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Induction of nitrate reductase activity and mRNA accumulation in rice

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Key words: mRNA accumulation, nitrate induction, rice

Abstrak

Analisis 'Western blot' dengan menggunakan antiserum NADH-nitrat reduktase menunjukkan bahawa ekstraksi daun yang diperoleh daripada anak padi yang telah diaruh dengan larutan nitrat, telah mengeluarkan jalinan polipeptid yang reaktif. Jalinan polipeptid ini didapati mempunyai molekul seberat 115 000 dalton. Ekstraksi yang sama juga telah didapati mengandungi kedua-dua aktiviti NADH- dan NAD(P)H-nitrat reduktase. Aktiviti NADH-nitrat reduktase telah dikesan 2 jam selepas aruhan nitrat dan meningkat dengan cepat selama 24 jam. Aktiviti ini terus meningkat secara perlahan sehingga 48 jam dan menurun kemudiannya. Pertambahan kandungan nitrat di dalam daun menunjukkan corak yang sama dengan aktiviti NADH-nitrat reduktase tetapi tidak sama dengan corak aktiviti NAD(P)H-nitrat reduktase. Aktiviti NAD(P)H-nitrat reduktase telah dikesan dalam masa 1 jam selepas diaruh, kemudiannya meningkat dengan mencapai maksimum dalam masa 3 jam. Kedua-dua klon pHBH1 dan pHBH2 menghibrid kepada mRNA yang berukuran 3.2 kb, tetapi kemunculan mRNA yang spesifik terhadap klon pHBH1 dan pHBH2 adalah pada jangka masa yang berlainan. Kajian tentang pengaruhan yang menggunakan jujukan DNA yang unik sebagai 'probe' telah menunjukkan bahawa klon pHBH1, mengesan mRNA yang berukuran 3.2 kb. Peningkatan mRNA yang berukuran 3.2 kb ini telah menunjukkan corak kinetik yang sama dengan aktiviti NADH-nitrat reduktase. Peningkatan mRNA yang berukuran 3.2 kb telah dikesan bermula selepas 1 jam pengaruhan dan menurun selepas 6 jam kemudian berakhir dari 12 jam hingga 24 jam. Sementara aktiviti NADH-nitrat reduktase meningkat dengan cepat semasa 4 jam pertama dan menurun selepas 48 jam pengaruhan nitrat. Klon pHBH2 telah mengesan mRNA yang berukuran 3.2 kb tetapi peningkatan mRNA ini mengikut corak kinetik yang sama dengan aktiviti NAD(P)H-nitrat reduktase. mRNA berukuran 3.2 kb telah dikesan sangat awal dan mencapai maksimum selepas 30 minit pengaruhan nitrat dan menurun dengan cepatnya. mRNA ini tidak dapat dikesan dari 6 jam hingga 24 jam selepas pengaruhan. Aktiviti NAD(P)H-nitrat reduktase meningkat dengan cepat mencapai maksimum dalam masa 3 jam dan berakhir selepas 5 jam. Keputusan ujikaji ini mencadangkan bahawa klon pHBH1 ialah gen yang bertanggungjawab bagi mengeluarkan enzim NADH-nitrat reduktase, sementara klon pHBH2 mengeluarkan enzim NAD(P)H-nitrat reduktase.

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Keputusan ini juga menandakan bahawa terdapat perbezaan susunan pada aras transkripsi antara NADH dan NAD(P)H-nitrat reduktase.

Abstract
Western blot analysis using barley NADH-nitrate reductase antisera revealed that nitrate-induced rice seedling leaf extracts contain a cross reacting polypeptide of 115 000 dalton. Nitrate-induced rice seedling leaf extracts appeared to contain both NADH- and NAD(P)H-nitrate reductase activity. The NADH-nitrate reductase activity was detected 2 h after the addition of nitrate and increased rapidly to 24 h. The activity continued to increase at a slower rate until about 48 h and declined there after. The results on the nitrate content in leaf showed a similar pattern to the NADH-nitrate reductase activity. The NAD(P)H-nitrate reductase activity was detected within 1 h, after induction with nitrate which increased rapidly reaching a maximum activity within 3 h. Both pHBH1, and pHBH2 clones (accompanying paper) hybridized to a 3.2 kb mRNA, but the appearance of the mRNA following nitrate induction was different. Induction studies using unique sequences of each clone as the probe revealed that clone pHBH1 hybridized to a 3.2 kb mRNA. Upon induction by nitrate, it showed kinetics similar to NADH-nitrate reductase activity. The 3.2 kb mRNA was detected after 1 h and peaked at 6 h after induction and declined from 12 h to 24 h. The NADH-nitrate reductase activity increased rapidly during the first 4 h and peaked at 48 h after nitrate induction. Clone pHBH2 hybridized to a 3.2 kb mRNA. Upon induction by nitrate, it showed kinetics similar to NAD(P)H-nitrate reductase activity. The 3.2 kb mRNA was detected very early, reached maximum 30 min after nitrate induction, and declined very rapidly. No 3.2 kb mRNA was detected from 6 h to 24 h after induction. The NAD(P)H-nitrate reductase activity was inducible by nitrate. The activity increased very rapidly reaching maximum within 3 h, declined after 5 h, and very little from 10 h to 72 h. These results suggested that clone pHBH1 codes for NADH-nitrate reductase while clone pHBH2 codes for NAD(P)H-nitrate reductase. The results also indicated that the regulation at transcriptional levels is different between the NADH and NAD(P)H-nitrate reductase.

Introduction

Rice plants are adapted to reductive soil conditions but it has been shown (Malavolta 1954) that they can utilize nitrate as the sole nitrogen source. Reduction of nitrate is catalyzed by nitrate reductase (NR) and the activity of nitrate induced NR has been reported in rice plants (Tang and Wu 1957; Oji and Izawa 1968; Shen 1969; Shawney and Naik 1973). NR is the rate limiting enzyme in the assimilation pathway of nitrate that is required for normal plant growth (Beevers and Hageman 1969). Nitrate simultaneously induces both NAD(P)H-NR and NADH-NR. The NAD(P)H-NR can accept electrons from NADH and NADPH. By using affinity chromatography on blue dextran sepharose, the two types of nitrate reductases in rice separated were NADH-NR and NAD(P)H bispecific-NR (Shen et al. 1976). NAD(P)H bispecific-NR utilizes NADPH directly as reductants and has preference for NADPH but reacts about half as well with

NADH (Shen et al. 1976). However, studies comparing the subspecies *indica* and *japonica* with respect to the rate of NR induction were limited (Ta and Ohira 1982; Zhenwu et al. 1984;). No study has been reported concerning the induction of NR at the mRNA level in rice. In barley (Cheng et al. 1986) and squash (Crawford et al. 1986) northern blot analyses showed that nitrate induction caused a marked increase in the steady state levels of NR mRNA.

There is little literature on the determination of the molecular weight of NR in rice. The molecular weight of native enzyme was reported to be 330 000 with six identical subunits of 57 000 (Zhenwu et al. 1984) and 240 000 each (Leong and Shen 1982). However, in most higher plants, the molecular weight of NADH-NR is approximately 200 000 with subunit size of approximately 100 000 (Notton and Hewitt 1979; Kuo et al. 1980; Redinbaugh and Campbell 1985). The deviations in reported NR subunit molecular weight in rice may be due to degradation of NR in vitro by proteolysis.

The objectives of this study were to determine the subunit molecular weight of NR from both *indica* and *japonica* rice subspecies by using antisera raised against barley NADH-NR, and to study the nitrate induction of NADH-NR and NAD(P)H-NR both at the activity and mRNA levels.

Materials and methods

Plant materials and growth

The cultivar M 201 of *Oryza sativa* L. subspecies *japonica* was obtained from the Department of Agronomy, University of California, Davis; the cultivar Labelle of subspecies *indica* was obtained from USDA, ARS, Southern Plain Areas, Beumont, Texas. The seeds were germinated for 2 days at room temperature in the dark and then transferred to a growth chamber with continuous light (300 $\mu E^{-1}m^{-1}s^{-1}$) at 26 °C. After 7 days growth on distilled water, NR was induced with 15 mM nitrate solution. Nitrate supply was renewed at 24 h intervals.

Determination of NR subunit molecular weight by western blot

Non-induced and 24-h nitrate induced leaves were extracted with 1 mL/gfw extraction buffer (Kuo et al. 1982) containing 250 mM Tris-HCl, pH 8.2, 1mM EDTA, 3 mM DTT, 1 μM Na_2MoO_4, 5 μm FAD, 250 μM PMSF, 1 μg/mL pepstatin, 10 mM antipain, and 2.5% (w/v) Bovine Serum Albumin (BSA). Crude extracts were adjusted to 4% (w/v) SDS and 5% (v/v) 2-mercaptoethanol SDS-PAGE of the samples was carried out in 1.5 mm gels [7.5% (w/v) acrylamide, 0.1% (w/v) N'-N'-methelyne bisacrylamide] at 4 °C (Laemmli 1970).

The protein were electroblotted to nitrocellulose filter according to Towbin et al. (1979). The nonspecific protein binding sites on the filters were blocked as described by Tsang et al. (1983) and Saravis (1984). The nitrocellulose sheets were treated for 6 h at room temperature with monospecific antiserum raised against barley NADH-NR (Sommers et al. 1983). The antiserum was diluted 1:200 in PBS (10 mM K phosphate, 150 mM NaCl, pH 7.3) containing 3% (v/v) liquid gelatin, 0.05% (v/v) Tween 20. The antigen-antibody complex was visualized by immunogold (Integrated Separation System) with 1:500 dilution in PBS containing 3% (v/v) liquid gelatin, 0.05% (v/v) Tween 20.

Nitrate reductase induction

Green leaves were sampled at different times after induction with 15 mM nitrate solution. Leaves were extracted with 6 mL/gfw of extraction buffer (Kuo et al. 1982). Leaf nitrate content was measured according to Lowe and Gillespie (1975),

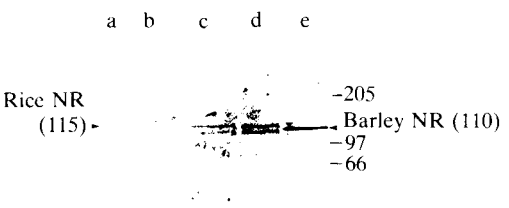

Figure 1. Two cross-reacting polypeptide bands of 115 kD and 105 kD from 24 h induced rice seedling extracts were observed in cv. Labelle subspecies ***indica*** *(d) and in cv. M 201 subspecies* ***japonica*** *(c). One cross-reacting polypeptide band of 110 kD was detected in barley cv. Steptoe (e). Cross-reacting bands were not detected from non-induced seedling leaf extracts in either cv. M 201 subspecies* ***japonica*** *(a) or cv. Labella subspecies* ***indica*** *(b)*

from an aliquot of the crude extracts. The NADH-NR activities were determined as described by Kuo et al. (1980). The NADPH-NR activities were determined as described by Dailey et al. (1982).

Induction of nitrate reductase mRNA

Total RNA was extracted from non-induced and nitrate-induced seedling leaves using guanidium/phenol procedure of Feramisco et al. (1982). Nitrate-induced total RNA was extracted at different times after induction. The high molecular weight RNA was precipitated with lithium chloride by the method of Wagoner et al. (1982). NR-mRNA accumulation was measured using the fragment *BglII/EcoRI* (5.6 kbp) of clone pHBH1 and fragment *ApaI/XhoI* (3.7 kbp) of clone pHBH2 (Hamat 1989) as hybridization probes in dot blot northern analysis (Thomas 1983). Each fragment was oligolabelled (Feinberg and Vogelstein 1983) with ^{32}P CTP. Hybridization and washing conditions were according to the methods of Church and Gilbert (1984).

Results

Rice nitrate reductase polypeptides

Western blot analysis using barley NADH-NR antisera revealed that nitrate induced rice seedling leaf extracts from both cv. Labelle subspecies *indica* and cv. M 201 subspecies *japonica* contained two cross-reacting polypeptides of 105 000 and 115 000 *(Figure 1)*. Cross-reacting polypeptide bands were not detected from non-induced seedling leaf extracts. One band of 110 000 was detected from nitrate-induced barley cv. Steptoe seedling leaf extracts. The two cross-reacting polypeptide bands from induced rice seedlings may represent two different rice nitrate reductases with homology to the barley NADH-NR. Alternatively the 105 000 band could be a degradation product of the 115 000 protein. However, this is not confirmed in this experiment.

Nitrate reductase activity induction

NADH-NR activity was detected 2 h after the addition of nitrate to 7-day-old rice seedlings and increased rapidly to 24 h in both cv. Labelle subspecies *indica* and cv. M 201 subspecies *japonica (Figure 2)*. The activity continued to increase at slower rate until about 48 h and declined after 72 h in both subspecies (data not shown). The activity was higher in cv. Labelle subspecies *indica* than cv. M 201 subspecies *japonica* at all the time intervals included in the experiment. Low NADH-NR activity was detected from non-induced seedlings in both subspecies. The nitrate content of the leaf showed a similar pattern to the NADH-NR activity *(Figure 2)*. The amount of nitrate in the leaf was identical in both cv. Labelle subspecies *indica* and cv. M 201 subspecies *japonica* for the first 6 h after nitrate induction. But from 6 h to 24 h the cv. Labelle subspecies *indica* was able to absorb more nitrate than cv. M 201 subspecies *japonica*.

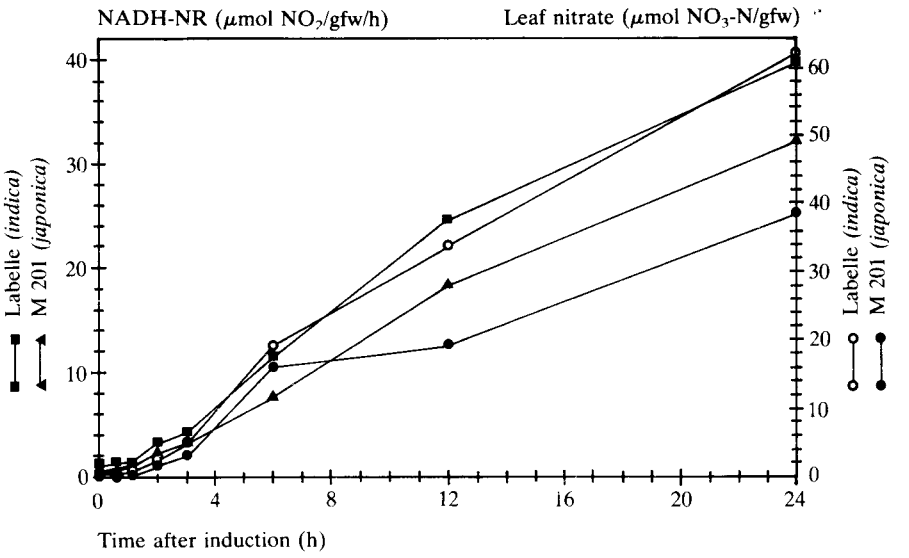

Figure 2. NADH-nitrate reductase activity and leaf nitrate content of rice cv. Labelle and M 201 after nitrate induction

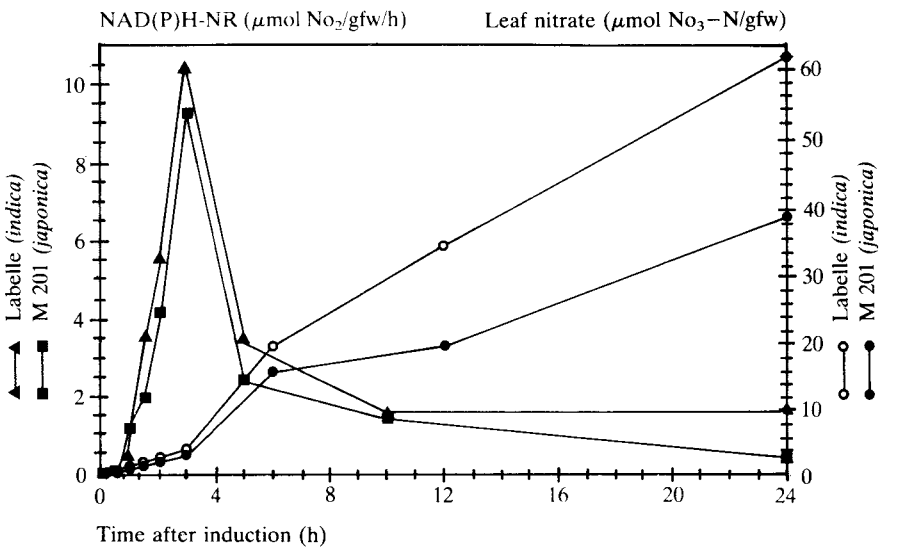

Figure 3. NAD(P)H-nitrate reductase activity and leaf nitrate content of rice cv. Labelle and M 201 after nitrate induction

The induction of NAD(P)H-NR activity was even more rapid reaching a maximum activity in both subspecies within the first 3 h of induction *(Figure 3)*. The NAD(P)H-NR activity was first detected 1 h after application of nitrate to the seedling roots. The maximum NAD(P)H-NR activity (10.2 μmol NO_2/gfw/h) was however, much lower than the maximum NADH-NR activity (42 μmol NO_2/gfw/h). The NAD(P)H-NR activity declined rapidly after 6 h and essentially very little activity was detected from 10 h to 72 h. NAD(P)H-NR was not detected from non-induced seedling leaf extracts of either subspecies. The NAD(P)H-NR activity did not follow nitrate content of the leaves *(Figure 3)*. However, NAD(P)H-NR responded to nitrate induction much earlier and at lower leaf nitrate concentrations as compared to NADH-NR. The nitrate induced NAD(P)H-NR activity was higher than the nitrate induced NADH-NR activity in the leaves for the first 3 h.

Induction of nitrate reductase mRNA

The NR-mRNA accumulation measured with *BglII/EcoRI* fragment of clone pHBH1 was very rapid for the first 6 h and declined also very rapidly thereafter *(Figure 4)*. The pattern of induction was similar for both cultivars but cv. Labelle subspecies *indica* showed higher level of mRNA accumulation at the various times sampled. At 6 h after induction the amount of mRNA accumulated was equivalent to 200 pg in cv. Labelle and 120 pg in cv. M 201 (data not shown).

The NR-mRNA accumulation measured with the *ApaI/XhoI* fragment of clone pHBH2 was very rapid reaching maximum within 30 min *(Figure 5)*. The mRNA declined abruptly at 2 h and no *ApaI/XhoI* specific mRNA was detected from 6 h to 24 h. The pattern of *ApaI/XhoI* specific mRNA induction was similar for both cv. Labelle subspecies *indica* and cv. M 201 subspecies *japonica*.

Discussion

Western blot analysis, using barley NADH-NR antisera, showed that nitrate-induced rice seedling leaf extracts contained two polypeptides of 105 000 and 115 000. These results suggest that rice may contain two different NRs with subunits of 105 000 and 115 000. Alternatively, the lower molecular weight band may represent a degradation product of the 115 000 polypeptide. The 105 000 polypeptide band was less intense and appeared diffused indicating that it may be a degradation product of 115 000 NR-polypeptide *(Figure 1)*. This possibility is in agreement with other reports (reviewed in Kleinhofs et al. 1985) suggesting that NR is very sensitive to proteolytic degradation. Most higher plant NADH-NR are homodimers with the estimated molecular weight of holoenzyme of 220 000 from barley 4+ Steptoe (Kuo et al. 1982), 200 000 from spinach (Notton and Hewitt 1979), *Nicotiana tabacum* (Mendel and Muller 1980), wheat (Jones and Mhuimhueachain 1985), and 230 000 from squash (Redinbaugh and Campbell 1985). The molecular weight of SDS subunits is about 110 000 for barley (Kuo et al. 1982); 115 000 for squash (Redinbaugh and Campbell 1985) and corn (Campbell and Remmler 1986). In rice, the molecular weight of NR was estimated to be 330 000 with six identical subunits of 57 000 each (Zhenwu et al. 1984) and 240 000 (Leong and Shen 1982). These workers however, did not include protease inhibitors in their extraction buffers and the smaller subunit molecular weight observed may be due to proteolytic degradation. In this work a crude extract was rapidly prepared in the presence of protease inhibitors thus minimizing any possible proteolytic degradation.

Very low levels of NADH-NR activity were detected in 7-day-old seedlings growing on distilled water. This observation was supported by RNA

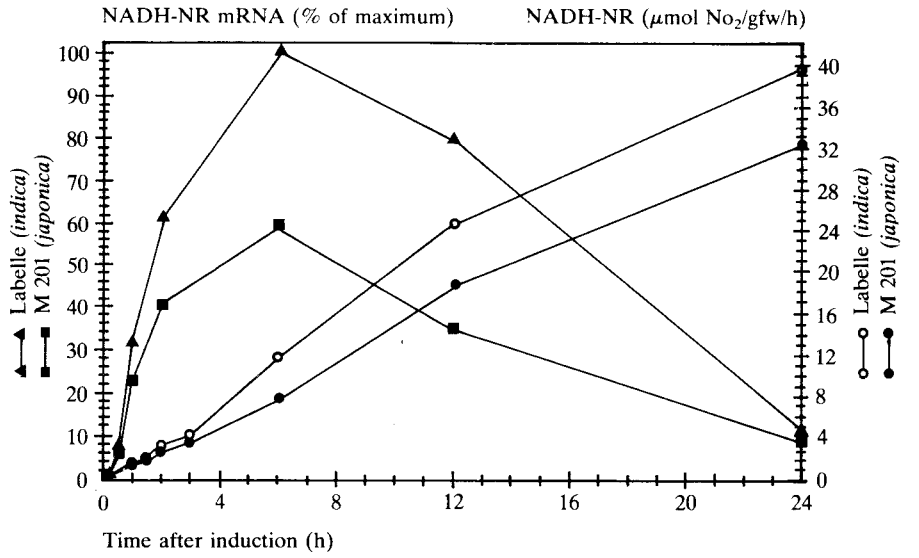

Figure 4. Nitrate reductase mRNA accumulation and NADH-nitrate reductase activity after nitrate induction with respect to time in rice cv. Labelle and M 201

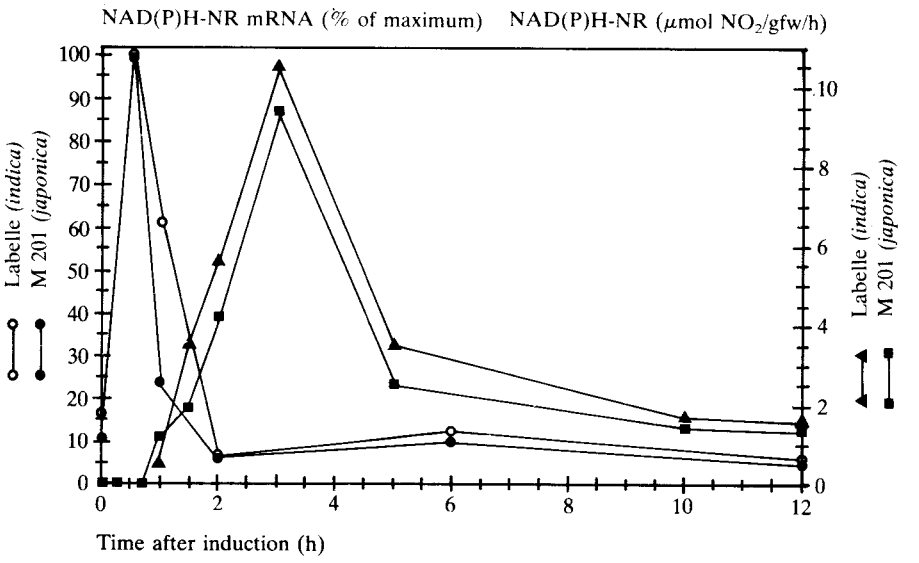

Figure 5. Nitrate reductase mRNA accumulation and NAD(P)H-nitrate reductase activity after nitrate induction with respect to time in rice cv. Labelle and M 201

accumulation assays that showed a low level of 3.2 kb mRNA from non-induced seedlings *(Figure 4)*. Others have reported constitutive NR in rice (Shen 1969). The amount of constitutive NADH-NR is very low when compared to the amount of induced NADH-NR.

Nitrate induced NADH-NR activity was detected within 2 h after addition of nitrate. The NADH-NR activity increased rapidly from 2 h to 12 h and showed a steady increase for at least 24 h *(Figure 4)*. Others have reported a lag period of 1 h (Sasakawa and Yamamoto 1978) or 2 h (Misra et al. 1980) in rice. Compared to barley, rice showed a quicker NADH-NR response to nitrate induction. In barley, NADH-NR activity and cross reacting materials were detected 2 h to 4 h after the addition of nitrate and increased to a maximum at 24 h (Sommers et al. 1983).

The NR-mRNA measured by hybridization with a probe from the pHBH1 clone was first detected after 30 min of nitrate induction and increased to a maximum at about 6 h. The accumulation curve of this mRNA was similar to the NADH-NR activity curve except for about a 3-h lag *(Figure 4)*. These data suggest that the pHBH1 clone codes for the NADH specific NR.

Upon induction with nitrate the NAD(P)H-NR activity increased rapidly reaching a maximum within 1 h. The very rapid induction of the NAD(P)H-NR suggested the possibility that activation of NR protein might be taking place. However, no NR specific protein was detected in rice seedlings before nitrate induction *(Figure 1)*. The NR-mRNA measured by hybridization with a probe from the pHBH2 clone, reached a maximum accumulation within 30 min after nitrate induction and then rapidly declined to near zero by 2 h *(Figure 5)*. These data suggested that the pHBH2 clone may code for the NAD(P)H-NR. The NAD(P)H-NR activity was measured only with NADPH as the electron donor. Therefore, it is not known if this is a NADPH specific enzyme or NAD(P)H bispecific enzyme. However, since no other NADPH specific NR are known in higher plants and an NAD(P)H bispecific NR has been previously reported in rice (Shen et al. 1976), it is suggested that this is an NAD(P)H bispecific NR.

The specific and rapid appearance and disappearance of the NAD(P)H-NR suggest some specific role for it in the plant development. What it might be is not clear at this time. The DNA sequence upstream of presumed 5' end of NAD(P)H-NR gene hybridized to a RNA band of 1.9 kb (Hamat 1989). However, this 1.9 kb RNA was not nitrate-induced. The possible regulatory role of the 1.9 kb RNA is not known. The regulatory mechanisms for turning on the NADH-NR and NAD(P)H-NR are clearly different. The appearance of NAD(P)H-NR activity at a very early stage of nitrate induction suggest that a trace amount of nitrate was able to induce NAD(P)H-NR *(Figure 3)*. A possible feedback inhibition might have taken place because increased accumulation of nitrate resulted in decreased NAD(P)H-NR activity at the later time points. On the other hand, the activity of NADH-NR showed almost identical pattern to the amount of nitrate in the leaf *(Figure 2)*. One could investigate these possibilities by studying the responses or the inducibility of NR promotor sequences by the different amount of inducer (nitrate) in the tissues.

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