Identification of proteins in leaf tissues of white clover using MALDI-TOF mass spectrometry

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Abstract

Protein extracts, made to leaves harvested from the stolons of the pasture legume white clover (*Trifolium repens* L.) at two developmental stages (newly initiated; onset of senescence) were purified further using reverse-phase and ion-exchange chromatography. Fractions enriched with the ethylene biosynthetic enzyme 1-aminocyclopropane-1-carboxylate (ACC) oxidase were selected for each stage and the final, partially purified fraction was subjected to two-dimensional gel electrophoresis (2DE). Antibodies raised against a recombinant ACC oxidase (ACO) from white clover (antiTR-ACO2) recognised a series of spots of differing pI suggesting that ACO undergoes post-translational modifications. Further, the pattern differed between the ACO proteins partially purified from newly initiated leaves with leaves at the onset of senescence suggesting that the environmental and developmental cues that operate in each tissue influences the type and/or degree of post-translational modifications of the ACO protein. MALDI-TOF mass spectrometry was used to identify protein spots associated with the ACO proteins. Protein with identities to an ACO isoform from *Oryza sativa,* and a phosphoribulokinase from *Arabidopsis thaliana* were identified in the 2DE separations from newly initiated leaves, while an isoflavone reductase from *Medicago sativa* was identified in the 2DE separation of the senescent leaf extract.

Additional key words: ACC oxidase, ethylene synthesis, isoflavone reductase, leaf development, phosphoribulokinase.

Introduction

The plant hormone ethylene is synthesised in plants *via* two committed enzyme steps from *S*-adenosyl methionine (SAM). The enzymes involved are 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) that converts SAM to ACC and 5´-methylthioadenosine (which is recycled back to methionine) and then ACC oxidase (ACO) that catalyses the conversion of ACC and O_2 to ethylene (and HCN and CO_2) (Kende 1993). In all plants studied thus far, ACS is represented by larger (0 - 12 member) gene families, with ten members characterised in *Arabidopsis thaliana,* comprising eight functional genes, one inactive protein and one pseudogene (Yamagami *et al.* 2003). Through evidence of the transcriptional regulation of the gene family and post-translational modifications of the protein products, ACS is widely considered to be the rate-determining step in the ethylene biosynthetic pathway, and regulates a number of developmental pathways (Wang *et al.* 2002, Lenochova *et al.* 2009). In the case of ACO, smaller gene families (3 - 5 members) have been identified but there is some evidence of transcriptional regulation of these gene families, including white clover (Hunter *et al.* 1999, Chen and McManus 2006). However, given the importance of ethylene in controlling many aspects of plant growth and development, it is probable that many tiers of control must operate to regulate both enzymes in the pathway to control the biosynthesis of the hormone.

In terms of post-translational modifications of the enzymes in the ethylene biosynthetic pathway, there is good evidence from *A. thaliana* that ACS undergoes phosphorylation which, indirectly through interaction with the ETO1 protein, regulates activity (Tatsuki and

Received 8 June 2009, *accepted* 15 March 2010.

Abbreviations: 2DE - two-dimensional gel electrophoresis (SDS-PAGE); ACC - 1-aminocyclopropane-1-carboxylate; ACO - ACC oxidase; IFR - isoflavone reductase; MALDI-TOF - matrix assisted laser desorption ionisation-time of flight; NI - new initiated; PRK- phosphoribulokinase; SE - senescent.

Acknowledgements: This research was funded from an Enterprise PhD scholarship to Z.D. supported by the Tertiary Education Commission of New Zealand and AgResearch Limited, and from the New Zealand Foundation for Research Science and Technology MeriNet programme C10X0404.

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Mori 2001, Wang *et al.* 2002, Kim *et al.* 2003, Liu and Zhang 2004). In addition to phosphorylation, there are a myriad of other post-translational modifications that occur to proteins (*e.g.* *N*- or *O*-linked glycosylation, acetylation, myristilation) that can also influence enzyme activity either directly or indirectly (Jensen 2004, Kwon *et al.* 2006).

In contrast to ACS, no evidence for post-translational modifications of ACO has been reported. While not widely considered rate-limiting in the ethylene biosynthetic pathway, it may be that such levels of control will operate. In this paper, as a prelude to studying post-translational modification of ACO, two-dimensional SDS-PAGE (2DE) and Western analysis is used to examine the fingerprint of the enzyme partially purified from developing leaf tissue of the pasture legume, white clover. The technique of 2DE has been used successfully to resolve complex mixtures of proteins (Mahmood *et al.* 2009) and in this study its use does provide the first evidence that ACO may undergo post-translational modification. Further, the degree of these modifications may be imparted by different developmental cues operating in each tissue. As well, MALDI-TOF mass spectrometry has been used to identify, in addition to ACO, two other metabolically-significant proteins in the leaf extracts of white clover.

Materials and methods

Plant growth conditions: Plants of white clover (*Trifolium repens* L.) cv. Grasslands Challenge, genotype 10F (AgResearch Grasslands, Palmerston North, New Zealand) were grown in a greenhouse at a minimum/maximum temperature of 15/25 °C and irrigated automatically twice daily. To initiate single stolons, apical cuttings comprising the apex and two or three nodes were excised from the stock plants, and all leaves, except those covering the terminal bud and the first fully unfolded leaf, were removed. The cuttings were placed in bark/nutrient potting mix and after establishment of roots (usually 4 weeks), the most homogeneous cuttings were transferred into trays containing fresh bark/nutrient mix with six plants per tray. Single stolons were trained out of the tray over a dry polythene surface to ensure that nodal roots did not develop. All axillary shoots and buds, flowers and unhealthy leaves were removed routinely (at 10-d intervals) to maintain single stolons attached to a basal root. Stolons were allowed to grow until they provided a consistent pattern of leaf developmental stages, ranging from initiation through to senescence (usually 3 months) (Hunter *et al.* 1999).

Hydrophobic and ion-exchange column chromatography: ACC oxidase was extracted from white clover leaf tissues as described in Gong and McManus (2000). Two stages of leaf development were followed: the newly initiated (NI) leaves and the senescent leaves (SE) at the point when the chlorophyll content just starts to decrease (Hunter *et al.* 1999, Yoo *et al.* 2003). To extract the protein, frozen leaf tissue was ground with liquid nitrogen in a pre-cooled mortar and pestle. Extraction buffer (100 mM Tris-HCl, pH 7.5, 10 % glycerol, 30 mM sodium ascorbate, 2 mM dithiotreitol, DTT, and 10 μM 1,10-phenanthroline (PA) was then added in a 3:1 (v/m) ratio to the ground frozen powder, the suspension incubated on ice with gentle stirring for 45 min, and then filtered through double layers of *Miracloth* (*Calbiochem-Novabiochem Corporation*, La Jolla, CA, USA). After centrifugation at 26 000 *g* for 20 min at 4 °C, the resulting supernatant (crude extract) was then adjusted to 40 % (m/v) with saturated (226 g dm^{-3} at 0 °C) ammonium sulfate. The mixture was incubated on ice with stirring for 40 min and then centrifuged again at 26 000 *g* for 20 min at 4 °C. The supernatant was transferred to a fresh pre-cooled beaker and adjusted to 80 % (m/v) with saturated ammonium sulfate. After 1 h incubation on ice with stirring, the protein was pelleted by centrifugation at 26 000 *g* for 30 min at 4 °C. The supernatant was discarded and the protein pellet redissolved in a minimal volume of pre-cooled resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 % glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10 μM PA). The protein solution was then desalted against the resuspension buffer using *Sephadex G-25*, and fractions containing protein were pooled and concentrated using *Amicon Centriprep-10* centrifugal concentrators (10 kDa cut-off, *NANOSEP*™, *Pall Filtron Corporation*, Ann Arbor, MI, USA) at 3 000 *g* at 4 °C.

For hydrophobic column chromatography, proteins after *Sephadex G-25* column chromatography were loaded onto a phenyl sepharose *HiLoad 26/10* column equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, containing 5.0 % glycerol, 30 mM sodium ascorbate, 2 mM DTT, 10 μM PA and 2.0 M ammonium sulfate). Total protein extract (400 - 600 mg), adjusted to 1.0 M ammonium sulfate, was loaded in a volume of 8 - 10 cm^3, and bound proteins were eluted in a decreasing gradient from 100 % buffer A: 0 % buffer B (buffer A without the ammonium sulfate) to 0 % buffer A: 100 % buffer B over a volume of 450 cm^3 at a flow rate of 5.0 cm^3 min^{-1}. Each fraction (16.0 cm^3) was assayed for protein content using the Bradford (1976) method. ACC oxidase was identified with Western analysis using the anti-TR-ACO2 antibody (Hunter *et al.* 1999). Fractions of interest were pooled and concentrated using a *Centriprep-10* centrifugal concentrator. For ion-exchange chromatography, a *Mono Q* prepacked *HR 5/5* strong anion exchange column (*Pharmacia Biotech*, Uppsala, Sweden) was pre-equilibrated with buffer A. Protein samples were loaded at flow rate of 0.5 cm^3 min^{-1} onto the column and bound proteins were eluted using a linear gradient of 100 % buffer A : 0 % buffer B (buffer A, pH 7.5, with 1.0 M NaCl) to 0 % buffer A : 100 % buffer B over a volume of 45 cm^3 at a

flow rate of 0.5 cm^3 min^{-1}. Fractions (1.2 cm^3) were assayed for protein content using the Bradford method and subjected to SDS-PAGE and Western analysis using the anti-TR-ACO2 antibody. Fractions of interest were pooled, concentrated and used for further experiments.

Two-dimensional SDS-PAGE (2DE) and Western analysis: To prepare samples for 2DE, the appropriate amount of protein sample was mixed with at least 3 volumes of ice-cold acetone at -20 °C for 3 h, the precipitated proteins were collected by centrifugation at 35 300 *g* for 12 min at 4 °C, and any residual acetone removed by air-drying. Prior to IPG strip rehydration, the protein pellet obtained was resuspended with rehydration buffer (8 M urea, 2 % 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfanate (CHAPS), 0.001 % bromophenol blue) to solubilize and denature proteins. Then the appropriate amount of IPG buffer was added to give a final concentration of *ca.* 0.5 % (m/v) of protein. *Immobiline DryStrips* (*Amersham-Pharmacia Biotech*; 70 mm linear, pH 3 - 10, or 180 mm linear, pH 4 - 7) were used and the samples applied as per the manufacturer's instructions using the *IPGphor* (*Amersham-Pharmacia Biotech*). Isoelectric focusing (IEF) was carried out automatically according to the programmed settings until *ca.* 45 000 V h was reached, and at the conclusion of IEF, the IPG gel strips were either stored between two sheets of plastic film at -80 °C or immediately equilibrated using the method of Görg *et al.* (1987).

For equilibration, gel strips were incubated on a rocking table for 15 min at room temperature in equilibration solution I (0.04 M Tris-HCl, pH 6.8, containing 6 M urea, 25 % glycerol, 2 % SDS and 0.01 % bromophenol blue, containing 10 mg cm^{-3} dithiotreitol, DTT), followed by 15 min in equilibration solution II (0.04 M Tris-HCl, pH 6.8, containing 6 M urea, 25 % glycerol, 2 % SDS and 0.01 % bromophenol blue, containing 2.5 % of iodoacetamide). The second dimension (SDS-PAGE) was performed in 15 % (m/v) acrylamide slab gels using either a *Mini-Protean II*® cell (*Bio-Rad*, Hercules, CA, USA), with electrophoresis conducted at 200 V for 50 to 60 min at room temperature, or larger gels (180 mm) run at 30 mA per gel constant current in a *Protean® II Cell* apparatus (*Bio-Rad*) until the dye front reached the bottom of the gel at 4 °C (about 6 h).

For protein spot visualisation using Coomassie blue staining, the gels were immersed in 0.1 % (m/v) Coomassie brilliant blue R-250, 40 % (v/v) methanol and 10 % (v/v) acetic acid) for 30 min (minigels) or overnight for larger gels with gentle shaking, followed by destaining in 30 % (v/v) ethanol until protein bands or spots became visible

For silver staining, the method described by Swain and Ross (1995) that was published earlier by Rabilloud *et al*. (1988) was used. After electrophoresis, the gel was fixed in 50 % (v/v) methanol and 10 % (v/v) acetic acid for 30 min, then washed in *Milli-Q* water (3 × 5 min) and then incubated in a solution of 0.01 % (m/v) sodium thiosulfate for 2 min followed by several washes with *Milli-Q* water. Then the gel was submerged in 0.2 % (m/v) silver nitrate for 20 min followed by thorough rinsing with *Milli-Q* water before being placed in 0.2 % (v/v) formalin, 0.004 % (m/v) sodium thiosulfate and 0.6 % (m/v) sodium carbonate. After the desired intensity of staining was achieved, the reaction was terminated by discarding the reagent, followed by washing the gel with cooled 5 % (v/v) methanol.

For Western analysis, the basic method described by Towbin *et al.* (1979) with some modifications was used. Proteins separated by 2DE gel were electrophoretically transferred from the gel to PVDF membrane (*Immobilin-P*, *Millipore Corporation*, Bedford, MA, USA) using 25 mM Tris, 190 mM glycine, pH 8.3 and 10 % (v/v) methanol as transfer buffer. Following electrophoretic transfer, the PVDF membrane was carefully peeled from the gel and placed protein-side up into a suitable clean container, and incubated in 0.2 % (m/v) *I-Block*™ (*Life Technologies*, Carlsbad, CA, USA) in 50 mM sodium phosphate, pH 7.4, containing 250 mM NaCl (PBSalt). This blocking solution was then discarded and the membrane rinsed with PBSalt containing 0.05 % (v/v) *Tween 20* (PBST) before incubation with the anti-TRACO2 rabbit IgG antibody at a 1 : 2000 dilution in 1× PBST at room temperature for 1 h. After washing, the membrane was incubated with anti-rabbit alkaline phosphatase conjugate IgG raised in goat (*Sigma Chemicals*, St. Louis, MO, USA) at a concentration of 1:10 000 in 1× PBST at room temperature for 1 h. Antibody recognition was determined using 150 mM Tris-HCl, pH 9.7, containing 0.01 % (m/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.02 % (m/v) ρ-nitro-blue tetrazolium chloride (NBT), 1 % (v/v) dimethyl sulfoxide (DMSO), and 8 mM $MgCl_2$ as substrate.

MALDI-TOF mass spectroscopy: For acquisition of mass spectrometric peptide maps of the proteins, some spots of interest were excised into small pieces with a sterile scalpel. The gel piece for each spot was washed by vortexing for 5 min in 0.4 cm^3 of sterile deionized water, the water discarded and the gel pieces dried in a vacuum centrifuge for 30 min (*Savant Speed Vac*, Holbrook, NY, USA). The gel pieces were then forwarded to the Australian proteome analysis facility (*APAF*; Macquarie University, Sydney, Australia) where they were trypsin-digested at 37 °C for 16 h, before the resulting peptides were extracted from the gel matrix with 50 % (v/v) aceto-nitrile, 1 % (v/v) trifluoroacetic acid (TFA). A 1 μm^3 aliquot was then spotted onto a sample plate with 1 μm^3 of matrix [0.8 % (v/v) α-cyano-4-hydroxy-cinnamic acid in 50 % (v/v) acetonitrile, 1 % (v/v) TFA] and allowed to air dry before MALDI-TOF mass spectrometry. A near point calibration was applied providing a typical mass accuracy of ~100 $\mu g\ g^{-1}$. The peak list supplied was then used to interrogate protein databases using *Aldente* in the *EXPasy* portfolio (http://ca.expasy.org), using the following parameters: trypsin digest; 1 missed cleavage; monoisotopic; mass tolerance of 25 $\mu g\ g^{-1}$.

Results

Partial purification of ACC oxidase isoforms from newly initiated and senescent leaves of white clover: For the NI leaf extract, the first separation utilized hydrophobic interaction column chromatography with a phenyl sepharose column (Fig. 1*A*). Using this method, ACC oxidase protein eluted in fractions 31 to 38 was determined by Western analysis (data not shown). With highest recognition by the anti-TR-ACO2 antibody a major protein of *ca.* 37 kDa was observed in fractions 33 and 34 (Fig. 1*A*). Using this hydrophobic interaction column, the majority of protein eluted first within a decreasing linear gradient of ammonium sulfate in the buffer, with a significant portion of protein eluting with no ammonium sulfate in the buffer. All fractions containing ACC oxidase (31 - 38) and designated NI-1, also eluted with no ammonium sulfate in the buffer (Fig. 1*A*).

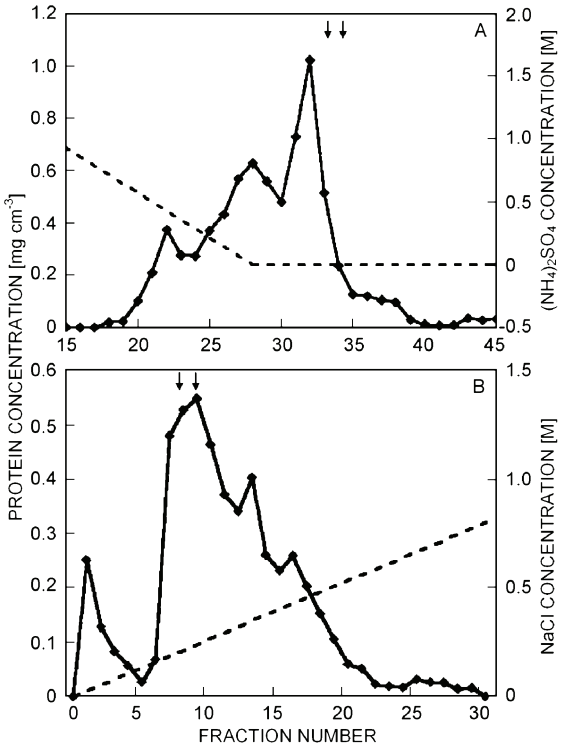

Fig. 1. Separation of a newly initiated green leaf protein extract, after ammonium sulfate precipitation and *Sephadex G-25* column chromatography, through a *Phenyl Superose* hydrophobic interaction column (*A*) and then selected fractions through a *Mono Q* anion exchange column (*B*). The *arrow* denotes fractions with maximum anti-TR-ACO2 antibody recognition. The separation of fractions 1 to 14 is not shown in *A*.

Anion exchange (*Mono Q*) column chromatography was used as the second purification step to further purify the NI-1 isoforms (Fig. 1*B*). Fractions 31 to 38 from the phenyl sepharose column were pooled, and then applied to a *Mono Q* anion exchange column and bound proteins were eluted using a linear increasing gradient of sodium chloride from 0 to 100 %. The most intense antibody recognition was observed in fractions 9 and 10 with one major protein of *ca.* 37 kDa recognized by the anti-TR-ACO2 antibody (Fig. 1B). These fractions with maximum ACC oxidase recognition eluted at concentration of 210 to 250 mM NaCl (fractions 9 and 10, Fig. 1*B*).

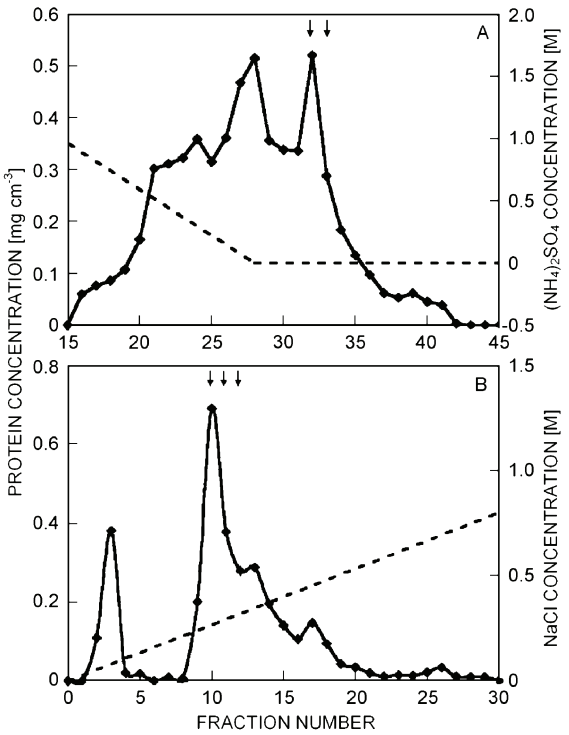

Fig. 2. Separation of a senescent leaf protein extract, after ammonium sulfate precipitation and *Sephadex G-25* column chromatography, through a *Phenyl Superose* hydrophobic interaction column (*A*) and then selected fractions through a *Mono Q* anion exchange column (*B*). The *arrow* denotes fractions with maximum anti-TR-ACO2 antibody recognition. The separation of fractions 1 to 14 is not shown in *A*.

Extracts from senescent leaves were also subjected to partial purification with hydrophobic interaction chromatography the first separation step utilised (Fig. 2*A*). Western analysis with anti-TR-ACO2 antibodies determined that the ACC oxidase protein eluted over fractions 32 to 36 (data not shown) with maximum recognition of fractions 32 and 33 (Fig. 2*A*; designated as SE-1). Fig. 2*A* showed that a large amount of protein, with no ACC oxidase, eluted within a decreasing linear gradient of ammonium sulfate, while the ACC oxidase proteins again eluted in the later fractions with no ammonium sulfate in the buffer.

Following hydrophobic interaction chromatography, the most intensely immuno-recognized fractions (fractions 32 to 36) were pooled and concentrated, and then loaded onto a *Mono Q* column (Fig. 2*B*). The more intense antibody recognition was observed in fractions 10 to 12, and in these fractions, a major protein of *ca.*

35 kDa was recognized by the TR-ACO2 antibodies (data not shown). Fractions with maximum ACC oxidase recognition eluted at a 260 to 320 mM NaCl (fractions 10 to 12, Fig. 2*B*).

2DE and MALDI-TOF mass spectroscopy: For the putative NI-1 ACC oxidase isoform, fractions 9 and 10 from the ion-exchange separation were subjected to 2DE and the separated proteins challenged with the anti-TR-ACO2 antibody (Fig. 3*A*). Western analysis indicated that the prominent ACC oxidase protein existed as at least three major variants differing in terms of pI (ranging from pH 5.1 to 5.4), but not in mass (*ca.* 37 kDa), with a minor protein with two pI variants (ranging from pH 5.0 to 5.2) with a mass of *ca.* 35 kDa. A series of identical 2DE separations were performed and proteins with positions which corresponded to those proteins recognised by the anti-TR-ACO2 antibody were excised for subsequent analysis by MALDI-TOF. Six spots in total (designated G1 to G6) were subjected to MALDI-TOF analysis with only two, G4 and G6 (Fig. 4*A*,*B*), returning significant identities with the database.

For the putative SE-1 ACC oxidase isoform, fractions 10, 11 and 12 from the ion-exchange separation was subjected to 2DE and the separated proteins challenged with the anti-TR-ACO2 antibody. Here, Western analysis revealed a more complex pattern of spots (Fig. 3*B*). Again, microheterogeneity exists for proteins at three different molecular masses with pI ranges of pH 5.1 to 5.3 for a *ca.* 34 kDa protein, pH 5.0 to 5.3 for a 35 kDa protein, and pH 5.1 to 5.4 for a 37 kDa protein. Again, identical separations were performed with five spots, designated S1, S2, S3, S4 and S5, subjected to MALDI-TOF with only one, S1 (Fig. 4*C*), returning significant identities with the database.

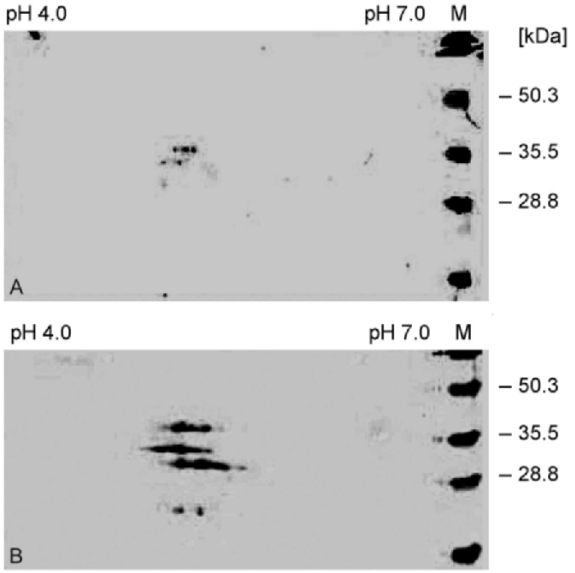

Fig. 3. Separations of NI-1 (*A*) and SE-1 (*B*), after hydrophobic interaction and anion exchange column chromatography, using 2-D-SDS-PAGE. The separated proteins were transferred to a PVDF membrane, challenged with anti-TR-ACO2 IgG and antibody recognition determined using alkaline-phosphatase-linked secondary antibodies and colorimetric substrates. *Lane* M is prestained molecular mass marker.

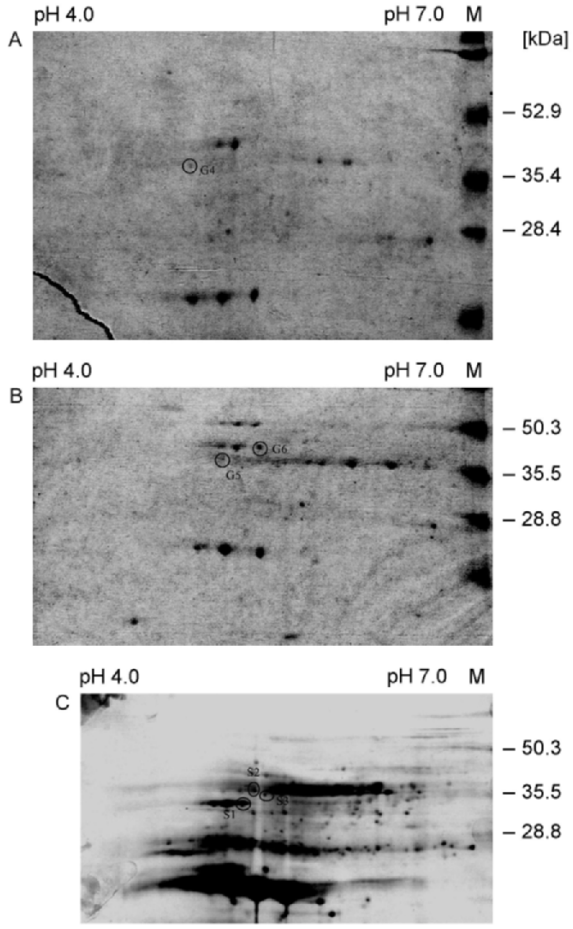

Fig. 4. Separations of NI-1 (*A*,*B*) and SE-1 (*C*), after hydrophobic interaction and anion exchange column chromatography, using 2-D-SDS-PAGE. Separated proteins were detected by Coomassie blue staining (*A*,*B*) or silver staining (*C*). *Lanes* M are prestained molecular mass markers. Proteins submitted for MALDI-TOF analysis as G4, G5 (*A*), G6 (*B*) and S1, S2, S3 (*C*) are indicated.

Interrogation of the database using the peptides excised from the 2DE separations was achieved using *Aldente* in the *ExPASY* portfolio and three significant alignments were revealed (Fig. 5). The first of these, phosphoribulokinase (from *Arabidopsis thaliana*), was identified from peaks derived from spot G4 (Fig. 4*A*; 42 peaks; mass range 804.4092 - 2979.013 Da) and from spot G6 (Fig 4*B*; 42 peaks; mass range 825.1693 - 2978.971 Da). The second, ACC oxidase (from *Oryza sativa*), was also identified from peaks derived from spot G4 (Fig 4*A*). The third protein, isoflavone reductase (from *Medicago sativa*), was identified from peak S1 (Fig. 4*C*; 27 peaks, mass range 756.5084 - 3047.167 Da). For each putatively identified protein, mass and pI data calculated from the database entry are compared with the calculation for each relevant spot after 2DE (Table 1).

A
Phosphoribulokinase – *Arabidopsis thaliana* – P52575
AQETIVIGLAADSGCGKSTFMRRLTSVFGGAAKPPK***GGNPDSNTLISDTTTVICLDDYHSLDR***YRKEQKVTALDPRANDFDLMYEQVKAL
KNGIAVEKPIYNHVTGLLDPPELIQPPKILVIEGLHPMFDER***VRDLLDFSIYLDISNEVK***FAWKIQR
DMAERGHSLESIKASIEARKPDFDAFIDPQKQYADAVIEVLPTTLIPDDNEGKVLRVRLIMKEGVK***YFSPVYLFDEGSTISWIPCGR***KLTCSY
PGIKFNYEPDSYFDHEVSVLEMDGQFDRLDELIYVESHLSNLSTK***FYGEVTQQMLK***HADFPGSNNGTGLFQTIVGLKIRDLYEQLIANKAT
ARAEAKA

B
ACC oxidase – *Oryza sativa* – A2Z1W9
MAPTSTFPVINMELLAGEERPAAMEQLDDACENWGFFEILNHGISTELMDEVEKMTKDHYKRVREQRFLEFASKTLKEGCDDVNK***AEKL***
DWESTFFVRHLPESNIADIPDLDDDYRRLMKRFAAELETLAER***LLDLLCENLGLEK***GYLTKAFRGPAGAPTFGTK***VSSYPPCPRPDLVKG***
LRAHTDAGGIILLFQDDSVGGLQLLKDGEWVDVPPMRHSIVVNLGDQLEVITNGRYK***SVMHRVVAQTDGNR***MSIASFYNPGSDAVISPAP
ALVKEEEAVVAYPK***FVFEDYMKLYVR***HKFEAKEPRFEAFKSMETETSNRIAI

C
Isoflavone reductase – *Medicago sativa* – P52575
MATENKILILGPTGAIGRHIVWASIK***AGNPTYALVRK***TPGNVNKPKLITAANPETKEELIDNYQSLGVILLEGDINDHETLVKAIKQVDIVICAA
GRLLIEDQVKIIKAIKEAGNVKK***FFPSEFGLDVDR***HEAVEPVRQVFEEKASIRR***VIEAEGVPYTYLCCHAFTGYFLR***NLAQLDTTDPPRDKV
VILGDGNVK***GAYVTEADVGTFTIR***AANDPNTLNKAVHIRLPENYLTQNEVIALWEKKIGKTLEKTYVSEEQVLKDIQESSFPHNYLLALYHS
QQIKGDAVYEIDPAKDIEASE AYPDVTYTTADEYLNQFV

Fig. 5. Deduced amino acid sequences of the three proteins with significant identities calculated from the masses of the peak list obtained from the excised gel spots after 2-D-SDS-PAGE. The italicised and underlined sequences are the matched peptides after a theoretical trypsin digest with the actual masses obtained from MALDI-TOF mass spectrometry of the excised gel spots. Details of coverage of each protein are given in Table 1.

Table 1. Summary data of identified protein spots ([a] - calculated from 2DE, [b] - theoretical from known protein sequence).

Spot	Score	Number of hits	Acc. No.	Identification	Mr^a [kD]	Mr^b [kD]	pI^a	pI^b	Coverage [%]
G4	10.94	6	P25697	phosphoribulokinase	37	39.2	5.1	5.16	25
G4	5.19	6	A2Z1W9	ACC oxidase	37	36.4	5.1	5.03	21
G6	13.20	8	P25697	phosphoribulokinase	38	39.2	5.4	5.16	26
S1	4.03	4	P52575	isoflavone reductase	34	35.4	5.0	5.23	19

Discussion

This study has sought to provide preliminary evidence that the enzyme ACC oxidase undergoes post-translational modification. While a number of post-translational modifications have now been identified to occur to plant proteins, many of these result in a change of surface charge or conformation of the native protein, a modification that is often manifested with a change in pI. For example, the 'pearls-on-a-string' obtained using 2DE is considered to be major diagnostic evidence of charge heterogeneity induced by phosphorylation (Jensen 2004). Thus 2DE has become one method of choice to detect post-translational modifications (Jensen 2004, Kwon *et al.* 2006).

For the two ACO isoforms under study, certainly evidence of protein modifications affecting pI is apparent with the 'pearls-on-a-string' pattern suggesting that phosphorylation may be one such modification. However, it should also be noted that the technique of iso-electric focussing can, itself, cause a range of protein modifications (Sarioglu *et al.* 2000, Xing *et al.* 2008). Precautions can be adopted to minimise such artificial modifications including the use a chaotrope (*e.g.* urea) to disrupt non-covalent interactions, non-ionic or zwitter-ionic detergents (*e.g.* CHAPS) to aid solubility and DTT to aid reduction prior to the IEF step (Rabilloud *et al.* 1988). In this study all of those constituents were included in the 2DE extraction buffer (Leimgruber 2005). Thus we can have confidence that the heterogeneity observed probably does represent post-translational modifications.

The 2DE study also revealed differences in the degree of modification between the two putative isoforms (NI-1 and SE-1). It is apparent that these proteins do display some charge heterogeneity as they eluted from the ion-exchange column at different concentrations along the salt gradient. Previous molecular studies with white clover have shown that distinct ACO genes are expressed at different stages of leaf ontogeny: *TR-ACO2* is expressed in newly initiated leaves, while *TR-ACO3* is expressed in senescent leaves (Hunter *et al.* 1999). One could speculate, therefore, that the different environmental and developmental cues that operate in these different tissues influence the type and/or degree of post-translational modifications of the ACO protein. As an initial step to determine such modifications, attempts were made to sequence the ACO proteins in each extract

using MALDI-TOF from stained and excised spots, although it should be noted that manual techniques were used to match immuno-recognised spots with their corresponding protein stained ones.

Peptide I:
O. sativa A E K L D W E S T F F V R
TR-ACO2 V K D M D W E S T F H L R
Peptide II:
O.sativa L L D L L C E N L G L E K
TR-ACO2 L L D L L C E N L G L E K
Peptide III:
O.sativa V S S Y P P C P R P D L V K G L R
TR-ACO2 V A N Y P P C P K P D L V K G L R
Peptide IV:
O. sativa S V M H R V V A Q T D G N R
TR-ACO2 S V E H R V I A Q S D G T R
Peptide V:
O. sativa F V F E D Y M K L Y V R
TR-ACO2 F V F E D Y M N L Y A G

Fig. 6. Alignment of the matched peptides after a theoretical trypsin digest with the actual masses obtained from MALDI-TOF mass spectrometry of the excised gel spots for the ACC oxidase protein from *Oryza sativa* with the corresponding peptides from TR-ACO2, as indicated. The residues underlined represent mismatches.

Using such procedures, the technique of MALDI-TOF mass spectrometry was successful in identifying an ACC oxidase protein and two other metabolic proteins. For the ACO identification, some identity to an ACC oxidase from rice (*Oryza sativa*) was determined for spot G4. Here, a coverage of 21 % was obtained with five peptides identified. With two proteins identified for spot G4 (ACO and phosphoribulokinase, PRK), it suggests that some protein contamination occurred particularly as manual techniques were used to align protein spots. However, if *BLAST* searches are performed with each of the ACO peptides identified, then hits against ACO are the only alignments obtained (with up to 50 alignments recorded; data not shown). This gives a degree of confidence that the identification of one of the proteins associated with spot G4 is an ACC oxidase. Further, very good agreement exists in terms of the theoretical pI and molecular mass (5.03 and 36.4 kDa) with the calculated values from the 2DE (5.1 and 37 kDa) and this also provides a degree of confidence. However, it is surprising that the sequence aligned is from rice given that ACO sequences from *T. repens* do occur in the database, as well as for *T. nigrescens* and seven other legume species. Thus a comparison of each of the five peptides from rice was made with the corresponding TR-ACO2 peptide (Fig. 6). For peptide I, 6 out of 13 residues differed, while no differences were observed for peptide II. For the other three peptides scores of 3/17 (III), 4/14 (IV) and 2/12 (V) were obtained. No significantly higher scores were obtained when comparisons were made with the deduced amino acid sequences corresponding from the other ACO genes expressed in the leaf, TR-ACO1 and TR-ACO3.

The first of the metabolic enzyme identified is the Calvin cycle-associated enzyme PRK, an example of an essential housekeeping enzyme in green, photosynthetically active leaf tissue. Given the source of the extract for the NI-1 separation such an identification is not surprising. Two spots were identified with good alignments to PRK (G4 and G6) with coverages of 25 and 26 %, respectively. The alignments were against the same protein from *A. thaliana* with a total of five distinct peptides identified from both spots. Further, good agreement of the calculated and theoretical pI values and molecular mass was obtained. It is interesting to note that two distinct spots after 2DE (G4 and G6) aligned to the same protein suggesting that PRK itself may also undergo post-translational modification. It is known that PRK forms a complex with the CP12 protein and with glyceraldehyde dehydrogenase (Graciet *et al.* 2004), and is also regulated by thioredoxin (Avilon *et al.* 2000). However, whether the protein itself undergoes post-translational modification prior to or during complex formation is still unknown (Gontero *et al.* 2006). The alignment with *A. thaliana* is not surprising as no PRK sequences from *T. repens* occur in the database, although sequences from *Medicago truncatula* and *Pisum sativum* do occur.

The second (non-ACO) protein identified is isoflavone reductase (IFR) (from *Medicago sativa*), an enzyme that functions in the *Leguminosae*, including alfalfa, in the isoflavonoid phytoalexin pathway (Oommen *et al.* 1994). A coverage of 19 % was achieved over four peptides with good agreement of the theoretical pI and molecular mass (5.23 and 35.4 kDa) with the calculated values from the 2DE separation (5.0 and 34 kDa). Again, no sequences from *T. repens* occur in the database, although sequences occur from eight other legume species. Of further interest is that transcripts of IFR are induced in infected leaves (*e.g.* bean) or elicitor-treated cell cultures (Dixon *et al.* 1995), and there is some evidence that pathogen-related proteins are induced during leaf senescence (Lers *et al.* 1988). Thus the identification of IFR in senescent leaves is of particular interest as it suggests that the protein may be expressed developmentally in this tissue in the absence of pathogen infection.

In the database searches performed in this study, the molecular mass tolerance was set at 25 $\mu g\ g^{-1}$ which is stringent and will give some confidence to the identified peptides. Clearly though, more definitive experiments are required to identify these proteins with more certainty including the use of tandem mass spectrometry techniques to obtain amino acid sequence data. With that level of resolution, then ACO sequences from *T. repens* may then be identified, and the determination of PRK and IFR, particularly in senescent leaf tissue, can be made with more certainty thus adding to our knowledge of these two proteins in an important member of the *Leguminosae*.

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