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Short Communication

Mutants with Low Nitrate Reductase Activity Selected from Seedlings Expressing Nitrogen Deficiency Symptoms in Rice (*Oryza sativa* L.)

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Nitrate reductase (NR)-deficient mutants have been used for studying nitrate assimilation in higher plants. In this experiment, screening for nitrogen deficiency symptoms was examined to select NR- or nitrate uptake-deficient mutants in rice. Of 48,000 M_2 seedlings mutagenized by gamma-ray (200 Gy), 386 seedlings exhibited pale green leaves as a symptom of nitrogen deficiency in the solution containing nitrate as a sole source of nitrogen. After determining the nitrate uptake and NADH-NR activity, 77 plants were identified as variants with a low NR activity and/or low nitrate uptake. Most of the variants were sterile, but 11 plants produced M_3 seeds. In the M_3 generation most of the variants exhibited wild-type levels of NR. Only two lines, NR676 and NR827, which were found to be defective in NR activity were identified as mutants with a low NR activity.

KEY WORDS: *Oryza sativa,* nitrate reductase, nitrate uptake, mutant

Introduction

Nitrate reductase (NR) is a key enzyme for nitrate assimilation. For the improvement of nitrate use efficiency in crop plants, genetical and biochemical investigation on nitrate reductase is important (Hasegawa *et al.* 1991). In higher plants, the structure-function relationships within the NR protein have been revealed using NR-deficient mutants. NR-deficient mutants are classified into two groups; apo-protein mutants and Mo-cofactor mutants. Several strategies for selecting mutants deficient in NR have been proposed. Interestingly, the selection strategy appears to play a role in determining the type of mutant recovered at the whole plant level (WRAY 1986). Screening for resistance to high chlorate concentration led to the recovery of only Mo-cofactor mutants in barley (BRIGHT *et al.* 1983), whilst apo-protein mutants were largely selected by screening for resistance to low chlorate concentration (WARNER and KLEINHOFS 1981, HASEGAWA *et al.* 1991, 1992). KLEINHOFS *et al.* (1980) demonstrated that selection by quantitative estimation of NR activity in individual seedlings enabled to a larger number of apo-protein mutants than Mo-cofactor mutants in barley. More recently, PELSY *et al.* (1991) screened seedlings for the inability to grow on nitrate in order to obtain mutants totally impaired in nitrate assimilation in *Nicotiana plumbaginifolia.* In this experiment, according to the modified strategy proposed by PELSY *et al.* (1991), we report the process of isolating NR-deficient mutants of rice; that is, the selection of plants showing nitrogen deficiency symptoms in the solution containing nitrate at a low concentration as a sole source of nitrogen.

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Materials and Methods

Screening of mutants was performed using M_2 seeds mutagenized by gamma-ray (200 Gy) in the M_1 generation in rice (*Oryza sativa* L. cv. Nipponbare, *Japonica* type).

Primary screening: M_2 seedlings were cultivated in tap-water for 10 days at 30°C and then transferred to a solution containing 50 μM nitrate as a sole source of nitrogen. On the 15th day after sowing, plants showing pale green leaves as a symptom of nitrogen deficiency were selected.

Secondary screening: The selected seedlings were placed in a 50 μM potassium nitrate and 500 μM calcium sulfate solution for 24h. Nitrate concentration in the solution was estimated by ultra-violet absorvance at 220 and 270 nm using a spectrophotometer (UBest 30, Japan Spectroscopic Co Ltd., Japan). Nitrate uptake was estimated by the depletion of nitrate from the solution. After the nitrate uptake measurement, all the surviving plants were transferred to soil culture. When the plants were 50- to 90-days-old, *in vitro* leaf NADH-NR activity was determined as described by HAGEMAN and REED (1980). Plants in which the NR activity or nitrate uptake was less than 50% of that of the wild type cultivar were selected as variants with a low NR activity and/or low nitrate uptake and were grown to maturity.

In the M_3 generation, seedlings were grown on a 30 μM potassium nitrate solution for 15 days and were then transferred to water culture with half strength KIMURA's B solution. Nitrate uptake and NR activity were determined using five seedling in each M_3 line on the 15th and 21st days after sowing, respectively, by the same methods as those applied in the M_2 generation.

Results

As shown in Table 1, 386 seedlings which showed symptoms of nitrogen deficiency on nitrate were selected from 48,000 M_2 seedlings. The frequency of the selected seedlings was 8×10^{-3} per M_2 seedlings.

Of the 386 seedlings selected in the primary screening, 136 seedlings showed an impaired nitrate uptake, but most of the seedlings with a low nitrate uptake died within a few days. Fifty to ninety days later, when NR analyses were performed, 61 of the 243 seedlings which survived were found to display a low NR activity (less than 50% of the wild type). These selection procedures enabled to identify 54 seedling with low NR activity, 16 with low nitrate uptake and seven with low NR activity and low nitrate uptake seedlings (Table 1) and these seedlings were grown to maturity. Most of the variants were sterile, but 11 plants produced M_3 seeds. Of the 11 plants, two were derived from the seedlings with a low NR activity and low nitrate uptake, and nine were from the seed-

Table 1. Screening for seedlings with low nitrate uptake and/or low nitrate reductase (NR) activity in M_2 generation

No. of seedlings screened	48,000
No. of seedlings showing nitrogen deficiency	386
No. of variants selected	
Low NR activity	54
Low nitrate uptake	16
Low NR and low nitrte uptake	7

lings with a low NR activity (Table 2).

In the M_3 generation, NR activities of NR676 and NR827 were about 40% of that of the wild type cultivar and thus both mutants were identified as a low NR activity (Table 2). Only one mutant deficient in nitrate uptake was obtained (NR688) which showed an impaired nitrate uptake at the young seedling stage but died before the NR activity could be measured. The other plants selected as variants with a low NR activity in the M_2 generation recovered the NR activity in the M_3 generation. NR676 and NR827 exhibited yellow green leaves and a reduced seedling height. The mutant character of NR827 was expressed in the following generations, but NR676 has not yet been characterized further.

Discussion

Selection pressure plays an important role in determining the types of NR-deficient mutants obtained at the whole plant level (Wray 1986). Strong pressure, such as selection for plants resistant to high chlorate concentraiton, tends to recover Mo-cofactor mutants (Bright *et al.* 1983). On the other hand, weak selection pressure, such as selection for plants resistant to low chlorate levels (0.1～1 mM) (Warner and Kleinhofs 1981, Hasegawa *et al.* 1991, 1992) or direct selection for plants with a low *in vivo* NR activity (Kleinhofs *et al.* 1980), produces a larger number of apo-protein mutants than Mo-cofactor mutants. In this experiment, nitrogen deficiency symptoms were used as an indicator of impaired nitrate assimilation and two mutants with a low NR activity, NR676 and NR827, were isolated. The strategy adopted in this experiment which is one of the weakest methods of selection pressure for isolating NR-mutants appears to be useful for selecting mutants with a low NR activity in rice. When seedlings were grown on nitrate as a sole source of nitrogen, symptoms of nitrogen deficiency were also a useful criteion for isolating nitrate uptake deficient mu-

Table 2. NADH-nitrate reductase activity and nitrate uptake of the selected variants in M_2 and M_3 generations by percentage of the wild type in two replications

Line No.	M_2 generation		M_3 generation	
	NR activity	nitrate uptake*	NR activity	Nitrate uptake
NR 676	2	0	42	90
NR 688	8	81	—**	35
NR 690	6	54	109	100
NR 715	11	74	122	83
NR 762	21	95	66	107
NR 827	35	62	42	93
NR 902	40	91	86	102
NR 903	39	89	112	102
NR 918	46	99	111	96
NR 933	23	38	92	98
NR 956	7	113	150	84

* Not replicated.

** All the M_3 seedlings died before NR analysis was performed.

tants (WALLSGROVE *et al.* 1989). However, no nitrate uptake mutants were obtained in the experiment reported here.

In most of the previously reported cases, the NR activity in the NR-deficient mutants was either zero or below 10% of that of the wild type. It is interesting to note that the NR activities in NR 676 and NR827 were intermediate between that of the wild type and that of the previously reported NR-deficient mutants. These mutants can be useful for investigating nitrate assimilation in higher plants. At present, the mechanism controlling the NR activity levels of these mutants has not been elucidated. Further biochemical characterization of NR827 is underway.

It has been reported that NR-deficient mutants can be selected with a relatively high frequency by screening M_2 seedlings; for example, values of 1.5×10^{-3} and about 5×10^{-3} per M_2 seedling were scored in barley (WARNER *et al.* 1977) and in *Nicotiana plumbaginifolia* (PELSY *et al.* 1991), respectively. However, only two mutants were selected from 48,000 in a M_2 population in the experiment reported here. The extremely low frequency of NR-deficient mutants (4×10^{-5} per M_2 seedling) obtained in this experiment with rice may be ascribed to the fact that rice displays both NADH- and NADPH-NR activity and that the NADPH-NR activity is high in a NADH-NR deficient mutant (HASEGAWA *et al.* 1992).

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窒素欠乏症を指標として選抜されたイネの低硝酸還元酵素活性突然変異体

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硝酸を唯一の窒素源とする水耕液でイネの幼植物を栽培し，葉色に窒素欠乏症が認められる個体を選ぶことにより，硝酸吸収欠失ならびに硝酸還元酵素（NR）欠失突然変異体の選抜を試みた．

品種「日本晴」の乾燥種子に 200 Gyのガンマ線を照射した後代の 48,000 のM_2種子を用い，硝酸を唯一の窒素源とする水耕液で 15 日間栽培したところ 386 個体に窒素欠乏症が現れた．これらの幼植物について硝酸吸収とNADH（還元型ニコチン酸アミドアデニンジヌクレオチド）－NR活性の測定を行ったところ，16 個体が低硝酸吸収を，54 個体が低NR活性を，さらに 7 個体が低硝酸吸収・低NR活性を示した．これらの個体の大部分は不稔であったが，低硝酸吸収・低NR活性を示した 2 個体と低NR活性を示した 9 個体の計 11 個体についてはM_3種子が得られた．M_3において 2 個体（NR676, NR827）の後代ではNR活性が原品種の約 40％で低NR活性突然変異体と認めた．NR676とNR827はM_3においてそれぞれ低硝酸吸収・低NR活性，低NR活性変異体として選抜されたものである．これら突然変異体の特徴はNR活性が従来報告されているNR欠失突然変異体と野生型の中間を示すことである．現在NR827の後代を育成して突然変異体の生化学特性を調べている．

NR欠失突然変異体を獲得するにあたっては選抜圧の差異により，得られる突然変異体のタイプが異なることが知られている．本実験で用いた選抜方法は最も弱い選抜圧であり，このことが低NR活性突然変異体を獲得できた一因であると考える．