

# Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species

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**A strategy for elucidating specific molecular targets of calcium and calmodulin in plant defense responses has been developed. We have used a dominant-acting calmodulin mutant (VU-3, Lys to Arg115) to investigate the oxidative burst and nicotinamide co-enzyme fluxes after various stimuli (cellulase, harpin, incompatible bacteria, osmotic and mechanical) that elicit plant defense responses in transgenic tobacco cell cultures. VU-3 calmodulin differs from endogenous plant calmodulin in that it cannot be methylated post-translationally, and as a result it hyperactivates calmodulin-dependent NAD kinase. Cells expressing VU-3 calmodulin exhibited a stronger active oxygen burst that occurred more rapidly than in normal control cells challenged with the same stimuli. Increases in NADPH level were also greater in VU-3 cells and coincided both in timing and magnitude with development of the active oxygen species (AOS) burst. These data show that calmodulin is a target of calcium fluxes in response to elicitor or environmental stress, and provide the first evidence that plant NAD kinase may be a downstream target which potentiates AOS production by altering NAD(H)/NADP(H) homeostasis.**

**Keywords:** active oxygen/calmodulin/elicitor/nicotinamide co-enzymes/tobacco

## Introduction

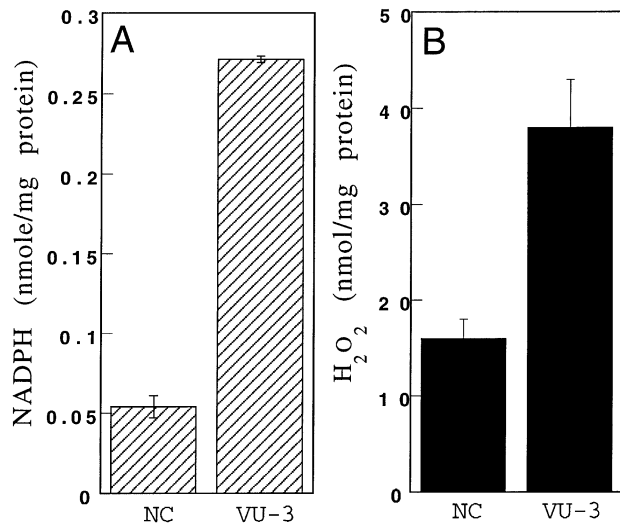
Part of plant defense responses to pathogen and environmental stresses is an oxidative burst reaction that generates active oxygen species (AOS) such as H<sub>2</sub>O<sub>2</sub> and superoxide (reviewed by Mehdy, 1994; Low and Merida, 1996). AOS production is proposed to be involved in several responses to these stimuli such as cell wall lignification (Halliwell, 1978), cross-linking of cell wall proteins (Bradley *et al.*, 1992; Brisson *et al.*, 1994) and the expression of defense response genes (reviewed by Lamb, 1994), and has been proposed to be a key signal co-ordinating these various responses (Chen *et al.*, 1993; Levine *et al.*, 1994; Price *et al.*, 1994). Several studies have implicated calcium fluxes in plant defense signaling pathways (Nürnbergger *et al.*, 1994; reviewed in Bush, 1993; Ebel and Cosio, 1994; Mehdy, 1994). Intracellular calcium reporters show that cytoplasmic free calcium increases following mechanical or elicitor stimulation (Knight *et al.*, 1991, 1992;

Messiaen *et al.*, 1993). Chelation of extracellular calcium, which would presumably quench intracellular calcium increases, also inhibits AOS (Schwake and Hager, 1992) and phytoalexin production (Stäb and Ebel, 1987; Bach *et al.*, 1993). In addition, inhibitor studies suggest that elicitors induce phospholipase C-mediated release of calcium from intracellular stores (Legendre *et al.*, 1993).

Although calcium is implicated in plant defense responses, the molecular targets of calcium action and how they influence downstream events are not well defined. In particular, little is known concerning the calcium receptor targets (e.g. calcium-modulated proteins such as calmodulin) and mechanisms of how these calcium regulatory proteins potentiate these responses. Approaches utilizing pharmacological reagents that inhibit calmodulin action *in vitro* have been used to attempt to demonstrate calmodulin involvement (Kurosaki *et al.*, 1987; Miura *et al.*, 1995). Although interesting effects have been observed with these reagents, they can also influence cellular processes not related to calcium and/or calmodulin signaling (reviewed in Roberts *et al.*, 1986a). Thus, whether calmodulin is a target for plant defense signaling and the specific calmodulin-dependent enzymes that are involved remains an open question.

It is becoming apparent that similarities exist between the oxidative burst reactions in animal and plant defense responses. AOS release in animal cells is preceded by the assembly and activation of a plasma membrane NADPH oxidase (reviewed in Cross and Jones, 1991; Heinecke and Shapiro, 1992; Henderson and Chappell, 1996). Although less defined, similar oxidase activities have been detected in plants (reviewed in Cross and Jones, 1991; Rubenstein and Luster, 1993; Mehdy, 1994; Low and Merida, 1996), and are elevated in response to stress and elicitor treatment (Doke, 1985; Auh and Murphy, 1995). Further, antibodies against the neutrophil oxidase subunits cross-react with similar proteins in plant extracts (Levine *et al.*, 1994; Dwyer *et al.*, 1996). AOS-producing neutrophils and sea urchin eggs also contain a calmodulin-dependent NAD kinase (Epel *et al.*, 1981; Williams and Jones, 1985) which catalyzes the conversion of NAD to NADP, and is proposed to contribute to the supply of NADPH for the oxidase. Calcium- and calmodulin-dependent NAD kinase is widely distributed in plants (reviewed in Roberts and Harmon, 1992), but its biological function has remained obscure. Its potential role during the oxidative burst response in plants has not yet been evaluated.

We report here that AOS levels are enhanced in transgenic plants that express a foreign, synthetic gene-derived calmodulin that hyperactivates NAD kinase. By using cell cultures derived from these plants, we further report that they exhibit a higher AOS burst reaction as well as an enhanced burst of NADPH production in response to



**Fig. 1.** NADPH and AOS levels in leaf tissue of transgenic tobacco plants. Fully expanded, upper leaves from 6-month-old tobacco plants were analyzed for (A) NADPH and (B) H<sub>2</sub>O<sub>2</sub> as described in Materials and methods. Histograms represent the mean of four independent determinations with error bars showing the standard error of the mean.

several environmental and elicitor stimuli, providing initial evidence that NAD kinase may play a metabolic role in plant defense.

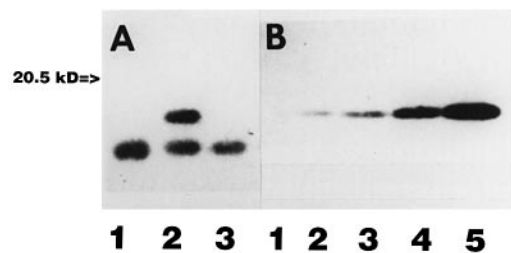
## Results

### **NADPH and AOS levels are elevated in transgenic tobacco expressing VU-3 calmodulin**

In previous work, we generated transgenic tobacco plants that express calmodulin derived from a synthetic gene (Roberts *et al.*, 1985) under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Roberts *et al.*, 1992). One set of transgenic plants was generated that expresses a calmodulin mutant (VU-3) with a lysine to arginine 115 substitution. This calmodulin is normal in all respects except that it is not able to be trimethylated post-translationally at Lys115 (Roberts *et al.*, 1992). Trimethylation attenuates the ability of calmodulin to activate calmodulin-dependent NAD kinase *in vitro* (Roberts *et al.*, 1986b). As a result, VU-3 calmodulin, which is incapable of methylation, hyperactivates NAD kinase (Roberts *et al.*, 1986b). Thus, the introduction of VU-3 calmodulin into plant tissues circumvents potential control by methylation and may result in a hyperactivation *in vivo* of NAD kinase.

Consistent with this proposal, analyses of leaf tissues of transgenic VU-3 plants showed 4-fold higher levels of NADPH than normal controls (Figure 1A), whereas the sum total of all nicotinamide co-enzymes did not differ substantially between the plant samples (data not shown). This argues that the changes are due to a shift in nicotinamide co-enzyme levels through the greater production of NADPH and could reflect a higher endogenous activation of NAD kinase.

Further analysis of the leaves of the transgenic plants (Figure 1B) show that H<sub>2</sub>O<sub>2</sub> levels were at least 2-fold higher in leaf tissue of VU-3 transgenic plants compared with control tobacco plants (Figure 1B). Thus, in addition to elevated NADPH, VU-3 plants show elevated H<sub>2</sub>O<sub>2</sub>



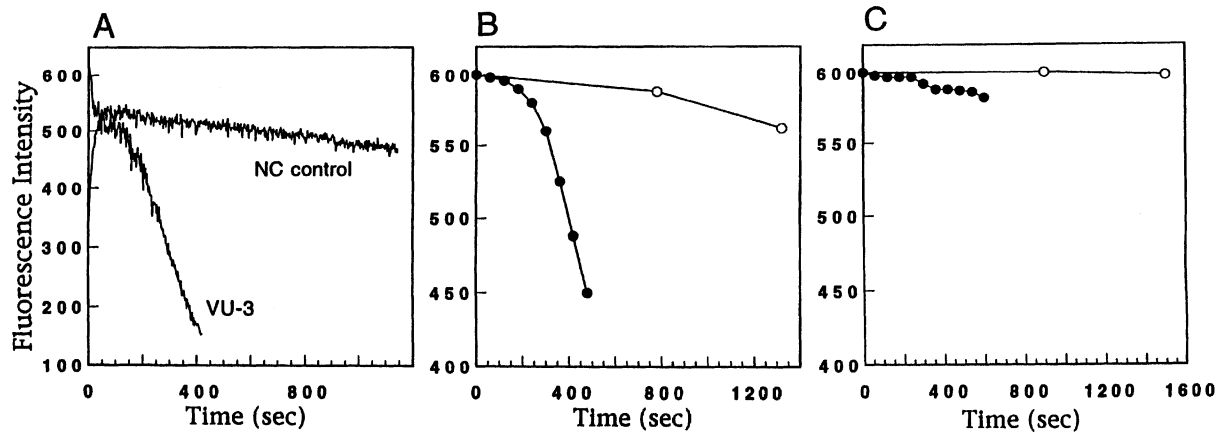
**Fig. 2.** Western blot analysis of steady-state calmodulin levels in suspension cell cultures. Extracts (10 µg protein/lane) were resolved by SDS-PAGE on 15% (w/v) polyacrylamide gels (Laemmli, 1970) in the presence of 1 mM EDTA and transferred (150 mA for 12 h) onto PVDF membranes. Western blot analysis was done with anti-calmodulin antibodies by using a chemiluminescent protocol as described in Materials and methods. (A) Extracts of 3-day-old suspension cell cultures of tobacco from NC cells, (lanes 1 and 3) and VU-3 cells, (lane 2). Each lane was loaded with 10 µg of soluble extract protein. VU-3 calmodulin shows slower mobility than the endogenous tobacco calmodulin (Roberts *et al.*, 1992). (B) Standard quantities of purified VU-1 calmodulin: (0.2, 0.4, 0.8, 1.2 and 1.6 pmol in lanes 1–5, respectively), to generate a standard curve.

levels. Because of the importance of AOS production in plant defense responses and the proposed role of calcium as a signal in mediating these responses, we investigated further changes in AOS and nicotinamide co-enzymes in response to treatments that elicit AOS production.

### **Generation of cell suspension cultures of transgenic tobacco lines**

To allow facile manipulation and rapid detection of responses to various stimuli, stable cell suspension cultures were established from various transgenic and control tobacco lines. Cell cultures were generated from several transgenic lines containing either the VU-3 calmodulin transgene (Roberts *et al.*, 1992) or transformed with a negative control (NC) construct (Zhang and Roberts, 1995). To eliminate the possibility of positional effects, cell suspensions generated from different VU-3 plant lines were evaluated in this study. Similar phenotypes were observed, and for the sake of convenience the data below are presented from results of representative VU-3 and NC cell lines. Both cell lines showed similar growth kinetics, which were essentially indistinguishable from the growth rates of cultures of untransformed tobacco (data not shown).

The expression levels of foreign VU-3 and endogenous tobacco calmodulins were investigated by Western blot analysis (Figure 2). VU-3 calmodulin possesses slight differences in sequence that result in differential mobility on SDS-PAGE compared with tobacco calmodulin (Roberts *et al.*, 1992). Thus, its expression can be assessed separately and compared with endogenous tobacco calmodulin. Consistent with this, VU-3 cell lines showed a second immunoreactive band characteristic of the foreign calmodulin, whereas NC control cells showed only one band characteristic of tobacco calmodulin (Figure 2A). Quantitation showed that total calmodulin levels (the sum of endogenous and VU-3 calmodulin) in VU-3 cells than in NC cells (200 pmol/mg protein) in VU-3 cells than in NC cells (100 pmol/mg protein). Thus, expression levels in the suspension cultures are elevated to an extent similar to that found with VU-3 transgenic plants (Roberts *et al.*, 1992).



**Fig. 3.** AOS production rates of transgenic tobacco cell lines in response to mechanical stimulation by rapid stirring. (A) Production of  $H_2O_2$  by 3-day-old VU-3 or NC cells was monitored continuously in a fluorimeter cuvette as described in Materials and methods. Pyranine was added ( $3 \mu\text{g}/\text{ml}$  suspension aliquot) at time 0, and the decrease in fluorescence intensity was monitored. Cells were suspended in the cuvette by a stir bar revolving at 150–200 r.p.m.. (B) Production of  $H_2O_2$  by unstirred VU-3 cells in flasks ( $\circ$ ) and by parallel cell samples stimulated mechanically with a stir bar ( $\bullet$ ). (C) Production of  $H_2O_2$  by unstirred NC cells in flasks ( $\circ$ ) and by parallel cell samples stimulated mechanically by stirring ( $\bullet$ ).

#### VU-3 cells show enhanced AOS production upon mechanical stimulation

$H_2O_2$  evolution by the suspension culture cell lines was monitored by assaying the  $H_2O_2$ -dependent degradation of the membrane-impermeant, fluorescent dye pyranine. In a real time assay in which the tobacco cells were suspended in a fluorimeter cuvette by stirring, VU-3 cells show an enhanced release of  $H_2O_2$  compared with NC cells (Figure 3A). This suggested that under the conditions of this assay, the VU-3 cells showed a higher basal evolution of  $H_2O_2$ . However, previous work (Legendre *et al.*, 1993; Yahraus *et al.*, 1995) has shown that mechanical stimulation, including vigorous stirring of cell suspensions, triggers  $H_2O_2$  evolution. To determine whether stirring was responsible for the enhanced evolution of  $H_2O_2$ , flask-adapted suspension cells were analyzed. In the absence of stirring, the basal level of AOS evolution was very low in VU-3 cells (Figure 3B). However, upon mechanical stimulation of the cells by stirring, a 10- to 15-fold enhancement in the rate of AOS production was observed (Figure 3B). NC control cells exhibited a much weaker response to stirring, and no  $H_2O_2$  was detected in the absence of stirring (Figure 3C). Thus, the VU-3 cells appear to be more sensitive to mechanical stimulation, and responded by producing a more rapid and intense release of  $H_2O_2$ . Consistent with this interpretation, other stimuli, such as hypoosmotic stimulation (Table I), that are proposed to cause a mechanical stimulation of the cells (Yahraus *et al.*, 1995), also show an enhanced response in VU-3 cells.

#### VU-3 cells show an enhanced response to various stimuli that elicit plant defense responses

Previous work has shown that tobacco suspension cells treated with cellulase exhibit calcium-dependent plant defense responses (Threlfall and Whitehead, 1988; Preisig and Moreau, 1994). Cellulase treatment of NC and VU-3 cells induced a burst of AOS release (Figure 4A). However, while an AOS burst was detected in both cell types, the kinetics of the response differed substantially. In particular, the onset of the burst was nearly instantaneous in VU-3 cells, while a lag of 6–10 min was observed with NC

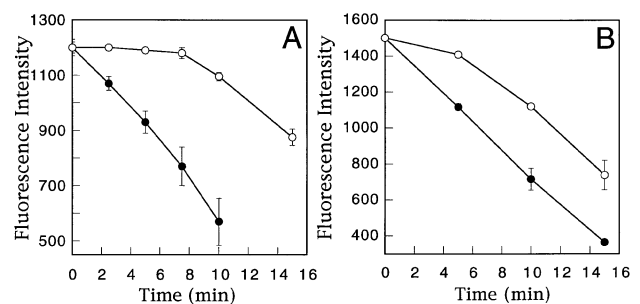
**Table I.** AOS production rates are enhanced in VU-3 cells

Treatment <sup>a</sup>	Pyranine fluorescence <sup>b</sup> Cell line	
	VU-3	NC
Basal	0.06 (0.03) <sup>c</sup>	0.01 (0.01)
Cellulase	1.39 (0.14)	0.74 (0.03)
<i>Pseudomonas</i>	0.37 (0.10)	0.19 (0.06)
Osmotic	0.24 (0.02)	0.13 (0.01)

<sup>a</sup>Basal, untreated cell suspensions in flasks ( $n = 3$ ); cellulase, cells treated with  $5 \mu\text{g}$  of cellulase/ml suspension ( $n = 3$ ); *Pseudomonas*, cells treated with  $10^7$  c.f.u. of HR<sup>+</sup> *P. syringae*, pathovar 61/ml ( $n = 5$ ); osmotic, cells diluted with 2 volumes of  $H_2O$  and changes in fluorescence calculated by difference with parallel flasks diluted with 2 volumes of isoosmotic media ( $n = 3$ ).

<sup>b</sup>The maximum rate of pyranine fluorescence decrease in 3-day-old cell suspension cultures containing 0.05 ml of settled cell volume/ml culture volume.

<sup>c</sup>Values represent the mean with standard errors shown in parentheses.



**Fig. 4.** AOS production rates of transgenic tobacco cell lines in response to cellulase and harpin. Tobacco cells (3–5 days after transfer to fresh media) were treated at time 0 with (A)  $5 \mu\text{g}$  of cellulase/ml cell suspension or (B)  $4 \mu\text{g}$  harpin<sub>ps</sub>/ml cell suspension under normal growth conditions in flasks. Pyranine ( $6 \mu\text{g}/\text{ml}$  suspension) was added 1 min prior to treatment, and  $H_2O_2$  was assayed by a decrease in fluorescence intensity in NC cells ( $\circ$ ) and VU-3 cells ( $\bullet$ ). Error bars represent the standard error of the mean of analyses of three independent biological samples.

cells (Figure 4A). In addition, the maximal AOS release rate was ~2-fold higher in VU-3 cells (Figure 4A and Table I).

**Table II.** AOS production rates of tobacco cell lines determined by pyranine fluorescence decrease are not limited by endogenous peroxidase

Cell line	$\Delta$ Fluorescence/min/ $\mu$ g protein	
	(+) <sup>a</sup>	(-)
VU-3	4.11 (0.38) <sup>b</sup>	4.48 (0.37)
NC	2.51 (0.34)	2.01 (0.30)

<sup>a</sup>(+): 0.1 U of exogenous horseradish peroxidase/ml suspension was included during the assay, (-): fluorescence decrease in the absence of added peroxidase.

<sup>b</sup>The changes in pyranine fluorescence were monitored in a fluorimeter cuvette as described in Materials and methods. AOS production was stimulated by addition of 5  $\mu$ g of cellulase/ml suspension. Cell suspensions were used 3–5 days after transfer to fresh media. Values represent the means and (standard error) of three independent determinations.

**Table III.** Enhanced uptake of O<sub>2</sub> by VU-3 cells following treatment with harpin or cellulase

Cell line	O <sub>2</sub> uptake <sup>a</sup> (nmol/mg protein/min)	
	Harpin	Cellulase
VU-3	70.0 (5.0) <sup>b</sup>	86.7 (5.8)
NC	22.8 (1.3)	31.3 (1.1)

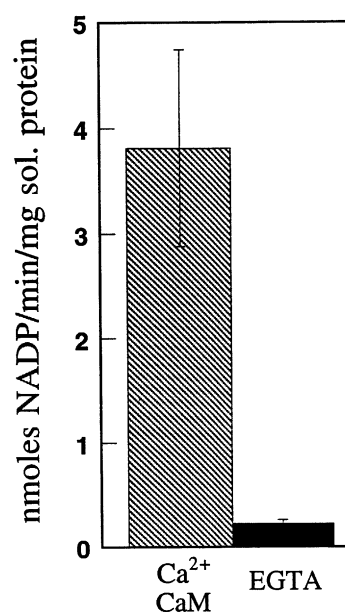
<sup>a</sup>Stimulation of oxygen uptake was calculated as the difference between O<sub>2</sub> uptake by untreated cells and by cells treated with 5  $\mu$ g of cellulase or 4  $\mu$ g of harpin/ml suspension as described in Materials and methods.

<sup>b</sup>Values show the means of four determinations with standard errors in parentheses.

Other molecules and microbes that elicit plant defense responses were also tested for their ability to trigger AOS release in VU-3 and NC control cells. The purified elicitor protein harpin from *Pseudomonas syringae* pathovar 61 (He *et al.*, 1993) showed results similar to those obtained with cellulase (Figure 4B), with VU-3 cells showing a faster rate and a quicker onset of AOS release than NC cells. VU-3 cells also showed a more enhanced response upon actual infection by *P.syringae* pathovar 61 (Table I). Overall, the data show that VU-3 cells exhibit an enhanced AOS release response to all stimuli tested (Figures 3 and 4 and Table I).

#### Enhanced responses of VU-3 cells are paralleled by an enhanced oxygen uptake

The pyranine assay for AOS is based on H<sub>2</sub>O<sub>2</sub>-dependent, cell wall peroxidase-catalyzed destruction of the fluorescent dye (Apostol *et al.*, 1989). To firmly establish that differences observed between VU-3 and NC cells are due to the rate of H<sub>2</sub>O<sub>2</sub> evolution and not to differences in extracellular peroxidases, two approaches were taken. First, it was found that addition of excess horseradish peroxidase to VU-3 and NC cells did not influence the rate of pyranine  $\Delta$ F (Table II), suggesting that the levels of AOS, and not the level of peroxidases, are the rate-limiting steps. Second, the oxidative burst reaction was assayed by monitoring a different parameter, the uptake of O<sub>2</sub> (Table III). The reduction of O<sub>2</sub> to O<sub>2</sub><sup>-</sup> and other AOS species during the oxidative burst results in an enhanced O<sub>2</sub> uptake (Dwyer *et al.*, 1996). Consistent



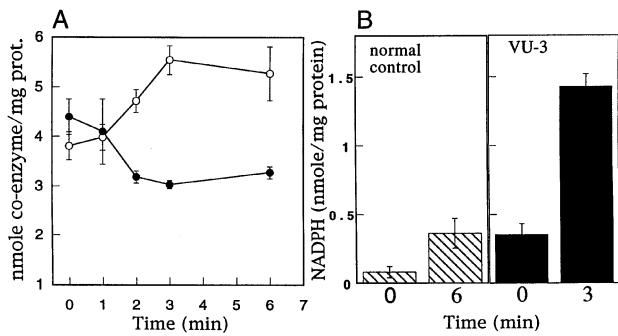
**Fig. 5.** NAD kinase activity in suspension tobacco cells is calcium and calmodulin dependent. Extracts of 3-day-old cultured suspension cells were prepared and NAD kinase activity was measured in the presence of 0.5 mM EGTA (solid bar); or 2.5 mM CaCl<sub>2</sub> and 2  $\mu$ M purified calmodulin (cross-hatched bar). The activity is standardized to total soluble protein in the extract. The data shown are from VU-3 cells; essentially identical results are obtained with NC cell extracts (data not shown). Error bars represent standard error of the mean of analyses of three independent biological samples.

with an enhanced AOS production based on pyranine fluorescence, VU-3 cells also show higher consumption of non-respiratory oxygen upon harpin or cellulase treatments (Table III).

#### Nicotinamide co-enzyme changes during the induction of AOS production

Since VU-3 calmodulin hyperactivates NAD kinase *in vitro* (Roberts *et al.*, 1986b), and because this enzyme has been implicated in nicotinamide fluxes preceding oxidative burst reactions in other systems (Epel *et al.*, 1981), this enzyme is a logical target that may be activated during the oxidative burst of plant cells. Analysis of tobacco cell extracts shows that virtually all detectable NAD kinase activity is calcium and calmodulin dependent (Figure 5). Consistent with the endogenous activation of NAD kinase, cellulase treatment of VU-3 tobacco cells results in a rapid increase in the sum total (reduced and oxidized) NADP co-enzyme pool and a concomitant decrease in total NAD co-enzymes (Figure 6A). The NADPH pool size increases 4-fold within 3 min of treatment (Figure 6B). Similar to the results with whole plants (Figure 1), steady-state levels of NADPH in NC cells were lower than those in VU-3 cells. However, cellulase treatment also resulted in an increase in NADPH in these cells (Figure 6B), although the magnitude was less and the rate of increase was slower (peaking at 6 min rather than 3 min). During the time course of these experiments, there were no detectable changes in the cellular levels of NAD kinase. Thus, the increase in NADP co-enzymes appears to result from enzyme activation (presumably by calmodulin) rather than a change in enzyme levels.

These differences in the rate and levels of NADPH



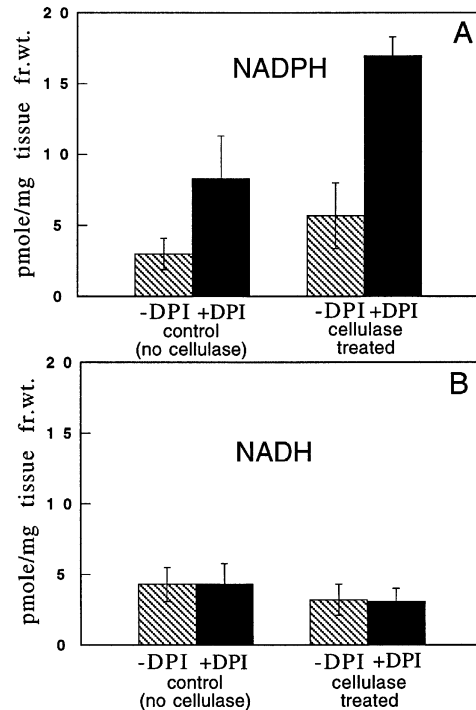
**Fig. 6.** Effect of cellulase treatment on nicotinamide co-enzyme levels. Flasks of 3-day-old cultured suspension cells were treated with 5  $\mu$ g cellulase/ml at time 0, and co-enzyme levels were analyzed as described in Materials and methods. Error bars represent standard error of the mean of analyses of three independent biological samples. (A) Total sum of reduced and oxidized NADP ( $\circ$ ) and NAD ( $\bullet$ ) levels in VU-3 cells. (B) Comparison of peak NADPH levels in the VU-3 (solid bars) and NC (cross-hatched bars) cells in response to cellulase treatment.

changes are consistent with the differences in the rate and magnitude of AOS release in these cell types, and argue that the activation of NAD kinase may be necessary for providing NADPH reductant for AOS production, similar to animal NADPH oxidase systems (Heinecke and Shapiro, 1992). Diphenyleneiodonium (DPI) is an inhibitor of the oxidative burst reaction in animal cells which covalently modifies the flavoprotein component of  $O_2^-$ -producing NADPH oxidases (Cross and Jones, 1986). DPI has been found also to inhibit the oxidative burst in plants (Dwyer *et al.*, 1996; Auh and Murphy, 1995), and the release of AOS exhibited in VU-3 and NC tobacco cells is abolished by micromolar DPI levels (data not shown). If NADPH is the principal reductant used for AOS production by an oxidase, one prediction is that DPI inhibition would decrease not only AOS production but also the flux of NADPH through the oxidase enzyme.

Consistent with this, treatment of cells with DPI results in an accumulation of NADPH levels whereas NADH levels are unaffected (Figure 7). This argues that the flux of electrons for AOS production proceeds through an NADPH-dependent pathway and that when this pathway is inhibited, NADPH accumulates in cells. Overall, the data suggest that NADPH levels are elevated rapidly through the activation of NAD kinase in stimulated cells and that the AOS-generating system in these cells preferentially utilizes NADPH as a reductant.

## Discussion

In the present study, a calmodulin (VU-3) which hyperactivates NAD kinase was used to evaluate the hypothesis that activation of calmodulin-dependent NAD kinase is part of the early responses of plants to environmental, microbe and elicitor stimulation. Further, this calmodulin was used to determine whether, similarly to certain animal cell models, enhanced NADP(H) production helps potentiate the generation of active oxygen species. In support of this proposal, we found that tobacco tissue expressing VU-3 calmodulin shows an enhanced ability to produce  $H_2O_2$ , as reflected by a quicker onset and more intense release, in response to all stimuli tested. This response



**Fig. 7.** Effect of DPI on levels of reduced nicotinamide co-enzymes. The concentration of (A) NADPH and (B) NADH were measured in flasks of VU-3 suspension cells in the absence (cross-hatched bars) or presence (solid bars) of 2  $\mu$ M DPI. Results from control (untreated) and cellulase-treated (5  $\mu$ g/ml suspension) cells are shown. Flasks were harvested 90 s after cellulase, cellulase/DPI or DPI addition, and nicotinamide co-enzyme levels were analyzed as described in Materials and methods. Error bars represent standard error of the mean of analyses of three independent biological samples.

was paralleled by an enhanced basal level of NADPH as well as a more rapid and higher accumulation of NADPH when challenged with a stimulus. Overall, the data provide strong evidence for a role for calmodulin and NAD kinase in metabolic changes associated with plant defense responses.

The introduction of gene products with modified protein sequences that result in dominant phenotypes has been an important and powerful approach for elucidating the components of signal transduction pathways. The strength of the present approach is that VU-3 calmodulin functions normally as a calmodulin and selectively shows one defect, the inability to be trimethylated post-translationally at residue 115 by the endogenous calmodulin *N*-methyltransferase (Roberts *et al.*, 1986b; Oh and Roberts, 1990). Endogenous plant calmodulin is found in mature tissues in a largely trimethylated form (Roberts and Harmon, 1992). Trimethylation does not affect most calmodulin activities but attenuates plant NAD kinase activation *in vitro* (Roberts *et al.*, 1986b). In contrast, VU-3 calmodulin hyperactivates NAD kinase to a level that is 4-fold higher than trimethylated calmodulin (Roberts *et al.*, 1986b). Thus, the use of VU-3 transgenic plants allows us to circumvent control by methylation and selectively evaluate the contribution of NAD kinase to plant responses.

The fact that most calmodulin isolated from plant tissues is highly methylated at Lys115 and that this selectively attenuates NAD kinase activation has led to the proposal that this post-translational modification may have a regu-

latory function (reviewed in Roberts and Harmon, 1992). Although most calmodulin isolated from plant tissues is largely methylated, calmodulin methylation appears to be subject to control, and can vary depending on the developmental state of the cells (Oh and Roberts, 1990; Oh *et al.*, 1992). In light of the present results, it will be of interest to determine whether endogenous calmodulin methylation modulates the level of NAD kinase activation, nicotinamide co-enzyme homeostasis and AOS production during development in wild-type cells.

Plant NAD kinase was the first calmodulin-dependent enzyme to be detected in plant cells (Muto and Miyachi, 1977; Anderson and Cormier, 1978), but its biological role has remained uncertain (Roberts and Harmon, 1992). The results of the present study provide evidence that NAD kinase may be among the targets of calmodulin in plant defense responses. In this regard, the results show some similarity to previous studies with NAD kinase and nicotinamide co-enzyme homeostasis in sea urchin eggs. Fertilization of sea urchin eggs is accompanied by a calcium flux, followed by the conversion of 25–50% of cell NAD to NADP by a calmodulin-dependent NAD kinase (Epel, 1964; Epel *et al.*, 1981). The resulting enhanced levels of NADPH are used as a reductant in an oxidative burst reaction that produces  $H_2O_2$ , leading to an altered cell coat architecture (Heinecke and Shapiro, 1992).

Similarly to sea urchin eggs, activated neutrophils also produce a calcium-dependent burst of AOS catalyzed by a plasma membrane oxidase that utilizes NADPH (reviewed in Cross and Jones, 1991; Heinecke and Shapiro, 1992; Henderson and Chappell, 1996). Recently, plant cell extracts have been shown to contain proteins that are immunologically related to the neutrophil NADPH oxidase (Dwyer *et al.*, 1996) and to have activities that are similar to the oxidase (reviewed in Cross and Jones, 1991; Rubenstein and Luster, 1993; Mehdy, 1994; Low and Merida, 1996). The fact that higher levels of NADPH in transgenic calmodulin cells lead to a more rapid and intense burst of AOS supports the proposed involvement of an NADPH oxidase. Further, we found that the AOS burst in the tobacco cells can be inhibited by DPI, an inhibitor which covalently modifies the flavoprotein component of animal NADPH oxidases (Cross and Jones, 1986). This is in agreement with previous reports that DPI blocks elicitor induction of the oxidative burst in soybean cells (Levine *et al.*, 1994; Auh and Murphy, 1995; Dwyer *et al.*, 1996; Murphy and Auh, 1996). It was also reported that DPI inhibited a plasma membrane oxidase/peroxidase activity that utilizes NADH (Auh and Murphy, 1995; Murphy and Auh, 1996). However, in our studies, NADPH alone accumulated when the AOS burst was inhibited by DPI, whereas no change in NAD/NADH co-enzymes was observed. This strongly suggests that the AOS burst is generated by a system utilizing NADPH as reductant. Further work, including the molecular characterization of the plant plasma membrane oxidase, will clarify further whether the plant enzyme is similar to the animal enzyme with respect to its function, assembly and regulation.

Other studies have provided support for calcium and calmodulin signaling in environmental and mechanical responses. For example, 10- to 100-fold increases in the abundance of mRNAs for calmodulin and calmodulin-like

proteins follow mechanical stimulation (Braam and Davis, 1990; Galaud *et al.*, 1993; Sistrunk *et al.*, 1994) and heat shock (Braam, 1992), suggesting an up-regulation of these calcium signaling pathways. Additionally, another calmodulin-dependent enzyme, glutamate decarboxylase (Ling *et al.*, 1994; Baum *et al.*, 1996), appears to be regulated following mechanical stimulation leading to accumulation of  $\gamma$ -aminobutyric acid in plant tissues (Wallace *et al.*, 1984). Thus, while the approach taken in the present work has allowed us to focus on one calmodulin target, NAD kinase, it is likely that the regulation of multiple target proteins by calmodulin, as well as calcium-modulated proteins distinct from calmodulin, will be required for a co-ordinated defense response.

## Materials and methods

### Transgenic plants and cell cultures

Transgenic tobacco plants expressing VU-3 calmodulin (Roberts *et al.*, 1992) or a negative control construct (Zhang and Roberts, 1995) ( $F_1$  generation seed) were germinated on MS agar media containing hygromycin and transferred and grown under greenhouse conditions as described previously (Roberts *et al.*, 1992). Cell suspension cultures were established from stem explants of 4-week-old tobacco seedlings by using the protocol of Smith (1986). Explants were cultured for 6–8 weeks on medium A [Murashige and Skoog basal salt-vitamin mix (Sigma), 1 mg of naphthalene acetic acid, 50  $\mu$ g of benzylaminopurine and 30 mg of hygromycin B per liter]. Calli were transferred to medium B (medium A lacking benzylaminopurine) and grown for 4–6 weeks. Friable calli (1 g) were selected to initiate 25 ml suspension cultures in medium C (medium B with 2 mg of 2,4-dichlorophenoxyacetic acid/l as the sole phytohormone). Cultures were maintained by weekly 1:5 dilution in medium C. Cell suspensions were diluted into fresh medium C 3 days before each experiment.

### Calmodulin expression in suspension cells

Cells were harvested, frozen in liquid nitrogen and were ground and extracted in 4 volumes of 50 mM Tris-HCl, (pH 7.5), 50 mM NaCl, 10 mM Na-ascorbate, 0.5 mM EGTA, 2  $\mu$ g/ml leupeptin, 0.3  $\mu$ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5% (w/v) polyvinylpyrrolidone (PVPP). After centrifugation at 20 000 g for 10 min at 4°C, the supernatant was removed and the pellet was re-extracted with 300  $\mu$ l of extraction buffer without PVPP. The protein content of pooled extracts was determined by the procedure of Bradford (1976). The calmodulin concentration was determined by a chemiluminescent Western blot protocol with anti-calmodulin antibodies (Oh and Roberts, 1990). Following SDS-PAGE, gels were soaked for 15 min in transfer buffer (25 mM potassium phosphate, pH 7.0) and proteins were transferred (150 mA for 12 h at 4°C) to polyvinylidene difluoride membranes as described by Hulen *et al.* (1991). Proteins were fixed for 30 min in transfer buffer containing 0.2% (v/v) glutaraldehyde (Van Eldik and Wolchok, 1984) and rinsed with phosphate-buffered saline (PBS, 8 mM sodium phosphate, 1 mM potassium phosphate, pH 7.2, 0.14 M NaCl, 3 mM KCl). Membrane filters were blocked for 1 h in PBS, 10% (w/v) non-fat dry milk. The blots were washed in PBS and were incubated with anti-calmodulin antibodies (1:1000 v/v) in PBS, 0.65 mM  $CaCl_2$ , 1% (v/v) goat serum. The blots were washed three times with PBS, 0.05% (v/v) Tween-20, and were incubated in 1:1000 (v/v) horseradish peroxidase-conjugated goat anti-rabbit IgG in PBS and 1% (v/v) goat serum. The blots were washed extensively at 37°C in PBS, 1% (v/v) goat serum, 0.1% (v/v) Tween-20, 0.5% (v/v) Triton X-100 and 0.1% (w/v) SDS, and calmodulin was visualized and quantitated by chemiluminescence detection (ECL kit, Amersham), using purified VU-1 calmodulin as a standard.

### Measurement of $H_2O_2$ and oxygen uptake

Fully expanded leaves from the upper third of 6-month-old plants were dissected, immediately frozen in liquid nitrogen and ground in ice-cold 5% (w/v) trichloroacetic acid.  $H_2O_2$  was extracted, and measured by the luminol chemiluminescence protocol of Warm and Laties (1982) with a Berthold LB9501 luminometer. Treatment of control samples with exogenous catalase (0.1 U/ml) prior to assay verified that the chemiluminescence signal was due to the  $H_2O_2$  content of the sample.

H<sub>2</sub>O<sub>2</sub> release in suspension cultures was quantitated by pyranine fluorescence (Apostol *et al.*, 1989). All measurements were performed on exponentially growing cells 3–5 days after dilution into fresh medium. Equivalent cell densities (based on cell protein and fresh weight) were used in all experiments, and was ~50 mg fresh weight/ml. Continuous, real time H<sub>2</sub>O<sub>2</sub> release was measured by adding 3 µg/ml pyranine (Molecular Probes, Eugene, OR) and monitoring the decrease in fluorescence ( $\lambda_{\text{ex}}$  405 nm,  $\lambda_{\text{em}}$  512 nm) in a Hitachi F-2000 fluorimeter equipped with a magnet-driven stir bar rotating at 150–200 r.p.m.. Addition of exogenous 0.1 U/ml catalase was used to verify that the pyranine fluorescence decrease ( $\Delta F$ ) was dependent on endogenous H<sub>2</sub>O<sub>2</sub> release. For treatments in flasks, suspension cultures were diluted to a cell density of 1.5 ml settled cell volume in 30 ml of medium C and were grown with gentle shaking (120 r.p.m.) for 3 days prior to treatment. Pyranine (6 µg/ml) was added 1 min prior to treatment. The release of H<sub>2</sub>O<sub>2</sub> was measured by harvesting aliquots of cells at fixed time points following treatment and measuring the decrease in pyranine fluorescence.

Oxygen uptake was measured with an oxygen monitor (YSI 5300, Yellow Springs Instruments) equipped with a YSI 5331 polarographic oxygen probe. Oxygen uptake rates were determined by monitoring cellulase-treated, harpin-treated and untreated control samples (3 ml of suspension cell aliquot per assay) for 15 min.

#### Elicitor, bacterial and osmotic treatments

Harpin<sub>ps</sub> was prepared from *Escherichia coli* DH5- $\alpha$  cells transformed with pSYH10 carrying the *hrpZ* open reading frame (a gift from Alan Collmer, Cornell University) as described previously (He *et al.*, 1993). Cellulase (*Trichoderma viride*, Sigma) and harpin treatments were done on cells in flasks at final concentrations of 5 and 4 µg/ml, respectively. *Pseudomonas syringae* pathovar *syringae*, strain 61 was grown for 25–30 h on King's medium (King *et al.*, 1954). Bacteria were resuspended in sterile deionized H<sub>2</sub>O and were added to tobacco cell suspensions at a titer of 10<sup>7</sup> c.f.u./ml suspension.

Hypoosmotic treatments were done by the general approach of Yahraus *et al.* (1995). Three-day-old cell suspensions were decanted to obtain a settled cell volume of 1.5 ml cells/10 ml culture, and were returned to flasks and allowed to equilibrate for 1 h. Pyranine (18 µg/ml) was added, and flasks were diluted either with two volumes of media (isoosmotic control) or two volumes of deionized water (hypoosmotic treatment). Following dilution, aliquots were removed at various times and pyranine fluorescence measured.  $\Delta F$  due to hypoosmotic treatment was determined by subtracting the  $\Delta F$  of the isoosmotic control cells from the  $\Delta F$  of the hypoosmotically treated cells.

#### Measurement of nicotinamide co-enzyme levels and NAD kinase activity

Nicotinamide co-enzymes were extracted and quantitated by the method of Matsumura and Miyachi (1980). For DPI experiments, DPI was added to suspension cultures to a final concentration of 2 µM DPI in 0.4% (v/v) dimethylsulfoxide (DMSO). Cells were incubated for 90 s prior to harvest and assay. Control cells contained an equivalent amount of DMSO without DPI. This treatment did not alter the nicotinamide co-enzyme levels (data not shown).

NAD kinase from frozen tissue powders was extracted (4 ml/g tissue) in 250 mM sucrose, 50 mM tricine-HCl (pH 7.8), 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 2 µg/ml leupeptin, 0.3 µg/ml pepstatin, 1 mM PMSF and 10% (w/v) PVPP. Homogenates were centrifuged at 20 000 g for 10 min at 4°C and NAD kinase was assayed in 250 mM sucrose, 3 mM ATP, 2 mM NAD, 5 mM MgCl<sub>2</sub>, 50 mM tricine-HCl (pH 7.8) by the procedure of Roberts *et al.* (1985). To determine the ratio of calcium/calmodulin-dependent to independent activities, assays were done in 2.5 mM CaCl<sub>2</sub> supplemented with excess (2 µM) VU-1 calmodulin to ensure saturation (calcium/calmodulin-dependent activity) or in the presence of 0.5 mM EGTA to remove all calcium from endogenous calmodulin (calmodulin-independent activity).

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