Protein tyrosine nitration in pea roots during development and senescence

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Abstract
Protein tyrosine nitration is a post-translational modification mediated by reactive nitrogen species (RNS) that is associated with nitro-oxidative damage. No information about this process is available in relation to higher plants during development and senescence. Using pea plants at different developmental stages (ranging from 8 to 71 days), tyrosine nitration in the main organs (roots, stems, leaves, flowers, and fruits) was analysed using immunological and proteomic approaches. In the roots of 71-day-old senescent plants, nitroproteome analysis enabled the identification a total of 16 nitrotyrosine-immunopositive proteins. Among the proteins identified, NADP-isocitrate dehydrogenase (ICDH), an enzyme involved in the carbon and nitrogen metabolism, redox regulation, and responses to oxidative stress, was selected to evaluate the effect of nitration. NADP-ICDH activity fell by 75% during senescence. Analysis showed that peroxynitrite inhibits recombinant cytosolic NADP-ICDH activity through a process of nitration. Of the 12 tyrosines present in this enzyme, mass spectrometric analysis of nitrated recombinant cytosolic NADP-ICDH enabled this study to identify the Tyr392 as exclusively nitrated by peroxynitrite. The data as a whole reveal that protein tyrosine nitration is a nitric oxide-derived PTM prevalent throughout root development and intensifies during senescence.

Key words: Nitration, nitric oxide, nitrotyrosine, peroxynitrite, plant development, reactive nitrogen species, senescence.

Introduction
Nitric oxide (NO) and related molecules such as S-nitrosothiol (GSNO) and peroxynitrite (ONOO−) belong to the group of compounds called reactive nitrogen species (RNS). Although the metabolism of RNS and their physiological implications have been intensively studied in relation to animal cells, less is known about plant cells. RNS can mediate post-translational modifications (PTMs) of proteins which could be pathological processes and/or mechanisms of cellular signalling (Radi, 2004; Dalle-Donne et al., 2005; Ischiropoulos and Gow, 2005; Corpas et al., 2007, 2009). These PTMs mediated by RNS include processes like binding to metal centres, nitrosylation of thiol and amine groups, and nitration of tyrosine and other amino acids (Gow et al., 2004). Nitrosylation of thiols to produce S-nitrosothiols and nitration of tyrosine residues in order to produce 3-nitrotyrosine (NO2-Ty) have been the subject of considerable study in relation to animal cells as they appear to be involved in signalling processes.

Protein tyrosine nitration is a covalent protein modification resulting from the addition of a nitro (-NO2) group to one of two equivalent ortho carbons in the aromatic ring of tyrosine residues (Gow et al., 2004). This process has routinely been used as a marker of pathological disease and oxidative stress in animal cells (Ischiropoulos, 2003). However,
tyrosine nitration of protein residues can alter protein function because the incorporation of large groups onto the aromatic ring lowers the pKa of the phenolic group and causes both steric and electronic perturbations that affect tyrosine's capacity to function in electron-transfer reactions and to maintain protein conformation (Eiserich et al., 1999). Thus, tyrosine nitration has been reported to lead to either gain or loss of function (Greenacre and Ischiropoulos, 2001; Radi, 2004). This NO-mediated PTM has also been studied in plants under stress conditions and has been proposed as a potential nitrosative stress marker (Corpas et al., 2007, 2013). A rise in protein tyrosine nitration has therefore been reported under biotic (Chaki et al., 2009) and abiotic stresses that include salinity (Valderrama et al., 2007; Corpas et al., 2009; Tanou et al., 2012), extreme temperature (Chaki et al., 2011; Airaki et al., 2012), and arsenic (Leterrier et al., 2012a).

However, not much information is available on protein tyrosine nitration during development and senescence in relation to higher plants. In this study, the protein profile of tyrosine nitration in the main organs of pea plants was studied. In addition, after studying the nitroproteome of senescent roots, this work discovered that cytosolic NADP-isocitrate dehydrogenase (ICDH) is a molecular target of this NO-mediated PTM which provoked its inhibition.

Materials and methods

Plant materials and growth conditions

Pea (Pisum sativum L., cv Lincoln) seeds, obtained from Royal Sluis (Enkhuizen, Holland), were germinated in vermiculite under optimum conditions (14/7 h 21/18 °C light/dark cycle, 190 µm2 s-1, 50–60% relative humidity). For senescence, pea seedlings of 14 days were transferred and then grown in aerated optimum nutrient solutions under greenhouse conditions (28/18 °C light/dark temperature; 80% relative humidity) for a total time of 71 days. Samples of roots, stems, and leaves were taken at 8, 12, 14, 16, and 71 days. Flowers and whole fruits were taken from plants aged 71 days.

Crude extracts of plant tissues

Tissues were frozen in liquid N2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 100 mM TRIS-HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM CHAPS, and 1 mM PMSF (1:2, w/v). Homogenates were centrifuged at 21,000 g for 20 min, and the supernatants were passed through Sephadex G-25 desalting columns (NP-10, Amersham) which eliminated low-molecular-weight substances (Mr <1000). The different flow-throughs were used for the assays.

Non-denaturing PAGE and detection of superoxide dismutase isoforms

Superoxide dismutase (SOD) isoforms were separated by native PAGE on 10% acrylamide gels and visualized by a photochemical method (Beauchamp and Fridovich, 1971). To identify the type of SOD isoform, gels were preincubated separately at 25 °C for 30–45 min in 50 mM K-phosphate (pH 7.8), in the presence or absence of either 5 mM KCN or 5 mM H2O2 (Corpas et al., 1998).

SDS-PAGE and Western blot

SDS-PAGE was done on 10% polyacrylamide gels as described by Laemmli (1970). Gels were stained with silver nitrate (Corpas and Trelase, 1998). For Western blot analysis, proteins were electroblotted to PVDF membranes by a semi-dry Trans-Blot cell (Bio-Rad). After transfer, membranes were used for cross-reactivity assays using polyclonal antibodies against nitrosotyrosine (1:8000 dilution) (Chaki et al., 2009), spinach cytosolic CuZnSOD (1:3000 dilution) (Kanematsu and Asada, 1989), and pea MnSOD (1:2000 dilution) (Corpas et al., 2006b). For immunodetection, an affinity-purified goat anti-rabbit IgG–horseradish peroxidase conjugate (BioRad) and an enhanced chemiluminescence kit (ECLPLUS, Amersham) was used. As positive control, commercial nitrated BSA (Sigma) was used. Immunoblot experiments were performed a minimum of three times, with similar results. Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA, USA), using BSA as a standard.

Detection of nitric oxide and peroxynitrite

Nitric oxide was detected with 10 µM 4,5-diaminofluorescein diacetate (DAF-FM-DA, Calbiochem) prepared in 10 mM TRIS-HCl (pH 7.4). Root cross-sections were incubated at 25°C for 1 h, in darkness, according to Corpas et al. (2006a). After incubation, samples were washed twice in the same buffer for 15 min each. Then root sections were embedded in a mixture of 15% acrylamide/bisacrylamide stock solution as described elsewhere (Corpas et al., 2006a), and 80–100 µm-thick sections, as indicated by the vibratome scale, were cut under 10 mM phosphate-buffered saline (PBS). Sections were then soaked in glycerol/PBS containing azide (1:1, v/v) and mounted in the same medium for examination by confocal laser scanning microscopy (CLSM, Leica TCS SL), using standard filters and collection modalities for DAF–2 green fluorescence (excitation 495 nm; emission 515 nm). Background staining, routinely negligible, was controlled with root sections unstained. As control, sections were preincubated 30 min at 25°C with 200 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxide, an NO scavenger.

Peroxynitrite was detected using the fluorescent reagent 3′-4′-ami- nophenyl) fluorescein (APF, Invitrogen) (Chaki et al., 2009). Pea root sections were incubated at 25°C for 1 h, in darkness, with 10 µM APF prepared in 10 mM TRIS-HCl (pH 7.4) and were processed as described by Chaki et al. (2009). Then, the samples were washed twice in the same buffer for 15 min each and mounted in a microscope slide for examination by CLSM using standard filters and collection modalities for APF green fluorescence (excitation 495 nm; emission 515 nm).

Immunolocalization of nitrotyrosine

Pea roots were cut into 4–5 mm pieces and fixed in 4% (w/v) p-formaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 3 h at room temperature. Then they were cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4°C. Serial sections, 60-µm thick, were obtained by means of a cryostat (2800 Frigocut E, Reichert-Jung, Vienna, Austria). The sections were incubated with a rabbit polyclonal antibody against 3-nitrotyrosine (NO2-Tyr) diluted 1:300 in TBSA-BSAT (5 mM TRIS buffer pH 7.2, 0.9% (w/v) NaCl containing 0.05% (w/v) sodium azide, 0.1% (w/v) BSA, 0.1% (v/v) Triton X-100) for 3 days at 4°C. After several washes, sections were incubated with Cy3-labelled anti-rabbit IgG (Amersham) diluted 1:1000 in PBS/glycerol (1:1). Controls for background staining, which was usually negligible, were performed by replacing the corresponding primary antisemum by preimmune serum. Root sections were examined by CLSM using standard filters for Cy3 and Cy2-streptavidin. Cross-sections of any section were examined by confocal laser scanning microscopy (CLSM, Leica TCS SL), using standard filters and collection modalities for Cy3 and Cy2 fluorescence (excitation 492 nm; emission, 510 nm).

Protein concentration of pea roots, 2D gel electrophoresis, and immunoblot analysis

Pea roots from 71-day-old plants were washed four times with tap water and twice with distilled water and drying with paper towel.
Tissues (45 g) were frozen in liquid N2 and ground in a mortar with a pestle. The powder was suspended in 125 ml of 50 mM HEPES (pH 7.6). The homogenate was filtered through four layers of nylon and centrifuged at 20,000 g for 15 min. To achieve the protein precipitation, three volumes of 100% acetone at −20 °C was added to the supernatants with stirring for 30 min (70% final concentration, v/v) and the mixture was centrifuged at 16,000 g for 15 min. The precipitate, which contained most of the proteins, was taken up in 3 ml of 50 mM HEPES and incubated overnight at 4 °C with agitation. Then, it was centrifuged at 39,000 g for 20 min. The supernatants were passed through Sephadex G-25 gel filtration columns (NAP-10, Amersham) which were equilibrated and eluted with 50 mM HEPES. The final concentration of the protein extract was 0.88 µg µl⁻¹ in 7 ml final volume (6 mg total yield).

Proteins were separated by two-dimensional gel electrophoresis (2-DE). Isoelectric focusing was carried out with precast IPG-gels pH 3–10. Each gel was loaded with 150 µg protein. The second-dimension separation was performed by glycine SDS-PAGE according to Laemmli (1970). Gels were SYPRO Ruby stained, scanned, and analysed with Bio-Rad PDQuest software. Western blot of the 2D gel was performed as described above with a minimum of three replicates with similar results, and then the immunoblot probed with a polyclonal antibody against nitrotyrosine (1:8000 dilution).

**In situ digestion of 2D spots and protein identification by MALDI TOF/TOF analysis**

Identified spots in the gel were automatically recovered using Investigator ProPeptide Protein Picking Workstation (Genomic Solutions). Then, they were digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomics Solutions). Briefly the procedure was as follows: denaturing with 40% acetonitrile/200 mM NH4HCO3 for 30 min (twice); wash with 25 mM NH4HCO3 for 5 min and 25 mM NH4HCO3/50% acetonitrile for 15 min, respectively (twice). Then, samples were dehydrated with 100% acetonitrile for 5 min and dried up. The samples were hydrated with 10 µl trypsin in 25 mM NH4HCO3 (12.5 ng µl⁻¹) at room temperature for 10 min then digested at 37 °C for 12 h. The reaction was stopped by adding 10 µl of 0.5% trifluoroacetic acid (TFA). Peptides were purified using a ProMS station (Genomic Solutions) using a column C18 (ZipTip, Millipore) eluted with 0.1% TFA in 70% acetonitrile/0.1% TFA in a MALDI plate (1 µl). After crystallization the samples were analysed by MALDI TOF/TOF mass spectrometry in a range mass-to-charge ratio (m/z) of 800–4000 Da using a spectrometer (4800, AB Sciex) to evaluate the quality of the sample. MALDI-TOF spectra were interpreted using a peptide mass fingerprinting mixture was analysed using a MALDI-TOF mass spectrometer (4800, AB Sciex) to evaluate the quality of the sample. MALDI-TOF spectra were interpreted using a peptide mass fingerprinting database search (Protein Prospector program). The database used for identification was Uniprot (release 2011_02). Then, sample was analysed by LC-MS/MS using a Velos-LTQ mass spectrometer equipped with a microESI ion source (ThermoFisher, San Jose, CA, USA). The sample was evaporated to dryness and diluted up to 40 µl with water containing 5% methanol and 1% formic acid. Then, the sample was loaded in a chromatographic system consisting in a C18 preconcentration column (Agilent Technologies, Santa Clara, CA, USA) connected to a 10 cm long, 150 µm i.d. C18 column (Ydac, IL, USA). The separation was done at 1 µl min⁻¹ in a 30 min acetonitrile gradient from 3 to 40% (solvent A: 1% formic acid; solvent B: acetonitrile/0.1% formic acid). The HPLC system was composed by an Agilent 1200 capillary pump, binary pump, a thermostat
microinjector, and a micro-switch valve. The Velos-LTQ instrument was operated in the positive ion mode with a spray voltage of 2 kV. The scan range of each full MS was m/z 400–2000. After each MS scan, a collection of targeted MS/MS spectra were obtained in order to identify both the unmodified and nitrated form of the expected tyrosine-containing peptides. The parent mass list of the targeted scan was selected to ensure maximum coverage of the tyrosine-containing tryptic peptides for NADP-ICDH. The list of targeted m/z values was obtained after *in silico* digestion of the proteins using nitrated tyrosine as a dynamic modification. The resulting list of predicted peptides (including both the nitrated and the unmodified forms) was filtered to exclude all peptides that did not contain tyrosine residues.

MS/MS spectra were searched using Proteome Discoverer software (ThermoFisher) with the following parameters: peptide mass tolerance 2 Da, fragment tolerance 0.8 Da, enzyme set as trypsin, and no missed cleavages. Dynamic modifications were cysteine carbamidomethylation (+57 Da), methionine oxidation (+16 Da), and tyrosine nitration (+45 Da). The searches were performed using a database containing all the proteins listed in Supplementary Table S1 (available at *JXB* online). Identifications were filtered with XCorr > 3, P(pep) < 15%. The MS/MS spectrum of the nitrated tyrosine was manually validated by comparison of the spectra obtained for the unmodified peptide and the nitrated peptide.

**Structural analysis of cytosolic NADP-ICDH**

The 3D structure of pea NADP-ICDH was modelled using the structure of the pig NADP-ICDH (PDB code access 1lwd_B) at a resolution of 1.85 Å and a homology of 69.3%. The analysis of the quality of the model resulted in a 93.1% residue with a 3D-1D average score higher than 0.2 and 87.7% in the Ramachandran plot. Determining the sites important for the structure and/or function of the protein was performed using the database available at www.uniprot.org and by homology with the structure of the pig NADP-ICDH (Ceccarelli *et al.*, 2002). Next, this study performed a sequence alignment of pea ICDH (AAS49171.1), rice (AAU44104.1), Arabidopsis (AEE30821.1), human (O75874.2), and pork (1lwd_B). In this way, it was determined that residues with significant relevance in the structure and/or function of the NADP-ICDH that were conserved in various species (Huang and Colman, 2005) and therefore can have a similar function in the pea ICDH. The location of these zones, together with the Tyr392 nitration identified as target, within the structure of the protein was done using the program Rasmol.

**Statistical analysis**

To estimate the statistical significance between means, the data were analysed by Student’s t-test.

**Results**

Fig. 1 shows the protein patterns analysed using silver stains and the corresponding tyrosine nitration protein patterns detected using an antibody against nitrotyrosine during development and senescence in the different pea organs of 8, 12, 14, and 16-day-old (young) and 71-day-old (senescent) plants (Pastori and del Río, 1997; Corpus *et al.*, 1999, 2004). In roots (Fig. 1A), the silver stain showed a general increase in the intensity of polypeptides, which reached very high levels in senescent plants (71 days old). On the other hand, roots showed a similar protein tyrosine nitration pattern in relation to all ages, with protein bands of 45, 52, 63, and 76 kDa, although its intensity increased with plant age and reached higher levels during senescence. There was also an additional band of 89 kDa which appeared to become tyrosine nitrated after 16 days and became more intense during senescence. In stems (Fig. 1B), the silver stain protein pattern was different to that found in the other organs. The nitration profile was also different, with the most prominent band being 89 kDa, with other less intense bands (25 and 59 kDa) being detected. Moreover, during senescence, a new and very clear protein band of 37 kDa appeared. In leaves (Fig. 1C), the most visible band was 89 kDa, with a 64 kDa nitrated protein becoming more intense at day 14, which, however, disappeared in Fig. 1.
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senescent 71-day-old leaves. In the case of flowers and whole fruits, the silver and nitration patterns were also different from the other organs.

As roots had the clearest intensification profile for protein nitration, these organs were selected as subjects of further research. Previous studies have shown that, during senescence, there is an increase in ROS generation and an induction of antioxidative enzymes such as SODs in leaves (Pastori and del Río, 1997). SOD activity and protein content were thus analysed to determine whether there is a similar behaviour pattern during root development and senescence (Fig. 2). In roots, three SOD isozymes were detected, one MnSOD and two CuZnSODs (I and II, in order of their electrophoretic mobility in the non-denaturing gel). Fig. 2A shows that the MnSOD and CuZnSODs increased during development, with the increase being more evident in 71-day-old plants. Moreover, these increases in SOD activities were accompanied by an enhancement in the protein content of MnSOD and both CuZnSODs (Fig. 2B and C).

Content and cellular localization of NO, ONOO–, and tyrosine nitration in young and senescent roots

This work studied the content and localization of NO, ONOO–, and tyrosine nitration in root cross-sections of 8-day-old (Fig. 3A, C, and E) and 71-day-old (Fig. 3B, D, F).
and F) pea plants. In young roots, an intense green fluorescence due to NO was observed in the epidermal cells and root hairs. In senescent roots, NO extended to some cortex cell layers closer to the epidermis. In the case of ONOO–, although localization in young roots was similar to that observed for NO, in senescent roots localization was also observed in vascular tissues. In the case of tyrosine nitration, the differences between young and senescent roots were greatest, as tyrosine nitration in senescent roots showed considerable intensification and a wide distribution in all cell types (epidermis, cortex, and vascular tissues).

**Proteomic identification of tyrosine-nitrated proteins in roots of senescent 71-day-old pea plants**

Fig. 4A shows the protein staining in a 2D gel of root samples from senescent pea plants and the corresponding immunoblot probed with an antibody against NO2-Tyr. A total of 16 immunoreactive spots were detected. Fig. 4B shows zoom boxes to illustrate in more detail which immunoreactive spots were more intense. All 16 of these spots were analysed by MALDI-TOF mass spectrometry after tryptic digestion using the MASCOT search engine to analyse MS data in order to identify proteins from primary-sequence databases (Perkins et al., 1999). The immunoreactive proteins identified are listed in Table 1, with the protein score confidence interval (CI) in almost all cases being over 99%, indicating that the proteins were not identified by random matches of peptide-mass data.

To gain a deeper understanding of the physiological importance of nitration in roots, the NADP-dependent isocitrate dehydrogenase (NADP-ICDH) was selected from among the proteins identified. NADP-ICDH, with a protein score CI of 100%, is an enzyme involved in cycling, redistribution, and export of amino acids, in addition to its primary function of GS/GOGAT-dependent nitrogen assimilation. NADP-ICDH also has the capacity to generate NAPDH which is essential for redox regulation and responding to oxidative stress (Valderrama et al., 2006; Leterrier et al., 2007, 2012a,b,c).

**Expression and purification of cytosolic NADP-ICDH: effect of ONOO–**

As a means of increasing knowledge of pea NADP-ICDH’s regulation mechanism, the recombinant protein was obtained by sequencing the pea clone and overexpression in *E. coli*. Fig. 5A shows 10% SDS-PAGE analysis of the different
fractions obtained after L YTRAP affinity column chromatography which obtained a recombinant cytosolic NADP-ICDH protein of approximately 66.0 kDa which is in line with the expected molecular mass of the cytosolic ICDH protein (45.0 kDa) plus the Ly-tag (21.28 kDa) predicted theoretically. The fractions E3–E7 show an adequate purity grade for this protein, with a NADP-ICDH activity of 10.1 ± 0.3 µmol NADPH min⁻¹ mg⁻¹ protein for the pharmacological in vitro assay with ONOO⁻, using the molecule SIN-1 as a peroxynitrite donor (Chaki et al., 2009). Fig. 5B depicts the effect of ONOO⁻’s inhibition of activity in a dose-dependent manner ranging from 65% with 0.1 mM SIN-1 to 100% with 5 mM SIN-1. The reliability of nitration by SIN-1 of the cytosolic NADP-ICDH was corroborated by immunoblot analysis of the purified protein using an antibody against NO₂-Tyr. Fig. 5C shows that the nitration grade of NADP-ICDH increased with the SIN-1 concentration.

Identification of the tyrosine nitration site in nitrated recombinant cytosolic NADP-ICDH

With the aim of identifying which of the 12 tyrosines present in the pea plant’s cytosolic NADP-ICDH are targets of this post-translational modification, non-nitrated and peroxynitrite-treated recombinant NADP-ICDHs were subjected to trypsin digestion followed by MALDI-TOF/TOF mass spectrometry examination. Supplementary Table S1 shows the list of peptides scanned and those identified

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### Table 1. Identified 3-nitrotyrosine proteins from pea roots of senescence plants.

| Spot no. | Identified protein | Acc. no. | Protein score%CI (no. of identified peptides) | MW (pI) | Functional grouping |
|----------|-------------------|----------|---------------------------------------------|---------|-------------------|
| 1        | Predicted protein Physcomitrella patens subsp. patens | gi|168066110 | 71.035 (9) | 41562.5 (5.24) | Pepin-like aspartate protease |
| 2        | Amidase, hydantoinase/carbamoylase family protein, expressed Oryza sativa Japonica group | gi|108862917 | 98.676 (10) | 40295.7 (5.62) | Peptidase dimerization domain |
| 3        | NADP-dependent isocitrate dehydrogenase Pisum sativum | gi|44921641 | 100 (13) | 46258.6 (6.2) | Catalyses the reversible conversion of isocitrate to 2-oxoglutarate |
| 4        | Mercuric reductase, putative Ricinus communis | gi|255589011 | 99.829 (10) | 48264.4 (7.15) | Pyridine nucleotide-disulphide oxidoreductase |
| 5        | Type 2 prolyl 4-hydroxylase Nicotiana tabacum | gi|215490183 | 86.137 (8) | 36522.9 (6.64) | Oxygenization of peptidyl proline |
| 6        | Wound-responsive protein-related Arabidopsis thaliana | gi|15237744 | 87.926 (10) | 44928.5 (6.97) | Nucleoside-diphosphate-sugar epimerases (cell-envelope biogenesis, outer membrane/carbohydrate transport and metabolism) |
| 7        | Putative aldo/keto reductase Oryza sativa (ISS. Ostreococcus tauri) | gi|116059968 | 88.988 (8) | 32271.8 (8.32) | Reduces aldehydes and ketones to primary and secondary alcohols |
| 8        | Alcohol dehydrogenase 1 Petunia × hybrida | gi|113363 | 86.452 (9) | 42288.1 (6.19) | Responsible for the conversion of alcohols to aldehydes in plants and is important for NAD metabolism during anaerobic respiration |
| 9        | Os04g0244400 Oryza sativa Japonica group | gi|115457404 | 78.528 (8) | 35479 (7.74) | Glutathione S-transferase |
| 10       | GBSSI Iochroma edule | gi|3755379 | 91.452 (8) | 28328.2 (7.59) | Glycosyltransferase GTB type |
| 11       | Putative vitamin B-12-independent methionine synthase Pisum sativum | gi|121053772 | 100 (3) | 34861.4 (5.75) | URO-D CIM-like protein superfamily |
| 12       | ANN6 (ANNEXIN Arabidopsis 6. A. thaliana | gi|15238094 | 94.086 (10) | 36661.1 (7.72) | Calcium ion-binding/calcium-dependent phospholipid binding |
| 13       | Peroxidase Pisum sativum | gi|62909963 | 100 (6) | 38649.4 (8) | Horseradish peroxidase and related secretory plant peroxidases |
| 14       | Fructose-bisphosphate aldolase, cytoplasmic isozyme 2 Pisum sativum | gi|1168410 | 100 (11) | 38638.1 (6.77) | Carbohydrate degradation, glycolysis |
| 15       | Endochitinase A2; Flags: Precursor Pisum sativum | gi|1705807 | 99.448 (3) | 35738.8 (7.33) | Catalyses the hydrolysis of the beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers |
| 16       | Predicted protein Populus trichocarpa | gi|224080749 | 100 (3) | 35304.5 (8.06) | Chitinase |
by LC-MS/MS. Among the identified peptides, only one contained a nitrated tyrosine with the amino acid sequence N389-EHYLNTEEFIDA V AAELK-C407. Fig. 6 shows the comparison of the nitrated (top) and unmodified (bottom) MS/MS spectra of the identified peptide from the cytosolic NADP-ICDH. The nitrated peptide EHYNTEEFIDAVAAELK (Z = 2) has a total of 18 amino acids and a mass of 2136 Da (2091 + 45 Da) which is compatible with the acquisition of a nitro group on Tyr392.

Fig. 7 shows the 3D model of cytosolic NADP-ICDH in pea plants showing Tyr392 and its potential interactions. Thus, Tyr392 is located at 8.6 Å of the Lys376 and His317 residues involved in the NADP-binding site (Huang and Colman, 2005). Arg111, Arg134, Tyr141, Lys214, Asp277, Asp281, and Glu306 appear to be the amino acids involved in isocitrate binding.

**NADP-ICDH activity in roots of young and senescent pea plants**

With the aim of investigating the physiological effects of the nitration of NADP-ICDH during senescence, its activity was analysed in the roots of young 8-day-old and senescent 71-day-old pea plants. Fig. 8 shows that NADP-ICDH activity fell by 75% in senescent roots. Catalase activity was also analysed and was observed to increase by 20%.

**Discussion**

In animal and plant cells, tyrosine nitration of proteins is a PTM mediated by NO-derived molecules that has been used as a marker of pathological processes and/or oxidative stress situations (Ischiropoulos, 2003; Dalle-Donne et al., 2005; Corpas et al., 2007). However, a body of new evidence indicates that tyrosine nitration could be involved in signalling processes (Gow et al., 2004) and alter the protein function (Alvarez and Radi, 2003; Radi, 2004; Lozano-Juste et al., 2011; Melo et al., 2011). As far as is known, there is no information available on protein tyrosine nitration in relation to plant development and senescence. In order to obtain basic information on tyrosine nitration in plant proteins, this modification in the main organs of plants during their development and senescence has been analysed using a proteomic approach with an antibody against nitrotyrosine (Chaki et al., 2009) which recognizes nitrated proteins that have undergone PTM mediated by NO-derived molecules. In this context, pea plants were selected as a model because basic information is available on the metabolism of NO (Leshem and Haramaty, 1996) which includes the characterization of l-arginine nitric oxide synthase (NOS) activity in different organs during seedling development and how NOS activity decreases in senescent leaves. Thus, various approaches have shown the presence of NOS activity (Barroso et al., 1999; Corpas et al., 2004) as well as the localization of NO in different organs of pea plants during their development (Corpas et al., 2006a; Kolbert et al., 2008) and the metabolism of RNS under environmental stress conditions (Barroso et al., 2006; Rodriguez-Serrano et al., 2006; Corpas et al., 2008).

The data indicate that, during the development of pea plants, each organ has a specific nitration pattern. However, among the different organs analysed, the root particularly showed a pattern that clearly increased during both development and senescence. This organ was therefore chosen in
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order to gain a deeper understanding of the role played by nitration in roots.

Roots are essential organs that undergo morphological changes during plant development such as elongation and

Fig. 6. Comparison of the nitrated (top) and unmodified (bottom) MS/MS spectra of the identified peptide (EHYLNTEEFIDAVAAELK) from the cytosolic pea NADP-isocitrate dehydrogenase.

Fig. 7. Three-dimensional model of cytosolic NADP-isocitrate dehydrogenase showing Tyr392 (red) and its potential interactions. Conserved residues implicated in the binding of Mg$^{2+}$ (green), isocitrate (blue), and NADP (yellow); catalytic residues are shown as purple or orange. The NADP-binding site includes Lys376 and His317 and is at 8.6 Å of the nitrated Tyr392 (this figure is available in colour at JXB online).

Fig. 8. NADP-isocitrate dehydrogenase and catalase activities in roots of young (8-day-old) and senescent (71-day-old) pea plants. Asterisks indicate significant differences from control values ($P < 0.05$).
the formation of secondary roots and hair roots to facilitate water and nutrient uptake and to anchor the plant body in the soil. In addition, under adverse growth conditions, root systems can also be subject to various changes in their architecture for adaptation purposes (de Dorlodot et al., 2007). Roots also undergo structural and functional modifications in order to adapt to nutrient- and water-deficient stresses as well as to pathogen attacks and interaction with beneficial micro-organisms (fungus or bacteria) (Barea et al., 2005; Mehta et al., 2008). All these heterogeneous events change during root development and some can result in the senescence or death of certain roots or the whole root system. Various aspects of NO’s role in root development have been studied that highlight its importance in roots. Thus, in pea plants, it has been demonstrated that there is a temporal correlation between root development and NO production from l-Arg-dependent NOS activity (Corpas et al., 2006a). Additionally, the presence of NOS activity in plant roots has been detected in several plant species (Cueto et al., 1996; Ribeiro et al., 1999). Moreover, the application of exogenous NO donors induces root tip elongation (Kopyra and Gwóźdź, 2003) and the formation of adventitious roots (Pagnussat et al., 2002, 2004).

Similar behaviour has been observed in tomato seedlings where the application of the NO donor sodium nitroprusside induced lateral root emergence and elongation, affecting the expression of cell cycle regulatory genes and modulated cellulose synthesis (Correa-Aragunde et al., 2004, 2006, 2008). It has also been reported that NO donors counteract the inhibitory effect of heavy metals and salinity on root growth, suggesting that NO detected in the root hairs of pea plants could also play a protective role under abiotic stress conditions produced by heavy metals (Cd, Pb) and NaCl (Kopyra and Gwóźdź, 2003). Under cadmium stress conditions, endogenous NO content is thus reduced in roots (Rodriguez-Serrano et al., 2006). In previous studies of pea plants, in senescent pea leaves, the current study group observed that NO content is reduced (Corpas et al., 2004), which was closely in line with previous data on pea and other plant species (Leshem and Haramaty, 1996; Leshem et al., 1998). However, in roots from senescent plants, an opposite behaviour pattern was observed, with a rise in NO content being recorded. Moreover, oxidative stress has been reported in previous studies carried out during and induced senescence of pea leaves (Pastori and del Río, 1997). Under the experimental conditions of the current study group, the activity and protein expression of all SOD isoenzymes were observed to increase, indicating an alteration in the ROS metabolism (Swanson and Gilroy, 2010). This was accompanied by a rise in peroxynitrite and tyrosine nitration as evaluated by CLSM. Unfortunately, as far as is known, there is no data available on protein tyrosine nitration during development and senescence in plant cells in order to make a comparative analysis.

In recent years, the use of proteomic analysis has started to contribute to the identification of new targets of NO-mediated PTMs, and the application of exogenous RNS (NO donors, peroxynitrite) could help to identify these target proteins. However, the identification of targets under physiological conditions is a challenge where different factors must be considered such as the relatively low abundance of tyrosine nitration and that this nitration process is generally induced under adverse stress conditions. Therefore, the results of this study of roots contribute to the knowledge of tyrosine nitration as some of the candidates identified are involved in the process of microbial interaction as well as the response to biotic and abiotic stresses (Table 1) where tyrosine residues play an essential role. For example, chitinases catalyse the hydrolysis of chitin, which is a major component of the cell wall of most fungi, and it has been shown that some chitinase isoforms can mediate the formation of mycorrhiza (Slezack et al., 2001) where NO accumulation has been described as a novel component in the signaling pathway that leads to the establishment of mycorrhizal symbiosis (Calcagno et al., 2012). Furthermore, the catalytic site of this enzyme involves an essential tyrosine residue (Verburg et al., 1992). Mercuric reductase is an inducible NADPH-dependent and flavin-containing disulphide oxidoreductase enzyme involved in environmental inorganic mercury detoxification. A putative mercuric reductase has been found in Ricinus communis (Chan et al., 2010) and, under the experimental conditions of the current study group, also detected in pea roots. A remarkable feature of this enzyme is the binding site for Hg⁺ involving two tyrosine residues (Rennex et al., 1994). Fructose-bisphosphate aldolase (also called aldolase) is involved in carbohydrate degradation. This enzyme accumulates in roots, especially in the apical region. Aldolase has also been shown to be physically associated with vacuolar H-ATPase in roots and may regulate the vacuolar H-ATPase-mediated control of cell elongation that determines root length (Konishi et al., 2004). In mammalian systems, this enzyme is subject to tyrosine nitration under different inflammatory conditions which causes activity loss (Koeck et al., 2004). However, the possible involvement of tyrosine nitration in each protein needs to be corroborated experimentally.

The present study aimed to extend knowledge of the nitration process during plant development and senescence and so have focused on the cytosolic NADP-dependent isocitrate dehydrogenase (NADP-ICDH). This enzyme catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate with the production of the reduced coenzyme NADPH (Gálvez and Gadal, 1995). Nitrination has been shown to provoke a loss of function with respect to different plant activities in vitro condition such as ascorbate peroxidase, catalase (Clark et al., 2000), S-Adenosyl homocysteine hydrolase (Chaki et al., 2009), ferredoxin-NAD reductase, carbonic anhydrase (Chaki et al., 2013), and O-acethylserine(thiol)lyase A1 (Alvarez et al., 2011). Thus, mass spectrometric identification of Tyr392 as nitrated tyrosine residues in NADP-ICDH after treatment with SIN-1 offers an effective means of gaining a greater insight into the modulation of the enzyme by tyrosine nitration. According to the enzyme’s model, Tyr392 is located at the active site, and nitration may account for the inhibition of enzymic activity. From a functional point of view, the location of Tyr392 suggests that its nitration may disrupt ICDH–coenzyme interaction and influence the enzyme’s catalytic activity.

The presence of NADP-ICDH in roots has been described in many plant species including pea plants (Chen et al., 1988),
alfalfa (Shorrosh and Dixon, 1992), soybean (Udvardi et al., 1993), pines (Palomo et al., 1998; Pascual et al., 2008), and Arabidopsis (Leterrier et al., 2012b) and it has always been associated with the metabolism of amino acids and ammonia (Fieuw et al., 1995; Gálvez and Gadal, 1995). However, the concomitant generation of NADPH opens up new avenues in the functioning of this enzyme given that this co-factor is necessary for different metabolic pathways including the ascorbate–glutathione cycle, the superoxide radical generated by NADPH oxidase, and NO generation through t-arginine-dependent NOS activity. It has been suggested that this enzyme could support antioxidative systems in response to adverse stress conditions (Valderrama et al., 2006; Leterrier et al., 2007, 2012c; Airaki et al., 2012) during leaf senescence (Corpas et al., 1999), development and ripening of fruit (Gallardo et al., 1995; Sadka et al., 2000; Mateos et al., 2009), and protection against oxidative stress in pea nodules (Marino et al., 2007). Under the current experimental conditions, roots were observed during senescence to undergo a general increase in the content of NO, ONOO⁻, and protein nitration. This was accompanied by an increase in antioxidative enzymes (SODs and catalase); however, NADP-ICDH nitration. This was accompanied by an increase in anti-oxidative enzymes (SODs and catalase); however, NADP-ICDH activity was downregulated, which closely correlates with the inhibition provoked by the nitration of Tyr392. Similar behaviour has been observed in Arabidopsis plants subject to arsenic stress which, in roots, provoked a rise in the content of NO, superoxide radicals, ONOO⁻, and protein nitration, although NADP-ICDH activity was observed to fall (Leterrier et al., 2012a).

In summary, the data indicate that each organ has a specific nitrotirosine protein pattern and that the intensity of the proteins which undergo this post-translation modification increases during the senescence of roots, stems, and leaves, with roots having the largest number of nitrated proteins. The nitrated proteins identified open up new perspectives where nitration of tyrosine could participate in regulating these proteins not only during development but also in the interaction of beneficial micro-organisms with roots (mycorrhizas, nodules) as well as responding to biotic and abiotic stresses. Specifically, during root senescence, cytosolic NADP-ICDH experiences specific nitration in Tyr392 that is involved in the binding site of NADPH, which provokes its inhibition. Thus, it is proposed that the inhibition of NADP-ICDH should contribute to the process of root senescence given that this enzyme is involved in the nitrogen metabolism and generates NADPH which is a key cofactor in cellular redox homeostasis.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Peptides scanned and peptides identified by LC MS/MS.

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