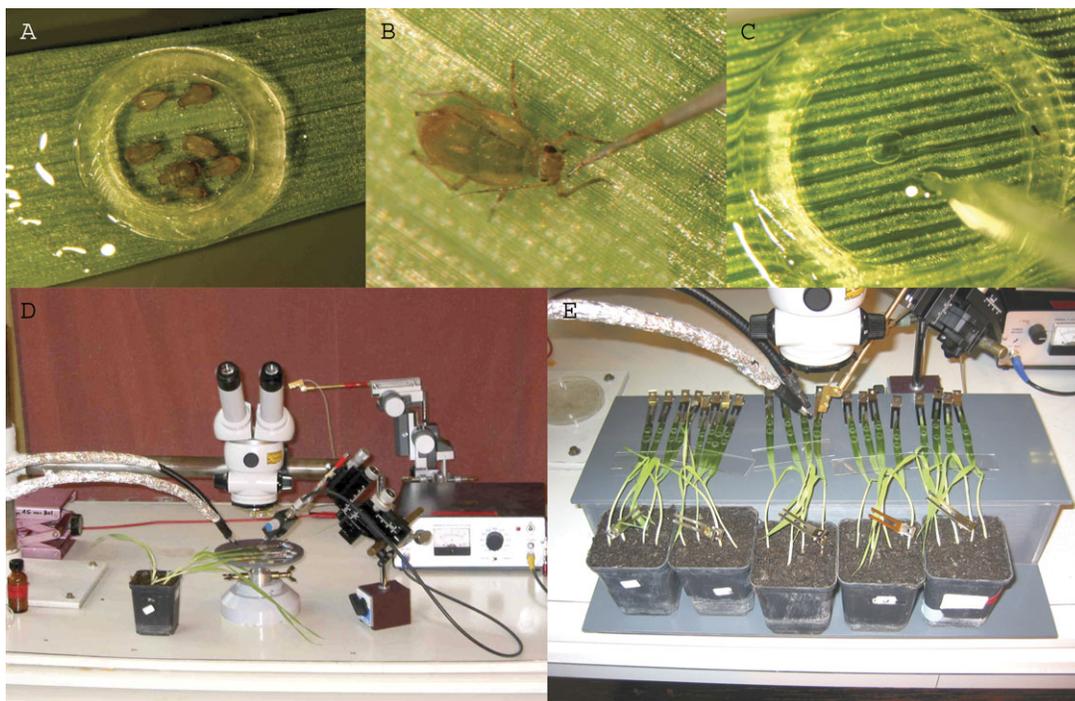
Fig. 1. Aphid stylectomy with barley. (A) Aphids (*Rhopalosiphum padi*) on a barley leaf. The cage was fixed and sealed with glue. (B) Cutting off the stylet of the aphids with a hot tungsten needle. (C) The cage flooded with silicon oil. Collection of exuding phloem sap with a capillary. (D) Work bench with binocular platform which can be raised and lowered, and turned, microcautery unit, and capillary (background). (E) Barley leaves with flooded cages placed on a platform for collection of phloem exudates.

cDNA-AFLP

Poly(A)$^+$ RNA was isolated from 5 µl of barley phloem sap using a Dynabeads Oligo (dT)$_{25}$ mRNA Purification Kit (Dynal, Hamburg, Germany) according to the manufacturer's instructions, and cDNA synthesis was performed with SUPERSCRIPT II RNase H$^-$ Reverse Transcriptase (Gibco-BRL, Karlsruhe, Germany). cDNA-amplified fragment length polymorphism (AFLP) was carried out following a protocol of R Bruggmann, University of Zürich, Switzerland. The method was originally published by Bachem *et al*. (1996) and applied to barley plants by Eckey *et al*. (2004).

cDNA from phloem mRNA was digested with the restriction enzymes *Sau*3AI and *Nco*I, and the following adaptors were ligated to the cut ends: *Sau*3AI adaptor top strand 5′-AGCGAT-GAGTCCTGAG; *Sau*3AI adaptor bottom strand 5′-TACTCAG-GACTCCTAG; *Nco*I adaptor top strand 5′-CCTGTAGACT-GCGTACAC; and *Nco*I adaptor bottom strand 5′-CATCTGACG-CATGTGGTAC. Primers against the adaptor sequences were used for pre-amplification of the cDNA pool: *Sau*3AI pre-amplification primer (S+0 primer) 5′-ATGAGTCCTGAGGATC; and *Nco*I pre-amplification primer (N+0 primer) 5′-AGACTGCGTACAC-CATGG. For selective amplification of a subset of the total cDNA pool, the unspecific N+0 primer was [γ-^{33}P]dATP end-labelled and combined with one of seven selective *Sau*3AI (S-) primers, which consisted of the nucleotides 5′-ATGAGTCCTGAGGATC+NN-3′, with NN representing +AG (S1 primer), +AC (S2), +TA (S3), +TT (S4), +TG (S5), +TC (S6), and +GG (S7). Conditions for pre-amplification PCR were 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C (30 cycles). Selective touch-down settings were 30 s at 94 °C, 30 s at 65 °C (–1 °C cycle^{-1}) and 2 min at 72 °C (nine cycles), 30 s at 94 °C, 30 s at 57 °C, and 2 min at 72 °C (25 cycles). After separation of amplification products on an 8% polyacrylamide gel, the gel was dried onto 3MM Whatman paper (Whatman,

Maidstone, UK) and exposed to Kodak Biomax MR film for 24 h. Bands were cut from the gel and cDNA was reamplified with the N+0 and S+0 primers. Subsequently, cDNAs were subcloned and sequenced. Sequences of transcript-derived fragments were compared with the entries of NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) and TIGR (The Institute for Genomic Research, Rockville, MD, USA) databases employing the BLAST (Basic Local Alignment Search Tool) algorithm tool (Altschul *et al*., 1997).

Protein determination, gel electrophoresis, and mass spectrometry

The protein concentration of 1 µl of barley phloem sap in a 500 µl sample volume was determined with the Bradford assay (Bradford 1976). Preparative one-dimensional electrophoresis of phloem proteins was done as described previously (Walz *et al*., 2002) and proteins were stained overnight using the Novex Colloidal Blue Staining Kit (Invitrogen, San Diego, CA, USA). Bands were cut, gel pieces destained, and proteins digested with trypsin. After extraction from the gel and desalting, peptides were analysed with an ESI-Q-TOF (electrospray ionization-quadrupole time-of-flight) mass spectrometer (Micromass, Altrincham, UK) as described in Walz *et al*. (2002).

SDS–PAGE and silver staining have been described previously in Gaupels *et al*. (2008). Two-dimensional electrophoresis with a phloem sap volume equivalent to 5 µg of protein was carried out in the Bioanalytics group, Institute of Molecular Biotechnology, RWTH Aachen, Germany, using the Protean IEF in combination with the Mini-Protean gel electrophoresis system (Bio-Rad, München, Germany). Proteins were visualized with silver stain. Further details can be found at www.molbiotech.rwth-aachen.de.

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Results and Discussion

Limitations in conducting phloem research with monocots are mostly caused by difficulties with phloem sampling. In contrast to various dicot species such as cucurbits, rice, yucca, lupin, or oilseed rape that allow the comparably easy collection of phloem sap exuding spontaneously from small incisions (Ziegler, 1975; Kehr and Rep, 2006), there seems to be only one monocot species, *Triticum aestivum*, showing spontaneous phloem exudation from grain pedicles in sufficient amounts for subsequent analyses (Fisher and Gifford, 1986; Fisher *et al.*, 1992). EDTA exudation and insect stylectomy offer two alternative ways of obtaining phloem samples from monocots, since they are mostly independent from the experimental plant species.

Facilitated exudation using the chelator EDTA to prevent calcium-dependent sieve tube occlusion by proteins and callose (King and Zeevaart, 1974) is a quick and easy method, but can lead to a significant amount of contamination. EDTA renders cell membranes permeable, causing contamination of the phloem sap by cell contents from surrounding tissues and, in addition, apoplastic contents can reach the exudates (Girousse *et al.*, 1991; van Bel and Gaupels, 2004). In contrast, insect stylectomy, where stylets of phloem-feeding insects are excised to collect the exuding phloem contents (Kennedy and Mittler, 1953; Fisher *et al.*, 1992), is regarded as a method that provides pure phloem samples from monocots (Fisher *et al.*, 1992; Sasaki *et al.*, 1998; Doering-Saad *et al.*, 2002). Gaupels *et al.* (2008) concluded from their studies with barley that stylectomy is the reference method for identification of phloem macromolecules from monocots. The purity of phloem exudates is particularly important for avoiding artefacts in mRNA analysis, since the copy number of phloem transcripts is probably very low (Doering-Saad *et al.*, 2002). Facilitated exudation may, however, be a useful tool for functional analyses of known phloem compounds in subsequent studies.

The main drawback of stylectomy is that it is time consuming and yields only small sample volumes in the nanolitre scale (Gaupels *et al.*, 2008). In order to circumvent this restriction, a set-up was developed for aphid stylectomy that allows the use of many plants and cut aphid stylets in parallel. The resultant barley exudates were analysed by cDNA-AFLP, one-dimensional SDS–PAGE followed by mass spectrometry, and two-dimensional electrophoresis. Eight transcripts previously unknown to be present in sieve tubes and seven proteins (two unknowns) could be identified, and their putative functions in the phloem are discussed.

Improved insect stylectomy

To carry out broad-spectrum analyses of the macromolecular content in barley phloem sap it was necessary to increase the efficiency of the previously described style-ctomy method (Gaupels *et al.*, 2008). In the improved experimental set-up, small plastic rings were fixed on the leaves and sealed with solvent-free glue, serving as cages for 5–10 aphids (Fig. 1A). The cages restrict aphids to a certain place on the leaf and at the same time allow easy access for cutting off the stylets. Modified cages can be easily attached to various plant organs including stems and petioles (not shown). Aphids were allowed to settle overnight and stylets were cut the next morning using a microcautery unit and a platform which could be raised and lowered, and turned (Fig. 1B, C). About five well-exposed stylets were cut per cage and immediately covered with water-saturated silicon oil to avoid evaporation and contaminations (Doering-Saad *et al.*, 2002). In water-saturated silicon oil DC 200, phloem exudates form spherical droplets on the inert chitin stylet stump (Fig. 1C). The exudate does not come into contact with air or the surface of the leaf. In this way, contamination or modifications (e.g. oxidation) of mRNAs and proteins can be reliably prevented. Leaves were then transferred to another platform for collection of exuding phloem sap using a microcapillary filled with silicon oil and connected to a pump (Fig. 1E).

Exposure to room temperature during the experiment did not seem to affect sample quality, probably because there is no mRNase activity in pure phloem exudates (Sasaki *et al.*, 1998; Doering-Saad *et al.*, 2002), and the presence of various protease inhibitors protects proteins from degradation (Kehr, 2006). Accordingly, no change in protein patterns or evidence for protein degradation could be detected even after 16 h of exudation under oil (Gaupels *et al.*, 2008).

From an average of 46 exuding stylets, ~10 µl of phloem sap could be obtained within 6 h of sampling (Table 1). The exudate volume per stylet was 0.22 µl and the estimated exudation rate was ~0.05 µl h^{-1} $stylet^{-1}$ (Table 1). This corresponds well to the 0.06–0.12 µl h^{-1} $stylet^{-1}$ reported in an earlier study with barley (Doering-Saad *et al.* 2002). A protein concentration of 0.4 µg $µl^{-1}$ (Table 1) was measured, which is somewhat higher than the reported 0.1 µg $µl^{-1}$ and 0.2 µg $µl^{-1}$ in wheat and rice phloem exudates, respectively (Schobert *et al.*, 1998). However, the exudation rate and protein concentration of

Table 1. *Sampling of barley phloem sap by aphid stylectomy—facts and numbers*

Number of leaves per experiment	20–30
Cages/leaf	2
Aphids/cage	10
Exuding stylets/experiment	46±10 (*n*=9)
Exudation volume/experiment	10±3 µl (*n* = 9)
Exudation volume/stylet	0.22 µl
Exudation volume h^{-1} $stylet^{-1}$	~0.05 µl
Protein concentration of exudates	0.40±0.16 µg $µl^{-1}$ (*n*=7)

phloem sap depend on the developmental stage and growth conditions of the plants, as shown for *R. communis* (Sakuth *et al*., 1993). Moreover, abiotic and biotic stress such as pathogen infection can have a significant impact on the yield of phloem samples (Gaupels *et al*., 2008; F Gaupels, unpublished observation).

In the present study, using up to 30 plants and 600 aphids in parallel, an average of 10 μl of pure phloem sap could be obtained within 6 h of sampling. Altogether, it was possible to collect and analyse >150 μl of barley phloem sap.

Quality control of phloem samples collected by aphid stylectomy

Previously, the purity of phloem samples was assessed by determination of the RNase activity (Sasaki *et al*., 1998; Doering-Saad *et al*., 2002). Addition of 40, 8, and as little as 1.6 ng ml^{-1} RNase A to a barley leaf RNA sample caused significant degradation of the RNA (Fig. 2), which could be visualized in an Agilent Bioanalyzer 2100. The RNase content in leaf extracts (0.5 g ml^{-1}) corresponded to the 40 ng ml^{-1} RNase A standard, while no RNase activity was detectable in phloem sap (Fig. 2). Similarly, RNases were absent in stylectomy samples from rice and barley (Sasaki *et al*., 1998; Doering-Saad *et al*., 2002). As
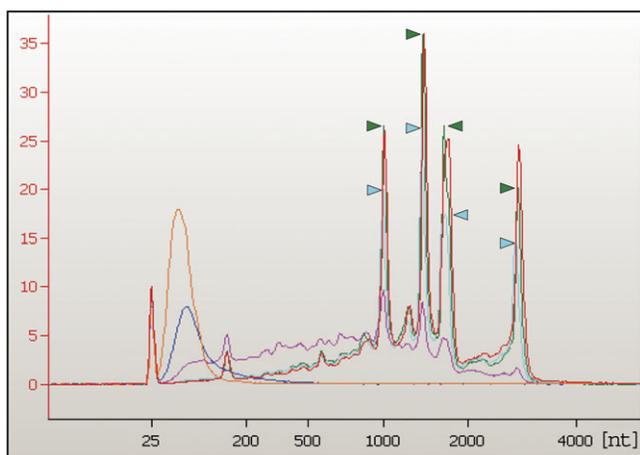

Fig. 2. Determination of RNase activity in stylectomy exudate of barley plants. RNase activity was tested with barley leaf total RNA. Samples were separated and visualized using an Agilent Bioanalyzer 2100. Phloem sap (green), leaf extract (blue), water (red), or 1.6 ng ml^{-1} (light blue), 8 ng ml^{-1} (pink), or 40 ng ml^{-1} (orange) RNase A standards were added to total RNA. RNase activity caused a decrease in the amount of high molecular weight rRNA—visible as four peaks between 1000 and 3600 nucleotides (nt)—and an accumulation of low molecular weight RNA fragments between 25 and 900 nt. Note that the peak maxima for the 1.6 ng ml^{-1} RNase A standard (light blue arrowheads) are consistently lower than the peak maxima for the phloem sample (green arrowheads) and the water control (red curve), whereas the light blue curve runs on top of the red and green (less pronounced) curves in the low molecular weight range. RNA concentration is depicted in arbitrary units and plotted versus the molecular weight in nucleotides determined by an internal RNA size marker.

a second quality control, phloem samples were analysed by RT-PCR with primers against the coding sequence for Rubisco small subunit (rbcS), because SEs are not photosynthetically active and should therefore not contain this protein (Ruiz-Medrano *et al*., 1999; Giavalisco *et al*., 2006). No specific rbcS product could be detected with 0.5 or 2 μl of barley phloem sap after 30, or even after 60 cycles of RT-PCR, while 30 cycles were sufficient to produce visible rbcS bands when using leaf extracts as template (results not shown).

Taken together, the results of both approaches indicate that the obtained samples are of high purity and not contaminated by the contents of leaf cells.

Identification of mRNAs in phloem exudates of barley by cDNA-AFLP

The first endogenous plant mRNA that was reliably found inside SEs and the plasmodesmata connecting SEs to CCs by immunolocalization was mRNA of the sucrose transporter 1 (SUT1) in *Solanaceae* (Kühn *et al*., 1997). Since then, mRNAs for thioredoxin h, cystatin, and actin have been detected by RT-PCR in the phloem from rice and *Brassica* (Sasaki *et al*., 1998; Giavalisco *et al*., 2006). Other approaches resulted in the collection of several functionally unrelated mRNAs from *Cucurbita maxima* exudate (Ruiz-Medrano *et al*., 1999) and a phloem-enriched cDNA library from *Ricinus* (Doering-Saad *et al*., 2006). There have been only two studies published regarding monocot species that found several transcripts, including SUT1, aquaporin, and a proton ATPase, in phloem sap of barley (Doering-Saad *et al*., 2002), and actin, cystatin, and thioredoxin from rice (Sasaki *et al*., 1998), by RT-PCR using gene-specific primers.

With small amounts such as samples from aphid stylectomy, RT-PCR has so far been the only option available to evaluate the presence of specific transcripts inside the phloem (Sasaki *et al*., 1998; Doering-Saad *et al*., 2002). To ensure that the phloem collection method is suitable for transcript analysis from barely phloem samples, the differential display technique cDNA-AFLP was applied, since this strategy, in contrast to RT-PCR with specific primers, represents an unbiased approach for transcript analysis. cDNA-AFLP allows both sensitive detection and identification of transcripts as well as analysis of gene expression upon treatment (e.g. infection) of plants (Bachem *et al*., 1996; Eckey *et al*., 2004). After extraction of poly(A)$^+$ RNA, reverse transcription, and amplification, cDNA was digested, yielding theoretically one adaptor-ligated cDNA fragment for each poly(A)$^+$ RNA species present in the original sample. Using primers against the adaptor sequence with a two base overhang specific for the fragment sequence, it was possible to divide the total cDNA pool into subsets of ^{33}P-labelled amplification products that were then

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separated on polyacrylamide gels. Subsequently, fragment cDNAs were extracted from the gel, sequenced, and mRNAs were identified by database searches.

The method used 5 μl of barley phloem sap with a combination of an unspecific N+0 primer and seven (out of 16) specific S-primers. Application of the N+0 primer led to unspecific detection of fragments with more than one primer combination [Table 2, see imidazoleglycerol-phosphate synthase (IGPS), carbonic anhydrase, and protein kinase]. An increase in the amount of sample and use of specific N-primers should prevent this problem in future experiments. A total of 23 bands were extracted from polyacrylamide gels. cDNAs were reamplified and yielded 15 sequences of good quality. Database searches with the 10 non-redundant sequences allowed identification of eight phloem mRNAs, whereas two sequences yielded no database hits (see Supplementary data available at *JXB* online).

None of these transcripts was described previously in the phloem of other plant species, and the translation product of only one mRNA (ferredoxin, electron transfer) has also been detected as a phloem protein in *B. napus* (Giaval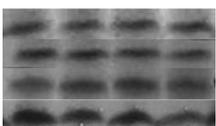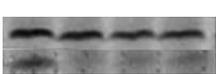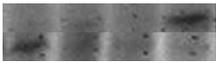isco *et al.*, 2006). However, transcripts from the same functional categories (metabolism, defence, signalling) have been found in earlier studies (Doering-Saad *et al.*, 2006; Omid *et al.*, 2007; Ruiz-Medrano *et al.*, 2007). For instance, IGPS is an enzyme of the histidine biosynthesis pathway, while elongin C is involved in protein processing by the ubiquitin system, which is a major functional category also found among *R. communis* and melon phloem mRNAs (Doering-Saad *et al.*, 2006; Omid *et al.*, 2007). Glycosyl transferases modify secondary metabolites, thereby regulating their storage and functions, for example in pathogen and herbivore defence (Gachon *et al.*, 2005).

Particularly interesting with respect to a putative role as phloem-translocated signals are the transcripts coding for a protein kinase, carbonic anhydrase, and a protein homologous to both WIR1A and a planthopper-inducible protein. These proteins are involved in signalling and defence reactions to pathogens and phloem-sucking insects (Bull *et al.*, 1992; Slaymaker *et al.*, 2002; Yuan *et al.*, 2004). Interestingly, after infection of pumpkin plants with cucumber mosaic virus, Ruiz-Medrano *et al.*, (2007) detected phloem mRNAs coding for the pathogen defence proteins PR1, a glucanase, and an F-box protein.

Table 2. *cDNA-AFLP with phloem sap of barley*
For fragment sequences, see Supplementary Table S1 at JXB online.

At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*.

Code[a]	cDNA-AFLP[b] bands 1 2 3 4	Length(bp)	Database search with BLASTN[c]: expected value, identities	Database search with BLASTX[c]: expected value, identities
S1-02 S5-14 S4-18 S7-23		92	BE213945 (*Hv*): 3e^{-44}, 100%	Imidazoleglycerol-phosphate synthase subunit H-like: NP568922 (*At*) 1e^{-04}, 70%
S2-03		134	BE421279 (*Hv*): 5e^{-62}, 97%	Putative ferredoxin: AC087851 (*At*) 3e^{-14}, 79%
S2-04		77	CD054184 (*Hv*): 4e^{-30}, 97%	Hom.[d]: WIR1A protein, planthopper-inducible protein-like
S2-05		97	CD056186 (*Hv*): 7e^{-45}, 97%	Putative regulatory protein: AA037940 (*Os*) 2e^{-10}, 100%
S2-07 S1-15		172	CD057212 (*Hv*): 4e^{-51}, 93%	Hom.: carbonic anhydrase
S3-09 S6-20		99	AV936683 (*Hv*): 1e^{-34}, 97%	Protein kinase family: NP17196 (*At*) 7e^{-13}, 100%
S5-13		82	AV939559 (*Hv*): 2e^{-23}, 90%	Hom.: putative glycosyl transferase
S6-19		88	CA024028 (*Hv*): 5e^{-36}, 96%	Hom.: elongin C

[a] Spezific S-primer (combined with unspecific N+0 primer)—serial number of the band.
[b] Four independent phloem samples.
[c] Homology search with BLASTN in EST- and with BLASTX in protein databases of NCBI and TIGR.
[d] Sequence of EST used for homology search with BLAST.

Such stress- and signalling-related mRNAs could be translated into proteins or could induce gene expression within the phloem or in distant target tissues. However, to date, it is completely unknown if these RNAs are localized in the phloem by accident or if they fulfil a specific function as signalling molecules.

SEs lack nuclei and a functional transcription and translation machinery. The SE–CC complexes are symplastically isolated, with only a few plasmodesmata connecting them to surrounding tissue. Hence, mRNAs in sieve tube sap most probably originate from CCs. The RNAs thus either could represent accidental RNA outflow from CCs to SEs, could fulfil unknown local duties, or could function in systemic signal transduction, as has been demonstrated for specific mRNAs. Phloem transport of a *CmNACP* mRNA from pumpkin could be directly demonstrated by heterograft experiments between pumpkin and cucumber plants, in which *CmNACP* transcripts from a pumpkin stock moved to cucumber scion tissues (Ruiz-Medrano *et al.*, 1999). Also movement of the transcript of a *KNOTTED1*-like homeobox gene has been demonstrated in tomato (Kim *et al.*, 2001), and a *BEL1*-like transcription factor was graft-transmissible in potato (Banerjee *et al.*, 2006). The same was observed for another gene, *GIBBERELLIC ACID INSENSITIVE* (GAI), in *Cucurbita maxima* and *Arabidopsis* gain-of-function mutants (Haywood *et al.*, 2005). In all these cases, the transport of mRNA led to observable phenotypic alterations, which indicates a physiological function for mRNA transport.

Future studies will be needed to establish functions for the growing set of as yet unknown mRNAs found in the phloem in the present and in previous studies.

Identification of barley phloem proteins by mass spectrometry after SDS–PAGE

One-dimensional electrophoresis was used to separate the proteins prior to trypsin digestion and identification by mass spectrometry. Despite the low amounts of protein loaded and the weak Coomassie staining (Fig. 3A), proteins could be assigned in seven protein bands (Table 3). Silver staining confirmed that the analysed bands represent major phloem proteins (Fig. 3B). Among the identified proteins were ubiquitin, thioredoxin h, cyclophilin, and a glycine-rich RNA-binding protein that were also found at detectable levels in phloem samples from other plant species (Barnes *et al.*, 2004; Giavalisco *et al.*, 2006; Aki *et al.*, 2008).

Thioredoxin h is thought to be part of the antioxidant defence machinery inside sieve tubes that is probably important to keep SE components intact over their complete, usually long, lifetime (Walz *et al.*, 2002). Cyclophilins have chaperone-like activity and are therefore likely to be involved in protein folding for import into and transport through SEs (Balachandran *et al.*, 1997). Also ubiquitin could be involved in protein sorting rather than in degradation (Giavalisco *et al.*, 2006). One of the most interesting protein classes are probably the various kinds of RNA-binding proteins also present in different species examined. Like CmPP16, the lectin PP2, or the small RNA-binding protein CmPSRP1, the glycine-rich RNA-binding protein present in barley phloem exudates might bind RNAs in order to facilitate their trafficking through plasmodesmata (Xoconostle-Cazares *et al.*, 1999; Barnes *et al.*, 2004; Gomez and Pallas, 2004; Yoo *et al.*, 2004; Giavalisco *et al.*, 2006; Aki *et al.*, 2008).
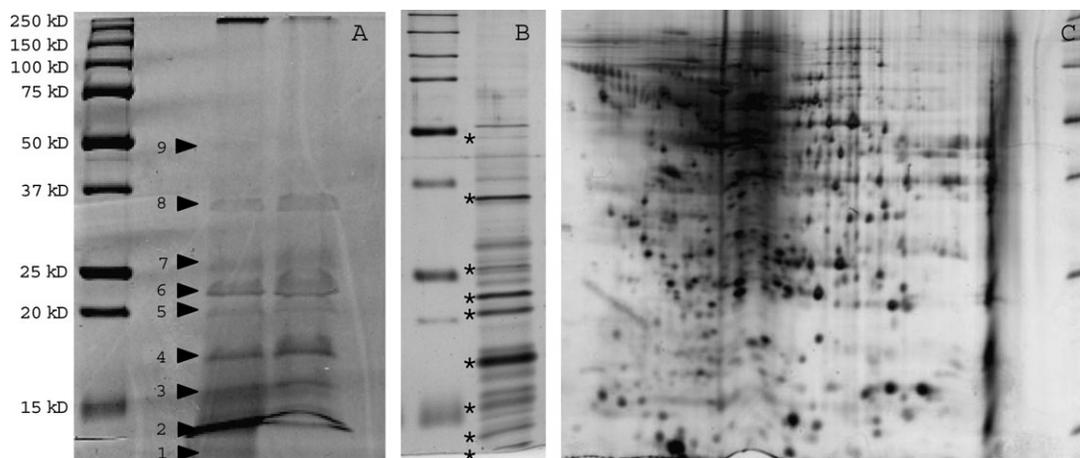
Fig. 3. Analysis of proteins in barley phloem exudates. (A) Preparative gel electrophoresis with 20 μl (left) and 30 μl (right) of barley phloem sap sampled by aphid stylectomy. The indicated Coomassie-stained bands were cut and analysed by mass spectrometry. (B) About 30 phloem proteins were visualized by SDS–PAGE and silver staining (5 μl of phloem sap). Major bands corresponding to the nine Coomassie-stained proteins in A are marked with asterisks. (C) More than 250 silver-stained phloem proteins can be distinguished after two-dimensional electrophoresis and silver staining.

Table 3. *Proteins in barley phloem sap*

Hv, *Hordeum vulgare*; M_{ox}, oxidized methionine; Mw_{ob} obtained mass in one-dimensional electrophoresis; Mw_{th}, theoretical mass; *Os*, *Oryza sativa*; *Ta*, *Triticum aestivum*. For band number, see Fig. 3A. Bold letters indicate amino acids that are identical to the respective database sequence.

Band no.	*m/z*	Sequence	Accession number	Plant	Annotation	Mw_{th} (Mw_{ob}) in kDa
1	–	No peptides detected				
2	534	**ESTLHLVLR**	gi|60499796	*Os*	Polyubiquitin	8.9 (~12)
2	882	**TITLEVESSDTIDNVK**	gi|60499796	*Os*		
2	596	**IM**$_{ox}$**APVFADLAK**	gi|32186042	*Hv*	Thioredoxin h isoform 2	13.2 (~12)
2	629	**VVGAIKEELTAK**	gi|32186042	*Hv*		
2	967	**VEAMPTFLFM**$_{ox}$**K**	gi|32186042	*Hv*		
3	1053	**GGGGGYGGGGGYG GQGGGYGGQGGGG YGGQGGGGYGGQR**	gi|728594	*Hv*	Glycine-rich/RNA-binding protein	16.8 (~16)
3	775	**GFVTFGSEESMR**	gi|728594	*Hv*		
3	783	**GFGFVTFGSEESM**$_{ox}$**R**	gi|728594	*Hv*		
4	712	**VFFDMTVGGAPAGR**	gi|13925737	*Ta*	Cyclophilin A-3	18.4 (~18)
4	720	**VFFDM**$_{ox}$**TVGGAPAGR**	gi|13925737	*Ta*		
4	822	**HVVFGEVVEGMDVVK**	gi|13925737	*Ta*		
5	454	**VVLAPANPK**	gi|66277464	*Ta*	Stress-responsive protein	22.2 (~20)
5	569	**ANPNYAM**$_{ox}$**SVR**	gi|66277464	*Ta*		
6	554	**DINAQTFVDNLKER**	gi|112821176	*Hv*	Hypothetical protein	23.0 (~22)
6	617	**SGVFGTPISEK**	gi|112821176	*Hv*		
6	625	**TNIGEGSTMNAR**	gi|112821176	*Hv*		
6	633	**TNIGEGSTM**$_{ox}$**NAR**	gi|112821176	*Hv*		
6	689	**DINAQTFVDNLK**	gi|112821176	*Hv*		
6	717	**NAQTFVDNLKER**	gi|112821176	*Hv*		
6	805	**LIYNATGATLSLAK**	gi|112821176	*Hv*		
6	839	**TVIATGEYKEPITQK**	gi|112821176	*Hv*		
6	995	**LANS**D**LNSTDKNYGYVSK**	gi|112821176	*Hv*		
7	839	**TVIATGEYKEPITQK**	gi|112821176	*Hv*	Hypothetical protein	23.0 (~26)
7	617	**GGLLDEK**	gi|112821176	*Hv*		
7	995	**LANS**D**LNSTDKNYGYVSK**	gi|112821176	*Hv*		
8	–	No peptides detected				
9	–	No peptides detected				

In addition to these known proteins, it was possible to identify two proteins formerly not found in phloem sap: a stress-responsive protein known from *T. aestivum* and a hypothetical protein from *H. vulgare* (Table 3). The stress-responsive protein is induced by abiotic stress and thus probably adds to the large family of stress- and defence-related phloem sap proteins already described (Giavalisco *et al.*, 2006; Kehr, 2006). The hypothetical protein is an interesting new candidate, because this protein has been shown to localize to scutellum and vascular bundles of barley seedlings and is induced by sugars, especially glucose and sucrose (Kidou *et al.*, 2006). Therefore, this protein has been associated with sugar mobilization and eventually sugar translocation from the seed into growing tissues, although its precise physiological function has as yet not been established (Kidou *et al.*, 2006). So far, sucrose transporters that mediate phloem sugar loading are known to occur in the plasma membrane of SEs in apoplastic loaders like barley (Kühn *et al.*, 1999). In addition, several enzymes from the glycolytic pathway have been found (Giavalisco *et al.*, 2006). Further analysis of this new sugar mobilization protein might allow new insights into sugar transport and metabolism inside the phloem tubes.

In the future, broad-spectrum analysis of proteins in barley phloem exudates will have to be performed. In these first experiments, silver-stained two-dimensional gels loaded with 5 µg of barley phloem proteins displayed >250 well separated spots (Fig. 3C). Very recently, Aki *et al.* (2008) applied nano-flow liquid chromatography/ mass spectrometry for the identification of 107 phloem proteins in rice phloem samples collected by stylectomy. Combined approaches utilizing the improved sampling techniques as described in the present report, and highly sensitive analysis methods would allow a comprehensive identification of phloem proteins from different monocots in the future.

Conclusions

A major barrier for molecular profiling of phloem sap from important monocot crop species is the small amount of phloem samples that can be obtained by insect stylectomy. The present experimental set-up overcomes this problem by allowing the collection of relatively large amounts of pure phloem sap from many barley plants in parallel. It is demonstrated that the obtained samples are

suitable for transcript and protein analysis by cDNA-AFLP and gel electrophoresis coupled to mass spectro-metric protein identification.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Sequences of transcript-derived fragments.

Acknowledgements

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