NO-mediated $[\text{Ca}^{2+}]_{\text{cyt}}$ increases depend on ADP-ribosyl cyclase activity in Arabidopsis

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SMA-A and CTH performed the experiments and analysed the data. MD and AND aided with the development of the assays. AGS and AARW supervised the research. SMA-A and AARW wrote the manuscript with contributions from all the other authors.

Funding
This work was supported by the Islamic Development Bank and the Cambridge Commonwealth Trust (SMA-A), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CTH) and BBSRC UK grant BB/D017904/1 (AND) awarded to AARW.

One sentence summary
ADP-ribosyl cyclase activity in Arabidopsis is upregulated by nitric oxide to increase the cellular concentration of cyclic ADP ribose and free Ca$^{2+}$. 
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ABSTRACT
cyclic ADP-ribose (cADPR) is a Ca^{2+}-mobilising intracellular second messenger synthesised from nicotinamide adenine dinucleotide (NAD) by ADP-ribosyl cyclases (ADPR cyclases). In animals, cADPR targets the ryanodine receptor (RyR) present in the sarco/endoplasmic reticulum (SR/ER) to promote Ca^{2+} release from intracellular stores to increases the concentration of cytosolic free Ca^{2+} ([Ca^{2+}]_{cyt}) in Arabidopsis. cADPR has been proposed to play a central role in signal transduction pathways evoked by the drought and stress hormone, abscisic acid (ABA) and the circadian clock. Despite evidence for the action of cADPR in Arabidopsis, no predicted proteins with significant similarity to the known ADPR cyclases have been reported in any plant genome database, suggesting either that there is a unique route for cADPR synthesis or a that homolog of ADPR cyclase with low similarity might exist in plants. We sought to determine whether the low-levels of ADPR cyclase activity reported in Arabidopsis are indicative of a bona fide activity that can be associated with the regulation of Ca^{2+} signalling. We adapted two different fluorescence-based assays to measure ADPR cyclase activity in Arabidopsis and found this activity has the characteristics of a nucleotide cyclase that is activated by nitric oxide to increase cADPR and mobilise Ca^{2+}.

INTRODUCTION
Cyclic adenosine diphosphate ribose (cADPR) is a signalling molecule that can evoke increase in the concentration of cytosolic-free Ca^{2+} ([Ca^{2+}]_{cyt}) in plant and animal cells (Hetherington & Brownlee, 2004; Zhang & Li, 2006). In animals, cADPR is synthesised by a class of NADases called the ADP-ribosyl cyclases. Metabolites of ADP-ribosyl cyclases (ADPR cyclase) including ADP-ribose (ADPR), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) are all signalling molecules involved in Ca^{2+} signalling (Guse & Lee, 2008; Lee, 2001 & 2006). In animals, both ADPR and cADPR stimulate Ca^{2+} influx through plasma membrane transient receptor potential (TRPM2).
channels (Sano et al., 2001; Perraud et al., 2001; Kraft et al., 2004). cADPR also mobilizes Ca\textsuperscript{2+} from the endoplasmic reticulum (ER) through an inositol 1,4,5 trisphosphate (InsP\textsubscript{3})-independent mechanism (Lee & Aarhus, 1991 & 1993; Galione, 1993 & 1994; Lee, 1993; Galione et al., 1991), which most likely involves modulation of ryanodine receptors (RyRs) (Li et al., 2001; Thomas et al., 2001, Ozawa, 2001). NAADP mobilizes intracellular Ca\textsuperscript{2+} from lysosomal and/or acidic stores and is active in a variety of mammalian cell types (Lee, 2005).

In plants, neither ADPR cyclase, nor an equivalent of the RyR have been identified in genomic databases, even though ADPR cyclase activity and cADPR-evoked Ca\textsuperscript{2+} release from vacuoles and ER have been reported (Allen et al., 1995; Muir & Sanders, 1996; Leckie et al., 1998; Navazio et al., 2000; Sanchez et al., 2004). cADPR injected in to guard cells causes stomatal closure (Leckie et al., 1998) and cADPR has been proposed to be involved in ABA-induced stomatal closure because 8-NH\textsubscript{2}-cADPR, a competitor of cADPR signalling, and nicotinamide, an inhibitor of ADPR cyclase activity both reduced ABA-induced stomatal closure (Leckie et al., 1998). The role of cADPR in ABA signalling also is supported by the statistically significant intersection between the sets of transcripts induced by ABA and cADPR (Sanchez et al., 2004). There is a similar intersection between transcript populations that are regulated by cADPR and the circadian clock, and together with circadian oscillations in the concentration of cADPR and an increased circadian period in the presence of nicotinamide, these data have led to the proposal that cADPR forms a feedback loop in the Arabidopsis circadian oscillator (Dodd et al., 2007).

The lack of orthologues for ADPR cyclase and RYR, and the limited characterisation of their activities, has led to uncertainty concerning whether plants have a bone fide ADPR cyclase activity associated with Ca\textsuperscript{2+}-signalling (Dodd et al., 2010). We sought to establish whether the reported ADPR cyclase-like activity in Arabidopsis has functional characteristics of an enzyme involved in the generation of cADPR to mobilize Ca\textsuperscript{2+} in plant signalling networks; specifically we investigated if the enzyme activity was correlated with stimulus-induced increases in cADPR and also [Ca\textsuperscript{2+}]\textsubscript{cyt}. We investigated the potential role of ADPR cyclase
activity in NO signaling because NO is a known regulator of the cADPR signaling pathway in animals (Galione et al., 1993; Willmott et al., 1996; Yu et al., 2000; Zhang & Li, 2006), and pharmacology suggests that NO-mediated increases in $[Ca^{2+}]_{cyt}$ are cADPR-dependent in *Vicia faba* (Garcia-Mata et al., 2003). We reasoned that if cADPR is associated with NO signaling as predicted by pharmacological studies, there might be NO-induced increases in Arabidopsis ADPR cyclase activity and NO-induced increases in the concentration of cADPR.

**RESULTS**

**Pharmacological identification of cADPR-dependent signalling pathways in Arabidopsis**

To investigate potential roles for cADPR in signalling in Arabidopsis we investigated the effects of an antagonist of cADPR signalling on stimulus-induced increases of $[Ca^{2+}]_{cyt}$ in response to cold, NaCl, H$_2$O$_2$ and NO. Cold treatment induced a transient increase of $[Ca^{2+}]_{cyt}$ in Arabidopsis that reached a peak of 440 ± 60 nM (mean ± se, Fig. 1A), almost three times higher than the touch response evoked by room temperature water (152 ± 9 nM; Fig. 1A). We selected nicotinamide as a suitable antagonist because it is a metabolic by-product of cADPR production that acts as an inhibitor through product inhibition and enzyme reversal described by basic Michaelis-Menten kinetics. This simple pharmacology is easier to interpret than that based on analogue compound chemistry and we have previously demonstrated dose-dependent inhibition of Arabidopsis ADPR cyclase activity by nicotinamide (Dodd et al., 2007). Nicotinamide inhibits also other NADases, including poly ADP ribose polymerases and SIRTUINS through the same product inhibition, however neither of those enzymes have known roles in Ca$^{2+}$ signalling and therefore an effect of nicotinamide on stimulus-induced $[Ca^{2+}]_{cyt}$ increases is indicative of ADPR cyclase activity (Galione, 1994). In the presence of 50 mM nicotinamide the cold-induced increase in $[Ca^{2+}]_{cyt}$ was slightly smaller, with the highest $[Ca^{2+}]_{cyt}$ peak of 358 ± 72 nM (Fig. 1A). A transient increase of $[Ca^{2+}]_{cyt}$ was detected in response to 10 mM H$_2$O$_2$ (peak $[Ca^{2+}]_{cyt}$ 673 ± 45 nM; Fig. 1B). Pre-incubation with
nicotinamide (50 mM) for two hours reduced and slightly delayed the \( \text{H}_2\text{O}_2 \)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase (peak \([\text{Ca}^{2+}]_{\text{cyt}}\) 429 ± 20 nM; Fig. 1B). NaCl at 150 mM induced a large, rapid increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) to a peak of 981 ± 229 nM (Fig. 1C) which was higher than cold water- and \( \text{H}_2\text{O}_2 \)-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) responses. A partial reduction of the NaCl-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) response was found when plants were incubated with nicotinamide (50 mM, peak \([\text{Ca}^{2+}]_{\text{cyt}}\) 662 ± 144 nM, Fig. 1C). S-nitroso-N-acetylpenicillamine (SNAP) acts as a NO donor and triggers increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in Arabidopsis (Neill et al., 2002). 300 μM SNAP elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) and the increase was stable for 400 s, which was more prolonged than those induced by cold water, \( \text{H}_2\text{O}_2 \), and NaCl. The peak for SNAP-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) increase was 368 ± 18 nM (Fig. 1D), which was achieved 160 s after SNAP treatment, compared to the rapid responses to cold water, \( \text{H}_2\text{O}_2 \) and NaCl in which the peak of \([\text{Ca}^{2+}]_{\text{cyt}}\) was induced within 15 - 30 seconds. Nicotinamide (50 mM) completely abolished SNAP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases (peak \([\text{Ca}^{2+}]_{\text{cyt}}\) 123 ± 4 nM; Fig. 1D).

**[Ca\(^{2+}\)]\text{cyt}** increases induced by NO are ADPR cyclase dependent

The inhibition of SNAP-induced increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) by nicotinamide was suggestive of a role for ADPR cyclase in the elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) by NO. We performed a further set of experiments to confirm that the effects of SNAP were linked to NO production and not with an unintended side effect. First, we tested the effect of an alternative NO donor, sodium nitroprusside (SNP; Neill et al., 2002). At 5 μM SNP induced sustained \([\text{Ca}^{2+}]_{\text{cyt}}\) increases that reached a plateau at 131.0 ± 6.6 nM (Fig. 2A). Increasing the concentration of SNP to 50 μM or 500 μM SNP had no further effect on \([\text{Ca}^{2+}]_{\text{cyt}}\), possibly because the experiment was performed in the dark which limits the effectiveness of SNP (Rico-Lemus and Rodríguez-Garay, 2014). At 150 μM, SNAP induced sustained increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) for 4 to 5 min until it reached a plateau at 195.1 ± 11.4 nM (Fig. 2B). Elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) by these two donors, suggested the effects were due to NO synthesis. This was confirmed by testing the effects of the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Neill et al., 2002).
Addition of 300 μM cPTIO 300 s after the addition of 150 μM SNAP decreased the $[\text{Ca}^{2+}]_{\text{cyt}}$ levels from the elevated value of $192.2 \pm 13.6$ nM to $115.3 \pm 7.6$ nM (Fig. 2C). Pre-incubation with 300 μM cPTIO reduced the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase evoked by 150 μM SNAP to $145.3 \pm 13.3$ nM (t-test against 150 μM SNAP without 300 μM cPTIO, P<0.01; Fig. 2D). Nicotinamide was equally effective in inhibiting NO-mediated increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ if added before or after the NO donor SNAP (Fig. 2E and 2F). Addition of 50 mM nicotinamide added 300 s after the addition of SNAP reduced $[\text{Ca}^{2+}]_{\text{cyt}}$ levels from $215.8 \pm 11.7$ nM to $121.6 \pm 5.6$ nM, however there was a long delay of over 60 s after the addition of nicotinamide before $[\text{Ca}^{2+}]_{\text{cyt}}$ decreased (Fig. 2E). This is supportive of the proposed role of nicotinamide inhibiting the production of cADPR, and possibly contributing to cADPR degradation by reversing the catalytic activity of ADPR cyclase to one of cADPR catalysis. SNAP addition after a prolonged incubation with nicotinamide resulted in a residual increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ only to $139.1 \pm 19.1$ nM (Fig. 2F) demonstrating that NO-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ might be almost completely dependent on cADPR. Osmotic effects of nicotinamide can be discounted since an equimolar concentration of mannitol was without effect (Fig. 2G). Pre-incubation for 300 s with GdCl₃ (the most effective blocker of Arabidopsis plasma membrane $\text{Ca}^{2+}$-influx channels (Demidchik et al. 2002)) at 1 mM, ten times higher than required to inhibit NaCl-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the same assay (Tracy et al. 2008), did not reduce the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase induced by 150 μM SNAP, which peaked at $198.7 \pm 17.2$ nM (t-test against 150 μM SNAP without 1 mM GdCl₃, P=0.81; Fig. 2H), suggesting that plasma membrane influx of $\text{Ca}^{2+}$ might not contribute to the response.

NGD- and NHD-based fluorescence spectrometer assays of Arabidopsis ADPR cyclase activity

The pharmacological manipulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ is strongly indicative of a nicotinamide-sensitive component being required for NO-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ in Arabidopsis. To test if this increase is mediated by the activation of an ADPR cyclase-like activity, we assayed for ADPR cyclase activity based on the conversion of non-fluorescent nucleotide analogues of NAD into
fluorescent cyclic nucleotides. Soluble total protein (TP) extracts of Arabidopsis have an enzymatic activity capable of converting the non-fluorescent NAD analogue, nicotinamide guanine dinucleotide (NGD), to the fluorescent cyclic GDP-ribose (cGDPR, Fig. 3A). The synthesis of the cGDPR was dependent on the presence of NGD in both Col-0 and plants heterologously expressing ADPR cyclase from the sea slug *Aplysia californica* (35S:Ac ADPR cyclase; Dodd *et al.*, 2007). Furthermore, the rate of fluorescence increase was higher in protein extracted from the 35S:Ac ADPR cyclase plants (Fig. 3A).

The conversion of NGD to cGDPR was inhibited by NAD. In our assay, equal concentrations of NGD and NAD reduced the activity to 0.5 fold, however an excess of NAD completely abolished the cyclisation of NGD (Fig. 3B). Whilst it is possible that NAD acts as a non-competitive inhibitor, the reduction of the conversion of NGD to cGDPR by NAD is an expected characteristic of a nucleotide cyclase activity that favors NAD as a substrate to generate cADPR as a product. Animal ADPR cyclase is reversible under standard conditions, so we tested whether inclusion of cADPR in the assay would inhibit the production of cGDPR from NGD. Addition of 25 µM cADPR reduced ADPR cyclase activity significantly (P=>0.001), however higher concentrations of cADPR (up to 75 µM) did not cause any further changes in activity (Fig. 3C). The ADPR cyclase-like activity was protein-dependent, being absent in boiled protein extracts (Fig. 3D). Based on these findings we considered the fluorescence intensity increase to be representative of a *bona fide* ADPR cyclase activity. To determine the specific activity we used commercial *Aplysia* ADPR cyclase to generate a standard curve (Supp. Fig. S1). This enabled us to estimate that the specific activity in extracts of unstimulated Arabidopsis Col-0 to be around 0.01 – 0.015 unit µg total protein⁻¹ min⁻¹ or unit µg protein⁻¹ min⁻¹ (Figure 3). An alternative assay based on the conversion of nicotinamide hypoxanthine dinucleotide (NHD) to cyclic inosine diphosphoribose (cIDPR) (Graeff *et al.*, 1996) resulted in a very similar estimate of Col-0 ADPR cyclase activity (Fig. 3E), whilst, as expected 35S:Ac ADPR cyclase plants had significantly higher ADPR cyclase activity of 0.027 ± 0.0008 unit µg protein⁻¹ min⁻¹ (P=<0.001; Fig. 3E).
**NO is a regulator of Arabidopsis ADPR cyclase activity**

NO treatment of whole plants significantly increased the extractable ADPR cyclase activity using either NGD or NHD as substrates (P =< 0.001) and the NO scavenger cPTIO significantly reduced the effect of SNAP on extractable ADPR cyclase activity (Fig. 4A and 4B). Similarly, adding SNAP to the extracted proteins also increased Arabidopsis ADPR cyclase activity, which was likewise reversed by cPTIO (Fig. 4B, P =<0.001). This demonstrates that NO can regulate ADPR cyclase activity in a cell-free manner.

To investigate whether physiologically relevant levels of NO can regulate ADPR cyclase activity in Arabidopsis, we measured it in lines carrying the calmodulin-like 24-4 allele which results in constitutively high NO (Tsai et al., 2007). Both cml24-4 and cml23-3 cml24-4 plants had significantly higher extractable ADPR cyclase activity compared with wild type Col-0 plants (P =<0.001; Fig. 4C).

ABA increases NO in guard cells (Neill et al., 2002) and therefore we tested the effect of this phytohormone on ADPR cyclase activity. Soluble protein extracts of Col-0 plants treated with 50 µM ABA had significantly higher ADPR cyclase activity of 0.026 ± 0.001 unit µg TP⁻¹ min⁻¹ (P =<0.001; Fig. 4D) compared with untreated protein extracts of Col-0 plants (Fig. 4D). This activation appears to be physiologically relevant because the activation by ABA was less than that due to the exogenous NO donor SNAP, which might be expected to cause very high levels of NO (P =<0.001; Fig. 4D). NO-induced ADPR cyclase activity was inhibited by nicotinamide in a dose-dependent manner, with complete inhibition being achieved at 50 mM nicotinamide (Supp. Fig. S2), consistent with the effect of nicotinamide on NO-induced increases in [Ca²⁺] cyt (Fig. 1D, Fig. 2E and 2F).

The activation of ADPR cyclase activity by NO was confirmed by measurement of [cADPR] in Arabidopsis treated with 300 µM SNAP or 0.5% methanol (MetOH) using a fluorescence-
based coupled assay (Dodd et al., 2007). Before treatment, [cADPR] was 0.72 ± 0.09 pmol μg protein⁻¹ (Fig. 5). [cADPR] levels in the plants treated with 0.5% MetOH control remained almost constant at all the time points varying from 0.47 ± 0.00 pmol μg protein⁻¹ 30 min after the treatment, to 0.89 ± 0.09 pmol μg protein⁻¹ 60 min after the treatment (Fig. 5). Addition of 300 μM SNAP caused a fast increase of [cADPR] in the first 5 min to 1.62 ± 0.34 pmol μg protein⁻¹ before slowly returning to resting levels at 60 min (0.77 pmol μg protein⁻¹, Fig. 5).

DISCUSSION

NO increases [Ca²⁺]cyt through a pathway that includes activation of ADPR cyclase

We found that NO-mediated [Ca²⁺]cyt increases were abolished by incubation with the NADase inhibitor nicotinamide, that NO increases ADPR cyclase activity, and that NO stimulates the production cADPR, a Ca²⁺ agonist. These data and the insensitivity of NO-mediated increases in [Ca²⁺]cyt to GdCl₃, an inhibitor of plasma membrane-mediated influx of Ca²⁺, lead us to conclude that the primary pathway by which NO increases [Ca²⁺]cyt in Arabidopsis is through cADPR-mediated Ca²⁺ release from the ER and/or the vacuole, dependent on the activity of ADPR cyclase.

The conservation of regulation of ADPR cyclase activity by NO between plants and animals could suggest a common ancestry for the pathway, or alternatively this might be an example of convergent evolution of signaling in the plant and animal lineages. However, the lack of obvious orthologues for ADPR cyclase and ryanodine receptors in the Arabidopsis and other plant genomes makes it challenging to confirm either of these hypotheses. Our adaptation of ADPR cyclase activity assays for Arabidopsis, and identification of both NO and cml24-4 mutants as activators of ADPR cyclase, provides a toolset that might aid isolation of the ADPR cyclase protein and identification of the corresponding gene. This might provide information concerning potential evolutionary features of cADPR-dependent NO-induced increases in [Ca²⁺]cyt. Our discovery that cml24-4 plants have higher ADPR cyclase activity provides a
potential genetic background to use in attempts to purify the enzyme. We have found that pharmacological tools can be used to activate and inhibit ADPR cyclase activity in a cell-free manner, which could be useful in confirming that a purified product represents a potential ADPR cyclase.

We found little evidence that cADPR signalling contributes to cold-, touch- and \( \text{H}_2\text{O}_2 \)-induced increases in \([\text{Ca}^{2+}]_{\text{cyt}}\). Cold-induced increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) are due to influx across the plasma membrane and efflux of \(\text{Ca}^{2+}\) from the vacuole (Knight et al. 1996), apparently through a cADPR-independent route. The analysis of \(\text{H}_2\text{O}_2\)-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) signals revealed that the initial increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) was partially suppressed by 50 mM nicotinamide (Fig. 1B). However, this effect was much less than observed for NO, and we conclude the bulk increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to \(\text{H}_2\text{O}_2\) is not ADPR cyclase-dependent. \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations in response to \(\text{H}_2\text{O}_2\) treatment arise primarily through activation of hyperpolarisation-activated \(\text{Ca}^{2+}\) permeable channels in the plasma membrane (Pei et al., 2000; Rental & Knight, 2004). NaCl elevates \([\text{Ca}^{2+}]_{\text{cyt}}\) within very short periods in plants (Knight et al., 1997; Kiegle et al., 2000; Knight, 2000; Moore et al., 2002). We also detected immediate rapid responses of \([\text{Ca}^{2+}]_{\text{cyt}}\) to NaCl. Nicotinamide had some inhibitory effects but did not abolish NaCl-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) increases (Fig. 1C). Based on this finding and studies with inhibitors of plasma membrane \(\text{Ca}^{2+}\)-influx (Tracey et al., 2008), it appears NaCl-induced increases involve both influx across the plasma membrane and involvement of cADPR-mediated \(\text{Ca}^{2+}\) release from ER or vacuole. Release of \(\text{Ca}^{2+}\) from multiple stores through different pathways might permit the oscillatory \([\text{Ca}^{2+}]_{\text{cyt}}\) signals induced by NaCl (Martí et al., 2013) which is a result of spatial heterogeneity (Tracy et al., 2008) and cell-specific dynamics (Martí et al., 2013).

**Nitric oxide modulates short-term \(\text{Ca}^{2+}\)-responses in Arabidopsis**

cADPR previously has been suggested to be involved in the NO signalling pathway in plants (Garcia-Mata et al., 2003; Lamotte et al., 2006; Zhang & Li, 2006), but measurements of NO regulation of ADPR cyclase activity and \([\text{cADPR}]\) have not been reported. By measuring ADPR
cyclase and cADPR levels it has been possible to observe that the elevation of cADPR in response to NO is transitory (Fig. 5) and that $[\text{Ca}^{2+}]_{\text{c}}$ returns rapidly to resting in the absence of cADPR synthesis (Fig. 2E). We conclude that cADPR-dependent NO-regulated $[\text{Ca}^{2+}]_{\text{c}}$ signalling is most likely to be involved in shorter-term responses that might occur in response to plant-pathogen interactions, symbiotic events or hormones (Mur et al., 2013). If these rapid, short-term NO-mediated increases in $[\text{Ca}^{2+}]_{\text{c}}$ are involved in than longer-term signalling such as the photoperiodic regulation of flowering, they are likely to be very early in the signalling cascade.

It is not known how NO regulates ADPR cyclase activity, however we have shown this to occur in a cell-free extract and therefore it is reasonable to suspect that the effect could be direct, through a mechanism such as nitrosylation. In mammals and sea urchins, NO increases the activity of ADPR cyclase through guanylate cyclase- and cGMP-dependent pathways (Galione, 1993 and 1994). There are many possible sources of NO in plants, including enzymatic and non-enzymatic (Bethke et al., 2004; Crawford, 2006). One of the most established sources of NO in plants is nitrate reductase (NR) which usually converts NO$_3^-$ in NO$_2^-$ but may also convert NO$_2^-$ into NO in anaerobic conditions and when NO$_2^-$ levels are high (Yamasaki et al., 1999; Rockel et al., 2002; Meyer et al., 2005; Crawford, 2006). Our data do not distinguish which of those are responsible for regulating ADPR cyclase, but we do demonstrate that high endogenous levels of NO, such as those achieved in cml24-4 mutants, are capable of increasing ADPR cyclase activity. Our identification of NO-regulated ADPR cyclase activity fills a missing link in the NO signal transduction chain. There are likely to be additional regulators of ADPR cyclase activity because the NO-induced cADPR increase was transient, contrasting with ABA-evoked cADPR increases which were sustained for at least an hour (Dodd et al., 2009; Sanchez et al., 2004).

**MATERIAL AND METHODS**
**Plant material and growth condition and measurement of [Ca]_{cyt}**

Experiments were performed with *Arabidopsis thaliana* ecotype Col-0, except where stated. Seeds were grown and [Ca]_{cyt} was measured using aequorin in plants carrying *CaMV35S:APOAEQUORIN* as described in Martí *et al* (2013). Seeds were sown in petri dishes and stratified in the dark at 4 °C for 2 - 3 days. Petri dishes were then transferred to a growth cabinet (12h light / 12h dark, 20 °C, 50 - 60 μmol m^{-2} s^{-1} irradiance) for 7 - 12 days for [Ca]_{cyt} measurement, three weeks for measuring [cADPR] and four to five weeks for ADPR cyclase activity measurement.

**Measurement of ADPR cyclase activity in protein extracts**

**Preparation of soluble protein extracts**

5 - 10 g whole rosette tissue, excluding roots, of 4 - 5 week old Arabidopsis plants were homogenised using a pestle and mortar at 4°C in solution A [340 mM glucose (Fisher Scientific, UK), 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma, UK), 1 mM MgCl_{2} (BDH Laboratory Supplies, UK), 50 g mL^{-1} soybean trypsin inhibitor (Sigma, UK), 10 µg mL^{-1} leupeptin (Sigma, UK) and 10 µg mL^{-1} aprotinin (Sigma, UK), pH 7.2, 3 mL g^{-1} FW]. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), and the resulting filtrate was centrifuged at 2000 g for 5 min at 4 °C to remove unbroken cell debris, tissues etc. The supernatant was transferred into 15 mL falcon tube and centrifuged at 12000 g for 15 min at 4°C (Beckman Coulter Avanti J-26XP centrifuge, UK). After centrifugation, the supernatant was collected carefully and ran through the PD-10 desalting column (GE Healthcare, UK) according to manufacturer’s protocols. Protein content was estimated with a protein assay kit (Bio-Rad Laboratories Inc., Germany) using bovine serum albumin (BSA; New England Biolabs, USA) as a standard.

**NGD/NHD assays of ADPR cyclase activity using a luminescence spectrometer**
145 μg protein of Col-0 plants were taken in 1200 μl solution A (pH 7.2) in quartz cuvettes and fluorescence intensity was measured for every single minute up to 10 min at 21 °C using a luminescence spectrometer (Perkin Elmer LS 55, UK) set with the excitation wavelength at 300 nm and emission wavelength at 410 nm. After 10 min, 60 μl of 4 mM NGD (prepared in solution A, final concentration 200 μM, Sigma, UK) was added to reactions and resultant fluorescence intensity was measured for another 10 - 15 min. Additionally, fluorescence intensity was measured for every single minute up to 10 - 15 min at 21 °C for 1200 μl reactions in solution A (pH 7.2) containing 145 μg total protein, 145 μg total protein + 200 μM NAD (Sigma, UK), 145 μg total protein + 200 μM NGD, 145 μg boiled protein (100 °C; 10-15 min) + 200 μM NGD. For NHD assay, 200 μM NHD (prepared in solution A, pH 7.2, Sigma, UK) was used in replace of NGD.

To test the effect of NO on ADPR cyclase activity in the protein extracts, four to five week old Col-0, cml24-4 or cml23-2 cml24-4 plants were incubated in the presence or absence of 300 μM SNAP (Calbiochem, UK), 300 μM SNAP and 300 μM cPTIO (Sigma, UK) for 40 – 50 min, separately. Alternatively, protein extracts of untreated Col-0 plants were incubated with 300 μM SNAP, 300 μM SNAP and 300 μM cPTIO for 40 – 50 min, separately. Total protein extract of Col-0 plants (four to five week old) was incubated with 50 μM ABA for 1 h.

Reverse cyclase assay for [cADPR] measurement

cADPR isolation and cADPR purification

The plant were dosed with 150 μM SNAP or 0.5% MeOH dosed by flooding for 1 min, after which all the liquid was taken out. Plants were harvested before the dosing (0 min) and 5 min, 10 min, 30 min and 60 min after. Only the aerial parts were harvested. Plants were pooled, frozen in liquid nitrogen and stored at -80 °C. Frozen samples were finely ground in liquid nitrogen. About 2 mg of frozen material was thawed and vortexed in 250 μl ice-chilled HPLC-H2O (about 4 °C, Fisher Scientific, UK). Protein quantification was performed on 25 μl of the
sample by Bradford assay. In order to precipitate proteins, 25 μl 7 M perchloric acid (Sigma, UK) was added to the samples and vortexed. 1 mL of ice-chilled 3:1 mix of 1,1,2-trichlorotrifluoroethane:tri-N-octylamine (TCFE/TO; Sigma, UK) was added to separate cADPR from the rest of plant extract. The mixture was vortexed and kept on ice until the precipitation of perchloric acid. Samples were centrifuged at 4 °C for 10 min at 1,500 g. After centrifugation, the samples had two-phases separated by a white film. The aqueous phase (upper) was taken off and 1 M NaPO₄ buffer (pH 8.0) was added to a final concentration of 20 mM NaPO₄. Contaminating nucleotides were removed from the isolated aqueous phase by enzymatic hydrolysis. 0.44 μg mL⁻¹ nucleotide pyrophosphatase (EC 3.6.1.9; Sigma, UK), 1.25 μg mL⁻¹ alkaline phosphatase (EC 3.1.3.1; isolated from bovine intestinal mucosa; Sigma, UK) and 0.06 μg mL⁻¹ NADase (EC 3.2.2.5; isolated from porcine brain; Sigma, UK) were added to the samples and incubated overnight at 37 °C. After the incubation, the enzymes were separated from the extract with 3000 MW cut-off filters (500 μl size; Millipore, UK) and spun at 4 °C for 30 min at 13,000 g. The final extract was diluted 1:1 with 200 mM phosphate buffer to a final concentration of 100 mM NaPO₄.

**Fluorescence-based cycling assay**

The cycling assay is based on a cycle of enzymatic conversions (Graeff & Lee, 2002): cADPR is first converted to NAD by ADPR cyclase (EC 3.2.2.5; from the marine sponge *Axinella polypoides*; gift from Prof. E. Zocchi and Dr S. Bruzzone, Universita’ di Genova, Italy) in the presence of high amounts of nicotinamide (Sigma, UK). Next, alcohol dehydrogenase (EC 1.1.1.1; extracted from *Saccharomyces cerevisiae*; Sigma, UK) converts EtOH and NAD into acetaldehyde and NADH. Finally, diaphorase (EC 1.8.1.4; extracted from *Clostridium kluyveri*; Sigma, UK) converts NADH and resazurin (Sigma, UK) into NAD and resorufin, a fluorescent substance that can be detected by a multifunctional microplate reader (FluoStar OPTIMA, BMG LabTech, Germany). To each well of black 96-well plates 100 μl of sample was added. First, 50 μl assay reagent (30 mM nicotinamide, 0.3 μg mL⁻¹ ADPR cyclase in 100 mM phosphate buffer, pH 8.0) was added, left for 15 min, then 100 μl cycling reagent [10 mM
nicotinamide, 20 μM resazurin, 10 μM riboflavin 5-monophosphate (Sigma, UK), 100 μg mL⁻¹ alcohol dehydrogenase, 10 μg mL⁻¹ diaphorase, 2% EtOH in 100 mM NaPO₄ buffer, pH 8.0] was added to each well and fluorescence was measured at 30 °C in every 5 min for 8 h at 540 ± 5 nm excitation and 590 ± 5 nm emission. Diaphorase is frequently contaminated with nucleotides so the enzyme was purified by incubation in 2% activated charcoal in 37 °C for 30 min followed by centrifugation at 5,000 g for 5 min. cADPR levels were estimated using 2 nM, 5 nM, 10 nM, 50 nM, 100 nM and 500 nM cADPR (Calbiochem, UK) as standards. As the cADPR isolation and purification steps in the samples might lead to cADPR losses, those steps were also performed in the cADPR standards.

Supplemental Data
The following supplemental materials are available.

Supplemental figure S1. Preparation of Aplysia standard curve.
Supplemental figure S2. ADPR cyclase activity in response to nicotinamide.

ACKNOWLEDGEMENTS
We are grateful to the Islamic Development Bank, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and the Cambridge Commonwealth Trust for financial support. We are also grateful to Dr María C. Martí and Mr Matthew Stancombe for their technical support.

Figure Legends

Figure 1. Nicotinamide abolished NO-induced [Ca²⁺]cyt increases in Arabidopsis. A. Effect of cold water (4 °C) on [Ca²⁺]cyt in the absence or presence of nicotinamide (50 mM). B. Effect of H₂O₂ on [Ca²⁺]cyt in the absence or presence of nicotinamide (50 mM). C. Effect of NaCl on [Ca²⁺]cyt in the absence or presence of nicotinamide (50 mM). D. Effect of NO (SNAP) on [Ca²⁺]cyt in the absence or presence of nicotinamide (50 mM). 12 day old aequorin-expressing individual seedlings were incubated for 2 hours with nicotinamide (50 mM). Cold water (4 °C), 10 mM H₂O₂, 150 mM NaCl
and 300 µM SNAP (NO donor) were added at 15 s and luminescence was measured for 896 s in a luminometer or multifunctional microplate reader. Data presented as the mean of 12 biological replicates from three independent experiments (n=12) and error bars represent standard error of the mean. Arrows indicate the time of stimulation.

**Figure 2. Nitric oxide evokes short-term [Ca$^{2+}$]$_{cyt}$ increases.**

A. SNP was added at 60 s and 360 s (n = 5 for each treatment) after the start of the experiment and [Ca$^{2+}$]$_{cyt}$ levels were measured for 600 s. B. SNAP added to provide a final concentration of 150 µM or 0.5% ethanol control was added 60 s after the start of the experiment and [Ca$^{2+}$]$_{cyt}$ levels were measured for 600 s (n = 19). C. SNAP to a final concentration of 150 µM was added at 60 s and 300 µM cPTIO was added at 360 s (n = 21). D. Seedlings were incubated with 300 µM cPTIO for 300 s before the start of the experiment when 150 µM SNAP was added at 60 s (n = 10). E. SNAP to a final concentration of 150 µM was added 60 s after the start of the experiment and 50 mM nicotinamide was added 300 s later (n = 20). F. Nicotinamide (50 mM) was added 60 s after the start of the experiment and 150 µM SNAP was added 300 s later (n = 8). G. SNAP to a final concentration of 150 µM was added 60 s after the start of the experiment and 50 mM mannitol was added 300 s later (n = 5). H. Seedlings were incubated for 300 s in 1 mM GdCl$_3$ before the start of the experiment. SNAP to a final concentration of 150 µM was added after 60 s (360 s; n = 13). Arrows indicate the time of each drug addition. Error bars represent standard error of the mean.

**Figure 3. Identification of ADPR cyclase activity in Arabidopsis.**

A. Time-course of ADPR cyclase activity in soluble protein extracts of leaves of Col-0 or plants transformed with 35S:Aplysia californica ADPR cyclase (35S:Ac ADPR cyclase). All components except substrate were added to the cuvette, then 200 µM NGD was added at 10 minutes and fluorescence intensity was measured for another 10 minutes. B. ADPR cyclase activity of extracts of Col-0 leaves in the presence of NAD. NAD reduced the activity in a concentration dependent manner. C. ADPR cyclase activity of Col-0 leaf extracts measured using NGD as a substrate (200 µM) in the presence of cADPR. D. The estimated ADPR cyclase activity in extracts of Col-0 based on the cyclisation of NGD in the absence or presence of 200 µM NGD, 200 µM NAD as an alternative substrate and when enzymatic activity had been inhibited by boiling for 10 minutes. E. The estimated ADPR cyclase activity in extracts of Col-0...
based on the cyclisation of NHD in the absence or presence of 200 µM NHD, 200 µM NAD as an alternative substrate and when enzymatic activity had been inhibited by boiling for 10 minutes. F. The activity of ADPR cyclase in the extracts of Col-0 and 35S:Ac ADPR cyclase plants calculated from a standard curve derived from Aplysia ADPR cyclase (Supp. Fig.1). Fluorescence is arbitrary units. Data are presented as the mean of three biological replicates of three independent experiments and error bars represent standard error of the mean.

Figure 4. ADPR cyclase activity in response to NO. A. ADPR cyclase activity in the soluble protein extract of SNAP (300 µM)-treated Col-0 plants. B. The effect of 300 µM SNAP on ADPR cyclase activity of the protein extracted from untreated Col-0 plants. C. ADPR cyclase activity in soluble protein extracts of cml24-4 and cml23-2 cml24-4 plants. D. ADPR cyclase activity in ABA (50 µM)-treated protein extracts of untreated Col-0 plants. Soluble protein extracts were prepared from four to five week old plants and equal amounts of protein (145 µg) were used to measure the ADPR cyclase activity by NGD assay or NHD assay. Data are presented as the mean of three biological replicates of three independent experiments and error bars represent standard error of the mean.

Figure 5. SNAP triggers [cADPR] accumulation in Arabidopsis. Three week old Arabidopsis seedlings grown in 12 h light/12 h dark were treated with 300 µM SNAP or methanol control (0.5% MeOH) by flooding the plates for 1 min. Each plate contained an average of 20 seedlings and all of them were harvested in each time point. Three independent replicates were harvested at the beginning of the time-course and 5 min, 10 min, 30 min and 60 min after drug treatment. [cADPR] was estimated by a coupled assay, each sample was measured at least twice. Error bars represent standard error of the mean.

LITERATURE CITED


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12 day old aequorin-expressing individual seedlings were incubated for 2 hours with nicotinamide (50 mM). Cold water (4 °C), 10 mM H$_2$O$_2$, 150 mM NaCl and 300 µM SNAP (NO donor) were added at 15 s and luminescence was measured for 896 s in a luminometer or multifunctional microplate reader. Data presented as the mean of 12 biological replicates from three independent experiments (n=12) and error bars represent standard error of the mean. Arrows indicate the time of stimulation.
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S1. Preparation of *Aplysia* ADPR cyclase standard curve. **A.** Changes in fluorescence intensity due to formation of cGDPR at different concentrations of NGD (0-200 µM NGD) with 12 units of *Aplysia* ADPR cyclase. **B.** Changes in fluorescence intensity at different units of *Aplysia* ADPR cyclase (0.5-12 units) with 200 µM of NGD. **C.** Standard curve of *Aplysia* ADPR cyclase for NGD assay. **D.** Standard curve of *Aplysia* ADPR cyclase for NHD assay prepared in a similar way. **E.** The ADPR cyclase activity (fluorescence unit/min) in the total protein extracts of Col-0 plants. For preparing standard curve, 200 µM NGD or 200 µM NHD was added to different units of *Aplysia* ADPR cyclase (0.5-12 units for NGD, 1-20 units for NHD) in solution A (pH 7.2, reaction volume 1.2 ml) and fluorescent intensity was measured for every single minute up to 10-15 min at 25 °C for each unit of *Aplysia* ADPR.
cyclase using luminescence spectrometer (Perkin Elmer LS 55, UK). The enzyme activity (fluorescent unit per min) was plotted against the amount of ADPR cyclase (expressed as unit per 1.2 ml of reaction volume). The linear regression was used to calculate plant endogenous ADPR cyclase activity. Data are presented as the mean of 3 biological replicates of 3 individual experiments and error bars represent standard error of the mean.

S2. ADPR cyclase activity in response to nicotinamide. Nicotinamide inhibits ADPR cyclase activity in a concentration dependent manner. 0-50 mM of nicotinamide was added to 145 µg total protein extracts of SNAP-treated Col-0 plants before measuring the activity. Data are presented as the mean of three biological replicates of three individual experiments and error bars represent standard error of the mean.