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Nitric oxide regulation of plant metabolism

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ABSTRACT

Nitric oxide (NO) has emerged as an important signal molecule in plants, having myriad roles in plant development. In addition, NO also orchestrates both biotic and abiotic stress responses, during which intensive cellular metabolic reprogramming occurs. Integral to these responses is the location of NO biosynthetic and scavenging pathways in diverse cellular compartments, enabling plants to effectively organize signal transduction pathways. NO regulates plant metabolism and, in turn, metabolic pathways reciprocally regulate NO accumulation and function. Thus, these diverse cellular processes are inextricably linked. This review addresses the numerous redox pathways, located in the various subcellular compartments that produce NO, in addition to the mechanisms underpinning NO scavenging. We focus on how this molecular dance is integrated into the metabolic state of the cell. Within this context, a reciprocal relationship between NO accumulation and metabolite production is often apparent. We also showcase cellular pathways, including those associated with nitrate reduction, that provide evidence for this integration of NO function and metabolism. Finally, we discuss the potential importance of the biochemical reactions governing NO levels in determining plant responses to a changing environment.

Key words: hypoxia, mitochondria, metabolism, nitric oxide, *S*-nitrosylation, pyridoxine, reactive nitrogen species, reactive oxygen species

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INTRODUCTION

Plants are sessile organisms exposed to an ever-changing environment and their metabolism must be sufficiently flexible to allow them to acclimate to changing conditions. Changes in light, temperature, mineral nutrients, or stress conditions, such as drought, flooding, and salinity, are all external factors that require such metabolic adjustments. As an example, flooding leads to hypoxia in submerged organs and the plant responds by changing its energy metabolism in those regions to fermentation as a replacement of oxidative phosphorylation in the mitochondria (Taiz et al., 2015). This requires signaling within and between the affected cells leading in the short term to modifications of key enzymes and in the long term to changes in the network of gene expression that controls the related metabolism. A number of small molecules are involved in this signal transduction, such as the plant hormones auxin and ethylene, in addition to NO. The purpose of this review is to summarize the current state-of-the-art regarding aspects of NO signaling that are integral to plant metabolism.

METABOLIC PATHWAYS ASSOCIATED WITH NO PRODUCTION AND REMOVAL

Plants utilize various oxidative and reductive pathways to generate NO (Gupta et al, 2011; Kolbert et al., 2019). Oxidative NO pathways operate under normoxic conditions and the enzymes responsible for NO production via these pathways have not been thoroughly investigated. These pathways are well studied in mammalian cells, with three central NO synthase (NOS) enzymes being described: neuronal NOS, inducible NOS, and endothelial NOS (Gupta et al., 2011; Kolbert et al., 2019). However, a previously reported plant NOS in *Arabidopsis* did not display significant sequence homology to any of the mammalian NOS isoforms (Guo et al., 2003). This *Arabidopsis* enzyme was found to lack NOS activity and to act instead as a GTPase and was therefore subsequently renamed Nitric

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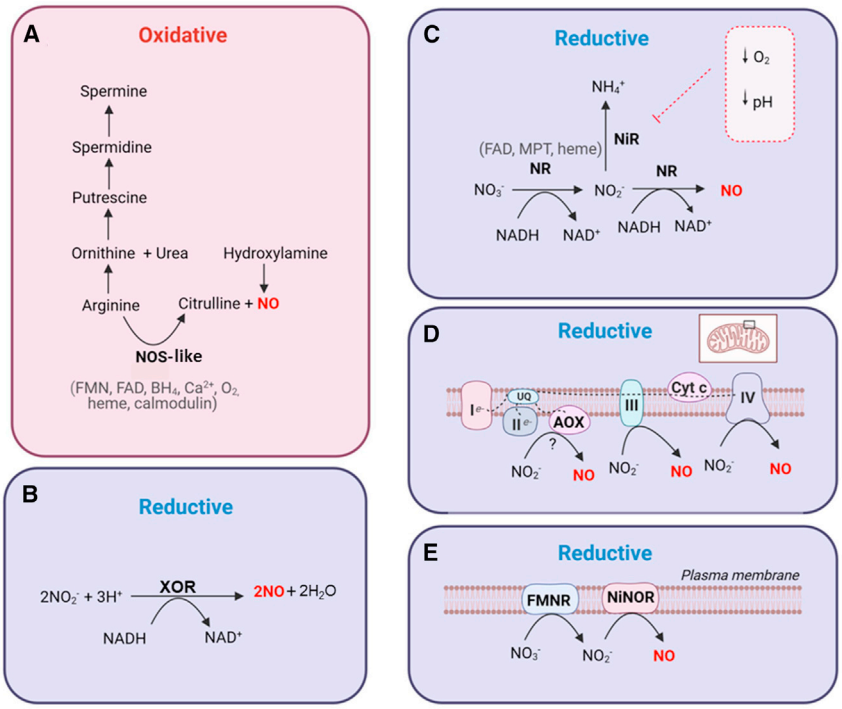

Figure 1. NO biosynthetic pathways in plants.

(A) NO is produced by oxidative pathways and reductive pathways. The former include a NOS-like enzyme, a polyamine-mediated pathway and a hydroxylamine pathway, while the latter include NR and xanthine oxidoreductase (XOR). A NOS-like enzyme may use L-arginine as substrate and produce L-citrulline and NO. This activity requires several cofactors such as BH_4, CaM (calmodulin), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), Ca^{2+}, and oxygen.

(B) XOR catalyzes the reduction of nitrite to NO using NADH or xanthine as reducing substrate.

(C) NR catalyzes reduction of nitrite (NO_2^-) to NO. The activity requires cofactors such as FAD, heme-iron, and molybdenum-molybdopterin (MPT). Under aerobic conditions, the cytoplasmic nitrate (NO_3^-) regulates NR activity, because nitrate competitively inhibits nitrite reduction. Thus, a lower nitrite concentration does not favor its reduction due to an increased K_m requirement. Under conditions such as hypoxia, and low pH, the NiR is inhibited, leading to an increased nitrite concentration and its concomitant removal.

(D) The nitrite generated under hypoxia is transported to mitochondria via a putative nitrite transporter. Under hypoxic conditions, nitrite reduction to NO takes place at complexes III and IV, and possibly AOX. cytochrome *c* oxidase (COX); inner mitochondrial membrane (IMM).

(E) NO is also generated by the combined action of a plasma membrane-bound nitrite-NO reductase (PM-NiNOR) and a plasma membrane-bound NR (PM-NR).

Oxide-Associated1 (Zemojtel et al., 2006). Nonetheless, NOS-like activity has been detected in peroxisomes and chloroplasts in plants (reviewed in Astier et al., 2018; Corpas and Barroso, 2017) and inhibitors of mammalian NOS (e.g. L-arginine analogs) are able to diminish peroxisomal NOS-like activity (Corpas and Barroso, 2017). To detect this activity, it was necessary to provide L-arginine and the co-substrates, NADPH and oxygen, together with multiple coenzymes, including flavin mononucleotide, and flavin adenine dinucleotide, as well as proteins such as calmodulin and tetrahydrobiopterin (BH_4) (Figure 1A) (Barroso et al., 1999; Corpas et al., 2004; Corpas and Barroso, 2017). Despite the conclusions of the aforementioned studies, the identification of proteins contributing to this NOS-like activity requires further analysis.

A recent bioinformatic study of the genomes of 1300 higher plant species failed to identify any NOS homologs (Jeandroz et al., 2016). This finding therefore raised the question of the identity of the enzymatic processes underlying the observed L-arginine-dependent NO biosynthesis route in *Arabidopsis* (reviewed in Corpas et al., 2009), suggesting that the enzyme responsible is not structurally related to mammalian NOS. Despite this, a mammalian NOS homolog was identified, and subsequently characterized, in the microalgae, *Ostreococcus tauri* (Foresi et al., 2010). This enzyme produces large amounts of NO during exponential growth, suggesting an important role for NO in the biology of microalgae. Lack of evidence of NOS in higher plants suggests that land plants may have lost this enzyme during evolution. The apparent absence of a NOS-like enzyme in higher plants has led to several other sources of NO being proposed, including polyamine- and hydroxylamine-based pathways. Polyamines (PAs), such as putrescine, spermine, and spermidine, have all been implicated in NO production (Tun et al., 2006; Zhou et al., 2016). For example, experimental evidence suggests that addition of putrescine, spermine, and spermidine significantly induced NO accumulation (Tun et al., 2006) and 2-aminoethyl-2-thiopseudourea, an inhibitor of animal NOS enzymes, inhibited polyamine-induced NO production. Copper amine oxidases (CuAOs) are enzymes of the polyamine pathways that regulate oxidation reactions. The loss of the CuAO1 and CuAO8 enzymes leads to reduced NO production in *Arabidopsis*, implying a potential polyamine-mediated NO production route in plants (Wimalasekera et al., 2011; Groß et al., 2017). Moreover, elicitor- or salt-induced NO production is lower in *Arabidopsis cuao1* and *cuao8* mutants compared with wild type supporting a role of these enzymes in NO metabolism (Groß et al., 2017). On the other hand, tobacco cell suspension cultures produced NO in an oxygen- and reactive oxygen species (ROS)-dependent reaction upon application of hydroxylamine (Rümer et al., 2009), but this NO biosynthetic pathway has not yet been fully characterized.

Cytosolic nitrate reductase (NR) is a well-known enzyme important in nitrogen metabolism and is widely implicated in NO production (Kolbert et al., 2019). NR mediates reduction of nitrate to nitrite using NADH as an electron donor, also requiring

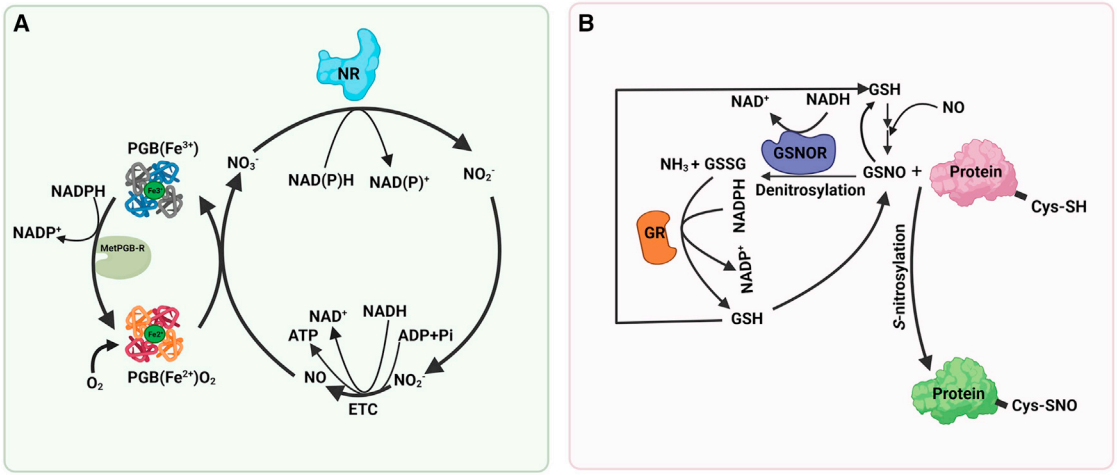

Figure 2. NO scavenging by PGB and GSNOR.

(A) The PGB–NO cycle operates via interconversion of nitrite, NO and nitrate. Under certain conditions, such as hypoxia, nitrate (NO_3^-) is reduced to nitrite (NO_2^-). The nitrite is reduced to NO at different sites (complexes III and IV, and possibly AOX). The produced NO diffuses to the cytosol where it is converted to nitrate (NO_3^-) by phytoglobin ($PGBO_2$), which then yields metphytoglobin (MetPGB), which is reduced by metphytoglobin reductase (MetPGBR). The produced NO_3^- will again become a substrate for NR. Operation of this cycle leads to the biosynthesis of a limited amount of ATP.

(B) The role of GSNOR in the regulation of GSNO homeostasis in plants. NO and reduced glutathione (GSH) react with each other to form *S*-nitrosoglutathione (GSNO). This product can be converted to oxidized glutathione (GSSG) and ammonia (NH_3) by GSNOR. In the process of transnitrosylation GSNO can also transfer NO to a reduced Cys residue of a given protein leading to protein *S*-nitrosylation.

molybdopterin, heme, and flavin adenine dinucleotide as coenzymes (Campbell, 2001). NR exhibits nitrite:NO reductase activity under certain conditions, such as hypoxia, anoxia, reduced pH, or pathogen infection (Yamasaki and Sakihama, 2000; Rockel et al., 2002), and when excess nitrite accumulation occurs due to inhibition of the nitrite reductase activity of NR (Figure 1C). It has been recently reported that an *Arabidopsis* nitrite reductase mutant obtained by CRISPR-Cas9 genome editing as well as transgenic plants overexpressing NR1 and NR2 proteins contained elevated endogenous NO levels under normoxic conditions (Costa-Broseta et al., 2020, 2021), thus supporting the relevance of reductive NR-dependent activity in the production of NO under normoxia. More specifically, under hypoxic or anoxic conditions, mitochondria can reduce nitrite to NO at the sites of complexes III and IV and possibly also at alternative oxidase (AOX) (Figure 1D). In addition, a plasma membrane-bound specific nitrite-NO reductase is capable of reducing nitrite to NO (Stöhr et al., 2001) (Figure 1E).

Recently, it was demonstrated that a dual system formed by the Amidoxime Reducing Component or Nitric Oxide-Forming Nitrite Reductase (ARC or NOFNiR) and NR molybdo-enzymes mediates nitrite-dependent NO production in the green algae, *Chlamydomonas reinhardtii* (Chamizo-Ampudia et al., 2016). NR and ARC are regulated both transcriptionally and at the protein activity level. This enzyme system is able to produce NO in the presence of nitrate. However, the role of ARC or NOFNiR in NO production is indirect, and it is not known whether a similar system operates in higher plants. Nitrite reduction to NO can also be catalyzed by the peroxisomal enzyme xanthine oxidoreductase (Figure 1B). This enzyme can generate uric acid and superoxide under aerobic conditions. Under anaerobic conditions, purified xanthine oxidoreductase has been shown to reduce nitrite to NO, with either xanthine as reducing substrate or NADH (Godber et al., 2000; Del Río et al., 2004).

In addition to NO biosynthesis, plants have evolved enzymes that can scavenge NO and convert this key signaling molecule into nitrate or *S*-nitrosoglutathione (GSNO). Consequently, net NO production is a function of biosynthesis minus scavenging. Two key proteins with known NO metabolism properties in plants are phytoglobins and *S*-nitrosoglutathione reductase (GSNOR) (Figures 2A and 2B) (Gupta et al., 2020). The phytoglobin (PGB)–NO cycle links cytosolic NR and mitochondria for enhanced energy production, as well as the protection of plants from excess NO due to the ability of this cycle to scavenge NO (Igamberdiev et al., 2005). NR converts nitrate to nitrite, which moves into the mitochondrion via an unknown transporter. Here it replaces oxygen as the terminal electron acceptor, leading to the reduction of nitrite to NO (Planchet et al., 2005). Since NO radicals are diffusible molecules they can readily move to the cytosol where PGB1 can oxidize NO to nitrate, which can subsequently be utilized by NR (Gupta et al., 2018). However, the full turn of the cycle is not very efficient at producing ATP (Hebelstrup and Møller, 2015) so, under most conditions, the cycle is likely to be more important for regulating the cellular NO level (Becana et al., 2020).

The PGB–NO cycle also plays a key role in anaerobic germination of rice (Kumari et al., 2021). Addition of nitrite results in enhanced expression of components of the PGB–NO cycle accompanied by enhanced ATP generation. In addition, the PGB–NO cycle functions in the establishment of nodulation via increased ATP production (Berger et al., 2020) and, furthermore, regulates NO generation during mycorrhizal associations (Martínez-Medina et al., 2019), underscoring the functional importance of this cycle.

The activity of mitochondrial AOX also indirectly reduces NO production (Cvetkovska and Vanlerberghe, 2012). Plants knocked down in AOX generated more superoxide and NO, whereas AOX-overexpressing lines produced less of these molecules.

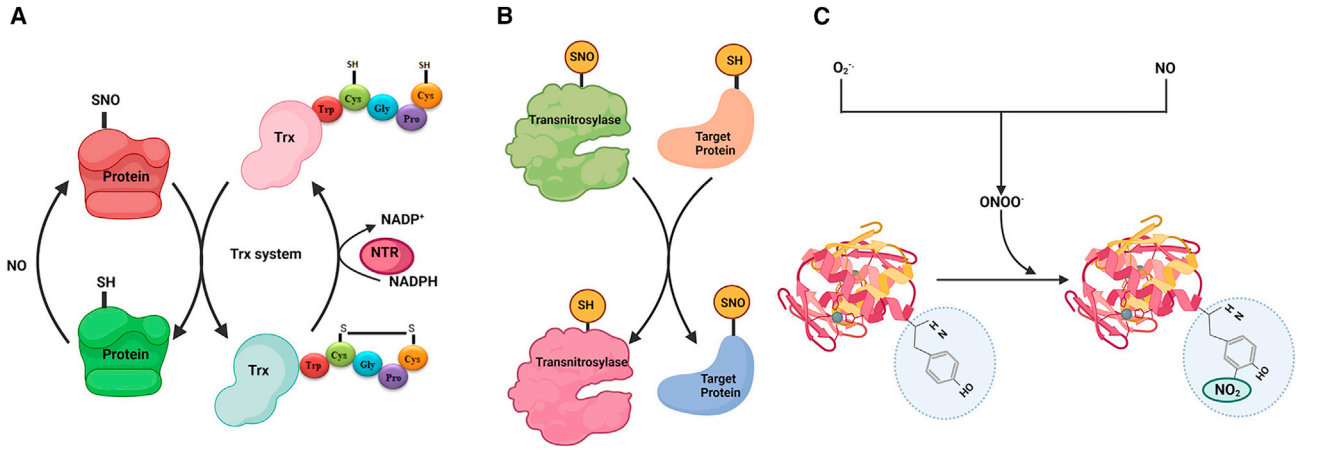

Figure 3. Chemistry of protein *S*-nitrosylation, denitrosylation, and transnitrosylation.

(A) Schematic drawing showing the process of *S*-nitrosylation and denitrosylation. *S*-Nitrosylation is a prominent post-translational modification in which the covalent addition of an NO group to a Cys thiol leads to formation of an *S*-nitrosothiol (SNO). The thioredoxin (Trx) system denitrosylates *S*-nitrosylated proteins via a dithiol moiety leading to formation of a reduced protein thiol (-SH) and oxidized Trx, which is subsequently reduced by NADPH-dependent Trx reductase (NTR).

(B) Transnitrosylation is catalyzed by a transnitrosylase carrying an SNO group that transfers the NO moiety to a target protein.

(C) A superoxide radical ($O_2^{\cdot-}$) reacts with NO leading to formation of peroxynitrite ($ONOO^-$) which can drive tyrosine nitration.

AOX prevents over-reduction of the electron transport chain (ETC) thereby lowering the electron leakage to oxygen or nitrite at complexes III and IV (Møller, 2001; Hebelstrup and Møller, 2015). Thus, the production of NO under biotic stress conditions, triggered by treatment of roots with the immune elicitor flg22, can be effectively prevented by AOX activity (Vishwakarma et al., 2018).

METABOLIC PATHWAYS ARE REGULATED BY *S*-NITROSYLATION

S-nitrosylation, the addition of an NO moiety to a reactive cysteine (Cys) thiol to form an *S*-nitrosothiol (SNO) (Figure 3A) (Lindermayr et al., 2005; Begara-Morales et al., 2014; Yun et al., 2016), is thought to be the dominant route for the mediation of plant NO bioactivity. The unique properties of the sulfur atom embedded within the amino acid, Cys, is key to enable the signaling outcomes associated with this modification (Umbreen et al., 2018). Thus, *S*-nitrosylation, as a prototypic redox-based post-translational modification (PTM), is conceptually similar to more established PTMs such as phosphorylation (Zhou et al., 2018; Gupta et al., 2020). In this context, *S*-nitrosylation can modulate protein function by regulating enzyme activity, protein localization, protein–protein interactions, protein degradation, and protein DNA binding (Yu et al., 2014; Albertos et al., 2015; Cui et al., 2018).

The accumulating data suggest that *S*-nitrosylation is a key switch to control important components of plant metabolism. Wang et al. (2009) demonstrated that a pathogen-triggered nitrosative burst mediates *S*-nitrosylation of Cys280 of Salicylic Acid-Binding Protein 3 (SABP3), suppressing binding to the key immune-related metabolite, salicylic acid (SA), and reducing the cognate carbonic anhydrase (CA) activity of this enzyme (Slaymaker et al., 2002). The CA function of SABP3 is essential for plant defense (Slaymaker et al., 2002). Hence, the inhibition of SABP3 CA function by *S*-nitrosylation may act as part of a negative feedback loop. On the other hand, NO accumulation promotes transcription of *SRG1*, which encodes a zinc finger transcription factor (Cui et al., 2018), which functions as a positive regulator of plant immunity, including the accumulation of the defense metabolite, SA. SRG1 is a transcriptional repressor; thus, to positively regulate immunity, this protein presumably represses an immune repressor. Accordingly, sustained NO accumulation resulted in *S*-nitrosylation of this protein at Cys87, which released SRG1 binding from its cognate *cis*-element and by extension, the associated SRG1 transcriptional repression activity. Subsequently, this may enable the expression of a negative regulator, subsequently curbing the immune response, including a decrease in SA biosynthesis (Cui et al., 2018).

S-nitrosylation also modulates ethylene biosynthetic pathways. For instance, methionine adenosyl transferase, an enzyme that plays a key role in the formation of *S*-adenosyl methionine (SAM), which is required for various methylation reactions and ethylene biosynthesis, has been shown to be *S*-nitrosylated (Pérez-Mato et al., 1999), leading to suppression of its activity (Lindermayr et al., 2006). Key antioxidant metabolic enzymes are also known to be regulated by *S*-nitrosylation. Yang et al. (2015) showed that this redox-based PTM modified ascorbate peroxidase 1 (APX1) at Cys32 enhancing its hydrogen peroxide-metabolizing activity, thereby reducing oxidative stress. In addition, *S*-nitrosylation of the *Arabidopsis* respiratory burst oxidase homolog D (RBOHD) at Cys890 reduced its capacity to generate ROS, curbing the oxidative burst and thereby limiting the extent of cell death associated with the hypersensitive response (Yun et al., 2011). Interestingly, Cys890 is evolutionarily conserved and *S*-nitrosylation of homologs of this RBOHD in flies and humans also reduces enzyme activity, indicating that this mechanism is conserved across kingdoms (Yun et al., 2011).

Peroxynitrite ($ONOO^-$) metabolism is also thought to play an important role in the development of pathogen-triggered hypersensitive cell death (Delledonne et al., 1998). In this context, it has been demonstrated that *S*-nitrosylation inhibits the hydrogen peroxide-metabolism (peroxidase) activity of peroxiredoxin II E (PrxII E). This protein has a key function in metabolizing $ONOO^-$. Thus, inhibition of PrxII E leads to increased $ONOO^-$ content, which can drive hypersensitive cell death development (Romero-Puertas et al., 2007).

The metabolism of glycine by the glycine decarboxylase complex is governed by a series of enzymes that are triggered in response to high concentrations of the amino acid glycine. The same set of enzymes is sometimes referred to as glycine synthase when it runs in the reverse direction to form glycine. The glycine cleavage system is composed of four proteins: T-protein, P-protein, L-protein, and H-protein (Kikuchi, 1973). Treatment of *Arabidopsis* cell cultures with the natural NO metabolite, GSNO, leads to *S*-nitrosylation of the glycine decarboxylase complex enzyme subunits P2 and H1, which contribute to the modulation of glycine and, by extension, photorespiration. In this vein, *S*-nitrosylation of the P2 and H1 subunits leads to the inhibition of this system, resulting in an increased ROS production (Palmieri et al., 2010).

It is also becoming increasingly appreciated that *S*-nitrosylation can regulate a number of more well-characterized PTMs (Gupta et al., 2020), significantly expanding the influence of NO over key cellular processes, including metabolism. Recently, it was shown that NO regulates conjugation of proteins with small ubiquitin-like modifier (SUMO), so-called SUMOylation, through *S*-nitrosylation of SUMO-conjugating enzymes (Skelly et al., 2019). SUMOylation has been shown to negatively regulate the deployment of plant immune responses, underpinned by SA accumulation. Pathogen recognition promotes NO accrual, which subsequently results in *S*-nitrosylation of SUMO-conjugating enzyme 1 (SCE1) at Cys139, reducing the activity of this enzyme and, by extension, decreasing global SUMO 1- and 2-dependent SUMOylation. The global reduction of SUMO 1/2 SUMOylation subsequently enables the attainment of maximal levels of the metabolite, SA, and subsequent SA-dependent immune responses. Significantly, the human homolog of SCE1, UBC9, is similarly regulated by *S*-nitrosylation of this conserved Cys residue, suggesting that this mechanism to control global SUMOylation is also conserved across kingdoms (Skelly et al., 2019).

PROTEIN DENITROSYLATION AND TRANSNITROSYLATION

An important feature of the addition of PTMs to their protein targets associated with cellular signaling is their selective reversal to disengage the given signal networks. In this context, it is possible that specificity in redox signaling is accomplished predominantly by reversal rates of Cys modifications, rather than by their formation, as rapidly degraded redox PTMs may have less impact than more persistent ones (Derakhshan et al., 2007). Thus, different protein-SNOs can have widely diverse biological lifetimes (Seth and Stamler, 2015). While a proportion of this can be attributed to the innate chemical stability of a given protein-SNO, this property is also influenced by potential non-enzymatic breakdown, for example, by either ascorbate or glutathione, key cellular antioxidants (Feechan et al., 2005; Masella et al., 2005; Benhar et al., 2008; Kneeshaw et al., 2014).

The metabolite, GSNO, can function as a natural NO donor and effectively acts as a relatively stable pool of NO bioactivity. Thus, increasing concentrations of GSNO in *Arabidopsis* promotes elevated levels of total protein *S*-nitrosylation. Conversely, decreasing GSNO concentrations result in reduced levels of this redox-based PTM (Feechan et al., 2005). GSNO can be metabolized by the activity of GSNOR, thus this enzyme indirectly controls the global levels of protein *S*-nitrosylation (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009). In the context of metabolism, *Arabidopsis* GSNOR, via its ability to regulate *S*-nitrosylation, has been shown to control the biosynthesis of the immune activator, SA (Feechan et al., 2005). Similar phenotypes to those of *Arabidopsis* have also been described in tomato GSNOR RNAi lines (Hussain et al., 2019), suggesting that the function of this enzyme is conserved across dicotyledonous species.

In phosphorylation, for example, a well-established signal transduction process, specificity is achieved via the result of a delicate poise between kinase and phosphatase activities. Recently, the conceptual equivalent of protein phosphatases associated with redox signaling has begun to emerge. Thioredoxins (Trxs) are present in all living organisms and their activity can be recycled by NADPH-dependent thioredoxin reductase (TrxR). Trx/TrxR-mediated denitrosylation has been uncovered as a key mechanism to control NO signaling in mammals (Benhar et al., 2008). Subsequently, *Arabidopsis* Trxh5 has emerged as a plant denitrosylase, which selectively denitrosylates the transcriptional co-activator, NPR1, which promotes SA biosynthesis and SA signaling during plant immunity (Kneeshaw et al., 2014) (Figure 3A). It is noteworthy, however, that NPR1 activity is also influenced by GSNOR function (Feechan et al., 2005; Tada et al., 2008), implying that the *S*-nitrosylation status of this co-activator may be controlled directly via Trxh5 and also indirectly via GSNOR. Moreover, Trxh5 and TrxR may denitrosylate a subset of the *Arabidopsis* SNO proteome directly and selectively *in vivo* (Kneeshaw et al., 2014). Consequently, the regulation of denitrosylation at additional Cys thiol residues embedded in regulatory proteins might also be mediated by Trxh5 and TrxR. Trx enzymes are encoded by a sizeable gene family in *Arabidopsis*, thus additional Trx family proteins may operate in conjunction with TrxR as direct and selective denitrosylases for a variety of other substrates.

In addition to Trxh5, and possibly other Trx proteins, *Arabidopsis* possesses two nucleoredoxins, NRX1 and NRX2. To date, NRX1 has unexpectedly been shown to be required for the protection of enzymes associated with ROS metabolism from oxidation within ROS-rich environments, including plant cells undergoing immune responses (Kneeshaw et al., 2017). Perhaps these two enzymes might also function as specific denitrosylases of key regulatory proteins within the plant nucleus.

Counterpoint to denitrosylation, the emerging evidence across life kingdoms suggests protein *S*-nitrosylation may occur within multiprotein macro-complexes, where an *S*-nitrosylated protein transfers an NO group directly to a target protein, a process termed transnitrosylation (Figure 3B) (Seth et al., 2018;

Chen et al., 2020). Here, the protein transferring the NO moiety, termed a nitrosylase, is increasing both the efficiency and specificity of this redox-based PTM in an enzyme-like fashion. It is likely that examples of transnitrosylation relevant to plant metabolism will also be uncovered in the near future.

Collectively, the current state-of-the-art suggests that Trx and TrxR enzymes, and possibly also NRX1 and NRX2, can function as direct and selective denitrosylases to regulate a subset of plant *S*-nitrosylated proteins associated with metabolic processes. In addition, our appreciation of how NO maybe transferred to target Cys residues relevant to metabolism by transnitrosylation, resulting from the activity of nitrosylases, is also set to increase.

PROTEIN TYR-NITRATION MODULATES METABOLIC PATHWAYS

An additional NO-based redox modification is tyrosine nitration, where protein tyrosine side chains are nitrated to give 3-nitrotyrosine (NO_2-Tyr) by peroxynitrous acid (HOONO), which is formed by the reaction of superoxide and NO, followed by a protonation (Chaki et al., 2014; Holzmeister et al., 2011) (Figure 3C). It is becoming clear that this is also a major regulatory PTM given that it appears to be involved in the control of a wide range of metabolic pathways. Many nitrated proteins and enzymes have been identified (Holzmeister et al., 2011; Chaki et al., 2014), but we will here only highlight examples relevant to metabolism, where the site of tyrosine nitration has been identified and the cognate effect established. Note to date this effect has always been inhibitory, which may point at a fundamental property of this type of metabolic regulation.

During senescence of pea roots, a total of 16 NO_2-Tyr proteins were identified. One of these, cytosolic NADP-isocitrate dehydrogenase, which is involved in amino acid interconversions and NADPH production, was shown to be inhibited by tyrosine nitration (Begara-Morales et al., 2013). Furthermore, the ascorbate-glutathione cycle detoxifies hydrogen peroxide in all the major subcellular compartments. Monodehydroascorbate reductase (MDAR) and APX are both also integral to this cycle and they are both inhibited by tyrosine nitration (Begara-Morales et al., 2014, 2015). Interestingly, mitochondrial manganese superoxide dismutase (SOD), which converts superoxide into hydrogen peroxide, is inhibited by peroxynitrate-mediated tyrosine nitration (Holzmeister et al., 2015). Thus, the superoxide formed by the mitochondrial ETC is not metabolized and therefore is free to react with any available NO to generate more peroxynitrate. In addition, peroxisomal hydroxypyruvate reductase (HPR1), part of the photorespiratory pathway, is prone to tyrosine nitration, which inhibits its activity (Corpas et al., 2013). The last step in the assimilation of sulfur (from sulfate) is catalyzed by the enzyme *O*-acetylserine(thiol)lyase. This enzyme is also inhibited by tyrosine nitration (Alvarez et al., 2010). Finally, in root nodules, glutamine synthetase (Melo et al., 2011) is another enzyme inhibited by tyrosine nitration, while leghemoglobin nitration is thought to act as a sink for potentially damaging nitrogen radicals thus protecting other proteins (Sainz et al., 2015).

A considerable limitation associated with a potential signaling role for NO_2-Tyr in metabolic regulation is that, while Cys *S*-nitrosylation, is readily reversible, there is no known pathway for the reversal of tyrosine nitration. Indeed, this PTM has been linked with protein degradation; for example, the turnover of ABA receptors following tyrosine nitration (Castillo et al., 2015). However, the degradation of tyrosine-nitrated proteins is an energetically expensive solution and may only be appropriate when the plant cell is closing down a metabolic process or is undergoing senescence.

INFLUENCE OF NO ON PEROXISOMAL ENZYMES LINKED TO OXIDATIVE METABOLISM

Peroxisomes are one of the major sites for ROS generation (Del Río and López-Huertas, 2016), and these organelles are also involved in NO production and constitute targets for NO (Corpas and Barroso, 2014; Begara-Morales et al., 2015). Peroxisomes contain antioxidant metabolic enzymes, including catalase (CAT), monodehydroascorbate reductase (MDAR), and manganeseSOD), to control the generation of ROS, mainly superoxide radicals and hydrogen peroxide (Corpas and Barroso, 2018; Rodríguez-Ruiz et al., 2019). Several lines of evidence suggest that CAT is inhibited by NO and $ONOO^-$ (Clark et al., 2000). Both *S*-nitrosylation and tyrosine nitration retard CAT activity in pea leaves and pepper fruits (Ortega-Galisteo et al., 2012; Chaki et al., 2015). However, the role of *S*-nitrosylation and tyrosine nitration-mediated inhibition of CAT requires further investigation to uncover the detailed molecular mechanisms associated with this PTM.

ROS and reactive nitrogen species (RNS) are essential for modulating peroxisomal function during pepper fruit (*Capsicum annuum* L.) ripening, especially under nitro-oxidative stress when CAT and other potential enzyme candidates are *S*-nitrosylated (Rodríguez-Ruiz et al., 2019). Purified recombinant MDAR from pea leaf peroxisomes is inhibited by both $ONOO^-$ and GSNO. Furthermore, MDAR undergoes nitration at Tyr345, in addition to *S*-nitrosylation at Cys68 (Begara-Morales et al., 2015). SOD is also prone to nitration at Tyr115 (Holzmeister et al., 2015), which leads to irreversible inhibition and increased superoxide accumulation (Corpas et al., 2019). HPR1, a peroxysomal enzyme, is also inhibited by Tyr-nitration (Corpas et al., 2013), while APX can be both activated by reversible *S*-nitrosylation and inactivated by irreversible nitration (Begara-Morales et al., 2014). Thus, numerous studies have now established NO as a ubiquitous regulator of antioxidant metabolic enzymes in the peroxisome.

NO AFFECTS MITOCHONDRIAL METABOLISM UNDER STRESS

Mitochondria are the energy powerhouses of the cell, but they also generate NO and contain protein targets for NO regulation (Møller, 2001; Hebelstrup and Møller, 2015; Gupta et al., 2018; Møller et al., 2020). All of the main complexes of the mitochondrial ETC interact, either directly or indirectly, with NO (Figure 4) (Gupta et al., 2018). For example, mutations in NADH dehydrogenase subunit 7 in *Nicotiana sylvestris* impaired NO production and resulted in cytoplasmic malesterility (Shah

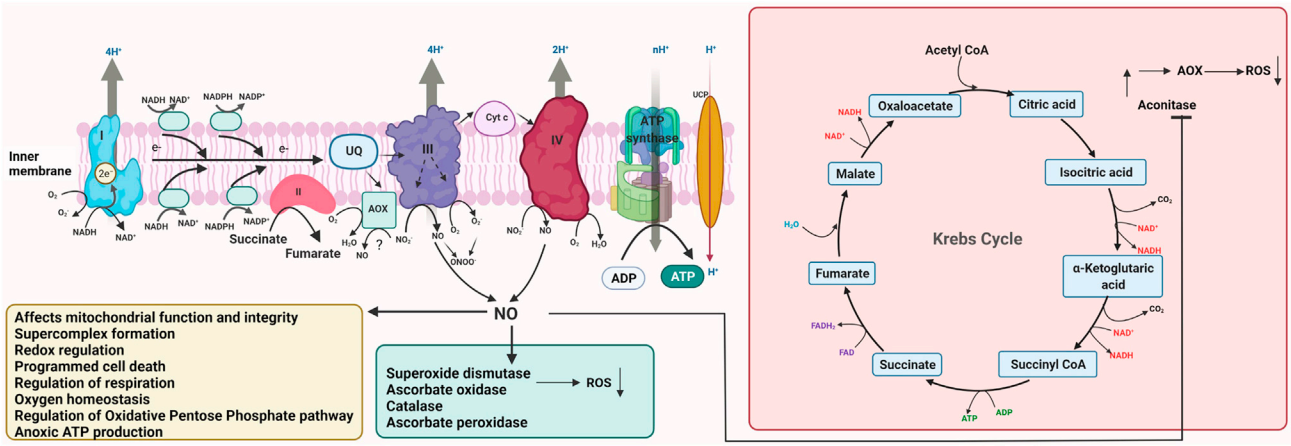

Figure 4. Under hypoxia or anoxia, nitrite can serve as an alternative electron acceptor in the mitochondrial electron transport chain leading to the generation of NO.

Mitochondrial electron transport components complexes III and IV, and possibly AOX, are involved in nitrite reduction to NO. The NO produced can inhibit aconitase leading to enhanced accumulation of citrate, which can activate AOX. The activated AOX lowers the reduction level of complexes I, III, and IV and therefore ROS production. NO also induces superoxide dismutase, ascorbate oxidase, catalase, and APX, which all help remove ROS. NO produced by the mitochondria affects mitochondrial function, integrity, formation of supercomplex formation, redox regulation, induction of programmed cell death, regulation of respiration, and oxygen homeostasis by inhibition of COX, regulation of the oxidative pentose phosphate pathway, and nitrite-driven ATP production.

et al., 2013). This mutant showed an increased expression of PGB1 under differential oxygen concentrations (Shah et al., 2013). It was recently demonstrated that nitrite protects mitochondrial structure and function under hypoxia (Gupta et al., 2017). Several complexes and super complexes are affected in this process. Interestingly, the specific activity of complex I was higher in the presence of NO. In addition, the supercomplex I + III_2 showed enhanced activity, suggesting a specific role of this supercomplex in mitochondrial protection. NO might also have a specific role in enhancing electron channeling via mitochondrial supercomplexes under hypoxia.

Complex II, which participates in both the tricarboxylic acid cycle and the electron transport chain (ETC), also interacts with NO. Simonin and Galina (2013) found that application of NO donors, such as *S*-nitroso-*N*-acetyl-DL-penicillamine or diethylenetriamine nonoate, led to a dramatic increase in the K_m for succinate, up to 45-fold under anoxic conditions.

Complex III is also both a site for the production of and a target for NO (Planchet et al., 2005; Alber et al., 2017). The site of NO production is the Q cycle, analogous to superoxide generation, where electron pressure in the Q cycle during stress leads to increased electron leakage and concomitant superoxide or NO production (Møller, 2001; Sun and Trumpower, 2003; Alber et al., 2017). The production of NO under anoxia by tobacco cell suspensions or tobacco roots is sensitive to myxothiazol, which inhibits the Q cycle of the bc1 complex of the mitochondrial ETC (Sun and Trumpower, 2003).

The cytochrome (COX) pathway is also sensitive to NO at complex IV (Millar and Day, 1996) and its inhibition leads to increased AOX abundance and engagement. In barley roots overexpressing PGB1 decreased the NO concentration and inhibited respiration, thereby increasing internal oxygen, reducing ROS production and subsequently enhancing metabolic flux via the oxidative pentose phosphate pathway (Gupta et al., 2014). Bulky tissues, such as germinating seeds, contain low internal oxygen levels, which can slow down germination. In this context, a recent discovery demonstrated that exogenously supplied NO stimulated germination of the slow germinating kabuli chickpea variety, which produces reduced levels of NO (Pandey et al., 2019). Application of NO led to increased internal oxygen concentrations and lowered ROS levels, which prompted germination. Chickpeas are a key crop on the Indian subcontinent, where they provide an important source of protein and fiber. Thus, breeding programs to enhance NO production in chickpeas might help in increasing the rate of seed germination of this important crop species (Pandey et al., 2019).

AOX catalyzes ubiquinol oxidation with a four-electron reduction of oxygen to water (Møller, 2001; Moore et al., 2013). Electron transfer via AOX does not lead to proton translocation via complexes III and IV, but plays a role in preventing over-reduction of the ubiquinone pool and concomitant ROS and NO production (Møller, 2001; Cvetkovska and Vanlerberghe, 2012). Both an AOX knockout mutant of tobacco and an AOX antisense line displayed higher NO accumulation than wild type (Cvetkovska and Vanlerberghe, 2012). Increased levels of NO inhibit COX and induce AOX activity, which may help to compensate for COX inhibition, as AOX is insensitive to NO (Millar and Day, 1996). This feature can convey an additional advantage to mitochondrial energy production in conditions such as hypoxia. Several lines of evidence suggest that *AOX* transcripts and protein are induced by NO. For example, the bacterial elicitor harpin leads to the accumulation of NO and the transcriptional activation of *AOX* (Huang et al., 2002). In addition, the pathogen-associated molecular pattern flg22, consisting of a 22-amino acid peptide from bacterial flagellin or hypoxia also resulted in the activation of *AOX* expression (Vishwakarma et al., 2018). As both of these cues elicit NO

production, this molecule might be a key signal for regulation of AOX.

NO produced by the ETC participates in the regulation of other aspects of mitochondrial metabolism (Møller et al., 2020). The tricarboxylic acid cycle enzyme, aconitase, is regulated by NO and ROS (Gupta et al., 2012). This enzyme contains an iron–sulfur (Fe–S) cluster, presumably targeted by NO, and is involved in the interconversion of three tricarboxylic acids (citrate, *cis*-aconitate, and isocitrate) (Navarre et al., 2000). Inhibition of aconitase by hypoxia-induced NO results in increased citrate levels and enhanced AOX activity (Gupta et al., 2012).

NO REGULATION OF CHLOROPLAST ENZYMES

In addition to mitochondria, another organelle linked with NO production and function is the chloroplast. Several lines of evidence suggests that chloroplasts are also a source of NO production (Galatro et al., 2013; Tewari et al., 2013). Using electron paramagnetic resonance spectroscopy together with the spin trap, iron (II)-*N*-methyl-D-glucamine dithiocarbamate ($Fe(MGD)_2$), it has been demonstrated that purified chloroplasts from soybean leaves can generate NO (Puntarulo et al., 2007). It was also found that both L-arginine- and nitrite-dependent pathways operate to generate NO in chloroplasts (Jasid et al., 2006). In addition, NO influences photophosphorylation, electron transport, and PSII activity (Misra et al., 2014). In this context, ETC components of PSII are targets of NO (Diner and Petrouleas, 1990): NO binding to the PSII component $Q_AFe^{2+}Q_B$ leads to a significant (10-fold) decrease of the electron transfer rate between Q_A and Q_B. Employing pulse amplitude modulation fluorescence coupled with flash oxygen evolution approaches on isolated pea thylakoid membranes, it was demonstrated that the electron donor site of PSII is the probable target of NO action (Vladkova et al., 2011). It was also found that several chloroplast proteins of *Arabidopsis* are *S*-nitrosylated in response to NO treatment including the Rubisco small chain 1a precursor, Rubisco activase, Rubisco large subunit, several PSII components, and the Rieske Fe-S protein (Lindermayr et al., 2005). In addition, a range of other proteins were found to undergo tyrosine nitration (Lozano-Juste et al., 2011). Investigation into the specific roles of tyrosine-nitrated and *S*-nitrosylated proteins in chloroplasts will provide information on NO function associated with metabolism linked to this organelle.

NO REGULATES AMINO ACID METABOLISM AND POLYAMINE PRODUCTION

NO accumulation correlates with increases in the levels of amino acids of the glutamate family (León et al., 2016). Furthermore, an increased level of γ-aminobutyric acid was observed coinciding with increased levels of γ-hydroxybutyrate and alanine, which play a role in conditions such as hypoxia (Rocha et al., 2010). NO accumulation also correlated with increased levels of proline, which can act as an osmolyte, antioxidant, and metal chelator (Hayat et al., 2012).

NO-enhanced metabolic flux via the oxidative pentose phosphate pathway and glycolysis plays a role in providing pyruvate for the tricarboxylic acid cycle to enhance energy production (Pandey et al., 2019). NO also enhanced the content of PAs, such as putrescine and spermidine, as well as agmatine (León et al., 2016). These PAs are thought to play an important role in stress tolerance (Khajuria et al., 2018). Increased polyamine levels were observed under nitrate nutrition, which resulted in enhanced NO production and associated increased plant disease resistance (Mur et al., 2019). NO-induced polyamine accumulation also plays a role in the delay of fruit ripening (Lokesh et al., 2019). Collectively, these findings suggest that NO is emerging as a key regulator for amino acid metabolism and associated polyamine biosynthesis.

A POTENTIAL ROLE OF NO IN VITAMIN B_6 METABOLISM

Recently, NO has been implicated in vitamin B_6 metabolism (Xia et al., 2014). Vitamin B_6 is a family of molecules, most notable among which is pyridoxal 5′-phosphate (PLP), known for its essential role as a coenzyme for numerous metabolic enzymes, with those involved in amino acid metabolism being among the best characterized. Other non-coenzyme forms of the vitamin B_6 family (pyridoxamine 5′-phosphate [PMP], pyridoxine 5′-phosphate [PNP]) and non-phosphorylated derivatives pyridoxal [PL], pyridoxamine and pyridoxine) are also emerging as potential key players in cellular metabolism and some are even touted as potent antioxidants (Mooney and Hellmann, 2010). A recent screen for NO hypersensitive mutants led to the isolation of a PL kinase (which phosphorylates PL to PLP) mutant termed *sno1* (*sensitive to nitric oxide 1*) (Xia et al., 2014). The *sno1* mutant is allelic to *sos4* (*salt overly sensitive 4*) isolated in a screen for sensitivity to sodium chloride (Shi and Zhu, 2002). NO is thought to play a signaling role in salt stress tolerance, through modulation of the Na^+ to K^+ ratio via the action of the Na^+/H^+ antiporter and the K^+ channel, (AKT1) (Campos et al., 2019). AKT1 activity was repressed in *sno1* plants as well as in the *nox1* mutant, which accumulates NO (Xia et al., 2014). However, the NO content was reportedly not increased in *sno1* plants (Xia et al., 2014). Instead, it was proposed that increased PLP levels measured in *sos4*/*sno1* lines inhibit AKT1 activity (Xia et al., 2014).

Conversely, it has recently been shown that PLP levels are decreased in the *sos4*/*sno1* mutants (Gorelova et al., 2021), which, while supportive of the role of SOS4 as a PL kinase, does not support the explanation for decreased AKT1 in the mutant lines. Therefore, the mechanism linking NO hypersensitivity, salt sensitivity, and misregulation of vitamin B_6 biosynthesis in *sno1*/*sos4* plants remains to be elucidated. Notably, this recent study demonstrated severe developmental defects in *sos4*/*sno1* mutants under standard growth conditions due to loss of vitamin B_6 homeostasis, which is suggested to render them hypersensitive to stress (Gorelova et al., 2021). Interestingly, loss of Pyridox(am)ine oxidase 3 (PDX3) function, another enzyme involved in vitamin B_6 metabolism, which oxidizes PMP/PNP to PLP, leads to a reduction in NR activity (Colinas et al., 2016). Therefore, NO levels may also be modulated in these plants and could be

implicated in the constitutive upregulation of defense-related genes observed in *pdx3* mutant lines (Colinas et al., 2016), but this remains to be deciphered. Nonetheless, given that members of the vitamin B_6 family of molecules are claimed to function as antioxidants and have been implicated in numerous abiotic stress responses (Colinas et al., 2016; Gorelova et al., 2021), it is possible that the imbalance derived from impairing enzymes of vitamin B_6 biosynthesis impact the level of ROS, as well as RNS. Indeed, an emerging theme is crosstalk between these reactive species (Lindermayr, 2017). For example, the GSNO pool, and thus level of SNO proteins, can be regulated by the direct effect of ROS on GSNOR (Kovacs et al., 2016). Thus, ROS/RNS homeostasis may be disrupted when there is also misregulation of vitamin B_6 homeostasis. A more rigorous study of vitamin B_6 metabolism and the role of specific vitamers (i.e. bioactive forms), particularly the non-coenzyme forms, will provide a clearer picture of the connection between the regulation of N metabolism, vitamin B_6 homeostasis, and ROS.

Interestingly, Tyr-nitration has been reported for the PLP synthase proteins PDX1.1 and PDX1.3 in *Arabidopsis* (Lozano-Juste et al., 2011) and needs to be investigated further to unravel biological context. NO signaling is also intimately connected with ethylene, as mentioned above, which in turn requires vitamin B_6 (i.e. PLP) as a coenzyme for its biosynthesis via 1-aminocyclopropane-1-carboxylic acid synthase activity (Boycheva et al., 2015). While the interplay between NO and ethylene may be synergistic, it is generally reported to be antagonistic. For example, ethylene mediates NO depletion during acclimation to flooding stress (Hartman et al., 2019). In addition, NO affects the levels of other hormones, e.g. auxin (Campos et al., 2019), which also requires PLP-dependent enzymes for its biosynthesis (Boycheva et al., 2015). Therefore, unravelling the interplay of vitamin B_6 with N metabolism and NO bioactivity may also require consideration of plant hormone function associated with these processes.

NO IS ASSOCIATED WITH FATTY ACID METABOLISM

NO is involved in fatty acid metabolism, which is an important pathway for maintenance of structural integrity and energy provision for various metabolic processes (Lim et al., 2017). Nitro fatty acids (NO_2-FAs) are formed in a reaction between either NO or $ONOO^-$ with unsaturated fatty acids (Aranda-Caño et al., 2019). In animal systems NO_2-FAs play important roles as signal molecules in protection against cardiac ischemic injury and are integral to inflammation cascades (Cui et al., 2006). Recent evidence also suggests that NO_2-FAs play a role in plant metabolism (Aranda-Caño et al., 2019; Mata-Pérez et al., 2020). These molecules can react with biological nucleophiles, such as glutathione, and can therefore indirectly modulate ROS homeostasis (Fazzari et al., 2014; Aranda-Caño et al., 2019). Furthermore, in *Arabidopsis*, endogenous nitro-linolenic acid (NO_2-Ln) was found at picomolar concentrations and was shown to be working as a signal molecule (Mata-Pérez et al., 2016b). Transcriptomic analyses showed that NO_2-Ln was involved in plant defense via induction of heat shock proteins by an unknown mechanism. NO_2-Ln can also modulate GSNO biosynthesis, suggesting that this metabolite plays a role in NO homeostasis. The NO-FA content is elevated under various stress conditions, such as osmotic stress, low temperature, wounding, and cadmium (Cd^{2+}) treatment (Mata-Pérez et al., 2016a). In *Arabidopsis*, NO_2-FAs are involved in ROS production and stomatal moments via modulating the activity of the superoxide-producing enzyme, NADPH oxidase (Di Palma et al., 2020). Therefore, generating further insights related to the role of NO in the control of fatty acid metabolism will be of significant value.

NO REGULATES ETHYLENE BIOSYNTHESIS AND POLYAMINE FUNCTION IN FRUIT RIPENING

Ethylene is a plant hormone extensively involved in several stages of plant development, including fruit ripening. NO modulates the ethylene biosynthetic pathway by transcriptional regulation, post translational regulation, and enzymatic regulation, and therefore influences ethylene production and fruit ripening. In the final step in ethylene biosynthesis, catalyzed by the aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACCO), ACC is oxidized to give ethylene (Pattyn et al., 2021). NO-mediated signal transduction can transcriptionally antagonize ethylene biosynthesis with impacts linked to fruit ripening (Manjunatha et al., 2010). It has also been reported that NO reacts with ACCO by binding to the active site of this enzyme (Tierney et al., 2005). Furthermore, the ethylene biosynthetic enzyme methionine adenosyltransferase is subjected to *S*-nitrosylation, leading to its inhibition (Lindermayr et al., 2006). NO and ACCO also form a complex, which is further chelated by ACC to produce a stable ternary ACC–ACCO–NO complex leading to ACCO inhibition, which negatively impacts ethylene biosynthesis (Tierney et al., 2005). In peach fruit, NO and/or $ONOO^-$ generated in a reaction between NO and ROS can retard ACCO activities via oxidative inactivation of their cofactors, leading to a decrease in ethylene levels (Zhu et al., 2006). Also related to fruit ripening, NO alters expression of enzymes responsible for cell wall metabolism, associated with both the lignification and pigmentation of fruits, thereby extending fruit shelf life (Manjunatha et al., 2010).

PAs, inducers of NO production, are involved in the delay of fruit ripening (Malik and Singh, 2004). The application of spermidine, the smallest polyamine with three amine groups, to peach fruit, slowed down ripening by impairing ripening-related gene expression of aminocyclopropane-1-carboxylate synthase (ACS1) (Torrigiani et al., 2012). Since spermidine application can lead to NO production (Tun et al., 2006), the observed delay of ripening mediated by spermidine most likely occurs via NO. Application of PAs to banana fruits caused a significant delay of the ripening processes including: softening, slowing of peel color transition, suppression of ethylene production, decreased mitochondrial respiration, and reduced ACCO activity (Purwoko et al., 2002). In *Arabidopsis*, NO inactivates SAMS1 by *S*-nitrosylation (Lindermayr et al., 2006). Therefore, it is likely that *S*-nitrosylation affects ethylene biosynthesis in plants by targeting multiple steps in this pathway.

SAM is a common precursor for both ethylene and PA biosynthesis. Recently, it has been shown that, in banana fruit, the

biosynthesis of PAs occurs via L-arginine-dependent pathways, but not via competitive diversion of SAM (Lokesh et al., 2019). Interestingly, NO fumigation of tomato fruits with NO gas reduced hydrogen peroxide-scavenging capacity, elevated the levels of antioxidants, such as ascorbate, and enhanced NO-mediated PTMs, such as protein *S*-nitrosylation (Zuccarelli et al., 2021). In addition, NO differentially affected a multitude of metabolic processes including carotenoid, tocopherol, and flavonoid production. Thus, a 60% higher flavonoid accumulation was found in NO-treated fruits relative to control untreated fruits. The content of several secondary metabolites, such as naringenin chalcone, naringenin glucoside, kaempferol rutinoside, quercetin diglycoside, and apigenin derivatives, were also elevated in NO-treated fruits (Zuccarelli et al., 2021).

NO treatment also regulates biochemical pathways related to tomato flavor, such as glutamate and aspartate production. In addition, GSNOR activity was down-regulated severalfold during ripening of pepper (*Capsicum annuum* L.) fruits accompanied by enhanced abundance of *S*-nitrosylated proteins (Rodríguez-Ruiz et al., 2019). Several enzymes involved in ROS production were differentially impacted by NO during the ripening process. Peroxisomal catalase activity was down-regulated by both tyrosine nitration and *S*-nitrosylation in sweet pepper (*Capsicum annuum* L.) fruits during ripening (Rodríguez-Ruiz et al., 2019). The RBOH, responsible for the production of extracellular ROS, was upregulated during ripening of pepper fruits (Chu-Puga et al., 2019), while NADP-malic enzyme activity was suppressed (Muñoz-Vargas et al., 2020). NO also regulates phenylpropanoid metabolism during ripening. In this context, NO treatment promoted enhanced activities of phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, and 4-coumaroyl-CoA ligase enzymes in peach fruit (Li et al., 2017). In addition, it has been shown that that the lipid metabolite, inositol 1,4,5-triphosphate, plays a major role in NO-induced chilling tolerance via enhanced activity of enzymes associated with antioxidant metabolism including SOD, peroxidase, CAT, APX, glutathione *S*-transferase, and glutathione reductase, leading to increased postharvest shelf life and enhanced disease resistance (Jiao et al., 2019).

NO therefore regulates ethylene biosynthesis in plants by targeting multiple steps in the biosynthetic pathway of this key gaseous hormone and by extension controls associated processes linked to ethylene function, including fruit ripening. NO gas treatment of fruits, such as tomato, and may thus provide novel future strategies for increasing fruit quality (Corpas et al., 2018; Zuccarelli et al., 2021).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The accumulating evidence indicates that NO plays a key role in regulating numerous metabolic enzymes, principally via *S*-nitrosylation. NO also orchestrates, either directly or indirectly, an array of responses to both biotic and abiotic stresses, and central to this ability is wide-ranging metabolic reprogramming, involving a plethora of metabolites from numerous pathways. The biosynthesis of several important nutrients, including amino acids, fatty acids, and perhaps vitamins, in addition to the key immune-related metabolite, SA, all appear to be regulated by NO. In addition, organelles, such as peroxisomes, chloroplasts, and mitochondria, are all thought to be sites of NO production and are functionally associated with plant metabolism. These organelles not only generate NO, but also ROS, and the interplay between NO and ROS in these organelles is important in regulating numerous metabolic processes.

However, a number of key questions remain to be addressed. Given the existing absence of clarity regarding the different possible enzymatic sources of NO in plants, these sources should be more rigorously characterized and their potential contribution to NO production in relation to plant metabolism carefully established. Also, it would be helpful to have a greater understanding of how NO production pathways might be manipulated both temporally and spatially to enable metabolic reprogramming. In addition, can redox switches, which enable key regulatory proteins to be controlled by cellular NO levels, be redesigned resulting in enhanced metabolic outputs? Might it be possible to modify plant NO levels and associated signaling through differential nitrogen-based feeding or modification of the enzymatic pathways involved in nitrogen assimilation? Furthermore, the interconversion of NO and its related N-oxides, occurring via different metabolic routes in plants, requires greater granularity. The emerging evidence also suggests that NO interacts with the deployment of other PTMs and signaling systems linked with plant metabolism. Detailed insights into the associated molecular mechanisms may help shape agriculturally relevant plant traits.

It is now well established that massive metabolite exchange occurs in plants during stress responses. It would be important to establish how NO orchestrates these exchanges together with deeper insights into the associated metabolic fluxes. A clear understanding of how NO influences the highly complex metabolic processes in plants is a crucial area, where progress might lead to novel strategies for plant breeding or crop design.

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