METABOLIC RESPONSES TO NITROGEN STARVATION IN *ARABIDOPSIS THALIANA*

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OVERVIEW

We have established a model of N-starvation on Arabidopsis thaliana plants cultivated under hydroponic conditions. A high mass-resolution GC-TOF approach was used to analyse the metabolic pattern of plants subjected to nitrogen starvation. Plants were grown for 5 weeks in a hydroponics device with a N supply non-limiting for growth. Total nitrogen starvation was applied for either two days or 10 days, allowing the analysis of intermediate and long term responses to nitrogen starvation. Roots and shoots were sampled separately and extracted for metabolite profiling.

Plant extracts were derivatised with methoxyamine hydrochloride and MSTFA and their metabolic patterns analysed in both EI and CI mode on a GCT Premier™ (Waters).

The data show the wide-ranging effects of plant metabolism to moderate and severe N starvation in roots and shoots.

EXPERIMENTAL

GC Conditions

GC	Agilent 6890N		
Column	J&W Scientific DB-5MS, 30m x 0.25mm i.d. x 0.25µm film		
Flow Rate	1.0mL/min Helium		
Injection Volume	1µL spilt 20:1		
Temperature Gradient	Temperature (°C)	Time (min)	Rate (°C/min)
	85	2	15
	320	5	
Transfer Line	280°C		
Solvent Delay	4.3 min		

MS Conditions

Parameter	Value
Ionisation Mode	EI and CI+
Source Temp	200°C
Electron Energy	70eV
Trap Current (EI+)	200mA
Emission Current (CI+)	200mA
Mass Range	50-1000
Scan Time	0.19 secs
Inter-scan delay	0.01 secs
Data	Centroid (DRE)
CI Reagent Gas	90:10 Methane: Ammonia
Lock Reference EI Exact Mass	Chloropentafluorobenzene 201.9609
Lock Reference CI+ Exact mass	2,4,6-Tris(Trifluoromethyl)-1,3,5-Triazine 286.0027

Figure 1. Waters GCT Premier™

INTRODUCTION

Nitrogen (N) is an essential element for plant growth and development. In natural soils N is often a significant factor limiting plant growth. Nitrogen stress triggers various responses at the level of metabolism, gene expression and development allowing the sessile plant to adapt by short and long term mechanisms. N starvation induces important changes in metabolism. N assimilated in biomolecules can be released back to inorganic nitrogen (NH_4) which can re-enter metabolism in various physiological processes as photorespiration and biosynthesis of phenylpropanoids. The impact of N metabolism on C metabolism has been described (Scheible et al)[1], but more detailed analysis of metabolite profiles are needed to understand the dynamic response of the metabolic network to N stress. Recent studies on potato plants (Urbanczyk-Wochniak and Fernie)[2] and Chlamydomonas (Bolling and Fiehn)[3] have demonstrated the immense changes in metabolite profiles after N starvation.

We have studied N starvation in Arabidopsis thaliana focussing on root and shoot metabolic profiles after moderate and severe N starvation. Moderate starvation was studied 2 days after N withdrawal. At this time point nitrate in roots dropped already to undetectable levels, whereas only after 10 days (severe starvation) leaf nitrate content was depleted. A detailed comparison of these two organs over a time course of starvation will therefore give further elements to understand the plant adaptive behaviour upon N stress.

METHODS

Sample Preparation

Arabidopsis thaliana plants were cultivated under hydroponic conditions using the method of Orsel et al.[4] The plants were grown for 5 weeks on 6mM nitrate in a hydroponic device using short days. Total nitrogen starvation was applied for either two days or 10 days and roots and shoots were sampled separately.

40 mg of Arabidopsis leaves and roots (fresh material) were extracted with 1 mL extraction buffer (Gullberg et al)[5]. The extraction buffer (chloroform / methanol / water (1:3:1 V/V/V at -20°C) allows the extraction of lipophilic and hydrophilic metabolites in one phase. All samples were vortex shaken for 3 minutes and then centrifuged for 10 minutes at 3000rpm and 4°C (glass vials). 300µL of supernatant was dried by speed vac concentration and the dried pellets were conserved under argon and stored at –80°C.

The dried extracts were derivatised using a 2-stage process based on the method of Fiehn et al.[6] 20µl of 40mg/mL methoxyamine hydrochloride in pyridine was added to the dried extracts and held at 28°C for 90 minutes. This was followed by the addition of 180µl of MSTFA for 30 minutes at 37°C.

RESULTS

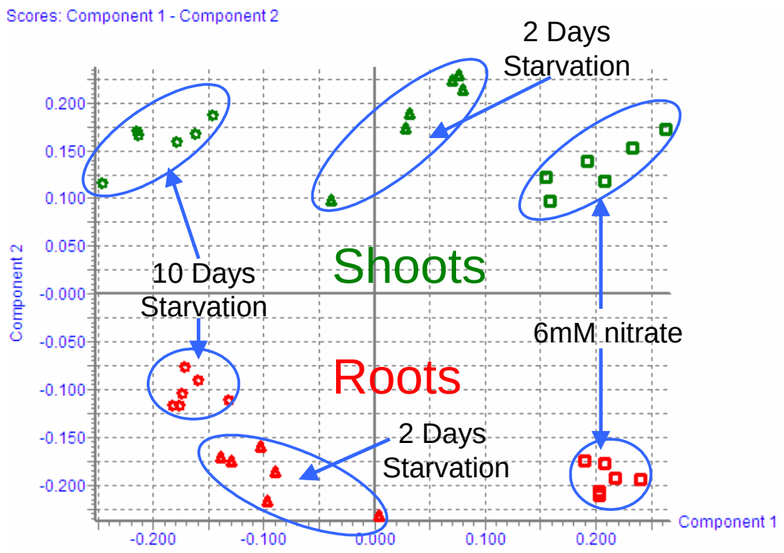

Figure 2. MarkerLynx PCA scores plot of EI data

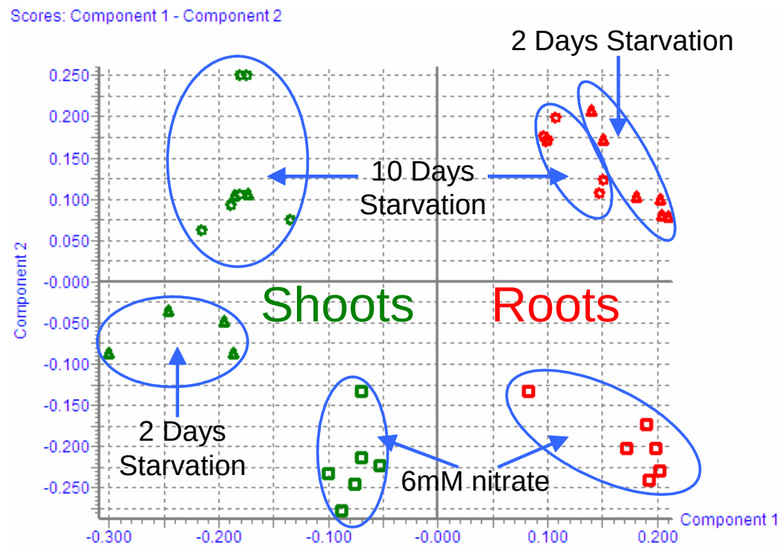

Figure 3. MarkerLynx PCA scores plot of CI data

	EI Results					CI Results		
RT (min)	Significant Ions m/z	Best Library Fit	Postulated Metabolites	Elemental Composition	Calculated Mass $[M+H]^+$	Measured Mass $[M+H]^+$	Error mDa	Error ppm
4.60	117, 147, 190, 219	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	Propanoic acid	$C_9H_{22}O_3Si_2$	235.1186	235.1192	0.6	2.6
5.09	116, 190	l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester	Alanine	$C_9H_{23}NO_2Si_2$	234.1346	234.1345	-0.1	0.4
7.18	70, 142	L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	Proline	$C_{11}H_{25}NO_2Si_2$	260.1502	260.1509	0.7	2.7
7.26	174, , 248, 276	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	Glycine	$C_{11}H_{29}NO_2Si_3$	292.1584	292.1587	0.3	1.0
9.98	84, 128, 156, 174, 230, 246	Glutamine, tris(trimethylsilyl)-	Glutamine	$C_{14}H_{33}NO_4Si_3$	364.1796	364.1816	2.0	5.5
10.76	73, 147, 205, 217, 307,319	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	Xylitol	$C_{20}H_{52}O_5Si_5$	513.2739	513.2750	1.1	2.1
11.46	147, 273, 347, 363, 375, 465	1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl	Isocitric/citric acid	$C_{18}H_{40}O_7Si_4$	481.1929	481.1945	1.6	3.3

Figure 4. Metabolites Showing a Significant Reduction in Shoots after Starvation

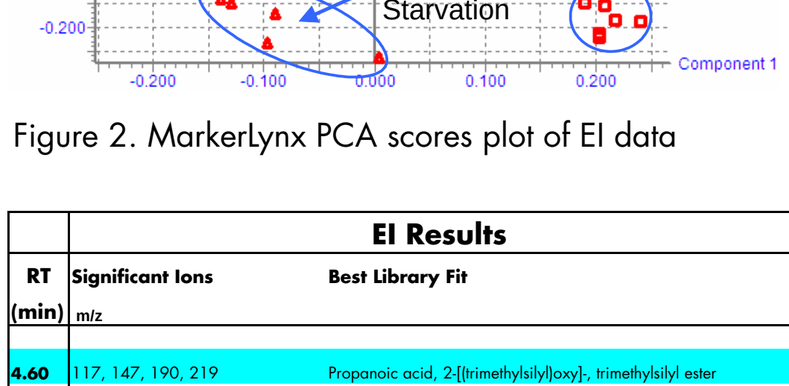

Figure 5. Representative TIC chromatograms from shoots with and without nitrogen starvation.

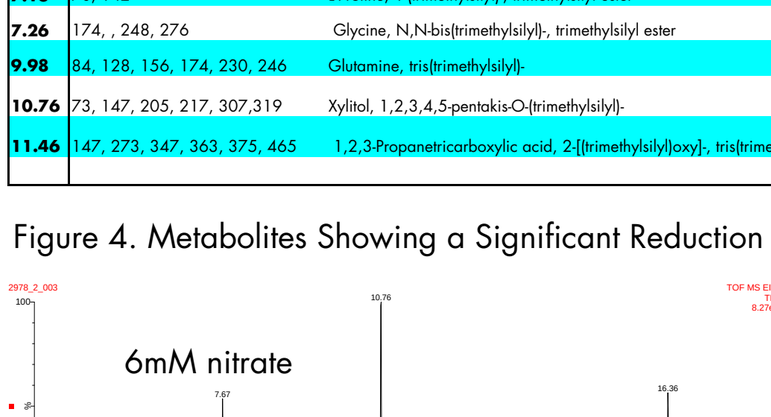

Figure 6. Representative trend plots for ions showing a significant decrease in shoots and roots after nitrogen

DISCUSSION

Clear separation was observed between the starved and non-starved root and shoot metabolite profiles with the root samples after 2 days starvation clustering closer to the 10 day starvation samples than the corresponding 2 day shoot samples which were closer to the non-starved samples. This corresponds to a more rapid depletion of the nitrogen pool in roots. The main discriminating compounds were found to be amino acids, sugars and organic acids although only the metabolites found to decrease significantly in the shoot samples are reported here (see Figure 4)

CONCLUSIONS

- MarkerLynx has been used to align and deconvolute the data prior to PCA analysis.
- PCA allowed clear discrimination between the different N starvation experiments (see Figures 2 and 3).
- The combination of EI with library searching and exact mass CI has aided in identification of the metabolites responsible for this discrimination.
- For shoots (leaves) a significant amount of this discrimination can be attributed to a reduction in the levels of some amino acids and organic acids (see Figure 4).

FUTHER WORK

- Further analysis of the data, including quantification of changes in the amount of known and unknown compounds will reveal more changes to metabolism as a result of N starvation.
- This information needs to be studied in combination with available global gene expression data. Changes in transcript or protein abundance may result from cascade regulatory processes which involve changes in metabolite content.
- On the other hand, changes of protein abundance in response to N stress will also change metabolite content.
- The global view of metabolite level, enzyme activities, protein and transcript abundance will in the future allow us to understand the adaptation process of plants to N limitation.

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