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Screening for Nitrate Reductase-Deficient Mutants in Rice (*Oryza sativa* L.)

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To obtain mutants deficient in nitrate reductase (NR) in rice, screening for chlorate resistance was carried out using the mutant lines maintained at the Institute of Radiation Breeding, NIAR, Japan. Of 437 lines tested, four chlorate resistant lines (M819, M821, M1004 and M1009) were selected. Differences in chlorate sensitivity were also observed among the four selected lines. The levels of *in vitro* NR activity of seedlings in M819 and M821 were below 30% of that of their parent, Norin 8. M819 and M821 were identified as low NR activity lines. NR activity in M1009 was about 80% of that of Norin 8. However, NR activity in M1004 was the same level as that in Norin 8. Seedlings of four selected lines took up nitrate to the same extent or a higher than Norin 8.

KEY WORDS: *Oryza sativa* , rice, mutants, chlorate resistance, nitrate reductase, nitrate uptake.

Introduction

Most higher plants use nitrate as a major source of nitrogen. Nitrate taken up into the cells is reduced to nitrite by nitrate reductase (NR). In order to improve the efficiency of nitrate utilization in crop plants, it is the most important to investigate the structure and function of NR, because NR is a key enzyme in nitrate assimilation (BRAY 1983). Genetical and biochemical studies on NR have been progressed using NR deficient mutants or cell lines from several higher plant species (reviewed in KLEINHOFS *et al.* 1985, WRAY 1986). However, we need further information on the genetical control of NR in various plant species.

NR deficient mutants can be selected as chlorate resistant mutants, because chlorate is taken up as an analog for nitrate into the cells and is reduced to toxic chlorite by NR. A number of mutants and cells resistant to chlorate have been selected in various plants and have been used for genetical and biochemical research on NR (WRAY 1986).

Since rice seedlings, unlike most crop plants, use ammonium as a major nitrogen source, we assume that rice may have a unique NR system and that rice mutants defective in NR may have a better chance of survival and seed production. This paper describes a screening technique for isolating chlorate resistant mutants and demonstrates that they are low NR activity lines.

Materials and Methods

Plant materials and culture of seedlings

Seeds of 437 rice mutant lines including 427M lines and 10HP lines were used. M lines are progenies of mutants altered in various kinds of morphological characters and HP lines

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are high protein mutants. All mutants whose original variety was Norin 8 (*japonica* type) were selected from the progenies mutagenized by gamma-rays in the 1960s and have been maintained at the Institute of Radiation Breeding, National Institute of Agrobiological Resources, Ibaraki, Japan.

Seeds were sown and cultured on a net float in a plastic container in a biotron (LH-200-RD, Nippon Medical and Chemical Instruments, Japan at 25±1°C with fluorescent light (6,000 lux, 16h photoperiod).

Primary screening

Seedlings were cultured with 0, 10^{-4} and 10^{-3}M potassium chlorate from sowing. Fourteen days after treatment, seedling damage induced by chlorate was visually evaluated on the basis of the reduction in seedling height and the extent of brown leaf spots (described as chlorate burn by KLEINHOFS *et al.* 1978). The grades of chlorate resistance were classified into four groups; R (resistance), R' (weak resistance), S (sensitive) and R/S (resistant seedlings segregating). Fifteen seedlings were used in each treatment.

Secondary screening

Seeds which had been classified as R at the primary screening were sown and cultured with 0, 5×10^{-5}, 10^{-4}, 5×10^{-4} and 10^{-3}M potassium chlorate under the same conditions as the primary screening. Seeds of Norin 8 were also used as control. Seedling height was measured as a parameter of chlorate injury on the 14th day after the sowing. Twenty seeds were used in each treatment.

Nitrate reductase activity

The *in vitro* NR activity of a 20-day-old seedling was determined in four lines of group R and Norin 8. For the analysis, seedlings were cultured with low concentration of culture solution including both nitrate and ammonium for 17 days and then were transferred into deionized water. Seedlings were placed in the 500μM potassium nitrate solution for 6h before NR assay to induce NR. NR was assayed by the method proposed by HAGEMAN and REED (1980). Five or ten seedings were used in each assay.

Nitrate uptake

Prior to the nitrate uptake measurement, seedlings were kept in a solution of 250μM potassium nitrate and 500μM calcium sulfate at 25±1°C for 24h. Fifteen days after sowing, seedlings were placed in a small beaker containing 50m*l* of a solution of 250μM potassium nitrate and 500μM calcium sulfate in a biotron at 28±1°C. After 6 hours, nitrate concentration of the solution was measured with a nitrate specific electrode (8201-06T, Horiba Ltd., Japan) with an ion meter (N-8F, Horiba Ltd., Japan). Net nitrate uptake into the seedlings was calculated from the depletion of the solution. Each experiment was replicated three times using 5 seedlings.

Results

When seedlings of Norin 8 were cultured with potassium chlorate at concentrations above 5×10^{-5}M, their growth was severely inhibited. Brown spots, indicative of chlorate-induced damage, were also found on the leaves. The number and size of the spots increased with the increase in chlorate concentration. From these observations, it was indicated that a

chlorate-resistant seedling could be easily distinguished from a chlorate-sensitive one.

Table 1 shows the classification of the lines tested into three groups based on extent of resistance. Of 437 mutant lines tested, four lines, M819, M821, M1004 and M1009, were classified as R, while fifteen lines were R'. In six lines classified as R/S, it was observed that a few well-grown seedlings were found among the sensitive ones. No chlorate resistant mutants were detected in HP lines.

The difference in chlorate sensitivity between Norin 8 and the four lines classified as R is shown in Fig. 1. Fig. 2 shows the responses to 10^{-3}M potassium chlorate in four chlorate resistant lines and Norin 8. In M819, M821 and M1004, reduction in seedling height was observed at a concentration of 5×10^{-5}M or higher, but no remarkable decrease in seedling height was found with increase in chlorate concentration except for the seedlings of M819 and M821 at 10^{-3}M chlorate. Differences in chlorate sensitivity were also observed between the lines classified as R. M819 and M821 were obviously resistant to chlorate. The chlorate resistance in both lines was confirmed from their small size of leaf spots. M1004 was the most resistant to chlorate when the resistance was evaluated by the reduction in seedling height, but the seedlings showed apparent brown leaf spots. On the other hand, chlorate resistance in M1009 was intermediate between three M lines and Norin 8.

Table 2 shows *in vitro* NR activity in the four lines classified as R and in Norin 8. Leaf NR activities of M819 and M821 were 29 and 28%, respectively, of that in Norin 8. Therefore, M819 and M821 were identified as low NR activity lines. On the other hand, leaf NR activity of M1004 was as almost the same as that in Norin 8. Leaf NR activity of M1009 was about

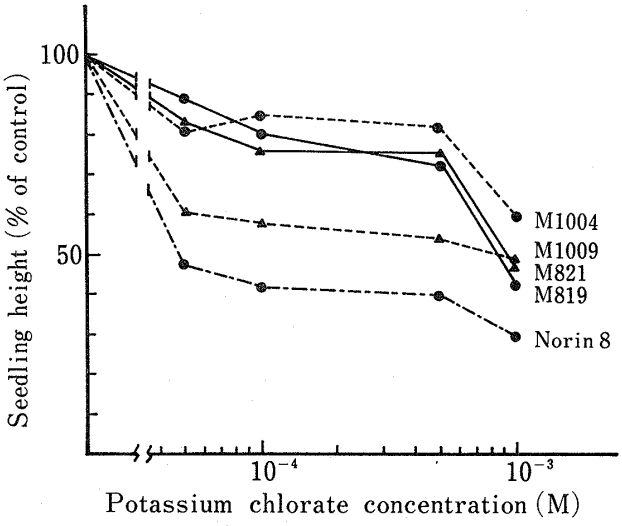

Fig. 1. Difference in chlorate sensitivity between four chlorate resistant lines and Norin 8. Seedling height was measured on 14th day after treatment.

Table 1. Chlorate resistant lines from 437 mutant lines

R	R'			R/S	
M 819	M 108	M 730	M1027	M 25	M1147
M 821	M 213	M 743	M1054	M 102	
M1004	M 643	M 828	M1082	M 689	
M1009	M 682	M1017	M1087	M 725	
	M 707	M1023	M1153	M 728	

R : Resistance
R' : Weak resistance
R/S : Resistant seedling(s) segregating

80% of that of Norin 8. Preliminary assay for NR activity was also carried out using the seedlings of the lines of group R', but no NR deficient mutants were found (data not shown).

As shown in Table 3, seedlings of both mutant lines and Norin 8 took up nitrate. Uptake of nitrate from the 250μM nitrate for 6h ranged from 9.2 to 10.5μ mol·g^{-1}·6h^{-1}. No significant difference in nitrate uptake was found between Norin 8 and the mutant lines except for M1009, where there was a significant difference at 5% level.

Discussion

Chlorate resistance has been used as a selective marker for isolating NR deficient mutant at both the cell and the whole plant level in many plant species (reviewed in WRAY 1986).

Table 2. Level of *in vitro* nitrate reductase activity (μmol NO_2^-·g fresh weight^{-1}·h^{-1}) of a 20-day-old seedling in four chlorate resistant lines and Norin 8

	Norin 8	M 819	M 821	M1004	M1009	Mutant/Norin 8
Assay 1[a]	3032±417	875±63	—	—	—	0.289
Assay 2[a]	3657±711	—	1004±201	—	—	0.275
Assay 3[b]	2941±360	—	—	2779±165	—	0.950
Assay 4[b]	2941±360	—	—	—	2264±138	0.770

[a] : Average of 10 seedlings
[b] : Average of 5 seedlings

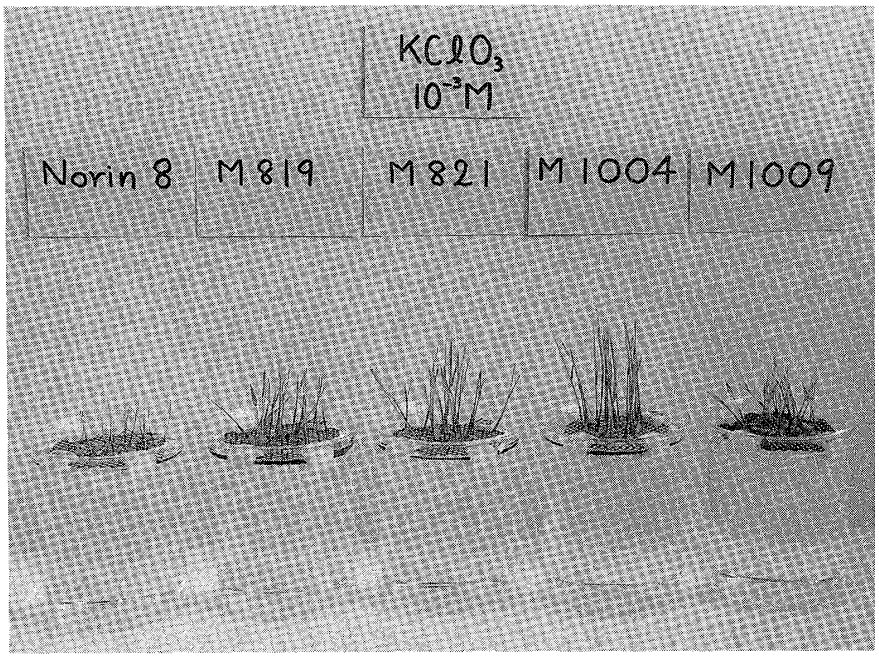

Fig. 2. Seedlings of chlorate resistant lines and Norin 8 cultured with 10^{-3}M potassium chlorate.

In this study, two chlorate resistant mutant lines, M819 and M821, were identified as low NR level ones. The levels of NR activity in M819 and M821 were higher than those in NR deficient barley mutants, which showed 0~10% of NR activities of their original varieties (WARNER *et al.* 1977, BRIGHT *et al.* 1983). NR activity of M1009 was about 80% of its parent. On the other hand, NR activity in M1004, which was also classified as chlorate resistant, were almost the same as in Norin 8. Seedlings of M1004 grew in the chlorate solution but showed apparent leaf spots. These facts suggest that the chlorate resistance in M1004 is controlled by a different mechanism from that in M819 and M821.

It was interesting that mutants deficient in NR were found among the lines which had been selected previously as mutants in other agronomic or morphological traits. At present, relationship between the low level of NR and the morphologically mutated character in M819 and M821 is unclear. The plants of M819 and M821 grew vigorously to maturity and produced seeds under the field condition fertilized with both nitrate and ammonium. This observation was different from that by KLEINHOFS *et al.* (1978), who reported that all chlorate resistant barley seedlings were lethal.

Since the 1980s, studies on NR in higher plants have entered into a new era because of the progress in genetical and immunological approaches (WRAY 1988, CAMPBELL 1989). The genes for NR have been identified in some plant species. In barley, some genes determining synthesis of either apoenzyme or molybdenum co-factor of NR have been identified by the genetical and biochemical characterization of NR deficient mutants (KLEINHOFS *et al.* 1980, BRIGHT *et al.* 1983). Similar researches have been conducted in *Nicotiana* species (MÜLLER. 1983, GABARD *et al.* 1988). In rice, WAKASA *et al.* (1984) established NR deficient cell lines, but whole plant mutants deficient in NR have not been reported. HAMAT *et al.* (1989) reported that rice NR was encoded by a small gene family and suggested that the NR system in rice was different from that of barley. Recently, the cDNA sequence of NR structural gene has been determined in *Arabidopsis* (CRAWFORD *et al.* 1988), rice (CHOI *et al.* 1989) and others. M819 and M821 have a potential value for the research on genetics and biochemistry in rice. Further studies on the chlorate resistant mutants selected in this experiment are now in progress.

Table 3. Nitrate uptake by 15-day-old seedlings of four mutant lines and Norin 8. The rates of nitrate uptake by rice seedlings were simulated from the decrease of nitrate from the root-bathing culture solution containing 250 μM KNO_3 + 500 μM $CaSO_4$

L. No.	Uptake (μmol·g fresh weight^{-1}[a]·6h^{-1}
Norin 8	9.6±0.29
M 819	9.8±0.59
M 821	9.2±1.00
M 1004	10.0±0.87
M 1009	10.5±0.30*

[a] : Whole plant fresh weight
* : Significantly different from Norin 8 at 5% level

It has been reported that some mutants resistant to chlorate may be deficient in nitrate uptake (OOSTINDIER-BRAAKSMA and FEENSTRA 1973). However, the seedlings of the mutant lines selected in this experiment took up nitrate to the same or higher extent than Norin 8. The fact that seedlings of the low NR level mutant lines, M819 and M821, took up nitrate to the same extent as their parent is similar to the reports on NR deficient *Arabidopsis* (DODDEMA *et al.* 1978) and pea (DEANE-DRUMMOND and JACOBSEN 1986). These observations support the view that nitrate uptake is independent of NR in higher plants (WARNER and HUFFAKER 1989).

Finally, it has been reported that a chlorate hypersensitive mutant in *Arabidopsis* shows both enhanced nitrate uptake and NR activity (WANG *et al.* 1988). In the mutant lines (M lines) tested in these experiments, some lines seemed to be more sensitive to chlorate than the parent. It may be possible to select mutants showing enhanced level of NR from the lines tested in this study.

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イネにおける低硝酸還元酵素活性突然変異体の選抜

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農業生物資源研究所放射線育種場において選抜され，維持されているイネ突然変異系統（原品種，農林8号）を用いて，塩素酸カリウム抵抗性指標とした硝酸還元酵素（NR）欠失突然変異体のスクリーニングを試みた．

各系統の種子を播種時より0，10^{-4}，および10^{-3}Mの塩素酸カリウム溶液で生育させた．播種後14日目における幼植物の草丈抑制程度ならびに葉の褐変斑の大きさにより可視的に塩素酸カリウム抵抗性の評価を行い，抵抗性程度をR（抵抗性），R'（弱い抵抗性），S（感受性）ならびにR/S（抵抗性個体が系統内に分離）に分類した．その結果，調査した437系統のうち，M 819，M 821，M 1004ならびにM 1009の4系統が塩素酸カリウム抵抗性（R）と認められた．またR'として15系統が，R/Sとして6系統が認められた（Table 1）．

次に塩素酸カリウム抵抗性4系統と農林8号の幼植物を0～10^{-3}Mの塩素酸カリウム溶液で生育させ，抵抗性の程度を再度調査した．M 819とM 821は草丈の抑制程度と葉の褐変斑の大きさの双方の基準から明瞭な塩素酸カリウム抵抗性を示した．一方，M 1004は草丈からは最も抵抗性を示したが葉には褐変斑が認められた．またM 1009はM 819，M 821と農林8号のほぼ中間の草丈抑制程度であった（Fig. 1, Fig. 2）．

播種後20日目の幼植物の葉における*in vitro*NR活性を調べたところ，M 819とM 821のNR活性は原品種の30%以下であり，両系統は低NR活性突然変異体であると判定された．一方，M 1009のNR活性は原品種の約80%であり，M 1004では原品種と同程度であることが明らかになった（Table 2）．塩素酸カリウム抵抗性は硝酸吸収欠損突然変異の指標としても有効である．4系統の硝酸吸収は農林8号と同程度あるいはそれ以上であり，吸収欠損突然変異体ではなかった．（Table 3）．

NRは植物の硝酸代謝において最も重要な役割をはたす酵素であり，その機能ならびに遺伝子支配の解明は，効率的な硝酸代謝を行なう作物育成の基礎として重要である．この解決にはNRにかかわる多様な突然変異の利用が有効であるので，本実験で明らかになった系統についてNRの遺伝生化学的調査を行なっている．