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THE EFFECT OF COLD SHOCK ON *A.THALIANA*: A PROTEOMICS STUDY FROM GEL SEPARATED ROSETTE LEAF HOMOGENATES USING LABEL FREE QUANTITATIVE LC-MS

Mark Ritchie(1), James Langridge (1), Keith Compson (1), Therese McKenna(1), Steffen Amme (2) Hans-Peter Mock (2).
(1)Waters MS Technology Centre, Manchester,M22 5PP, UK
(2) Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, D-06466 Gatersleben, Germany

OVERVIEW

- The effect of cold shock on rosette leaves from *Arabidopsis thaliana* is studied using a label free LC-MS technique.
- Rosette leaf protein extracts were separated by 1D SDS PAGE before being subjected to simultaneous quantitative and quantitative proteomic analysis.
- Responses in protein expression were observed by comparison to non cold shocked controls.

INTRODUCTION

- Plants deal with a wide range of stresses during their life cycle and have developed a variety of defence mechanisms in order to survive.
- Characterisation of various plant systems has revealed considerable overlap of responses when challenged with different stresses.
- Quantitative proteomics plays a role in determining protein based responses. Here we show the application of a label free LC-MS method for the assessment and characterisation of responses to cold shock in rosette leaves in *Arabidopsis thaliana*.

METHODS

Plant Growth and Harvesting

- 5 week old (2 weeks germination, 3 weeks growth) *Arabidopsis thaliana* (ecotype Col-o) plants were split into two groups; control and cold stress
- Cold stressed plants were kept at 6°C for one week while the control remained at 20°C
- Rosette leaves from half the plants in each group were harvested and directly frozen in liquid nitrogen
- The remaining plants were kept at 20°C for a further week before their rosette leaves were also harvested and frozen

Protein Extraction and Gel Separation

- Plant material was ground under liquid nitrogen then suspended in 10%TCA and 0.07% 2-mercaptoethanol in acetone (1)
- Precipitated material was washed in acetone then dried in a vacuum centrifuge.
- Proteins were solubilised in in 8M Urea, 2% CHAPS 30mM Tris-HCl pH 8.5
- 20ug samples from each condition were diluted in 1xDTE buffer, then loaded on to a 12.5% Acrylamide gel and run at a constant voltage of 175V for 1h.
- The gel was stained with colloidal Coomassie G250 (GelCode Blue Stain Reagent, Pierce 24592, Rockford, USA) for 1 h, washed with water for 5 min and stored in water
- Gel bands were excised as shown in fig 1, and proteins were extracted and digested manually (2).

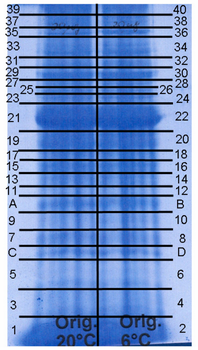

Figure 1. *Coomassie blue stained gel of rosette leaf protein extracts from the control; (left hand lane) and cold shocked experiments (right hand lane). Excised bands are shown and numbered. Bands labelled A-D were used to optimise the extraction procedure.*

LC-MS

- Liquid chromatography was carried out using a nanoACQUITY UPLC™ System (Waters, Milford, MA) with a 75um analytical column packed with BEH dC18 1.7µM stationary phase.
- A 90 minute gradient of 0-40% ACN containing 0.1% formic acid was used to separate peptides.
- The Q-Tof™ Premier (Waters, Manchester, UK) was set to acquire LC-MS data in an alternating normal and elevated collision energy mode as described in Bateman et al (3)
- Proteins were identified and relative quantification was carried out using Waters® Protein Expression System informatics (WPES)(Waters, Manchester, UK) as described previously (4)

RESULTS

- Cultivation of Arabidopsis plants under cold stress conditions leads to reduced growth, as shown in figure 1
- Other than differential growth rates, no obvious morphological differences were observed between the experimental groups.

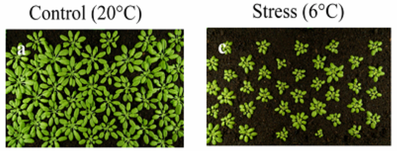

Figure 2 *- Differences in growth rate due to different temperature regimes.*

LC-MS Analysis

- Gel extracts analysed revealed moderate complexity in the bands
- Typically 10-20 proteins were identified per band depending on density of staining
- Peptides were identified across approx. 3 orders of intensity.

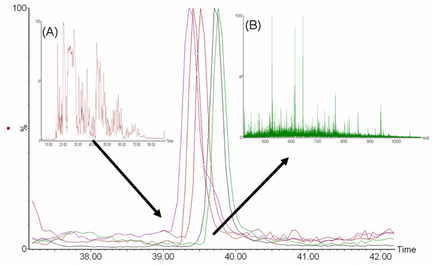

Figure 3. *Chromatogram from one gel extract. The main plot shows an overlaid mass chromatogram for 5 ions eluting between 39 and 40 mins. By use of HPLC these peptides can be partially separated, and each has a unique profile. This chromatographic profile is used in the matching of fragment ions to precursors. Sample complexity is illustrated chromatographically (inset A) and spectrally (inset B).*

Qualitative Proteomic Analysis

- Protein species present in each gel extract were identified using the Waters Protein Expression Informatics (ProteinLynx Global SERVER 2.2.5), searching against an *Arabidopsis thaliana* protein database obtained from the European Bioinformatics Institute (www.ebi.ac.uk).
- Results were filtered using the following criteria; Each protein had to contain at least one unique peptide at 100% confidence (i.e. that peptide could not be accounted for by any other protein in the database) and be present in 2/3 replicates.
- Over 230 different validated proteins were identified from all bands (see appendix). Some proteins spanned multiple bands.

Quantitative Proteomic Analysis

- Expression changes were measured on a protein basis, with all identified peptides contributing to the fold change of a particular protein (5).
- 20 proteins were identified to have clear regulation changes, but the fold change could not be measured. These are assigned as "unique" to the condition in which they were identified.

Acc No	Description	score	sample
544607.1	Catalase 3 SEN2	26.92	control
541933.1	Glycine rich RNA binding protein GRP7	42.24	control
538794.1	Arabidopsis thaliana genomic DNA chro	30.33	control
657055.1	Similar to 40S ribosomal protein SA R	29.77	cold
548649.1	Dehydrin ERD10	48.36	cold
548427.1	Plastid lipid associated protein PAP	74.62	cold
548261.1	Proteasome subunit alpha type 5 B	23.61	cold
547342.1	Expressed protein	24.19	cold
542534.1	Sulfite reductase	32.3	cold
541999.1	Protein kinase family protein	24.58	cold
534676.1	Encodes a cysteine synthase isomer	58.61	cold
532111.2	40S ribosomal protein SA 1	29.75	cold
529380.1	Glutamate 1 semialdehyde 2 1 aminomuta	49.13	cold
527199.1	40S ribosomal protein S20	76.13	cold
520209.1	Splice Isoform 1 of Proteasome subunit	30.88	cold
520177.1	Plastocyanin major isoform chloroplas	26.79	cold
519439.1	Coproporphyrinogen III oxidase chloro	28.32	cold
518644.1	Sulfate adenylyltransferase 1	28.27	cold
518377.2	Outer mitochondrial membrane protein p	42.82	cold
518094.1	DEAD box RNA helicase	26.46	cold

Figure 4. *Proteins identified as "unique" in one condition*

- Around 40 proteins were identified to have significant and quantifiable fold changes. These expression ratios ranged from a four fold down regulation, to a three fold up regulation under cold shock, Figure 5.

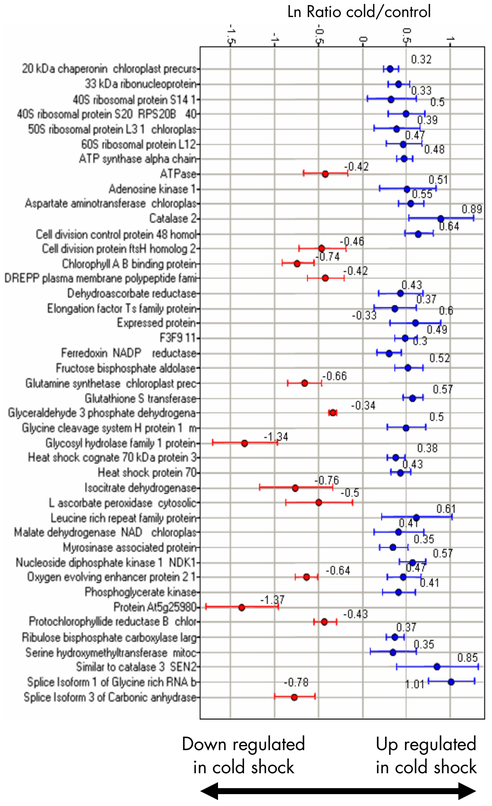

Figure 5. *Protein expression changes between control and cold shock experiments. Fold changes are expressed as Ln ratios with error bars. All fold changes are statistically significant to at least 97% confidence. For more explanation of the quantitation method see ref 5*

- Both cell division and energy production appear to be down regulated (ATP'ase, ftsH), correlating well with the decreased growth observed.
- Ribosomes appear to be up-regulated, indicating a change in protein production, as does catalase which may indicate that cold shock also brings oxidative stress.
- A number of observations (e.g Dehydrin) agree with a complementary study on the same samples, quantifying from staining in 2D Gels (publication in press)

CONCLUSION

- In this study we have used a combination of 1D PAGE separation and exact mass LC-MS to study cold shock induced changes in the protein complement of *A. thaliana*.
- This method has been able to confidently identify over 230 proteins and simultaneously highlight approximately 40 individual fold changes in this experiment.
- A further 20 proteins were to be unique in expression, either in the control or under conditions of cold shock.

REFERENCES

1. Damerval *et al.*1986,Electrophoresis ***7***, 52-54
2.MassPrep users guide, Waters, Manchester UK
3. Bateman R et al, J Am Soc Mass Spectrom. 2002 Jul;13(7):792-803
4. McKenna *et al.* A Novel Approach to Protein Identification: A Direct Comparison to Traditional Mass Spectrometric Techniques. 52nd meeting of the ASMS. Poster presentation (2004)
5. Silva et al Anal chem. 2005, *77*, 2187-2200.

APPENDIX

Accession	Description	Score	Bands	Accession	Description	Score	Bands	Accession	Description	Score	Bands
529270.1	ATP synthase beta chain	53.14	22,24	543135.1	Elongation factor EF-2	49.13	33,34	537428.1	Cell division protein ftsH homolog 2	26.98	27,28
532095.1	Phosphoglycerate kinase	77.41	17,18,19,20	546616.1	Glutamine synthetase	48.01	33,34	548649.1	Heat shock protein 17	26.81	31,32
538260.1	Chaperonin 60 beta subunit chloroplast	57.36	23,24,25,26	548676.1	Catalase 3 SEN2	26.92	130,131,132	532095.1	Phosphoglycerate kinase	77.41	17,18,19,20
538260.1	Cpn60 beta 1 of Cyclophorin 60 Cyc	57.36	23,24,25,26	548676.1	Similar to catalase 3 SEN2	48.08	130,131,132	529380.1	Glutamate 1 semialdehyde 2 1 aminomuta	49.13	5,6
529270.1	ATP synthase alpha chain	50.13	22,24,25,26	543675.1	60 kDa chaperonin protein	46.44	33,32	538260.1	Chaperonin 60 beta subunit	57.36	23,24,25,26
544292.1	Similar to subunit binding protein beta 1	144.85	25,26	532167.1	DREPP plasma membrane polypeptide	43.67	33,32,34	538676.1	Chaperonin factor Ts family protein	58.68	30,32,33,34
538260.1	Cpn60 beta 1 of Cyclophorin 60 Cyc	57.36	23,24,25,26	538260.1	Chaperonin 60 beta subunit chloroplast	57.36	23,24,25,26	543135.1	Elongation factor EF-2	49.13	33,34
529270.1	Cpn60 beta 1 of Cyclophorin 60 Cyc	57.36	23,24,25,26	543135.1	Elongation factor Ts family protein	48.01	33,32,34	537428.1	Cell division protein ftsH homolog 2	26.98	27,28
529270.1	Cpn60 beta 1 of Cyclophorin 60 Cyc	57.36	23,24,25,26	541125.1	Elongation factor Tu family protein	42.48	35,36	537428.1	Cell division protein ftsH homolog 2	26.98	27,28
529270.1	ATP synthase alpha chain	50.13	22,24,25,26	543135.1	Elongation factor Ts family protein	48.01	33,34	537428.1	Cell division protein ftsH homolog 2	26.98	27,28
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