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Molecular mapping of the *cnx2* locus involved in molybdenum cofactor biosynthesis in rice (*Oryza sativa* L.)

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Abstract Molybdenum cofactor (Moco) is essential for nitrate reductase (NR), xanthine dehydrogenase (XDH), and aldehyde oxidase to perform their catalytic functions in plants. Moco biosynthesis is a complex process involving many genes. Little is known about the genetics and molecular aspects of Moco biosynthesis in plants and other eukaryotes. In rice, we previously isolated a Moco mutant C25 with a mutation in the *CNX2* gene from a mutagenized *indica* cultivar IR30 and characterized its biochemical properties. This mutant was crossed with a *japonica* cultivar, Norin 8, to investigate the linkage of *cnx2* to restriction fragment length polymorphism (RFLP) and cleaved amplified polymorphic sequence (CAPS) markers. Chlorate resistance was used to trace the *cnx2* mutation because of its cosegregation with the loss of NR and XDH activities observed earlier. RFLP and CAPS analyses show the location of the *cnx2* locus on the long arm of chromosome 4. It is mapped between RFLP markers C513 and C377 with a distance of 9.5 and 13.1 cM, respectively. It is also linked with CAPS marker RA0738 at a distance of 30.3 cM.

Keywords Molybdenum cofactor (Moco) · Nitrate reductase (NR) · RFLP · Cleaved amplified polymorphic sequences (CAPS) · Rice (*Oryza sativa* L.)

Introduction

Molybdenum (Mo) is an essential micronutrient for plant growth, development, and yield (Hewitt 1983). Biologically active Mo is complexed by a cofactor, molydopterin. Moco, the molybdenum cofactor, combines with apoproteins to interact with other components of the enzyme for electron transport. In higher plants, lack of Mo in the soil, or the plant's inability to take up Mo, to synthesize molydopterin, or to bind Mo to molydopterin, interferes with metabolic functions leading to plant death (Mendel 1997).

So far, there are three molybdoenzymes reported in plants: (1) NR, which catalyzes the first step of nitrate assimilation, (2) XDH involved in purine catabolism, and (3) aldehyde oxidase, which catalyzes the last step in the biosynthesis of indoleacetic acid and abscisic acid (Mendel and Schwarz 1999). The occurence of sulfite oxidase, which is involved in the formation of sulfate, has yet to be confirmed in plants (Zimmer and Mendel 1999). These enzymes are homodimeric proteins that anchor electron transport chains involving different prosthetic groups (FAD, heme or Fe-S, and Moco). Since they share a common Moco, Moco-deficient mutants in plants show pleiotropic loss of molybdoenzyme activities. Although Moco biosynthesis in *Escherichia coli* (Rajagopalan and Johnson 1992; Rajagopalan 1996) and *Arabidopsis thaliana* (Hoff et al. 1995; Stallmeyer et al. 1995; Mendel 1997) have been investigated, relatively little is known in other plants and eukaryotes. Due to instability of the cofactor and its precursor, the establishment of the biosynthetic pathway by direct detection and analysis of the intermediates are difficult. However, isolation and analysis of mutants affected in Moco biosynthesis have helped provide an understanding of Moco biosynthesis in plants.

NR has a crucial role in nitrate assimilation, a pathway that is of key importance for plant nutrition. It has been intensively investigated to describe properties relevant to its catalytic efficiency and regulation (for reviews see Kleinhofs and Warner 1990; Solomonson and Barber 1990; Crawford 1995; Campbell 1996, 1999; Mendel and Schwarz 1999). Moco-deficient mutants have been isolated as NR mutants on the basis of chlorate resistance in many organisms (Hoff et al. 1994). In plants, the

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toxicity of chlorate is almost completely dependent on NR activity because NR simultaneously catalyzes the reduction of chlorate to toxic chlorite. Thus chlorate-resistant plants usually have a defective NR. Two main types of NR mutants are known in higher plants: *nia* and *cnx* or Moco mutants. The *nia* mutants are defective in the structural gene for NR. They have low or deficient NR activity but normal XDH activity. The *cnx* or Moco mutants have defects in the Moco. They are low or deficient in both NR and XDH activities. Hoff et al. (1994, 1995) described Moco mutants in various organisms.

Like other crop species, rice uses nitrate or ammonium as a nitrogen source. Rice can assimilate nitrate even in anaerobic and flooded conditions (Barlaan et al. 1998). Nitrate taken up by plants induces and regulates NR in rice (Reggiani et al. 1993; Barlaan and Ichii 1996). Recently, NR-deficient mutants in rice were isolated. A *nia* mutant, M819 (Hasegawa et al. 1992; Ichii et al. 1995), and six *cnx* mutants, C25, C27, C32, C33, C290, and C384 (Sato et al. 1996; Sato and Ichii 1998), have been characterized. At least three genes (*cnx1*, *cnx2* and *cnx3*) were found to be involved in Moco biosynthesis in rice. These are monogenic recessive but their functions in Moco synthesis are not clearly defined. C25, a *cnx2* mutant, is deficient in NADH-NR, NADPH-NR, and XDH activities and is thought to be involved in an earlier step of Moco biosynthesis. Neither of these loci have been analyzed for their location in the rice genome. Here we report the linkage of the *cnx2* locus with RFLP and CAPS markers.

Materials and methods

Plant materials

C25, a *cnx2* mutant, was isolated on the basis of chlorate resistance from the original *indica* cultivar IR30 mutagenized with sodium azide (Sato et al. 1996). The M_5 generation of this mutant line was crossed as female with Norin 8, a *japonica* cultivar. The F_2 population was grown in an irrigated lowland ricefield at Kagawa University, Japan. For DNA analysis, a total of 107 F_2 plants were randomly selected. Leaf material for DNA isolation was collected before heading. F_3 seeds from these 107 F_2 plants were used for a test of chlorate resistance. Due to sterility of the interspecific cross, limited F_2 and F_3 progenies were available for evaluation.

Assessment of chlorate resistance

Chlorate resistance, NR activity, and XDH activity were reported to show parallelism in segregation in the F_2 population of the cross between C25 and IR30 (Sato et al. 1996). It was not feasible to analyze the NR and XDH activities in many individual plants in F_3 families to determine the segregation of these traits. Thus we used chlorate resistance as the basis for mapping the *cnx2* locus in the rice genome. About 50 seeds of each F_3 family were sown in a 1 mM potassium chlorate solution in a growth chamber at 30°C under natural daylength (12 h). After 5 days, reactions to chlorate in each family were evaluated. Chlorate-sensitive seedlings showed brown spots or burns in the leaves with significantly reduced plant height and root length. Chlorate-resistant individuals showed no symptoms or else the same symptoms to a lesser degree. Families were scored either as homozygous or heterozygous for resistance or sensitivity to chlorate. A chi-square test was used to evaluate the segregation ratio.

RFLP analysis

DNA clones for RFLP analysis were generously provided by the Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF) Institute, Tsukuba, Japan. The RFLP markers obtained were tested in F_2 progeny of a Nipponbare×Kasalath cross. RFLP markers were mainly composed of genomic clones and cDNA clones from calli and roots. These clones were amplified by transformation (as described by Sambrook et al. 1989) or the polymerase chain reaction (PCR), or both. Probes detecting polymorphism between C25 and Norin 8 with at least one enzyme were used to detect the genotype of each F_2 plant.

Total DNAs of the parents and F_2 individuals were extracted by the CTAB method (Murray and Thompson 1980) and digested with the following restriction enzymes: *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, and *Kpn*I. The procedure for Southern-blot analysis described by Kurata et al. (1994) was followed with some modifications. The digest was electrophoresed in an 0.8% agarose gel for 16 h at 20 V. After electrophoresis, the gel was washed with 0.25 N HCl, alkaline solution (0.5 M NaOH, 1.5 M NaCl) and neutralizer reagent [1.5 M NaCl, 1 M Tris HCl (pH 8)] and blotted onto a positively charged nylon membrane (Boeringer, Manheim) saturated with 20×SSC for 14 h. The membrane was washed with 2×SSC, air dried, and baked at 80°C for 2 h. The DNA probes were labeled and hybridized using enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham) according to the manufacturer's instructions. The ECL of hybridized filters was detected on x-ray film for 3 h.

CAPS analysis

We used the same DNAs of parents and F_2 individuals for RFLP and CAPS analysis. DNAs of RFLP probes were sequenced and converted into CAPS markers. The primer sequences were kindly provided by the STAFF Institute. To confirm the position of *cnx2* determined by RFLP and to evaluate whether or not these CAPS markers could be used in linkage mapping, CAPS analysis was limited to markers on chromosome 4. The procedures of CAPS analysis described by Konieczny and Ausubel (1993) were used with few modifications. To survey restriction endonucleases that generate polymorphism, PCR was performed in a volume of 50 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM dNTPs, 500 ng of each primer, 100 ng of genomic template, and 2.5 U Ampli*Taq* Gold. PCR conditions were: denaturation at 94°C for 30 s, annealing at 50–61°C for 1 min, polymerization at 72°C for 1 min, and extension at 72°C for 10 min through 35 cycles. The PCR products for C25 and Norin 8 were digested with the four to five base-cutter restriction endonucleases *Acc*II, *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Rsa*I, *Sau*3AI, *Taq*I, and *Scr*FI. Subsequently, these were size-fractionated on 1% agarose gels and visualized by staining with ethidium bromide. PCR reactions in 57 F_2 individuals were scaled down to a volume of 25 µl.

Linkage analysis

Linkage of chlorate resistance, RFLP, and CAPS markers was analyzed using MAPMAKER v. 2.0 (Lander et al. 1987). A LOD of 3.0 was declared and the distances between markers were presented in Kosambi cM units.

Results

Segregation of chlorate resistance

In the F_3 population, 29 families were sensitive, 54 were heterozygous, and 24 were resistant to chlorate (Table 1). The segregation fits a 1:2:1 genotypic ratio, which con-

Table 1 Segregation of chlorate resistance in 107 F_3 families from the cross C25×Norin8

Item	Homozygous sensitive	Heterozygous	Homozygous resistant	χ^2 (1:2:1)	*P*
No. of families	29	54	24	0.476	>0.95

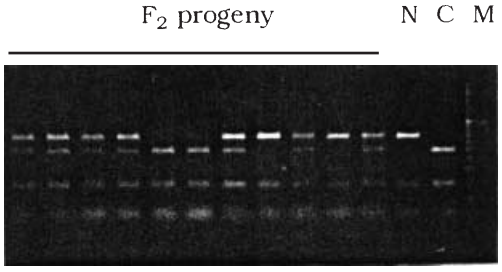

Fig. 1 CAPS analysis of RA0738 linked to the *cnx2* locus. DNAs of F_2 progeny and parents (*N*) Norin 8 and (*C*) C25 were PCR-amplified with RA0738 primers (forward 5´ATCCTGGAGAAAACACTAAG3´ and reverse 5´ATACGCATTTGTCTACGCTA3´) with an annealing temperature of 55°C (see Materials and methods). PCR products were digested with *Hae*III, separated on a 1% agarose gel, and stained with ethidium bromide. The 200-bp DNA ladder is shown in lane *M*

firmed the monogenic and recessive control of chlorate resistance in C25 as described previously by Sato et al. (1996).

Magnitude of polymorphism

Of the 147 selected RFLP markers, 78% showed polymorphism between C25 and Norin 8. However, we used only codominant markers so that we could determine heterozygotes in the F_2 population. In cases where no polymorphism was observed between parents in selected markers, we tested additional markers to fill the gaps. The RFLP markers, genotyped with 107 F_2 individuals, encompassed the rice genome with an average interval of about 20 cM between marker loci (data not shown). About 19% of the RFLP markers showed skewed segregations from the theoretical ratio of 1:2:1 ($P<0.05$). The segregation distortions were found in chromosomes 3, 4, 5, 6, 11, and 12.

In CAPS, after PCR of primers of the 14 markers in chromosome 4, five markers produced single bands, six expressed multiple bands, and three showed no amplification. After digestion with endonucleases, two markers exhibited codominant bands and two markers produced a single band in either C25 or Norin 8. Figure 1 shows the codominant bands in parents and F_2 individuals of CAPS marker RA0738, which showed linkage to *cnx2*. One marker, CK0513, produced codominant bands after PCR but the results were not reproducible in F_2 progenies.

Linkage mapping

Linkage analysis shows that the *cnx2* locus is located on the long arm of chromosome 4 and mapped between

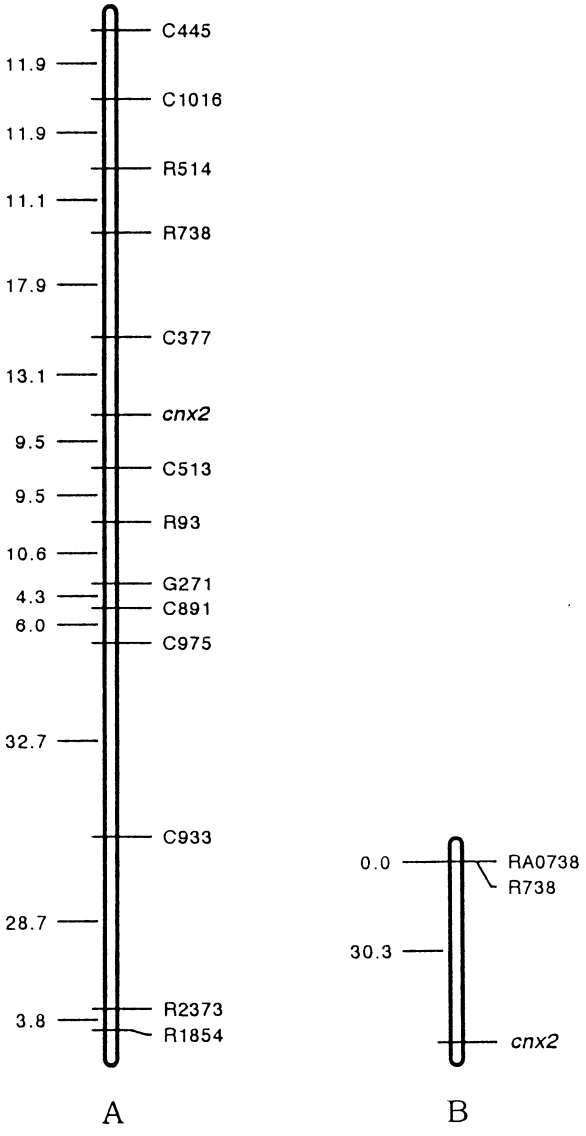

Fig. 2 Linkage of *cnx2* with (**A**) RFLP markers and (**B**) CAPS marker RA0738 and RFLP marker R738 on chromosome 4. Distances between markers are shown in Kosambi cM to the left of each map

RFLP markers C513 and C377 with a distance of 9.5 and 13.1 cM, respectively (Fig. 2A). When the map of chromosome 4 was compared with the map of Kurata et al. (1994) and Harushima et al. (1998), it was found that most of the tested markers were located with the expected order. However, the distances between markers varied, most likely due to differences in population size and the number of probes tested.

CAPS analysis showed the location of *cnx2* on chromosome 4 by its linkage with RA0738 at a distance of

30.3 cM (Fig. 2B). CAPS marker RA0738 was converted from RFLP marker R738. Integrating these two markers along with *cnx2* using 57 F_2 individuals resulted in a similar distance or position on the same chromosome. RFLP markers C513 and C377, and other markers on chromosome 4, were converted to CAPS with primers ranging from 17 to 22 bp oligomers. Unfortunately, PCR products of more than half of the CAPS markers had multiple bands or did not amplify. We tried to manipulate PCR conditions and tested additional restriction endonucleases. Again, we were unable to obtain the desired results; thus, we failed to map other CAPS markers on chromosome 4.

Discussion

Moco biosynthesis in higher plants is a complex process and involves multiple steps controlled by different genes. In rice, there is still a long way to go to identify the genes involved in the pathway. So far, at least three loci (*cnx1*, *cnx2* and *cnx3*) have been identified. The *cnx1* mutants C27, C32, and C33 were involved in Mo transfer, while *cnx2* mutant C25 and *cnx3* mutants C290 and C384 were believed to be involved in early steps of biosynthesis (Sato et al. 1996, Sato and Ichii 1998). Mendel (1997) proposed a model for Moco biosynthesis in *A. thaliana*, which includes three stages involving seven genes: (1) conversion of the guanosine derivative into precursor Z catalyzed by *cnx2* and *cnx3*; (2) incorporation of sulfur into precursor Z and conversion of precursor Z to molybdopterin catalyzed by molybdopterin synthase, which is a product of the genes *cnx5*, *cnx6* and *cnx7*; and (3) transfer of Mo to molydopterin by a series of reactions such as the uptake and processing of molybdate and intracellular transfer by the product of *cnx1*. The *cnx4* gene is assumed to be involved in the vesicle transport system. This model, however, was based on the Moco biosythetic pathway in *E. coli* where five Moco-specific operons (designated *moa*, *mob*, *mod*, *moe*, and *mog*) comprise more than 15 genes (Rajagopalan and Johnson 1992; Rajagopalan 1996). In other plants, six genetic loci in *Nicotiana* (Gabard et al. 1988) and nine loci in barley (Kleinhofs et al. 1989; Mendel and Schwarz 1999) were reported to be involved in Moco biosynthesis. The multitude of genes involved shows the complexity of the process in higher plants, which merits additional investigation.

The specific role that the mutation in C25 plays in Moco biosynthesis is not clear. This needs further study and confirmation once *cnx2* is cloned in rice. Hoff et al. (1995) cloned the genes *cnx2* and *cnx3* in *A. thaliana* by functional complementation of *E. coli* Moco mutant genes involved in early steps of Moco biosynthesis. The expressions of these genes in *A. thaliana* were abundant in roots. However, the precise functions of both proteins in the early steps are still unknown. Although these genes have been cloned, there is a need for further investigation to reveal not only the functional correspondence of *cnx* loci between rice and *A. thaliana* but also the difference or similarity of gene expression in plant organs.

An earlier study showed that the rice *cnx2* mutant C25 had impaired Moco biosynthesis based on NAD(P)H, XDH, and *Neurospora crassa nit*-1 assays. Also, its chlorate resistance cosegregated with XDH activities (Sato et al. 1996). The close association of these traits might be due to pleiotropy of defective genes or the tight linkage of genes controlling these traits. In this regard, we used chlorate resistance as the parameter in mapping *cnx2* in rice. RFLP and CAPS analyses revealed the location of *cnx2* on chromosome 4. However, the distance between *cnx2* and the RFLP or CAPS markers was not close enough for positional cloning. We tried to investigate additional RFLP probes located in chromosome 4, but we obtained neither linkage nor polymorphism between the parents. This might be due to differences in the mapping populations (Lin et al. 1996) or in the population size between the mapping populations (Antonio et al. 1996). Further evaluation of additional RFLP markers from the updated linkage map of rice (Harushima et al. 1998) or the use of a different population with a larger population size may allow successful mapping.

In CAPS, of 14 converted RFLP markers on chromosome 4, only RA0738 was found to be linked with *cnx2*. That is attributed to the absence of amplification and the generation of multiple bands in PCR products, no polymorphism after digestion, and no linkages. This situation is commonly observed in CAPS analysis, therefore, CAPS primer sequences need reconstruction (Shikanai et al. 1996). Moreover, the CAPS markers, which had been tested in Nipponbare and Kasalath, may not be suitable to our mapping population because of differences in the target sites for amplification or endonuclease restriction. Another implication is that CAPS markers from one population may not be a good alternative source for comprehensive linkage mapping in another population. This restriction may not be overcome unless the primer sequences and appropriate restriction endonucleases are thoroughly tested. This hypothesis, however, needs to be examined in different populations with many CAPS markers on different chromosomes. In our experience, from the CAPS markers we evaluated in chromosome 4 alone, only 14% showed polymorphism and only 7% showed linkage, in contrast to more than 70% exhibiting both polymorphism and linkage using RFLPs. It would be, therefore, unrealistic to pursue comprehensive screening and linkage mapping in the rice genome using CAPS analysis in our population.

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