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Research Paper

Nitric oxide triggers specific and dose-dependent cytosolic calcium transients in Arabidopsis

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Calcium (Ca^{2+}) transients have been shown to take place in response to diverse developmental and physiological cues. Also, it is involved in biotic and abiotic stress signaling. Nitric oxide (NO) is an important signaling molecule that plays a crucial role in plant growth and development, starting from germination to flowering, ripening of fruit and senescence of organs. Moreover, it plays a pivotal role in several biotic and abiotic stress signaling processes. In the present work, the ability of NO to trigger increases in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) was investigated. For this purpose, transgenic Arabidopsis seedlings constitutively expressing the luminescent Ca^{2+}-sensitive protein apoaequorin (*35S::APOAEQUORIN*) was employed. In chemiluminescence and in vivo Ca^{2+} imaging assays, the NO-donor sodium nitroprusside (SNP) triggered a strong, instantaneous, reproducible, and dose-dependent rise in $[Ca^{2+}]_{cyt}$. Moreover, the observed rise in $[Ca^{2+}]_{cyt}$ was shown to be NO-specific and not associated with decomposition products of SNP, as the NO-scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (C-PTIO) significantly blunted the observed NO-mediated spike in $[Ca^{2+}]_{cyt}$. Interestingly, preincubation of *35S::APOAEQUORIN* Arabidopsis seedlings with the plasma membrane channel blocker lanthanum chloride resulted in partial concentration-dependent blocking of the NO-specific Ca^{2+} transient. This observation indicates that, in addition to the mobilization of $[Ca^{2+}]_{cyt}$, as an external source in response to NO treatment, there also exists an appreciable contribution of an as yet unidentified internal pool.

Introduction

Calcium (Ca^{2+}) is deemed to be one of the most important second messengers in many signal transduction networks in all living organisms, including animals and plants. In animal cells, Ca^{2+}-mediated signal transduction pathways have been shown to be enormously versatile and complex in the temporal and spatial organisation of the signal, as well as in the proteins that produce and intercept it.[1] Equally, plants possess signal transduction networks that are highly conserved in terms of components which are also as sophisticated as that in their mammalian counterparts.[2] In the resting (un-stimulated) state, the cytosolic free Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ is ~200 nM and most of the cellular calcium is sequestered in cytoplasmic organelles such as the vacuole (100 mM), endoplasmic reticulum (ER) (1 mM), or the cell wall (1 mM).[3] This difference of many orders of magnitude in free Ca^{2+} concentrations between the cytoplasm and cytosolic compartments creates a large electrochemical Ca^{2+} gradient (200–300 mV), across the plasma membrane (PM), ER, and tonoplast that favours the energy-independent (downhill) movement of Ca^{2+} into the cytosol (influx). This movement is controlled by a diverse set of specialized Ca^{2+} channels and pores. Conversely, the movement of Ca^{2+} against this electrochemical gradient, from the cytoplasm into the stores (efflux), is energy-dependent, and, requires ATP or proton motive force and is provided by specialized Ca^{2+}-ATPase pumps. Both types of proteins, channels and pumps, are abundant in the PM and tonoplast and also occur in the ER, mitochondria and chloroplasts.[3,4]

The channels that are responsible for Ca^{2+} influx into the cytosol are gated by either voltage, stretch, inositol triphosphate (IP3), cADPR or G-proteins. Ca^{2+}-mediated signaling takes place when some of these channels open, allowing Ca^{2+} to move down the electrochemical gradient.[4] A single channel can conduct 10^6 Ca^{2+} atoms per second; thus, $[Ca^{2+}]_{cyt}$ can increase rapidly. On the other hand, subsequent activation of membrane-bound Ca^{2+}-ATPases (or Ca^{2+}/H^+ exchange proteins) in the ER, vacuolar, mitochondrial, or PM ensures that most $[Ca^{2+}]_{cyt}$ signals are transients.[3,5] In plants, Ca^{2+} or Ca^{2+} fluxes function in many biochemical and processes including: red light, abscisic acid, gibberellin, salinity/drought, hypo-osmotic stress, touch, cold, heat shock, and nodulation factors.[6-8] Moreover, Ca^{2+} fluxes play a pivotal role in developmental and physiological processes such as: egg cell fertilisation,[9] pollen tube elongation,[10] circadian rhythms,[11] oxidative stress[12,13] and pathogen infection.[14]

The ability of a plant cell to differentiate among various Ca^{2+} signals to activate the correct set of cellular responses is intriguing. Each signal creates its own Ca^{2+} fingerprint that is unique in the lag period, frequency and amplitude of the Ca^{2+} wave, and its spatial distribution. The targets of Ca^{2+} signal transduction can be divided into two categories: primary sensors and downstream substrates. Phosphorylation cascades regulated by protein kinases

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and phosphatases represent the primary transduction route interpreting the $[Ca^{2+}]_{cyt}$ signal. Ca^{2+}-dependent protein kinase (CDPK), Ca^{2+}/Calmodulin (CaM)-dependent protein kinase (CaM kinase) and a protein kinase C-type enzyme have all been shown to be present in plants with primary role in Ca^{2+} sensing and decoding. When bound to Ca^{2+}, CaM activates over 100 proteins, most of which have not been characterized. The activities of several enzymes including myosin V, kinesin, Ca^{2+}-ATPase, Ca^{2+} channels, glutamate carboxylase, NAD kinase, and a number of protein kinases are all calmodulin dependent in plant cells.[15,16]

In animal cells, nitric oxide (NO) is a well-known potent inducer of calcium transients. It has been demonstrated the ability of sodium nitroprusside (SNP), an NO donor, and to elicit dose-dependent Ca^{2+} fluxes in human neutrophiles.[17] Moreover, it has been indicated that exogenous NO donor SNP induced the release of Ca^{2+} from intracellular IP(3) receptorsensitive stores of respiratory burst neutrophils via S-nitrosylation.[18] To this end, NO was found to play a crucial role in plant growth and development, starting from germination to flowering, ripening of fruit and senescence of organs. Also in case of environmental stress hazard, caused by both abiotic and biotic factors, enhanced NO generation is observed in different plant species and organs.[19-23]

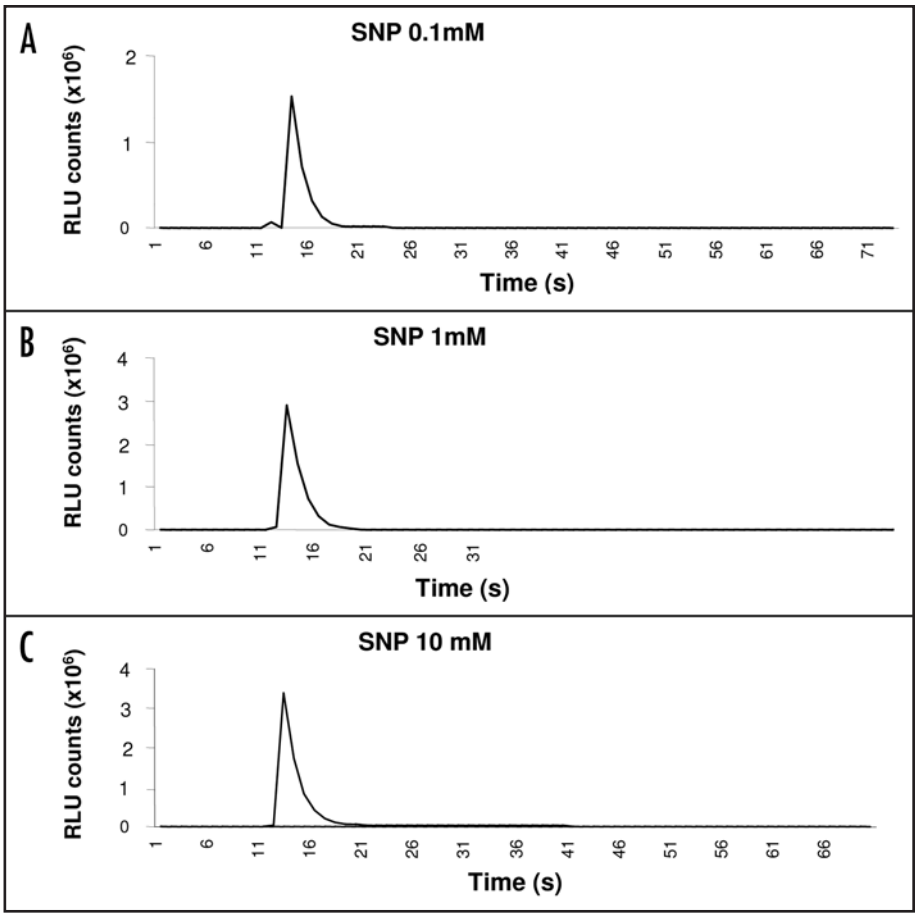

Figure 1. Dose dependency of the Ca^{2+} transient induced by the NO donor SNP. SNP 0.1 mM (A), 1 mM (B) and 10 mM (C). Traces shown best represent the average response for each concentration Treatments were repeated at least three times with similar results.

Taken together, an increasing body of evidence points to a role for NO in plant development, stress responses, and programmed cell death,[19-23] although its situation within any one signal cascade is still poorly understood. Moreover, in plant systems, a comprehensive knowledge on the ability of NO to induce Ca^{2+} fluxes and the potential contributing cellular sources is incomplete. Therefore, this work was primarily focused on the investigation of the ability of NO to induce Ca^{2+} transients via the utilisation of transgenic *Arabidopsis thaliana* seedlings, expressing the Ca^{2+}-sensitive reporter apoaequorin. The obtained results are discussed in the light of the specificity, dose-dependence and the possible cellular sources of the obtained NO-mediated Ca^{2+} transients.

Results

Ca^{2+} transients follow a dose-dependent fashion in response to NO. Arabidopsis seedlings were employed to study the changes in $[Ca^{2+}]_{cyt}$ levels in response to the NO-donor SNP. Elevations in luminescence responses or relative luminescence units (RLU) counts were interpreted as a faithful reporter of increases in $[Ca^{2+}]_{cyt}$ levels. Figure 1 illustrates the results obtained from the dose-response experiments between SNP and luminescent light. An instantaneous spike, with a maximum RLU value of ~1500000, was observed when a 0.1 mM SNP solution was injected (Fig. 1 A). Moreover, the observed RLU counts almost doubled when the reconstituted aequorin seedlings were treated with a ten-time more concentrated SNP solution (i.e., 1.0 mM) (Fig. 1 B). Furthermore, the increase in the RLU value in response to increasing SNP concentrations was maintained, albeit to a lesser extent (only ~1/6 increase), when a solution of 10 mM SNP was administered (Fig. 1C). The observed dose-dependency was reproducible in several independent experiments. Irrespective of NO concentration, a single instantaneous transient spike of Ca^{2+}-dependent luminescence was observed. Moreover, the overall increase in $[Ca^{2+}]_{cyt}$ was transient, lasting between 1 and 2 s, even though the seedlings remained immersed in the solution of SNP.

To examine whether the observed Ca^{2+} spike is due to a direct reaction between aequorin and SNP, apoaequorin was in vitro reconstituted and its bioluminescence counts were integrated over 10 s upon treatment with 10 mM SNP. Typically, the reconstituted aequorin gave a luminescence value of ~150000 when treated with 25 mM $CaCl_2$, whereas a background luminescence value averaging around 30 was obtained when the reconstituted aequorin was treated with sterile water at room temperature (data not shown). Moreover, when the reconstituted aequorin was treated with a 10 mM solution of SNP, luminescence values around 100 were consistently obtained. Furthermore, the 10 mM SNP solution in the absence of aequorin gave similar values to that obtained in the presence of aequorin (i.e., 100) (data not shown).

SNP-induced $[Ca^{2+}]_{cyt}$ increase is partially contributed by PM channels. Lanthanum chloride (La^{3+}) is a well known plasma

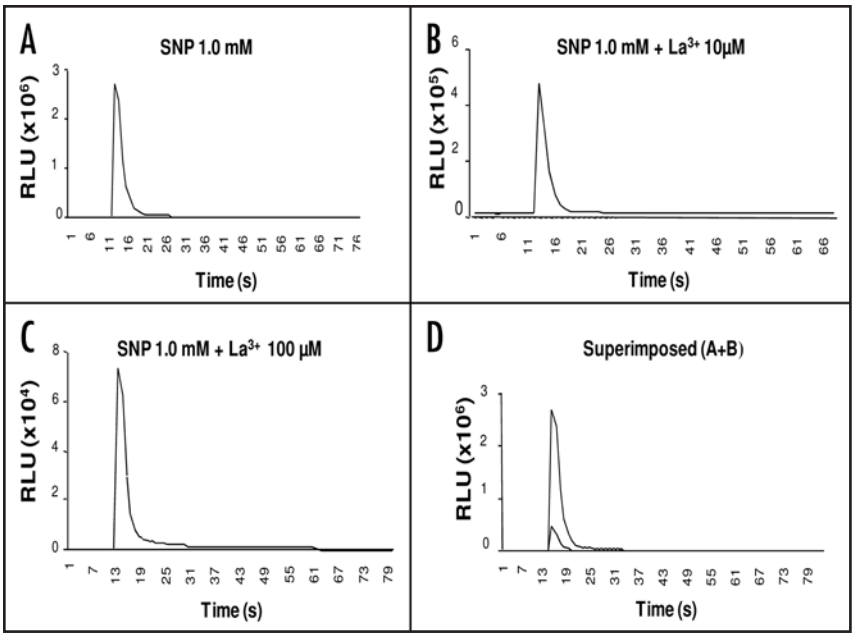

Figure 2. La^{3+} significantly diminishes the SNP-induced Ca^{2+} transient. Leaves were incubated with La^{3+} 10 μM (B) and 100 μM (C) for 4 hrs before SNP injection. (D) The superimposition of (A and B).

membrane channel blocker.[26] To investigate the contribution of PM channels towards the observed elevation in $[Ca^{2+}]_{cyt}$, in response to SNP challenge *35S::AEQUORIN* seedlings were pretreated with increasing concentrations of La^{3+}. Figure 2 represents luminescence traces obtained upon La^{3+}-pretreated seedlings that were subsequently challenged with a 1.0 mM SNP solution. A one order-of-magnitude reduction was observed when seedlings were pretreated with a 10 μM La^{3+} (Fig. 2B). Moreover, a two order-of-magnitude inhibition was obtained when seedlings were treated with a ten-time more concentrated La^{3+} solution (100 μM) (Fig. 2C). No significant inhibitions in $[Ca^{2+}]_{cyt}$ spike's magnitude were observed when more concentrated La^{3+} solutions were tested (data not shown).

$[Ca^{2+}]_{cyt}$ increase induced by SNP is NO-specific. To investigate whether the observed increase in $[Ca^{2+}]_{cyt}$ is associated with NO and not with SNP, we tested the impact of the SNP non-functional analogue potassium ferrocyanide $\{K_4Fe(CN)_6\}$, on the induction of Ca^{2+} spikes. Treatment with a 1.0 mM solution of $\{K_4Fe(CN)_6\}$ induced only a weak spike that was three order-of-magnitudes weaker than that induced by 1.0 mM SNP (Fig. 3A and B). Moreover, we tested the effect of the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) on the observed SNP-induced Ca^{2+} spike. A significant two-order-reduction in the magnitude of the 1.0 mM SNP-induced Ca^{2+} spike was obtained when Arabidopsis seedlings were pretreated with 150 μM C-PTIO (Fig. 3C and D). Approximately, a three order-of-magnitudes reduction was observed when a two-time more concentrated C-PTIO solution (300 μM) was used (data not shown). Moreover, in vivo Ca^{2+} imaging experiments indicated that infiltrating leaves of adult Arabidopsis plants with 1 mM SNP strongly induced a bioluminescence response. The observed bioluminescence was strongly blunted when leaves were preinfiltrated with 150 μM C-PTIO (Fig. 3E and F). Leaves infiltrated with sterile water exhibited an undetectable bioluminescent response (image not shown).

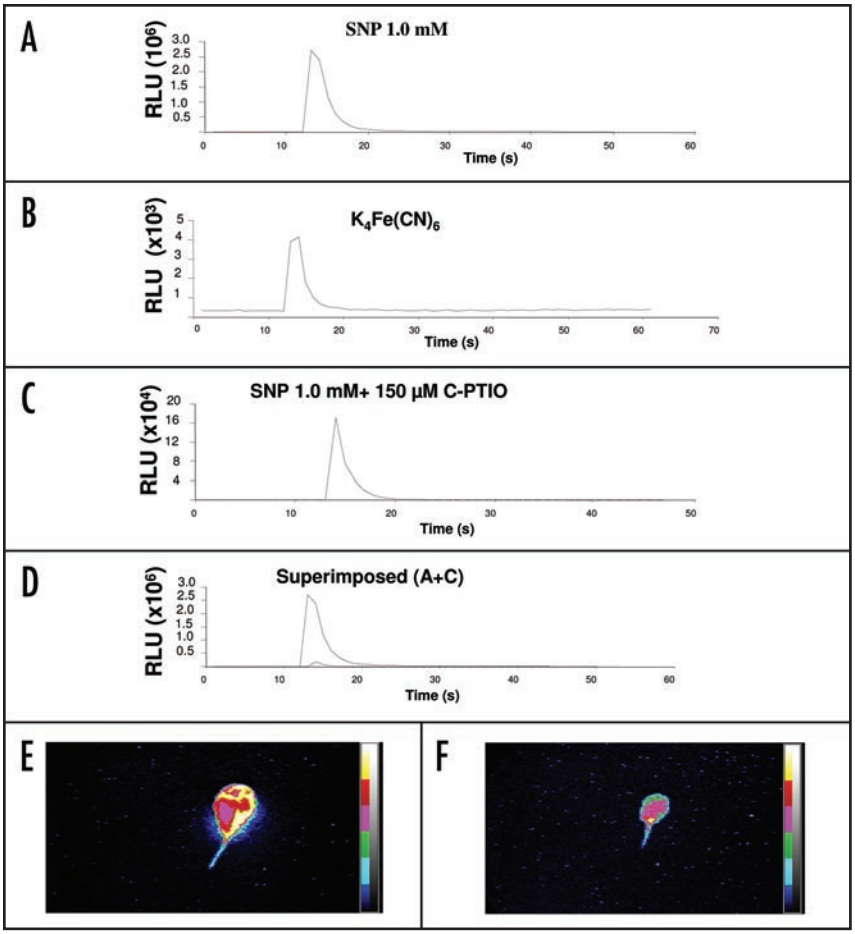

Figure 3. The effect of NO scavengers non-functional analogues on SNP-mediated Ca^{2+} transient. Treatment with 1.0 mM $\{K_4Fe(CN)_6\}$ results in weak Ca^{2+} transient (B) when compared with the strong SNP-mediated Ca^{2+} transient (A). Seedlings preincubated with 150 μM C-PTIO (C). In vivo Ca^{2+} imaging, with an ultra low light camera, of a leaf treated with 1.0 mM SNP (E) and SNP treatment of a leaf that was pretreated with 150 μM C-PTIO (F).

Discussion

The use of transgenic plants that have been transformed with the soluble, calcium-sensitive, luminescent protein aequorin has significantly contributed to the advancement of our knowledge of the signals that trigger increase in $[Ca^{2+}]_{cyt}$ levels.[27] Aequorin is a Ca^{2+}-activated photoprotein normally produced by the marine jellyfish *Aequoria victoria*. The protein consists of a single polypeptide chain, apoaequorin.

Coelenterazine, a hydrophobic luminophore, converts apoaequorin into an active calcium-sensitive aequorin. When Ca^{2+} ions bind to this protein, the lumniphore is discharged (oxidized) and a finite amount of blue light is emitted in a dose-dependent manner.[26] In this work, apoaequorin-expressing Arabidopsis plants were employed to examine the involvement of Ca^{2+} in NO signal transduction. Several chemical compounds have previously been reported to act as donor for NO. These include S-nitrosothiols (e.g., SNAP) and nitroprussides (e.g., SNP). The SNP anion $[Fe(CN)_5(NO)]^{2-}$, with an NO^+ nitrosyl group, is an effective nitrosating agent to S and N nucleophiles at neutral pH values. The product may then decompose to release NO and oxidized thiol.[28] Thus, SNP has been previously used as a potential NO donor to study NO-mediated signaling in mammalian and plant systems.[17,29,30] Therefore, SNP was utilized as the NO-donor in all experiments. Previously, luminescence response has been successfully used as a satisfactory parameter for qualitative analysis of changes in $[Ca^{2+}]_{cyt}$ in response to oxidative signals.[12] Therefore, in this work we solely relied on the use of relative luminescence units (RLU) counts. Results indicated that NO is capable of triggering a strong monophasic Ca^{2+} pulse in a dose-dependent manner (Fig. 1). Interestingly, the average amplitude of the bioluminescent signal exceeded that produced in response to both cold shock and hydrogen peroxide (H_2O_2) treatments. Precisely, the average amplitude of the Ca^{2+} spike obtained upon treatment with 10 mM SNP was approximately 500 and 60 times greater than that observed upon either H_2O_2 or cold shock treatments, respectively.[12] However, despite being different in amplitude, the patterns of the induced $[Ca^{2+}]_{cyt}$, in response to H_2O_2, cold shock and NO, are similar in that they are all monophasic. Conversely, it has previously been reported that hypo-osmotic shock induces a distinctively different elevation in $[Ca^{2+}]_{cyt}$, which is biphasic.[31] Moreover, the observed $[Ca^{2+}]_{cyt}$ spike in response to NO challenge was similar to that obtained upon cold shock treatment, in the fact that it was instantaneous, with no apparent lag period (Fig. 1). The reported $[Ca^{2+}]_{cyt}$ spike obtained upon H_2O_2 treatment has been shown to follow a definite lag period of 20–40 s lasting between 1–2 min.[12] Furthermore, the decay time in the NO-induced luminescent signal was comparable with a shorter transient signal lasting only between 3–5 s. Taken together, it is envisaged that these apparent qualitative and quantitative differences in $[Ca^{2+}]_{cyt}$ dynamics, between different stimuli, underline the uniqueness of each $[Ca^{2+}]_{cyt}$ transient in transducing a distinct signal. This observation conforms to the current knowledge that the lag period length before the transient starts, the rise time to the transient peak and the decay time back to the resting level are all unique parameters to each Ca^{2+} signal.[16]

It was conceivable that the observed luminescence emission was not brought about by NO but rather via a direct chemical modification of aequorin by SNP or one of its byproducts. To evaluate this possibility, a combination of a SNP non-functional analogue and NO scavenger was employed. Any direct interaction between SNP and aequorin was ruled out by demonstrating that SNP was not capable of inducing any significant light emissions when added to in vitro reconstituted aequorin. Moreover, the SNP non-functional analogue $\{K_4Fe(CN)_6\}$ was shown to induce negligible luminescence in chemiluminometer assay (Fig. 3). Furthermore, the NO scavenger C-PTIO significantly blunted the SNP-induced $[Ca^{2+}]_{cyt}$ spike in both chemiluminometer and in vivo Ca^{2+} imaging assays (Fig. 3). Taken together, these results suggest that the observed SNP-mediated spike in $[Ca^{2+}]_{cyt}$ is specific to NO. Thus, based on the obtained experimental evidence (Figs. 1 and 3), the NO capacity of inducing $[Ca^{2+}]_{cyt}$ spikes in plants was clearly indicated. These results are in agreement with previously reported studies providing strong evidence that NO regulates cytosolic Ca^{2+} homeostasis in plant cells.[22]

Previously, pharmacological studies have been successfully employed to identify the sources of observed Ca^{2+} transients induced in response to several stimuli.[12,21,31] In this work, the use of a Ca^{2+} channel inhibitor proved to be useful. The PM-channel blocker La^{3+} was utilized, which acts as a potent Ca^{2+} antagonist, to assess the contribution of external Ca^{2+} pools towards the observed NO-induced $[Ca^{2+}]_{cyt}$ spike. La^{3+} treatment caused a concentration-dependent inhibition in $[Ca^{2+}]_{cyt}$ spike. However, complete abolition of the luminescent signal was not evident (Fig. 2). Moreover, La^{3+} concentrations above 100 μM resulted in no further significant inhibition. Thus, it can be concluded that the NO-mediated elevation in $[Ca^{2+}]_{cyt}$ is only partially contributed by an external Ca^{2+} pool or source. Taken together, it can be concluded that the observed elevation in $[Ca^{2+}]_{cyt}$ can partially be accounted for by a contribution from external Ca^{2+} pool and might possibly be from PM channels. Moreover, the observation that the inhibition in spike's magnitude is only partial suggests that there are, as yet unidentified, other sources of Ca^{2+} giving rise to the observed SNP-induced increases in $[Ca^{2+}]_{cyt}$. In contrast, employing a combination of La^{3+} and ruthenium red (a mitochondrial channel blocker), it has been shown that H_2O_2 is capable of mobilizing Ca^{2+} from both external and internal pools, with a major contribution from the internal pools.[12] Conversely, cold shock has been reported to mobilize Ca^{2+} primarily from external pools.[12] Interestingly, hypo-osmotic shock has been reported to induce a biphasic $[Ca^{2+}]_{cyt}$ influx. The first influx has been shown to derive from external Ca^{2+} stores, whereas the second influx derives from internal ones [31]. These results suggest that NO, H_2O_2, cold shock and hypo-osmotic shock mobilize different Ca^{2+} pools. Elegant studies employing *Nicotiana plumbaginifolia* cells expressing the Ca^{2+} reporter apoaequorin subjected to hyperosmotic stress, showed that NO was able to activate both PM and intracellular Ca^{2+}-permeable channels via signaling cascades; involving PM depolarization, cADPR, and protein kinases.[21] Recently, Lamattina and co-workers, via employing guard cell systems of Arabidopsis and cucumber, have made significant contributions that deepened our understanding of the involvement of NO in Ca^{2+} signaling. ABA has been shown to trigger a complex sequence of signaling events that lead to concerted modulation of ion channels at the plasma membrane of guard cells and solute efflux to drive stomatal closure in plant leaves. In this context, recent work has indicated that (NO) and its synthesis are a prerequisite for ABA signal transduction and stomatal closure in Arabidopsis and *Vicia faba* guard cells.[32] Latest results in *Vicia faba* guard cells have revealed that both Ca^{2+}- and NO-mediated signaling pathways are implicated in the observed ABA inhibition of light-induced stomatal opening.[33] Moreover, in *Vicia faba* guard cells it has been shown that NO selectively regulates Ca^{2+}-sensitive ion channels by promoting release from intracellular stores to raise $[Ca^{2+}]_{cyt}$. via a cADPR-dependent endomembrane Ca^{2+} channels, mediated through the action of a cGMP-dependent cascade.[34] Interestingly, protein kinase has been implicated as an essential component for intracellular Ca^{2+} release and ion

channel control by NO and ABA in guard cells. In this context, it has been demonstrated that NO-sent signals can be modulated through protein phosphorylation, located upstream of intracellular Ca^{2+} release.[35] Similarly, it has been reported that Ca^{2+} and Ca^{2+}-dependent protein kinases are involved in NO- and auxin-induced adventitious root formation in cucumber.[36]

In the future, it will be interesting to investigate the contribution of internal Ca^{2+} pools towards the observed NO-induced $[Ca^{2+}]_{cyt}$ spike.

Materials and Methods

Aequorin chemiluminescence measurement. Transgenic Col-0 Arabidopsis plants genetically transformed with a chimeric gene in which the cauliflower mosaic virus 35S promoter was fused to the apoaequorin coding region were used. Seeds of this transgenic reporter line were a kind gift of Prof. Tony Trewavas (University of Edinburgh). Two-week old homozygous *35S::APOAEQUORIN* seedlings were aequorin-reconstituted by floating into a cuvette containing 0.5 ml of 10 μM coelenterazine solution (prepared in sterile water) at room temperature in the dark overnight.[24] Four seedlings per cuvette were systematically used for each treatment. Then, reconstituted seedlings, floating in the cuvette, were treated with the NO donor sodium nitroprusside (SNP) at different concentrations. Increasing concentrations of SNP, between 0.1–10 mM, prepared in water, were injected via a syringe, fitted with a special needle, into the cuvette through a designated rubber-wrapped luminometer port. Chemicals such as lanthanum chloride ($LaCl_3$) (a general plasma membrane channel blocker), potassium ferrocyanide $\{K_4Fe(CN)_6\}$, and 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (C-PTIO) (Calbiochem®), were added to the coelenterazine-reconstituted seedlings 4 h before the treatment with SNP. To check whether SNP interacts with apoaequorin producing inappropriate luminescence, apoaequorin was reconstituted in vitro. Apoaequorin was in vitro reconstituted and extracted from *35S::APOAEQUORIN* Arabidopsis seedlings in a buffer containing 0.5 M NaCl, 5 mM b-mercaptoethanol, 5 mM EDTA, 0.1% gelatine (w/v), 10 mM Tris-HCl pH 7.4 and 10 μM coelenterazine in the darkness for 4 h. Luminescence measurements were made using a digital chemiluminometer with a photomultiplier, model 9757 AM (THORN EMI Electron Tubes Limited, Ruislip, Middlesex, UK) at 1 kV with a discriminator.[25] In order to minimize background counts, temperature of the cuvette chamber was continuously kept at approximately -30°C by means of an installed cooler. The cuvette temperature was kept at room temperature. Luminescence counts were measured every 1 s for the duration of the experiment (~2 min).

In vivo Ca^{2+} imaging. Four-week old Arabidopsis *35S::APOAEQOURIN* plants were reconstituted by infiltrating the abaxial side of the leaf with 10 μM coelenterazine solution and incubation in the dark overnight. The next day, reconstituted leaves were infiltrated with SNP. Immediately after infiltration, leaves were detached and placed in the dark box for imaging. Leaves were imaged within 1 min after infiltration and placement in the camera darkbox. Imaging was performed using an ultra low light imaging camera system, EG&G Luminograph 980, (Berthold, London, UK). Images were collected over a three-minute accumulation period and integrated for 1.5 min. Images were processed using Confocal Assistant and PaintShop Pro software.

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