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NO news is good news for plants

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The organization of redox signaling and the use of nitric oxide (NO) to transmit information, modulate biological processes or create cellular damage are highly complex. Recent reports provide an exceptional picture of NO production, of the regulation of NO bioactivity through detoxification reactions and of biochemical events by which NO transduces signals into cellular responses, in particular during disease resistance. Furthermore, other exciting reports on NO function in germination, growth and reproduction support the view that NO is a 'do it all' molecule that plays a crucial role during the entire lifespan of the plant.

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Introduction

Small, simple, and highly toxic, nitric oxide (NO) is a gas with a broad chemistry that involves an array of inter-related redox forms with different chemical reactivities. The discovery and elucidation of its biological functions in the 1980s came as a surprise. NO was named 'Molecule of the Year' in 1992 by the journal *Science*, a Nitric Oxide Society was founded, and a scientific journal devoted entirely to NO was created.

As a pollutant, NO is produced by both automobile engines and power stations. NO is also emitted from plants under stress situations, such as herbicide treatment or pathogen attack, as well as under normal growth conditions [1]. As a mediator of physiological processes, NO has an incredible number of beneficial effects; for example, it functions as a messenger in immune responses. But it can become very toxic under certain complex conditions determined, for example, by its rate of production and diffusion and the redox state of the cell [2].

NO is involved in diverse physiological processes in plants. As a developmental regulator, it promotes germination, leaf extension and root growth, and also delays leaf senescence and fruit maturation [3]. As modulator of disease resistance, it triggers hypersensitive cell death and activates the expression of several defense genes [4]. This wide variety of effects reflects the basic signaling mechanisms that are utilized by virtually all living organisms. In this review, I discuss recent progress in understanding the function of NO in plant responses to biotic stresses and in plant development. I explore the mechanisms through which plants regulate NO bioactivity, and speculate on the biochemical events through which NO transduces signals into cellular responses.

NO production during biotic stress

Several assays are now available for NO. The fluorescent NO-specific dyes 4,5-diaminofluorescein (DAF-2) and 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) are suitable for the direct measurement of NO [5,6]. More-accurate measurements of NO concentration can be provided by direct assays, such as spin-trapping electron paramagnetic resonance (EPR) [7], or by methods that measure NO emission in the gas phase by chemiluminescence [8], photoacoustic laser detection [9] and mass spectrometry [10]. However, the variability in NO concentration as the redox state of the cell changes [11] and the large discrepancies that are sometimes observed when NO is measured by different methods in the same system still call for an improvement of the available methodology. For example, photoacoustic laser detection revealed that tobacco leaves challenged with incompatible *Pseudomonas syringae* accumulated NO in a monophasic manner [9], a pattern of NO accumulation that is similar to that demonstrated by DAF-2 staining of tobacco leaves and cell suspensions challenged with cryptogeiin [5,12*] and tobacco cell suspensions challenged with elicitor [13]. By contrast, when NO accumulation was measured by membrane inlet mass spectrometry, both soybean and tobacco cells challenged with incompatible *P. syringae* were found to emit a rapid NO burst that was maximal approximately 1 h after treatment, followed by a second burst of NO at 4–8 h after treatment. This pattern of NO accumulation is similar to that detected by the oxyhemoglobin assay of soybean cell suspensions challenged with an incompatible strain of *P. syringae* [14].

Most of the experimental data available on NO detection during plant–pathogen interactions come from studies of infections by biotrophic pathogens [4], during which the accumulation of NO has been shown to occur under

conditions that are often concomitant with the avirulent gene-dependent oxidative burst that occurs immediately before the onset of hypersensitive cell death [14]. However, plants also possess the ability to recognize basal and more general elicitors of plant defense, such as lipopolysaccharides (LPS) on the cell-surface of Gram-negative bacteria. In *Arabidopsis thaliana*, LPS from plant and animal pathogens have been found to induce a strong and rapid NO burst, which was largely dependent on the activity of *AtNOS1*, the only plant gene that encodes a nitric oxide synthase (NOS) identified to date [15**].

Plant responses to necrotrophic infection share only a few cellular and molecular mechanisms in common with their responses to biotrophic pathogens. However, infection by necrotrophic pathogens has also been shown extensively to be associated with oxidative stress and the death of host cells [16]. Reactive oxygen species (ROS) that are generated as a result of an oxidative burst during infection mediate hypersensitive cell death as part of the host resistance mechanism. ROS also enhance disease resistance in several ways, however, for example by having direct toxic effects on pathogens and by causing the cross-linking of cell-wall proteins so as to render the cell wall more resistant to attack by pathogen enzymes [4]. By contrast, necrotrophic pathogens might induce toxic levels of ROS in the host thereby promoting infection [17]. Recently, *Botrytis elliptica*, a necrotrophic pathogen that has a very narrow host range, has been shown to kill lily cells by stimulating their production of both ROS and NO, but only during compatible interactions. A partially purified *B. elliptica* culture filtrate triggered ROS and NO accumulation as well as cell death, and made incompatible *Botrytis* species pathogenic on lily [18*]. These data further support the existence of an interplay among signaling networks in both pathogens and hosts that has evolved to allow successful infection by the pathogen and host resistance to disease.

Only a small amount of conflicting information is available regarding the production of NO during the defensive response to herbivorous insects. For example, wounding tomato leaves does not cause an increase in NO production [19], whereas significant amounts of NO are produced after wounding sweet potato or *A. thaliana* plants [20,21]. In addition, NO has been shown to activate early wounding-related genes in *A. thaliana* plants, although it does not appear to be a key player in this response because the induction of these genes after wounding is not affected by NO scavengers [21]. NO can also function as a negative regulator of some wound-related signals as NO donors can either inhibit or delay the expression of wound-dependent genes, such as proteinase inhibitors in tomato or ipomoelin in sweet potato [20], and can reduce the production of wound-induced H₂O₂ [19]. However, when analyzed by either DAF-2 staining or EPR analysis, NO was recently found to accumulate rapidly in epider-

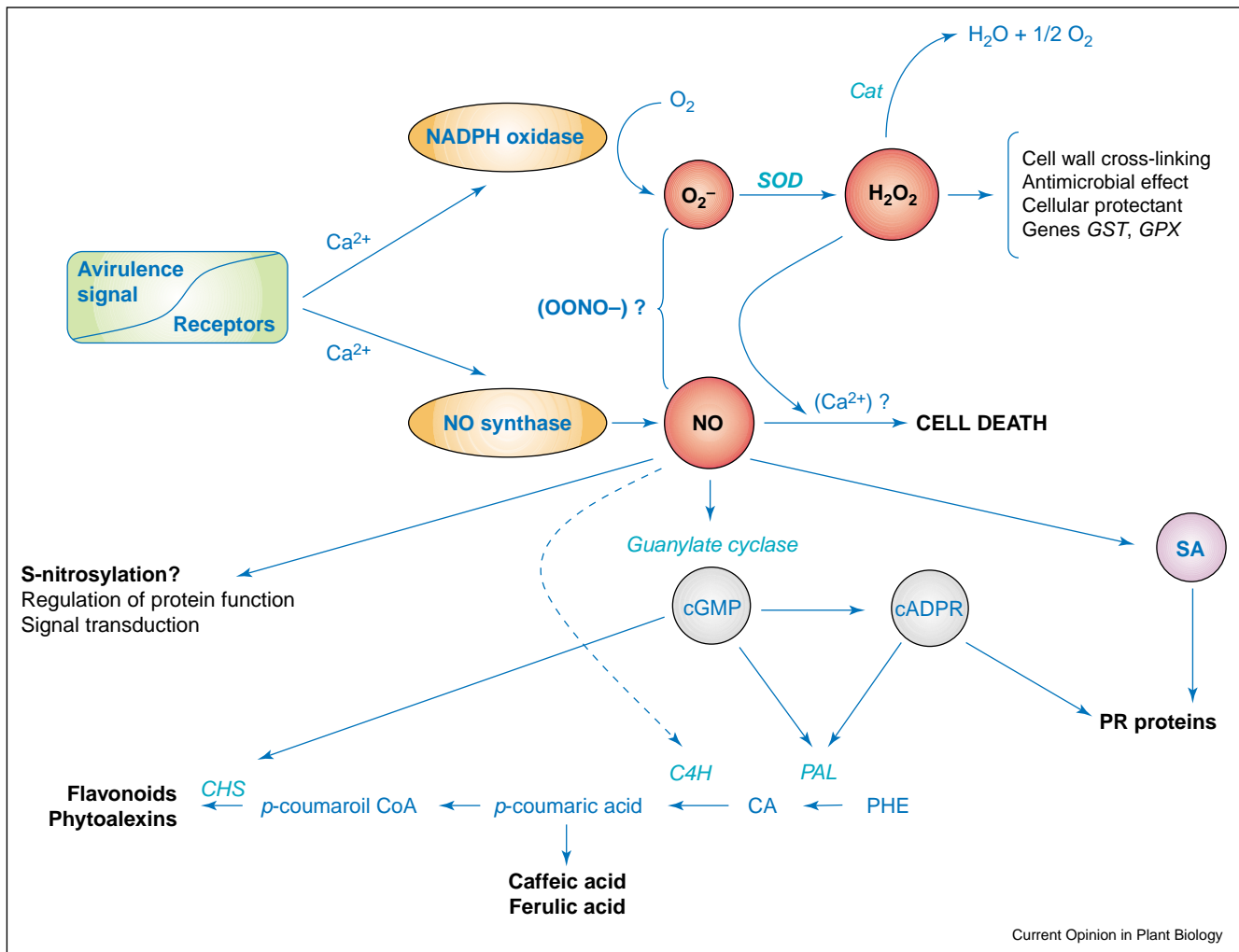
mal cells of wounded *A. thaliana* leaves [21]. Surprisingly, jasmonic acid (JA) treatment resulted in strong NO production, suggesting a self-amplifying JA–NO loop. NO is known to induce the accumulation of salicylic acid (SA), the crucial mediator for the establishment of the systemic resistance response. NO treatment was also found to induce both JA accumulation and JA-responsive genes, such as defensin (*PDF1.2*) and JA-induced protein (*JIP*), but only in SA-deficient NahG plants [21]. Therefore, the role of NO in responses to biotic stresses appears to be fine-tuned by SA, which can inhibit JA biosynthesis and antagonize the activation of the normal defense response.

NO-mediated signal transduction during the resistance response

The plant hypersensitive disease resistance response (HR) is characterized by the rapid accumulation of ROS and NO. This signaling system has been shown to trigger localized hypersensitive cell death, to induce sets of defense genes, and to mediate a network that is involved in the establishment of systemic acquired resistance (Figure 1; [14,22]). Recent reports suggest that NO might also play a crucial role as an intracellular signal that functions in the cell–cell spread of the HR. Pharmacological studies have indicated that NO is required for the onset of apoptotic cell death in adjacent cells during the defense response of oat to avirulent *Puccinia coronata* [23]. Moreover, *A. thaliana* plants that were challenged with avirulent *P. syringae* were found to initially accumulate NO exclusively in the extracellular space before NO appeared in the cytoplasm of nearby cells; many of these cells died soon after [6].

The signaling functions of NO depend on many complex conditions that affect its reactivity. These include the rate of NO production and diffusion and the redox state of the cell, with ROS being the key mediator in channeling NO into the cell death pathway [24*] through an unknown mechanism [25]. NO-induced cell death requires RNA processing and protein synthesis [26], and it triggers an active process in which proteases appear to play a crucial role. Cystatin-sensitive proteases have been found to be critical regulators of hypersensitive cell death in soybean cell suspensions, [27] and genes that encode cysteine proteases are induced by NO in *A. thaliana* [28]. In addition, caspase-specific protein fragmentation has recently been revealed during the HR in tobacco plants that were infected with tobacco mosaic virus (TMV) [29]. Furthermore, Ac-YVAD-CMK (acetyl-tyrosyl-valyl-alanyl-aspartyl-chloromethylketone), an irreversible inhibitor of mammalian caspase-1, has been shown to block NO-induced cell death [26]. The requirement of cGMP synthesis, a well-known second messenger of NO in animal systems [26], for programmed cell death is additional evidence of the involvement of NO in executing this process. By activating different types of Ca²⁺ channels, cGMP can both directly and indirectly regulate various physiological functions [30]. NO is involved in the

Figure 1



Representation of NO signaling functions during the hypersensitive response. CA, cinnamic acid; Ca^{2+} , calcium influx; cADPR, cyclic ADP ribose; Cat, catalase; C4H, cinnamic acid-4-hydroxylase; CHS, chalcone synthase; cGMP, cyclic GMP; GPX, glutathione peroxidase; GSNO, S-nitroso-L-glutathione; GST, glutathione S-transferase; NOS, nitric oxide synthase; ONOO⁻, peroxynitrite; PAL, phenylalanine ammonia lyase; PHE, phenylalanine; PR, pathogenesis-related proteins; SA, salicylic acid; SOD, superoxide dismutase.

mobilization of intracellular Ca^{2+} , thereby establishing Ca^{2+} channels as putative NO targets in signal transduction mechanisms [12^{*}]. NO also affects the expression of numerous plant genes [31], including those that encode phenylalanine ammonia lyase (PAL) and pathogenesis-related 1 (PR1) [22]. Because the accumulation of PAL and PR1 has been detected in tobacco cell suspensions that were treated with a membrane-permeable analogue of cGMP, and taking into account that the induction of PAL in tobacco suspension cells by NO can be suppressed by several inhibitors of guanylate cyclase, the activation of at least some NO-dependent defense genes is thought to be mediated by cGMP [22].

The existence of multiple mechanisms for NO action makes the dissection of specific pathways difficult, but

might explain the incomplete inhibition observed when individual steps in specific NO-mediated pathways are blocked [26]. Some of these mechanisms might depend on the high reactivity of NO with amino acids, which results in the posttranslational modification of proteins. Tyrosine nitration and methionine oxidation can introduce irreversible protein modifications that lead to loss of protein function, whereas cysteine nitrosylation is a reversible modification that can modulate protein function [32]. Protein cysteinyl thiol groups can be nitrosylated by low-molecular-weight nitrosothiol (*S*-nitroso-glutathione [GSNO]) or by transfer of a NO group from another *S*-nitrosylated protein. *S*-nitrosylation, often called *S*-nitrosation in recent literature, is a protein- and cysteine-specific modification. Only a precise subset of cysteine residues are nitrosylated. The remarkable

specificity of the reaction of *S*-nitrosylation is conferred by both the subcellular compartmentalization of NO sources and proteins targets and by the presence of consensus motifs flanking the cysteine residues [33]. *S*-nitrosylated proteins can be easily denitrosylated because the *S*-NO bond is labile in a reductive micro-environment. Therefore, *S*-nitrosylation shares many features with phosphorylation, the prototype posttranslational modification involved in signal transduction. Recently, proteomic approaches that have been developed to identify *S*-nitrosylated proteins have finally begun to reveal the potential targets for *S*-nitrosylation in plants [34^{••}]. These include stress-related proteins, signaling or regulating proteins, redox-related proteins, cytoskeleton proteins and metabolic enzymes, further confirming that NO-regulated processes in plants and animals have common features.

NOt only pharmacology

Most of the current information about the function of NO in plants has come from pharmacological studies using NO scavengers and NOS inhibitors. This approach cannot, however, reproduce the concentration and localization of NO inside cells or tissues that have been exposed to different treatments. As a consequence, it is often difficult to discriminate between the physiologically relevant and pharmacological effects derived from NO reactivity. Owing to a better definition of NO signaling mechanisms and reactivity in plants, and to the availability of *A. thaliana* plants that have altered NOS activity [35^{••}], additional genetic-based approaches aimed at confirming the role of NO are finally being used. Compared to wild-type plants, *A. thaliana* *Atmos1* mutant plants are dramatically more susceptible to virulent *P. syringae* and show enhanced bacterial growth and a faster and much more severe development of disease symptoms [15^{••}]. Furthermore, the expression of genes encoding bacterial flavohemoglobins, which possess a strong nitric oxide denitrosylase activity, in *A. thaliana* plants as well as in avirulent *P. syringae* revealed that the removal of NO from either inside or outside the host cell reduces hypersensitive cell death, attenuates the induction of *pal* transcript, and delays the expression of *PR-1* [36[•]]. Furthermore, the hypothesis that NO mediates hypersensitive cell death by acting in partnership with H₂O₂ has been validated: transgenic *A. thaliana* plants overexpressing a thylakoidal ascorbate peroxidase that scavenges H₂O₂ showed significantly increased resistance to NO-induced cell death compared to control plants [24[•]].

A 'do it all' molecule

In 1997, Keeley and Fotheringham [37] found that nitrous oxide at concentrations present in biomass smoke was highly effective in inducing the germination of dormant seeds. Researchers have identified dozens of smoke-germinated species worldwide and have isolated several compounds from smoke that are believed to be potential

triggers for germination. Recent reports indicate that NO disrupts dormancy and stimulates seed germination in several species [38,39[•]]. Furthermore, EPR analysis revealed that endogenous NO accumulates in germinating sorghum seeds [40]. Both nitrate reductase and NOS activities appear to be required for the burst of NO during germination, although the production of NO by the non-enzymatic reduction of apoplastic nitrite under acidic conditions is also likely [41^{••}]. One possible mechanism of action of NO during germination is based on the activation of β -amylase, which is involved in the early stages of seed germination that have been shown to be triggered by NO in several species [38]. However, other mechanisms that are based on the modulation of the activity of regulatory proteins and biosynthetic enzymes by NO, probably through nitration/nitrosylation and *S*-nitrosylation, are likely to await discovery.

Additional recent reports support the view that NO is a 'do it all' molecule that plays a crucial role during the entire lifespan of the plant. NO regulates several growth and developmental processes. For example, in roots, it operates in the auxin signaling pathway for adventitious root development through cGMP-dependent and cGMP-independent mechanisms, the latter involving the activation of a mitogen-activated protein kinase cascade [42[•],43]. In vascular tissue, NO regulates programmed cell death and lignification during xylogenesis: it accumulates in tracheary elements during the early processes of xylem differentiation and is required for secondary cell-wall formation and cell autolysis [44]. Downregulation of endogenous NO promotes the shift from vegetative to reproductive growth, whereas its upregulation represses the flowering photoperiod response, affects the expression of regulatory genes in flowering pathways, and thus suppresses the floral transition [35^{••}]. NO is also involved in fertilization, in which it functions as a pollen-tube growth modulator that induces the growth orientation required for pollen navigation on the pistil [45[•]]. A lack of NO accumulation at the tip of the pollen tube permits its growth, whereas the generation of NO behind the tip causes growth arrest through a cGMP-dependent mechanism. Peroxisomes are an important source of NO [46], and their distribution behind the tip of the pollen tube determines the direction of its growth [45[•]]. Strikingly, a similar phenomenon has been observed in roots, in which gravistimulation induces an auxin-dependent asymmetric accumulation of NO on the lower side of horizontal roots and hence gravitropic bending [47[•]]. Once again, NO production induces cGMP synthesis, which is required for a full gravitropic response. The involvement of cGMP has in various plant signal transduction pathways been clearly demonstrated. However, NO does not appear to be the physiological activator of cGMP accumulation as the activity of the only plant guanylate cyclase identified to date is not NO dependent [48].

How to get rid of NO

The combustion caused by human activity has drastically increased the production of NO. Since NO exerts important signaling functions, atmospheric NO might interfere with basal metabolism and alter several crucial physiological processes. This crucial 'side effect' became clear in 1996, when pharmacologists at the Free University of Berlin got a number of unexpected results in experiments with guanylate cyclase [49]. Because this enzyme is activated by NO, they began to wonder if their sample was being exposed to exogenous NO. When enzyme activity was measured in close proximity to the Berlin inner-city circular highway, they found a strong correlation with atmospheric NO concentration [49].

Mammals regulate NO bioactivity through hemoglobins either by detoxification or by delivery through transnitrosylation reactions [50]. They control internal NO levels very tightly through fine regulation of the activity of the various NOS isoforms. Plants must deal with atmospheric NO as well as with the internal leakage of NO that accumulates under normal growth conditions because of its production from nitrite [51^{••}]. Therefore, plants must block interference from either endogenous or atmospheric NO by activating NO detoxification mechanisms. Recent studies have challenged the common perception that the widespread presence and long evolutionary history of plant hemoglobins is related to oxygen transport. Non-symbiotic hemoglobins possess a high affinity for oxygen and slow oxygen dissociation rate constants, they are therefore unlikely to function as oxygen transporters [52]. However, many plant non-symbiotic hemoglobins are upregulated during hypoxia, a stress condition that generates copious amounts of NO [53], and their expression is directly associated with protection against hypoxic challenge [54^{••},55]. Plant non-symbiotic hemoglobins from *A. thaliana* [54^{••}], barley [56[•]] and alfalfa [57] are now known to detoxify NO to nitrate in an NAD(P)H-dependent manner. The methemoglobin intermediate might then be reduced directly by NADPH, as is hemoglobin from *A. thaliana* [54^{••}], or by a methemoglobin reductase, as has been demonstrated in alfalfa root cultures [56[•]].

Although the oxidation of NO to nitrate by hemoglobin is probably the major metabolic route for NO, other enzymes such as xanthine oxidase, glutathione peroxidase and GSNO reductase are reported to break down NO-related species [58–60]. However, the physiological relevance of these enzymes to the metabolism of NO is unclear, and no enzymes that serve a metabolic NO-oxidizing function *in vivo* or that are induced specifically by a nitrosative stress have been identified. Nevertheless, the transcriptional regulation of *A. thaliana* GSNO reductase in response to JA and SA supports its function in modulating NO signals that are associated with plant defense [60].

Conclusions

The impressive increase in the number of publications in the field of nitric oxide signaling in plants is undoubtedly providing a more comprehensive understanding of the biology of NO in plants. NO participates in the regulation of several physiological processes, but the molecular mechanisms by which NO operates are still largely unknown. A great effort is now needed to identify and characterize the direct targets of NO. The recent identification of plant proteins that are potential targets for *S*-nitrosylation *in vivo* [34^{••}] is a promising starting point.

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Infection by necrotrophic pathogens has been extensively shown to be associated with oxidative stress in the host that promotes infection. This paper reports that *Botrytis elliptica*, a necrotrophic pathogen that has a very narrow host range, kills lily cells by stimulating their production of both ROS and NO, but only during purified compatible interactions. The authors also show that a partially purified *B. elliptica* culture filtrate was able to trigger ROS and NO accumulation as well as cell death, and to confer pathogenicity on lily to incompatible *Botrytis* species.

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Using a genetic approach, the authors validated the hypothesis that H₂O₂ mediates hypersensitive cell death upon pathogen attack by acting in partnership with NO as a trigger of cell death. They show that NO represses the accumulation of thylakoidal ascorbate peroxidase, a scavenger of H₂O₂, and that overexpression of thylakoidal ascorbate

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Proteomic studies represent a powerful complement to transcriptomic studies because they allow evaluation of the expressed proteins and of their potential post-transcriptional modification by S-nitrosylation. In this work, extracts from *A. thaliana* were treated with NO and subjected to the biotin-switch method, converting S-nitrosylated cysteines to biotinylated cysteines. Biotin-labeled proteins were purified and analyzed using nano-scale liquid chromatography in combination with mass spectrometry. This led to the identification of proteins that are candidates to undergo S-nitrosylation, including examples involved in stress, redox reactions, cellular signaling or regulation, the cytoskeleton, and metabolism.

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This work provides pharmacological, physiological, molecular and genetic data that demonstrate that NO represses the photoperiod and autonomous floral pathways. The authors also report the identification of *nox1*, a mutant that displays an elevated level of endogenous NO. Treatment of *A. thaliana* plants with a NO donor enhanced the vegetative growth of the plants and significantly delayed flowering in a dose-dependent manner. Although the precise molecular action of NO remains to be determined, the analysis of genetic mutants that have altered levels of NO revealed that NO affects genes that control both the environmentally sensitive pathways and the autonomous pathways that lead to flowering.
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