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TITLE: Protein tyrosine nitration in plants: present kNOwledge, computational prediction and future perspectives

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ABSTRACT

Nitric oxide (NO) and related molecules (reactive nitrogen species) regulate diverse physiological processes mainly through posttranslational modifications such as protein tyrosine nitration (PTN). PTN is a covalent and specific modification of tyrosine (Tyr) residues resulting in altered protein structure and function. In the last decade, great efforts have been made to reveal candidate proteins, target Tyr residues and functional consequences of nitration in plants. This review intends to evaluate the accumulated knowledge about the biochemical mechanism, the structural and functional consequences and the selectivity of plants' protein nitration and also about the decomposition or conversion of nitrated proteins. At the same time, this review emphasizes yet unanswered or uncertain questions such as the reversibility/irreversibility of tyrosine nitration, the involvement of proteasomes in the removal of nitrated proteins or the effect of nitration on Tyr phosphorylation. The different NO producing systems of algae and higher plants raise the possibility of diversely regulated protein nitration. Therefore studying PTN from an evolutionary point of view would enrich our present understanding with novel aspects. Plant proteomic research can be promoted by the application of computational prediction tools such as GPS-YNO_2 and iNitro-Tyr software. Using the reference *Arabidopsis* proteome, Authors performed *in silico* analysis of tyrosine nitration in order to characterize plant tyrosine nitroproteome. Nevertheless, based on the common results of the present prediction and previous experiments the most likely nitrated proteins were selected thus recommending candidates for detailed future research.

Keywords: *Arabidopsis;* computational prediction; GPS-YNO_2; iNitro-Tyr; plant; tyrosine nitration

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1. Biochemical mechanism of protein tyrosine nitration

In general, attach of a nitro group (-NO_2) to a chemical compound through a chemical reaction is known as nitration. In biological systems, fatty acids, nucleic acids and proteins can be targets of such modifications. Despite the fact that in proteins several amino acids such as tyrosine, tryptophan, cysteine and methionine can be affected by nitration, tyrosine nitration got particular attention in both animals and plants partly because besides nitro-tyrosine, the formation of phospho-, chloro-, sulfatyrosine is also feasible (Feeney and Schöneich 2012).

After the discovery of phosphorylation, in 1992, Ischiropoulos and co-workers first demonstrated the *in vivo* occurrence of protein tyrosine nitration (PTN). Interestingly, as opposed to tyrosine phosphorylation, nitration does not involve enzymatic activity. Regarding the biochemical mechanism, the covalent addition of a nitro group in the *ortho* position of the aromatic ring in tyrosine (Tyr) molecule happens in two steps. The initial step is the formation of tyrosil radical ($Tyr^{\bullet}$) during the one-electron oxidation of the aromatic ring. The main Tyr oxidants are hydroxyl ($OH^{\bullet}$) and carbonate ($CO_3^{\bullet-}$) radicals derived from peroxynitrite through at least three pathways (Fig 1): (1) at suitable pH unstable peroxynitrous acid (ONOOH) is formed by protonation of peroxynitrite, which homolyzes to $OH^{\bullet}$ and $^{\bullet}NO_2$; (2) at physiological carbon dioxide concentration (1.3 mM) in aqueous environment peroxynitrite reacts with CO_2 generating nitroso-peroxocarboxylate ($ONOOCO_2^-$) which decomposes to carbonate radical and nitrogen dioxide radical ($^{\bullet}NO_2$); and (3) NO can be oxidized to nitrite (NO_2^-) which together with hydrogen peroxide (H_2O_2) can be metabolized by peroxidases to generate $OH^{\bullet}$ and $^{\bullet}NO_2$. The oxidation is followed by a radical-radical nitration reaction in which the nitrogen dioxide radical is added to the $Tyr^{\bullet}$ and 3-nitrotyrosine (YNO_2) is being formed (Souza et al. 2008). All the direct *in vivo* oxidants (mainly carbonate and hydroxyl radicals) and nitrating agents ($^{\bullet}NO_2$) derive from peroxynitrite ($ONOO^-$), which itself is only an indirect contributor to PTN (Yeo et al. 2015; Radi 2013). Peroxynitrite is formed in the fast reaction between superoxide anion ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$); therefore, peroxynitrite derives from NO and consequently it belongs to the group of NO-originated molecules, the reactive nitrogen species (RNS, Patel et al. 1999). Figure 1 summarizes the chemical reactions leading to the formation of 3-nitrotyrosine.

Superoxide radical anion ($O_2^{\bullet-}$) has a remarkably shorter biological half-life compared to NO (Table 1, Vranova et al. 2002) and because of its negative charge at physiological pH; its

diffusion across membranes depends on the presence of anion channels (Denicola et al. 1998). The different diffusion properties of $O_2^{\bullet-}$ and NO suggest that in biological systems, the non-radical anion, peroxynitrite generates close to the sites of $O_2^{\bullet-}$ formation where NO produced at distant cellular spaces arrives (Denicola et al. 1998). Peroxynitrite itself shows longer half-life compared to the other discussed ROS (Siegel et al. 2015, Table 1), but it is more reactive than NO. Regarding the diffusion distance of peroxynitrite, it is similar to that of H_2O_2 and $O_2^{\bullet-}$, but it is shorter compared to NO (Denicola et al. 1998). The direct nitrant, $^{\bullet}NO_2$ radical has a relatively short half-life and diffusion capability compared to the other reactive nitrogen species (Ford et al. 2002).

2. Fate of nitrated proteins

In order to influence signal transduction independently from phosphorylation routes, tyrosine nitration has to be reversible. This thermodynamically stable modification has earlier been considered to be irreversible but later reductant-dependent and reductant- independent denitrase mechanisms were described in animals (Kuo et al. 1999). Recently, denitrase activity has been characterized in animals (Deeb et al. 2013) and non-enzymatic denitration has also been revealed in case of 8-Nitro-cGMP (Akaike et al. 2010). In plants, denitrase enzyme has not been identified so far, thus the reversibility of tyrosine nitration remained uncertain. The reduction of the nitro group to amino group resulting 3-aminotyrosine is also conceivable and such reactions may involve nitroreductase activity. Although, bacterial or mammalian nitroreductases proved to be incapable of reducing nitro-tyrosine (Lightfoot et al. 2000). Formation and accumulation of proteasome-resistant protein aggregates can also be conceivable (Hyun et al. 2003). Nitration enhances the susceptibility of the protein for degradation by the proteasome implicating that proteasome functioning is critical for the removal of nitrated proteins (Souza et al. 2000). In plants, it was speculated that nitrated proteins of the roots may be more willing to degrade in 20S proteasomes (Tanou et al. 2012). Castillo et al. (2015) provided recent experimental evidence regarding the role of proteasomes in the degradation of nitrated proteins. In their work, nitrated abscisic acid receptor PYR/PYL/RCAR was polyubiquitylated and consequently it underwent proteasome-regulated degradation.

3. Consequences of tyrosine nitration

Regarding the functional consequences (Fig 2) of PTN, it leads to the decrease of pK_a of the hydroxyl moiety in the tyrosine residue (from 10-10.3 to 7.2-7.5, Creighton 1993). Furthermore, nitration of tyrosine enhances the hydrophobicity of the residue and consequently induces structural changes (Souza et al. 2008). A further spatial consequence of PTN originates from the fact that nitrotyrosine is more spacious than tyrosine, which can lead to steric restrictions (Savvides et al. 2002). In plant cells, the available data show that PTN usually causes functional loss of the particular enzyme protein (see Table 2); however the *in vitro* activity of pea glutathione reductase was not affected by this modification (Begara-Morales et al. 2015). In animal systems, PTN-triggered activation, inactivation or no change of activity has been evidenced (Yeo et al. 2015). At the same time, the presence of nitrated tyrosine(s) in a protein is not necessarily the cause of the functional loss, because all biological nitrating agents are also able to exert oxidative effects on amino acids like cysteine or methionine (Alvarez and Radi 2003).

Another consequence of PTN is the positive or negative impact on tyrosine phosphorylation (Fig 2), influencing cell signalling as it was observed in non-plant systems (Gow et al. 1996; Kong et al. 1996; Brito et al. 1999; Aburima et al. 2010). In plants, there is no convincing evidence regarding the relationship between tyrosine phosphorylation and nitration. However, recent bioinformatic studies revelaed the presence of tyrosine-specific kinases in the *Arabidopsis* proteome (Carpi et al. 2002), their existence is still controversial (Kovaleva et al. 2013). Both the alteration of tyrosine phosphorylation and nitration causes disturbances in microtubule organization and root hair morphology (Sheremet et al. 2012, Blume et al. 2008) indicating a link between tyrosine phosphorylation and nitration of α-tubulin. It is possible that nitration competes with phosphorylation of α-tubulin for the binding sites (Blume et al. 2008, 2013). Another indirect evidence for the interplay between the two covalent Tyr modifications has been provided by Galetskiy et al. (2011) who revealed that conversely regulated protein phosphorylation and nitration levels control the stability of photosynthetic complexes under high light condition.

4. Specificity and selectivity of tyrosine nitration

Interestingly, only 1-2% of the total tyrosine pool may be the target of *in vivo* nitration (Bartesaghi et al. 2007), suggesting the highly selective nature of the process. This is supported by the low number of YNO_2 sites in plant enzymes containing several tyrosine amino acids (e.g. methionine synthase or monodehydroascorbate reductase, Lozano-Juste et al. 2011 and Begara-Morales et al. 2015, respectively). This also means that the overall yield of nitration (millimole of 3-nitrotyrosine/mole tyrosine) is low, what is more in sunflower grown under physiological conditions, nitration yield proved to be in the order of μmol 3-nitro-tyrosine/mol tyrosine (Chaki et al. 2009). This raises questions regarding the biological relevance of PTN (Souza et al. 2008). Is tyrosine nitration only an inevitable consequence of stress or it actively regulates protein pool size?

The question is partly answered by the fact that in the proteome of healthy, unstressed plants, a certain degree of nitration can be detected, meaning that they have physiological nitroproteome. Presumably, this is an inactivated part of the whole protein pool and has significance in the regulation of its size. Physiological nitroproteomes were published in the organs of several plant species such as *Brassica juncea, Brassica napus, Pisum sativum, Lotus japonicus, Citrus aurantium, Capsicum annum* (Feigl et al. 2015, 2016; Lehotai et al. 2016; Corpas et al. 2009; Signorelli et al. 2013; Tanou et al. 2012, Chaki et al. 2015); although, the nitroproteins were identified only in some of these works. In a large-scale study, Lozano-Juste et al. (2011) identified 127 nitroproteins in wild-type *Arabidopsis thaliana* grown under normal conditions. Additionally, 21 proteins were found to be nitrated in sunflower hypocotyls (Chaki et al. 2009), 26 nitrated proteins were evidenced in the roots of non-stressed *Citrus* plants (Tanou et al. 2012) and 16 candidates were determined in senescent pea root (Begara-Morales et al. 2013a).

Protein tyrosine nitration is associated also with processes of growth and development such as ripening (Chaki et al. 2015), senescence (Begara-Morales et al. 2013a), cell growth and division (Jovanović et al. 2012). Recently, apoplastic proteins like peroxidases, enolase, extracellular glycoproteins were shown to be susceptible for nitration during control circumstances and under osmotic stress as well (Szuba et al. 2015). Krasuska et al. (2016) detected and identified tyrosine nitrated proteins such as legumin A-like proteins and poly ADP-ribose polymerases in apple embryos. During the normal metabolism of root nodules, leghemoglobin suffers nitration which decreases during senescence (Sainz et al. 2016).

According to Castillo et al. (2015) PYR/PYL/RCAR ABA receptors are inactivated by tyrosine nitration leading to the NO-induced decrease of ABA sensitivity during germination.

Furthermore, stress-induced intensification of tyrosine nitration has been widely proven in different plant species (reviewed by Corpas et al. 2013). E.g. tyrosine nitration was recently found to be intensified by leaf wounding in pumpkin (Gaupels et al. 2016), by salt stress in sunflower seedlings (David et al. 2016), by cadmium exposure in soybean root (Gzyl et al. 2016) by selenite in pea (Lehotai et al. 2016) or by zinc treatment in *Brassica* species (Feigl et al. 2015, 2016). These recent results indicate the general occurrence of tyrosine nitration as the effect of stress situations, which assigns nitration as biomarker of secondary nitrosative stress. At the same time, the existence of physiological nitroproteomes supposes regulatory function for nitration.

Tyrosine nitration can be considered as a selective process but consensus sequence within the target protein does not ensure this specificity. Instead, several factors provide selectivity and specificity such as the centrifugal-centripetal position of the tyrosine residue within the 3D structure of the protein, the subcellular location of the target protein, and also the secondary structure of the protein are important determinants of the nitration (Yeo et al. 2015). Despite the lack of a consensus sequence in the protein primary structure, some common features have been revealed, such as the presence of acidic residues (glutamic or aspartic residues) neighbouring to the YNO_2 site, cysteine or methionine next to the target Tyr and the presence of loop-forming amino acids such as proline or glycine (Souza et al. 2008).

5. Evolutionary considerations

Since the conservation of signalling pathways throughout evolution can be considered as a hallmark of their relevance in the homeostasis of an organism (Bottari 2015), we have to raise the question whether protein nitration in the plant kingdom is conserved or not. Mammalian-like nitric oxide synthase (NOS) enzymes are present in algae but seems to be absent in land plants (Jeandroz et al. 2016, Santolini et al. 2016) where NO production is based mainly on nitrate/nitrite reduction (Kumar et al. 2015). At the same time, L-arginine dependent NOS-like activities were detected in higher plants, which can be explained by the possible existence of cooperating complexes of NO producing enzymes having the same requirements like mammalian NOS (Corpas and Barroso, 2016). The difference in NO producing systems in marine green algae and in higher land plants raises the possibility of diverse processes of protein

nitration. In the photosynthetic prokaryote (*Calothrix* BI22 cyanobacterium) endogenous peroxynitrite generation was detected (Pérez et al. 2016), while in *Anabaena* 7120, PII signal protein involved in nitrogen metabolism was shown to be nitrated at Tyr-51 which was hypothesized to cause gain of function (Zhang et al. 2007). In photosynthetic eukaryote algae, NO is synthetized by NOS enzymes (Foresi et al. 2010) but there is no evidence for endogenous peroxynitrite formation. What's more, some algal species are able to produce substances involved in peroxynitrite detoxification (Chung et al. 2001, Seo et al. 2004). Till this date, there is no direct experimental evidence showing that algal species undergo protein nitration. Therefore, detection and identification of nitrated proteins (if any) in algae may be a promising future research task.

6. Tools for detecting tyrosine nitration in higher plants: immune-affinity based approaches and bioinformatics

The experimental detection of 3-nitrotyrosine in biological systems proved to be problematic, partly due to the low abundance of the nitrotyrosine-containing proteins. The 1D and 2D gel electrophoresis followed by immunoblotting probed with anti- 3-nitrotyrosine antibodies are widely used techniques in plant studies. The nitrated proteins are identified by mass spectrometry and the nitration site(s) within the protein quaternary structure can be determined by MALDI-TOF MS and LC-MS/MS (Yeo et al. 2015). To date, most of the performed plant studies applied immune-affinity based approaches to identify tyrosine nitrated-proteins (e.g. Corpas et al. 2008, Lozano-Juste et al. 2011, Cecconi et al. 2009, Tanou et al. 2012, Begara-Morales et al. 2013ab). Although, non-specific antibody binding may result in false positive detection and the identified protein occasionally does not match in the protein database (Corpas et al. 2013). Great efforts are being made to eliminate the above mentioned technical problems through the continuous improvement of mass spectrometry assays (Ng et al. 2013). In Table 2, the few plant proteins are listed in which nitrated Tyr residues have been identified so far. These results have been achieved over the past five years parallel to the improvement of analytical techniques.

In the last decade, the demand for the cognition of exact PTN sites increased, which motivated the development of specific computational tools. In contrast to the lengthy and often technically problematic proteomic approaches, these software tools are capable of rapidly generate extensive information for further experiments. The Group-based Prediction System

YNO_2 (GPS) was the first algorithm developed for predicting nitrated tyrosine residues based on the biochemical properties of neighbouring amino acids (Liu et al. 2011). Using cross-validation, the algorithm showed promising performance (accuracy of 76.51%, sensitivity of 50.09%, specificity of 80.18%). Predictions can be performed at three different threshold levels (low, medium, high). Moreover, whole proteome analysis can easily be carried out with the help of "Batch Predictor" tool of GPS-YNO_2. The software contains also a "Domain Graph" tool with which domain structures of proteins can be drawn, and YNO_2 sites can be indicated. Recently, Xu et al. (2014) has developed novel predictor software called iNitro-Tyr. It is based on the incorporation of the position-specific dipeptide propensity into the general pseudo amino acid composition which makes possible to discriminate the nitrotyrosine sites from the non-nitrated positions. Besides the length of the submitted amino acid sequence, this algorithm represents the total number of tyrosine residues within the protein sequence which is useful information. Also, iNitro-Tyr is capable of performing whole proteome predictions. Both software, GPS-YNO_2 and iNitro-Tyr are easy to handle and freely available on-line (http://yno2.biocuckoo.org/ and http://app.aporc.org/iNitro-Tyr/, respectively).

In the present study, the nitration sites were predicted in proteins presented in Table 2 using GPS-YNO_2 and iNitro-Tyr software. In total, 26 YNO_2 sites were experimentally determined in the eleven proteins, and both programs predicted similar number of YNO_2 sites (22 and 23, respectively) meaning that ~ 84-86 % of the total number of tyrosine nitration sites were successfully indicated by the algorithms. This is similar to the human proteome, where ~85% of the experimentally identified YNO_2 sites were predicted *in silico* using GPS-YNO_2 software (Ng et al. 2013). Furthermore, from ten of the eleven nitrated proteins justified by mass spectrometry, nitration sites were forecasted by GPS-YNO_2 program. Regarding iNitro-Tyr, only eight from the total eleven proteins proved to be predicted as candidates. Although, it should not be ignored that there are only four predicted nitration sites in the eleven proteins that was experimentally evidenced meaning that the nitration sites predicted and experimentally determined show slight match. The relative big difference between the actual YNO_2 sites and the predicted ones may originate partly from the fact that MALDI based methods applied for the identification of nitrated tyrosine residues have some disadvantages. The modified peptide may decompose during the ionization and may form several decay products which makes these techniques challenging and often unreliable (Ytterberg and Jensen 2010). On the other hand, prediction algorithms like GPS-SNO or GPS-YNO_2 do not consider the second or three dimensional protein structures (Chaki et al. 2014), which can be another reason for the moderate correlation between the predicted and actual YNO_2 sites.

Using on-line software tool, the whole *Arabidopsis* tyrosine nitroproteome can be predicted. From the TAIR database, 27 416 amino acid sequences were downloaded and the YNO_2 sites were determined in them using the "Batch Predictor" tool of GPS-YNO_2 1.0. Of these, 26 592 proteins (97%) contain at least one tyrosine residue, in total 122 403 tyrosines were identified in *Arabidopsis* proteome. Using computational prediction, 38% of all tyrosine residue (46 623 nitrated sites) was found to be nitrated in 19 901 proteins (74.8% of the whole tyrosine proteome) meaning that 72.5% of all *Arabidopsis* proteins can be candidates for tyrosine nitration. Consequently, *Arabidopsis thaliana* supposedly has a tyrosine nitroproteome containing approximately 20,000 proteins. Interestingly, in the human proteome, similar number of YNO_2 sites (41 623) was predicted in fewer protein candidates (14 454) (Ng et al. 2013). In case of the other NO-related posttranslational modification (PTM), S-nitrosylation, 60% of the cysteine proteome was predicted to be affected (Chaki et al. 2014). This means that the size of predicted tyrosine nitroproteome is 16% bigger than the cysteine nitrosoproteome in *Arabidopsis* (Fig 3). Considering the possibility that a protein containing both tyrosine and cysteine residues is able to be modified by both NO-related PTMs, S-nitrosylation sites were predicted in nitrated protein candidates using GPS-SNO 1.0 algorithm. From the 19 901 nitrated candidates, 11 917 was found to be susceptible also for S-nitrosylation, thus ~60% of the tyrosine nitroproteome may be S-nitrosylated as well. This suggests a considerable overlap between the two NO-dependent redox PTMs, which beside the common features (e.g. NO-dependence, redox nature) have several differences (e.g. affected amino acid residue, reversibility) as well. There are only few experimental evidences for proteins affected by both PTMs. For instance, ascorbate peroxidase (APX) was shown to be induced by S-nitrosylation of a particular cysteine residue, while down-regulated by nitration of Tyr_{235} (Begara-Morales et al. 2013b). Interestingly, GPS-SNO (medium threshold) did not identify this protein as candidate for S-nitrosylation (data not shown). Also the abscisic acid receptors PYR/PYL/RCAR are under dual regulation, since the nitration of three tyrosine residues resulted in their inhibition, while S-nitrosylation caused their activation (Castillo et al. 2015). Although, neither GPS-YNO_2 (medium threshold) nor iNitro-Tyr predicted nitration sites in these proteins (Table 2).

In *Arabidopsis*, 127 nitrated proteins have been previously identified by LC-MS/MS (Lozano-Juste et al. 2011). In this large-scale study, the exact sites of tyrosine nitration of most proteins were not experimentally determined. We carried out further *in silico* analysis in order to compare the experimental data with the predictions (Table S1). From 126 experimentally

identified nitroproteins, 115 proteins (91.2%) were predicted by GPS-YNO_2 program (medium threshold), which means good efficiency. From the total 2245 tyrosine residues being present in the 126 nitroproteins, 422 were predicted to be nitrotyrosine. The highest number of YNO_2 sites relative to the total number of tyrosine residues was predicted in the following proteins: actin-2, actin-7, heat shock 70 kDa, malate dehydrogenase and adenine phosphoribosyltransferase. To determine the proteins in which the prediction has the highest certainty, score/cutoff values for each clusters were calculated. Twelve proteins (10% of all) with the highest score/cutoff values were selected and considered as 10% highest confident candidates as described by Chaki et al. 2014. Among them e.g. ABC transporters, heat shock proteins and tubulins can be found (Table S2). According to our knowledge, there is no detailed study regarding the modification of plant ABC transporters by tyrosine nitration, which makes these proteins potential candidates for further research. In neurons, the nitration of a single tyrosine in heat shock 90 proteins (Hsp 90) protein has been reported to result in cell death (Franco et al. 2013) and the nitration of Hsp 90 in cancer cells down-regulated mitochondrial activity (Franco et al. 2015). In plant systems, similar, detailed study on the effect of tyrosine nitration on Hsp chaperons has not been conducted so far. Similarly to ABC transporters and Hsp70s, both serine glyoxalate aminotransferase (At2g13360) acting in photorespiration and in asparagine metabolism (Liepman and Olsen 2001) and germin-like protein (subfamily 2 member 5, At5g26700) playing a role in plant defence are highly confident nitroprotein candidates. Apart from their Tyr modification further details (such as number and position of YNO_2 residues, functional effect) have not been revealed yet. Contrary, the fact and the exact site of tyrosine nitration in ascorbate peroxidase has already been revealed. Moreover, the inactivation of APX as the consequence of nitration was supposed to contribute to the accumulation of reactive oxygen species (ROS) and oxidative stress (Begara-Morales et al. 2013b). Considering that the above mentioned proteins are highly confident candidates in computational prediction and their tyrosine nitration has not been experimentally verified, these proteins seem to be excellent objects for detailed future research.

7. Conclusions and future objectives

Using the search expression “protein nitration in plant”, Scopus listed 130 papers published in 2016 reflecting that this is an intensely developing area of plant biology. Nevertheless, there are numerous unanswered questions which this paper intended to draw up and summarize appointing new research directions regarding tyrosine nitration of plant

proteome. Still, there is uncertainty about the existence of plant denitrases and consequently about the reversibility/irreversibility of PTN. Similarly, the possible involvement of proteasomal degradation in the removal of nitrated proteins needs to be strengthened. Moreover, the examination of nitration in evolutionary point of view may provide interesting new clues. Revealing the functional consequences of tyrosine nitration thus its role in the regulation of protein activity should be top priority later on. The cost-efficient computational analyses presented in this review can be used for establishing and completing time-consuming and expensive proteomic work but it is clear that the computational mapping tools cannot substitute the experimental procedures. Similarly to cysteine S-nitrosylation, tyrosine nitration is an enzyme-independent covalent amino acid modification, which is therefore influenced by several factors providing biological selectivity (e.g. subcellular location of the protein, the concentration of nitrating agents in the microenvironment). More importantly, tyrosine residues located in loop structures have higher affinity to nitration; therefore the secondary structure of the protein supposedly plays pivotal role in determining the intramolecular position of nitration (Yeo et al. 2015). The prediction algorithms have been developed so far, are based on the primary protein structure, which can be the reason for the moderate correlation between the predicted and actual YNO_2 sites.

Based on the above, it has to be admitted that both the *in silico* predicting tools and the experimental approaches must be developed in the near future in order to achieve more accurate knowledge about the mechanism and the significance of protein tyrosine nitration in plant systems.

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FIGURE LEGENDS

Fig 1 Biochemistry of tyrosine nitration.

Production of direct tyrosine oxidant ($OH^{\bullet}$ and $CO_3^{\bullet-}$) and tyrosine nitrant ($^{\bullet}NO_2$) radicals from peroxynitrite *via* three (1,2,3) chemical pathways involving the formation of peroxynitrous acid (ONOOH, 1), nitroso-peroxocarboxylate ($ONOOCO_2^-$, 2) or nitrite (NO_2^-, 3). The production of peroxynitrite from $^{\bullet}NO$ and $O_2^{\bullet-}$ is also depicted. Direct oxidants like $OH^{\bullet}$ and $CO_3^{\bullet-}$ are involved in the formation of tyrosil radical, while $^{\bullet}NO_2$ catalyses the addition of a nitro group and the consequent formation of 3-nitrotyrosine.

Fig 2. Fates and consequences of PTN.

Possible mechanisms regulating nitrated protein pool (left) and possible functional, signalling consequences of tyrosine nitration (right). The nitro group in tyrosine residue can be reduced, but neither enzymatic nor non-enzymatic reductants have been identified in plants or in animals. Denitrase activity has been characterized in animals but not in plants consequently the reversibility of tyrosine nitration is still questionable. Formation and accumulation of proteasome-resistant protein aggregates can also be conceivable. Nitrated proteins can be targeted for polyubiquitination and for proteasomal degradation as it was evidenced by Castillo et al. 2015. Nitration may cause structural and consequently functional modifications (inactivation, activation) in proteins. In plants, evidences available for PTN-triggered functional loss or unaffected activity (Begara-Morales et al. 2015). Moreover, nitration of tyrosine amino acid may interfere with phosphorylation. In plants, the converse regulation of Tyr phosphorylation and nitration has been evidenced which raises the possibility of competition between the two post-translational regulatory processes (modified from Souza et al. 2008).

Fig 3. Predicted nitroso- and nitroproteome of *Arabidopsis*.

Schematic representation of the size of predicted *Arabidopsis* cysteine (nitroso)proteome (the data are published by Chaki et al. 2014) and tyrosine (nitro)proteome. The GPS-YNO_2 software was downloaded at yno2.biocuckoo.org/ and used to predict tyrosine nitration sites (Liu et al. 2011). In total, 27 416 amino acid sequences were extracted from the Arabidopsis Information Resource (TAIR, www.arabidopsis.org/ TAIR10_pep_20110103_representative_gene_model). The data were collected in an Excel file for further analysis. Amino acid sequences (27 416) in FASTA format were submitted to the software and the prediction was performed using medium

threshold and “Batch Predictor” tool. The prediction results (position, peptide, score, cutoff, cluster) were extracted into an Excel file for further analysis.

Table 1 Comparison of some physiochemical properties of reactive intermediates involved in tyrosine nitration.

Table 2 List of plant proteins in which the nitrated tyrosine residues have been experimentally identified. The effects of tyrosine nitration on the activity of the affected proteins are also described. “n.d” not determined. Nitration sites in the listed proteins were computationally predicted using GSP-YNO_2 1.0 and iNitro-Tyr software. „Y” in bold matched tyrosine residue, „-„ non-predicted site.

Table S1 Number of nitrated sites from experimentally identified nitrated proteins predicted by GPS-YNO_2 1.0. Tyrosine nitrated proteins (126) in wild-type *Arabidopsis thaliana* published by Lozano-Juste et al. (2011) were analysed using GPS-YNO_2 1.0 software. „-„ non-predicted site. (.doc)

Table S2 Predicted nitrated proteins with the highest score/cutoff values reflecting highest prediction confidence. Prediction was performed using GPS-YNO_2 1.0 program. (.doc)

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Table 1 Comparison of some physiochemical properties of reactive intermediates involved in tyrosine nitration.

	Superoxide anion ($O_2^{\cdot-}$)	**Nitric oxide ($^{\cdot}NO$)**	**Peroxynitrite ($ONOO^-$)**	**Nitrogen dioxide ($^{\cdot}NO_2$)**
Radical character	yes	yes	no	yes
Charge	negatively charged	non-charged	negatively charged	non-charged
Half-life (ms)	0.002-0.004	5000-15 000	< 10	< 0.01
Diffusion distance (μm)	~ x 10	100-200	4	~0.2

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Table 2 List of plant proteins in which the nitrated tyrosine residues have been experimentally identified. The effects of tyrosine nitration on the activity of the affected proteins are also described. "n.d" not determined. Nitration sites in the listed proteins were computationally predicted using GSP-YNO_2 1.0 and iNitro-Tyr software. „Y" in bold matched tyrosine residue, „-„ non-predicted site.

					Tyr-NO2 sites predicted		
Protein name	**Accession number (UniProt)**	**Total number of Tyr**	**Consequence of Tyr nitration**	**Tyr-NO2 experimentally identified**	**GPS-YNO2 1.0**	**iNitro-Tyr**	**Reference**
Metionine synthase	O50008	26	decreased activity	Y_{287}	Y_{463}, Y_{469}, Y_{698}	Y_{141}, Y_{623}, Y_{650}	Lozano-Juste et al. 2011
O-acetylserine(thiol)-lyase	P47998	7	decreased activity	Y_{302}	Y_{158}	-	Álvarez et al. 2011
Photosystem II protein D1	P83755	12	Monomerization of PSII dimers	Y_{262}	Y_{73}, Y_{107}, Y_{237},Y_{246}	Y_{246}	Galetskiy et al. 2011
Isocitrate dehydrogenase [NADP]	Q6R6M7	14	decreased activity	Y_{392}	Y_{69}, Y_{210}, Y_{221}, Y_{274}	Y_{172}, Y_{185}, Y_{221}, Y_{233}, Y_{241}, Y_{259}, Y_{274}	Begara-Morales et al. 2013a

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L-ascorbate peroxidase, cytosolic	P48534	7	decreased activity	**Y_5**, Y_{235}	**Y_5**	**Y_5**, Y_{93}	Begara-Morales et al. 2013b
Hydroxypyruvate reductase, peroxisomal	Q9C9W5	11	decreased activity	Y_{97}, **Y_{108}**, Y_{198}	Y_{10}, **Y_{108}**, Y_{150}	Y_{10}, Y_{150}, Y_{251}	Corpas et al. 2013
Abscisic acid receptor PYR1	O49686	4	decreased activity	Y_{23}, Y_{58}, Y_{120}	-	-	Castillo et al. 2015
Superoxide dismutase [Mn] 1, mitochondrial	O81235	10	decreased activity	Y_{38}, Y_{40}, **Y_{63}**, Y_{67}, Y_{198}, Y_{199}, Y_{202}	**Y_{63}**, Y_{226}	**Y_{63}**, Y_{67}, Y_{226}	Holzmeister et al. 2015
Leghemoglobin-1	P02232	3	peroxynitrite scavanging, protection of bacteroids	Y_{25}, Y_{30}, Y_{133}	Y_{134}	-	Sainz et al. 2015
Monodehydroascorbate reductase I	Q66PF9	22	decreased activity	Y_{213}, **Y_{292}**, Y_{345}	Y_{154}, Y_{340}	Y_7, Y_{192}, **Y_{292}**	Begara-Morales et al. 2015
Oxygen-evolving enhancer protein 1-1, chloroplastic	P23321	8	n.d	Y_9	Y_{94}, Y_{102}, Y_{328}	Y_{236}	Takahashi et al. 2015

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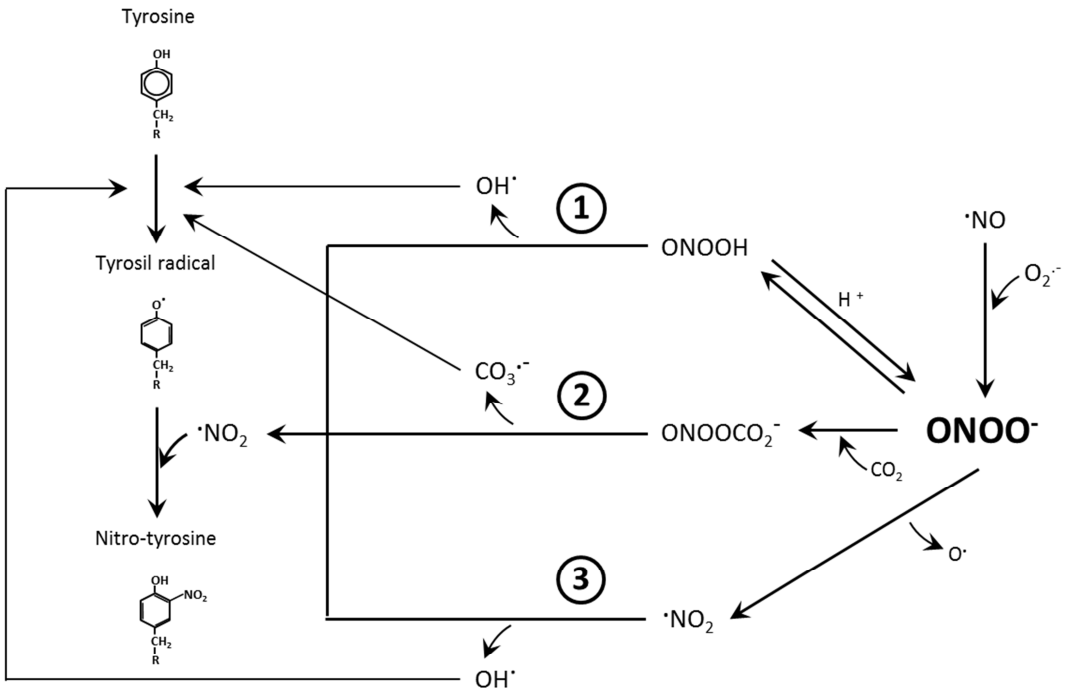

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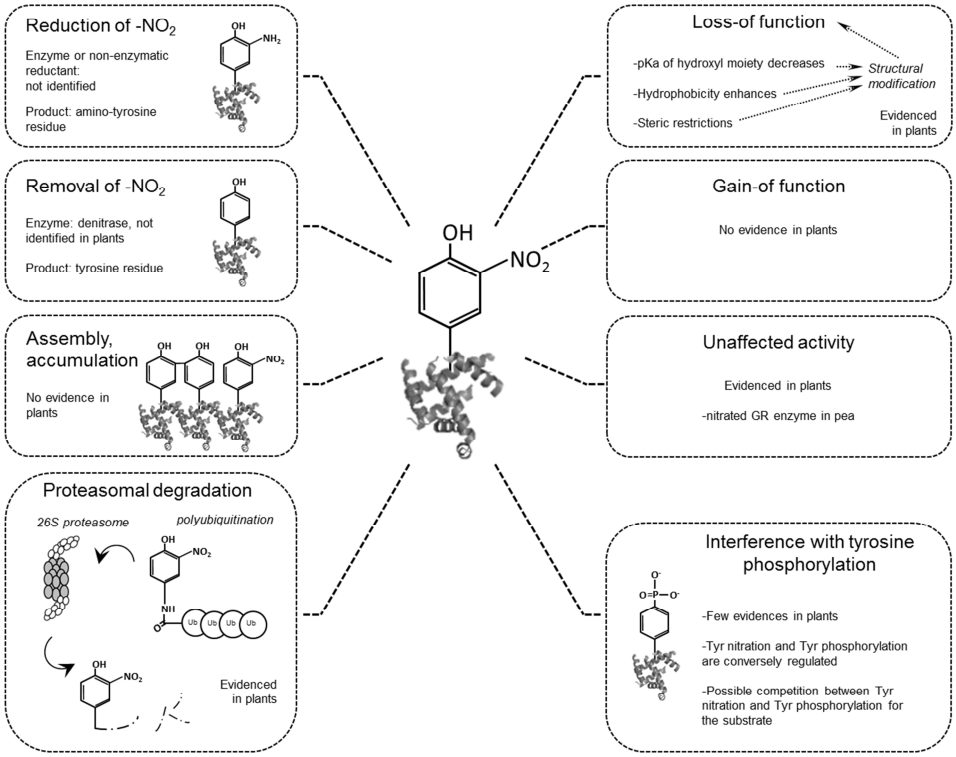
Possible fates of tyrosine nitrated proteins

Possible consequences of protein tyrosine nitration



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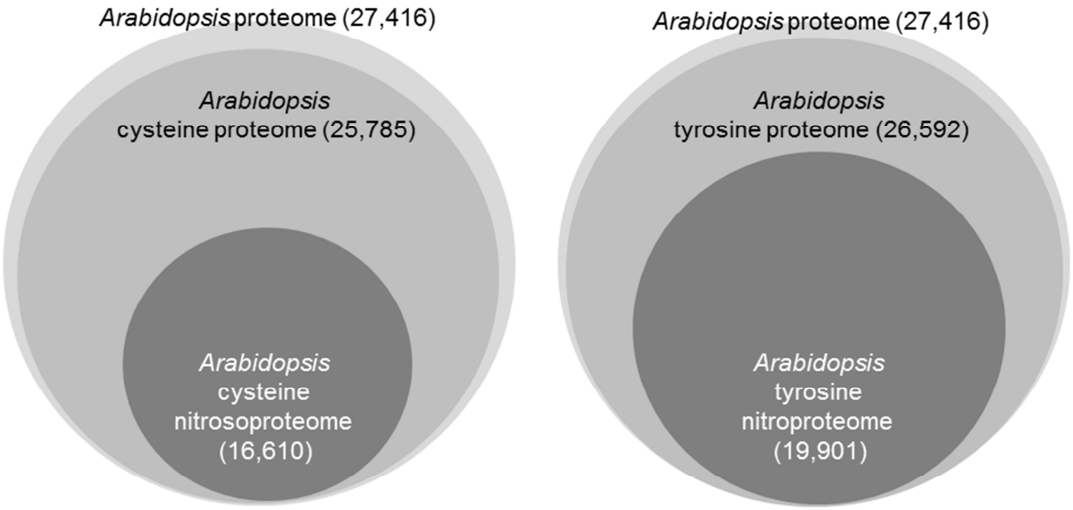

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HIGHLIGHTS

- Protein tyrosine nitration (PTN) causes functional loss in plant proteins
- Reversibility and evolutionary conservation of plant PTN are still questionable
- Predicted nitroproteome of *Arabidopsis* consists of ~20,000 proteins

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CONTRIBUTION

Hereby, I declare that the manuscript was prepared by Zsuzsanna Kolbert with the contribution of László Erdei. Gábor Feigl, Ádám Bordé and Árpád Molnár carried out the computational prediction.

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