NO-Mediated $[\text{Ca}^{2+}]_{\text{cyt}}$ Increases Depend on ADP-Ribosyl Cyclase Activity in Arabidopsis

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Cyclic ADP ribose (cADPR) is a $\text{Ca}^{2+}$-mobilizing intracellular second messenger synthesized from NAD by ADP-ribose cyclases (ADPR cyclases). In animals, cADPR targets the ryanodine receptor present in the sarcoplasmic/endoplasmic reticulum to promote $\text{Ca}^{2+}$ release from intracellular stores to increase the concentration of cytosolic free $\text{Ca}^{2+}$ in Arabidopsis (Arabidopsis thaliana), and cADPR has been proposed to play a central role in signal transduction pathways evoked by the drought and stress hormone, abscisic acid, 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 that a 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 $\text{Ca}^{2+}$ signaling. We adapted two different fluorescence-based assays to measure ADPR cyclase activity in Arabidopsis and found that this activity has the characteristics of a nucleotide cyclase that is activated by nitric oxide to increase cADPR and mobilize $\text{Ca}^{2+}$.

Cyclic ADP ribose (cADPR) is a signaling molecule that can evoke increases in the concentration of cytosolic free $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_{\text{cyt}}$) in plant and animal cells (Hetherington and Brownlee, 2004; Zhang and Li, 2006). In animals, cADPR is synthesized by a class of NADases called the ADP-ribose cyclases (ADPR cyclases). Metabolites of ADPR cyclase, including ADP-ribose (ADPR), cADPR, and nicotinic acid adenine dinucleotide phosphate, are all signaling molecules involved in $\text{Ca}^{2+}$ signaling (Lee, 2001, 2006; Guse and Lee, 2008). In animals, both ADPR and cADPR stimulate $\text{Ca}^{2+}$ influx through plasma membrane transient receptor potential channels (Perraud et al., 2001; Sano et al., 2001; Kraft et al., 2004). cADPR also mobilizes $\text{Ca}^{2+}$ from the endoplasmic reticulum (ER) through an inositol 1,4,5-trisphosphate-independent mechanism (Galione et al., 1991; Lee and Aarhus, 1991, 1993; Galione, 1993, 1994; Lee, 1993), which most likely involves the modulation of ryanodine receptors (Li et al., 2001; Ozawa, 2001; Thomas et al., 2001). Nicotinic acid adenine dinucleotide phosphate mobilizes intracellular $\text{Ca}^{2+}$ 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 ryanodine receptor has been identified in genomic databases, even though ADPR cyclase activity and cADPR-evoked $\text{Ca}^{2+}$ release from vacuoles and ER have been reported (Allen et al., 1995; Muir and Sanders, 1997; Leckie et al., 1998; Navazio et al., 2000; Sánchez et al., 2004). cADPR injected into guard cells causes stomatal closure (Leckie et al., 1998), and cADPR has been proposed to be involved in abscisic acid (ABA)-induced stomatal closure because 8-NH$_2$-cADPR, a competitor of cADPR signaling, and nicotinamide, an inhibitor of ADPR cyclase activity, both reduced ABA-induced stomatal closure (Leckie et al., 1998). The role of cADPR in ABA signaling also is supported by a statistically significant intersection between the sets of transcripts induced by ABA and cADPR (Sánchez 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 (Arabidopsis thaliana) circadian oscillator (Dodd et al., 2007). The lack of orthologs for ADPR cyclase and RYR, and the limited characterization of their activities, have led to uncertainty concerning whether plants have a bona fide ADPR cyclase activity associated with Ca²⁺ signaling (Dodd et al., 2007). 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 Ca²⁺ in plant signaling networks; specifically, we investigated if the enzyme activity was correlated with stimulus-induced increases in cADPR and also [Ca²⁺]cyt. We investigated the potential role of ADPR cyclase activity in nitric oxide (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 and Li, 2006) and pharmacology suggests that NO-mediated increases in [Ca²⁺]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 Signaling Pathways in Arabidopsis
To investigate potential roles for cADPR in signaling in Arabidopsis, we investigated the effects of an antagonist of cADPR signaling on stimulus-induced increases of [Ca²⁺]cyt in response to cold, NaCl, hydrogen peroxide (H₂O₂), and NO. 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 analog compound chemistry, and we previously demonstrated dose-dependent inhibition of Arabidopsis ADPR cyclase activity by nicotinamide (Dodd et al., 2007). Nicotinamide also inhibits other NADases, including poly-ADP ribose polymerases and SIRTUINS, through the same product inhibition; however, neither of those enzymes has a known role in Ca²⁺ signaling, so an effect of nicotinamide on stimulus-induced [Ca²⁺]cyt increases is indicative of ADPR cyclase activity (Galione, 1994). Cold treatment induced a transient increase of [Ca²⁺]cyt in Arabidopsis that reached a peak of 440 ± 60 nM (mean ± SE; Fig. 1A), almost 3 times higher than the touch response evoked by room temperature water (152 ± 9 nM; Fig. 1A). In the presence of 50 mM nicotinamide, the cold-induced increase in [Ca²⁺]cyt was slightly smaller, with the highest [Ca²⁺]cyt peak of 358 ± 72 nM (Fig. 1A). A transient increase of [Ca²⁺]cyt was detected in response to 10 mM H₂O₂ (peak [Ca²⁺]cyt of 673 ± 45 nM; Fig. 1B). Preincubation with nicotinamide (50 mM) for 2 h reduced and slightly delayed the H₂O₂-induced [Ca²⁺]cyt increase (peak [Ca²⁺]cyt of 429 ± 20 nM; Fig. 1B). NaCl at 150 mM induced a large, rapid increase in [Ca²⁺]cyt to a peak of 981 ± 229 nM (Fig. 1C), which was higher than cold water- and H₂O₂-mediated [Ca²⁺]cyt responses. A partial reduction of the NaCl-induced [Ca²⁺]cyt response was found when plants were incubated with nicotinamide.
Cyclase Dependent

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only to 139.1 [Ca\textsuperscript{2+}] cyt levels from 215.8

nicotinamide 300 s after the addition of SNAP reduced
cPTIO 300 s after the addition of 150

increases that reached a plateau at 131

by nicotinamide was suggestive of a role for ADPR cy-

[Ca\textsuperscript{2+}] cyt Increases Induced by NO Are ADPR

m

m

neither cold water, H\textsubscript{2}O\textsubscript{2}, nor NaCl. The peak for
SNAP-mediated [Ca\textsuperscript{2+}] cyt increase was 368 ± 18 nm (Fig.

1D), which was achieved 160 s after SNAP treatment,

with the rapid responses to cold water, H\textsubscript{2}O\textsubscript{2},

and NaCl, in which the peak of [Ca\textsuperscript{2+}] cyt was

within 15 to 30 s. Nicotinamide (50 mM) completely abol-
ished SNAP-induced [Ca\textsuperscript{2+}] cyt increases (peak [Ca\textsuperscript{2+}] cyt of

123 ± 4 nm; Fig. 1D).

[Ca\textsuperscript{2+}] cyt Increases Induced by NO Are ADPR
Cyclase Dependent

The inhibition of SNAP-induced increases in [Ca\textsuperscript{2+}] cyt
by nicotinamide was suggestive of a role for ADPR cy-
class in the elevation of [Ca\textsuperscript{2+}] cyt by NO. We performed a
further set of experiments to confirm the effects of
SNAP were linked to NO production and not to an
unintended side effect. First, we tested the effect of an
alternative NO donor, sodium nitroprusside (SNP; Neill
et al., 2002). At 5 \mu M, SNP induced sustained [Ca\textsuperscript{2+}] cyt
increases that reached a plateau at 131 ± 6.6 nm (Fig. 2A).

Increasing the concentration of SNP to 50 or 500 \mu M SNP
had no further effect on [Ca\textsuperscript{2+}] cyt, possibly because the
experiment was performed in the dark, which limits the
effectiveness of SNP (Rico-Lemus and Rodriguez-Garay,
2014). At 150 \mu M, SNP induced sustained increases in
[Ca\textsuperscript{2+}] cyt for 4 to 5 min until it reached a plateau at 195.1 ±

11.4 nm (Fig. 2B). Elevation of [Ca\textsuperscript{2+}] cyt by these two donors
suggested that 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 \mu M
cPTIO 300 s after the addition of 150 \mu M SNP decreased the
[Ca\textsuperscript{2+}] cyt levels from the elevated value of 192.2 ±

13.6 nm to 115.3 ± 7.6 nm (Fig. 2C). Preincubation with
300 \mu M cPTIO decreased the [Ca\textsuperscript{2+}] cyt increase evoked by
150 \mu M SNP to 145.3 ± 13.3 nm (Student’s t test against
150 \mu M SNP without 300 \mu M cPTIO, P < 0.01; Fig. 2D).

Nicotinamide was equally effective in inhibiting NO-
mediated increases in [Ca\textsuperscript{2+}] cyt if added before or after
the NO donor SNAP (Fig. 2, E and F). Addition of 50 mM
nicotinamide 300 s after the addition of SNAP reduced the
[Ca\textsuperscript{2+}] cyt levels from 215.8 ± 11.7 nm to 121.6 ± 5.6 nm;
however, there was a long delay of over 60 s after the ad-
dition of nicotinamide before [Ca\textsuperscript{2+}] cyt decreased (Fig. 2E).

This is supportive of the proposed role of nicotinamide
in inhibiting the production of cADPR and possibly
contribution to cADPR degradation by reversing the
catalytic activity of ADPR cyclase to one of ADPR ca-
taly sis. SNAP addition after a prolonged incubation with
nicotinamide resulted in a residual increase in [Ca\textsuperscript{2+}] cyt
only to 139.1 ± 19.1 nm (Fig. 2F), demonstrating that
the NO-induced increase in [Ca\textsuperscript{2+}] 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). Preincubation for
300 s with GdCl\textsubscript{3} (the most effective blocker of Arabidopsis
plasma membrane Ca\textsuperscript{2+} influx channels; Demidchik et al.,
2002) at 1 mM, 10 times higher than required to inhibit NaCl-
induced increases in [Ca\textsuperscript{2+}] cyt in the same assay (Tracy et al.,
2008), did not reduce the [Ca\textsuperscript{2+}] cyt increase induced by
150 \mu M SNAP, which peaked at 198.7 ± 17.2 nm (Student’s t test against
150 \mu M SNAP without 1 mM GdCl\textsubscript{3}, P = 0.81; Fig. 2H), suggesting that plasma membrane
influx of Ca\textsuperscript{2+} might not contribute to the response.

Nicotinamide Guanine Dinucleotide- and Nicotinamide
Hypoxanthine Dinucleotide-Based Fluorescence
Spectroscopy Assays of Arabidopsis ADPR
Cyclase Activity

The pharmacological manipulation of [Ca\textsuperscript{2+}] cyt is
strongly indicative of a nicotinamide-sensitive compo-
nent being required for NO-induced increases in [Ca\textsuperscript{2+}] 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 conver-
sion of nonfluorescent nucleotide analogs of NAD into
fluorescent cyclic nucleotides. Soluble total protein
extracts of Arabidopsis have an enzymatic activity capa-
bles of converting the nonfluorescent NAD analog, nicotina-
mide guanine dinucleotide (NGD), to the fluorescent cy-
clic GDP-ribose (cGDRP; Fig. 3A). The synthesis of
the cGDRP was dependent on the presence of NGD in both
ecotype Columbia-0 (Col-0) and plants heterologously
expressing ADPR cyclase from the sea slug A. californica
(355:Ac ADPR cyclase; Dodd et al., 2007). Furthermore,
the rate of fluorescence increase was higher in protein
extracted from the 355:Ac ADPR cyclase plants (Fig. 3A).

The conversion of NGD to cGDRP was inhibited by
NAD. In our assay, equal concentrations of NGD and
NAD reduced the activity to 0.5-fold; however, an ex-
cess of NAD completely abolished the cyclization of
NGD (Fig. 3B). While it is possible that NAD acts as a
noncompetitive inhibitor, the reduction of the conver-
sion of NGD to cGDRP by NAD is an expected char-
acteristic of a nucleotide cyclase activity that favors
NAD as a substrate to generate cADPR as a product.
Animal ADPR cyclase is reversible under standard con-
ditions, so we tested whether the inclusion of cADPR in
the assay would inhibit the production of cGDRP from
NGD. Addition of 25 \mu M cADPR reduced ADPR cyclase
activity significantly (P < 0.001); however, higher con-
centrations of cADPR (up to 75 \mu M) 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 find-
ings, we considered the fluorescence intensity increase to be
representative of a bona fide ADPR cyclase activity. To
determine the specific activity, we used commercial
A. californica ADPR cyclase to generate a standard curve
(Supplemental Fig. S1). This enabled us to estimate the
specific activity in extracts of unstimulated Arabidopsis
Col-0 to be around 0.01 to 0.015 units \mu g\textsuperscript{-1} total protein
min\textsuperscript{-1} or units \mu g\textsuperscript{-1} protein min\textsuperscript{-1} (Fig. 3). An alternative
assay based on the conversion of nicotinamide hypoxanthine dinucleotide (NHD) to cyclic inosine diphosphoribose (Graeff et al., 1996) resulted in a very similar estimate of Col-0 ADPR cyclase activity (Fig. 3E), while, as expected, 35S:Ac ADPR cyclase plants had significantly higher ADPR cyclase activity of 0.027 ± 0.0008 units μg⁻¹ protein 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 substrate (P ≤ 0.001), and the NO scavenger cPTIO significantly reduced the effect of SNAP on extractable ADPR cyclase activity (Fig. 4, A and B). 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-like24-4 allele, which results in constitutively high NO (Tsai et al., 2007). Both cml24-4 and cml23-3 cml24-4 plants had significantly higher extractable

Figure 2. NO evokes short-term [Ca²⁺]cyt increases. A, SNAP was added at 60 and 360 s (n = 5 for each treatment) after the start of the experiment, and [Ca²⁺]cyt levels were measured for 600 s. B, SNAP was 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 [Ca²⁺]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 at 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 at 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 GdCl₃ before the start of the experiment. SNAP at 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 SE.
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); 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 units mg⁻¹ total protein 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 (Supplemental Fig. S2), consistent with the effect of nicotinamide on NO-induced increases in [Ca²⁺]cyt (Figs. 1D and 2, E and F).

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

**DISCUSSION**

**NO Increases [Ca²⁺]cyt through a Pathway That Includes the 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 Ca\(^{2+}\) agonist. These data and the insensitivity of NO-mediated increases in \(\left[Ca^{2+}\right]_{cyt}\) to GdCl\(_3\), an inhibitor of the plasma membrane-mediated influx of Ca\(^{2+}\), lead us to conclude that the primary pathway by which NO increases \(\left[Ca^{2+}\right]_{cyt}\) in Arabidopsis is through cADPR-mediated Ca\(^{2+}\) release from the ER and/or the vacuole, dependent on the activity of ADPR cyclase.

The conservation of the regulation of ADPR cyclase activity by NO between plants and animals could suggest a common ancestry for the pathway; alternatively, this might be an example of the convergent evolution of signaling in the plant and animal lineages. However, the lack of obvious orthologs 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 the identification of both NO and cml24-4 mutants as activators of ADPR cyclase, provide a tool set that might aid in the isolation of the ADPR cyclase protein and the identification of the corresponding gene. This might provide information concerning potential evolutionary features of cADPR-dependent NO-induced increases in \(\left[Ca^{2+}\right]_{cyt}\). 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 signaling contributes to cold-, touch-, and H\(_2\)O\(_2\)-induced increases in \(\left[Ca^{2+}\right]_{cyt}\). Cold-induced increases in \(\left[Ca^{2+}\right]_{cyt}\) are due to influx across the plasma membrane and efflux of Ca\(^{2+}\) from the vacuole (Knight et al., 1996), apparently through a cADPR-independent route. The analysis of H\(_2\)O\(_2\)-mediated \(\left[Ca^{2+}\right]_{cyt}\) signals revealed that the initial increase of \(\left[Ca^{2+}\right]_{cyt}\) was partially suppressed by 50 mM nicotinamide (Fig. 1B). However, this effect was much...
less than observed for NO, and we conclude that 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 the activation of hyperpolarization-activated \(\text{Ca}^{2+}\)-permeable channels in the plasma membrane (Pei et al., 2000; Rentel and 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 (Tracy et al., 2008), it appears that NaCl-induced increases involve both influx across the plasma membrane \(\text{Ca}^{2+}\) influx (Tracy et al., 2008), it appears that NaCl-induced increases involve both influx across the plasma membrane and cADPR-mediated \(\text{Ca}^{2+}\) release from the 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 (Marti et al., 2013), which is a result of spatial heterogeneity (Tracy et al., 2008) and cell-specific dynamics (Marti et al., 2013).

**NO Modulates Short-Term \(\text{Ca}^{2+}\) Responses in Arabidopsis**

\(\text{cADPR}\) previously has been suggested to be involved in the NO signaling pathway in plants (Garcia-Mata et al., 2003; Lamotte et al., 2006; Zhang and Li, 2006), but measurements of NO regulation of ADPR cyclase activity and \([\text{cADPR}]\) have not been reported. By measuring ADPR cyclase and \(\text{cADPR}\) levels, it has been possible to observe that the elevation of \(\text{cADPR}\) in response to NO is transitory (Fig. 5) and that \([\text{Ca}^{2+}]_{\text{cyt}}\) returns rapidly to resting in the absence of \(\text{cADPR}\) synthesis (Fig. 2E). We conclude that ADPR-dependent NO-regulated \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling is most likely 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 \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling are involved in the photoperiodic regulation of flowering, they are likely to be very early in the signaling cascade.

It is not known how NO regulates ADPR cyclase activity, but we have shown this to occur in a cell-free extract: 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, 1994). There are many possible sources of NO in plants, including enzymatic and nonenzymatic (Bethke et al., 2004; Crawford, 2006). One of the most established sources of NO in plants is nitrate reductase, which usually converts NO\(^2\) into NO\(^\cdot\) but also may convert NO\(^-\) into NO in anaerobic conditions and when NO\(^2\) levels are high (Yamasaki et al., 1999; Rockel et al., 2002; Meyer et al., 2003; 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 cm124-4 mutants, are capable of increasing ADPR cyclase activity. Our identification of NO-regulated ADPR cyclase activity fills a gap in the NO signal transduction chain. There are likely to be additional regulators of ADPR cyclase activity, because the NO-induced \(\text{cADPR}\) increase was transient, contrasting with ABA-evoked \(\text{cADPR}\) increases, which were sustained for at least 1 h (Sánchez et al., 2004).

**MATERIALS AND METHODS**

**Plant Material, Growth Conditions, and Measurement of \([\text{Ca}^{2+}]_{\text{cyt}}\)**

Experiments were performed with Arabidopsis (Arabidopsis thaliana) ecotype Col-0, except where stated. Seeds were grown and \([\text{Ca}^{2+}]_{\text{cyt}}\) was measured using aequorin in plants carrying CaMV35S:APAOEQLORIN as described by Marti et al. (2013). Seeds were sown in petri dishes and stratified in the dark at 4°C for 2 to 3 d. Petri dishes were then transferred to a growth cabinet (12 h of light/12 h of dark, 20°C, 50–60 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) irradiance) for 7 to 12 d for \([\text{Ca}^{2+}]_{\text{cyt}}\) measurement, 3 weeks for measuring \(\text{cADPR}\), and 4 to 5 weeks for ADPR cyclase activity measurement.

**Measurement of ADPR Cyclase Activity in Protein Extracts**

**Preparation of Soluble Protein Extracts**

Five to 10 g of whole rosette tissue, excluding roots, of 4- to 5-week-old Arabidopsis plants was homogenized using a pestle and mortar at 4°C in solution A (340 mM Glc [Fisher Scientific], 20 mM HEPES [Sigma], 1 mM MgCl\(_2\) [BDH Laboratory Supplies], 50 g mL\(^{-1}\) soybean trypsin inhibitor [Sigma], 10 \(\mu\)g mL\(^{-1}\) leupeptin [Sigma], and 10 \(\mu\)g mL\(^{-1}\) aprotinin [Sigma], pH 7.2, 3 mL g\(^{-1}\) fresh weight). The homogenate was filtered through two layers of Miracloth (Calbiochem), and the resulting filtrate was centrifuged at 2,000 \(\times\) g for 10 min at 4°C. The supernatant was transferred to a 15-mL falcon tube and centrifuged at 12,000 \(\times\) g for 15 min at 4°C (Beckman Coulter Avant J-26XP centrifuge). After centrifugation, the supernatant was collected carefully and run through the PD-10 desalting column (GE Healthcare) according to the manufacturer’s protocols.

**NGD/NHD Assays of ADPR Cyclase Activity Using a Luminescence Spectrometer**

A total of 145 \(\mu\)g of protein from Col-0 plants was taken in 1,200 \(\mu\)L of solution A (pH 7.2) in quartz cuvettes, and fluorescence intensity was measured for every minute up to 10 min at 21°C using a luminescence spectrometer (Perkin Elmer LS 55) set with the excitation wavelength at 300 nm and emission wavelength at 420 nm. After 10 min, 60 \(\mu\)L of 4 mM NGD (prepared in solution A, final concentration of 200 \(\mu\)M Sigma) was added to reactions, and the resultant fluorescence intensity was measured for another 10 to 15 min. Additionally, fluorescence intensity was measured for every minute up to 10 min at 21°C for 1,200 \(\mu\)L reactions in solution A (pH 7.2) containing 145 \(\mu\)g of total protein, 145 \(\mu\)g of total protein + 200 \(\mu\)M NAD (Sigma), 145 \(\mu\)g of total protein + 200 \(\mu\)M NAD and 145 \(\mu\)g of boiled protein (100°C, 10–15 min) + 200 \(\mu\)M NAD. For the NHD assay, 200 \(\mu\)M NHD (prepared in solution A, pH 7.2, Sigma) was used in place of NGD.

To test the effect of NO on ADPR cyclase activity in the protein extracts, 4–5-week-old Col-0, cm124-4, or cm123-2 cm124-4 plants were incubated in the presence or absence of 300 \(\mu\)M SNAP (Calbiochem) or 300 \(\mu\)M SNAP and 300 \(\mu\)M cPTIO (Sigma) for 40 to 50 min, separately. Alternatively, protein extracts of untreated Col-0 plants were incubated with 300 \(\mu\)M SNAP or 300 \(\mu\)M SNAP and 300 \(\mu\)M cPTIO for 40 to 50 min, separately. Total protein extract of Col-0 plants (4–5 weeks old) was incubated with 50 \(\mu\)M ABA for 1 h.

**Reverse Cyclase Assay for [cADPR] Measurement**

**cADPR Isolation and cADPR Purification**

Plants were dosed with 150 \(\mu\)M SNAP or 0.5% methanol by flooding for 1 min, after which all the liquid was taken out. Plants were harvested before
dosing (0 min) and 5, 10, 30, 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 of ice-chilled HPLC-water (about 4°C; Fisher Scientific). Protein quantification was performed on 25 μL of the sample by Bradford assay. In order to precipitate proteins, 25 μL of 7 μL perichloric acid (Sigma) was added to the samples and vortexed. One milliliter of ice-chilled 3:1 mix of 1,1,2-trichlorotrifluoroethane/n,N-cyclamylene (Sigma) was added to separate cADPR from the rest of the plant extract. The mixture was vortexed and kept on ice until the precipitation of perichloric acid. Samples were centrifuged at 4°C for 10 min at 1,500 g. After centrifugation, the samples might lead to cADPR losses, those steps also were performed in the 4°C; Fisher Scientific. 

5 min. cADPR levels were estimated using 2, 5, 10, 50, 100, and 500 nM cADPR standards. As the cADPR isolation and purification steps in the samples might lead to cADPR losses, those steps also were performed in the 4°C; Fisher Scientific. Samples were taken off, and 1M NaPO4 buffer (pH 8) was added to a 25 μL 10°C; Fisher Scientific. 

1M NaPO4 buffer (pH 8) was added to the samples and incubated overnight at 37°C. After incubation, the enzymes were separated from the extract with 3,000 molecular weight cutoff filters (500 μL; Millipore) and spun at 4°C for 30 min at 13,000g. The final extract was diluted 1:1 with 200 μL phosphate buffer to a final concentration of 100 μM NaPO4.

**Fluorescence-Based Cycling Assay**

The cycling assay is based on a cycle of enzymatic conversions (Graef and Lee, 2002). cADPR is first converted to NAD by ADP cyclase (EC 2.5.2.5; from the marine sponge Axinella polypoides; a gift from E. Zocchi and Dr. S. Bruzzone, Università di Genova) in the presence of high amounts of nicotinamide (Sigma). Next, alcohol dehydrogenase (EC 1.1.1.1; extracted from Saccharomyces cerevisiae; Sigma) converts ethanol and NAD into acetaldehyde and NADH. Finally, diaphorase (EC 1.8.1.4; extracted from Clostridium kluyveri; Sigma) converts NADH and resorcin (Sigma) into NAD and resorcinol, a fluorescent substance that can be detected by a multifunctional microplate reader (FluoStar OPTIMA; BMG LabTech). To each well of black 96-well plates, 100 μL of sample was added. First, 50 μL of assay reagent (30 μM nicotinamide and 0.3 μM CaCl2; isolated from porcine brain; Sigma) were added to the samples and incubated overnight at 37°C. After incubation, the enzymes were separated from the extract with 3,000 molecular weight cutoff filters (500 μL; Millipore) and spun at 4°C for 30 min at 13,000g. The final extract was diluted 1:1 with 200 μL phosphate buffer to a final concentration of 100 μM NaPO4.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Preparation of the *A. californica* standard curve.

**Supplemental Figure S2.** ADPR cyclase activity in response to nicotinamide.

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**LITERATURE CITED**


