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NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants?

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Polyamines, such as spermine, spermidine and putrescine, are ubiquitous polycationic compounds that are produced by almost all living organisms, including plants, animals, fungi and bacteria. Polyamines are multifunctional and interact with polyanionic biomolecules such as DNA or protein. However, despite their potential significance, the polyamine-dependent signal transduction system has not been revealed yet. Ni Ni Tun and colleagues have recently reported a possible linkage between polyamine and nitric oxide (NO), another ubiquitous signalling molecule.

Old but new

Polyamines (PAs) carry a positive charge on each nitrogen atom at neutral pH and can interact with polyanionic molecules, such as DNA, within the cell. Putrescine, spermidine and spermine (Figure 1) are the major PAs that can be found in most living organisms [1]. The names of spermine and spermidine reflect their discovery in human semen in 1678, whereas the term putrescine originates from its contribution to the odour of putrefying flesh. More than 300 years since their discovery, PAs are still attracting much interest from researchers because of their wide spectrum of physiological functions [1,2].

A new linkage between ubiquitous molecules

Because plants do not seem to have specific receptors for PAs, it is particularly interesting to investigate how PAs perform diverse functions in plant cells. The involvement of secondary messenger(s) could account for their versatile actions: polyamine oxidase activity generates the secondary messenger H_2O_2 (a reactive oxygen species), which is associated with plant defence, including programmed cell death, and with abiotic stress responses [1,2].

A recent publication by Ni Ni Tun and colleagues [3] presents pharmacological evidence that, in addition to H_2O_2, PAs induce the production of nitric oxide (NO), a gaseous signalling molecule, in various tissues within seedlings of *Arabidopsis thaliana* [3]. The effect is rapid and there is no lag phase between the addition of spermine and increased fluorescence from the dye diaminorhodamine-4M (DAR-4M), a fluorescent probe for NO. Spermidine and spermine stimulate increases in DAR-4M fluorescence whereas putrescine and another PA, arginine, do not. This response can be abolished by adding 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide (cPTIO), a commonly used NO scavenger. They have also demonstrated that 2-aminoethyl-2-thiopseudourea, an inhibitor of mammalian NO synthase (NOS), inhibits spermine-induced DAR-4M fluorescence [3]. These results suggest the presence of an unidentified pathway for NO production in response to PAs.

It is well known that supplemental PA provides an anti-senescence effect in many plant species. The anti-senescence effect of PAs can be partially mimicked by NO [4]. Tun and colleagues had previously presented similar pharmacological evidence for cytokinin-induced NO generation in various plant cell cultures [5], a finding that might provide the clue needed to understand the functional overlap between NO and cytokinin and PAs.

Plant NO biology in a state of flux

Currently, the most confounding issue in plant NO biology is the mechanism(s) for NO production. Until only a few years ago, the source of arginine-dependent NO production in plants had been presumed to be a mammalian-type NOS. However, to date, no such homologue has been found in plant genomes. In 2003, two types of NOS (iNOS and AtNOS1) were reported that do not share sequence similarity to mammalian-type NOS but were thought to have unique mechanisms for arginine-dependent NO synthesis [6]. One year later, two papers describing iNOS were retracted

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[7], and now the NOS activity of AtNOS1 seems to be in doubt [8]. Although it is still possible that a unique plant NOS might exist, the loss of two strong candidates for catalysis of an arginine-dependent NO pathway in plants has left a gaping hole in the field. Problems with NO detection methodologies could account for some of the present confusion [9].

As an enzymatic source of NO in plants, nitrate reductase (NR) is now the only protein whose NO-producing activity has been confirmed. Although it seems unlikely that cytosolic assimilatory NR functions as the sole source of NO signals, PAs can down-regulate nitrate assimilation, and probably also NO production from nitrite, by interacting with the binding protein 14-3-3 [10]. Although further confirmation of PA-induced NO production is necessary [11], the observations reported by Tun *et al.* [3] could imply the presence of an unknown enzyme responsible for direct conversion of PAs to NO.

Communication with a microbial world

Unlike vertebrate animals that have evolved nervous systems, plant systems for sensing and responding to abiotic and biotic stimuli are less complex morphologically but resist simple mechanistic explanations [6]. In general, plant growth and development are closely linked to the surrounding environment, in particular to the activities of soil microorganisms. The ubiquity of PAs and NO leaves open the possibility that plants and microbes share these same communication molecules under natural conditions. For example, PA production by a *Streptomyces* species in soil confers significant improvements to bean plants that are not observed when a non-producing mutant derivative is present instead [12]. Cytokinins are also commonly produced by plant-associated bacteria. Plant responses to PAs and cytokinins might have evolved in part as a result of the selective advantage in associating with bacteria, such as *Streptomyces*, that can outcompete potential pathogens and assist plants in obtaining nutrients such as iron and phosphate. Elevated levels of NO from various sources in the rhizosphere are likely to promote acclimation of bacteria to this prooxidative environment [13,14]. NO has cytotoxic potential, but PA synthesis in *Escherichia coli* protects cells against NO toxicity by an unknown mechanism that does not seem to be direct chemical quenching or reduction in mutation frequency [15].

Traffic jam?

PAs can form a variety of conjugates *in vivo*. Trypanothione (which is formed from two glutathione molecules and the PA spermidine; Figure 1) acts as an antioxidant for enzymatic scavenging of H_2O_2 in the parasitic protozoan *Trypanosoma* [16]. In plants, phenylpropanoid–PA conjugates (Figure 1) have been reported to accumulate in response to stress conditions [1]. Most phenylpropanoids are potent antioxidants against reactive oxygen species and reactive nitrogen species and, in conjunction with peroxidase activity, phenylpropanoid phytophenolics can destroy H_2O_2 [17]. Under anoxic conditions, NO can react with PAs to produce NONOates. Spermine NONOate (Figure 1) has been favoured as a chemical NO donor because it spontaneously releases NO in aqueous solution;

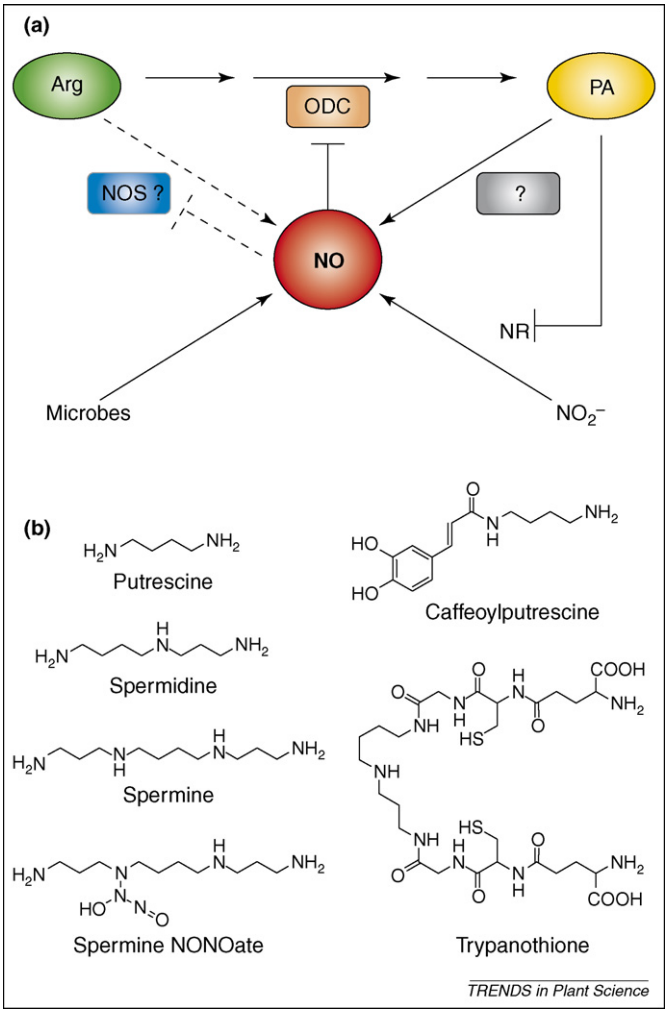

Figure 1. Polyamines in relation to NO production. **(a)** NO can be produced in plant cells by nitrite-dependent NR-catalysed and non-enzymatic (not shown) mechanisms [6] and by unknown arginine-dependent mechanisms. NO down-regulates its own synthesis by direct and indirect means, possibly to prevent NO cytotoxicity. Negative feedback on PA accumulation is also likely because several enzymes involved in PA biosynthesis are inhibited by NO-mediated *S*-nitrosylation [19]. **(b)** The molecular structures of common PAs and PA conjugates found *in vivo,* and a synthetic NO-donating NONOate. Abbreviations: Arg, arginine; PA, polyamine; NO, nitric oxide; ODC, ornithine decarboxylase; NR, nitrate reductase; NOS, NO synthase.

this shows a direct chemical link between PA and NO [18]. As reported by Tun *et al.* [3], there are many potential links between PA and NO, but none have been verified. In addition to molecular approaches, future research needs to be conducted with a comprehensive view of chemical biology to provide a solution for the traffic jam at the crossroads.

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Letters

Plant nitric oxide synthase: a never-ending story?

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In their report 'Identification of a plant nitric oxide synthase gene involved in hormonal signaling' [1], Nigel Crawford and colleagues reported on the discovery of a distinct nitric oxide synthase (AtNOS1) in *Arabidopsis thaliana* that regulates growth and hormonal signaling in plants. *Arabidopsis* mutants (*Atnos1*) were found to be impaired in NO production and organ growth. Although the AtNOS1 sequence has no similarities to any mammalian NOS isoform, the cloned and purified AtNOS1 protein was demonstrated to use the substrates arginine and nicotinamide adenine dinucleotide phosphate to produce NO. AtNOS1 activity was dependent on Ca^{2+} and calmodulin, which has also been shown for other NOSs.

Recent results from our laboratories raise critical questions regarding the function of AtNOS1. Over the past 2 years, we and several other groups have confirmed that basal NO production is impaired or that AtNOS1 mutants have a reduced capacity to mount a NO burst, although in some cases the picture was unclear [2–4]. Inspired by the original report by *Crawford* and colleagues [1], we cloned AtNOS1 (Q66GP9) and the orthologous genes from rice (Q6YPG5) and maize (AY110367). Unfortunately, after purification of the recombinant proteins as described in Ref. [1] we failed to detect any NOS activity with an [^{3}H]-arginine and Greiss reagent-based NOS assays [1]. Moreover, given that AtNOS1 was identified as a member of a novel evolutionary conserved GTP-binding protein family, with members in organisms ranging from bacterium to human [5], we also tested mammalian orthologs for NOS activity (NP_062810, NP_115689), again without success.

If AtNOS1 is not a real NOS, what could be an alternative explanation for the observed NO-deficient mutant phenotype? We initially hypothesized that AtNOS1 might be only a subunit of a larger NO-making complex. We did not entirely rule out this possibility even though our reconstitution experiments of purified *Arabidopsis*, rice and maize proteins with plant extracts did not result in NOS activity. However, one could envision other scenarios, for example, AtNOS1 could be anywhere

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