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Expression proteomics identifies biochemical adaptations and defense responses in transgenic plants with perturbed polyamine metabolism

Marina Franceschetti, Barry Perry, Benjamin Thompson, Colin Hanfrey, Anthony J. Michael*

Division of Food Safety Science, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK

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Abstract Soluble proteins from leaves of transgenic tobacco plants with perturbed polyamine metabolism, caused by *S*-adenosylmethionine decarboxylase overexpression, were analysed by comparative proteomics. A group of proteins was found to be increasingly repressed, in parallel with the degree of polyamine perturbation, in each of the three independent transgenic lines. These were identified as isoforms of chloroplast ribonucleoproteins, known to be involved in chloroplast mRNA stability, processing and translation. Another group of eight proteins strongly induced in the most metabolically perturbed line was identified as multiple, uncharacterised isoforms of the defense protein PR-1, a known marker for systemic acquired resistance.

Keywords: Proteomic; *S*-Adenosylmethionine decarboxylase; Polyamine; Chloroplast ribonucleoprotein; PR-1

1. Introduction

Polyamines are small organic polycations found in almost all cells and organisms [1] and are associated with core physiological processes such as chromatin formation, RNA function and protein synthesis [2]. In the model plant *Arabidopsis thaliana*, polyamines are required for normal growth and cell elongation [3]. Spermidine is synthesised from the precursor polyamine putrescine (1,4-diaminobutane) by the addition of an aminopropyl group derived from decarboxylated *S*-adenosylmethionine (dcAdoMet). Spermine is synthesised from spermidine by the symmetrical addition of a second aminopropyl group. Thus, the formation of dcAdoMet by AdoMet decarboxylase (AdoMetDC) is a key step in polyamine biosynthesis. Plant AdoMetDC mRNAs contain highly conserved overlapping upstream open reading frames [4] that translationally regulate AdoMetDC. Overexpression of a translationally deregulated Arabidopsis AdoMetDC in tobacco plants resulted in increased AdoMetDC activity, accumulation of dcAdoMet and perturbation of polyamine levels [5], which were decreased in response to the excess AdoMetDC activity. This resulted in stunted growth, wrinkled leaves and inhibition of cell expansion [5].

To obtain insight into how the transgenic plants adapt to AdoMetDC overexpression and polyamine disruption, an expression proteomics analysis was performed to assess changes to the transgenic leaf proteome. Proteins were separated by 2D gel electrophoresis and identified by mass spectrometry. Three independent transgenic tobacco lines overexpressing AdoMetDC at different levels were examined. Only one set of proteins was found to be changed in all three lines, with a decrease in abundance, and these corresponded to isoforms of chloroplast ribonucleoproteins. This suggests that mRNA translation in chloroplasts might be particularly affected by polyamine perturbation. In the transgenic line with the highest level of AdoMetDC overexpression, a group of proteins accumulated that corresponded to eight uncharacterised isoforms of the PR-1 defense protein, a marker for systemically acquired resistance (SAR).

2. Materials and methods

2.1. Preparation of leaf protein extracts

Young leaves from control null segregant (syngenic) and transgenic plants were harvested simultaneously and immediately flash frozen in liquid nitrogen and ground to a fine powder. The powder was mixed immediately with 2 volumes of 25% w/v TCA in 50 ml polyallomer oakridge tubes, to precipitate protein over a period of more than 60 min on ice. After centrifugation, the pellets were drained and washed and centrifuged twice with acetone, followed by a final diethylether step, to allow the pelleted material to be air dried before holding at −20 °C.

To obtain a protein extract, weighed samples (~30 mg) of the dried leaf powder in 1.5 ml microfuge tubes were hydrated with 1.3 ml, pH 7.9, liquid phenol (Sigma–Aldrich Product #P4557) and 13 μl β-mercaptoethanol (98% v/v). Samples were then allowed to extract, with intermittent mixing, over 24 h at 20 °C. Liquid extracts were recovered by centrifugation. From this extract, samples were back extracted with a solution of 50 mM Tris–HCl (pH 8.0)/1% sodium lauryl sulfate (Tris/SDS).

By this procedure, the phenol phase was cleaned and reduced in volume, enabling proteins to be precipitated out from the phenol by addition of >5 vol acetone (1.3 ml) and holding on ice for >2 h, before recovery of the precipitated protein by centrifugation at 6 °C for 45 min at 20 000 × *g*. This pelleted material was again washed by resuspension and centrifugation under the same conditions, 2× with 1.3 ml

* Corresponding author. Fax: +44-1603-255288.
E-mail address: tony.michael@bbsrc.ac.uk (A.J. Michael).

Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; MALDI-ToF MS, matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry; Q-ToF MS, quadrupole time-of-flight mass spectrometry

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acetone, followed by 1× with 1.0 ml diethylether. The pellet was carefully broken up prior to air drying at 20 °C.

Appropriate samples of this pelleted material were finally prepared for isoelectric focussing by extraction for 24 h at 4–10 °C with a rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 1% IPG buffer, pH 4–7 (Amersham Biosciences). Insoluble material was removed by centrifugation at 6 °C for 45 min at $20\,000 \times g$ and from the supernatant 400 μl was used to rehydrate each 18 cm Immobiline DryStrip, pH 4–7 (Amersham Biosciences), with the addition of 1 μl bromophenol blue marker solution and 6.5 μl of dithiothreitol (DTT) in water (200 mg/500 μl) immediately before use.

2.2. Electrophoresis

For electrophoresis and the immediately preceeding step, all solutions were made from electrophoresis grade reagents. Rehydration of the Immobiline DryStrips was performed overnight at 18 °C in a pHaser from Genomic Solutions (Huntingdon, UK). The strips were also covered with 'plusone' DryStrip cover fluid from Pharmacia Biotech (Amersham Biosciences) to prevent dessication and prior to focussing wetted paper electrode wicks were placed at each end of the strips to ensure contact between the focussing gel matrix and the electrode wire. Isoelectric focussing was performed at 18 °C on a Genomic Solutions chiller unit using a Genomic Solutions Investigator 5000 Programmable Power Supply, which delivered a total of 85 kVh over a period of 23–24 h. Immobiline DryStrips were then removed and placed individually into 5 ml polystyrene disposable serological pipettes (Corning Inc, Corning, USA) and stored at −70 °C.

For the second dimension SDS–polyacrylamide gel electrophoresis (PAGE), separation DryStrips were thawed and rinsed with water to remove residual cover fluid before placing in a 45 μm filtered solution of 7 M urea, 2 M thiourea and 2% CHAPS with bromophenol blue marker and containing DTT (400 mg/50 ml). After 40 min gentle shaking on a rotating platform, the strips were then transferred into a similar solution without DTT, which contained iodoacetamide (1.25 g/50 ml) and again shaken for 40 min. Strips were then loaded onto the top of homemade 1 mm thick Duracryl gels (Genomic Solutions, Ann Arbor, USA). The Duracryl stock solution contains 30% acrylamide and 0.65% *N*,*N*-methylenebisacrylamide and the gels were made in a multiple forming cassette using a Tris–HCl/SDS buffer polymerised with ammonium persulfate and TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine). The second dimension upper tank buffer was 0.2 M Tris, 0.2 M Tricine and 0.4% SDS and the lower tank buffer was 25 mM Tris acetate.

After electrophoresis, gels were fixed overnight in 400 ml of 40% methanol and 10% acetic acid prior to staining overnight in 330 ml of SYPRO Ruby stain (BioRad Laboratories, Hercules, USA). Post-staining was in a solution of 10% methanol, 6% acetic acid prior to fluorescence imaging using a Perkin–Elmer ProEXPRESS Imaging System (Perkin–Elmer, Cambridge, UK). Gel images were analysed using Proteomeweaver® software (Definiens AG, Munich, Germany). The Matrix-Assisted Laser Desorption/Ionisation Time of Flight (MALDI-ToF) mass spectrometric analysis of peptides was performed with a Reflex III MALDI-ToF MS (Bruker Daltonics, Coventry, UK) and the Quadrupole Time of Flight (Q-ToF) MS analysis was performed with an electrospray-ToF mass spectrometer (Micromass UK Ltd., Manchester, UK). Protein database serching was carried out with an in-house copy of the search tool Mascot (www.matrixscience.com). Mass spectrometric analysis was performed at the joint John Innes Centre-Institute of Food Research proteomics facility by Andrew Bottrill and Michael Naldrett.

3. Results and discussion

Young leaves, where polyamine metabolism is more active, were harvested from transgenic plants derived from the self-fertilisation of three independent transgenic lines of common tobacco (*Nicotiana tabacum*), overexpressing the *A. thaliana* AdoMetDC1 cDNA [5], and from their corresponding null segregants (syngenic control plants). Each pool of transgenic and syngenic leaf material analysed for line 754 was derived from 16 different plants of the T_1 generation. The T_2 generation transgenic and syngenic pools of line 850 were derived from 245 and 155 plants, respectively, and for line 756 from 211 and 144 plants, respectively. For each line, the transgenic material was a mix of homozygous and heterozygous plants. The three transgenic lines overexpressed AdoMetDC in the order: 756>754>850, which also reflected the order of metabolic and growth perturbation [5]. To identify significant changes to the leaf proteome caused by AdoMetDC overexpression, soluble proteins were separated by 2D PAGE and the transgenic and syngenic samples compared for each line. We focused on two groups of proteins (Fig. 1). Group 1 proteins were notable because they were repressed in all the transgenic lines and group 2 proteins were markedly induced in line 756. The 756 line shown in Fig. 1 was the most biochemically and developmentally affected line with a relatively large number of protein spot changes seen only in this line.

Protein spots were picked, trypsin digested and analysed by MALDI-ToF MS. The group 1 proteins were putatively identified on the basis of their peptide mass fingerprints as nuclear-encoded chloroplast ribonucleoproteins (see Table 1). It has been proposed that the tobacco chloroplast ribonucleoproteins (cpRNPs) act as stabilising factors for non-ribosome-bound mRNAs in the chloroplast stroma [6]. In wood tobacco (*N. sylvestris*), there are five characterised cpRNPs, possessing a conserved C-terminal region: cp28, cp29A, cp29B, cp31 and cp33 [6]. In contrast, cp29A and cp29B show 68% amino acid identity across the entire protein. In each of the AdoMetDC-overexpressing lines, the group1 spot 1 corresponded to *N. sylvestris* cp29A and spots 2 and 3 to *N. sylvestris* cp29B (Fig. 2). The MALDI-ToF MS analysis provided the highest match for spots 4 and 5 to the curly leaf tobacco (*N. plumbaginofolia*) cp31 and the second place match was for the *N. sylvestris* cp29B. To clarify the identification of spots 2–5, peptides from the spots were sequenced by Q-ToF MS. For both spots 2 and 3, the same two peptide sequences were obtained: (1) AAEQQFNGYE(L/I)DGR, identical to a sequence in cp29B from *N. sylvestris* (with one mismatch in position three to the same peptide sequence in cp29A) and (2) VA(L/I)SDFDQ(L/I)EDDVEVSEQPR, which differs in the two underlined amino acids from the *N. sylvestris* cp29B, whereas the same region in cp29A differs by another two additional positions and also includes a four amino acid insertion (Fig. 3). For spots 4 and 5, the same three peptide sequences were obtained: (1) DSAA(L/I)AG(L/I)FER, identical only to cp29B of *N. sylvestris*, (2) AGNVEMVEV(L/I)YDK, present in both cp29A and cp29B of *N. sylvestris*, and (3) V(L/I)(L/I)SDFDQ(L/I)EDDVEVAEQPR present in cp29B of *N. sylvestris* with two changes to the amino acids underlined (Fig. 3); this region is not conserved in the cp31 of *N. sylvestris*.

It is clear from the Q-ToF MS analysis that spots 2–5 most closely resemble the *N. sylvestris* cp29B protein. Peptide (1) of spots 2 and 3 and peptide (3) of spots 4 and 5 are exactly the same region of the *N. sylvestris* cp29B (amino acids 63–82), but they both have two amino acids that differ from the *N. sylvestris* cp29B and they both differ from each other by two amino acids. There are two probable explanations for the differences in amino acid sequence and peptide mass fingerprints between spots 2 and 3 on the one hand and spots 4 and 5 on the other. Common tobacco (*N. tabacum*) is an allotetraploid with two diploid parental genomes: *N. sylvestris* and *N. tomentosiformis* [7]. This is an explanation for differences between the tobacco spots analysed here and *N. sylvestris* cp29B, i.e., the protein spots come from genes encoded by the

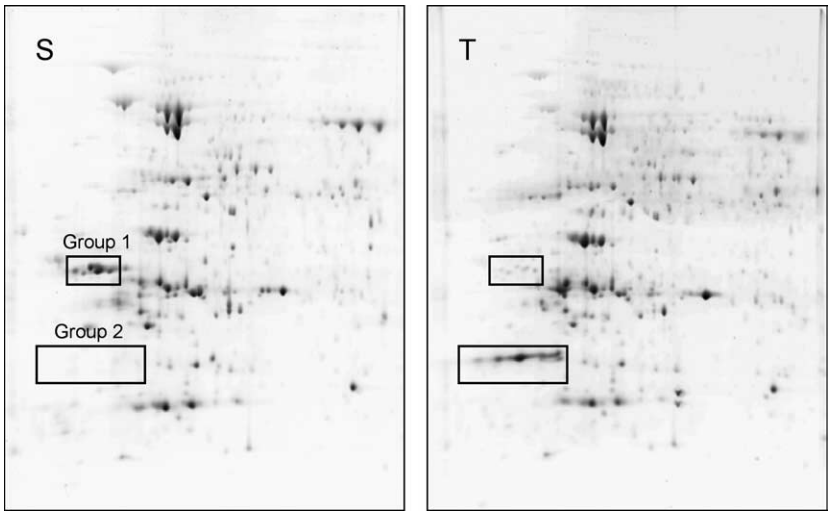

Fig. 1. 2D gels (pH 4–7) of soluble proteins extracted from young leaves of control (syngenic, S) and transgenic (T) tobacco plants of line 756. Leaves were pooled from 144 syngenic and 211 transgenic plants and gels were stained with the fluorescent stain SYPRO Ruby.

Table 1
Relative abundance of chloroplast RNPs in transgenic plants

Chloroplast RNP	Transgenic/control protein ratio		
	Line 756	Line 754	Line 850
CP29BI (spot 2)	0.06	0.27	0.67
CP29BI (spot 3)	0.08	0.34	0.50
CPB29II (spot 4)	0.06	0.30	0.93
CP29BII (spot 5)	0.16	0.33	0.49
CP29A (spot 1)	0.20	0.55	0.94

2D gel spot intensity ratio between transgenic and corresponding syngenic plants for different chloroplast RNP isoforms. Results are means of three gels each for 754 and 850 and two gels for 756.

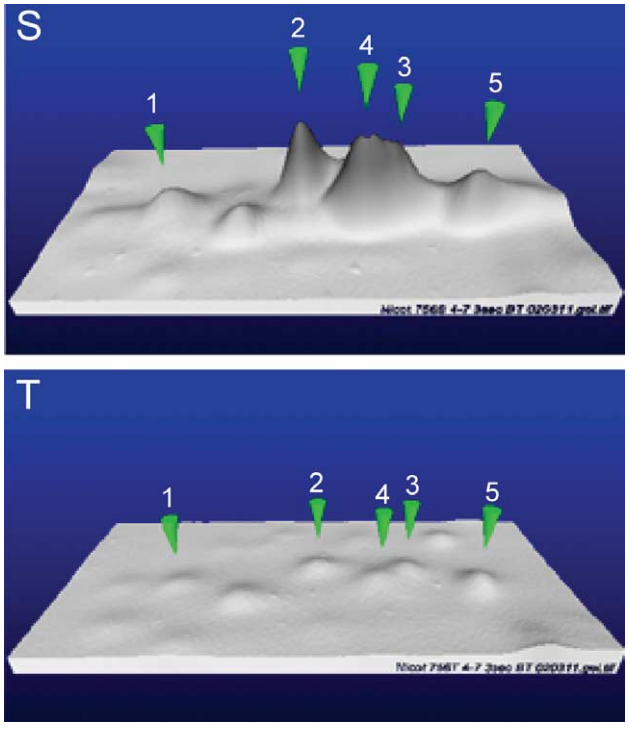

Fig. 2. 3D representation of the sections of the 2D gels corresponding to the group 1 box of Fig. 1, visualised by the Proteomeweaver® software. (S, syngenic control and T, transgenic).

MASSSVSSLQFLFVTPQTPSSLKPNSTLSFFSLPS
SSLNLSLSSSSTGLCSIKPFESSFSTR**VALSGFDQ**
spots 2&3
LEDDVEVAEQPRFSEDLKLFVGNLPFSV**DSAALAG**
pep.2; spots 4&5 pep.3 spots 4&5
LFER|**AGNVEMVEVIYDK**LTGRSRGFGFVTMSTKE
pep.1| spots 4&5 pep.2
EVE**AAEQQFNGYEIDGR**AIRVNAGPAPAKRENSSF
spots 2&3 pep.1
GGGRGGNSSYGGGRDGNSSFGGARGGRSVDSSNRV
YVGNLSWGVDDLALKELFSEQGNVVDAKVVYDRDS
GRSRGFGFVTYSSSKEVNDAIDSLNGVDLDGRSIR
VSAAEERPRRQF

Fig. 3. Alignment of group 1 peptide sequences identified by Q-ToF MS with the *N. sylvestris* cp29B amino acid sequence (Accession No. Q08937). Sequences in bold represent the position of the tryptic peptides sequenced by Q-ToF MS, with the corresponding protein spot and tryptic peptide indicated underneath.

N. tomenosiformis parental genome. However, the differences between spots 2 and 3 and then spots 4 and 5 are probably due to the presence of additional gene family members of cp29B from either parental genome. It is therefore likely that spots 2 and 3 represent two isoforms of one cp29B family member and spots 4 and 5 represent two isoforms of another cp29B gene. The reason for two spots for each cp29B gene-specific member is likely to be post-translational modification, alternative splicing or proteolysis.

The eight group 2 proteins (Fig. 1) could not be unequivocally identified by MALDI-ToF MS and only one peptide sequence for each protein spot could be determined by Q-ToF MS. Each spot furnished the same sequence of QPSQQDY(L/I)DAHNTAR and this sequence is present in each of the pathogenesis related protein PR-1a, PR-1b and PR-1c isoforms from common tobacco except for N instead of P in position two, which may again reflect the allotetraploid nature of tobacco. An additional peptide sequence was obtained for spot 3 (the most abundant spot): VQCNNGGYVVSCNYDPPGNYR corresponding exactly to the PR-1a precursor. It is possible that all eight group 2 proteins are the PR-1a protein. The nature of the differences between the different isoforms of the

chloroplast ribonucleoproteins (cpRNPs) and also the PR-1 protein isoforms detected by 2D PAGE in this study remains unsolved. As only the 756 line exhibited the accumulation of the PR-1 isoforms, the correlation between polyamine disruption and PR-1 expression should be viewed as tentative.

The tobacco cpRNPs exist as complexes with ribosome-free chloroplast-encoded mRNAs in the stroma and are thought to stabilise the nonribosome-bound mRNAs, contributing to RNA stability and processing [6]. Dissociation of the cpRNPs from the mRNA is required for ribosome binding and translation of the mRNA. What might be the reason for lower cpRNP levels in plants with lower polyamine levels? Most cellular polyamines exist as polyamine-RNA complexes [8] and polyamines are required for efficient translation of mRNAs. The AdoMetDC-overexpressing plants have greatly reduced levels of putrescine and a substantial reduction in spermine content. In *Escherichia coli*, polyamines stimulate protein synthesis from some mRNAs through structural changes in the Shine Dalgano sequence and the AUG initiation codon [9]. The prokaryotic-like translational machinery in chloroplasts and the Shine Dalgano and AUG regions of the chloroplast-encoded mRNAs might be particularly sensitive to disruption of polyamine levels. Therefore, the observed depletion of cpRNPs might facilitate translation of chloroplast-encoded mRNAs in response to sub-optimal polyamine levels.

Identification of accumulated multiple isoforms of the defense protein PR-1 is indicative of a SAR response to AdoMetDC overexpression. The fitness cost associated with such a constitutive expression of defense responses can lead to growth inhibition [10], possibly due to resource allocation from growth to defense and the consequent metabolic burden. Part of the growth inhibition observed with line 756 could be explained by the constitutive SAR response. It is not evident as to why PR-1 is induced in the transgenic plants but polyamines are known to act as free radical scavengers [11]. A reduction in polyamine levels (and free radical scavenging) could lead to increased lipid peroxidation, which is associated with induction of PR-1 expression [12]. Alternatively, changes to chloroplast physiology could bring about changes in cellular redox environment, leading to activation of the transcription factor NPR1, a key mediator of PR-1 gene expression [13].

In conclusion, expression proteomic analysis of AdoMetDC-overexpressing plants has identified an impact on chloroplast physiology and in the case of high level overexpression, a constitutive SAR response is observed.

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References

[1] Cohen, S. (1998) A Guide to the Polyamines. Oxford University Press, Oxford. pp. 1–543.
[2] Igarashi, K. and Kashiwagi, K. (2000) Biochem. Biophys. Res. Commun. 271, 559–564.
[3] Hanzawa, Y., Takahashi, T., Michael, A.J., Burtin, D., Long, D., Pineiro, M., Coupland, G. and Komeda, Y. (2000) EMBO J. 19, 4248–4256.
[4] Franceschetti, M., Hanfrey, C., Scaramagli, S., Torrigiani, P., Bagni, N., Burtin, D. and Michael, A.J. (2001) Biochem. J. 353, 403–409.
[5] Hanfrey, C., Franceschetti, M., Mayer, M.J., Illingworth, C. and Michael, A.J. (2002) J. Biol. Chem. 277, 44131–44139.
[6] Nakamura, T., Ohta, M., Sugiura, M. and Sugita, M. (2001) J. Biol. Chem. 276, 147–152.
[7] Gregor, W., Mette, M.F., Staginnus, C., Matzke, M.A. and Matzke, A.J. (2004) Plant Physiol. 134, 1191–1199.
[8] Watanabe, S.-I., Kusama-Eguchi, K., Kobayashi, H. and Igarashi, K. (1991) J. Biol. Chem. 266, 20803–20809.
[9] Yoshida, M., Meksuriyen, D., Kashiwagi, K., Kawai, G. and Igarashi, K. (1999) J. Biol. Chem. 274, 22723–22728.
[10] Yang, S. and Hua, J. (2004) Plant Cell 16, 1060–1071.
[11] Ha, H.C., Sirisoma, N.S., Kuppusamy, P., Zweier, J.L., Woster, P.M. and Casero, R.A. (1998) Proc. Natl. Acad. Sci. USA 95, 11140–11145.
[12] Anderson, M.D., Chen, Z. and Klessig, D.F. (1998) Phytochemistry 47, 555–566.
[13] Mou, Z., Fan, W. and Dong, X. (2003) Cell 113, 935–944.