Characterization of Rice Mutants Deficient in the Formation of Crown Roots

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To investigate the genetic mechanism regulating crown root formation, we identified two recessive rice mutants (odm 202 and BRX334). The odm 202 mutant was detected in a MNU-mutagenized M₃ population of rice (cv. Taichung 65) and the BRX334 mutant in a γ-ray mutagenized M₂ population of rice (cv. Blue Rose). The number of crown roots of the odm 202 and BRX334 seedlings was significantly lower than that of the respective wild types and this mutation type was designated as *crown rootless* with the gene symbol *crl*. Allelism test indicated that the two mutant genes were not allelic and the *CRL* loci of odm 202 and BRX334 were designated as *CRL1* and *CRL2*, respectively. Histological observations suggested that the initiation of crown root primordia was impaired in the *crl1* mutant, while in the *crl2* mutant both the initiation and subsequent growth of crown root primordia were impaired. In addition, the *crl1* mutant grew normally until maturity except for the difference in the number of crown roots from the wild type, whereas the *crl2* mutant showed many abnormal morphological characters besides the defect in the formation of crown roots. These facts suggest that *CRL1* regulates specifically the initiation of crown root primordia but is not involved in the radicle and lateral root initiation or shoot development, while *CRL2* regulates various processes of plant development.

Key Words: *Oryza sativa*, crown root, mutation, root formation, root initiation.

Introduction

Many soil resources are unevenly distributed, or are subject to localized depletion. Therefore, the spatial deployment of the root system, that is, root architecture, to a large extent determines the ability of a plant to exploit those resources. A plant root system generally consists of several component roots with different characteristics. Root architecture is the result of initiation, emergence, extension growth, senescence or death of different component roots, and the plasticity of these processes in response to environ-

mental conditions (Lynch 1995, Yamauchi *et al.* 1996). It is therefore important to understand how the developmental characteristics of different component roots are genetically regulated.

Generally, the root system of most of the dicot plants develops from the radicle, while monocot plants have a so-called fibrous root system which is characterized by many adventitious (crown) roots (Klepper 1992). For example, the root system of a field-grown rice plant usually has several hundreds (sometimes over one thousand) of crown roots (Kawata *et al.* 1978, Kawashima 1988). Recently, many mutants affecting root development have been identified in dicots such as *Arabidopsis thaliana* and tomato and are contributing to our understanding of the genetic mechanisms of root development (Zobel 1991, Benfey and Sciefelbein 1994, Dolan and Roberts 1995, van den Berg *et al.* 1998). In monocots, however, these mechanisms are poorly documented mainly because considerably fewer root developmental mutants have been identified in monocots than in dicots. For the mutational analysis of root development in monocots, we attempted to isolate rice mutants and two mutants with a defect in the formation of crown roots (odm 202 and BRX334) were obtained.

In this paper, we characterized these mutants phenotypically and genetically and then we analyzed the two mutations at the histological level. Because it was reported that the plant hormone auxin is associated with the promotion of adventitious root formation in a variety of plant species (Blakesley *et al.* 1991), we also investigated the effects of auxin treatment on the crown root formation of these mutants.

Materials and Methods

Screening for mutants

Screening for the mutants was performed using a M₃ progeny of rice (cv. Taichung 65) treated with *N-methyl-N-nitrosourea* (MNU) as described by Hong *et al.* (1995). Although these materials were prepared to isolate embryo mutants originally, we also used them to isolate root developmental mutants in this study. In addition, a M₂ progeny of rice (cv. Blue Rose) derived from 200 Gy γ-ray-irradiated seeds was used. After the MNU-mutagenized M₃ seedlings were grown in 16 cm long × 15 cm wide seed-pack growth pouches (Vaughan's Seed Company, USA) and the γ-ray-mutagenized M₂ seedlings were grown in water culture without nutrients for two weeks, we selected

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seedlings which apparently differed in the root number, length and diameter from the respective wild types. Seedlings of these putative mutants were then transplanted to plastic pots (19.5 cm in height, 15.8 cm in diameter) filled with soil and grown until maturity. After obtaining selfed seeds for each mutant to confirm the reappearance of such root characteristics, two-week-old seedlings of these lines and respective wild types were grown in the same way as for each previous generation. Similarly, the reappearance of each mutant characteristic was tested for further next generations.

Inheritance of mutant characters and allelism test

First, to examine the inheritance modes of the *crl* mutants, segregation of the phenotype in the progenies derived from selfed heterozygous plants for the two mutant lines was determined. Next, to determine whether the mutations identified in these lines affected the same or different genes, odm 202 was crossed with BRX334 and the F_2 progeny was examined. Since BRX334 was sterile, heterozygous plants were used for the allelism test.

Characterization of mutants

After seeds of the *crl* mutants and respective wild types were surface-sterilized and germinated, the seeds were placed in 0.8% agar medium to raise seedlings, which were kept in a plant growth chamber maintained at $30 \pm 1^\circ\text{C}$ under a 12 h photoperiod ($320 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) regime. After two weeks, the seedlings were sampled to measure their plant height, plant age (leaf number), length of seminal roots, diameter of seminal roots, number of crown roots and branching density of lateral roots on the seminal root axis.

For the histological observations, node-containing stem regions excised from two-week-old seedlings were fixed in FAA (formalin: acetic acid: 70% ethanol (1 : 1 : 18)). Excised stem regions were dehydrated in a graded ethanol/tert-butanol series, embedded in paraffin and sectioned into $10 \mu\text{m}$ thick sections. Paraffin sections were stained with Delafield's hematoxylin and observed under a light microscope.

Effects of auxin on crown root formation

Seedlings of the *crl* mutants and respective wild types were grown in a 0.8% agar medium supplemented with 10^{-6} , 10^{-7} , or 10^{-8} M α -naphthaleic acid (α -NAA) in a plant growth chamber maintained at $30 \pm 1^\circ\text{C}$ under a 12 h photoperiod ($320 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) regime. The number of crown roots was counted for two-week-old seedlings.

Results

Isolation and genetic analysis of mutants

M_2 or M_3 progenies were screened to isolate mutants showing abnormal root development and two mutants with a defect in the formation of crown roots (odm 202,

BRX334) were obtained. This type of mutation was designated as *crown rootless* with the gene symbol *crl*. The odm 202 mutant was detected in a *MNU*-mutagenized M_3 population of rice (cv. Taichung 65) and the BRX334 mutant in a γ -ray mutagenized M_2 population of rice (cv. Blue Rose). These mutant phenotypes were found to be stable for at least three generations. Segregation of phenotype in the progenies derived from selfed heterozygous plants for the two mutant lines fitted the 3 wild type : 1 mutant (Table 1), indicating that both mutant phenotypes are controlled by a single recessive gene, respectively. In the F_2 progeny from the cross of odm 202 $\times$ BRX334, a wild type and mutant segregation was also consistent with the 9 : 7 ratio (Table 2). These results indicate that the two mutant genes were not allelic. The *CRL* loci of the odm 202 and BRX334 mutants were designated as *CRL1* and *CRL2*, respectively.

Morphological characterization of crown rootless mutants

As shown in Fig. 1 and Table 3, the number of crown roots of the *crl* mutants was significantly lower than that of the respective wild types. Except for the difference in the number of crown roots, no significant difference was detected between the *crl1* mutant and wild type seedlings for the other characteristics (Table 3). Under adequate water and fertilizer conditions, the *crl1* mutant grew vigorously and was fertile (Fig. 2). However, the *crl2* mutant showed many abnormal morphological characters besides the defect in the formation of crown roots. Compared with the wild type, the mutant shoot was smaller, the seminal root was thicker and longer, and the branching density of the lateral roots was lower (Table 3). Although some of *crl2* mutant plants continued to grow and formed young panicles under sufficient water and fertilizer application, in all the plants shoot growth was markedly reduced and abnormal through out the growing period, resulting in sterility (Fig. 3).

To determine at which stage of root development the defect in crown root formation became apparent in the *crl* mutants, we examined serial cross-sections of the node-con-

Table 1. Segregation of phenotype in the progenies derived from selfed heterozygous plants for the two mutant lines (odm 202, BRX334)

Lines	Phenotype		χ^2 (3 : 1)	P
	Normal	Mutant		
odm 202 ¹⁾	134	38	0.78	0.25-0.50
BRX334 ²⁾	106	40	0.45	0.50-0.75

¹⁾ M_4 progeny was investigated.

²⁾ M_3 progeny was investigated.

Table 2. Segregation of phenotype in the F_2 progeny of the cross between odm 202 and BRX334

Phenotype		χ^2 (9 : 7)	P
Normal	Mutant		
151	91	3.72	0.05-0.10

Table 3. Morphological characteristics of *crl* mutants (*crl1*, *crl2*) and respective wild types (Taichung 65, Blue Rose) in two-week-old seedlings

Character	Taichung 65	<i>crl1</i> mutant	Blue Rose	<i>crl2</i> mutant
Plant height (cm)	13.3 ± 0.1	14.1 ± 0.2 ^{ns}	14.1 ± 0.4	9.2 ± 0.8**
Plant age (leaf number)	3.1 ± 0.0	3.1 ± 0.1 ^{ns}	2.9 ± 0.0	2.5 ± 0.2 ^{ns}
Length of seminal roots (cm)	18.4 ± 0.3	19.1 ± 0.1 ^{ns}	17.2 ± 0.4	21.2 ± 0.7**
Diameter of seminal roots (μm) ¹⁾	368.4 ± 6.6	356.4 ± 9.2 ^{ns}	364.2 ± 10.8	599.1 ± 15.5**
Number of crown roots	7.3 ± 0.5	0.5 ± 0.3**	5.3 ± 0.3	0.8 ± 0.5**
Branching density of lateral roots (cm ⁻¹) ²⁾	9.1 ± 0.4	8.0 ± 0.1 ^{ns}	11.1 ± 0.3	6.3 ± 0.6**

Data show means ± S. E. (n = 4).

¹⁾ This value was determined at 1.0 cm behind the root tip.

²⁾ Number of first order lateral roots on seminal root axis/length of seminal root axis.

ns, *, **: not significant, significant at 5% and 1% levels from the wild type, respectively.

Fig. 1. Two-week-old seedlings of wild type (A: Taichung 65, C: Blue Rose) and *crl* mutants (B: *crl1*, D: *crl2*). Arrows indicate seminal roots. Bars = 2 cm.

taining stem regions of these mutants and the respective wild type seedlings. In the wild type seedlings, crown roots were endogenously formed close to the peripheral vascular cylinder of the stem (Figs. 4A, C, 5A, C). These findings were in agreement with the observation made by Kaufman (1959) and Kawata *et al.* (1972). On the other hand, the *crl1* mutant seedlings did not produce crown root primordia (Figs. 4B, D), which indicates that the initiation of crown root primordia was impaired in the *crl1* mutant. In contrast, the *crl2* mutant seedlings produced crown root primordia, although the number was lower than that of the wild type (Figs. 5B, D). However, the growth was arrested after this formation (Figs. 5B, D). The results show that both initiation and subsequent growth of crown root primordia were impaired in the *crl2* mutant.

Effects of auxin treatment on crown root formation

To investigate the effects of auxin on crown root formation in the *crl* mutants and the respective wild types, the seedlings were grown in an agar medium containing different concentrations of α -NAA. The number of crown roots significantly increased in the *crl1* mutant and the wild type seedlings treated with 10^{-6} M α -NAA (Table 4). In contrast, α -NAA scarcely affected crown root formation in the *crl2* mutant seedlings regardless of the concentration (Table 4).

Discussion

Our results showed that the initiation of crown root primordia was impaired in the *crl1* mutant, while in the *crl2* mutant both initiation and subsequent growth of crown root primordia were impaired. However, two-week-old seedlings

Fig. 2. Plants of wild type (Taichung 65) and *crl1* mutant at the grain filling stage (four months after planting). A: wild type, B: *crl2* mutant. Bar = 15 cm.

of both *crl1* and *crl2* mutants formed crown roots, although in a significantly lower number than the wild type. These facts indicate that neither mutants completely lost their ability for the initiation and subsequent growth of crown root primordia. In addition, the *crl1* mutant grew normally until maturity except for the difference in the number of crown roots from the wild type, whereas the *crl2* mutant showed many abnormal morphological characters besides the defect in the formation of crown roots. These facts suggest that *CRL1* regulates specifically the initiation of crown root primordia but is not involved in radicle and lateral root initiation or shoot development, while *CRL2* regulates various processes of plant development. In addition, the defect in the function of formation of crown roots in the *crl1* mutant was alleviated by the supply of α -NAA, unlike that of the *crl2* mutant, suggesting that the function of *CRL1* differs from that of *CRL2*.

The *crl* mutants found in this study were different from the adventitious root-deficient mutants reported by other researchers. The *alf4-1* mutant of *Arabidopsis* reported by Celenza *et al.* (1995) did not form lateral roots or adventitious roots, while the *crl* mutants were capable of forming

Fig. 3. Plants of wild type (Blue Rose) and *crl2* mutant. A: wild type, B: *crl2* mutant (five months after planting). Bar = 15 cm. C: leaves (*crl2*), D: panicle (*crl2*). Bar = 2 cm.

Table 4. Number of crown roots of *crl* mutants (*crl1*, *crl2*) and respective wild types (Taichung 65, Blue Rose) grown on agar medium that contained different concentrations of α -NAA

α -NAA concentration (M)	Number of crown roots			
	Taichung 65	<i>crl1</i> mutant	Blue Rose	<i>crl2</i> mutant
0	7.3 $\pm$ 0.3	0.5 $\pm$ 0.3	5.3 $\pm$ 0.3	0.8 $\pm$ 0.5
10 ⁻⁸	7.5 $\pm$ 0.3 ^{ns}	0.8 $\pm$ 0.3 ^{ns}	5.5 $\pm$ 0.3 ^{ns}	0.5 $\pm$ 0.3 ^{ns}
10 ⁻⁷	8.0 $\pm$ 0.4 ^{ns}	1.3 $\pm$ 0.3 ^{ns}	6.3 $\pm$ 0.5*	1.0 $\pm$ 0.4 ^{ns}
10 ⁻⁶	11.5 $\pm$ 0.3**	3.5 $\pm$ 0.3**	7.5 $\pm$ 0.3**	0.8 $\pm$ 0.5 ^{ns}

Data show means $\pm$ S. E. (n = 4).

ns, *, **: not significant, significant at 5% and 1% levels from 0 M, respectively.

Fig. 4. Cross-sections through the coleoptile and the first nodes of the wild type (Taichung 65) and *crl1* mutant in two-week-old seedlings. A: coleoptilar node of the wild type, B: coleoptilar node of the *crl1* mutant, C: first node of the wild type, D: first node of the *crl1* mutant. Arrows indicate crown roots and the primordia, and arrowheads the peripheral vascular cylinder. Bars = 300 μ m.

lateral roots. Lund *et al.* (1996) reported that cuttings of a tobacco mutant, *rac*, underwent cell division in response to exogenous auxin application, but never formed adventitious root primordia. In contrast, the *crl1* mutant produced crown roots in response to exogenous auxin treatment and the *crl2* mutant formed crown root primordia without auxin application. The *rtcs* mutant of maize reported by Hetz *et al.* (1996) had a defect in the function of initiation of crown root primordia like the *crl1* mutant. However, there were also different characteristics between these two mutants. As mentioned above, two-week-old *crl1* mutant seedlings formed a small number of crown roots, while even in the 50-day-old *rtcs* mutant plants, no crown root formation was observed, and in addition, the *rtcs* mutant did not form crown roots in response to exogenous auxin application (Hetz *et al.* 1996). Such new characteristics of the *crl* mutants, therefore, may enable to obtain further information about the role of individual genes involved in the adventitious (crown) root formation.

Hetz *et al.* (1996) further reported that, after treatment with auxin, the *rtcs* mutant formed mesocotyl roots, indicating that the ability to form roots on the mesocotyl was still present in the *rtcs* mutant in spite of their inability to form crown roots. Similarly, a number of mutants deficient in the

ability to initiate either radicle or adventitious (crown) roots have been identified (Berleth and Jürgens 1993, Celenza *et al.* 1995, Hong *et al.* 1995, Lund *et al.* 1996). These facts suggest that the initiation mechanism of different types of roots is under different genetic control. On the other hand, the lack of ability for both adventitious and lateral root initiation in the *alf4-1* mutant suggests that some mechanisms regulating root initiation may be common to both types of roots. Morphological, anatomical, and embryological differences or similarities in different types of roots have been well described (e. g., Waisel and Eshel 1991, Yamauchi *et al.* 1996), although genetic aspects are poorly documented. As mentioned above, it is likely that *CRL1* regulates specifically crown root initiation, while *CRL2* regulates various processes of plant development, including crown and lateral root formation. Therefore, further phenotypic characterization and evaluation of the hormonal regulation of the *crl* mutants may enable to elucidate how crown root initiation is regulated by genes that are specific to crown roots and are commonly involved in other types of roots. Furthermore, because of the specific *CRL1* gene action, the *crl1* mutant could also be a useful material for studying the effect of a markedly reduced root system on shoot physiology.

As mentioned above, the defect in the function of for-

Fig. 5. Cross-sections through the coleoptile and the first nodes of the wild type (Blue Rose) and *crl2* mutant in two-week-old seedlings. A: coleoptilar node of the wild type, B: coleoptilar node of the *crl2* mutant, C: first node of the wild type, D: first node of the *crl2* mutant. Arrows indicate crown roots and the primordia, and arrow-heads the peripheral vascular cylinder. Bars = 300 μ m.

mation of crown roots in the *crl1* mutant was alleviated by the supply of α -NAA. The plant hormone auxin has been implicated in the regulation of almost every aspect of plant development (King 1988). In fact, tobacco plants transformed with the *iaaL* gene that induces a decrease of the free IAA level displayed reduced apical dominance, abnormal leaf growth and inhibition of vascular differentiation as well as reduced root development (Romano *et al.* 1991). Therefore, taking into account the specific *CRL1* gene action on crown root initiation, it is unlikely that the mutation of *CRL1* would reduce the whole level of free IAA and thus inhibit crown root initiation. It has been suggested that the accumulation of IAA in the adventitious root-forming part of the cutting may act as the triggering factor for root initiation (Blakesley 1994). On the other hand, Trewavas (1991) suggested that in many cases, sensitivity to plant growth substances is more important than absolute concentrations of the plant growth substances. For example, the expression of the *rolB* gene of *Agrobacterium rhizogenes* is considered to induce adventitious root formation through the increase of the sensitivity of transformed cells to auxin (Shen *et al.* 1988, Maurel *et al.* 1994). Therefore, although other explanations are possible, the failure of crown root initiation in

the *crl1* mutant may be caused by the lack of ability for auxin accumulation or for increasing the sensitivity in the adventitious root-forming part.

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