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Characterization of a rice (*Oryza sativa* L.) mutant deficient in the heme domain of nitrate reductase

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Summary. Biochemical and genetical characterization of a rice nitrate reductase (NR)-deficient mutant, M819, which had been isolated as a chlorate-resistant mutant, was carried out. In M819, leaf NADH-NR activity was found to be about 10% of that of the wild-type cv 'Norin 8', while NADPH-NR activity was higher than that in the wild-type; $FMNH_2$-NR and MV-NR activities were also 10% of those of the wild type; BPB-NR activity was higher than that of the wild type; and xanthine dehydrogenase activity was revealed to be present in both. These results suggest that the mutant line M819 lacks the functional heme domain of the NADH-NR polypeptide due to a point mutation or a small deletion within the coding region of the structural gene. Chlorate resistance in M819 was transmitted by a single recessive nuclear gene.

Key words: *Oryza sativa* L. – Nitrate reductase – Deficient mutant – Chlorate resistance – Heme domain

Introduction

The regulation of nitrate reductase (NR), the first enzyme of the nitrate assimilation pathway, is of agronomic importance as it is directly involved in the improvement of the efficiency of nitrate utilization in crops. The structure and function of NR in higher plants have been investigated using NR-deficient mutants in several plant species such as barley, *Nicotiana* species and *Arabidopsis thaliana* (reviewed in Kleinhofs et al. 1985; Wray 1986). Recently, Chérel et al. (1990) evaluated 65 mutants of *Nicotiana plumbaginifolia* that had undergone alterations in the NR structural gene using immunological reaction analysis, NR reduction with artificial electron donors and cytochrome c reduction with NADH, and they demonstrated that the mutants could be classified into four classes. The nucleotide sequence of the cDNA of the NR structural gene has been determined in several higher plants including rice. The organization of plant NR genes has been found to be very similar (Caboche and Rouzé 1990). At present, however, the genetical control of the structure-function relationship of NR in higher plants is still unclear. It is necessary to collect and characterize more mutants having alterations in their NR structure gene.

Rice (*Oryza sativa* L.) seems to have some advantages for the study of NR. The fact that rice contains both NADH- and NADPH-NR and prefers ammonium as a nitrogen source to nitrate suggests that rice mutants deficient in NR may have a better chance of survival (Hasegawa et al. 1991). Furthermore, immunological analysis of NR has indicated that the rice NR system is different from that of barley (Hamat et al. 1989). In a previous paper (Hasegawa et al. 1991) we reported that two NR-deficient rice mutant lines had been isolated as chlorate-resistant mutants. In this paper, the biochemical and genetical characterization of a NR-deficient mutant line, M819, is reported.

Materials and methods

Plant materials

A NR-deficient rice mutant line, M819, and wild-type cv 'Norin 8' (*Japonica* type) were used. M819 was isolated as a chlorate-resistant mutant line from the γ-ray induced mutant

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Abbreviations: NR, Nitrate reductase; NiR, nitrite reductase; $FMNH_2$, reduced flavin nucleotide; MV, reduced methyl viologen; BPB, reduced bromphenol blue; XDH, xanthine dehydrogenase

lines maintained at the Institute of Radiation Breeding, Japan (Hasegawa et al. 1991). Seedlings were cultured in distilled water at 28 °C for 10 days and then transferred to hydroponic culture in which the nutrient solution contained 0.45 m*M* nitrate and 0.36 m*M* ammonium in a biotron (type LH-200-RD, Nippon Medical and Chemical Instruments, Japan) at 25 °C under fluorescent light (6,000 lux, 16-h photoperiod).

Enzyme assays

NR. Crude extracts were prepared from 6-week-old seedlings according to the procedure of Kleinhofs et al. (1986). The crude extracts were fractionated with ammonium sulfate (10–50% saturation), and the precipitates were desalted by passage through Sephadex G-25 columns equilibrated with the extraction buffer. The eluates were brought to a final concentration of 50% with glycerol. NADH- and NADPH-NR activities and the partial catalytic activities ($FMNH_2$-NR, MV-NR) were assayed by the methods described by Kleinhofs et al. (1986). BPB-NR was determined by the procedure of Campbell (1986).

NiR. MV-linked nitrite reductase was assayed in the crude extracts for the NR assays according to Ida and Mikami (1986).

XDH. The preparation of crude extracts, gel electrophoresis and staining were according to the procedure of Mendel and Müller (1976) for 10-week-old plants.

Genetical study of chlorate resistance

The F_2 seeds from the cross M819 × 'Norin 8' were cultured in 0.1 m*M* potassium chlorate solution upon sowing in a biotron (type LH-200-RD Nippon Medical and Chemical Instruments, Japan) at 25 °C under fluorescent light (6,000 lux, 16-h photoperiod). Fourteen days after sowing, chlorate resistance was evaluated visually from the reduction in seedling height and the extent of brown spots on the leaves (Hasegawa et al. 1991). One hundred and eighty seeds were used.

Results

NR and NiR activities

Both NADH-NR and NADPH-NR were detected in the leaves of M819 and the wild-type cv 'Norin 8'. In the latter the level of NADH-NR activity was 484.0 nmol NO_2^-/g fw per minute, while NADPH-NR activity was 2.9 NO_2^-/g fw per minute, less than 1% of the NADH-NR activity (Table 1). NADH-NR activity in M819 was 52.2 nmol NO_2^-/g fw per minute, about 10% of that of the wild type, suggesting that M819 is an NADH-NR deficient mutant. On the other hand, NADPH-NR activity in M819 was higher than that observed for the wild type. NADPH-NR activity in M819 was 15.4 nmol NO_2^-/g fw per minute, about 30% of the NADH-NR activity. NADPH-NR activity in M819 was higher than that of the wild type.

As shown in Table 1, NiR activity in M819 was 223.7 nmol NO_2^-/g fw per minute, which was about twice that observed in the wild type (103.8 nmol NO_2^-/g fw per minute).

Table 1. NADH-nitrate reductase (NR), NADPH-NR and nitrite reductase (NiR) activities in 'Norin 8' and NR-deficient mutant M819

Line	Activity		
	NADH-NR [a]	NADPH-NR	NiR [c]
Norin 8	484.0 (100 [b])	2.9 (100)	103.8 (100)
M 819	52.2 (10)	15.4 (527)	223.7 (216)

[a] nmol NO_2^-/g fresh weight (fw) per minute
[b] Percent of the wild type
[c] nmol NO_2^-/g fw per minute

Table 2. The partial catalytic activities of 'Norin 8' and M819

Line	Activity (nmol NO_2^-/g fresh weight per minute)		
	$FMNH_2$-NR	MV-NR	BPB-NR
Norin 8	294.6 (100 [a])	274.4 (100)	132.6 (100)
M 819	26.0 (9)	20.0 (6)	417.8 (315)

[a] Percent of the wild type

Effect of artificial electron donors on NR

In order to characterize the nature of the mutation, three artificial electron donors for NR, $FMNH_2$, MV and BPB were tested in an assay of partial enzymatic activity (Table 2). In the wild type, the activities of $FMNH_2$-NR, MV-NR and BPB-NR were 294.6, 274.4 and 132.6 nmol NO_2^-/g fw per minute, respectively. In M819, differential responses to three artificial electron donors were observed. $FMNH_2$-NR and MV-NR activities were less than 10% of those of the wild type; these decreases in activity correspond to that found for terminal NR activity. On the other hand, an increased level of BPB-NR activity (about 3 times that of the wild type) was observed in M819.

XDH activity

To determine whether the NR deficiency of M819 is due to the lack of the Mo cofactor or not, XDH activity was assayed. As shown in Fig. 1, the same banding patterns were found in both M819 and the wild type. This result showed that M819 was not a Mo-cofactor type (*cnx* type) mutant.

Genetical analysis of chlorate resistance

Table 3 shows the segregation of chlorate resistant seedlings in the F_2 population from the cross of M819 × 'Norin 8'. Of the 166 seedlings germinated, 47 were resistant and 119 were sensitive. This ratio fitted well to the

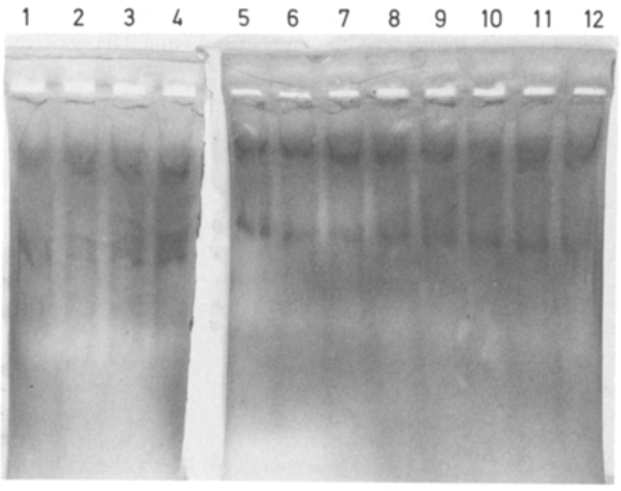

Fig. 1. Detection of xanthine dehydrogenase activity in 'Norin 8' (lanes 1–4) and NR-deficient mutant M819 (lanes 5–12) by gel electrophoresis

Table 3. Segregation of chlorate resistant seedlings in F_2 progeny from the cross M 819 and 'Norin 8'

Cross	Number of resistant seedlings	Number of sensitive seedlings	χ^2 (1:3)	Probability
M 819 × Norin 8	47	119	0.80	$0.25 < P < 0.50$

1:3 ratio ($\chi^2 = 0.80$, $0.25 < P < 0.50$), indicating that the mutant trait was transmitted by a single recessive nuclear gene.

Discussion

Hasegawa et al. (1991) reported that two NR-deficient rice mutants had been isolated as chlorate-resistant ones. In resistant M819, leaf NADH-NR activity was about one-tenth that of the wild type. XDH activity was detected in both M819 and the wild type, indicating that M819 is deficient in the NADH-NR apoenzyme and is therefore a *nia*-type mutant. With respect to the inheritance of NR deficiency in barley, codominant and recessive genes have been identified in the apoenzyme mutant and the Mo-cofactor mutant, respectively (Kleinhofs et al. 1980). Chlorate resistance in M819 was inherited as a single recessive nuclear gene. In view of the fact that in *Arabidopsis thaliana* the chlorate resistant gene *CHL3* has been identified to be the NR structural gene *NIA2* (Wilkinson and Crawford 1991), it is reasonable to conclude that chlorate resistance in M819 results from a NR deficiency.

NR has been shown to be a homodimer, with each polypeptide composed of three domains: FAD, heme and Mo cofactor. The location of the mutation in the NR apoenzyme-deficient mutant can be identified from analysis of the partial enzymatic activity of NR. Chérel et al. (1990) showed that *nia*-type NR-deficient mutants in *Nicotiana plumbaginifolia* could be classified into four classes according to their immunological reaction and difference in partial enzymatic activities. In this experiment, NR reduction with three electron donors, $FMNH_2$, MV and BPB, was measured. In M819, $FMNH_2$-NR and MV-NR activities were about one-tenth of those of the wild type, while BPB-NR activity in M819 was higher than that of the wild type. Another experiment (Ichii et al., in preparation) showed that in M819 the activity of cytochrome c reductase (the diaphorase activity of NR) of the crude extracts was one-half of that observed in the wild type. Although an immunological assay has not been carried out in this experiment, our results indicate that M819 could be classified into 'Class 4' of NR-deficient mutants designated by Chérel et al. (1990); that is, M819 was identified as a mutant deficient in the heme domain of NR. Mutants deficient in the heme domain of NR seem to be relatively rare. In *Nicotiana plumbaginifolia*, Chérel et al. (1990) reported that only 3 mutants out of 65 were classified as heme domain-deficient mutants. Therefore, M819 may be useful material for studying the function of the heme domain of NR.

The DNA nucleotide sequence of the NR structural gene has been determined in some plant species. A high degree of homology of the sequence of the NR structural gene has been reported among the higher plants (Caboche and Rouzé 1990). Choi et al. (1989) reported that in rice the nucleotide sequence of the *nia-1* gene carries an open reading frame of 916 amino acids (101,482 Da). In the NR structural gene of higher plants, the length of the sequence encoding the heme and FAD domain was found to be about 1,300 bp (Caboche and Rouzé 1990). Therefore, it may be concluded that the NR deficiency of M819 may be due to a point mutation or a small deletion within the coding region of the heme domain. These findings imply that the NR gene is a useful marker for the detection of mutations at the molecular level.

In plant species containing both NADH-NR and NADPH-NR, Kleinhofs et al. (1989) suggested that chlorate resistance is not a useful selective marker for isolating NADH- or NADPH-NR-deficient mutants. Wray (1986) suggested that selection pressure plays a role in the appearance of the type of NR-deficient mutant at a whole plant level. In barley, screening for chlorate resistance at high concentration led to the recovery of only Mo-cofactor mutants (Bright et al. 1983), but the direct selection of seedlings showing decreased levels of NR led to the recovery of more apoenzyme mutants than Mo-cofactor mutants (Kleinhofs et al. 1980). Pelsy et al. (1991) also reported that the majority of NR-deficient mutants selected as chlorate-resistant ones from a M_2 population were of the Mo-cofactor type in *Nicotiana plumbaginifolia*. Hasegawa et al. (1991) demonstrated that in M819, which was selected as a chlorate-resistant mutant, seedling growth was reduced at a concentration of 1 m*M* chlorate. In a barley mutant deficient in NR apoenzyme, the seedlings grown on nitrate and treated with chlorate were slightly more resistant to chlorate than the control (Warner and Kleinhofs 1981). Thus, it might be possible that selection for marginal resistance to chlorate results in the isolation of NR apoenzyme mutants.

Finally, it is interesting that NADPH-NR and NiR activities in M819 were higher than those of the wild type. In M819, an increased level of BPB-NR activity was also observed. In view of the fact that M819 grew normally (Hasegawa et al. 1991), we suggest that both

growth and nitrate assimilation of M819 are supported by increased levels of NADPH-NR and NiR. An increase in the NADPH-NR/NADH-NR ratio has been reported in an NADH-NR deficient barley mutant (Warner et al. 1987). Elevated NiR activity has also been observed in NR-deficient barley (Kleinhofs et al. 1980) and *Arabidopsis thaliana* (Braaksma and Feenstra 1982). In the *Arabidopsis* mutants, increased levels of NiR were explained by the high nitrate concentration, since nitrate is an inducer of NiR. Further studies are necessary for investigating nitrate assimilation in the mutant in relation to increased NADPH-NR and NiR activities.

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