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Nitric oxide and gene regulation in plants

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Abstract

There is increasing evidence that nitric oxide (NO), which was first identified as a unique diffusible molecular messenger in animals, plays an important role in diverse physiological processes in plants. Recent progress that has deepened our understanding of NO signalling functions in plants, with special emphasis on defence signalling, is discussed here. Several studies, based on plants with altered NO-levels, have recently provided genetic evidence for the importance of NO in gene induction. For a general overview of which gene expression levels are altered by NO, two studies, involving large-scale transcriptional analyses of *Arabidopsis thaliana* using custom-made or commercial DNA-microarrays, were performed. Furthermore, a comprehensive transcript profiling by cDNA-amplification fragment length polymorphism (AFLP) revealed a number of *Arabidopsis thaliana* genes that are involved in signal transduction, disease resistance and stress response, photosynthesis, cellular transport, and basic metabolism. In addition, NO affects the expression of numerous genes in other plant species such as tobacco or soybean. The NO-dependent intracellular signalling pathway(s) that lead to the activation or suppression of these genes have not yet been defined. Several lines of evidence point to an interrelationship between NO and salicylic acid (SA) in plant defence. Recent evidence suggests that NO also plays a role in the wounding/jasmonic acid (JA) signalling pathway. NO donors affect both wounding-induced H_2O_2 synthesis and wounding- or JA-induced expression of defence genes. One of the major challenges ahead is to determine how the correct specific response is evoked, despite shared use of the NO signal and, in some cases, its downstream second messengers.

Key words: *Arabidopsis*, gene expression, microarray, nitric oxide, signal transduction.

Introduction

Despite the widespread medicinal use of nitroglycerine since the late 1940s, nitroglycerine-derived NO was only suggested as the pharmacologically active agent as recently as 1977 by Ferid Murad (Katsuki *et al*., 1977). In 1987, the endothelial-derived relaxing factor was shown to be NO by Louis Ignarro (Ignarro *et al*., 1987) and Salvador Moncada (Palmer *et al*., 1987), leading to the massive burst of medicinal NO publications persisting to this day. Plant researchers did not realize the enormous meaning of these findings and only focused on NO as an atmospheric pollutant until the mid-1990s. Data presenting NO as an inducer of leaf expansion, root growth, and phytoalexin production (Leshem, 1996; Noritake *et al*., 1996) and the identification of plants as active NO producers (Wildt *et al*., 1997) suddenly brought NO into the focus of plant scientists.

Today, the participation of NO in a great number of plant signalling pathways is common knowledge and almost weekly new data on NO-derived effects in plants are published. Nevertheless, this situation resembles that of the 19th century doctors: there is an astonishing gap between the abundant involvement of NO in different plant cell signalling pathways and the actual knowledge about its direct targets or inductive or repressive effects on gene expression level. The high reactivity and janus-faced character of NO complicates the situation and delays the development of a model about its role in the cellular signalling networks.

This review starts with a short description of two whole-genome approaches and will further focus on single NO-mediated stress responses and signal transduction pathways of physiological importance.

Whole genome approaches

The 64 000 dollar question is which genes are directly modulated by NO. Despite the rapid spread of microarray

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technology in recent years and the fact that microarray technology was used in several small-scale experiments (as described later) only two whole-genome approaches were started. Polverari *et al.* (2003) investigated the changes of expression profiles of *A. thaliana* after infiltration with the NO donor sodium nitroprusside (SNP) by cDNA-amplification fragment length polymorphism (AFLP) transcript profiling. The respective SNP concentration led to leaf tissue collapse after 24 h. With this approach the expression level of 2500 transcripts was checked at different time points and NO-derived alterations could be detected for 120 transcripts. Sequence analysis of 71 differentially expressed cDNAs and their comparison to microarray results in public databases showed that most NO-modulated genes are also affected in other abiotic or biotic stress-related conditions. They belong to the functional categories of signal transduction, defence or cell death, ROS generation and removal, photosynthetic processes, cellular trafficking, and basic metabolism. Almost one-third of them consist of unclassified proteins. Astonishingly, only a few of them are seen as specifically stress-related, but belong to a great number of different physiological processes. Only two of the 71 identified genes were repressed (lipoyl transferase, putative leucine-rich repeat transmembrane protein kinase) while all the others were shown to be NO-inducible.

Expression profiling by microarrays has developed into a powerful method in plant research. This more sophisticated approach was used by Parani *et al.* (2004), who investigated the NO-induced alteration of the *A. thaliana* expression profile by using a whole-genome microarray (MicroArray Suite 5.0, Affymetrix, Inc.) representing approximately 24 000 genes. *Arabidopsis* roots were treated with 0.1 mM and 1.0 mM of sodium nitroprusside (SNP), respectively. 342 up-regulated (162 with a dose-dependent increase) and 80 down-regulated genes were observed after treatment with SNP. The dose-dependent-induced transcripts could be classified into plant defence, protection against oxidative stress, iron homeostasis, signal transduction and transcription factors. The transcript level of several typical pathogen-induced genes (e.g. *NBS-LRRs*, *NDR1*) and genes coding for disease resistance proteins was induced by the NO donor SNP. In addition to the findings of Polverari *et al.* (2003), the transcript level of several plant defence response modulating transcription factors, like WRKYs, EREBPs (ethylene responsive element-binding proteins) several zinc finger proteins, and dehydration responsive element binding proteins (DREB1 and DREB2), were also induced by SNP. Other interesting induced transcripts were coding for oxidative stress-related proteins (GSTs, ABC transporters), iron homeostasis proteins (e.g. ferritin genes), signal transduction factors (e.g. members of the defence-related MAP kinase modules), and plant development. Perhaps due to the different methods of NO-application, the number of genes found to

be induced by AFLP- and the microarray approach is astonishing low. Furthermore, the application of NO donors probably does not reflect any spatio-temporal aspects of NO signalling in plants.

Thus in *Arabidopsis* the expression level of a relatively low number of genes is influenced by NO. Nevertheless, these genes belong to a wide range of different physiological functions. This is mirrored by the large diversity of signal transduction pathways with NO involvement, as described in the following chapters.

Regulation of gene expression by NO/*S*-nitrosylation

How does NO induce the expression of the genes described above? The activities of a variety of nuclear regulatory proteins are affected dramatically by NO. In this context, the formation of *S*-nitrosylated proteins seems to be an especially important mechanism in the regulation of the function/activity of transcription factors. *S*-nitrosylated proteins are created when a cysteine thiol reacts with NO in the presence of an electron acceptor to form an S-NO bond. Under physiological conditions this post-translational modification affects the function of a wide range of cellular proteins, like stress-related proteins, signalling proteins, metabolic proteins, and nuclear regulatory proteins. The latter group include hypoxia-inducible factor I (Palmer *et al.*, 2000), nuclear factor-κB (Marshall and Stamler, 2001), stimulating proteins 1 and 3 (Zaman *et al.*, 2002), and the prokaryotic transcription factors OxyR (Hausladen *et al.*, 1996) and SoxR (Ding and Demple, 2000). Until now no plant transcription factor is described to be regulated by *S*-nitrosylation, but proteomic studies identified promising candidates for *S*-nitrosylated regulatory proteins (Lindermayr *et al.*, 2005). Next to the direct *S*-nitrosylation of transcription factors, gene expression can also be regulated by *S*-nitrosylation of proteins which are part of a signalling cascade, for example, nuclear factor-κB kinase (Reynaert *et al.*, 2004) or protein tyrosine phosphatase 1B (Li and Whorton, 2003). In addition, the degradation of nuclear proteins can be regulated by protein *S*-nitrosylation. Next to the reaction with cysteine residues NO can form metal nitrosyls by binding to the haem moiety of proteins or it can react with tyrosine residues (protein nitration) representing further important NO-dependent regulation mechanisms (Pfeilschifter *et al.*, 2001).

NO's role in stress responses

Transcript profiling on the genome-wide level seems to give clear answers on the question of NO-induced gene expression (Table 1). Nevertheless, further investigation of specific cases is absolutely necessary to avoid misinterpretation of NO-dependent gene induction. Gene induction

Table 1. *Stress-related genes modulated by NO*

Colour-code is red for NO-induced gene expression and green for NO-repressed gene expression.

Plant system	Elicitor/Treatment	Gene expression	Citation
Wounding			
A. thaliana plants	Wounding	*AOS, LOX2, OPR3*	Huang *et al.*, 2004
Ipomoea batatas plants	Wounding/MeJA.	*IPO*	Imanishi *et al.*, 1997
	Wounding + SNP	(*IPO*: Delayed compared to wounding alone)	Jih *et al.*, 2003
Elicitor molecules			
A. thaliana plants and cell suspensions WT versus *AtNOS1* mutant	LPS	*AtNOS1*-dependent: ABC-transporters, cytochrome P450 genes, glutathione-complex-related genes, PR-genes and other oxidative stress- or defence-related genes	Zeidler *et al.*, 2004
Nicotiana tabacum (Xanthi) cell suspension cultures	Cryptogein	Cryptogein-induced and NO-dependent expression of ACC synthase and sHSP. Expression of *LOX1*, *Pr-3*, *GST-1a*, and *Pal* is NOT inhibited by cPTIO	Lamotte *et al.*, 2004
Microorganisms			
A. thaliana plants WT versus NO-deficient *hmp8* plants (heterologous expression of a bacterial NOD)	Avirulent *Ps. syringae* pv. *tomato* WT versus NO-deficient *hmpX*. (heterologous expression of a bacterial NOD)	NO-dependent:. *Pal, Pr-1*	Zeier *et al.*, 2004
NO and other kinds of oxidative stress			
A. thaliana Col-0 plants: AFLP analysis	SNP infiltrated in leaves	2500 cDNAs 69 fragments: functional categories: signal transduction, resistance and cell death, ROS-related, chloroplast, transport, basic metabolism, unknown proteins 2 fragments: lipoyl transferase, putative leucine-rich repeat transmembrane proteine kinase	Polverari *et al.*, 2003
A. thaliana Col-0 plants Microarray analysis	SNP applied via the roots	24 000 genes 342 genes up-regulated (162 dose-dependent): functional categories: plant defence response, protection against oxidative stress, signal transduction, transcription factors 80 genes downregulated	Parani, 2004
N. tabacum cv. Xanthi plants and suspension cultures	Mammalian NOS, GSNO, SNAP	*Pal* *Pr-1*	Durner *et al.*, 1998
Glycine max cell suspension cultures	SNP	*Pal* *CHS*	Delledonne *et al.*, 1998
A. thaliana Col-0 plants *A. thaliana* Col-0 suspension cells	Gaseous NO NOR-3	*AOX1a*, glutathione peroxidase, glutathione S-transferase, glutaredoxins	Huang *et al.*, 2002
A. thaliana Col-0 plants	Gaseous NO	*AOS, LOX2, OPR3* (*PDF1.2* and *JIP* only in the absence of SA)	Huang *et al.*, 2004
A. thaliana Col-0 plants: WT, mutants overexpressing tAPX	SNP	*tAPX*	Murgia *et al.*, 2004*b*

Abbreviations: Substances: cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl; JA, jasmonic acid; LPS, lipopolysaccharide; MeJA, methyl jasmonate; NO, nitric oxide; NOR-3, (E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexene-amide; SA, salicylic acid; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.
Genes and proteins: *AOS*, allene oxide synthase; *AOX*, alternative oxidase; *AtNOS*, *A. thaliana* NO synthase; *Chs*, chalcone synthase; *GST*, gluthathion S-transferase; *IPO*, ipomoelin; *JIP*, jasmonic acid induced protein; *LOX*, lipoxygenase; *NOD*, nitric oxide dioxygenase; *OPR3*, 12-oxophytodienoate reductase; *Pal*, Phe-ammonia lyase; *PDF1.2*, plant defensin; *Pr*, pathogenesis related; *tAPX*, thylakoidal ascorbate peroxidase.
Others: WT, wild type.

alone does not necessarily induce metabolic change and, therefore, a physiological reaction of the cell. The great multitude of different signal factors interacting directly or indirectly with NO makes the existence of a simple cause-and-effect chain very unlikely and results in the conflicting data and hypothesis about NO's role as described below. Modulation of hypersensitive cell death and defence gene activation by NO was presented at first by Delledonne *et al.* (1998) and Durner *et al.* (1998). Actually, NO is involved in almost every stress response analysed for NO so far.

Interaction of NO with different plant hormones during wounding-induced stress

Wounding-derived gene expression in different plant species is a convincing example of the discrepancy between the effect of NO on gene expression level and the actual result of this gene expression at the cellular and physiological level. In *Arabidopsis* an NO burst was demonstrated to be triggered within minutes after wounding of the leaf epidermis. When plants were treated with NO directly, northern analysis revealed that key enzymes of the octadecanoid pathway, like *AOS*, *LOX2*, or *OPR3*, were highly induced (Huang *et al.*, 2004). Surprisingly this induction did not result in elevated jasmonic acid (JA) levels, and therefore JA responsible genes, like *PDF1.2*, were not induced. Substrate limitation would be one possible explanation for this paradox. Another possible explanation is the demonstrated antagonistic role of salicylic acid (SA) (Farmer *et al.*, 1998; Glazebrook, 2001). It was shown that NO increases the SA level (Durner *et al.*, 1998; Durner and Klessig, 1999; Huang *et al.*, 2004). In SA-deficient *NahG* plants, NO treatments led to elevated JA levels followed by the induction of *PDF1.2* and *JIP*, which were non-responsive in wild-type plants. Nevertheless, SA does not always play a role in NO-induced gene expression. In the late 1990s Durner *et al.* (1998) presented evidence for the increase of total SA levels and the induction of *Pr-1-* and *Pal*-expression in NO-treated tobacco leaves. Astonishingly, the induction of *Pr-1* was shown to be SA-dependent, whereas *Pal*-expression was not.

Besides the model plant *Arabidopsis*, several economically important crop plants were analysed for their wound-induced responses. The expression of the *Ipomoelin* gene (*IPO*) in sweet potato was shown to be enhanced by methyl jasmonate (MeJA) and mechanical wounding (Imanishi *et al.*, 1997). Although NO and H_2O_2 accumulation were both enhanced, SNP-derived NO delayed wounding-induced *IPO* expression (Jih *et al.*, 2003). The authors suggest two important wound-response-related effects of NO: initiation of the cell death cycle together with hydrogen peroxide, and delay of *IPO*-expression. Nevertheless, this interpretation is in direct contrast to the data of Tada *et al.* (2004), who see NO as an important factor for propagation, but not initiation, of cell death in oat plant cells directly infected with avirulent crown rust fungus (see below).

The results described above are in contrast to respective data from tomato plants (Orozco-Cárdenas and Ryan, 2002). Neither wound-induced NO burst could be demonstrated here, nor NO-induced elevation of endogenous SA levels. Moreover, H_2O_2 accumulation and expression of the proteinase inhibitors *Inh1*, *Inh2*, cathepsin D inhibitor (*CDI*), and metallocarboxypeptidase inhibitor (*CPI*) were inhibited by SNP-derived NO, but not the expression of *AOS* or *LOX*. Thus the authors suggest that NO is inhibiting signalling downstream from JA, but still upstream from ROS generation. These contradictory results correlate with several reports on the basic differences in wound-induced signalling pathways in *Arabidopsis* and those in the Solanaceae (Leon *et al.*, 2001). Nevertheless, they demonstrate clearly that the accumulation of one signalling substance alone is not sufficient to induce any physiological changes.

NO's role in plant–pathogen interaction

Plants growing in the grasslands of the temperate zone are confronted with roughly estimated 10^6 individuals of bacteria, fungi, protozoa, and algae, 6000 nematodes, plus 100 or more microarthropods, oligochaeta, and earthworms in their habitat. Plant defence mechanisms against this great abundance of potentially dangerous species can be subdivided into 'innate immunity' consisting of unspecific mechanical or biochemical mechanisms, and the specific or cultivar resistance for which a specific interaction of host- and pathogen-specific gene products is characteristic. NO is involved in both mechanisms.

Innate immunity

Invariant pathogen-associated molecular patterns (PAMPs) are recognized and trigger innate immunity. The high conservation of structures responsible for the flagellin perception in plants and animals was demonstrated (Gomez-Gomez and Boller, 2002; O'Neill, 2002; Zipfel *et al.*, 2004). Recently Zeidler *et al.* (2004) presented data that one of the most prominent animal innate immunity features, the LPS-mediated NO burst, also appears in plants. Interestingly, they gave strong evidence for the plant NO synthase *AtNOS1* (previously described by Guo *et al.*, 2003) as the source of the LPS-mediated NO burst, while nitrate reductase is not involved in this process. An application-based microarray comparison of LPS-induced gene expression in wild-type plants and *AtNOS1* SALK insertion mutants impaired in NO synthase activity clearly demonstrated the role of NO as an important signal substance. The induction of ABC transporters, cytochrome P450 genes, glutathione-complex-related genes, Pr-genes,

and several other oxidative stress- or defence-related genes occurred only in wild-type plants, but not in the mutants impaired in *AtNOS1* activity. Nevertheless, the role of NO has to be investigated for every single gene in detail.

Incompatible plant–pathogen interaction

Manipulating the plants' NO level by the application of pharmacological active substances like NO donors or NO scavengers aggravates the differentiation between real NO-derived effects and side-effects of the carrier molecules. For example, treatment of plant cell cultures with different NO donors like SNP, SNAP, or NOC-18, respectively, results in different cellular responses (Murgia *et al*., 2004*a*; see below). This general problem is also discussed in an excellent review by Shapiro (2005). For this reason mutants like *AtNOS1* proved to be an effective research tool, although some of them show a strong phenotype. Another non-pharmacological research approach was used by Zeier *et al*. (2004). They investigated NO burst and NO-induced gene expression in an incompatible plant–pathogen interaction system consisting of *Arabidopsis* and *Pseudomonas* both heterologously expressing two NO dioxygenases of different origin (NOD, *hmp* and *hmpX,* respectively, both under the control of a DEX-inducible promotor) converting NO to nitrate. The authors demonstrated an attenuated pathogen-induced NO burst, a strong inhibition of *Pal* and a delay in the expression of *Pr-1*. SA accumulation and hypersensitive response (HR) were also diminished. The UV-light-induced expression of *Pal* and *Chs* was also strongly repressed in *hmp-Arabidopsis* plants. Surprisingly the intensity of several of the described effects increased when NOD was expressed not only in *Arabidopsis* alone, but also in the bacterial part of the system. Nevertheless, side-effects cannot be excluded in this system as the NOD activity leads to decreased H_2O_2 levels during the oxidative burst. The authors themselves discussed other additional possible side-effects in their paper.

Gene induction during the hypersensitive response and cell death as a result of a fine-tuned NO/reactive oxygen species balance

During the oxidative burst preceding the hypersensitive response, a correlating accumulation of reactive oxygen species such as H_2O_2 or O_2^- and NO is often observed (Delledonne *et al*., 1998; Krause and Durner, 2004; Tada *et al*., 2004). The first evidence that NO, in combination with other reactive oxygen species, is required for cell death was given by Delledonne *et al*. (1998). Actually it is still controversial, whether NO itself is sufficient to initiate cell death or whether it only plays a role in cell death propagation (C Zhang *et al*., 2003; Tada *et al*., 2004; Casolo *et al*., 2005).

NADPH-oxidase derived O_2^- is seen as a substrate for the SOD-catalysed H_2O_2 formation during the cellular response. NO reacts with O_2^- to the non-HR-inducing peroxynitrite ($ONOO^-$) in an extremely fast chemical reaction (Hippeli and Elstner, 1998). The importance of the fine tuning of this NO/H_2O_2 balance was demonstrated using thylakoidal ascorbate peroxidases (*tAPX*) mutants with enhanced (Murgia *et al*., 2004*b*) or reduced (Tarantino *et al*., 2005) *tAPX* expression. In plants overexpressing *tAPX*, SNP clearly reduces *tAPX* transcript accumulation. The role of peroxynitrite in cell death is still controversial (Alamillo and García-Olmedo, 2001; Delledonne *et al*., 2001; Lamotte *et al*., 2004). Following these arguments one should keep in mind that peroxynitrite itself can serve as a NO scavenger via the formation of nitrogen dioxide (Daiber *et al*., 2002).

Although NO alone is not sufficient for the induction or propagation of cell death it influences gene expression. NO-derived induction of defence-related genes was shown for *Pr-1* and *Pal* in tobacco (Durner *et al*., 1998) and *Pal* and *Chs* in soybean (Delledonne *et al*., 1998).

NO as a signal affecting plant cell organelles

Mitochondria

Animal cell death pathways can be subdivided into two components, either involving death receptors or mitochondria (Brune, 2003). NO is seen as a signalling factor in the latter. NO inhibits the activity of the last enzyme in the mitochondrial respiratory electron transport chain, the cytochrome *c* oxidase (COX) leading to the generation of superoxide O_2^- due to the dramatically reduced ubiquinone pool. Plant mitochondria are also a target of NO (Zottini *et al*., 2002), but possess another terminal oxidase, the alternative oxidase, AOX. In *Arabidopsis* five isoforms are known (Thirkettle-Watts *et al*., 2003). Electron transport from ubiquinol to AOX is non-phosphorylating and releases energy as heat.

Application of the NO donor NOR-3 to *Arabidopsis* cell suspension culture resulted in the induction of several genes (Huang *et al*., 2002). Mircoarray analysis showed the increased expression of glutathione peroxidase, glutathione *S*-transferase, glutaredoxins, and other antioxidant genes. Of special interest is the highly induced alternative oxidase AOX1a which is localized in the mitochondria. In *Arabidopsis*, expression of *AOX1a* is not only induced by NO but also by several kinds of biotic stresses like *Ps. syringae* pv. *tomato* (Simons *et al*., 1999) or the proteinaceous bacterial elicitor Harpin (Krause and Durner, 2004). AOX's enzyme activity is not affected by NO (Millar and Day, 1996), so that the protein is able to re-oxidize the over-reduced ubiquinone pool during NO treatment. Therefore AOX's role for the NO tolerance of higher plants is discussed (Millar and Day, 1997). Using the SA-signalling mutants (*pad4*, *npr1*) and the SA-deficiency mutant (*NahG*) Huang *et al*. (2002) demonstrated the SA independence of the NO-induced *AOX1a* transcription.

Chloroplasts

UV-light has an effect on many different life processes of plants (Shi *et al.*, 2005). These effects are mediated by the UV-sensitivity of a great abundance of different cellular macromolecules. Besides damaging macromolecules, UV-stress leads to an increase in ROS accumulation. The antioxidative effects of NO due to its chemical reactions with different reactive oxygen species have already been discussed in earlier reports (Beligni and Lamattina, 1999; Beligni *et al.*, 2002).

Recently, NO's protective role against UV-stress was investigated in several plants. Bean leaves treated with UV-B show increased ion leakage and damage in the photosynthetic apparatus as a consequence of photo-oxidative stress (Shi *et al.*, 2005). NO, given as SNP, was thought to attenuate these damages by decreasing the H_2O_2 content through enhanced activities of antioxidant enzymes like superoxide dismutases, ascorbate peroxidases, and catalases. Whether these enhanced activities followed from increased gene expression or from post-translational modification of the respective proteins was not investigated.

NO in non-stress-related signalling pathways

NO-derived signalling is not restricted to plant responses to abiotic and biotic stress, but is involved in many other different plant signal transduction pathways as well (Table 2). A recently published example is the NO-induced increase in expression of the *LjHb1* gene coding for non-symbiotic haemoglobin in *Lotus japonicus* (Shimoda *et al.*, 2005). NO's role in plant adaptation to hypoxia is presented in a review by Igamberdiev and Hill (2004), and

new mechanisms to modulate NO-dependent bioactivities in plants are summarized in Crawford and Guo (2005). Due to the series of recently published papers, this review focuses on plant growth and development, iron uptake, and ABA-induced stomatal closing.

Sexual reproduction

NO was shown to be involved in many different steps of the sexual reproduction process. First of all floral transition is an excellent example for the use of mutants to learn more about the developmental processes in plants. The first evidence for the involvement of NO in the sexual reproduction of plants was demonstrated by analysing *AtNOS1*-deficient *Arabidopsis* plants which flower earlier than wild-type plants (Guo *et al.*, 2003). Nevertheless, the inflorescence was reduced and fertility was low. Correlating with this *Arabidopsis* mutant line, NO-overproducing plants (*nox1*) flowered later than the wild type (He *et al.*, 2004). This overproduction is the result of the accumulation of *ATNOS1*'s substrate L-arginine by the disruption of the activity of a plastidial phosphoenolpyruvate/phosphate translocator. NO affects flowering time by reducing the amplitude, but not the rhythm of signalling derived from the circadian clock to the photoperiod pathway. NO suppressed the expression of *Constans* and *Gigantea*, but also enhanced *Flowering locus C* (FLC) expression. In spite of the identified target genes NO's direct targets remain unknown. A short summary of He's results has been given by Simpson (2005).

Besides floral transition, NO is involved in the growth regulation of pollen tubes (Prado *et al.*, 2004), programmed cell death in the aleurone (Fath *et al.*, 2001; Beligni *et al.*, 2002), and in the breaking of seed dormancy (Bethke

Table 2. *NO and gene expression in processes of physiological relevance*

Colour-code is red for NO-induced gene expression and green for NO-repressed gene expression.

Plant system	Process	Gene expression	Citation
Iron homeostasis			
A. thaliana leaves	NO-dependent ferritin expression	*Atfer1*	Murgia *et al.*, 2002*a*
A. thaliana Landsberg cell cultures	NO-dependent ferritin expression	*Atfer1* (induction only by SNP, not by other tested NO-donors)	Murgia *et al.*, 2004*a*
Zea mays	NO's role in the internal metabollically active iron availability in plant tissue	*rbcL, psbA* in iron deficient plants by SNP	Graziano *et al.*, 2002
Sexual reproduction			
A. thaliana plants, WT or *nox1* (=*cue1*) with elevated endogenous NO-levels	Floral decision	*Constans, Gigantea* *Flowering locus C*	He *et al.*, 2004
Hordeum vulgare	Programmed cell death in aleurone layers	GA-repression of *Cat2* expression slightly delayed by SNAP GA-induced decline of *SOD* mRNA delayed by SNP or SNAP	Beligni *et al.*, 2002
Symbiosis			
Lotus japonicus	Symbiosis between *Lotus japonicus* and rhizobia	*LjHb1*	Shimoda *et al.*, 2005

Abbreviations (see also Table 1): *Atfer, Arabidopsis ferritin; Cat, catalase; cue1*, encodes a chloroplastic phosphoenolpyruvate/phosphate translocator; GA, gibberilin; *LjHb1, Lotus japonicus* non-symbiotic haemoglobin; *nox1*, see *cue1; rbcL*, Rubisco large subunit; *psbA*, D1 protein; *SOD, superoxide dismutase*.

et al., 2004; Li et al., 2005). Until now no analysis of these processes was done at the level of gene expression.

Plant growth and development

Evidence for NO as an inducer of leaf expansion, root growth and phytoalexin production was given 10 years ago (Leshem, 1996; Noritake et al., 1996). NO is known to be involved in vegetative growth processes of the shoot (M Zhang et al., 2003; An et al., 2005), cell division (Ötvös et al., 2005), xylem differentiation (Gabaldon et al., 2005), root system development (Pagnussat et al., 2002, 2003; Guo et al., 2003; Correa-Aragunde et al., 2004), plant–rhizobacterium interaction (Creus et al., 2005), and gravitropic bending (Hu et al., 2005). Nevertheless, the nature of NO's real targets in these developmental and growth processes still remains unclear. Hypotheses vary from NO as an unspecific antioxidant protecting IAA from oxidation to NO-specific targets as cell-cycle genes or enzymes involved in IAA-dependent signal transduction. Analysis of the respective gene expression is still lacking.

Modulation of other gene expression by NO

Stomatal closure

Stomatal closure is regulated by abscisic acid (ABA)-derived guard cell membrane transport to promote osmotic solute loss. Desikan et al. (2002), demonstrated, by using the double mutant *nia1/nia2*, that nitrate reductase-produced NO, but not NOS-derived NO is required for the ABA-regulated stomatal closure in *Arabidopsis*. Other reports demonstrate the involvement of NOS (Guo et al., 2003). The role of nitrate reductase is discussed in more detail in Garcia-Mata and Lamattina (2003). With ABA-insensitive mutants *abi1* and *abi2*, the phosphatases positions in ABA signal transduction cascade were localized downstream of NO. Nevertheless, direct targets of NO were unknown until Garcia-Mata et al. (2003) demonstrated that, in *Vicia faba*, Ca^{2+}-sensitive ion channels were regulated by NO-derived calcium–release from intracellular stores. This release is cGMP-dependent. NO is discussed to be involved only in a subset of an ABA-enlisted signalling pathway. One year later, researchers of the same team (Sokolovski and Blatt, 2004) published data indicating that NO directly regulates outward rectifiying K^+ channels ($I_{k,out}$) or a closely associated protein, perhaps by protein *S*-nitrosylation.

Iron homeostasis

Iron ions as components of functional enzymes are involved in an abundance of plant metabolic pathways. Iron homeostasis strongly depends on the iron storage protein ferritin. Ferritins of plants and animals consist on 24 sub-
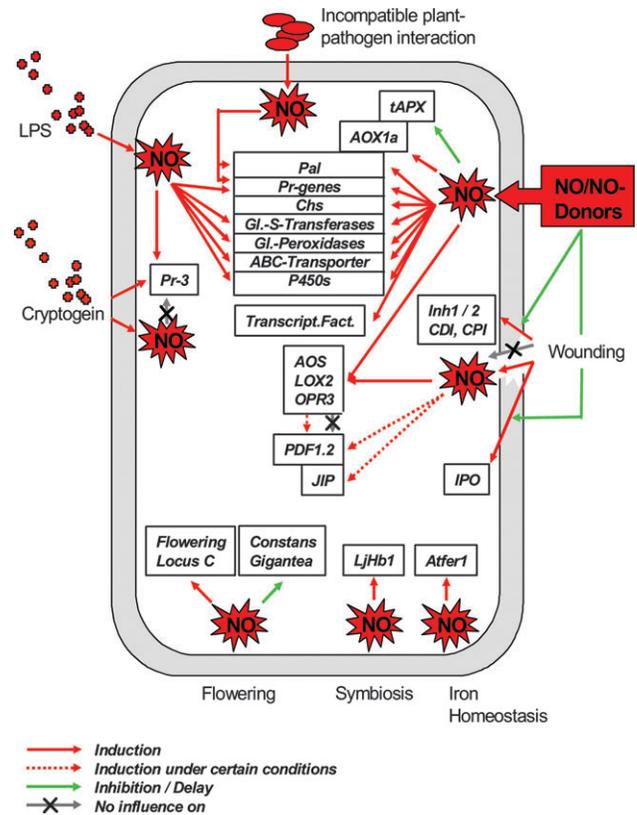

Fig. 1. Overview of selected genes and gene families regulated by NO. Gene names are explained in the respective chapters of the text.

units forming a protein coat for the storage of iron ions (Murgia et al., 2002). Both an excess of iron and many different factors eliciting or mimicking oxidative stress are able to induce the accumulation of the iron storage molecule. Murgia et al. (2002) demonstrated the induction of ferritin expression by SNP-derived NO on mRNA and protein levels in *Arabidopsis* leaves, emphasizing the former. Moreover they identified NO as factor essential for the iron-induced ferritin induction. Under low iron supply the iron-dependent regulatory sequence (IDRS) of the *Arabidopsis* ferritin gene promoter (*Atfer*) is responsible for transcriptional repression of ferritins. Using mutant *Arabidopsis* plants with GUS reporter constructs, IDRS was identified as the target sequence for NO-modulated gene expression as well. The authors postulated a proteic factor directly binding to the DNA sequence and itself modulated by NO. Nevertheless, these effects depend on the applied NO donor (Murgia et al., 2004a). The NO donors SNAP and NOC-18 do not induce the expression of *Atfer1* which means that artificial NO donor-derived data sets have to be interpreted carefully. Further evidence for the NO involvement in iron homeostasis of plant cells was given by Graziano et al. (2002) and Vanin et al. (2004). A summary of NO–iron interaction in plants is given in a recent review by Graziano and Lamattina (2005).

Perspectives

NO, at first glance a simple molecule consisting of only two different atoms, was shown to be a 'global player' in plant cell signalling networks (Fig. 1). Its important role is mirrored by the large amount of NO-specific publications in the last few years in all the research areas of biology and the medical sciences. Oxidative and antioxidative, deleterious and protective, repressive and inductive: the more we learn about this small molecule the more its chameleon character becomes apparent. Experimental limitations and high re-activity combined with a huge number of potential reaction partners make it impossible to develop a 'theory of every-thing' about NO's role in the plant signal transduction net-works. We now face two big challenges: developing better experimental systems and finding the real targets of NO.

Acknowledgements

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