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Protein Tyrosine Nitration in Abiotic Stress in Plants

Ankita Sehrawat • Renu Deswal*

Department of Botany, University of Delhi, Delhi -110007, India

Corresponding author: * **rdeswal@botany.du.ac.in**

ABSTRACT

Research in the last two decades has proven, without a doubt, that nitric oxide (NO) is a cytotoxic as well as a signaling molecule in biological systems. NO is one of the nitrogen oxides present in air and being a free radical it is very reactive. It combines readily with all major macromolecules whether it is lipids, nucleic acids or proteins. Lipid and nucleic acid modification by NO are relatively less extensively investigated more so in plants. Proteins are mostly post-translationaly modified by $NO^{\cdot}$ and its derivatives, which together constitute reactive nitrogen species (RNS). Although recently, good progress has been made regarding 'NO' signaling in plants but focus has been more on nitrosylation (a covalent addition of NO to free thiol group in a protein). Another modification, which has received relatively little attention is 'nitration', which is the addition of a nitro group (NO_2) to an amino acid, preferable tyrosine. Abiotic stress conditions contributes to 'NO' production enhancing the nitrosative stress. In animal system 'tyrosine nitration' is shown to be a 'nitrosative stress marker'. Current studies in NO signaling hints at a similar scenario in plants. About 150 tyrosine nitrated proteins are known. A generalized increase in nitration by abiotic stress was observed in many plants including *Arabidopsis thaliana* and *Helianthus annuus*. Mechanisms and signaling of nitration are being deciphered. Therefore, 'tyrosine nitration' with reference to abiotic stress is reviewed in the present review to describe this very new and relatively unexplored research area in plants.

Keywords: nitric oxide, post translational modifications, signaling, reactive nitrogen species
Abbreviations: CLSM, confocal laser-scanning microscopy; **NO**, nitric oxide; **$ONOO^-$**, peroxynitrite; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species, **Tyr**, tyrosine

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INTRODUCTION

Nitric oxide (NO) is an air pollutant and contributes to acid rain. In plants, NO can be produced both enzymatically (by nitric oxide synthase (NOS); Guo *et al.* 2003; Talwar *et al.* 2012, nitrate reductase (NR); Desikan *et al.* 2002 and nitrite: NO-oxido-reductase (NI-NOR); Stöhr *et al.* 2001) as well as non-enzymatically (by decomposition of nitrous acid to NO and the carotenoid-mediated NO production, Cooney *et al.* 1994).

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Invited Review

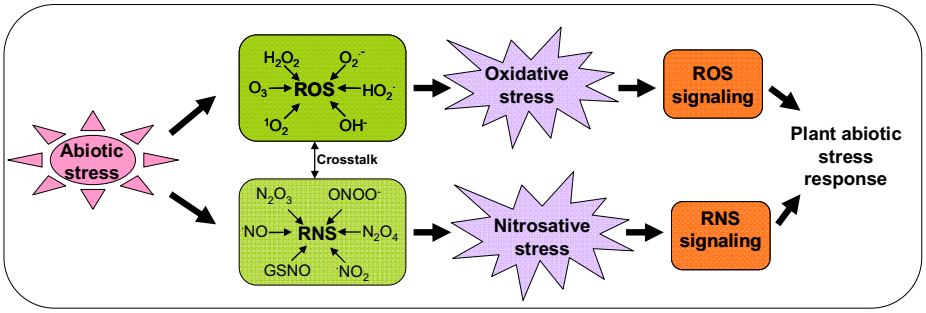

Fig. 1 A schematic model depicting oxidative and nitrosative signaling mechanism during abiotic stress.

NO is a highly reactive free radical and has attracted the attention of many researchers as it regulates broad spectrum of functions by altering numerous physiological and developmental processes in the entire life span of plant from seed germination (Šírová *et al.* 2011) to flower development (Simpson *et al.* 2005) and senescence (Mishina *et al.* 2007) during normal as well as in the stress conditions (Abat *et al*. 2008a). NO is an important signaling molecule in many physiological processes (Lamattina *et al.* 2003; Wilson *et al.* 2008; Yemets *et al.* 2011). "Reactive nitrogen species" (RNS) refers to NO and a group of NO-related molecules like peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), S-nitrosoglutathione (GSNO), nitrogen dioxide ($^{\cdot}NO_2$), nitrosyl cation (NO^+), S-nitrosothiols (RSNOs) etc. (Hausladen and Stamler 1999). RSNOs production involves the reaction of nitrosonium (NO^+) with a thiol group. The GSNO is the result of the reaction between $^{\cdot}NO$ and reduced glutathione (GSH). It can also be produced by transnitrosation from other RSNOs with GSH. Both RSNOs and GSNO act as a reservoir and vehicle for NO (Singh *et al.* 1996). $ONOO^-$ is produced by the rapid reaction between superoxide radicals ($O_2^{\cdot-}$) and $^{\cdot}NO$. In abiotic stress a cross talk between reactive oxygen species (ROS) and RNS signaling has been demonstrated (Zaninotto *et al.* 2006, **Fig. 1**). All these reactions results in the initiation of defense response in plants and animals.

NO works at both transcriptional and posttranslational level. It regulates the expression of kinases, transcription factors and various signaling molecules like salicylic acid and jasmonic acid. One of the NO mediated post translational modification (PTMs) is protein tyrosine nitration which involves the addition of nitro group to the ortho-positon of tyrosine (Tyr) residues of the protein resulting in 3-nitrotyrosine formation.

Protein tyrosine nitration has been reported mainly in animal systems (Bhattacharjee *et al.* 2009; Fan *et al.* 2011). In recent years focus has shifted to plants. First report of nitration was in tobacco with impaired nitrite reductase activity (Morot *et al.* 2002). Nitrated proteins were first identified in sunflower using immunological and proteomic approaches (Chaki *et al.* 2009). All the above mentioned reports were restricted to the identification of *in vitro* protein nitration in plants, but nothing was mentioned regarding the sites of nitration. Recently, identification of *in vivo* nitration sites were reported in *Arabidopsis thaliana* (Lozano *et al.* 2011). Protein tyrosine nitration can lead to structural and conformational modification in the proteins which can further effect activity which in turn could alter the susceptibility of proteins to protease digestion and signaling processes. For example in purified CDC2 (cell cycle kinase), nitration of single Tyr residue prevents its phosphorylation. Local concentration of $ONOO^-$ plays a critical role in determining whether Tyr residues will nitrate or phosphorylate.

In field conditions, plants are exposed to extreme environmental conditions such as high and low temperature, high salt, high light intensity, drought, ultraviolet (UV) radiation, heavy metal and mechanical injury which affect the growth and development. Abiotic stress affects plant productivity either by altering major carbon metabolic pathways like photosynthesis and respiration or by affecting the proteins and nucleic acids metabolism. Enough information is available on the physiological role of NO during abiotic stress (Corpas *et al.* 2011; Siddiqui *et al.* 2011), but relatively less is known about the role of protein nitration in stress. Abiotic stress-induced protein nitration has been demonstrated in *Taxus cuspidata* (Gong and Yuan 2006), *Olea europaea* (Valderrama *et al.* 2007), *Pisum sativum* (Corpas *et al.* 2008), *Arabidopsis thaliana* (Corpas *et al.* 2009b; Leterrier *et al.* 2012), *Helianthus annuus* (Chaki *et al.* 2011a, 2011b) and *Capsicun annuum* (Airaki *et al.* 2012). As abiotic stress induces protein tyrosine nitration,

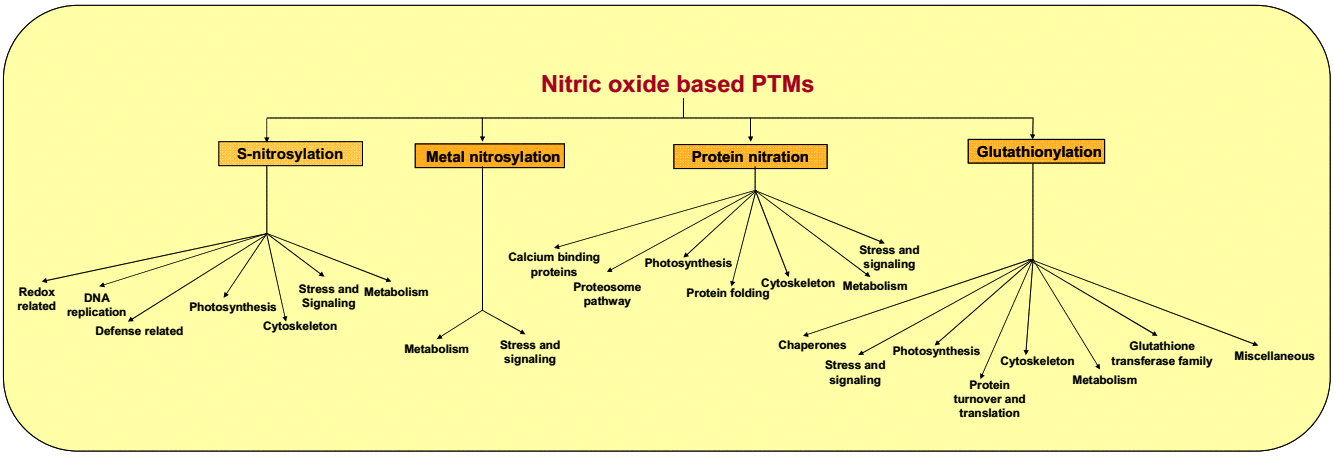

Fig. 2 A schematic diagram showing NO based post translational modifications (PTMs) in plants. NO can modify proteins by post translationally modifying them by S-nitrosylation, metal nitrosylation, protein tyrosine nitration and glutathionylation. Proteins associated with different functional categories like photosynthesis, metabolism and stress/signaling are effected by these PTMs. NO based PTMs either directly alters the physiological response by affecting the proteins activity (e.g. Rubisco) or causes the alteration in gene expression by modulating the signaling pathway (e.g. R2R3 MYB).

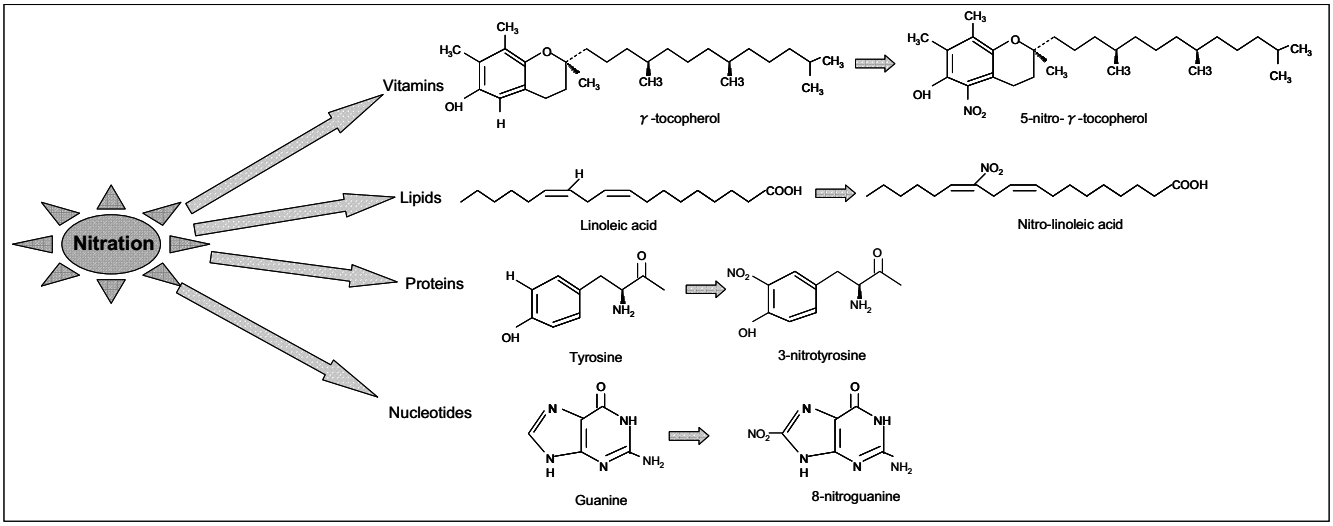

Fig. 3 Nitration in different biomolecules.

this modification is regarded as a marker of nitrosative stress.

The goal of this review is to provide the latest findings on plant tyrosine nitration with emphasis on its role in abiotic stress.

NO-ASSOCIATED POST TRANSLATIONAL MODIFICATIONS OF PROTEINS

NO is synthesized in plants by nitrite/nitrate dependent and L-arginine dependent pathway (Negi *et al.* 2011). Once produced, NO acts mainly via PTMs like S-nitrosylation, metal nitrosylation, glutathionylation and nitration (**Fig. 2**).

S-nitrosylation

S-nitrosylation is a reversible modification which involves the formation of a covalent bond between NO and reactive cysteine residues (Astier *et al*. 2011). S-nitrosylation plays a very important role in regulation of various metabolic pathways (Lindermayr *et al*. 2005; Abat *et al*. 2008b; Abat and Deswal 2009). Denitrosylation occurs both enzymatically as well as non enzymaticaly with the help of enzymes like thioredoxin or thioredoxin reductase (Benhar *et al*. 2008).

Metal nitrosylation

Similar to S-nitrosylation, metal nitrosylation is a reversible PTM. NO being a free radical can donate electrons and thus can react with transition metals. Covalent interaction of NO with proteins having metal centres like heme, zinc finger proteins can results in either an increase or a decrease in the activity. The most studied protein regulated by metal nitrosylation in animals is soluble guanylate cyclase (sGC, Denninger and Marletta 1999). Binding of NO to sGC heme increases the activity of sGC and thus cause many fold increase in 3, 5-cyclic GMP (cGMP, a second messenger) synthesis which further binds to kinases and influence downstream pathways (Cary *et al.* 2006).

Glutathionylation

In glutathionylation, glutathione (GSH) molecule attaches to the proteins via disulfide bond. Using mitochondria as a subcellular organelle, 11 proteins including glycine decarboxylase complex (GDC) and ATP synthase subunit α were identified to be glutathionylated and/or S-nitrosylated in *Arabidopsis thaliana* leaves (Palmieri *et al.* 2010).

Nitration

Nitration is a chemical process which involves the addition of a nitro group into a chemical compound or biomolecule. As the focus of this mini-review is protein nitration, it would be discussed in details.

NITRATION OF BIOMOLECULES AND ITS PHYSIOLOGICAL IMPLICATIONS

Nitration modifies proteins, lipids, nucleotides and other biomolecules (**Fig. 3**). Protein tyrosine nitration can occur at amino acids like tyrosine, tryptophan, cysteine and methionine. Nitrated proteins and lipids are key players in redox based signaling events. Lots of work has been done in the field of lipid nitration in humans like identification, detection and quantification of nitrated fatty acids in human plasma (Lima *et al.* 2002). Oleic acid and linolenic acid are reported to be nitrated in humans (Lima *et al.* 2002; Cui *et al.* 2006). Various pathways are proposed to explain lipid nitration (Trostchansky and Rubbo 2007; Rubbo and Radi 2008) which includes 1) reaction of $^{\cdot}NO_2$ with unsaturated lipids and lipid radicals which results in formation of nitroalkene and nitrohydroxy derivatives. Involvement of $ONOO^-$ in mediating fatty acid nitration is also proposed as it can readily diffuse through membranes (Rubbo *et al.* 1994), 2) nitroalkene formation by electrophilic substitution at double bond of unsaturated fatty acid by NO_2^+ moiety and 3) reaction of $^{\cdot}NO_2$ with a carbon-centered radical (Rubbo *et al.* 1994). Nitrolipids not only play an important role in mediating signal transduction cascades (Freeman *et al.* 2008) but are also reported to act as footprints of pathophysiological processes (Rubbo and Radi 2008; Trostchansky and Rubbo 2008). Till now no information is available about lipid nitration in plants.

Apart from proteins and lipids, nucleotides can also undergo nitration. 8-nitroguanosine is nitratively modified nucleoside and its role in inflammation-associated mutagenesis and carcinogenesis is reported (Kaneko *et al.* 2008). Recently, the role of 8-Nitroguanosine 3', 5'-cyclic monophosphate (8-nitro-cGMP), a nitrated derivative of cGMP (cyclic nucleotide from GTP) as an electrophilic second messenger in the regulation of ROS signaling was reported in rat C6 glioma cells (Ahmed *et al.* 2012). $ONOO^-$ and myeloperoxidase dependent oxidation of nitrite in the presence of hydrogen peroxide were shown to be the two major pathways for guanine nitration. The role of mitochondria derived superoxide in nitration of guanine is also demonstrated.

Vitamin E is commonly known as tocopherols and tocotrineols. α-Tocopherol and γ-tocopherol are two types of tocopherols found in plants. α-Tocopherol is found in green

Table 1 Comparison of Tyrosine and Tryptophan nitration mechanism (Nuriel *et al.* 2011)

Steps	Tyrosine nitration	Tryptophan nitration
1. Formation of radical intermediate during nitration	Tryosine nitration in presence of $ONOO^-$ results in formation of tyrosyl radical which can later reacts with NO_2 to form 3-nitrotyrosine.	Formation of tryptophanyl radicals in response to $ONOO^-$ treatment hints that Trp modification by $ONOO^-$ also involves the formation of radical intermediate like tyrosine nitration
2. Site for modification	Tyrosine nitration can occur only at the single carbon on its benzene ring.	Tryptophan contain the indole side-chain which has many reactive sites like 1-nitrogen and 2-, 4-, 5-, 6-, 7- carbon which results in the formation of 1-, 2-, 4-, 5-, 6-, and 7-nitrotryptophan.
3. Reaction with peroxynitrite	Tyrosine residues do not directly reacts with $ONOO^-$.	Tryptophan residues can react directly with $ONOO^-$.

tissue whereas γ-tocopherol is mainly found in seeds, fruits and nuts. γ-Tocopherol affects seedling germination by influencing the NO content (Desel and Krunpiska 2005). The nitration of vitamin E is reported in humans (Morton *et al*. 2002) as well as in plants (Desel *et al*. 2007). The nitration of γ-tocopherol is reported in *Arabidopsis thaliana* seeds where it reacts with nitrogen dioxide to generate 5-nitro-γ-tocopherol (5-NγT) (Desel *et al.* 2007). 5-nitro-γ-tocopherol (5-NγT) can act as RNS scavenger during abiotic stress indicating the physiological relevance of nitration.

Phenolics are potential antioxidants and are known to scavenge ROS and RNS, (Heijnen *et al.* 2001). Phenolics are characterized by the presence of at least one aromatic ring (C6) with one or more hydroxyl groups. Mechanism of phenolics nitration involves the free radical reaction between phenoxyl radical ($Phe^{\cdot}$) and nitrogen dioxide radical ($NO_2^{\cdot}$). Hydroxycinnamic acids (HCA) are polyphenolic compounds and are known to be nitrated by suppressing the $ONOO^-$ mediated tyrosine nitration because of structural similarity between tyrosine and plant HCAs. Hemeperoxidases like horseradish peroxidase (HRP) mediated nitration of p-coumaric acid (p-CA, a plant phenolic) was shown by Sakihama *et al.* (2003). Apoplastic localization of HRP/ nitrite-dependent plant phenolics nitration was suggested, indicating the role of nitration in stress and signaling (Sakihama *et al.* 2003).

PROTEIN NITRATION

$ONOO^-$ is known to modify many aromatic (Try, Trp, Phe and His) and sulfur-containing amino acids (Cys and Met) (Alvarez and Radi 2003). To date, research on the nitration of the proteins in the plants has focussed mainly on tyrosine nitration. However, in the animal system there is growing evidence of tryptophan nitration playing a significant role in regulating cellular signaling mechanism in stress. In a recent review by Nuriel *et al.* (2011), the role of tryptophan nitration in nitrosative stress was discussed. Proteins like acidic fibroblast growth factor (FGF-1, at Trp121), creatine kinase (at Trp 264 and Trp 268) and lysozyme (at Trp 62, Trp 63 and Trp 63 and Trp 123) showed $ONOO^-$ induced tryptophan nitration. A comparative analysis of tyrosine and tryptophan nitration is presented in **Table 1**.

TYROSINE NITRATION

Tyrosine nitration is a PTM which involves the addition of a nitro group in *ortho* position of the phenolic hydroxyl group of Tyr residues of the protein. Nitration causes physiochemical changes in the modified amino acid due to addition of nitro group which may further modulate protein function.

Mechanism of tyrosine nitration

Mechanism of tyrosine nitration involves free radical chemistry. Tyrosine nitration is a two-step process. In the first step, the phenolic ring of tyrosine is oxidised to produce tyrosyl radical. Few *in vivo* oxidants are $CO_3^{\cdot-}$, $^{\cdot}OH$, $^{\cdot}NO_2$ and compound I of peroxidases. Second step involves radical-radical termination reaction in which $^{\cdot}NO_2$ reacts with the tyrosine.

1. Peroxynitrite-mediated tyrosine nitration

$ONOO^-$ is highly reactive and can modify proteins, lipids and DNA. It is involved in diseases and can cause cell damage (Szabó *et al.* 2007). It may also interfere with $NO^{\cdot}$-mediated signaling (Beckman and Koppenol 1996; Salvemini *et al.* 2006). Factors affecting $ONOO^-$-mediated tyrosine nitration includes presence of transition metal ions like Cu^{2+}, Fe^{3+}, Fe^{2+}, protoporphyrin IX and ethylene diamine tetraacetic acid (EDTA) (Beckman *et al.* 1992; Bian *et al.* 2003). In the case of $ONOO^-$ mediated tyrosine nitration, $ONOO^-$ reacts with carbon dioxide (CO_2) to form $CO_3^{\cdot-}$ and $^{\cdot}NO_2$ which further results in the formation of tyrosyl radical in the aqueous medium. Tyrosyl radical react with $^{\cdot}NO_2$ to form 3-nitrotyrosine (Rubbo and Radi 2008). The formation of 3-nitrotyrosine induces a shift in the local pKa of hydroxyl group from 10.07 (tyrosine) to 7.50 (nitrotyrosine) due to the addition of a nitro group at the ortho-position adjacent to the hydroxy group. This changes the protein function. $ONOO^-$-mediated nitration is maximal at physiological pH (about pH 7.4), and its yield decreases quickly in more acidic or basic conditions. GSH is known to inhibit protein tyrosine nitration by scavenging carbonate and nitrogen dioxide radicals. It also inhibits the repair reaction of tyrosyl radicals.

2. Heme Peroxidase-dependent tyrosine nitration

Heme peroxidase enzymes like mylenoperoxidases and horseradish peroxidases in presence of nitrite and hydrogen peroxide can cause protein nitration via simultaneous oxidation of nitrite and tyrosine to nitrogen dioxide radical and tyrosyl radical respectively. Reaction between these two radicals yields nitrotyrosine (Turko and Murad 2002; Peluffo and Radi 2007). Heme/iron catalyzed tyrosine nitration was shown in the presence of H_2O_2 and nitrite in heart and skeletal muscles of male CF-1 mice. H_2O_2 helps in the protein oxidation whereas nitrite is important for heme (Fe), H_2O_2 and the NO_2^- mediated nitration (Bian *et al.* 2003).

3. Transition metals-mediated tyrosine nitration

It involves the interaction of tyrosyl radicals with $^{\cdot}NO$ resulting in 3-nitrosotyrosine formation. On oxidation, 3-nitrosotyrosine yields 3-nitrotyrosine (Radi 2004; Bartesaghi *et al.* 2007). Proteins like prostaglandin endoperoxide H synthase-2 involve transition metals during nitration. $ONOO^-$ is found to increase tyrosine nitration in metal binding proteins like manganese superoxide dismutase (MnSOD, Quijano *et al.* 2001).

Factors affecting tyrosine nitration

1. Selection of proteins and tyrosine residues in proteins

Tyrosine nitration is a selective process and does not occur at random. Neither the protein nor the tyrosine residue abundance are the criteria for tyrosine nitration (Souza *et al.* 1999; Ischiropoulos 2003). Out of 3-4 mol % of tyrosine in plants only one or two of these tyrosines are nitrated (Corpas *et al*. 2009a). For e.g. human serum albumin contains 18 tyrosine residues but only 2 are known to be nitrated.

Table 2 Comparative analysis of methodologies utilized for detection and identification of tyrosine nitration.

Technique	Principle	System	Advantage	Disadvantage	References
Immunological studies	Detection of nitrated proteins by western blotting using anti-nitrotyrosine antibodies	*Pisum sativum*	User friendly and inexpensive	Non specific binding of antibody	Corpas *et al.* 2008
2-Dimensional electrophoresis and MS analysis of nitrotyrosine-containing proteins and peptides	Nitrated proteins resolved on 1D or 2-D gels are transferred to NC/PVDF membrane. Membrane is probed with monoclonal anti-nitrotyrosine antibody and the immunocomplexes are detected by chemiluminescence.	*Arabidopsis thaliana*	Identification of nitrated proteins	Dominance of abundant proteins during identification	Lozano *et al.* 2011
Immunoprecipitation	3-nitrotyrosine containing proteins from total proteome are immunoprecipitated using anti-nitrotyrosine antibodies	*Arabidopsis thaliana*	Identification of very acidic or basic proteins	Non specific binding of antibody	Lozano *et al.* 2011
Immunohistochemistry	Immunohistochemistry is carried out by confocal analysis of immunofluorescence-stained sections using antibody against NO_2-Tyr.	*Helianthus annuus*	Detection of stress mediated changes can be easily performed	Nitrated proteins can not be identified	Chaki *et al.* 2011b
Derivatization assay	Involves transformation of the nitro-group to a sulfhydryl-group which are enriched using thiopropyl-beads.	C57BL/6J inbred strain male mouse	Highly specific method	Sample loss during derivatization and clean up steps.	Zhang *et al.* 2007
Gas chromatography/negative chemical ionization tandem mass spectrometry	Nitrotyrosine is reduced to aminotyrosine followed by total protein hydrolysis and amino acid analysis using gas chromatography/negative chemical ionization tandem mass spectrometry.	Mesencephalon cultures	Quantitative assessment of degree of nitration	Site of nitration can not be identified	Larsen *et al.* 2008
Combined fractional diagonal chromatography (COFRADIC)	Involves two consecutive high performance liquid chromatography (HPLC) with an in between modification step resulting in a shift in the retention time of the peptides.	Bovine serum albumin	Easy and reproducible for identification of tyrosine nitrated proteins and the nitration sites in complex samples.	Still unexplored, need of a unautomated protocol for nitrated peptide analysis in complex samples	Larsen *et al.* 2011

2. Protein structure, localization and its interaction with other molecules

Secondary structure of the protein and the local environment of the tyrosine residue affect tyrosine nitration (Souza *et al.* 1999; Ischiropoulos 2003). Most of the nitrated tyrosines are located in the loop structures. Presence of a glutamate residue in the near vicinity of tyrosine favours nitration. Cysteine and methionine can react with the nitrating agents and act as scavengers by decreasing tyrosine nitration. The unsaturated to saturated fatty acids ratio is shown to influence the yield of nitration.

3. Medium of nitration

Aqueous or hydrophobic environment can affect this PTM either by influencing the rate or yield of nitration. For example, N-t-BOC L-tyrosine tert butyl ester (BTBE) was shown to be nitrated in a hydrophobic environment (Bartesaghi *et al.* 2006).

Effect of tyrosine nitration on proteins

1. Protein function

Tyrosine nitration may affect proteins by inhibiting/favouring their activity (Souza *et al.* 2008). Nitrated enzymes showing activity inhibition includes MnSOD, nitric oxide synthase 2, glutathione reductase and cytochrome P450 (Schopfer *et al.* 2003). Oxidative stress is considered to be one of the causes of proteins inactivation via tyrosine nitration. Contrary to this, tyrosine nitration also activates certain enzyme like glutathione *S*-transferase (Ji *et al.* 2006).

2. Proteolytic degradation

In animals, it is reported that tyrosine nitrated proteins like nitrated bovine Cu, Zn superoxide dismutase are more susceptible to proteolysis than non nitrated proteins due to their faster degradation by the proteasomes (Souza *et al.* 2000). In plants, detail(s) regarding nitration mediated protein degradation are not yet reported. Thus, further work is needed to build the relationship between tyrosine nitration and protein degradation in plants.

3. Phosphorylation

Tyrosine nitration can interfere with tyrosine phosphorylation. Phosphorylation is an important PTM which plays an important role in signal transduction associated with various cellular responses. Tyrosine nitration might affect cellular function(s) by interfering with tyrosine phosphorylation. Nitration and phosphorylation are two competing chemical reactions at a given tyrosine residue. Phosphorylation together with tyrosine nitration modulates signal transduction (Shi *et al.* 2007; Monteiro *et al.* 2008).

Methods for tyrosine nitration detection

Many methods have been developed which allow the detection of the 3-nitrotyrosine, identification of the nitrated proteins and the site of nitration. Identification of *in vivo* tyrosine nitrated proteins is important as these will indicate the actual physiological relevance of nitration. Several approaches as detailed in the following section have been developed to investigate it (**Table 2**).

1. Immunological studies

Majority of nitration studies in plants involve the immunological methods using anti-nitrotyrosine antibodies. Using antibody against 3-nitrotyrosine, nitration was detected in *Pisum sativum* (Corpas *et al.* 2008) and *Arabidopsis thaliana* (Corpas *et al.* 2009b) in abiotic stress. To remove false positive results due to non specific binding of anti-3-nitrotyrosine antibodies, control experiment are performed in which the NC/PVDF membrane is reduced with sodium dithionite before western blotting. This step reduces nitrotyrosine to aminotyrosine and allows the detection of false positive spots which corresponds to nonspecific immunoreactivity of the antibody. The nitrated protein or the site of modification cannot be identified by this method.

2. 2-Dimensional electrophoresis and MS analysis of nitrotyrosine-containing proteins and peptides

As one-dimensional SDS–PAGE has low resolution capacity, the nitrated proteins are detected using 2D-PAGE and western blotting followed by identification of nitrated proteins and site of modification by mass spectrometry (MS). Nitrated proteins can be resolved on 1D or 2D gels and then transferred to NC/PVDF membrane. Membrane is then probed with monoclonal anti-nitrotyrosine antibody and the immunocomplexes are detected by chemiluminescence. An approach based on 2-Dimensional electrophoresis (2-DE) combined with western blot using an anti 3-nitrotyrosine antibody is reliable for the detection of the nitrated proteins. MS identification can further be confirmed by performing western blotting of the identified proteins with their specific antibodies in the samples immunopurified by precipitation with anti-3-nitrotyrosine antibodies. In *Arabidposis*, 127 *in vivo* nitrated proteins were identified by resolving immunopurified fractions on 2D SDS-PAGE following LC-MS/MS (Lozano *et al.* 2011). Success in the identification of large number of nitrated proteins is achieved using MS but MS/MS spectrum with a good MASCOT score is still not obtained for nitrated peptides. In *Arabidopsis thaliana* only six proteins out of 22 showed MALDI-ToF spectra for nitrated peptides (Lozano *et al.* 2011). Limitations of 2-DE approach for the identification of nitrated proteins are 1) mostly abundant proteins are identified 2) proteins with poor solubility and extreme pI values are missed during resolution 3) poor recovery of nitrotyrosine containing peptides from the gels.

3. Immunoprecipitation

Immunoprecipitation of 3-nitrotyrosine containing proteins from crude protein extracts using anti 3-nitrotyrosine antibodies followed by analysis on 1D or 2D SDS-PAGE is done. In *Arabidposis*, 450 spots were detected on resolving the immunoprecipitated fraction on 2D SDS-PAGE (Lozano *et al.* 2011). But no unequivocal nitration sites were found by this method which could be either due to low abundance of tyrosine residues or specific selectively of only one or two tyrosine residues dung tyrosine nitration. This method helps in the identification of very acidic or basic proteins which are difficult to identify by 2-DE and MS. However, immunoprecipitation is a lengthy process (16 h incubation) and requires more antibodies in comparison with the western blotting. Also, non-specific binding of antibody to non nitrated proteins can give false positive results.

4. Immunohistochemistry

The cellular location and level of NO_2-Tyr can be detected by staining the tissue sections using specific antibody against NO_2-Tyr and CLSM. It is a useful technique for analyzing the difference in the distribution of NO_2-Tyr in stress conditions. In *Helianthus annuus*, the control hypocotyl sections showed distribution of NO_2-Tyr in cortex and vascular tissue whereas after mechanical wounding fluorescence intensification was observed in all cell types (Chaki *et al*. 2011b).

5. Other approaches utilized to detect nitration in animals

5.1 Derivatization assay

It is a four-step procedure involving transformation of the nitro-group into a sulfhydryl-group that could be enriched using thiopropyl-beads. It is a highly specific method but sample losses are observed during derivatization and clean up steps.

5.2 Reduction process

5.2.1. Derivatized using dansyl-chloride

The nitro-group is reduced to an amino-group and then derivatized using dansyl-chloride to create a product which forms a specific reporter ion at m/z 170 and allows the identification of nitrated peptides. Low yield of dansylchloride reaction makes the technique less user friendly.

5.2.2. Gas chromatography/negative chemical ionization tandem mass spectrometry

In this method nitrotyrosine is reduced to aminotyrosine followed by a step of total protein hydrolysis and amino acid analysis using gas chromatography/negative chemical ionization tandem mass spectrometry after derivatization. Quantitative assessment of degree of the nitration can be done but the site of nitration cannot be identified using this method.

5.3 Combined fractional diagonal chromatography (COFRADIC)

This method involves two consecutive high performance liquid chromatography (HPLC) with modification step in between which results in a shift in the retention time of the peptides. COFRADIC is an easy and reproducible method for the identification of tyrosine nitrated proteins and the nitration sites in complex samples. Recently, using COFRADIC in combination with off-line nano-LC-MALDI, Larsen *et al*. (2011) identified six nitrated peptides from *in vitro* nitrated bovine serum albumin, to prove suitability of the procedure for the detection of the nitrated peptides.

Is tyrosine nitration relevant in cellular signal transduction pathways?

NO signaling takes place either by NO mediated activation of guanylate cyclase resulting in formation of cGMP (second messenger) or through NO based PTMs like S-nitrosylation (Abat and Deswal 2011), tyrosine nitration (Abat and Deswal 2010; Moreau *et al.* 2010). Recently, nitrotyrosine is not only considered as an indicator of $ONOO^-$ formation but also as a reflector of RNS (Arasimowicz and Floryszak 2011). During tyrosine nitration, the pK_a shift of tyrosine occurs due to the addition of a nitro group. These changes results in signal modulation which can further lead to loss of function (GSNOR) or gain of function (cytochrome c) (Moreau *et al.* 2010). Among the identified tyrosine nitrated targets in sunflower, putative serine/threonine-protein kinase, 14-3-3-like protein, calmodulin-like protein are involved in signal transduction thus, reflecting the role of tyrosine nitration in signaling in plants (Chaki *et al.* 2009). S-adenosyl-homocysteine hydrolase (SAHH), involved in amino acid biosynthesis is regulated by S-nitrosylation and RNS, showed activity inhibition after tyrosine nitration. The physiological relevance of tyrosine nitration during stress in plants is well documented (Leitner *et al.* 2009). The role of tyrosine nitration in signaling can be justified by the fact that it influences tyrosine phosphorylation or dephosphorylation signaling pathways via modulation of $ONOO^-$ concentration (Liaudet *et al.* 2009). Tyrosine nitration being a reversible PTM can play important role in signaling (Souza *et al.* 2008). Like phosphorylation/dephosphorylation, a controlled and specific nitration/denitration reactions process is required for regulation as reported by Koeck *et al.* (2004). Monterio *et al.* (2008) suggested that in normal conditions a balance is maintained between nitration/denitration and phosphorylation/dephosphorylation processes but in oxidative and nitrosative stress, nitration of proteins and other compounds increases over denitration and signaling is initiated.

Inhibition of tyrosine nitration by antioxidants

Antioxidants are the molecules which inhibits the oxidation of other molecules. They are known to scavenge $ONOO^-$. $ONOO^-$ is a well known strong oxidizing as well as nitrating agent and it is involved in oxidative and nitrosative stress in different biological systems (Arasimowicz *et al.* 2011). One of the mechanism by which tyrosine undergoes nitration includes formation of 3-nitrotyrosine in the presence of $ONOO^-$. Sinapic acid or sinapinic acid, a naturally occurring hydroxycinnamic acid has antioxidant properties. Zou *et al.* (2002) demonstrated $ONOO^-$ scavenging activity of SA in *Brassica juncea*. In this study the disappearance of a characteristic peak of nitrotyrosine at 430 nm was shown on the exposure of tyrosine with sinapic acid prior to $ONOO^-$ exposure. Apart from the inhibition of $ONOO^-$-mediated tyrosine nitration, sinapic acid also inhibited $ONOO^-$-mediated LDL (low-density lipoprotein) and BSA nitration. Hydroxyl group present on the sinapic acid structure is reported to be responsible for its $ONOO^-$ scavenging activity. However, the mechanism of $ONOO^-$ scavenging is still not clear but the involvement of electron donation is suggested to be involved in this process. This study provided a potential scavenger of $ONOO^-$ which can be used to prevent various $ONOO^-$-mediated diseases.

Subcellular protein tyrosine nitration

Subcellular studies on protein tyrosine nitration during abiotic stress are important to understand the molecular mechanism operative in a plant to withstand stress. This information can contribute to the development of stress tolerant crops. In animals many studies have reported subcellular nitration in mitochondria. Mitochondrial proteins like acyl-CoA dehydrogenase, *β*-ketothiolase, malate dehydrogenase and aconitase are known to be nitrated (Koeck *et al.* 2005). Recently, Lee *et al.* (2009) identified smoke (generated by burning aspen wood shavings) induced nitrated mitochondrial proteins (F1-ATP synthase alpha subunit, voltage dependent anion channel 1) belonging to diverse functions like ion transport and ATP production in rat brain. Although the information about subcellular nitration in plants is limited, recently an important contribution was made by Gaketskiy *et al.* (2011). They identified 126 tyrosine and 12 tryptophan nitration sites in 164 nitrated peptides in thylakoid membrane of *Arabidopsis thaliana* chloroplasts. Increase in the tyrosine and tryptophan nitration in the reaction centre of photosystem II (PSII) proteins and the oxygen-evolving complex while a decrease in the nitration in light harvesting proteins of PSII in light stress reflected the significance of protein nitration as an important regulatory PTM in photosynthesis during abiotic stress.

PROTEIN TYROSINE NITRATION AS A FOOTPRINT OF ABIOTIC STRESS

Plants are exposed to different environmental conditions like high and low temperature, light, drought, salinity and wounding stress which affects the physiology of the plants (Potters *et al.* 2007). All stress conditions whether biotic and abiotic contribute to yield loss. Abiotic stress can cause overproduction of ROS such as $O_2^{\cdot-}$, hydrogen peroxide, and hydroxyl radicals ($OH^{\cdot}$), which further causes oxidative damage to biomolecules. The information on the role of NO and NO-derived molecules during abiotic and biotic stress is still scanty. Mechanism on how these RNS interact with proteins and affect their function in plants is still unknown. Tyrosine nitration is viewed as a marker for cellular stress in animals. In humans, animals and cell systems an increase in protein nitration was observed at the onset as well as with the progression of the disease. Tyrosine nitration is considered as a nitrosative stress marker in cardiovascular diseases in humans (Peluffo and Radi 2007; Rubbo and Radi 2008). The field of research dedicated to plant tyrosine nitration in stress has been extremely informative in past decade and has helped scientists to develop new concepts about the role of tyrosine nitration in abiotic stress. In plants, several reports have shown the accumulation of nitrated proteins in different environmental stress conditions some of these are described in the following section.

High temperature stress

High temperature (HT) affects vegetative as well as reproductive growth of the plants. Sunflower seedlings were used as model plants by Chaki *et al.* (2011a) to understand the function of the NO and NO-derived molecules during HT as HT above 40°C can reduce seed yield, oil and protein content in sunflower (Hewezi *et al.* 2008). HT is also involved in the overproduction of ROS, which further triggers defense responses (Locato *et al*. 2008). ROS provoke membrane damage due to lipid hydroperoxidation. HT showed a 48% increase in lipid hydroperoxide content (Chaki *et al*. 2011a), indicating that abiotic stress provokes oxidative stress in plants. Induction of heat shock 70 proteins (Hsp70) during HT (38°C, 4 h) in sunflower seedlings indicated HT induced overexpression of heat responsive proteins. In HT stress, a 2.5-fold increase in the NO_2-Tyr content was observed in comparison with the control. A total of 22 immunoreactive tyrosine nitrated spots were detected in HT stress on probing the immunoblots with anti-NO_2-Tyr antibodies. Although only one novel spot was detected in comparison with the healthy condition, an increase in the intensity of spots from 10 to 22 was observed. The novel spot was identified as carbonic anhydrase. Ferredoxin–NADP reductase (FNR), one of the HT induced nitrated proteins showed 31% activity inhibition in HT. Also, with an increase in SIN-1 (a $ONOO^-$-generating system) concentration a decrease in the FNR activity in sunflower hypocotyl samples was observed with almost 91% activity inhibition with 5 mM SIN-1 indicating that $ONOO^-$-mediated tyrosine nitration could be responsible for its activity inhibition.

Low temperature

Low temperature (LT) stress affects plant growth by influencing photosynthesis and uptake of water and nutrients. Alternation in these important functions further influence crop production and quality. LT stress is known to regulate the expression of many genes (Medina *et al.* 2011), causes a change in protein abundance (Rinalducci *et al.* 2011) and metabolites leakage (Sharma *et al.* 2005). In contrast to salinity stress, NO content showed a reduction in *Capsicum annum* leaves during cold stress (8°C for 1 to 3 days) conditions (Airaki *et al.* 2012). NO localization studies using DAF-FM DA as fluorescent probe, showed a slight decrease in the green fluorescence in palisade, mesophyll, vascular tissues like xylem and phloem, epidermal cells after 1 day (d) LT stress (8°C) in contrast with the salt stress where an increase in NO was observed in stress (Valderrama *et al.* 2007). $ONOO^-$ detected using fluorescent probe 3'-(*p*-aminophenyl) fluorescein (APF) showed an increase in the intensity of the green florescence in the vascular tissue after 1 d LT stress (8°C). Tyrosine nitration analyzed at 3 different time periods (1, 2 and 3 d at 8°C), showed an increase in the intensity of four polypeptides of 45, 71, 91 and 97 kDa on the immunoblot after 1 d LT stress (8°C) in comparison with 4 weak polypeptides of same molecular masses in the control leaves. After 2 days (8°C), the intensity of tyrosine nitrated polypeptides decreased and the detection was almost negligible at the 3rd d indicating cold acclimation. A similar observation was recorded in LT (8°C) treated pea plants analyzed for protein tyrosine nitration studies (Corpas *et al.* 2008). Six polypeptides of 29 to 59 kDa were found to be nitrated in LT with the intensification of three immunoreactive polypeptides (59, 42 and 29 kDa) and an appearance of new immunoreactive polypeptide of 73 kDa which was absent in the control plants.

Salinity stress

Salinity stress alters the plant productivity by affecting the important physiological processes like photosynthesis, metabolism and respiration (Hasegawa *et al.* 2000). Salinity stress is reported to cause a severe reduction in plant growth, photosynthetic rate and chlorophyll content in plants. Also, it is shown to induce oxidative stress by creating an imbalance between ROS production and antioxidant defence. Valderrama *et al.* (2007) showed overproduction of NO and its products in *Olea europaea* after salinity stress. In olive leaves, a significant increase in the intensity of the green fluorescence measured using CLSM and DAF-2A as fluorescent probe was observed in the vascular tissues, epidermis and spongy mesophyll cells in comparison with the control leaves indicating an enhanced NO accumulation in the salinity stress. In an interesting study by Corpas *et al.* (2009b), the role of peroxisomes in cytosolic NO accumulation during salinity stress in *Arabidopsis thaliana* was demonstrated. In salinity stress (100 mM NaCl), a 6.4-fold increase in the NO production measured by CLSM in *A. thaliana* root cells was observed. Also, in saline conditions a significant increase (4.8-fold) in NO production was observed in *A. thaliana.* A 3.6-fold increase in L-arginine-dependent NOS activity measured using ozone chemiluminiscence method was observed in the olive plants in salinity. Similarly, RSNOs level was shown to increase by 2-fold in salt stress. Increased accumulation of superoxide radicals was observed in salt stress. These superoxide radicals react with NO to produce $ONOO^-$ (Radi 2004). A 5.6-fold increase in $ONOO^-$ generation was observed in *A. thaliana* roots measured by CLSM using 3-(*p*-aminophenyl) fluorescein (APF as the fluorescence probe) in salinity stress. $ONOO^-$ is known to mediate tyrosine nitration (Rubbo and Radi 2008). Salinity stress showed an increase in the number and intensity of polypeptides (40 to 60 kDa) undergoing tyrosine nitration in olive leaves as detected by immunoblotting using anti-tyrosine antibody. An increase in the intensity of tyrosine nitration in vascular tissues, epidermis and spongy mesophyll cells in olive leaves after salinity stress was observed by analyzing the leaf section treated with fluorescent anti-nitrotyrosine antibodies and observing the sections in CLSM (Valderrama *et al.* 2007). A similar pattern was observed in *A. thaliana* roots grown in salinity stress (100 mM), where intensity of a 55 kDa polypeptide increased with appearance of an additional polypeptide at 42 kDa during tyrosine nitration (Corpas *et al.* 2009b), thus, correlating the results of an increase in $ONOO^-$ dependent tyrosine nitration during salinity stress which can act as nitrosative stress marker in plants. These significant findings appear to indicate the role of peroxisomes in increasing protein tyrosine nitration during salinity stress by acting as a NO source in cytosol or peroxisomes to initiate the process of $ONOO^-$ mediated nitrosative stress. Peroxins are suggested to be involved in the import of enzymes involved in NO generation to peroxisomes.

Light stress

NO content and localization studies by Corpas *et al.* (2008) during high light (HL), continuous light (CL) and darkness (D) showed different results in *Pisum sativum*. Continuous light and darkness induced the decrease in the NO content in all cell types (adaxial, andabaxial epidermis, main vein, palisade and spongy mesophyll) whereas high light intensity did not cause any significant change in NO content in comparison with control leaves of pea plant. Protein tyrosine nitration analyzed on immunoblot using 3-nitrotyrosine (NO_2-Tyr) in HL, CL and D showed intensification of the immunoreactive polypeptides from 29 to 59 kDa on the blot. HL stress also showed an appearance of a new immunoreactive band of 73 kDa which was absent in the control.

Wounding stress

Mechanical wounding (MW) provokes long-distance signals. The injury/damage caused to plants from wounding can result in oxidative and nitrosative stress (Lin *et al.* 2011). NO localization studies done by CLSM using green fluorescent probe DAF-2 DA showed its presence mainly in the epidermal cells and the vascular tissue in cross section of control sunflower hypocotyls. Although after mechanical wounding (4 h) the localization of NO was similar to control but with decreased fluorescence intensity (Chaki *et al.* 2011b). Immunolocalization of NO_2-Tyr using rabbit polyclonal antibody against NO_2-Tyr showed intensification of bright green fluorescence after mechanical wounding in all cell types in comparison with the control hypocotyls sections where an increase in fluorescence intensity was observed only in cortex cells and vascular tissues.

Shear stress

Mechanical stress is reported to induce NO production in *Taxus* (Pedroso *et al.* 2000). After 5 h shear stress, a 16.9 times increase in NO concentration was reported in *Taxus cuspidata* cells (Gong and Yuan 2006). NO localization experiments using DAF-2DA showed an increase in the intensification of fluorescence after shear stress thus, confirming shear stress induced NO generation in *T. cuspidate* suspension cultures. 3-nitrotyrosine content showed 31% increase in its content after 4 h shear stress. NO-mediated tyrosine nitration was confirmed by addition of cPTIO which showed only 13.2% increase in 3-nitrotyrosine. Role of NO-mediated tyrosine nitration in decreasing the GST activity during shear stress is also suggested.

Arsenic stress

Arsenic has become a major environmental contaminant which is known to cause several health problems to humans and affect the physiology of the plants. Recently, RNS and ROS metabolism was analyzed in arsenic stress treated *Arabidopsis thaliana* plants (Leterrier *et al.* 2012). In this study an increase in the NO content was demonstrated using DAF-FM DA as fluorescent probe on treatment of the primary roots with 500 µM and 100 µM potassium dihydrogen arsenate (KH_2AsO_4) for seven days. Arsenic stress also caused an increase in the protein tyrosine nitration of 40 - 90 kDa proteins which were analyzed on immunoblot probed with 3-nitrotyrosine antibodies.

Experimental data indicates an increase in either number or the intensification of polypeptides on immunoblot during tyrosine nitration detection in abiotic stress. Thus, abiotic stress induced increase in the protein tyrosine nitration can be regarded as a reliable marker for nitrosative stress and can be considered as a footprint of nitrosative stress as in animals (Corpas *et al.* 2007). A comparative analysis of NO, RNS and tyrosine nitration in abiotic stress is presented in **Table 3**. NO levels measured using fluorescent probes showed a differential modulation in different abiotic stress as LT, MW showed both an increase as well as a decrease whereas salinity and shear stress showed an increase. HT showed a decrease in NO level. An increase in NOS activity was observed in all abiotic stresses except MW in *Helianthus annuus* where 54% decrease in activity was observed. CL and D showed no significant difference in NOS activity in comparison with the control. NR activity and nitrate and nitrite content showed insignificant changes in comparison with the control in abiotic stress indicating that L-arginine dependent pathway might be playing a major role in NO production during abiotic stress. In all abiotic stress an increase in SNO content was observed indicating that the NO generated during stress might be responsible in contributing to the SNO pool of the cell. An increase in tyrosine nitration was observed in all abiotic stress. On the basis of observations from **Table 3**, a schematic model is presented emphasizing on the mechanism of abiotic stress

Table 3 Comparative analysis of NO, SNO, $ONOO^-$ content and tyrosine nitration in abiotic stress.

Stress	Plant system	NO	NOS activity	NR activity and total nitrite and nitrate	RSNO and SNO content	GSNOR activity	$ONOO^-$ content	Tyrosine nitration	Reference
HT	*Helianthus annuus*	↓	-	↔	↑	↓	↑	↑	Chaki *et al.* 2011
	Pisum sativum	↓	↔	↔	↑	↑	-	↑	Corpas *et al.* 2008
LT	*Capsicun annuum*	↓	-	-	↑	↑	↑	1d- ↑ 2d- ↓ 3d -↓	Airaki *et al.* 2012
	Pisum sativum	↑	↑	↔	↑	↑	-	↑	Corpas *et al.* 2008
MW	*Helianthus annuus*	↓	↓	↔	↑	↓	-	↑	Chaki *et al.* 2011
	Pisum sativum	↑	↑	↔	↑	↑	-	↑	Corpas *et al.* 2008
S	*Arabidopsis thaliana*	↑	-	-	-	-	↑	↑	Corpas *et al.* 2009b
	Olea europaea	↑	↑	-	↑	-	-	↑	Valderrama *et al.* 2007
L	*Pisum sativum*	HL- ↔ CL - ↓ D – ↓	HL- ↑ CL - ↔ D – ↔	↔	↑	↑	-	↑	Corpas *et al.* 2008
SH	*Taxus cuspidata*	↑	↑	-	-	-	-	↑	Gong *et al.* 2006
As(V)	*Arabidopsis thaliana*	↑	-	-	-	↑	-	↑	Leterrier *et al.* 2012

↔ - Results similar to control
↑ - Increase in comparison with control
↓ - Decrease in comparison with control
- - Not detected
HT - high temperature, LT - low temperature, MW - mechanical wounding, S - salinity, L - light, HL - high light, CL - continuous light, D - darkness, SH - shear, AS(V) – arsenic, 1d - one day, 2d - two day and 3d - three day low temperature stress

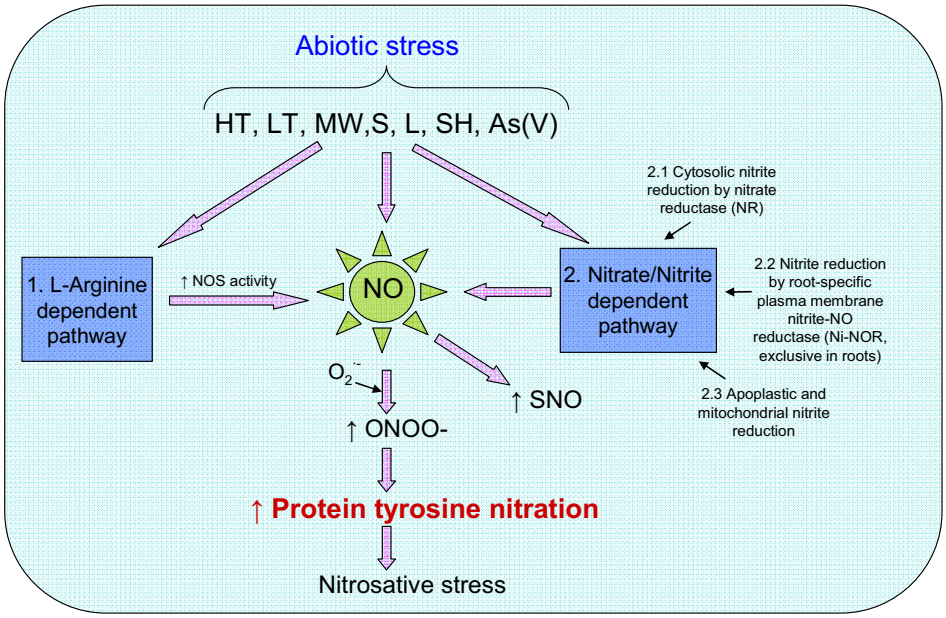

Fig. 4 Schematic model showing effect of abiotic stress on tyrosine nitration. NO can be produced in plants either by L-Arginine dependent pathway involving NOS-like enzyme or by reduction of nitrite in cytosol, apoplast and mitochondria. NO reacts with superoxide radical (O_2^-) to produce peroxynitrite ($ONOO^-$) which increases tyrosine nitration and contributes to nitrosative stress. HT-high temperature, LT-low temperature, MW-mechanical wounding, S-salinity, L-light, SH-shear stress, As(V)- arsenic stress, SNO-S-nitrosothiols, NOS-Nitric oxide synthase.

induced tyrosine nitration (**Fig. 4**). Model suggests that during abiotic stress, NO is produced either via L-arginine dependent pathway (enhanced NOS activity) or nitrate/nitrite dependent pathway. NO reacts with superoxide radicals (O_2^-) to produce $ONOO^-$ which in turn is responsible for enhanced protein tyrosine nitration in plants. These further results in nitrosative stress in plants.

IN VIVO NITRATION IN *ARABIDOPSIS THALIANA*

Tyrosine nitration modulates protein function in plants and animals. Although many studies in the past have emphasized on *in vivo* nitration, but little is known about the *in vivo* tyrosine nitrated targets and their relevance in the plants. Identification of *in vivo* tyrosine nitrated proteins was attempted in *Arabidopsis thaliana*. In *Arabidopsis thaliana*, using immunprecipitation with anti-3-nitroY antibodies and LC-MS/MS identification, 127 *in vivo* tyrosine nitrated proteins were identified in *Arabidopsis thaliana* (Lozano *et al.* 2011). Among the identified nitrated proteins, 35% had homologue counterparts in animals, thus indicating the functional relevance of tyrosine nitration. Among the identified nitrated Y residues, all had acidic residues in vicinity and basic amino acid in the primary sequence flanking the Y residue. With the exception to Y337 and Y135 from transketolase and putative mannitol dehydrogenase respectively, most of the nitrated tyrosine showed less than 70 accessible solvent areas. Interestingly, all the identified protein targets have sub-cellular localization like mitochondria, apoplast, chloroplasts. More than 60% of identified targets were metabolic proteins reflecting the role of tyrosine nitration in regulation of cellular metabolic pathways. Also, many proteins involved in gluconeogenesis including GAPDH and methionine biosynthesis were the target for nitration which further strengthens the regulatory role of tyrosine nitration.

GRAY AREA AND FUTURE PROSPECTS

The role of nitration in abiotic stress and signaling mecha-

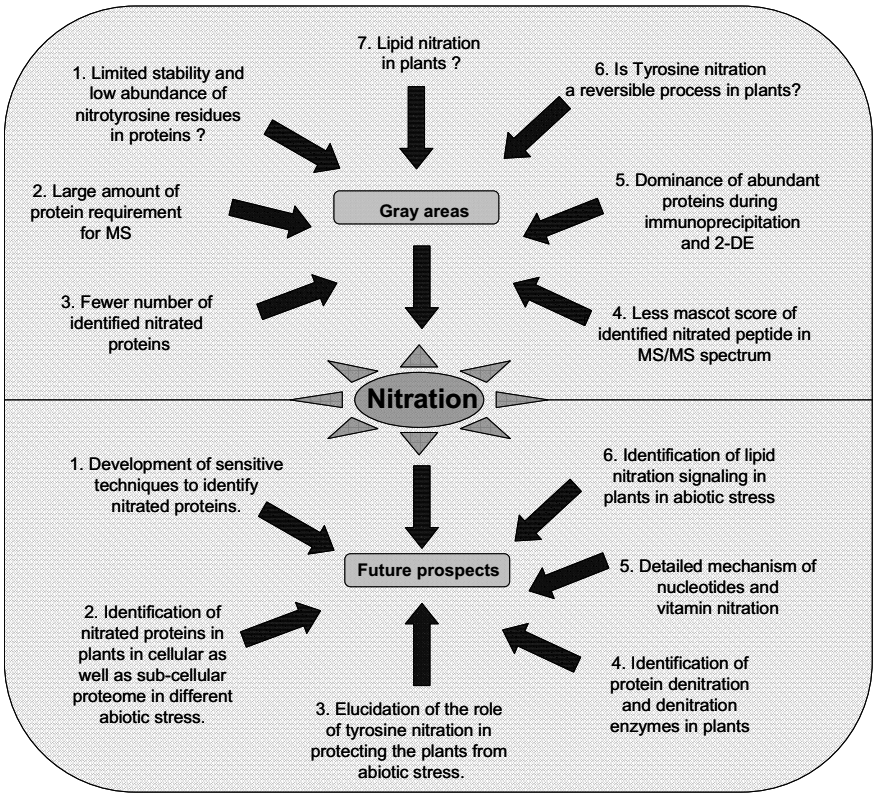

Fig. 5 Schematic representation of grey areas and future prospects in nitration.

nism is still not fully explored and has many loopholes. A few problems which need detailed investigations are summarized next (**Fig. 5**).

1. Nitrotyrosine residues in proteins are low abundant and they show limited stability. Therefore, high amount of protein is required for the identification of nitrated peptides. Although, a large number of nitrated proteins are identified but yet MS/MS spectrum with a good mascot score is yet to be obtained for nitrated peptides. To overcome all these problems there is a need to develop more sensitive identification techniques which can identify low abundant nitrated proteins.

2. The identified tyrosine nitrated proteins are still fewer in comparison with another NO based PTM like S-nitrosylation. Thus, there is an urgent need to identify all nitrated protein to understand the role of nitration in totality in plants. Once all the potential targets of tyrosine nitration are identified, the next step should be to elucidate the role of nitration in abiotic stress. Role of tyrosine nitration in subcellular state in different abiotic stress requires analysis. Only few reports are available about tryptophan nitration in plants. October 2012 Pubmed literature showed 4478 published manuscripts with nitrotyrosine in either title or abstract whereas only 27 with nitrotryptophan. This explains the need for research in this area so that an overall effect of nitration of all amino acids and their cross talk can be analyzed.

3. Till now, neither the nitrated peptide and nor the corresponding nitration site are unequivocally identified and future work is needed in this area in plants.

4. Although a lot of work has been done in the field of lipid nitration but no information is available about lipid nitration in plants. Thus, there is need to identify targets of lipid nitration and the mechanism regulating this PTM in the plants to understand effect of this modification in lipid signaling. Similarly, nucleotides and vitamin nitration and their mechanism still need to be explored.

CONCLUSIONS

The present review focussed on the impact of abiotic stress on tyrosine nitration. A crosstalk between ROS and RNS during abiotic stress in plants is suggested. In this mini-review recent technologies utilized for the detection and identification of tyrosine nitration in plants and animals are summarized which allowed us to connect the links between different NO based PTMs. Current perspective of nitration in different biomolecules is presented but further knowledge in the field of lipid and nucleotide nitration in plants would be of interest to understand the complicated network of NO signaling in plants. The evidences presented hints at the role of $ONOO^-$ as an important regulator of signaling in the plants. In this review, latest contributions in tyrosine nitration are summarized to delineate the importance of protein tyrosine nitration in abiotic stress which will help in understanding the signaling mechanism operative in abiotic stress. An increase in tyrosine nitration in high and low temperature, salinity, light, mechanical wounding and shear stress indicates it to be a marker of nitrosative stress in plants. Identification and functional categorization of *in vivo* and HT-induced tyrosine nitrated proteins in *Arabidopsis thaliana* and *Helianthus annuus* respectively showed that the nitrated targets belongs to different functional categories including photosynthesis, metabolism, proteolysis, stress and signaling and thus, reflected the physiological relevance of tyrosine nitration in plants and provided new insight into the role of tyrosine nitration in different cellular mechanisms. Examination of recent work on tyrosine and tryptophan nitration in subcellular proteome of *Arabidopsis thaliana* chloroplast in light stress suggested the regulatory role of nitration in plants. Nevertheless, many questions regarding detailed mechanism of tyrosine nitration and its physiological relevance in abiotic stress still needs to be answered. Further the identification of tyrosine nitration in other organelle in stress would be important for elucidating the role of nitration in stress and signaling. Future work should focus on the development of improved techniques to identify low abundant nitrated targets to completely understand the crosstalk between NO-based modifications in different plant species in stress.

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