Environmental and Experimental Botany 161 (2019) 228–241

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Drought stress triggers the accumulation of NO and SNOs in cortical cells of *Lotus japonicus* L. roots and the nitration of proteins with relevant metabolic function☆

Santiago Signorelli[a,b,*], Francisco J. Corpas[c], Marta Rodríguez-Ruiz[c], Raquel Valderrama[d], Juan B. Barroso[d], Omar Borsani[a], Jorge Monza[a]

[a] Laboratorio de Bioquímica, Departamento de Biología Vegetal, Facultad de Agronomía, Universidad de la República, CP 12900 Montevideo, Uruguay
[b] Molecular Plant Biology, Biology Department, Faculty of Science, KU Leuven, 3001 Leuven, Belgium
[c] Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, C/Profesor Albareda 1, Granada E, 18008, Spain
[d] Group of Biochemistry and Cell Signaling in Nitric Oxide, Department of Biochemistry and Molecular Biology, Campus 'Las Lagunillas', University of Jaén, Jaén E, 23071, Spain

ARTICLE INFO

Keywords:
Drought
Lotus
Nitric oxide
Protein nitration
Abiotic stress
ROS

ABSTRACT

Drought is considered one of the abiotic stresses with significant implications on plant productivity. Previously, we have shown that water deficit produces a differential nitro-oxidative stress in roots and leaves of *Lotus japonicus* L. plants. Using this model legume, we studied the nitro-oxidative stress in drought-stressed roots by complementary biochemical, cellular and proteomic approaches. Cellular analyses of root cross-sections by confocal laser scanning microscopy (CLSM) using specific fluorescent probes for superoxide radical ($O_2^{\cdot -}$), nitric oxide (NO), peroxynitrite ($ONOO^-$) and *S*-nitrosothiols (SNOs) showed that drought stress causes a differential cellular localization of these reactive species. Mainly, $O_2^{\cdot -}$ and $ONOO^-$ had a wide distribution in almost all root cell types (xylem, parenchyma, and peridermis), whereas NO and SNOs accumulated in cortical cells (peridermis). Liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) analyses showed that the content of ascorbate, *S*-nitrosoglutaathione (GSNO), and reduced glutathione (GSH) in drought-stressed roots was drastically diminished. Nitroproteome analysis by two-dimensional gel electrophoresis and mass spectrometry allowed to identify 13 tyrosine-nitrated proteins such as methionine synthase, Hsp70, adenosyl-homocysteinase, peroxidase, alcohol dehydrogenases, glutamine synthetase, fructokinase, 1,3-beta-glucanase, chitinases, endochitinase, among others which are directly (24%) or indirectly (74%) related to plant defense. Taken together, these results indicate that drought-stressed roots have an active metabolism of reactive oxygen and nitrogen species (ROS and RNS) characterized by an increase of protein nitration and accumulation of NO and SNOs in cortical cells. The possibility of autophagy taking place in the stressed roots is also discussed.

1. Introduction

Drought is considered one of the major abiotic stresses affecting plant productivity due to their negative effects on plant growth (Fahad et al., 2017). According to the data provided by the United Nations Food and Agriculture Organization, drought is the abiotic stress affecting crop production which occurs in nearly all regions. In fact, drought has affected more people worldwide in the last 40 years than any other natural threat. For example, throughout 2017 Africa underwent a long period of drought resulting in the suffering of many millions of people (http://www.fao.org/3/a-bs902e.pdf).

Under drought conditions plants undergo evident morphological changes such as the reduction in the growth of shoots (leaves and stem) and roots suffer modification in diameter and length which disrupts plant-water relations and reduces water-use efficiency (Bechtold, 2018; Feller, 2016; Yordanov et al., 2000). At cellular level, drought induces

☆ This article is part of a special issue entitled is "Revisiting the role of ROS and RNS in plants under a changing environment" published at the journal Environmental and Experimental Botany 161.

* Corresponding author at: Laboratorio de Bioquímica, Departamento de Biología Vegetal, Facultad de Agronomía, Universidad de la República, CP 12900 Montevideo, Uruguay.
E-mail address: ssignorelli@fagro.edu.uy (S. Signorelli).

https://doi.org/10.1016/j.envexpbot.2018.08.007
Received 25 May 2018; Received in revised form 27 July 2018; Accepted 7 August 2018
Available online 16 August 2018

the production of reactive oxygen species (ROS) and alteration of antioxidative systems (Khan and Khan, 2017; Signorelli et al., 2013a; Ye et al., 2018). However, less is known about how reactive nitrogen species (RNS) are affected by this environmental condition. Nitric oxide (NO) can be accumulated both enzymatically and non-enzymatically. There are some reports about how it modules photosynthesis, stomata movement or ABA-mediated responses (Fan and Liu, 2012; Signorelli and Considine, 2018; Wang et al., 2016). Given its regulatory capacity, understanding the sites of accumulation and the effect of certain conditions, such as droughts, is important to predict its potential role under such specific condition. In plants a NOS-like activity has been described (Corpas et al., 2009). However, the lack of a single gene confiding for this activity in plants makes difficult to predict where and when NO is going to be accumulated, and thus the biochemical determination of this molecule becomes more relevant. On the other hand, plant roots exposed to drought stress undergo morphological changes such as root diameter and length, but beyond these adaptations, there are biochemical and physiological processes which in many cases have associated overproduction of ROS but also of RNS, mainly produced in chloroplasts, mitochondria, and peroxisomes. ROS are also generated in the extracellular site, mostly through the Respiratory burst oxidase homologs (Rbohs) activity, which produce superoxide ($O_2^{\cdot -}$) and results in the formation of apoplastic hydrogen peroxide (H_2O_2). At high concentrations, ROS and RNS can damage the cells through the oxidation of lipids, proteins, and DNA (Sofo et al., 2015; You and Chan, 2015; Zhang et al., 2017). When this happens due to an overproduction of ROS, researchers usually refer to an oxidative stress condition; whereas nitrosative stress is associated to an overproduction of RNS, that results in nitrosative damage. Protein tyrosine nitration (3-NO_2-Tyr) has long been considered the best marker of nitrosative damage (Corpas et al., 2007; Radi, 2004). In fact, these stresses are often mixed, and when this happens we name it nitro-oxidative stress (Corpas and Barroso, 2013; Signorelli et al., 2013b). In this context, it is important to evaluate how NO and derived molecules such as nitrosothiols (SNOs) or peroxynitrite ($ONOO^-$) are affected considering its production and cellular distribution in specific plant organs. Understanding this could provide valuable information because in many cases the response can be local or broad (Houmani et al., 2018).

In a previous report, we showed that drought produces nitro-oxidative stress in *L. japonicus* plants (Signorelli et al., 2013b). In particular, we found a main nitrosative stress component in roots; however, those plants were grown in Leonard jars (hydroponic system), and drought was imposed by removing the water from the jar. That stress condition might not be so representative of what happens in soils. Therefore, in this study, we first evaluated whether Leonard jars are a suitable approach to assess drought responses and then we further investigated the accumulation and cellular localization of ROS and RNS in roots. Moreover, by a proteomic approach, we identified potential protein targets of tyrosine nitration in roots, finding out that these are proteins with relevant metabolic functions.

2. Material and methods

2.1. Plant material and growth conditions

Lotus japonicus (Regel) Larsen cv. Gifu seeds were surface-sterilized as described in Signorelli et al., (2013b) and germinated at 25 °C for 2 days. Plants were grown for 35 days under controlled conditions: 16/8 h light/dark cycle at 24/18 °C with photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Two different growth systems were used, pots and Leonard jars, containing a mix of sand:vermiculite (1:1) as substrate and supplied with Rigaud and Puppo nutrient solution supplemented with 1 mM KNO_3 (Rigaud and Puppo, 1977).

Potted plants were irrigated every two days to sustain a gravimetric water content (GWC) near 30%. Control plants were kept in this condition, whereas drought plants were deprived of watering during 5 days. In the case of plants grown in Leonard jars the roots were completely submerged into the nutrient solution, which was replaced every 2 weeks. For the drought stress imposition, the nutrient solution was discarded, the jars were sealed with Parafilm® to prevent an excessive dehydration of the root system, and the plants were allowed to grow for additional 5 days in this condition (Signorelli et al., 2013b). At the end of the drought imposition, the GWC of soil and the relative water content (RWC) of leaves were determined. GWC was determined as: [(weight of wet soil − weight of dry soil)/weight of dry soil] × 100, while RWC was determined as described in Signorelli et al. (2013b).

2.2. Determination of ascorbate and glutathione species (GSH, GSSG and GSNO) by liquid chromatography-electrospray/mass spectrometry (LC-ES/MS)

Root and leaf samples were frozen in liquid N_2 and ground with a mortar and pestle in the presence 0.1 M HCl in a ratio 1:3 (w/v). Homogenates were centrifuged at 21,000 *g* for 20 min at 4 °C and the supernatants were collected, filtered through 0.45 μm nylon filters and immediately analyzed by LC-ES/MS system which consisted of a Waters Allience 2695 HPLC system connected to a Micromass Quattro micro API triple quadrupole mass spectrometer, both obtained from the Waters Corporation. LC was carried out using an Atlantis® T3 3 μm 2.1 x 100 mm Column obtained from the Waters Corporation. The Micromass Quattro Micro API mass spectrometer was used in positive electrospray ionization mode for simultaneous detection and quantification of ascorbate, GSH, GSSG and GSNO (Airaki et al., 2011; Corpas et al., 2016).

2.3. Protein extraction

Roots and leaves were frozen in liquid N_2 and ground in a mortar. The powder was suspended in a ratio 1:2 (w/v) in the extraction buffer containing 50 mM Tris-HCl, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.2% (v/v) glycerol. The ground tissue was mixed with the extraction buffer using micro pestles into micro-tubes for 1 min each. Micro-tubes were placed on ice during all procedure. Homogenates were centrifuged at 20,000 *g* for 20 min at 4 °C and supernatants were placed in new micro-tubes and immediately used for assay or stored at − 20 °C.

2.4. SDS-PAGE, Western blotting and immune-detection

Protein samples were separated either by 12% SDS-PAGE or mini-protean® TGX TM 4–20% precast polyacrylamide gels using a Mini-Protean electrophoresis cells (Bio-Rad, Hercules, CA, USA). For immunoblot analysis, proteins were transferred onto 0.45-μm PVDF membranes using a semidry blot system (Bio-Rad). After transfer, the membranes were used for cross-reactivity assays with a rabbit polyclonal antibody against nitro-tyrosine (Sigma, ref N 0409) (dilution 1:5000) (Signorelli et al., 2016) and FeSOD (diluted 1:3000) (Houmani et al., 2016). For immunodetection, an affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (diluted 1:2,000, Bio-Rad Laboratories) and an enhanced chemiluminescence kit (Clarity™ Western ECL Substrate, BioRad) were used.

2.5. Histochemical detection of ROS and RNS by confocal laser scanning microscope (CLSM) in root sections

The detection of the different ROS and RNS was done in 2.5 mm-long transversal sections of *L. japonicus* roots that were incubated in darkness with specific fluorescent probes (Chaki et al., 2011, 2009). Roots coming from at least three different plants were used. Then, these samples were embedded in a mixture of 15% acrylamide-bisacrylamide stock solution as described previously elsewhere (Valderrama et al., 2007) and at least ten 100-μm-thick sections were cut under 10 mM

phosphate-buffered saline (PBS) and observed by CLSM. Briefly, for $O_2^{\cdot -}$, the root sections were incubated with 10 μM dihydroethidium (DHE, Sigma-Aldrich-Fluka) (excitation 488 nm; emission 520 nm). Potential background staining was evaluated by the pre-incubation of root sections with the $O_2^{\cdot -}$ scavenger TMP (1 mM) for 1 h. Nitric oxide (NO) was detected with 10 μM 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Calbiochem) prepared in 10 μM Tris − HCl (pH 7.4) (excitation 495 nm; emission 515 nm). As control, sections were pre-incubated with 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger, at 25 °C for 30 min. Peroxynitrite ($ONOO^-$) was detected with 10 μM 3′-(paminophenyl) fluorescein (APF, Invitrogen) prepared in 10 mM Tris − HCl at pH 7.0. Root sections were incubated with APF at 25 °C for 1 h in darkness (excitation 495 nm; emission 515 nm). As a control, sections were preincubated for 2 h at 25 °C with 20 μM Ebselen ($ONOO^-$ scavenger) (Chaki et al., 2009; Corpas and Barroso, 2014). To detect *S*-nitrosothiols (SNOs), root samples were incubated with 100 μM diethylenetetraminepentaacetic acid (DTPA) plus 10 mM N-ethyl-maleimide NEM prepared in ethanol, which blocks free sulfhydryl groups, then sections were incubated with the fluorescent reagent Alexa fluor 488 Hg-link phenylmercury (excitation 495 nm; emission 519 nm). As control, samples were pre-incubated in the presence of reductants (1 mM ascorbate, 10 μM CuCl and 200 μM cPTIO) (Chaki et al., 2011). All samples were examined with a confocal laser scanning microscope system (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). Background staining, routinely negligible, was controlled with root sections unstained.

2.6. Two-dimensional (2D) gel electrophoresis and immunoblot analysis

Root samples were separated by two-dimensional gel electrophoresis. Isoelectric focusing was carried out with precast IPG gels pH 3-10. Each gel was loaded with approximately 150 μg of proteins. Second-dimensional separation was performed by glycine-SDS-PAGE according to Laemmli, (1970). Gels were SYPRO Ruby stained, scanned, and analyzed with Bio-Rad PD Quest software. Western blot of 2D gels was performed as described above (Chaki et al., 2009).

2.7. In situ digestion of 2D spots and protein identification by MALDI TOF-TOF analysis

By comparison of the protein partner spots obtained in the 2D gel and its corresponding immnoblot, it was possible to identified protein spots in the gel. The selected protein spots were automatically recovered using Investigator ProPic Protein Picking Workstation (Genomic Solutions). Then, the protein spots were digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomics Solutions). The procedure was as follows: spot destaining with 40% acetonitrile/200 mM NH_4HCO_3 for 30 min (twice); washing with 25 mM NH_4HCO_3 for 5 min and further with 25 mM NH_4HCO_3/50% acetonitrile for 15 min, respectively (twice). Then, samples were then re-dehydrated with 100% acetonitrile for 5 min and dried. The samples were hydrated with 10 μL trypsin in 25 mM NH_4HCO_3 (12.5 ng μL^{-1}) at room temperature for 10 min and 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) with a C18 column (ZipTip, Millipore) and eluting with α-cyano-4-hydroxycinnamic acid (3 mg mL^{-1}) in 70% acetonitrile/0.1% TFA in a MALDI plate (1 μL). After crystallization the samples were analyzed using a MALDI TOF-TOF Mass Spectrometer in a range mass-to-charge ratio (*m/z*) of 800–4000 Da using a spectrometer 4700 Proteomics Analyser (Applied Biosystems) in automatic mode. Internal calibration of the mass spectrums was performed using the *m/z* of the peptides from porcine trypsin autolysis (mass MH^+ = 842.509, mass MH^+ = 2211.104), given a precision in the *m/z* ratio of 20 ppm. From each sample, the three spectrums with the highest *m/z* ratios were selected. The protein was identified by combining the MS spectrum with the corresponding MS/MS using the MASCOT program from the database of MatrixScience (http://www.matrixscience.com/). The following search parameters were applied, limiting the taxonomic category to green plants: a mass tolerance of 100 ppm and one incomplete cleavage were allowed; complete alkylation of cysteine by carbamidomethylation and partial oxidation of methionine (Begara-Morales et al., 2013; Chaki et al., 2009). After analyses, approximately 60 spots were identified, containing a rank of potential proteins, the protein score, the molecular weight (MW) and point isoelectric (PI) among other information (**Table S1**). To determine the candidate protein in *L. japonicus*, first it was verified that the suggested MW and PI were concordant with the position found in the 2D gel. When the first protein of the rank was already in *L. japonicus* and their MW and PI were coherent with the observed in the 2D-gel, the protein was reported as it is. When the first protein of the rank was from other species, the homologous protein in *L. japonicus* was identified by performing a blastp at NCBI (https://www.ncbi.nlm.nih.gov/). Once all the proteins were identified, the presence and number of tyrosine were confirmed in those identified as candidate nitrated proteins. UniProt (uniprot.org) was used to assign the biological function of the proteins and classify them.

2.8. Other assays

The protein concentration was determined with the Bio-Rad protein assay, using BSA as a standard. The analyses of the variance were performed with data from at least three independent replicates and means from the results were compared using Tukey's test at the $p \leq 0.05$ level. Computational prediction of tyrosine nitration sites in proteins were done with the GPS-YNO2 program.

3. Results

3.1. Drought induces a nitro-oxidative response in roots, independently of the experimental approach used (Leonard jars or pots) to impose the stress

Considering that in a previous report the experimental approach used to impose the drought/water stress was on "Leonard jars", first, the imposition of drought in this system was evaluated against pots. Fig. 1**A** shows the appearance of *L. japonicus* plants grown in pots subjected to drought for 5 days until a wilting phenotype became visible. At that time, both relative water content (RWC) in leaves and gravimetric water content (GWC) in soil substrate were drastically reduced under drought conditions in pots (Fig. 1B). These data are in a good agreement with those reported previously in Leonard jars (Signorelli et al., 2013b). Additionally, we evaluated by immunoblot assays, the content of two previously observed markers, nitrated proteins, and Fe-SOD. Fig. 1**C** shows the content of nitrated proteins assessed with an antibody against nitro-tyrosine being observed an increase of nitrated proteins in roots of plants exposed to drought stress in pots. Also an increase of nitrated protein was observed in roots of plants grown and exposed to drought stress using Leonard jars (Signorelli et al., 2013b), although the profile was not exactly the same.

Iron superoxide dismutase (FeSOD) is part of the family of superoxide dismutases including CuZnSODs and MnSODs, which are differentially modulated under different oxidative stress conditions (Houmani et al., 2016) including drought stress (Faize et al., 2011; Selote and Khanna-Chopra, 2010). Fig. 1**D** shows the content of FeSOD, analyzed by immunoblot, in roots of *L. japonicus* plants exposed to drought stress using both pots and Leonard jars. In both systems, the FeSOD content increased under drought stress being more evident in roots of plant growth in Leonard jars.

3.2. GSH and ascorbate content is diminished in roots under drought stress

In our previous study, the antioxidant enzymes such as glutathione

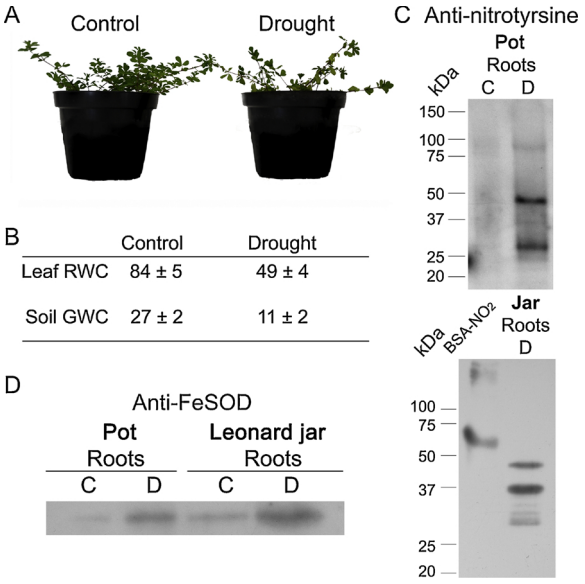


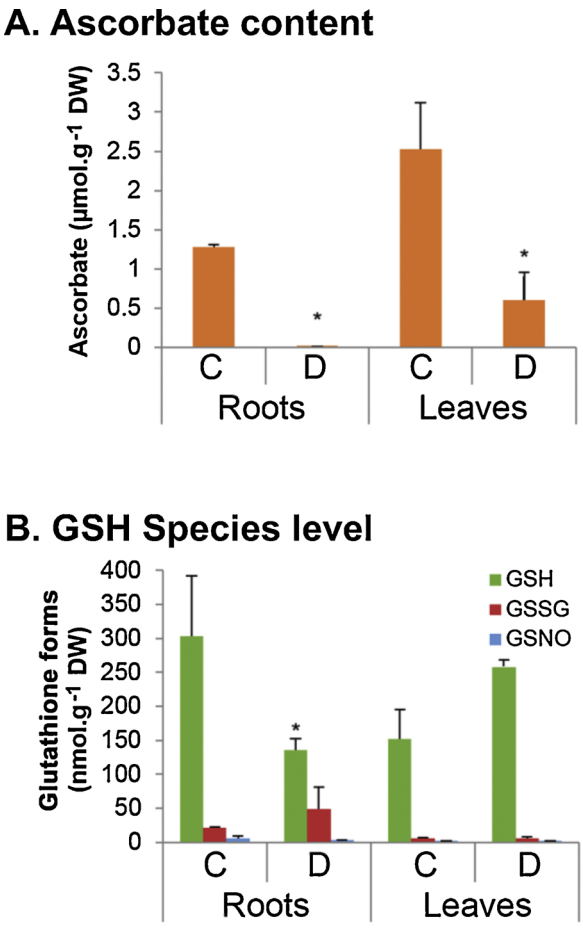
Fig. 1. Water content, Fe-containing superoxide dismutase (FeSOD) and protein nitration in *L. japonicus* L. plants grown in pots under drought conditions. A. Phenotype of plants growth under optimal conditions (C, control) and drought (D) stress. **B.** Relative water content (RWC) in leaves of *L. japonicus* and gravimetric water content (GWC) of control and drought soils. **C.** Comparison of nitrated protein in roots of plants grown in pots and Leonard jars **D.** Fe-SOD immunoblot of roots and leaves extracts. C, control, and D, drought. The protein load was 20μg of protein per line.

reductase (GR) and ascorbate peroxidase (APX), as well as the NADPH-generating systems and GSNO reductase were analyzed in roots of *L. japonicus* under drought stress (Signorelli et al., 2013b) being significantly affected. To get more profound insights, complementary analyses have been carried out by LC-ES/MS including the content of ascorbate (ASC) and glutathione (GSH) which are considered the most abundant soluble antioxidants in plant cells (Foyer and Noctor, 2011), as well as the content of oxidized glutathione (GSSG), and *S*-nitrosoglutathione (GSNO), being this last molecule a natural NO reservoir in the cell (Airaki et al., 2011; Kailasam et al., 2018). Fig. 2**A** shows the analysis of ASC content in roots and leaves of plants under drought conditions. ASC content was significantly reduced in both roots and leaves under drought stress suggesting that this situation affects the antioxidant status in whole plants. A similar response has been found in cucumber and soybean plants under drought stress (Fan and Liu, 2012; Seminario et al., 2017) as well in Arabidopsis under arsenic stress (Corpas et al., 2016).

On the other hand, GSH content in roots was diminished by 55% whereas increased in leaves (Fig. 2B). This decrease of GSH has also been described in cucumber plants (Fan et al., 2014). However, GSSG did not show statistically significant differences either in roots and leaves under drought conditions. The GSH/GSSG ratio was determined to be 11.7 and 2.9 in roots and 26.1 and 41.6 in leaves, under control and drought conditions respectively. GSNO is produced by the *S*-nitrosylation of GSH and its contents were 3–7 folds higher in roots than in leaves (Fig. 2C), but under drought conditions the GSNO content was unaffected. These data are in part in line with earlier observations showing that GR activity is 3-folds greater in leaves than roots, whereas roots have a more active GSNO metabolism since the GSNOR activity was 2.5-folds higher in roots than in leaves (Signorelli et al., 2013b).

3.3. Drought stress triggers the accumulation of NO and SNOs in cortical cells of L. japonicus L. Roots

As part of the metabolism of ROS and RNS, the content and distribution of relevant molecules including $O_2^{\cdot -}$, NO, $ONOO^-$ and SNOs



C. GSNO content

	Roots		Leaves	
	C	D	C	D
Means	6.1^{a}	3.4^{ab}	0.8^{b}	1.1^{b}
SD	2.7	0.2	1.4	0.3

Fig. 2. Content of ascorbate, glutathione (reduced and oxidized), S-nitrosoglutathione (GSNO) in roots and leaves of *L. japonicus* plants subjected to drought stress. A and B, Data represents the mean ± SD of at least three different experiments. DW, dry weight. Asterisk indicate significant differences ($P < 0.05$). C, GSNO data are expressed as nmol.g-1 DW. The different letters indicate significant differences ($P < 0.05$).

were analyzed by CLSM using specific fluorescent probes. Fig. 3**A** shows cellular content and localization of $O_2^{\cdot -}$ analyzed by CLSM using the fluorescence probe dihydroethidium (DHE) in roots cross-section of *L. japonicus* plants under control and drought stress (upper panels) and its corresponding merge with the bright-field images (lower panels). Thus, the green fluorescence corresponding to $O_2^{\cdot -}$ was imperceptible in control roots but under drought stress, it was intensified in almost all root cell types including vascular tissues (xylem), parenchyma and cortical cells. As control of the $O_2^{\cdot -}$ production, root sections from plants under drought stress were pre-incubated with 1 mM TMP (a $O_2^{\cdot -}$ scavenger), and the green fluorescence was sharply reduced. Fig. 3**B** shows similar analyses but in this case to detect NO using DAF-FM DA as specific fluorescence probes (Corpas et al., 2006). Whereas the level of NO was almost undetectable in control roots, under drought stress, the green fluorescence was mainly located in cortical cells (Cc). As control of the NO production, root sections from plants under drought stress were pre-incubated with cPTIO (a NO scavenger) and the green

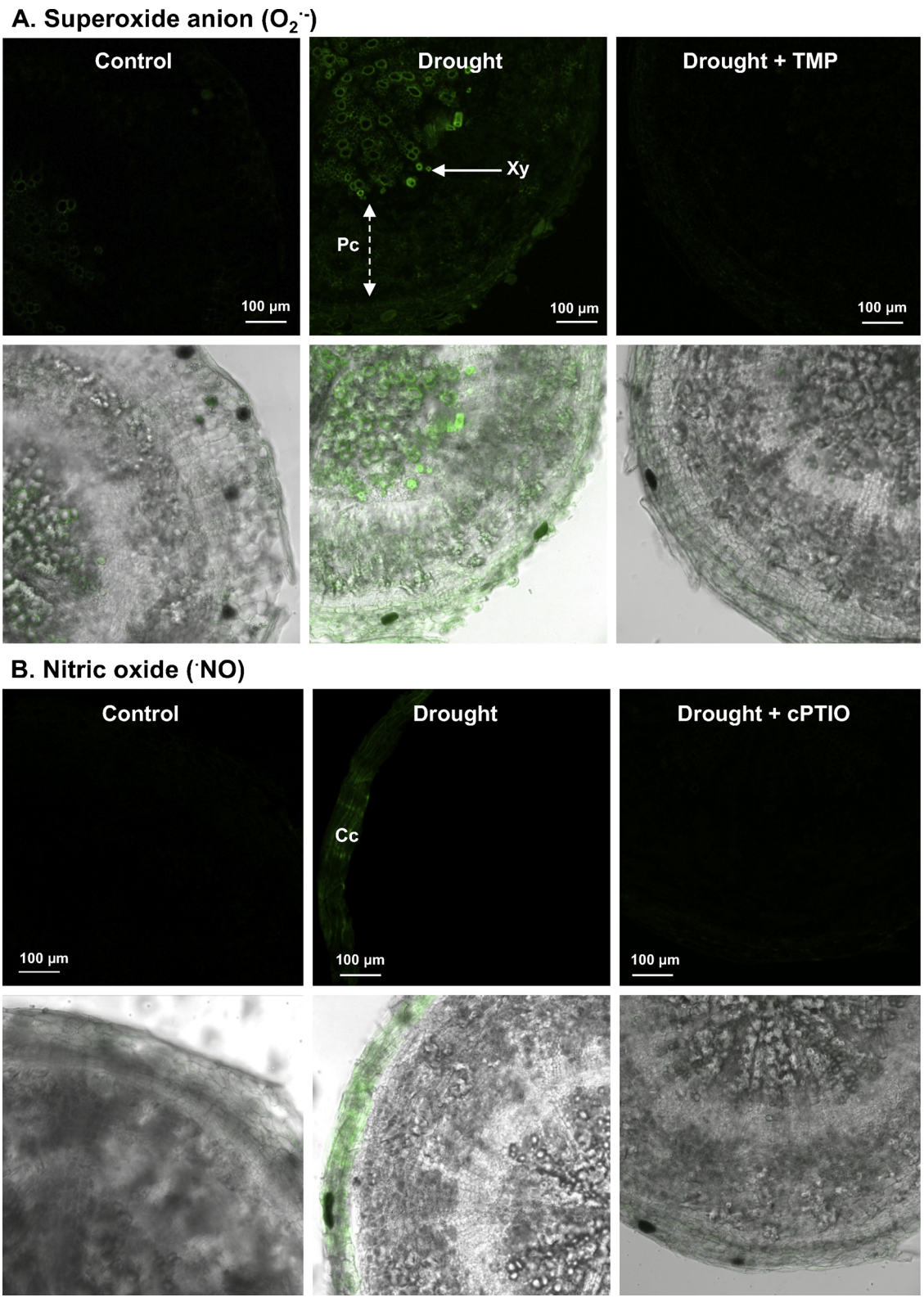


Fig. 3. Cellular CLSM detection of (A) superoxide anion (O2·-) and (B) nitric oxide (NO) in cross sections of roots of *L. japonicas* subjected to drought stress. The bright green fluorescence corresponds to the detection of each reactive species with the corresponding fluorescence probe: DHE for O2·- and DAF-FM DA for NO. The specificity of each fluoresce probes was evaluated by specific scavenger: TMP for $O2^{\cdot}-$ and cPTIO for NO. Cc cortical cells (peridermis); Pc parenchyma cells of the cortex, Xy xylem. Bright-field plus merged images of corresponding samples. At least 3 roots were employed and 10 sections per roots were analyzed under the microscope. The most representative images for each condition is shown.

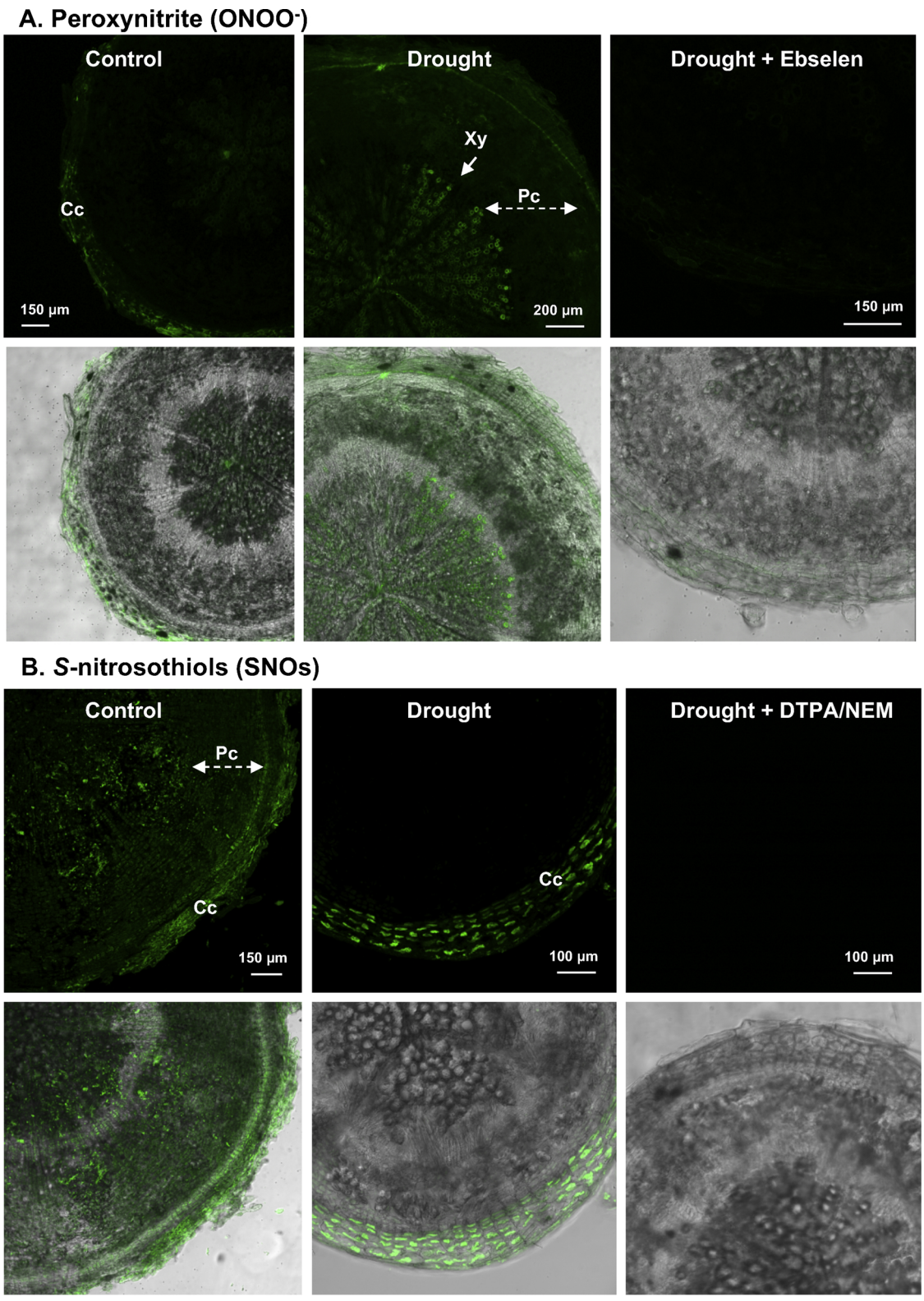


Fig. 4. Cellular CLSM detection of (A) peroxynitrite ($ONOO^-$) and (B) *S*-nitrosothiols (SNOs) in cross sections of roots of *L. japonicus* subjected to drought stress. The bright green fluorescence corresponds to the detection of each reactive species with the corresponding fluorescence probe: APF for $ONOO^-$ and Alexa fluor 488 Hg-link phenylmercury for SNOs. The specificity of each fluoresce probes was evaluated by specific scavenger: ebselen for $ONOO^-$ and in the presence of reductants (NEM, N-ethylmaleimide; DTPA, diethylenetetraminepentaacetic acid) for SNOs (see material and methods for details). Cc cortical cells; Pc parenchyma cells of the cortex, Xy xylem. Bright-field plus merged images of corresponding samples. At least 3 roots were employed and 10 sections per roots were analyzed under the microscope. The most representative images for each condition is shown.

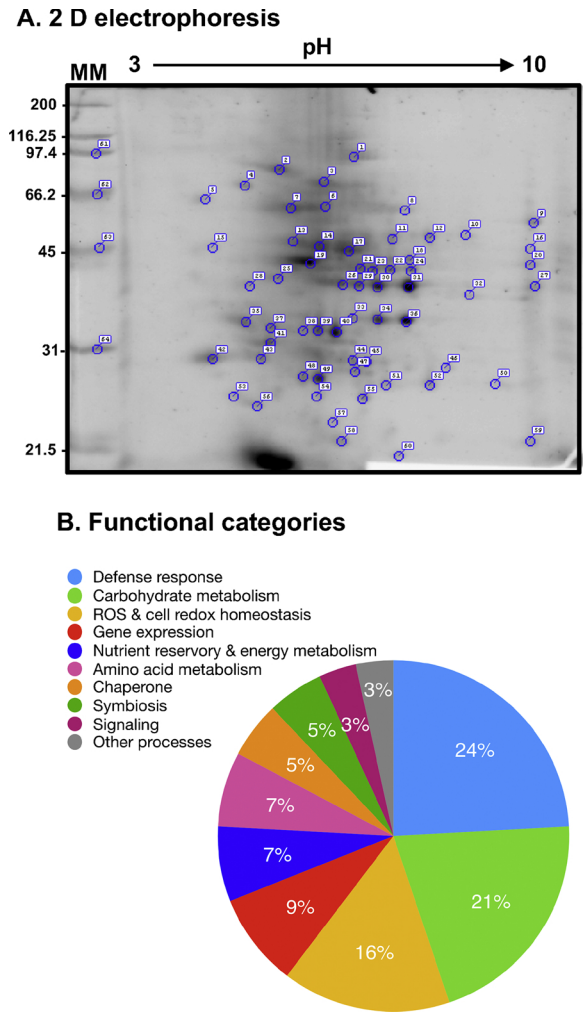


Fig. 5. Proteomic analysis of *L. japonicus* roots under stress conditions. (A) Representative 2D electrophoresis (pH 3–10 for the first dimension) of *L. japonicus* L. root samples stained with Sypro Ruby. Molecular-mass standards are indicated on the left in kDa. Approximately 150 µg protein was loaded per gel. **(B)** Functional category of the most abundant root proteins of *L. japonicus* L. subjected to drought.

fluorescence was substantially reduced. Fig. 4**A** illustrates the cellular detection of $ONOO^-$ using the fluorescent probe APF (Chaki et al., 2011). In control roots, $ONOO^-$ was mainly found in cortical cells, whereas under drought stress the $ONOO^-$ was significantly increased in all cell types. As control of the $ONOO^-$ production, root sections from plant under drought stress were pre-incubated with Ebselen (a $ONOO^-$ scavenger) (Chaki et al., 2009), and the green fluorescence was strongly reduced. Fig. 4**B** illustrates the histological detection of SNOs using the fluorescent probe Alexa Fluor 488 Hg-link, which can react with SNOs via the Saville reaction (Chaki et al., 2009; Corpas et al., 2008; Valderrama et al., 2007). While SNOs had a widespread distribution along all cell types with considerably high levels in control roots, under drought, stress the SNOs were almost localized in cortical cells. As control of the SNOs production, root sections from plants under drought stress were pre-incubated with DTPA and NEM which avoid the SNOs interaction with the fluoresce probes (Chaki et al., 2009) and consequently the green fluorescence was undetectable.

3.4. Proteomic analysis of roots of L. japonicus, plants subjected to drought

Root samples were analyzed by 2D gel electrophoresis (Fig. 5A) and then identified by MALDI-TOF mass spectrometry after trypsin digestion using the MASCOT search engine to analyze MS data, and identify proteins from primary sequence databases. A total of 60 proteins were identified and Table 1 lists the identified proteins (see **Sup** Table 1 for information related to their biological processes and potential sub-cellular localization). When an uncharacterized protein was

Table 1
Proteins identified in roots of *L. japonicus* subjected to drought. MW, molecular weight expressed in daltons. PI, point isolectric. The MW and PI correspond to the suggested protein by the database, not necessarily to *L. japonicus* protein.

Spot	Protein	MW	PI
1	Methionine synthase	84861	6.02
2	Heat Shock Protein 70-family	71589	5.17
3	Uncharaterized P (2,3-bisphosphoglycerate-independent phosphoglycerate mutase)	60728	5.43
4	Protein disulfide isomerase	56191	4.84
5	Uncharacterized P (potentially a Calreticulin)	50140	4.5
6	Adenosylhomocysteinase	53809	5.49
7	ATP synthase subunit beta	45917	5.32
8	Catalase - *Lotus japonicus*	57256	6.67
9	Elongation factor 1-alpha	49404	9.2
10	At2g07360-like protein (fragment)	18327	8.87
11	Peroxidase	28752	6.83
12	Peroxidase	28752	6.83
13	Beta-actin	41834	5.31
14	Alcohol dehydrogenase 1	41716	6.03
15	Uncharacterized P (Glutamyl-tRNA (Gln) amidotransferase)	18716	5.14
16	Aspartate aminotransferase	45543	8.84
17	Alcohol dehydrogenase ADH1 Lj	41653	6.03
18	Fructose-bisphosphate aldolase Lj	34469	8.81
19	Glutamine synthetase cytosolic Lj	39293	5.49
20	Predicted protein (class III peroxidase)	36532	9.31
21	Glyceraldehyde 3-phospahte dehydrogenase	36751	6.55
22	Glyceraldehyde 3-phospahte dehydrogenase	36479	6.67
23	Glyceraldehyde 3-phospahte dehydrogenase	36479	6.67
24	Glyceraldehyde 3-phospahte dehydrogenase	36568	7.06
25	Fructokinase 4	35546	5.29
26	Isoflavone reductase	35531	5.69
27	Ribosomal protein S3 (chloroplastic)	25861	10.7
28	No data		
29	1,3-beta-D-glucanase	35422	5.93
30	1,3-beta-D-glucanase	35422	5.93
31	1,3-beta-D-glucanase	36958	6.22
32	ATP synthase epsilon chain	38491	6.95
33	Chitinase class I	34918	6.28
34	Chitinase	35927	6.77
35	14.3.3 like protein (regulatory)	29241	4.7
36	Endochitinase	36333	8.43
37	Uncharacterized P. (Legume lectin beta domain protein)	29489	5.66
38	GTP-binding protein	27070	6.85
39	Predicted P - Cytosolic Glutathione reductase (fragment)	26082	5.91
40	Acid phosphatase class IIIB protein	32848	5.71
41	Legume lectin beta domain protein	29441	5.56
42	Pre-mRNA splicing factor domain-containing protein	18633	4.38
43	L-type lectin-domain containing receptor kinase	24839	4.97
44	Glutathione-s-transferase theta	24378	6.18
45	Dehydroascorbate reductase (Glutathione-s-transferase)	23817	7.7
46	Putative NtPRp27-like protein	25362	7.77
47	Uncharacterized P (NADPH-quinone oxidoreductase)	21766	6.21
48	Thaumatin-like protein Pathogenesis-related-5b (fragment)	26900	5.26
49	Thaumatin-like protein Pathogenesis-related-5b (fragment)	26900	5.26
50	Uncharacterized P (mitochondrial substrate carrier family)	23968	8.49
51	Germin-like protein subfamily 1	23856	6.81
52	Germin-like protein subfamily 1	23940	7.71
53	Uncharacterized P (Kunitz trypsin inhibitor)	24026	4.85
54	Uncharacterized protein (Chitinase Pathogenesis-related)	23150	5.31
55	Pi1 protein (fragment)	15293	6.62
56	Uncharacterized P (Kunitz trypsin inhibitor)	24061	4.98
57	Uncharacterized P (Kunitz trypsin inhibitor)	23735	6.3
58	Uncharacterized P (Pathogenesis-related protein bet V I family protein)	18048	5.63
59	Peptidyl-prolyl cis-trans isomerase	18250	9.18
60	Uncharacterized P (Blasted sequences do not match with this MW)	21805	5.85

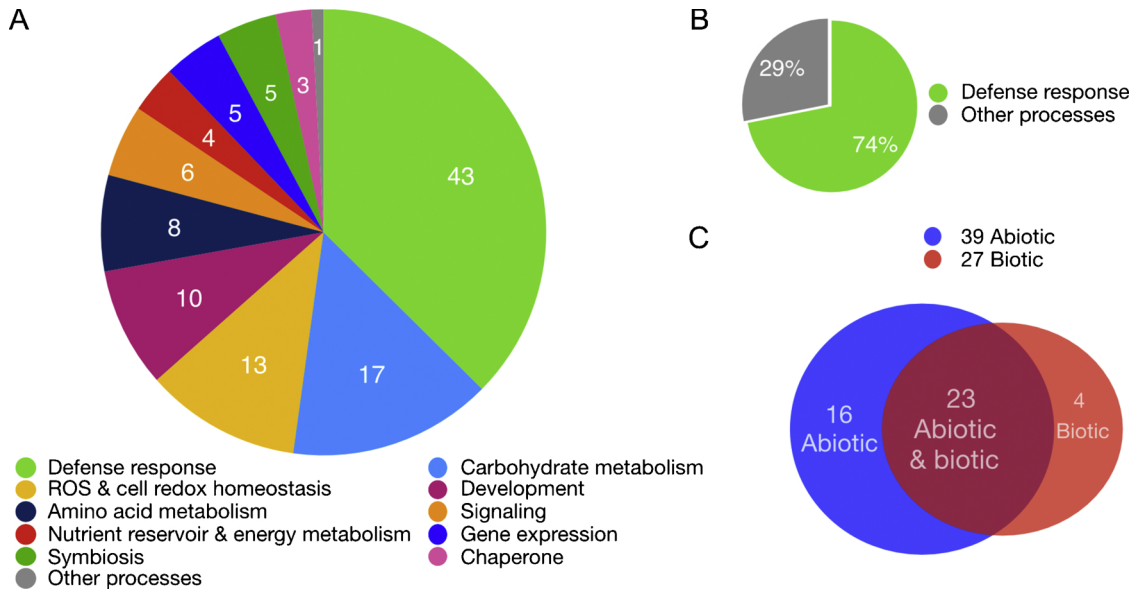


Fig. 6. Biological processes in which the identified proteins has been related to. (A) Number of proteins that have been related to the different biological processes. Most proteins account for more than one process and thus the values together exceed the number of proteins identified. **(B)** Percentage of proteins that have been related to defense response. **(C)** Venn diagrams showing the distribution of the defense- responsive-proteins involved in different types of stresses.

suggested as the best match, the other matches for the same spot were taken into account, or a blast of the peptides was performed in *L. japonicus* database to identify the candidate protein. These proteins were divided into 10 groups (Fig. 5B) including defense response (24%), carbohydrate metabolism (21%), ROS and cellular redox homeostasis (16%), gene expression (9%), nutrient reservoir and energy metabolism (7%), amino acid metabolism (7%), molecular chaperone (5%), symbiosis (5%), signaling (3%), and others (3%). Based in this division, in the discussion section (Section 4.2) we described the identified proteins separately by functional category, compare the results with other works in which the differential expression between control and drought has been studied, and discuss their potential role under drought stress.

Despite the fact that the identified proteins were classified into different functional groups accordingly to their primary function, most of these proteins were suggested to be involved in other responses. When considering all the processes in which every protein has been proposed to play a role, we found that 43 out of 58 identified proteins (i.e., 74%) were previously connected to defense responses (Fig. 6**A and B**). Among these, 39 proteins had been related to abiotic stress responses and 27 proteins to biotic stress responses (**Table S1**, Fig. 6C). From them, 16 proteins were exclusively related to abiotic stress, 23 to both biotic and abiotic stress and only 4 solely related to biotic stress (Fig. **6C**). Interestingly, development was the fourth most represented category (Fig. 6A) which it was not even represented when the primary function of these enzymes were considered (Fig. 6).

3.5. Identification of nitrated proteins

With the goal of knowing which of the identified proteins could undergo a process of nitration, a western-blot of the 2D-electrophoresis of root samples of drought-stressed *L. japonicus* was done and probed with an antibody against nitro-tyrosine (dilution 1:1000). The immunoreactive spot pattern was used to select these spots in the 2D gel stained with SYPRO Ruby and picked them up (Fig. 7). These spots were analyzed by MALDI-TOF mass spectrometry after trypsin digestion using the MASCOT search engine to analyze MS data to identify proteins from primary sequence databases. A total of 13 immunoreactive spots were observed, and the putative tyrosine-nitrated proteins are listed in Tables 2 and **S3.** This includes a methionine synthase, a Hsp70, an adenosyl-homocysteinase, a peroxidase, two alcohol

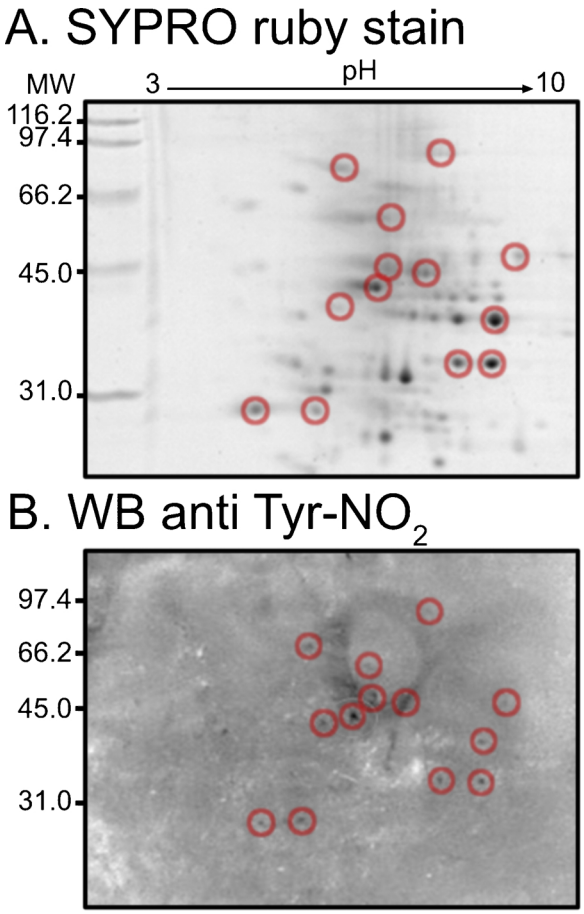


Fig. 7. Detection of nitrated proteins in *L. japonicus* roots of plants exposed to drought stress by 2-D electrophoresis and immunoblot. (A) Representative 2D electrophoresis (pH 3–10 for the first dimension) of the concentrated hypocotyl extracts stained with Spypro Ruby. Molecular-weight (MW) standards are indicated on the left in kDa. **(B)** The corresponding Western blot of root samples probed with a polyclonal antibody against nitrotyrosine (dilution 1:10,000). Approximately 150 µg of proteins were loaded per gel. The circles indicate all the immunoreactive spots.

Table 2
Nitrated proteins in drought-stressed roots of *L. japonicus*. The spot numbers refer to the numbers in the 2D-electrophoresis (Fig. 2S). Note that in *L. japonicus* database (Lj ID), some genes are represented by more than one ID number. Computational prediction of tyrosine nitration sites in proteins were done with the GPS-YNO2 program. UN, unidentified.

Spot	Proteins	Tyr	Predicted position of nitrated Tyr(s)	Lj ID
1	Methionine synthase	9	4, 203	Lj0g3v0222539.1
2	HeatShock Protein 70-family	15	314,408,423,801	Lj1g3v4550110.1, Lj3g3v3188570.1
6	Adenosyl-homocysteinase	9	222,254,263	Lj6g3v0270780.1
12	Peroxidase	6	UN	Lj5g3v1925220.1
14	Alcohol dehydrogenase 1	8	53	Lj1g3v0913280.1
17	Alcohol dehydrogenase ADH1 Lj	8	53	Lj1g3v0913270.1
19	Glutamine synthetase cytosolic Lj	18	73	Lj6g3v1887790.1
25	Fructokinase 4	7	147,174	Lj3g3v0381590.1
31	1,3-beta-D-glucanase	6	62	Lj0g3v0213009.1
34	Chitinase	6	UN	Lj0g3v0247049.1
36	Endochitinase	16	UN	Lj0g3v0247049.1
42	Pre-mRNA splicing factor domain-containing protein	15	72	Lj0g3v0100099.1, Lj0g3v0011739.1
43	L-type lectin-domain containing receptor kinase	1	168	Lj6g3v1693730.1

dehydrogenases, a glutamine synthetase, a fructokinase 4, an 1,3-beta-glucanase, a chitinase, an endochitinase, a pre-mRNA splicing factor domain-containing protein and a L-type lectin-domain containing receptor kinase. Several of these proteins have a similar molecular weight which explains why fewer bands are observed in one-dimension electrophoresis. In some cases, the protein suggested by MS was already a *L. japonicus* protein and in that cases the corresponding protein ID was used. In the other cases in which the best match referred to other species, the sequence of the suggested protein was found and blasted in *L. japonicus* database to obtain the *L. japonicus* ID.

4. Discussion

L. japonicus is a legume taxonomically related to economically-important legumes but with a smaller and sequenced genome. These advantages placed *L. japonicus* as a model legume, frequently employed to evaluate plant responses to environmental stresses (Calzadilla et al., 2016; Signorelli and Monza, 2017). In a previous study using this legume as a model, water/drought stress was observed to generate a spatial distribution of nitro-oxidative stress in the plant; with the oxidative stress component being higher in leaves and the nitrosative stress one higher in roots (Signorelli et al., 2013b). In fact, the increase of protein nitration in roots has also been described in response to toxic metals (Feigl et al., 2015), senescence (Begara-Morales et al., 2013) and salinity (David et al., 2015; Signorelli et al., 2016; Tanou et al., 2012).

The present study is an additional step to get deeper knowledge in the nitrosative stress component in roots, specifically to find out the identity of nitrated protein drought-stressed roots of *L. japonicus* through proteomic analyses. The system selected to investigate this stress was the Leonard jars because the sampling of roots is easier and avoids possible mechanical damages. In addition, the use of a responsive marker to drought, such as the Fe-SOD suggested that it is possible to induce a drought-like response in Leonard jars (Fig. 1D).

4.1. Drought produces nitro-oxidative stress in roots of L. japonicus

Our results about the antioxidant pool showed that ASC levels are dramatically affected by drought, suggesting that GSH becomes an essential piece of the antioxidant system, having to compensate for that lack of ASC. Looking at the GSH/GSSG ratio as indicator of the redox poise, it is observed that in roots drought produces a decrease from 11.7 to 2.9, which indicates a more oxidized state. However, in leaves no decrease is observed and the values are much greater, indicating a more reductive condition. Regarding the levels of GSNO, we found they represents less than 1% of the total pool of GSH in roots, supporting its potential signaling function. In this sense, GSNO is considered a natural reservoir of NO in plant cells and can mediate signaling pathways throughout specific post-translational modification of redox-sensitive proteins by trans-nitrosylation reactions (Corpas et al., 2013a,b) or even regulating gene expression (Begara-Morales et al., 2014).

The analysis of ROS and RNS in roots (Figs. 3 and 4) support that drought triggers the activation of their metabolism in roots. This general response has also been described under other stresses where roots are directly involved such as salinity or heavy metals (Feigl et al., 2015; Manai et al., 2014; Sun et al., 2017). In fact, this observation has been used for some authors as a strategy to improve the mechanism of response to a stress by the pre-exposure of roots to either NO or H_2O_2 to induce acclimation against a subsequent stress (Kharbech et al., 2017; Molassiotis et al., 2016; Singh et al., 2009; Xiong et al., 2009). Interestingly, it is remarkable the differential cellular distribution of each analyzed molecule in roots of plant growth under optimal conditions in comparison to the roots of plants under drought stress. Thus, whereas $O_2^{-\cdot}$ and $ONOO^-$ showed a wide distribution in all root cell types under drought stress which is reasonable considering the nitro-oxidative stress previously described (Signorelli et al., 2013b), the behaviour of NO and SNOs was entirely different because they were preferably located in cortical cells. This behaviour could be associated to a local response considering that these root cells are the first ones affected under drought stress. It should be mentioned that the observed differences between NO content and distribution in roots under optimal conditions (NO almost undetectable) and under drought stress (NO mainly located in cortical cells) is not unusual. For instance, in a previous report of pea plants exposed to six different stresses, it was reported drastic differences of the NO content and distribution in leaves depending of the stress. While NO was almost absent in leaves of pea plants grown under dark conditions, NO was overproduced with a general cellular distribution in leaves under mechanical wounding (Corpas et al., 2008). Comparable consideration could be made with SNOs where a similar distribution of SNOs has been reported in sunflower hypocotyls under biotic stress (Chaki et al., 2009) which was associated to a mechanism of protection against the pathogen in the site of penetration.

4.2. Identified proteins of roots of L. japonicus described by functional categories

4.2.1. Proteins assigned to defense response

Among the most abundant proteins in *L. japonicus* stressed roots, the most represented category was related to pathogen response. This category includes three chitinase isoforms and one endochitinase, two thaumatin-like protein pathogenesis-related-5b, three Kunitz trypsin inhibitor, a Pi1 protein, a pathogenesis-related protein bet V I family, a putative NtPRp27-like protein and two isoforms of germin-like protein subfamily 1. Although the chitinases can be considered as part of the

sugars metabolism too, plants do not have chitin but produce chitinases as a pathogen response to degrade insects and fungus cell walls. The induction of biotic stress responses by abiotic stress is well documented (Greco et al., 2012). For instance, short-term drought stress was reported to produce the up-regulation of chitinase protein levels in soybean roots (Alam et al., 2010). In sugarcane leaves subjected to drought, the increase of thaumatin-like proteins was also reported (Jangpromma et al., 2010). Osmotic stress imposed by PEG 10% also produced the accumulation of Kunitz trypsin inhibitor and pathogenesis-related proteins in roots of soybean seedlings (Toorchi et al., 2009). This agreement in the literature suggests that the accumulation of proteins involved in pathogen response is likely to be under the regulation of master regulators of stress response that triggers both abiotic and biotic stress responses.

The NtPRp27-like protein and the two germin-like proteins are extracellular proteins with ion binding functions; however, the NtPRp27-like can bind different metals whereas the germin-like proteins bind only manganese. The NtPRp27-like proteins are known to contribute to pathogen resistance, mainly by antimicrobial apoplastic activity (Xie and Goodwin, 2014). The germin-like proteins are glycoproteins with diverse functionally. Although their function is not completely clear, they have been reported to increase abiotic and biotic stress tolerance in many plant species (Kumar et al., 2016).

4.2.2. Proteins assigned to carbohydrate metabolism

2,3-bisphosphoglycerate-independent phosphoglycerate mutase, fructose-bisphosphate aldolase, four isoforms of glyceraldehyde 3-phosphate dehydrogenase, and fructokinase 4, which are involved in the glycolytic pathway, and two isoforms of alcohol dehydrogenase, which are implicated in fermentation, were detected in drought-stressed roots of *L. japonicus*. Besides, three isoforms of 1,3-beta-D-glucanase, which are involved in the hydrolysis of beta-glucans, were also identified.

Other proteomic studies have shown the induction of the 2,3-bisphosphoglycerate-independent phosphoglycerate mutase in response to drought/osmotic stress in roots of Soybean (Alam et al., 2010; Mohammadi et al., 2012a; Toorchi et al., 2009); the glyceraldehyde 3-phosphate dehydrogenase in rapeseed (*Brassica napus*) roots (Mohammadi et al., 2012b), and roots of soybean seedlings (Mohammadi et al., 2012a); the fructokinase and fructose-bisphosphate aldolase in roots of soybean seedlings (Mohammadi et al., 2012a) in response to drought stress. This coincidence suggests that the induction of enzymes involved in the glycolytic pathway is also a conserved response of plants to drought.

4.2.3. Proteins related to ROS and cellular redox homeostasis

High levels of disulfide isomerase, catalase, three peroxidases, including a class III peroxidase, cytosolic glutathione reductase, glutathione-S-transferase theta, dehydroascorbate reductase and NADPH-quinone oxidoreductase were observed in roots of *L. japonicus* under drought stress. Disulfide isomerases play a role in the rearrangement and formation of protein disulfide bonds. Osmotic stress induced these proteins in roots of soybean seedlings (Toorchi et al., 2009) and watermelon plants (Yoshimura et al., 2008). Plants have many peroxidases that play a direct role in detoxifying H_2O_2 and other organic hydroperoxides such as lipid peroxides. Giving the increase of ROS generated in roots (Fig. 3A) and of lipid peroxidation (Signorelli et al., 2013b) it is not surprising to find several peroxidases within the most abundant proteins of *L. japonicus* roots. Many peroxidases have been observed to be induced by drought stress in roots of rapeseed (Mohammadi et al., 2012b), roots of soybean seedlings (Mohammadi et al., 2012a), and roots of watermelon plants (Yoshimura et al., 2008).

Two of the most abundant enzymes identified here participate in the glutathione-ascorbate cycle, the glutathione reductase (GR) and the dehydroascorbate reductase (DHAR). This cycle is an important piece to control the oxidative damage. In *L. japonicus* plants, it was previously found that the GR activity increased by drought in roots and leaves (Signorelli et al., 2013b). Other proteomic studies have identified the DHAR as one of the proteins responsive to drought (Mohammadi et al., 2012b; Yoshimura et al., 2008).

The glutathione *S*-transferases are known to participate in detoxification processes. In particular, these enzymes bind GSH to different xenobiotic, which then can be transported to the vacuole. These enzymes were also linked to response to stress response and oxidative stress (Marrs, 1996). Indeed, in other proteomic studies, they were found to be over-expressed by drought in roots (Mohammadi et al., 2012b; Yoshimura et al., 2008).

NADPH-quinone oxidoreductase catalyzes the reduction of quinones to semiquinones consuming NADPH and was suggested to have $O_2^{\cdot -}$ scavenger activity (Siegel et al., 2004). These proteins have been demonstrated to be over-expressed in drought-stressed roots of watermelon (Yoshimura et al., 2008) and soybean seedlings (Mohammadi et al., 2012a).

4.2.4. Proteins related to gene expression

An elongation factor 1-alpha, an At2g07360-like protein, a glutamyl-tRNA (Gln) amidotransferase, a ribosomal protein S3, and a pre-mRNA splicing factor domain-containing protein were also found among the most abundant proteins in stressed roots of *L. japonicus*. Elongation factors were found to be responsive to drought in roots of rapeseed (Mohammadi et al., 2012b) as well as watermelon plants (Yoshimura et al., 2008). Different ribosomal proteins were found to be up-regulated as well just a few hours after drought imposition in roots of watermelon plants (Yoshimura et al., 2008). The adenosylhomocysteinase was also found among the most abundant proteins of *L. japonicus* roots. Although it was classified into the amino acid metabolism-related proteins, this protein has been involved in the control of gene silencing by methylation (Rocha et al., 2005).

4.2.5. Proteins related to nutrient reservoir and energy metabolism

Two ATP synthase subunits, the beta and the epsilon chain, an acid phosphatase class IIIB, and a mitochondrial substrate carrier family were found in drought-stressed *L. japonicus* roots. The ATP synthase produces ATP using the proton motive force as the source of energy. Most of the few proteomic works focused on drought-stressed roots showed the over-expression of ATP synthase subunits (Jangpromma et al., 2010; Mohammadi et al., 2012a, 2012b). The mitochondrial substrate carrier family is suggested to participate in the ADP and AMP transport, as well as phosphate ion transmembrane transport. On the other hand, the acid phosphatase plays a role in the hydrolysis and mobilization of inorganic phosphate. Together the high amount of these proteins suggests that the ATP metabolism is active in stressed roots.

4.2.6. Proteins related to the folding of proteins and chaperone activity

A heat shock protein 70-family (Hsp70), a calreticulin, and a peptidyl-prolyl cis-trans isomerase were found among the most abundant proteins with chaperone activity. Although disulfide isomerase was classified within the proteins regulating the redox homeostasis it could have been also grouped in this section because their function contributes to ensuring the correct folding of proteins. The Hsp70 are proteins with chaperone activity and responsive to diverse stresses but also were shown to suppress apoptosis (Beere et al., 2000). Other studies showed that the expression of these proteins is induced in roots of several species subjected to drought (Mohammadi et al., 2012a, 2012b; Yoshimura et al., 2008). Calreticulin is a Ca^{2+} binding protein of the endoplasmic reticulum which contributes to the protein folding. However, in plants, diverse functions have been assigned to this protein with significance in biotic and abiotic stress responses (Jia et al., 2009). Finally, peptidyl-prolyl cis-trans isomerase plays a role in the folding of newly synthesized proteins, in particular in the cis-trans isomerization of peptide bonds preceding proline (Shaw, 2002).

4.2.7. Proteins assigned to amino acid metabolism

In drought-stressed *L. japonicus* roots the methionine synthase, adenosylhomocysteinase, aspartate aminotransferase, and cytosolic glutamine synthetase were identified among the most abundant proteins and related to the amino acid metabolism. Both adenosylhomocysteinase and methionine synthase participate in the metabolism of methionine. Almost every work evaluating the enrichment of proteins by drought/osmotic stress in roots have found the methionine synthase (Alam et al., 2010; Mohammadi et al., 2012a, 2012b; Toorchi et al., 2009; Yoshimura et al., 2008). This evidence suggests that methionine synthase over-expression is a conserved response of plants to drought.

The aspartate aminotransferase participates in the biosynthesis of aspartate, and it is crucial to control the carbon/nitrogen ratio. The glutamine synthetase is also a key enzyme in the nitrogen metabolism, in particular in the assimilation of nitrogen. This enzyme was observed to be accumulated in roots of soybean seedlings subjected to drought (Mohammadi et al., 2012a), roots of watermelon plants subjected to drought for 0, 1 and 3 days (Yoshimura et al., 2008), roots of soybean subjected to short-term drought stress (Alam et al., 2010) and roots of rapeseed (*Brassica napus*) under drought stress (Mohammadi et al., 2012b).

4.2.8. Proteins related to symbiosis

Three legume lectin proteins were found, two beta domains and a domain containing receptor kinase. These are glycoproteins which participate in the attachment of nitrogen-fixing bacteria to legumes and also have been suggested to directly interact with nod factors (Etzler et al., 1999).

4.2.9. Proteins related to signaling

A 14.3.3-like protein and a GTP-binding protein were found among the most abundant roots proteins of drought-stressed *L. japonicus* roots. The 14.3.3-proteins are conserved proteins capable of binding several signaling proteins such as kinases, phosphatases, and transmembrane receptors and thus have an important regulatory role (Fu et al., 2000). The GTP-binding proteins, also known as GTPase, are proteins able to bind GTP and hydrolyze it. These proteins are considered molecular switches due to their capacity to change from an 'ON conformation' to 'OFF conformation' depending on the presence of GTP (Bourne et al., 1990). It is worth mentioning that other proteins previously cited here could have also been assigned here, as the calreticulin, which is involved in Ca^{2+} signaling and the three legume lectins, which participate in the legume-rhizobia interaction.

4.2.10. Proteins with other function

Among the unclassified proteins, the β-actin was found. Interestingly, when referring to the literature, most of the works evaluating proteomics in roots under drought condition have reported an over-expression of this protein in response to drought (**Table S2**). Actin has also been linked to having a role in root development (**Table S1**), which is likely to be necessary in a drought condition, to respond as a mechanism to find water.

Overall, few works have focused on the study of proteomics in roots in response to abiotic stress and less in particular to drought (for revision see Ghosh and Xu, 2014). However, the majority of the proteins that we found in *L. japonicus* drought-stressed roots have also been identified in those works to be responsive to drought stress (**Table S2**). The fact that enzymes involved in the vacuolar transport (*S*-glutathione transferase), the sugar catabolism and chaperone activity, where found at high concentration suggest the possibility that autophagy is taking place. Autophagy is a process by which the cells recycle their components, in the vacuole, upon a stress condition. There are many types of stress conditions able to induce autophagy in plants, and some of them are abiotic stress and oxidative damage. It has been shown that the protein kinases SnRK1 and TOR, both master regulators of plant growth and development (Baena-González et al., 2007; Meitha et al., 2018; Signorelli et al., 2018), participate in the control of plant autophagy (Pu et al., 2017). Further research is required to see whether these nitrated proteins are directly involved in plant autophagy. Also, it would be interesting to evaluate if there is a cross-talk between the SnRK1 and TOR kinases and the molecular switches controlling kinases, such as the 14.3.3-like protein and the GTP-binding protein that we found here.

4.3. Nitrated proteins in L. japonicus roots under drought stress

Protein nitration is a post-translational modification mediated by NO-derived molecules which increase is associated with stress situations (Corpas et al., 2013a,b). Previously, we showed by immunoblot analysis that in control roots the level of protein nitration was almost undetectable; however, under drought stress, some immunoreactive bands were induced (see Fig. 1**C** here and Signorelli et al., 2013b). Considering that $ONOO^-$ is recognized as one of the molecules which can mediate protein nitration (Ferrer-Sueta et al., 2018; Rubbo and Radi, 2008), this result is in good agreement with the $ONOO^-$ increase observed in Fig. 4**A** under drought stress, such as it has been described in other stress conditions (Corpas and Barroso, 2014; Feigl et al., 2015; Houmani et al., 2018). Here we identified 13 putative nitrated proteins. Some of the proteins we identified, such as methionine synthase, peroxidase, alcohol dehydrogenase and endochitinase were also identified as nitrated in a previous study in roots of pea (*Pisum sativum L.*), where (Begara-Morales et al., 2013). Thus, methionine synthase, was the protein that has been found over-expressed in response to drought (**Table S2**), suggesting that its function is vital in stress conditions. Interestingly, methionine synthase is known to be inactivated by nitration (Nicolaou et al., 1996). If its role were relevant in stress conditions, the nitration would attenuate the plant response. However, the possibility that a compensatory mechanism is working here cannot be ruled out. Another of the putative nitrated proteins identified in roots of *L. japonicus*, the adenosyl-homocysteinase, was also found to be inactivated by nitration in sunflower (Chaki et al., 2009). Two isoforms of alcohol dehydrogenases were found to be potentially nitrated (Table 1). This enzyme was also demonstrated to be inactivated *in vitro* by nitration (Crow et al., 1995; Daiber et al., 2013). The cytosolic glutamine synthetase was found to be potentially nitrated here, and this enzyme has been reported to be inactivated by nitration in roots nodules of *Medicago truncatula* (Melo et al., 2011).

On the other hand, no evidence was found about the effect of nitration on the functionality of Hsp70, peroxidase, fructokinase, 1,3-beta-glucanase, chitinase and endochitinase, Pre-mRNA splicing factor domain-containing protein, and L-type lectin-domain containing receptor kinase. Though, most of them have been shown to be susceptible to nitration by *in vitro* assays, confirming that these proteins are targets of nitration. Further studies should be carried out to see whether the nitration affects their functionality. It is important to remark that some of these proteins, such as the pathogen response proteins and the thaumatin-like protein are extracellular proteins, which suggests that nitrating agents, like $ONOO^-$, are also present in the apoplast.

It is interesting also to point out that the nitration of the 1,3-β-glucanase could have beneficial implications for the plants in a negative loop as proposed in Fig. 8. This "beneficial effect" is because 1,3-β-glucans are known to protect against $ONOO^-$ (Saluk-Juszczak et al., 2010). Given that the 1,3-β-glucanase hydrolyzes the 1,3-β-glucans, the inactivation of this degradation by $ONOO^-$ would result in more protection for the plant. Here we demonstrated that the nitration of 1,3-β-glucanase takes place in physiological conditions, however, to confirm this loop the inactivation of this enzyme by nitration should be demonstrated. So far, all the enzymes demonstrated to be nitrated here that have been functionally analyzed showed a reduction in their activity. Hence, it is not unthinkable to consider this loop.

Interestingly, proteins well represented here as the glyceraldehyde-3-phosphate dehydrogenase for which 4 isoforms were found (**Table S1**) and which was shown to be nitrated by $ONOO^-$ generators

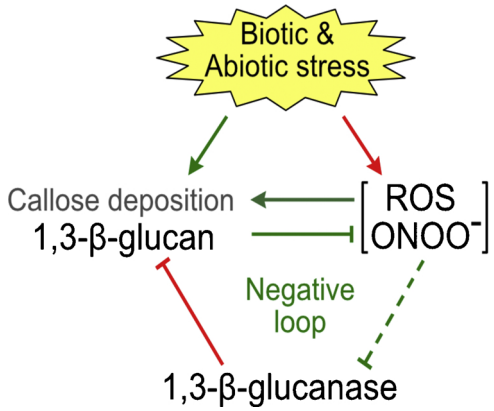


Fig. 8. Proposed negative loop to control peroxynitrite ($ONOO^-$) levels. Elements in red and green indicate processes with negative and positive implications for the plant defense respectively. 1,3-betaglucan is known to have antioxidative and antinitrative activities (Saluk-Juszczak et al., 2010). 1,3-βglucanase activity degrades 1,3-βglucan. The attack of peroxynitrite to the 1,3-betaglucanase is demonstrated here, however, it is not know yet if it produces the inactivation of the enzyme. Thus this process is represented with a dashed line.

(Lozano-Juste et al., 2011), was not identified as nitrated in drought stress. Also, pathogenesis-related proteins and thaumatin-like proteins were also found in *L. japonicus* roots, but not nitrated. However, several of these proteins were determined to be nitrated when nitrogen dioxide ($\cdot NO_2$) is applied to plant (Takahashi et al., 2016). Together, this shows the relevance of studying *in vivo* protein nitration by environmental stresses. Here we report that some enzymes known to be inactivated by nitration, such as methionine synthase, adenosyl-homocysteinase, glutamine synthetase, two isoforms of alcohol dehydrogenases, are also determined as putative nitrated by environmental stress. Finally, the profile of NO and SNOs found in cortical cells of stressed roots, strongly suggest a malfunction of these cells under stress, because the *S*-nitrosylation and trans-nitrosylation of Cys residues of proteins involved in signaling pathways of these cells is likely to take place and this has been extensively reported to block signaling pathways and the plant response, ultimately leading to cell death. Given some proteins, expressed at high concentration in *L. japonicus* roots, were not nitrated in a drought condition, it might be possible that their functionality is still relevant in such condition for cell survival.

Author statements

Santiago Signorelli, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Review and Editing, Visualization, Project Administration. **Francisco J. Corpas**, Conceptualization, Methodology, Formal analysis, Resources, Writing - Review and Editing, Visualization, Supervision, Funding Acquisition, Project Administration. **Marta Rodríguez-Ruiz**, Formal analysis. **Raquel Valderrama**, Formal analysis, Investigation. **Juan B. Barroso**, Visualization, Resources. **Omar Borsani**, Resources, Writing - Review and Editing, Supervision, Funding Acquisition. **Jorge Monza,** Resources, Writing - Review and Editing, Visualization, Supervision, Funding Acquisition

Acknowledgements

LC-ES/MS was carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (CSIC), Granada, confocal laser-scanning microscopy (CLSM) analyses were carried out at the Technical Services of the University of Jaén, and proteomic analyses were made at the Proteomic Service (SCAI) at the University of Córdoba, Spain. Researchin FJC´s laboratory is supported by an European Regional Development Fund (ERDF) grant co-financed by the Ministry of Economy and Competitiveness (AGL2015-65104-P). SS, OB, and JM were partially supported by PEDECIBA (Programa de Desarrollo de Ciencias Básicas, Uruguay). SS, OB, and JM are associate and active members of the National System of Researchers (SNI, Uruguay).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.08.007.

References

Airaki, M., Sanchez-Moreno, L., Leterrier, M., Barroso, J.B., Palma, J.M., Corpas, F.J., 2011. Detection and quantification of S-nitrosoglutathione (GSNO) in pepper (Capsicum annuum L.) plant organs by LC-ES/MS. Plant Cell Physiol. 52, 2006–2015. https://doi.org/10.1093/pcp/pcr133.

Alam, I., Sharmin, S.A., Kim, K.H., Yang, J.K., Choi, M.S., Lee, B.H., 2010. Proteome analysis of soybean roots subjected to short-term drought stress. Plant Soil 333, 491–505. https://doi.org/10.1007/s11104-010-0365-7.

Baena-González, E., Rolland, F., Thevelein, J.M., Sheen, J., 2007. A central integrator of transcription networks in plant stress and energy signalling. Nature 448, 938–942. https://doi.org/10.1038/nature06069.

Bechtold, U., 2018. Plant life in extreme environments: how do you improve drought tolerance? Front. Plant Sci. 9, 543. https://doi.org/10.3389/fpls.2018.00543.

Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M., Green, D.R., 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat. Cell Biol. 2, 469–475. https://doi.org/10.1038/35019501.

Begara-Morales, J.C., Chaki, M., Sanchez-Calvo, B., Mata-Perez, C., Leterrier, M., Palma, J.M., Barroso, J.B., Corpas, F.J., 2013. Protein tyrosine nitration in pea roots during development and senescence. J. Exp. Bot. 64, 1121–1134. https://doi.org/10.1093/jxb/ert006.

Begara-Morales, J.C., Sanchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Perez, C., Lopez-Jaramillo, J., Padilla, M.N., Carreras, A., Corpas, F.J., Barroso, J.B., 2014. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. J. Exp. Bot. 65, 527–538. https://doi.org/10.1093/jxb/ert396.

Bourne, H.R., Sanders, D.A., McCormick, F., 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125–132. https://doi.org/10.1038/348125a0.

Calzadilla, P.I., Signorelli, S., Escaray, F.J., Menéndez, A.B., Monza, J., Ruiz, O.A., Maiale, S.J., 2016. Photosynthetic responses mediate the adaptation of two *Lotus japonicus* ecotypes to low temperature. Plant Sci. 250, 59–68. https://doi.org/10.1016/j.plantsci.2016.06.003.

Chaki, M., Valderrama, R., Fernandez-Ocana, A.M., Carreras, A., Lopez-Jaramillo, J., Luque, F., Palma, J.M., Pedrajas, J.R., Begara-Morales, J.C., Sanchez-Calvo, B., Gomez-Rodriguez, M.V., Corpas, F.J., Barroso, J.B., 2009. Protein targets of tyrosine nitration in sunflower (Helianthus annuus L.) hypocotyls. J. Exp. Bot. 60, 4221–4234. https://doi.org/10.1093/jxb/erp263.

Chaki, M., Valderrama, R., Fernandez-Ocana, A.M., Carreras, A., Gomez-Rodriguez, M.V., Lopez-Jaramillo, J., Begara-Morales, J.C., Sanchez-Calvo, B., Luque, F., Leterrier, M., Corpas, F.J., Barroso, J.B., 2011. High temperature triggers the metabolism of S-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin-NADP reductase by tyrosine nitration. Plant Cell Environ. 34, 1803–1818. https://doi.org/10.1111/j.1365-3040.2011.02376.x.

Corpas, F.J., Barroso, J.B., 2013. Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. New Phytol. https://doi.org/10.1111/nph.12380.

Corpas, F.J., Barroso, J.B., 2014. Peroxynitrite ($ONOO^-$) is endogenously produced in arabidopsis peroxisomes and is overproduced under cadmium stress. Ann. Bot. 113, 87–96. https://doi.org/10.1093/aob/mct260.

Corpas, F.J., Barroso, J.B., Carreras, A., Valderrama, R., Palma, J.M., León, A.M., Sandalio, L.M., Del Río, L.A., 2006. Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. Planta 224. https://doi.org/10.1007/s00425-005-0205-9.

Corpas, F.J., del Rio, L.A., Barroso, J.B., 2007. Need of biomarkers of nitrosative stress in plants. Trends Plant Sci. 12, 436–438. https://doi.org/10.1016/j.tplants.2007.08.013. https://doi.org/S1360-1385(07)00192-6 [pii].

Corpas, F.J., Chaki, M., Fernandez-Ocana, A., Valderrama, R., Palma, J.M., Carreras, A., Begara-Morales, J.C., Airaki, M., del Rio, L.A., Barroso, J.B., 2008. Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. Plant Cell Physiol. 49, 1711–1722. https://doi.org/10.1093/pcp/pcn144.

Corpas, F.J., Palma, J.M., Del Rio, L., Barroso, J.B., 2009. Evidence supporting the existence of l-arginine-dependent nitric oxide synthase activity in plants. New Phytol. 184, 9–14. https://doi.org/10.1111/j.1469-8137.2009.02989.x.

Corpas, F.J., Alché, J.D., Barroso, J.B., 2013a. Current overview of S-nitrosoglutathione (GSNO) in higher plants. Front. Plant Sci. 4, 1–3. https://doi.org/10.3389/fpls.2013.00126.

Corpas, F.J., Palma, J.M., Del Rio, L.A., Barroso, J.B., 2013b. Protein tyrosine nitration in

higher plants grown under natural and stress conditions. Front. Plant Sci. 4, 29. https://doi.org/10.3389/fpls.2013.00029.
Corpas, F.J., Aguayo-Trinidad, S., Ogawa, T., Yoshimura, K., Shigeoka, S., 2016. Activation of NADPH-recycling systems in leaves and roots of Arabidopsis thaliana under arsenic-induced stress conditions is accelerated by knock-out of Nudix hydrolase 19 (AtNUDX19) gene. J. Plant Physiol. 192, 81–89. https://doi.org/10.1016/j.jplph.2016.01.010.
Crow, J.P., Beckman, J.S., McCord, J.M., 1995. Sensitivity of the Essential Zinc-Thiolate Moiety of Yeast Alcohol Dehydrogenase to Hypochlorite and Peroxynitrite. Biochemistry 34, 3544–3552. https://doi.org/10.1021/bi00011a008.
Daiber, A., Daub, S., Bachschmid, M., Schildknecht, S., Oelze, M., Steven, S., Schmidt, P., Megner, A., Wada, M., Tanabe, T., Münzel, T., Bottari, S., Ullrich, V., 2013. Protein tyrosine nitration and thiol oxidation by peroxynitrite-strategies to prevent these oxidative modifications. Int. J. Mol. Sci. 14, 7542–7570. https://doi.org/10.3390/ijms14047542.
David, A., Yadav, S., Baluška, F., Bhatla, S.C., 2015. Nitric oxide accumulation and protein tyrosine nitration as a rapid and long distance signalling response to salt stress in sunflower seedlings. Nitric Oxide - Biol. Chem. 50, 28–37. https://doi.org/10.1016/j.niox.2015.08.003.
Etzler, M.E., Kalsi, G., Ewing, N.N., Roberts, N.J., Day, R.B., Murphy, J.B., 1999. A nod factor binding lectin with apyrase activity from legume roots. Proc. Natl. Acad. Sci. 96, 5856–5861. https://doi.org/10.1073/pnas.96.10.5856.
Fahad, S., Bajwa, A.A., Nazir, U., Anjum, S.A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M.Z., Alharby, H., Wu, C., Wang, D., Huang, J., 2017. Crop production under drought and heat stress: plant responses and management options. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.01147.
Faize, M., Burgos, L., Faize, L., Piqueras, A., Nicolas, E., Barba-Espin, G., Clemente-Moreno, M.J., Alcobendas, R., Artlip, T., Hernandez, J.A., 2011. Involvement of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress. J. Exp. Bot. 62, 2599–2613. https://doi.org/10.1093/jxb/erq432.
Fan, Q.-J., Liu, J.-H., 2012. Nitric oxide is involved in dehydration/drought tolerance in Poncirus trifoliata seedlings thorugh regulation of antioxidant systems and stomatal response. Plant Cell Rep. 31, 145–154.
Fan, H.F., Ding, L., Du, C.X., Wu, X., 2014. Effect of short-term water deficit stress on antioxidative systems in cucumber seedling roots. Bot. Stud. 55. https://doi.org/10.1186/s40529-014-0046-6.
Feigl, G., Lehotai, N., Molnár, Á., Ördög, A., Rodríguez-Ruiz, M., Palma, J.M., Corpas, F.J., Erdei, L., Kolbert, Z., 2015. Zinc induces distinct changes in the metabolism of reactive oxygen and nitrogen species (ROS and RNS) in the roots of two Brassica species with different sensitivity to zinc stress. Ann. Bot. 116, 613–625. https://doi.org/10.1093/aob/mcu246.
Feller, U., 2016. Drought stress and carbon assimilation in a warming climate: reversible and irreversible impacts. J. Plant Physiol. https://doi.org/10.1016/j.jplph.2016.04.002.
Ferrer-Sueta, G., Campolo, N., Trujillo, M., Bartesaghi, S., Carballal, S., Romero, N., Alvarez, B., Radi, R., 2018. Biochemistry of Peroxynitrite and Protein Tyrosine Nitration. Chem. Rev. https://doi.org/10.1021/acs.chemrev.7b00568.
Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2–18. https://doi.org/10.1104/pp.110.167569.
Fu, H., Subramanian, R.R., Masters, S.C., 2000. 14-3-3 proteins: structure, function, and regulation. Annu. Rev. Pharmacol. Toxicol. 40, 617–647. https://doi.org/10.1146/annurev.pharmtox.40.1.617.
Ghosh, D., Xu, J., 2014. Abiotic stress responses in plant roots: a proteomics perspective. Front. Plant Sci. 5. https://doi.org/10.3389/fpls.2014.00006.
Greco, M., Chiappetta, A., Bruno, L., Bitonti, M.B., 2012. In Posidonia oceanica cadmium induces changes in DNA methylation and chromatin patterning. J. Exp. Bot. 63, 695–709. https://doi.org/10.1093/jxb/err313.
Houmani, H., Rodríguez-Ruiz, M., Palma, J.M., Abdelly, C., Corpas, F.J., 2016. Modulation of superoxide dismutase (SOD) isozymes by organ development and high long-term salinity in the halophyte Cakile maritima. Protoplasma 253, 885–894. https://doi.org/10.1007/s00709-015-0850-1.
Houmani, H., Rodríguez-Ruiz, M., Palma, J.M., Corpas, F.J., 2018. Mechanical wounding promotes local and long distance response in the halophyte Cakile maritima through the involvement of the ROS and RNS metabolism. Nitric Oxide - Biol. Chem. 74, 93–101. https://doi.org/10.1016/j.niox.2017.06.008.
Jangpromma, N., Kitthaisong, S., Lomthaisong, K., Kaen, K., 2010. A proteomics analysis of drought stress-responsive proteins as biomarker for drought-tolerant sugarcane cultivars. Am. J. Biochem. Biotechnol. 6, 89–102. https://doi.org/10.3844/ajbbsp.2010.89.102.
Jia, X.Y., He, L.H., Jing, R.L., Li, R.Z., 2009. Calreticulin: Conserved protein and diverse functions in plants. Physiol. Plant. 136, 127–138. https://doi.org/10.1111/j.1399-3054.2009.01223.x.
Kailasam, S., Wang, Y., Lo, J.C., Chang, H.F., Yeh, K.C., 2018. S-Nitrosoglutathione works downstream of nitric oxide to mediate iron-deficiency signaling in Arabidopsis. Plant J. 94, 157–168.
Khan, M.I.R., Khan, N.A., 2017. Reactive oxygen species and antioxidant systems in plants: role and regulation under abiotic stress. Reactive Oxygen Species and Antioxidant Systems in Plants: Role and Regulation Under Abiotic Stress. https://doi.org/10.1007/978-981-10-5254-5.
Kharbech, O., Houmani, H., Chaoui, A., Corpas, F.J., 2017. Alleviation of Cr(VI)-induced oxidative stress in maize (Zea mays L.) seedlings by NO and H2S donors through differential organ-dependent regulation of ROS and NADPH-recycling metabolisms. J. Plant Physiol. 219, 71–80. https://doi.org/10.1016/j.jplph.2017.09.010.
Kumar, P., Kumar, V., Chattopadhyay, T., 2016. Gene Cloning, Recombinant Expression and Purification of a Germin-Like Protein from Tomato (Solanum lycopersicum L.). J. Crop Improv. 30, 595–607. https://doi.org/10.1080/15427528.2016.1207122.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lozano-Juste, J., Colom-Moreno, R., Leon, J., 2011. In vivo protein tyrosine nitration in Arabidopsis thaliana. J. Exp. Bot. 62, 3501–3517. https://doi.org/10.1093/jxb/err042.
Manai, J., Gouia, H., Corpas, F.J., 2014. Redox and nitric oxide homeostasis are affected in tomato (Solanum lycopersicum) roots under salinity-induced oxidative stress. J. Plant Physiol. 171, 1028–1035. https://doi.org/10.1016/j.jplph.2014.03.012.
Marrs, K.A., 1996. The functions and regulation of glutathione-S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–158. https://doi.org/10.1146/annurev.arplant.47.1.127.
Meitha, K., Agudelo-Romero, P., Signorelli, S., Gibbs, D.J., Considine, J.A., Foyer, C.H., Considine, M.J., 2018. Developmental control of hypoxia during bud burst in grapevine. Plant Cell Environ. 41, 1154–1170. https://doi.org/10.1111/pce.13141.
Melo, P.M., Silva, L.S., Ribeiro, I., Seabra, A.R., Carvalho, H.G., 2011. Glutamine Synthetase Is a Molecular Target of Nitric Oxide in Root Nodules of Medicago truncatula and Is Regulated by Tyrosine Nitration. Plant Physiol. 157, 1505–1517. https://doi.org/10.1104/pp.111.186056.
Mohammadi, P.P., Moieni, A., Hiraga, S., Komatsu, S., 2012a. Organ-specific proteomic analysis of drought-stressed soybean seedlings. J. Proteomics 75, 1906–1923. https://doi.org/10.1016/j.jprot.2011.12.041.
Mohammadi, P.P., Moieni, A., Komatsu, S., 2012b. Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F1 line under drought stress. Amino Acids 43, 2137–2152. https://doi.org/10.1007/s00726-012-1299-6.
Molassiotis, A., Job, D., Ziogas, V., Tanou, G., 2016. Citrus plants: a model system for unlocking the secrets of NO and ROS-Inspired priming against salinity and drought. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00229.
Nicolaou, A., Kenyon, S.H., Gibbons, J.M., Ast, T., Gibbons, W.A., 1996. In vitro inactivation of mammalian methionine synthase by nitric oxide. Eur. J. Clin. Invest. 26, 167–170. https://doi.org/10.1046/j.1365-2362.1996.122254.x.
Pu, Y., Luo, X., Bassham, D.C., 2017. TOR-dependent and -Independent pathways regulate autophagy in Arabidopsis thaliana. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.01204.
Radi, R., 2004. Nitric oxide, oxidants, and protein tyrosine nitration. P. Natl. Acad. Sci. USA 101, 4003–4008.
Rigaud, J., Puppo, A., 1977. Effect of nitrite upon leghemoglobin and interaction with nitrogen fixation. Biochim. Biophys. Acta 497, 702–706 https://doi.org/0304-4165(77)90291-4 [pii].
Rocha, P.S.C.F., Sheikin, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B., Wagner, C., Vaucheret, H., Furner, I., 2005. The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING1 Gene Codes for an S-Adenosyl-L-Homocysteine Hydrolase Required for DNA Methylation-Dependent Gene Silencing. Plant Cell 17, 404–417. https://doi.org/10.1105/tpc.104.028332.
Rubbo, H., Radi, R., 2008. Protein and lipid nitration: role in redox signaling and injury. Biochim. Biophys. Acta - Gen. Subj. https://doi.org/10.1016/j.bbagen.2008.03.007.
Saluk-Juszczak, J., Krolewska, K., Wachowicz, B., 2010. beta-glucan from Saccharomyces cerevisiae as a blood platelet antioxidant. Platelets 21, 451–459. https://doi.org/10.3109/09537101003780032.
Selote, D.S., Khanna-Chopra, R., 2010. Antioxidant response of wheat roots to drought acclimation. Protoplasma 245, 153–163. https://doi.org/10.1007/s00709-010-0169-x.
Seminario, A., Song, L., Zulet, A., Nguyen, H.T., González, E.M., Larrainzar, E., 2017. Drought stress causes a reduction in the biosynthesis of ascorbic acid in soybean plants. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.01042.
Shaw, P.E., 2002. Peptidyl-prolyl isomerases: a new twist to transcription. EMBO Rep. 3, 521–526. https://doi.org/10.1093/embo-reports/kvf118.
Siegel, D., Gustafson, D.L., Dehn, D.L., Han, J.Y., Boonchoong, P., Berliner, L.J., Ross, D., 2004. NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. Mol. Pharmacol. 65, 1238–1247. https://doi.org/10.1124/mol.65.5.1238.
Signorelli, S., Considine, M.J., 2018. Nitric oxide enables germination by a four-pronged attack on ABA-induced seed dormancy. Front. Plant Sci. 9, 296. https://doi.org/10.3389/fpls.2018.00296.
Signorelli, S., Monza, J., 2017. Identification of Δ1-pyrroline 5-carboxylate synthase (P5CS) genes involved in the synthesis of proline in *Lotus japonicus*. Plant Signal. Behav. 12. https://doi.org/10.1080/15592324.2017.1367464.
Signorelli, S., Casaretto, E., Sainz, M., Díaz, P., Monza, J., Borsani, O., 2013a. Antioxidant and photosystem II responses contribute to explain the drought-heat contrasting tolerance of two forage legumes. Plant Physiol. Biochem. 70, 195–203. https://doi.org/10.1016/j.plaphy.2013.05.028.
Signorelli, S., Corpas, F.J., Borsani, O., Barroso, J.B., Monza, J., 2013b. Water stress induces a differential and spatially distributed nitro-oxidative stress response in roots and leaves of *Lotus japonicus*. Plant Sci. 201–202, 137–146. https://doi.org/10.1016/j.plantsci.2012.12.004.
Signorelli, S., Imparatta, C., Rodríguez-ruiz, M., Borsani, O., Corpas, F.J., Monza, J., 2016. In vivo and in vitro approaches demonstrate proline is not directly involved in the protection against superoxide, nitric oxide, nitrogen dioxide and peroxynitrite. Funct. Plant Biol. 43, 870–879. https://doi.org/10.1071/FP16060.
Signorelli, S., Agudelo-Romero, P., Considine, M.J., Foyer, C.H., 2018. Roles for light, energy and oxygen in the fate of quiescent axillary buds. Plant Physiol. 176, 1171–1181. https://doi.org/10.1104/pp.17.01479.
Singh, H.P., Kaur, S., Batish, D.R., Sharma, V.P., Sharma, N., Kohli, R.K., 2009. Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of Oryza sativa (rice). Nitric Oxide - Biol. Chem. 20, 289–297. https://doi.org/10.1016/j.niox.2009.02.004.

Sofo, A., Scopa, A., Nuzzaci, M., Vitti, A., 2015. Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. Int. J. Mol. Sci. https://doi.org/10.3390/ijms160613561.

Sun, C., Liu, L., Zhou, W., Lu, L., Jin, C., Lin, X., 2017. Aluminum induces distinct changes in the metabolism of reactive oxygen and nitrogen species in the roots of two wheat genotypes with different aluminum resistance. J. Agric. Food Chem. 65, 9419–9427. https://doi.org/10.1021/acs.jafc.7b03386.

Takahashi, M., Shigeto, J., Izumi, S., Yoshizato, K., Morikawa, H., 2016. Nitration is exclusive to defense-related PR-1, PR-3 and PR-5 proteins in tobacco leaves. Plant Signal. Behav. 11. https://doi.org/10.1080/15592324.2016.1197464.

Tanou, G., Filippou, P., Belghazi, M., Job, D., Diamantidis, G., Fotopoulos, V., Molassiotis, A., 2012. Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. Plant J. 72, 585–599. https://doi.org/10.1111/j.1365-313X.2012.05100.x.

Toorchi, M., Yukawa, K., Nouri, M.Z., Komatsu, S., 2009. Proteomics approach for identifying osmotic-stress-related proteins in soybean roots. Peptides 30, 2108–2117. https://doi.org/10.1016/j.peptides.2009.09.006.

Valderrama, R., Corpas, F.J., Carreras, A., Fernández-Ocaña, A., Chaki, M., Luque, F., Gómez-Rodriguez, M.V., Colmenero-Varea, P.A., del Río, L., Barroso, J.B., 2007. Nitrosarive stress in plants. FEBS Lett. 581, 453–461.

Wang, H., Huang, J., Li, Y., Li, C., Hou, J., Liang, W., 2016. Involvement of nitric oxide-mediated alternative pathway in tolerance of wheat to drought stress by optimizing photosynthesis. Plant Cell Rep. 35, 2033–2044. https://doi.org/10.1007/s00299-016-2014-y.

Xie, W., Goodwin, P.H., 2014. A PRp27 gene of Nicotiana benthamiana contributes to resistance to Pseudomonas syringae pv. tabaci but not to Colletotrichum destructivum or Colletotrichum orbiculare. Funct. Plant Biol. 36, 351–361. https://doi.org/10.1071/FP08241.

Xiong, J., An, L., Lu, H., Zhu, C., 2009. Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. Planta 230, 755–765. https://doi.org/10.1007/s00425-009-0984-5.

Ye, H., Roorkiwal, M., Valliyodan, B., Zhou, L., Chen, P., Varshney, R., Nguyen, H.T., 2018. Genetic diversity of root system architecture in response to drought stress in grain legumes. J. Exp. Bot. https://doi.org/10.1093/jxb/ery082.

Yordanov, I., Velikova, V., Tsonev, T., 2000. Plant responses to drought, acclimation, and stress tolerance. Photosynthetica. https://doi.org/10.1023/A:1007201411474.

Yoshimura, K., Masuda, A., Kuwano, M., Yokota, A., Akashi, K., 2008. Programmed proteome response for drought avoidance/tolerance in the root of a C3 xerophyte (wild watermelon) under water deficits. Plant Cell Physiol. 49, 226–241. https://doi.org/10.1093/pcp/pcm180.

You, J., Chan, Z., 2015. ROS Regulation During Abiotic Stress Responses in Crop Plants. Front. Plant Sci. 6, 1092. https://doi.org/10.3389/fpls.2015.01092.

Zhang, G., Zhang, M., Zhao, Z., Ren, Y., Li, Q., Wang, W., 2017. Wheat TaPUB1 modulates plant drought stress resistance by improving antioxidant capability. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-08181-w.