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Article

Alteration in Pyridine Nucleotide Status in Cells as an Adaptive Response to Water Stress in Rice (*Oryza sativa* L.) Seedlings

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An adaptive measure of photosynthetic cells to a condition identified with a reduction of cellular energy charge, caused by water deficit-induced impairment of photosynthetic ATP production, was investigated using hydroponically cultured rice seedlings. Water stress treatment of the seedlings resulted in a marked decrease in cellular ATP level, a significant increase in the content of NAD(H) and concurrent decrease in that of NADP(H) in shoots, which accompanied a decrease in the activity of NAD kinase (EC 2.7.1.23) that specifically converts NAD(H) to NADP(H). The decline in the enzyme activity was particularly evident in the Ca^{2+}/calmodulin-dependent kinase, the major form of NAD kinase in plants, whereas the level of active calmodulin remained unchanged during water deficit. The ratio of $NADP^+$ to NADPH was maintained nearly constant and no increases were seen in the level of H_2O_2 and the activities of superoxide/H_2O_2-detoxifying enzymes in shoots stress-treated for two days. Based on these results, it may be suggested that rice plants take a strategy to cope with an adverse situation of limited photophosphorylation created by water deficit in that cells facilitate ATP production through glycolysis and oxidative phosphorylation; in doing so, rice cells suppress NAD kinase activity, consequently up-sizing the NAD(H) pool at the expense of the NADP(H) pool. Several parameters associated with the stress symptoms are also of implicative that there is no overproduction of superoxide radical or the related active oxygen at least in rice seedlings.

Key words : *water stress, pyridine nucleotide, NAD kinase, rice* (Oryza sativa *L.*).

Water stress refers to physiological dysfunctions in mesophytic plant species encountering water-limited environments. Although there is hardly a physiological process which is not affected by water stress, many lines of evidence indicate that photosynthesis is very sensitive to a water deficit condition.[1] This may be intuitive, because leaves situated in a severe shortage of water supply, close their stomata in order to suppress transpiration, preventing further water loss, which leads to a marked reduction of the CO_2 influx and consequently a decrease in the CO_2 fixation rate. The reduced activity of PCR in the stroma can in turn give rise to a reduced activity of PET in thylakoids; for the regeneration of $NADP^+$, the final electron acceptor of PET, through the PCR action is limited. The PET system itself may also be impaired by water stress, independently of the decreased PCR activity.[2] Regardless of whether the impairment of PET is associated with the limited CO_2 fixation or not, it would cause a substantial decrease in the photophosphorylation rate,[3] resulting in lowering the ATP level in cells. It has been speculated that, as the photosynthetic ATP production decreases, leaf cells respond to such adverse situation of the mitigated energy charge by enhancing oxidative phosphorylation coupled to mitochondrial electron transport and probably substrate-level phosphorylation linked to glycolysis.[4] If such is the case, cellular pyridine nucleotide status should be significantly affected in that the balance of NAD(H) to NADP(H) is shifted in favor of the former. This concept was subjected to experimental scrutiny in the present study.

Paddy rice is a semiaquatic plant that requires high levels of soil moisture for proper growth and development, and thus known to be among the most drought-sensitive

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Abbreviations: APOX ascorbate peroxidase; CaM, calmodulin; DW, dry weight; Cat, catalase; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; NAD(H), NAD^+ plus NADH; NADP(H), $NADP^+$ plus NADPH; PCR, photosynthetic carbon reduction; PET, photosyntheitc electron transport; PMSF, phenylmethylsulfonyl fluoride; POX, nonspecific peroxidase; PVPP, polyvinyl polypyrrolidone; rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TSP, total soulble protein.

cultivated plant species.[5] Due to this reason, rice has provided appropriate plant materials for physiological and biochemical investigations regarding water stress.[6] Using the seedlings, we conducted measurements of some parameters which are indicative of the occurrence of stress symptoms at molecular levels as well as the alteration in pyridine nucleotide status in cells responding to the decreased ATP production under water stress conditions. Mechanistic implications of the results are discussed, particularly focusing on changes in ATP, NAD(H), NADP(H) and Ca^{2+}/ CaM-dependent and -independent NAD kinase (EC 2.7.1.23).

Materials and Methods

Plant cultivation and water stress treatment. Rice (*Oryza sativa* L., cv. Daeya) seedlings were grown in a plastic tray ($20\times20\times15$ cm^3) under continuous illuminations (30 W/m^2) in a growth chamber, maintained at relative humidity of 60~65% and at 25 ± 1°C. Each tray was supplied with Hoagland solution just enough to cover the seeds once a day. On the 12th day of growth, rice seedlings were subjected to stress treatments by immersing roots in 30% PEG 6000 solution. After the stress treatments, shoots were harvested and used either immediately or stored at -60°C until use for various assays. The RWC of the shoot tissues was determined, as described by Smart and Bingham.[7]

Metabolite analyses. Samples were prepared by grinding freshly harvested and liquid nitrogen-frozen shoots in a mortar with addition of suitable ice-cold extraction media. ATP, extracted with 6% perchloric acid and neutralized by addition of 17 M ammonia and 0.1 M Hepes, was measured by an enzymatic end point method as in Brooks *et al.*[8] Pyridine nucleotide, extracted with 0.1 M KOH (for NADH and NADPH) or 0.1 M HCl (for NAD^+ and $NADP^+$), heated at 90°C for 10 min and neutralized with 0.1 M Tris (pH 8.0), was measured by the enzyme cycling method of Wagner and Scott.[9] Chlorophyll was spectrophotometrically measured in 80% acetone extract, as in Lichtenthaler.[10] H_2O_2, extracted in 5% trichloroacetic acid, was measured against catalase-treated samples by use of its reaction with 4-(2-pyridylazo)resorcinol-titanium (IV), as described by Patterson *et al.*[11] Lipid peroxidation was estimated by spectrophotometrically measuring the content of TBARS in shoot homogenates, prepared in 10% trichloroacetic acid containing 0.25% 2-thiobarbituric acid and heated at 95°C for 25 min, as in Heath and Packer.[12]

Enzyme assays. NAD kinase was extracted with ice-cold buffer A (50 mM Tris-Cl, 1 M KCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.5 mM PMSF and 2.5% PVPP, adjusted to pH 7.5), as in Anderson *et al.*[13] and assayed according to the procedure of Harmon *et al.*[14] with a slight modification. The reaction mixture for the assay of total NAD kinase activity comprised NAD^+ (2 mM), ATP (3 mM), $MgCl_2$ (5 mM), $CaCl_2$ (2 mM) CaM (9 μg/ml) and Tris-Cl (50 mM, pH 7.5) in addition to the enzyme extract. For Ca^{2+}/CaM-independent NAD kinase assay, EGTA (2 mM) was added to the reaction mixture in place of $CaCl_2$ and CaM. Parallel blanks were also prepared with the enzyme extract preheated in a boiling water bath for 2 min. The mixtures were incubated at 37 °C for 30 min and the reaction was terminated by heating in a boiling water bath for 2 min. After cooling and centrifugation, the content of NADP(H) in the supernatant was determined by the enzymatic cycling method as described above. Ca^{2+}/CaM-dependent NAD kinase activity was estimated by subtracting the Ca^{2+}/CaM-independent activity from total NAD kinase activity.

Antioxygenic enzymes were extracted with ice-cold 50 mM K-phosphate buffer (pH 7.0) and spectrophotometric assays were carried out at 25°C by continuously monitoring changes in absorbance for up to 30 s. SOD (EC 1.15.1.1) activity was determined by measuring the rates of reduction of acetylated cytochrome C by O_2^-, which was supplied by the xanthine-xanthine oxidase (EC 1.1.3.22) system, in the presence and absence of the extract, as in McCord and Fridovich.[15] One unit of SOD was defined as the amount of enzyme that lowered the rate of the cytochrome C reduction by 50%. APOX (EC 1.11.1.11) assay was based on the method of Nakano and Asada,[16] correcting the reaction rate for H_2O_2-independent oxidation of ascorbate. POX (EC 1.11.1.7) was assayed as in Amako *et al.*,[17] using pyrogallol as substrate, correcting for H_2O_2-independent formation of purprogallin. The activity of CAT (EC 1.11.1.6) was determined by following the disappearance of H_2O_2, as described by Beers and Sizer.[18]

Rubisco (EC 4.1.1.39) was extracted with 100 mM Hepes buffer (pH 7.8) containing 0.25 mM EDTA, 5 mM dithiothreitol, 1 mM ascorbate and 0.35 mM diethylthiocarbamic acid, and assayed following the procedures of Loza-Tavera *et al.*,[19] using $NaH^{14}CO_3$ (3.7×10^6 Bq/mmol).

Assay of CaM. CaM extract was prepared by homogenizing rice shoots, with ice-cold buffer B (50 mM Tris-Cl, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.5 mM PMSF and 1% PVPP, adjusted to pH 7.5), briefly boiling the homogenate and then centrifuging to obtain supernatant. CaM was assayed as in Harmon *et al.*,[14] using its ability to activate the Ca^{2+}/CaM-dependent NAD kinase in the reaction mixture consisting of 2 mM NAD^+, 3 mM ATP, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM Tris-Cl (pH 7.5), the CaM extract and partially purified Ca^{2+}/CaM-dependent NAD kinase. Parallel blanks lacking NAD kinase were also prepared. The content of NADP(H) was determined by employing the same procedure as for the NAD kinase assay. Total protein was quantified by the

Lowry method with bovine serum albumin as the standard in the same extract without heat treatment.

The Ca^{2+}/CaM-dependent NAD kinase was partially purified to a CaM-deficient state by use of anion exchange chromatogaphy according to Anderson *et al.*[13] Briefly describing, the enzyme was extracted from rice shoots with ice-cold buffer A and then subjected to $(NH_4)_2SO_4$ fractionation (30~55% saturation). The resulting pellet was dissolved in a minimal volume of buffer C (50 mM Tris-Cl, 0.1 M KCl, 1 mM EGTA, 2 mM $MgCl_2$ and 0.5 mM PMSF, adjusted to pH 7.5) and desalted by use of a Sephadex G-25 column. The enzyme solution was passed through a DEAE-Sephacel column equilibrated with buffer C so as to remove CaM.

A calibration curve for the assay of CaM was made with CaM, which had been purified from rice germs to homogeneity, as described by Gopalakrishina and Anderson.[20] Protein extraction was done with buffer B and the purification protocol utilized $(NH_4)_2SO_4$ fractionation, isoelectric precipitation at pH 4.0, affinity chromatography on phenyl sepharose CL-4B and DEAE Sephacel chromatography using a NaCl gradient (0 to 500 mM). The yield of CaM from rice germs was ca. 2.5 mg/kg fresh weight. From the comparative standpoint, bovine CaM was also prepared by employing essentially the same method, giving the final yield of ca. 70 mg/kg fresh weight of brain. The purity of CaM was assessed by sodium dodecyl sulphate-polyacylamide gel electrophoresis using the gel prepared by Laemmli's formulation.[21]

Results and Discussion

When hydroponically cultured rice seedlings were subjected to water stress treatment, rapid dehydration of shoot occurred, which accompanied a substantial increase in the formation of TBARS, a widely accepted index for membrane lipid peroxidation, as shown in Fig. 1. This is apparently pertinent to the concept that water stress is by nature oxidative stress.[22,23] It has frequently been proposed that the enhanced production of superoxide radical ($O_2^{\cdot-}$) and other reactive oxygen species originated from $O_2^{\cdot-}$, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), in cells by water deficit is the direct cause for damage-initiating cellular processes leading to physiological dysfunctions in plants.[5] In order to ascertain whether such was the case in rice seedlings, two approaches were made. Firstly, changes in the steady state level of H_2O_2 in the shoots upon exposure to water deficit were measured because this active oxygen is the immediate dismutation product of $O_2^{\cdot-}$. Secondly, enzyme assays were conducted for antioxygenic enzymes which are known to be involved in eliminating $O_2^{\cdot-}$ and H_2O_2 in plant cells. As summarized in Table 1, the H_2O_2 level was not risen in the shoots, but rather remarkably lowered by water deficit: consistent with this, no increases were seen in the activities of SOD, APOX, POX and CAT. In spite of the occurrence of oxidative processes in cells caused by water deficit, the lack of positive responses of those $O_2^{\cdot-}$ and H_2O_2-detoxifying enzymes to the stress treatment as well as the decreased production of H_2O_2 may lead one to rule out the possibility that overproduction of $O_2^{\cdot-}$ and $O_2^{\cdot-}$-originated active oxygen is associated with water deficiency-induced oxidative stress, at least, in rice seedlings. If this holds true, a certain activated chemical species other than $O_2^{\cdot-}$ and H_2O_2 may be assumed to be formed to an excessive extent in the stressed plant cells. An investigation on this possibility is currently in progress in this laboratory.

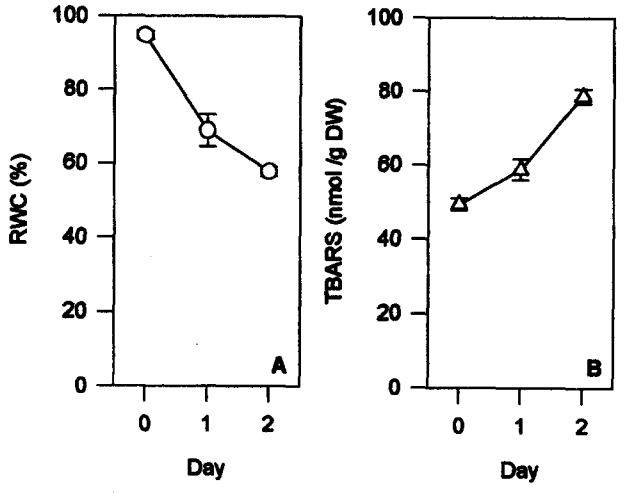

Fig. 1. Changes in RWC (A) and the content of TBARS (B) during water deficit in rice shoots. Water stress treatment was done by immersing roots in 30% PEG 6000 solution under continuous illumination of white light at 30 W/m^2. Data are expressed as means SD (n=4).

Effects of water deficit in the photosynthetic apparatus were checked by measuring changes in the content of chlorophyll and the activity of rubisco as the respective indices of structural and functional integrity of the light and dark phase reaction systems of chloroplasts. Fig. 2 shows that both chlorophyll content and rubisco activity in shoots decreased to a sizable extent during the stress treatment. For the moment, although no information is

Table 1. Alterations in the steady state level of H_2O_2 (μmol/g DW) and the activities of superoxide/H_2O_2-detoxifying enzymes (units/g DW) in rice shoots by water deficit*.

Parameters	Control	Stressed	Stressed/Control (%)
H_2O_2 content	3.08±0.28	1.18±0.11	38.3
SOD activity	5770±615	5440±587	94.3
APOX activity	119±12.1	110±6.34	92.4
POX activity	757±49.7	648±39.6	85.6
CAT activity	17900±763	10600±598	59.2

*Water stress treatment was done for 2 days and data are presented as means SD (n=4).

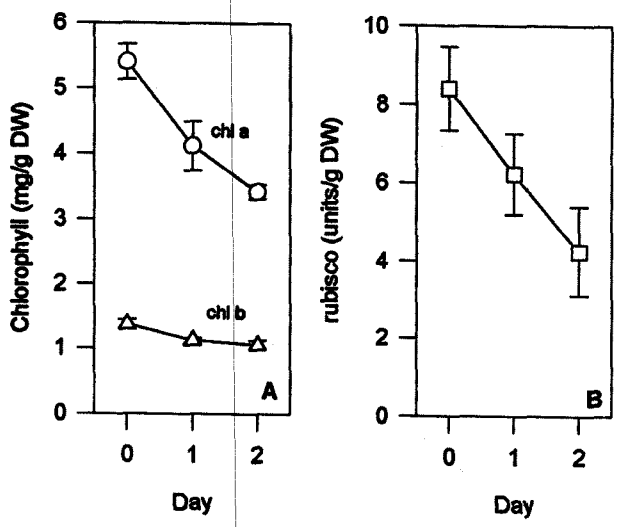

Fig. 2. Changes in the content of chlorophyll (A) and the activity of rubisco (B) during water deficit in rice shoots. The stress treatment and the data presentation are the same as in Fig. 1.

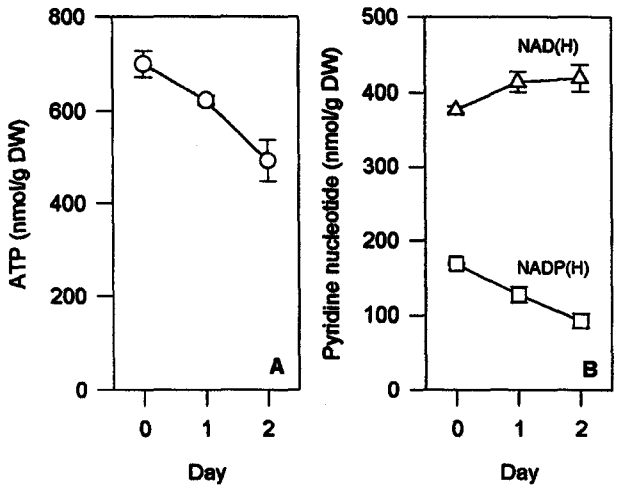

Fig. 3. Changes in the content of ATP (A) and pyridine nucleotide (B) during water deficit in rice shoots. The stress treatment and the data presentation are the same as in Fig. 1.

available as to whether the decrease in chlorophyll content was due to the oxidative degradation of the pigment in thylakoid membranes, it can be said that the machinery of the light-dependent phase of photosynthesis is indeed significantly affected by water deficit in cells.[24] It is not either certain whether the loss of rubisco activity resulted from oxidative protein degradation or reversible inactivation. However, a speculation may be made that a decrease in the light-driven H^+ uptake into intrathylakoid space and the accompanying efflux of Mg^{2+}, which are directly linked to the PET activity of thylakoid membranes, play a role in inactivating rubisco in chloroplasts stressed by water deficit (note that this enzyme of the PCR cycle *in situ* is regulated by light).[25,26]

In photosynthetically active leaves, the energy requirement for biological works is assumed to be largely met by the ATP produced by functioning chloroplasts. The impairment of photosynthesis would therefore result in a decrease in the steady state level of ATP in leaf cells. Under such circumstances, the cells may mobilize other energy-yielding machineries such as those in mitochondria and cytosol, stimulating the carbon-flux from reserve carbohydrate through glycolysis to the tricarboxylic acid cycle.[4] To facilitate these catabolic pathways, increased concentrations of NAD(H) should be naturally in demand. Results shown in Fig. 3 are in line with such notion. The ATP level in rice shoots gradually decreased with increasing duration of the stress treatment, which accompanied decreases in NADP(H) content and increases in NAD(H) content. The ratio of NADP(H) to total pyridine nucleotide was found to be ca. 30% in unstressed shoots but reduced to ca. 17% in the shoots stress-treated for 2 days. Even though the increment of NAD(H) content was not precisely in agreement with the decline in NADP(H) content, total content of NAD(H) plus NADP(H) could be said to be remain more or less constant during the stress treatment. Another interesting observation was that the ratio of NADPH to $NADP^+$ remained virtually unchanged by water deficit, being ca. 34% in rice shoots.

Because NAD kinase is the only enzyme in cells that converts NAD(H) to NADP(H) and thus responsible for adjusting cellular balance between these two classes of pyridine nucleotide under varied physiological conditions,[27] and because the increase in NAD(H) content of the stressed shoots appeared to achieve at the expense of NADP (H), it would be reasonable to expect that the enzyme activity is reduced by water stress. Results shown in Fig. 4 conform to this expectation. Apart from animal tissues, there are two forms of NAD kinase in plants; Ca^{2+}/CaM-dependent and -independent enzymes, the former being the major form.[28] Interestingly enough, the activity of the former which functions only in the presence of CaM, a protein along with Ca^{2+} involved in signal transduction processes in biological systems, showed much higher sensitivity to water deficit in tissues, as compared with Ca^{2+}/CaM-independent NAD kinase activity. In order to ascertain whether the high susceptibility of the Ca^{2+}/CaM-dependent enzyme to the stress-induced inactivation was due to a decrease in CaM concentration, CaM present in the shoots was monitored during stress treatment, finding out that there were virtually no changes in CaM content as in TSP content (see panels B and C of Fig. 4). The CaM quantification was performed by using the same protein purified from rice germs to homogeneity: rice CaM has an apparent molecular weight of 18 kDa, but 16 kDa when bound to Ca^{2+}, with somewhat higher mobility on gels compared with bovine CaM, as shown in Fig. 5, and is distinctive from the latter in UV absorption characteristics, demonstrating one tyrosine residue in rice CaM *versus* two in bovine CaM (data not shown). The cellular level of CaM in shoots of rice seedlings

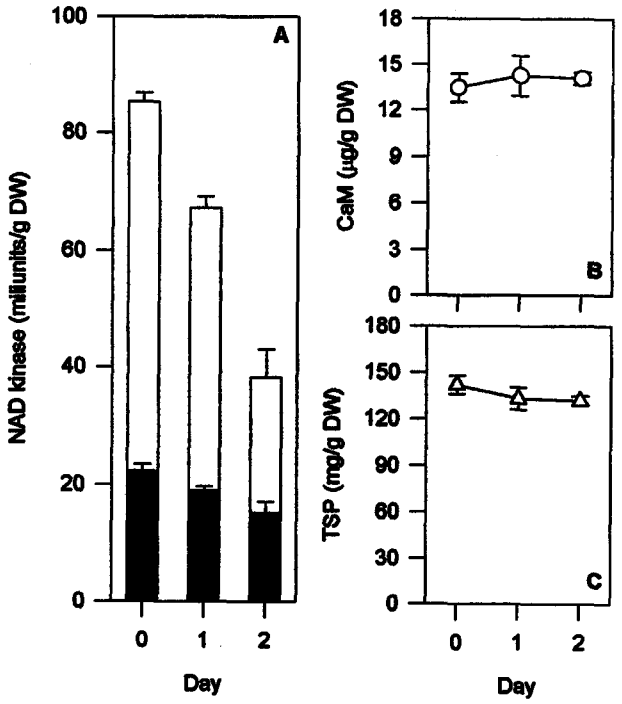

Fig. 4. Changes in the activity of NAD kinase (A), and the content of CaM (B) and TSP (C) in rice shoots during water deficit. In panel A, the share of the Ca^{2+}/CaM-independent enzyme in NAD kinase activity is represented by darkened regions of the respective bars. The stress treatment and the data presentation are the same as in Fig. 1.

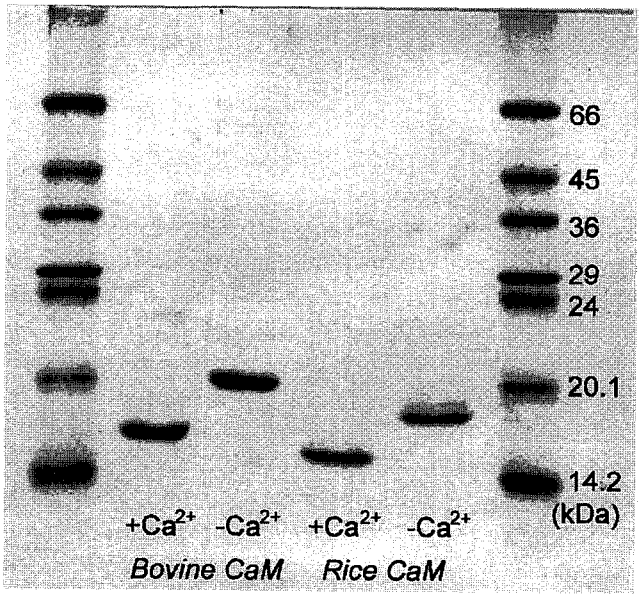

Fig. 5. Electrophoresis of CaMs purified from rice germs and bovine brain. The proteins were separated on 15% polyacrylamide gel containing 0.1% sodium dodecyl sulphate in the presence of 2 mM $CaCl_2$ or the presence of 2 mM EGTA, and were stained with Coomassie Blue.

was estimated to be ca. 1.8 μg/m*l* without consideration of its cellular compartmentation. Since this concentration is high enough to fully activate NAD kinase, CaM in the stress treated shoots is not thought to limit the activity of the target enzyme.

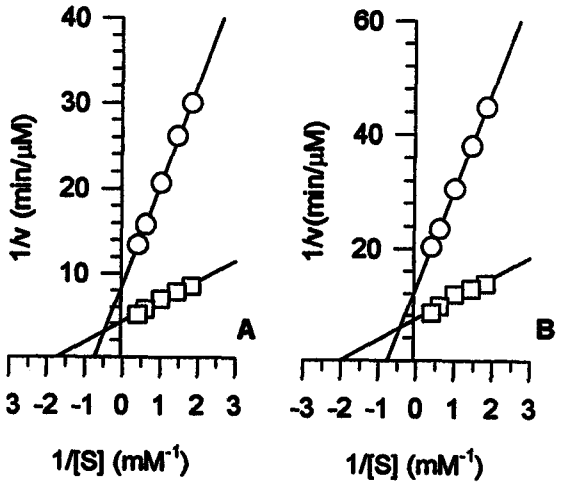

Fig. 6. The double reciprocal plots for the activity of NAD kinase measured with varied cocentrations of NAD^+ using crude extracts from the control (A) and 2 day water-stressed rice plants (B). The assay was done at constant concentration of ATP (3 mM) in the presence of 2 mM EGTA for Ca^{2+}/CaM-independent NAD kinase (○) and in the presence of 2 mM $CaCl_2$ plus 9 μg/ml rice CaM for total NAD kinase activity (□).

The decrease in the activity of NAD kinase during water deficit could be ascribed to the conformational change of the enzyme caused by a low water status and a high osmotic potential in leaf cells. This point was checked by examining enzyme kinetic properties of both Ca^{2+}/CaM-dependent and -independent forms; K_m values for NAD^+ were estimated from the double reciprocal plots, as shown in Fig. 6. It turned out that the values are 1.30 mM and 1.35 mM for the Ca^{2+}/CaM-independent activity and 0.49 mM and 0.58 mM for the total NAD kinase activity (Ca^{2+}/CaM-independent plus Ca^{2+}/CaM-dependent activity) in the stressed and unstressed rice shoots, respectively, indicating no significant difference in K_m values between two shoot samples. Based on this observation, the possible association of the structural alteration of NAD kinase by water stress with the reduction of its activity may be ruled out: instead it is tentatively assumed that the enzyme activity in cells decreased probably due to a slowdown of the enzyme synthesis under water stress conditions.

In conclusion, regarding cellular events at molecular level occurring in cells of rice shoots exposed to water deficiency the following inference may be drawn from the results presented herein. (1) There is no overproduction of O_2^- and O_2^--originated active oxygen in chloroplasts of water stressed rice plants. It is neither likely that the redox components of photosynthetic electron transport including $NADP^+$ are excessively reduced, as has been hypothesized as the cause of presumably increased formation of reactive oxygen species and thus oxidative damage to cells by the toxic oxygen.[5] (2) A decrease in

the phosphorylation rate and the resultant decline in cellular ATP level stimulate the energy-yielding processes linked to the NAD(H) pool using reserve carbohydrate at the expense of anabolism connected to the NADP(H) pool in the stressed tissues. This is probably one of the adaptive measures of plants to an adverse physiological condition caused by limited water supply. (3) A stress signal is supposedly transmitted to the NAD kinase synthesizing system. Responding to the signal, this system adjusts its activity to reduce cellular content of NAD kinase, resulting in suppression of the conversion of NAD (H) to NADP(H).

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