Metabolic networks of *Cucurbita maxima* phloem

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Abstract

Metabolomic analysis aims at a comprehensive characterization of biological samples. Yet, biologically meaningful interpretations are often limited by the poor spatial and temporal resolution of the acquired data sets. One way to remedy this is to limit the complexity of the cell types being studied. *Cucurbita maxima* Duch. vascular exudates provide an excellent material for metabolomics in this regard. Using automated mass spectral deconvolution, over 400 components have been detected in these exudates, but only 90 of them were tentatively identified. Many amino compounds were found in vascular exudates from leaf petioles at concentrations several orders of magnitude higher than in tissue disks from the same leaves, whereas hexoses and sucrose were found in far lower amounts. In order to find the expected impact of assimilation rates on sugar levels, total phloem composition of eight leaves from four plants was followed over 4.5 days. Surprisingly, no diurnal rhythm was found for any of the phloem metabolites that was statistically valid for all eight leaves. Instead, each leaf had its own distinct vascular exudate profile similar to leaves from the same plant, but clearly different from leaves harvested from plants at the same developmental stage. Thirty to forty per cent of all metabolite levels of individual leaves were different from the average of all metabolite profiles. Using metabolic co-regulation analysis, similarities and differences between the exudate profiles were more accurately characterized through network computation, specifically with respect to nitrogen metabolism.
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1. Introduction

One of the most important keys for plant growth and survival is the accurate control over signal, nutrient, and assimilate transport between its organs, i.e. composition and flux in plant xylem and phloem. Classically, analyses of sink-source interactions have largely focused on loading and unloading mechanisms, assuming that assimilates are not dramatically altered during transport. This may not hold true for a variety of species. Symplastic loaders such as cucurbits have long been known for their ability to transform primary assimilates before long distance transport in phloem veins. Symplastic loaders are primarily defined by the high number of plasmodesmatal connections between specialized intermediary cells (Turgeon, 1996; Hoffmann-Thoma et al., 2001) and the surrounding tissues, i.e. parenchyma and bundle sheath cells, that are again symplastically connected to leaf mesophyll cells. Further, the ratio of raffinose family oligosaccharides (raffinose, stachyose, and verbascose) to other neutral sugars is far higher in symplastic than in apoplastic loaders, which primarily load sucrose as major form of carbon transport to sink tissues. It has been shown that the source of Stachyose synthesis in cucurbits is the intermediary cell/sieve element complex (IC/SE) of minor veins (Hendrix, 1977; Holthaus and Schmitz, 1991; Beebe and Turgeon, 1992; Ayre and Turgeon, 2000; Oparka and Turgeon, 1999) which is performed by elongation of sucrose using galactinol and release of myo-inositol. A 'size trapping' hypothesis was proposed to explain this sucrose conversion in the loading step. This mechanism might inhibit size-increased oligosaccharides to flow back into mesophyll tissues. However, such strict control of size exclusion limits is hard to understand (Schaffer et al., 1996) since it is known that a range of far larger molecules may pass plasmodesmata (Alachandran et al., 1997), and basic physiological considerations (Stitt, 1996) do not back this hypothesis. Phloem concentrations of

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raffinose family oligosaccharides (RFO) in collecting minor veins were shown to be in the range of hundreds of mM using microdissection (Schmitz, 1970; Haritatos et al., 1996) or aphid exudates (Knop et al., 2001) for phloem collection whereas monosaccharides and sucrose were only present at rather low amounts.

However, seemingly conflicting data were obtained from groups focusing on transport phloem exudates that were gathered by EDTA exudation, stem incisions (Mitchell et al. 1992; Mitchel and Madore, 1992) or cuts (Richardson et al., 1982; Tolstikov and Fiehn, 2002) at leaf petioles, stem apexes, stem internodes, peduncles, or fruit stems. Although RFOs were still present in larger amounts than other neutral sugars, total sugar concentration was found only at the low mM range, whereas the sum of amino and hydroxy acids was far higher (Richardson et al., 1982).

The vascular system of plants and, specifically, of cucurbits is long known not to be a uniform string of companion cell/sieve element complexes (Kempers et al., 1998) (CC/SE) but spatially distinguishable into external, internal (Golecki et al., 1999), and extrafascicular phloem veins (Crafts, 1932). Therefore, cuts or incisions might cause a mixture of the contents of all these veins, thereby losing spatial resolution. In the following, such total vascular exudates will be summarized to 'phloem'. In this work, a metabolomic approach was used to study metabolic effects observable in changes of chemical composition of phloem exudates. Metabolomics has been defined in recent reviews in analogy to proteomics or transcriptomics (Fiehn, 2001,2002) as the analysis of the total complement of individual metabolites of a given biological sample. This is specifically in contrast to metabolic fingerprinting techniques which do not aim at analysing individual components, but rather gather physical spectra (e.g. ^{1}H NMR, or infusion mass spectra) of unseparated complex mixtures. Therefore, the general purpose of fingerprinting is to rapidly classify numerous samples according to relative distances by multivariate statistics. Such classifications can also easily achieved by metabolomic analyses, which, in turn, may be used for an important further step: to get to the level of individual metabolites in order to analyze causes of differences in biochemical networks in a detailed way. This paper describes the use of metabolomics to unravel the hidden structure of metabolic regulations in biochemical networks of phloem.

2. Results

In a previous study using LC/ion trap MS we observed that in *C. maxima* phloem saps sampled from petiole cuts, only low mM amounts of known sugars (glucose, fructose, sucrose, raffinose, stachyose, verbascose,

and UDP-glucose) were found (Tolstikov and Fiehn, 2002). Most strikingly large peaks of highly uncommon metabolites were detected, among them 3-*O*-[β-D-xylopyranosyl-(1-6)-β-glucopyranosyl]-(3*R*)-octen-3-ol, 1,4-dideoxy-1,4-imino-D-arabinitol, UDP-*N*-acetylglucosamine and a series of nitrogen-containing *O*-glycans. Due to the low amounts of common sugars and the uncommon nature of the secondary metabolites we identified, it was highly unlikely that this observation was caused by contamination of wounded ground tissue cells. Further, these metabolites were not observable in any non-vascular tissue analyzed so far. This implies that metabolic control in IC/SE and CC/SE complexes might be going far beyond simple housekeeping of glycolysis (Lehmann, 1973a,b), sugar metabolism (Lehmann, 1981), or antioxidative response (Walz et al., 2002). These experiments were thus repeated using less invasive sampling techniques, i.e. 450 µm OD petiole recesses and 50 µM OD microcapillary sampling (Arlt et al., 2001), finding the same LC/MS profiles as before. This finding further supported the assumption of limited contamination problems from wounded non-vascular cells.

In order to test phloem composition in a metabolomic way, these LC/MS profiles were complemented by analysis of small polar components using established GC/MS profiling methods (Fiehn et al., 2000b). Phloem chromatograms obtained by GC/MS again resulted in complex profiles that were analysed in a novel way by applying automated mass spectral deconvolution and identification software (AMDIS). AMDIS finds even low abundant peaks that are not revealed by visible inspection of total ion chromatograms, and it purifies successfully mass spectra of overlapping and severely co-eluting peaks (Stein, 1999). These purified mass spectra result in enhanced mass spectra match factors if compared with mass spectra libraries. AMDIS spectra purification thus improves the reliability of identifications of minor compounds and enhances the feasibility of detecting chemicals at trace levels, making full use of the three-dimensional information included in GC/MS data files (retention times, mass spectra, and intensities). Co-elutions of adjacent peaks could be resolved in all cases in which retention time differences were at least two scans (1 s). Subsequently, all peak detections were manually checked for false positive and false negative assignments. These mass spectra were then compared to own and commercially available mass spectral libraries. However, of the 400 phloem components, only a minor part could directly be identified this way. Therefore, denovo identification of GC/MS peaks was utilized using TBS-silylation and combination of mass re-calibrations and isotope ratio calculations that was recently published (Fiehn et al., 2000a). Among others, 2-methylserine and *S*-methylcysteine have been identified in phloem of *C. maxima* for the first time this way. *S*-

Methylcysteine might act as additional source of sulfur transport (Bourgis et al., 1999), but also other biological roles have been discussed for this particular compound (Maw, 1982). Biological roles of 2-methylserine in vascular exudates are completely unclear. Unknown compounds were manually classified according to characteristic ions and patterns of their mass spectra. Fig. 1 exemplifies this strategy by depicting differences between a typical leaf tissue disk extract and a vascular exudate from the same leaf. It can clearly be seen that only small amounts of mono-, di- and trisaccharides are detectable in *C. maxima* vascular exudates, whereas numerous low molecular weight metabolites were found to be more abundant in exudates compared to leaf disks. By applying external calibration curves, phloem concentrations were determined for a number of classical amino acids and sugars (Table 1). Using 4 phloem replicates of 25 individual plants of *C. maxima*, average levels were comparable to earlier results. Interestingly, the ratio of amino acids and sugars remained remarkably constant: in this study, an amino acid/carbohydrate ratio of 2.7 was found, in the previous a ratio of 2.9, whereas for individual metabolites up to 20-fold differences were found (e.g. threonine). This might indicate a stable property in vascular metabolism compared to high flexibility in individual amounts.

Next, metabolites were searched that were apparently phloem-specific. From a single mature *C. maxima* leaf, 6 phloem sap samples garnered from petiole recesses were compared to six 25 mg FW leaf disks from the same leaf. By visual inspection of GC/MS chromatograms

dramatic differences were observed. A massive decrease of mono-, di- and trisaccharides was accompanied with a number of high abundant, yet unknown metabolites that were eluting in the range of amino acids and monosaccharides. In order to compare the samples semi-quantitatively, a rough calculation was carried out based on the premise that symplastic connections between the cytosol of leaf mesophyll cells and IC/SE complexes exist. The cytosol in mesophyll cells accounts for about 10% of the total cell volume, so any difference smaller than 10-fold in comparison between leaf disks and phloem contents might be due to intracellular compartmentation. Vice versa, putative contamination from vascular cells might be more relevant for phloem than for leaf tissue samples, so here, a relevance threshold of 10-fold difference was assumed. After normalization to absolute volume, carbohydrates—especially glucose and fructose—were indeed found to be largely decreased whereas unknown amino compounds were almost exclusively found in phloem (Table 2). This was supportive evidence for the hypothesis that CC/SE complexes in transport phloem possess a very distinct metabolism.

Using small recesses instead of petiole incisions or cuts enables repetitive phloem sampling from the same leaf. Therefore, the hypothesis of distinct metabolic patterns in transport phloem was followed using the idea of metabolic snapshots (Kell and Mendes, 2000). Assuming that phloem composition was primarily altered by assimilate production rates, phloem from eight mature leaves of four *C. maxima* plants was sampled every 4 h over 4.5 days. No obvious impact of the
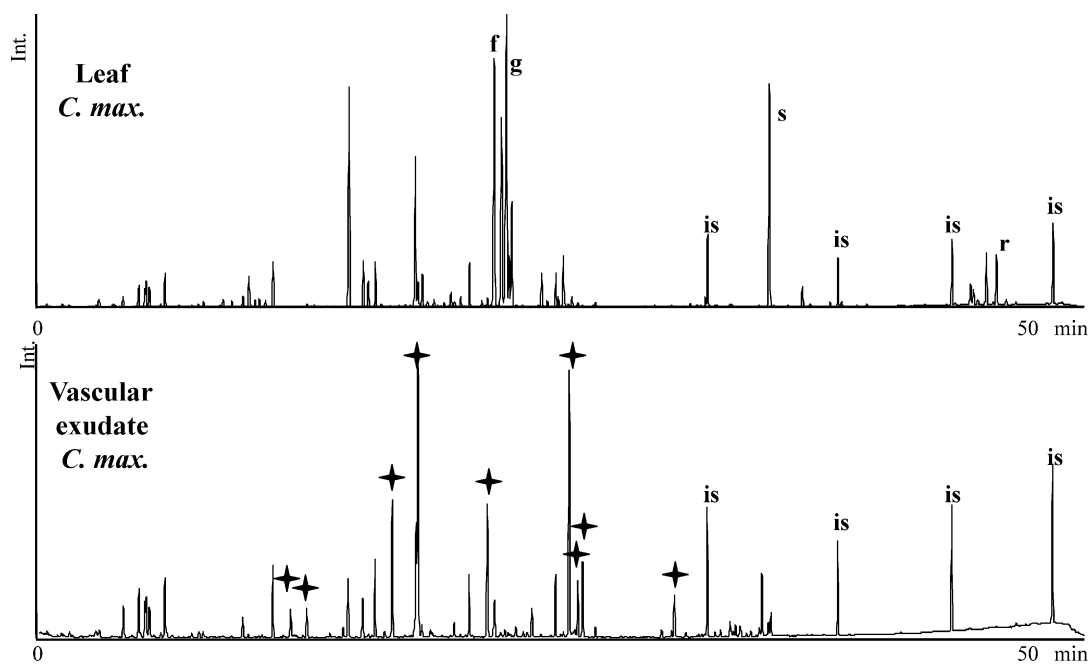

Fig. 1. Comparison of phloem and leaf GC/MS profiles. Abundant phloem metabolites that are virtually absent in leaf disks are marked by symbols. f = Fructose, g = glucose, s = sucrose, r = raffinose, is = internal standards.

repeated phloem recesses were observed over this time with respect to exudation volumes or total metabolite concentrations, although later on, the studied leaves showed earlier senescence than control leaves of the same plants. Using such metabolic snapshots, the majority of the studied phloem profiles should follow the same pattern if day–night rhythms or circadian clocks directly controlled metabolite levels. However, only for some metabolites in select phloem profiles such patterns were found, e.g. for hexose phosphates in plant 1 leaf 1 (P1L1, Fig. 2). The profiles for hexose phosphates in other leaves were clearly different, showing either no diurnal rhythm or in parts even opposite regulations. The same differences were found for other metabolites, concluding that with this refined sampling and metabolic analysis method, an earlier report on putative diurnal rhythms in cucurbit phloem compositions (Mitchell et al., 1992) could not be verified.

Instead, the acquired data sets were investigated in detail to learn more about differences and similarities between phloem profiles of *C. maxima* plants. First, metabolic phenotypes (Fiehn et al., 2000b) from phloem snapshots were computed by principle component analysis (PCA). PCA uses linear combination of all metabolic

Table 1
Comparison of metabolite levels (mM) in petiole vascular exudates of *Cucurbita maxima* plants in this study (*n* = 100, 25 plants) by GC/MS and stem cut exudates in a previous study (*n* = 10). Stachyose contents in this study were determined by LC/MS by a pool of 12 plants

	This study	Richardson et al., 1982		This study	Richardson et al., 1982		This study	Richardson et al., 1982		This study	Richardson et al., 1982
Sucrose	0.8	0.8	Ala	0.3	3.4	Ile	0.13	0.8	Malate	1.6	42.9
Raffinose	0.09	0.01	Arg	0.34	0.6	Leu	0.09	0.8	Succinate	0.18	1.8
Stachyose	5.0	7.4	Asp	6.5	21.1	Lys	2.1	0.7	Citrate	2.1	1.6
Melibiose		0.4	Cys		0.4	Met	0.19	0.7	Lactate	1.6	0.9
Glucose	2.0	12.0	GABA	0.8	0.0	Meser	2.3		Pyruvate		0.2
Fructose	2.7	5.5	Gln	0.36	0.0	Norval	0.03		Oxalate		0.1
Galactose		1.1	Glu	6.0	11.6	Phe	0.26	0.6	Malonate		2.9
Inositol	0.4	0.0	Gly	0.25	2.7	Pro		7.0	Fumarat	0.21	0.3
F6P	0.1		His		3.9	Ser	0.8	9.5	Akg	0.24	1.0
G6P	0.5		Hser	11.4		Thr	0.17	11.8	Glutarate		0.1
						Tyr		0.6			

Compounds with empty cells were not included in quantification in one of the studies. Three-letter codes for amino acids are used. GABA = 4-aminobutyric acid, meser = 2-methylserine, norval = norvaline, G6P = glucose-6-phosphate, F6P = fructose-6-phosphate, akg = α-ketoglutarate.

Table 2
Comparison of metabolite ratios between petiole vascular exudates of *Cucurbita maxima* plants (*n* = 6) and tissue disks from the same leaf (*n* = 6), P/L ratio

Metabolite	P/L ratio	Metabolite	P/L ratio	Metabolite	P/L ratio	Metabolite	P/L ratio
Y84 amine m	2402	Y96	179	Y106	65	2-*O*-Glyl-α-gal	14
Y63 m	2307	Y19 amine	173	Y12 amine	54	Y43	13
Y98 m	1590	Y93 m	166	Y60 amine	53	Y18 amine	13
Y91	1208	Y68 m	150	Y110	48	Y120	12
Y51	938	*S*-Methylcysteine	146	Y23 amine	39	Y59 amine	12
Y83 amine m	873	Y62	142	6-P hexonate	38	Palmitate	12
Y100	842	Y29 amine m	139	spermidine	37	Y41	11
Y65 amine m	770	Y89 amine	133	Y35 amine	34	Lysine	10
Y54 m	679	Y104	131	Y20 amine m	33	Y22	0.09
Y95	359	Y88 hexP	128	Y61 phosphate	28	Threonate	0.09
Y52	331	Y87	124	Y72	26	Inositol	0.08
Y37 amine	321	Y32 amine	117	Y28	24	Sucrose	0.07
Y30 amine m	305	Y64	108	Sinapinate	23	Y105	0.06
Y66 amine m	252	Y94 amine m	97	2-Methylserine	22	Galactinol	0.06
Y67	235	Aminobutandiol	84	Y11 aa	21	Trehalose	0.06
Y107	232	Y25	83	Y82 amine	21	Galactose	0.05
Y33 amine	228	Y92	81	Phosphate	21	Fumarate	0.03
Y26 m	222	Homoserine	79	Y71 amine	19	Glucose	0.008
Y45 m	210	2,4-Diaminobutyrate	78	Alloxanate	16	Fructose	0.006
Y31 m	181	Y8 amine	70	Y117	15		

Unknown compounds are marked with a Y, a number and chemical classification guided by mass spectral interpretation. Small additions '*m*' marks peaks that were appearing as major peaks in phloem GC/MS profiles. *aa* = amino acid, 6-P hexonate = 6-phosphohexonic acid, hexP = hexose phosphate, 2-*O*-glyl-α-gal = 2-*O*-glyerol-α-galactoside.

data to generate new vectors that best explain overall variance in the data set, without prior assumption how and if these data might cluster. Plotting these overall metabolic snapshots, it was immediately clear that each plant (Fig. 3A) and, more precisely, each leaf (Fig. 3B) had its own distinct metabolic phenotype. Such observations of distinct metabolic phenotypes are regularly observed when analyzing distinct plant genotypes (Taylor et al., 2002; Roessner et al., 2001) but so far, it was never observed for individual organs from the same plants. In terms of statistics, phloem profiles from the different leaves can therefore be treated as independent populations, at least if garnered from different plants.

Student's t-test comparisons between these independent leaf exudates populations and the total average over all leaves showed that for each leaf, 30–40% of all detected metabolites were significantly different from an assumed average *C. maxima* phloem metabolite composition (Table 3). This also explains differences found between average metabolite levels given in Table 1: averages of exudates measurements will largely depend on the size of the sampled phloem collection and on the biological state of the individual leaves from which the samples were garnered.

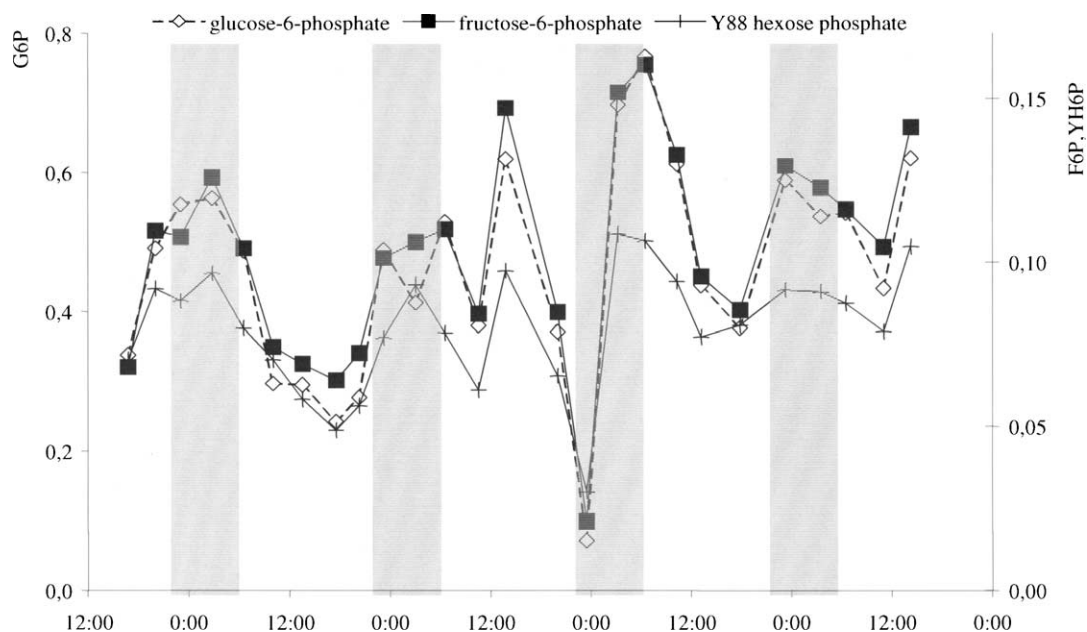Apart from average levels, further insights can be obtained using a data analysis method that implicitly is

Fig. 2. Course of phloem snapshots along day/night rhythms in plant 1 leaf 1. Relative metabolite levels are given in arbitrary units after normalisation to inorganic phosphate. Patterns of phloem courses were different for hexose phosphates in other leaves.
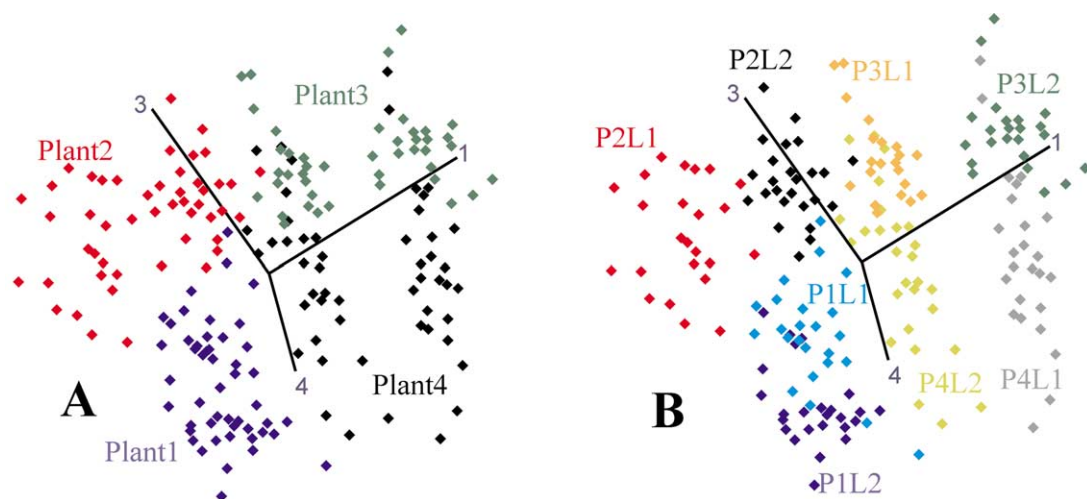

Fig. 3. Principle component analysis using vectors 1, 3 and 4 for clustering all collected phloem snapshots according to inherent metabolic variance. A, phloem profiles colored according to individual plants. B, phloem profiles according to individual leaves numbering plant 1 leaf 1 and P1L1 and onwards.

already given in Fig. 2: some metabolites such as hexose phosphates will show very strong co-regulation whereas other metabolite pairs will have less or no such correlations. Strong correlation was indeed observed not only for glucose-6-phosphate and fructose-6-phosphate from phloem of plant1 leaf1 but also for all other leaves in the diurnal test data set (Fig. 4). This can easily be explained by the impact of the glycolytic enzyme glucose-6-phosphate isomerase (EC 5.3.1.9) which was shown to act in cucurbit phloem as early as 1973 (Lehmann, 1973a,b). However, also other regulatory mechanisms might lead to pair-wise correlations, e.g. influenced by increased or decreased fluxes through linear pathways, or transcription factors controlling different pathways simultaneously. Therefore, the complete data set was comprehensively analyzed for metabolic co-regulations using Pearson correlation assessments and applying a relevance threshold of $r_{xy} > 0.80$ (Kose et al., 2001). Long tables of metabolite:metabolite correlations are resulting from such calculations, and a network overview may be given by graph visualization (Fig. 5). In these graphs, each node represents a certain metabolite, and its number and the degree of its correlations with all other metabolites determine its location on the graph. Surprisingly, the overall shapes of these networks were highly similar for all phloem metabolic phenotypes, despite the large differences in average levels of particular metabolites. Inspecting such metabolic correlation networks in more detail, some further observations can be derived. For example, members of the two main clusters (Fig. 6A and B) were similar for all leaves analyzed. Both clusters contained different forms of amino compounds, m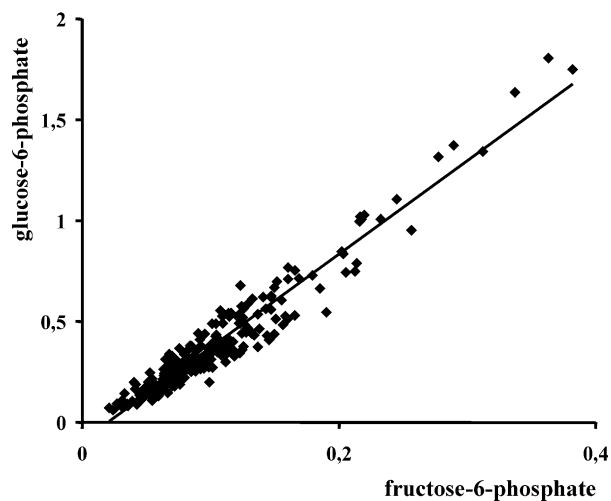ost of them non-identified. The larger cluster primarily contained compounds indicative for strong co-regulation of nitrogen metabolism. Polyamines (spermidine, 2,4-diaminobutyric acid) were correlated with aromatic amino compounds (xanthine, uric acid, pipecolic acid), compounds involved in primary nitrogen metabolism (glutamate, aspartate) and rather uncommon compounds (2-methylserine, 2-O-glycerol-α-glycoside). It is interesting that metabolites belonging to completely separate pathways seem to be closely co-regulated and cluster together in a nitrogen metabolism building block. The smaller cluster (Fig. 6B) included several small hydroxy acids (lactic acid, 2-oxobutyric acid, 3-hydroxypropanoic acid, glyceric acid), export products indicative for beginning leaf senescence (palmitic acid, stearic acid, hydroxystearic acid) and further (unfortunately unknown) amines. Common amino acids were connecting and loosely surrounding these two major clusters in most cases. Even clearer clustering of metabolic correlations with respect to known biological and biochemical building blocks was observed for vascular exudates of two of the eight investigated leaves. For example, strong correlations between snapshot levels of sucrose, hexose phosphates, fructose and glucose were observed in plant 4 leaf 2 (Fig. 6C), in which average sucrose contents were also decreased by a factor of 2 compared to the average of all phloem snapshots. This might indicate a stronger influence of primary carbohydrate metabolic pathways including sucrose breakdown. Similarly, strong clustering of amino acids was perceived only from exudates collected from plant 3 leaf 2 that had an average decrease of amino acid levels of about 2–3 fold (Fig. 6D). This sub-cluster was located quite distantly from the main nitrogen-metabolism cluster. Such reduced links of commonly known amino acid biosynthetic pathways might be due to increase amino acid degradation. However, for both examples, the underlying reason why primary metabolism in phloem exudates was found different in vascular exudates of these leaves remained necessarily unknown.

3. Discussion

There is a massive flux of assimilates exported from leaves to sink organs in cucurbits, resulting in a net increase in dry weight of fruits from 0.1–2 g/h and phloem velocities of about 60–240 cm/h (Schaffer et al., 1996; Crafts, 1932). Therefore, a huge gradient in osmotic pressure must be present. This gradient was in fact found for *C. pepo* callus for collecting phloem (Lackney and Sjolund, 1991) as well as could be calculated from high amounts of RFO sugars found again for collecting phloem (Haritatos et al., 1996) and from aphid exudates (Knop et al., 2001) although for some species, no pressure gradients in collecting phloem was observed (Turgeon and Medville, 1998). It is an intri-

Fig. 4. Correlation scatter plot of relative G6P and F6P levels over all collected phloem profiles. Metabolite levels are given in arbitrary units.
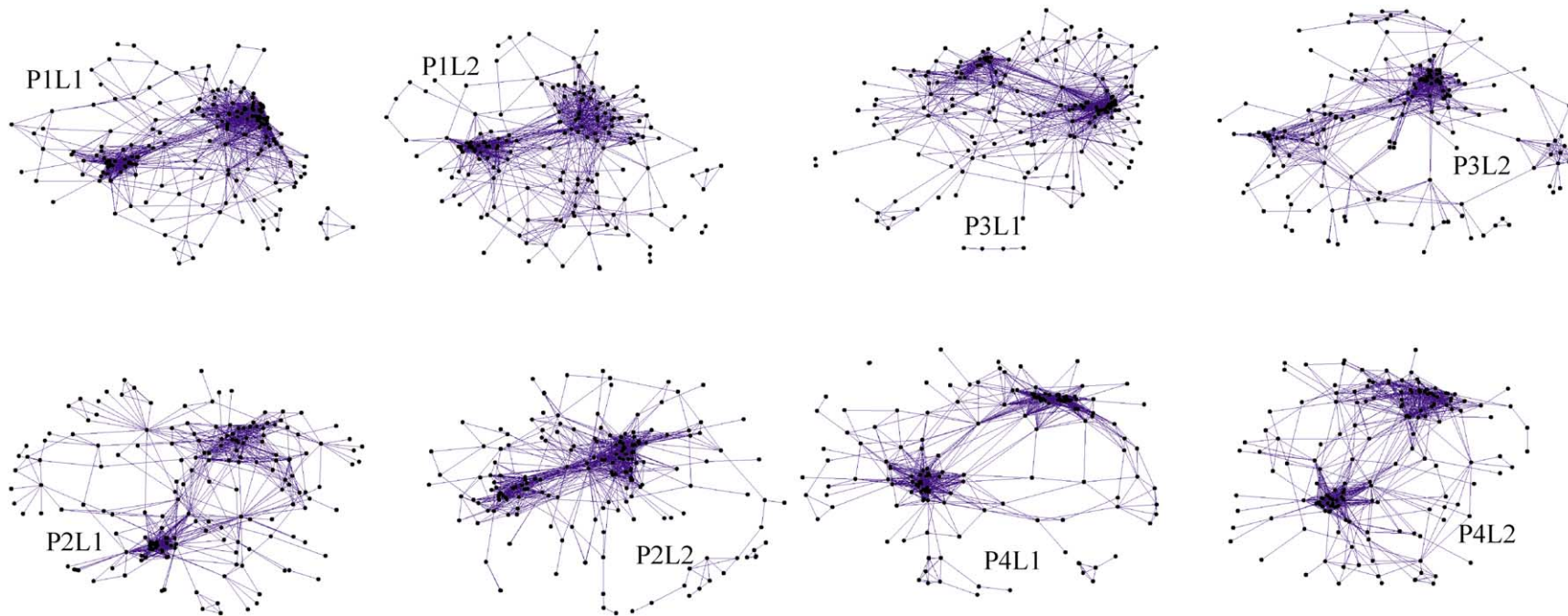
Fig. 5. Metabolic correlation networks obtained from comprehensive pair-wise computation of Pearson correlations for all individual leaves. Nodes in the graph visualization mark individual metabolites that are connected via edges with other metabolites in clusters.
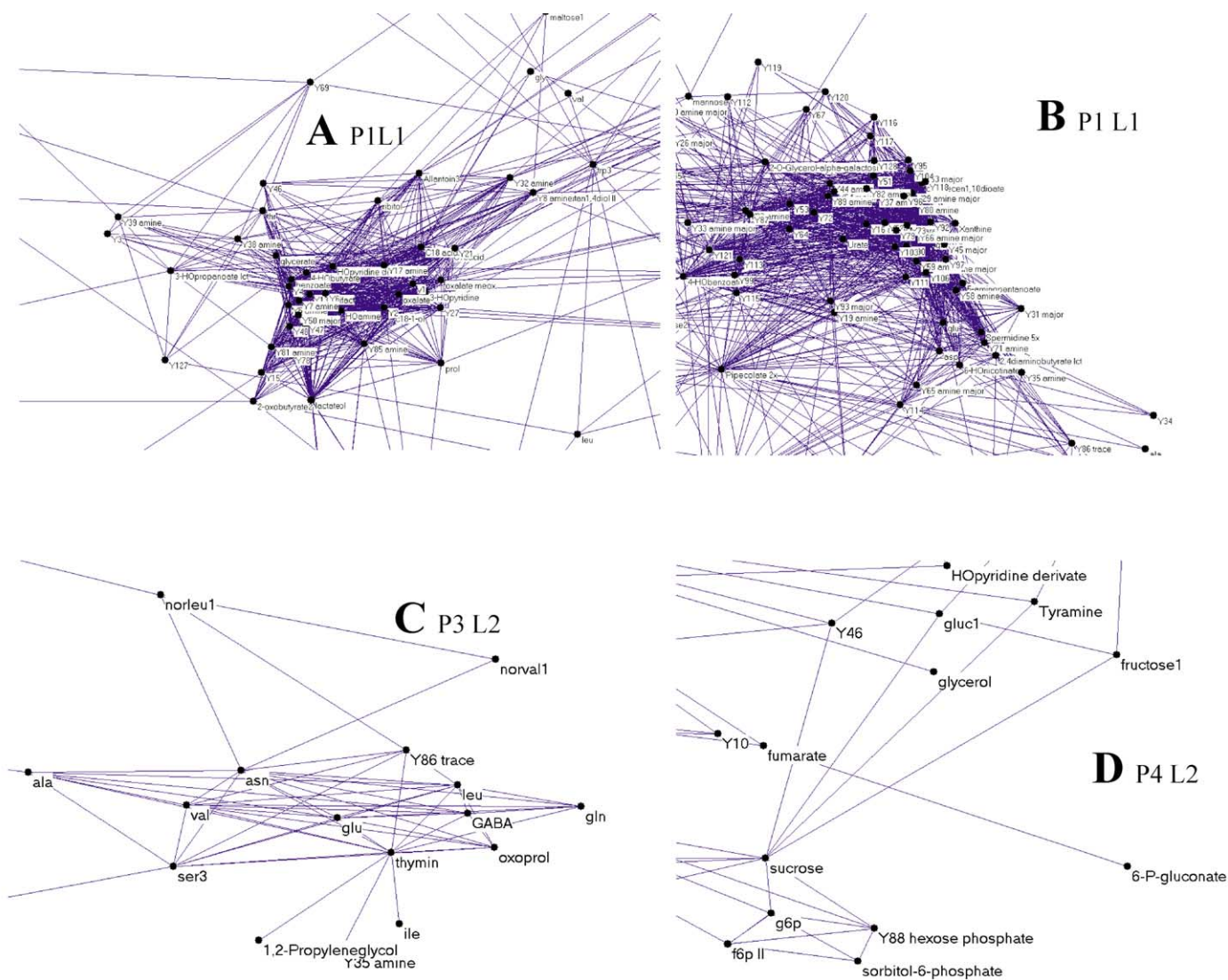
Fig. 6. Enlarged view of sub-clusters of metabolic correlation networks. (A) and (B) are found as major clusters in all phloem networks, here given for plant 1 leaf 1. Distinct sub-clusters are found in phloem obtained from plant 3 leaf 2 (C) for amino acids, and for primary sugar metabolism in plant 4 leaf 2 (D).

guing idea that the low concentrations of RFO sugars observed from total vascular exudates might be due to some kind of dilution process, for example by xylem sap (Schaffer et al., 1996) or water channels (Tyerman et al., 2002). However, a dilution of vascular exudates by two orders of magnitude is somewhat unlikely to be caused by xylem fluxes, since xylem is usually under-pressurized. Average lengths of mature transport sieve elements are about 30 μm per cell, thus even using 50 μm OD microcapillaries might cause major ruptures in the vascular tissue, causing a massive release of turgor pressure and subsequent co-extraction of extrafascicular and internal phloem vein contents. There is shallow indication that extrafascicular phloem might be specialized for amino acid transport (Leegood et al., 1997) but it is hard to believe that fluxes of these streams should be able to dilute total vascular exudates that far. At least, this would pose a possible location for biosynthesis of the number of amino compounds that were found in this study, and it would support the common perception that RFO sugars are responsible for rapid fruit growth in cucurbits (Chrost and Schmitz, 1997; Gao et al., 1999).

Alternatively, the idea could be considered that RFO sugars are *not* the major transport form of carbon towards sink tissues but only account for the major part of neutral sugars in transport phloem veins. Some evidence for this hypothesis is gained through ^{14}C import studies carried out by Richardson et al. almost 20 years ago (Richardson et al., 1984). Using labelling with ^{14}C carbon dioxide, sucrose, raffinose, or stachyose it was found that even for short term (15 min) labelling periods, 44–76% of the total radioactivity in total vascular exudates was not found within the neutral sugar fraction but in acidic or basic fractions which would fit to the number of uncommon amino compounds presented here. It would be a very interesting experiment to repeat this study but then analyze external, internal, and extrafascicular phloem contents by microdissections rather than stem incisions. The idea of rapid metabolism in transport phloem rather than simple RFO transport through non-interactive sieve elements is further supported by high activity of metabolic enzymes (Lehmann, 1973a,b), the complexity of proteins found in vascular exudates of cucurbits (Haebel and Kehr, 2001; Kehr et al., 1999; Walz et al., 2002) and the high concentrations of sugar nucleotides (Lehmann, 1981) in phloem saps. The number and amount of small molecular weight molecules might be sufficient to explain loss of RFO sugars and gain in osmotic pressure, in order to result in the observed high phloem velocities in cucurbits. Unfortunately, unknown compounds cannot be accurately quantified, thus calculation of the osmolarity caused by these small molecules are yet undoable. It cannot yet be decided if high metabolic turnovers or spatial phloem specifications or dilution and con-

tamination may better explain experimental findings. Whatever might be the final answer, at least explanations must be found for the clear finding of the high number of uncommon low molecular weight amino compounds detected in vascular exudates presented in this study.

It is an interesting finding that overall structure of metabolic networks may be highly similar despite large variation in average metabolite contents. Analysis of metabolic networks was yet limited to detailed analysis of small networks, often carried out by isotopomer analysis (Szyperski, 1998; Möllney et al., 1999). The approach of large correlation networks presented here already provided insights into differences in metabolic regulation between different plants and organs. In order to make full use of such approaches, major bottlenecks have to be solved or circumvented: first, the number of unidentified compounds is simply too high. However, de novo identification of metabolites is a slow and tedious process and hard to be automated. Therefore, international cooperation must be pursued building up mass spectral libraries with standardized instrument parameters and retention indices for both, LC/MS and GC/MS. So far, such large programs have not been started in the public area.

After improved metabolite identification, the next bottleneck is to link the derived network information to background knowledge and to further experimental data, e.g. proteomic approaches. Applying such combinations would map metabolite correlation networks more easily to biochemical pathways and theoretical models like metabolic control analysis (MCA) (Fell, 1997). Correlations are highly similar to co-response analyses (Hofmeyr et al., 1993) derived from MCA, yet they are different with respect to the unknown reasons why these correlations occur and to what underlying changes they respond to.

Last, robustness and flexibility of networks are essential parameters in terms of theoretical biology

Table 3
Percentage of significantly different metabolites comparing phloem profiles from a specific leaf to all collected profiles ($P < 0.05$)

	Total	Up	Down
P1L1	33	26	7
P1L2	42	36	5
P2L1	43	18	25
P2L2	35	10	25
P3L1	23	12	11
P3L2	35	17	18
P4L1	32	22	10
P4L2	33	18	15

'Up' means increased, 'down' means reduced average level. The individual leaves are counted onwards from P1L1 for plant 1 leaf 1.

approaches. Computational methods need to be developed and to be applied that calculate cluster coefficients and similarities between metabolic networks in relation to subtle changes in environmental, biochemical or developmental conditions. Obviously, the analysis of day-night rhythms of vascular exudates gave not sufficient resolution to link plant compartmentalization ('phloem') and timed events ('assimilation') to directly understand differences in networks. Instead, it was clear that the superposition of the biological state of the individual plants and their individual organs was resulting in larger effects than the anticipated control by light and relaxation times. It might be interesting to further study these theoretical aspects in synchronized unicellular organisms to avoid some of the aforementioned problems.

4. Conclusions

Metabolomic analysis has revealed astonishing facts about the complexity and flexibility of plant vascular exudates. Compositional analysis confirmed absolute amounts of classical sugars, acids, and amino acids that have been reported earlier. In addition, dozens of uncommon amino acids, amino sugars and simple amines have been detected in comparatively large amounts that have not been reported before but that could at least be classified via mass spectral characterization. Diurnal rhythms of phloem metabolites could not be confirmed. Instead, large differences between individual plants and individual leaves were observed by unsupervised clustering methods and metabolic network computation. It can be foreseen that the latter method may lead to detailed biological information once more metabolites have been identified and once networks can be linked to quantitative proteomics.

5. Experimental

Seeds from *C. maxima* Duch. cv. Gelber Zentner were purchased from Julius Wagner Saatzucht (Heidelberg, Germany) and were grown in GS 90 standard soil (Einheitserdewerk W. Tantau, Uetersen, Germany) under controlled conditions (16 h light, 8 h darkness, 20 °C, 60% relative humidity). Seventy days after sowing, phloem exudates of two different mature, fully expanded source leaves of four plants were taken at the petiole using a modification of the stem cut exudation method (Kehr et al., 1999) by punching small incisions of 450 μm OD recesses at the top of the petiole. Incisions were immediately dried with paper tissue. Subsequently exuding phloem sap (0.1–1 μl) was collected into Eppendorf tubes containing 500 μl water and 300 μl chloroform. The tubes were vortexed for 10 s to precipitate proteins. After centrifugation at 14000 *g* for 30

s, 450 μl of the upper aqueous layer were given into fresh tubes and lyophilized to complete dryness in a SpeedVac concentrator. Leaf disk samples were prepared as previously reported (Fiehn et al., 2000b). The chloroform phase containing lipophilic compounds was discarded for both phloem and leaf disk samples. Derivatization of polar leaf extracts were done as reported. Carbonyl moieties were protected either by methoximation using 10 μl of a 20 mg/ml solution of methoxyamine hydrochloride in pyridine at 37 °C for 90 min. Afterwards, acidic protons were derivatized with 10 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 30 °C or 10 μl *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) at 70 °C for 30 min (Godber and Parsons, 1998). All chemicals were purchased from Sigma-Aldrich-Fluka (SAF, Deisenhofen, Germany). 2 μl of these solutions were injected at a split ratio of 1:25 into a GC/MS system consisting of an AS 2000 autosampler, a GC 8000 gas chromatograph, and a Voyager quadrupole mass spectrometer (all Thermo-Quest, Manchester, GB). Injection temperature was 230 °C, the interface was set to 250 °C and the ion source was adjusted at 200 °C. Helium flow was 1 ml min^{-1}. After a 5 min solvent delay time at 70 °C, oven temperature was increased at 5 °C min^{-1} to 310 °C, 1 min isocratic, cool down to 70 °C and additional 5 min delay, afterwards. Peak finding was performed using AMDIS (http://chemdata.nist.gov/mass-spc/amdis/). Integration of extracted ion traces was done using the MassLab FindTarget method. Identification of unknown metabolites was carried out by recalibration of quadrupole mass spectrometric data as recently reported (Fiehn et al. 2000a). Mass spectra can be downloaded from http://www.mpimp-golm.mpg.de/mms-library/index-e.html. Data were log scaled before calculation of Pearson's correlations in order to down weight the impact of outliers. Network graph layout was done using the 2D Fruchtermann-Reingold algorithm in Pajek (http://vlado.fmf.uni-lj.si/pub/networks/pajek/)

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