MARDI Res. J. 17(1) 1989: 1–11

Molecular cloning and characterization of nitrate reductase genes in rice (*Oryza sativa* L.)

H. B. Hamat*

Key words: nitrate, nitrate reductase genes, cloning, rice

Abstrak

Satu klon nitrat reduktase cDNA klon yang diperoleh daripada tanaman barli telah digunakan sebagai 'probe' dalam ujikaji penghibridan untuk mengenalpasti penyusunan gen nitrat reduktase di dalam daun padi cv. M 201 (subspesies *japonica*) dan Labelle (subspesies *indica*). Tiga jalur *EcoRI* (18, 14 dan 12 kbp) telah dikesan di dalam cerakinan DNA daripada tanaman padi cv. Labelle dan empat jalur (18, 14, 10 dan 3 kbp) di dalam cv. M 201. Analisis 'southern dot blot' telah menunjukkan bahawa lapan gen nitrat reduktase per haploid genom didapati dalam cv. Labelle dan enam di dalam cv. M 201. Koleksi DNA diperoleh daripada daun padi cv. M 201 yang disepara potong oleh *EcoRI* dan disimpan di dalam vektor lambda Charon 35, telah ditapis bagi memisahkan dan mencirikan gen nitrat reduktase. Klon yang mengandungi serpihan *EcoRI*, 14 dan 18 kbp telah dikecilkan dan dikenalpasti sebagai lambda AR1 dan lambda BR1. Serpihan 8.2 kbp *EcoRI/BamHI* lambda AR1 dan serpihan 13.5 kbp *EcoRI/BamHI* lambda BR1 adalah homologus kepada 'probe' cDNA barli dan disubklonkan ke dalam pUC8 untuk membentuk plasmid rekombinan pHBH1 dan pHBH2. Satu peta endonuklease bagi klon-klon pHBH1 dan pHBH2 telah dibentuk dan lokasi 3' penghujung gen nitrat reduktase padi telah dikenalpasti di dalam setiap plasmid rekombinan. Kedua-dua klon ini didapati berlainan. Klon pHBH1 mengandungi jujukan DNA yang unik, 5.6 kbp, yang disisikan oleh ketinggian ulangan jujukan DNA pada 3' tetapi tidak pada 5' penghujung. Kemungkinan klon ini tidak mengandungi 5' penghujung gen nitrat reduktase padi. Walau bagaimanapun, pHBH2 mengandungi jujukan unik 6.5 kbp diiringi oleh ketinggian ulangan jujukan DNA pada kedua-dua 5' dan 3' penghujung. Hasil penyelidikan ini mencadangkan bahawa klon pHBH2 mengandungi gen nitrat reduktase padi yang sempurna. Sebagai hasil daripada ujikaji penghibridan, yang menggunakan setiap serpihan DNA dari pHBH1 hibridisasi yang positif telah ditunjukkan terhadap RNA yang diperoleh daripada anak-anak padi yang telah diaruhkan oleh nitrat. Hasil kajian ini menyokong kesimpulan bahawa klon pHBH2 mengandungi gen nitrat reduktase yang sempurna sementara pHBH1 tidak.

Abstract

A barley Nitrate Reductase cDNA clone was used as a hybridization probe to investigate the nitrate reductase gene organization in rice cv. M 201 (subspecies *japonica*) and Labelle (subspecies *indica*). Three *EcoRI* bands

*Central Research Laboratories Division, MARDI, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Author's full name: Halim bin Hamat

(18, 14, and 12 kbp) were detected in cv. Labelle DNA and four (18, 14, 10 and 3 kbp) in cv. M 201. Southern dot blot analysis indicated that there were eight nitrate reductase gene copies in cv. Labelle and six in cv. M 201 per haploid genome. A partial *EcoRI* digested cv. M 201 DNA library in lambda Charon 35 vector was screened in order to isolate and characterize the rice nitrate reductase genes. Clones containing the 14 kbp and 18 kbp *EcoRI* fragments were isolated and designated lambda AR1 and lambda BR1 respectively. The 8.2 kbp *EcoRI/BamHI* fragment of lambda AR1 and the 13.5 kbp *EcoRI/BamHI* fragment of lambda BR1 were homologous to the barley cDNA probe, and were subcloned into pUC8 to form recombinant plasmids pHBH1 and pHBH2, respectively. The restriction endonuclease maps of pHBH1 and pHBH2 clones were constructed, and the 3' end of the rice nitrate reductase gene in each recombinant plasmid was determined. The two clones were different. pHBH1 contained unique DNA sequence of 5.6 kbp which was flanked by highly repetitive DNA sequence at the 3' but not at the 5' end. This clone might not contain the 5' end of the gene. However, pHBH2 contained unique sequences of 6.5 kbp flanked by highly repetitive DNA sequences at both the 3' and 5' ends. These results suggested that pHBH2 clone contains a complete nitrate reductase gene. Results from hybridization of each DNA fragment from pHBH1 and pHBH2 to total RNA isolated from nitrate induced rice seedling further supported the conclusion that pHBH2 clone contained a complete nitrate reductase gene while pHBH1 did not.

Introduction

Rice is the most important food crop in the tropics, occupying nearly 140 million hectares and most of the rice grown on them is rainfed (Barker and Herdth 1979). In rainfed situations, the soil may dry and become oxidized. Nitrate is particularly susceptible to loss through denitrification if the soil is reflooded or if the nitrate moves to a reduced soil zone. Several researchers (Khind and Ponnamperuma 1981, Fillery and Vleck 1982) have suggested that rice plants are efficient sinks for nitrate and that they greatly reduce the significant denitrification losses.

Nitrate reductase (NR) catalyzes the initial reduction of nitrate to nitrite and is considered a rate limiting step in the assimilation of nitrate (Beevers and Hageman 1969; Hewitt 1975). NR is a complex enzyme containing FAD, cytochrome b_{557}, and molybdenum as prosthetic group (Kleinhofs et al. 1985). Nitrate induced *de novo* synthesis of NR has been demonstrated in barley (Sommers et al. 1983) and in tobacco XD cells (Zielky and Filner 1971). In rice, NR is inducible by nitrate (Shen 1969) and NR regulation is influenced by parameters such as light (Tang and Wu 1957), nitrate (Shen 1969), NR inhibitors (Leong and Shen 1982), and hormones (Jiangming et al. 1986).

Understanding NR regulation at the molecular level would be a significant step towards improving nitrate assimilation in rice. Before the study of NR regulation can be conducted, the DNA suquences involved must be identified and isolated. This paper describes the molecular cloning and characterization of the nitrate reductase genes in rice.

Materials and methods

Southern blot analysis of nitrate reductase gene organization

Total rice DNA was prepared from 7-day-old green leaves of cultivars M 201, subspecies *japonica* and Labelle, subspecies *indica* according to the procedure of Murray and Thompson (1980). DNA samples (10 μg) were digested with *EcoRI*, and the resulting DNA fragments were fractionated in 0.4% agarose gels and transferred onto nylon 66 filters according to Thomas (1980). Hybridization was carried out at 68 °C for 18 h using a barley NR cDNA probe (1.1 kbp) (Cheng et al. 1986) radioactively labelled by oligolabelling (Feinberg and Vogelstein 1983). Hybridization buffers, hybridization and washing conditions were according to Church and Gilbert (1984). The stringency of the final wash was at (Tm − Ti) = 12 °C (Beltz et al. 1983). The G + C contents of rice chromosomal DNA from subspecies *japonica* and *indica* are 41.7% and 44.4% respectively (Iyengar et al. 1979). Filters were exposed for 24 h to Kodak XAR5 x-ray films at −70 °C with double intensifying screens.

Total rice DNA samples (0.25−1 μg) from cultivars M 201 and Labelle were hybridized with the 1.1 kbp barley NR cDNA probe to determine the copy number equivalent per haploid genome of rice. Copy number equivalent was calculated based on rice genome size of 0.6 pg or 5 x 10^8 bp (Iyengar and Sen 1978). Copy number equivalent was constructed using a barley NR cDNA plasmid clone, bNRp10 (3.7 kpb) (Cheng et al. 1986). A dot blot method of Kafatos et al. (1979) was employed.

Isolation of nitrate reductase genomic clones

Lambda phage, Charon 35, was grown on *Escherichia coli* strain KH802, ($hsdR^-$, $hsdM^+$, lac^-, gal^-, met^-, supE) (Wood 1966). *E. coli* DH1 (F^-, recA1, endA1, gyrA96, thi1, hsdR17 (r_k^-, m_k^-), supE44, relA1?, lambda$^-$) (Hanahan 1983), was the recipient strain for plasmid transformation. The rice genomic library consisted of partial *EcoRI* digested rice cultivar M 201 DNA ligated into Charon 35 vector.

Approximately 40 000 phages were grown per plate, and a total of 200 000 plaques were screened using NR cDNA clone of barley as the probe. The positive plaques were picked, replated and rehybridized until a pure positive clone was isolated. Phage DNA was prepared according to Puhler (1984). Preliminary restriction endonuclease mapping of the DNA from positive isolates indicated that two different recombinant lambda clones harbouring 14 kbp and 18 kbp were recovered. Subsequently, the 8.2 kbp *EcoRI/BamHI* and the 13.5 kbp *EcoRI/BamHI* fragments that were homologous to the barley NR cDNA were subcloned into pUC8 and named pHBH1 (10.8 kbp) and $pHBH_2$ (16.1 kbp).

Single and double digestions of pHBH1 and pHBH2 DNA with restriction endonuclease enzymes that recognize hexanucleotide sequences were used to construct the restriction maps of pHBH1 and pHBH2. The 3' end of the NR gene was determined by hybridizing restriction endonuclease digested DNA blots with the 3' end of the barley NR cDNA.

Determination of unique and repeated DNA sequences

pHBH2 DNA was digested into four fragments (*ApaI/XhoI*, 3.7 kbp; *XhoI/BssHII*, 2.8 kbp; *BssHII/BglII*, 2.7 kbp; and *BglII/EcoRI*, 3.5 kbp), and pHBH1 DNA into three fragments (*BamHI/BglII*, 2.6 kbp; *BglII/EcoRV*, 3.4 kbp; and *EcoRV/EcoRI*, 2.2 kbp). Each of these fragments was ^{32}P-labelled by oligolabelling and used as a probe for hybridization with *EcoRI* digested total rice genomic DNA from cv. M 201, subspecies *japonica*.

Determination of the functional regions of the clones

Total RNA from cultivar M 201 was extracted from non-induced and nitrate-induced seedling leaves using guanidium/phenol RNA extraction procedure of Feramisco et al. (1982). The high molecular weight RNA was precipitated with lithium chloride by the method of Wagoner et al. (1982). Total RNA samples (10 μg) were denatured with glyoxal and dimethysulfoxide at 50 °C for 1 h and electrophoresed in 1% agarose gels under denaturing conditions (Maniatis et al. 1982). Denatured RNA was transferred to nylon 66 filters which were then baked for 2 h at 80 °C (Thomas 1980).

Each of the four fragments of pHBH2 (*ApaI/XhoI*, 3.7 kbp; *XhoI/BssHII*, 2.8 kbp; *BssHII/BglII*, 2.7 kbp; *BglII/EcoRI*, 3.5 kbp) and each of the four fragments of pHBH1 (*BamHI/BglII*, 2.6 kbp; *BglII/EcoRV*, 3.4 kbp; *EcoRV/EcoRI*, 2.2 kbp; *EcoRI/SmaI*, 1.4 kbp) was used as a probe in Northern blot analysis (Church and Gilbert 1984). Filters were washed under conditions where there was minimal cross hybridization at the DNA-DNA level. The final wash was at 75 °C with 40 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA. The stringency was at Tm − Ti = 5 °C (Beltz et al. 1983).

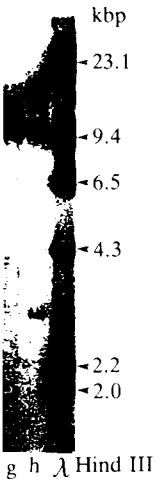


Figure 1. Southern blot analysis of restriction enzyme ***EcoRI*** *digested total rice and the isolated lambda clones DNA. Ten-μg total DNA samples of rice cv. Labelle subspecies* ***indica*** *(g), and cv. M 201 subspecies* ***japonica*** *(h) were digested with* ***EcoRI*** *and probed with barley NADH reductase cDNA (1.1 kbp).*

Results

Nitrate reductase gene organization

Southern blot analysis showed that the barley NR cDNA probe hybridized to three *EcoRI* bands of 18, 14, and 12 kbp in cv. Labelle, subspecies *indica* and four *EcoRI* bands of 18, 14, 10 and 3 kbp in cv. M 201, subspecies *japonica* (*Figure 1*). These results suggest that there may be at least three different NR genes in rice. The 3 and 10 kbp fragments in cv. Labelle, subspecies *indica*, may be equivalent to the 12 kbp fragment in cv. M 201, subspecies *japonica*. Dot blot analysis

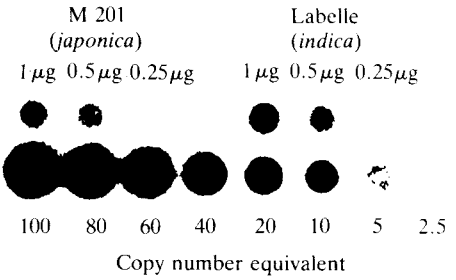


Figure 2. Estimation of nitrate reductase gene copies in rice cv. Labelle and M 201. The copy equivalent was calculated based on 1 μg total rice DNA

showed that there were six nitrate reductase gene copies in cv. M 201, subspecies *japonica*, and eight in cv. Labelle, subspecies *indica*, per haploid genome of rice (*Figure 2*).

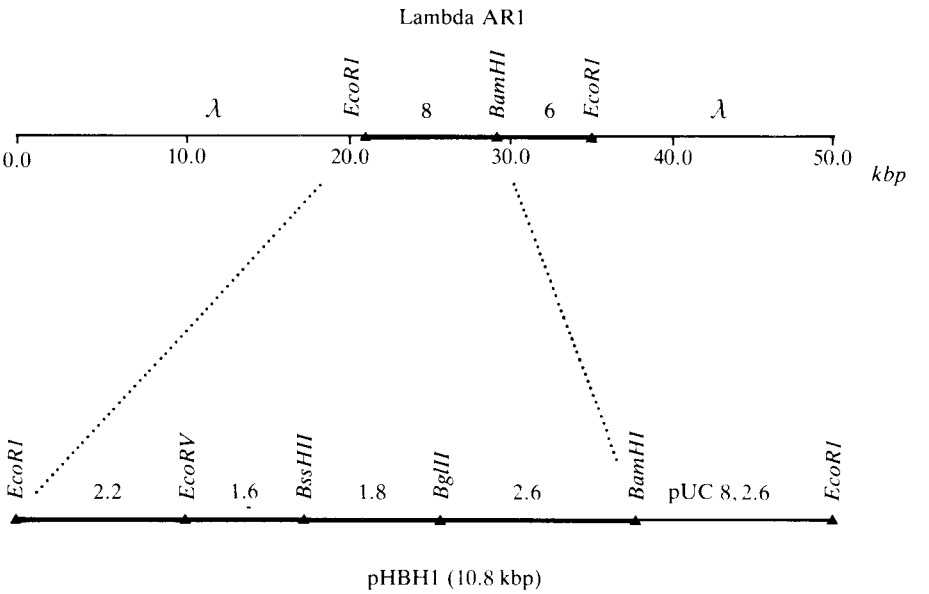


Figure 3. Partial restriction enzyme maps of clone lambda AR1 and the derived plasmid pHBH1. The heavy line indicates the rice DNA insert and the thin line indicates vector DNA

Isolation and characterization of genomic clones

Ten recombinant lambda clones were isolated by screening the cv. M 201 genomic library with the barley NR cDNA probe. Two of these, lambda AR1 and lambda BR1, contained the 14 and 18 kbp *EcoRI* fragments from cv. M 201, respectively (*Figure 3* and *Figure 4*). The 8.2 kbp *EcoRI/BamHI* fragment of lambda AR1 and 13.5 kbp *EcoRI/BamHI* fragment of lambda BR1 contained the region of homology to the barley cDNA and were subcloned into pUC8. The resultant hybrid plasmid clones were designated pHBH1 (10.8 kbp) and pHBH2 (16.1 kbp) (*Figure 3* and *Figure 4*). Even though there were two *BamHI* sites, 10 kbp apart, located on the insert DNA in pHBH2, an identical fragment of 10.2 kbp obtained from lambda BR1 and pHBH2 hybridized strongly to the probe (fragment *ApaI/XhoI*, 3.7 kbp, derived from pHBH2) indicating that the two *BamHI* sites were intact during subcloning.

Restriction endonuclease maps of pHBH1 and pHBH2 are shown in *Figure 5* and *Figure 6* respectively. Results of hybridization between restriction endonuclease digested DNA of pHBH1 and pHBH2 with the barley NR cDNA probe showed that the 1.8 kbp DNA fragment (*BglII/BssHII*) of pHBH1 (*Figure 5*) and the 3.7 kbp fragment (*ApaI/XhoI*) of pHBH2 (*Figure 6*) were homologous to the barley NR cDNA. The 3' end of the NR gene was located at the *BglII* site of pHBH1 and at the *ApaI* site of pHBH2. Since the probe used was not a full length NR cDNA the results showed that the NR gene may be in the region from the *BglII* site extending to or beyond the *EcoRI* site in pHBH1 (*Figure 5*), and from the *ApaI* site extending to or beyond the *EcoRI* site in pHBH2 (*Figure 6*).

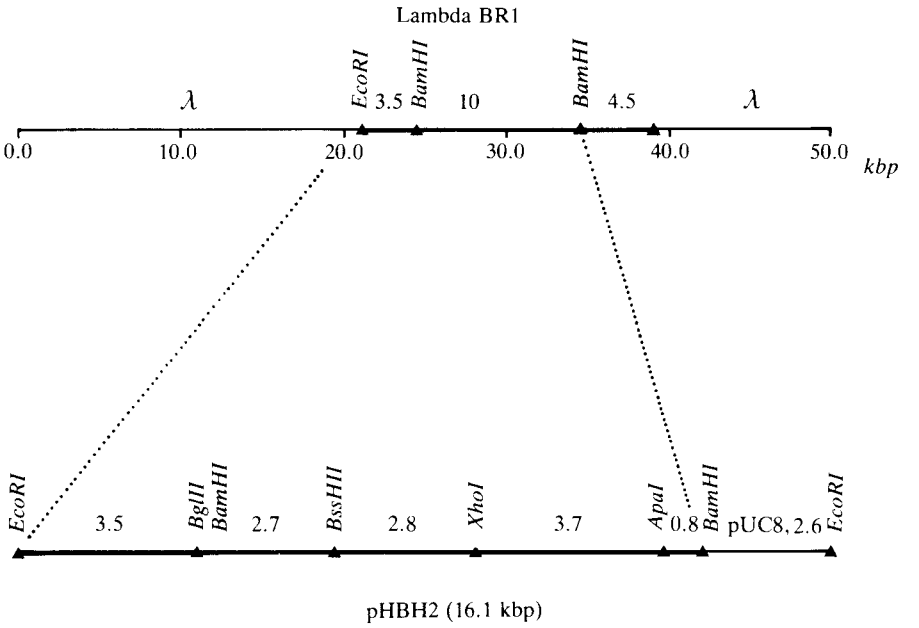
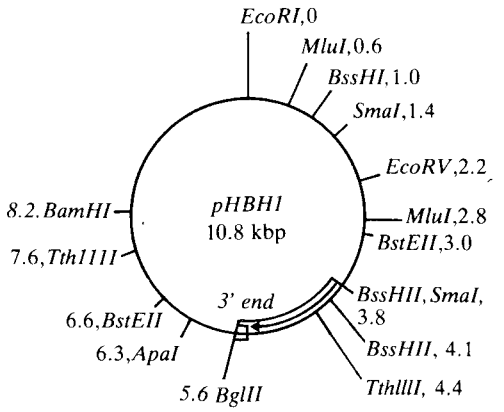


Figure 4. Partial restriction enzyme maps of clone lambda BR1 and the derived plasmid pHBH2. The heavy line indicates the rice DNA insert and the thin line indicates the vector DNA



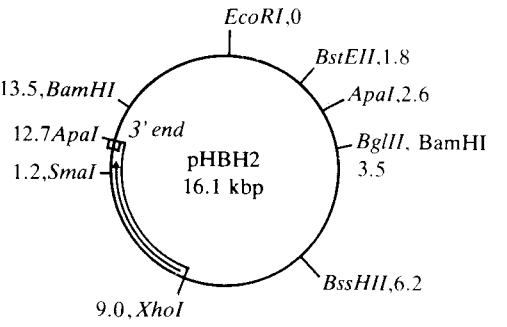


Figure 5. Restriction endonuclease enzyme map of genomic clone pHBH1. The double line indicates the region of homology to the barley nitrate reductase cDNA. The arrow indicates the presumed direction of transcription terminating at the 3' end of the gene determined by homology to the 3' end of the barley nitrate reductase cDNA. The 5' end of the gene was not located. The pUC8 vector DNA is from ***BamHI*** *to* ***EcoRI*** *(2.6 kbp)*

Figure 6. Restriction endonuclease enzyme map of genomic clone pHBH2. The double line indicates the region of homology to the barley nitrate reductase cDNA. The arrow indicated the presumed direction by homology to the 3' end of the barley nitrate reductase cDNA. The 5' end of the gene was not located. The pUC8 (2.6 kbp) vector is from ***BamHI*** *(13.5 kbp) to* ***EcoRI***

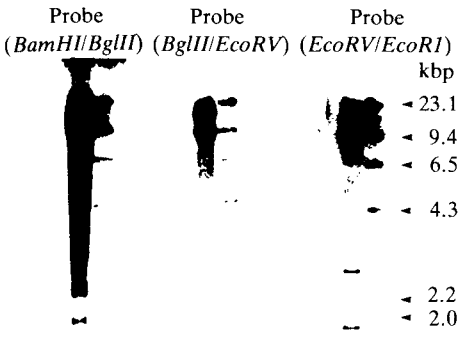


Figure 7. Southern blot analysis to determine the region of repetitive and non-repetitive DNA sequences on genomic pHBH1

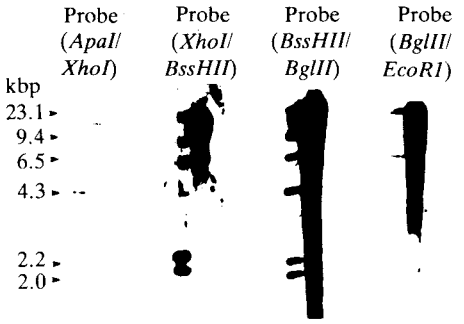


Figure 8. Southern blot analysis to determine the region of repetitive and non-repetitive DNA sequences on genomic pHBH2

Unique and repeated DNA sequence regions of nitrate reductase genomic clones

The nature of the DNA sequences of the NR genomic clones was investigated. Fragments *BglII/EcoRV* (3.4 kbp) and *EcoRV/EcoRI* (2.2 kbp) of pHBH1 (*Figure 7*) and fragments *ApaI/XhoI* (3.7 kbp) and *XhoI/BssHII* (2.8 kbp) of pHBH2 (*Figure 8*) hybridized to distinct bands on Southern blots of total DNA of cv. M 201, indicating that these fragments represented unique or low copy number sequences. However, the fragment *BamHI/BglII* (2.6 kbp) of pHBH1 (*Figure 7*) and fragments *BssHII/BglII*

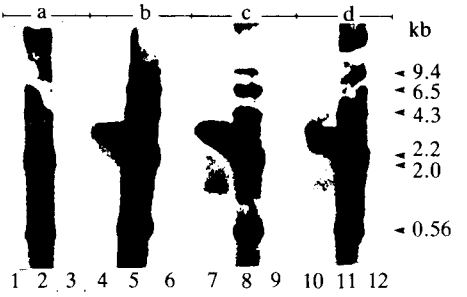


Figure 9. Northern blot analysis to identify the region of the genomic clone pHBH1 which codes for nitrate reductase mRNA. Total RNA samples from 12-h nitrate induced (lanes 1, 4, 7, 10) and non-induced (lanes 3, 6, 9, 12) rice seedlings were used. Lanes, 2, 5, 8, 11 are HindIII digested lambda DNA markers. Fragments ***BglII/EcoRV*** *(3.4 kbp) (column b),* ***EcoRV/EcoRI*** *(2.2 kbp) (column c),* ***SmaI/ EcoRI*** *(1.4 kbp) (column d) strongly hybridized to a mRNA band of 3.2 kb from 12-h nitrate induced seedlings (lanes 4, 7, 10). Faint bands of the same size (3.2 kbp) were observed from non-induced seedlings (lanes 6, 9, 12). The fragment* ***BamHI/BglII*** *(2.6 kbp) (column a) did not hybridize to any band from either 12 h nitrate induced or non-induced seedlings*

(2.7 kbp) and *BglII/EcoRI* (3.5 kbp) of pHBH2 (*Figure 8*) hybridized to multiple bands and formed a smear suggesting that they represented highly repeated DNA sequences.

Functional regions of nitrate reductase genomic clones

Northern blot analysis was conducted to identify the NR coding regions of the NR clones. Fragments *BamHI/BglII* (2.6 kbp), *BglII/EcoRV* (3.4 kbp), *EcoRV/ EcoRI* (2.2 kbp), and *EcoRI/SmaI* (1.4 kbp) of pHBH1 were used to probe the leaf total RNA from non-induced and 12 h nitrate induced rice cv. M 201 seedlings. *Figure 9* shows that except for the *BamHI/ BglII* fragment (2.6 kbp), the other three

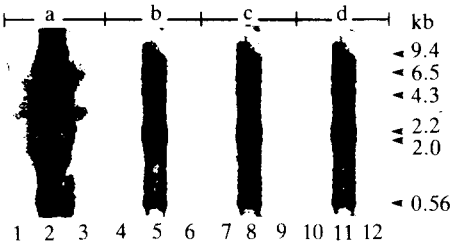


*Figure 10. Northern blot analysis to identify the region of the genomic clone pHBH2 which codes for nitrate reductase mRNA. Total RNA samples extracted from non-induced (lanes 3, 6, 9, 12), and 12-h nitrate induced (lanes 1, 4, 7, 10) rice seedlings were used. Lanes, 2, 5, 8, 11 are HindIII digested lambda DNA markers. The fragments (**ApaI/XhoI** 3.7 kbp column a: **XhoI/BssHII** 2.8 kbp column b: **BssHII/BglII,** 2.7 kbp column c: and **BglII/EcoRI**, 3.5 kbp, column d) did not hybridize to RNA bands from either non-induced or 12 h nitrate induced samples.*

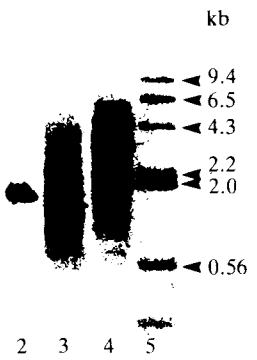


*Figure 11. Northern blot analysis of total rice seedling RNA isolated 30 min after nitrate induction using clone pHBH2 DNA fragments as hybridization probe. A 3.2 kb mRNA band was detected by fragment **ApaI/XhoI** (lane 4). The fragment **XhoI/BssHII** hybridized to two mRNA bands of 3.2 kb and 1.9 kb respectively (lane 3), while the fragment **BssHII/BglII** hybridized to a single mRNA band of 1.9 (lane 2). The fragment **BglII/EcoRI** did not hybridize to any mRNA band (lane 1), lane 5 is HindIII digested lambda DNA markers*

fragments of pHBH1 detected a RNA band of 3.2 kb in the total RNA samples from 12 h nitrate induced rice seedlings. Bands were not detected by any of the fragments in the total RNA from non-induced rice seedlings (*Figure 9*). *Figure 10* shows that none of the four fragments (*ApaI/XhoI*, 3.7 kbp; *XhoI/BssHII*, 2.8 kbp; *BssHII/BglII*, 2.7 kbp; and *BglII/EcoRI*, 3.5 kbp) of pHBH2 hybridized to any RNA from the same non-induced and 12 h nitrate induced rice seedlings. The results showed that the *EcoRI/BglII* fragment (5.6 kbp) of pHBH1 contained sequences that hybridized to a mRNA of 3.2 kb which is presumed to be the NR gene mRNA. The pHBH2 apparently did not contain any sequence capable of hybridizing with this mRNA at the conditions used.

However, when the experiment was repeated using total RNA isolated from rice cv. M 201 seedlings which were induced with nitrate for 30 min, fragments *ApaI/XhoI* (3.7 kbp), *XhoI/BssHII* (2.8 kbp) and *BssHII/BglII* (2.7 kbp) of pHBH2 detected a 3.2 kb RNA band, two RNA bands of 3.2 kb and 1.9 kb, and a RNA band of 1.9 kb, respectively (*Figure 11*). No RNA band was detected by the *BglII/EcoRI* fragment (3.5 kbp) (*Figure 11*). The results showed that pHBH2 contained sequences homologous to a NR mRNA of approximately the same size as that detected by pHBH1. This NR mRNA is, however, present only very early after nitrate induction. pHBH2 may harbour the NADPH NR gene which is expressed only very early after nitrate induction, and pHBH1 may harbour the NADH NR gene which is expressed later after nitrate induction (Hamat 1989).

Discussion

Dot plot analysis indicated that there were eight NR gene copies in cv. Labelle, subspecies *indica* and six in cv. M 201, subspecies *japonica* per haploid genome

of rice. Southern blot analysis of *EcoRI* digested total rice DNA showed three bands (18, 14 and 12 kbp) in cv. Labelle, subspecies *indica* and four bands (18, 14, 10 and 3 kbp) in cv. M 201, subspecies *japonica*. These data suggest that there are at least three different NR genes in rice. The 3 kbp and 10 kbp fragments in cv. Labelle, subspecies *indica* may be equivalent to the 12 kbp fragment in cv. M 201, subspecies *japonica*. In barley, only two to three NR gene copies were detected (Kleinhofs, Washington State Uni. pers. comm.). The results from this study are in agreement with the findings of Shen et al. (1976) that there are two types of nitrate reductase enzyme in rice (NADH-nitrate reductase and NAD(P)H-nitrate reductase). In barley, only NADH-nitrate reductase was detected (Kleinhofs et al. 1985).

Two different NR clones were isolated from a genomic library of cultivar M 201, subspecies *japonica*. These clones contained the 14 kbp and 18 kbp *EcoRI* fragments. The 10 kbp *EcoRI* fragment was not recovered from the library after 600 000 plaques have been screened. The 3 kbp *EcoRI* fragment would have been too small to be packaged by the Charon 35 vector used to construct the library (Leonen and Blattner 1983). Other vector and strategy may be used to clone the 3 kbp and 10 kbp *EcoRI* fragments. Detailed restriction enzyme mapping was conducted to compare the two rice NR clones with each other and with the barley NR cDNA clone which was used as the probe to identify the rice NR clones. The data showed that the two rice NR clones were different from one another. Although both rice NR clones showed strong homology to the barley NR cDNA clone, no region of similar restriction enzyme sites could be identified. The DNA fragments homologous to the barley NR cDNA were identified by Southern blot analysis. The 3' ends of the rice NR genes were also identified by Southern blot analysis using the 3' ends of the barley NR cDNA clone as hybridization probe.

These results showed that the NR gene in pHBH1 ends around the *BglII* site and extends anticlockwise towards the *EcoRI* site which is the end of the rice DNA insert (*Figure 5*). The 5' end of the gene has not yet been determined, but may be beyond the end of the insert. This conclusion is suggested, based on the observations that the DNA region from the *BglII* to the *EcoRI* sites is unique or low copy DNA (*Figure 7*) and hybridizes to a RNA band that is presumed to be the NR mRNA (*Figure 9*). The pHBH1 NR clone probably codes for the NADH specific rice NR. This conclusion is based on the similarity of mRNA and NADH NR induction kinetics (Hamat 1989).

In clone pHBH2, the results indicated that the 3' end of NR gene is around the *ApaI* site. The 5' end of the gene has not been conclusively defined. However, the data from Southern analysis suggested that the 5' end of NR gene may be around the *BssHII* site. This inference is based on the occurrence of highly repeated DNA sequences beyond the *BssHII* site (*Figure 8*), and the absence of homology between this DNA sequence to the presumed NR mRNA (*Figure 11*). The *XhoI/BssHII* fragment and *BssHII/BglII* fragment hybridized to another RNA band of 1.9 kb (*Figure 11*). An experiment was conducted to determine if the 1.9 kb RNA were inducible by nitrate (data not shown). the fragment *XhoI/BglII* (5.6 kbp) of pHBH2 was used as a probe to monitor the intensity of the 1.9 kb RNA bands in total RNA extracted from rice seedlings at 30, 60 and 90 min after the addition of nitrate. The 1.9 kb bands showed no difference in intensity throughout the induction period, indicating the 1.9 kb mRNA was not inducible by nitrate. Therefore, it may not have any relationship to the nitrate reductase gene. The clone of pHBH2

probably codes for the NADPH rice NR. This conclusion is based on the similarity of mRNA and NADPH NR induction kinetics (Hamat 1989).

Acknowledgement

The author wishes to thank Mr Ismail Hassan for his technical assistance.

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Accepted for publication on 12 October 1988