PROTEOME ANALYSIS OF COLD STRESS RESPONSE IN *ARABIDOPSIS THALIANA*: A LABEL-FREE QUANTITATIVE PROTEOMIC STUDY

Waters

Mark Ritchie(1), James Langridge (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* has been studied using a label free exact mass LC-MS technique.
- Protein extracts were digested with trypsin, to enable a bottom-up proteomics approach
- Relative response in protein expression was observed by comparison to appropriate controls.

INTRODUCTION

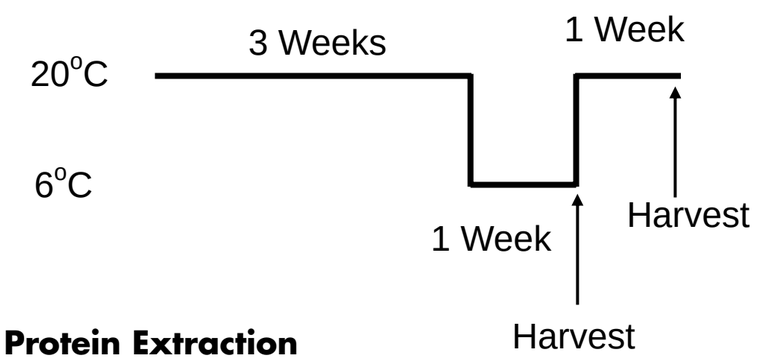
- Plants deal with a wide range of stresses during their life cycle and have developed a variety of defence mechanisms in order to survive.
- Characterisation of various plant systems has revealed considerable overlap of responses when challenged by different types of stress.
- Quantitative proteomics can play a crucial role in determining the protein level response to stress
- Here we present an LC-MS method used for the preliminary characterisation of responses to cold shock in rosette leaves from *Arabidopsis thaliana*.

Arabidopsis thaliana

METHODS

Plant Growth and Harvesting

- 5 week old *Arabidopsis thaliana* plants were split into two groups
- 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

- 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

Protein Digestion

- Protein extracts were diluted 1/10 and digested in 0.1% Rapigest® (Waters, Milford MA) with sequencing grade trypsin at 37 °C overnight
- Rapigest was precipitated out by acidification and centrifugation.
- Samples were diluted to a protein concentration of 0.5 µg/µl and analysed in triplicate.

LC-MS

- Liquid chromatography was carried out using a nanoACQUITY UPLC™ system (Waters, Milford, MA) with a 75µmx100 mm Atlantis dC18 3.0µM analytical column.
- Gradient; 0-40% acetoniltrile containing 0.1% formic acid over 120mins.
- The Q-Tof Premier (Waters, Manchester, UK) was set to acquire LC-MS data into two functions, alternating between low and elevated collision energy, as we have described previously [2]
- Proteins were identified and relative quantification was carried out using Waters Protein Expression System informatics.

RESULTS

- Cultivation of Arabidopsis plants under cold stress conditions, leads to reduced growth as illustrated in figure 1
- The delay in growth is also visible one week after re-shifting cold shock plants to control conditions (20 °C).

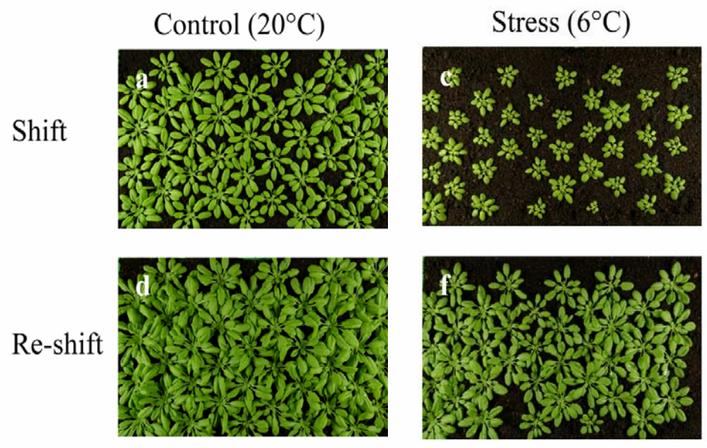


Fig 1 - Comparison of Arabidopsis under different temperature regimes; 20 °C and 6 °C.

Quantitative Proteomic Analysis

- Approximately 5,000 individual peptide components were automatically detected from each LC-MS data set.
- Relative fold changes were calculated by normalising the data and comparing the cold shock data to the control sample, and also the 1 week recovery to its appropriate control.
- The majority of peptides, and hence, proteins detected in the sample remained static under cold shock conditions, however, several clearly underwent expression changes.
- COR6, a cold response kinogen, was seen to be up regulated during cold shock (approx 3.5 times)
- After recovery at 20°C, expression levels of COR6 drop to normal levels, as seen in Figure 2

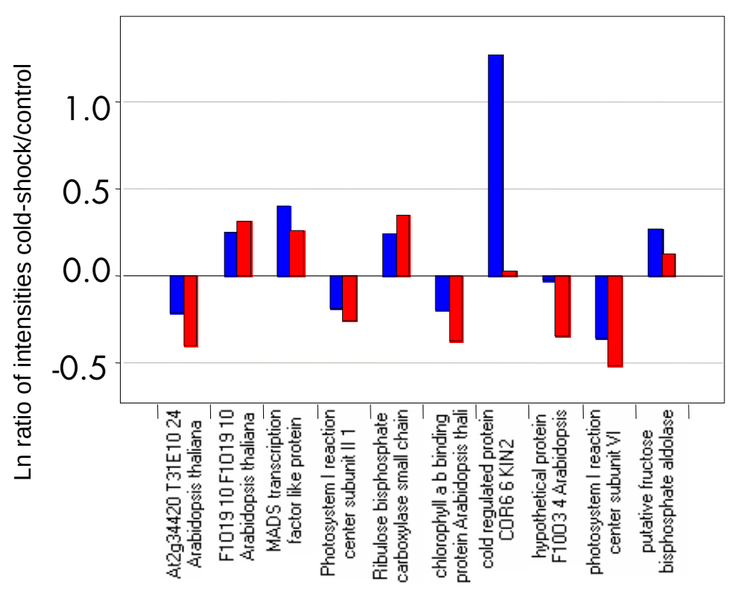


Fig 2– Expression change of Arabidopsis proteins under cold shock (blue bars) and after recovery (red bars). Ratios are expressed as a natural log against their relevant 20°C control.

Example Data - identification and regulation of COR6

- The identification of COR6 protein from 4 peptides using PLGS2.2 is shown below, along with one of the elevated-energy MS spectra, annotated with the amino acid sequence from the databank.
- These 4 peptides amounted to 71% coverage of this small protein

Peptide Matches	Coverage	mW	pI	Description	Average Mass Error
4	71.2121	6547.335	9.7824	stress responsive protein KIN2 stress induced	2.9009
4	71.2121	6547.335	9.7824	Stress induced KIN2 protein Cold induced COR6 6 p	2.9009
4	71.2121	6547.335	9.7824	cold regulated protein COR6 6 KIN2 Arabidopsis	2.9009
4	71.2121	6547.335	9.7824	cold regulated protein COR6 6 KIN2 Arabidopsis	2.9009
4	71.2121	6547.335	9.7824	cor6 6 Arabidopsis thaliana	2.9009
4	71.2121	6547.335	9.7824	cold regulated protein kin2 COR6 6 Arabidopsis	2.9009
4	71.2121	6547.335	9.7824	cold regulated protein COR6 6 KIN2 Arabidopsis	2.9009

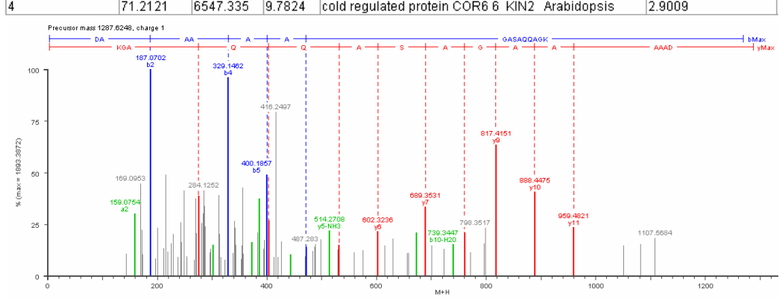


Fig 3 - Identification of COR6 from the exact mass LC-MS data

- Mass chromatograms for the $[M+2H]^{2+}$ ion of the tryptic peptide (with the best match against the COR6 sequence above) from the cold shock and control samples were plotted and the associated peak areas calculated.
- Figure 4 shows the LC-MS chromatograms, where the peak areas for the COR6 peptide are 3 times greater in the cold shock sample than in the control.
- The overall fold change is calculated taking into account the change in regulation of all the identified peptides, (n=4).

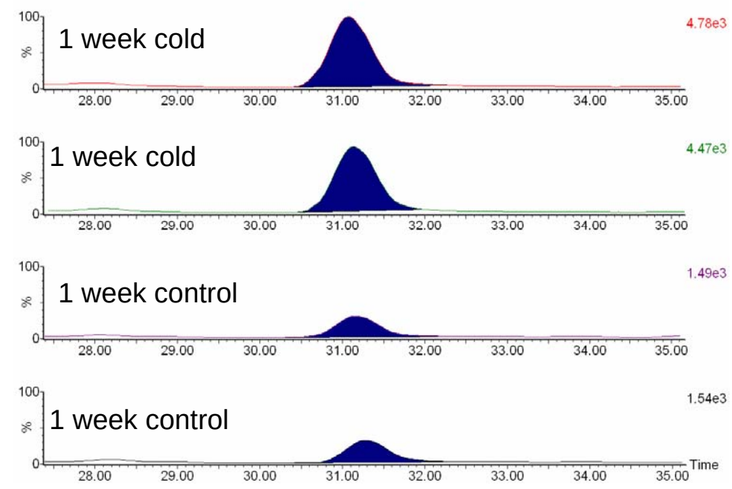


Fig 4 - Mass chromatograms for the $[M+2H]^{2+}$ ion from peptide DAAAAAGASAQQAGK (COR6). Integrated peak areas are displayed on the right hand side, allowing comparison of cold shock and the control samples.

Peptide level analysis

- Data was also quantitatively compared at the peptide level, using the exact mass retention time (EMRT) route as described by Silva et al [4].
- Peptides from RNA binding protein were observed to be around 2.7 times up regulated after cold shock compared to the control (Figure 5).
- This compares to a 2.3 fold up-regulation observed by 2D gel based analysis of the same sample.

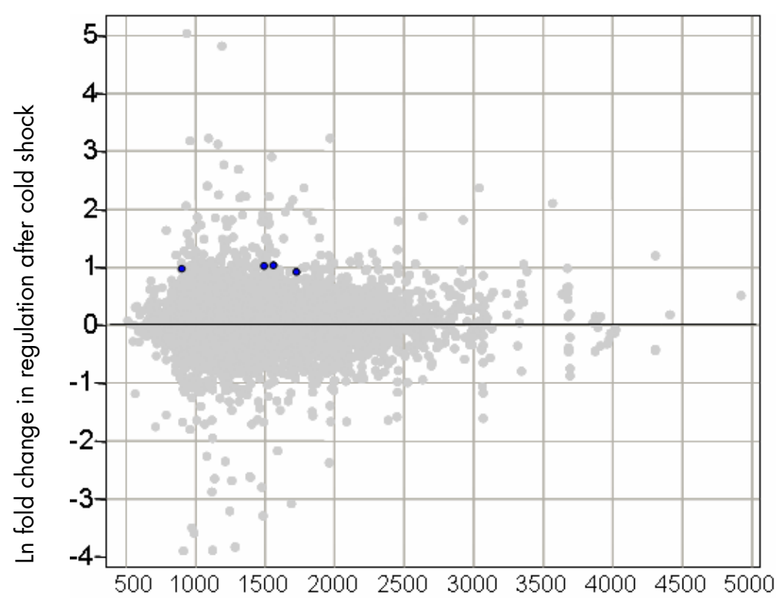


Fig 5– EMRT plot showing up regulation of four peptides from RNA binding protein after cold shock. The peptides high-lighted in blue all originate from the RNA binding protein.

- This RNA-binding protein (CP29) has been shown to be localized in chloroplasts and is a subunit of photosystem II. In maize, cold-resistant and sensitive lines differed in the phosphorylation of CP29. In the non-phosphorylating genotype, cold stress was followed by photo-inhibitory damage
- Peptides from Glycine Rich RNA Binding protein also showed up regulation of around 2.5 times, (data not shown) compared to the 2.7-3.7 up regulation observed in the gel based route.
- Previous studies [5] have shown that this Glycine rich RNA binding protein increases the freezing tolerance of *Arabidopsis*.

SUMMARY

- In this work we have used LC-MS to study protein expression changes in *Arabidopsis thaliana*.
- Under conditions of cold shock the majority of proteins identified remained un-changed, as might be expected.
- Changes in cold shock proteins were observed, notably in COR6, which underwent a 3.5 times up regulation after cold shock. These responses diminished after 1 week of recovery at 20°C.
- More subtle changes were characterised using the EMRT route, and revealed regulation changes in good agreement with gel based results
- Future studies will look at a comprehensive study of cold shock response in *Arabidopsis*, using a 1-D gel based separation in combination with an LC-MS strategy.

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