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A New Locus *cnx* 3 Involved in Molybdenum Cofactor Biosynthesis in Rice (*Oryza sativa* L.)

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Summary

Two molybdenum cofactor (MoCo)-deficient mutants, C290 and C384, were isolated from 204,500 M_2 seedlings of rice (*Oryza sativa* L., ssp. *indica*, cv. IR30) by means of chlorate-resistance. The two mutations were monogenic and recessive. Biochemical analysis showed that the activities of nitrate reductase (NR) and xanthine dehydrogenase (XDH) in the mutants were considerably lower than in the wild type IR30, which indicated that the two mutants were actually MoCo-deficient types. On the other hand, nitrite reductase (NiR) activity and nitrate content in the two mutants were higher than IR30. An F_1 strain of cross between the mutants was mutant, indicating that the mutations were allelic. However, the mutants were able to complement the defects in two previously isolated mutants involved in the biosynthesis of MoCo, *cnx*1 and *cnx*2. Therefore, genes carried by C290 and C384 define a new locus, which was designated cofactor for nitrate reductase and xanthine dehydrogenase 3 (*cnx* 3). In contrast to *cnx*1 mutant, adding 0.5 mM molybdate into growth medium did not recover NADH-NR activity in C290 and C384. Therefore, the *cnx* 3 gene presumably encodes a different step from *cnx*1 in the MoCo biosynthetic pathway.

Key Words : molybdenum cofactor, mutant, nitrate reductase, *Oryza sativa*, rice, xanthine dehydrogenase.

Introduction

The essential trace element molybdenum functions as a catalytically active metal in molybdoenzymes, such as nitrogenase, NR, XDH, sulfite oxidase, aldehyde oxidase and formate dehydrogenase (Stallmeyer *et al.* 1995, Hoff *et al.* 1995). In both prokaryotes and eukaryotes, these enzymes are important in several pathways, including: nitrogen assimilation, sulfur and purine metabolism, and hormone biosynthesis (Hoff *et al.* 1995). All known molybdoenzymes, with an exception of nitrogenase, contain molybdenum as MoCo, whose basic structure is a complex of 6-alkyl pterin (named molybdopterin) and molybdenum atom (reviewed in Rajagopalan and Johnson 1992). The basic structure of MoCo is common in all organisms studied to date; however, in eubacteria a nucleotide is attached to molybdopterin (Rajagopalan and Johnson 1992). Although the structure of MoCo has been well-characterized, very little information is available on its biosynthetic pathway, in higher plants or other eukaryotes, because of the instability of the cofactor and its precursors (Hoff *et al.* 1995).

One approach to study the MoCo biosynthetic pathway is with genetics, through the isolation and the ensuing characterization of MoCo-deficient mutants. In *Nicotiana plumbaginifolia*, at least seven loci have been identified that correspond to seven enzymatic steps in the pathway (Gabard *et al.* 1988, Leydecker *et al.* 1995). In rice, two loci (*cnx* 1 and *cnx* 2) in the pathway have been found (Sato *et al.* 1996). In this report, we describe the isolation and characterization of two new MoCo-deficient mutants of rice, C290 and C384. Complementation analysis with other rice MoCo-deficient mutants, C27 and C25 affected in *cnx* 1 and *cnx* 2, respectively, indicated that C290 and C384 define a third locus in the biosynthetic pathway for MoCo.

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Materials and Methods

Screening of chlorate-resistant mutants

A total of 204,500 M_2 seeds of rice (*Oryza sativa* L., ssp. *indica*, cv. IR30), which had been mutagenized by sodium azide or ethyleneimine in the M_1, were sown in 0.1 mM potassium chlorate inside a greenhouse (natural daylight and 30℃). Between 12 to 31 days after sowing, chlorate-resistant seedlings were isolated that had neither small brown spots nor reduced plant height (symptoms of chlorate toxicity; Hasegawa *et al.* 1991). The isolated seedlings were transferred to soil culture and grown to maturity. Chlorate-resistance was retested in M_3 progeny from the putative chlorate-resistant mutants by the same method as that applied to the M_2 generation. Lines showing resistance in the second screen were selected and propagated for further analysis.

Plant growth

Seedlings were cultured for 7 days hydroponically in half strength Kimura's B solution (Yoshida *et al.* 1972) inside a growth chamber with additional light to assure a 16 hours photo-period at 30℃ . If the material was to be used for detection of XDH activity, shoots were harvested and stored at −80 ℃ until required. For the assays of NR, NiR and nitrate content, 7-day-old

seedlings were further treated with NR induction solution (2 mM potassium nitrate and 0.5 mM calcium sulfate) for 24 hours, and leaf blades were harvested and stored at − 80 ℃ until required.

NR and NiR assays

Preparation of crude extracts from plant material and measurement of NADH-NR and NADPH-NR activities were carried out as described by Kleinhofs *et al.* (1986). NiR activity in the same crude extracts was measured according to Ida and Mikami (1986).

Determination of nitrate content

Extraction of nitrate from plant material and spectrophotometric-determination of nitrate concentration were performed in accordance with the method of Cataldo *et al.* (1975).

XDH assay

Preparation of crude extracts from plant material, gel electrophoresis and gel staining followed the procedure of Mendel and Müller (1976).

Restoration of NR activity by molybdate

Seedlings were cultured in Kimura's B solution (Yoshida *et al.* 1972) for 7 days under natural daylight at 30 ℃ and then treated with the modified Kimura's B solution containing 0.5 mM sodium molybdate for 2 days. Shoots were harvested and stored at − 80 ℃ until required. NADH-NR activity was assayed as described above.

Genetic analysis

Two mutants, C290 and C384, were crossed with wild type IR30. Both NADH-NR and NADPH-NR activities were measured in F_1 strain as the method mentioned above. In the F_2 population, the segregation ratio of chlorate-resistance was scored. The F_2 seeds were sown in 1 mM potassium chlorate solution, then chlorate-resistance was evaluated 7 days after sowing, as described earlier.

To test for allelism, C290 was crossed with C384. Additionally, C290 and C384 were crossed with other two mutants in the MoCo biosynthetic pathway (Sato *et al.* 1996): C27 (genotype: *cnx* 1/*cnx* 1) and C25 (genotype: *cnx* 2/*cnx* 2), both in the IR30 background. NADH-NR and XDH activities were investigated in each F_1 strain as described above.

Results

Screening of chlorate-resistant mutants

Out of 204, 500 M_2 seedlings screened, 845 of them were chlorate-resistant. These seedlings were transferred to soil culture where 668 of them produced M_3 seeds. When chlorate resistance of the 668 M_2 seedlings was retested in their M_3 lines, 26 chlorate-resistant mutant lines were identified by their capacity to grow on 0.1 mM chlorate (Fig. 1). Among the 26 lines, only two

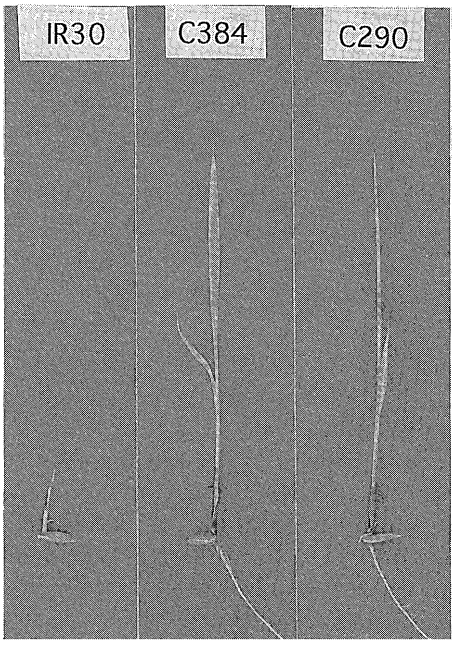

Fig. 1. Growth of chlorate-resistant mutants and their wild type IR30 on 0.1 mM potassium chlorate for 7 days. Among 26 chlorate-resistant mutant lines, C290 and C384 are representative.

lines are derived from ethyleneimine mutagenesis and the remaining 24 lines (including C290 and C384) come from sodium azide mutagenesis. Since C290 and C384 were sufficiently propagated, we focused our analysis on these. The results are presented here.

NR and NiR assays

NADH-NR, NADPH-NR and NiR activities were assayed in the leaves of C290, C384 and their wild type IR30 (Table 1). NADH-NR activities of C290 and C384 were 386 and 394 n mol NO_2^-/ min / g fresh weight, respectively, both of which corresponded to 34% of the wild type activity. Likewise, NADPH-NR activity was low in the mutants, less than 50% of the wild type activity for both lines. These data show that C290 and C384 are mutants with low NR activity.

NiR activity of the mutants was almost same as that of

Table 1. NADH-NR, NADPH-NR and NiR activities in chlorate-resistant mutants, C290 and C384, and their wild type IR30

	Line	NADH-NR[2]	NADPH-NR[2]	NiR[2]
A[1]	IR30	1110 ± 154 (100)[3]	16.5 ± 8.0 (100)[3]	137 ± 37 (100)[3]
	C290	386 ± 81 (34)	7.7 ± 8.0 (46)	158 ± 39 (115)
B[1]	IR30	1151 ± 387 (100)[3]	20.2 ± 19.5 (100)[3]	153 ± 20 (100)[3]
	C384	394 ± 111 (34)	9.1 ± 13.6 (44)	190 ± 39 (124)

Each activity averaged over five replicates. At least 20 seedlings were used per enzyme extraction.

[1] Experiments A and B were carried out separetely,

[2] n mol NO_2^-/ min / g fresh weight,

[3] Percentage of wild type.

Table 2. Nitrate content in the mutnats, C290 and C384, and IR30

Line	Nitrate content[1)]
IR30	583 (100)[2)]
C290	769*(131)
C384	861*(147)

Means of five replicates are shown. At least 250 seedlings were used per experiment.
[1)] μ M NO_3^- / g dry weight,
[2)] Percentage of wild type,
*) Singificantly different from IR30 at 5% level according to t test.

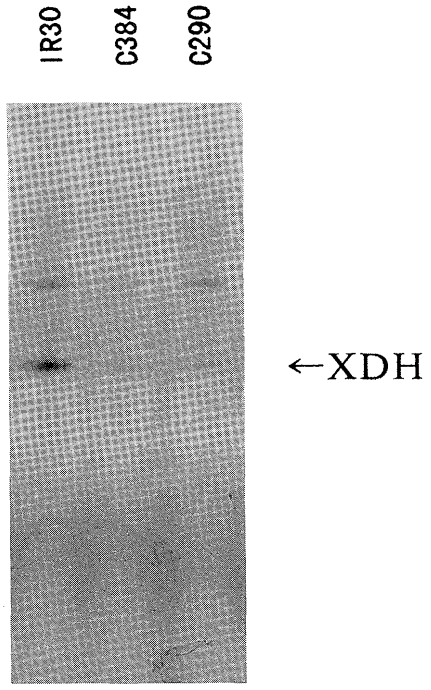

Fig. 2. Detection of xanthine dehydrogenase activity by native PAGE in the two mutants, C290 and C384, and their wild type IR30. At lest 20 seedlings of each line were used for XDH extraction. The upper bands are non-specific: they have a different color than the bands of XDH (lower).

the wild type. However, a slight elevation of NiR activity in the mutants was observed; namely, the activities in C290 and C384 were 115 and 124% of the wild type activity, respectively (Table 1).

Determination of nitrate content

In Arabidopsis NR-deficient mutants, the higher NiR activity than wild type was explained by their elevated nitrate content, because nitrate induces NiR (Braaksma and Feenstra 1982a). We measured nitrate content in our mutants (Table 2). Indeed the nitrate contents in C290 and C384 were more than 30% higher than in wild type as for the Arabidopsis NR-deficient mutants (Braaksma and Feenstra 1982a). Thus, the elevated NiR activity in our mutants may be explained by the elevated nitrate.

XDH assay

MoCo is a common prosthetic group of NR and XDH, and it is essential for the activity of both enzymes (Mendel and Müller 1976). Therefore, mutants affected in the MoCo biosynthetic pathway lack the activity of both NR and XDH (Gabard *et al.* 1988, Sato *et al.* 1996). To identify whether our mutants are MoCo-deficient type, XDH activity in the mutants was determined by native PAGE (Fig. 2). There was a strong band showing XDH activity in IR30, however only a faint band was observed in C290 and C384. Thus, XDH activity in the two mutants was deficient, which identifies them as being of the MoCo-deficient type.

Restoration of NR activity by molybdate

MoCo-deficient mutants are divided into two groups: one that recovers NR activity upon addition of excess molybdate to the growth medium while the other does not (Mendel *et al.* 1981, Steffen and Schieder 1984, Gabard *et al.* 1988, Sato *et al.* 1996). To classify our MoCo-deficient mutants, 7-day-old seedlings were treated with Kimura's B solution containing 0.5 mM sodium molybdate for 2 days and NADH-NR activity was measured. The medium containing 0.5 mM molybdate was toxic, because rice seedlings grown on the medium from sowing were yellow and had a reduced growth rate within a week (data not shown). To minimize the toxicity, we treated seedlings with the medium for only two days. As shown in table 3, molybdate addition decreased the NADH-NR activity in IR30 to about 40% of the control, which was probably due to the molybdate-toxicity. Likewise, activities in C290 and C384 decreased to 52% and 65% of the control, respectively. On the other hand, another MoCo-deficient mutant, C27, increased its NADH-NR activity to 273% of the control (Table 3). Thus, in contrast to C27, C32 and C33 (Sato *et al.* 1996), C290 and C384 belong to the group whose NR activity can not be recovered by exogenous molybdate.

Table 3. Recovery of NADH-NR activity by molybdate in the mutants, C290 and C384, and IR30

Line	NADH-NR[1)]	
	$-$ Na_2MoO_4	+0.5 mM Na_2MoO_4
IR30	1337 ± 201	561 ± 107 (41)[2)]
C290	330 ± 55	170 ± 39 (51)
C384	404 ± 105	263 ± 47 (65)
C27	170 ± 18	465 ± 79 (273)

Data are mean ± S. D. from five replicates. At leatst 20 seedlings were used per enzyme extraction.
[1)] n mol NO_2^- / min / g fresh weight,
[2)] Percentage of control.

Genetic analysis

NADH-NR activities in the two F_1 strains derived from C290/IR30 and C384/IR30 were at least 10% higher than the wild type activity (Table 4). Also, in every F_2 population derived from mutant/IR30 crosses, chlorate-sensitive (IR30 type) and resistant (mutant type) seedlings segregated in accordance with a 3 :1 ratio (Table 5). Thus, these results indicate that each mutation of C290 and C384 is controlled by a single recessive gene.

An allelism test showed that C290 did not complement either NADH-NR or XDH deficiencies of C384. Namely, the NADH-NR activity in F_1 strain derived from C384/C290 was no more than 35% of the wild type, and only a faint XDH band was observed in extracts from the strain (Table 6, Fig. 3). Allelism was also checked between the two mutants and other two MoCo-deficient mutants, C27 (genotype: *cnx* 1/*cnx* 1) and C25 (genotype: *cnx* 2/*cnx* 2). In the four kinds of F_1 strains, namely C27/C290, C27/C384, C25/C290 and C25/C384, NADH-NR activity was recovered to more

Table 4. NADH-NR activity in F_1 strains from C290/ IR30 and C384/ IR30, and in their parents

	Line	NADH-NR[2]
A[1]	IR30	632 ± 222 (100)[3]
	C290	128 ± 20 (20)
	(C290/IR30)F_1	733 ± 216 (115)
B[1]	IR30	776 ± 114 (100)[3]
	C384	299 ± 214 (38)
	(C384/IR30)F_1	877 ± 440 (112)

Data are mean ± S. D. from five replicates. At leatst 20 seedlings were used per enzyme extraction.
1) Experiment A and B were carried out separately,
2) n mol NO_2^- / min / g fresh weight,
3) Percentage of wild type.

Table 5. Segregation of chlorate-resistance in F_2 populations between MoCo-deficient mutants and IR30

Line	Chlorate		Expected ratio	χ^2 (3:1)	Probability
	Sensitive	Resistant			
IR30	20	0			
C290	0	20			
C384	0	20			
(C290/IR30)F_2	73	31	3:1	1.282	0.2~0.3
(C384/IR30)F_2	87	32	3:1	0.289	0.5~0.7

Table 6. NADH-NR activity in F_1 strains between MoCo-deficient mutants

	Line	NADH-NR[2]
A[1]	IR30	1459 ± 180 (100)[3]
	C290	371 ± 79 (25)
	C384	482 ± 33 (33)
	(C384/C290)F_1	513 ± 24 (35)
B[1]	IR30	1071 ± 139 (100)[3]
	C27	58 ± 8 (5)
	C290	235 ± 36 (21)
	C384	284 ± 100 (26)
	(C27/C290)F_1	775 ± 111 (72)
	(C27/C384)F_1	857 ± 168 (80)
C[1]	IR30	1184 ± 136 (100)[3]
	C25	18 ± 3 (1)
	C290	241 ± 36 (20)
	C384	391 ± 90 (33)
	(C25/C290)F_1	978 ± 113 (82)
	(C25/C384)F_1	881 ± 174 (74)

Data are mean ± S. D. from five replicates. At leatst 20 seedlings were used per enzyme extraction.
1) Experiment A, B and C were carried out separately,
2) n mol NO_2^-/ min / g fresh weight,
3) Percentage of wild type.

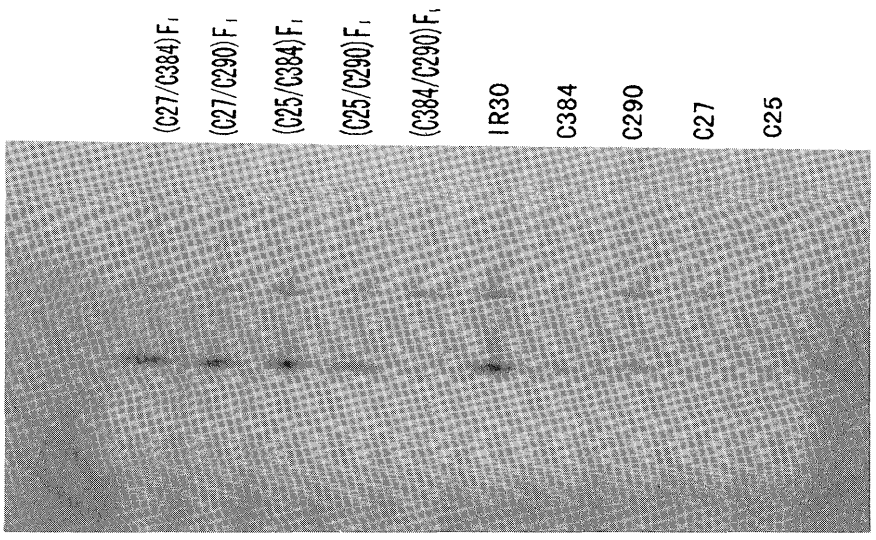

Fig. 3. XDH electrophoretic pattern of F_1 strains from crosses between mutants.

than 70% of the wild type activity (Table 6). Also, XDH activity of all four F_1 types was the same level as that of their parents (Fig. 3). Thus, we conclude that the mutant locus in both C290 and C384 is different from *cnx* 1 and *cnx* 2 loci. We have designated the new locus "cofactor for nitrate reductase and xanthine dehydrogenase 3 (*cnx* 3)".

Discussion

Molybdenum deficiency has reduced the yield of rice (Fujiwara and Tsutsumi 1955), and has lead to the 'whiptail' disorder of cauliflower and broccoli (Hewitt 1983) and to damage from low temperatures in winter wheat grown on acidic soils (Vunkova-Radeva *et al.* 1988). Therefore, analysis of the molybdenum assimilation with mutants is of agronomic importance. Mutants affected in the MoCo biosynthetic pathway have typically been isolated as chlorate-resistant mutants (Braaksma and Feenstra 1982b, Steffen and Schieder 1984, Gabard *et al.* 1988, Schoenmaker *et al.* 1991, Sato *et al.* 1996). Owing to the pleiotropic loss of NR activity, MoCo-deficient mutants are resistant to chlorate. Mutants lacking NR activity can no longer reduce the nitrate analogue chlorate to the toxic compound chlorite (Hoff *et al.* 1994). In our work, we isolated 26 chlorate-resistant mutants from 204,500 M_2 seedlings of rice. Among 26 mutants, since C290 and C384 were well-propagated, these mutants were further characterized. The remaining 24 mutants are now under propagation. In C290 and C384, the activities of 3 MoCo-containing enzymes (NADH-NR, NADPH-NR and XDH) were significantly lower than in the wild type IR30, which indicated that both mutants were MoCo-deficient. Although the two MoCo-deficient mutants were 'leaky mutants' in that the activity of the enzymes was reduced but not eliminated, the phenotypes were stable among M_3 ~M_5 generations (data not shown) and were easy to distinguish from the wild type. We decided to characterize them further.

In *Nicotiana plumbaginifolia*, a genetic analysis of MoCo-deficient mutants indicated that at least six loci, corresponding to six independent enzymatic steps, were involved in the MoCo biosynthetic pathway (Gabard *et al.* 1988). Additionally, a new locus *aba*1 has been identified recently by the analysis of a hormone-resistant mutant of *Nicotiana plumbaginifolia*, which involved in the last step of the MoCo biosynthesis shared by XDH and ABA aldehyde oxidase (Leydecker *et al.* 1995). Also in barley, six loci involved in MoCo biosynthesis have been found (Hoff *et al.* 1994), and one of the these loci (*nar* 2) was mapped on chromosome 7 by linkage analysis (Melzer *et al.* 1988). Previously, we reported on four MoCo-deficient mutants of rice (*Oryza sativa* L., cv. IR30), which were divided into two complementation groups (Sato *et al.* 1996). The mutated loci corresponding to each of the two complementation groups were designated as *cnx* 1 and *cnx* 2, respectively (Sato *et al.* 1996). In the present report, genetic analysis of C290 and C384 revealed that each phenotype of the mutants was controlled by a single recessive gene, which was different from *cnx* 1 and *cnx* 2. Thus, we conclude that there are at least three loci involved in the MoCo biosynthetic pathway in rice. We are now mapping the three loci.

The rice mutants affected at *cnx* 1 recovered their phenotypes under unphysiologically high (0.5 mM) molybdate (Sato *et al.* 1996). Also, in other species, MoCo-deficient mutants recovered their phenotypes in the presence of excess molybdenum (Mendel *et al.* 1981, Mendel and Müller 1985, Gabard *et al.* 1988, Steffen and Schieder 1984). In *Nicotiana plumbaginifolia*, it was suggested that the *cnx* A locus, which may correspond to the rice *cnx* 1, inserted molybdenum into molybdopterin at a late step of the MoCo biosynthetic pathway (Mendel and Müller 1985, Gabard *et al.*1988). Recently, the deduced *cnx* A gene was cloned in Arabidopsis (Stallmeyer *et al.* 1995). In this report, we added molybdate to the growth medium and measured NADH-NR activity in C290 and C384. However, none of the two mutants at the *cnx* 3 locus recovered their NADH-NR activities. Therefore, it is reasonable to conclude that *cnx* 3 gene encodes a different step from *cnx* 1 in the MoCo biosynthetic pathway: e. g., molybdate uptake or molybdopterin biosynthesis. Also in Arabidopsis, two genes which coded activities in molybdopterin biosynthesis were cloned (Hoff *et al.* 1995). Investigating the relationship between these genes and *cnx* 3 locus may help us identify the precise function of the locus.

Finally, it is interesting that NiR activities in C290 and C384 were slightly higher than that of the wild type. The elevated NiR activity in NR-deficient mutants has been repeatedly observed in barley, Arabidopsis and rice (Kleinhofs *et al.* 1980; Braaksma and Feenstra 1982 a, b; Hasegawa *et al.* 1992; Sato *et al.* 1996). The high NiR activity in NR-deficient mutants of Arabidopsis was explained by an elevated nitrate content, because nitrate induces NiR (Braaksma and Feenstra 1982a). In C290 and C384, the nitrate content was higher than that of the wild type. Also in C27 and C25, which are other rice MoCo-deficient mutants with reduced NR activity, elevated nitrate content has been observed (Sato and Ishizaka, unpublished data). Thus, it is likely that the high NiR activity of rice MoCo-deficient mutants can be explained by the elevated nitrate, as previously suggested for Arabidopsis NR-deficient mutants (Feenstra and Braaksma 1982a).

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