Comparative proteomics of cucurbit phloem indicates both unique and shared sets of proteins

Rosa M. Lopez-Cobollo, Ioannis Filippis, Mark H. Bennett and Colin G.N. Turnbull*

Department of Life Sciences, Imperial College London, London SW7 2AZ, UK

*corresponding author: c.turnbull@imperial.ac.uk

Running title: Cucurbit phloem proteomes

Key words: Phloem; proteomics; *Cucurbita maxima*; *Cucumis sativus*; extrafascicular; fascicular; multiple reaction monitoring.

SUMMARY

Cucurbits are well studied phloem biology models but unusually possess both fascicular phloem (FP) within vascular bundles and additional extrafascicular phloem (EFP). Although the functional differences between the two systems are not yet clear, sugar analysis and limited protein profiling previously established that FP and EFP have divergent composition. Here we report a detailed comparative proteomics study of FP and EFP in two cucurbits, pumpkin and cucumber. We re-examined the sites of exudation by video microscopy, and confirmed that in both species, the spontaneous exudate following tissue cutting derives almost exclusively from EFP. Comparative gel electrophoresis and mass spectrometry-based proteomics of exudates, sieve element contents and microdissected stem tissues established that EFP and FP profiles are highly dissimilar, and that there are also species differences. Searches against cucurbit databases enabled identification of more than 300 FP proteins from each species. Few of the detected proteins (~10%) were shared between sieve element contents of FP and EFP, and enriched Gene Ontology categories also differed. To explore quantitative differences in the proteomes, we developed multiple reaction monitoring methods for cucumber proteins that are representative markers for FP or EFP and assessed exudate composition at different times after tissue cutting. Based on failure to detect FP markers in exudate samples, we conclude that FP is blocked very rapidly and therefore contributes minimally to the exudates. Overall, the highly divergent contents of FP and EFP indicate that they are substantially independent vascular compartments.

INTRODUCTION

The phloem is a living tissue in vascular plants that functions primarily in transporting organic nutrients from source tissues (photosynthetic leaf cells) to sink tissues (roots, developing flowers, fruit, immature leaves, etc.). Phloem is composed of several cell types including sclerenchyma, parenchyma, sieve elements (SE) and companion cells (CCs). Sugars, amino acids, organic acids, proteins, mRNA, small RNA and hormones are translocated in the highly specialized SE (Walz *et al*., 2004; Gomez *et al*., 2005; Kehr, 2006; Aki *et al*., 2008; Buhtz *et al*., 2008; Guelette *et al*., 2012). These elongated cells are joined at their ends by perforated walls called sieve plates to form the sieve tubes (Esau, 1969). During SE development, there is a highly selective degradation of many cellular organelles including nucleus, Golgi bodies, ribosomes and vacuole. The remaining modified plastids, endoplasmic reticulum, mitochondria, and phloem-specific structural proteins (P-proteins), occupy a parietal position thus leaving a relatively unimpeded central path for mass flow of water and solutes in the translocation stream (Esau, 1969; van Bel and Knoblauch, 2000). Sieve element and companion cell pairs are closely associated as SE-CC complexes, with extensive plasmodesmata connections acting as cytoplasmic bridges. Since mature enucleate SEs have lost their capacity for protein synthesis, their adjoining CCs play a life-support role by providing new proteins, and indeed there is extensive exchange of proteins, metabolites, and signaling molecules (van Bel and Knoblauch, 2000). Surrounded by parenchyma cells and other specialized cells such as phloem fibres that provide structural support, SE transport cells are difficult to isolate.

Most proteomic studies of phloem have instead focused on analysis of either spontaneous sap exudate, as in castor bean (*Ricinus communis*) (Barnes *et al.,* 2004), rape (*Brassica napus*) (Giavalisco *et al*., 2006), pumpkin (*Cucurbita maxima*) (Haebel and Kehr 2001; Lin *et al*., 2009), cucumber (*Cucumis sativus*) (Walz *et al*., 2004; Fan *et al*., 2015) and melon (*Cucumis melo*) (Malter and Wolf, 2011; Serra-Soriano *et al.,* 2015); or sap collected via stylectomy, as in rice (*Oryza sativa*) (Aki *et al*., 2008) and barley (*Hordeum vulgare*) (Gaupels *et al*., 2008); or via EDTA-facilitated phloem sap exudate, as in Arabidopsis (Batailler *et al*., 2012) and rice (Du *et al.,* 2015). Overall, several hundred phloem sap proteins have now been identified.

Those investigations have identified phloem sap proteins involved in redox regulation, response to stress, defense and calcium regulation. However, there are fewer studies

of the proteins present in the entire phloem tissue. Laser microdissection (Schad *et al*., 2005) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Koroleva and Cramer, 2011) have been used in Arabidopsis to identify proteins from phloem tissue. Similarly, differential extraction methods combined with LC-MS-MS allowed the identification of 379 proteins from broccoli (*Brassica oleracea*) phloem strands (Anstead *et al*., 2013). Such approaches can provide new information on membrane-associated or complexed non-mobile SE proteins, or proteins present in other phloem cell types.

Cucurbits have long been used as model plants for phloem physiology and biochemistry because of the large amount of sap exudate that can readily be collected from cut petioles or stems (van Bel and Gaupels, 2004; Lin *et al*., 2009; Atkins *et al*., 2011; Zhang *et al*., 2012; Hu *et al*., 2016). By this approach, over 1200 non-redundant proteins were identified from pumpkin exudate (*Cucurbita maxima*) (Lin *et al*., 2009). Nevertheless, because of the unusual phloem anatomy of the Cucurbitaceae family, cucurbit exudates collected after incision are not necessarily representative of vascular plants in general. Specifically, cucurbits have two spatially separated phloem systems: fascicular phloem (FP) and extrafascicular phloem (EFP) (Crafts, 1932). The FP is located bicollaterally, both internal and external to the xylem, whereas EFP is distributed as scattered elements throughout the ground tissue (cortex), peripheral to the vascular bundles and both outside and inside of the sclerenchyma ring. Lateral commissural EFP elements form a complex network by connecting cortical strands with those neighboring the vascular bundles. In recent years, evidence has accumulated that, although physically adjacent, EFP and FP have different compositions and thus are likely to have divergent functions (Zhang *et al*., 2010; Zhang *et al*., 2012; Gaupels *et al*., 2012). In contrast to FP, the EFP does not accumulate effective callose plugs upon wounding, instead exuding profusely from cut stems and petioles (van Bel and Gaupels, 2004; Turgeon and Oparka, 2010; Zhang *et al*., 2010; Zhang *et al*., 2012). Cucurbit exudate contains vastly less sugar (10 to 30 mM) than predicted for assimilate transport and from comparison with other species (~1 M) (Richardson *et al*., 1982; Fiehn, 2003; Zhang *et al*., 2012). Indeed, raffinose family oligosaccharide (RFO) sugars characteristic of cucurbits are predominant in FP tissue, with estimated total concentration of approx. 0.5 to 1 M (Haritatos *et al*., 1996; Zhang *et al*., 2010). Moreover, the FP proteome, assessed from protein plugs of individual FP sieve elements (Zhang *et al*., 2010) or aphid stylectomy (Gaupels *et al*., 2012) is distinct from that of the phloem exudate.

Here, we analysed FP-enriched tissues and cell contents of FP sieve elements (FP-SE) to study the phloem proteomes of pumpkin (*Cucurbita maxima*) and cucumber (*Cucumis sativus*). Microscopy facilitated visual discrimination of the different tissues within the vascular bundles, enabling accurate microdissection of relatively pure tissue types. Proteins from the enriched tissues were compared with those in FP-SE and phloem sap exudate using LC-MS-MS analysis, and revealed unique and shared proteins in both FP and EFP. Targeted MS methods for FP- and EFP-specific proteins enabled more precise definition of the origins of exudate.

RESULTS

Sampling of cucurbit vascular tissue and cell types

The fascicular phloem is difficult to sample as the vascular bundles contain multiple adjacent cell types embedded in the ground tissue (Figure 1a,b). In an attempt to isolate FP proteins from pumpkin and cucumber, amido-black-stained coagulated protein strands from within external FP sieve elements were collected using hypodermic needle tips (Figure 1c), as described by Zhang *et al* (2010). The resultant SDS-PAGE protein profile shows the dominant band corresponding to the reported major protein in FP, FPP1 (Zhang *et al*., 2010), a member of the sieve element occlusion-related (SEOR) family (Figure 1f). Despite these samples being highly enriched for FP sieve element proteins, the protein quantity was low, with only 57 and 37 proteins identified for pumpkin and cucumber, respectively (Data S1 and S2). To increase coverage of FP proteins and also to identify proteins present throughout the FP tissue, we took advantage of the visually distinct features of each tissue type (fascicular phloem, xylem and ground tissue) within and adjacent to the vascular bundles of cucurbits when observed by stereo microscopy of fresh transversely cut stems (Figure 1a). Stem sections were subsequently cut longitudinally to isolate the vascular bundles (Figure 1b). Staining with amido black further aids recognition of the different cell and tissue types (Figure 1b,c). Because some cross-contamination between tissues is inevitable as the micro-dissection cannot be totally precise, we refer to each dissected tissue type as being enriched rather than pure. Comparison of SDS-PAGE protein profiles from FP-SE contents and microdissected pumpkin FP samples showed very similar major bands (Figure 1f). The greater total amount of protein derived by microdissection led to a larger number of identified FP proteins: 326 and 359 for pumpkin and cucumber, respectively. As expected, most (76% to 89%) of the proteins identified from FP-SE samples were also detected in the microdissected FP

tissues (Figure 2a,b). Alternatively, comparing the non-redundant proteins with homologs in the Arabidopsis proteome, 100% (pumpkin) and 96% (cucumber) of the detected FP-SE proteins were also found in microdissected FP (Figure S1).

We next compared the proteomes of enriched xylem and ground tissues with the equivalent FP tissue data. Xylem cells are elongated with thick walls, distinct from other tissues and easy to isolate due to higher tissue hardness. Ground tissue surrounding vascular bundles was separately collected. After dissection, the three tissues - FP, xylem and ground tissue - retain very different appearances (Figure 1d). We also collected exudates, previously shown to represent EFP cell contents, from cut stems (Figure 1e). Proteins extracted from each tissue type or cell contents had distinct SDS-PAGE band patterns for both pumpkin and cucumber (Figure 1g) and the profiles for corresponding tissues also display some species-specific differences. The major EFP exudate components were phloem proteins 1 and 2 (PP1 and PP2), which account for >80% of the total proteins in EFP (Gaupels *et al.,* 2012), and were much less abundant in all other tissues. In contrast, the SEOR band was confirmed as predominant in both FP sample types (microdissected tissue and SE contents), with much lower abundance in all other samples.

Protein identification

Comparative analysis by LC-MS-MS revealed both unique and shared proteins across all the four sample types: EFP, microdissected FP, ground tissue and xylem (Figure 3a–c; Data S3 and S4). Only a small proportion of detected proteins was common to FP and EFP in both pumpkin (4% of total) and cucumber (5%), with more sharing among xylem, ground tissue and FP. Although ground tissue contains many scattered EFP elements, the overlap between ground tissue and EFP proteomes was not greater than between EFP and the other tissue types (Figure 3a,b,d,e). Very few detected proteins were common to all sample types: 1% of total protein list for pumpkin and 2% for cucumber. Similar results were obtained from an equivalent comparison of only the proteins identified from FP-SE content against the EFP, ground tissue and xylem proteins (Figure 3d–f; Data S1 and S2).

Targeted analysis of unique protein markers for FP and EFP

Although there are several recent papers on cucurbit phloem, the origin of the droplets exuding from cut tissues has been disputed. Separate sampling of pumpkin exudate and microdissected FP revealed large differences in sugar and protein composition,

enabling attribution of the exudate exclusively to EFP (Zhang *et al*., 2010). The different protein pattern between pumpkin EFP and FP sieve tube contents was confirmed by aphid stylectomy for collection of pure phloem sap which showed a protein profile very similar to that of dissected FP and unlike that of EFP exudate (Gaupels *et al*., 2012). Zhang *et al*. (2012) argue that pumpkin exudate is pure EFP but in other cucurbits such as cucumber, the exudate is a mixture derived from both EFP and FP. Using the protein data sets obtained from our microdissected and exudate samples, we developed multiple reaction monitoring (MRM) methods for cucumber proteins that are representative markers for FP or EFP. We selected Cucsa.386600, a member of the SEOR family, and Cucsa.395940, a non-membrane-bound member of the leucine repeat rich family, as markers for FP, as they are both abundant with multiple unique peptides detected (Figure 4 and Table S1). The MRM results clearly show that both proteins are present in FP (microdissected and SE content) but virtually absent in EFP exudates. Conversely, the proteins selected as exudate (EFP) markers, Cucsa.151370, annotated as a cystatin/monellin superfamily protein and commonly known as phloem protein 1 (PP1), and Cucsa.091340, a lipoxygenase (LOX5), are virtually absent from FP samples (Figure 4). Together, these targeted data provide further strong evidence that FP and EFP have highly divergent contents.

Phloem exudation time series in cucumber

We next attempted to visually resolve the question of the origin of phloem exudates in cucumber by undertaking video microscopy analysis similar to the pumpkin study of Zhang *et al.* (2010). As with pumpkin, the initial flow of exudate from cut cucumber stems is too profuse to allow the cellular origins to be clearly determined. Individual droplets rapidly enlarged and merged together to spread over the entire cut surface. However, after removing the exudates several times with filter paper over a period of 3 to 5 minutes, the flow decreased significantly, allowing visualisation of discrete droplets that initiated mainly around the edge of the vascular bundles and in the sclerenchyma ring, i.e. the principal sites where the EFP is located (Figure 5 and Movie S1).

These results indicate that in cucumber, as in pumpkin, the exudates originate from EFP. However, the source of the droplets can be confidently assigned only after repeatedly removing the initial rapid exudate flow, leaving the possibility that the very first exudation comes from a mix of FP and EFP. We therefore analysed the proteome of exudates collected from both species at different times after cutting. The initial exudates (t0) were collected immediately for the first 30 seconds after cutting, without

any cleaning of the tissue. Subsequent samples were taken within one minute of cutting but after removal of the initial exudate by washing and filter paper cleaning of the tissue (t1) or in the following two minutes after the t1 sample had been collected (t3) (Figure S2). The resulting SDS-PAGE protein profiles were very similar at all three different sampling times, but very different from the FP profile (Figure 6a,b). Analysis of MRMs for Cucsa.386600 and Cucsa.395940 (FP markers) and Cucsa.151370 and Cucsa.091340 (EFP markers) showed that the FP markers were undetectable in all exudate samples including those collected immediately after cutting (Figure 6c,d). These results indicate that FP is indeed very rapidly blocked, resulting in essentially no flow out of wounded FP sieve elements and thus not contributing to the exudate. In contrast, abundance of the EFP markers in exudates was very similar across all sampling times (Figure 6e,f).

Of the 326 and 359 FP proteins identified in pumpkin and cucumber, respectively, 280 and 322 had significant matches in Arabidopsis (Data S5 and S6). Although the EFP gel banding patterns at different exudation times look very similar (Figure 6) and overall more than 50% of the proteins were shared across all three exudation times (Figure S3), some proteins were detected only at one sampling time, as predicted from relatively low proteome coverage in each individual sample. To compare proteins from FP microdissected and SE content samples with those from the exudate time series, we combined the exudate EFP t1 and t3 protein lists, but excluded the EFP t0 proteins, to be consistent with previous studies where cut surfaces were always cleaned before collection of exudate samples. Less than 9% of the total identified proteins are shared between microdissected FP and EFP exudate in pumpkin and cucumber (Figure 2c,d), broadly in agreement with the results of our preliminary study (4 and 5%; Figure 3). These values drop to 5% and 4% when FP-SE content is compared with EFP exudate in pumpkin and cucumber, respectively (Figure 2e,f), again in agreement with the results of our preliminary study (3 and 6%; Figure 3). Moreover, proteins from FP-SE samples had only 4% and 3% overlap with those from the EFP t0, as a proportion of the total proteins in pumpkin and cucumber, respectively (Figure S4). Overall, our data indicate that FP and EFP are independent, with some shared but many unique proteins. We found no evidence that the exudate is a mixture between FP and EFP, even in cucumber. The phloem exudates derive almost exclusively from EFP and maintain very similar composition for several minutes after tissue cutting.

Functional analysis

Analysis of Gene Ontology (GO) annotations in Arabidopsis and MapMan categories (BAR) for molecular functions and biological processes showed that the FP proteomes of pumpkin and cucumber were very similar (Figure 7a and Tables S2a,b). Comparing GO Slim annotation categories of proteins with known gene functions between the FP-enriched proteomes and the whole Arabidopsis genome, the main over-representations were in structural molecule activity, nucleotide and protein binding, other binding and other enzyme activity. In contrast, there was under-representation for kinase activity, nucleic acid binding and transcription factor activity. Comparing GO Slim categories for known biological processes, GO terms for electron transport or energy pathways, responses to abiotic or biotic stimulus, response to stress, cell organization and biogenesis and transport were most significantly over-represented, while annotations for transcription or DNA-dependent processes were significantly under-represented. Although the results were closely matched between pumpkin and cucumber, proteins related to secondary metabolism, minor carbohydrate metabolism and gluconeogenesis/glyoxylate cycle were absent from the pumpkin FP dataset, while no proteins involved in the biodegradation of xenobiotic compounds were found in cucumber FP (Table S3a,b and Figure S5).

For both EFP proteomes, GO Slim categories for known biological processes were over-represented in electron transport or energy pathways, responses to abiotic or biotic stimulus, response to stress, cell organization and biogenesis, and protein metabolism, but depleted for transcription and DNA dependent processes (Figure 7b and Tables S2a,b). Comparing GO Slim molecular function categories for the EFP proteomes against the whole Arabidopsis genome shows the main enrichment was in annotations for nucleotide and protein binding, other binding and other enzyme activity.

Gene categories for FP and EFP proteomes were compared (Figure S5 and Table S3a,b). Some similarities were evident, including enrichment of both datasets for: response to abiotic and biotic stimulus, response to stress, electron transport, cell organization and biogenesis, protein metabolism and other biological process, but depletion for transcription and DNA-dependent process, kinase activity and nucleic acid binding. There were also several differences between EFP and FP. Structural molecule activity was highly enriched from FP samples (microdissected and SE contents) compared with EFP (Figure S6). Enzymes involved in photosystems, electron transport/ATP synthesis, N-metabolism, and major carbohydrate metabolism were found only in FP samples, consistent with FP's known high sugar content (Zhang *et al*.,

2010) and primary function of phloem in carbohydrate translocation. Phloem-specific enzymes involved in sugar metabolism (Barratt *et al*., 2009, 2011) were found in FP but not EFP, including sucrose synthase (SUS5-At5g37180 and SUS6-At1g73370 homologs) and a starch branching enzyme (SBE2-At5g03650). Signal transduction proteins were less represented in EFP compared with FP, with the latter containing G proteins, annexins, leucine-rich repeat proteins, calreticulin1a, calcium signalling proteins and 14-3-3 proteins. Of these, EFP contained only G proteins. One class almost exclusive to EFP samples was biodegradation of xenobiotics.

Discussion

Phloem sap and phloem tissue proteomes

Cucurbits have long been popular models for phloem proteomics, and indeed the greatest numbers of proteins identified in exudate of any species are in cucurbits, with over 1100 non-redundant proteins recorded (Lin *et al*., 2009; Hu *et al.,* 2016). However, because of the complexity of cucurbit phloem, having both fascicular and extrafascicular systems, these species may have proteomes that are not generically representative of angiosperms. A debate has continued on whether cucurbit exudate comes from EFP alone or a combination of FP and EFP. Here we aimed to resolve this question by analysing essentially pure FP-SE protein samples, collected as coagulated sieve element protein strands from external FP, as described by Zhang *et al* (2010). These authors also analysed internal FP, finding that the sugar and protein composition of both was very similar. Because internal FP is much smaller and more difficult to dissect than external FP, we focussed on the latter. Comparisons of proteins detected in FP-SE with those in exudate show low proportions of proteins in common (4% and 5% in cucumber and pumpkin, respectively), even in the first seconds of exudation immediately after cutting tissues (Figures 6 and S4). In both species, the exudate contains essentially undetectable levels of the major FP-SE proteins, and we therefore conclude that it derives almost entirely from EFP.

FP of cucumber and pumpkin rapidly blocks after cutting

Taken together, our results from imaging, video recording and protein profiles clearly show that FP blocks almost immediately after cutting in both cucumber and pumpkin. However, the mechanism by which this happens remains unclear. Although direct evidence is lacking, we suggest that the blocking may be attributable to the SEOR proteins that represent some of the most abundant proteins in FP, as first identified

from pumpkin by Zhang *et al*. (2010). True sieve element occlusion (SEO) genes encode subunits of forisomes, readily visible proteinaceous structures found uniquely in Fabaceae sieve elements (Rüping *et al*., 2010). Forisomes undergo reversible conformational changes after wounding, from a condensed to an expanded (so-called "dispersed") state, thereby blocking the sieve tube. In many non-Fabaceae species lacking forisomes, SEO and SEO-related (SEOR) homologs have been identified, including 10 family members in cucumber (Ernst *et al.,* 2011). Here we detected 6 and 7 SEOR proteins from FP-enriched tissues and 4 and 5 in the case of SE contents from cucumber and pumpkin, respectively. However, the role of these proteins in blocking the SE has been questioned (Knoblauch *et al*., 2014) and conclusive evidence is lacking that they are necessary or sufficient for blocking of SE following wounding. In particular, the plugs formed on sieve plates after wounding may not impede translocation of the phloem stream (Knoblauch *et al*., 2014). Nonetheless, other evidence does point to a protein-based FP blocking as we see protein plugs clearly evident at the cut surfaces of stems and petioles, and FP contents are virtually absent from exudates of both cucumber and pumpkin. The EFP exudates, in contrast, gel slowly over a period of a few minutes largely due to the well-studied P proteins (PP1 and PP2) (Alosi *et al*., 1988, Golecki *et al*., 1999).

FP-specific proteome

Our intention here was to compare the main proteins that distinguish FP and EFP, rather than generating high-coverage, exhaustive protein lists. The latter would include large numbers of low abundance proteins, which can represent truly rare proteins from FP or EFP, but equally can derive from the small amounts of cross contamination during sampling. To avoid this ambiguity, we focussed on the higher abundance subsets of proteins readily detected from these tissue and sap sources. Nonetheless the needle tip method yields relatively low amounts of SE proteins and limits studies to proteins present in the sap, many of which potentially translocate in the phloem stream of intact plants. Many additional non-mobile proteins are present in sieve elements and in other cell types within the phloem tissues. We therefore used microdissected tissues, similar to an approach used for broccoli phloem (Anstead *et al*., 2013), to extend coverage of the FP proteome to over 300 proteins from each species, and to enable comparison of FP-SE contents with those in FP tissue as a whole. Protein profiles by SDS-PAGE showed that the major bands in FP-SE and FP tissue were broadly similar (Figure 1f), consistent with most of the identified FP-SE proteins also featuring in the

FP tissue datasets. Although each tissue type was readily distinguishable by dissecting microscope, there is inevitably some contamination from adjacent tissues, and from EFP strands peripheral to vascular bundles. Cross-contamination appears somewhat greater in cucumber than in pumpkin, based on the consistently higher numbers of shared proteins among cucumber tissues, and may be attributable due to the smaller size of cucumber stems making accurate dissection more challenging.

FP versus EFP: Functional analysis

Arabidopsis GO and MapMan genome annotations were used to infer the molecular function and biological processes associated with proteins from pumpkin and cucumber FP-enriched tissues, and thus provide indications of specialized functions distinct from EFP and from the rest of the plant. Several GO terms were significantly enriched in FP: under Gene Function, the most notable category was structural molecule activity and in Biological Process, energy pathways, response to abiotic and biotic stimuli, and stress were elevated (Figure 7, Tables S2a,b).

Of the many proteins associated with structural molecule activity present in the FP data, several were ribosomal proteins (homologs of RPS6A (At4g31700), RPS6B (At5g10360) and RPL3B (At1g61580)), most likely derived from cell types other than mature SE which are enucleate and lack protein synthesis machinery. There were also members of the actin and tubulin families from both species. Actin has been previously found in phloem exudate of several species (Schobert *et al*., 1998; Walz *et al*., 2004; Giavalisco *et al*., 2006; Lin *et al*., 2009; Anstead *et al*., 2013). Hafke *et al.* (2013) demonstrated a fully developed parietal actin meshwork in SEs. We also found clathrin (BBH At3g11130) in both pumpkin and cucumber, previously reported from pumpkin exudates (Lin *et al*., 2009) and broccoli phloem strands (Anstead *et al*., 2013). In contrast, the only structural molecule activity identified in both pumpkin and cucumber EFP data sets was Actin7.

As might be predicted from the major functions of phloem in sugar loading/unloading and translocation, several enzymes involved in primary metabolism are present in the FP-enriched tissues. They included enzymes of photorespiration, the oxidative pentose phosphate cycle (OPP) and gluconeogenesis. Interestingly, photosystem proteins, rubisco and sucrose synthase proteins were identified in FP-enriched tissues but not in EFP exudates of either species. Similarly, although previously reported to be present in pumpkin exudate (Lin *et al.,* 2009), we identified aconitase (At2g05710) only in FP-

enriched tissues. This enzyme, which catalyses the conversion of citrate to isocitrate, has been suggested also to play a role in mediating oxidative stress and regulating cell death in Arabidopsis and *Nicotiana benthamiana* (Moeder *et al.,* 2007).

We found 14-3-3 proteins in FP-enriched tissues but not in EFP exudates. Members of this family have already been reported from phloem of Arabidopsis (Batailler *et al.,* 2012; Guelette *et al.,* 2012), poplar (Dafoe *et al.,* 2009) and from pumpkin exudate (Lin *et al.,* 2009; Gaupels *et al.,* 2012). The 14-3-3 proteins are a family of conserved eukaryotic regulatory proteins that function in protein-protein interactions and in mediating signal transduction pathways, especially those associated with plant responses to hormones as well as biotic and abiotic stresses (Gökirmak *et al.,* 2010; Denison *et al*., 2011; Jaspert *et al.*, 2011).

Stress-related proteins were identified in both FP and EFP, including scavengers of reactive oxygen species, such as ascorbate peroxidase (APX1), together with other peroxidases and lipoxygenase (LOX). The functions of LOX relate to synthesis of a number of different compounds with signalling functions (Creelman and Mullet, 1997; Parchmann *et al*., 1997), antimicrobial activity (Croft *et al*., 1993; Weber *et al*., 1999), or to the development of the HR (Rustérucci *et al*., 1999). Enzymes of primary metabolism such as phosphoglycerate kinase (PGK) and malate dehydrogenase also participate in stress responses, with the latter involved in maintenance of redox balance and in responses to abiotic stresses such as drought and salinity (Sairam and Tyagi 2004; Hebbelmann *et al*., 2012). Some stress-related proteins were detected only in FP, such as prohibitin, which functions in mitochondrial biogenesis and protection against senescence (Ahn *et al*., 2006). Likewise, one of the key enzymes in ethylene biosynthesis, 1-aminoacyclopropane-1-carboxylate oxidase (ACO4 - At1g05010) was found only in FP-enriched tissues.

FP-enriched tissues and EFP exudate versus other phloem proteomes

Using high stringency cutoff points, we identified representative sets of proteins from FP-enriched tissues: 322 and 280 non-redundant proteins for cucumber and pumpkin, respectively, that have homologs in the Arabidopsis proteome. For reasons discussed above, these lists are likely not exhaustive. To enable resolution of whether the two phloem types have distinct compositions, and whether cucurbit exudates represent only EFP or a mixture of EFP and FP, we also developed a list of 60 non-redundant proteins for cucumber and pumpkin from EFP t1 and t3 time course samples. Many

other proteins have been reported from EFP (Lin *et al.,* 2009), most of which are relatively minor components, and it is likely that there are further undiscovered minor proteins of FP.

We compared our FP and EFP proteome lists with data for pumpkin phloem exudates (Lin *et al*., 2009), Arabidopsis EDTA-facilitated phloem exudates (Batailler *et al*., 2012), Brassica phloem exudates (Giavalisco *et al*., 2006) and Brassica dissected phloem tissue (Anstead *et al*., 2013). The strongest similarities, as expected, are between our EFP (t1+t3) samples and the pumpkin exudates of Lin *et al*. (2009), with 78% of our pumpkin proteins and 55% of our cucumber proteins in common with the published data. For the FP-enriched tissues, the strongest correspondences are with the brassica dissected phloem tissue (28% for cucumber and 31% for pumpkin) (Table 1). This is not surprising given the similar sampling methodology used in both studies. Even in closely related species, comparison of *B. napus* phloem sap proteome with *B. oleracea* phloem tissue proteome shows only 31% overlap. The imperfect correspondence is attributable in part to incomplete coverage of the proteome in all experiments, and also to the substantial difference between phloem tissue and phloem sap composition. In general, phloem exudate and EDTA-facilitated exudate provide mainly soluble proteins, whereas dissected tissues of both broccoli and our FP-enriched tissues contained many additional proteins. Using MapMan annotations, it was notable that the majority of the significantly enriched and depleted categories in FP corresponded with trends reported for the Arabidopsis phloem exudate proteome (Figure S7a; Batailler *et al.,* 2012), but with a lower correspondence between EFP and Arabidopsis phloem (Figure S7b).

Conclusions

Reports in the past few years have highlighted the many differences between FP and EFP contents (Zhang *et al.,* 2010; Gaupels *et al.,* 2012; Zhang *et al.,* 2012). Despite this evidence, some papers continue to describe cucurbit exudate as the sieve element assimilate translocation stream, moving via the vascular phloem (FP), and not as representing EFP (Hu *et al*., 2016). In our view, it is important to comment on the interpretations made by these authors, who do not attempt to clarify whether exudate comes from FP or EFP. Although absolute concentrations are not reported, their metabolite profile data indicate that exudate does not contain the high levels of RFO sugars (raffinose, stachyose, verbascose) expected of the assimilate stream, and exudate was not enriched in RFO sugars relative to the bulk vascular bundle tissue

analysed (Hu *et al.*, 2016).. Low RFO sugars in exudate are consistent with many previous findings that also report presence of monosaccharides, the latter being atypical of phloem, (Fiehn, 2003; Zhang *et al.*, 2010, 2012). These exudate sugar profiles are in stark contrast to molar concentrations of RFOs measured in tissue samples of cucurbit leaf minor vein loading phloem (Haritatos *et al.*, 1996) and stem FP (Zhang *et al.*, 2010). By gel analysis, the protein profiles of exudate and FP are very different, whether the latter was sampled from dissected tissue (Zhang *et al.*, 2010) or via aphid stylectomy (Gaupels *et al.*, 2012), likewise pointing to the exudate not originating from the FP translocation stream. Our present results further establish that FP and EFP are largely independent systems with highly divergent content. Moreover, exudates in cucumber and pumpkin both emanate almost exclusively from EFP. A key remaining question is whether cucurbit FP and EFP evolved to share the functions normally conducted by the single phloem of non-cucurbit species or whether FP is equivalent to the phloem in species with only a single phloem system. If the latter is true, then EFP would have functions largely or exclusively different from normal phloems including FP. Indeed, it has been proposed that EFP instead has more similarities to latex-containing laticifer systems than to phloem (Gaupels and Ghirardo, 2013). Based on FP rather than EFP showing the closer correspondence to phloems of other species, we propose that FP is essentially a normal, complete phloem system, but we cannot rule out that some functions, for example in defence and stress responses, may have been duplicated or shifted into the EFP system.

EXPERIMENTAL PROCEDURES

Plant Materials and sampling

Cucurbita maxima cv. Ghost Rider (pumpkin) and *Cucumis sativus* cv. Marketmore 76 (cucumber) were grown in a 4:1 (v/v) mixture of peat-based compost and vermiculite at 23°C day/18°C night under a 16 h photoperiod in a greenhouse supplemented with Son-T lights at 120 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation). Three-month-old mature plants containing fully developed stem phloem were used.

Extrafascicular phloem contents (“exudates”) were normally collected from stems directly into protein extraction buffer, after cutting the tissue, cleaning the cut surface with filter paper and discarding the first exuded droplets. For time course series, exudate was collected directly for the first 30 s after cutting without any cleaning or washing of the tissue surface (t0 sample); a second sample involved cutting, washing

and cleaning the tissue surface with a filter paper, then collecting exudate for the following minute (t1); and a third sample was subsequently collected after the t1 sample and a further cleaning step, with exudate taken during the two following minutes (t3) (Figure S1).

Fascicular phloem, ground tissue and xylem tissues were microdissected from chilled fresh stem segments under a stereo microscope. Stems were cut to ~0.5 cm lengths and stained with 0.1% (w/v) amido black in $CaCl_2$ (pH ~6.8) to visualize the coagulated contents of FP sieve elements. After slicing stem pieces longitudinally to leave three to six vascular bundles intact, each bundle was dissected into ground tissue, xylem and fascicular phloem, or to collect coagulated FP sieve element (FP-SE) contents with hypodermic needle tips. Tissue types were discerned by characteristic colour, shape, and cell sizes. Internal fascicular phloem was incised close to the xylem, and visible pith was removed by razor blade. The xylem was then removed and collected separately. External phloem strands of 3-5 mm length were then stripped out with a razor blade and forceps. The surrounding tissue was collected as ground tissue. Samples were then transferred to sample tubes and frozen immediately in liquid nitrogen. Fascicular phloem, xylem and ground tissue from ~50 stem sections from at least 3 individual plants were pooled as one sample (200–500 mg). Samples were kept at -80°C until protein extraction.

FP-SE contents were collected directly, using a method based on Zhang *et al.* (2010) Briefly, stem segments were sliced and stained with amido black as described above. Under the stereomicroscope, stained fascicular phloem proteins in large sieve elements were clearly visible as blue strands (see Figures 1b,c) and were collected using fine hypodermic needle tips. The needle tip was then dipped directly into protein extraction buffer then samples were stored at -80°C until extraction.

Microscopic observation and video recording

Phloem exudation from cut stems was observed by bright-field stereo microscope (Navitar) with a Pixelink PL-A6XX image capture system operating at 10 frames per second. To enable observation immediately after cutting, focus was pre-adjusted before the stem was cut transversely with a clean scalpel. For continued observation of exudation, it was necessary to remove further stem slices of ~1–10 mm and/or clean the tissue surface with filter paper.

Protein Extraction

Phloem exudates and FP-SE contents were collected directly into tubes containing extraction buffer (4% w/v SDS, 2% w/v β mercaptoethanol, 20% w/v glycerol, 2 mM PMSF, 100 mM Tris-HCl pH8.5, 10 mM DTT, 1% Sigma plant protease inhibitor cocktail) and immediately processed or frozen in liquid nitrogen and stored at −80°C. For tissue samples, ~200 mg of frozen microdissected fascicular phloem-, ground tissue- or xylem-enriched tissues were ground in liquid nitrogen with a micro-pestle, directly in the microcentrifuge tube to avoid loss of material, and extracted in 1 ml of extraction buffer. Proteins were then collected by acetone precipitation (Zhang *et al.,* 2010). Protein concentrations were determined by Bradford protein assay with catalase standard. Proteins were separated by 1D SDS-PAGE and visualized by Coomassie staining.

Mass Spectrometry

To improve coverage, protein samples were first separated by a short run on SDS PAGE (4 cm separation), then lanes were cut into 4-6 gel slices which were each subjected to in-gel trypsin digestion prior to analysis by LC-MS-MS. A shotgun non-targeted proteomics approach was used (Wu and MacCoss, 2002). The resultant peptide fragment ion spectra together with the cognate precursor peptide masses allow the identification of proteins from which they are derived using protein database searches.

Targeted protein analysis was also applied for a selection of proteins of interest and potential tissue-specific markers. For this, LC-MS-multiple reaction monitoring (MRM) was used in triple quadrupole mode on an Applied Biosystems QTrap 6500 system, with a predefined set of proteotypic peptides (Picotti and Aebersold, 2012). Initially proteins in solution were trypsin digested and used to optimise LC-MS-MRM methods.

Trypsin digested samples were analysed on an AB-SCIEX 6500 QTrap MS coupled to an Eksigent Nano LC400 system via an Ion Drive Turbo V source. The LC system was configured to load samples (20 µl) onto a trap column (Agilent Zorbax SB 5 µm x 0.3 mm x 35 mm) using the loading pump; the trap column was washed and then switched into the capillary flow; peptides were separated at 40°C on a capillary column (Eksigent C18CL 3 µm 0.3 mm x 150 mm). The loading/washing solvent was H_2O containing 0.2% CHOOH, 0.02% TFA at a flow rate of 30 µl min^{-1} and the resolving solvent was a gradient system of 0% B to 40% B over 45 min at a flow rate of 6 µl min^{-1} [(A)94.9% H_2O, 5% CH_3CN, 0.1% CHOOH; (B) 94.9% CH_3CN, 5% H_2O, 0.1% CHOOH]. The

valve was switched to direct the flow from the trap into the resolving column after a 5 min wash.

The MS, configured with high mass enabled, was used in “Trap” mode to acquire Enhanced Product Ion (EPI) scans for peptide sequencing and “Triple Quadrupole” mode for Multiple Reaction Monitoring (MRM). For qualitative discovery mode analysis, an Information Dependent Acquisition (IDA) method consisting of a survey scan (300-1200 m/z) followed by an Enhanced Resolution scan and then Enhanced Product Ion scans (230-1500 m/z) of the “top 5” was used. Dynamic background subtraction and rolling collision energy were used as default parameters. MS parameters were: Curtain Gas 35 psi, GS1 15 psi, GS2 30 psi, Interface heater on, TEM 125°C, DP 80, CXP 13. The MS source values were retained for the MRM analysis but the CAD gas was reduced from high to medium. MRM data analysis was performed using Analyst software 1.6.2 (AB SCIEX).

Proteotypic peptides. Signature peptides for the targeted analysis were determined from trial MRM-MS runs. The typical workflow to select the best signature peptides was to perform an in silico tryptic digest using Skyline (MacLean *et al.*, 2010), and a cucurbit library (see ‘proteome analysis’ below) was used to look for potential non-unique peptides. In addition to in silico analysis, peptide selection was assisted by data from IDA experiments and from daughter ion fragment information from targeted EPI scans. The combined information was used to derive MRM transition sets for the different peptides; typically 3-4 transitions per peptide were used; CE (collision energy) was set according to either empirically determined values or that estimated by Skyline software.

MS parameters were further optimised by testing against typical samples, with final methods containing MRM transitions to analyse four marker proteins, two each from FP and EFP, with a minimum of two peptides per protein (Table S1).

Proteome Analysis and GO enrichment

Analysis of MS-MS (IDA) data was carried out using ProteinPilot 4.5 (AB Sciex). Peptides were searched against a Cucurbit database comprising of translated predicted proteins from the cucumber genome (Huang *et al*., 2009); pumpkin phloem proteome sequences (Lin *et al*., 2009) and further translated ESTs from NCBI and proteins from UniProtKB, together with sequences of common contaminants (AB Sciex). The software was configured to allow for gel-based modifications, biological

modifications and amino acid substitutions. Proteins with a ProteinPilot Unused ProtScore >2 (99% CI) and at least 2 peptides were then submitted to further analysis. Putative orthologues were identified with BLASTP searches (E-value threshold $1e^{-5}$) against the Arabidopsis and rice proteomes. Although the pumpkin predicted proteome is not complete due to lack of available genomic sequence, the reciprocal best BLAST hit was also examined. The following database and software versions were used: NCBI BLASTP 2.2.26, TAIR10 protein database (TAIR10_pep_20101214) and MSU Rice release 7 protein database (all.pep). An in-house Bash script was developed to perform this analysis. To compare the proteins identified in the different tissues and between FP and EFP time courses, Venn diagrams were generated in Venny (Oliveros, 2007; http://bioinfogp.cnb.csic.es/tools/venny/).

Identified proteins were assigned to functional classes according to the GO and MapMan categories on TAIR (http://www.arabidopsis.org/) and BAR (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). Normed frequencies were calculated as follows: $(Nb_inClass_{input_set}/Nb_Classified_{input_set})/(Nb_in_Class_{reference_set}/Nb_Classified_{reference_set})$, with the mean and standard deviations calculated for 100 bootstraps of the input data set (Provart and Zhu., 2003). *P*-value of the hypergeometric distribution was calculated as: $P = BC(M,x) * BC(N-M, n-x) / BC(N,n)$, with BC as the binomial coefficient calculated as: $BC(n,k) = n! / (k! * (n-k)!)$; x as the number of input genes with the selected classification; n as the total number of input genes; M as the number of genes with the selected classification in the database (GO or MapMan); N as the total number of genes in this database. Values of $P<0.05$ were considered as significant.

ACKNOWLEDGEMENTS

This research was supported by the Biotechnology and Biological Sciences Research Council of the UK, grant number BB/I021930/1. We are grateful for the expert horticultural assistance of Martin Selby.

The authors declare no conflict of interest.

SUPPORTING INFORMATION LEGENDS

Figure S1. Venn diagrams for microdissected FP and FP-SE, using Arabidopsis homologs as reference for identification

Figure S2. Scheme of time series for exudate collection**.**

Figure S3. Venn diagrams for phloem proteins from EFP time series.

Figure S4. Venn diagrams indicating EFP protein overlaps at different time points and between EFP and SE content.

Figure S5. Frequencies of MapMan functional categories for FP and EFP proteins

Figure S6. Frequencies of MapMan functional categories for microdissected FP, FP-SE content and EFP proteins.

Figure S7. Comparison of MapMan functional categories between Arabidopsis and cucurbit phloem proteomes.

Table S1. Summary of unique peptides for FP and EFP markers.

Table S2a. Summary and statistical analysis of GO annotations for pumpkin phloem proteomes.

Table S2b. Summary and statistical analysis of GO annotations for cucumber phloem proteomes.

Table S3a. Summary and statistical analysis of MapMan functional annotations for pumpkin phloem proteomes.

Table S3b. Summary and statistical analysis of MapMan functional annotations for cucumber phloem proteomes.

Data S1. Comparison of pumpkin proteomes from FP-SE content and tissues within the vascular bundle.

Data S2. Comparison of cucumber proteomes from FP-SE content and tissues within the vascular bundle.

Data S3. Comparison of pumpkin proteomes from microdissected FP and other stem tissues.

Data S4. Comparison of cucumber proteomes from microdissected FP and other stem tissues.

Data S5. Comparison of pumpkin FP and EFP proteomes.

Data S6. Comparison of cucumber FP and EFP proteomes.

Movie S1. Phloem exudation from cucumber stem.

REFERENCES

Ahn C.S., Lee J.H., Reum Hwang A., Kim, W.T. and Pai, H.S. (2006) Prohibitin is involved in mitochondrial biogenesis in plants. *Plant J.* **46**, 658–667.

Aki, T., Shigyo, M., Nakano, R., Yoneyama, T. and Yanagisawa, S. (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-like proteins in phloem and xylem and sap from rice. *Plant Cell Physiol.* **49**, 767–790.

Alosi, M.C., Melroy, D.L. and Park, R.B. (1988) The regulation of gelation of phloem exudate from *Cucurbita* fruit by dilution, glutathione, and glutathione reductase. *Plant Physiol.* **86**, 1089–1094.

Anstead, J.A., Hartson, S.D. and Thompson, G.A. (2013) The broccoli (*Brassica oleracea*) phloem tissue proteome. *BMC Genomics* **14**, 764-785.

Atkins, C.A., Smith, P.M. and Rodriguez-Medina, C. (2011) Macromolecules in phloem exudates: a review. *Protoplasma* **248**, 165-72.

Barnes, A., Bale, J., Constantinidou, C., Ashton, P., Jones, A. and Pritchard, J. (2004) Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. *J. Exp. Bot.* **55**, 1473–1481.

Barratt, D.H.P., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., Feil, R., Simpson, C., Maule, A.J. and Smith, A.M. (2009) Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. *Proc. Natl. Acad. Sci. USA* **106**, 13124-13129.

Barratt, D.H.P., Kolling, K., Graf, A., Pike, M., Calder, G., Findlay, K., Zeeman, S.C. and Smith, A.M. (2011) Callose synthase GSL7 is necessary for normal phloem transport and inflorescence growth in Arabidopsis. *Plant Physiol.* **155**, 328-341.

660 **Batailler, B., Lemaitre, T., Vilaine, F., Sanchez, C., Renard, D., Cayla, T., Beneteau,**
661 **J. and Dinant, S.** (2012) Soluble and filamentous proteins in Arabidopsis sieve
662 elements. *Plant Cell Environ.* **35**, 1258-1273.
663 **Buhtz, A., Springer, F., Chappell, L., Baulcombe, D.C. and Kehr, J.** (2008)
664 Identification and characterization of small RNAs from the phloem of *Brassica*
665 *napus*. *Plant J.* **53**, 739–749.
666 **Crafts, A.S**. (1932) Phloem anatomy, exudation, and transport of organic nutrients in
667 cucurbits. *Plant Physiol*, **7**, 183–225.
668 **Creelman, R.A. and Mullet, J.E.** (1997) Biosynthesis and action of jasmonates in
669 plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 355–381.
670 **Croft, K.P.C., Juttner, F. and Slusarenko, A.J.** (1993) Volatile products of the
671 lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated
672 with *Pseudomonas syringae* pv *phaseolicola*. *Plant Physiol.* **101**, 13–24.
673 **Dafoe, N.J., Zamani, A., Ekramoddoullah, A.K.M., Lippert, D., Bohlmann, J. and**
674 **Constabel, C.P.** (2009) Analysis of the poplar phloem proteome and its response
675 to leaf wounding. *J. Proteome Res.* **8**, 2341-2350.
676 **Denison, F.C., Paul, A.L., Zupanska, A.K. and Ferl R.J.** (2011) 14-3-3 proteins in
677 plant physiology. *Semin. Cell Dev. Biol.* **22**, 720–727.
678 **Du, B., Wei, Z., Wang, Z.Q., Wang, X.X., Peng, X.X., Du, B., Chen, R.Z., Zhu, L.L.**
679 **and He, G.C.** (2015) Phloem-exudate proteome analysis of response to insect
680 brown plant-hopper in rice. *J. Plant Physiol.* **183**, 13-22.
681 **Ernst, A.M., Rüping, B., Jekat, S.B., Nordzieke, S., Reineke, A.R., Müller, B.,**
682 **Bornberg-Bauer, E., Prüfer, D. and Noll, G.A.** (2011) The *sieve element*
683 *occlusion* gene family in dicotyledonous plants. *Plant Signal. Behav.* **6**, 151-153.
684 **Esau, K.** (1969) The Phloem. In: Handbuch der Pflanzenanatomie (W. Zimmermann,
685 P. Ozenda, and M. D. Wulfe, ed.), vol.**5**, part 2. Borntraeger, Berlin.
686 **Fan, H.F., Xu, Y.L., Du, C.X. and Wu, X.** (2015) Phloem sap proteome studied by
687 iTRAQ provides integrated insight into salinity response mechanisms in
688 cucumber plants. *J. Proteomics* **125**, 54-67.
689 **Fiehn, O.** (2003) Metabolic networks of *Cucurbita maxima* phloem. *Phytochemistry* **62**,
690 875–886.
691 **Gaupels, F., Buhtz, A., Knauer, T., Deshmukh, S., Waller, F., van Bel, A.J.E.,**
692 **Kogel, K.H. and Kehr, J**. (2008) Adaptation of aphid stylectomy for analyses of
693 proteins and mRNAs in barley phloem sap. J. Exp. Bot. **59**, 3297-3306.

694 **Gaupels, F. and Ghirardo, A.** (2013) The extrafascicular phloem is made for fighting.
695 *Front Plant Sc*i. **4**, 187.
696 **Gaupels, F., Sarioglu, H., Beckmann, M., Hause, B., Spannagl, M., Draper, J.,**
697 **Lindermayr, C. and Durner, J.** (2012) Deciphering systemic wound responses
698 of the pumpkin extrafascicular phloem by metabolomics and stable isotope-coded
699 protein labeling. *Plant Physiol.* **160**, 2285-2299.
700 **Giavalisco, P., Kapitza, K., Kolasa, A., Buhtz, A. and Kehr, J.** (2006) Towards the
701 proteome of *Brassica napus* phloem sap. *Proteomics* **6**, 896-909.
702 **Gökirmak, T., Paul, A.L. and Ferl, R.J.** (2010) Plant phosphopeptide-binding proteins
703 as signalling mediators. *Curr. Opin. Plant Biol.* **13**, 527-532.
704 **Golecki, B., Schulz, A. and Thompson, G.A.** (1999). Translocation of
705 structural P proteins in the phloem. *Plant Cell* **11**, 127-140.
706 **Gomez, G., Torres, H. and Pallas, V.** (2005) Identification of translocatable RNA-
707 binding phloem proteins from melon, potential components of the long-distance
708 RNA transport system. *Plant J.* **41**, 107–116.
709 **Guelette, B., Benning, U.F. and Hoffmann-Benning, S.** (2012) Identification of lipids
710 and lipid-binding proteins in phloem exudates from *Arabidopsis thaliana*. *J. Exp.*
711 *Bot.* **63**, 3603-3616.
712 **Haebel, S. and Kehr, J.** (2001) Matrix-assisted laser desorption/ionization time of flight
713 mass spectrometry peptide mass fingerprints and post source decay: a tool for
714 the identification and analysis of phloem proteins from *Cucurbita maxima* Duch.
715 separated by two-dimensional polyacrylamide gel electrophoresis. *Planta* **213**,
716 586–593.
717 **Haritatos, E., Keller, F. and Turgeon, R.** (1996) Raffinose oligosaccharide
718 concentrations measured in individual cell and tissue types in *Cucumis melo* L.
719 leaves: implications for phloem loading. *Planta* **198**, 614-622.
720 **Hafke, J.B., Ehlers, K., Föller J., Höll, S-R., Becker, S. and van Bel, A.J.E.** (2013)
721 Involvement of the sieve element cytoskeleton in electrical responses to cold
722 shocks. *Plant Physiol*. **162**, 707–719.
723 **Hebbelmann, I., Selinski, J., Wehmeyer, C., *et al.*** (2012) Multiple strategies to
724 prevent oxidative stress in Arabidopsis plants lacking the malate valve enzyme
725 NADP-malate dehydrogenase. *J. Exp. Bot.* **63**, 1445-1459.
726 **Hu, C., Ham, B-K., El-shabrawi, H.N., Alexander, D., Zhang, D., Ryals J. and**
727 **Lucas, W.J.** (2016) Proteomics and metabolomics analyses reveal the cucurbit

728 sieve tube system as a complex metabolic space. *Plant J.* Accepted article doi:
729 10.1111/tpj.13209.
730 **Huang**, **S., Li, R., Zhang**, **Z., *et al*.** (2009) The genome of the cucumber, C*ucumis*
731 *sativus* L. *Nature Genetics* **41**, 1275-1283.
732 **Jaspert, N., Throm, C. and Oecking, C.** (2011) *Arabidopsis* 14-3-3 proteins:
733 fascinating and less fascinating aspects. *Front. Plant Sci.* **2**, 96.
734 **Kehr, J.** (2006) Phloem sap proteins: their identities and potential roles in the
735 interaction between plants and phloem-feeding insects. *J. Exp. Bot.* **57**, 767–774.
736 **Knoblauch, M., Froelich, D.R., Pickard, W.F. and Peters, W.S.** (2014) SEORious
737 business: structural proteins in sieve tubes and their involvement in sieve
738 element occlusion. *J. Exp. Bot.* **65**, 1681-1688.
739 **Koroleva, O.A. and Cramer R.** (2011) Single-cell proteomic analysis of glucosinolate-
740 rich S-cells in *Arabidopsis thaliana*. *Methods* **54**, 413-423.
741 **Lin, M-K., Lee, Y-J., Lough, T.J., Phinney, B.S. and Lucas W.J.** (2009) Analysis of
742 the pumpkin phloem proteome provides insights into angiosperm sieve tube
743 function. *Mol. Cell. Proteomics* **8**, 343-356.
744 **MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen,**
745 **B., Kern, R., Tabb, D. L., Liebler, D.C. and MacCoss M.J.** (2010) Skyline: an
746 open source document editor for creating and analyzing targeted proteomics
747 experiments. *Bioinformatics* **26**, 966–968.
748 **Malter, D. and Wolf, S.** (2011) Melon phloem-sap proteome: developmental control
749 and response to viral infection. *Protoplasma* **248**, 217-224.
750 **Moeder, W., del Pozo, O., Navarre, D.A., Martin, G.B. and Klessig, D.F.** (2007)
751 Aconitase plays a role in regulating resistance to oxidative stress and cell death
752 in Arabidopsis and *Nicotiana benthamiana*. *Plant Mol. Biol.* **63**, 273–287.
753 **Oliveros, J.C.** (2007) VENNY. An interactive tool for comparing lists with Venn
754 Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.
755 **Parchmann, S., Gundlach, H. and Mueller, M.J.** (1997) Induction of 12-oxo-
756 phytodienoic acid in wounded plants and elicited plant cell cultures. *Plant Physiol.*
757 **115**, 1057–1064.
758 **Picotti, P. and Aebersold, R.** (2012) Selected reaction monitoring-based proteomics:
759 workflows, potential, pitfalls and future directions. *Nature Methods* **9**, 555–566.
760 **Provart, N.J. and Zhu, T.** (2003) A browser-based functional classification
761 SuperViewer for Arabidopsis genomics. *Currents in Computational Mol. Biol.*
762 2003, 271-272.

763 **Richardson, P.T., Baker, D.A. and Ho, L.C.** (1982) The chemical composition of
764 cucurbit vascular exudates. *J. Exp. Bot.* **33**, 1239–1247.
765 **Rüping, B., Ernst, A.M., Jekat, S.B., Nordzieke, S., Reineke, A.R., Müller, B.,**
766 **Bornberg-Bauer, E., Prüfer, D. and Noll, G.A.** (2010). Molecular and
767 phylogenetic characterization of the sieve element occlusion gene family
768 in Fabaceae and non-Fabaceae plants. *BMC Plant Biol.* **10**, 219.
769 **Rustérucci, C., Montillet, J-L., Agnel, J-P., *et al.*** (1999) Involvement of
770 lipoxygenase-dependent production of fatty acid hydroperoxides in the
771 development of the hypersensitive cell death induced by cryptogein of tobacco
772 leaves. *J. Biol. Chem.* **274**, 36446–36455.
773 **Sairam, R.K. and Tyagi, A.** (2004) Physiology and molecular biology of salinity stress
774 tolerance in plants *Curr. Sci*. **86**, 407–421.
775 **Schad, M., Lipton, M.S., Giavalisco, P., Smith, R.D. and Kehr, J.** (2005) Evaluation
776 of 2-DE and LC-MS/MS for tissue-specific protein profiling of laser
777 microdissected plant samples. *Electrophoresis* **26**, 2729-2738.
778 **Schobert, C., Baker, L., Szederkényi, J., Großmann, P., Komor E., Hayashi, H.,**
779 **Chino, M. and Lucas. W.J.** (1998) Identification of immunologically related
780 proteins in sieve-tube exudate collected from monocotyledonous and
781 dicotyledonous plants. *Planta* **206**, 245-252.
782 **Serra-Soriano, M., Navarro, J.A., Genoves, A. and Pallas, V.** (2015) Comparative
783 proteomic analysis of melon phloem exudates in response to viral infection. *J.*
784 *Proteomics*, **124**, 11-24.
785 **Turgeon, R. and Oparka, K.** (2010) The secret phloem of pumpkins. *Proc. Natl. Acad.*
786 *Sci. USA* **107**, 13201-13202.
787 **van Bel, A.J.E. and Knoblauch, M.** (2000) Sieve element and companion cell: the
788 story of the comatose patient and the hyperactive nurse. *Australian J. Plant*
789 *Physiol.* **27**, 477–487.
790 **van Bel, A.J.E. and Gaupels, F.** (2004) Pathogen-induced resistance and alarm
791 signals in the phloem. *Mol. Plant Pathol*. **5**, 495–504.
792 **Walz, C., Giavalisco, P., Schad, M., Juenger, M., Klose, J. and Kehr, J.** (2004)
793 Proteomics of curcurbit phloem exudate reveals a network of defence proteins.
794 *Phytochemistry* **65**, 1795–1804.
795 **Weber, H., Chételat, A., Caldelari, D. and Farmer, E.E.** (1999) Divinyl ether fatty acid
796 synthesis in late blight-diseased potato leaves. *Plant Cell* **11**, 485–493.

Wu, C.C. and MacCoss, M.J. (2002) Shotgun proteomics: Tools for the analysis of complex biological system. *Curr. Opin. Mol. Ther.* **4**, 242-250.

Zhang, B., Tolstikov, V., Turnbull, C., Hicks, L. and Fiehn, O. (2010) Divergent metabolome and proteome suggest functional independence of dual phloem transport systems in cucurbits. *Proc. Natl. Acad. Sci. USA* **107**, 13532-13537.

Zhang, C.K., Yu, X.Y., Ayre, B.G. and Turgeon, R. (2012) The origin and composition of cucurbit “phloem” exudate. *Plant Physiol.* **158**, 1873–1882.

TABLE

Table 1 Proportion of shared proteins among the different described phloem proteomes. Percentages above the diagonal are calculated relative to values in column header and values below the diagonal are relative to those for row headers. Values in brackets indicate number of non-redundant proteins for each phloem proteome that have homologs in the Arabidopsis proteome. C, cucumber; P, pumpkin.

	FP C (322)	EFP C (60)	FP P (280)	EFP P (60)	EFP sap (874)	Arabidopsis (377)	Brassica (81)	Broccoli (374)
FP C (322)		50%	70%	40%	12%	17%	26%	24%
EFP C (60)	9%		9%	42%	4%	4%	11%	4%
FP P (280)	61%	40%		53%	12%	18%	21%	23%
EFP P (60)	9%	42%	11%		5%	5%	11%	5%
EFP sap (874)[1]	33%	55%	37%	78%		16%	31%	21%
Arabidopsis (377)[2]	20%	25%	24%	30%	7%		28%	22%
Brassica (81)[3]	7%	15%	6%	15%	3%	6%		7%
Broccoli (374)[4]	28%	25%	31%	32%	9%	22%	31%	

[1] Lin *et al.,* 2009; [2] Batailler *et al.,* 2012; [3] Giavalisco et al., 2006 [4] Anstead *et al.,* 2013

Figure Legends

Figure 1. Micro-dissection and protein profiles of different tissue and cell types from cucumber and pumpkin stems. (a) Transverse free-hand section of pumpkin stem, showing a vascular bundle. (b) Longitudinal free-hand section of pumpkin vascular bundle stained with 0.1% amido black. (c) Close-up of boxed region in (b). Red asterisks indicate protein strands (dark blue) from fascicular phloem (FP). (d) Final dissected pumpkin tissues. (e) Phloem exudate (EFP) from cut cucumber stem. (f) Protein profiles from pumpkin microdissected FP tissue (FP_{MD}) and FP-SE contents. (g) Protein profiles from pumpkin and cucumber EFP, ground tissue (G), xylem tissue (X) and fascicular phloem tissue (FP). Coomassie-stained SDS-PAGE. Arrows indicate major proteins: yellow, PP1; brown, PP2 and black, SEOR. FP_{ext}, external fascicular phloem; FP_{int}, internal fascicular phloem; M, molecular weight markers, run on the same gels but irrelevant lanes omitted in (f). See Figure 6 for additional comparisons of FP and EFP profiles.

Figure 2. Overlap among proteins identified in FP and EFP from pumpkin and cucumber. Shared and unique proteins for: microdissected FP tissue (FP) and FP sieve elements (FP-SE) in pumpkin (a) and cucumber (b); microdissected FP tissue and EFP in pumpkin (c) and cucumber (d); and FP-SE and EFP in pumpkin (e) and cucumber (f). Cut-off for identified proteins was set at 99% CI (ProteinPilot Unused ProtScore >2) and at least 2 peptides each**.** Phloem EFP exudate is combined from t1 and t3 samples.

Figure 3. Overlap among proteins identified in different tissue types and exudate of pumpkin and cucumber. Shared and unique proteins for phloem exudate (EFP), microdissected FP tissue (FP), ground tissue (G) and xylem tissue (X) in pumpkin (a) and cucumber (b). (c) Numbers of unique and total proteins in each tissue type. Shared and unique proteins for phloem exudate (EFP), SE content (FP-SE), ground tissue (G) and xylem (X) in pumpkin (d) and cucumber (e). (f) Numbers of unique and total proteins in each tissue type. Cut-off for identified proteins was set at unused score of >2 (99% CI) and at least 2 peptides each**.**

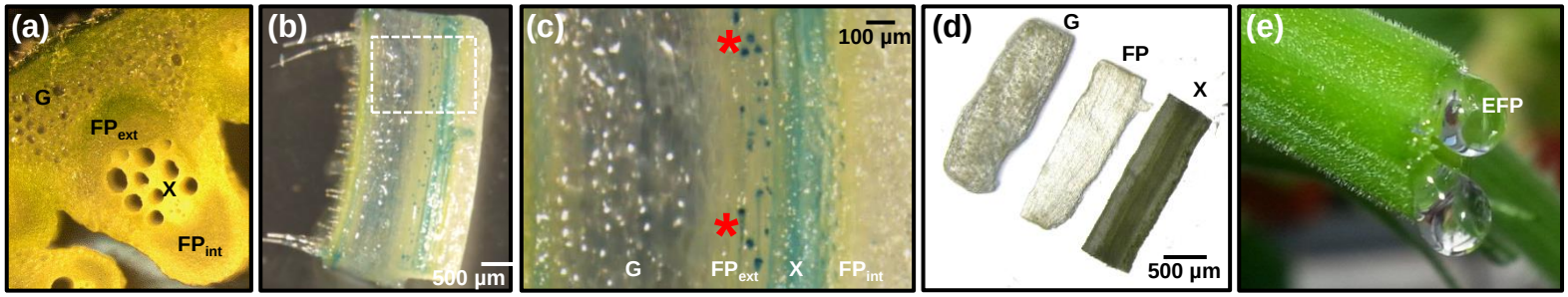
Figure 4. Cucumber FP and EFP marker proteins showing presence or absence in microdissected FP and EFP samples. Each panel contains representative LC-MS-MRM traces for chromatograms for multiple peptides from each of the markers: Cucsa.386600, Cucsa.395940, Cucsa.151370 and Cucsa.091340. Upper row, FP

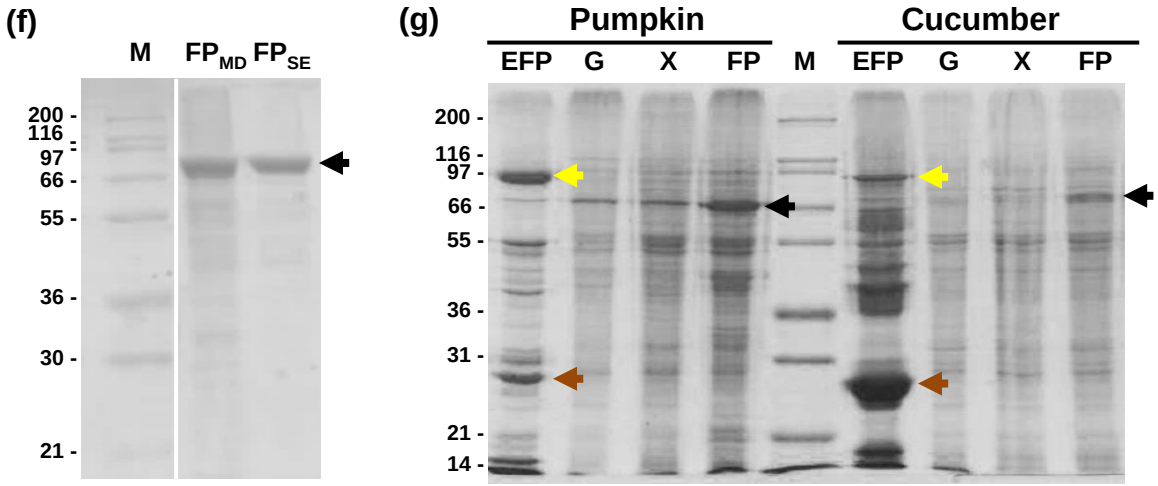
846 samples; lower row, EFP samples. Several peptides are detected for each protein and
847 each MRM channel is individually coloured. Multiple colours at a single retention time
848 represent different MRMs for a single peptide. See Table S1 for MRM details.

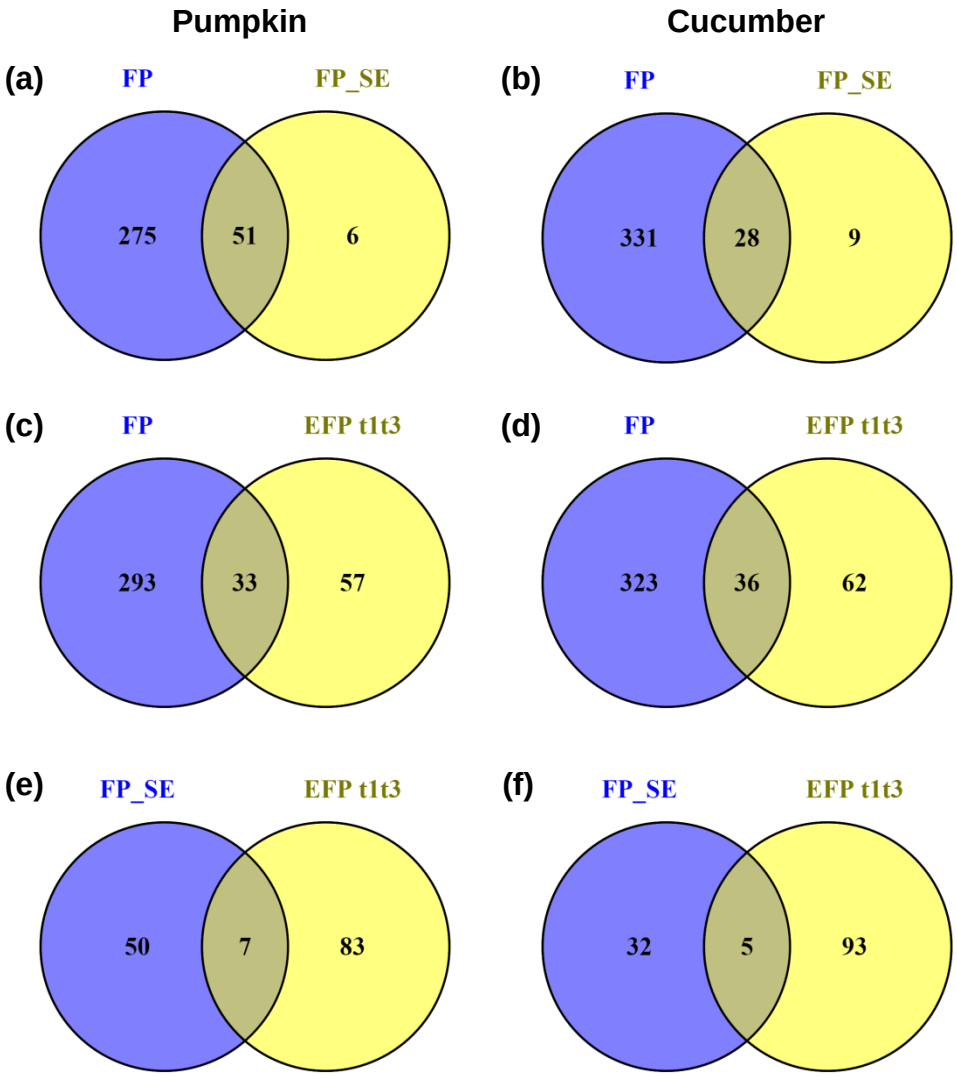
849 **Figure 5.** Direct observation of exudation from stem of cucumber. Image series starts
850 from approximately 30 s after cutting and removal of sap with filter paper, when
851 exudation has slowed. (a–c) Images of exudation at 30 s (a), 60 s (b) and 90 s (c). (d)
852 Close-up of vascular bundle from (a) showing droplets of exudate (arrowed) emanating
853 from extrafascicular phloem sites surrounding fascicular phloem region. (e) Location of
854 vascular tissue types within and around vascular bundle, and in cortex; see Movie S1.
855 Image brightness and contrast have been modified for enhanced clarity.

856 **Figure 6.** Time series collection of exudate from pumpkin and cucumber showing
857 minimal change in composition. Comparison of FP micro-dissected tissues with
858 exudate (EFP) collected at different times (0 to 3 min) after cutting. Coomassie-stained
859 SDS-PAGE for pumpkin (a) and cucumber (b). Arrows indicate major proteins: yellow,
860 PP1; red, PP2 and black, SEOR. M, molecular weight markers. EFP, extrafascicular
861 phloem; FP, fascicular phloem. MRM chromatograms from the different EFP exudation
862 times for cucumber FP markers Cucsa.386600 (c) and Cucsa.395940 (d), and EFP
863 markers Cucsa.151370 (e) and Cucsa.091340 (f).

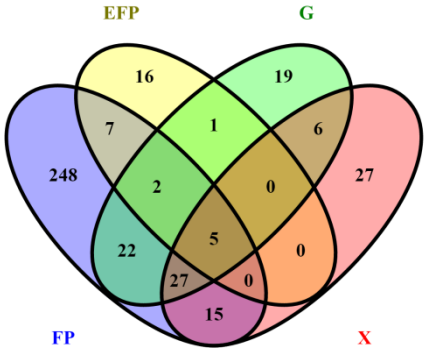
864 **Figure 7.** Frequencies of GO annotations for the cucumber and pumpkin phloem
865 proteomes. Biological Process and Molecular Function categories are shown as
866 normed frequencies for the proteomes of cucumber and pumpkin microdissected FP
867 enriched tissue (a) and EFP exudate (b). Frequencies significantly above or below the
868 values for the *Arabidopsis* reference set are indicated by * and °, respectively (*P* value
869 < 0.05). C, cucumber; P, pumpkin.


(a)
G
FP$_{ext}$
X
FP$_{int}$
(b)
500 µm
(c)
100 µm
G
FP$_{ext}$
X
FP$_{int}$
(d)
G
FP
X
500 µm
(e)
EFP

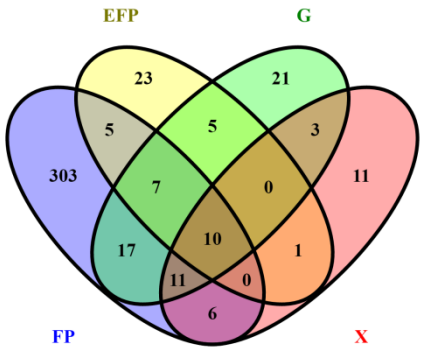

(f)
M
FP$_{MD}$
FP$_{SE}$
200
116
97
66
55
36
30
21
(g)
Pumpkin
Cucumber
EFP
G
X
FP
M
EFP
G
X
FP
200
116
97
66
55
36
31
21
14


Pumpkin
Cucumber
(a)
FP
FP_SE
275
51
6
(b)
FP
FP_SE
331
28
9
(c)
FP
EFP t1t3
293
33
57
(d)
FP
EFP t1t3
323
36
62
(e)
FP_SE
EFP t1t3
50
7
83
(f)
FP_SE
EFP t1t3
32
5
93

(a) Pumpkin



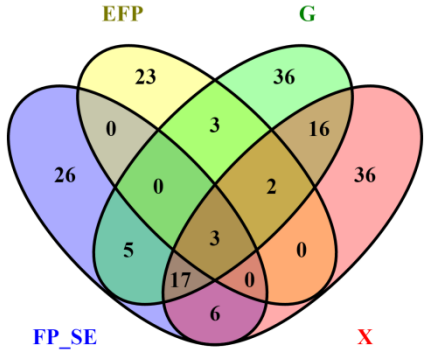
(b) Cucumber



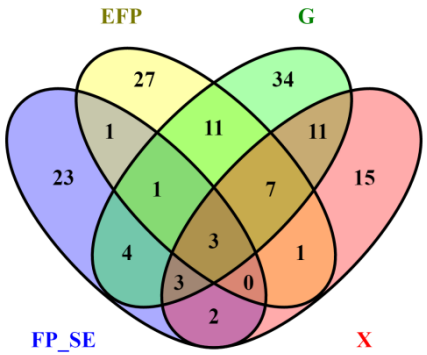
(c)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
EFP	31	16	51	23
FP	326	248	359	303
Ground tissue	82	19	74	21
Xylem	80	27	42	11

(d) Pumpkin

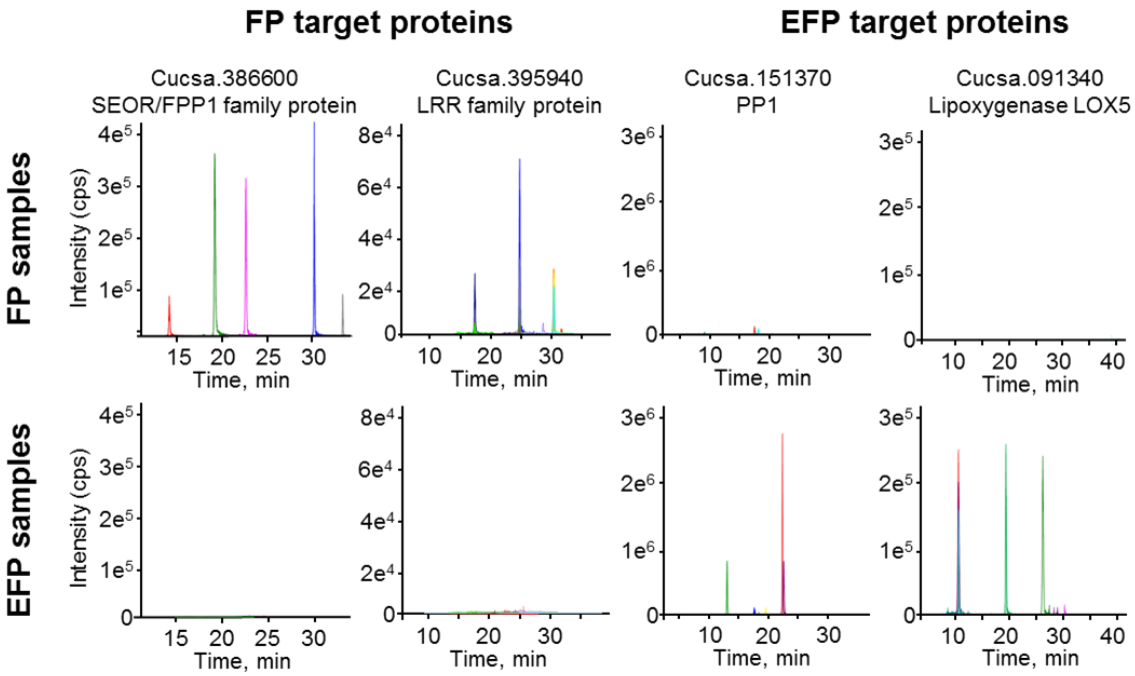


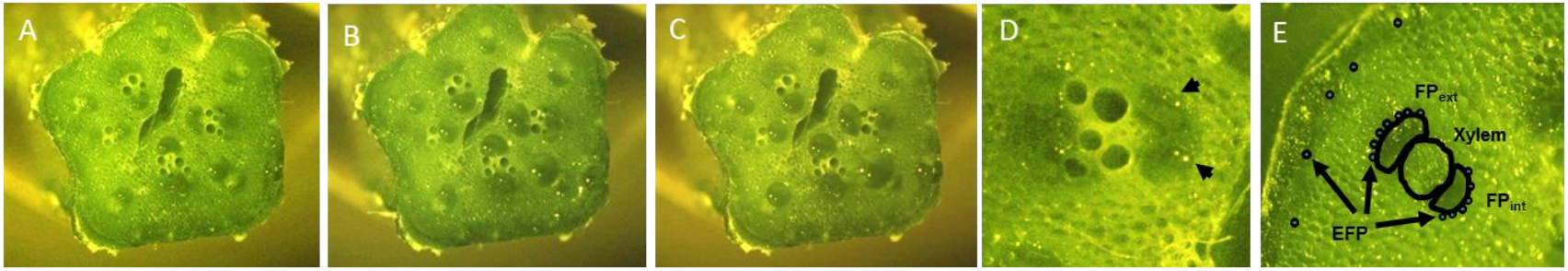
(e) Cucumber

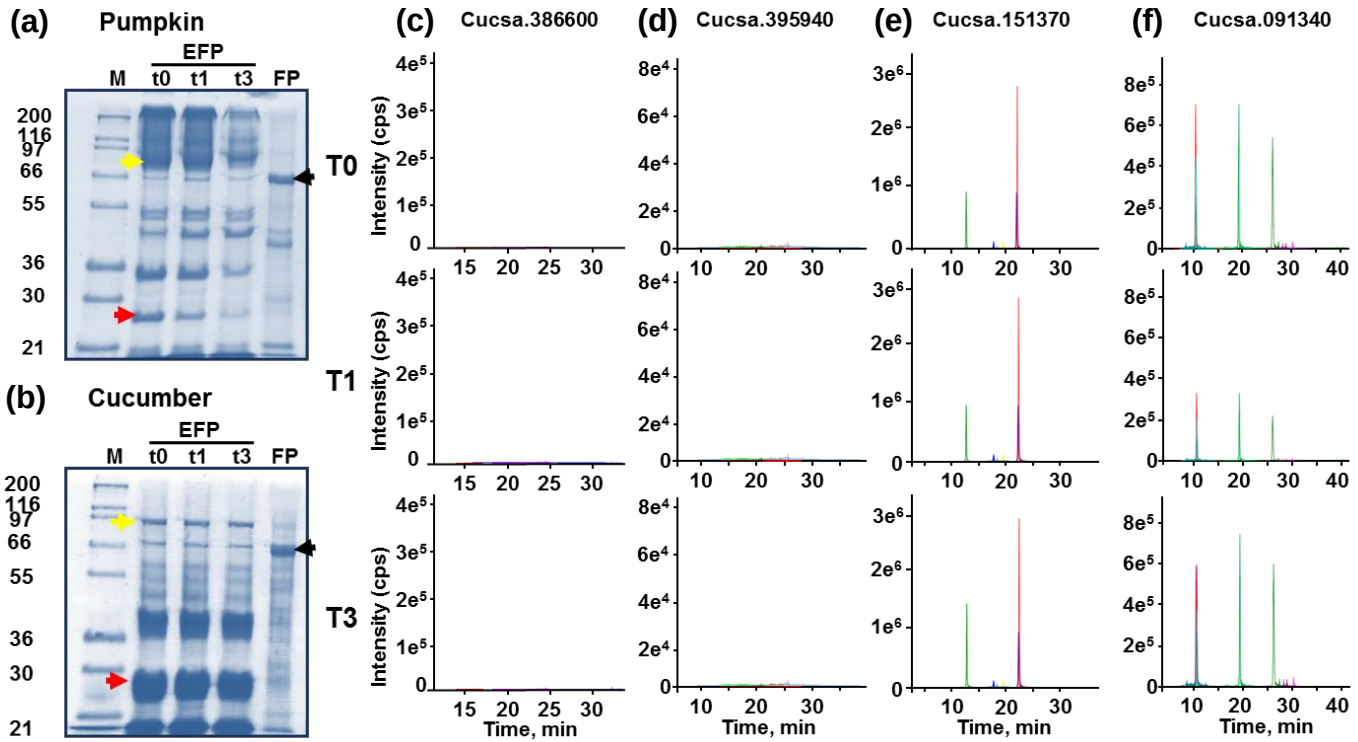


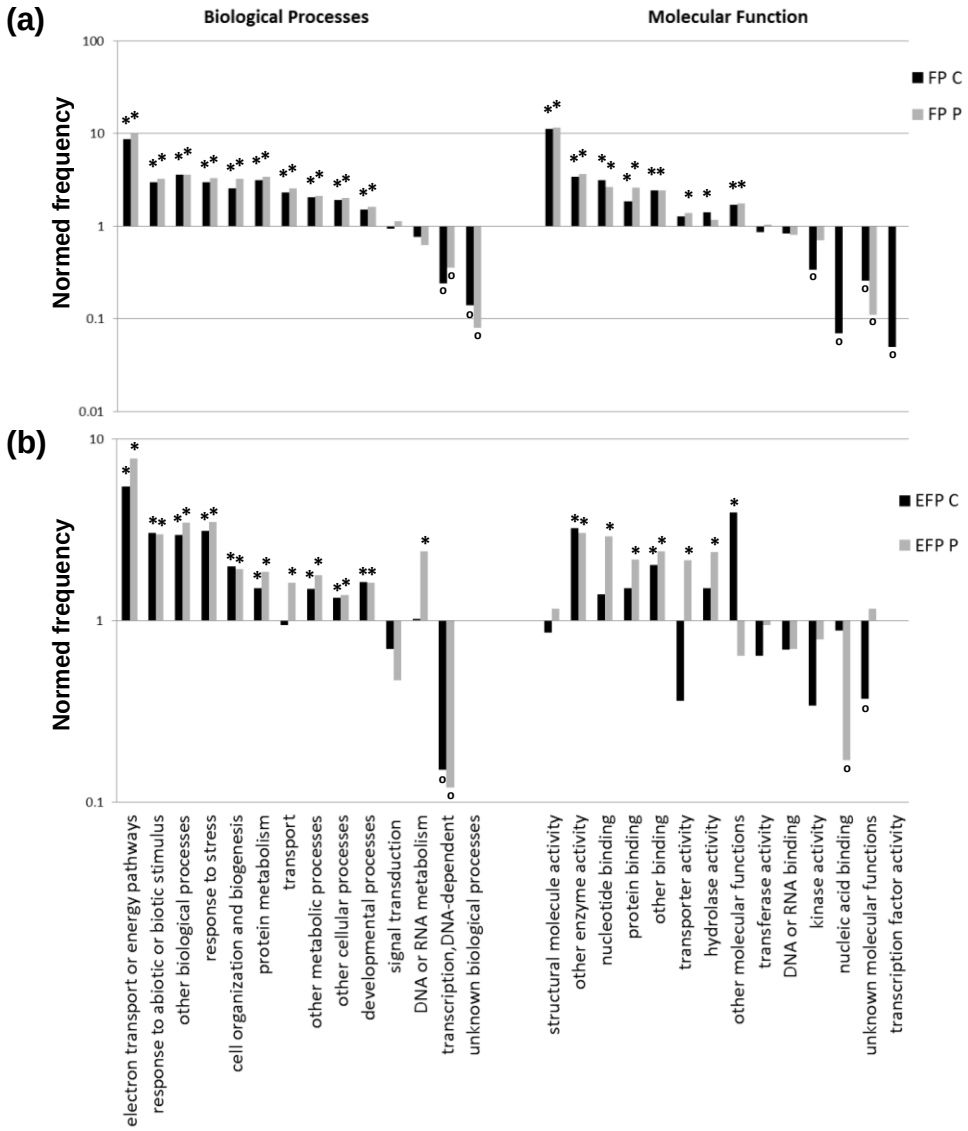
(f)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
EFP	31	23	51	27
FP_SE	57	26	37	23
Ground tissue	82	36	74	34
Xylem	80	36	42	15


FP target proteins
EFP target proteins
Cucsa.386600
SEOR/FPP1 family protein
Cucsa.395940
LRR family protein
Cucsa.151370
PP1
Cucsa.091340
Lipoxygenase LOX5
FP samples
EFP samples
Intensity (cps)
Time, min


A
B
C
D
E
FP$_{ext}$
Xylem
FP$_{int}$
EFP


(a)
Pumpkin
EFP
M
t0
t1
t3
FP
200
116
97
66
55
36
30
21
(b)
Cucumber
T0
T1
T3
(c)
Cucsa.386600
(d)
Cucsa.395940
(e)
Cucsa.151370
(f)
Cucsa.091340
Intensity (cps)
Time, min


(a)
Biological Processes
Molecular Function
Normed frequency
100
10
1
0.1
0.01
FP C
FP P
(b)
Normed frequency
10
1
0.1
EFP C
EFP P
electron transport or energy pathways
response to abiotic or biotic stimulus
other biological processes
response to stress
cell organization and biogenesis
protein metabolism
transport
other metabolic processes
other cellular processes
developmental processes
signal transduction
DNA or RNA metabolism
transcription,DNA-dependent
unknown biological processes
structural molecule activity
other enzyme activity
nucleotide binding
protein binding
other binding
transporter activity
hydrolase activity
other molecular functions
transferase activity
DNA or RNA binding
kinase activity
nucleic acid binding
unknown molecular functions
transcription factor activity

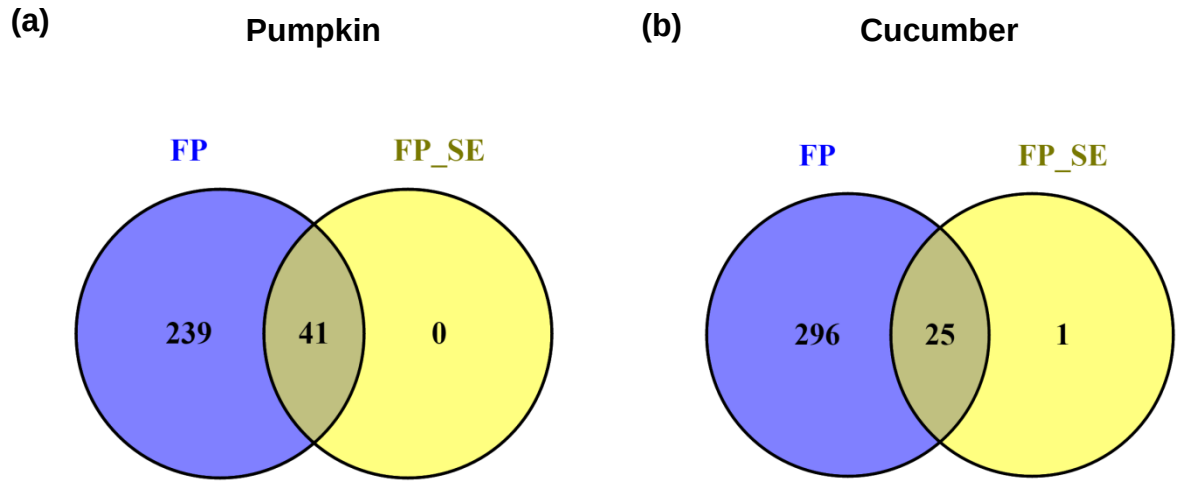
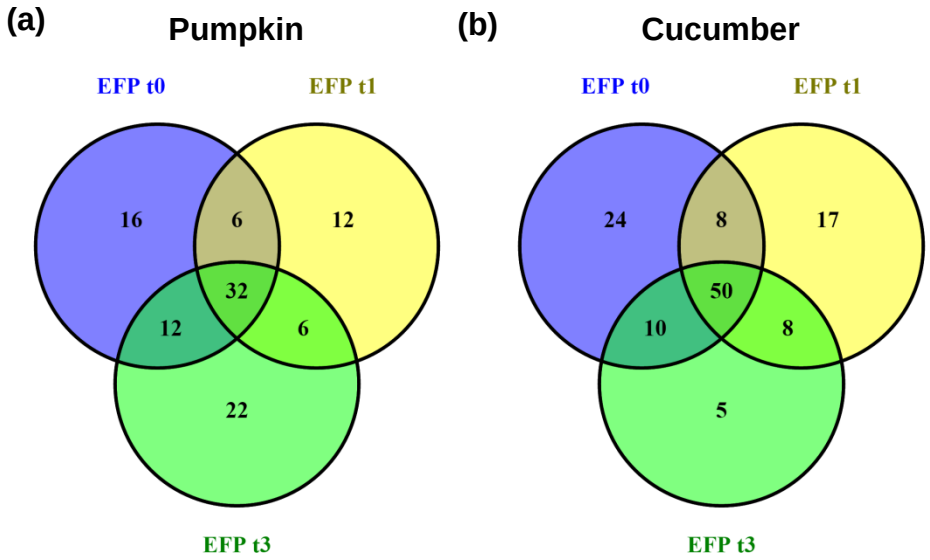


Figure S1. Overlap among number of non-redundant proteins for microdissected FP tissue (FP) and FP sieve elements (FP-SE) in pumpkin and cucumber that have homologs in the Arabidopsis proteome. Shared and unique proteins for pumpkin (a) and cucumber (b). Cut off for identified proteins was set at 99% CI (ProteinPilot Unused ProtScore >2) and at least 2 peptides each.

t0	Cut tissue 	Collect for 30” 			
t1	Cut tissue 	Clean tissue	Collect for 1’ 		
t3	Cut tissue 	Clean tissue	Wait 1’ 	Clean tissue	Collect for 2’ 

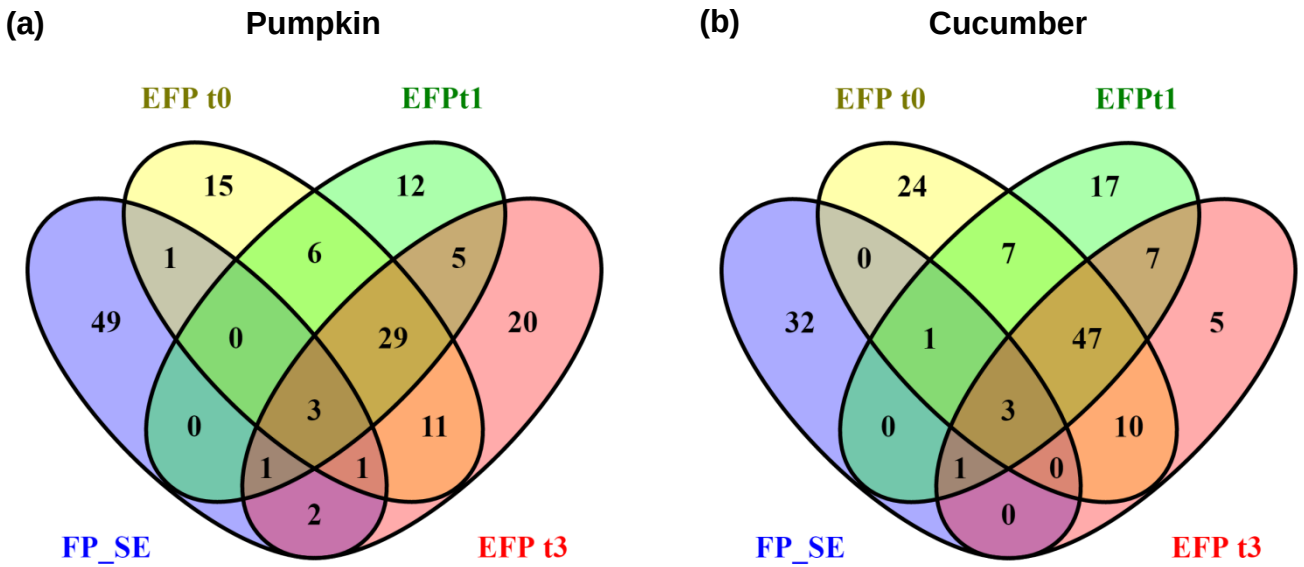
Figure S2. Scheme of the time series for exudate collection from pumpkin and cucumber**.**



(c)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
EFP t0	66	16	92	24
EFP t1	56	11	83	17
EFP t3	72	22	73	5

Figure S3. Venn diagrams summarising phloem proteins identified in the time series exudate collection for pumpkin and cucumber. Shared and unique proteins between phloem exudate (EFP) at different collection times in pumpkin (a) and cucumber (b). (c) Unique and total numbers of proteins detected in pumpkin and cucumber phloem exudate (EFP) at different collection times. Cut off of the identified proteins was set at unused score of >2 (99.9 % CI) and at least 2 peptides each.



(c)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
FP_SE	57	49	37	32
EFP t0	66	15	92	24
EFP t1	56	12	83	17
EFP t3	72	20	73	5

Figure S4. Venn diagrams summarising phloem proteins identified in the time series exudate collection for pumpkin and cucumber and compared with SE content. Shared and unique proteins for SE content (FP_SE) and EFP collected at different time points (t0, t1 and t3) in pumpkin (a) and cucumber (b). (c) Numbers of unique and total proteins in FP_SE and EFP at different exudation times. Cut off for identified proteins was set at unused score of >2 (99.9 % CI) and at least 2 peptides each.

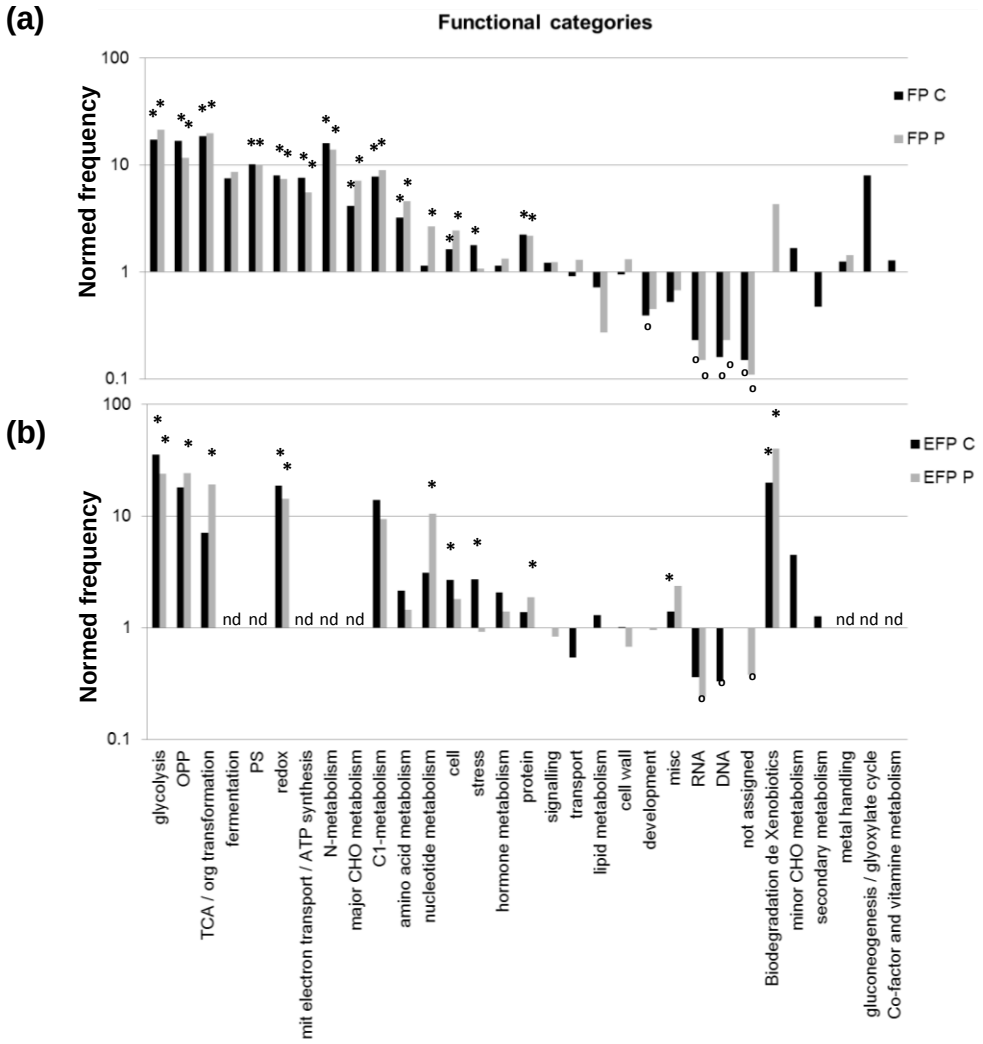


Figure S5. Frequencies of BAR MapMan functional categories for the cucumber and pumpkin proteins compared to the Arabidopsis genome. Normed frequencies for the proteome of cucumber and pumpkin FP-enriched samples (a) and EFP exudate (b). Frequencies significantly above or below the values for the *Arabidopsis* reference set are indicated by * and °, respectively (P <0.05). nd, not detected; C, cucumber; P, pumpkin; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; PS, light reaction photosystems.

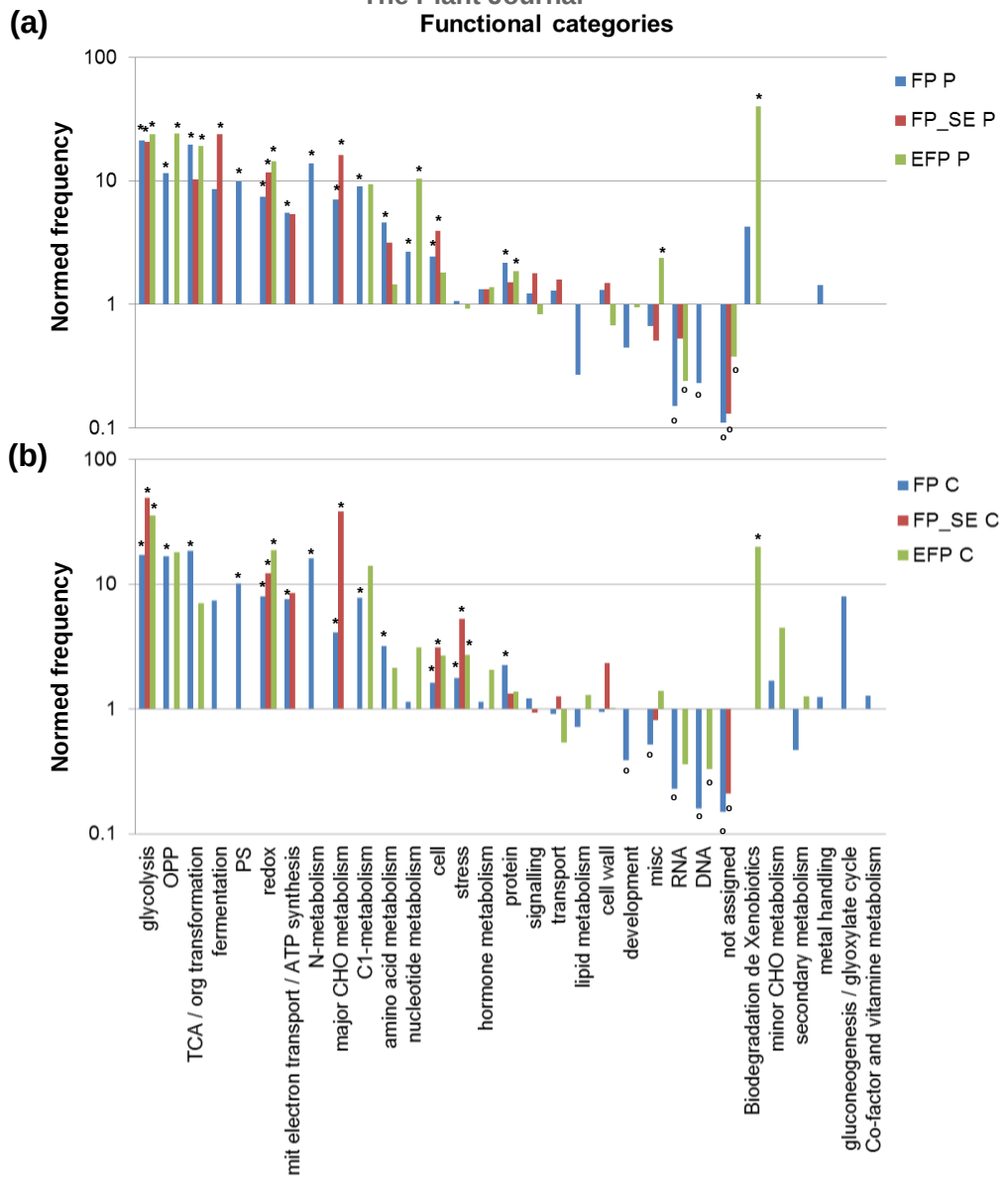


Figure S6. Frequencies of BAR MapMan functional categories for the cucumber and pumpkin proteins compared to the Arabidopsis genome. Normed frequencies (*bootstrap StdDev*) for the proteome of A) cucumber and B) pumpkin FP enriched samples, SE content and EFP exudate. Frequencies significantly above or below the values for the *Arabidopsis* reference set are indicated by * and °, respectively (*P* value < 0.05). N.d., not detected; C, cucumber; P, pumpkin; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; PS, light reaction photosystems

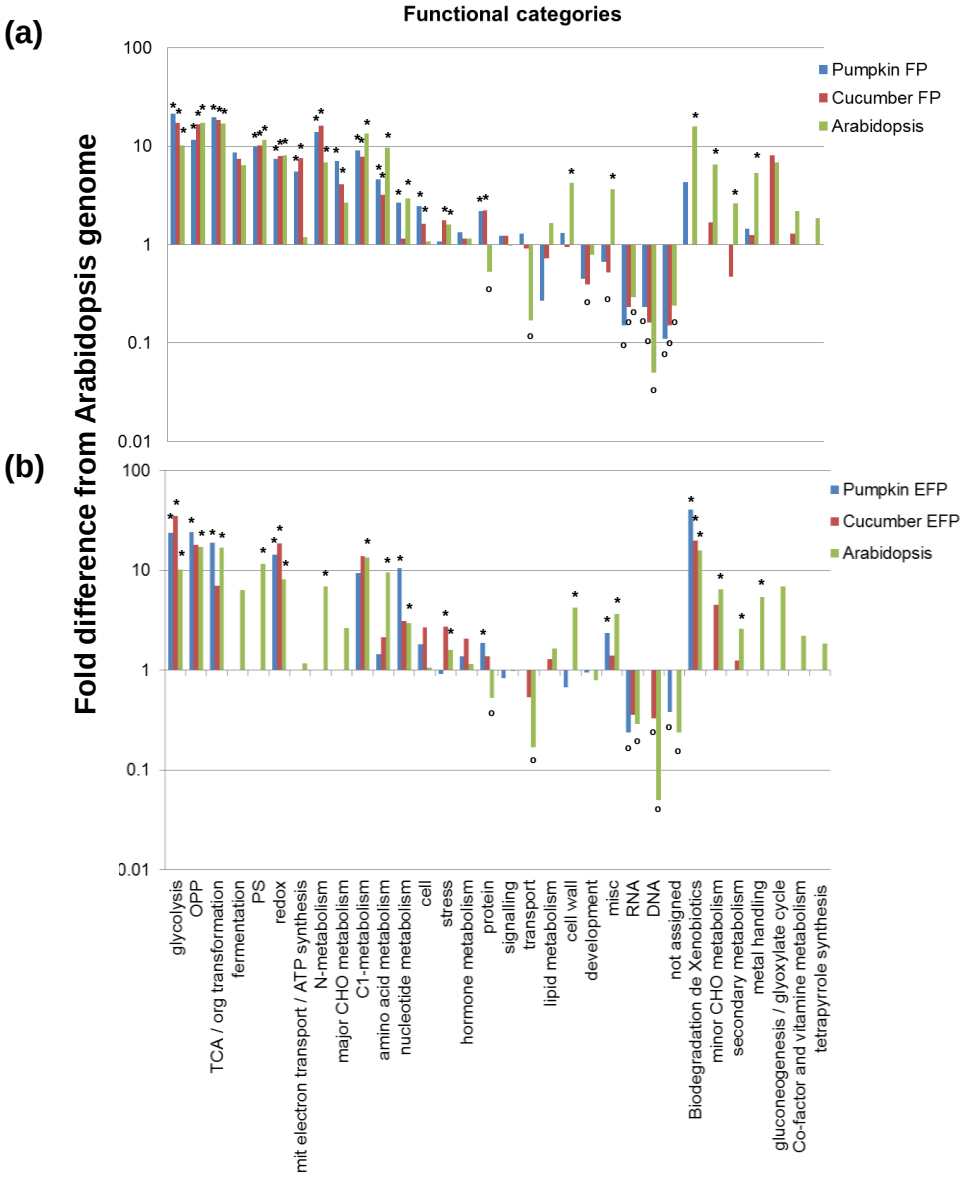
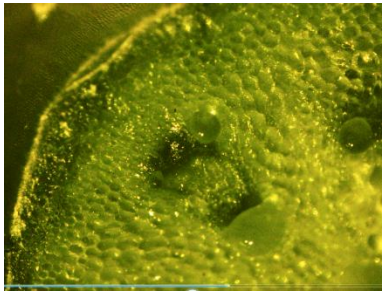


Figure S7. Comparison of frequencies of BAR MapMan functional categories between Arabidopsis and cucurbit phloem proteomes. Analyses are derived from data in Table S3 and Batailler et al. (2012). (a) Comparison of Arabidopsis phloem and FP proteomes. (b) Comparison of Arabidopsis phloem and EFP proteomes. For clarity, error bars are omitted. *,° significantly above and below, respectively, the frequency in Arabidopsis genome ($P < 0.05$).



Movie S1. Phloem exudation in cucumber. Exudation recorded after removal of initial very rapid flow by filter paper. Dark tissues within vascular bundle in centre of view are external and internal fascicular phloem (FP). The main exudate droplets originate from extrafascicular phloem (EFP) around the periphery of FP but not from the FP tissue itself. See also Figure 5.

Table S1. Unique peptides for FP and EFP markers

protein ID (marker for)	Q1	Q3	rt (min)	peptide. charge/ion	DP	CE
Cucsa.091340.1 (EFP)	408.7141	361.183	10.4	GVAVEDAR.+2y3	60.9	23.6
	408.7141	490.2256	10.4	GVAVEDAR.+2y4	60.9	23.6
	408.7141	589.294	10.4	GVAVEDAR.+2y5	60.9	23.6
	408.7141	660.3311	10.4	GVAVEDAR.+2y6	60.9	23.6
	430.5422	474.2671	19.7	HASDELYLGER.+3y4	62.5	21
	430.5422	637.3304	19.7	HASDELYLGER.+3y5	62.5	21
	430.5422	750.4145	19.7	HASDELYLGER.+3y6	62.5	21
	549.3137	373.2809	26.2	EQALPADLIK.+2y3	71.2	28.6
	549.3137	656.3978	26.2	EQALPADLIK.+2y6	71.2	28.6
	549.3137	769.4818	26.2	EQALPADLIK.+2y7	71.2	28.6
	549.3137	840.5189	26.2	EQALPADLIK.+2y8	71.2	28.6
Cucsa.151370.1 (EFP)	494.2245	539.246	13	YAVDDYNK.+2y4	67.1	26.6
	494.2245	654.2729	13	YAVDDYNK.+2y5	67.1	26.6
	494.2245	753.3414	13	YAVDDYNK.+2y6	67.1	26.6
	494.2245	824.3785	13	YAVDDYNK.+2y7	67.1	26.6
	379.2445	470.3337	22.3	SLSPILK.+2y4	58.8	22.5
	379.2445	557.3657	22.3	SLSPILK.+2y5	58.8	22.5
	379.2445	670.4498	22.3	SLSPILK.+2y6	58.8	22.5
Cucsa.386600.1 (FP)	391.2033	491.2824	14	EVSVHESLSGK.+3y5	59.6	18.9
	391.2033	620.325	14	EVSVHESLSGK.+3y6	59.6	18.9
	391.2033	757.3839	14	EVSVHESLSGK.+3y7	59.6	18.9
	550.2704	718.3883	19.1	HYSDEVVTSHIYAK.+3y6	71.2	27.5
	550.2704	819.4359	19.1	HYSDEVVTSHIYAK.+3y7	71.2	27.5
	550.2704	918.5043	19.1	HYSDEVVTSHIYAK.+3y8	71.2	27.5
	550.2704	1017.573	19.1	HYSDEVVTSHIYAK.+3y9	71.2	27.5
	450.7561	545.3194	22.2	LQNWIAR.+2y4	64	25.1
	450.7561	659.3624	22.2	LQNWIAR.+2y5	64	25.1
	450.7561	787.4209	22.2	LQNWIAR.+2y6	64	25.1
	554.8217	527.33	30.5	ELPELPAALR.+2y5	71.6	28.8
	554.8217	640.4141	30.5	ELPELPAALR.+2y6	71.6	28.8
	554.8217	769.4567	30.5	ELPELPAALR.+2y7	71.6	28.8
	554.8217	866.5094	30.5	ELPELPAALR.+2y8	71.6	28.8
	559.8264	589.3192	34.6	IGFVLAELEK.+2y5	71.9	29
	559.8264	702.4032	34.6	IGFVLAELEK.+2y6	71.9	29
	559.8264	801.4716	34.6	IGFVLAELEK.+2y7	71.9	29
	559.8264	1005.562	34.6	IGFVLAELEK.+2y9	71.9	29
Cucsa.395940.1 (FP)	645.8493	570.3358	17.4	QPINNPGPLANR.+2y5.light	78.2	32.1
	645.8493	724.41	17.4	QPINNPGPLANR.+2y7.light	78.2	32.1
	645.8493	838.453	17.4	QPINNPGPLANR.+2y8.light	78.2	32.1
	645.8493	952.4959	17.4	QPINNPGPLANR.+2y9.light	78.2	32.1
	573.3193	630.357	24.9	FTGPIPSTIGR.+2y6.light	72.9	29.5
	573.3193	743.441	24.9	FTGPIPSTIGR.+2y7.light	72.9	29.5
	573.3193	840.4938	24.9	FTGPIPSTIGR.+2y8.light	72.9	29.5
	573.3193	897.5152	24.9	FTGPIPSTIGR.+2y9.light	72.9	29.5
	442.2373	536.2827	30.4	LSFVDFR.+2y4.light	63.4	24.8
	442.2373	683.3511	30.4	LSFVDFR.+2y5.light	63.4	24.8
	442.2373	770.3832	30.4	LSFVDFR.+2y6.light	63.4	24.8
	rt-retention time, DP -declustering potential, CE- collision energy					

Table S2a: Summary and statistical analysis of GO annotations for phloem proteomes in pumpkin. Values in bold are deemed significant ($P < 0.05$). FP, microdissected-FP; FP_{SE}, FP-SE; EFP, phloem exudate.

	Pumpkin								
	FP			FP_{SE}			EFP t1t3		
Biological Processes	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**
electron transport or energy pathways	10	1.263	**8.04E-43**	8.92	2.735	**2.31E-06**	7.82	1,908	**4.60E-11**
response to abiotic or biotic stimulus	3.27	0.238	**6.96E-36**	4.01	0.594	**9.19E-10**	3	0.352	**4.10E-10**
other biological processes	3.62	0.214	**8.76E-46**	3.65	0.606	**4.17E-08**	3.46	0.426	**5.90E-13**
response to stress	3.32	0.226	**3.44E-39**	3.85	0.579	**1.95E-09**	3.49	0.324	**7.50E-16**
cell organization and biogenesis	3.23	0.264	**2.53E-30**	1.85	0.526	**2.80E-02**	1.92	0.322	**1.70E-03**
protein metabolism	3.44	0.214	**4.33E-44**	2.22	0.506	**2.36E-03**	1.86	0.239	**1.30E-04**
transport	2.55	0.26	**4.83E-15**	2.05	0.53	**1.70E-02**	1.62	0.321	**0.012**
other metabolic processes	2.14	0.07	**2.16E-46**	1.87	0.2	**2.53E-05**	1.78	0.078	**9.60E-13**
other cellular processes	2.03	0.058	**1.50E-47**	1.74	0.17	**6.13E-05**	1.38	0.096	**1.20E-04**
developmental processes	1.63	0.177	**4.49E-05**	1.15	0.433	0.163	1.61	0.303	**0.011**
signal transduction	1.14	0.24	0.082	0.86	0.57	0.271	0.47	0.255	0.074
DNA or RNA metabolism	0.63	0.263	0.096	0.86	0.745	0.368	2.41	0.879	**0.018**
transcription,DNA-dependent	0.36	0.116	**3.07E-04**	0.62	0.431	0.211	0.12	0.094	**1.60E-03**
unknown biological processes	0.08	0.03	**1.93E-31**	0.08	0.085	**1.48E-05**	nd	nd	nd
	FP			FP_{SE}			EFP t1t3		
Molecular Functions	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**
structural molecule activity	11.69	1.389	**1.21E-40**	9.01	3.158	**4.53E-05**	1.16	0.775	0.268
other enzyme activity	3.65	0.369	**5.67E-21**	2.59	0.847	**0.012**	3.04	0.398	**2. 8E-10**
nucleotide binding	2.64	0.524	**2.45E-05**	2.45	1.303	0.089	2.92	0.457	**1.20E-08**
protein binding	2.61	0.333	**4.27E-11**	3.95	0.979	**2.27E-05**	2.17	0.43	**1.10E-03**
other binding	2.43	0.21	**1.45E-13**	3.8	0.683	**3.979E-07**	2.4	0.229	**1.10E-11**
transporter activity	1.38	0.312	**0.046**	1.76	0.905	0.152	2.15	0.42	**3.30E-04**
hydrolase activity	1.18	0.246	0.053	0.89	0.456	0.228	2.39	0.804	**8.40E-03**
other molecular functions	1.76	0.534	**0.038**	1.33	0.954	0.358	0.64	0.247	0.081
transferase activity	1.04	0.218	0.085	1.23	0.548	0.188	0.94	0.269	0.13
DNA or RNA binding	0.81	0.155	0.055	1.85	0.641	**0.047**	0.7	0.356	0.184
kinase activity	0.7	0.235	0.072	nd	nd	nd	0.79	0.332	0.178
nucleic acid binding	nd	nd	nd	nd	nd	nd	0.17	0.066	**2.10E-09**
unknown molecular functions	0.11	0.037	**5.62E-28**	0.08	0.087	**2.26E-05**	1.16	0.775	0.268
transcription factor activity	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table S2b: Summary and statistical analysis of GO annotations for phloem proteomes in cucumber. FP, microdissected-FP; FP_{SE}, FP-SE; EFP, phloem exudate.

	Cucumber								
	FP			FP_{SE}			EFP t1t3		
Biological Processes	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**
electron transport or energy pathways	8.69	0.904	**4.85E-39**	7.32	2.384	**1.80E-03**	5.46	2,021	**8.70E-05**
response to abiotic or biotic stimulus	2.98	0.186	**1.11E-32**	3.88	0.668	**4.01E-06**	3.05	0.539	**1.40E-07**
other biological processes	3.59	0.209	**1.85E-51**	3.29	0.657	**1.43E-04**	2.97	0.525	**8.90E-07**
response to stress	3	0.182	**8.09E-35**	4.02	0.653	**8.60E-07**	3.11	0.508	**1.00E-08**
cell organization and biogenesis	2.55	0.226	**5.14E-19**	2.36	0.747	**1.60E-02**	1.99	0.504	**5.10E-03**
protein metabolism	3.16	0.218	**3.58E-41**	2.24	0.656	**1.30E-02**	1.51	0.321	**0.022**
transport	2.3	0.236	**4.85E-13**	2.25	0.752	**3.00E-02**	0.94	0.36	0.156
other metabolic processes	2.06	0.069	**2.02E-46**	1.64	0.285	**9.28E-03**	1.49	0.129	**1.40E-04**
other cellular processes	1.91	0.058	**1.13E-41**	1.48	0.256	**2.30E-02**	1.34	0.127	**2.90E-03**
developmental processes	1.52	0.159	**1.97E-04**	1.57	0.556	0.116	1.63	0.469	**0.025**
signal transduction	0.94	0.25	0.096	nd	nd	nd	0.7	0.372	0.186
DNA or RNA metabolism	0.77	0.286	0.117	nd	nd	nd	1.02	0.622	0.275
transcription,DNA-dependent	0.24	0.114	**2.41E-06**	nd	nd	nd	0.15	0.078	**1.30E-07**
unknown biological processes	0.14	0.039	**9.92E-31**	0.13	0.112	**2.07E-03**	nd	nd	nd
	FP			FP_{SE}			EFP t1t3		
Molecular Functions	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**	**Normed to Freq. in Arab set**	**Std Dev**	**p-value**
structural molecule activity	11.31	1.542	**2.46E-44**	7.38	3.895	**6.84E-03**	0.86	0.701	0.367
other enzyme activity	3.41	0.365	**1.30E-20**	2.43	0.918	0.056	3.22	0.534	**2.30E-08**
nucleotide binding	3.13	0.505	**2.85E-08**	1.34	1.071	0.361	1.39	0.386	0.077
protein binding	1.85	0.278	**3.03E-05**	4.32	1.23	**2.62E-04**	1.51	0.54	0.076
other binding	2.45	0.203	**8.82E-16**	2.56	0.885	**1.10E-02**	2.02	0.277	**2.20E-05**
transporter activity	1.27	0.342	0.061	2.89	1.368	0.065	0.36	0.424	0.177
hydrolase activity	1.41	0.229	**7.50E-03**	0.49	0.357	0.265	1.51	0.49	0.057
other molecular functions	1.7	0.549	**0.037**	2.02	0.963	0.087	3.95	1,045	**1.50E-04**
transferase activity	0.86	0.177	0.068	0.43	0.355	0.226	0.64	0.278	0.121
DNA or RNA binding	0.84	0.164	0.055	nd	nd	nd	0.69	0.297	0.122
kinase activity	0.34	0.13	**2.13E-03**	nd	nd	nd	0.34	0.221	0.16
nucleic acid binding	0.07	0.061	**2.49E-05**	nd	nd	nd	0.88	0.504	0.225
unknown molecular functions	0.26	0.054	**1.61E-20**	0.57	0.257	0.079	0.37	0.127	**2.40E-04**
transcription factor activity	0.05	0.041	**5.40E-08**	nd	nd	nd	nd	nd	nd

Table S3a: Summary and statistical analysis of BAR MapMan functional annotations for phloem proteomes in pumpkin. FP, microdissected FP; FP_{SE}, FP sieve element contents; EFP, phloem exudate.

	Pumpkin								
Functional categories	**FP**	**FP StdDev**	**FP p-value**	**FP_{SE}**	**FP_{SE} StdDev**	**FP_{SE} p-value**	**EFP t1t3**	**EFP t1t3 StdDev**	**EFP t1t3 p-value**
glycolysis	21.26	4.656	**4.16E-15**	20.74	14.503	**4.09E-03**	23.89	10,636	**2.2E-06**
OPP	11.61	5.819	**2.04E-03**	nd	nd	nd	24.35	15,118	**3.0E-03**
TCA / org transformation	19.74	5.393	**1.10E-13**	10.37	8.361	0.088	19.11	8,644	**5.7E-05**
fermentation	8.57	6.922	0.105	23.87	8.031	**1.80E-07**	nd	nd	nd
PS	9.9	2.547	**1.92E-12**	nd	nd	nd	nd	nd	nd
redox	7.42	2.275	**2.48E-08**	11.7	5.37	**2.03E-03**	14.38	4,862	**8.8E-08**
mit electron transport / ATP synthesis	5.56	2.312	**2.46E-04**	5.42	4.751	0.154	nd	nd	nd
N-metabolism	13.84	7.264	**1.23E-03**	nd	nd	nd	nd	nd	nd
major CHO metabolism	7.12	2.861	**1.85E-04**	16.22	9.952	**6.54E-03**	nd	nd	nd
C1-metabolism	9	4.762	**4.16E-03**	nd	nd	nd	9.43	8,289	0.096
amino acid metabolism	4.61	1.421	**5.92E-05**	3.15	2.546	0.233	1.45	1,429	0.348
nucleotide metabolism	2.66	1.343	**0.047**	nd	nd	nd	10.48	4,598	**1.1E-04**
cell	2.45	0.591	**4.28E-04**	3.94	1.585	**0.015**	1.81	0.918	0.108
stress	1.07	0.304	0.119	nd	nd	nd	0.92	0.573	0.226
hormone metabolism	1.33	0.531	0.13	1.33	0.926	0.257	1.39	0.913	0.248
protein	2.18	0.192	**1.81E-13**	1.52	0.414	0.064	1.87	0.341	**8.2E-04**
signalling	1.23	0.358	0.081	1.8	0.983	0.148	0.83	0.421	0.216
transport	1.29	0.417	0.086	1.6	1.196	0.227	nd	nd	nd
lipid metabolism	0.27	0.202	0.098	nd	nd	nd	nd	nd	nd
cell wall	1.31	0.509	0.132	1.49	1.084	0.347	0.68	0.593	0.342
development	0.45	0.235	0.065	nd	nd	nd	0.95	0.59	0.274
misc	0.67	0.198	0.06	0.51	0.399	0.279	2.37	0.724	**6.1E-03**
RNA	0.15	0.076	**6.35E-08**	0.53	0.218	0.166	0.24	0.154	**8.2E-03**
DNA	0.23	0.086	**9.47E-07**	nd	nd	nd	nd	nd	nd
not assigned	0.11	0.034	**2.94E-37**	0.13	0.076	**4.70E-06**	0.38	0.091	**2.8E-06**
Biodegradation de Xenobiotics	4.28	3.109	0.186	nd	nd	nd	40.45	22,833	**5.5E-05**
minor CHO metabolism	nd	nd	nd	nd	nd	nd	nd	nd	nd
secondary metabolism	nd	nd	nd	nd	nd	nd	nd	nd	nd
metal handling	1.44	1.373	0.349	nd	nd	nd	nd	nd	nd
gluconeogenesis / glyoxylate cycle	nd	nd	nd	nd	nd	nd	nd	nd	nd
Co-factor and vitamine metabolism	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table S3b: Summary and statistical analysis of BAR MapMan functional annotations for phloem proteomes in cucumber. FP, microdissected FP; FP_{SE}, FP sieve element contents; EFP, phloem exudate.

	Cucumber								
Functional categories	**FP**	**FP StdDev**	**FP p-value**	**FP_{SE}**	**FP_{SE} StdDev**	**FP_{SE} p-value**	**EFP t1t3**	**EFP t1t3 StdDev**	**EFP t1t3 p-value**
glycolysis	17.17	5.529	**6.5E-13**	49.07	21.031	**3.1E-05**	35.44	15173	**3.06E-04**
OPP	16.83	7.132	**1E-05**	nd	nd	nd	18.06	15269	0.053
TCA / org transformation	18.49	4.307	**2.8E-14**	nd	nd	nd	7.08	5939	0.123
fermentation	7.45	6.418	0.118	nd	nd	nd	nd	nd	nd
PS	10.13	1.975	**1.3E-14**	nd	nd	nd	nd	nd	nd
redox	7.95	2.017	**2.2E-10**	12.3	6.787	**0.011**	18.66	5782	**9.43E-08**
mit electron transport / ATP synthesis	7.6	2.295	**2.3E-07**	8.55	7.688	0.104	nd	nd	nd
N-metabolism	16.05	8.152	**1.00E-04**	nd	nd	nd	nd	nd	nd
major CHO metabolism	4.13	2.155	**0.013**	38.38	18.883	**6.4E-05**	nd	nd	nd
C1-metabolism	7.82	4.065	**6.05E-03**	nd	nd	nd	14	10767	0.067
amino acid metabolism	3.21	1.272	**2.82E-03**	nd	nd	nd	2.15	1595	0.294
nucleotide metabolism	1.15	0.668	0.267	nd	nd	nd	3.11	2.51	0.234
cell	1.63	0.501	**0.028**	3.11	2.14	0.109	2.69	1268	**0.045**
stress	1.78	0.413	**4.05E-03**	5.26	2.067	**1.95E-03**	2.73	1064	**0.016**
hormone metabolism	1.15	0.41	0.153	nd	nd	nd	2.07	1163	0.179
protein	2.24	0.149	**1.7E-16**	1.33	0.532	0.156	1.38	0.359	0.064
signalling	1.22	0.293	0.074	0.94	0.586	0.375	nd	nd	nd
transport	0.91	0.335	0.13	1.26	0.956	0.365	0.54	0.362	0.295
lipid metabolism	0.72	0.348	0.191	nd	nd	nd	1.3	0.892	0.359
cell wall	0.95	0.42	0.177	2.35	1.914	0.281	1.02	0.819	0.371
development	0.39	0.214	**0.036**	nd	nd	nd	nd	nd	nd
misc	0.52	0.197	**0.016**	0.81	0.529	0.366	1.4	0.697	0.162
RNA	0.23	0.081	**3.1E-07**	nd	nd	nd	0.36	0.236	0.059
DNA	0.16	0.067	**6.5E-09**	nd	nd	nd	0.33	0.111	**2.80E-05**
not assigned	0.15	0.039	**3.7E-37**	0.21	0.123	**1.24E-03**	nd	nd	nd
Biodegradation de Xenobiotics	nd	nd	nd	nd	nd	nd	20	16926	**0.048**
minor CHO metabolism	1.68	1.003	0.217	nd	nd	nd	4.51	4856	0.178
secondary metabolism	0.47	0.273	0.127	nd	nd	nd	1.26	1174	0.362
metal handling	1.25	0.949	0.362	nd	nd	nd	nd	nd	nd
gluconeogenesis / glyoxylate cycle	8.02	7.82	0.111	nd	nd	nd	nd	nd	nd
Co-factor and vitamine metabolism	1.28	1.091	0.36	nd	nd	nd	nd	nd	nd

Significance Statement

Cucurbits are phloem model plants partly because sap exudate is easy to collect, but they unusually possess both fascicular phloem (FP) within vascular bundles and additional extrafascicular phloem (EFP), leading to hot debates on the origins of the exudate and uncertainty about the underlying differences between these two systems. Here, comparison of the two proteomes reveals little overlap, suggesting not only spatial separation but functional divergence.

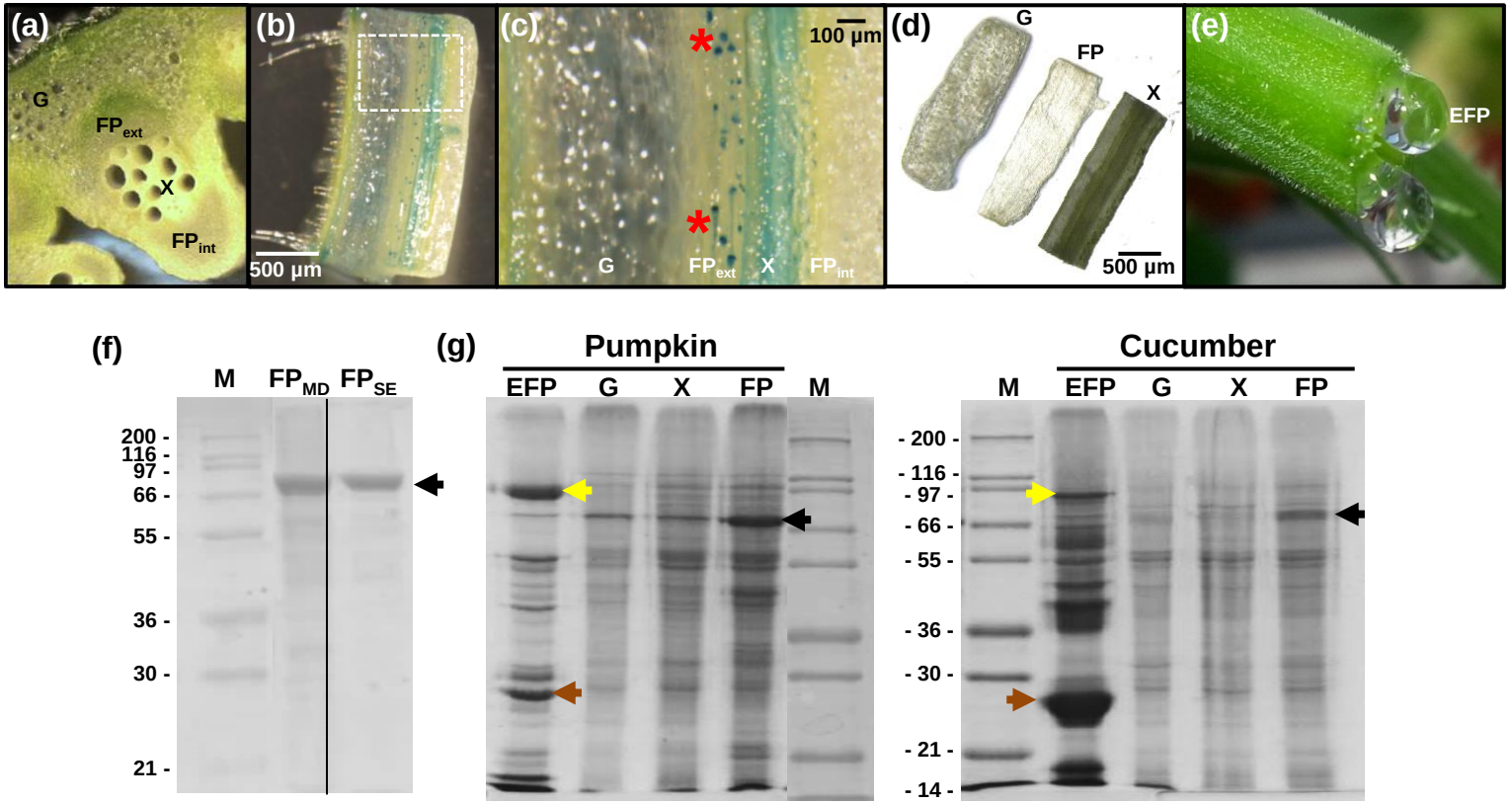


Figure 1. Micro-dissection and protein profiles of tissue and cell types from cucumber and pumpkin stems. (a) Transverse free-hand section of pumpkin stem, showing a vascular bundle. (b) Longitudinal free-hand section of pumpkin vascular bundle stained with 0.1% amido black. (c) Close-up of boxed region in (b). Red asterisks indicate sieve element protein strands (dark blue) from fascicular phloem (FP). (d) Final dissected pumpkin tissues. (e) Phloem exudate (EFP) from cut cucumber stem. (f) Protein profiles from pumpkin microdissected FP tissue (FP_{MD}) and FP-SE contents. (g) Protein profiles from pumpkin and cucumber EFP, together with microdissected ground tissue (G), xylem tissue (X) and fascicular phloem tissue (FP). Coomassie-stained SDS-PAGE. Arrows indicate major proteins: yellow, PP1; brown, PP2 and black, SEOR. FP_{ext}, external fascicular phloem; FP_{int}, internal fascicular phloem; M, molecular weight markers.

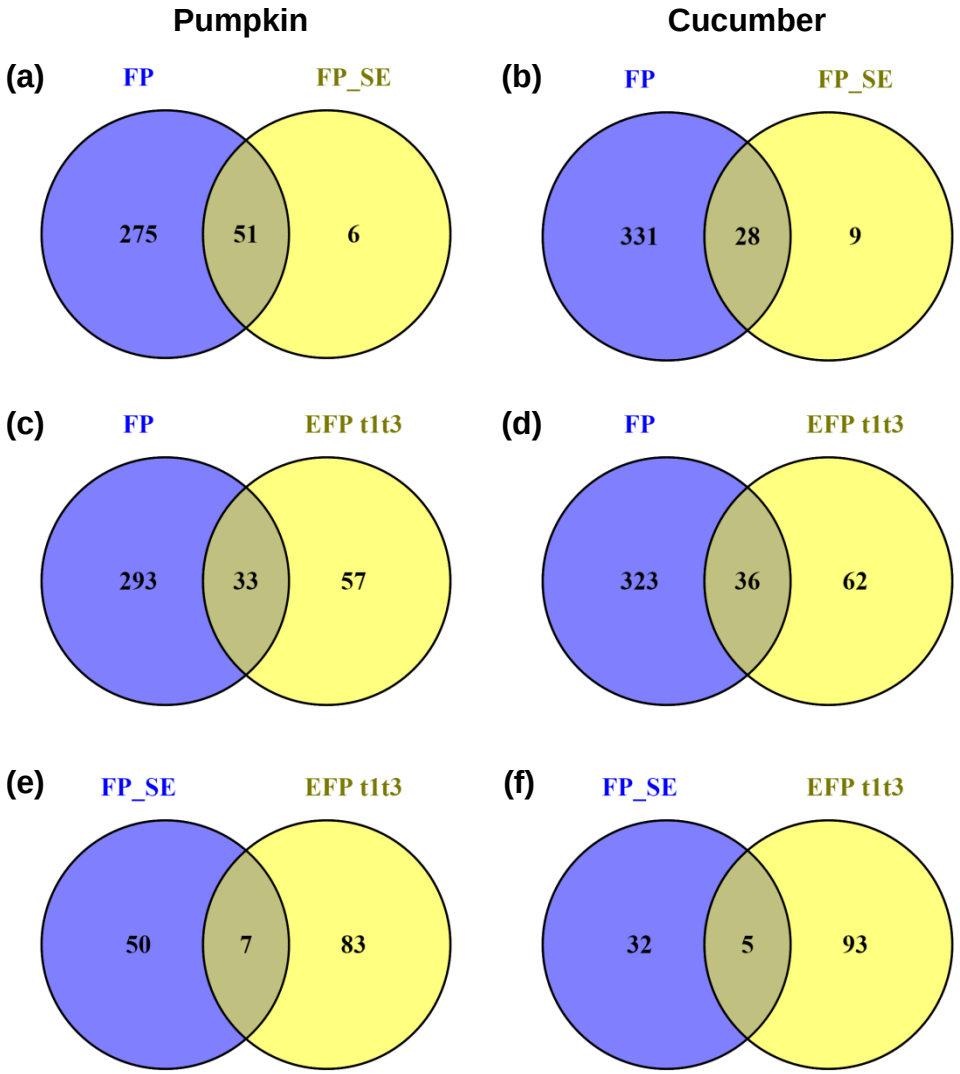
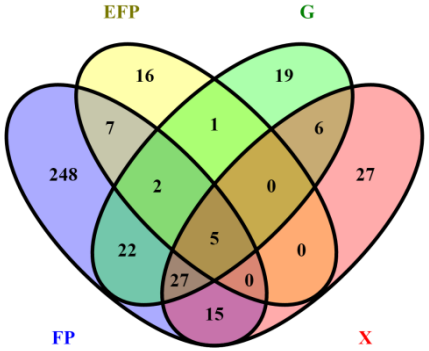
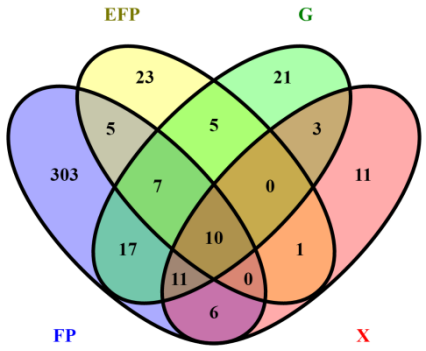


Figure 2. Overlap among proteins identified in FP and EFP from pumpkin and cucumber. Shared and unique proteins for: microdissected FP tissue (FP) and FP sieve elements (FP-SE) in pumpkin (a) and cucumber (b); microdissected FP tissue and EFP in pumpkin (c) and cucumber (d); and FP-SE and EFP in pumpkin (e) and cucumber (f). Cut-off for identified proteins was set at 99% CI (ProteinPilot Unused ProtScore >2) and at least 2 peptides each**.** Phloem EFP exudate is combined from t1 and t3 samples.

(a) Pumpkin



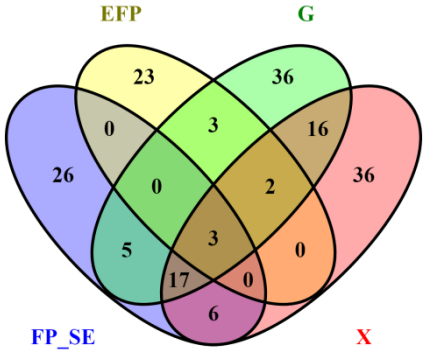
(b) Cucumber



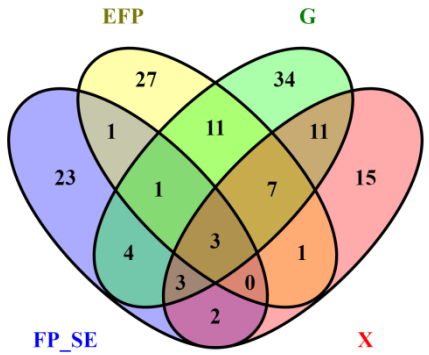
(c)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
EFP	31	16	51	23
FP	326	248	359	303
Ground tissue	82	19	74	21
Xylem	80	27	42	11

(d) Pumpkin



(e) Cucumber



(f)

	Pumpkin		Cucumber	
	Total	Unique	Total	Unique
EFP	31	23	51	27
FP_SE	57	26	37	23
Ground tissue	82	36	74	34
Xylem	80	36	42	15

Figure 3. Overlap among proteins identified in different tissue types and exudate of pumpkin and cucumber. Shared and unique proteins for phloem exudate (EFP), microdissected FP tissue (FP), ground tissue (G) and xylem tissue (X) in pumpkin (a) and cucumber (b). (c) Numbers of unique and total proteins in each tissue type. Shared and unique proteins for phloem exudate (EFP), SE content (FP-SE), ground tissue (G) and xylem (X) in pumpkin (d) and cucumber (e). (f) Numbers of unique and total proteins in each tissue type. Cut-off for identified proteins was set at unused score of >2 (99% CI) and at least 2 peptides each.

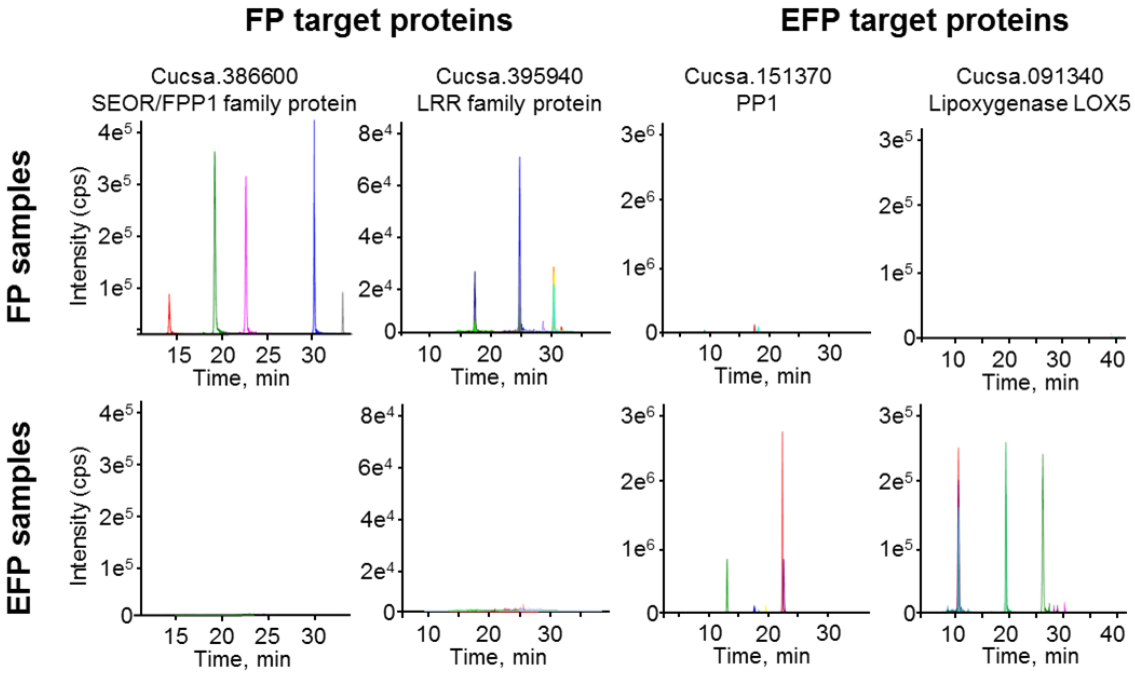


Figure 4. Cucumber FP and EFP marker proteins showing presence or absence in microdissected FP and EFP samples. Each panel contains representative LC-MS-MRM traces for chromatograms for multiple peptides from each of the markers: Cucsa.386600, Cucsa.395940, Cucsa.151370 and Cucsa.091340. Upper row, FP samples; lower row, EFP samples. Several peptides are detected for each protein and each MRM channel is individually coloured. Multiple colours at a single retention time represent different MRMs for a single peptide. See Table S1 for MRM details.

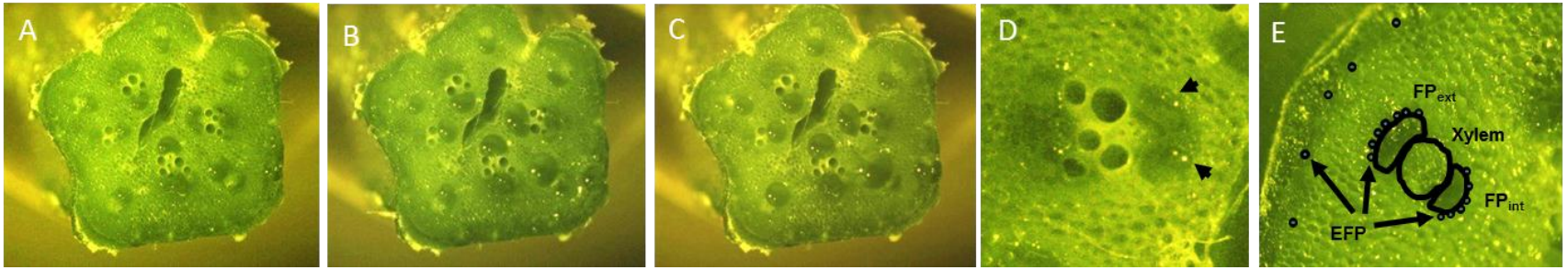


Figure 5. Direct observation of exudation from stem of cucumber. Image series starts from approximately 30 s after cutting and removal of sap with filter paper, when exudation has slowed. (a–c) Images from video record (Movie S1) of exudation at 30 s (a), 60 s (b) and 90 s (c). (d) Close-up of vascular bundle showing droplets of exudate (arrowed) emanating from extrafascicular phloem sites surrounding fascicular phloem region. (e) Location of vascular tissues. Image brightness and contrast have been modified for enhanced clarity.

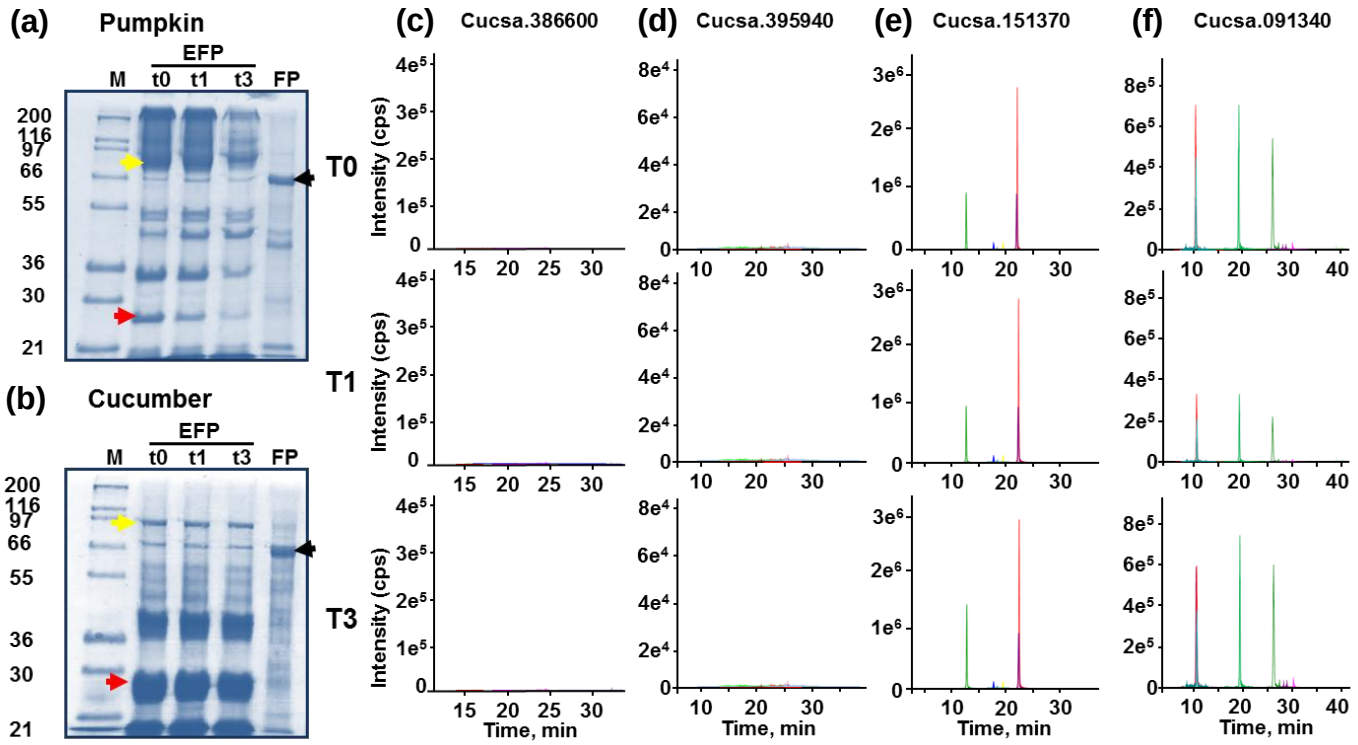


Figure 6. Time series collection of exudate from pumpkin and cucumber showing minimal change in composition. Comparison of FP micro-dissected tissues with exudate (EFP) collected at different times (0 to 3 min) after cutting. Coomassie-stained SDS-PAGE for pumpkin (a) and cucumber (b). Arrows indicate major proteins: yellow, PP1; red, PP2 and black, SEOR. M, molecular weight markers. EFP, extrafascicular phloem; FP, fascicular phloem. MRM chromatograms from the different EFP exudation times for cucumber FP markers Cucsa.386600 (c) and Cucsa.395940 (d), and EFP markers Cucsa.151370 (e) and Cucsa.091340 (f).

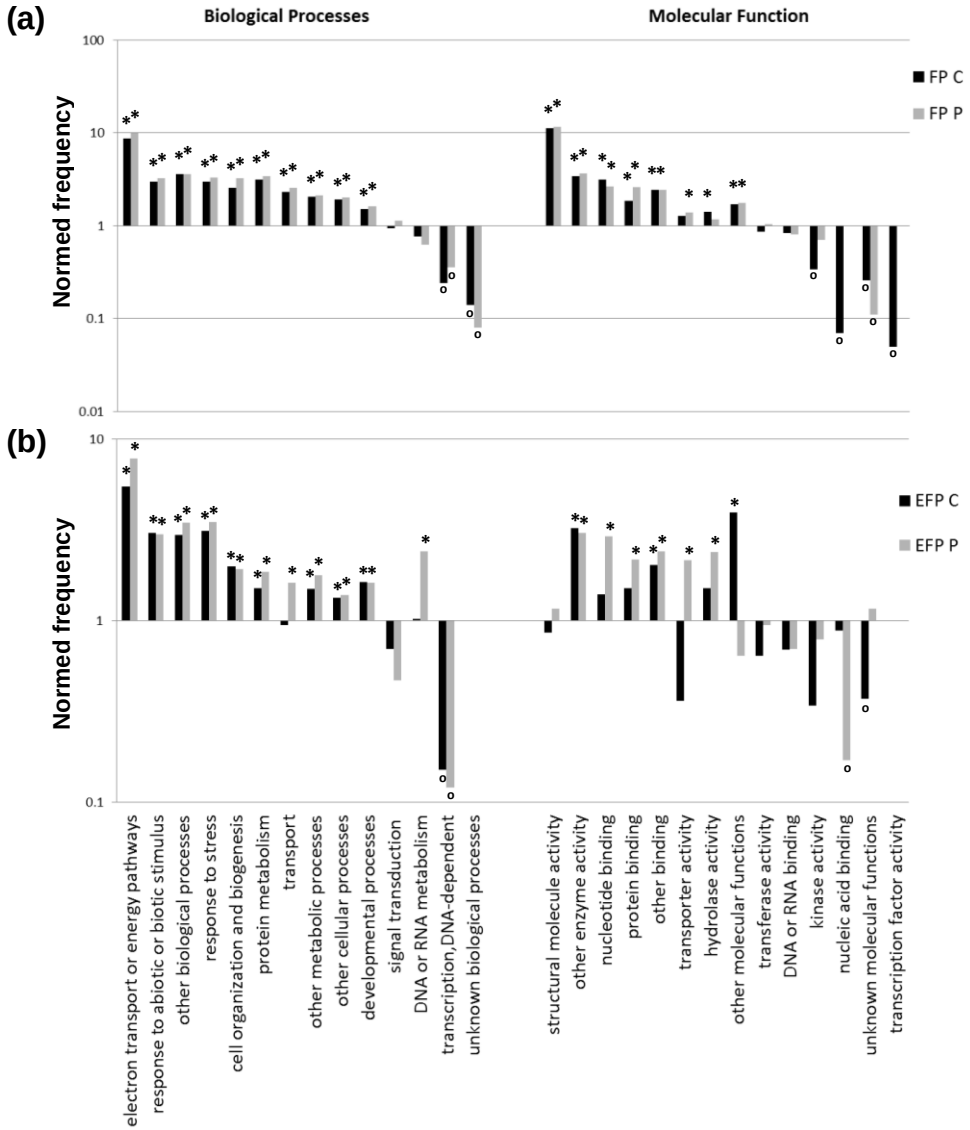


Figure 7. Frequencies of GO annotations for the cucumber and pumpkin phloem proteomes. Biological Process and Molecular Function categories are shown as normed frequencies for the proteomes of cucumber and pumpkin microdissected FP enriched tissue (a) and EFP exudate (b). Frequencies significantly above or below the values for the *Arabidopsis* reference set are indicated by * and °, respectively (*P* value < 0.05). C, cucumber; P, pumpkin.