Metabolite Profiling and Assessment of Metabolome Compartmentation of Soybean Leaves using Non-Aqueous Fractionation and GC-MS Analysis

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In the present study, non-aqueous fractionation (NAQF) and GC-MS were used to obtain a spatially resolved view of metabolism in mature leaves of soybean (*Glycine max* Merr.). NAQF of lyophilized soybean leaves was performed using CCl_4-*n*-heptane and ultracentrifugation that yielded a gradient comprised of six fractions. Chlorophyll content, and marker enzyme activities, phosphoenolpyruvate carboxylase (PEPC) and α-mannosidase, were utilized as stroma, cytosol and vacuole markers, respectively. GC-MS analyses of each fraction resulted in the identification of around 100 different metabolites. The distribution of these identified compounds showed a decreasing order from the vacuole to cytosol to chloroplast stroma. In other words, a greater number of identified compounds were found in the vacuole when compared to the cytosol or stroma.

Levels of sugars, organic acids and fatty acids showed greater relative abundances in the vacuole with 50%, 55% and 50% of the respective pools. A greater relative abundance of amino acids was observed in the cytosol where 45% of the total of amino acids content was recorded. The relatively large pool of sugars and phenolic acids in the vacuole compartment implies high levels of starch metabolism and phenylpropanoid biosynthesis. The low amino acids pool, on the other hand, suggests low nitrogen accumulation in the leaves of soybean. Hierarchical cluster analysis on the most abundant metabolites revealed three clusters containing 10, 20 and 2 of the 32 selected metabolites. The data were discussed in term of NAQF and GC-MS analysis of soybean mature leaves, and also in term of distribution and compartmentation of metabolites at subcellular levels.

KEY WORDS: Compartmentation; GC-MS; Metabolome; Non-aqueous fractionation; Metabolite Profiling; Soybean (*Glycine max* Merr.)

1. Introduction

A principal goal of metabolomics is the measurement of the complete range of primary and secondary metabolites present within, or transported from, a biological system as a means to better understand the precise biological role or function of that system at a defined developmental stage or under specific environmental factors. The search for new trait-enhanced crops, of course, renders it necessary to continue to develop new insights into the response of plants to genetic and environmental changes induced by biotic or abiotic stresses. Metabolite profiling and metabolic pathways mapping, can be considered as critical steps (Fiehn *et al*., 2000; Kell, 2004) in providing such information.

A metabolomics experiment typically extends from sampling and sample preparation through analytical strategies for data acquisition to statistical and bioinformatics approaches to raw data processing and data analysis (Brown *et al*., 2005). Given the diverse physico-chemical nature of different metabolites, no single analytical technology can suffice to cover the entire metabolome. However, the range of current comprehensive technologies, including chromatographic techniques coupled to mass-spectrometry, currently facilitate extensive coverage of a wide array of metabolites associated with important biosynthetic pathways and processes (Dunn and Ellis, 2005). Yet a somewhat underrepresented approach to metabolomic studies relates to assessments of the relative distribution of metabolites in different plant compartments. Whilst clearly a technical challenge, approaches to sub-cellular fractionation that minimize biochemical degradation during sample preparation are known. In principle, these can be combined with current developments in metabolic profiling to more accurately reflect the distribution of

metabolic processes within a cell or tissue. We have initiated preliminary evaluations of this approach when applied to soybean leaf and this is the focus of this paper.

Compartmentalization of metabolites and metabolic process is an important aspect of metabolic regulation; metabolite compartmentalization constitutes a principal regulatory characteristic of plant metabolism (Fridman and Pichersky, 2005, Goodacre *et al*., 2004; Lunn, 2007). Typically, metabolic pathways are highly segregated in different subcellular organelles (Browsher and Tobin, 2001; Lunn, 2007). Thus, a fundamental understanding of plant metabolism requires a qualitative and quantitative description of the metabolome within different compartments of the cell. The chloroplast, cytosol and vacuole constitute the main compartments of photosynthetic and other metabolic activities, including biosynthetic and hydrolytic activities, in the leaves of plants. Knowledge of metabolic networks and their regulation can therefore be further developed based upon increased understanding of relative distributions of metabolites in these compartments as determined by experimental analyses. A cellular fractionation process which minimizes loss or degradation of metabolites is clearly of immense value as a prelude to such compartmentation analysis. Given the dynamic and highly labile nature of most metabolites, it is essentially that such fractionation processes are conducted under as inert an environment as possible. One of the most promising approaches to the study of compartmentalized metabolism in leaf tissue is the non-aqueous fractionation (NAQF) procedure used to separate subcellular compartments under biochemically and enzymatically inactive conditions (Gerhardt and Heldt, 1984; Stitt *et al*., 1989). There have been many investigations in soybean leaf including for example studies on

carbohydrate metabolism (Allen et al., 1998) that could, at least in principle, benefit from spatially resolved metabolic profiling. Therefore the primary objectives of this study were to (i) effectively implement NAQF for soybean leaves, (ii) profile the soybean leaf metabolome using GC-MS, and (iii) determine compartmentation at the subcellular level of the profiled metabolites, and their relative abundances in each compartment. This information will serve as an important prelude to detailed comparative experiments (unpublished studies ongoing).

2. Materials and Methods

2.1. Plant material

Soybean (*Glycine max* Merr. cv. Kitamusume) was grown in Hokkaido University Farm, Sapporo, Japan, from May to August. Leaves were sampled from mature (10 weeks) plants early morning (from 8 to 9 a.m) and immediately plunged in liquid nitrogen to quench further metabolism. Afterwards, leaves were freeze-dried for 5 days. The major veins were removed, and samples were re-dried for two additional days for complete drying. Freeze-dried samples were stored in air-tight tubes at - 80ºC until use. Three set of plants were used for NAQF and metabolite analysis. The NAQF was run on each set of plants to provide one biological replicate.

2.2 Non-aqueous fractionation

NAQF of plant material was carried out, with slight modification, by the method of Gerhardt and Heldt (1984). All operations were carried out on ice and/or 4ºC unless otherwise stated. A sample of 300-350 mg of freeze-dried leaves was ground in a mortar

and 20 ml carbon tetrachloride (CCl_4) and *n*-heptane mixture (d = 1.28), dried using molecular sieve beads (4A, Merck) at 10:1 v/w ratio, was added to the sample. The mixture was sonicated for 30 s × 3 times with an interval of 30 s during which samples were cooled with ice to avoid overheating. The homogenate was filtered through a layer of quartz wool to discard coarse material and one volume of *n*-heptane was added to the filtrate, vortexed and centrifuged at 3000 × *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 3 ml of CCl_4-*n*-heptane mixture (d = 1.28). Two samples of 250 µl each were withdrawn for enzyme marker and metabolite analyses. The remainder was used for NAQF. An exponential gradient of CCl_4-*n*-heptane mixtures was made by layering, from the bottom to the top, 1 ml of the following densities: 1.59 (pure CCl_4), 1.54, 1.49, 1.44, 1.40, 1.36, 1.32, and 1.28. The remainder (~2.5 ml) was layered on the top of the gradient and centrifuged at 25,000 × *g* for 3 h (Beckman Coulter, model Optima L-XP). The content of the tube was collected as 6 fractions, one of ~1.5 ml and five of ~2 ml each. From each fraction, 500 µl was withdrawn for enzymatic analysis. To each portion one volume of *n*-heptane was added and microcentrifuged for 3 min at room temperature. The supernatant was discarded and the pellet was dried overnight under low pressure in a desiccator containing silica gel and paraffin. For chlorophyll content and enzyme marker activities, 500 µl of 100 mM Bicine (pH. 7.8), 50 µl of 5 mM $MgCl_2$ and 50 µl of 1 mM EDTA were added to the sample. After a 30 s sonication pulse, samples were left for 5 min at room temperature, then microcentrifuged for 5 min. The aqueous supernatant was collected and used for marker enzyme assays, while the pellet was used for chlorophyll determination.

2.3. Enzymes and chlorophyll content assays

Chlorophyll content (stroma marker) was determined by the method of Wintermans and De Mots (1965). Phosphoenolpyruvate carboxylase (cytosol marker) was assayed spectrophotometrically by following the rate of NADH oxidation as described by Lane *et al.* (1969). The assay of α-mannosidase (vacuole marker) was determined spectrophotometrically by the method of Li (1967) based on the utilization of ρ-nitrophenyl-α-D-mannoside as substrate and the liberation of ρ-nitrophenol.

2.4. Extraction and derivatization for GC-MS analysis

The six NAQF fractions were dried and the metabolites derivatized, prior to GC-MS analysis, as described by Broeckling *et al.* (2005) with some modifications. The dried portions of the NAQF fractions were resuspended in 500 µl of water containing ribitol (10 µl ml^{-1}) as internal standard, vortexed and sonicated in a sonication-bath until total resuspension of the dry pellet. The samples were then dehydrated under a low stream of nitrogen until dryness. Afterwards, the metabolites of the dry pellet were first derivatized by methoxyamination using 60 µl of 15 mg ml^{-1} of methoxyamine-HCl in pyridine (dried prior to use over molecular sieve beads), vortexed and incubated at 50ºC for 1 h. Subsequently, metabolites were then derivatized with 60 µl of N-methyl trimethylsilylfluoroacetamide (MSTFA) + 1% trichlormethylchlorsilane (TMCS) for 1h at 50ºC. The samples were then transferred to a 300 µl glass insert and analyzed by GC-MS.

2.5. GC-MS and mass spectra analyses

Samples were analyzed by GC-MS (HP 6890 Gas chromatograph coupled with an HP 5973 MS detector and HP 6890 Series autosampler, Agilent Technologies Inc.) as described previously (Broeckling *et al*., 2005; Roessner *et al.*, 2000). The mass spectral data were then analyzed using the AMDIS software (Automated Mass Spectra Deconvolution and Identification System, http://chemdata.nist.gov/mass-spectra/amdis/) and comparison was made using commercial NIST 02 Mass Spectral Library or a custom internal database generated from authentic compounds. The data files were aligned, and quantitative and qualitative data extracted using MET-IDEA software (Noble Foundation, Ardmore. OK, USA) (Broeckling *et al*., 2006).

2.6. Data and statistical analysis

The data and calculation were obtained from the measurements of three density gradient of the same leaf material. The evaluation of the subcellular compartmentation of metabolites was performed using the deconvolution method of Riens *et al*. (1991) which is based upon the assumption that the metabolites are confined to the stroma, cytosol and vacuole designated by the distribution of the corresponding marker enzymes in the six fractions collected after NAQF.

Correlation analysis was performed with GraphPad Prism 4.03 software (GraphPad Software Inc., San Diego, CA, USA). Hierarchical cluster analysis (HCA) was performed with SPSS 10.0 software (SPSS Inc., Chicago, IL. USA). In order to ensure that groups share good correlation, the Pearson correlation coefficients were selected as the measurement between the three compartments, and the cluster method in use was furthest neighbor.

3. Results and Discussion

3.1. Fractionation of subcellular organelles

Subcellular fractionation, which consists of disruption of the cellular organization (homogenization) followed by fractionation of the homogenate to separate the different organelles, was applied to plant material by Gerhardt and Heldt (1984) and developed later by Stitt *et al*. (1989). This methodology has been consistently improved and many solvents have been recommended instead of CCl_4, such as tetrachloethylene (Riens *et a*l., 1991) or dichlorobromoethane (Hartwell *et al*., 2002). The former solvent was suggested because of its low toxicity, while the latter was shown to allow effective separation of vacuolar from cytosol material when applied to mature leaves of *Bryophyllum*.

In Figure 1A, the chloroplastic material appeared, as expected, mainly in the middle density fractions, while the cytosolic material appeared mainly in the middle or heaviest fractions. On the other hand, the vacuolar material was concentrated in the highest density fractions which contain more than 70% of the total activity of α-mannosidase. The linear regression analysis of the fractionated material and collection of six fractions showed that the goodness of fit was 0.93 (Figure 1B). The calculated P value was high with a low deviation from zero intercept, indicating good reproducibility and separation into 6 fractions (data not shown). This good distribution is likely due to the number of fractions collected. Although previous studies made no recommendation on the number of density fractions to be collected, we suggest that six or seven fractions should be considered very appropriate for the calculation based on the model of Riens *et al.* (1991). Although we did not observe significant difference in the distribution of the markers when 8 fractions were

collected (data not shown), however, a high ($n \geq 8$) or low ($n \leq 5$) number of fractions could falsify criteria for the best fit for the calculation of the distribution of metabolites between the compartments. The reader is referred to Riens *et al*. for details of calculations used in assessing compartmental distribution of metabolites and on goodness-of-fit criteria used. Overall, the distribution of the chlorophyll and the marker enzymes in the six fractions is comparable with many other previous works carried out on leaves of spinach (Gerhardt and Heldt, 1984; Riens *et al*., 1991; Stitt *et al*., 1989; Weiner *et al*., 1989) and pea (Sharkey and Vanderveer, 1989). More specifically, Sharkey and Vanderveer (1989) suggested chlorophyll as a chloroplastic marker because they did not find significant differences between this marker and glyceraldehyde-3-P-dehydrogenase). They also noted high proportion of chlorophyll in fractions two and three (lightest fractions) which may reflect challenges in fractionating leaf material since a typical plant cell contains fragile subcellular organelles, while being surrounded by a strong cell wall.

3.2. Spatially profiling of metabolites by GC-MS

To our knowledge, there are few published reports on the metabolic profiles of soybean leaves. Our GC-MS analyses of the fractionated soybean leaf material in this study identified over 200 resolved peaks. Approximately 50% of these peaks could be identified as discrete metabolites with known chemical structure (Table 1). These identified metabolites represent numerous metabolic pathways and photosynthates involved in biosynthesis and breakdown, including processes such as starch biosynthesis (e.g. hexoses and phosphorylated metabolites), starch breakdown (e.g. maltose), phenolic and related compound metabolism (e.g. shikimic acid). The identification of cyclitols (e.g. pinitol and

inositol) suggests also that our plant material was exposed to environmental stress since plant leaves were harvested at maturity during a drought period during summer, although samples were collected early morning. We also noted the presence of compounds, such as ribonic acid and pipecolic acid, which are considered as intermediary moieties of specific pathways such as alkaloid biosynthesis. Thus, from a purely fundamental point of view, our metabolite profiling approach should be able to rapidly and accurately identify diverse metabolic changes and the data can contribute to understanding physiological processes that operate as a response to developmental, genetic and/or environmental changes (Lange, 2006; Weckwerth and Fiehn, 2002).

In addition to reporting on a large number of identified metabolites, the relative ratios of profiled metabolite classes were estimated. Approximately 30% of identified components were soluble sugars and sugar alcohols, while around 4% were represented by inositol, myo-inositol, ascorbic acid and dehydroascorbic acid. Organic acids, primarily comprised of TCA cycle intermediates, represented 20% of the identified metabolites, fatty acids 12%, amino acids 16% and other compounds (e.g. glucaric acid, galactonic acids, saccharic acid) represented 5%, while phenolic compounds represented around 10%. We also noted that the most relatively abundant metabolites in stroma, cytosol and vacuoles (see later) are: citric, succinic, malic and fumaric acids, glucose, fructose and sucrose, pinitol and myo-inositol, and, β-alanine and glycine.

3.3 Compartmentation Analysis

The compartmentation of the major classes of metabolite was estimated by the three compartmentation analysis method of Riens *et al*. (1991). As illustrated by Figure 2, sugars, organic acids and fatty acids had a greater relative abundance in the vacuole with 50%, 55% and 50%, respectively, whereas amino acids showed a greater relative abundance in the cytosol with 45%. Based on the calculation method adopted here, it is apparent that the compartmental distribution showed a decreasing order from vacuole to chloroplasts: vacuole > cytosol > stroma. Whilst we must of course consider the interconnected issues of synthesis, transport and catabolism this decreasing order of metabolite abundance suggests, at least in the soybean leaf studied here, that photosynthates accumulate in compartments associated with biosynthetic activities i.e. the vacuole and cytosol, to a greater extent than in the chloroplasts.

It was of considerable interest to note that not only major TCA cycle intermediates accumulate in the vacuole compartment, but also other primary metabolites such as maltose and mannitol, derived from starch breakdown or physiological stress, do also. The presence of a high proportion of phenolic acids (p-coumaric, caffeic acid and ferulic acid) in the vacuole also implies consistent phenylpropanoid biosynthesis suggesting metabolism induced by one or more biotic or abiotic stresses (Dixon and Pavla, 1995; Wingler *et al*., 2000). The phenylpropanoid pathway leads to lignin biosynthesis suggesting, at least in part, the beginning of senescence. The low levels of glutamic acid and alanine in the vacuolar compartment (Table 2) and the high portion of glycine in chloroplast compartment (Table 2) may suggest low storage of nitrogen in leaves, and the ready export

of amino acid, as it has been earlier reported that amino acids accumulation is greater in the sink than in the source in soybean leaves (VerNooy and Lin, 1986).

These and other findings highlight the complexity of metabolism where all pathways are interconnected and plant tissues are comprised of more than one cell type giving heterogenic organelle preparations (Lunn, 2007). Only through greater spatial resolution can we understand the role of subcellular synthesis and different transport phenomena in plants. As such, this highlights the value in studying metabolome compartmentation. This can be illustrated by considering sucrose which, as we all know, has a central role in carbohydrate metabolism. A vast literature reports that sucrose metabolism takes place in the cytosol (Baroja-Fernández *et al*., 2001; Koch, 1996). Its distribution in the cytosol was shown to be 100% in spinach leaves (Riens *et al*., 1991), while in potato tubers the figure is only 17% (Farré *et al*., 2001). New findings in the regulation of carbohydrate continue apace. For example, it was recently hypothesized that the presence of maltose during light periods is a bridge between transitory starch breakdown and the plant's adaptation to change in environmental conditions. Since the biochemical pathways of synthesis and degradation of transitory starch is still not fully understood (Lu and Sharkey, 2006; Zeeman *et al*., 2004), a role for spatial metabolic profiling methods is clearly suggested.

Hierarchical cluster analysis (HCA) was also performed on the highest abundant metabolites from the total identified metabolites. The distribution of these metabolites is shown in Table 2. We noted that first cluster (A) contains compounds which are present at higher relative abundances in the cytosol, while cluster B and cluster C comprise the most

abundant compounds in the vacuole and stroma, respectively. Surprisingly and given the variability of the different metabolites, we did not observe from one compartment any value for a given metabolite less than 5% of the overall abundance. Values ≤ 20% represented only 17% of the total values reported in Table 2. Given this variability within the calculated values, we might suggest that it is not appropriate to consider a distribution value below 5% and this value could constitute the limit of detection required to presume the presence of a given metabolite.

Whilst the compounds in the cluster A are relatively abundant in the cytosol, their relative distribution in stroma and vacuole still represents over 20% of total abundance. Cluster A also included stress related compounds such as pinitol and myo-inositol. We noted that a great portion of glutamic acid and alanine was compartmentalized in the cytosol, while a greater portion of glycine was observed in the stroma (Cluster C). The greater portion of glutamic acid and alanine in the cytosol is likely due to the difficult situation where no appropriate separation of the mitochondria and the cytosol compartments has been achieved using NAQF procedure (Gerhardt and Heldt, 1984). The reactions of amino acids are compartmentalized mainly in mitochondria (Oaks and Bidwell, 1970) although there is evidence that many enzymes involved in amino acid biosynthesis are found also in the cytosol (Miflin and Lea, 1977).

The compounds in cluster B are abundant and preferrentially compartmentalized in the vacuole, while their relative abundances in the stroma very low except for glucose. Cluster B included the main part of sugars and almost total secondary metabolites indicating that major carbohydrate, flavonoid and phenylpropanoid accumulation occurs in the vacuole during this mature stage. Major part of TCA cycle organic acids were found to be in

vacuole, and this suggests that there is a large pool of organic acids located in this organelle, while a relatively small one in other organelles. This high amount of organic acids in vacuole has also been reported in many species (Martinoia and Rentsch, 1994, Martinoia *et al*., 2000). As for organic acids, major sugars also accumulate in vacuole, because it is generally admitted that these organelles are storage compartments of a large variety of organic compounds, and have multiple functions ((Martinoia *et al*., 2007; Matile, 1977).

Cluster C is minor and the two compounds are distributed one half in stroma and one half is almost equally divided between cytosol and vacuole. Biologically, no specific metabolic pathway, such as glycolate pathway, suggests itself. We did not confirm the presence of glyoxylate, easily oxidized to oxalic acid (Richardson and Tolbert, 1961), although glycolate is known to play a role as an activator of fatty acids biosynthesis (Castelfranco *et al*., 1955).

4. Concluding Remarks

In conclusion, a procedure for NAQF and GC-MS analysis of soybean leaf tissue as described above has been described. A large number of metabolites have been identified and our prelimnary analysis suggest we can demonstrate the degree of subcellular compartmentalization of these metabolites. This can, at least in principle, lead to hypotheses on the role of compartmentalization in the regulation of metabolite synthesis. Comparative analyses of plants exposed to different abiotic and biotic stresses will prove particularly useful and unpublished studies are pending.

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Table 1 Metabolites identified from non aqueous fractionated sample of soybean leaves.

Organic acids	Amino acids	Sugars + Sugar alcohols	Fatty acids	Phenolics	Cyclitols
1 2-Ketoglutaric acid	20 Arginine	41 Arabinose	70 Aconitic acid-trans	81 Caffeic Acid	90 Inositol
2 3-PhosphoGlyceric acid	21 Glutamic acid	42 Arabitol	71 Allantoin	82 Calystegine	91 Myo-Inositol
3 Ascorbic Acid	22 Alanine	43 Cellobiose	72 alpha-Linoleic Acid	83 Coumaric Acid	92 Phytol
4 Benzoic acid	23 Asparagine	44 D-Mannitol	73 Butyric acid	84 Gentistic acid	93 Pinitol
5 Citric Acid	24 Aspartic acid	45 D-Mannose	74 Decanoic Acid	85 Quinic acid	
6 Dehydroascorbic acid	25 Glutamine	46 Erythritol	75 Eicosanoic acid	86 Salycilic acid	
7 Erythronic acid	26 Glycine	47 Fructose	76 Erythronic acid	87 Shikimic acid	
8 Fumaric Acid	27 Homoserine	48 Fructose-6-Phosphate	77 Linoleic acid	88 Sinapinic acid	
9 Glyceric acid	28 Isoleucine	49 Galactose	78 Propanoic Acid	89 trans-Ferulic acid	
10 Glycolic Acid	29 Leucine	50 Gentiobiose	79 Stearic Acid		
11 Hexonic acid	30 Lysine	51 Galactitol	80 Steric acid		
12 Malic Acid	31 Methionine	52 Glucose			
13 Malonic Acid	32 Norleucine	53 Glucose-6-Phosphate			
14 Oxalic Acid	33 Ornitine	54 Glucitol			
15 Phosphoric acid	34 Phenylalanine	55 Glycerol			
16 Pipecolic acid	35 Proline	56 Gulose			
17 Ribonic acid	36 Putrescine	57 Maltose			
18 Succinic acid	37 Serine	58 Maltotriose			
19 Threonic acid	38 Threonine	59 Melezitose			
	39 Tyrosine	60 Melibiose			
	40 Valine	61 Rhamnose			
		62 Ribose			
		63 Sorbose			
		64 Sucrose			
		65 Tagatose			
		66 Trehalose			
		67 Xylitol			
		68 Xylose			
		69 Xylulose			

Table 2 Subcellular distribution of the relatively most abundant and important metabolites profiled in mature soybean leaves. Estimation was calculated using the three-compartment calculation as described in Materials and Methods.

			Stroma	Cytosol	Vacuole
Cluster A	3	Succinic acid	27	38	35
	28	Pinitol	27	39	34
	11	Propionic acid	26	40	34
	29	Myo-inositol	32	38	31
	13	Glucose	29	31	40
	9	Glutamic acid	21	50	29
	19	Ribose	26	49	25
	12	Stearic acid	18	57	24
	15	Sucrose	25	55	21
	8	Alanine	30	61	9
	21	Xylulose	36	48	16
	1	Oxalic acid	52	29	18
	7	Glycine	44	29	27
Cluster B	14	Fructose	12	27	61
	23	Xylitol	11	26	64
	5	Malic acid	12	24	62
	4	Fumaric acid	11	23	66
	25	α ketoglutaric acid	10	32	58
	26	Ascorbic acid	12	30	59
	16	Maltose	7	35	58
	17	Galactose	24	27	49
	22	Arabinose	22	31	47
	10	Butyric acid	14	36	50
	20	Cellobiose	12	42	46
Cluster C	6	Citric acid	5	17	78
	18	Mannose	7	17	76
	31	Ferulic acid	nd	22	78
	24	Mannitol	nd	nd	100
	27	Dehydroascorbic acid	nd	nd	100
	2	Malonic acid	nd	8	92
	32	Caffeic acid	nd	9	91
	30	ρ-Coumaric acid	nd	12	88

Legends to Figures

Figure 1. Distribution and fractionation (**A**), and comparative regression correlations (**B**) of marker enzymes and chlorophylls in a non aqueous gradient obtained from dried mature soybean leaves.

Figure 2. Subcellular distribution of the relatively most abundant profiled metabolites in mature soybean leaves evaluated from six fraction and calculated as described in Materials and Methods.

Figure 3**.** Dendogram showing the Hierarchical Cluster Analysis (HCA) of the relatively most abundant and important profiled metabolites in mature soybean leaves. HCA was done on extracts obtained from non-aqueous fractionated plant material and subcellular distributed metabolites.

Figure 1

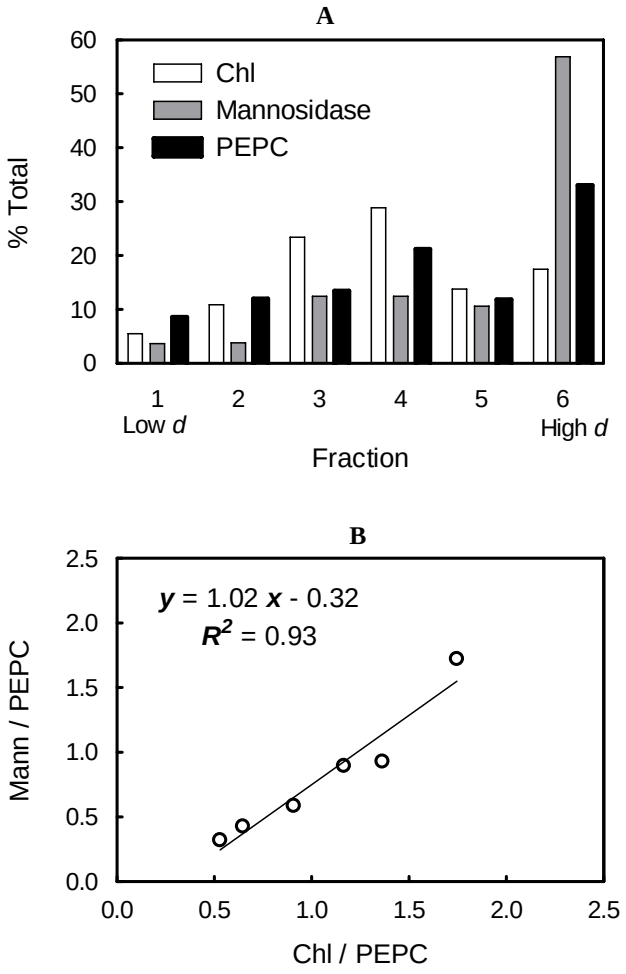

A
60
50
40
30
20
10
0
% Total
Chl
Mannosidase
PEPC
1
Low d
2
3
4
5
6
High d
Fraction
B
2.5
2.0
1.5
1.0
0.5
0.0
Mann / PEPC
y = 1.02 x - 0.32
R2 = 0.93
0.0
0.5
1.0
1.5
2.0
2.5
Chl / PEPC

Figure 2

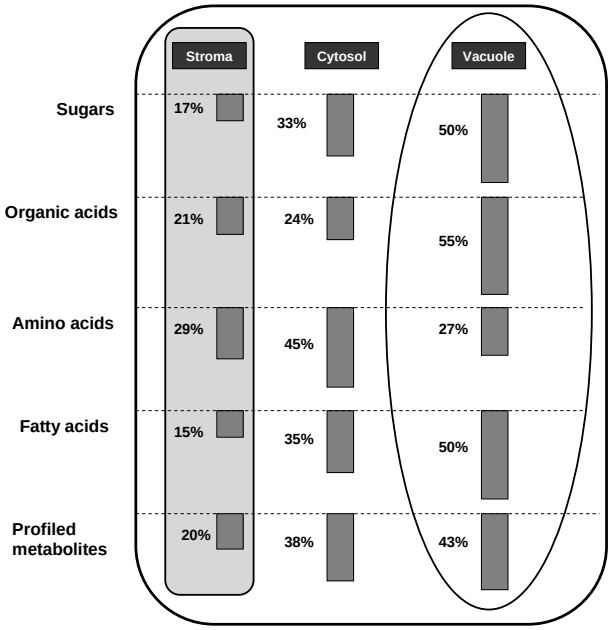
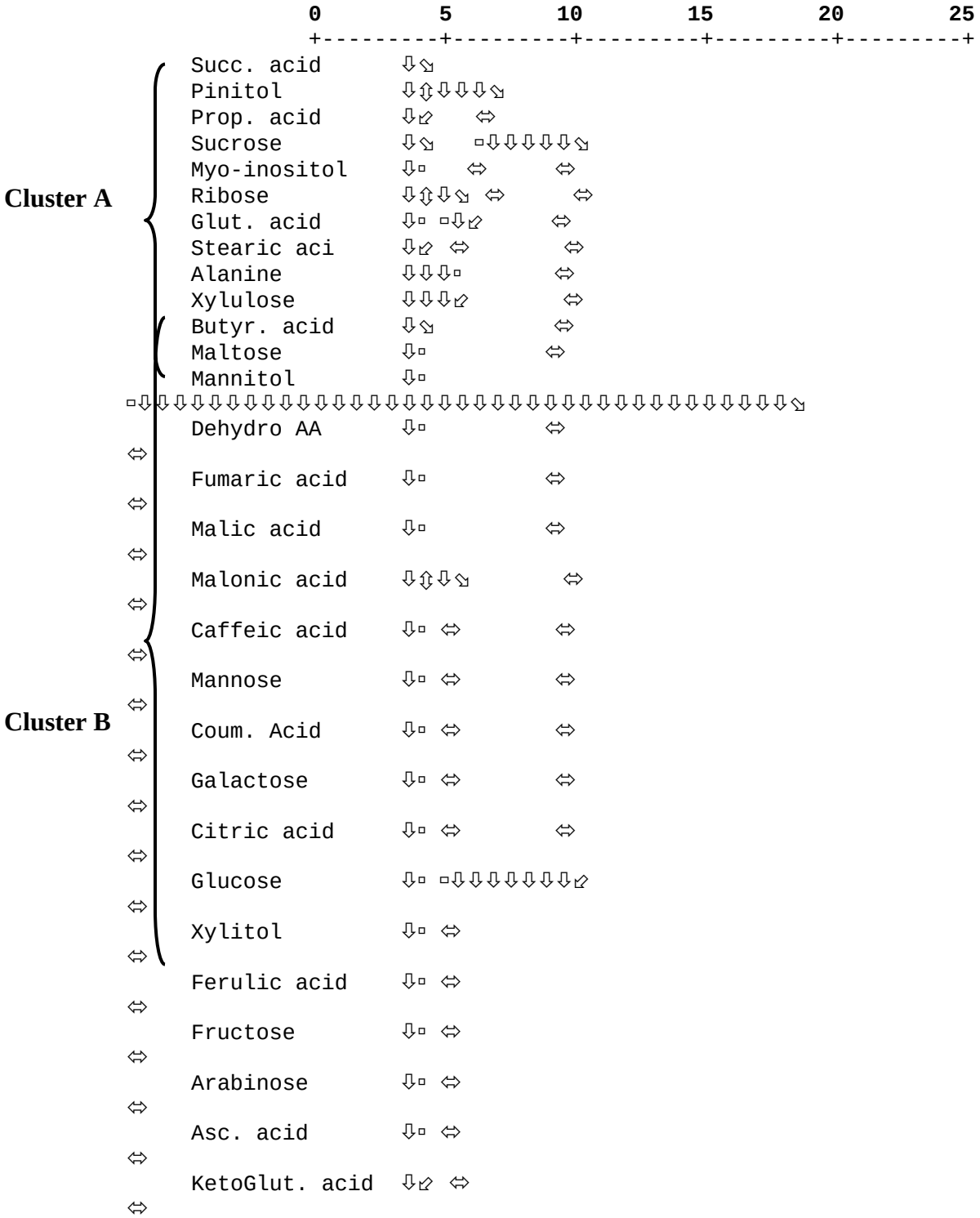
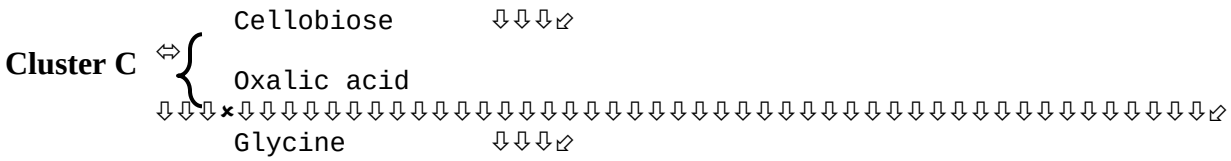

Stroma
Cytosol
Vacuole
Sugars
Organic acids
Amino acids
Fatty acids
Profiled metabolites
17%
33%
50%
21%
24%
55%
29%
45%
27%
15%
35%
50%
20%
38%
43%

Figure 3


0
5
10
15
20
25
Cluster A
Cluster B
Succ. acid
Pinitol
Prop. acid
Sucrose
Myo-inositol
Ribose
Glut. acid
Stearic aci
Alanine
Xylulose
Butyr. acid
Maltose
Mannitol
Dehydro AA
Fumaric acid
Malic acid
Malonic acid
Caffeic acid
Mannose
Coum. Acid
Galactose
Citric acid
Glucose
Xylitol
Ferulic acid
Fructose
Arabinose
Asc. acid
KetoGlut. acid


Cellobiose
Cluster C
Oxalic acid
Glycine