

Protein tyrosine nitration in higher plants grown under natural and stress conditions

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Protein tyrosine nitration is a post-translational modification (PTM) mediated by reactive nitrogen species (RNS) that is linked to nitro-oxidative damages in plant cells. During the last decade, the identification of proteins undergoing this PTM under adverse environmental conditions has increased. However, there is also a basal endogenous nitration which seems to have a regulatory function. The technological advances in proteome analysis have allowed identifying these modified proteins and have shown that the number and identity of the nitrated proteins change among plant species, analysed organs and growing/culture conditions. In this work, the current knowledge of protein tyrosine nitration in higher plants under different situations is reviewed.

Keywords: nitric oxide, nitroproteome, peroxyntirite, reactive nitrogen species (RNS), tyrosine nitration

INTRODUCTION

Protein tyrosine nitration is a post-translational modification (PTM) mediated by nitric oxide-derived molecules. It is the result of the addition of a nitro ($-\text{NO}_2$) group to one of two equivalent ortho carbons in the aromatic ring of tyrosine residues (Gow et al., 2004). This process can alter protein function because the incorporation of this nitro group into the aromatic ring lowers the pKa of the phenolic group from 10.1 of the tyrosine to 7.2 in the nitrotyrosine (Sokolovsky et al., 1967; Abello et al., 2009). This provokes both steric and electronic perturbations that affect Tyr's capacity to function in electron-transfer reactions and to maintain protein conformation (van der Vliet et al., 1999). Tyrosine nitration can change the function of the protein in several ways: function gain; no effect on function; and inhibition of function, the latter being the most common consequence of tyrosine nitration (Greenacre and Ischiropoulos, 2001; Radi, 2004). On the other hand, tyrosine nitration may influence many signal transduction pathways because this modification prevents phosphorylation of tyrosine and consequently affects one regulatory mechanism (Galetskiy et al., 2011).

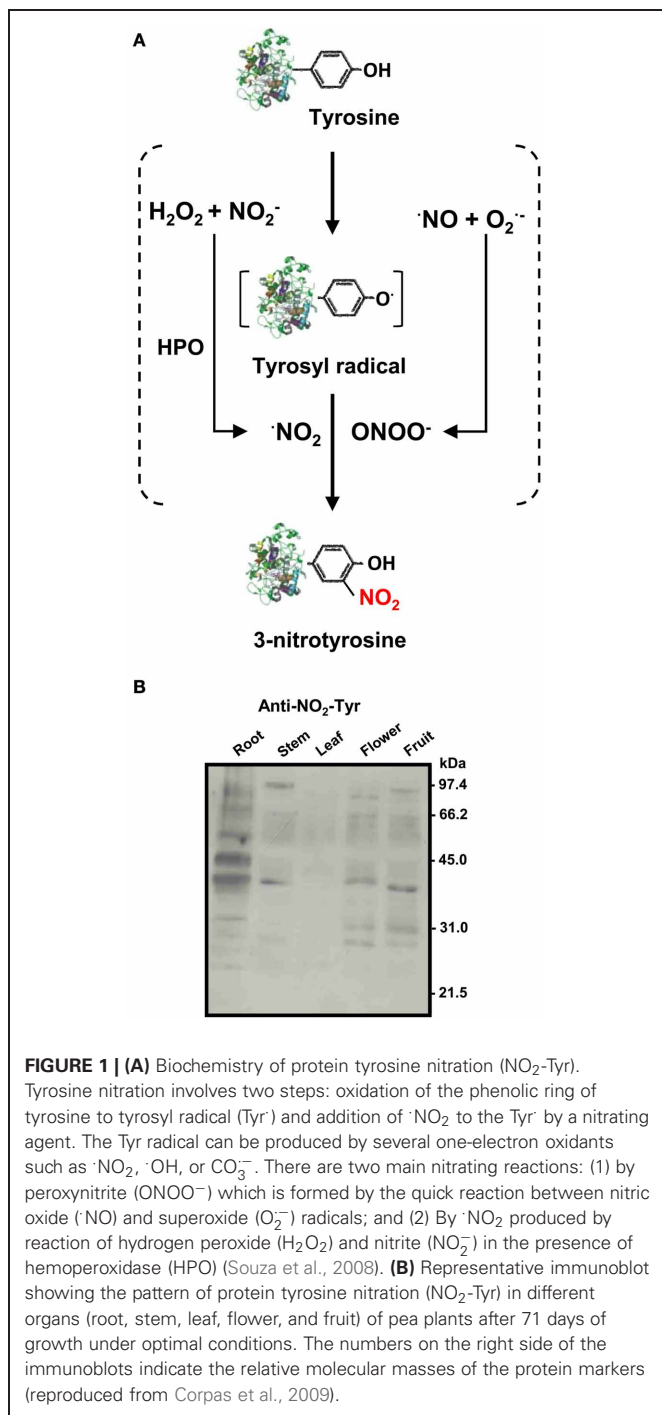
The presence of nitrotyrosine has been considered as a footprint of the occurrence of the strong nitrating agent peroxyntirite (ONOO^-), a reactive nitrogen species (RNS), which is formed usually under stress conditions by the chemical reaction between two radicals, nitric oxide ($\cdot\text{NO}$) and superoxide anion ($\text{O}_2^{\cdot-}$) (Ischiropoulos, 2003; Chaki et al., 2009a; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). However, there is another potential mechanism involving a hemoperoxidase that, in the presence of hydrogen peroxide (H_2O_2) and nitrite (NO_2^-), can

generate the radical nitrogen dioxide ($\cdot\text{NO}_2$) which acts as a nitrating agent (Souza et al., 2008) (Figure 1A). A relevant aspect is that tyrosine nitration is not a random process because not all Tyr residues are susceptible to nitration which depends on their accessibility to the solvent. Tyrosine has a hydropathy index of -1.3 being a mildly hydrophilic amino acid and, therefore, most likely exposed to the aqueous environment.

In most proteins, the number of tyrosine residues is around 3–4% out of the primary structure, but only few of these tyrosines may become preferentially nitrated. A good example is the human serum albumin which has 18 Tyr residues, and under *in vitro* nitration by peroxyntirite only 2 Tyr are susceptible of nitration. In animal organisms, the number of nitrotyrosine-containing proteins identified by proteomics approaches ranges from few proteins to over 100, depending on the organ (brain, heart, liver, etc.), subcellular compartment, and physiological conditions (normal or stress situations) (Abello et al., 2009).

In vivo DETECTION OF PROTEIN TARGETS OF TYROSINE NITRATION IN PLANTS

In higher plants, tyrosine nitration was initially detected using 1D gel electrophoresis followed by immunoblotting probed with antibodies against 3-nitrotyrosine. Thus, in tobacco leaf extracts and BY2 cell cultures it was observed that the exogenous application of nitrating agents such as peroxyntirite provoked a rise of immunoreactive proteins (Morot-Gaudry-Talarmain et al., 2002; Saito et al., 2006). Later, a similar behavior in the profile of nitrated proteins was observed in leaves of olive plants grown with 200 mM NaCl which induced both oxidative and



nitrosative stresses (Valderrama et al., 2007). After detecting the proteins which underwent tyrosine nitration, the next step was to identify those proteins which were targets of this PTM. Thus, the combination of proteomic techniques (two-dimensional polyacrylamide gel electrophoresis) followed by immunoblotting or immunoprecipitation, gel tryptic digestion and mass spectrometry have become powerful tools to develop nitroproteome studies in higher plants. Although there might be some technical problems like those due to the unspecific recognition of the antibody used against nitrotyrosine, the low number of nitrotyrosines

in a given protein, the low abundance of the nitrotyrosine-containing proteins, and that the isolated protein occasionally does not match in the protein database, it must be pointed out that these approaches have allowed establishing a basic background of knowledge in this research area (Dekker et al., 2012). In any case, after a specific protein has been identified as a putative nitration target, a necessary additional step is to identify the nitration site(s) within the protein quaternary structure by MALDI-TOF MS and LC-MS/MS (Ytterberg and Jensen, 2010). Finally, *in vitro* analyses of the physiological effects of nitration on the specific proteins must be developed. **Table 1** summarizes some of the identified nitrated proteins in higher plants where the locus for the nitrated tyrosine residue have been identified and the physiological effect of this PTM established.

NITRATION AND ENVIRONMENTAL STRESS SITUATIONS

Tyrosine nitration has been mainly studied in plants under stress conditions and it is assumed that a rise in the protein tyrosine nitration is a reliable marker of nitro-oxidative stress (Corpas et al., 2007). A significant number of data in different organs and plant species support this idea since increases of some specific nitrated proteins under different abiotic and biotic stresses have been reported. Thus, this PTM event occurs in leaves from pea plants subjected to different abiotic stresses including low and high temperature (HT), continuous light, and high light intensity (Corpas et al., 2008), in olives leaves, in *Arabidopsis* roots and *Citrus* leaves under salinity stress (Valderrama et al., 2007; Corpas et al., 2009; Tanou et al., 2012), in sunflower hypocotyls infected by the pathogen *Plasmopara halstedii* (Chaki et al., 2009a), in *Prunus* genotypes under high bicarbonate and high pH (Cellini et al., 2011), in pepper leaves under low temperature (Airaki et al., 2012), in *Arabidopsis* seedlings under arsenic stress (Letierrier et al., 2012), and in *Lotus japonicus* roots and leaves exposed to water stress (Signorelli et al., 2013). However, there are different environmental conditions (hypoxia, UV radiation, ozone, etc.) where protein tyrosine nitration has not been studied yet.

The nitroproteome analysis under certain stress conditions has allowed the identification of the induced proteins. Thus, in *Arabidopsis* leaves, up to eight different proteins undergoing Tyr-nitration and mainly involved in photosynthesis were identified (Cecconi et al., 2009). After infection with an avirulent bacterial pathogen (*Pseudomonas syringae* pv. Tomato), a general rise in the expression of those nitrated proteins was observed. Besides, three new nitrated proteins were detected, although they could not be identified (Cecconi et al., 2009). High light conditions induced specific tyrosine nitration in the protein PSBA(D1) of PhotosystemII (PSII) which provoked a dissociation of the PSII dimers and PSII-LHCII supercomplexes and a possible subsequent degradation of damaged protein subunits (Galetskiy et al., 2011). The exposure of sunflower seedlings to HT caused both oxidative and nitrosative stress, and nitroproteome analysis showed an increase in 12 tyrosine-nitrated proteins compared to control plants, plus the detection of a newly nitrated protein, a carbonic anhydrase (CA) (Chaki et al., 2011). Among the tyrosine-nitrated proteins under HT stress, two of them were investigated in more detail, ferredoxin-NADP reductase (FNR) and CA, since both enzymes are involved in photosynthetic

Table 1 | Examples of proteins identified in higher plants which are targets of tyrosine nitration, and the effect of this PTM on their function.

Protein	Plant species	Subcellular localization	Effects	Identified nitrated Tyr	References
S-adenosyl homocysteine hydrolase (SAHH)	Sunflower	Nucleus	Decreased activity	Tyr-448 ^a	Chaki et al., 2009a
Ferredoxin-NADP reductase	Sunflower	Chloroplast	Decreased activity	ND	Chaki et al., 2011
Carbonic anhydrase (β -CA)	Sunflower	Chloroplast	Decreased activity	Tyr-205 ^a	Chaki et al., 2013
PSBA(D1) of Photosystem II complex	<i>Arabidopsis</i>	Chloroplast	Disassembly of PSII dimers	Tyr-262 ^b	Galetskiy et al., 2011
Methionine synthase	<i>Arabidopsis</i>	Cytosol	Decreased activity	Tyr-287 ^b	Lozano-Juste et al., 2011
Glutamine synthetase	<i>Medicago truncatula</i>	Cytosol Chloroplast	Decreased activity	ND	Melo et al., 2011
O-acetylserine(thiol) lyase A1	<i>Arabidopsis</i>	Cytosol	Decreased activity	Tyr-302 ^b	Álvarez et al., 2011
Glyceraldehyde-3-phosphate dehydrogenase	<i>Arabidopsis thaliana</i>	Cytosol Chloroplast	Decreased activity	ND	Lozano-Juste et al., 2011
NADP-isocitrate dehydrogenase	Pea	Cytosol	Decreased activity	Tyr-392 ^b	Begara-Morales et al., 2013
α -Tubulin	Rice and tobacco cell cultures	Microtubules	Mitosis inhibition	ND	Jovanović et al., 2010

^aIn silico identification.

^bMass spectrometric techniques (LC-MS/MS).

ND: Not determined.

carbon assimilation, a process very sensitive to HT. Under HT stress the activities of FNR and CA were inhibited by 31% and 43%, respectively. This inhibition was corroborated under *in vitro* conditions, where their respective activities were determined in the presence of peroxyxynitrite as nitrating agent (Chaki et al., 2011, 2013).

Nitration can also provoke a loss of function as observed for different plant enzyme activities analyzed *in vitro* including ascorbate peroxidase and catalase (Clark et al., 2000), S-adenosyl homocysteine hydrolase (Chaki et al., 2009b), and O-acetylserine(thiol)lyase A1 activities (Álvarez et al., 2011).

NITRATION AND PLANT DEVELOPMENT

As indicated above, tyrosine nitration has been associated with situations of nitro-oxidative stress. However, some data illustrate the existence of a physiological protein nitration, which is not directly related to specific adverse conditions. Thus, in sunflower seedlings grown under optimal conditions, nitroproteome analysis of hypocotyl samples allowed the identification of 21 nitrotyrosine-immunopositive proteins involved in photosynthesis, and antioxidative, ATP, carbohydrate, and nitrogen metabolisms (Chaki et al., 2009b). More recently, nitroproteome analysis of *Citrus* roots revealed 26 potential candidate proteins to nitration (Tanou et al., 2012). Moreover, comparison of the nitroproteomes from green and red mature pepper (*Capsicum annuum* L.) fruits allowed identifying, by 2D gel and immunoblot, the profile of nitrated proteins which changed from 21 immunoreactive spots to 31 during fruit ripening. This could mean that protein tyrosine nitration could be used as an indicator of the ripening process in fruits. In rice seedlings and in tobacco BY-2 suspension cells grown under normal conditions, tyrosine nitration of α -tubulin may inhibit cell division and consequently cell growth as mitosis is inhibited (Jovanović et al., 2010).

Recently, nitration analysis during development and senescence of different organs from 8, 12, 14, and 16-day-old (young), and 71-day-old (senescent) pea plants has shown that each organ

has its own protein nitration pattern (Figure 1B). In the case of roots, it was observed that the intensity of nitrated proteins increased with root age. Roots of senescent pea plants contained 16 nitrotyrosine-immunoreactive proteins. Among the identified proteins, cytosolic NADP-isocitrate dehydrogenase, an enzyme involved in carbon and nitrogen metabolism, redox regulation, and response to oxidative stress, was studied to determine the effect of nitration during root senescence, a developmental stage which produced a significant decrease of its activity (Begara-Morales et al., 2013).

CONCLUSIONS

In higher plants, there is a growing interest in the analysis of protein tyrosine nitration as well as the identification of *in vivo* nitrated proteins. These studies are difficult to accomplish since tyrosine nitration is a low-abundance PTM. For example, in animal organisms under inflammatory conditions the estimated frequency of tyrosine nitration is 1 out of 10,000 tyrosines (Radi, 2004). The available data in higher plants are generally based on 2D gel electrophoresis and immunoblotting, what have provided some specific nitroproteomes including 21 proteins in sunflower cotyledons (Chaki et al., 2009a), 16 proteins in pea roots (Begara-Morales et al., 2013), and 127 proteins in *Arabidopsis* whole seedlings (Lozano-Juste et al., 2011). The identification of specific nitroproteomes from different plant organs under natural and stress conditions will be an exciting challenge for incoming research. Additionally, the study of the nitroproteome at subcellular level can provide fundamental data on cell proteomics of plants under different conditions.

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