

The complexity of nitric oxide generation and function in plants

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ABSTRACT: Plants are exposed to environmental stress, in natural and agricultural conditions. Nitric oxide (NO), a small gaseous molecule which plays important roles in plants, has been involved in many physiological processes, and emerged as an important endogenous signaling molecule in the adaptation of plants to biotic and abiotic stress. NO is produced from a variety of enzymatic and non enzymatic sources, which are not yet fully understood. Also, NO and reactive nitrogen species (RNS) can produce posttranslational modifications affecting protein function. Nitrate reductase, a key enzyme in the nitrogen metabolism, is a proposed source of NO in plants which could be affected by posttranslational modifications. Thus, different pathways seem to be involved and can also regulate NO synthesis in the plant cell under physiological or stress conditions. However, how the levels of NO are reached in such time and place to fulfill its functions, are still puzzles to elucidate.

Plants are frequently exposed to environmental stress, both in natural and agricultural conditions. Acclimation to environmental stress results from an integrated series of events, occurring from the anatomical and morphological levels to the cellular, biochemical, and molecular levels (Taiz and Zeiger, 2010). As abiotic stress conditions can lead to growth restriction, acclimation responses become the plant priority. Stress induced-plant growth reduction may be followed by an alteration in the redox state, leading to oxidative and/or nitrosative stress, due to an increase in reactive oxygen species (ROS), or in reactive nitrogen species (RNS) respectively. Also, ROS and RNS can produce protein posttranslational modifications (PTM) affecting their function. Nitrated proteins have been detected in plants of tobacco, soybean, and *Arabidopsis* (Morot-Gaudry-Talarmain *et al.*, 2002; Jasid *et*

al., 2009; Lozano-Juste *et al.*, 2011). Moreover, nitration and S-nitrosylation have been involved in acclimation to salinity stress. Tanou *et al.* (2012) showed that ROS/RNS-mediated protein post-translational modifications are a key molecular strategy for signaling transduction and salinity acclimation.

Nitric oxide (NO) is a small gaseous molecule which plays important roles in plants. It has been involved in many physiological processes and emerged as an important endogenous signaling molecule in the adaptation of plants to biotic and abiotic stress. A role for NO, and in some cases S-nitrosothiols (SNOs), has been suggested in a variety of stress responses, including drought, salt, heat, cold and heavy metal stress (Yu *et al.*, 2014).

Most of the published studies demonstrated accumulation of NO under stress conditions (Saxena and Shekawat, 2013). However, it cannot be considered a general stress response. During plant responses to cadmium stress, NO was increased or decreased, acting as inducer or inhibitor of stress tolerance (Arasimowicz-Jelonek *et al.*, 2011). Also,

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iron deficiency triggered NO signaling in *Arabidopsis thaliana* (Chen *et al.*, 2010) but repressed basal NO synthesis in *Zea mays* (Kumar *et al.*, 2010).

It is possible that NO may confer abiotic stress tolerance in part by functioning as an antioxidant, as has been reported by several authors (Laspina *et al.*, 2005; Hasanuzzaman *et al.*, 2011; Verma *et al.*, 2013). The relevance of NO in stress-induced redox signaling was investigated by treatment of plants with NO donors before or during exposure to abiotic stress conditions. NO treatments either reversed the stress-induced decline, or even further amplified up-regulation of the antioxidant defense system, concomitantly with a reduction in H₂O₂ accumulation and lipid peroxidation (Hasanuzzaman *et al.*, 2010).

In plants, NO is produced from a variety of enzymatic and non enzymatic sources, which are not yet fully understood, and are still under study (Table 1). Higher plants seem to have lost nitric oxide synthases (NOSs) in the course of evolution (Fröhlich and Durner, 2011). NOSs are present in almost all known organisms except plants, where neither the gene nor any protein with high sequence similarity to known NOS have been found (Lamattina *et al.*, 2003). However, a NOS-like activity L-Arginine (L-Arg) dependent, has been highly reported in plants, together with a nitrate reductase (NR) dependent pathway.

Rasul *et al.* (2012) have suggested that L-Arg and NR pathways are co-involved in NO production and do not work independently. Part of the NO produced by L-Arg dependent pathway could be oxidized to nitrite, providing substrate for NR-dependent NO synthesis. Soybean cotyledons, growing in the presence of ammonia (without nitrate), were

able to produce similar amounts of NO showing that different sources could operate for NO accumulation in soybean cotyledons (e.g. nitrite- and L-Arg-dependent sources). It is likely that under different physiological or stress conditions, one pathway could result more operative depending on the substrate availability to maintain or increase NO generation supporting the required levels (Galatro *et al.*, 2014). However, NO generation was reduced in ammonium-fed tobacco plants where nitrogen assimilation bypassed the NR step, and compromised immune responses (Gupta *et al.*, 2013).

NO is a free radical which diffuses readily through biological membranes and has a biological half-life ranging from 5 to 15 s (Gupta *et al.*, 2011). This short half-life reflects the highly reactive nature of the molecule: it reacts with metal complexes and other radicals, and with biomolecules such as nucleic acids, proteins and lipids (Gupta *et al.*, 2011). Prolonged exposure to stress may result in enhanced production of NO and its derivatives, resulting in nitrosative stress. The nitrosylation of lipids, proteins and nucleic acids leads to severe metabolic impairment and degradation of cellular metabolites leading to programmed cell death (Krasnylenko *et al.*, 2010; Misra *et al.*, 2011). S-nitrosylation of proteins, also known as S-nitrosation, constitutes the most studied and described NO-dependent posttranslational modification in plants. It refers to the reversible covalent binding of an NO moiety to the thiol group of a cysteinyl residue (Cys) of a target protein, to produce an S-nitrosothiol (SNO) (Astier *et al.*, 2012). S-nitrosylation may be integral to NO function during a variety of cellular processes (Simontacchi *et al.*, 2013). Depending on the target protein concerned, this PTM will lead to a modification of its enzymatic activity or its protein function.

TABLE 1

Proposed sources of NO generation in plants

Oxidative pathways	Reductive pathways (Nitrite dependent)
<ul style="list-style-type: none"> • L-Arg-dependent (Jasid <i>et al.</i>, 2006; Galatro <i>et al.</i>, 2004; Corpas <i>et al.</i>, 2006) • PAs-mediated (Tun <i>et al.</i>, 2006; Yamasaki and Cohen, 2006) • Hydroxylamine-mediated (Rümer <i>et al.</i>, 2009) 	<ul style="list-style-type: none"> • Nitrate reductase (Yamasaki and Sakihama, 2000; Rockel <i>et al.</i>, 2002) • Root-specific, membrane-bound NiNOR (Stöhr <i>et al.</i>, 2001) • Thylakoids supplemented with nitrite (Jasid <i>et al.</i>, 2006) • Mitochondrial electron transfer chain (Gupta <i>et al.</i>, 2011)¹ • Peroxisomal enzyme XOR (Godber <i>et al.</i>, 2000; Whang <i>et al.</i>, 2010) • Non-enzymatic reduction of nitrite (Bethke <i>et al.</i>, 2004)²

PAs, Polyamines; NiNOR, nitrite NO reductase; XOR, xanthine oxidoreductase

¹ Under low oxygen concentration.

² In the apoplast.

NR, a key enzyme in the nitrogen metabolism and, as mentioned before, a source of NO in plants, may be affected by posttranslational nitrosative modifications that could modify its functionality. In wheat leaf segments exposed to sodium nitroprusside (SNP) or S-nitrosoglutathione (GSNO), NR activity was significantly reduced to different degrees between 3 and 21 h of treatment, whereas its activity was partially recovered in the presence of the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (Rosales *et al.*, 2011). Neither NR protein expression nor Tyr nitration, were affected by SNP in wheat segments (Rosales *et al.*, 2011). In this sense, it will be interesting to study how NO could regulate its NR-dependent formation by S-nitrosative PTM. Frungillo *et al.* (2014) demonstrated that NO and SNO modulate nitrogen assimilation by differentially inhibiting nitrate uptake and reduction. Also, NO regulates GSNO reductase (GSNOR1) through post-translational modification (S-nitrosylation). GSNOR catalyses the reduction of GSNO to oxidized glutathione and ammonium. These results suggest a novel mechanism by which NO controls its own bioavailability (Frungillo *et al.*, 2014).

Polyamines (PAs), nitrogenous aliphatic compounds, also appear to be involved in the regulation of NR activity. PAs form H₂O₂ during their catabolism, and are also NO producers by still unknown mechanisms (Yamasaki and Cohen, 2006). NO mediates spermine-induced reduction in root elongation in wheat plants (Groppa *et al.*, 2008). The PAs putrescine, spermidine and spermine induced a biphasic response in NR activity, inhibiting the enzyme activity at short incubation times (3h) and stimulating it at longer exposition times (21h) (Rosales *et al.*, 2012). NO is involved in this response, which could be reverted employing the NO scavenger cPTIO.

In addition to the multiple metabolic pathways that lead to NO formation, they can occur in different cell compartments (Table 1). Chloroplasts are proposed sites of NO generation under physiological and stress conditions (Foissner *et al.* 2000; Arnaud *et al.*, 2006; Jasid *et al.*, 2006; Galatro *et al.*, 2013; Tewari *et al.*, 2013). Gas *et al.* (2009) proposed that chloroplasts are key players for the control of NO levels in the plant cell. It was shown that chloroplast function positively affects NO levels not only in this organelle, but also in the whole tissue (Galatro *et al.*, 2013). NO detection goes along with maximum chlorophyll content, and quantum yield of photosystem II (ΦPSII) in soybean cotyledons highlighting a role for chloroplast functionality in NO generation as it was previously proposed (Galatro *et al.*, 2013).

Thus, different pathways are involved, work together, and also modulate NO production in the plant cell under physiological or stress conditions. However, how the levels

of NO are reached in such time and place to fulfill their functions, and how NO can regulate its own synthesis, are still puzzles to elucidate.

NO metabolism in plants is still a challenge. It is useful to identify the ways and sources of NO formation as well as the entry points of NO at the signaling metabolic network during normal or stress physiology. Other intriguing point is how PAs contribute to NO generation, and the physiological significance of other proposed sources (as hydroxylamine or non-enzymatic NO generation).

The elucidation of NO multiple pathways in plants will help to understand plant strategies to withstand stress and, in this way, to contribute to develop plants species with higher tolerance to stress.

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References

- Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Gwozdz EA (2011). The message of nitric oxide in cadmium challenged plants. *Plant Science* **181**: 612-620.
- Arnaud N, Murgia I, Boucherez J, Briat J-F, Cellier F, Gaymard F (2006). An iron induced nitric oxide burst precedes ubiquitin-dependent protein degradation for *Arabidopsis* AtFer1 ferritin gene expression. *Journal of Biological Chemistry* **281**: 23579-23588.
- Astier J, Kulik A, Koen E, Besson-Bard A, Bourque S, Jeandroz S, Lamotte O, Wendehenne D (2012). Protein S-nitrosylation: what's going on in plants? *Free Radical Biology and Medicine* **53**: 1101-1110.
- Bethke PC, Badger MR, Jones RL (2004). Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* **16**: 332-341.
- Chen WW, Yang JL, Qin C, Jin CW, Mo JH, Ye T, Zheng SJ (2010). Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in *Arabidopsis*. *Plant Physiology* **154**: 810-819.
- Corpas FJ, Barroso JB, Carreras A, Valderrama R, Palma JM, León AM, Sandalio LM, del Río LA (2006). Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. *Planta* **224**: 246-254.
- Foissner I, Wendehenne D, Langebartels C, Durner J (2000). In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. *Plant Journal* **23**: 817-824.
- Fröhlich A, Durner J (2011). The hunt for plant nitric oxide synthase (NOS): is one really needed? *Plant Science* **181**: 401-404.

- Frungillo L, Skelly MJ, Loake GJ, Spoel SH, Salgado I (2014). S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. *Nature Communications* **5**: 5401.
- Galatro A, Simontacchi M, Puntarulo S (2004). Effect of nitric oxide exposure on antioxidant capacity of soybean leaves. *Current Topics in Plant Biology* **5**: 69-79.
- Galatro A, Puntarulo S, Guiamet JJ, Simontacchi M (2013). Chloroplast functionality has a positive effect on nitric oxide level in soybean cotyledons. *Plant Physiology and Biochemistry* **66**: 26-33.
- Galatro A, Puntarulo S (2014). An update to the understanding of nitric oxide metabolism in plants. In: *Nitric Oxide in Plants: Metabolism and Role in stress Physiology* (MN Khan, M Mobin, F Mohammad, FJ Corpas, eds.), p. 3-15. Springer International, Switzerland.
- Gas E, Flores-Pérez Ú, Sauret-Güeto S, Rodríguez-Concepción M (2009). Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism. *Plant Cell* **21**: 18-23.
- Godber BLJ, Doel JJ, Sapkota GP, Blake DR, Stevens C R, Eisenthal R, Harrison R (2000). Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *Journal of Biological Chemistry* **275**: 7757-7763.
- Groppa MD, Rosales EP, Iannone MF, Benavides MP (2008). Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots. *Phytochemistry* **69**: 2609-2615.
- Gupta KJ, Igamberdiev AU, Manjunatha G, Segu S, Moran JF, Neelawarne B, Bauwe H, Kaiser WM (2011). The emerging roles of nitric oxide (NO) in plant mitochondria. *Plant Science* **181**: 520-526.
- Gupta KJ, Brotman Y, Segu S, Zeier T, Zeier J, Persijn ST, Cristescu SM, Harren FJ. M, Bauwe H, Fernie AR, Kaiser WM, Mur LAJ (2013). The form of nitrogen nutrition affects resistance against *Pseudomonas syringae* pv. *phaseolicola* in tobacco. *Journal of Experimental Botany* **64**: 553-568.
- Hasanuzzaman M, Hossain MA, Fujita M (2010). Physiological and biochemical mechanism of nitric oxide induced abiotic stress tolerance in plants. *American Journal of Plant Physiology* **5**: 295-324.
- Hasanuzzaman M, Hossain MA, Fujita M (2011). Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. *Plant Biotechnology Reports* **5**: 353-365.
- Jasid S, Simontacchi M, Bartoli CG, Puntarulo S (2006). Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiology* **142**: 1246-1255.
- Jasid S, Galatro A, Villordo JJ, Puntarulo S, Simontacchi M (2009) Role of nitric oxide in soybean cotyledon senescence. *Plant Science* **176**: 662-668.
- Krasylenko YA, Yemets AI, Blume YB (2010) Functional role of nitric oxide in Plants. *Russian Journal of Plant Physiology* **57**: 451-461.
- Kumar P, Tewari RK, Sharma PN (2010) Sodium nitroprusside mediated alleviation of iron deficiency and modulation of antioxidant responses in maize plants. *AoB PLANTS* plq002.
- Lamattina L, Garcia-Mata C, Graziano M, Pagnussat G (2003). Nitric Oxide: The versatility of an extensive signal molecule. *Annual Review of Plant Biology* **54**: 109-136.
- Laspina NV, Groppa MD, Tomaro ML, Benavides MP (2005). Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. *Plant Science* **169**: 323-330.
- Lozano-Juste J, Colom-Moreno R, León J (2011). *In vivo* protein tyrosine nitration in *Arabidopsis thaliana*. *Journal of Experimental Botany* **62**: 3501-3517.
- Misra AN, Misra M, Singh R (2011) Nitric oxide ameliorates stress responses in plants. *Plant Soil and Environment* **57**: 95-100.
- Morot-Gaudry-Talarmin Y, Rockel P, Moureaux T, Quilleré I, Leydecker MT, Kaiser WM, Morot-Gaudry J (2002). Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. *Planta* **215**: 708-715.
- Rasul S, Wendehenne D, Jeandroz S (2012). Study of oligogalacturonides-triggered nitric oxide (NO) production provokes new questioning about the origin of NO biosynthesis in plants. *Plant Signaling and Behavior* **7**: 1031-1033.
- Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *Journal of Experimental Botany* **53**: 103-110.
- Rosales EP, Iannone MF, Groppa MD, Benavides MP (2011). Nitric oxide inhibits nitrate reductase activity in wheat leaves. *Plant Physiology and Biochemistry* **49**: 124-130.
- Rosales EP, Iannone MF, Groppa MD, Benavides MP (2012). Polyamines modulate nitrate reductase activity in wheat leaves: involvement of nitric oxide. *Aminoacids* **42**: 857-865.
- Rümer S, Gupta KJ, Kaiser WM (2009). Plant cells oxidize hydroxylamines to NO. *Journal of Experimental Botany* **60**: 2065-2072.
- Saxena I, Shekhawat GS (2013). Nitric oxide (NO) in alleviation of heavy metal induced phytotoxicity and its role in protein nitration. *Nitric Oxide* **32**: 13-20.
- Simontacchi M, García Mata C, Santa María GE, Bartoli CG, Lamattina L (2013). Nitric oxide as a key component in hormone-regulated processes. *Plant Cell Reports* **32**: 853-866.
- Stöhr C, Strube F, Marx G, Ullrich WR, Rockel P (2001). A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* **212**: 835-841.
- Taiz L, Zeiger E. 2010. *Plant Physiology* (5th Edition), Sinauer Associates Inc, Sunderland, Massachusetts U.S.A.

- Tanou G, Filippou P, Belghazi M, Job D, Diamantidis G, Fotopoulos V, Molassiotis A (2012). Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. *Plant Journal* **72**: 585-599.
- Tewari RK, Prommer J, Watanabe M (2013). Endogenous nitric oxide generation in protoplast chloroplasts. *Plant Cell Reports* **32**: 31-44.
- Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Segal Floh EI, Scherer G FE (2006). Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. *Plant and Cell Physiology* **47**: 346-354.
- Verma K, Mehta S, Shekhawat G (2013). Nitric oxide (NO) counteracts cadmium induced cytotoxic processes mediated by reactive oxygen species (ROS) in *Brassica juncea*: cross-talk between ROS, NO and antioxidant responses. *Biometals* **26**: 255-269.
- Wang BL, Tang XY, Cheng LY, Zhang AZ, Zhang WH, Zhang FS, Liu JQ, Cao Y, Allan DL, Vance CP, Shen JB (2010). Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin. *New Phytologist* **187**: 1112-1123.
- Yamasaki H, Sakihama Y (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters* **468**: 89-92.
- Yamasaki H, Cohen MF (2006). NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? *Trends in Plant Science* **11**: 522-524.
- Yu M, Lamattina L, Spoel SH, Loake GJ (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytologist* **202**, 1142-1156.