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NaCl and wounding induced changes in NAD reductase in hypocotyls and root tips of *Phaseolus vulgaris* L.

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Abstract

The effect of 300 mM NaCl and wounding on the nicotinamide adenine dinucleotide (NAD) kinase, revealed by the guanosine triphosphate-dependent NAD reductase activity, was studied in two differently resistant bean cultivars using densitometric analysis of electrophoretic gels. In the presence of NaCl the total activity of NAD reductase was increased, in hypocotyls and root tips of resistant cultivar. The contribution of each of NAD reductase isoforms to the total activity was not significantly different between cultivars. Conversely, after wounding the hypocotyl, an increase could be observed in both cultivars and differences were demonstrated in the contribution of the different isoforms.

Key words: Isoenzymes, NAD kinase, stress resistance.

Introduction

NAD kinase (EC 2.7.1.23.) catalyses the synthesis of NADP(H) from NAD(H) and ATP (or other phosphoryl donors such as GTP), in the presence of Mg^{2+}. NADP(H) is the reducing power used in the most of biosynthetic pathways. The presence of this ubiquitous enzyme has been well established in various plant tissues (for review see *e.g.* McGuiness and Butler 1985). In plants, the NAD kinase could be located in cytoplasm (Marmé and Dieter 1982), in mitochondria (Dieter and Marmé 1984), and in chloroplast in soluble and membrane bound forms (Cormier *et al.* 1981). Some

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Abbreviations: CaM - calmodulin; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; GTP - guanosine triphosphate; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD - nicotinamide adenine dinucleotide; PES - phenazine ethosulfate; PMSF - phenylmethylsulfonyl fluoride; PVP - polyvinylpyrrolidone.

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forms of the enzyme are stimulated by the Ca^{2+}-CaM complex (Anderson and Cormier 1978).

In some plants submitted to exogenous stimuli or abiotic stresses, variations of NAD kinase activity were observed. Low temperatures induced the activation of NAD kinase in winter rape (Maciejewska 1990), while water deficit stimulated the CaM-dependent form of the enzyme in drought-adapted wheat genotypes (Zagdańska 1990). Under aluminium stress, a CaM-independent form seemed to be involved in the tolerance mechanism (Slaski 1989), since differential responses in NAD kinase activity were observed in aluminum-sensitive and aluminum-tolerant wheat genotypes. Plant NAD kinase activity can also be affected by mechanical stresses: the effect of slicing in potato tubers was shown to greatly enhance the enzyme activity (Harada *et al.* 1980).

In pea, Allan and Trewavas (1985) described two forms of NAD kinase (CaM-dependent and CaM-independent form), evolving differently during root development. When physiological changes affect organisms, variations of the enzyme activity occurred that result in distinct individual molecular forms.

In an attempt to reveal NAD kinase activity on electrophoretic polyacrylamide gels, Jalouzot *et al.* (1994) have recently described the presence of three different isoforms revealed by a GTP-dependent NAD reductase activity in the dry seeds of common bean. The existence of different forms of enzyme in this plant material justified their potential exploitation as genetic markers of resistance to abiotic stresses. The purpose of the present work was to examine the effect of high NaCl concentration and wounding on the activities of the three different isoenzymes in the hypocotyl and root apex of two common bean genotypes displaying different degrees of tolerance to environmental stresses.

Materials and methods

Plant material: Two genotypes of common bean (*Phaseolus vulgaris* L.) were used: "Plant Introduction no 165426" (Pi), originating from South America and resistant to environmental stresses; and "Fins de Bagnols" (Ba), cultivated in France, with no known resistance against environmental stresses.

Seeds were sterilized with 1 % sodium hypochlorite, rinsed extensively with distilled water, and germinated for 3 d at 20 °C, on filter paper with a 16/8 h photoperiod (Sylvania lamps), irradiance 80 - 210 $\mu mol\ m^{-2}\ s^{-1}$.

Stress application: Three experiments were performed: (*1*) - seedlings were grown in the presence of 300 mM NaCl or in distilled water (control); (*2*) - after excision of the root part of 3-d old seedlings grown on distilled water, the remaining plants were disposed for 3 h in distilled water in Petri dishes; uncut seedlings, simultaneously grown, were used as control; (*3*) - an identical experiment than that described in (*2*) was carried out, but the sectioned hypocotyls were for 3 h in contact with 300 mM NaCl.

Preparation of the extracts: Root tips [2 - 3 mm long, experiment (*1*)] and hypocotyl pieces [2 mm long in the experiment (*1*), and 2 mm long above the cutting zone in the experiments (*2*) and (*3*)] were ground in a mortar in the presence of liquid nitrogen. Extracts were prepared as previously described (Jalouzot *et al.* 1994), with a 100 mM Tris-HCl buffer (pH 7.4) containing 2.4 mM EDTA, 10 % glycerol, 0.2 % *Triton X100*, 1 % PVP (just before use 14 mM 2-mercaptoethanol, 5 mM DTT and 2 mM PMSF were added), with a powder-extraction buffer ratio of 1:2 (m/v). The protein concentrations in the 12 500 *g* supernatants were estimated according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Electrophoresis: Gel electrophoresis was carried out using an *SE600 Hoeffer* apparatus (*Biorad*, Iury, France), native gels (14 × 16 cm × 1.5 mm) were composed of 112 mM Tris-acetate buffer (pH 6.4) and of a 10 - 14 % polyacrylamide gradient. 25 mM Tris - 192 mM glycine (pH 8.3) was used as running buffer. The plant extract supernatants were adjusted to 6.5 g dm^{-3} of total protein, and 0.03 cm^3 were layered at the cathodic end of the gel. The electrophoresis was run at 4 °C for about 5 h (250 V, 10 mA).

Activity staining: After electrophoresis, the NAD reductase activity observed during the search of the NAD kinase was revealed using a staining solution containing: 30 cm^{-3} of 120 mM Tris-HCl buffer, pH 7.5, 7 mM $MgCl_2$, 2 mM $CaCl_2$ and 2.5 mM of both NAD and GTP, 1 mM PES and 0.42 mM MTT (Jalouzot *et al.* 1994). Gels were incubated at 32 °C for 12 h, destained and simultaneously fixed for 4 h in a solution of 10 % acetic acid and 5 % glycerol.

Gel analysis: The staining density of the bands was evaluated by scanning the gels in a flat bed apparatus (*Color One Scanner*, *Apple*, Paris, France). The data were analysed using the computer programme *NIH Image 1.52*, which allows peak area calculations (in arbitrary units). Results were expressed either as the total activity of the detected GTP-dependent reductase activities (by summation of all peak areas for a given extract), or as the relative contributions (expressed in percentages of the total activity of a given extract) of each of the individual peaks.

Results

NAD reductase activity in the hypocotyls and root tips of the cultivars Pi and Ba grown on water or 300 mM NaCl: NAD reductase electrophoretic patterns revealed 3 main isoforms, that were indicated isoform 1 (Rf 0.57), isoform 2 (Rf 0.49) and isoform 3 (Rf 0.40), in hypocotyls (Fig. 1A), as well as in root tips (Fig. 1B) of 3-d-old seedlings from Pi and Ba cultivars. In the control hypocotyl (Fig. 2A) and root tip extracts (Fig. 2B), a higher total activity was noticed in Ba (about 1.5 to 2.5-fold the total activity found in Pi comparable control organs). In both cultivars, isoform 1 accounted for 40 - 60 %, isoform 2 for 30 - 40 % and isoform 3 for about 10 - 20 %

in hypocotyls (Fig. 3A and 3B); in root tips, isoform 1, 2 and 3 represented 50 - 70, 30 - 40 and 1 - 10 %, respectively (Fig. 3C and 3D).

After germination on 300 mM NaCl, the staining intensity of the band pattern on gel increased significantly in the Pi cultivar, both in the hypocotyls and in the root tips (Fig. 1A and 1B). In the hypocotyls, the total activity of the NAD reductase isoforms was approximately 2 times that of the control (Fig. 2A) and up to 4-fold in the root tips (Fig. 2B). However, after NaCl treatment of Pi seedlings, the contribution of isoform 1 to total activity decreased by 30 - 40 % in the hypocotyls (Fig. 3A), as well as in the root tips (Fig. 3C) and isoform 2 did not vary in a

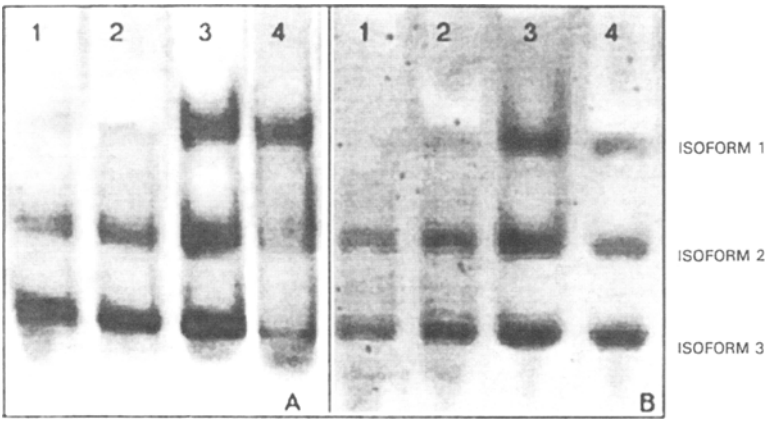

Fig. 1. Electrophoretic patterns of NAD reductase in extracts from hypocotyls (*A*) and root tips (*B*) of seedlings of two common bean cultivars (Pi and Ba), grown for 3 d on distilled water (control) or 300 mM NaCl. The figure corresponds to computer scannings of the gels. *1* - Pi, control; *2* - Ba, control; *3* - Pi, 300 mM NaCl; *4* - Ba, 300 mM NaCl.

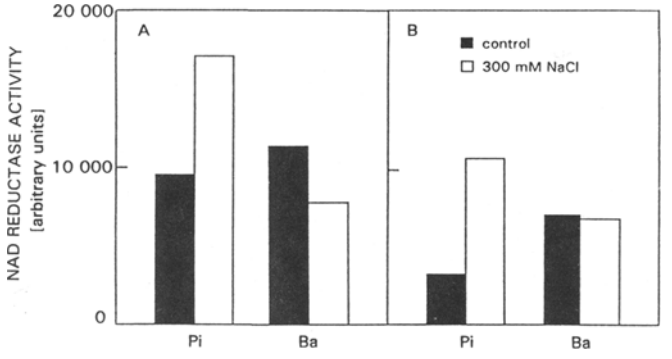

Fig. 2. Total NAD reductase activity (expressed in arbitrary units and calculated from densitometric analysis of gels presented in Fig. 1), in seedlings of the two common bean cultivars Pi and Ba grown either on distilled water (control) or in the presence of NaCl (300 mM). *A* - hypocotyls; *B* - root tips.

significant manner. Conversely, the NaCl strongly enhanced the contribution of isoform 3 in Pi (up to 2.5 - 3 and 15 times, in the hypocotyls and the root tips respectively). Thus, in NaCl treated Pi seedlings, the increase of total activity was mainly due to isoform 3.

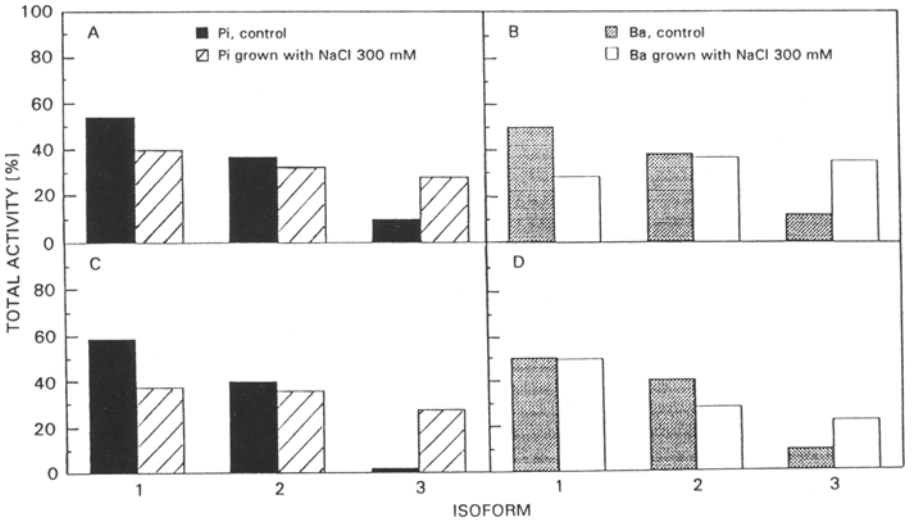

Fig. 3. Effect of NaCl (300 mM) on the contribution of each isoform of NAD reductase (expressed in % of total activity). *A* - Pi, hypocotyls; *B* - Ba, hypocotyls; *C* - Pi, root tips; *D* - Ba, root tips.

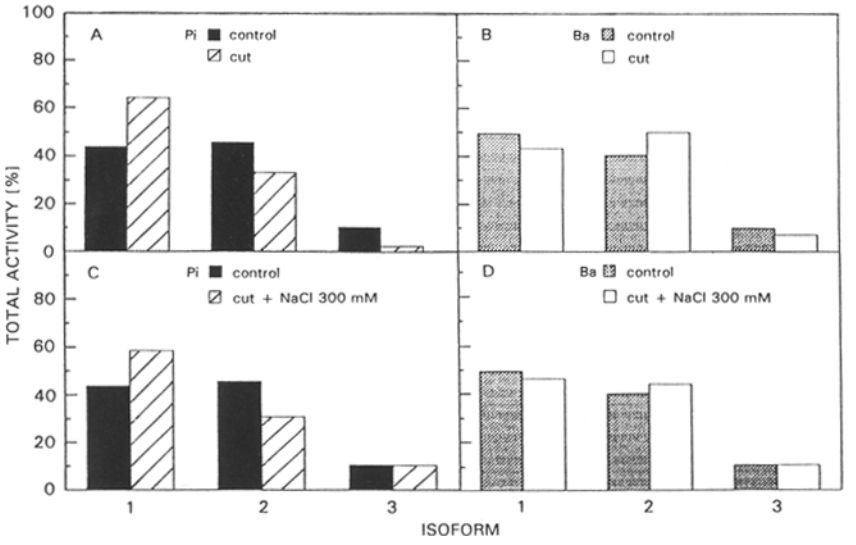

Fig. 4. Effect of a hypocotyl cutting on the contribution of each isoform of NAD reductase (expressed in % of total activity) in hypocotyl extracts of the common bean cultivars Pi and Ba. Cuttings followed by a 3-h contact with distilled water (*A* - Pi; *B* - Ba) or cuttings followed by a 3-h contact with 300 mM NaCl (*C* - Pi; *D* - Ba).

In NaCl-treated Ba seedlings, densitometric data showed that total activity of NAD reductase decreased by 40 % in the hypocotyls (Fig. 2A) but remained nearly unchanged in the root tips (Fig. 2B). The relative contributions in activity of the three isoforms in Ba (Fig. 3B and 3D), evolved roughly as described for Pi isoforms. Thus, for the cultivar Ba, the remarkable increase of the contribution of isoform 3 in NaCl-treated hypocotyls (Fig. 3B) or root tips (Fig. 3D) did not led to an increase of total activity, as it was in Pi seedlings.

NAD reductase activity in the hypocotyls of the Pi and Ba cultivars, 3 h after wounding: 3 h after cutting, the total activity of NAD reductase increased in the hypocotyls by factors about 1.5 in Pi and up to 2.5 in Ba (data not presented). The contribution of isoform 1 increased by 10 - 50 % in Pi (Fig. 4A), while it remained stable or diminished slightly in Ba (Fig. 4B). The contribution of isoform 2 decreased by 10 - 30 % in Pi, but increased by 10 - 25 % in Ba. The isoform 3 exhibited a drastic decrease in both cultivars: 30 - 75 % in Pi and about 30 % in Ba. The increase of total activity observed after wounding can consequently be correlated to an increase of activity of isoform 1 in Pi and isoform 2 in Ba.

NAD reductase activity after wounding plus NaCl treatment: After a mechanical stress (cutting) followed by a chemical one (300 mM NaCl), the total activity of NAD reductase increased by factors 1.5 - 2.5 in Pi and about 1.5 in Ba hypocotyls (data not presented). In both cultivars, NAD reductase activity related to isoform 1 and 2 were similar after either the sole cutting (Fig. 4A and 4B) or the double stress (Fig. 4C and 4D). The percentages of activity of isoform 3, which were depressed by wounding in Pi and Ba, were approximately readjusted to the control values when an additional NaCl treatment was applied on the wound (Fig. 4C and 4D) - or even slightly enhanced (results not shown). In both lines, the increase of total activity, due to the stress caused by wounding and NaCl, can be associated to the contribution of two isoforms in each of the cultivars: isoforms 1 and 3 in Pi, and isoforms 2 and 3 in Ba.

Discussion

Although the presented electrophoretic study did not estimate directly the NAD kinase activity, but revealed an associated GTP-dependent NAD reductase, it allowed to distinguish different responses to stresses between the two bean cultivars. After growth of the seedlings in the presence of 300 mM NaCl, the total activity greatly arose in the bean genotype known as being tolerant to environmental stresses. Comparable results were obtained after treatment by 2 mM cadmium chloride (data non shown). In agreement with these results, Slaski (1989) pointed out that aluminum stimulates much more NAD kinase activity in an aluminum-tolerant wheat genotype than in an aluminum-sensitive one (increases by factors 6 and 2.5, respectively).

On the other hand, a mechanical stress, stimulated the total activity of NAD reductase in both bean lines. However, the three isoforms of NAD reductase,

previously described in common bean (Jalouzot *et al.* 1994), did not contribute the same way to the increases in total activities in the two lines. During development of different species, evolution in the ratios of different isoforms of NAD kinase had already been evidenced (Afanasieva *et al.* 1982). The nature of the stress applied on bean seedlings determined differential and specific responses of the three isoforms (Table 1). Other studies have shown that plant resistance to environmental stresses (aluminum or drought) is correlated to a modified proportion of Ca^{2+}-CaM dependent and Ca^{2+}-CaM independent forms of NAD kinase (Slaski 1989, Zagdańska 1990). The Ca^{2+}-CaM complex could then be involved in the signal transduction of stresses *via* the intracellular regulation of Ca^{2+} in plant cells (Trewavas and Gilroy 1991). In addition exogenous Ca^{2+} can ameliorate the adverse effect of NaCl (Abd El-Samad 1993, Hamada 1994). The increase of GTP-dependent NAD reductase total activity in response to stresses particularly well demonstrated for the resistant bean line (Pi), could correspond to a *de novo* synthesis of the enzyme or to an activation of a pre-existing pool by Ca^{2+}-CaM. It will therefore be of special interest to determine whether the three isoforms described here, and possibly related to NAD kinase, are susceptible or not to the Ca^{2+}-CaM complex and if such a regulation could be associated with a mechanism of resistance in our plant material.

Table 1. Effect of 300 mM NaCl and cutting on NAD reductase total activities and on the contributions (in percentage of total activity) of each isoform 1, 2 and 3, in the cultivars Pi and Ba of common bean: – decrease of the activity, + increase of the activity, = control level, H - hypocotyls, R - root tips.

	NaCl				Cutting		Cutting + NaCl	
	Pi		Ba		Pi	Ba	Pi	Ba
	H	R	H	R				
Total activity	+	+	=	–	+	+	+	+
Isoform 1	–	–	–	=	+	– =	+	– =
Isoform 2	=	=	=	–	–	+	–	+
Isoform 3	+	+	+	+	–	–	= +	= +

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