

# Nitric oxide is involved in growth regulation and re-orientation of pollen tubes

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## Summary

Nitric oxide (NO) controls diverse functions in many cells and organs of animals. It is also produced in plants and has a variety of effects, but little is known about their underlying mechanisms. In the present study, we have discovered a role for NO in the regulation of pollen tube growth, a fast tip-growing cellular system. Pollen tubes must be precisely oriented inside the anatomically complex female ovary in order to deliver sperm. We hypothesized that NO could play a role in this guidance and tested this hypothesis by challenging the growth of pollen tubes with an external NO point source. When a critical concentration was sensed, the growth rate was reduced and the growth axis underwent a subsequent sharp reorientation, after which normal growth was attained. This response was abrogated in the presence of the NO scavenger CPTIO and affected by drugs interfering in the cGMP signaling

pathway. The sensitivity threshold of the response was significantly augmented by sildenafil citrate (SC), an inhibitor of cGMP-specific phosphodiesterases in animals. NO distribution inside pollen tubes was investigated using DAF2-DA and was shown to occur mostly in peroxisomes. Peroxisomes are normally excluded from the tip of pollen tubes and little if any NO is found in the cytosol of that region. Our data indicate that the rate and orientation of pollen tube growth is regulated by NO levels at the pollen tube tip and suggest that this NO function is mediated by cGMP.

Movies available online

Key words: Pollen, NO, cGMP, Peroxisome, Guidance, *Arabidopsis*

## Introduction

Unraveling the molecular mechanism of pollen tube guidance is a central issue in sexual plant reproduction and there is a general agreement that directional growth should depend on physical and chemical signals that are exchanged between the male and female gametophytes (Pruitt, 1999; Palanivelu and Preuss, 2000; Cheung and Wu, 2001; Johnson and Preuss, 2002; Lord and Russel, 2002). Despite intense research efforts in the past two decades aimed at defining a mechanistic explanation of the process, consensus around a central or conserved theory of pollen tube guidance is still lacking. Chemotropic roles have been suggested for diverse style extracts, sugars, Ca<sup>2+</sup>, available water, and, more recently, short-range effects have been described for lipids, arabinogalactan-proteins and adhesins (Wolters-Art et al., 1998; Wu et al., 1995; Mollet et al., 2000) (reviewed by Johnson and Preuss, 2002). Genetic evidence has also accumulated for long-distance guidance cues, mostly on the basis of mutant screening for reproductive defects (Hülkamp et al., 1997; Ray et al., 1997). In one of the best-characterized physiological models (*Torenia fournieri*), diffusible signals from the synergid cells of the embryo sac are effective at distances of 100-200 µm (Higashiyama et al., 2003). On a different experimental basis, it was estimated that in the *maa*

mutant of *Arabidopsis*, the ovule entrance (mycophyle) can direct pollen tube growth over distances of 50-90 µm (Shimizu and Okada, 2000). Recently, the GABA molecule, a neurotransmitter in animals, was proposed to be a part of this navigation system, presumably through the formation of a continuous gradient towards the ovule that would be sensed and acted upon by the growing pollen tube (Palanivelu et al., 2003). The fact that pollen tube guidance frequently fails in crosses between relatively closely related species implies that at least some of the signals must be species specific. However, this does not rule out a role for more universal simple molecules such as GABA; it may simply mean that the specificity of the guidance cues comes from differential response or differential sensitivity to a common signal (Johnson and Preuss, 2002).

However, given the biological relevance of fertilization, it is plausible that evolution has created functional redundancy or co-functionality for different molecules. In fact, theoretical arguments have been raised that a single chemical gradient could hardly be responsible for guidance in most species, which led Lush et al. (Lush et al., 1998) to propose mechanical/structural stringencies as co-operative mechanisms in the guidance of pollen tubes. Classical experiments show that directionality of growth along the pistil/ovary can in

principle occur in more than one direction, restricting the guidance cue necessity to just a few crucial steps along the pollen tube path and overruling positive single molecule chemotropism as the sole mechanism of guidance (reviewed by Heslop-Harrison, 1987; Mascarenhas, 1993; Lord and Russel, 2002).

In an effort to bridge the gap between *in vitro* and *in vivo* experiments of pollen tube growth manipulation, our attention was drawn to NO as a possible communication molecule in this system on the basis of a number of well-known characteristics derived from studies in animals (reviewed by Ignarro, 2000; Stamler, 1994): (1) NO diffuses freely across cell membranes; (2) it is known to act as an intra and inter-cellular messenger in a number of regulation mechanisms; (3) it is known to act as positional cue diffusing from point sources; and (because it is a gas) (4) it acts on minimal thresholds over considerable distances. In plants, NO has been proposed as a regulator of growth and developmental processes (Lamattina et al., 2003), as exemplified in roots, where NO mediates the response to indole acetic acid during adventitious root formation (Pagnussat et al., 2003), in senescence by downregulating ethylene emission (Leshem et al., 1998) and through the stimulation of seed germination (Beligni and Lamattina, 2000). NO also promotes adaptive responses against drought stress operating downstream from ABA (Mata and Lamattina, 2001), and it has been implicated in the establishment of legume *Rhizobium* symbiosis (Hérouart et al., 2002). In plant disease resistance, NO plays a role by enhancing the induction of hypersensitive response (Delledonne et al., 1998; Durner et al., 1998; Huang et al., 2002).

We present data to indicate that NO can function as a pollen tube growth modulator by inducing growth re-orientation, the crucial cellular response to pollen navigation on the pistil. Pollen tubes respond to threshold concentrations of NO by sharp re-orientation, and this reaction is totally abrogated by adding the NO scavenger CPTIO to the medium. Furthermore, we provide data to indicate that this response is mediated through a cGMP pathway, and that NO is primarily synthesized in peroxisomes. On the basis of these data, we propose an NO-based regulatory growth mechanism that could account for the basic curvature needed for ovule targeting by pollen tubes.

## Materials and methods

### Pollen germination and tube growth NO assays

Fresh *Lilium longiflorum* pollen was germinated in 1.6 mM  $\text{H}_3\text{BO}_3$ , 1.0 mM KCl, 500  $\mu\text{M}$   $\text{CaCl}_2$ , 6% sucrose and 50  $\mu\text{M}$  MES, pH 6. Healthy, growing tubes were challenged with an artificial NO source (aNOs), a micropipette (20–30  $\mu\text{m}$  tip diameter), tip-filled with 1% agarose and 10 mM SNAP (s-nitroso-acetylpenicillamine, Sigma). Control for reactive oxygen intermediates was done carried out by addition of 100 U  $\text{ml}^{-1}$  SOD+Catalase. NO specificity was assayed by perfusion of 200  $\mu\text{M}$  of the NO-scavenger CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide,  $\text{K}^+$ -salt, Calbiochem] in presence of an aNOs. Sildenafil citrate (SC; Viagra<sup>TM</sup>, Pfizer) was assayed from 319 to 339  $\mu\text{M}$  with a diluted aNOs (5–5.8 mM SNAP in 1% agarose). Randomly chosen pollen tubes were challenged with diluted aNOs. Absence of re-orientation response was interpreted as indicating a sub-threshold sensitivity of that pollen tube to the imposed gradient. Under these conditions, SC was perfused and the response recorded by time-lapse video.

### NO imaging

The presence of NO in pollen tubes was assayed and visualized with 10  $\mu\text{M}$  4,5-diaminofluorescein diacetate (DAF-2DA, Molecular Probes). Pollen tubes longer than 200  $\mu\text{m}$  were grown in a glass coverslip coated with 0.01% PLL (poly-L-lysine hydrobromide,  $M_r$  331; Sigma), incubated for 5 minutes and perfused to wash excess fluorophore. Imaging was carried out using confocal (488 nm) or two-photon excitation (890 nm) on a BioRad MRC1024MP with a Coherent Mira/Verdi Ti-Sa laser, using a Nikon PlanFluo NA1.3 lens. Emission was collected with a 522DF35 filter. Images were processed with Metamorph (MM; Universal Imaging Corporation, v. 6.1). Kymographs were produced by averaging pixel intensity along a linescan of the whole pollen tube at each time point. Pollen tube length is represented on the horizontal axis of the kymograph and time on the vertical axis. The tip boundary was aligned on the right side of the kymograph by applying a custom-made journal under MM.

### NO flux measurements

Carbon fiber microelectrodes were built and operated as previously described for NO flux measurements (Cahill and Wightman, 1995; Friedman et al., 1996; Porterfield et al., 2001). Electrodes were polarized for NO detection at +9.0 V (versus Ag/AgCl half cell connected to the solution by a 0.5% agarose/3 M KCl bridge) and calibrated by dilution of a standard 2 mM NO solution (Gevantman, 1995). To characterize the NO gradients created by SNAP, an artificial NO source (aNOs) was immersed in medium and allowed to reach equilibrium. The self-referencing polarographic NO vibrating-electrode was stepped linearly from the aNOs tip at 10  $\mu\text{m}$  intervals and NO fluxes measured at each point. The diffusion of NO is described by Fick's Law ( $J = -D\Delta C/\Delta r$ ), where  $J$  is expressed as  $\text{pmol cm}^{-2} \text{s}^{-1}$ ,  $D$  is the diffusion coefficient for NO ( $2.6 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$ ),  $\Delta C$  is the concentration difference between two electrode positions and  $\Delta r$  is the excursion path of the electrode (10  $\mu\text{m}$ ). The conversion of the electrode signal to a concentration differential followed a previously established protocol (Porterfield and Smith, 2000). Data acquisition, processing and control of electrode movements were accomplished using a 3D stepper micropositioner and amplifier (www.applicableelectronics.com) controlled by ASET software (www.ScienceWares.com) (Shipley and Feijó, 1999).

### Subcellular characterization

Pollen tubes loaded with DAF2-DA were co-incubated with specific probes for mitochondria (Rhodamine 1,2,3; 10  $\mu\text{M}$ , Molecular Probes), acidic organelles (LysoTrackerRed, 100  $\mu\text{M}$ , Molecular Probes) and Golgi (Bodipy-TR, 1  $\mu\text{M}$ , Molecular Probes) and observed by confocal microscopy. Peroxisomes were imaged by transient expression of an ECFP-Peroxi construct (6931-1, Clontech). This vector contains a fusion between ECFP and the peroxisomal targeting signal 1 (PTS1), which was extracted and cloned into a construct containing the LAT52 pollen-specific promoter (Twell et al., 1990) in a pBluescript SKII vector (Chen et al., 2002). Tungsten particles (1.1  $\mu\text{m}$ , BioRad, Hercules, CA) were coated with this construct and bombarded into *Lilium* pollen using the biolistic PDS-1000/He system (BioRad). After bombardment, pollen was germinated in coverslip-bottom Petri dishes coated with 0.01% poly-L-lysine hydrobromide,  $M_r$  331 and imaged 10 hour after germination with a Leica Confocal microscope TC-SP2/AOBS (excitation at 458 nm to a spectral gate ranging from 469 to 500 nm).

## Results

### Lily pollen tubes show a negative tropic response in the presence of a nitric oxide point source

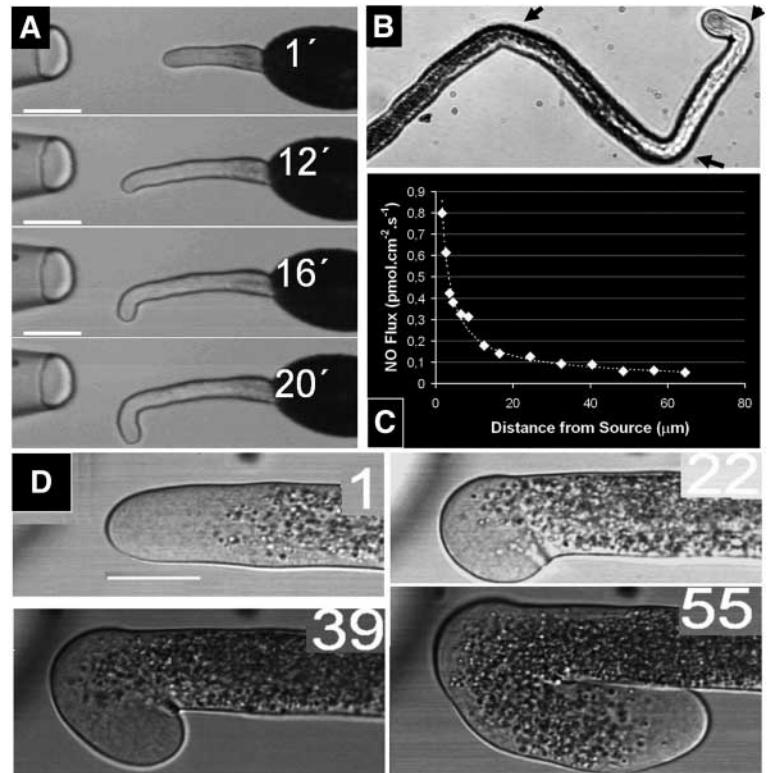
Pollen tube growth is restricted to the pollen tube tip (Feijó et al., 2001). To address if NO plays any effect on *Lilium longiflorum* pollen tube growth we designed an *in vitro* system

to deliver NO specifically to the tip. A glass micropipette was loaded with an agarose solution containing the NO donor SNAP (s-nitroso-acetylpenicillamine), allowing the molecule to diffuse to the medium and establishing a gradient in the vicinity of the growing pollen tube tip. The point diffusion gradient was allowed to settle in liquid germination medium and the growing pollen tubes were then placed 60  $\mu\text{m}$  away facing the pipette tip and growth was recorded by time-lapse videography. As pollen tubes move into the gradient, their growth is reduced or, in some cases, completely abrogated. After 12–15 minutes pollen tube growth resumes, but with the growth axis sharply rotated by an average angle of  $97.7 \pm 3.6^\circ$  (mean  $\pm$  s.e.m.;  $n=28$ ), an angle that is similar to the curvatures observed when pollen tubes target ovules in lily (Janson et al., 1994) and *Arabidopsis* (Shimizu and Okada, 2000), but remarkably sharper than that produced by any other treatment to lily pollen tubes. After the new growth axis is re-established, pollen tubes achieve a normal growth rate (Fig. 1A). The same pollen tube could be induced to re-orient its growth axis several times by repeated exposure to exogenous NO (Fig. 1B; arrows indicate the position of the source). The formation of an NO gradient by SNAP was confirmed by measurement with a self-referencing vibrating polarographic microelectrode selective for NO. A typical exponential-decay diffusion field from the point source was observed (Fig. 1C). These measurements indicate that the extracellular activation threshold for the reorientation response is on the order of 5–10  $\text{nmol l}^{-1}$ , or a flux of 0.1–0.2  $\text{pmol cm}^{-2} \text{s}^{-1}$ , values well within the physiological range of NO action (Ignarro, 2000; Lamattina et al., 2003). The re-orientation was maintained after addition of catalase and superoxide dismutase (SOD), excluding the possibility of chemical reactions between NO and reactive oxygen species (ROS), and the subsequent secondary production of NO-derived molecules, such as peroxynitrate.

More importantly, the re-orientation was totally abrogated by addition of the specific NO scavenger CPTIO, a condition in which pollen tubes were observed to grow at normal rates inside the SNAP-containing pipette (intracellular data on the CPTIO effect below).

### cGMP mediates pollen tube re-orientation response

The re-orientation of the growth axis in the presence of an external gradient lead us to further investigate the existence of downstream messengers of NO. In animal cells, NO effects can be mediated by cGMP-independent signaling pathways (Ignarro, 2000), and it is well established that this second messenger conveys NO signaling in a number of physiological conditions. cGMP levels are modulated by NO in animal cells, and equilibrium concentrations of cGMP are dependent on NO-activated guanylate cyclases (GC) and breakdown activity of phosphodiesterases (PDEs). Although these enzymes have not been well characterized in plants, we tested the effects of a



**Fig. 1.** (A) Time-lapse sequence of a *Lilium longiflorum* (lily) pollen tube growing facing an extracellular NO point-source (SNAP on agarose; left on the image). Pollen tube slows as it moves into the NO-gradient, but direction proceeds unchanged for ~12 minutes. A new growth axis then starts to be defined, forming a sharp right angle from the original axis ( $97.7 \pm 3.6^\circ$ ,  $n=28$ ). The pollen tube then regains normal growth rate (16–20 minutes). Scale bar: 30  $\mu\text{m}$ . (See Movie 1 at <http://dev.biologists.org/supplemental>) (B) Lily pollen tube showing three consecutive re-orientation responses induced by moving the same source to the locations marked with arrows. The growth axis changed reproducibly by right angles after each challenge by the NO source in front of the pollen tube tip. (C) Artificial NO source measurements using a vibrating self-referenced polarographic probe to NO. The graph shows a typical exponential NO gradient decay from the point source at different step distances. Although variations between sources were detected, these measurements show that within the effective distance (see A) the NO concentration is in the range of 5–10  $\text{nmol l}^{-1}$ , and the NO flux is in the range of 0.1–0.2  $\text{pmol cm}^{-2} \text{s}^{-1}$  (values well within the physiological range accepted for NO action). (D) Time-lapse sequence of a pollen tube being challenged with a diluted NO artificial source in the presence of sildenafil citrate (Viagra<sup>TM</sup>) (numbers in the top right-hand corner represent minutes after detection of the response). Using these diluted sources, most pollen tubes do not show any response, often growing into the pipette. For this experiment, pollen tubes were first incubated on standard medium and challenged with the diluted NO source. If a pollen tube showed no response, i.e. if it was demonstrated to be insensitive to such low amounts of NO, the medium was perfused with sildenafil citrate and the same pollen tube is challenged with the same NO source. Despite the lower amount of NO, reverse re-orientation angles were observed in the presence of sildenafil citrate ( $109.8 \pm 9.8^\circ$ ,  $n=9$ ) showing a sensitization effect, from unresponsive to the peak response (see movie 1 at <http://dev.biologists.org/supplemental>).

number of described effectors of its activity: IBMX, a general inhibitor of the PDE family, and sildenafil citrate (SC; VIAGRA<sup>TM</sup>), a drug that inhibits cGMP degrading phosphodiesterases (PDE5 and PDE6) in mammals (Corbin and Francis, 1999). The use of IBMX at different



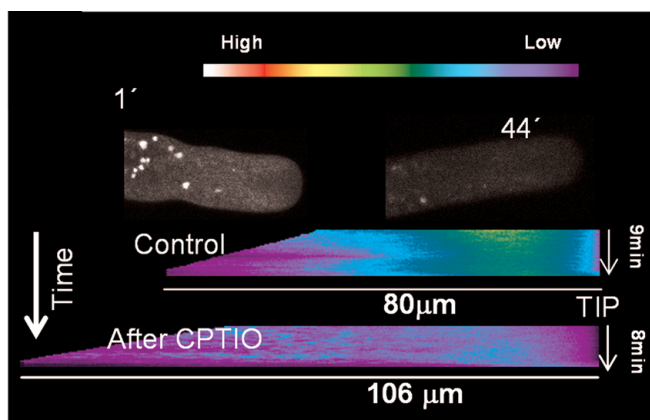
concentrations promoted the occurrence of diverse tip abnormal or subnormal morphologies, pointing to the occurrence of pleiotropic effects (data not shown). Although the drug clearly disrupted growth regulation, the pleiotropic responses made it difficult to isolate or test its specificity to the cGMP hypothesis.

SC, however, has recently been shown to delay flower senescence (Leshem, 2000). If the re-orientation response of pollen tubes to exogenous NO involves cGMP and if SC inhibited cGMP degradation in plants, the drug should sensitize pollen tubes to NO and/or prolong the re-orientation response. To test this possibility we exposed pollen tubes to suboptimal doses of SNAP. The criterion to validate this experiment was the detection of pollen tubes insensitive to a less concentrated NO source. Thus, pollen tubes that were previously shown not to re-orient its growth axis on a lower SNAP concentration were submitted to a SC final concentration of 339  $\mu\text{M}$ . Under these conditions, eight out of nine pollen tubes ( $n=9$ ) showed re-orientation after addition of SC, and some even re-oriented to previously never observed angles of  $180^\circ$  (Fig. 1D). We further confirmed this result by measuring an increase of the average re-orientation angle to  $120^\circ \pm 12$  ( $n=9$ ). This angle is 25% steeper than the control but, more significantly, is obtained with much lower concentrations of NO. Dose-response curves of SC also showed a dose-dependent stimulatory effect of  $\sim 30\%$  on the growth rate of pollen tube at 50  $\mu\text{M}$ . This finding suggests that the effect of

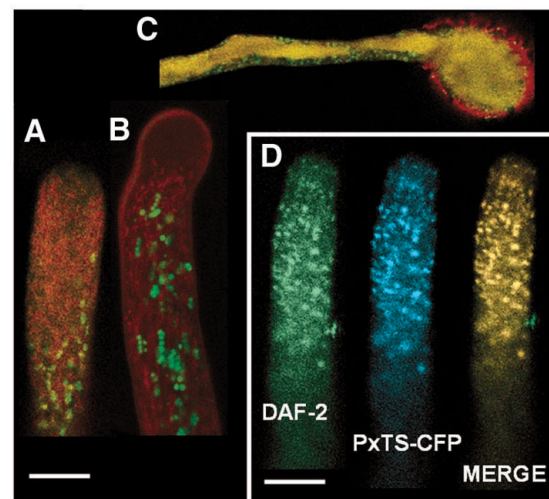
exogenous NO on pollen tube growth is mediated by cGMP, and that this second messenger is in the signaling cascade that affects the growth regulation mechanism.

### Nitric oxide is produced in peroxisomes

Once a re-orientation response takes place, we asked whether this means that the extracellular NO challenge induces this response by modulating the intracellular NO levels. To address this question, we searched to see if NO was endogenously produced in pollen tube. Live cells were loaded with the NO sensitive fluorophore, DAF2-DA (4,5-diaminofluorescein diacetate) and imaged by confocal or two-photon microscopy (Fig. 2). This probe was previously shown to be NO-specific in plant tissues (Foissner et al., 2000). Fluorescence was found throughout the cytosol, although in the region subjacent to the tip it was very low (Fig. 2, 1'). A very strong signal was found in round organelles of about 2  $\mu\text{m}$  diameter (Fig. 2, 1'). The spatiotemporal dynamics of intracellular NO ( $\text{iNO}$ ) are shown in the form of kymographs in which we averaged an active representative region inside each pollen tube at each time-point as a color-coded line, and plotted these lines as a function of time (YY' axis) and pollen tube length (XX' axis) (Fig. 2). For the sake of clarity, the pollen tube tips were right-side aligned, and therefore the slope on the left side of the kymograph reflects the pollen tube growth rate. In non-challenged pollen tubes, no significant variation over time is seen. NO levels are very low in the tip and highest in the subapical domain (control; Fig. 2). To validate the specificity of the observed signal, the NO scavenger CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide] was applied in



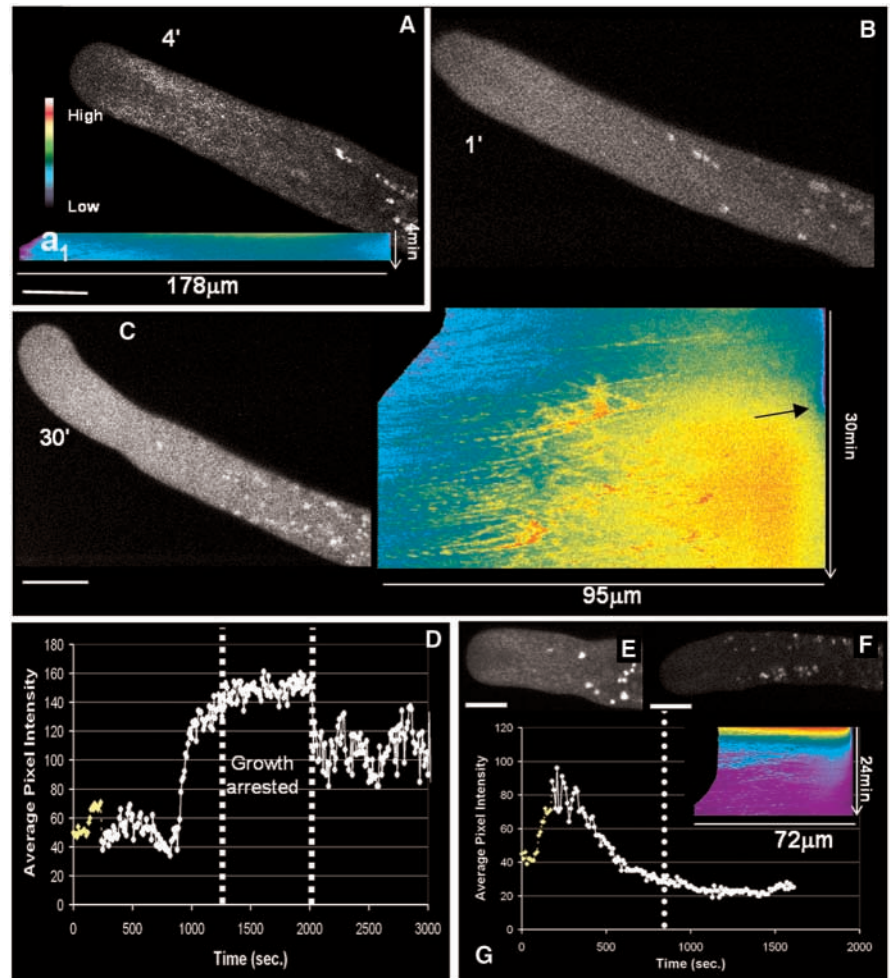
**Fig. 2.** Detection of intracellular NO in a growing pollen tube of lily using the NO-specific fluorophore DAF2-DA (1'). Fluorescence is seen in the cytosol, with less intensity in the apical domain, and is very bright on round cytoplasmic organelles. The spatiotemporal dynamics of intracellular NO is shown in the form of kymographs in which we averaged an active representative region inside each pollen tube at each time-point as a color-coded line (see top wedge), and plotted these lines as a function of time (y-axis) and pollen tube length (x-axis). For the sake of clarity, the pollen tube tip was aligned with the right side, and therefore the slope on the left side of the kymograph reflects the growth rate. The chronological order of each time point is read from top to bottom as illustrated by the arrow on the y-axis. In a non-challenged pollen tube (control), no significant variation along time is seen. Apical depletion and subapical accumulation of NO are clearly visible. Incubation with the NO-scavenger CPTIO (44') almost suppressed the signal from the cytosol, but the round organelles are still distinguishable. Kymograph analysis shows the overall decrease after CPTIO addition, but the apical/subapical pattern, polarity and dimensions are maintained.



**Fig. 3.** The DAF-2DA-positive organelles are peroxisomes. (A-D) Confocal images of growing lily pollen tubes incubated in DAF-2DA (green) and in organelle-specific dyes (red). No co-localization was found in the endomembrane system (A, BodipyTR), mitochondria (B, Rhodamine 123) or acidic organelles (C, LysoTrackerRed). (D) Peroxisomes were then tagged by transient transformation of pollen grains with a construct containing the pollen-specific LAT52 promoter driving an ECFP-peroxisome targeting signal (PxtS) fusion. Pollen tubes were observed 10 hours after germination. The NO-producing organelles (DAF-2, green) show an almost complete co-localization with the ECFP signal (PxtS-CFP, blue), as shown in the merged image. Scale bars: 15  $\mu\text{m}$ .

continuous perfusion. The fluorescence signal was always reduced in tubes that were perfused with CPTIO. Typically, cytosolic NO was almost completely eliminated, confirming the presence of NO inside pollen tubes and the specificity of the probe. Even though the cytosolic NO signal decayed, the round organelles still showed a clear signal even after 44 minutes, suggesting that they continued to generate NO at a high rate (Fig. 2, 44' and lower kymograph). These brightly fluorescent organelles visible after DAF2-DA exposure have a diameter of about 2  $\mu\text{m}$  ( $2.17 \pm 0.17$ ). We performed double-labeling experiments using different organelle-specific probes and DAF-2DA probe to determine their identity. Double labeling with DAF2-DA (green) and selective, color complementing dyes (red) for Golgi and ER (Bodipy-TR, Fig. 3A), mitochondria (rhodamine 123, Fig. 3B) and acidic organelles (LysoTracker, Fig. 3C) showed no co-localization. As peroxisomes and plastids are still within this size range, we transiently transformed pollen tubes with a peroxisomal targeting signal (PxTS) fused to ECFP under the control of the pollen-specific promoter LAT52. LAT52 is a very late-acting promoter in monocots, and thus we imaged pollen tube 10 hours after germination. The double labeling with ECFP and DAF2-DA co-localized to the same organelles (Fig. 3D) and morphometric analysis (diameter and circularity index) indicated that the double-stained organelles did not include plastids. These results identify peroxisomes as the NO-producing organelles.

In pollen tubes, growth is regulated in the tip (Feijó et al., 2001; Hepler et al., 2001), and the low abundance of endogenous NO in this region may be a prerequisite for pollen tube growth. The growth arrest induced by exogenous NO would then probably be due to the diffusion of NO into this region. To demonstrate directly the diffusion of exogenous NO into the tip region, time-course experiments were performed with DAF-2DA-loaded pollen tubes exposed to exogenous gradients of NO as previously described (Fig. 4). Pollen tubes were allowed to grow for 4 minutes, in order to adjust the basal signal magnitude (Fig. 4A) and then brought to the vicinity of the NO source (Fig. 4B). The re-orientation response is preceded by an increase of NO in the tip region and a reduction in the pollen tube growth rate (Fig. 4C and inserted kymograph). During this period, the subapical region of low NO concentration is no longer



**Fig. 4.** An increase in intracellular NO precedes re-orientation. The time-lapse sequence (A–C) shows the changes in intracellular NO after challenge with an extracellular point source, as reported by DAF-2DA fluorescence. In A, a DAF-2DA loaded pollen tube was followed for 4 minutes. The inserted kymograph shows the typical NO pattern, and no significant variation with time. (B) Challenging with an external source produces a rise in fluorescence within 1 minute. After ~10 minutes, the low NO concentration domain disappears (arrow) simultaneously with growth arrest (slope on the left side of the kymograph) and soon after the NO concentration peaks. (C) As the concentration stabilizes, the negative NO tip gradient starts to be defined and re-orientation occurs. Scale bars: 15  $\mu\text{m}$ . (D) The average pixel intensity variations of the DAF-2DA signal plotted as a function of time at the tip of a growing pollen tube before (yellow) and after extracellular NO challenge (white). Accumulation of intracellular NO is obvious soon after the pollen tube moves into the gradient, but builds up strongly from a threshold point. When the peak point is reached, growth is arrested. As soon as growth is regained in the new axis, the level of NO drops to a stable value, which is about twice that seen before challenge. Addition of the NO-scavenger CPTIO totally inhibited the re-direction response as illustrated in E–G. A DAF-2DA stained tube (E) was challenged with an extracellular NO point source in the presence of CPTIO. While the signal decreased as in Fig. 2, the growth of the tube slowed but slowly regained normal growth without any change of direction (F). Evolution of intracellular NO shows that after some initial increase, this reaction is immediately followed by a decrease to levels below the initial level (G and inserted kymograph). Scale bar: 16  $\mu\text{m}$ .

discernable (arrow in the kymograph). Approximately 10 minutes after challenge with the NO donor, there is an overall sustained increase of the signal, coincident with growth arrest (arrow). The re-orientation response then takes place and growth resumes at a normal rate, but iNO remains at higher



levels than before the extracellular challenge with the NO source (Fig. 4D). Interestingly, growth is preceded by the re-definition of the subapical NO-depleted domain. Fig. 4D traces the evolution of NO along the whole sequence (yellow points plot values before NO challenge). Diffusion of NO is traceable by the fluorescent signal increase, and growth arrest occurs when the maximal cytosolic values are reached. More importantly, the re-orientation and re-start of normal growth are concomitant with a sharp decrease of NO.

A possible explanation for this could lie on the following sequence of events: NO from the point-source diffuses into the tip, causes growth arrest and stimulates endogenous NO production in this region. Thus, much of the NO measured would be endogenously produced, and subsequently decreased upon resumption of normal growth. Based on the fluorescence intensity, after growth is resumed, the basal level of  $i$ NO remained stable around a value that is, on average, twice the pixel-intensity than before the response. This change suggests a different steady-state condition at the end of the process, which could underlie some sort of molecular memory to the first exposure to external NO. Whatever the reason, pollen tubes remain sensitive to NO and are capable of re-direction again and again (Fig. 1B). Therefore, if some memory is retained, it must be mediated by an increase in sensitivity, which could be dependent on higher background levels of cGMP.

We also monitored the NO levels after perfusion with CPTIO (Fig. 4E-G). Challenge with the NO-source provoked a slight increase in NO but immediately after, it decreased progressively to levels below the initial basal concentration. This level then remained constant (inserted kymograph). Under these conditions, no re-orientation took place, and growth rate slowly recovered. Together these results show a direct relationship between  $i$ NO and the regulation of cell growth and re-orientation.

## Discussion

Our data prompt us to propose a new role for NO in plant biology. NO has been previously suggested to act as messenger in plant developmental processes, stress, defense responses and symbiosis establishment (Lamattina et al., 2003). We directly demonstrate a role for NO in pollen tube growth and guidance. In animals, NO is synthesized by NO synthases (NOS) and its signaling function is mediated, at least in part, by soluble guanylyl cyclases (sGC) that generate cGMP and by phosphodiesterases (PDEs) that hydrolyze cGMP (Ignarro, 2000). In plants, no sequences homologous to mammalian NOS, sGC and PDEs have been found namely in the *Arabidopsis* genome. Yet, there is evidence for cGMP function in plants (Penson et al., 1996; Durner et al., 1998). In agreement with a role for cGMP in plants, one functional plant guanylate cyclase was identified in *Arabidopsis*, though it shows an unusual domain organization (Ludidi and Gehring, 2003). There are also reports of NO production (Barroso et al., 1999; del Rio et al., 2002). The enzyme nitrate reductase appears to produce NO necessary for stomata closure (Lamattina et al., 2003). More importantly, two recent reports describe new enzymes with NOS activity, one identified as an inducible NOS with a sequence variant to a glycine decarboxylase (Chandok et al., 2003) and a constitutive NOS

form (NOS1) with no sequence similarities to any mammalian isoform (Guo et al., 2003). Consistent with our data, the *nos1* mutants show a reduced reproductive growth and fertility, indicating that NO might participate in these events.

We also show that NO is synthesized in the peroxisomes, a hypothesis previously proposed in plants (Barroso et al., 1999; del Rio et al., 2002).

Our findings indicate that NO is a negative regulator of growth, at least in pollen tubes. We show that endogenously generated NO is low or absent at the tip of pollen tube, but is present at higher levels behind the tip, where the presumed NO-generating peroxisomes are located. We suggest that this pattern of NO production is permissive for pollen tube growth at the tip and may, in the absence of an exogenous NO source, act as a positive feedback reinforcement of elongation in a straight growth axis. The ubiquitous presence of NO in the medium, by addition of SNAP, prevents germination and inhibits pollen tube growth rate. However, pointed application of an NO donor near the pollen tube tip results in transient growth arrest, which is followed by re-directed growth. This effect is mediated by NO that diffuses into the tip. Growth arrest is then associated with changes in cell polarity and peroxisome redistribution, as shown by the extension of the streaming lanes into the tip. We assume that it is the localization of peroxisomes and thus the site of endogenous NO production, which eventually determines the direction into which pollen tube growth resumes. Peroxisomes are the plant cell oxidative organelles and there are reports that highlight the relevance of this organelles in the production of signal molecules, i.e. NO and reactive oxygen species. These organelles have a rich enzymatic machinery and are reported to participate in developmental processes such as photomorphogenesis in *Arabidopsis* (Hu et al., 2002; Barroso et al., 1999; del Rio et al., 2002).

Although our data show that endogenous NO production is correlated with the regulation of pollen tube growth, the *in vivo* confirmation of this is made difficult by various experimental obstacles. Real-time imaging of pollen tube guidance *in vivo* would imply the possibility of optical sectioning closed flowers, which implies demanding technical conditions (two-photon excitation and water-immersion, long working distance objectives) far from optimized for this specific application. Excitation-derived photo-damage of cells or, more dramatically, any sort of ovary dissection or injury of the ovary is not an option because it will generate stress-induced bursts of NO production (Lamattina et al., 2003). A possibility for overcoming these obstacles will be either the use of pollen tubes expressing highly fluorescent reporter genes to closely monitor the pollen tube-pistil interaction, or, otherwise, the use of floral mutants with open ovaries and exposed, yet functional, transmitting tissue and ovules.

Another problem is related to the high reactivity of NO, with an half life depending on the redox status of the surrounding environment, namely when ROS are present (Ignarro, 2000; Thomas et al., 2001). This makes it difficult to gauge the amount of NO being produced *in vivo*, so no invasive techniques for NO can easily quantify a putative signal from the female tissue. A self-referencing NO selective electrode could be used, but again tissue accessibility is a limiting factor.

Several difficulties arise when interpreting chemical cues identified in different plant species: it can be argued that

general mechanisms do not assure species specificity to avoid widespread cross-fertilization (Johnson and Preuss, 2002). One possible explanation could be related to different threshold sensitivities operating for a given molecule from species to species. Otherwise a different species could use similar mechanism, but with derivatised molecules within a single chemical family, which would be transduced into different effects. Given the diversity of molecules shown to have guidance effects on pollen tubes, and predicting that more will be uncovered through successive genetic screens, it is likely that chemical signaling between the pollen tube and pistil could convey specificity by using universal molecules in various combinations.

This finding indicates the need for further cues from the pistil. Whether or not NO takes part in communication cannot be deduced from in vitro studies, but the striking re-orientation response warrants for further investigation. In the context of our data, a feasible NO-guidance mechanism would be possible if there were specialized female tissues acting as NO 'hot spots', for example, at the base of the funiculus, where a sharp change in pollen tube growth direction is required, or near the embryo sac after fertilization, in order to prevent secondary pollen tube from penetrating the micropyle. The indication that *nos1*, the only bona fide NO-producing mutant so far described, shows fertility deficiencies is a positive indication that NO may be involved in pollen tube guidance.

In past research, pollen tube guidance could not be fully explained by the actions of positive guidance cues. In addition, it remains debatable that tracking down a molecule will overcome questions related to pollen tube path length and thickness (Lush et al., 1998). Yet, a gaseous molecule may overcome these barriers easily. In proposing NO, a diffusible gas, as a candidate for pollen tube guidance, we may address a controversial aspect of pollen tube guidance. In *Arabidopsis*, Hülskamp et al. (Hülskamp et al., 1995) propose that each ovule guides the pollen tube by chemotatic gradients with ~100 µm range of action at the junction of the ovule with the placenta. However, wild-type *Arabidopsis* pollen tubes make a sharp turn to enter the micropyle in 10 µm of this area (Shimizu and Okada, 2000). Surface localized diffusion of chemotatic signals effective through 50 cells diameters would require signal molecules of less than one kDa (Ray et al., 1997; Crick, 1970). The ability of NO to function as a messenger across cell layers and to trigger cellular processes is nowadays well established in animals (Ignarro, 2000). The negative chemotropism described here for NO is reminiscent of the effects of semaphorins on axon guidance in animals: these proteins function as chemorepellents, which prompt axons to make right angle turns within an environment that contains both attractants and repellents (Tessier-Lavigne and Goodman, 1996). Similarly, NO acts as negative effector on the retinal patterning of the optical lobe in *Drosophila*, where NO prevents further extension of axons beyond their target neurons (Gibbs and Truman, 1998). NO function as a guidance cue implies that (1) it is able to form a concentration gradient, (2) it produces a specific response, (3) it remains stable for a given period of time, and (4) it varies in effectiveness with distance to the target (Palanivelu and Preuss, 2000). Our in vitro data support these criteria. We were able to detect an artificially generated external NO gradient to which the pollen tubes respond in a specific way (re-orientation growth axis),

the gradient is maintained in time as a single pollen tube can undergo consecutive re-orientation responses with the same NO source. In addition, the response can be prevented if the gradient is perturbed or annihilated by an NO scavenger.

The events downstream of NO seem to be, at least in part, mediated by cGMP. Support for this assertion comes from our finding that, among other tested chemicals, sildenafil citrate, a drug that inhibits cGMP-selective PDEs of mammals, facilitated the redirected growth of pollen tubes in response to low doses of NO donors that were themselves ineffective. Previous studies with cyclic nucleotide analogues also suggest that cGMP and cAMP are involved in pollen tube growth control (Moutinho et al., 2001; Elias et al., 2001). A likely target downstream of cGMP is a family of cyclic nucleotide-gated channels (CNGs) (Leng et al., 1999), also represented in the pollen transcriptome (Becker et al., 2003). Directly or coupled with other transporters, CNGs may regulate the flux of ions such as  $\text{Ca}^{2+}$ ,  $\text{H}^{+}$  and  $\text{Cl}^{-}$  that are known to be involved in pollen tube growth control (Feijó et al., 2001; Becker et al., 2003; Feijó et al., 1999; Zonia et al., 2002). Cyclic nucleotide balance, modulation of  $\text{Ca}^{2+}$  channels and the control of nerve growth bi-directional axon guidance have recently been linked (Nishiyama et al., 2003). These findings encourage further efforts to characterize the various components of the NO signal pathway in plants and to endue in genetic and biochemical approaches.

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