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Transgenic Tobacco Plants Expressing a Mutant VU-4 Calmodulin Have Altered Nicotinamide Co-Enzyme Levels and Hydrogen Peroxide Levels

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In order to understand the biological role of calmodulin in plants, transgenic tobacco plants expressing a calmodulin mutant (VU-4 calmodulin, lys to ile-115) gene have been analyzed. SDS-PAGE and Western-blot analyses showed that the foreign calmodulin mutant is stably and highly expressed in the transgenic tobacco plants. The levels of H_2O_2 were elevated approximately 2-fold in the transgenic plants. Furthermore, the transgenic tobacco plants have more than 6-fold higher levels of NADPH compared to control tobacco plants. The present findings, combined with previous data showing differences in the susceptibility of the transgenic tobacco seeds and normal tobacco seeds to fungal contamination (Oh and Yang, 1996), suggest that the expression of the calmodulin derivative gene in tobacco plants could increase resistance to infection by fungal pathogens.

Keywords: Active oxygen species, Calmodulin, Nicotinamide co-enzymes, Transgenic plants.

Introduction

Calcium plays a key role as a signal molecule in the regulation of intracellular events in eukaryotes (Hepler and Wayne, 1985; Berridge, 1990; Han *et al.*, 1995). Calcium serves as an intracellular signal transducer by reversibly interacting with specialized calcium-binding proteins known as calcium-modulated proteins (Poovaiah and Reddy, 1987; Roberts and Harmon, 1992). Calmodulin is a highly conserved calcium-modulated protein that has been found in all eukaryotic cells examined and which interacts with and regulates a variety of enzymes (Cohen and Klee, 1988; Roberts and Harmon, 1992; Oh *et al.*, 1995). Plants have been shown to possess Ca^{2+}/calmodulin-stimulated NAD kinase which catalyzes the phosphorylation of NAD to NADP using ATP as a co-substrate (McGuinness and Butler, 1985). In most animal tissues, NAD kinase is not a calmodulin-stimulated enzyme. However, an exception to this is the Ca^{2+}/calmodulin-stimulated NAD kinase from human neutrophils (Williams and Jones, 1990). NADPH is utilized by NADPH oxidase in the generation of active oxygen species (AOS) for bactericidal activity in the neutrophils (Baggiolini and Wymann, 1990; Park and Ahn, 1995). Therefore, the elevation of NADP by Ca^{2+}/calmodulin-stimulated NAD kinase could be necessary to provide a rapid increase in substrate NADPH for reductive processes.

The production of AOS in plants has been proposed to be part of the rapid defense response to environmental stress conditions including plant pathogens and elicitors (Apostol *et al.*, 1989; Mehdy, 1994). Interestingly, many of the same stresses that stimulate AOS production in plants also cause fluxes in cytosolic Ca^{2+} (Doke, 1985; Braam and Davis, 1990). From these, it can be proposed that Ca^{2+}/calmodulin modulation of NAD kinase may serve *in vivo* as a regulatory mechanism for the oxidative burst response of plants to pathogens (Mehdy, 1994). In this regard, it is interesting to note that transgenic tobacco plants that have twice as much calmodulin as untransformed plants showed enhanced production of AOS (Harding *et al.*, 1997). In order to test further how the elevation of calmodulin affects nicotinamide co-enzyme homeostasis and AOS production, we have analyzed transgenic tobacco expressing a calmodulin mutant with an isoleucine-115 substitution (VU-4 calmodulin). Previously, it was found that VU-4 calmodulin retains calmodulin activity and

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activates NAD kinase to an activity that is 4-fold higher than trimethylated calmodulin (Roberts *et al.*, 1990). Further, it is resistant to methylation of calmodulin at position 115. Therefore, the introduction of this calmodulin mutant into higher plants may change the calmodulin/NAD kinase regulatory system. In the present study, we have focused on nicotinamide co-enzyme and hydrogen peroxide contents in the leaves of transgenic tobacco. We found that the transgenic tobacco plants expressing VU-4 calmodulin gene have elevated hydrogen peroxide levels. Furthermore, comparisons of nicotinamide co-enzyme levels in the transgenic and control tobacco plants show a higher ratio of NADPH to NADH, suggesting the possibility of the involvement of calmodulin-dependent NAD kinase.

Materials and Methods

Materials PVDF-membrane and AG1-X8 resin were purchased from Bio-Rad (Hercules, USA) and Western blotting detection reagents were from Amersham (Buckinghamshire, England). Other chemicals were purchased from Sigma (St. Louis, USA). Transgenic tobacco plants expressing the VU-4 calmodulin gene under the control of the CaMV 35S promoter were generated as previously described (Roberts *et al.*, 1992; Oh and Yang, 1996). For transformation, leaves from tobacco plants (*Nicotiana tabacum* L. cultivar Wisconsin 38) were used. F_1 seeds were imbibed and germinated on MS agar (Murashige and Skoog, 1962) containing 50 μg/ml hygromycin. Plants were transferred and grown under standard greenhouse conditions as described previously (Roberts *et al.*, 1992).

Analysis of calmodulin protein levels Calmodulin expression was analyzed by Western blot analysis with anti-calmodulin antibodies (Oh and Roberts, 1990). Tobacco leaves were extracted by using the method described by Roberts *et al.* (1992) with a modified extraction buffer consisting of 100 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA, 20 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, and 10% (w/v) polyvinylpolypyrrolidone. Total protein was measured by the Bradford method (1976). Twenty μg of total protein extract was separated by SDS-PAGE on 15% (w/v) polyacrylamide gels in the presence of 1 mM EGTA as previously described (Roberts *et al.*, 1992). Proteins were electroblotted onto PVDF membranes in 25 mM potassium phosphate buffer, pH 7.2, at 150 mA for 12 h at 4°C (Harding *et al.*, 1997). Western blot analysis was done with anti-calmodulin antibodies (Oh and Roberts, 1990) by using a chemiluminescence protocol (Amersham).

Measurement of nicotinamide co-enzyme levels Extraction of oxidized and reduced NAD and NADP coenzymes was done by the method of Matsumura and Miyachi (1980) with some modifications. Tobacco leaves were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. One hundred mg of powder was extracted with 1.3 ml of cold acetone and centrifuged at 14,000 rpm in a microcentrifuge at 4°C for 10 min. Pellets were suspended in either 0.5 ml of 0.2 N HCl (for oxidized co-enzyme analysis) or 0.5 ml of 0.2 N NaOH (for reduced co-enzyme analysis). The mixtures were placed in a boiling water bath for 1 min, degassed, and incubated for an additional 4 min in the boiling water bath. The samples were centrifuged at 14,000 rpm in a microcentrifuge at 4°C for 10 min. Supernatants were collected and neutralized with 1 ml of 1 M Bicine-NaOH, pH 8.0. Nicotinamide co-enzymes were quantitated enzymatically as described by Matsumura and Miyachi (1980).

Measurement of hydrogen peroxide levels Extraction of tobacco leaves for H_2O_2 measurement was done as previously described (Warm and Laties, 1992; Chen *et al.*, 1993) with some modifications. Leaf tissue (3.5 g) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was transferred to 14 ml of 5% (w/v) trichloroacetic acid and further homogenized with a homogenizer (Cole-Parmer Instrument, Vernon Hills, USA). The homogenates were centrifuged for 30 min at 12,000 $\times$ g at 4°C. A 0.5 ml aliquot of the supernatant was applied to a 1 ml column of AG1-X8 resin (Bio-Rad, Hercules, USA). The column was eluted with ice cold deionized water and three fractions of 0.5 ml each were collected. H_2O_2 was measured as described by the luminol-based chemiluminescence protocol of Chen *et al.* (1993). Chemiluminescence was measured using a Lumat LB9501 luminometer (Berthold, Pittsburgh, USA). To verify specificity, selected samples (32 μl) were neutralized with 0.2 N NH_4OH to a final pH 7.0 and treated with 400 units of bovine liver catalase (Sigma) in darkness at room temperature for 10 min prior to reading the chemiluminescence.

Results and Discussion

In previous work, we have generated transgenic tobacco plants expressing the calmodulin mutant with isoleucine at position 115 (VU-4 calmodulin) (Oh and Yang, 1996). To determine the level of calmodulin in the transgenic tobacco tissues, a chemiluminescent Western blot protocol (Harding *et al.*, 1997) using anti-calmodulin antibodies (Oh and Roberts, 1990) was used. VU-4 calmodulin possesses slight differences in sequence that result in differential mobilities on SDS-PAGE compared with tobacco calmodulin (Roberts *et al.*, 1990; Oh and Yang, 1996). Thus, we were able to selectively analyze the relative levels of foreign calmodulin protein by Western blot. Analysis of transgenic plants by Western blot after SDS-PAGE shows two bands; a higher mobility band corresponding to endogenous tobacco calmodulin and a lower mobility band corresponding to foreign VU-4 calmodulin (Fig. 1). The analysis showed that the calmodulin mutant is stably and highly expressed in the transgenic plants (Fig. 1). Since ile mutant calmodulin hyperactivates NAD kinase (Roberts *et al.*, 1990), and is incapable of post-translational methylation, the expression of this calmodulin mutant could result in the alteration of nicotinamide co-enzyme homeostasis *in vivo*. In order to assess the effect of foreign calmodulin expression on nicotinamide co-enzyme levels, we analyzed 13 separate transgenic lines of VU-4 tobacco plants, and the

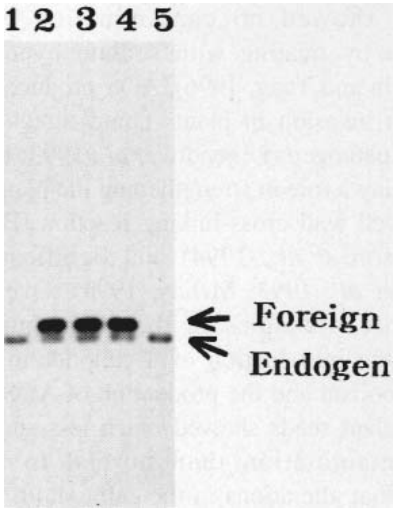

Fig. 1. Western blot analysis of calmodulin levels in leaves of transgenic tobacco. Twenty micrograms of protein from tobacco leaf extracts were applied on 15 % (w/v) SDS-polyacrylamide gel containing 1 mM EGTA to separate proteins. Proteins were blotted onto PVDF-membrane. After incubation with anti-calmodulin polyclonal antibodies, anti-rabbit-IgG-peroxidase was used as a secondary antibody. For chemiluminescence detection, solutions from Amersham were used. Lanes 1 and 5, calmodulins from control tobacco plants; lanes 2, 3, and 4, calmodulins from transgenic tobacco plants (separate transformed line 4-1, 4-2, 4-5). The foreign calmodulin mutant shows slower mobility than the endogenous calmodulin. Foreign, the position of foreign VU-4 calmodulin; Endogen, the position of endogenous tobacco calmodulin.

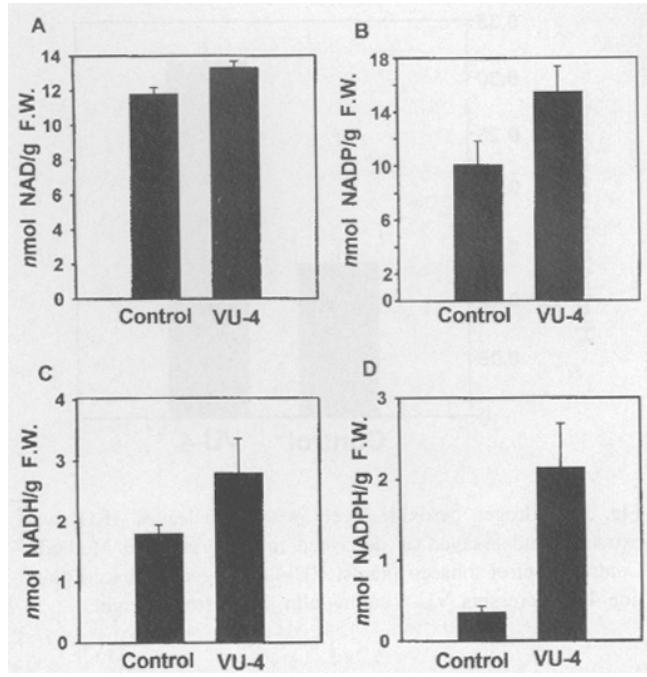

Fig. 2. Comparison of nicotinamide co-enzyme levels between VU-4 tobacco and control tobacco leaves. Nicotinamide co-enzymes were prepared and assayed as described in Materials and Methods. Values are the averages of three separate determinations, with error bars showing the standard deviations. Control, control tobacco plants; VU-4, transgenic tobacco plant line 4-1 expressing VU-4 calmodulin. F.W., fresh weight.

comparison data between a representative transgenic line and control tobacco plants is shown in Fig. 2. The analyses showed higher levels of all nicotinamide co-enzyme levels in leaf tissues of the transgenic tobacco than the levels in control plant tissues (Fig. 2). In particular, the level of NADPH was more than 6-fold higher in VU-4 plant leaves than in control plant leaves (Fig. 2D). The ratios of NADPH/NADH determined from the co-enzyme analyses were 4-fold higher in transgenic plants than in control plants (Table 1). In previous work, it was found that the VU-4 calmodulin shows hyperactivation of plant NAD kinase *in vitro* (Roberts *et al.*, 1990). Thus, these results may suggest that the regulation of calmodulin-dependent NAD kinase is changed in the cells of transgenic tobacco and the kinase can be responsible for the increased levels of NADPH. Since nicotinamide co-enzyme metabolism is altered during AOS production in other model systems (Baggiolini and Wymann, 1990; Shapiro, 1991), we investigated whether the oxidative burst metabolism of transgenic tobacco that overexpressed the isoleucine-115 mutant calmodulin is altered, by using a luminol-based chemiluminescent assay (Warm and Laties, 1992; Chen *et al.*, 1993). Basal levels of H_2O_2 were elevated approximately 2-fold in VU-4 tobacco plants compared to control plants (Fig. 3). This increase in chemiluminescence

Table 1. Ratios of nicotinamide co-enzymes from transgenic tobacco plants.

	tobacco plants	
	control[b]	VU-4
NADP/NAD[a]	0.86 (0.09)	1.16 (0.12)
NADPH/NADH	0.20 (0.04)	0.77 (0.05)

[a] Ratios of NADP/NAD and NADPH/NADH were determined from co-enzyme determinations done as shown in Fig. 2. Standard errors are given in parentheses.

[b] Control, untransformed tobacco plants; VU-4, transgenic tobacco plant line 4-1 (Oh and Yang, 1996) expressing VU-4 calmodulin.

was apparently due to elevated H_2O_2 levels since catalase treatment reduced the signal to background levels (Fig. 4). The data suggest that the elevation of calmodulin levels in VU-4 tobacco plants results in a perturbation of endogenous signalling pathways that control H_2O_2 production. The production of active oxygen species such as H_2O_2 is proposed to be involved in plant defense responses to pathogen infection and environmental stresses (reviewed by Mehdy, 1994). Calcium-fluxes are involved in these responses (Knight *et al.*, 1991; 1992; 1993), and calmodulin is a logical target (Oh and Yang, 1996). In this

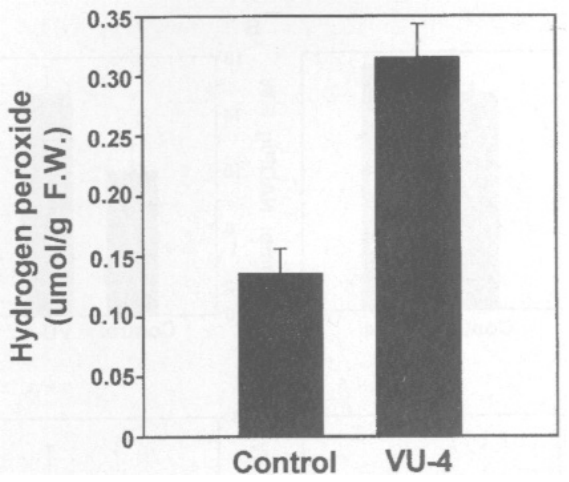

Fig. 3. Hydrogen peroxide levels in tobacco leaves. H_2O_2 was extracted and assayed as described in Materials and Methods. Control, control tobacco plants; VU-4, transgenic tobacco plant line 4-1 expressing VU-4 calmodulin. F.W., fresh weight.

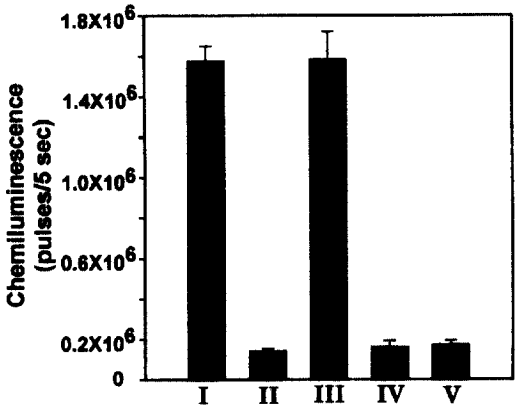

Fig. 4. Test for the specificity of the chemiluminescence for hydrogen peroxide. To verify the specificity, 400 units of catalase from bovine liver (Sigma) was added to 32 μl of samples buffered with 0.2 N NH_4OH, pH 9.5 to pH 7.0. The reaction mixtures were kept at room temperature in dark conditions for 10 min and then placed on ice. The chemiluminescence was recorded using 50 μl of reaction solution. VU-4 samples were obtained from AG1-X8 chromatography of an extract of VU-4 transgenic tobacco leaves. I, untreated VU-4 sample; II, VU-4 sample treated with catalase; III, 5.0 nmol of standard hydrogen peroxide; IV, 5.0 nmol of standard hydrogen peroxide treated with catalase; V, background luminescence from reagents without added samples.

regard, it is of interest to note that seeds obtained from VU-4 transgenic tobacco plants showed no fungi contamination with normal germination by treating with sterilized water alone (Oh and Yang, 1996). In contrast, seeds from control tobacco plants showed severe contamination with fungus by treating with sterilized water alone and showed no contamination with normal germination by treating with sodium hypochlorite (2% chlorine) (Oh and Yang, 1996). AOS produced in response to pathogen invasion in plants could directly damage or destroy the pathogen (Legendre *et al.*, 1993; Mehdy, 1994) and could play a role in strengthening the plant cell wall by aiding the cell wall cross-linking reaction (Bradley *et al.*, 1992; Brisson *et al.*, 1994) and lignification reaction (Legendre *et al.*, 1993; Mehdy, 1994). Overall, the data suggests that alterations in the calmodulin signalling system by the introduction of a calmodulin mutant alter redox metabolism and the production of AOS. Further, the transgenic plant seeds showed much less susceptibility to fungal contamination than normal tobacco seeds, suggesting that alterations in the calmodulin pathway also affect plant defence responses to pathogen infection. Future studies with this system may provide insight into the mechanism of defence of plants during pathogen infection and environmental stresses.

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