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Short Communication

Pyridine nucleotides induce changes in cytosolic pools of calcium in Arabidopsis

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Abstract

NAD is a pyridine nucleotide that is involved in cell metabolism and signaling of plant growth and stress. Recently, we reported on the multifaceted nature of NAD-inducible immunity in Arabidopsis. We identified NAD as an integral regulator of multiple defense layers such as production of ROS, deposition of callose, stimulation of cell death and modulation of defense metabolism including the defense hormones SA, JA and ABA, and other defense-associated metabolites. Altogether, NAD-induced immune effects confer resistance to diverse pathogenic microbes. Our addendum to this work further demonstrates an impact of NAD on the cytosolic calcium pool, a well-known component of early plant defense response.

Keywords

NAD, calcium, reactive oxygen species, phytohormones, defense response

Abbreviations

ABA abscisic acid

ADPr ADP ribose

Arabidopsis Arabidopsis thaliana

H_2O_2 hydrogen peroxide

JA jasmonic acid

NAD(P) nicotinamide adenine dinucleotide (phosphate)

ROS reactive oxygen species

SA salicylic acid

TEXT

The redox cofactor NAD is important for plant metabolism and signaling during growth and stress. [1--3] Previously, we demonstrated that an inducible accumulation of NAD in Arabidopsis conferred resistance to the hemibiotrophic avirulent bacteria Pst-AvrRpm1 by modulating SA signaling at the transcripts and metabolite level.[4] Recently, we have detailed the NAD-inducible defense responses that are effective against biotrophic and necrotrophic pathogens.[5] Our study reveals that NAD^+ acts as an elicitor by stimulating basal defenses (i.e. ROS production, callose deposition, cell death) and influencing hormonal balance.[5] Metabolomics further demonstrated similarities between plants treated with NAD^+ and the fungal elicitor chitin.

Recent data suggest the involvement of a coordinated integration of ROS and calcium (Ca^{2+}) waves in plants under pathogen infections.[6] In addition, NAD and Ca^{2+} signaling potentially interact during stress responses.[3] In fact, NAD^+ and its phosphate derivative $NADP^+$ could trigger the release of intracellular Ca^{2+} considering its potential role as an ADP ribose (ADPr) donor for cyclic ADPr and nicotinic acid adenine dinucleotide phosphate (NAADP). Thus far, however, direct evidence of effects of NAD^+ on Ca^{2+} fluxes are missing.

To investigate the Ca^{2+}-related component of NAD signaling, we tested cytosolic Ca^{2+} concentrations (denoted as $[Ca^{2+}]_{cyt}$) in response to NAD^+ by luminometry using 2 weeks-old transgenic apoaequorin

Arabidopsis seedlings (pMAQ2).[7,8] Upon NAD^+ addition at physiological concentration (1 mM) [2,3], $[Ca^{2+}]_{cyt}$ transiently increased as compared to the control (water treatment) in plants incubated for 10 min, 20 min and 40-50 min (**Fig. 1A**). $[Ca^{2+}]_{cyt}$ then dropped after 60 min suggesting either a consumption to replenish non-cytoplasmic calcium pools or an important binding of cytoplasmic Ca^{2+}. This agrees with previous results showing an upregulation of genes associated with Ca^{2+} response and binding.[4] Hence, direct NAD^+ treatment is able to induce changes in $[Ca^{2+}]_{cyt}$. To get further insights into the specificity of the $[Ca^{2+}]_{cyt}$ response, we then conducted pharmacological treatments using pyridine nucleotides and their derivatives at 1 mM (**Fig. 1B**). We focused on the decrease in $[Ca^{2+}]_{cyt}$ observed after 60 min of incubation (**Fig. 1A**) to investigate the long term effect. Under our conditions, quinolinate (Q), nicotinate (NA), nicotinamide (NAM) and isonicotinate (INA) failed to substantially change $[Ca^{2+}]_{cyt}$ at 60 min after treatment compared to water application. Furthermore, application of SA did not change $[Ca^{2+}]_{cyt}$. Conversely, all pyridine nucleotides (NAD^+, $NADP^+$, NADH and NADPH) significantly decreased $[Ca^{2+}]_{cyt}$ at 60 min after treatment, with a more pronounced impact for $NADP^+$ (**Fig. 1B**). Changes in Ca^{2+} appeared to be independent of the reduction state of pyridine nucleotides (e.i. NAD^+ vs NADH, and $NADP^+$ vs NADPH), suggesting that reduced and oxidized pools similarly impact Ca^{2+} signaling under our conditions. This variation in $[Ca^{2+}]_{cyt}$ was as pronounced as the positive controls ADPr and H_2O_2 (**Fig. 1B**). Quite noticeably, increasing concentrations of NAD^+, $NADP^+$ and NADH led to dose-dependent changes in $[Ca^{2+}]_{cyt}$, with a more important effect of $NADP^+$ (**Fig. 1C**). Hence, luminometry assays indicate a dose-dependent and specific effect of pyridine nucleotides on Ca^{2+} cytosolic pools.

During defense responses, Ca^{2+}-signaling acts upstream of R gene mediated-hypersensitive cell death and ROS production associated with SAR regulation.[6,9] After infection with Pst-AvrRpm1 and upon NAD build-up, we detected enhanced cell death and ROS levels.[5] Furthermore, $[Ca^{2+}]_{cyt}$ levels regulate SA-, JA-, and ABA-mediated stress responses in plants.[6] In fact, cycam mutants affected in $[Ca^{2+}]_{cyt}$ showed increased levels of SA, ABA and bioactive JA (JA-isoleucine) with or without infection with the necrotrophic fungus Alternaria brassicae.[10] Whilst cytosolic Ca^{2+} peaks are potentiated by elicitors treatments such as the elicitor peptide PEP1,[11,12] AtPEP1 and Ca^{2+}-responsive genes are upregulated by higher NAD.[4] In addition, trapping of Ca^{2+}

by EGTA prevents the induction of Pathogen Related (PR) genes after treatment with exogenous NAD(P).[13] Hence, NAD and Ca^{2+}-mediated signal transductions are possibly related.[1,3] Here, we provide evidence for changes in $[Ca^{2+}]_{cyt}$ caused by pyridine nucleotides (**Fig. 1**). This is accompanied by the accumulation of SA, JA and ABA as has been shown in NAD^+-infiltrated leaves.[5] $[Ca^{2+}]_{cyt}$ response was dose-dependent, could be mimicked by the positive controls ADPr/H_2O_2 and was particularly intense upon $NADP^+$ application, thus suggesting its effect was explained by the donation of its ADPr moiety to form cyclic ADPr and NAADP so as to release Ca^{2+} from intracellular compartments.[1--3]

In conclusion, our study revealed that pyridine nucleotides modulated Ca^{2+} fluxes in Arabidopsis. This strengthens the paradigm of a multifaceted nature of NAD-inducible immunity. Still, the precise mechanisms by which pyridine nucleotides regulate Ca^{2+} signaling are open questions that require further investigations such as the analysis of earlier responses in Ca^{2+} fluxes and the use of inhibitors of internal stock calcium release.

Experimental measurements of $[Ca^{2+}]_{cyt}$ by luminometry

$[Ca^{2+}]_{cyt}$ were determined from aequorin-based luminescence of 2 week-old transgenic pMAQ2 Arabidopsis plantlets grown on 0.8% full strength MS medium.[7,8,14] Prior to luminometry measurements, pMAQ2 seedlings were incubated overnight in the dark at 21°C in 20 mL of distilled water supplemented with 10 µL of 5 mM coelenterazine to reconstitute functional aequorin. For each treatment and each time point, 16 biologically replicated pMAQ2 seedlings were incubated in the chemicals at mentioned concentrations and for the corresponding time (n = 16), then rinsed in distilled water. Relative luminescence units were then detected for each time point on a Berthold luminometer (Lumat LB9507, Germany) for 10 s, and at the end of each measurement, 1 mL of 2 M $CaCl_2$ (20% ethanol) was used to discharge excess of aequorin. $[Ca^{2+}]_{cyt}$ were calculated by applying the equation below, as already reported:[8]

pCa = 0.332588(-log k) + 5:5593,

where k = luminescence counts s^{-1}/total luminescence counts remaining.

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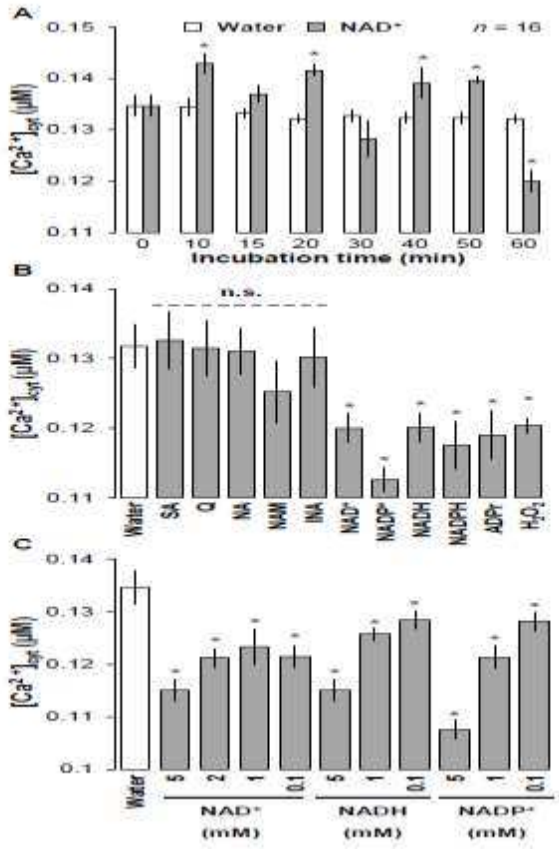

Figure 1. Calcium pools dynamically fluctuate after pyridine nucleotides treatment in a dose dependent manner. **A**, quantification of cytosolic calcium pools expressed in $[Ca^{2+}]_{cyt}$ (µM) after treatment with water or 1 mM NAD^+. For each time point, seedlings were incubated in water or NAD^+, rinsed and $[Ca^{2+}]_{cyt}$ was measured by luminometry. **B**, impact of pharmacological treatments on $[Ca^{2+}]_{cyt}$ after 60 min of incubation. **C**, dose dependent response of pyridine nucleotides treatments on $[Ca^{2+}]_{cyt}$ (60 min). Data are means of 16 seedlings ± SE. ADPr, ADP ribose; INA, isonicotinic acid; NA, nicotinic acid; NAM, nicotinamide; Q, quinolinic acid; SA, salicylic acid. Asterisks indicate statistically significant differences compared to the water treatment ($P < 0.05$, t-test). n.s., not statistically significant.