UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

LUCAS SCORZA FRUNGILLO LIMA

SELF CONTROL OF NITRIC OXIDE BIOAVAILABILITY IN PLANTS: MOLECULAR MECHANISMS AND INTERPLAY WITH NITRATE ASSIMILATION

AUTORREGULAÇÃO DA BIODISPONIBILIDADE DO ÓXIDO NÍTRICO EM PLANTAS: MECANISMOS MOLECULARES E RELAÇÃO COM O PROCESSO DE ASSIMILAÇÃO DE NITRATO

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2015

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“Self control of nitric oxide bioavailability in plants: molecular mechanisms and interplay with nitrate assimilation”

“Autorregulação da biodisponibilidade do óxido nítrico em plantas: mecanismos moleculares e relação com o processo de assimilação de nitrato”

Supervisor/*Orientadora*: Profa. Dra. Ione Salgado

PhD Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Functional and Molecular Biology in Biochemistry.

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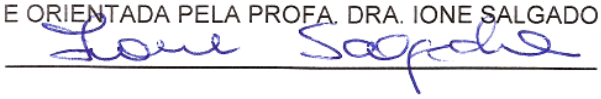

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"Olhe só como a natureza é caprichosa."
Milton Frungillo

"Observe how whimsical nature is."
Milton Frungillo

To those enthusiasts of nature,
especially to my wife Livia.

Dedico este trabalho a todos entusiastas da natureza,
em especial à minha esposa Livia.

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RESUMO

FRUNGILLO, L. **Autorregulação da biodisponibilidade do óxido nítrico em plantas: mecanismos moleculares e relação com o processo de assimilação de nitrato.** Tese (Doutorado) - Instituto de Biologia, Universidade Estadual de Campinas, Campinas/SP, Brasil, 2015.

Este trabalho de Tese avalia mecanismos moleculares envolvidos na ação sinalizadora do radical óxido nítrico (NO) e sua relação com o controle da assimilação de nitrato na planta modelo *Arabidopsis thaliana.* Os objetivos desta Tese foram atingidos através do cultivo de plantas de *A. thaliana* selvagem e mutantes cognatos em condições controladas de disponibilidade de nutrientes, seguido da análise de expressão gênica por qPCR, determinação de atividades enzimáticas e conteúdo de metabólitos por espectro e fluorimetria e fracionamento de metabólitos por HPLC, bem como construção de transgênicos de interesse e análise de modificação pós traducional de proteína.

No ***Capítulo I*** desta Tese apresento uma abrangente revisão científica publicada no periódico *Brazilian Journal of Botany (DOI: 10.1007/s40415-013-0013-6)* na qual mecanismos estabelecidos de produção, degradação e sinalização do NO são detalhados. Neste capítulo há um breve levantamento histórico sobre a compreensão científica da ação sinalizadora do radical NO em sistemas celulares e uma análise crítica e comparativa de como a homeostase deste radical é diferencialmente atingida em animais e plantas. Neste capítulo são introduzidas as informações necessárias no campo da sinalização redox mediada por NO que serviram de base para o desenvolvimento do trabalho experimental apresentado no capítulo a seguir.

No ***Capítulo II*** apresento evidências experimentais originais de que a ação sinalizadora do radical NO em plantas impacta em sua própria degradação, através da regulação da atividade enzimática da GSNOR1, e síntese, através do processo de assimilação de nitrato. Este capítulo é apresentado conforme manuscrito publicado no periódico científico *Nature Communications (DOI: 10.1038/ncomms6401)*. Neste trabalho mostramos que *S*-nitrosotióis suprimem a captação e redução do nitrato por transportadores e redutases específicos. Ainda, apresentamos um robusto conjunto de evidências que indica o controle da atividade enzimática da GSNOR1 através de uma *S*-nitrosilação inibitória. Concluímos, por tanto, que um novo mecanismo de autorregulação da biodisponibilidade do NO esteja envolvido na regulação da assimilação de nitrato em plantas e propomos um modelo que sumariza nossos achados.

No ***Capítulo III*** apresento um texto submetido à publicação na forma de capítulo de livro no qual são analisados criticamente recentes avanços no campo da sinalização redox mediada por NO e do processo de assimilação de nitrato em plantas. Em especial, nossos achados experimentais são balizados em relação ao atual conhecimento científico, dessa forma podendo ser visto como uma extensão e aprofundamento à discussão apresentada no *Capítulo II*. Apresento neste capítulo uma detalhada descrição do processo de assimilação do nitrato e sua íntima associação à síntese e degradação do radical NO em plantas. Ao longo de todo o texto são destacados objetivos que considero promissores para o avanço de pesquisas na área de nutrição vegetal, em especial relacionadas à sinalização redox.

Como contribuição científica desta Tese, propomos um novo mecanismo de autorregulação da biodisponibilidade do NO em plantas com relevante impacto no

processo de assimilação de nitrato. Espero que essas novas propostas substanciem pesquisas e práticas agrícolas com o objetivo de aumentar a produção vegetal, mitigar perdas econômicas e reduzir a poluição ambiental causada pelo uso excessivo de fertilizantes.

Palavras chave: Sinalização celular; Modificação pós traducional; Nitrato redutases (NR); *S*-nitrosoglutationa redutase (GSNOR); Transportadores de nitrato (NPF/NRT).

ABSTRACT

FRUNGILLO, L. **Self control of nitric oxide bioavailability in plants: molecular mechanisms and interplay with nitrate assimilation.** PhD Thesis - Institute of Biology, University of Campinas, Campinas/SP, Brazil, 2015.

This Thesis assesses the molecular mechanisms involved in the nitric oxide (NO)-mediated redox signalling, with a focus on the control of the nitrate assimilation process in the model plant *Arabidopsis thaliana.* To achieve the objectives of this Thesis, wild-type *A. thaliana* plants and cognates mutants were cultivated under nutrient controlled conditions and subjected to gene expression analysis by qPCR technique, determination of enzyme activities and metabolite content by spectrometry and fluorimetry, and metabolite fractionation by HPLC, as well as construction of transgenic lines of interest and analysis of protein post-translational modification.

In ***Chapter I*** of this Thesis is presented a comprehensive scientific review published in the *Brazilian Journal of Botany (DOI: 10.1007/s40415-013-0013-6)* in which the established mechanisms of NO production, scavenging and signalling are detailed. In this chapter it is briefly described how the NO perception in biological systems has evolved, as well as a critical and comparative review of how NO homeostasis is achieved between plants and animals. Importantly, in *Chapter I*, the background technical and scientific information concerning the NO-mediated redox signalling that supports the experimental work presented in the following chapter is introduced.

Chapter II presents a set of original experimental evidence indicating that in plants NO-mediated redox signalling impacts its own scavenging, through the regulation of GSNOR1 activity, and synthesis, through the nitrate assimilatory

process. This chapter is organized as published in the scientific journal *Nature Communications (DOI: 10.1038/ncomms6401).* We provide evidence that *S*-nitrothiols feedback regulate nitrate uptake and reduction. Additionally, we present a robust set of evidence that GSNOR1 is directly inhibited by NO through *S*-nitrosylation. We conclude that a novel mechanism of NO self-control of bioavailability is involved in the fine-tuning of nitrate assimilation in plants. We then propose a model that summarizes our findings.

Chapter III contains a manuscript submitted for publication as a book chapter in which a critical review is presented of recent advances in the NO signalling field, together with that of research on the nitrate assimilatory process. Especially, our recent findings are discussed in face of current knowledge, so this chapter can be read as an extension of the discussion presented in *Chapter II.* Along *Chapter III,* a detailed description of the nitrate assimilatory process and its intimate interplay with NO synthesis and scavenging in pants can be found. Throughout the text is highlighted what I consider promising objectives to the scientific progress in the field of plant nutrition, specially related with the redox signalling.

As a scientific contribution of this Thesis, we propose a novel molecular mechanism of NO control of its own bioavailability with a significant impact on the nitrate assimilatory process in plants. I expect these new proposals to substantiate scientific research and agriculture practices aiming to raise crop yield and mitigate economic and environmental losses due to the excessive use of fertilizers.

Key words: Cell signalling; Post-translational modification; Nitrate reductase (NR); -*S*-nitrosoglutathione reductase (GSNOR); Nitrate transporters (NPF/NRT).

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µM	Micromolar	*Micromolar*
35S::FLAG-GSNOR1	Overexpressed FLAG epitope-*tagged GSNOR1 (genotype)*	*Superexpressão da GSNOR1 marcada com FLAG (genótipo)*
ABA	Abscisic acid	*Ácido abscísico*
Ala	Alanine	*Alanina*
ANR1	Arabidopsis nitrate regulated 1	*"Arabidopsis nitrate regulated 1"*
AOX	Alternative oxidase	*Oxidase alternativa*
Arg	Arginine	*Arginina*
Asn	Asparagine	*Asparagina*
Asp	Aspartate	*Aspartato*
cGMP	Cyclic guanosine monophosphate	*Monofosfato cíclico de guanosina*
CHL1	chlorate resistant 1	*Resistente à clorato 1*
CHS	Chalcone synthase	*Sintase de chalcona*
CLC	Chloride channel	*Canal de cloreto*
COX	Cytochrome c oxidase	*Citocromo c oxidoredutase*
cue1-6	Chlorophyll a/b binding protein underexpressed 1 (genotype)	*Proteína ligadora de clorofila a/b não expressa 1 (genótipo)*
Cys	Cysteine	*Cisteína*
EDRF	Endothelium-derived relaxing factor	*Fator de relaxamento derivado do endotélio*
Gaba	Gama-aminobutiric acid	*Ácido gama-aminobutírico*
GADPH	Glyceraldehyde 3-phosphate dehydrogenase	*Desidrogenase de gliceraldeído 3-fosfato*
Gc	Guanylate cyclase	*Ciclase de guanilato*
Gln	Glutamine	*Glutamina*
Glu	Glutamate	*Glutamato*
Gly	Glycine	*Glicina*
GMP	Guanosine monophosphate	*Monofosfato de guanosina*
GOGAT	Glutamine synthetase/glutamine-2-oxoglutarate transaminase	*Sintase de glutamina/ Transaminase de glutamina-2-oxoglutarato*

GS	Glutamine synthetase	*Sintetase de glutamina*
GSH	Reduced glutathion	*Glutationa reduzida*
GSNO	S-nitrosoglutathione	*S-nitrosoglutationa*
GSNOR	S-nitrosoglutathione reductase	*Redutase de S-nitrosoglutationa*
gsnor1	GSNOR1 mutant (genotype)	*Mutante para GSNOR1 (genótipo)*
GSSG	Oxidized glutathione	*Glutationa oxidada*
HATS	High Affinity Transport System	*Sistema de transporte de alta afinidade*
Hb	Hemoglobin	*Hemoglobina*
His	Histidine	*Histidina*
HR	Hypersensitive response	*Resposta hipersensitiva*
Ile	Isoleucine	*Isoleucina*
JA	Jasmonic acid	*Ácido jasmônico*
Km	Michaelis constant	*Constante de Michaelis*
LATS	Low Affinity Transport System	*Sistema de transporte de baixa afinidade*
Leu	Leucine	*Leucina*
Lys	Lysine	*Lisina*
Met	Metionine	*Metionina*
mM	Milimolar	*Milimolar*
N	Nitrogen	*Nitrogênio*
NADH	Nicotinamide adenine dinucleotide	*Dinucleótido de nicotinamida e adenina*
NADPH	Nicotinamide adenine dinucleotide phosphate	*Fosfato de dinucleótido de nicotinamida e adenina*
NH_3	Ammonia	*Amônia*
NH_4^+	Ammonium	*Amônio*
Ni:NOR	Nitrite:NO reductase	*Redutase de nitrito:óxido nítrico*
NIA	Nitrate reductase (gene)	*Redutase de nitrato (gene)*
nia1nia2	NR-double mutant (genotype)	*Duplo mutante para NR (genótipo)*
NiR	Nitrite reductase	*Redutase de nitrito*
NO	Nitric oxide	*Óxido nítrico*
NO_2^-	Nitrite	*Nitrito*
NO_3^-	Nitrate ion	*Íon nitrato*
NOS	Nitric oxide sinthase	*Óxido nítrico sintase*
nox1	Nitric oxide overproducer	*"Nitric oxide overproducer"*

NPF	Nitrate transporter/peptide transporter	*Transportador de nitrato e peptídeo*
NPR	Nitrate primary response	*Resposta primária ao nitrato*
NPR1	Non-expressor of pathogenesis-related protein 1	*Não expressor de proteína relacionada à patogenicidade 1*
NR	Nitrate reductase	*Redutase de nitrato*
NRT	Nitrate transporter	*Transportador de nitrato*
O_2	Molecular oxygen	*Oxigênio molecular*
O_2^-	superoxide anion	*Ânion superóxido*
$ONOO^-$	Peroxynitrite	*Peroxinitrito*
PAL	Phenylalanine ammonia lyase	*Liase de fenilalanina amônia*
par2-1	Paraquat resistant 2-1 (genotype)	*Resistente à paraquat 2-1 (genótipo)*
Phe	Phenilalanine	*Fenilalanina*
pM	Picomolar	*Picomolar*
PR	Pathogenesis-related	*Relacionado à patogênese*
Protein-SNO	S-nitrosilated protein	*Proteína S-nitrosilada*
Prx	Peroxyrredoxin	*Peroxirredoxinas*
RNS	Nitrogen reactive species	*Espécies reativas de nitrogênio*
ROS	Oxygen reactive species	*Espécies reativas de oxigênio*
SA	Salicylic acid	*Ácido salicílico*
SAB3	Salicylic acid-binding 3	*Ligadora de ácido salicílico 3*
Ser	Serine	*Serina*
SLAC1/SLAH	Slow chloride channel 1	*Canal*
SNO	S-nitrosothiol	*S-nitrosotiol*
Thr	Threonine	*Treonina*
TIR1	Transport inhibitor response 1	*"Transport inhibitor response 1"*
Tyr	Tyrosine	*Tirosina*
UV	Ultraviolet	*Ultra violeta*
Val	Valine	*Valina*
WT	Wild type	*Tipo selvagem*

SUMMARY

(*SUMÁRIO*)

INTRODUCTION

About the biochemical strategies of cellular responses to stimulus and cell signalling.
About the scientific contribution of this Thesis.

All living organisms, regardless life strategy, must be able to respond biochemically to external stimulus to ensure survival. Roughly, the stimulus may have biotic (from another organism) or abiotic (from the surrounding environment) origin. It is possible to note the organisms' ability to respond to a stimulus looking at, for instance, the break of seed dormancy by cold exposition or bolting associated with climate changes. Responses to environmental cues are strategies evolved to allow organisms to overcome challenges imposed in their lives, and then survive and perpetuate. Despite the apparent simplicity and mechanicity, every response is triggered appropriately to ensure the organism will overcome the challenge. There is truly a biochemical coordination at the cellular level, when countless structures and molecules are recruited to act coordinately and specifically in response to a stimulus. Therefore, in other words, the coordination of different molecular mechanisms recruited in distinct subcellular sites confers the organism with the ability to sense, read and respond biochemically to virtually all kinds of challenges. In Biology, the process comprised by the sense of a stimulus (or *signal*) by an organism and its *transduction* to a chemical-based response is designated *signal transduction.* In this sense, one can assume rightly that a proper and accurate signal sense is needed to a similarly proper and accurate cell response, enabling the organism to develop and grow. For example, seed germination following hydration, the development and growth of the radicle and its interaction with microorganisms in the soil soil, root foraging for water and nutrients needed for the development and growth of the whole plant until the production of new seeds, and then the cycle of life can start again, are all events that are governed through molecular mechanisms of signal transduction. The complex process of coordination of molecular mechanisms

at the cellular level in response to a given signal is designated *cell signalling*. It is *cellular signalling* that this Thesis is about.

The classical molecular description of a cell signalling process comprises the joint action of a hormone and its respective receptor. In this view, a hormone, produced in adjacent cells or even in distant tissues, binds specifically to the extracellular portion of a cellular transmembrane receptor. On binding the receptor, the hormone frequently induces a conformational modification in the receptor which in turn leads to a chemical reaction. It is at this moment that *signal transduction* occurs. As a consequence of this first chemical reaction triggered by the hormone-receptor complex, other chemical reactions are triggered in a non stoichiometric proportion. The sequential increase in magnitude of the chemical cell response is designated *signalling cascade*, a cornerstone process that determines the extension of cell response. It is, for example, the basic principle behind the scenes of the differential growth of a stem in response to directional light. Although correct and largely found in textbooks, this description of cell signalling is incomplete. Currently it is well accepted that not only hormones are able to trigger a signalling process in biological systems, but also other organic or inorganic molecules that are not sensed through a receptor. As discussed next, this is the case of *free radicals*.

Free radicals are highly reactive atoms or molecules in terms of Gibbs free energy in thermodynamics. This means that reactions with free radicals are highly favorable energetically, or in other words, they are spontaneous. The high reactivity of the free radicals is due to their unpaired valence electron, a feature that defines them in the broadest sense. Despite the spontaneity, the assumption that the reaction occurs at high speed is not at all times correct. Sometimes the kinetics of the reaction is not favored. An illustrative example is the glucose oxidation by the diradical molecular oxygen. Although it is thermodynamically favored, the glucose oxidation by molecular oxygen does not occur appreciably without the aid of enzymes due the requirement of high activation energy. The molecular oxygen is a diradical classified in a wide group of molecules known as Reactive Oxygen Species (ROS). ROS is a term relatively known due its constant appearance in the media, frequently as an unhealthy deleterious-causing agent. ROS may be generated in the cell through exposure to external cues, such as UV light , or as a consequence of the normal metabolism. Importantly, the notion of ROS as merely deleterious is restricted. ROS are an integral part of healthy processes, such as disease resistance.

Another relevant class of reactive species that has been raising great scientific interest lately is the Reactive Nitrogen Species (RNS), of which I highlight the radical *nitric oxide* (NO). The RNS are intimately implicated in a broad range of developmental and physiological process in animals and plants. In plants, for instance, evidence indicates that the treatment with NO donors induce changes in subcellular localization of target proteins and gene expression in plant defense against pathogen challenge. Additionally, an artificial NO atmosphere looks a promising strategy to antagonize the phytohormone ethylene and increase the shelf life of fruits. Thus it is clear by now how relevant reactive species are as signalling agents in living organisms. However, how is it possible to signal without receptors?

The molecular mechanism of the transient and active molecules, the free radicals, to act as cell signals is through its commitment in *oxi-reduction*, or simply *redox*, reactions with organic molecules. The redox reactions are characterized by the electron transfer from a molecule to another. Due to the high electron affinity, or tendency to gain electrons, of the oxygen atoms, it is said that the molecule that looses an electron is oxidized, while the one that gains an electron is reduced. In biological systems, redox modifications occur in *target sites of proteins.* The addition of a chemical group through redox reaction frequently triggers conformational change in proteins that leads to alterations in its biological activity. A target of redox modification in proteins is the *thiol group,* found in cysteine residues. The reduced thiol group consists of a sulfur atom bounded to hydrogen (-S-H). Due its relatively high pKa, thiol groups are easily ionized to thiolates and become highly susceptible to oxidizing agents, such as ROS and RNS. Among the different possible oxidation states of the thiol group, a prominent redox modification is S-*nitrosylation. S*-nitrosylation is the addition of a NO moiety (a RNS) to a thiol group to form a *S*-nitrosothiol (*protein-SNO*). *S*-nitrosylation frequently alters protein conformation, activity and localization. The S-NO bond can be easily broken in the presence of divalent cations, UV light or through enzyme activity, such as specific denitrosylases. The versatility of redox states of thiol groups, in particular the specificity and reversibility of S-nitrosylation, is a biochemical feature that places them as an important convergence point of molecular mechanisms involved in cell signalling. In this sense, the understanding of the molecular mechanisms committed in the NO-mediated redox signalling, especially those concerning protein-SNO homeostasis, is the key to allow us to manipulate the associated cell responses.

Given the biochemical features and role in a wide spectrum of biological process, it is becoming accepted that the formation of protein-SNO is the main signalling event in transduction NO bioactivity. Proteomic approaches have revealed an extensive list of protein targets of *S*-nitrosylation. Different lines of evidence indicate that the formation of protein-SNO is pivotal during the initial steps in cell signalling events, as well as necessary to sustain and modulate cell responses. In fact, all biological events described throughout this Introduction are, at least at some level, controlled by NO-mediated redox signalling. Genetic and biochemical evidence indicate that the control of NO signalling is possible through the control of its synthesis and/or scavenging. In cellular systems, NO may react with glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), considered the major biological NO reservoir. Both NO and GSNO are able to *S*-nitrosylate proteins, acting as a signalling agent. In cells, the control of the GSNO pool is mainly achieved through the activity of the enzyme *S*-nitrosoglutathione reductase 1 (GSNOR1), which catalysis GSNO reduction to oxidized glutathione (GSSG) and ammonium (NH_4^+). On the other hand, the synthesis of NO seems to be an output of different pathways working synergistically. Roughly, NO may be synthesized through reductive or oxidative pathways. In plants, among the oxidative pathways greater attention has been drawn to the oxidation of L-arginine to L-citrulline yielding NO, whereas among the reductive pathways the reduction of nitrite (NO_2^-) to NO is a common step. Interestingly, the availability of L-arginine as well as of NO_2^- to NO synthesis is closed linked with the nitrate (NO_3^-) assimilatory pathway flux. Primarily, the nitrate assimilatory pathway is responsible for the N acquisition in land plants, an essential nutrient to build up central molecules for life such as amino acids and nucleotides. In plants, nitrate availability in soil is directly related to growth and crop yield. Despite the potential economic relevance, only recently have molecular mechanisms been revealed that are committed to the control of nitrate assimilation in plants. Several lines of evidence suggest the operation of a *negative feedback* mechanism in the control of nitrate assimilation. However, the identity of the *signal* committed to and the molecular mechanism involved in the feedback control of nitrate assimilation has remained obscure for almost two decades.

As I hope it will become clear in the next chapters of this Thesis, the nitrate assimilatory process may be considered a very elucidative example of how plants sense and respond to environmental cues through reactive species-mediated

signalling. In this Thesis, I show original experimental evidence (*Chapter II*) that indicate a mechanism of NO self-control of its bioavailability in plants. The proposed mechanism relies on the control of NO synthesis through the nitrate assimilatory process and its degradation by GSNOR1. The cellular signalling mechanism proposed here comprises the post-translational modification of proteins by *S*-nitrosylation and regulation of gene expression. Concatenating our original findings, a novel NO signalling-nitrate assimilation feedback mechanism is proposed and discussed. Still, we raise important considerations concerning how specificity in NO signalling is achieved, which I personally consider the next step towards the understanding of molecular mechanisms committed in NO-mediated signalling.

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HYPOTHESIS

About the hypothesis tested in this Thesis, the starting point of the study.

With regard to the proposal of this Thesis, two hypotheses were tested and the molecular mechanism that integrate them investigated. Firstly, it was hypothesized that *the free radical nitric oxide is a key actor in the feedback regulation of nitrate assimilation in plants.* Next, it was hypothesized that *nitrate assimilation impacts nitric oxide homeostasis through its synthesis and degradation.* Further, aiming to concatenate the two working hypotheses of this Thesis, the following question was formulated: *Which molecular mechanism underpins the control of synthesis and scavenging of nitric oxide in plants?*

OBJECTIVES

About the objectives of this Thesis: general and specific.

General Objective

The general objective of this Thesis was to study the impact of nitrate availability in soil as the mineral source of N on plant growth and nitric oxide (NO)-mediated redox signalling in *Arabidopsis thaliana.* It was also the objective of this Thesis to study the role of NO-mediated signalling on the nitrate assimilatory pathway, as well as the molecular mechanisms involved in the control of NO synthesis and scavenging in plants.

Specific Objectives

To get an insight into the general objective of this Thesis, the following specific objectives were set:

- By using *A. thaliana* wild-type, and cognate transgenic and mutant plants, to assess the impact of NO-mediated redox signalling at key points of the nitrate assimilatory pathway, namely, control of gene expression of nitrate transporters and control of gene expression and enzymatic activity of reductases, as well as its consequences on plant growth.

- To assess the impact of nitrate availability in soil and the nitrate assimilatory process flux on NO homeostasis (synthesis and scavenging) by feeding experiments with transgenic and mutants plants of *A. thaliana*.

- Investigate the molecular mechanism of NO self-control of bioavailability through GSNOR1 and its impact on fine-tuning nitrate assimilation.

INTRODUÇÃO

Sobre estratégias bioquímicas de resposta celular a estímulos e sinalização celular.
Sobre as contribuições científicas desta Tese.

Todos os organismos vivos, independentemente da estratégia de vida, devem ser capazes de responder bioquimicamente a estímulos externos para garantirem sua sobrevivência. De uma maneira geral, esses estímulos externos podem ter origem biótica, provenientes de outros organismos, ou abiótica, do ambiente que os cercam. Podemos observar a capacidade dos organismos em responder a estímulos ambientais quando, por exemplo, a dormência de uma semente é quebrada pela exposição ao frio ou quando observamos o florescimento de uma planta associado a mudanças climáticas. Respostas a estímulos ambientais são estratégias selecionadas ao longo do curso da evolução biológica que permitem aos organismos vivos superarem os desafios que viver lhes impõe e perpetuar a espécie. Apesar da aparente simplicidade e mecanicidade, para que cada uma das inúmeras possíveis respostas seja disparada de forma a tornar o organismo apto a superar o desafio imposto, há uma complexa coordenação bioquímica em nível celular, quando inúmeras estruturas e moléculas celulares são recrutadas e agem de forma conjunta, coordenada e específica em resposta ao estímulo. Portanto, em outras palavras, a coordenação de diferentes mecanismos moleculares recrutados em diferentes compartimentos celulares equipa o organismo com a habilidade de perceber, interpretar e responder bioquimicamente aos mais variados desafios. Na Biologia, ao processo que envolve a percepção de um *estímulo* (ou também podemos dizer *sinal*) por um organismo e *tradução* deste estímulo para uma resposta baseada em reações químicas é dado o nome de *transdução de sinal*. Dessa forma, podemos então assumir corretamente que a precisa e adequada percepção desse *sinal* é imprescindível para que seja desencadeada uma resposta celular da mesma forma precisa e adequada, garantindo então o desenvolvimento e crescimento do organismo. São mecanismos moleculares de transdução de sinal que governam, por exemplo, a germinação de uma semente quando esta recebe água, o crescimento da radícula e sua interação com microrganismos presentes no

solo, o forrageamento da raiz em busca de água e nutrientes para o desenvolvimento e crescimento de uma planta até a produção de novas sementes, e assim o ciclo pode começar novamente. Ao complexo processo de coordenação dos mecanismos moleculares em nível celular envolvidos nas respostas à sinais dá-se o nome de *sinalização celular*. É sobre *sinalização celular* que esta Tese trata.

A mais clássica descrição de uma via de sinalização celular envolve a ação conjunta de um hormônio e seu receptor específico. Neste caso, um hormônio produzido em células adjacentes ou mesmo em tecidos distantes do seu local de ação liga-se especificamente à região extracelular de um receptor celular transmembrana. A ligação do hormônio no sítio ativo do receptor frequentemente induz uma *modificação conformacional* no receptor que desencadeia uma reação química. É nesse momento que o processo de transdução de sinal ocorre. Em consequência dessa reação química desencadeada pela ligação hormônio-receptor, outras reações químicas são disparadas em uma proporção não estequiométrica. A esse aumento sequencial de magnitude das respostas celulares é dado o nome de *cascata de sinalização*, processo imprescindível para a determinação da amplitude da resposta celular. É esse o mecanismo molecular básico que governa, por exemplo, o crescimento diferencial do caule de uma planta em resposta ao estímulo direcional de luz. Apesar de correta e largamente vinculada em livros textos, esta descrição de sinalização celular está incompleta. Atualmente é sabido que não apenas hormônios são capazes de desencadear um processo de sinalização em sistemas biológicos, mas também outras moléculas orgânicas ou inorgânicas que não são interpretadas por receptores. Esse é o caso dos *radicais livres*, como discuto a seguir.

Radicais livres são átomos ou moléculas altamente reativas segundo os conceitos termodinâmicos de energia livre de Gibbs. Isso significa que reações que envolvam radicais livres são altamente favoráveis energeticamente, ou seja, são reações espontâneas. A alta reatividade dos radicais livres se dá pelo desemparelhamento de seus elétrons na camada de valência, característica que os definem quimicamente em seu sentido mais amplo. Apesar da espontaneidade das reações, a presunção de que essas ocorrem em alta velocidade nem sempre está correta. Em alguns casos a cinética da reação é desfavorecida. Um bom exemplo disso é a reação de oxidação da glicose na presença do birradical oxigênio molecular. Apesar da oxidação da glicose pelo oxigênio ser termodinamicamente

favorável, esta não ocorre de maneira apreciável sem o concurso de enzimas, pois para que seja iniciada é necessária uma alta energia de ativação. O oxigênio molecular é um birradical classificado em um grupo de moléculas conhecido como espécies reativas de oxigênio (ROS). ROS é um termo relativamente conhecido pelo público em geral devido à sua constante exposição na mídia como moléculas que devem ser combatidas através da alimentação e hábitos de vida saudáveis. As ROS podem ser geradas nas células por ação de fatores externos como a radiação UV presente na luz solar ou durante processos metabólicos rotineiros como a respiração celular. Muito é falado sobre os malefícios causados pelos ROS, frequentemente sua produção é ligada a processos como o envelhecimento e danos ao DNA. Porém o reconhecimento das ROS como agentes meramente deletérios é limitado. ROS também estão envolvidas em processos benéficos à manutenção do organismo, como por exemplo, nos processos celulares de defesa às infecções. Outra classe relevante de espécies reativas que vem despertando o interesse de pesquisadores são as *espécies reativas de nitrogênio* (RNS), dentre as quais destaco o radical *óxido nítrico* (NO). As RNS estão intimamente implicadas em inúmeros processos do desenvolvimento e fisiologia animal e vegetal. Em plantas, por exemplo, estudos indicam que o tratamento com liberadores de NO induzem a mudança de localização subcelular de proteínas específicas e expressão de diversos genes relacionados à resposta de defesa vegetal. Ainda, a criação de uma atmosfera de NO parece ser uma estratégia biotecnológica promissora para suprimir os efeitos do fitohormônio etileno no processo de amadurecimento de frutos e assim aumentar o tempo de prateleira. Torna-se claro então a relevância da ação sinalizadora de espécies reativas em seres vivos. No entanto, como é possível essa ação sinalizadora sem a participação de receptores?

O mecanismo molecular de ação das espécies transitórias e ativas, os radicais livres, é através de seu envolvimento em reações de *óxido-redução*, ou *redox*, com moléculas orgânicas. As reações redox são caracterizadas pela transferência de elétrons entre as moléculas envolvidas na reação. Devido a alta eletroafinidade do átomo de oxigênio, ou seja, sua tendência em ganhar elétrons, diz-se que a molécula que perde elétrons foi oxidada, enquanto que a que ganha elétrons é reduzida (devido a redução no número de oxidação pela carga negativa do elétron). Em sistemas biológicos, as modificações redox ocorrem em *sítios alvos específicos em proteínas*. A adição de um novo grupamento frequentemente

desencadeia uma alteração conformacional na proteína impactando em sua atividade. Um importante sítio alvo de modificações redox presente em proteínas são *grupamentos tióis* em resíduos de cisteínas. O grupamento tiol reduzido é constituído de um átomo de enxofre ligado a um átomo de hidrogênio (-S-H). Devido ao seu relativo alto valor de pKa variando entre 8-9, o grupamento tiol de cisteínas forma facilmente grupamentos tiolatos ionizados altamente susceptíveis a ação de oxidantes, como ROS e RNS. Dentre os diferentes estados de oxidação, uma proeminente modificação redox de grupamento tiol é a S-*nitrosilação*. A *S*-nitrosilação é a ligação de um radical NO (uma espécie pertencente à classe das RNS) à um grupamento tiol de um resíduo de cisteína em um dada proteína, formando um *S*-nitrosotiol (*proteína-SNO*). Frequentemente a *S*-nitrosilação altera a conformação, atividade e localização das proteínas alvo. A ligação S-NO é facilmente reversível através de catálise não enzimática, na presença de cátions divalentes ou radiação UV, ou enzimáticas, por ação de enzimas denitrosilases específicas. A versatilidade dos estados redox de grupamentos tióis, em especial a reversibilidade e especificidade da *S*-nitrosilação, são características bioquímicas que o tornam um importante ponto de convergência molecular de diferentes mecanismos de sinalização celular. Dessa forma, a compreensão dos mecanismos moleculares envolvidos no controle da sinalização redox mediada por NO, especificamente envolvidos na homeostase de proteína-SNO, torna-se crucial para a manipulação de respostas celulares.

Dado suas características bioquímicas e envolvimento em diversos processos biológicos, acredita-se que a formação de proteínas-SNO seja a principal via de sinalização do NO em organismos. Diversos trabalhos proteômicos revelaram uma vasta gama de proteínas alvo de *S*-nitrosilação em plantas. Diversas linhas de evidência indicam que a formação de proteínas-SNO representa um importante evento nos processos iniciais de sinalização celular em diversos processos fisiológicos, assim como necessário para a manutenção e controle das respostas celulares. De fato, todos os processos metabólicos e fisiológicos descritos ao longo dessa Introdução são, em algum ponto, controlados por sinalização redox mediada por NO. Evidências genéticas e bioquímicas sugerem que seja possível controlar a sinalização do NO em sistemas biológicos através do controle de sua degradação e síntese. O NO pode reagir com o antioxidante celular glutationa (GSH) para formar a *S*-nitrosoglutationa (*GSNO*), considerado o principal reservatório de NO em sistemas

celulares. Ambos, NO e GSNO, são capazes de *S*-nitrosilar proteínas e, portanto, agir como sinalizadores celulares. Em células, o controle do nível de GSNO é realizado pela enzima *S*-nitrosoglutationa redutase (GSNOR1), que reduz o GSNO a glutationa oxidada (GSSG) e amônio (NH_4^+). Apesar da ação indireta, a GSNOR1 tem se mostrado a principal via de degradação do NO em células. Em contrapartida, a síntese de NO em plantas parece ser atingida pelo consórcio de diferentes vias, comumente divididas entre vias oxidativas e redutivas. Dentro das vias oxidativas, mais atenção tem se dado à síntese de NO através da oxidação do aminoácido L-arginina, enquanto que a redução do nitrito (NO_2^-) a NO é o ponto comum nas vias redutivas de produção de NO em plantas. Interessantemente, a disponibilidade tanto de L-arginina quanto de NO_2^- para a produção de NO em plantas está intimamente ligada à via de assimilação de nitrato (NO_3^-). Primariamente, a via de assimilação de nitrato é a principal fonte de obtenção de N para plantas terrestres, nutriente essencial para os seres vivos por constituir biomoléculas centrais como aminoácidos e nucleotídeos. A disponibilidade de nitrato no solo está diretamente relacionada ao crescimento e produção vegetal. Apesar da potencial relevância econômica que a compreensão da regulação do processo de assimilação de nitrato possa ter, apenas recentemente começamos a desvendar mecanismos moleculares envolvidos no controle deste processo. Diversas linhas de evidências indicam a existência de um mecanismo de *feedback negativo* controlando o fluxo da assimilação de nitrato em plantas. No entanto, a identidade do *sinal* e o mecanismo molecular envolvido neste feedback permaneciam obscuros há aproximadamente duas décadas.

Como busco mostrar nessa Tese, o processo de assimilação do nitrato pode ser considerado como um excelente exemplo de como as plantas percebem e respondem à estímulos externos através da sinalização celular mediada por espécies reativas. Nesta Tese apresento dados originais (*Capítulo II*) que indicam a operação de um mecanismo de autorregulação da biodisponibilidade do NO em plantas. O mecanismo proposto envolve o controle de sua síntese através do processo de assimilação do nitrato e sua degradação, através do controle da atividade da enzima GSNOR1. O mecanismo de sinalização celular aqui proposto envolve modificação pós-traducional de proteínas por *S*-nitrosilação e regulação da expressão gênica. Integrando nossos resultados um novo ciclo de regulação da sinalização NO/assimilação de nitrato é proposto e discutido. Ainda, levantamos importantes considerações a cerca de como é atingida a especificidade em

processos de sinalização redox mediada pelo NO, o que considero o próximo passo importante na compreensão dos mecanismos moleculares de sinalização redox do NO.

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HIPÓTESES

Sobre as hipóteses testadas nesta Tese, o ponto de partida do estudo.

Para o desenvolvimento desta Tese foram testadas duas hipóteses e investigado o mecanismo molecular que as integram metabolicamente. Primeiramente foi hipotetizado *o envolvimento do radical óxido nítrico em um mecanismo de feedback negativo que regula o processo de assimilação de nitrato em plantas.* Em seguida hipotetizamos que *o processo de assimilação de nitrogênio impacta na homeostase do óxido nítrico através de sua síntese e degradação.* Enfim, para conciliar as duas hipóteses de trabalho desta Tese, a seguinte pergunta foi feita: *Por qual mecanismo molecular se dá a relação entre o controle da síntese e da degradação do óxido nítrico em plantas?*

OBJETIVOS

Sobre os objetivos definidos para esta Tese, geral e específicos.

Objetivo Geral

O objetivo geral desta Tese foi avaliar o efeito da disponibilidade de nitrato como fonte de nutrição mineral de N e seu impacto no crescimento vegetal e mecanismos de sinalização redox mediada por óxido nítrico (NO) utilizando como modelos de estudo diferentes genótipos da planta *Arabidopsis thaliana*. Fez parte ainda do objetivo desta Tese a avaliação da ação sinalizadora do NO sobre o processo de assimilação do nitrato, bem como a investigação do mecanismo molecular envolvido no controle da síntese e degradação do NO em plantas.

Objetivos Específicos

Para atingir os objetivos gerais desta Tese, os seguintes objetivos específicos foram estabelecidos:

- Através da utilização de plantas mutantes e transgênicas de *A. thaliana,* avaliar o efeito da sinalização mediada por NO em pontos chave do processo de assimilação de nitrato, a saber, controle da expressão de transportadores e expressão e atividade de redutases de nitrato e seu consequente impacto no crescimento vegetal.

- Avaliar a influência da disponibilidade de nitrato no solo e do fluxo da via de assimilação de nitrato na homeostase (síntese e degradação) do radical NO através do cultivo de *A. thaliana* em condições de disponibilidade de nutrientes controlada e utilização de diferentes genótipos produzidos através de mutações dirigidas e transgenias.

- Investigar o mecanismo molecular de autorregulação da degradação do NO via GSNOR1 com impacto na regulação da assimilação de nitrato.

CHAPTER I

About the background in nitric oxide signalling in plants.
Basic foundations of the experimentations presented in Chapter II.

Nitric oxide signaling and homeostasis in plants: a focus on nitrate reductase and *S*-nitrosoglutathione reductase in stress-related responses

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Abstract

Studies in the last two decades have firmly established that the gaseous free radical nitric oxide (NO) is an intracellular and intercellular mediator of signal transduction pathways controlling plant growth and development, as well as plant responses to biotic and abiotic stresses. The underlying mechanisms of NO action may rely on its reactivity with different kinds of biomolecules, leading to modulation of enzymatic activities, and of gene transcription, with profound impact on metabolism and signal transduction pathways. NO homeostasis depends on the appropriate coordination of NO synthesis and degradation under different physiological conditions. The mechanisms by which NO is synthesized *de novo* in plants are still a matter of controversy, although in the last years, the key role of the enzyme nitrate reductase (NR) in plants NO production has been widely accepted. In addition, S-nitrosoglutathione (GSNO), which formed by spontaneous reaction of NO with glutathione, is likely a major NO reservoir and NO donor in plant cells. GSNO levels are controlled by the enzyme GSNO reductase that has emerged as the main enzyme responsible for the modulation of S-nitrosothiol pool. The number of plant processes influenced/modulated by NO has dramatically increased in the last years. This review particularly emphasizes the roles of NR and GSNOR enzymes in NO homeostasis and NO-mediated plant responses to environmental challenges.

Introduction

The gaseous free radical nitric oxide (NO) has emerged as an important signaling molecule in plant biology. In the last few years it has been demonstrated that many plant physiological and developmental processes, as well as plant responses to various biotic and abiotic stresses, require or are mediated by NO (reviewed by 1–4).

Early studies on NO were restricted to the environmental field, due to its toxic effects as a pollutant agent. NO is generated by vehicle engine and industrial combustion, accumulating in the air and causing acid rain and ozone layer destruction[5]. The important finding, in 1987, that NO has a key role in mammals as endothelium-derived relaxing factor (EDRF) raised enormous interest and stimulated NO research in all biological systems[6]. Actually, it is well known that NO exerts a broad range of effects on various metabolic and physiological processes in mammals, such as muscle contractility, platelet aggregation, neuronal activity, and immune responses (reviewed by 7). Additionally, impaired NO homeostasis has been associated to a number of pathological situations, such as tumors, asthma, neurodegenerative diseases, diabetes, hypertension, and other cardiovascular dysfunctions.

In 1979, it was demonstrated that NO can be emitted into the atmosphere by plants[8], but the interest of researchers on plant NO production was only stimulated after the discovery of the NO crucial roles in mammals. Thus, it was discovered that NO regulates seed germination, root growth, leaf senescence, and fruit ripening (reviewed by 9). Moreover, NO production was shown to be required for plant disease resistance and drought-stress tolerance (reviewed by 10,11), and for control of plant mitochondrial respiration[12]. Further on, the involvement of NO in additional plant biological processes was progressively enlarged, and nowadays it is known that NO plays a role in the control of flowering, pollen tube growth, gravitropism, xylem development, breaking of seed dormancy, and establishment of symbiotic interactions, among others (reviewed by 13). NO is also involved in plant responses to stress conditions, such as wounding, hypoxia, UV radiation, salinity, low and high temperatures, and heavy metals (reviewed by [2,4]).

The involvement of NO in such a wide variety of plant biological processes has been attributed, at least in part, to cross-talks with plant hormone signaling pathways. In a pioneer work, the influence of NO on auxin-induced growth of maize root

segments was proposed[14]. Moreover, NO was shown to modulate abscisic acid (ABA)-induced stomatal closure and ethylene-, salicylic acid (SA)-, and jasmonic acid (JA)-signaling pathways (reviewed by 9,11). NO was also reported to mediate ABA-induced up-regulation of Crassulacean acid metabolism in pineapple plants[15]. There is also evidence of NO involvement in cytokinins and giberellins signaling pathways[9], and it was recently shown that NO and cytokinins can react chemically, modulating in this way each other's homeostatic levels and bioactivity[16].

Although NO exerts important beneficial effects on biological systems, an excess of NO may be toxic to the cells, leading to apoptosis or necrotic cell death (reviewed by 17). Thus, for this radical to exert properly its signaling functions, mechanisms controlling the appropriate NO levels under different physiological or stressful situations are essential. Endogenous NO levels are maintained by a balance of synthesis and degradation rates (Fig. 1). The major proposed routes for NO biosynthesis are either oxidation of L-arginine or reduction of nitrite, through various biosynthetic pathways. On the other hand, mechanisms of NO degradation occur by NO reaction with molecular oxygen (O_2), superoxide anion (O_2^-), glutathione (GSH), or hemoglobin (Hb).

Despite the widely accepted importance of NO as a signaling molecule in plants, identification of the NO-mediated pathways under either physiological and/or adverse conditions is still a major task. In this paper, we review recent advances on our understanding about mechanisms of NO synthesis and degradation in plants, both under physiological and adverse environmental situations. We also discuss NO-mediated physiological responses to various types of stress, with special emphasis on plant defense against pathogens.

Nitric oxide synthesis in plants

Although the molecular mechanisms responsible for NO synthesis in plants are still a subject of controversy, L-arginine (Arg) and nitrite (NO_2^-) are considered the main precursors of NO (Fig. 1). In mammals, the major route for NO production is the NADPH-dependent oxidation of Arg to L-citrulline, catalyzed by the enzyme nitric oxide synthase (NOS) (reviewed by 18). However, no genes with homology to the mammalian NOS have been found in the wholly sequenced Arabidopsis genome. Strikingly, inhibitors of mammalian NOS have been successfully used to block NO

production in plants, and formation of L-citrulline from Arg has been demonstrated (reviewed by 19,20).

Actually, nitrite (NO_2^-) reduction is considered the major source of NO in plants (Fig. 1). Nitrite reduction can occur by both non-enzymatic and enzymatic mechanisms. Non-enzymatic nitrite reduction occurs spontaneously in the apoplast, due to the acidic conditions or to the presence of ascorbic acid or phenols[21]. The proposed nitrite reduction by enzymatic mechanisms includes the reaction catalyzed by nitrite:NO reductase (Ni:NOR) in the plasma membrane[22], and the reaction catalyzed by nitrate reductase (NR) in the cytosol[23,24].

The primary role of NR in plants is nitrogen assimilation, through the NAD(P)H-dependent reduction of nitrate to nitrite, which is subsequently reduced to ammonium by the nitrite reductase (NiR). The ammonium is then incorporated into amino acids and other nitrogen-derived compounds through the glutamine synthetase/glutamine-2-oxoglutarate transaminase system[25]. In addition to this role, some authors propose that NR can catalyze the reduction of nitrite to NO[23,24]. The efficiency of NR in NO production, however, is low, and requires low oxygen tensions and high nitrite levels[23,24], raising the possibility that this activity is not relevant under physiological conditions. Thus, an alternative view suggests that the main role of NR in NO synthesis is the generation of nitrite, which will be subsequently reduced to NO by electrons leaked from the mitochondrial respiratory chain[26]. A nitrite-reducing mitochondrial activity was observed in the alga *Chlorella sorokiniana*, in tobacco suspension cells, and in mitochondria isolated from yeast and animal cells (27 and references therein), as well as from various plant species[26,28]. These results suggest the existence of a common Arg-independent mechanism for NO production in living organisms. In addition to the mitochondrial respiratory chain, nitrite reduction to NO by the electron transporters of chloroplasts has been reported[29].

More recently, it was shown that polyamines induce NO production in plants[30]. Thus, Arg, as a biosynthetic precursor of polyamines, may indirectly affect NO generation independently of NOS activity (Fig. 1), as revealed by the study of Arabidopsis mutants devoid of arginase activity[31]. Another suggested mechanism for plant NO biosynthesis involves hydroxylamines[32]. Although exogenous supply of hydroxylamine to tobacco cell cultures resulted in large amounts of NO released, the physiological significance of this effect remains unclear, since natural occurrence of hydroxylamines in plants has not yet been proven unequivocally (reviewed by 20).

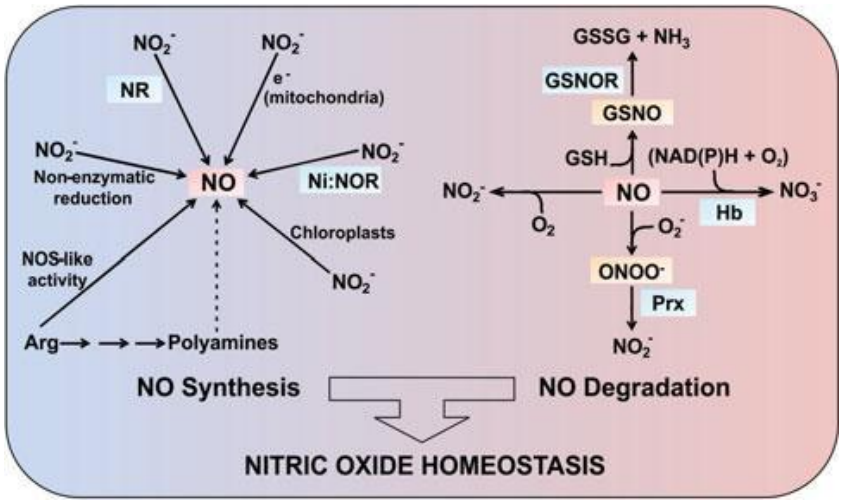

Fig. 1 Proposed routes for NO synthesis and degradation in plants, controlling NO homeostasis. Abbreviations: Arg L-arginine, GSH reduced glutathione, GSSG oxidized glutathione, GSNO S-nitroso-glutathione, GSNOR S-nitrosoglutathione reductase, Hb class-1 non-symbiotic hemoglobin, Ni:NOR nitrite:NO reductase, NOS NO synthase, NR nitrate reductase, Prx peroxiredoxin

Nitric oxide reaction with biomolecules

NO is a gaseous free radical, uncharged, and with a relatively long half-life (approximately 5 s) when compared to other radicals[33]. As one of the smallest diatomic molecules, NO exhibits a good diffusion rate through hydrophobic and hydrophilic compartment[33], facilitating its interaction with biomolecules and other compounds in the surrounding cells. In particular, NO and its derivatives can react with thiols, tyrosine residues, metal centers, and reactive oxygen species (reviewed by 33,34), as discussed below.

Protein S-nitrosylation

Protein *S*-nitrosylation is the covalent and reversible attachment of NO to thiol side chains of reduced Cys residues (reviewed by 35). Proteomics analysis has revealed the existence of numerous protein candidates for *S*-nitrosylation in plants[36,37]. Recently, *S*-nitrosylation has emerged as the prototypic redox-based post-translational modification of proteins required for plant immunity (reviewed by 38).

Key proteins (see below) involved in the induction of programmed cell death and in the transcriptional reprogramming of host cells during plant immunity responses have been reported to be *S*-nitrosylated on specific Cys residues. Additionally, Arabidopsis cytosolic glyceraldehyde 3-phosphate dehydrogenase (GADPH) and metacaspase 9 were identified as targets for *S*-nitrosylation in plants, and *S*-nitrosylation of methionine adenosyltransferase 1, involved in ethylene biosynthesis, was shown to cause inhibition of ethylene production (reviewed by 39). NO also enhances desiccation tolerance of recalcitrant seeds via *S*-nitrosylation[40], and influences auxin signaling through *S*-nitrosylation of TIR1 (transport inhibitor response 1), one of the main intracellular auxin receptors[41].

It is currently believed that the likelihood of *S*-nitrosylation of a particular protein is a reflection of the intracellular levels of *S*-nitrosoglutathione (GSNO), which has been proposed to be the major NO reservoir and donor in cells[33,36]. GSNO formation results from the spontaneous reaction of NO with reduced glutathione (GSH). GSNO can transfer the NO moiety directly to other thiol groups, and this process is called transnitrosylation[42]. Intracellular GSNO levels are controlled mainly through the activity of the evolutionary conserved enzyme GSNO reductase (GSNOR), which catalyzes the reduction of GSNO to GSSG and NH_3 (Fig. 1). This enzyme controls GSNO levels in plants, animals, and bacteria, as well as the likelihood of protein *S*-nitrosothiol (SNO) formation[42].

Nitric oxide reaction with superoxide anion

NO can react non-enzymatically with superoxide anion to form peroxynitrite ($ONOO^-$), and this is an important mechanism for NO degradation[43] (see Fig. 1). Recent studies have shown that external NAD(P)H dehydrogenases from plant mitochondria are important sources of superoxide formation by electron leakage, thereby promoting mitochondrial NO degradation[44,45].

$ONOO^-$ can be enzymatically degraded to nitrite by peroxyredoxins (Prx; see Fig. 1). PrxII E activity is inhibited by *S*-nitrosylation, thus resulting in inhibition of $ONOO^-$ degradation, suggesting that *S*-nitrosylation controls the levels of intracellular $ONOO^-$ (46).

Protein tyrosine nitration

Protein tyrosine nitration is a NO-mediated posttranslational modification, in which $ONOO^-$ transfers a nitro (NO_2) group to the aromatic ring of a Tyr residue (reviewed by 34). This type of modification can lead to profound structural and functional changes in proteins, some of which contribute to altered cell and tissue homeostasis (reviewed by 34). Recent studies have suggested that nitration of Tyr residues may be a relevant regulatory mechanism in the plants responses to both abiotic and biotic stress. Increased levels of Tyr nitration have been described after light, salt, and shear stress in pea plants, olive leaves, and *Taxus cuspidate* suspension cultures, respectively, and during the progression of the hypersensitive response in *Arabidopsis thaliana* and in tobacco BY2 suspension cells treated with fungal elicitin (47 and references therein). Moreover, proteomic analysis identified a large number of putative nitrated proteins in different plant species (see 48 and references therein).

Tyr nitration has been well studied in animal systems, but little is known about its functional effects in plants[49]. It was recently shown that glutamine synthetase (GS), a key enzyme for nitrogen assimilation in the root nodules, is regulated by Tyr nitration[50]. It is long known that NO is produced in the nodules, where it inhibits nitrogenase leading to a concomitant decrease of ammonium generation. The inhibition of GS activity by Tyr nitration would shut down the ammonium assimilation pathway, in conditions of low or null nitrogenase activity[50]. Glutamate (Glu), a substrate for GS activity, is also a precursor for GSH synthesis. Thus, upon NO-mediated GS inhibition, Glu could be channeled for the synthesis of GSH, which is known to play a major role as antioxidant in root nodules.

Formation of metal nitrosyl complexes

One of the earliest identified reactions of NO in animal cells is with transition metals present in target molecules, to form nitrosyl complexes. The high affinity binding of NO to reduced iron of the heme group in the guanylate cyclase (GC), stimulates production of cyclic GMP (cGMP), which in turn affects intracellular calcium levels that modulate many cellular activities. This NO-cGMP-Ca^{2+}-signaling pathway is the major NO-mediated signalling pathway in animal cells (reviewed by

51). In an analogous manner, plants accumulate cGMP in response to NO, although a NO-sensitive GC enzyme has not been found in plants (reviewed by 39). Additionally, several studies have confirmed that NO is capable of modulating the activity of plant Ca^{2+} channels; increased Ca^{2+} levels lead to stimulation of NO synthesis in several situations (reviewed by 52). However, whether these NO- and Ca^{2+}-mediated processes in plants are transduced via GC is not yet known.

NO also binds to Fe^{2+} of the heme a3 group of cytochrome c oxidase (COX), in competition with O_2. COX is the terminal enzyme of the mitochondrial respiratory chain, and the NO binding results in a reversible inhibition of mitochondrial respiration[12]. In mammals, it has been proposed that the binding of nanomolar concentrations of NO to COX correlates with reduction of O_2 utilization, thus increasing O_2 availability for the cells located far from blood vessels[53]. In plants, NO-mediated COX inhibition might represent a mechanism to prevent strict anoxia in tissues with low oxygen supply (reviewed by 54). However, prolonged NO treatments cause plant cell death by affecting normal mitochondrial functions[55].

NO may also bind to the heme group of plant hemoglobins. It has been shown that oxygenated class-1 non-symbiotic hemoglobins catalyze the conversion of NO to nitrate[56] (see Fig. 1). A negative correlation between the expression of hemoglobins and NO emission has been observed in diverse plant species (57 and references therein). Therefore, non-symbiotic plant hemoglobins may play important roles in NO detoxification, in particular under hypoxic conditions[56]. In symbiotic root nodules, plant leghemoglobins, and bacterial proteins may play similar roles in the modulation of NO levels[58].

Control of gene expression by nitric oxide

As part of the NO important roles in plant physiological functions, there is genetic evidence of NO-dependent control of gene expression. Large-scale transcriptomic analysis have demonstrated that NO modulates the expression of large set of genes involved in diverse cellular functions, such as defense, signal transduction, transport, basic metabolism, and antioxidant response[59,60]. It is noteworthy that a high number of NO-modulated genes encode proteins functioning in stress-related responses (around 30%) (reviewed by 1).

Pioneering studies in soybean, Arabidopsis, and tobacco cells, demonstrated that NO donors induce the expression of defense-related genes that are also up-regulated by salycilic acid (SA)[61,62]. Further studies using plants with altered NO-levels and large-scale transcriptional analysis corroborated the importance of the NO-mediated modulation of SA- and JA-dependent pathways during plant defense responses to herbivore and pathogen attack (reviewed by 63; see also 64).

NO is also involved in the transcriptional modulation of plant responses to diverse types of abiotic stress (reviewed by 63,65). In particular, NO mediates the induction of antioxidant enzymes involved in plant tolerance to abiotic stress, some of which are ROS-scavenging enzymes[65]. In addition, NO upregulates the expression of alternative oxidase (AOX)[66], an important mitochondrial protein that, by decreasing the pool of reduced ubiquinone, reduces ROS production from the respiratory chain. Moreover, AOX is not inhibited by NO (differently to cytochrome c oxidase), and thus allows mitochondrial respiration to occur in the presence of NO[12].

The NO-mediated control of gene expression is not restricted to stress responses, but also affects processes such as lateral root formation, flowering, symbiosis, and iron homeostasis (reviewed by 63). NO was identified as a repressor of floral transition, through the upregulation of the key floral repressor gene FLOWERING LOCUS C, which suppresses the expression of the floral promotive genes LEAFY, CONSTANS, and GIGANTEA[67]. Therefore, *nox1* mutant that overproduces NO, as well as NO-treated plants, show delayed flowering, whereas *Atnoa1* mutant that is NO deficient, shows early flowering in comparison to wild-type plants[67]. Further studies with *nia1nia2* double mutant, and with Arabidopsis plants that have been engineered for gene silencing or gene overexpression of a non-symbiotic hemoglobin, corroborated the negative correlation between NO levels and flowering time[57,68].

Nitric oxide is a key signal in plant disease resistance

Plants resistance to diseases is determined by mechanisms activating a broad range of defenses, including crosslinking of cell wall proteins, generation of reactive oxygen species (ROS), local programmed cell death, and activation of pathogenesis-related (PR) genes both at local and systemic sites. This set of defenses involves

specific plant receptors recognizing different signals released by the pathogens, and protects the entire plant tissues from subsequent invasions by a broad range of pathogens (plant immunity) (reviewed by 69,70).

The hypersensitive response (HR) is characterized by the rapid cell death in plant tissues surrounding the infection site, and requires production of ROS and NO[61]. ROS are mainly produced by activation of NADPH oxidases present in the plasma membrane, which catalyze O_2^- formation from O_2. Importantly, NADPH activity is inhibited by *S*-nitrosylation, suggesting a negative feedback loop for the attenuation of ROS generation to limit the HR spread[71].

Accumulation of salicylic acid (SA) is another important signaling molecule in plant defense (reviewed by 70). A key transcription factor in the SA-mediated pathways is NPR1 (non-expressor of pathogenesis-related protein 1). NPR1 activity is redox-sensitive and the oxidized oligomeric form of NPR1 resides in the cytoplasm[72]; increase of SA upon pathogen infection triggers reduction of Cys residues, leading to NPR1 monomerization and rapid translocation to the nucleus. In the nucleus, NPR1 interacts with co-transcription factors of the TGA family, inducing transcription of defense genes (reviewed by 70). In addition, NPR1 is regulated by *S*-nitrosylation (controlled by NO/GSNO levels). *S*-nitrosylation promotes NPR1 oligomerization, and thus contributes to maintain the NPR1 cytoplasmic pool[73]. However, other authors showed that NO promotes NPR1 translocation to the nucleus, where it interacts with *S*-nitrosylated TGA1, enhancing TGA1 DNA-binding activity[74]. To conciliate these disparate results, it has been proposed that the *S*-nitrosylation-mediated oligomerization might not have an inhibitory effect on NPR1 activity, but may constitute a step prior to monomer accumulation, favoring the idea of a positive effect of NO/GSNO on plant defense. Additionally, Lindermayr et al.[74] propose that a secondary, activating *S*-nitrosylation of NPR1 might occur once this protein is already in the nucleus.

The SA-binding protein SAB3, which is a positive regulator of plant immunity, is also a target for *S*-nitrosylation. In this case, SAB3 post-translational modification abolishes its SA-binding capacity and strongly reduces its carbonic anhydrase activity, which is required for immune signalling[75].

As discussed above, NO also activates directly the expression of defense-related genes, such as those encoding phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), two enzymes of the phenylpropanoid pathway involved in

the synthesis of flavonoids with antimicrobial activity. Studies in potato tubers, soybean cotyledons, and wheat plants confirmed the importance of NO in the production of antimicrobial compounds in plant–pathogen interactions (reviewed by 76).

Role of NR in stress-triggered nitric oxide production

Early studies on NO function in plants proposed the existence of a NOS-like enzyme as the main responsible for NO production in plant defense[61]. However, it was later shown that in *A. thaliana–Pseudomonas syringae* interactions NO production was dependent on nitrite accumulation derived from NR activity[26]. This was corroborated by using the NR-deficient Arabidopsis *nia1nia2* double mutant, which exhibits lower NO emission and impaired HR when inoculated with avirulent strains of *P. syringae*[26,77,78] or in the presence of the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*[79]. Arabidopsis *nia1nia2* mutants also have reduced levels of amino acids; treatments with glutamine were able to restore the wild-type amino acid levels, but not the resistance to avirulent bacteria[80], thus excluding that plant susceptibility resulted from defects in nitrogen metabolism. Differently, infiltration of *nia1nia2* leaves with nitrite causes increased NO emissions and activation of HR in pathogen-challenged plants[26,77], supporting the idea that NR is only required to generate the nitrite necessary for NO production (Fig. 2). Nitrite reduction to NO during *A. thaliana–P. syringae* interactions is thought to be carried out by the mitochondrial respiratory chain[26].

NR-dependent NO synthesis in plants has also been reported to occur in response to endogenous and pathogen elicitors, and in other stressful situations, such as those that induce stomatal closure, in roots hypoxic response, cold acclimation, and freeze tolerance, and also in nitrogen-fixing nodules ([81,82] and references therein).

GSNOR controls nitric oxide homeostasis and signaling under stress

It has been reported that plants with null or reduced expression of GSNOR show increased levels of total *S*-nitrosothiols (SNO), and conversely, that GSNOR

over-expressing plants show reduced SNO content[83,84]. This role of the GSNOR enzyme in modulating intracellular SNO levels has important consequences on plant immunity. Using an anti-sense strategy, Rustérucci et al.[84] reported that plants with decreased GSNOR activity (50%) showed enhanced basal resistance and enhanced induced systemic acquired resistance (SAR), whereas GSNOR overexpressing plants showed increased susceptibility to pathogens compared to wild type plants. Strikingly, other authors showed that null mutants for the AtGSNOR1 gene were compromised in both basal and pathogen-induced (gene-for-gene) resistance[83]. A remarkable difference in these two types of mutants was that the content of SA, also necessary for plant immunity, was not modified in the antisense plants[64,84], but was drastically reduced in GSNOR null mutants. Moreover, GSNOR null mutants were also insensitive to exogenous SA[71,83]. The apparently contradictory results obtained with null[83] and antisense[84] mutants might be conciliated if the complex regulation of NPR1, is considered. Thus, GSNOR knockout mutants might entirely hinder activation of the NPR1/TGA1-signalling pathway by their inability to remove the over accumulation of GSNO, whereas diminished levels of GSNOR activity in the antisense plants might favor the existence of the appropriate ratio of *S*-nitrosylated/ NPR1/TGA1 forms, with a positive effect on plant defense, as has been postulated in Espunya et al.[64].

It has also been reported that GSNOR might be a key regulator of systemic defense responses, both in wounding and pathogenesis (Fig. 2). GSNOR is transcriptionally regulated by wounding, SA and JA, both at local and systemic sites[85]. Espunya et al.[64] showed that GSNO levels increased rapidly and uniformly in injured Arabidopsis leaves, whereas in systemic leaves GSNO was first detected in vascular tissues and later spread over the parenchyma. These results suggest that GSNO is involved in the transmission of the wound mobile signal through the vascular tissue. Moreover, GSNO accumulation is required to activate the JA-dependent wound responses, whereas the alternative JA-independent wound-signalling pathway does not involve GSNO.

GSNOR also modulates SNO levels in response to various abiotic stresses, and this is important for resistance and acclimation. Mutation of the AtGSNOR1 gene results in plants resistant to the herbicide PARAQUAT[86] and reduction of GSNOR activity was shown to be necessary to overcome cadmium stress[87]. On the other hand, null GSNOR mutants were unable to acclimate to high temperatures[88].

Moreover, GSNOR activity increases by arsenic stress in *A. thaliana* and by low temperatures in pea seedlings and pepper plants (reviewed by [89]). Recently, analysis of oxygen uptake in isolated mitochondria from Arabidopsis cultured cells uncovered the role of GSNOR in modulating the activity of mitochondrial respiratory chain and energy conservation[90]. Experiments performed under optimal growth conditions or under nutritional stress showed that mitochondrial complex I and external NADH dehydrogenase were inhibited under stress conditions in cells overexpressing GSNOR, whereas NADH dehydrogenase was constitutively activated in GSNOR antisense cells. Furthermore, GSNOR over-expressing cells were unable to activate the enzyme alternative oxidase (AOX), and GSNOR antisense cells did not present inhibition of uncoupling protein (UCP) activity under stress. Altogether, these findings suggest that GSNOR activity may control the cellular redox state by affecting mitochondrial bioenergetics under adverse situations.

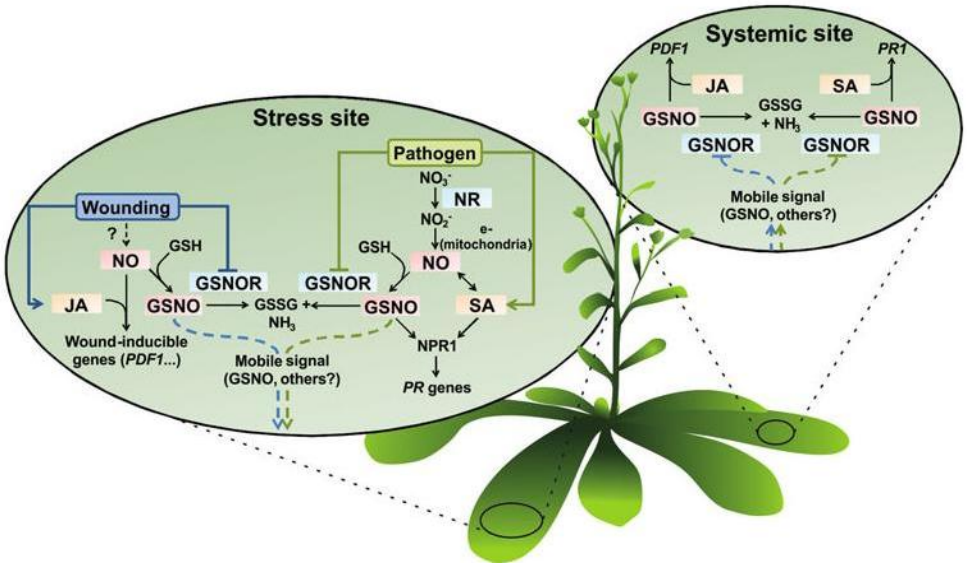

Fig. 2 Proposed model of action for GSNOR and NR in NO homeostasis and plant immunity. After pathogen challenging, activation of NR at the local site leads to accumulation of NO_2^-, which is then reduced to NO by the mitochondrial electron transport system. New synthesis of NO rapidly raises the concentration of GSNO and other nitrosothiols; moreover, the transcriptional inhibition of GSNOR also contributes to maintain the enhanced GSNO pool. Then, GSNO together with SA modulates the activity of the transcription factor NPR1 in the SA-dependent pathway, and together with JA activates the wound-inducible responses. GSNO and/or other signaling molecules are transported to systemic sites, increasing the systemic GSNO pool (to which also contributes the transcriptional inhibition of GSNOR, among other possible mechanisms) and activating SA- or JA-dependent pathways.

Conclusions and future perspectives

The interest on NO as a signaling molecule in plants has increased exponentially over the last few years, opening new horizons in various aspects of plant physiology and metabolism, both under normal and adverse conditions. The great ability of NO to interact with and modify different targets within the plant cells may explain the multiple roles of this radical in plant biology. An important goal for the future will be to fully identify the mechanisms responsible for NO synthesis in plants, which still remain elusive. The efforts of many laboratories to unveil the role and source of NO during plant–pathogen interactions have been quite successful, and nowadays it is known that NR and GSNOR are important regulators of these processes. Our understanding of the NO-mediated mechanisms in response to other environmental stresses, and in plant physiology and development must be extended in the future. Understanding how the various metabolic pathways that control NO homeostasis are tunneled for the regulation of specific processes in plants must be one of the major objectives of NO research in the near future.

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CHAPTER II

About the original experimental contributions of this Thesis.
About the intimate interplay between nitric oxide signalling and nitrate assimilation in plants.

S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway

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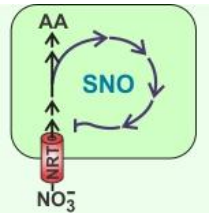

Abstract

Nitrogen assimilation plays a vital role in plant metabolism. Assimilation of nitrate, the primary source of nitrogen in soil, is linked to the generation of the redox signal nitric oxide (NO). An important mechanism by which NO regulates plant development and stress responses is through *S*-nitrosylation, that is, covalent attachment of NO to cysteine residues to form *S*-nitrosothiols (SNO). Despite the importance of nitrogen assimilation and NO signalling, it remains largely unknown how these pathways are interconnected. Here we show that SNO signalling suppresses both nitrate uptake and reduction by transporters and reductases, respectively, to fine tune nitrate homeostasis. Moreover, NO derived from nitrate assimilation suppresses the redox enzyme *S*-nitrosoglutathione Reductase 1 (GSNOR1) by *S*-nitrosylation, preventing scavenging of *S*-nitrosoglutathione, a major cellular bio-reservoir of NO. Hence, our data demonstrates that (S)NO controls its own generation and scavenging by modulating nitrate assimilation and GSNOR1 activity.

Introduction

Nitrogen is a conspicuous building block of many central biomolecules, such as nucleic acids, amino acids and cofactors. The primary source of nitrogen available to land plants is inorganic nitrate (NO_3^-), the concentration of which can vary from micromolar to millimolar amounts in soils[1]. To cope with such large fluctuations in nitrate availability, higher plants have evolved sophisticated high-affinity and low-affinity transport systems[2,3]. These systems rely mainly on two families of membrane-bound nitrate transporters (NRTs) of which NRT2 members are high affinity, while most members of NRT1 are low-affinity NRTs[2,3]. NRT2.1 and NRT1.1 (firstly identified as CHL1, for chlorate resistant 1) are particularly important for nitrate uptake by roots of Arabidopsis thaliana plants[4]. AtNRT2.1 is a pure high-affinity NRT that is repressed by high nitrate levels and activated under low nitrate conditions[5]. AtNRT1.1 is an exception in the NRT1 family being a dual-affinity NRT: it normally has low-affinity uptake but can change to the high-affinity mode under low nitrate levels[6,7]. The switch from low- to high-affinity transport is mediated by phosphorylation at Thr101 residue of NRT1.1, which enhances its affinity to nitrate[8,9], as well as by transcriptional downregulation of NRT1.1 and upregulation of NRT2.1 allowing scavenging of available nitrate[2,7].

Once taken up by roots, nitrate is mainly transported to shoots for further assimilation and in leaves it is reduced to nitrite (NO_2^-) by the activity of NAD(P)H-dependent cytosolic nitrate reductases (NR). Nitrite, in turn, is promptly removed from cells or transported to chloroplasts where it is reduced by nitrite reductase into ammonium (NH_4^+) for further assimilation into organic compounds by the glutamine synthetase/glutamine-2-oxoglutarate aminotransferase system[1,10,11].

In Arabidopsis, the catalytic activity of NR, which is considered limiting to nitrogen assimilatory pathways[12,13], is conferred by the genes *NIA1* and *NIA2*. Double mutant *nia1nia2* plants display poor growth on media with nitrate as the sole nitrogen source, which is in part due to the lack of nitrogen incorporation into amino acids[14]. Curiously, gene expression of *NRT1.1* and *NRT2.1* transporters is constitutively upregulated in roots of *nia1nia2* plants, suggesting that NR activity or a nitrogen-containing metabolite derived from nitrate reduction feedback regulate uptake systems[15]. However, the identity of this regulatory metabolite remains obscure[15,16].

In addition to its reduction to NH_4^+, nitrite can be reduced to nitric oxide (NO) via non-enzymatic as well as various enzymatic pathways [17,18]. High levels of nitrite allow NR to reduce this assimilate into NO[19,20], although genetic evidence suggests that the main role of NR in NO biosynthesis is the production of nitrite[21,22]. L-arginine, polyamines and hydroxylamines are also potential sources for NO synthesis in higher plants; however, the molecular mechanisms responsible for these activities have not been identified so far[17,18,23].

NO is a free radical with a wide range of important signalling functions in all eukaryotes. Accordingly, Arabidopsis NO-over-producing *nox1* (also known as *cue1-6*) mutants accumulate elevated levels of NO and exhibit defects in floral transition, root apical meristems and pathogen-induced programmed cell death[24–26]. The underlying mechanisms of NO action rely on its physicochemical properties that allow reactivity with different kinds of biomolecules, thereby altering the redox state of their active groups. NO and its derivatives can react with thiols, tyrosine residues, metal centres and reactive oxygen species[17,27]. Particularly, addition of NO to cysteine thiols results in the formation of *S*-nitrosothiols (SNO), which have been shown to alter the activity, localization or conformation of target proteins[27,28].

NO may also react with glutathione (GSH) to form S-nitrosoglutathione (GSNO), which is thought to be a major cellular reservoir of NO capable of generating protein-SNO. Cellular GSNO levels are controlled by the evolutionary conserved, cytosolic enzyme GSNO reductase 1 (GSNOR1), which catalyses the NADH-dependent reduction of GSNO to oxidized GSH and ammonium[29–31]. Arabidopsis plants with impaired GSNOR1 function display elevated levels of protein-SNO and exhibit deficiencies in development, immunity and thermotolerance, indicating that GSNOR1 indirectly controls the level of biologically active protein-SNO[31–36]. Taken together, these studies clearly indicate that the generation and scavenging of NO is connected to molecular pathways of nitrogen assimilation. However, it remains unclear if nitrate or other nitrate-derived metabolites directly affect NO signalling, and vice versa, if NO signalling influences nitrogen homeostasis[37]. Here we provide genetic and biochemical evidence for intimate interplay between nitrate assimilation and NO signalling. We identified novel NO-mediated feedback pathways that regulate the transcription of NRTs and enzymatic activities of NR as well as GSNOR1 by redox-based post-translational modification.

Our data reveal that nitrate assimilation and NO signalling are connected in unexpected ways, allowing plants to fine tune NO generation and scavenging.

Results

NO signalling feedback regulates nitrogen assimilation

Because significant amounts of NO and derived SNO result from the nitrogen assimilation pathway, we considered that NO may feedback regulate this pathway. Nitrogen assimilation commences by the uptake of nitrate by low- and high-affinity transport systems, in which the *NRT1.1* and *NRT2.1* transporter genes play key roles[2,3]. We assessed the expression of these genes in roots of wild-type (WT) plants as well as the NO and SNO signalling mutants, *nox1* (ref. 24) and *gsnor1* (ref. 34) (also known as *par2-1*). While nox1 plants overproduce free NO, gsnor1 plants accumulate high levels of GSNO [28], a more stable redox form of NO. Compared with WT plants grown under moderate nitrate availability, the expression of *NRT2.1* was strongly suppressed in both *nox1* and *gsnor1* mutants, whereas the expression of NRT1.1 remained unchanged (Fig. 1a). Accordingly, exogenous treatment of WT plants with GSNO or the alternative NO donor, DEA/NO, also led to the inhibition of *NRT2.1* expression but left *NRT1.1* expression unaltered (Fig. 1b). These findings suggest that elevated NO and SNO levels induce a switch from high- to low-affinity nitrate transport.

Once taken up into the root, nitrate is mainly transported to the shoots where it is assimilated at the expense of photosynthetic reducing power[2]. In leaves, nitrate is reduced to nitrite by the cytosolic enzyme NR. To examine if NO also regulates this rate-limiting step in nitrogen assimilation, we measured NR activity in leaves of the genotypes with altered (S)NO homeostasis (Fig. 1c). Compared with WT, *gsnor1* mutant plants exhibited strongly reduced NR activity, while GSNOR1-overexpressing plants (35S::FLAG-GSNOR1, Supplementary Fig. 1a,b) displayed enhanced NR activity. Surprisingly, however, mutant *nox1* plants, which only accumulate 30–40% more SNO than WT plants under basal conditions[26], did not exhibit altered NR activity (Fig. 1c). Together with the fact that expression of the NR gene *NIA2* was indifferent in all mutants (Supplementary Fig. 1c,d), these data suggest that GSNO and free NO differentially affect NR activity. To understand the cumulative

consequences of (S)NO-modulated nitrate transport and reduction, we also measured nitrate contents of mutant leaves and compared them with WT and NR double mutant *nia1nia2* leaves, the latter of which is known to accumulate high levels of nitrate due to lack of NR activity[15]. Figure 1d shows that *nox1* plants accumulated significantly less nitrate than the WT, likely caused by partial switching to the low-affinity transport system in these mutants (Fig. 1a). However, in *gsnor1* plants, activation of the low-affinity transport system in conjunction with reduced NR activity apparently resulted in relatively normal nitrate levels. Instead, a regulatory role for GSNO only became apparent in 35S::FLAG-GSNOR1 plants, which accumulated elevated levels of nitrate (Fig. 1d). Taken together, these findings indicate that NO and SNO modulate nitrogen assimilation by differentially inhibiting nitrate uptake and reduction.

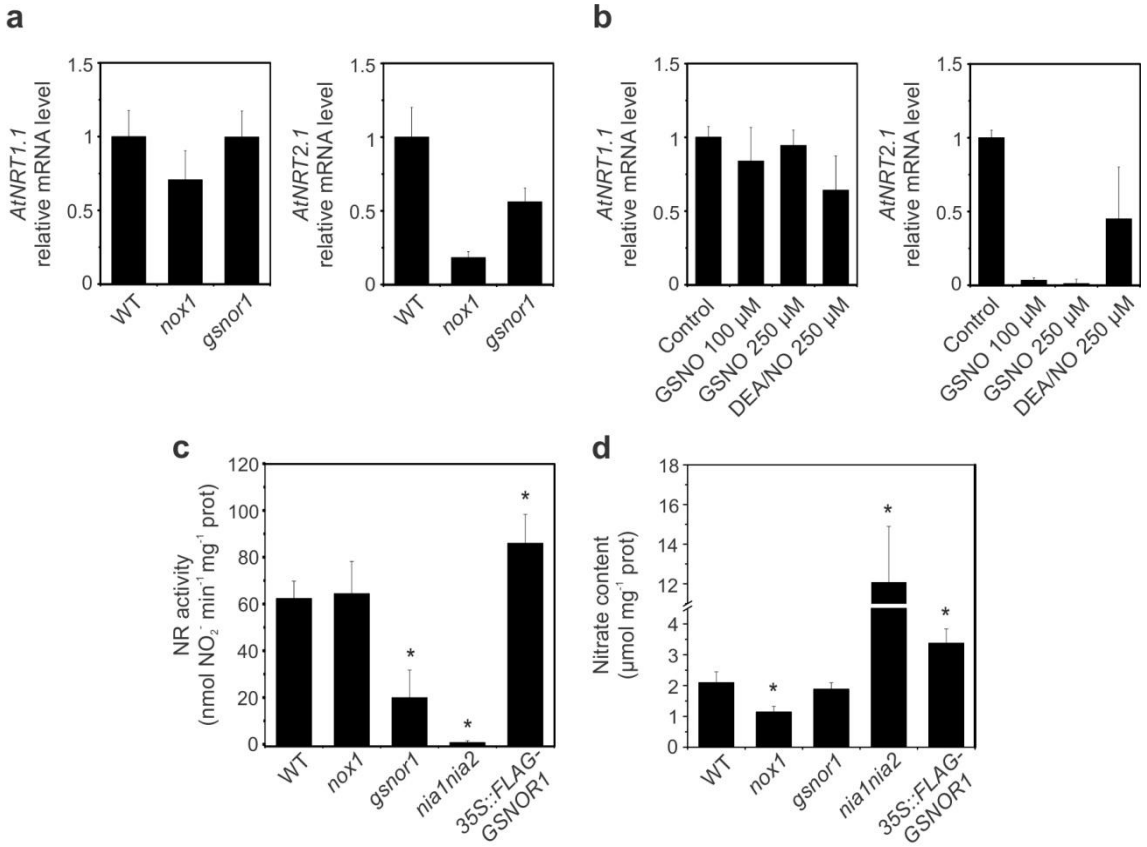

Figure 1 Nitrate uptake and reduction in plants with altered NO signalling. (a) Expression of the NRT marker genes NRT1.1 and NRT2.1 in the roots of WT, *nox1* and *gsnor1* plants was determined by quantitative reverse transcriptase-PCR (qRT–PCR) and normalized to expression of *ACT2*. Error bars represent s.d. (n = 3). (b) Effect of GSNO on

nitrate-induced expression of NRT genes in roots. WT seedlings grown in half-strength MS medium (9.4 mM KNO_3 and 10.3 mM NH_4NO_3) were incubated for 3 h in water with 1 mM nitrate (KNO_3), in the absence or presence of GSNO or DEA/NO. NRT expression was determined by qRT–PCR and normalized to expression of *ACT2*. Error bars represent s.d. (n = 3). (c) NR activity and (d) nitrate (NO_3^-) content determined in leaf extracts of WT plants and genotypes with enhanced (*nox1* and *gsnor1*) or impaired (*nia1nia2* and 35S::FLAG-GSNOR1) (S)NO homeostasis, after 6 hours of light. Data points represent means ± s.d. of three independent experiments. Asterisks indicate statistical differences from the WT (Student's t-test, $P<0.05$).

To assess the biological impact of (S)NO on nitrate assimilation, we analysed the vigour of (S)NO signalling mutants by measuring growth and biomass accumulation parameters (Fig. 2a–d). As expected, the inability of *nia1nia2* plants to reduce nitrate led to reduced leaf area and a decrease in dry shoot weight compared with WT. Like *nia1nia2*, mutant *nox1* and *gsnor1* plants also displayed strongly decreased growth vigour. Conversely, leaf area and biomass growth tended to increase, albeit not always statistically significant, in GSNOR1-overexpres-sing 35S::FLAG-GSNOR1 plants (Fig. 2a–d). These findings suggest that (S)NO-mediated suppression of nitrate assimilation may have dramatic effects on plant growth. To confirm the poor growth vigour phenotypes of (S)NO mutants were due to decreased nitrate assimilation, we sought to bypass this pathway by the exogenous addition of glutamine (Gln), the main end product of nitrate assimilation. Addition of Gln to WT plants did not further improve growth compared with nitrate-replete conditions (Supplementary Fig. 2). Importantly, however, irrigation of *nox1* and *gsnor1* mutants in the presence of Gln recovered growth vigour of *gsnor1*, but not that of *nox1*, to levels comparable to those of WT and 35S::FLAG-GSNOR1 plants (Fig. 2a–d). Thus, bypassing both nitrate uptake and reduction by feeding Gln rescued the *gsnor1* phenotype, while suppression of N assimilation may not be the only cause for lack of growth vigour in *nox1* plants. In contrast to GSNOR1 that is directly involved in NO homeostasis, the metabolic changes in *nox1* that lead to an increase in NO production are indirect. The *nox1* mutant is defective in a phosphoenolpyruvate/phosphate translocator that imports phosphoenolpyruvate for the synthesis of aromatic amino acids and related compounds through the shikimate pathway[38]. Consequently, *nox1* mutants display an overall marked increase in the levels of free amino acids, including L-arginine, a precursor of NO[24] (Supplementary

Fig. 3). In addition, this mutant exhibits an imbalance of aromatic versus non-aromatic amino acids and a marked reduction in secondary phenolic compounds that are dependent on the shikimate pathway for precursors, severely compromising the establishment of photoautotrophic growth[38]. Thus, alterations of amino acid levels as a direct consequence of the *nox1* mutation are not expected to be complemented by simply adding Gln, as other imbalances are not corrected by this treatment. Accordingly, the effect of the *nox1* mutation on biomass is much more severe than the *nia1nia2* knockout mutation (Fig. 2a–d), indicating that mechanisms unrelated to nitrate assimilation underpin the *nox1* phenotype. In contrast, our data indicate that mutation of GSNOR1 affected plant growth by inhibiting nitrate uptake and assimilation (Fig. 1), and accordingly, this phenotype can be rescued by addition of Gln (Fig. 2a–d).

To further establish that suppressed nitrate assimilation underpins the poor primary productivity phenotype of *gsnor1* plants, we assessed the global accumulation of amino acids in this mutant (Supplementary Fig. 3). Particularly the accumulation of glutamine (Gln), glutamate (Glu), asparagine (Asn), and aspartate (Asp) are informative for nitrogen homeostasis, because they represent the primary transported amino acids derived from ammonium[11]. Several observations made in *gsnor1* mutants support the notion that (S)NO-mediated suppression of nitrate assimilation affects primary productivity. First, low nitrogen conditions stimulate the formation of Gln and Glu because of their comparatively lower nitrogen-to-carbon ratios (2N:5C for Gln and 1N:5C for Glu). Importantly, even in the presence of high nitrate, *gsnor1* plants accumulated more Gln and Glu compared with the WT (Fig. 2e,f), indicating that these mutants were suffering from nitrogen shortage. Second, compared with Gln and Glu, the amino acid Asn is rich in nitrogen (2N:4C ratio) and its production is therefore avoided under low nitrogen availability. Despite the presence of 25 mM nitrate, mutant *gsnor1* plants contained decreased levels of Asn compared with WT (Fig. 2e,f), further indicating that these mutants experience a shortage in nitrogen. Finally, in both WT and *gsnor1* plants, exogenous addition of 5 mM Gln led to an expected rise in endogenous Gln and also increased Asn content, while the levels of Glu and Asp remained largely unchanged compared with the low nitrate regime (Fig. 2e–g, Supplementary Fig. 3). Taken together, these data indicate that (S)NO are important regulators of nitrate assimilation and thus, plant growth and development.

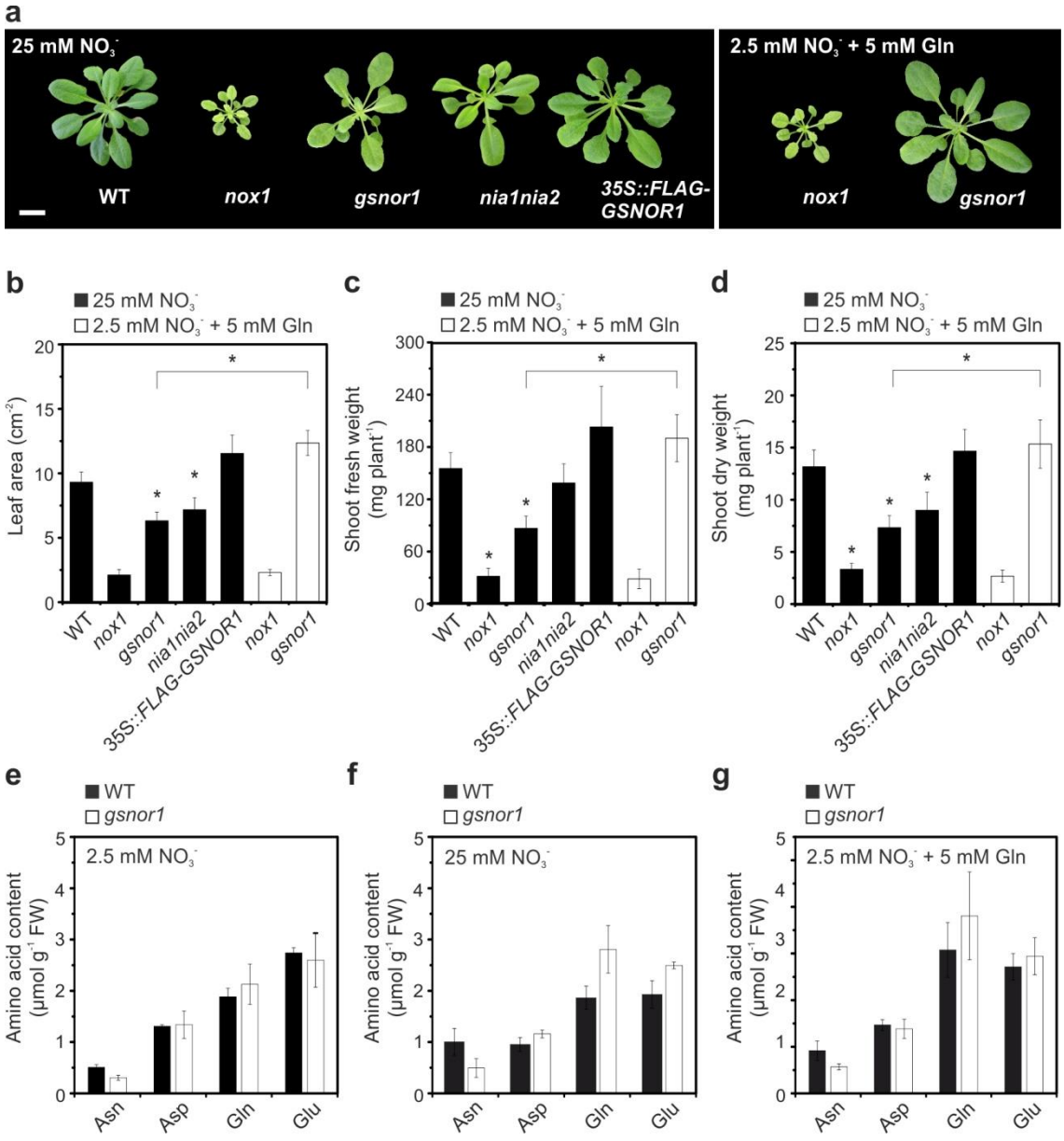

Figure 2 Growth vigour and amino acid content of genotypes with impaired and enhanced (S)NO signalling. (a) Phenotype, (b) leaf area, (c) shoot fresh weight and (d) shoot dry weight of 4-week-old plants grown on perlite:vermiculite (1:1) under 12 h/12 h light/dark and irrigated three times a week with a MS nutrient solution containing 25 mM nitrate (half KNO_3 and half NH_4NO_3) (black bars) or with 2.5 mM nitrate and 5 mM glutamine (white bars). Scale bar, 1 cm. Data points represent means±s.d. (n = 15 plants). (e–g) Contents of primary transported amino acids in WT and *gsnor1* leaves. Asn, asparagine; Asp, aspartate; Gln, glutamine; Glu, glutamate. Data represent means ± s.d. of three independent analyses. Asterisks indicate statistically significant differences from WT (Student's t-test, $P<0.05$).

Nitrogen metabolism regulates GSNOR1 activity

Given the impact of GSNOR1 on nitrate assimilation, we considered that GSNOR1 activity may be feedback regulated by nitrate. To examine this possibility, we grew WT plants under high nitrate availabilities that caused good growth vigour (25 and 40 mM), as well as lower nitrate availabilities (1 and 2.5 mM) that resulted in poor biomass accumulation (Supplementary Fig. 2). Because the irrigated nutrient solution was not compensated with any other nitrogen source, nitrate concentrations lower than 2.5 mM appeared undesirably detrimental for plant growth and development. Therefore we selected 2.5 and 25 mM nitrate concentrations for further experimentation. In addition, as the nitrate concentration of nutrient solutions was composed of half KNO_3 and half NH_4NO_3, we checked the possibility that the effects observed could partially be attributed to NH_4^+. However, when the concentration of NH_4^+ in the nutrient solution was reduced by 10 times (from 12.5–1.25 mM), parameters of biomass growth of WT plants were comparable (Supplementary Fig. 4), indicating that the effect of ammonium in determining plant growth vigour was negligible under our conditions. Interestingly, increasing nitrate availability from 2.5 to 25 mM reduced mean GSNOR1 activity by 35% ± 8% while significantly enhancing NR activity (Fig. 3a,b). Additional increase in nitrate availability to 40 mM did not suppress GSNOR activity any further, while intermediate nitrate levels (12.5 mM) reduced GSNOR activity by ~10% (Supplementary Fig. 5). These data suggest that nitrate levels may regulate GSNOR1 activity. However, mutant *nia1nia2* plants that are void of NR activity (Figs 1c and 3b) and accumulate elevated levels of endogenous nitrate[15] (Fig. 1d), did not exhibit reduced but rather slightly elevated GSNOR1 activity (Fig. 3a). Thus, instead of nitrate, a metabolite downstream of NR-catalysed nitrate reduction may be responsible for inhibition of GSNOR1 activity.

Nitrite is the first reductive metabolite downstream of nitrate and in elevated concentrations can be converted into NO[21,22,39]. Therefore, we measured NO emission in plants grown under low and high nitrate availability. High concentrations of nitrate promoted NO emission in WT plants (Fig. 3c and Supplementary Fig. 6). Despite having elevated endogenous nitrate levels, *nia1nia2* plants did not show elevated NO emission when grown under higher nitrate, indicating that high nitrate availability leads to NR-mediated generation of NO.

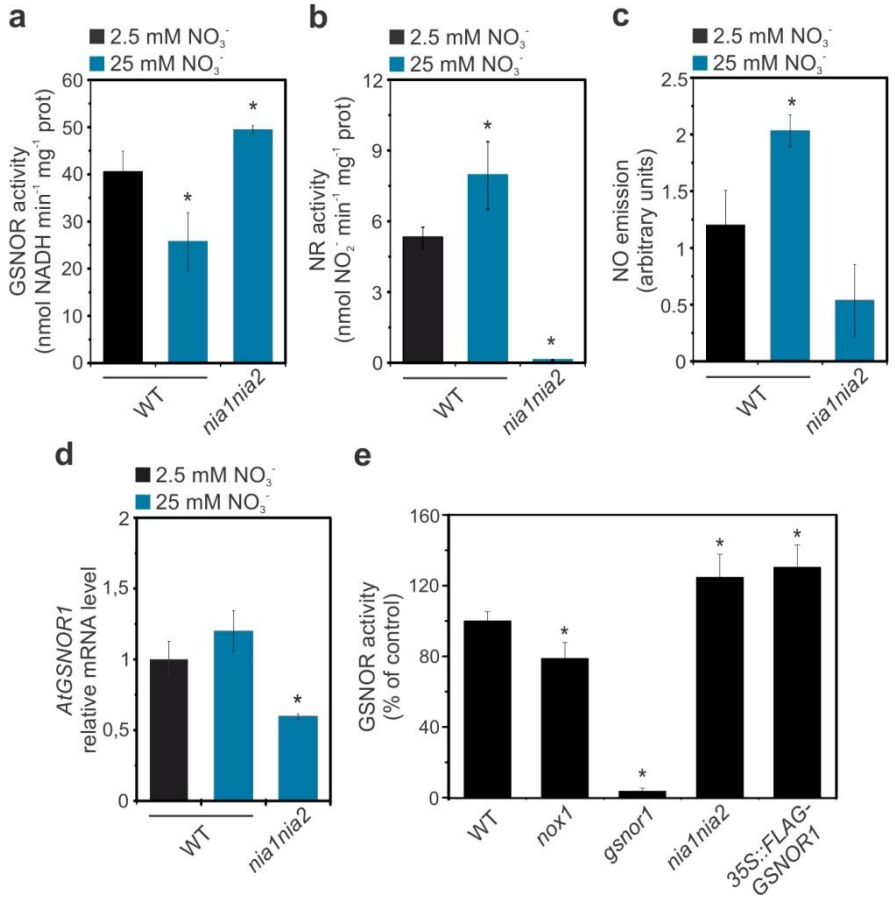

Figure 3 Nitrate-derived NO suppresses activity of GSNOR1. (a) GSNOR activity, (b) NR activity, (c) NO emission and (d) GSNOR1 gene expression measured in leaf extracts of WT and *nia1nia2* plants grown under low or high nitrate availability. Plants were grown in perlite:vermiculite (1:1) under a 12/ 12 h light/dark period and irrigated three times a week with MS nutrient solution containing 2.5 or 25 mM nitrate (half KNO_3 and half NH_4NO_3). All measurements were taken 6 hours post light exposure. Relative expression of GSNOR1 in (d) was determined by quantitative reverse transcriptase-PCR and normalized to expression of *ACT2*. (e) GSNOR activity in genotypes with impaired and enhanced (S) NO signalling. Plants were grown in soil with a photoperiod of 16/8 h light/dark and irrigated with water as needed. Data points represent means ± s.d. of three independent experiments. Asterisks indicate significant differences from the WT (Student's t-test, $P<0.05$).

Because nitrate-induced, NR-mediated NO production was associated with reduced enzymatic activity of GSNOR1 (Fig. 3a–c) but not gene expression (Fig. 3d), we considered a more direct role for NO in regulating the GSNOR1 enzyme. We examined this in genotypes with impaired and enhanced (S)NO signalling. Importantly, NO-overproducing *nox1* mutants displayed significantly reduced GSNOR1 activity (Fig. 3e). Conversely, *nia1nia2* double mutants that cannot synthesize NO through the NR pathway, exhibited increased GSNOR1 activity to

similar levels as 35S::FLAG-GSNOR1 plants. Collectively, these data suggest that the nitrogen assimilatory pathway inhibits GSNOR1 by a post-transcriptional, NO-dependent mechanism.

NO-induced S-nitrosylation inhibits GSNOR1

To further investigate if GSNOR1 is inhibited directly by NO or by other nitrogen assimilates, we measured its *in vitro* activity in pharmacological assays. Addition of the redox-active NO donors diethylamine NONOate (DEA/NO) and Cys-NO to WT leaf extracts resulted in dose-dependent inhibition of GSNOR1 with 15–30% decrease in activity already at only 50 mM of NO donors and over 60% at 250 mM (Fig. 4a). In contrast, the redox-active molecules, GSH and L-Cysteine that do not donate NO, had relatively little effect on GSNOR1 activity. Similarly, incubation with physiologically relevant concentrations of nitrogen assimilates (nitrate, nitrite and ammonium) did not affect GSNOR1 activity with the exception of high concentrations of the NO-related signal molecule peroxynitrite ($ONOO^-$), which is formed by the reaction of NO and O_2^- (Fig. 4b)[40]. Furthermore, when WT plants were fumigated for 12 h with 60 p.p.m. of NO gas, GSNOR activity in leaves was nearly 40% lower when compared with those exposed to normal air (44.6±2.6 versus 71.7±5.4 nmol NADH min^{-1} per mg protein) (Fig. 4c). Taken together, these data demonstrate a direct inhibitory effect of NO on GSNOR activity. NO has been well documented to regulate protein function by *S*-nitrosylation[24,41]. Therefore, we employed the biotin switch technique to examine if GNSOR1 is subjected to *S*-nitrosylation. This technique relies on specific reduction of SNO groups by ascorbate followed by their labelling with biotin[42]. Extracts of plants expressing 35S::FLAG-GSNOR1 (Supplementary Figs 1 and 7) were treated with or without the NO donor Cys-NO and subjected to the biotin switch technique. Figure 4d shows that Cys-NO induced strong *S*-nitrosylation of FLAG-GSNOR1 protein that was completely dependent on addition of ascorbate during biotin switching, indicating that GSNOR1 can be *S*-nitrosylated *in vitro*.

Next, we assessed if GSNOR1 is also *S*-nitrosylated *in vivo* by examining SNO modifications in NO-overproducing *nox1* plants that exhibit reduced GSNOR1 activity (Fig. 3e). To that end, we crossed 35S::FLAG-GSNOR1-expressing plants with *nox1* mutants and applied the biotin switch technique on the resulting homozygous progeny in which FLAG-GSNOR1 protein accumulated to comparable

levels as the parent line (Supplementary Fig. 7). Whereas most of the FLAG-GSNOR1 protein was unmodified in WT plants, it was significantly *S*-nitrosylated in *nox1* mutants (Fig. 4e). Taken together, these data indicate that nitrate-derived NO prevents scavenging of its major storage form by inhibitory *S*-nitrosylation of GSNOR1.

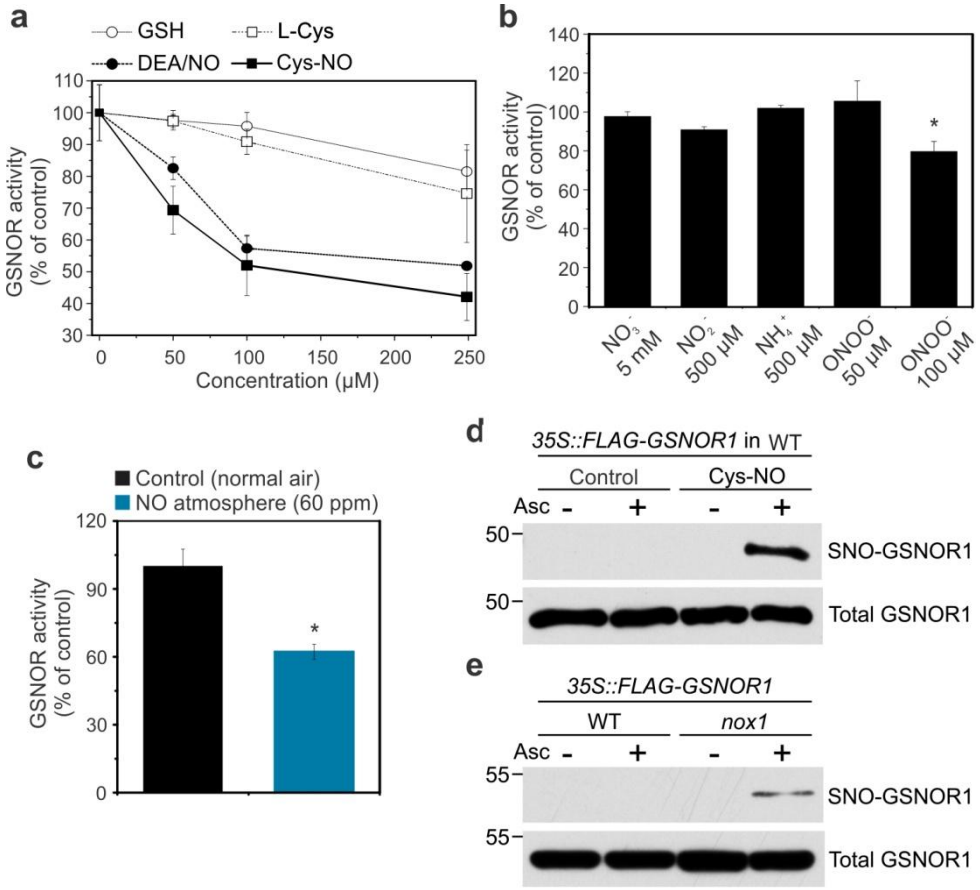

Figure 4 NO inhibits GSNOR1 by S-nitrosylation. (a) Dose response of GSNOR activity in WT leaf extracts supplemented with the *S*-nitrosylating agents DEA/NO and Cys-NO or the redox-active molecules GSH and L-Cys. (b) GSNOR activity in WT leaf extracts supplemented with indicated intermediates of the nitrogen assimilation pathway. (c) GSNOR activity in leaf extracts of WT plants fumigated with NO gas (60 p.p.m.) or with normal air for 12 h. (d) Cys-NO induced *S*-nitrosylation of GSNOR1 *in vitro*. Leaf extracts from 35S::FLAG-GSNOR1 plants in WT background were exposed to Cys-NO and subjected to the biotin switch technique. Total GSNOR1 protein ensures equal protein loading. The position of a 50-kDa marker is indicated. (e) *In vivo S*-nitrosylation of GSNOR1 using a 35S::FLAG-GSNOR1 construct in WT and *nox1* background. SNO-GSNOR1 was analysed and detected as in d. The position of a 55-kDa marker is indicated. Data points from a to c represent means ± s.d. of at least three independent experiments. Asterisks indicate significant differences from the controls (Student's t-test, P<0.05).

Discussion

As immobile organisms, plants have evolved to cope with environmental fluctuations by fine tuning metabolic pathways. Nitrogen metabolism is of particular importance as its intermediates influence plant development and responses to stress. Our study shows that NO, one of the end products of nitrogen metabolism, feedback regulates flux through nitrate assimilation pathways and controls its bioavailability by modulating its own consumption as depicted in Fig. 5.

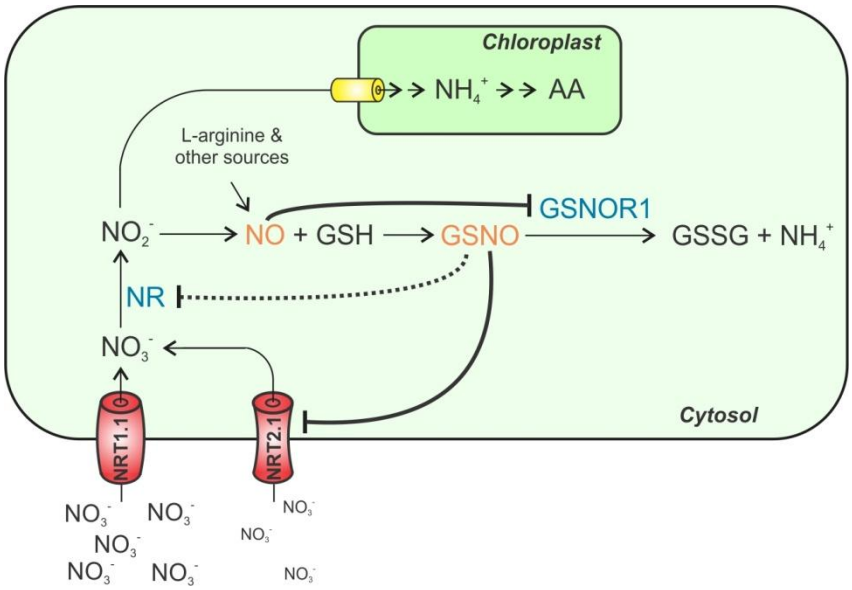

Figure 5 Schematic model for the control of nitrogen assimilation in plants through NO signalling. Nitrate (NO_3^-) is taken up by NRT in roots and reduced in leaves to nitrite (NO_2^-) by NR. Besides the transport to chloroplasts where it is reduced to ammonium (NH_4^+) and incorporated into amino acids (AA), nitrite can be reduced to NO by any favourable reducing power. NO is also thought to be generated from other sources, such as L -arginine. NO reacts with reduced glutathione (GSH) producing S-nitrosoglutathione (GSNO), the major cellular reservoir of NO. The levels of GSNO are controlled by the enzyme GSNO reductase (GSNOR1), which catalyses the reduction of GSNO to oxidized glutathione (GSSG) and ammonium (NH_3). GSNO inhibits nitrate uptake and reduction and NO *S*-nitrosylates and inhibits GSNOR1 preventing GSNO degradation. In this way NO, one of the end products of nitrogen metabolism, feedback regulates flux through nitrate assimilation pathway and controls its bioavailability by modulating its own consumption.

Previously it has been suggested that a metabolite resulting from nitrate reduction may feedback regulate nitrate uptake systems, but the identity of this metabolite remained unknown[15,16,23]. Genetic manipulation of NO signalling in our

experiments illustrated that NO controls flux through nitrogen assimilatory pathway by modulating the expression of NRTs and activity of NR (Fig. 1). Mutants that accumulate NO or GSNO displayed a classical switch in gene expression from high- to low-affinity transport, which is typically associated with decreased uptake of exogenous nitrate[4]. Moreover, genetically elevated levels of GSNO inhibited the activity of NR, while reduced levels promoted its activity. We show that the cumulative effects of perturbed NO signalling on nitrate uptake and reduction determined leaf nitrate content (Fig. 1), homeostasis of primary transport amino acids (Fig. 2e–g and Supplementary Fig. 3) and affected plant growth vigour (Fig. 2a–d). This NO-dependent mechanism may ensure the adjustment of plant growth according to nitrate availability.

In higher plants, NO is probably generated through a variety of mechanisms, including NO synthase-like activities, polyamine biosynthetic pathways and mitochondrial or peroxisomal pathways[20,23]. Notably, however, significant amounts of NO are also thought to be generated through a NR-dependent process, which may be particularly important in root architecture[43], floral transition[44], responses to abiotic stresses[45,46] and immune responses[22,47]. Thus, by suppressing nitrate uptake and reduction, NO may not only regulate nitrogen assimilation fluxes, it probably also feedback regulates its own generation.

Remarkably, NR activity was coupled to the level of functional GSNOR1 (Figs 1c and 3e). As GSNO often regulates enzyme activity through *S*-nitrosylation, it is tempting to speculate that NR is also subject to this post-translational modification. Indeed, NR is known to be regulated by other post-translational mechanisms, including phosphorylation and degradation. NIA2 was shown to interact with mitogen-activated protein kinase 6, resulting in site-specific phosphorylation that promoted NR activity[43]. Furthermore, phosphorylation of a distinct residue was shown to recruit inhibitory 14-3-3 proteins and may also promote NR proteolysis[48–50]. While we observed impaired NR activity in *gsnor1* plants that are deficient in functional GSNOR1, no effect was seen in NO-overproducing *nox1* plants (Fig. 1). Because NO donor stereochemistry and structure as well as allosteric effectors have a large influence on SNO reactivity[51], these data imply that NO and GSNO do not always modify the same target proteins. Indeed, NO radicals are thought to *S*-nitrosylate proteins directly through a radical-mediated pathway or indirectly via higher oxides of NO, whereas GSNO trans-nitrosylates cysteine residues[52].

Feeding experiments not only confirmed the previously described ability of nitrate to promote NR activity[53], they also demonstrated that elevated nitrate levels suppress GSNOR1 activity (Fig. 3). GSNOR1 plays an important role in controlling the cellular levels of GSNO, which is thought to be the main NO reservoir in cells. Accordingly, the mutation of GSNOR1 leads to elevated levels of protein SNO[31], indicating that GSNO functions as a potent cellular NO donor. As high NR activity promoted generation of NO (Fig. 3b,c), inhibition of GSNOR1 may be necessary to amplify SNO signals. Indeed, storing NO as GSNO dramatically prolongs its half-life[54], perhaps enabling plants to utilize NO more efficiently while curbing loss due to emission. Taken together, our data illustrate that nitrate availability promotes formation of a more stable pool of NO, which in turn feedback regulates nitrate assimilation, allowing plants to finely tune nitrogen homeostasis. They also indicate that nitrogen-based nutrient availability may influence a variety of NO-mediated signalling events. This is supported by recent reports showing that the form of nitrogen assimilation determines NO-mediated immune responses[55,56]. Arabidopsis *nia1nia2* mutants are susceptible to the bacterial pathogen *Pseudomonas syringae*, even after amino acid recovery by feeding with Gln[56]. Furthermore, treatment of WT tobacco plants with ammonium bypassed NR-mediated generation of NO and consequently compromised immune responses[55]. In contrast, application of nitrate or nitrite promoted both NO formation and immune-induced hypersensitive cell death, a process that restricts pathogen growth and is known to be stimulated by SNO[26,55,56].

It should be noted that although nitrate-induced NO emission was strongly reduced in absence of functional NR, residual NR-independent NO emission is still observed in *nia1nia2* (Fig. 3c, Supplementary Fig. 6). Taken together with the fact that NR-independent NO overproduction in *nox1* mutants decreased nitrate content in part by suppressing nitrate transport (Fig. 1), these data indicate that NR-independent NO production may also contribute to nitrate homeostasis (Fig. 5).

We showed that *in vitro* application of intermediates of nitrate assimilation did not affect GSNOR1 activity, whereas application of NO donors specifically blocked its activity even at low dosage (Fig. 4a,b). Accordingly, genetic manipulation of NO levels in *nox1* and *nia1nia2* plants (Fig. 3e) and direct fumigation of NO gas on WT plants (Fig. 4c) also impacted GSNOR1 activity *in planta*. Remarkably, the inhibitory effect of NO was associated with *S*-nitrosylation of GSNOR1 both *in vitro* and *in vivo* (Fig. 4d,e), indicating that this NO-scavenging enzyme is itself subject to direct

regulation by NO. So how does *S*-nitrosylation inhibit the activity of GSNOR1? Elucidation of the crystal structure of tomato GSNOR1 indicated the presence of a number of important cysteine residues that might serve as sites for *S*-nitrosylation[57]. Two clusters of cysteine residues coordinate binding of two zinc atoms with catalytic and structural roles. The catalytic zinc atom may be necessary for coordination of the substrate and coenzyme NAD^+ . Thus, *S*-nitrosylation of any cysteine residue within the catalytic cluster could prevent coordination of zinc and disrupt the substrate or NAD^+ binding pockets. Alternatively, *S*-nitrosylation of cysteines within the structural cluster may prevent GSNOR1 from folding appropriately. The exact site of *S*-nitrosylation of GSNOR1 and associated inhibitory mechanism remain to be determined. Regardless of these details, our data show NO directly regulates GSNOR1 through post-translational modification and suggest a novel mechanism by which NO controls its own bioavailability (Fig. 5).

Taken together with our biochemical and genetic evidence that nitrate assimilation is feedback repressed by NO, we conclude that NO is at the centre of fine tuning nitrogen homeostasis in plants. These findings raise important considerations for the impacts of nitrogen-based fertilizers on redox-mediated traits in agricultural crops. Nitrogen is a major nutrient required for plant growth and development and for this reason insufficient N in soil severely restricts the use of potential agricultural lands. To circumvent this limitation, application of nitrate-based fertilizers has been the most widely used method to increase crop yields. However, the unutilized nitrate in agricultural fields is one of the main sources of environmental N pollution, as well economic losses[58]. Therefore, understanding the physiological basis involved in the adjustment of plant growth in response to nitrate availability is essential for the development of crop plants either adapted to N-limiting conditions or with high efficiency in nitrogen assimilation[59]. The present identification of NO as a key element for adjustment in plant growth according to nitrate availability generates an important basis for future research programs to attain higher yields and promote a reduction in fertilizer-based environmental pollution.

Methods

Plant materials, growth conditions and treatments

Arabidopsis thaliana Columbia-0 WT and the mutants *nia1nia2* (ref. 14), *gsnor1* (par2-1)[34] and *nox1* (*cue1-6*)[24], as well as the transgenic lines 35S::FLAG-GSNOR1 in WT and *nox1* backgrounds were grown in soil in a controlled environmental chamber at 20–22 LC, 65% relative humidity and a photoperiod of 16/8 h light/dark. The form and content of N in the soil was not determined and the plants were irrigated with water as needed. Where indicated nitrate availability was controlled by growing plants in perlite:vermiculite (1:1) under a 12/12 h light/dark period. Plants were irrigated with Murashige–Skoog (MS)[60] nutrient solution three times a week. In these treatments, the composition of inorganic N was altered from the original one in a way that nitrate supply was composed of half KNO_3 and half NH_4NO_3. Four-week-old plants were used for the experiments.

For analysis of gene expression in roots, seeds were surface sterilized with 10% bleach for 5 min, washed three times with sterile water and sown aseptically in petri dishes containing half-strength MS medium (in which the N source is composed of 9.4 mM KNO_3 and 10.3 mM NH_4NO_3). Petri dishes were maintained vertically in a photoperiod of 16/8 h light/dark at 20–22 LC. After 15 days, the seedlings were gently lifted from petri dishes using forceps and analysed immediately or incubated in 10 ml of sterile deionized water containing 1 mM KNO_3 supplemented with or without GSNO or DEA/NO at room temperature for 3 h. Roots were then separated from the shoot with the aid of a scalpel and RNA extraction was carried out as described below.

Construction of transgenic 35S::FLAG-GSNOR1 plants

The full-length GSNOR1 gene was multiplied from cDNA and TOPO cloned into the Gateway compatible pENTR/SD/D-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The resulting pENTR/GSNOR1 clone was linearized with the restriction enzyme MluI to prevent subsequent transformation of the entry vector into *E. coli*. Using LR clonase (Invitrogen), the GSNOR1 sequence flanked by the entry vector's attL recombination sites were recombined into the plant

transformation vector pEarleyGate 202 (ref. 61), which contains an amino-terminal FLAG epitope tag driven by a cauliflower mosaic virus 35S promoter. The resulting pEarleyGate 202/35S::FLAG-GSNOR1 vector was transformed into *Agrobacterium tumefaciens* strain GV3101(pMP90), which was subsequently used to transform WT plants by floral dipping[62]. Transgenic plants were selected on soil by repeated spraying with glufosinate ammonium. A homozygous transgenic line with appropriate transgene expression was isolated by immunoblotting for FLAG and crossed into nox1 mutants.

Plant NO fumigation

WT plants grown in perlite:vermiculite (1:1), irrigated with MS nutrient solution containing 12.5 mM nitrate and maintained in a growth chamber with a 12 h photoperiod were fumigated with NO gas[63]. Briefly, plants were transferred to an acrylic fumigation chamber for 12 h. Exposure to NO (60 p.p.m.) was performed by bubbling of NO gas with a continuous flow of 90 ml min^{-1} (200 ppm diluted in N_2) plus 210 ml min^{-1} of commercial air. In the control assays, a total flow of 300 ml min^{-1} of air was applied. Subsequently, fumigated leaves were collected and prepared for measurement of GSNOR activity.

Measurement of GSNO reductase activity

GSNO reductase activity in leaf extracts was measured spectrophotometrically as the rate of NADH oxidation in the presence of GSNO[31]. Briefly, total leaf protein was extracted in 20 mM HEPES buffer (pH 8.0), 0.5 mM EDTA and proteinase inhibitors (50 mg ml^{-1} TPCK; 50 mg ml^{-1} TLCK; 0.5 mM PMSF). Protein concentrations were measured with a Bio-Rad Protein Assay Kit II (Bio-Rad, Hercules, CA) according to the manufacturer's instructions and adjusted to either 62.5 mg (for *nia1nia2*, which have very low protein content) or 125 mg (for all remaining genotypes). Protein extracts were incubated in 1 ml of reaction buffer containing 20 mM HEPES buffer (pH 8.0), 350 mM NADH and 350 mM GSNO. GSNO reductase activity was determined by subtracting NADH oxidation in the absence of GSNO from that in the presence of GSNO. All samples were protected from light during the assay and tested for linearity. Where indicated, protein extracts were preincubated for 20 min

with intermediates of nitrogen metabolism or NO signalling molecules at the stated concentrations before addition of the reaction buffer.

Measurement of NR activity

NR activity was measured as the rate of NO_2^- production[64]. Briefly, total leaf protein was extracted in 20 mM HEPES (pH 8.0), 0.5 mM EDTA, 10 mM FAD, 5 mM Na_2MoO_4, 6 mM $MgCl_2$ and proteinase inhibitors (50 mg ml^{-1} TPCK; 50 mg ml^{-1} TLCK; 0.5 mM PMSF). A total of 50 mg protein was incubated in 300 ml of extraction buffer supplemented with 10 mM KNO_3 and 1 mM NADH. Nitrite production was determined by adding equal volumes of 1% sulphanilamide and 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride in 1.5 N HCl, and absorbance measured at 540 nm on a spectrophotometer. The obtained values were compared with those of a standard curve constructed using KNO_2 and normalized by protein content. All samples were protected from light during the assay.

Interplay between NO signalling and nitrate assimilation

Nitrate content was determined by nitration of salicylic acid[65]. Briefly, leaves were ground in liquid nitrogen and resuspended in 20 mM HEPES (pH 8.0). After centrifugation at 10,000g for 10 min at 4 ºC, aliquots of 5 ml of supernatant were mixed with 45 ml of 5% (v/v) salicylic acid in sulfuric acid for 20 min. The solution was neutralized by slowly adding 950 ml of NaOH (2 N). Absorbance was determined at 410 nm and the values obtained were compared with those of a standard curve constructed using KNO_3 and normalized by protein content.

Measurement of NO emission

NO emission by leaves was determined by fluorometric analysis using 4,5-diamino-fluorescein-2 (ref. 66). Briefly, leaf samples of WT and *nia1nia2* plants were incubated in the dark with 10 mM 4,5-diamino-fluorescein-2 dissolved in 10 mM phosphate buffer, pH 8.0. After 1 h incubation, fluorescence emission at 515 nm under an excitation at 495 nm was recorded using an F-4500 spectrofluorometer (Hitachi, Tokyo, Japan). As negative control, leaves were incubated in the presence

of 200 mM of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide and the residual fluorescence subtracted.

Analysis of amino acid content

Leaf free amino acids were determined by reversed-phase high-performance liquid chromatography[56] after derivatization with o-phthaldialdehyde (OPA)[67]. Leaves were ground in liquid nitrogen and resuspended in methanol/chloroform/water (12:5:3, v/v). After incubation at room temperature for 24 h, the homogenate was centrifuged at 1,500g for 30 min and the resulting supernatant mixed with chloroform/water (4:1:1.5, v/v/v). After decanting for 24 h, the aqueous phase was separated and subjected to derivatization by mixing with 50 mM OPA, 1% 2-mercaptoethanol in 400 mM borate buffer pH 9.5 (1:3, v/v) for 2 min. The OPA derivatives content were determined by reverse-phase HPLC (Shimadzu Corporation, Kyoto, Japan) using a Waters Spherisorb ODS2 C-18 column (4.6 mm, 4.6 x 250 mm) eluted at 0.8 ml min^{-1} by a linear gradient formed by solutions A (65% methanol) and B (50 mM sodium acetate, 50 mM disodium phosphate, 1.5 ml acetic acid, 20 ml tetrahydrofuran, 20 ml methanol in 1 l water, pH 7.2). The gradient increased the proportion of solution A from 20 to 60% between 0 and 25 min, 60 to 75% from 25 to 30 min and 75 to 100% from 30 to 50 min. The column effluent was monitored by a Shimadzu fluorescence detector (model RF-10AXL) operating at an excitation of 250 nm and emission of 480 nm. Amino acids were identified by their respective retention times and values compared with those of an amino acid standard solution (AA-S-18, Sigma Aldrich, plus 250 mM asparagine, glutamine and gamma-aminobutyric acid) and normalized by fresh weight of leaf tissue.

Gene expression analysis

For real-time PCR analysis, total RNA was extracted with Trizol (Invitrogen) and ethanol precipitation, and subsequently treated with Amplification Grade DNAse I (Invitrogen). The cDNA was synthesized using Im-Prom II reverse transcriptase (Promega, Fitchburg, WI), as recommended by the manufacturer. Gene expression analysis was carried out using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) in a Real-Time PCR System 7500 (Applied Biosystem, Foster City, CA).

Gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method[68] with *actin2* as internal standard[69]. All gene-specific primers used in this study are shown in the Supplementary Table 1.

In vitro *and* in vivo S-*nitrosylation assays*

Leaf extracts from 35S::FLAG-GSNOR1 in WT or *nox1* plants were mock-treated or *S*-nitrosylated *in vitro* with 500 mM of Cys-NO for 20 min in the dark. Excess Cys-NO was removed using Zeba desalting columns (Thermo Fisher Scientific) and proteins subjected to the biotin switch technique as described previously[42]. Biotinylated proteins were pulled down with streptavidin agarose CL-6B (Thermo Fisher Scientific) and FLAG-GSNOR1 protein detected by western blotting with an anti-Flag M2 clone antibody (1:2,000 or 1:2,500, Sigma Aldrich, cat. no. F3165) (Supplementary Fig. 8).

Acknowledgements

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Author contributions

L.F. performed the enzymatic assays, determination of nitrate, amino acid content and NO emission, analysis of growth vigour and wrote the manuscript. M.J.S. was under the supervision of G.J.L. and performed biotin switch technique and western blot assays together with L.F. Both L.F. and S.H.S. determined gene expression. I.S.

and S.H.S. designed and supervised the study and wrote the manuscript. All authors contributed to the discussion of the results.

Competing financial interests: The authors declare no competing financial interests.

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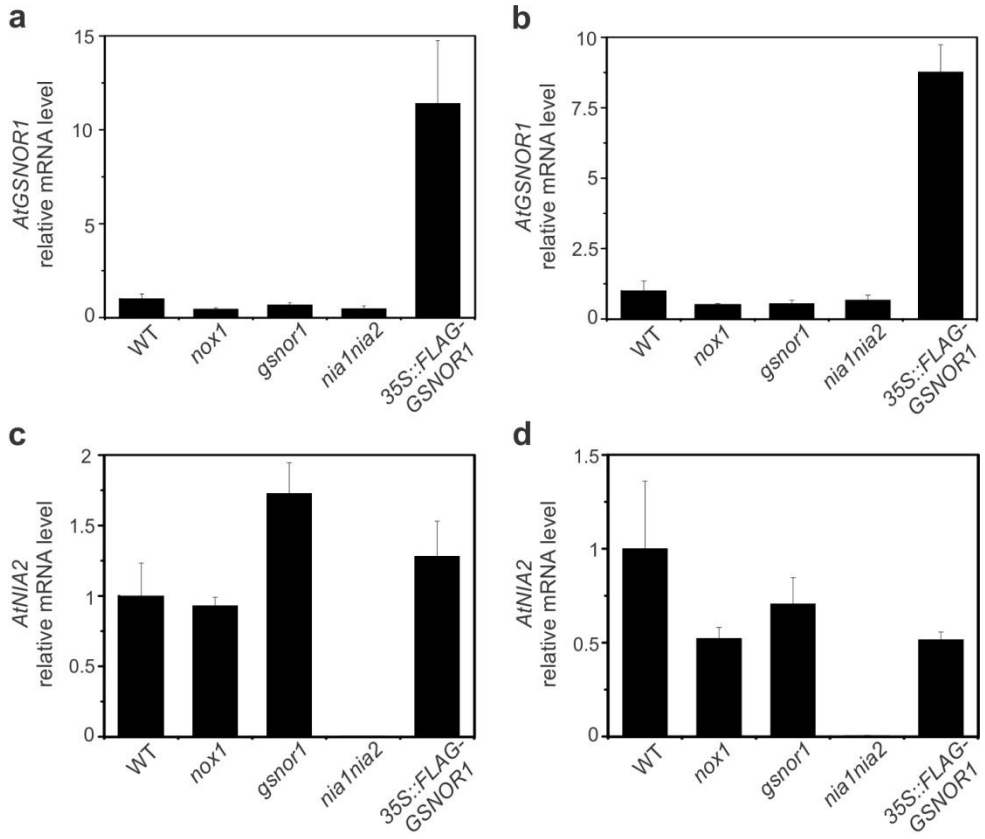
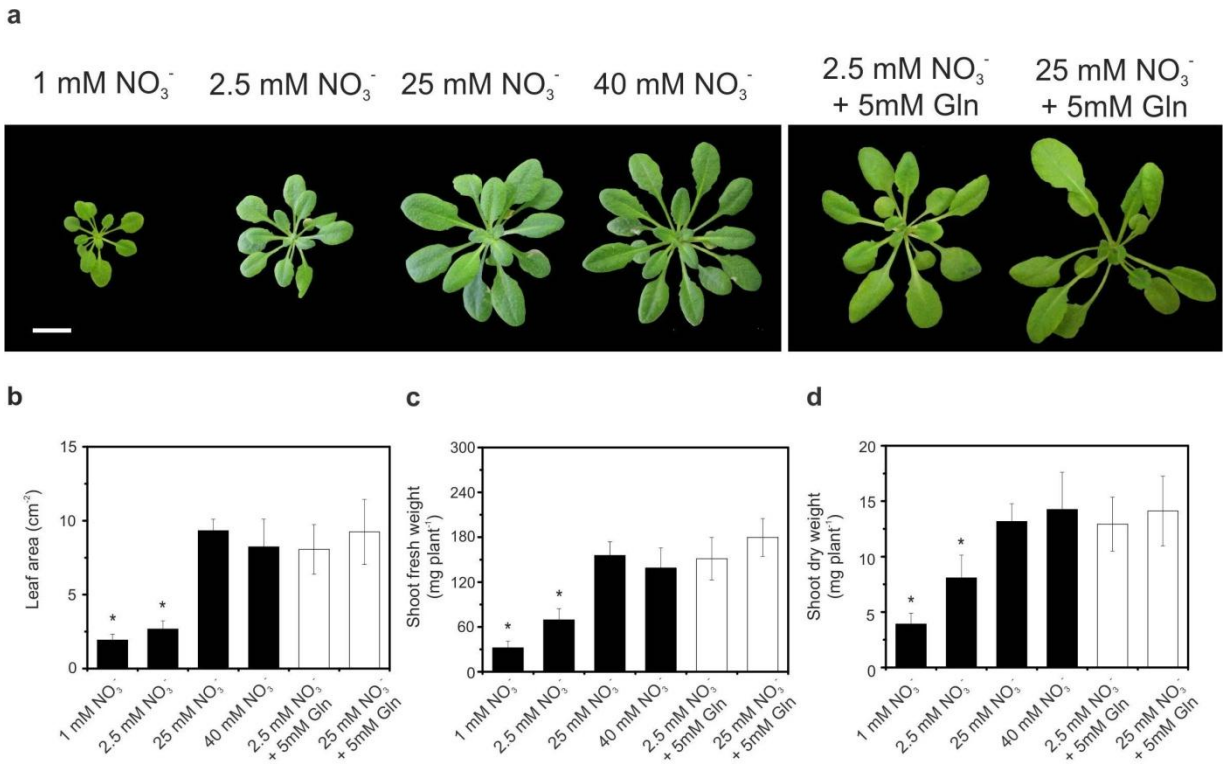
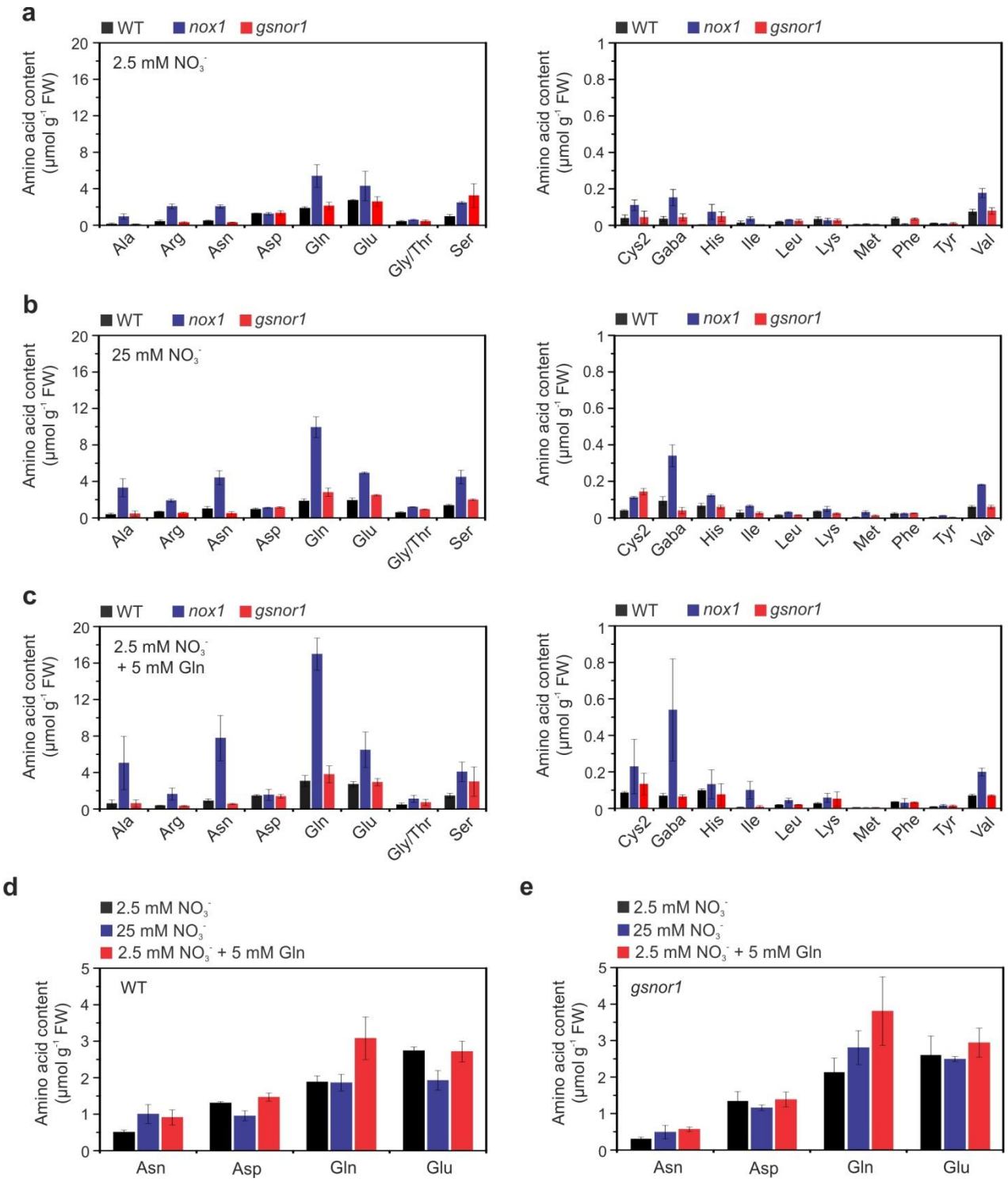
Supplementary Information



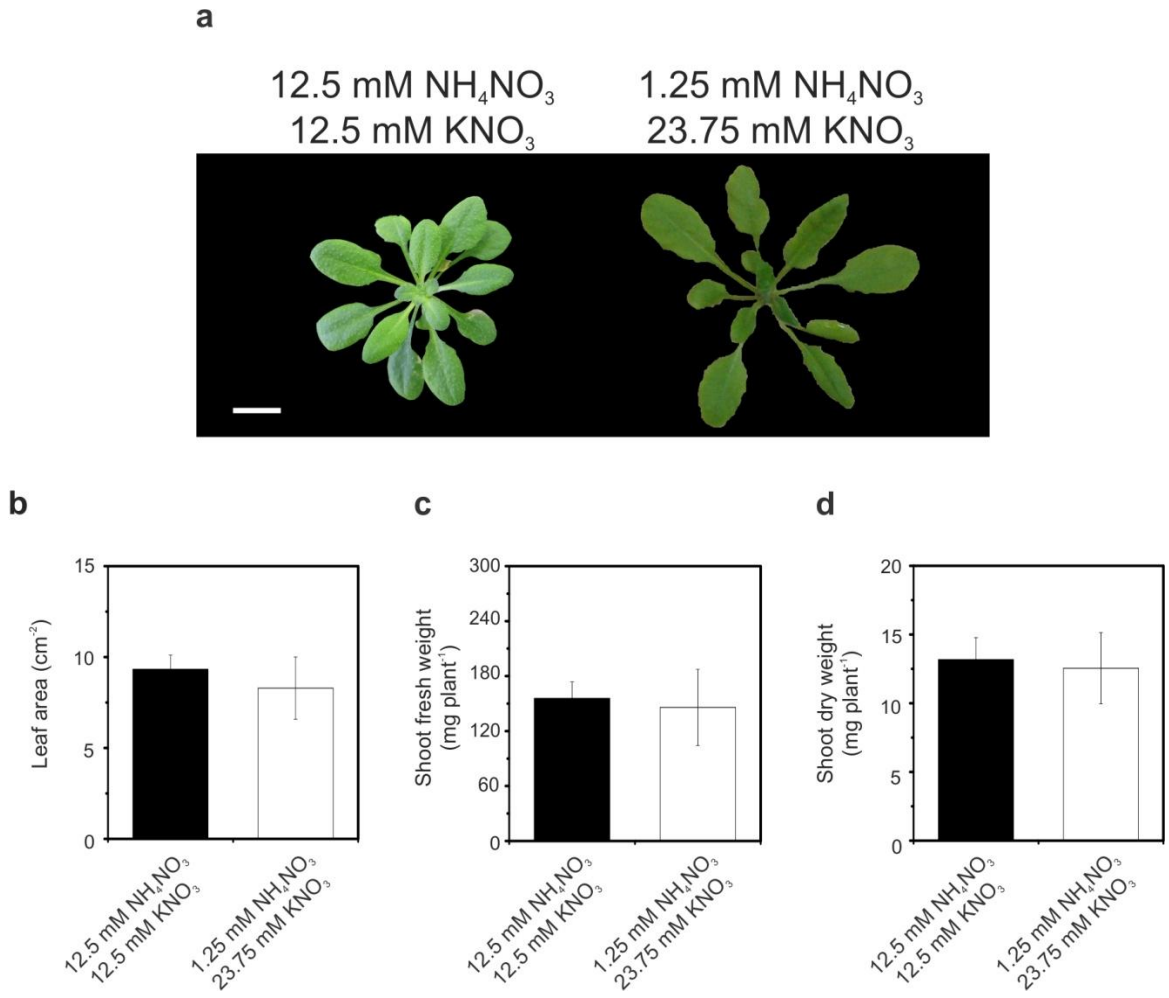
Supplementary Figure 1. Gene expression analysis of GSNOR1 and NIA2 in genotypes with altered NO signalling. Relative expression of *GSNOR1* in leaves (a) and roots (b) and *NIA2* in leaves (c) and roots (d) of WT plants and genotypes with enhanced (*nox1* and *par2-1*) or impaired (*nia1nia2* and 35S::FLAG-GSNOR1) (S)NO homeostasis determined by qRT-PCR and normalized to expression of *ACT2*. Analysis of gene expression in roots was carried out on 15 days-old plants grown in petri dishes containing half-strength MS medium (9.4 mM KNO_3 and 10.3 mM NH_4 NO_3). For analysis of gene expression in leaves plants were grown in soil and the form and content of N in the soil was not determined. Error bars represent SD (n = 3).



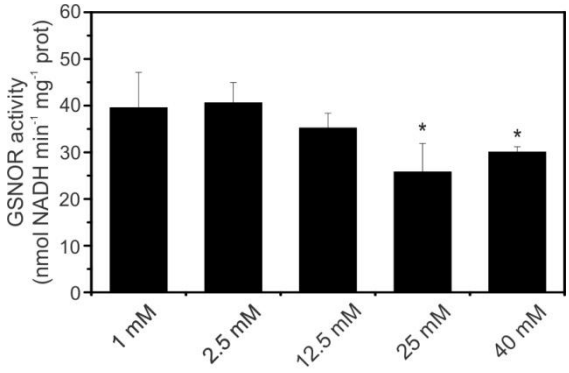
Supplementary Figure 2. Effect of nitrate availability and glutamine on growth vigour of wild-type plants. (a) Phenotype, (b) leaf area, (c) shoot fresh weight and (d) shoot dry weight of four-week-old plants grown on perlite:vermiculite (1:1) under 12h/12h light/dark and irrigated three times a week with a MS nutrient solution containing nitrate (half KNO_3 and half NH_4NO_3) and glutamine as indicated. In (a) scale bar, 1 cm. In (b-d) data points represent means ± SD (n=15). Asterisks indicate statistically significant difference from 25 mM ($P < 0.05$, Student's t test).



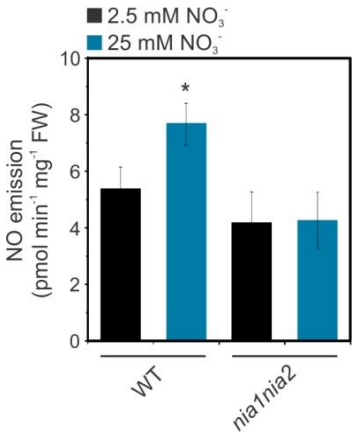
Supplementary Figure 3. Free amino acid contents in leaves: effect of nitrate and glutamine availability. (a-c) Free amino acid profile in WT, *nox1* and *gsnor1* and (d,e) contents of primary transported amino acids in WT and *gsnor1* leaves of four-week-old plants irrigated with MS nutrient solution containing 2.5 mM nitrate, 25 mM nitrate or 2.5 mM nitrate and 5 mM glutamine, as indicated. Data represent means ± SD of three independent analyses. Cys2, cystine; Gaba, gamma-aminobutiric acid.



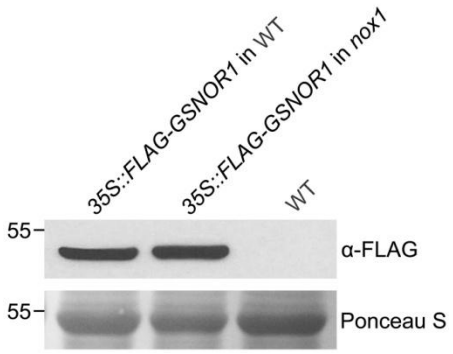
Supplementary Figure 4. Growth vigour of WT plants irrigated with different concentrations of NH_4^+ in the nutrient solution. Phenotype (a), leaf area (b), shoot fresh weight (c) and shoot dry weight (d) of four-week-old plants grown on perlite:vermiculite (1:1) under 12h/12h light/dark and irrigated three times a week with a MS nutrient solution containing 12.5 mM NH_4 NO_3 and 12.5 mM KNO_3 or 1.25 mM NH_4NO_3 and 23.75 mM KNO_3 , as indicated. In (a) scale bar, 1 cm. In (b-d) data points represent means ± SD (n = 15 plants).



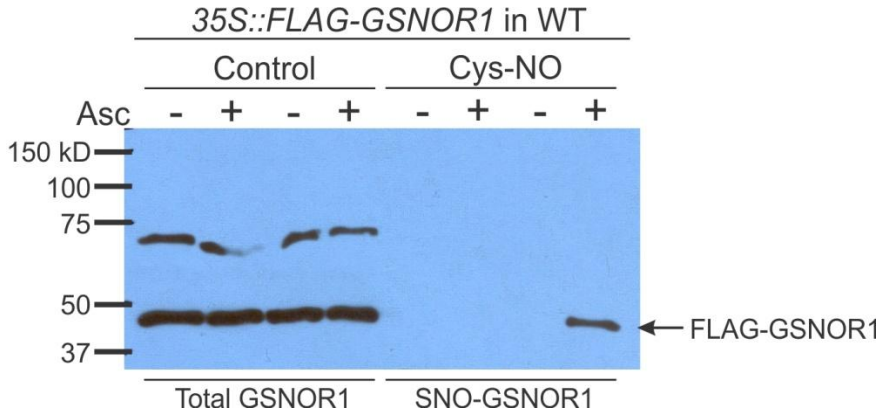
Supplementary Figure 5. GSNOR activity in wild-type plants cultivated under different nitrate availability. Plants were grown on perlite:vermiculite (1:1) under 12h/12h light/dark and irrigated three times a week with a MS nutrient solution containing nitrate as indicated (half KNO_3 and half NH_4 NO_3). Asterisks indicate statistically significant difference from 2.5 mM ($P<0.05$, Student's t test).



Supplementary Figure 6. NO emission by leaves of wild-type (WT) and* nia1nia2 *plants cultivated under low (2.5 mM) and high (25 mM) nitrate availability. NO concentration was measured using the electrochemical sensor ISO-NOP connected to a free radical analyser Apollo 4000 (World Precision Instruments, Sarasota, FL). Leaves (200 mg) were harvest and washed tree times with deionized water before incubation in 0.5 mM phosphate buffer (pH 7.8) and 5% DMSO. Analyses were carried out after electrode stabilization. Signal from 3 to 5 min of capture were used to construct a linear fit. The NO electrode was calibrated with S-nitroso-N-acetyl-penicillamine (SNAP) in 0.1 M CuCl 2 according to Zhang (2004, Front. Biosci. 9: 3434). Data points represent means ± SD of three independent experiments. Asterisks indicate significant differences from the WT grown at low nitrate (Student's t test, $P < 0.05$).



Supplementary Figure 7. Accumulation of FLAG-GSNOR1 protein in both wild-type and* nox1 *backgrounds. Protein was extracted from wild-type Col-0 and *nox1* plants that were untransformed or transformed with 35S::FLAG-GSNOR1. Proteins were analysed by SDS-PAGE and western blotting using an anti-FLAG antibody. Ponceau S staining confirmed equal loading. The position of a 55 kDa marker is indicated.



Supplementary Figure 8. In vitro and in vivo S-nitrosylation of GSNOR1. Full scans of the blots presented in Figs. 4d (left) and 4e (right) of the main text.

Supplementary Table 1. Primers sequences.

ACT2F	CGTACAACCGGTATTGTGCTGG
ACT2R	CTCTCTCTGTAAGGATCTTCATG
GSNOR1F	GGTCTCTTTCCTTGTATTCTAG
GSNOR1R	GCATTCACGACACTCAGCTTG
NIA2F	CTCAGTACCTAGACTCTTTGC
NIA2R	ACCGTGAACCGTGAAACTAC
NRT1.1F	ACACGCTCATGGTCCAACAG
NRT1.1R	AGATTAACGCTTCGCCGATACC
NRT2.1F	GCTTGCACGTTACCTGTGACC
NRT2.1R	GCGTCCACCCTCTGACTTGG

CHAPTER III

About the nitrate assimilatory process and nitric oxide signalling.
Proposed theoretical advances based on the findings presented in Chapter II.

Control of nitrogen assimilation in plants through *S*-nitrosothiols

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Key words: Nitrogen assimilation; Nitrate transporter (NRT); High affinity transport system (HATS); Low affinity transport system (LATS); Nitrate Reductase (NR); Nitric oxide synthase-like (NOS-like); Nitric oxide (NO); *S*-nitrosothiol (SNO); *S*-notrosoglutathione (GSNO); *S*-nitrosoglutathione Reductase (GSNOR1).

Abstract

The inorganic ion nitrate is the primary source of nitrogen for land plants, and the availability of this nutrient in the soil represents a bottleneck in crop yield. To assimilate nitrate, plants employ a variety of transporters and reductases expressed in different tissues and organs to transport and catalyse the sequential reduction of assimilates. Nitrate assimilation is a high-energy consuming process subjected to tight metabolic control, which is not yet fully understood. Nitrate assimilation has been recently demonstrated as a feedback mechanism regulated through the free radical nitric oxide (NO). NO primarily acts through covalent attachment to the thiol groups in Cys residues, causing *S*-nitrosylation, a reversible post-translational protein modification. Previous evidence has indicated that *S*-nitrosylation feedback regulates nitrate transporters and reductases in a novel mechanism involving the production and scavenging of NO. In this review, we will discuss recent advances in the field of nitrate assimilation, focusing on the interplay between this process and NO-mediated redox signalling pathways in plants.

Introduction

Nitrogen (N) is an essential nutrient required for plant growth, development and yield. To meet nutritional needs, plants have evolved a highly specialized transport system for N uptake in various forms available in soil. N can be acquired through the roots as inorganic ions (nitrate, nitrite, ammonium and dinitrogen) and organic molecules (urea and amino acids), and availability largely differs depending on the region and type of soil[1]. Among the different N forms available to plants, nitrate (NO_3^-) is the most abundant source for annual crops[2]. Millions of tons of nitrate-containing fertilizers are applied annually as an agricultural practice to ensure N supply and support plant productivity[3]. Despite massive fertilization, crops are frequently challenged with N deprivation, reflecting the high mobility of nitrate ions leached from the soil, eventually leading to river eutrophication[4]. Thus, an understanding of how plants respond to and assimilate available nitrate is essential to increase N use efficiency, avoid environmental impact and prevent economic losses.

Recently, cross-talk between the nitrate assimilatory pathway and nitric oxide (NO)-mediated redox signalling has been revealed in the model plant *Arabidopsis thaliana*[5]. NO is a free radical that plays key roles in various physiological processes during plant growth, development and defence against environmental cues[6–8]. The broad range of effects of NO or related molecules in plants primarily reflect the effects of this molecule on gene expression and post-translational protein modification[7,9–11]. Despite its importance in plant biology, it remains unclear how NO homeostasis and signalling specificity are achieved.

In this review, we discuss how NO homeostasis might control the uptake and reduction of nitrate in plants. We also critically examine potential links between the NO-mediated control of nitrate assimilation and other metabolic processes that facilitate prompt responses to environmental and cellular fluctuations in N status and mediate adjustments in growth and development accordingly.

Nitrate uptake and transport

To achieve N homeostasis and sustain development and growth, land plants are equipped with a complex apparatus to uptake nitrate from the soil (reviewed by [2,12]).

Four gene families in *Arabidopsis thaliana* encode nitrate transporters: NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER (NRT1/PTR), NITRATE TRANSPORTER 2 (NRT2), CHLORIDE CHANNELS (CLC) and SLOW CHLORIDE CHANNEL 1 HOMOLOGUES (SLAC1/SLAH). Most members of the NRT1/PTR family were initially named according to the first identified substrate. However, several members of this group transport more than one substrate, and the analysis of sequence homologies showed no correlation with substrate selectivity. Recent efforts to develop a practical, straightforward and unified nomenclature for NRT1/PTR proteins have suggested NPF (NTR1 PTR FAMILY) to designate this group of plant proteins[13]. Biochemical and phylogenetic analyses have also lead to the identification of eight different clades in the NPF family (NPF1 to NPF8) (13). Thus, for the sake of clarity, when citing a member of the NPF family (the new proposed nomenclature), the respective former name (NRT) is also provided. Currently, among the 73 genes grouped in these gene families, 24 genes have been characterized and implicated in nitrate transport, comprising influx and/or efflux throughout different cellular compartments in plants[12]. Increased attention has been directed to NPF6.3/NRT1.1 and NRT2.1, as these proteins play roles in nitrate influx and signalling in the roots.

Nitrate is actively taken up through the roots via specific transporter systems differentially recruited depending on availability[14,15]. The concentration of nitrate in the soil largely varies from less than 1 mM to 70 mM[1]. Hence, to ensure adequate N acquisition in plants, the nitrate transport system in roots has evolved to cope with large variations in nitrate availability through switching sensitivity to nitrate[16]. Nitrate uptake through the roots is based on the activity of the High Affinity Transport System (HATS, *Km* in the µM range) and Low Affinity Transport System (LATS, *Km* in the mM range)[15] (Figure 1). For instance, at high availability, nitrate uptake is performed through the LATS, in which the main effector is the product of *NPF6.3/NRT1.1* gene expression[17,18]. In contrast, low nitrate availability induces *NRT2.1* expression and the phosphorylation of the NPF6.3/NRT1.1 transporter, thereby increasing affinity to nitrate[14,15]. Thus, while NRT2.1 is a pure high-affinity transporter, NPF6.3/NRT1.1 is a dual-affinity transporter, involved in nitrate uptake through LATS and HATS, depending on the post-translational state.

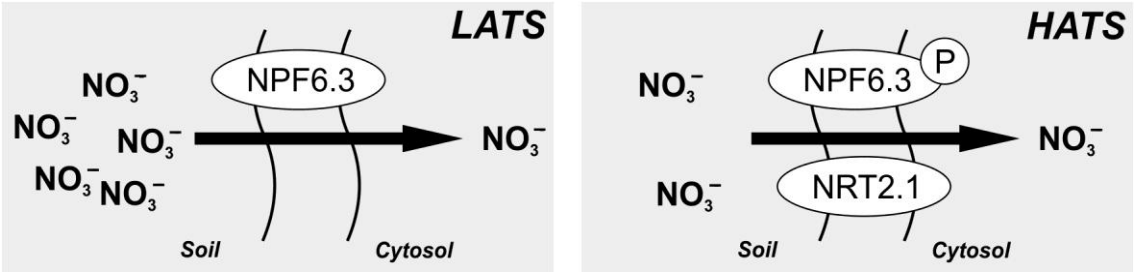

Figure 1. Systems of nitrate (NO_3^-) transport in plant roots. The recruitment of transporters and post-translational state might differ depending on nitrate availability in the soil. Low Affinity Transport System (LATS): under nitrate abundance, nitrate transport primarily relies on NPF6.3/NTR1.1 activity (left panel). High Affinity Transport System (HATS): under nitrate shortage, NPF6.3/NRT1.1 exhibits increased affinity to nitrate through phosphorylation (P) at Tyr101, and the pure high-affinity NRT2.1 is recruited (right panel).

Recently, two crystallographic studies have shed light on how phosphorylation at the Thr101 residue in NPF6.3/NRT1.1 alters nitrate transport[19,20]. Both studies examined the inward portion of the NPF6.3/NRT1.1 protein, comprising 12 transmembrane helices. Together these studies have indicated that the phosphorylation of NPF6.3/NRT1.1 induces dimer-to-monomer modification, thereby increasing protein flexibility and ultimately, reducing the *Km* to nitrate[19,20]. However, it remains unknown whether this conformational modification increases the binding affinity for nitrate or merely improves transport capacity through accelerating nitrate shuttle.

Several lines of evidence have indicated a role for NPF6.3/NRT1.1 in nitrate assimilation far beyond the acquisition of this nutrient through the roots. It has been suggested that the post-transcriptional modification of NPF6.3/NRT1.1 not only controls transport capacity, but is also critical for the plant response to nitrate availability[18]. Plants rapidly trigger the gene expression of the nitrate assimilatory pathway in response to nitrate supply, a process called the Nitrate Primary Response (NPR) (18,21,22). Notably, NPR is observed in mutants impaired in the initial reductive steps of nitrate assimilation, indicating that the signal to this response is nitrate itself[23–25]. NPR is characterized by two distinct levels of gene induction, depending on the nitrate concentration: under nitrate availability higher than 1 mM, the induction of nitrate responsive genes are approximately twice as strong as

induction under nitrate levels below the mM range[18,26]. The *npf6.3/nrt1.1* mutant exhibits the trace expression of *NRT2.1*, a HATS component marker gene for NPR. Interestingly, replacing Thr101 with an Asp, mimicking phosphorylation, suppressed the upregulation of the *NPF6.3/NRT1.1* gene under nitrate availability above mM concentrations. Conversely, replacing the Thr101 residue with an Ala induced a stronger response to nitrate, regardless of nitrate concentration[18]. Taken together, these data indicate that rather than simply acting as a transporter, NPF6.3/NRT1.1 is a transceptor, acting as a receptor that senses nitrate availability and coordinates plant responses through critical phosphorylation. In accordance to the observation that NPR is triggered as fast as 3 minutes, the nitrate transport activity of NPF6.3/NRT1.1 can be decoupled from its sensing activity[18], suggesting that NPF6.3/NRT1.1 is responsible for signalling nitrate availability.

NPF7.3/NRT1.5 and NPF7.2/NRT1.8, members of the subfamily NPF7, have been implicated in nitrate loading in the xylem through the control of the efflux and influx of nitrate, respectively, from xylem vessels[27–29]. The observation that *NPF6.3/NRT1.1* is co-expressed with NPF7.3/NRT1.5 and NPF7.2/NRT1.8 in the mature parts of the roots, including the endodermis and stele[30,31], suggested a role for NPF6.3/NRT1.1 in nitrate translocation to the shoots[32]. Accordingly, the phenotype analysis indicated that *npf6.3*/*nrt1.1* mutants display unaffected nitrate uptake through the roots[33], with an unexpected delay in nitrate distribution to the shoots[32]. Furthermore, after loading *Xenopus* oocytes expressing *AtNPF6.3/NRT1.1* with ^{15}N-labeled nitrate and subsequently measuring the appearance of ^{15}N-labeled nitrate in the incubation buffer, Léran and colleagues demonstrated that NPF6.3/NRT1.1 mediates nitrate efflux, even with no favourable gradient. These results suggest that the NPF6.3/NRT1.1 transporter synergistically functions with NPF7.2/NRT1.8 for nitrate translocation through the plant. However, the molecular mechanism determining whether NPF6.3/NRT1.1 acts in the influx or efflux of nitrate and the role of this bidirectional transport in root nitrate uptake remain unknown.

In addition to a role in transporting and sensing nitrate, NPF6.3/NRT1.1 has also been implicated in shaping the root architecture[31,34]. The formation of lateral roots from the primary root is an important mechanism through which plants forage for water and nutrients. Lateral roots are initiated as mitotically active cells in the pericycle of primary roots that protrude through the epidermis after a few days. Lateral root formation in nitrate-rich patches is strikingly associated with lateral root

elongation. The MADS box gene *ANR1* encodes a transcription factor and is required for lateral root elongation in Arabidopsis[31]. Interestingly, *npf6.3/nrt1.1* mutant plants displayed reduced *ANR1* levels and accordingly, reduced root colonization, a phenotype that restrains plant growth. Additionally, NPF6.3/NRT1.1 transports the hormone auxin from the developing lateral root, thereby negatively impacting lateral root elongation through the reduction of the auxin concentration[34]. Collectively, these findings clearly indicate that NPF6.3/NRT1.1 is a key element in orchestrating biochemical and morphological responses in plants through nitrate transport and sensing.

Nitrate assimilation

The incorporation of the N atom from inorganic ion nitrate into the carbon skeleton to form N-containing organic molecules, such as amino acids, proteins and nucleotides, is one of the most energy-consuming biochemical pathways in nature[1]. Initiated through N uptake from the soil, the nitrate assimilatory pathway comprises several redox reactions that together consume 12 ATPs per N atom assimilated[35]. In plants, once taken up through the root, nitrate is transported to the leaves where this molecule is stored in vacuoles or effectively assimilated into organic compounds. The first reductive step in nitrate assimilation is the reduction to nitrite (NO_2^-) through the activity of the cytosolic enzyme nitrate reductase (NR). This reaction involves the transfer of two electrons donated from NADPH or NADH, depending on the NR isoform[36]. In *Arabidopsis*, NR is encoded by two structural genes, *NIA1* and *NIA2,* of which NIA2 accounts for the majority of NR activity in the shoots[25]. Due to high reactivity and potential toxicity, nitrite is promptly removed from the cells[37] or transported to chloroplasts in the leaves or plastids in the roots for further reduction. Nitrite is then reduced to ammonium through nitrite reductase (NiR) at the expense of 6 electrons donated from reduced Ferredoxin (Fd) (1). The final process in nitrate assimilation is the conversion of ammonia into amino acids in chloroplasts and plastids through a two-step pathway (Figure 2). First, glutamine synthetase (GS) incorporates ammonia into glutamate, forming glutamine. Sequentially, glutamine oxoglutarate aminotransferase (GOGAT) transfers the amide group of glutamine to the organic acid oxoglutarate to yield two glutamate molecules. After this step,

several transaminases mediate the production of other amino acids through transamination reactions[38].

Due to the requirement of the C skeleton to incorporate the N atom from nitrate into organic molecules, it is likely that nitrate assimilation is closely associated with the photosynthetic process in plants. Indeed, several lines of evidence have supported the idea that the plant C status is associated with nitrate assimilation through a stimulatory effect in nitrate uptake, at least in the short term[2]. For example, while examining non-nodulated soybean plants (*Glycine max*), Delhon and colleagues showed that the diurnal variation of nitrate transporter expression was associated with plant C status[39]. Interestingly, feeding experiments with glucose and sucrose as a source of C were effective in stimulating nitrate transporter expression and nitrate uptake in soybeans and Arabidopsis plants[39–42]. Thus, it has been suggested that C initially assimilates in the leaves and is subsequently transported to roots for the stimulation of nitrate uptake in plants[39,40]. In addition to physiological interplay, photosynthesis and nitrate assimilation are also associated at the biochemical level. The maintenance of the pool of reduced Fd, necessary for the nitrite to ammonia reduction, is achieved through chloroplastic electron transport in photosystem I (PSI) during photosynthesis in the leaves and the oxidative pentose phosphate pathway (OPPP) in the roots[1]. Conversely, nitrate supply and sensing regulates the expression of OPPP-related genes[22,24,43], indicating that nitrate assimilation also impacts C metabolism. Taken together, these data indicate a complex C:N regulatory network that profoundly impacts plant metabolism. Fluctuations in the rate of photosynthesis or nitrate assimilation might be sensed by each other, although the underlying molecular mechanisms are not yet fully understood.

Association between nitrate assimilation and nitric oxide (NO) formation

According to the role of NR in the nitrate assimilation pathway, NR-double-deficient (*nia1nia2*) *A. thaliana* plants are unable to reduce nitrate to nitrite and consequently

show a drastic reduction in leaf amino acid levels, reduced biomass and typical pale leaves[5,25,44]. Additionally, *nia1nia2* plants display severe susceptibility symptoms when challenged with the avirulent strain *Pseudomonas syringae* pv. *maculicola*[44,45]. Interestingly, feeding experiments with the end products of the N assimilation pathway, such as L-arginine or L-glutamine, effectively restored the *nia1nia2* leaf amino acid content to levels compatible with WT, but failed to rescue disease resistance[44]. Still, *nia1nia2* mutants are defective in seed germination, seedling establishment, seedling development, secondary metabolite synthesis, drought stress adaptation, and floral induction[46–48]. These data indicate that in addition to a role in N homeostasis, the nitrate assimilation pathway is required for proper development and responses to biotic and abiotic stimuli.

Several studies have demonstrated that *nia1nia2* mutant plants show reduced nitric oxide (NO) production and emission[5,44–46,49], indicating an association between nitrate assimilation and NO production. The active redox molecule NO is a free radical with signalling action in all living organisms. Particularly in plants, NO has been implicated in growth and development and responses to biotic and abiotic cues (reviewed by [50]). Despite the relevance of NO signalling in plant biology, the synthesis of NO remains a matter of debate (Figure 2). In mammals, NO is synthesized through a family of nitric oxide synthase (NOS) enzymes that catalyse the oxidation of the guanidine nitrogen of L-arginine to produce L-citrulline and NO in a reaction dependent on molecular oxygen (O_2) and NADPH[51]. Several lines of evidence have demonstrated NOS activity in plants. For example, NO production, estimated through the oxidation of L-arginine to L-citrulline in plant extracts, has been reported[52–55]. Importantly, human NOS inhibitors suppress NO production through L-arginine-dependent NOS activity in several plant species[53,56,57]. Using a genomic approach, Foresi and colleagues identified an enzyme with 45% similarity to human NOS in the unicellular green algae *Ostreococcus tauri*[58]. Molecular characterization revealed that OtNOS shares structural and kinetics similarities to human NOS. Additionally, *Escherichia coli* transformed with recombinant OtNOS displayed increased NO production in response to L-arginine treatment and oxidative challenge[58]. Intriguingly, however, until recently, genomic analyses have not identified homologs of mammalian NOS in higher plants. It has also been suggested that NO synthesis in plants occurs through polyamine and hydroxylamine oxidation[59–61]. Despite evidence showing that NO production through these sources is

physiologically relevant, the precise mechanism underlying these pathways has not been resolved (Figure 2).

In addition to the oxidative routes, NO can be synthesized through at least four different nitrite reductive pathways (Figure 2). In tobacco (*Nicotiana tabacum*) roots, nitrite was reduced to NO through the activity of membrane-bound nitrite:NO reductase (Ni:NOR). Ni:NOR-mediated NO production plays a role in mycorrhizal fungus interactions in a nitrate-dependent manner[62].

At the cellular level, under low O_2 tensions, nitrite accumulates through NR activation and the partial inhibition of plastidic nitrite reduction[63,64]. Under these conditions, nitrite can be reduced to NO through the activity of peroxisomal xanthine oxidoreductase (XOR), mitochondrial inner membrane-bounded cytochrome *c* oxidase (Cyt C-oxidase) and cytosolic NR. The nitrite to NO reduction via XOR occurs at the expense of NADH or xanthine as reducing agents (Figure 2), potentially representing the local interaction of NO with reactive oxygen intermediates (ROIs) (65,66).

Oxygen, the final electron acceptor in the mitochondrial respiratory chain, can be partially reduced through electron leakage, resulting in the generation of the superoxide anion (O_2^-) and representing a significant mechanism of ROI production in mitochondria, particularly under oxygen shortage[67]. Interestingly, under hypoxia, nitrite acts as an alternative electron acceptor in the respiratory chain in the mitochondria of mammals[68,69] and plants[70,71]. The mitochondrial reduction of nitrite to NO alleviates the stress induced under low oxygen tension via the flow of electrons through the mitochondrial respiratory chain and the maintenance of needful ATP generation[70,72]. Moreover, reduction of nitrite to NO through mitochondrial electron transport was significant during the incompatible interaction of *A. thaliana* with *Pseudomonas syringae*[49].

In addition to a major role in nitrate assimilation, NR has also been implicated in the reduction of nitrite to NO[73,74] (Figure 2). However, the reduction of nitrite to NO through NR catalysis is dependent on high concentrations of nitrite and low oxygen tensions[70,74], and this mechanism might be physiologically relevant under specific conditions. Additionally, in the presence of nitrite, the rate of *in vitro* NO production in leaf homogenates of *A. thaliana* plants defective in the two NR structural genes (*NIA1* and *NIA2*) was similar to that in wild-type plants[49]. Importantly, this NR-independent reduction of nitrite to NO was abolished using inhibitors of mitochondrial respiration,

suggesting that NO production might be derived from electron leakage in the mitochondrial respiratory chain[49]. The production of NO in the nitrogen-fixing nodules of *M. trunculata* might also result from a two-step mechanism involving NR, followed by mitochondrial electron transport[75]. Thus, in contrast to direct involvement in nitrite-to-NO catalysis, the primary role of NR in NO homeostasis is the production of nitrite, i.e., providing the substrate for NO production[49,50].

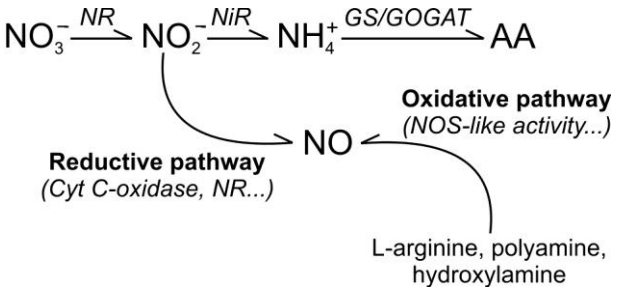

Figure 2. Proposed links between nitrate (NO_3^-) assimilation and nitric oxide (NO) production in plants. As depicted in the schematic representation, the substrates for either the reductive or oxidative pathways in NO production are directly or indirectly provided through nitrate assimilation. NO_2^-, nitrite; NH_4^+, ammonium; Cyt C-oxidase, mitochondrial inner membrane-bounded cytochrome *c* oxidase; NiR, nitrite reductase; GS/GOGAT, glutamine synthetase/ glutamine oxoglutarate aminotransferase; AA, amino acids; NOS, nitric oxide synthase.

Redox signalling of NO through protein modification

Particularly in plants, NO-mediated redox post-translational modification alters protein function and localization in a wide range of situations (reviewed by [7,8,50]). Effective redox-mediated signalling mechanisms rely on specific and fully reversible post-translational modification that alters protein function. Based on these features, *S*-nitrosylation is pivotal for the molecular mechanism of NO bioactivity transfer (Figure 3). *S*-nitrosylation involves the addition of an NO moiety to a specific biologically

active thiol group in cysteine residues in proteins, forming an *S*-nitrosothiol (protein-SNO) (76). *S*-nitrosylation also alters protein localization, activity and function in plants[5,77,78]. This reaction is dependent on O_2 and might be limited to aerated and hydrophobic microenvironments[76,79]. Alternatively, NO reacts with glutathione (GSH), a major antioxidant in cells, forming *S*-nitrosoglutathione (GSNO)[80]. GSNO acts as an NO carrier, increasing the half-life of this compound in biological systems[81,82], and has been implicated in NO signalling through the formation of protein-SNO. By acting as an *S*-nitrosylation agent, GSNO transfers an NO moiety to thiol groups in proteins through *S*-transnitrosylation[83] (Figure 3).

Important advances in the field of NO-mediated redox signalling have been achieved through the recognition of the enzymatic control of protein-SNO. The intracellular level of GSNO is controlled through the evolutionary conserved cytosolic enzyme *S*-nitrosoglutathione reductase (GSNOR1)[80,84]. GSNOR1 primarily reduces GSNO to oxidized glutathione and NH_4^+, thereby reducing the likelihood of protein *S*-nitrosylation[80,85–87] (Figure 3). *GSNOR1*-deficient plants exhibit increased global levels of protein-SNO, which negatively correlates with pathogen resistance[77,84,88], herbicide resistance[86], and heat acclimation[85], revealing the biological relevance of GSNOR1 in controlling plant responses to environmental cues. Recently, new layers of complexity have been demonstrated in NO signalling in plants. While GSNOR1 globally reduces protein-SNO formation through a reduction of the intracellular GSNO pool, genetic and biochemical characterization of the Thioredoxin-*h*5 (TRX*h*5) system suggests a function for these oxidoreductases in the control of specific pools of protein-SNO[88]. TRX*h*5 exhibits denitrosylating activity and selectively discriminates protein-SNO, representing the first demonstration of the selective reversion of protein-SNO in plants.

In addition to a role in protein *S*-nitrosylation, NO reacts with O_2^- through rate-limiting diffusion to yield the potent oxidant peroxynitrite ($ONOO^-$). In biological systems, $ONOO^-$ permanently reacts with Tyr residues in proteins to form nitrotyrosine, a process referred to as Tyr-nitration[89]. Tyr-nitration plays a crucial role in hypersensitive responses and abiotic stress responses[78,90]. *In vitro* and *in vivo* evidence has indicated that the nitrotyrosine level is controlled through the $ONOO^-$ detoxification activity of Peroxiredoxin II E (PrxII E) in plant cells[78]. Interestingly, PrxII E has been identified as a target of *S*-nitrosylation. The S-nitrosylation of PrxII E in plants challenged with avirulent pathogens inhibit the $ONOO^-$ detoxifying activity of

this enzyme, leading to a marked increase in nitrotyrosine content and resulting in nitrosative stress[78].

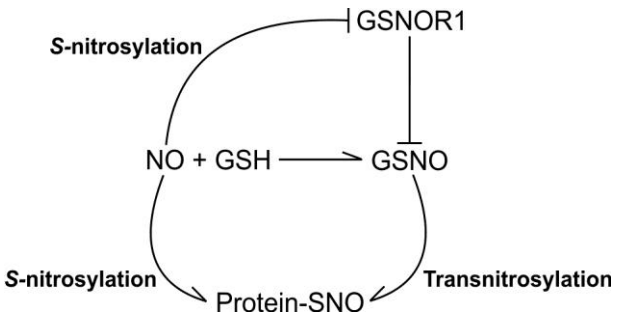

Figure 3. Kinetics of nitric oxide (NO) signalling through *S*-nitrosylation. In cellular systems, NO might react with glutathione (GSH) to form *S*-nitrosoglutathione (GSNO). GSNO levels are controlled through the enzymatic activity of GSNO reductase 1 (GSNOR1). NO regulates its own bioavailability through the control of GSNOR1 activity through inhibitory *S*-nitrosylation. Both NO and GSNO form *S*-nitrosothiols (protein-SNO), and although they overlap, recent studies have indicated that NO and GSNO target different sets of proteins for *S*-nitrosylation.

The different mechanisms of NO-mediated post-translational protein modification are responsible for the plasticity of NO as a cellular signal. Specifically, *S*-nitrosylation is important in NO signalling, as confirmed in reports of targeted proteins and the reversibility of this process[91,92]. Moreover, the recent identification of a previously unrecognized mechanism for the control of specific branches of protein-SNO in plants[88] has shed light on how NO modulates a wide range of effects in biological systems. Additionally, NO-mediated Tyr-nitration and S-nitrosylation might be interconnected at the molecular level, as exemplified through the inhibition of the $ONOO^-$ detoxifying activity of PrxII E through *S*-nitrosylation[78].

The role of NO in nitrate assimilation pathway

Despite increasing knowledge concerning the multiple pathways involved in NO homeostasis, coordination among NO synthesis and scavenging has only recently been revealed. Frungillo and colleagues have proposed that NO controls self-generation and scavenging through the control of nitrate assimilation pathways and GSNOR1 activity[5] (Figure 4).

Previous genetic and biochemical analysis of nitrate uptake in the roots of *A. thaliana* plants demonstrated that the high affinity transporter NRT2.1, a marker component of HATS, is upregulated at the transcriptional level under nitrate starvation[40]. The transcriptional regulation of NRT2.1 in response to nitrate availability enables plants to circumvent shortages in nitrate supply and ensure adequate nitrate uptake. Remarkably, the NR-double mutant plant, *nia1nia2*, exhibits the disrupted regulation of *NRT2.1* expression, which is overexpressed, even when nitrate is adequately supplied[40]. Although these observations strongly suggest the feedback repression of nitrate uptake[33,40], the identity of the metabolite implicated in the control of nitrate assimilation and the molecular mechanism remained largely unknown. Recently, it was proposed that NO fine-tunes nitrate assimilation through the regulation of nitrate uptake and reduction through the control of its own bioavailability[5]. The gene expression analysis of *nitric oxide overproducer1* (*nox1*) in plant roots and the *gsnor1* mutant revealed the repression of *NRT2.1* expression in these genotypes compared with wild-type (WT) plants. Consistently, the pharmacological treatment of WT roots with GSNO or the NO donor DEA/NO was effective in suppressing *NRT2.1* gene expression. Importantly, whereas *NRT2.1* expression was affected through NO and GSNO, the expression of NPF6.3/*NRT1.1* remained unchanged[5]. These observations suggested a switch from high- to low-affinity nitrate transport through NO/GSNO, which might impact nitrate uptake through the roots[5,16] (Figure 4). Considering that NPF6.3/NRT1.1 plays a key role in sensing nitrate availability and controlling the switch between LATS and HATS, it is tempting to speculate that NO signalling is involved in the nitrate-sensing activity of NPF6.3/NRT1.1. Still, in addition to transcriptional control, recent studies have suggested that NRT2.1 is also subjected to post-translational modification[93]. However, the molecular mechanism underlying the control of NRT2.1 activity remains

unclear. Thus, it could be fruitful to determine whether NO or related molecules exert a role in controlling the activity of NRTs.

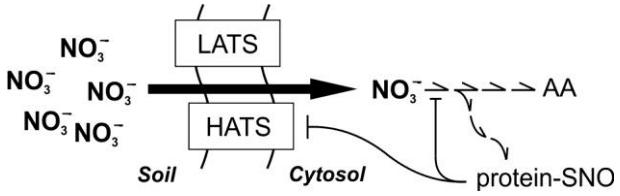

Figure 4. Proposed control of nitrate (NO_3^-) assimilation through *S*-nitrosothiols (protein-SNO) in plants. Protein-SNO is produced as a consequence of nitrate assimilation. Evidence has indicated that protein-SNO feedback regulates nitrate assimilation through the inhibition of transporters and reductases.

The analysis of *in vitro* enzymatic activity showed reduced NR activity in *gsnor1* mutant plants compared with WT plants[5]. Conversely, plants with presumably reduced protein-SNO levels resulting from the overexpression of *GSNOR1* under the 35S promoter showed increased NR activity (Figure 4). Thus, SNO has been suggested to negatively regulate nitrate uptake and reduction through a feedback mechanism and consequently impair plant growth. Accordingly, while the leaf area and biomass accumulation were markedly decreased in *gsnor1* plants, plant vigour increased in *GSNOR1*-overexpressing plant lines. Remarkably, when *gsnor1* plants were fed L-glutamine, as the primary end product of nitrate assimilation, plant vigour was rescued to WT levels, suggesting that the (S)NO-mediated feedback mechanism of nitrate assimilation might significantly undermine plant growth. Collectively, these data indicate that the nitrate assimilation pathway is regulated through NO signalling[5]. However, how appropriate adjustments in nitrate assimilation are achieved has only been revealed in studies of GSNOR1 activity. In feeding experiments with controlled nitrate availability, GSNOR1 activity was inversely associated with nitrate supply, indicating crosstalk between nitrate assimilation and NO signalling[5]. Previous studies have suggested that plant GSNOR1 might be the target of post-translational modification, which could impact the activity of this enzyme[85] (Figure 3). Indeed, measurements of *in vitro* enzymatic activity in the presence of different NO donors and by products of the nitrate assimilation pathway

indicated that GSNOR1 could be directly inhibited by NO. The application of the well-established biotin switch technique using leaf extracts transformed with epitope-tagged 35S::FLAG-GSNOR1 in WT and *nox1* backgrounds showed that GSNOR1 is the target of inhibitory *S*-nitrosylation *in vitro* and *in vivo*, thereby preventing GSNO catabolism[5].

Studies indicating that GSNOR1 activity is directly inhibited through NO-mediated post-translational modification are intriguing, as an increase in the denitrosylating activity of GSNOR1 is expected under NO production. Nevertheless, this observation is finely circumvented based on evidence that, although they might overlap, the redox-active molecules NO and GSNO control different subsets of protein-SNO[5,88,94]. Indeed, the observation that GSNOR1 is a target of *S*-nitrosylation is an elucidative example of disparate subsets of protein-SNO.

The inhibitory *S*-nitrosylation of GSNOR1 might represent a molecular mechanism through which NO bioavailability is controlled and nitrate assimilation is adjusted according to the N demand in plants[5]. Additionally, GSNOR1 activity could be slightly inhibited through the *in vitro* addition of $ONOO^-$ (5), suggesting that different NO-mediated mechanisms might control GSNOR1 activity at the post-transcriptional level. However, additional studies are needed to determine the biological relevance of this process.

Evidence suggests that NR activity might also be subjected to NO-mediated redox control at the post-transcriptional level. NR is a homodimer cytoplasmic enzyme involved in different regulatory strategies (reviewed by [95,96]). Although variations in the NR mRNA levels have been reported, particularly under stress situations[97], NR has long been known to be regulated through phosphorylation/dephosphorylation modification[1]. The recruitment of 14-3-3 proteins through NIA2 phosphorylation at Ser534 inhibits NR activity and promotes protein degradation[98–100]. However, phosphorylation at Ser627 through mitogen-activated protein kinase 6 (MPK6) stimulates NIA2 activity. This mechanism for the regulation of NR activity plays a role in NO production under oxidative stress[101]. Additionally, *AtSIZ* positively regulates Arabidopsis NR through small ubiquitin-related modifier (SUMO) proteins via E3 SUMO ligase activity. Consistent with NR activation through *AtSIZ*, *siz1-2* mutants displayed reduced NO production and a dwarf phenotype[102]. Furthermore, evidence suggests that NR activity might also be subject to NO-mediated redox control at the post-transcriptional level. Intriguingly, this NO-mediated

redox control of NR activity might be associated with nitrate supply. The roots of tomato plants (*Solanum lycocarpum*) exposed to different NO donors in nutrient solution containing high nitrate levels (5 mM) showed marked inhibition of NR activity. Conversely, NO stimulates NR activity in plants fed low nitrate (0.5 mM), and this effect was reversed after NO removal from the medium[103]. These results suggest that NR is the target of liable NO-mediated modification, such as *S*-nitrosylation. Thus, it might be interesting to investigate whether NR is indeed *S*-nitrosylated and if so, it might be worthwhile to determine the potential crosstalk between different NR post-translational modifications.

NO might also exert indirect effects on nitrogen assimilation through metabolically interconnected pathways. For example, sunflower (*Helianthus annuus* L.) plants subjected to high temperature stress show downregulated GSNOR1 gene expression and activity, finely correlated with an increase in protein-SNO and Tyr-nitration levels[90]. Chaki and colleagues proposed that protein-SNO acts as NO reservoirs during heat stress to mediate the generation of $ONOO^-$. Nitroproteome analysis identified 22 proteins as targets of Tyr-nitration under temperature stress, including ferredoxin-NADP oxidoreductase (FNR) (90). FNR catalyses the electron transfer from reduced ferredoxin to $NADP^+$ during the final step of photosynthesis[104,105]. *In vitro* assays indicated that FNR activity was inhibited through the $ONOO^-$ donor SIN-1, suggesting a role for NO-derivates in controlling photosynthesis[90]. Together with the fact that *in vitro* GSNOR1 activity is also inhibited in the presence of $ONOO^-$ (5) and photosynthesis is closely associated with nitrogen assimilation (see below), it is tempting to speculate that $ONOO^-$-mediated redox signalling impacts nitrogen assimilation through the post-transcriptional control of FNR and GSNOR1 activities.

One of the well-established targets of NO is the enzyme aconitase. Aconitase is involved in the stereoisomerization of citrate to isocitrate in the cytosol and mitochondrial matrix. In both animals and plants, aconitase is inhibited through NO in a reversible manner[106,107]. In addition to the involvement of this enzyme in the citric acid cycle and cellular energy metabolism, the regulation of aconitase activity might also be key for the provision of the C skeleton to amino acid biosynthesis[108]. The roots of Arabidopsis plants under hypoxia show NO production through a NR-dependent pathway, resulting in the significant inhibition of aconitase activity and a consequent increase in citrate levels[109]. Considering that NR activity and amino acid

levels are markedly increased under hypoxia, the NO-dependent inhibition of aconitase leads to a shift towards amino acid biosynthesis[109].

Taken together, the multiple roles of NO-mediated signalling in N metabolism, as discussed in this review, suggest that specificity during plant responses to environmental cues might be achieved through a balance between the synthesis and scavenging of NO and related molecules in a stimulus-specific manner. Importantly, different sources of NO and newly described pathways of NO degradation in plants, and the molecular associations of these features should be addressed in future studies of plant NO-mediated redox systems.

Conclusions and future remarks

Particularly in plants, NO synthesis is achieved through the operation of multiple oxi-reductive routes. These different pathways for NO synthesis have represented a trammel in the genetic manipulation of NO signalling in plants. Attempts to identify the primary source of NO in plants frequently generate discrepant results, and together with the fact that different mechanisms for NO production occur in distinct subcellular sites, have indicated that NO homeostasis depends on the specificity of the stimulus and the triggered cellular response. Alternatively, the molecular mechanisms underlying NO degradation have only recently been revealed[5,88] and might lead to significant advances in NO research field.

Recently, a novel NO-mediated feedback mechanism for the fine-tuning of nitrate assimilation has been proposed[5]. Importantly, the redox control of GSNOR1 activity through *S*-nitrosylation has been suggested as a point of convergence in the control of nitrate assimilation and NO signalling in plants. Investigation of the exact site of *S*-nitrosylation in GSNOR1 should be the next step towards understanding the role of the post-transcriptional control of this enzyme. These data might provide information concerning different protein modifications that control GSNOR1 activity.

Although the interdependency between nitrate assimilation and photosynthesis has been firmly established, the underlying molecular mechanisms remain poorly understood. Considering that key photosynthetic proteins are targets of NO, it is tempting to speculate about the coordination of C and N metabolism mediated

through NO. Future studies focusing on the NO-mediated post-translational modification of proteins in N and C metabolism might be interesting.

Recent advances in the redox control of plant metabolism, particularly those concerning the NO-mediated post-translational modification of key enzymes, might foster future efforts to improve N use efficiency in agriculture and reduce the cost and environmental impact of fertilization.

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CONCLUSION AND FUTURE REMARKS

About the final considerations.

As immobile organisms, plants have evolved to overcome environmental challenge through the fine tune of metabolic processes. Despite the extensive knowledge concerning the metabolic processes triggered and the resulting phenotypes in response to abiotic cues, how these responses are signalled and modulated is, to the same extent, unclear. In this Thesis we present a robust set of genetic and biochemical evidence indicating an intensive interplay between nitric oxide (NO) signalling and nitrate assimilation with the regulation of the enzymatic activity of GSNOR1 as a convergence point in plants.

The process of nitrate uptake by roots is mediated by the activity of transport systems precisely regulated metabolically. Several lines of evidence discussed throughout this Thesis strongly suggest that NO/GSNO plays a role as an integral signal of a negative feedback of nitrate uptake and reduction. It is noteworthy, as originally proposed in this Thesis, NO and GSNO act as metabolic signals committed in the fine-tuning of nitrate assimilation. Taking into consideration, however, the relative short half-life and scanty concentration of NO in biological systems, the proposition that NO/GSNO feedback represses nitrate assimilation raises a question:

How can fluctuations at the cellular NO level as a consequence of nitrate assimilation trigger a relevant biological effect that culminates in inhibition of nitrate uptake and reduction?

This question is finely resolved by the counterintuitive finding that GSNOR1 activity is directly modulated by NO through an inhibitory *S*-nitrosylation. This is the first report of a post-translational modification of GSNOR1 in plants. In the proposed metabolic path, the inhibition of GSNOR1 activity represents a strategic point of amplification and sustaining of the signal aiming to feedback regulate nitrate assimilation. This metabolic path may also represent a strategy of NO self-control of bioavailability, avoiding toxicity and futile signalling. Furthermore, in *Chapter III* of this Thesis our findings are comprehensively framed with the current knowledge in the field of NO signalling and nitrate assimilation aiming to highlight new insights and future research perspectives.

I hope that the findings presented in this Thesis substantiate agriculture practices in order to improve crop yield. On the premise that N availability is directly correlated with crop production, tons of fertilizers are indiscriminately applied to farming annually. However, as experimental evidence shows in this Thesis, a negative feedback mechanism of nitrate assimilation suppresses nitrate assimilation and consequently plant biomass accumulation. Genetic and biochemical engineering of the key point in this novel mechanism seems promising to improve nitrate use efficiency in plants without nitrate saturation of soil. The reduction in the use of fertilizers would have economic and environmental benefits, as would reduce economic losses and eutrophication due nitrate leaching to rivers and groundwater.

In addition to the proposals concerning agriculture practices, our findings evidence molecular mechanisms of specificity in cellular signalling. The observation that the enzyme GSNOR1, responsible for the catalysis of GSNO (adduct of NO and GSH), is directly and post-transcriptionally inhibited by NO, strongly implies the existence of different branches of proteins targeted by *S*-nitrosylation by NO and GSNO. Although evidence indicates that these groups overlap, I hope this work influences future research efforts towards an understanding of how specificity in NO-mediated redox signalling is achieved in cellular systems.

CONCLUSÃO E PERSPECTIVAS

Sobre as considerações finais.

Como organismos imóveis, plantas evoluíram para superar desafios impostos pelo ambiente através do ajuste fino de processos metabólicos. Apesar do extensivo conhecimento de quais os processos metabólicos disparados e dos fenótipos resultantes de estímulos ambientais, como essas respostas são sinalizadas e moduladas ainda permanece largamente desconhecido. Nesta Tese apresentei um robusto conjunto de evidências genéticas e bioquímicas de uma intensiva interação entre a sinalização do óxido nítrico (NO) e a assimilação do nitrato em plantas tendo a atividade da GSNOR1 como um importante ponto de convergência.

O processo de captação de nitrato do solo pelas raízes das plantas é realizado por sistemas de transporte finamente controlados metabolicamente. Diversas linhas de evidencias apresentadas ao longo dessa Tese sugerem fortemente que o NO/GSNO apresente uma importante função como sinalizador integrante de um mecanismo de feedback negativo que reprime a assimilação de nitrato através de sua captação e redução. Importante, é proposto originalmente nessa Tese que o NO e GSNO ajam como sinais metabólicos que regulam a assimilação de nitrato. Considerando, no entanto, a curta meia vida do NO e sua baixa concentração em sistemas celulares, a proposição de que o NO/GSNO inibam a assimilação de nitrato leva a um questionamento:

Como as alterações no nível de NO durante o processo de assimilação do nitrato podem gerar um efeito biológico relevante a ponto de inibir a captação e redução do nitrato?

Esta questão é elegantemente respondida através da contraintuitiva descoberta de que a GSNOR1 pode ser diretamente inibida pelo NO através da *S*-nitrosilação. Esta é a primeira vez que é mostrado que a atividade da GSNOR1 de plantas está submetida a controle pós-traducional. No ciclo de regulação da assimilação do nitrato proposto, a inibição da atividade da GSNOR1, por tanto, representa um ponto estratégico de amplificação e sustentação do sinal gerado para o retrocontrole da assimilação de nitrato. Este ciclo pode também representar um mecanismo de autocontrole da biodisponibilidade do NO em plantas, evitando assim toxicidade e sinalização de eventos fúteis. Ainda, nesta Tese nossos dados são compreensivamente balizados com recentes avanços nos campos de sinalização redox mediada por NO e controle da assimilação do nitrato com o objetivo de indicar perspectivas do que acredito representar os futuros desafios nessas áreas.

Espero que os achados apresentados nessa Tese substanciem práticas agrícolas com o objetivo de aumentar a eficiência de produção vegetal. Partindo da premissa de que o aumento da disponibilidade de fontes de N no solo leva a um aumento da produção vegetal, anualmente toneladas de fertilizantes são aplicadas à lavoura de forma indiscriminada. No entanto, conforme evidências experimentais apresentadas nessa Tese, um mecanismo de retroalimentação negativo do processo de assimilação de nitrato age, em última instância, limitando o acúmulo de biomassa. A manipulação bioquímica e genética de pontos de regulação estratégicos deste novo mecanismo de regulação apresenta-se como uma possível estratégia

promissora para aumentar a eficiência de assimilação de nitrato em plantas sem que haja a necessidade de saturação de nitrato no solo. A redução do uso de fertilizantes, além de mitigar perdas econômicas, previne a eutrofisação de lençóis freáticos e rios pela lixiviação do nitrato do solo; dessa forma apresentando vantagens econômicas e ambientais.

Adicionalmente às novas propostas relacionadas a práticas agrícolas, nossos dados estabelecem mecanismos moleculares de especificidade em processos de sinalização celular. A observação de que a enzima GSNOR1, responsável pela catálise de GSNO (aduto entre GSH e NO), é diretamente inibida pós tradicionalmente por NO, fortemente implica na existência de grupos diferentes de proteínas alvos de *S*-nitrosilação para NO e GSNO. Apesar de evidências apontarem uma sobreposição entre estes conjuntos de proteínas, espero que esse trabalho de Tese influencie futuras pesquisas em busca da compreensão de como a especificidade da sinalização redox mediada pelo NO é atingida em ambiente celular.

APPENDIX

(APÊNDICE)

Biosefety certificate/*Certificado de biossegurança*

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INFORMAÇÃO

INFORMO que o projeto Projeto CIBio No.: 2008/05 – "Estudo do efeito da elevação dos níveis endógenos de óxido nítrico nas características funcionais de mitocôndrias vegetais", cuja pesquisadora responsável é a Profa. Dra.Ione Salgado, encontra-se devidamente aprovado e regularizado junto a CIBio/IB-UNICAMP e a CTNBio, conforme legislação vigente. Informamos ainda que o Doutorando Lucas Frungillo é membro da equipe executora do projeto.

Cidade Universitária "Zeferino Vaz",
12 de junho de 2015.

Profa. Dra. HELENA COUTINHO FRANCO DE OLIVEIRA
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