1 **Title:** *In vivo* protein tyrosine nitration in *Arabidopsis thaliana*

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17 **Date of submission**: January 27, 2011

18 **Number of tables**: 3

19 **Number of figures**: 4

20 **Running title**: Protein nitration in Arabidopsis

21

Abstract

Nitration of tyrosine (Y) residues of proteins is a low abundant post-translational modification that indeed modulates protein function or fate in animal systems. However, very little is known about the *in vivo* prevalence of this modification and its corresponding targets in plants. Immunoprecipitation, based on an anti-3-nitroY antibody, was performed to pull down potential *in vivo* targets of Y nitration in *Arabidopsis thaliana* proteome. Further shotgun LC-MS/MS proteomic analysis of the immunoprecipitated proteins allowed identifying 127 proteins. Around 35 % of them corresponded to homologues of proteins that have been previously reported as Y-nitrated in other non-plant organisms. Some of the putative *in vivo* Y-nitrated proteins were further confirmed by Western-blot with specific antibodies. Furthermore, MALDI-TOF analysis of protein spots, separated by 2-DE from immunoprecipitated proteins, led to the identification of seven nitrated peptides corresponding to six different proteins. However, *in vivo* nitration sites among putative targets could not be identified by MS/MS. Nevertheless, a MS/MS spectrum with 3-aminoY318 instead of expected 3-nitroY was found for cytosolic glyceraldehyde-3-phosphate dehydrogenase. Reduction of nitroY to aminoY during MS-based proteomic analysis together with the *in vivo* low abundance of these modifications made the identification of nitration sites difficult. In turn, *in vitro* nitration of methionine synthase, which was also found in our shotgun proteomic screening, allowed identifying unequivocally a nitration site at Y287.

Key words: Arabidopsis, post-translational modification, protein nitration, nitrotyrosine, nitroY, aminoY, nitric oxide.

Introduction

During the last twenty years, NO has been characterized as an essential regulator of many physiological processes in animals. More recently, NO has been characterized as a signal molecule regulating plant defence against pathogens (Romero-Puertas *et al*. 2004; Mur *et al*. 2006), resistance to abiotic stress (Zhang *et al*. 2006), and different developmental processes including seed dormancy and germination (Bethke *et al*. 2006; Liu *et al*. 2007), floral transition (He *et al*. 2004; Simpson 2005) and leaf senescence (Mishina *et al*. 2007). NO acts as a regulator of gene expression at the transcriptional level by regulating disease resistance processes (Polverari *et al.* 2003), the expression of stress-related transcription factors and signalling-related kinases (Parani et al. 2004), and by the interaction with other signalling molecules such as salicylic acid (SA) and jasmonic acid (JA) (Grün *et al*. 2006).

Some of the regulatory properties of NO are exerted through NO-mediated post-translational modifications including nitrosylation of thiol groups and nitration of tyrosine (Y) residues (Gow *et al*. 2004). This is thought to affect the activity, the stability or the intracellular location of proteins thus potentially altering their functions and eventually cell signalling. The regulation of protein function at the levels of NO-related post-translational modifications represent a new area of research in plant biology, and it will help to elucidate the mode of action of NO in regulating many processes in plants. Recent reports suggest that S-nitrosylation is specific and regulated (Lindermayr *et al*. 2005; Romero-Puertas *et al*. 2008), and it may play a

1 regulatory role on central processes in plants such as ethylene biosynthesis
2 (Lindermayr *et al*. 2006). The interaction between NO and superoxide leads to the
3 formation of peroxynitrite, a reactive molecule with strong nitrating activity (Szabó
4 *et al*. 2007). It has been reported the production of peroxynitrite under physiological
5 conditions in plants (Bechtold *et al*., 2009; Chaki *et al*., 2009). Some proteins are
6 targets of peroxynitrite and the nitration of Y residues to 3-nitrotyrosine represents a
7 hallmark of post-translational protein modification associated with human
8 pathologies and biological aging (Hong *et al*. 2007). Although well characterized in
9 mammals, scant information is available on nitration of proteins in Y residues in
10 plants. Detection of nitrated proteins was first reported in tobacco plants with
11 reduced nitrite reductase activity (Morot-Gaudry-Talarmain *et al*. 2002). After that, it
12 has been reported the detection of *in vivo* nitrated proteins in plants treated with
13 exogenous nitrating reagents (Saito *et al*. 2006) as well as under physiological
14 conditions in both unstressed conditions (Chaki *et al*. 2009) or upon pathogen
15 challenge (Romero-Puertas *et al*. 2007; Cecconi *et al*. 2009). However, in all these
16 recent reports there is no data about unequivocal identification of nitrated peptides or
17 proteins (i.e. nitration sites). Here we report on the identification of potential *in vivo*
18 nitration sites of some Arabidopsis proteins. Drawbacks in proteomic approaches to
19 identify Y-nitration post-translational modification under physiological conditions
20 are also discussed. The analysis of the regulatory functions of Y-nitration of proteins
21 in any plant biological process will require, after initial identification of potential
22 targets, a case-by-case analysis. Recent proteomic approaches based on the
23 protection of primary amino group by acetylation followed by the reduction of
24 nitroY to aminoY residues, and further derivatization of amino group from aminoY
25 residues (Chiapetta *et al*. 2009; Tsumoto *et al*. 2010) will help to overcome some of

the difficulties found due to low abundance and limited stability of nitroY residues in proteins found as potentially nitrated *in vivo* in this work.

Materials and Methods

Plant growth conditions

Seeds of Col-0 wild type accession of *Arabidopsis thaliana* were sown in moistened soil and grown under photoperiodic conditions (cycles of 8 h day and 16 h night for short days, at 22 ºC and 20 ºC respectively) as mentioned in different experiments. Plants were illuminated with 150 µE m^{-2} s^{-1} cool-white fluorescent lamps and grown under 60 % relative humidity. Alternatively, surface sterilized seeds were germinated and grown in sterile liquid or agar-supplemented Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands) with 1 % (w/v) sucrose.

Protein extraction and immunoprecipitation

Two week-old seedlings were frozen and ground in liquid nitrogen. Proteins were extracted by adding extraction buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % (v/v) protease inhibitor cocktail from Sigma, USA] and briefly vortexing. Protein extracts were obtained by 13000 x g centrifugation at 4 ºC. Protein extracts (4 x 1 mg) were each pre-cleared with 50 µL of Protein A-agarose (EZView Sigma, USA) for 8 hours at 4 ºC. The unbound fractions were each incubated overnight with 0.1 µg of monoclonal anti-3-nitroY antibody (Cayman, USA) at 4 ºC. To recover 3-nitroY-containing proteins, 60 µL of Protein A-agarose were added and incubated for 8

1 hours at 4 ºC. After extensive washing with extraction buffer, proteins were eluted at
2 95 ºC with elution buffer (1% SDS, 100 mM DTT, 50 mM Tris-HCl pH=7.6) three
3 times. After removing agarose beads with a 0.2 µm filter (Costar Corning, NY,
4 USA), the proteins were precipitated, combined and processed with 2D-Clean Up Kit
5 (GE, UK) for subsequent 2-DE and LC-MS/MS analysis.
6
7 *2-DE and image analysis*
8
9 Protein samples (100 µg) were dissolved in DeStreak Rehydration solution (GE, UK)
10 before electrophoresis. For first dimension, 18cm 3-10NL strips were passively
11 rehydrated overnight at room temperature. Then the set-up of the IPGphor3 (GE,
12 UK) was 1 hour 50V step-and-hold, 1 hour 150 V gradient, 1hour 30 minutes 500 V
13 gradient, 1 hour 30 minutes 1000 V gradient, 2 hours 4000 V gradient, 2 hours 8000
14 V gradient and 7 hours 8000 V step-and-hold. The strips were then treated with 1 mg
15 ml^{-1} of DTT for 15 min and then alkylated with 25 mg ml^{-1} of iodoacetamide for 15
16 min in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % glycerol, 2 %
17 SDS and 0.002 % bromophenol blue), and the focused proteins were then separated
18 in 12.5 % acrylamide gels in the EttanDalt six electrophoresis unit (GE, UK) as
19 recommended by manufacturers for an overnight run. The gels were stained with
20 DeepPurple (GE, UK) or PlusOne™ Silver Staining Kit (GE, UK), digitalised with
21 Typhoon (GE, UK) and analyzed by using Image Master Platinum 5.0 (GE, UK)
22 software.
23
24 *MS analysis*
25

1 Samples were digested with sequencing grade trypsin (Promega, USA). Peptide
2 separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC
3 Packings) and a QSTAR XL Q-TOF hybrid mass spectrometer (MDS Sciex-Applied
4 Biosystems). Samples (5 µL) were delivered to the system using a FAMOS
5 autosampler (LC Packings) at 40 µL min^{-1}, and the peptides were trapped onto a
6 PepMap C18 pre-column (5 mm, 300 m i.d; LC Packings). Peptides were then eluted
7 onto the PepMap C18 analytical column (15 cm, 75 m i.d; LC Packings) at 200 nl
8 min^{-1} and separated using a 55 min gradient of 15–50% ACN (120 min for the
9 mixtures). The QSTAR XL was operated in information-dependent acquisition
10 mode, in which a 1-s TOF MS scan from 400–2000 m/z, was performed, followed by
11 3-s product ion scans from 65–2000 m/z on the three most intense doubly or triply
12 charged ions. Database search on Swiss-Prot and NCBInr databases was performed
13 using MASCOT search engine (Matrix- Science). Searches were done with tryptic
14 specificity allowing one missed cleavage and a tolerance on the mass measurement
15 of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of C
16 was used as a fixed modification and oxidation of M, deamidation of D and E, and
17 nitration or amination of Y as variable modifications.
18
19 *Western-blot*
20
21 Protein extracts (10 µg) were separated by SDS-PAGE, blotted onto nitrocellulose
22 membrane, stained with Ponceau-S and probed with antibodies at the followed
23 dilutions: monoclonal anti-3-nitroY (Cayman Chemicals) 1:1000 and anti-5xHis
24 (QIAGEN) 1:8000, and polyclonal anti-GAPDH 1:10000; anti-CA 1:3000; anti-PKL
25 1:5000; anti-FBPase 1:2000; anti-GRP 1:2500. Secondary antibody was anti-mouse

1 or anti-rabbit, for monoclonal or polyclonal primary antibodies respectively, coupled
2 to horseradish peroxidase (GE, UK) at 1:10000 dilution, and ECL kit (GE, UK) was
3 used for visualization of proteins.
4
5 *GAPDH activity*
6
7 Proteins were extracted in 50mM Tris-HCl pH=7.4 and quantified. GAPDH activity
8 of the extracts was assayed according to Muñoz-Bertomeu et al (2009) with minor
9 modifications. Briefly, 50 µg of protein extrats from plants treated or not with 2 mM
10 SIN-1 or 0.5mM SNP were incubated in reaction buffer (10 mM Tris-HCl pH=7.4,
11 20 mM Arsenate, 2 mM NAD, 0.5 mM DTT) and the reaction was initiated by the
12 addition of 2 mM of DL-glyceraldehyde-3-phosphate in a final volume of 1mL.
13 GAPDH activity was measured following the conversion of NAD to NADH at 340
14 nm during 4 minutes.
15
16 *Synthesis, purification and nitration of His-tagged methionine synthase AtMS1*
17
18 A plasmid containing *AtMS1* cDNA fused to 6xHIS tag (Dixon *et al*. 2005) was used
19 to transform BL21(DE3) competent cells (Sigma-Aldrich) for recombinant protein
20 production. For protein induction, cell cultures with OD = 0.7 were treated with
21 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours. Recombinant
22 protein production was checked by SDS-PAGE and Western-blot analysis.
23 Recombinant protein purification was carried out with the QIAexpress Ni-NTA Fast
24 Start Kit (Qiagen) following manufacturers recommendations. Purified AtMS1 were
25 treated or not with a nitrating buffer as described previously (Chen *et al*. 2008).

1 Briefly, 10 µL of purified protein were incubated with 500 µM H_2O_2 and 500 µM

2 $NaNO_2$ in 0.1 M pH = 7.2 potassium phosphate buffer for NO_2 radical-mediated

3 protein nitration at 37 ºC in dark for 2 hours in a total volume of 500 µL. To clean

4 nitrated protein, the nitrating reaction volume was filtered trough a 10 kDa cut-off

5 filter (Microcon, Ambion). Proteins were then analyzed by SDS-PAGE and Western-

6 blot. Protein nitration was confirmed with anti-3nitroY antibody (Cayman) and the

7 anti-5xHis antibody supplied by the manufacturers (QIAGEN). A duplicate gel was

8 run and stained with coomassie-blue, the bands excised, trypsin digested and further

9 analyzed by LC-MS/MS as described above.

10

11 *Protein modelling and structural analysis*

12

13 3-D protein models were generated by homology modelling at the SWISS-MODEL

14 workspace (Arnold *et al*. 2006) using the coordinates of GAPDH from rat (PDB code

15 2VYN), Serine Hydroxymethyltransferase from *Mycobacterium tuberculosis* (PDB

16 code 3H7F), Transketolase from Maize (PDB code 1ITZ), RuBisCO form Spinach

17 (PDB code 1IR1) and Mannitol Dehydrogenase from *Cladosporium harbarum* (PDB

18 code 3GDF) as templates. For Methionine synthase, crystal structure from

19 *Arabidopsis thaliana* was used (PDB code 1U1J). Model qualities were evaluated by

20 ANOLEA, Verify3D and Procheck (Melo and Feytmans, 1998; Bowie *et al*. 1991,

21 Laskowski *et al*. 1996, respectively). 3-D models were visualized and manipulated

22 with Yasara (www.yasara.org) or PyMol (www.pymol.org). Distance between

23 residues in Amstrongs (Å) and the presence of hydrogen bonds was analyzed with

24 both programs using default settings.

25

1

2 **Results**

3

4 Crude protein extracts from *Arabidopsis thaliana* plants contained a number of
5 proteins spanning the whole range of molecular weights that cross-react with
6 antibodies against 3-nitroY in Western blot analysis (Fig. 1A). The specific cross-
7 reaction of antibodies with 3-nitroY residues of those target proteins was checked by
8 on-membrane protein reduction of 3-nitroY to 3-aminoY with sodium dithionite, as
9 previously reported (Miyagi *et al*. 2002), thus resulting in no cross-reaction with the
10 specific antibodies (Fig. 1A). Upon antibody validation, anti-3-nitroY antibodies
11 were used as a specific immunoprecipitation reagent together with Protein A-agarose
12 to pull down 3-nitroY-containing proteins from crude Arabidopsis seedling extracts.
13 Figure 1B shows that a small number of proteins present in the crude extracts, less
14 than twenty bands as detected by Coomassie staining, were recovered in the
15 immunoprecipitated fraction. Those proteins were further checked to cross-react in
16 Western blots with anti-3-nitroY antibodies (Fig. 1B). A moderate enrichment in
17 nitrated proteins was thus observed in the immunoprecipitated fraction (Fig. 1B).
18 Considering the low resolution capacity of one dimension SDS-PAGE, the
19 complexity of the immunopurified samples was further assessed by 2-DE and the
20 more sensitive silver staining, resulting in the separation of around 450 spots with
21 isoelectric point in the 3 to 10 range (Fig. 1C).

22 To identify potential *in vivo* targets of Y nitration in Arabidopsis, the
23 immunopurified fraction was then analyzed by MS following two different strategies.
24 First, a shotgun analysis, based on LC-MS/MS of the immunoprecipitated proteins,
25 was performed. Comparison of MS generated data with SwissProt database by

1 specifying taxonomy for Arabidopsis allowed us to identify 127 proteins with a
2 statistically significant Mascot score of at least 35 and more than two matched
3 peptides (Table 1). Among identified proteins, 35 % have homologue counterparts
4 that have been previously reported as nitrated in non-plant organisms (Table S1),
5 thus supporting the usefulness of the immunoprecipitation approach to enrich the
6 purified fractions in potential Arabidopsis nitrated proteins. To further validate our
7 proteomic identification, we proceed to detect several of the identified proteins by
8 Western blots with specific antibodies in the samples immunopurifed by
9 precipitation with anti-3-nitroY antibodies. We selected some proteins identified
10 with Mascot score higher than 200, such as chloroplastic glyceraldehyde-3-
11 phosphate dehydrogenase (GAPDH), carbonic anhydrase (CA) or fructose-1,6-
12 bisphosphatase (FBPase) and some other with lower score such as the glycine-rich
13 RNA-binding protein 7 (GRP7, score 66) and the CHD3-type chromatin remodelling
14 factor PICKLE (PKL, score 58). All of them cross-reacted with proteins in the 3-
15 nitroY-immunoprecipitated samples (Fig. 2), making the proteomic identification
16 reliable. For proteins such as GAPDH or PKL showing no signal in the supernatant,
17 most of the corresponding proteins were associated to IP resin and a significant
18 proportion further recovered after washings in the IP. By contrast, the
19 immunoprecipitation is far less efficient for others such as FBPase or CA showing
20 similar amount of protein in the supernatant and CE, thus suggesting the
21 corresponding nitrated forms should not be abundant in the total protein population
22 of crude extracts. Most of the proteins tested gave complex patterns of cross-reacting
23 bands both in crude extracts and immunopurified samples (Fig. 2, Fig. S5). This is
24 likely due to different cross-reactive isoforms that are the result of potential post-
25 translational modifications or to unespecific cross-reaction of the antibody.

1 Despite the success in identifying a large number of potentially nitrated
2 proteins, no MS/MS spectrum with good enough MASCOT score was obtained for
3 nitrated peptides, thus preventing the identification of unequivocal nitration sites. To
4 overcome this, and because the amount of protein required for the identification of
5 nitrated peptides is often a limitation in the method, the most abundant proteins in 2-
6 DE gels from 3-nitroY-immunoprecipitated proteins were excised from the gels,
7 digested with trypsin and further analyzed by MALDI-TOF. Table S2 summarizes
8 the identified proteins, their MASCOT scores, the number of non-redundant peptides
9 and the corresponding sequence coverage. Twenty two proteins were identified with
10 MASCOT score above 59 considered as significant in the proteomic analysis.
11 Unfortunately, we could not obtain any MS/MS spectra with high enough score
12 corresponding to a *bona fide* Y-nitrated peptide. However, six out of 22 identified
13 proteins showed MALDI-TOF spectra for potentially nitrated peptides with a
14 signal/noise ratio above 25 considered as significant in the analysis. The
15 simultaneous identification of nitrated peptides and their unmodified forms in
16 addition with the length of the nitrated peptides identified ($\geq$ 7 amino acid residues)
17 makes the identification more reliable (Stevens *et al*. 2008). Table 2 shows the
18 identity of those proteins and the corresponding nitrated peptides with signal/noise
19 ratio, the molecular mass of the unmodified and modified peptides and the
20 corresponding +45 shift to the modification of Y to nitroY. Three out of those six
21 proteins (Rubisco, Rubisco activase and transketolase) showed nitrated peptides
22 containing a single Y residue and a +45 mass shift, thus allowing the assignment of a
23 putative nitration site for those proteins. For Serine hydroxymethyltransferase, the
24 nitrated peptide contained two Y residues and a mass shift of +90 compatible with
25 two Y nitration sites. Finally, for the other two proteins, a cytosolic GAPDH and a

putative mannitol dehydrogenase, the nitrated peptides contained two Y residues and a mass shift of +45 corresponding to a single nitration event, so no nitration site could be proposed for these proteins (Table 2). Y residues contained in the nitrated peptides were checked to fulfill the previously characterized factors determining the selectivity of Y-nitration in proteins. These factors include the proximity of a basic amino acid within the primary sequence, the exposure of the aromatic ring to the surface of the protein, the location of the Y residue on a loop structure, its association with a neighbouring negative charge, and the proximity of the proteins to the site of nitrating agents generation (Souza *et al*. 1999; Ischiropoulos 2003; Chaki *et al*. 2009). With the exception of Rubisco activase, for which no structural model is available, the structures of the proteins were modelled as indicated in Materials and Methods. All putative nitrated Y residues had acidic residues close enough (less than 10 Å from Y target) and all of them have basic amino acids in the primary sequence flanking the Y residue (Table 3). However, only Y337 and Y135 from transketolase and putative mannitol dehydrogenase, respectively, were located in loops, and most of them showed Accesibility Solvent Area (ASA) indexes below 70 (Table 3), thus having low probability to be efficiently exposed to the solvent. Regarding the proximity of the proteins to the site of generation of nitrating agents, all the proteins identified are located at subcellular compartments previously characterized as sites of NO and superoxide production in plants such as apoplast, mitochondria and chloroplast (Jasid *et al*. 2006; Flores-Pérez *et al*. 2008; Corpas *et al*. 2001; Bethke *et al*., 2004; Gupta *et al*. 2005; Igamberdiev and Hill 2009). In addition, the fact that some of Y residues found as potentially nitrated are highly conserved Y residues in proteins functionally homologous in other organisms (Fig. S1), confer potential functional relevance to this post-translational modification as a regulatory

1 mechanism of their activity/function. Regarding this, we have confirmed that
2 treatment of seedlings with the peroxynitrite donor, such as SIN-1, led to inhibition
3 of GAPDH activity (Fig. 3A).
4 Despite efforts made to identify *in vivo* Y-nitration sites among proteins
5 immunoprecipitated with anti-3-nitroY, not a single MS/MS spectrum corresponding
6 to a nitrated peptide was identified. Two causes might explain this lack of success.
7 First, nitrated form of the identified proteins could be naturally very low abundant in
8 the analyzed samples, thus making MS/MS-based identification extremely difficult.
9 Second, the lack in detecting nitrated peptides may be the result of the unstable
10 nature of nitroY under the conditions used to process samples by MS. Regarding the
11 latter, it has been proposed that the nitro group linked to Y residues of proteins can
12 be reduced to amino group (Sarver *et al*. 2001; Tsumoto *et al*. 2010). When crude
13 proteomic data from 2-DE gels-excised spots were searched for aminoY instead of
14 nitroY post-translational modification, a MS/MS fragmentation spectrum
15 corresponding to LVSWYDNEWGYSSR peptide (monoisotopic mass of neutral
16 peptide of 1776.7631; ion score 43; expect 0.00088) was found for cytosolic
17 glyceraldehyde-3-phosphate dehydrogenase (G3PC). This spectrum included a +15
18 shift compatible with an amino modification of Y318 (Fig. 3B). These data suggest
19 that from two Y residues found as potential targets to be nitrated in G3PC (Table 2),
20 part of the 3-nitroY318 residues in the protein population might undergo reduction to
21 3-aminoY318 under the conditions used for MS analysis. Since reduction may occur
22 with any nitroY, the crude data from LC-MS/MS shotgun analysis were searched for
23 aminoY modification, and found 51 putative aminoY-containing peptides with ion
24 scores above15 that corresponded to 47 different proteins (Table S3). Comparison of
25 nitroY and aminoY searches led to only five peptides that were detected with Y

1 nitrated or aminated in the same residue, but all of them had ion scores below 10,
2 thus suggesting the partial reduction of nitroY may lower the abundance of both
3 modifications making MS identification even more difficult.

4 To overcome the low abundance of nitrated forms of proteins found *in vivo*,
5 one of the proteins identified in our screen as potentially nitrated, 5-methyl
6 tetrahydropteroyltriglutamate-homocysteine S-methyltransferase or methionine
7 synthase 1 (AtMS1) was expressed as a 6xHis tagged version in bacteria. The tagged
8 recombinant protein was expressed to moderately high levels by 5 h after induction
9 with IPTG (data not shown). Crude recombinant extracts were checked for AtMS1
10 protein content by Western blot with anti-5xHis tag antibodies and, subsequently,
11 purified with Ni-resin. Purified protein was then split into two equivalent samples,
12 being one of them nitrated *in vitro* whereas the other was used as control of no
13 exogenous nitration. The efficiency of nitration was then checked by Western blot
14 with anti-3-nitroY. No cross-reacting band was detected in control protein but a
15 strong signal was observed in nitrated recombinant AtMS1 protein (Fig. 4A). Both
16 samples had comparable levels of recombinant protein as confirmed by Western blot
17 with anti-5xHis antibodies (Fig. 4A). A duplicate 1D-SDS-PAGE gel was stained
18 with Coomassie and the bands corresponding to nitrated and not nitrated proteins
19 were excised from the gel, digested in gel with trypsin and further analyzed by LC-
20 MS/MS. We found the same YLFAGVVDGR peptide from control not nitrated
21 protein (m/z 1096.58, Score 85) and nitrated protein (m/z 1141.54, Score 53),
22 showing a shift of 44.96 equivalent to the typical shift of a single nitration (Fig. 4C).
23 The MS/MS spectrum of nitrated peptide showed most of the peaks corresponding to
24 the y and b series and also the immonium ion of nitrated Y287 residue (Fig. 4B).
25 These data allowed identifying an unequivocal site of nitration in AtMS1 in the

Y287. Whether this post-translational modification of AtMS1 may alter its activity, stability, subcellular location or further post-translational modifications will require more work. Nevertheless, Y287 is conserved in plant methionine synthases but not in yeast MSs (Fig. S2A), and it is located in a loop on the external surface of the protein far from the 5-methyl tetrahydropteroyltriglutamate (THG) and homocysteine binding sites inside catalytic pocket (Fig. S2B). Y287 forms hydrogen bonds with two proximal residues T262 and F264, which may be important to keep a suitable folding of the protein but that is not certainly interfering directly with substrate binding or cofactor function. However, it has been described that MS activity is regulated by NO. NO treatments impairs MS activity in different models both *in vitro* (Brouwer et al., 1996, Nicolaou et al., 1996, 1997) and *in vivo* (Danishpajooh et al., 2001) suggesting that tyrosine nitration might be the responsible of the NO-dependent reduction of MS activity.

Discussion

Although several reports regarding proteomic approaches for the identification of nitrated proteins in mammals have been published recently (Suzuki *et al*. 2005; Sultana *et al*. 2006; Hong *et al*. 2007; Zhang *et al*. 2007) and the detection of nitrated proteins in pathogen-challenged plants was also reported (Romero-Puertas *et al*. 2007), the first two reports focused on general proteomic approaches of nitrated plant protein identification were not published till last year (Cecconi *et al*. 2009; Chaki *et al*. 2009). Both groups described the use of anti 3-nitroY antibodies for detecting plant putatively nitrated proteins in western-blot and the subsequent identification of the immunoreactive proteins by MALDI-TOF/TOF.

1 A total of 8 and 21 proteins were identified in both reports (Cecconi *et al*. 2009;
2 Chaki *et al*. 2009), respectively. However, no nitrated peptides and consequently
3 nitration sites were identified in any of those reports, probably due to the low level of
4 nitration under non stressed condition (Chaki *et al*. 2009) and technical limitations
5 (Cecconi *et al*. 2009), as described by the authors. In this work, we have used a
6 proteomic methodology to purify and identify proteins nitrated *in vivo* at Y residues
7 in *Arabidopsis thaliana*. The method is based on the purification of nitrated proteins
8 by immunoprecipitation with well characterized anti-3-nitroY antibodies (Schmidt *et*
9 *al*. 2003; Gokulrangan *et al*. 2007), and further identification by LC-MS/MS. This
10 method has been previously reported as useful in identifying nitrated proteins in
11 mammals (Turko *et al*. 2003; Zhan and Desiderio, 2009; Liu *et al*. 2009). The
12 procedure was sensitive enough to identify 127 potentially nitrated proteins from
13 Arabidopsis seedlings. These results are in the range of the best proteomic methods
14 reported in animal systems (Suzuki *et al*. 2005; Sultana *et al*. 2006; Hong *et al*. 2007;
15 Zhang *et al*. 2007), and it represents the description of the widest potential *in vivo*
16 nitroproteome of a plant to date. A literature search showed that around 35 % of the
17 identified Arabidopsis Y-nitrated proteins were previously described as Y-nitrated in
18 other organisms (Table S1 and references therein), which support the reliability of
19 the method in identifying potentially Y-nitrated proteins. Moreover, a large
20 proportion of the proteins reported to be potential targets of nitration in the two
21 previous reports on plants (Cecconi *et al*. 2009; Chaki *et al*. 2009) were also
22 identified as putatively nitrated in our work. Moreover, we have technically validated
23 some of our MS-based protein identifications by detection of the corresponding
24 proteins in the immunopurified samples by Western blot with specific antibodies
25 (Fig. 2). Although the methodology presented in this work seems to be reliable and

1 robust enough to be considered a good starting point for the characterization of Y-
2 nitrated plant proteins, no unequivocal nitration sites were found by MS/MS. Due to
3 the low abundance of Y residues in proteins and because the nitration sites were
4 usually restricted to one or two Y per protein (Abello *et al*. 2009) a low occurrence
5 of Y nitration is expected. The most abundant protein spots in 2-DE gels from anti 3-
6 nitroY-immunoprecipitated proteins were thus analyzed and searched for Y-nitration
7 modification. Nitrated peptides for GAPDH, Ribulose Bisphosphate Carboxylase
8 large chain, RuBisCO activase, Mannitol Dehydrogenase and Transketolase were
9 identified (Table 2). The identifications are based on peptide mass fingerprinting data
10 obtained by MALDI-TOF because no good fragmentation MS/MS spectra were
11 obtained. Only molecular ions with signal-to-noise ratio greater than 25 and
12 difference between the experimental and calculated masses < 0.15 were selected.
13 Furthermore, *in silico* analysis of potentially nitrated peptides showed that most of
14 them fulfilled most of the criteria to be nitration targets: Y residues were located in
15 loops with a large solvent accessibility area and had a basic amino acid in the vicinity
16 and a proximal negative charge (Table 3). Gene Ontology tools for the analysis of the
17 potentially Y-nitrated identified proteins showed a significant over-representation of
18 proteins located in chloroplast, peroxisome, mitochondria and apoplast, subcellular
19 compartments that have been proposed as source of NO and superoxide anion in
20 plants (Jasid *et al*. 2006; Flores-Pérez *et al*. 2008; Corpas *et al*. 2001; Bethke *et al*.
21 2004; Gupta *et al*. 2005; Igamberdiev and Hill 2009), thus representing cellular
22 domains were the nitrating reagent peroxynitrite is produced (Szabó *et al*. 2007).
23 These data support the previously proposed idea that the proximity of proteins to the
24 site of generation of nitrating agents is a main factor in directing protein nitration
25 (Ischiropoulos 2003).

1 When we used the Gene Ontology tools for the analysis of the Y-nitrated
2 identified proteins, we found that more than 60 % were involved in primary
3 metabolism. Post-translational nitration of key enzymes and the subsequent alteration
4 of their catalytic properties may represent a new level of regulation of the primary
5 metabolism. Noteworthy, one of the proteins identified as putatively nitrated in this
6 work (S-adenosyl homocysteine hydrolase, Table 1) has been also reported to be
7 nitrated in sunflower hypocotyls (Chaki et al. 2009). The activity of the enzyme was
8 inhibited upon nitration (Chaki et al. 2009), thus suggesting the activity of the
9 Arabidopsis counterpart may be also regulated through nitration. Moreover, Rubisco
10 activase, ATP synthase subunit α and glutamine synthetase 2 has been also identified
11 as putative nitrated proteins in pathogen-challenged Arabidopsis (Cecconi et al.
12 2009). It has been discussed that nitration of these proteins may be a way to
13 modulate defense-related responses including the hypersensitive response (Cecconi
14 et al. 2009). Alternatively, nitration of abundant proteins such as those involved in
15 photosynthesis and carbon metabolism may represent just a non-selective scavenging
16 system for reactive nitrogen and oxygen species produced under standard or stress-
17 related conditions. Moreover, the functional relevance of this post-translational
18 modification on these targets is further supported by the fact that most of the
19 identified nitrated Y residues are strictly conserved in the amino acid sequence of
20 homologous proteins from other organisms (Fig. S1), thus supporting a potential
21 functional effect of this post-translational modification. In the case of
22 glyceraldehyde-3-phosphate dehydrogenase, the two Y residues identified as nitrated
23 in peptide LVSWY*DNEWGY*SSR were not only conserved in the rabbit GAPDH
24 but it were actually also identified as nitrated LISWY*DNEFGY*SNR, resulting in
25 complete loss of catalytic activity (Palamalai and Miyagi, 2010). GAPDH models for

1 rat and Arabidopsis were highly overlapping throughout the molecule and
2 particularly on nitrated Y residues (Fig. S3). In addition, as reported for yeast and
3 mammals (Buchczyk *et al*. 2000; Palamalai and Miyagi, 2010), Arabidopsis GAPDH
4 activity was also inhibited by peroxynitrite (Fig. 3A). Notwithstanding, several
5 proteins participating with GAPDH in the gluconeogenesis conversion of malate to
6 sucrose were also identified as nitrated forms in Arabidopsis (Table 1 and Fig. S4),
7 thus suggesting a potential for Y-nitration as a significant regulatory level on this
8 principal metabolic pathway. Interestingly, among potential targets of Y-nitration in
9 Arabidopsis were also three enzymes involved in the biosynthesis of methionine, the
10 5-methyl tetrahydropteroyltriglutamate homocysteine methyltransferase, also called
11 methionine synthase, the S-adenosylmethionine synthetases 1 and 2 and S-
12 adenosylhomocysteinase 1 (Fig. S4). It has been previously reported that NO likely
13 inhibits mammalian MS activity by reaction with cobalt-containing cobalamin
14 cofactor (Brouwer *et al*. 1996; Nicolaou *et al*. 1997; Danishpajooh *et al*., 2001).
15 Nevertheless, in the light of the results we have obtained this mode of action for NO
16 is anyway compatible with the mechanism of control of MS activity through nitration
17 of key Y residues of the protein. Moreover, the fact that not only a key regulatory
18 step but most of the enzymes involved in methionine biosynthesis are potentially
19 nitrated in Arabidopsis suggests that Y-nitration may represent an important
20 regulatory level to control the biosynthesis of this amino acid in plants. Furthermore,
21 nitration of S-adenosylmethionine synthetases could represent also an interesting
22 regulatory point on ethylene production. Regarding this, it has been recently reported
23 the S-nitrosylation of S-adenosylmethionine synthetase 1 resulting in reduced
24 activity and decreased ethylene production in Arabidopsis (Lindermayr *et al*. 2006).

1 The fact that neither in this work nor in the two previous reports on protein
2 nitration in plants (Cecconi *et al*. 2009; Chaki *et al*. 2009) were unequivocally
3 identified any nitrated peptide and the corresponding nitration site needs further
4 discussion. It is well known that Y-nitration is a very low abundant post-translational
5 modification as compared to other protein modifications such as phosphorylation
6 (Abello *et al*. 2009). In fact, only 0.033 to 0.43 mmol of nitro-Y per mol of Y has
7 been detected in plant proteins, depending on the tissue or specie studied (Bechtold
8 et al. 2009, Chaki et al. 2009). Moreover, it is also likely that under non-stressed
9 conditions, when only basal levels of NO and superoxide and thus low amounts of
10 peroxynitrite is generated by cells, even lower abundance is expected. Nevertheless,
11 because the presented methodology enriched samples in potentially Y-nitrated-
12 containing proteins by immunoprecipitation with a specific anti-3-nitroY antibody,
13 we should expect the identification of some nitrated peptides by MS/MS. A survey of
14 the literature on identification of nitrated proteins in different organisms point to a
15 very low number of nitrated sites identified, thus suggesting the existence of
16 technical difficulties intrinsically associated to MS-based analysis of this kind of
17 protein modification. A possible explanation for the lack of nitroY signatures could
18 be related to alterations produced by the treatments done before mass spectrometry
19 analysis or during the ionization of the protein samples. It has been reported that the
20 treatment of nitrated proteins with DTT and elevated temperature, as used for trypsin
21 digestion, can reduce the nitro-Y to amino-Y or other related species (Söderling *et al*.
22 2007). Moreover, the ionization energy for MALDI or ESI technologies are too
23 aggressive for the nitrated-Y residues, and it has been reported the laser-induced
24 photochemical decomposition of nitro-Y to amino-Y during MALDI-MS analysis
25 (Sarver *et al*. 2001). Therefore, a conversion of nitro-Y to amino-Y in our samples

1 during sample processing before MS analysis may explain the lack of detection of
2 nitrated peptides. To validate this hypothesis we searched our proteomic experiments
3 for amino-Y modification instead of nitro-Y. By selecting amino-Y as a variable
4 modification in the MASCOT data analysis in MALDI-TOF/TOF experiments, a
5 fragmentation MS/MS spectrum corresponding to a peptide containing a 3-aminoY
6 residue was found in the protein spot corresponding to glyceraldehyde-3-phosphate
7 dehydrogenase (Fig. 3). More precisely we found a peptide containing aminoY318
8 suggesting that from the two Y residues found as potential targets to be nitrated in
9 G3PC (Table 2), part of the 3-nitroY318 residues in the protein population might
10 undergo reduction to 3-aminoY318 under the conditions used for MS analysis.
11 Moreover, although we did not get more MS/MS spectra corresponding to aminoY-
12 containing peptides, we found around 50 additional putative aminoY-containing
13 peptides with ion score greater than 15 (Table S3). This confirms the hypothesis that
14 the lack in identification of nitrated peptides in this work, and likely in others, may
15 be due to the conversion of the nitro-Y to amino-Y. Such a conclusion leads to
16 propose that future analysis of Y nitration of proteins should be based on a
17 simultaneous search for both nitroY and aminoY variable modifications. Eventually,
18 the chemical reduction of all nitroY to aminoY by means of a strong reducing
19 reagent such as sodium dithionite may represent an advantage in further proteomic
20 analysis either searching directly for aminoY or after derivatization of aminoY
21 (Ghesquière *et al*. 2009; Abello *et al*. 2010).
22 The proteomic method described in this work represents a tool to identify
23 proteins undergoing *in vivo* Y-nitration in plants. The application of this
24 methodology, with the improvements discussed above, to the analysis of different
25 biological processes in plants will allow the identification of Y-nitration protein

targets. Because of the low abundance and limited stability of this post-translational modification, obtained data suggest that after identification of *in vivo* targets, the confirmation of the modification sites and the functional consequences have to be addressed through *in vitro* assays with larger amounts of modified protein. Anyway, these Y-nitrated proteins may represent nodes for a new unexplored level of regulation of proteins exerted by NO through post-translational modification. Further characterization of the identified Y-nitrated proteins will provide key information about new regulatory features of NO in many aspects of plant growth, development and defence.

Supplementary data

Supplementary data are available at *JXB* online.

Acknowledgements

We thank Rafael Ruiz-Partida (CIPF) for the valuable help with protein modelling. We also thank Renate Scheibe (Universitaet Osnabrueck, Germany), Dorothee Staiger (University of Bielefeld, Germany), Mariam Sahrawy (EEZ-CSIC, Granada, Spain), Joe Ogas (Purdue University, USA) and Dominique Rumeau (Université de la Méditerranée, France) for their kind donation of antibodies against GAPDH, GRP7, fructose bisphosphatase, PICKEL and carbonic anhydrase, respectively. The AtMS1 cDNA fused to 6xHis tag was kindly donated by David Dixon (University of Durham, UK). MS-based protein identification was performed by the Proteomic Service of CIPF-PROTEORED (Valencia, Spain). This work was supported by

1 Ministerio de Educación y Ciencia from Spain and FEDER funds from EU grants
2 GEN2003-20477-C02-02, BIO2005-00222, BIO2008-00839 and CONSOLIDER
3 TRANSPLANTA CSD2007-00057 (to J.L.) and a fellowship of the Bancaja-CSIC
4 Programme (to J.L-J.) and a contract of the I3P Programme of CSIC (co-financed
5 with FEDER funds of the EU, to R.C-M.).

References

Abello N, Barroso B, Kerstjens HA, Postma DS, Bischoff R. 2010. Chemical labeling and enrichment of nitrotyrosine-containing peptides. *Talanta* **80**, 1503-1512.

Abello N, Kerstjens HA, Postma DS, Bischoff R. 2009. Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. *Journal of Proteome Research* **8**, 3222-3328.

Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**,195-201.

Arutyunov DY, Muronetz VI (2003) The activation of glycolysis performed by the nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase in the model system. *Biochemistry and Biophysics Research Communications* **300**, 149–154

Bacharach AD, Markland FS, Pellino A, Weber BH. 1977. Modification of yeast 3-phosphoglycerate kinase: isolation and sequence determination of a nitrated active-site peptide and isolation of a carboxyl modified active-site peptide. *Biochemistry and Biophysics Research Communication* **74**, 165-171.

Bechtold U, Rabbani N, Mullineaux PM, Thornalley PJ. 2009. Quantitative measurement of specific biomarkers for protein oxidation, nitration and glycation in Arabidopsis leaves. *The Plant Journal* **59**, 661-671.

Bethke PC, Badger MR, Jones RL. 2004. Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* **16**, 332-41.

Bethke PC, Libourel IG, Jones RL. 2006. Nitric oxide reduces seed dormancy in Arabidopsis. *Journal of Experimental Botany* **57**, 517-526.

Bowie JU, Lüthy R, Eisenberg D. 1991 A method to identify protein sequences that fold into a known three-dimensional structure. *Science* **253**, 164-170.

Brouwer, M., Chamulitrat, W., Ferruzzi, G., Sauls, D. L., and Weinberg, J. B. 1996. Nitric oxide interactions with cobalamins: biochemical and functional consequences.*Blood* **88,** 1857–1864

Buchczyk DP, Briviba K, Hartl FU, Sies H. 2000. Responses to peroxynitrite in yeast: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a sensitive intracellular target for nitration and enhancement of chaperone expression and ubiquitination. *Biological Chemistry* **381**, 121-126.

Casoni F, Basso M, Massignan T, Gianazza E, Cheroni C, Salmona M, Bendotti C, Bonetto V. 2005. Protein nitration in a mouse model of familial

amyotrophic lateral sclerosis: possible multifunctional role in the pathogenesis. *Journal of Biological Chemistry* **280**, 16295-16304.

Cecconi D, Orzetti S, Vandelle E, Rinalducci S, Zolla L, Delledonne M. 2009. Protein nitration during defense response in *Arabidopsis thaliana*. *Electrophoresis* **30**, 2460-2468.

Chaki M, Valderrama R, Fernández-Ocaña AM, Carreras A, López-Jaramillo J, Luque F, Palma JM, Pedrajas JR, Begara-Morales JC, Sánchez-Calvo B, Gómez-Rodríguez MV, Corpas FJ, Barroso JB. 2009. Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. *Journal of Experimental Botany* **60**, 4221-4234.

Chang GG, Huang TM. 1980. Involvement of tyrosyl residues in the substrate binding of pigeon liver malic enzyme. *Biochimica et Biophysica Acta* **611**, 217-226.

Chen H-J C, Chang C-M, Lin W-P, Cheng D-L, Leong M-I. 2008. H2O2/Nitrite-Induced Post-translational Moodifications of Human Hemoglobin Determined by Mass Spectometry: Redox Regulation of Tyrosine Nitration and 3-Nitrotyrosine Reductions by Antioxidants. *ChemBioChem* **9**, 312-323.

Chiappetta G, Corbo C, Palmese A, Galli F, Piroddi M, Marino G, Amoresano A. 2009. Quantitative identification of protein nitration sites. *Proteomics* **9**,1524-1537.

Corpas FJ, Barroso JB, del Río LA. 2001. Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends in Plant Science* **6**, 145-150.

Danishpajooh IO, Gudi T, Chen Y, Kharitonov VG, Sharma VS, Boss GR. 2001. Nitric oxide inhibits methionine synthase activity in vivo and disrupts carbon flow through the folate pathway. *Journal of Biological Chemistry.* **20**, 27296-27303.

DiCola D, Polidoro G, Di Ilio G, Del Boccia C, Politi L, Scandurra R. 1976. Role of tyrosine residues in cytoplasmic aspartate aminotransferase from beef kidney. *Molecular and Cellular Biochemistry* **11**, 97-101.

Dixon DP, Skipsey M, Grundy NM, Edwards, R. 2005. Stress-Induced Protein S-Glutathionylation in Arabidopsis. *Plant Physiology* **138**, 2233-2244.

Fiore G, Di Cristo C, Monti G, Amoresano A, Columbano L, Pucci P, Cioffi FA, Di Cosmo A, Palumbo A, d'Ischia M. 2006. Tubulin nitration in human gliomas. *Neuroscience Letters* **394**, 57-62.

Flores-Pérez U, Sauret-Güeto S, Gas E, Jarvis P, Rodríguez-Concepción M. 2008. A mutant impaired in the production of plastome-encoded proteins uncovers a mechanism for the homeostasis of isoprenoid biosynthetic enzymes in Arabidopsis plastids. *Plant Cell* **20**, 1303-1315.

Ghesquière B, Colaert N, Helsens K, Dejager L, Vanhaute C, Verleysen K, Kas K, Timmerman E, Goethals M, Libert C, Vandekerckhove J, Gevaert K. 2009. In vitro and in vivo protein-bound tyrosine nitration characterized by diagonal chromatography. *Molecular and Cellular Proteomics* **8**, 2642-2652.

Ghosh S, Janocha AJ, Aronica MA, Swaidani S, Comhair SA, Xu W, Zheng L, Kaveti S, Kinter M, Hazen SL, Erzurum SC. 2006. Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. *Journal of Immunology* **176**, 5587-5597.

Gokulrangan G, Zaidi A, Michaelis ML, Schöneich C. 2007. Proteomic analysis of protein nitration in rat cerebellum: effect of biological aging. *Journal of Neurochemistry* **100**, 1494-1504.

Görg B, Bidmon HJ, Keitel V, Foster N, Goerlich R, Schliess F, Häussinger D. 2006. Inflammatory cytokines induce protein tyrosine nitration in rat astrocytes. *Archives of Biochemistry and Biophysics* **449**, 104-114.

Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. 2004. Biological significance of nitric oxide-mediated protein modifications. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **287**, L262-L268.

Grün S, Lindermayr C, Sell S, Durner J. 2006. Nitric oxide and gene regulation in plants. *Journal of Experimental Botany* **57**, 507-516.

Gupta KJ, Stoimenova M, Kaiser WM. 2005. In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. *Journal of Experimental Botany* **56**, 2601–2609.

He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM, Pei ZM. 2004. Nitric oxide represses the Arabidopsis floral transition. *Science* **305**, 1968-1971.

Hong SJ, Gokulrangan G, Schöneich C. 2007. Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry. *Experimental Gerontology* **42**, 639-651.

Igamberdiev AU, Hill RD. 2009. Plant mitochondrial function during anaerobiosis. *Annals of Botany* **103**, 259-268.

Ischiropoulos H. 2003. Biological selectivity and functional aspects of protein tyrosine nitration. *Biochemistry and Biophysics Research Communication* **305**, 776-783.

Ito K, Hanazawa T, Tomita K, Barnes PJ, Adcock IM. 2004. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression:

role of tyrosine nitration. *Biochemistry and Biophysics Research Communication* **315**, 240-245.

Jasid S, Simontacchi M, Bartoli CG, Puntarulo S. 2006. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiology* **142**, 1246-1255.

Knyushko TV, Sharov VS, Williams TD, Schöneich C, Bigelow DJ. 2005. 3-Nitrotyrosine modification of SERCA2a in the aging heart: a distinct signature of the cellular redox environment. *Biochemistry* **44**, 13071-13081.

Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM. 1996. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *Journal of Biomolecular NMR* **8**, 477-486.

Layne PP, Najjar VA. 1979. Evidence for a tyrosine residue at the active site of phosphoglucomutase and its interaction with vanadate. *Proceedings of the National Academy of Science of USA* **76**, 5010-5013.

Lindermayr C, Saalbach G, Bahnweg G, Durner J. 2006. Differential Inhibition of Arabidopsis Methionine Adenosyltransferases by Protein S-Nitrosylation. *Journal of Biological Chemistry* **281**, 4285-4291.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic Identification of S-Nitrosylated Proteins in Arabidopsis. *Plant Physiology* **137**, 921-930.

Liu B, Tewari AK, Zhang L, Green-Church KB, Zweier JL, Chen YR, He G. 2009. Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target. *Biochimica et Biophysica Acta* **1794**, 476-485.

Liu HY, Yu X, Cui DY, Sun MH, Sun WN, Tang ZC, Kwak SS, Su WA. 2007. The role of water channel proteins and nitric oxide signaling in rice seed germination. *Cell Research* **17**, 638-649.

Melo F, Feytmans E. 1998. Assessing protein structures with a non-local atomic interaction energy. *Journal of Molecular Biology* **277**, 1141-1152.

Mishina TE, Lamb C, Zeier J. 2007. Expression of a nitric oxide degrading enzyme induces a senescence programme in Arabidopsis. *Plant Cell & Environment* **30**, 39-52.

Miyagi M, Sakaguchi H, Darrow RM, Yan L, West KA, Aulak KS, Stuehr DJ, Hollyfield JG, Organisciak DT, Crabb JW. 2002. Evidence that light modulates protein nitration in rat retina. *Molecular and Cellular Proteomics* **1**, 293-303.

Morot-Gaudry-Talarmain Y, Rockel P, Moureaux T, Quilleré I, Leydecker MT, Kaiser WM, Morot-Gaudry JF. 2002. Nitrite accumulation and nitric

oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. *Planta* **215**, 708-715.

Muñoz-Bertomeu J, Cascales-Miñana B, Mulet JM, Baroja-Fernández E, Pozueta-Romero J, Kuhn JM, Segura J, Ros R. (2009) Plastidial glyceraldehyde-3-phosphate dehydrogenase deficiency leads to altered root development and affects the sugar and amino acid balance in Arabidopsis. *Plant Physiology* **151**, 541-558.

Mur LA, Carver TL, Prats E. 2006. NO way to live; the various roles of nitric oxide in plant-pathogen interactions. *Journal of Experimental Botany* **57**, 489-505.

Nicolaou, A., Kenyon, S. H., Gibbons, J. M., Ast, T., and Gibbons, W. A. 1996. In vitro inactivation of mammalian methionine synthase by nitric oxide. *Eur. J. Clin. Invest.* **26,** 167–170.

Nicolaou, A., Warefield, C. J., Kenyon, S. H., and Gibbons, W. A. 1997 The inactivation of methionine synthase in isolated rat hepatocytes by sodium nitroprusside.*Eur. J. Biochem.* **244,** 876–882.

Palamalai V, Miyagi M. 2010. Mechanism of glyceraldehyde-3-phosphate dehydrogenase inactivation by tyrosine nitration. *Protein Science* **19**, 255-262.

Parani M, Rudrabhatla S, Myers R, Weirich H, Smith B, Leaman DW, Goldman SL. 2004. Microarray analysis of nitric oxide responsive transcripts in Arabidopsis. *Plant Biotechnology Journal* **2**, 359-366.

Petersen B, Petersen TN, Andersen P, Nielsen M, Lundegaard C. 2009. A generic method for assignment of reliability scores applied to solvent accessibility predictions. *BMC Structural Biology* **9**, 51.

Polverari A, Molesini B, Pezzotti M, Buonaurio R, Marte M, Delledonne M. 2003. Nitric oxide-mediated transcriptional changes in *Arabidopsis thaliana*. *Molecular Plant Microbe Interactions* **16**, 1094-1105.

Romero-Puertas MC, Campostrini N, Mattè A, Righetti PG, Perazzolli M, Zolla L, Roepstorff P, Delledonne M. 2008. Proteomic analysis of S-nitrosylated proteins in Arabidopsis thaliana undergoing hypersensitive response. *Proteomics* **8**, 1459-1469.

Romero-Puertas MC, Laxa M, Mattè A, Zaninotto F, Finkemeier I, Jones AM, Perazzolli M, Vandelle E, Dietz KJ, Delledonne M. 2007. S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. *Plant Cell* **19**, 4120-30.

Romero-Puertas MC, Perazzolli M, Zago ED, Delledonne M. 2004. Nitric oxide signalling functions in plant-pathogen interactions. *Cell Microbiology* **6**, 795-803.

Saito S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K. 2006. Peroxynitrite Generation and Tyrosine Nitration in Defense Responses in Tobacco BY-2 Cells. *Plant and Cell Physiology* **47**, 689–697.

Sarver A, Scheffler NK, Shetlar MD, Gibson BW. 2001. Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ ionization mass spectrometry. *Journal of American Society of Mass Spectrometry* **12**, 439-448.

Schmidt P, Youhnovski N, Daiber A, Balan A, Arsic M, Bachschmid M, Przybylski M, Ullrich V. 2003. Specific nitration at tyrosine 430 revealed by high resolution mass spectrometry as basis for redox regulation of bovine prostacyclin synthase. *Journal of Biological Chemistry* **278**, 12813-12819.

Simpson GG. 2005. NO flowering. *Bioessays* **27**, 239-241.

Söderling AS, Hultman L, Delbro D, Højrup P, Caidahl K. 2007. Reduction of the nitro group during sample preparation may cause underestimation of the nitration level in 3-nitrotyrosine immunoblotting. *Journal of Chromatography B* **851**, 277-286.

Souza JM, Daikhin E, Yudkoff M, Raman CS, Ischiropoulos H. 1999. Factors determining the selectivity of protein tyrosine nitration. *Archives of Biochemistry and Biophysics* **371**, 169-178.

Stevens SM Jr, Prokai-Tatrai K, Prokai L. 2008. Factors that contribute to the misidentification of tyrosine nitration by shotgun proteomics. *Molecular and Cellular Proteomics* **7**, 2442-2451.

Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, Markesbery WR, Butterfield DA. 2006. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. *Neurobiology of Disease* **22**, 76-87.

Suzuki Y, Tanaka M, Sohmiya M, Ichinose S, Omori A, Okamoto K. 2005. Identification of nitrated proteins in the normal rat brain using a proteomics approach. *Neurological Research* **27**: 630-633.

Szabó C, Ischiropoulos H, Radi R. 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nature Reviews on Drug Discovery* **6**, 662-680.

Tsumoto H, Taguchi R, Kohda K. 2010. Efficient identification and quantification of peptides containing nitrotyrosine by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry after derivatization. *Chemical & Pharmaceutical Bulletin* **58**, 488-494.

Turko IV, Li L, Aulak KS, Stuehr DJ, Chang JY, Murad F. 2003. Protein tyrosine nitration in the mitochondria from diabetic mouse heart. Implications to dysfunctional mitochondria in diabetes. *Journal of Biological Chemistry* **278**, 33972-33977.

Zhan X, Desiderio DM. 2009. Mass spectrometric identification of in vivo nitrotyrosine sites in the human pituitary tumor proteome. *Methods in Molecular Biology* **566**, 137-163.

Zhang Q, Qian WJ, Knyushko TV, Clauss TR, Purvine SO, Moore RJ, Sacksteder CA, Chin MH, Smith DJ, Camp DG, Bigelow DJ, Smith RD. 2007. A method for selective enrichment and analysis of nitrotyrosine-containing peptides in complex proteome samples. *Journal of Proteome Research* **6**, 2257-2268.

Zhang Y, Wang L, Liu Y, Zhang Q, Wei Q, Zhang W. 2006. Nitric oxide enhances salt tolerance in maize seedlings through increasing activities of proton-pump and Na+/H+ antiport in the tonoplast. *Planta* **224**, 545-555.

Zisapel N. 1978. The reactivity of a functional tyrosyl residue in carboxypeptidase B. Nitration of the cadmium enzyme. *European Journal of Biochemistry* **90**, 199-203.

Table 1. Immunopurified Y-nitrated proteins identified in *Arabidopsis thaliana* seedlings by a shotgun LC-MS/MS approach. Those proteins that have been previously reported as nitrated in other plant systems have been marked with * (Chaki *et al*. 2009) or § (Cecconi *et al*. 2009).

SwissProt Locus	AGI code	Description	Mascot score	Peptides matched (n.r.)	Best two peptides (ion Score)
ATPB_ARATH	AtCg00480	ATP synthase subunit beta	1150	23	R.FVQAGSEVSALLGR.M (85) K.IGLFGGAGVGK.T (80)
METE_ARATH	At5g17920	Methionine synthase 1	1014	27	K.DEALFSANAAALASR.R (97) K.MLAVLEQNILWVNPDCGLK.T(91)
G3PB_ARATH	At1g42970	GAPDH B, chloroplast	884	22	K.IVDNETISVDGK.L (85) R.KDSPLEVVVLNDSGGVK.N (75)
G3PA_ARATH	At3g26650	GAPDH A, chloroplast	831	18	R.VPTPNVSVVDLVVQVSK.K (68) K.KVIITAPGK.G (60)
RCA_ARATH	At2g39730	§ RuBisCo activase, chloroplast	761	20	R.GLAYDTSDDQQDITR.G (81) R.VQLAETYLSQAALGDANADAIGR.G (72)
GOX1_ARATH	At3g14415	Probable peroxisomal Glycolate oxidase1	670	16	R.AASAAGTIMTLSSWATSSVEEVASTGPGIR.F (101) K.DIQWLQTITNMPILVK.G (58)
GOX2_ARATH	At3g14420	Probable peroxisomal Glycolate oxidase2	651	16	R.AASAAGTIMTLSSWATSSVEEVASTGPGIR.F (101) R.IPVFLDGGVR.R (52)
SAHH1_ARATH	At4g13940	* Adenosyl homocysteinase 1	581	18	K.VALLHLGK.L (55) R.DSAAVFAWK.G (54)
PGKH_ARATH	At1g56190	Phosphoglycerate kinase, chloroplast	542	14	K.LASLADLYVNDAFGTAHR.A (77) K.FAAGTEAIANK.L (75)
ATPA_ARATH	AtCg00120	§ ATP synthase subunit alpha	504	12	R.EAYPGDVFYLHSR.L (64) R.EQHTLIIYDDLSK.Q (62)
EFTU_ARATH	At4g20360	Elongation factor Tu, chloroplast	491	13	K.KYDEIDAAPEER.A (72) R.SYTVTGVEMFQK.I (54)
G3PC_ARATH	At3g04120	GAPDH C, cytosolic	479	13	R.VPTVDVSVVDLTVR.L (71) K.KVVISAPSK.D (52)
CAHC_ARATH	At3g01500	Carbonic anhydrase 1, chloroplast	475	13	K.YGGVGAAIEYAVLHLK.V (64) R.EAVNVSLANLLTYPFVR.E (60)
EF1A_ARATH	At1g07940	Elongation factor 1-alpha	450	11	R.EHALLAFTLGVK.Q (103) K.FHINIVVIGHVDSGK.S (82)
ACT7_ARATH	At5g09810	Actin-7	448	12	K.SEYDESGPSIVHR.K (75) K.NYELPDGQVITIGAER.F (57)
ACT2_ARATH	At3g18780	Actin-2	430	12	K.NYELPDGQVITIGAER.F (57) K.AGFAGDDAPR.A (52)
KPPR_ARATH	At1g32060	Phosphoribulokinase chloroplast	418	13	R.LDELIYVESHLSNLSTK.F (55) K.ILVIEGLHPMFDER.V (52)
RUBB_ARATH	At1g55490	RuBisCO large subunit beta	389	13	R.GYISPYFVTDSEK.M (71) K.YEDLMAAGIIDPTK.V (52)
CAH2_ARATH	At5g14740	Carbonic anhydrase 2	379	11	R.EAVNVSLANLLTYPFVR.E (60) K.VENIVVIGHSACGGIK.G (59)
TBA6_ARATH	At4g14960	Tubulin alpha-6 chain	358	11	R.AVFVDLEPTVIDEVR.T (67) R.LVSQVISSLTASLR.F (50)
METK1_ARATH	At1g02500	S-adenosyl methionine synthetase 1	334	11	R.FVIGGPHGDAGLTGR.K (73) K.IIIDTYGGWGAHGGGAFSGK.D (64)
RUBA_ARATH	At2g28000	RuBisCO large subunit alpha, chloroplast	331	11	K.VVNDGVTIAR.A (60) K.TNDSAGDGTTTASILAR.E (56)
METK2_ARATH	At4g01850	S-adenosyl methionine synthetase 2	326	11	R.FVIGGPHGDAGLTGR.K (73) K.IIIDTYGGWGAHGGGAFSGK.D (64)

GLNA2_ARATH	At5g35630	§ Glutamine synthetase, chloroplast/ mitochondrial	314	10	K.VSGEVPWFGIEQEYTLLQQNVK.W (76) K.HETASIDQFSWGVANR.G (42)
SGAT_ARATH	At2g13360	Serine-glyoxylate aminotransferase	306	10	R.AALDLIFEEGLENIIAR.H (61) K.VFFDWNDYLK.F (42)
RBS1A_ARATH	At1g67090	RuBisCO small chain 1A chloroplast	299	9	K.LPLFGCTDSAQVLK.E (71) K.EVDYLIR.N (46)
TBA3_ARATH	At5g19770	Tubulin alpha-3/alpha-5 chain	284	8	R.AVFVDLEPTVIDEVR.T (67) R.LISQIISSLTTSLR.F (65)
PORB_ARATH	At4g27440	Protochlorophyllide reductase B	263	12	R.LLLDDLKK.S (53) K.GYVSETESGKR.L (46)
RBS1B_ARATH	At5g38430	RuBisCO small chain 1B chloroplast	254	7	K.LPLFGCTDSAQVLK.E (71) K.EVDYLLR.N (46)
ILV5_ARATH	At3g58610	Ketol-acid reductoisomerase, chloroplast	240	9	K.VSLAGYEEYIVR.G (44) K.APVSLDFETSVFK.K (43)
TBB4_ARATH	At5g44340	Tubulin beta-4 chain	226	8	K.LAVNLIPFPR.L (54) R.YLTASAVFR.G (35)
HSP71_ARATH	At5g02500	* Heat shock cognate 70 kDa protein 1	217	10	R.MVNHFVQEFK.R (40) K.ATAGDTHLGGEDFDNR.M (35)
F16P1_ARATH	At3g54050	Fructose-1,6-bisphosphatase	214	10	R.TLLYGGIYGYPR.D (58) R.VLDIQPTEIHQR.V (42)
TBB2_ARATH	At5g62690	Tubulin beta-2/beta-3 chain	203	9	K.LAVNLIPFPR.L (54) R.AVLMDLEPGTMDSLR.S (35)
TBB1_ARATH	At1g75780	Tubulin beta-1 chain	193	8	K.LAVNLIPFPR.L (54) R.AVLMDLEPGTMDSIR.S (35)
PGMP_ARATH	At5g51820	Phosphoglucomu tase, chloroplast	173	9	K.SLPTKPIEGQK.T (30) K.LPFFEVPTGWK.F (26)
P2SAF_ARATH	At5g23120	Photosystem II stability/assembly factor HCF136	172	8	R.ADGGLWLLVR.G (40) K.GTGITEEFEEVPVQSR.G (34)
HSP73_ARATH	At3g09440	* Heat shock cognate 70 kDa protein 3	172	7	R.MVNHFVQEFK.R (40) K.ATAGDTHLGGEDFDNR.M (35)
APX1_ARATH	At1g07890	L-ascorbate peroxidase 1, cytosolic	161	5	K.EGLLQLVSDK.A (44) K.QMGLSDKDIVALSGAHTLGR.C (35)
MTDH_ARATH	At4g39330	Probable mannitol dehydrogenase	139	5	K.NYGGYSENIVVDQR.F (47) K.NYGGYSENIVVDQR.F (34)
CD48A_ARATH	At3g09840	Cell division control protein 48 A	120	6	R.KGDLFLVR.G (29) R.IVSQLLTLMDGLK.S (29)
GME_ARATH	At5g28840	GDP-mannose 3,5-epimerase	112	5	R.SFTFIDECVEGVLR.L (43) K.KLPIHHIPGPEGVR.G (31)
GBLP_ARATH	At1g18080	Guanine nucleotide-binding protein subunit beta	103	4	R.LWDLAAGVSTR.R (42) K.DGVVLLWDLAEGK.K (27)
CLPP_ARATH	AtCg00670	ATP-dependent Clp protease	99	2	R.SPGEGDTSWVDIYNR.L (70) R.TGKPIWVISEDMER.D (30)
GCST_ARATH	At1g11860	Aminomethyltransfe rase, mitochondrial	99	5	K.GGDVSWHIHDER.S (25) R.AEGGFLGADVILQQLK.D (24)
AAT5_ARATH	At4g31990	Aspartate aminotransferase, chloroplast	98	5	K.ATAELLFGAGHPVIK.E (27) R.VATIQGLSGTGSLR.L (24)
ACA9_ARATH	At3g21180	Ca-transporting ATPase 9, plasma membrane	98	7	R.VAIDSMAK.N (28) R.QAALVLNASRR.F (21)
RH56_ARATH	At5g11200	DEAD-box ATP-dependent RNA helicase 56	97	5	K.LSEMEKNR.K (30) K.VSVFYGGVNIK.I (25)
ENO_ARATH	At2g36530	Enolase	96	6	K.AGAVVSGIPLYK.H (30) K.LAMQEFMILPVGAASFK.E (30)
MRP7_ARATH	At3g13100	Multidrug resistance-associated protein 7	86	7	R.YGPHLPMVLRGLTCTFR.G (20) R.GIEAGWLK.K (17)

AFB3_ARATH	At1g12820	AUXIN SIGNALING F-BOX 3	84	6	R.LWILDSIGDK.G (23) R.LMSCAPQLVDLGVGSYENEPDPESFAK.L (17)
PDX13_ARATH	At5g01410	Pyridoxal biosynthesis protein	79	4	K.VGLAQMLR.G (43) R.NMDDDEVFTFAK.K (14)
PDX11_ARATH	At2g38230	Pyridoxal biosynthesis protein	75	3	K.VGLAQMLR.G (43) K.IAAPYDLVVQTK.E (20)
EFTM_ARATH	At4g02930	Elongation factor Tu, mitochondrial	75	2	R.GSALSALQGTNDEIGR.Q (49) K.LMDAVDEYIPDPVR.V (26)
MDR11_ARATH	At3g28860	Multidrug resistance protein 11 (P-glycoprotein 19)	73	6	K.SSVIAMIER.F (24) R.AVLKNPTVLLLDEATSALDAESECVLQEALERLMR.G (22)
MDHP_ARATH	At3g47520	Malate dehydrogenase, chloroplast	70	3	K.DVNVVVIPAGVPR.K (35) K.LFGVTTLDVVR.A (22)
SR54C_ARATH	At5g03940	Signal recognition particle 54 kDa protein, chloroplast	70	5	R.GVKPDQQLVK.I (16) R.QEDAEDLQKK.I (16)
MDHG1_ARATH	At5g09660	Malate dehydrogenase, glyoxysomal	70	3	R.TGAEEVYQLGPLNEYER.I (31) K.LLGVTTLDVAR.A (30)
TAF1B_ARATH	At3g19040	Transcription initiation factor TFIID subunit 1-B	69	7	R.ENLKQLNSDARGR.L (20) K.EIGTPICQMKKILK.E (17)
TYW23_ARATH	At4g04670	tRNA wybutosine-synthesizing protein	69	5	R.ADPLNILNDVWR.L (24) K.RVIIAIRCSIR.M (15)
CATA3_ARATH	At1g20620	Catalase-3	69	3	R.LGPNYLQLPVNAPK.C (32) K.GFFEVTHDISNLTCADFLR.A (28)
KASC1_ARATH	At5g46290	3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplast	68	3	K.LLSGESGISLIDR.F (53) R.ADGLGVSSCIER.C (9)
ATPG1_ARATH	At4g04640	ATP synthase gamma chain 1, chloroplast	68	2	R.ALQESLASELAAR.M (52) R.ASSVSPLQASLRELR.D (16)
GRP7_ARATH	At2g21660	Glycine-rich RNA-binding protein 7	66	1	R.ALETAFAQYGDVIDSK.I (66)
FDH_ARATH	At5g14780	Formate dehydrogenase, mitochondrial	66	5	R.QAVVDAVESGHIGGYSGDVWDPQPAPK.D (18) R.LQMAPELEK.E (17)
HSP83_ARATH	At5g56010	* Heat shock protein 81-3	62	5	K.GIEVLYMVDAIDEYAIGQLK.E (21) K.EGQNDIFYITGESK.K (16)
TGA2_ARATH	At5g06950	Transcription factor TGA2	61	4	K.LTQLEQELQR.A (19) R.LQTLQQMIR.V (15)
TCPA_ARATH	At3g20050	T-complex protein 1 subunit alpha	61	6	R.NKIHPTSIISGYR.L (19) R.GANDYMLDEMER.A (15)
CAPP3_ARATH	At3g14940	Phosphoenolpyruvate carboxylase 3	60	4	K.LLVSEDLWAFGEKLR.A (22) K.RLVSDLGK.S (15)
WRK19_ARATH	At4g12020	WRKY transcription factor 19	60	6	K.CTYLGCPSKRK.V (19) K.LCQVEGCQKGAR.D (16)
THI4_ARATH	At5g54770	Thiazole biosynthetic enzyme, chloroplast	59	2	K.HAALFTSTIMSK.L (33) K.ALDMNTAEDAIVR.L (26)
OMT1_ARATH	At5g54160	Quercetin 3-O-methyltransferase 1	59	2	K.NPEAPVMLDR.I (34) K.VLMESWYHLK.D (25)
IF5A2_ARATH	At1g26630	Eukaryotic translation initiation factor 5A-2 (eIF-5A)	59	2	K.LPTDDGLTAQMR.L (33) K.CHFVAIDIFTAK.K (26)
PKL_ARATH	At2g25170	PICKLE chromatin-remodeling factor	58	6	K.GLLHPYQLEGLNFLR.F (19) K.AYKSNHRLK.T (14)
Y1934_ARATH	At1g09340	Uncharacterized protein chloroplast	57	3	K.SSLSAEGFDVVYDINGR.E (26) R.FIGLFLSR.I (16)
VIN3_ARATH	At5g57380	VERNALIZATION-INSENSITIVE 3	56	5	R.GIVNRLSSGVHVQKLCSQAMEALDK.V (27) R.NEIMKIICAEMGKER.K (14)
PME4_ARATH	At2g47030	Pectinesterase-4	54	6	K.AVQGICQSTSDKASCVK.T (16)

		(VANGUARD1-like protein 1)			K.NTAGPMGHQAAAIRVNGDRAVIFNCR.F (12)
APT1_ARATH	At1g27450	Adenine phosphoribosyltransferase 1 (APRT 1)	54	3	R.AIIIDDLIATGGTLAAAIR.L (35) K.DTIALFVDR.Y (15)
DRL19_ARATH	At1g63350	Putative disease resistance protein At1g63350	54	4	R.NAELQRLCLCGFCSKSLTTSYR.Y (17) K.MCLLYCALFPEDAK.I (16)
FABG_ARATH	At1g24360	3-oxoacyl-[acyl-carrier-protein] reductase, chloroplast	54	3	K.WGTIDVVVNNAGITR.D (25) K.ILGTIPLGR.Y (19)
BSL1_ARATH	At4g03080	Ser/thr-protein phosphatase BSL1	53	4	K.IICMHGGIGR.S (16) R.HGAASVGIRIYVHGGLR.G (16)
PER9_ARATH	At1g44970	Peroxidase 9	52	3	K.AYAEDERLFFQQFAK.S (26) K.EPRMAASLLR.L (13)
UPL1_ARATH	At1g55860	E3 ubiquitin-protein ligase UPL1	52	5	K.LLSDIVLMYSHGTSVILR.R (20) R.LIDFDNKKAYFR.S (16)
HDA5_ARATH	At5g61060	Histone deacetylase 5	51	3	R.KVGLIYDETMCK.H (24) K.LQLAGVSQR.C (18)
HAC12_ARATH	At1g16710	HAC12 Histone acetyltransferase	51	5	K.LTTHPSLADQNAQNK.E (14) K.ASGQSDFSGNASK.D (13)
MRP14_ARATH	At3g59140	Multidrug resistance-associated protein 14	50	7	R.IATFLEAPELQGGERRR.K (16) R.VVAVENPTKPVK.E (11)
ASHH2_ARATH	At1g77300	Histone-lysine N-methyltransferase ASHH2	50	6	K.ILPRPRPR.M (13) K.SPSENGSHLIPNAKKAK.H (13)
ATM_ARATH	At3g48190	Serine/threonine-protein kinase ATM (PI3Kc_related)	47	8	R.RVLLQILGCEKCTMQHLLQSASLLR.K (14) K.QIPMAQLHENEGRK.S (11)
FBX10_ARATH	At1g51290	Putative F-box only protein 10	47	4	R.LVICCYDETQQVYIYIVRR.N (16) K.YVIGYDNKK.R (14)
PSBP1_ARATH	At1g06680	Oxygen-evolving enhancer protein 2-1, chloroplast	45	3	K.TNTDFLPYNGDGFK.V (25) K.EIEYPGQVLR.F (12)
CHLD_ARATH	At4g18480	Magnesium-chelatase subunit chlD, chloroplast	45	3	K.IYKAGMSLLVIDTENK.F (26) R.VAAVGIATQFQERCNEVFR.M (22)
FBK38_ARATH	At2g29800	Putative F-box/Kelch-repeat	44	3	K.MANFGGKLVILGCYR.S (20) R.HLRNMKR.D (16)
GLYM_ARATH	At4g37930	Serine hydroxymethyltransferase mitochondrial	44	4	R.GFVEEDFAK.V (22) K.VLEAVHIASNK.N (11)
SCP37_ARATH	At3g52010	Serine carboxypeptidase-like 37	44	3	K.AIHANTTK.L (19) K.KLPGQPSGVSFR.Q (18)
COL14_ARATH	At2g33500	CONSTANS-LIKE 14	44	3	K.LCLPCDQHVHSANLLSR.K (20) K.SNNIPAAIHSHK.S (14)
SYV_ARATH	At1g14610	Valyl-tRNA synthetase	43	7	K.SDLFKADAK.S (16) K.INLDILRVVGYR.Q (13)
DRP1D_ARATH	At2g44590	Dynamin-related protein 1D	43	3	R.MQCAKRLELYK.K (22) R.MGSEYLAK.L (14)
VATB_ARATH	At1g76030	Vacuolar ATP synthase subunit B	43	3	R.NIFQSLDLAWTLLR.I (16) R.KFVMQGAYDTR.N (15)
SIZ1_ARATH	At5g60410	E3 SUMO-protein ligase SIZ1	42	5	K.WQCPICLK.N (15) R.HRSLNKICIILCAGK.N (12)
HAC2_ARATH	At1g67220	HAC2 Histone acetyltransferase	42	4	R.ACTGCYTKNRTLR.H (16) K.LGTVVDIIEPMKCDER.S (11)
TMK1_ARATH	At1g66150	Putative receptor protein kinase TMK1 precursor	42	4	K.GNDPCTNWIGIACSNGNITVISLEK.M (18) K.VVNLTNNHLQGPVPVFK.S (12)
SYM_ARATH	At4g13780	Probable methionyl-tRNA synthetase	42	3	R.LVEGSCPFEGCNYDSAR.G (26) K.CKVCQNTPR.I (12)
WEE1_ARATH	At1g02970	Wee1-like protein kinase	41	3	R.AMPPPCLK.N (19) K.LPLLPGHSLQLQQLLK.T (15)

ARR12_ARATH	At2g25180	Two-component response regulator	41	5	-.MTVEQNLEALDQFPVGMR.V (17) R.HCQYHVTTTNQAQK.A (9)
CESA4_ARATH	At5g44030	Cellulose synthase A catalytic subunit 4	41	4	K.KAGAMNAMVR.V (22) K.SSLMSQKNFEKR.F (12)
AUR2_ARATH	At2g25880	Ser/thr-protein kinase Aurora-2	41	3	R.LYGYFYDQKRVYLILEYAVR.G (18) M.LYQAASEAAQK.R (14)
Y1838_ARATH	At1g18380	Uncharacterized protein At1g18380	41	3	R.YIMEDKACR.R (32) R.SSDSDEGCMKYAEIPMLR.S (8)
2AAA_ARATH	At1g25490	Ser/thr-protein phosphatase 2A regulatory subunit A alpha	41	4	R.LAGGEWFAAR.V (17) R.RAAASNLGK.F (11)
FBK84_ARATH	At4g19865	F-box/kelch-repeat protein At4g19865	40	3	K.IEFGNVNEMCAYDTKLCK.W (20) K.IYVMGGCQGLKDEPWAEVFNTK.T (10)
MSH3_ARATH	At4g25540	DNA mismatch repair protein MSH3	40	4	R.LVNAGYKIGVVK.Q (17) R.LVNAGYK.I (13)
DCDA1_ARATH	At3g14390	Diaminopimelate decarboxylase 1, chloroplast	39	1	R.DAAVLMIEYIDEIR.R (39)
GL25_ARATH	At5g26700	Probable germin-like protein subfamily 2-5	39	3	R.IDYAPNGLNPPHVHPR.A (17) K.LPGLNTLGLSMSR.I (14)
CYSK1_ARATH	At4g14880	Cysteine synthase (OAS-TL A)	39	3	K.IDGFVSGIGTGGTITGAGK.Y (21) R.IGFSMISDAEK.K (15)
MRP13_ARATH	At1g30410	Multidrug resistance-associated protein 13	39	4	R.KKYYNCVLGLLACYCVVEPVLR.L (22) R.SVLIKQEER.E (14)
ERG11_ARATH	At5g24150	Squalene monooxygenase 1,1	39	3	R.RLLQPLSNLGNAQK.I (18) R.LFGLAMKMLVPHLK.A (13)
DPOLA_ARATH	At5g67100	DNA polymerase alpha catalytic subunit	38	4	K.NGCNVLSIENSERALLNRLFLELNK.L (14) R.KRSGILSHFTVVR.N (13)
CWP17_ARATH	At2g06850	23 kDa cell wall protein	38	3	-.IPCRKAIDVPFGTR.Y (19) R.KAIDVPFGPR.Y (13)
MOCOS_ARATH	At1g16540	Molybdenum cofactor sulfurase (ABA3)	38	7	K.LLKSLTPSAIWMHTTSLSIYVK.K (12) R.YEIDEKR.Q (10)
ALA11_ARATH	At1g13210	Phospholipid-transporting ATPase 11	38	5	K.SLTYALEDDFKK.K (18) R.SMAMRSNGSSLVGDDLDVVVDQSGPK.I (10)
TAP1_ARATH	At1g70610	Antigen peptide transporter-like 1, chloroplast	38	3	R.GCFFGIANMILVKRMR.E (16) R.QRIGYVGQEPK.L (12)
AGO1_ARATH	At1g48410	Protein argonaute	37	2	R.INLLDEEVGAGGQR.R (36) R.GYGQPPQQQQQYGGPQEYQGRGR.G (4)
FBK19_ARATH	At1g32430	Putative F-box/Kelch-repeat protein At1g32430	37	2	K.VEVRELTLNNPGLK.A (22) R.CIKLEVNEPSLDFLGIGYDNNK.R (14)
LUMI_ARATH	At4g02560	LUMINIDEPENDENS	37	2	K.KHMLGSNPSYNK.E (21) K.HDSSTHPYWNQNK.R (18)
CAPP1_ARATH	At1g53310	Phosphoenolpyruvate carboxylase 1	36	2	K.LEELGSVLTSLDPGDSIVIAK.A (23) K.GIAAGLQNTG.- (14)
WBC16_ARATH	At3g55090	Probable white-brown complex homolog protein 16	36	2	K.TIIGDEGHR.G (29) R.ILFYLCLLLGSKNK.R (8)
CNGC4_ARATH	At5g54250	Cyclic nucleotide-gated ion channel 4	36	3	R.IGLTCGGR.R (36) R.GVDECEMVQNLPEGLR.R (5)
U496I_ARATH	At2g18630	UPF0496 protein At2g18630	36	2	K.INSEYTEHLSSYER.A (21) K.YEKVVRGQK.E (13)
ARFM_ARATH	At1g34170	Auxin response factor 13	36	2	K.FVDAMNNNYIVGSR.F (20) K.FVDAMNNNYIVGSRFR.M (16)
CYSKM_ARATH	At3g59760	Cysteine synthase, mitochondrial (OAS-TL C)	35	3	K.IQGIGAGFIPK.N (15) R.IGYSMVTDAEQKGFISPGK.S (15)

Table 2. Putative Y-nitrated peptides identified by MALDI-TOF from 2D gel-excised spots.

Samples containing 3-nitroY immunopurified proteins were separated by 2-DE and identified by MALDI-TOF as described in Materials and Methods. The AGI identifiers for each identified protein are included along with the corresponding Y-nitrated peptide sequence (underlined appeared the residues susceptible for Y nitration and bold font indicates unequivocally nitration of Y), Error (difference between the experimental and calculated masses), Signal-to-noise ratio, Relative Molecular mass (Mr) observed for the modified and the corresponding unmodified peptide that appeared in the same Mascot search. Values in parentheses indicate the absence of the unmodified peptide. The Mass Shift (Shift) and the modifications of the corresponding peptide with their respective mass increases are also shown. Those proteins that have been previously reported as nitrated in other plant systems have been marked with * (Chaki *et al*. 2009) or § (Cecconi *et al*. 2009).

Descripiton	**AGI Identifier**	**Peptide Sequence**	**Error**	**Signal /Noise**	**Mr (obs) (unmodified)**	**Mr (obs)**	**Shift**	**Modification**
Rubisco activase, chloroplast precursor	At2g39730	$_{351}$R.V**Y**DDEVR.K$_{359}$ $_{72}$R.GLA**Y**DTSDDQQ DITR.G$_{88}$	0.01 -0.05	110 25	895.34 1697.66	940.41 1744.66	+45.07 +46.97	Nitro-Y (+45) 2 Deamination (+2) Nitro-Y (+45)
Serine Hydroxymethyl transferase	At4g13930	$_{160}$K.VNFTTG**Y**ID**Y**D KLEEK.A$_{177}$	0.03	60	1934.83	2025.92	+91.09	Deamination (+1) 2 Nitro-Y (+90)
* Transketolase, putative	At3g60750	$_{333}$K.ANS**Y**SVHGAA LGEKEVEATR.N$_{354}$	0.15	57	(2090.15)	2135.15	(+45)	Nitro-Y (+45)
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	At3g04120	$_{313}$K.LVSWYDNEW GYSSR.V$_{328}$	-0.06	50	1761.72	1806.72	+45	Nitro-Y (+45)
Probable mannitol dehydrogenase	At4g39330	$_{133}$K.NYGGYSENIV VDQR.F$_{148}$	-0.04	27	1613.63	1658.70	+45.07	Nitro-Y (+45)
§ Rubisco large chain precursor	AtCg00490	$_{236}$K.GH**Y**LNATAGT CEEMIK.R$_{253}$	0.04	25	(1794.84)	1839.84	(+45)	Nitro-Y (+45)

Table 3. Structural features of potential Y targets of nitration in MALDI-TOF-identified proteins.

Protein annotation and AGI code along with putative nitrated Y are indicated. Parameters were calculated as described in Materials and Methods. Accesibility Solvent Area (ASA) was calculated by NetSurfP software (Petersen *et al*. 2009).

Protein/AGI	**Putative Nitrated Y**	**Distance to D/E**	**Proximal basic amino acids in primary sequence**	**Location in loop**	**ASA**
Rubisco activase, chloroplast precursor_ At2g39730	Y353 Y76	(no model)	R351, R358, K359 R72	(no model)	5.45 79.13
Serine Hydroxymethyl transferase_ At4g13930	Y167 Y170	5.99 Å to E342 5.04 Å to D197	K160, K172, K176 K160, K172, K176	No No	62.38 23.35
Transketolase, putative_ At3g60750	Y337	9.01 Å to D268	K333, H340, K347	Yes	66.16
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic_ At3g04120	Y318 Y324	6.08 Å to D319 6.61 Å to E321	K313, R327 K313, R327	No No	7.35 19.17
Probable mannitol dehydrogenase_ At4g39330	Y135 Y138	4.31 Å to E8 3.75 Å tu D53	K133, R147 K133, R147	Yes No	34.66 13.55
Rubisco large chain precursor_ AtCg00490	Y239	6.33 Å to E158	K236, H238, K252, R253	No	6.37

Legends for figures

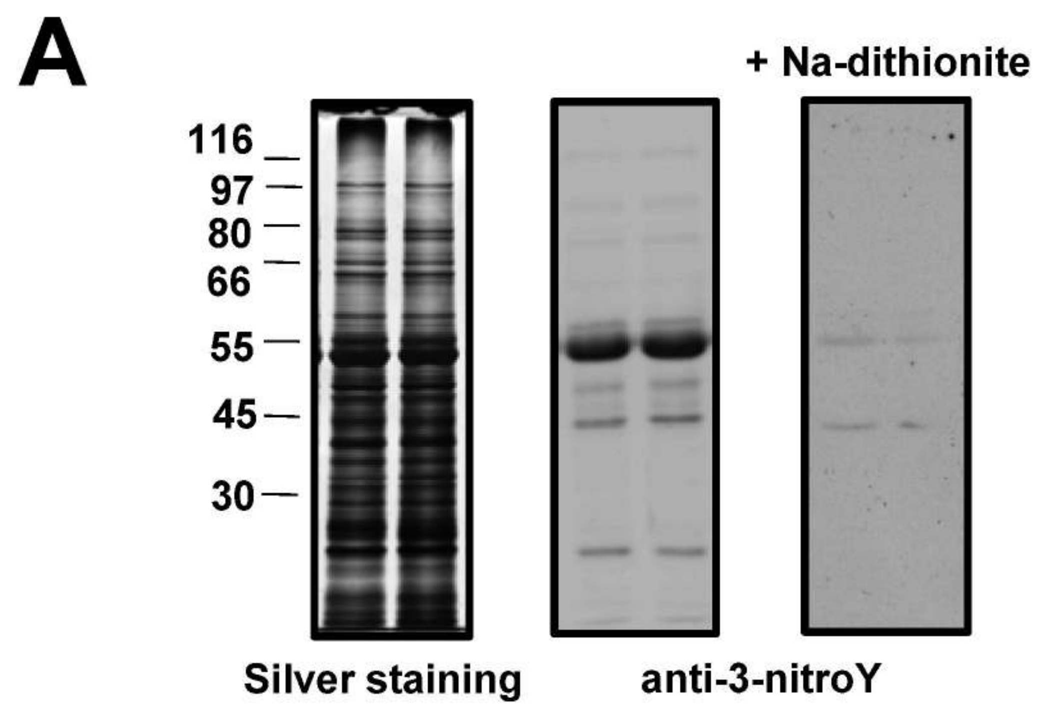
Fig. 1. Detection of 3-nitroY-containing proteins. (A) Crude protein extracts (10 μg per lane) were separated through 10 % SDS-PAGE in duplicate. Left panel shows the silver stained gel with the position of a molecular weight protein ladder. Central panel shows the corresponding Western blot performed with anti-3-nitroY primary antibody, and the right panel the corresponding Western blot after reduction of 3-nitroY to 3-aminoY with 100 mM sodium dithionite (SDT) for 30 min. (B) *In vivo* immunoprecipitation of Arabidopsis 3-nitroY-containing proteins. Crude extracts (CE) were immunoprecipitated with antibody against 3-nitroY. The resulting supernatants (Sup) and immunoprecipitated proteins (IP) alongside CE were separated by 1D-SDS-PAGE in duplicate and either Coomassie stained (left panel) or transferred to nitrocellulose membrane and probed with anti-3-nitroY antibodies by Western blot (right panel). Immunoprecipitated proteins detected in 1D-SDS-PAGE are marked with black arrowheads. The protein A which is released from the resin in the immunoprecipitates is marked with a gray arrowhead. (C) Immunoprecipitated proteins (0.1 mg) were separated by 2-DE with an isoelectric focusing in the range of pH 3 to 10 NL and a second dimension 10 % gel. The identification of spots corresponding to nitrated proteins was performed by comparing four independent sets of 2-DE gels corresponding to biologically independent replicates with similar spot patterns. Molecular mass marker positions are indicated in kDa on the left side. Proteins were silver-stained.

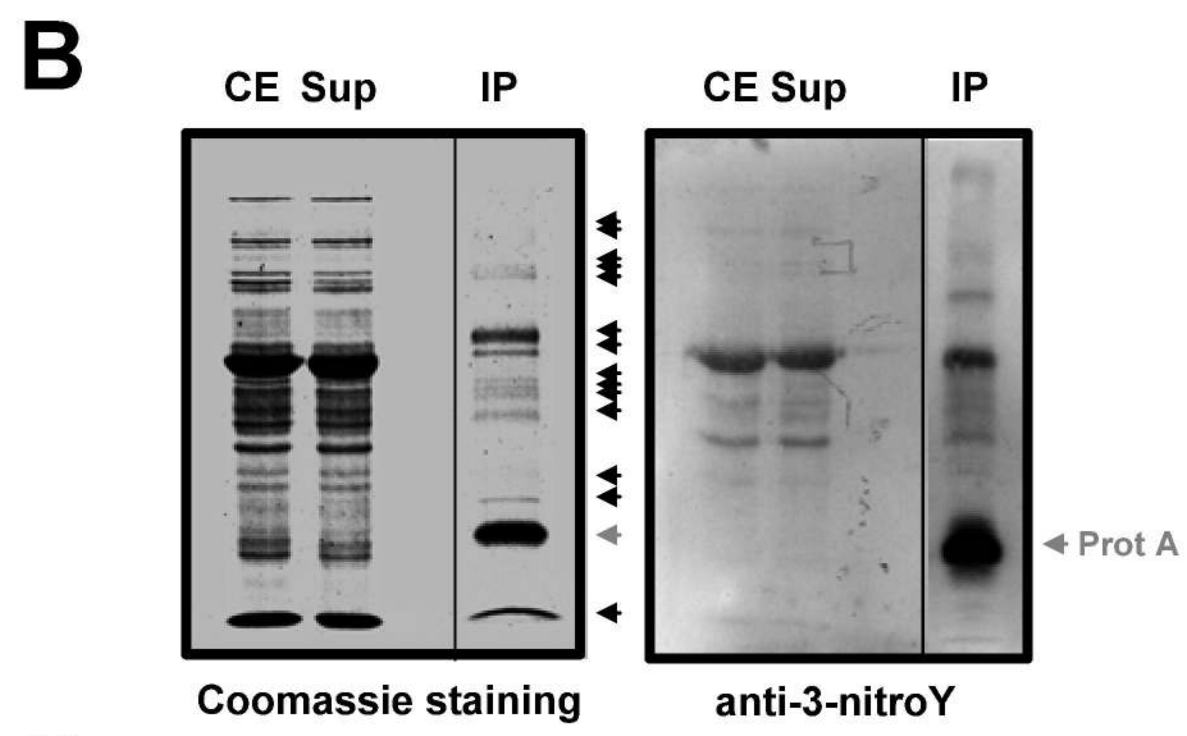
Fig. 2. Confirmation of the presence of proteins identified through shotgun proteomic analysis in the immnunopurified nitroproteome. Crude protein extracts (CE) were immunoprecipitated (IP) with anti-3-nitroY (anti-3-NY) antibodies. The CE, supernatant and IP were separated by 12 % SDS-PAGE, transferred to nitrocellulose membrane and probed with specific antibodies raised against chloroplastic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glycine-rich protein 7 (GRP7), fructose-1-3-bisphosphatase (FBPase), PICKEL (PKL) or carbonic anhydrase (CA). The procedure started from 1 mg of total protein in the crude extract that was immunoprecipitated as described in the corresponding Materials and Methods section and then, the whole IP was loaded in the gel along with 1 % of the CE input and the corresponding supernatant.

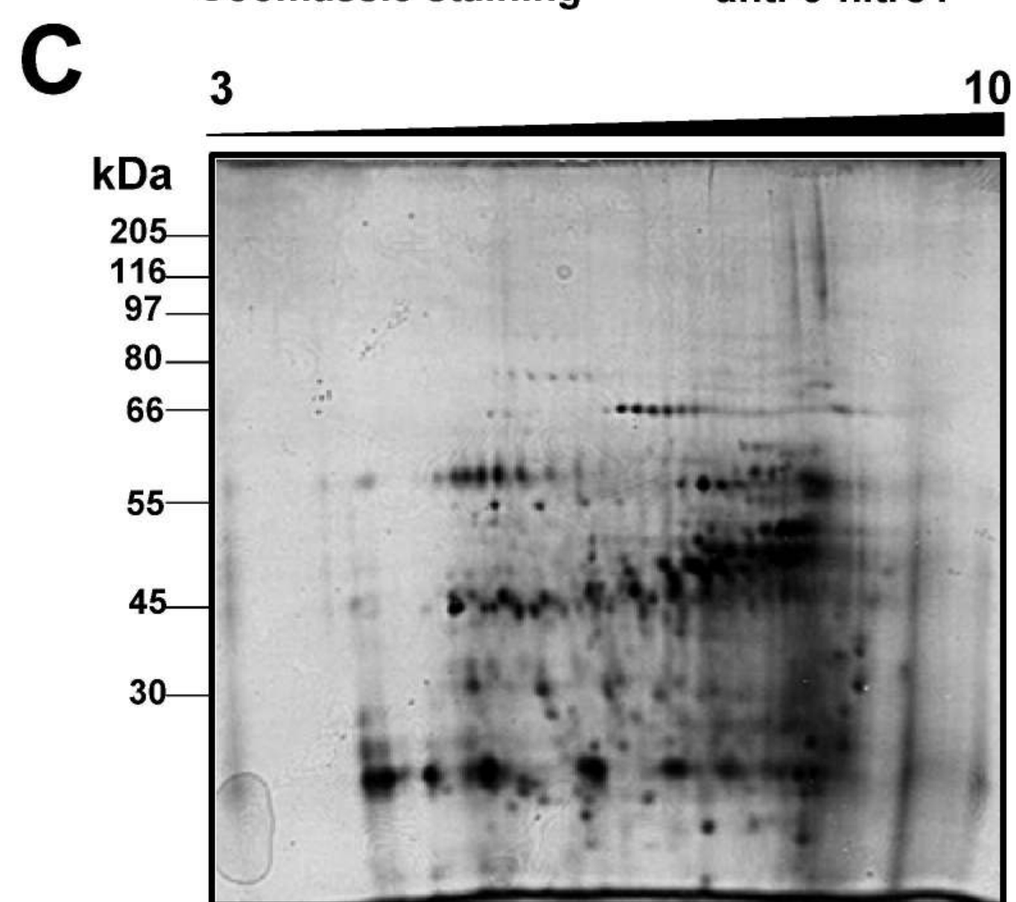
Fig. 3. Effect of nitration on GAPDH. (A) Arabidopsis seedlings were treated with SIN-1 (light grey) or SNP (dark grey) at the indicated concentrations. After indicated times, the GAPDH activity levels were measured in crude protein extracts from whole seedlings as described in Materials and Methods. Measurements for activity were performed by triplicate and the average values ± SD are shown. (B) MS/MS spectrum of aminated LVSWYDNEWGYSSR peptide from Arabidopsis glyceraldehyde-3-phosphate dehydrogenase. Detected peaks of y and b series as well as immonium ions of L, Y and W are indicated.

Fig. 4. Identification of the nitration site in recombinant tagged methionine synthase 1 from Arabidopsis. (A) Equal amounts (5 µg) of recombinant AtMS1 protein were nitrated (+) or not (-), separated by 1D-SDS-PAGE and blotted onto nitrocellulose to be probed by Western blot with anti-3-nitroY (anti-3NY) antibodies. After stripping,

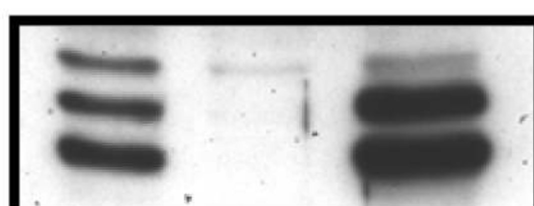
membranes were further probed with anti-5xHis antibodies. Molecular size markers are shown in the left side of panels. (B) MS/MS spectrum of nitrated YLFAGVVDGR peptide from AtMS1. The insert shows the detected y and b series as well as a detail of the spectrum showing the immonium ion corresponding to nitrated Y. (C) Table summarizing data from MS analysis of the nitrated and not nitrated peptides in AtMS1.

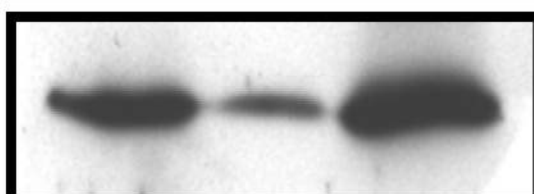

A
+ Na-dithionite
116
97
80
66
55
45
30
Silver staining
anti-3-nitroY

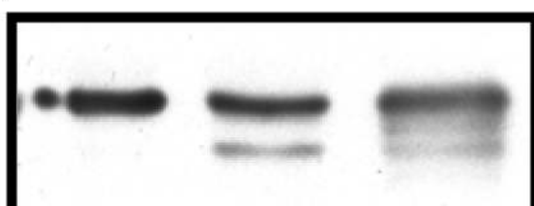

B
CE Sup
IP
CE Sup
IP
Prot A
Coomassie staining
anti-3-nitroY


C
3
10
kDa
205
116
97
80
66
55
45
30

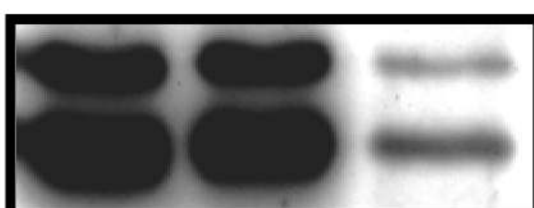
CE
Supernatant
IP (anti-3NY)


GAPDH

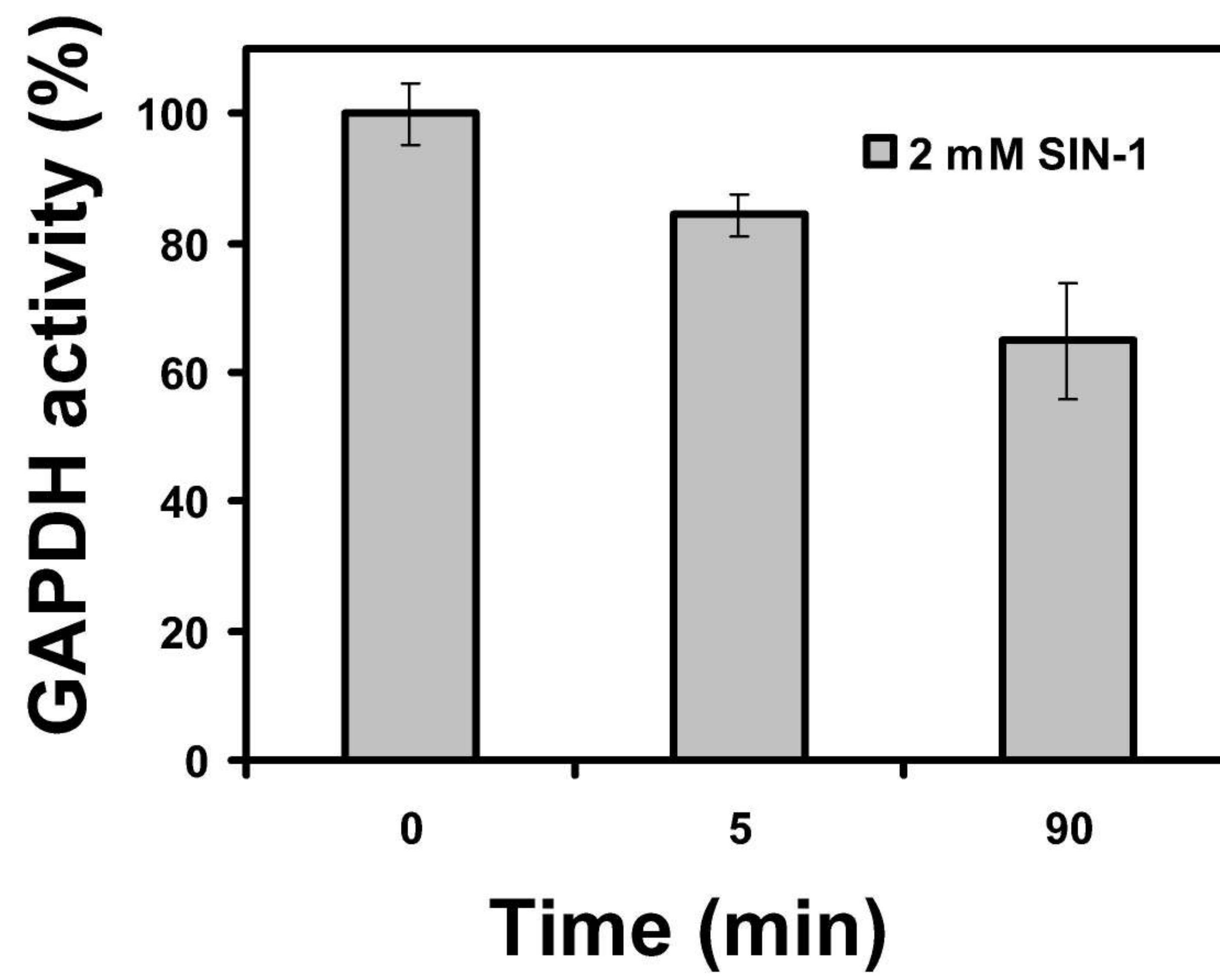

GRP7


FBPase

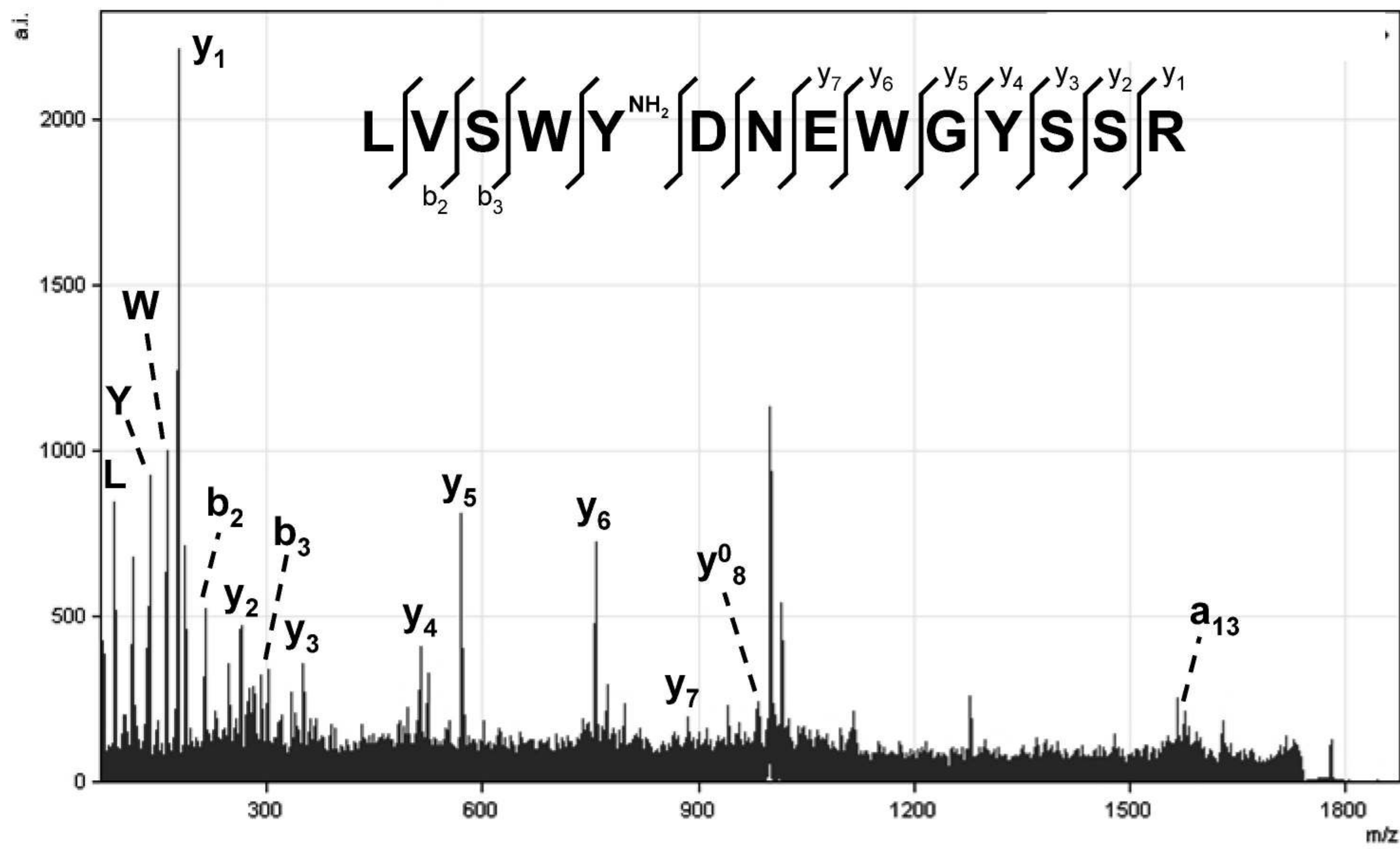
PKL


CA

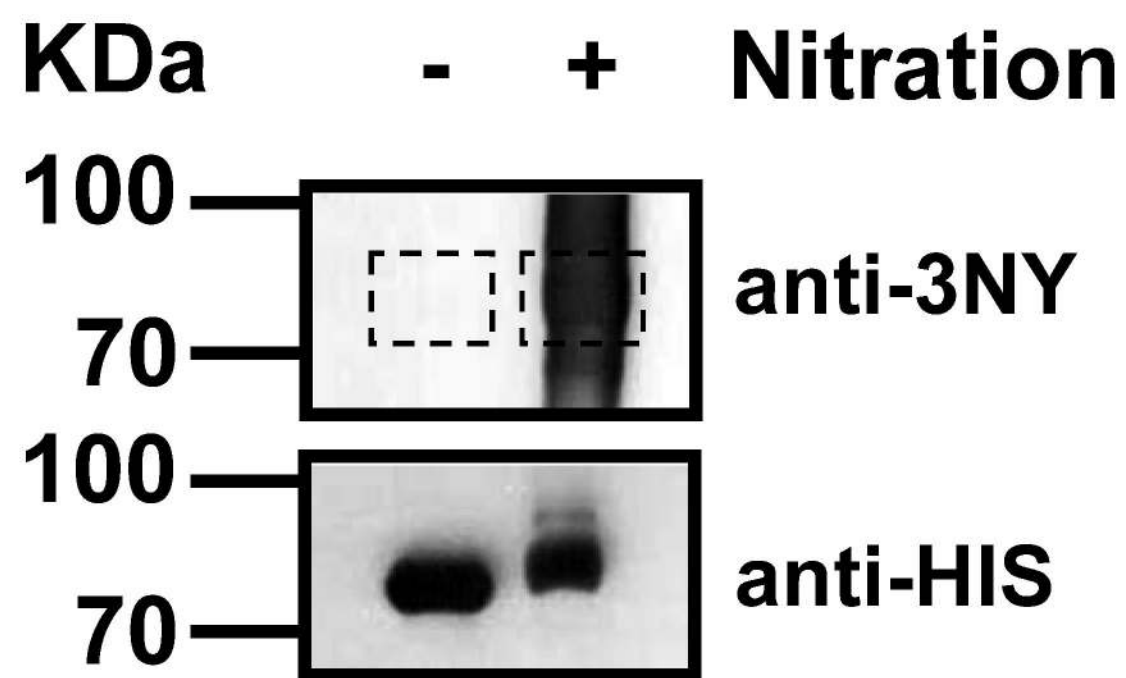
A



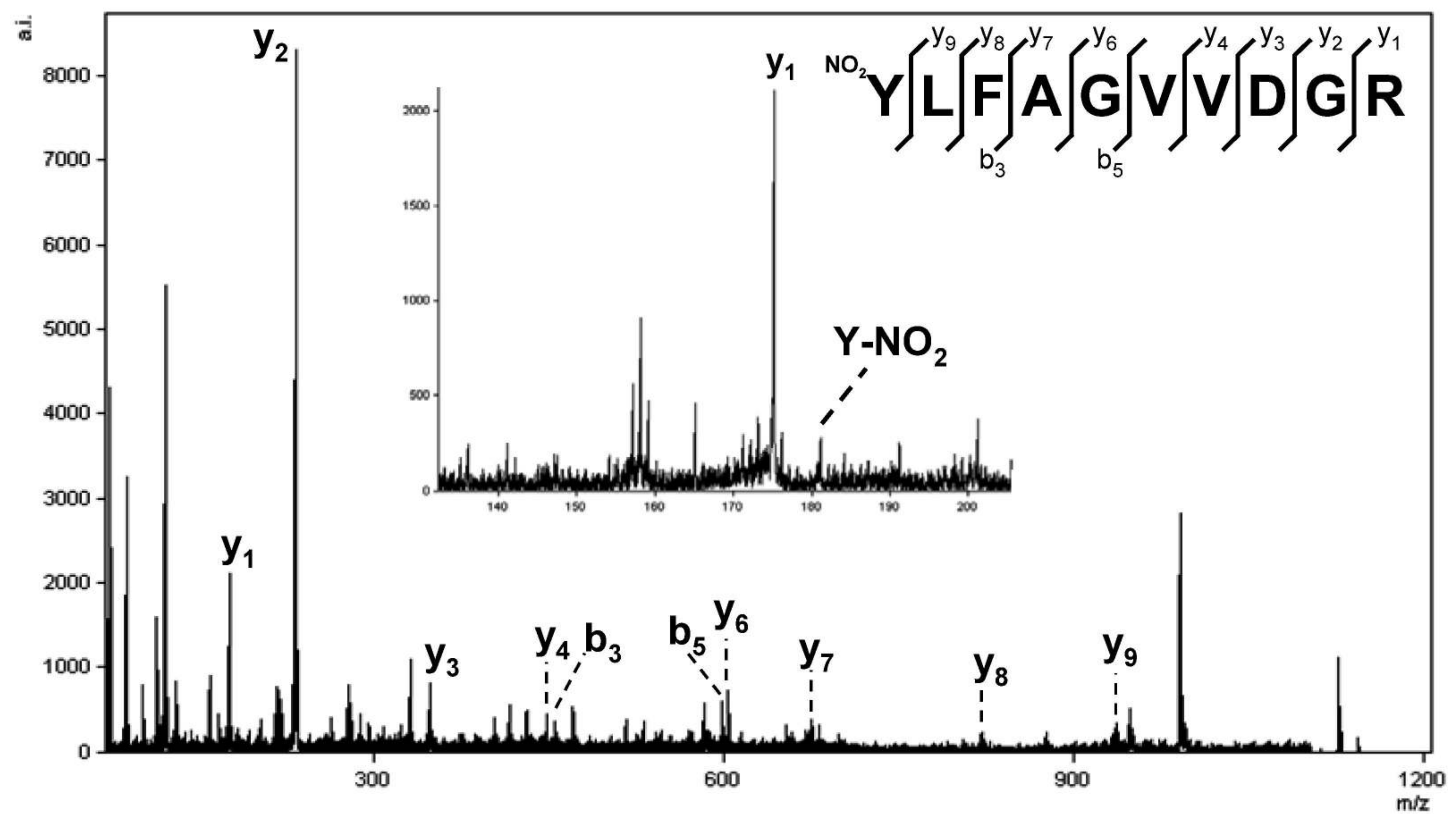
B



A



B



C

Peptide	Start-End (aa)	Score	Expect	Mass ion (m/z)	Shift	Treatment
YLFAGVVDGR	286-296	85	$4.7\ 10^{-8}$	1096.58		Not nitrated
YLFAGVVDGR	286-296	53	$2.4\ 10^{-4}$	1141.54	45	Nitrated