Mechanical Stimulus–Sensitive Mutation, rrl3, Affects the Cell Production Process in the Root Meristematic Zone in Rice

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Abstract: Genetic studies on the response of plant root to environmental stimuli are important for elucidating the mechanism of the stress tolerance of plants. We isolated and characterized a recessive rice mutant, rrl3, which was highly sensitive to mechanical stimulus and has short roots. No significant difference was observed between the seminal roots of rrl3 mutant and wild type in the number, length and diameter of mature cortical cells. In addition, the rrl3 mutant and the wild type did not differ in sensitivity to ethylene, IAA or ABA. These results suggest that the RRL3 gene specifically regulates the cell production process in the root meristematic zone under a mechanically impeded condition and does not regulate the sensitivities to ethylene, IAA and ABA.

Key words: Cell flux, Mechanical stimulus, Mutation, Oryza sativa, Root apical meristem, Root elongation.

The response of plant roots to environmental stimuli has been studied in relation to stress tolerance of plants (O'Toole and Bland, 1987; Kono et al., 1987; Yamauchi et al., 1996). It is important to understand how such a response to stimuli is genetically regulated, and the mechanism of the gravitropic response has been studied by isolating and analyzing the mutants with aberrant morphological phenotypes in gravity–response (Okada and Shimura, 1992; Hobbie and Estelle, 1995; Fukaki et al., 1997). However, the genetic mechanism of the response to other stimuli was rarely studied.

In the previous paper, we reported on isolation and characterization of rice mutants deficient in the formation of crown roots (Inukai et al., 2001a), or in the elongation of seminal roots (Inukai et al., 2001b). During the screening for these mutants, we found a mutant (BRX180) whose roots showed a specific response to environmental stimulus. In water culture without bubbling for aeration, the seminal root growth of BRX180 did not differ from that of the wild type, but in water culture with bubbling, the seminal root growth of BRX180 was restrained by the high concentration of dissolved oxygen in the solution and/or mechanical stimulus caused by bubbling that hit the roots. In this study, we first characterized the BRX180 phenotypically and genetically, and then analyzed the role of this mutation gene in the histological response of the root to the mechanical stimulus.

Mechanical stress generally suppresses elongation and promotes radial expansion of roots, and increasing evidence suggests the involvement of plant hormones in the response of the root to external stress (Goss and Russell, 1980; Atwell, 1993; Bengough et al., 1997). A correlation between mechanically induced growth change and ethylene evolution from the tissue led to the hypothesis that tissue response is mediated by the action of ethylene (Goeschl et al., 1966; Kays et al., 1974; Sarquis et al., 1991). On the other hand, Lachno et al. (1982) and Moss et al. (1988) reported that the IAA content of roots was increased 3.5 times by the mechanical impedance, but ABA content was not. However, Tardieu et al. (1992) and Mulholland et al. (1996) reported that ABA content increased significantly under a mechanically impeded root of maize and barley. Therefore, we compared BRX180 with wild type in their response to these three hormones and their inhibitors to examine involvement of the action of plant hormones in this mutation.

Materials and Methods

1. Screening for mutant

We speculated that the seminal root growth of BRX180 was restrained by the high concentration of dissolved oxygen in the solution and/or mechanical stimulus caused by bubbling that hit the roots. We supposed that the seminal root growth of BRX180 did not differ from that of the wild type, but in water culture with bubbling for aeration, the seminal root growth of BRX180 did not differ from that of the wild type, but in water culture with bursting, the seminal root growth of BRX180 was restrained by the high concentration of dissolved oxygen in the solution and/or mechanical stimulus caused by bursting that hit the roots. In this study, we first characterized the BRX180 phenotypically and genetically, and then analyzed the role of this mutation gene in the histological response of the root to the mechanical stimulus.
the screening, we found a mutant, BRX180, that showed a specific response to environmental stimuli.

2. Morphological characterization of mutant
The seeds of BRX180 and the wild type were surface-sterilized and germinated. The seedlings were grown by water culture with bubbling that slightly shook the roots. Plants were grown in a growth chamber maintained at 30±1°C under 12 h photoperiod (320 μmol photons m⁻² s⁻¹) regime. After one week, the seedlings were sampled to measure their plant height, plant age in leaf number, the length and diameter of seminal root, the number of crown roots, and the number and length of lateral roots on the seminal root.

3. Genetic analysis
The segregation of the BRX180 phenotype in the M₃ progeny derived from selfed M₂ heterozygous plants was determined by growing the M₃ progeny by a water culture with bubbling.

In a previous paper (Inukai et al. 2001b), we isolated four recessive rice mutants with reduced seminal root length. Complementation tests revealed that these mutations resided in two complementation groups. The first locus was named REDUCED ROOT LENGTH1 (RRL₁) and the other was RRL₂. To determine whether the mutant gene of BRX180 is located on the same loci or not, we crossed BRX180 with rrl1 and rrl2−1 mutants, and examined the phenotypes of F₁ progenies.

4. Identification of environmental factor triggering short root trait expression of mutant
The seeds of BRX180 and wild type were surface-sterilized and germinated. Then four seeds of each type were grown in 25-, 6- or 1-L deionized water with or without shaking as follows. Plants were grown in a growth chamber maintained at 30±1°C under 12 h photoperiod (320 μmol photons m⁻² s⁻¹). The seminal root length of seedlings and the concentration of dissolved oxygen in the water were measured daily for a week.

(1) Control of the concentration of dissolved oxygen
In the water without bubbling, the concentration of dissolved oxygen decreases gradually during the culture period due to root respiration. The seedlings of BRX180 and wild type were grown for one week in 25-, 6- and 1-L deionized water (in 25x40x30 cm, 25x20x15 cm and 6x14x25 cm containers, respectively). At the end of the culture period, the mean concentrations of dissolved oxygen in 1- and 6- L water were 1.66 and 0.95 μL L⁻¹ lower than that in 25 L water, respectively (Fig. 1A), and thus these waters were designated as high dissolved oxygen (HDO), moderately dissolved oxygen (MDO) and low dissolved oxygen (LDO) water, respectively.
various concentrations as follows or to 500 ml distilled water in an airtight container with ethylene at various concentrations. Another group of the seedlings was also grown in 1-L water with shaking, but together with eight wild type seedlings, which had been grown for two weeks in the same water without shaking. Since the concentration of dissolved oxygen in the water in the first group was always higher than that in the second group (Fig. 1B), the former water was designated as high dissolved oxygen and shaken (HDOS) water, and the latter as low dissolved oxygen and shaken (LDOS) water.

5. Histological observations and estimation of cortical cell flux
Seminal root segments were excised from the seedlings grown in the water with bubbling for three days and fixed in FAA (formalin: acetic acid: 70% ethanol (1:1:18)). These segments were rinsed for 30 minutes twice in 0.1 M phosphate buffer (pH 7.0), dehydrated in a graded acetone series, embedded in a water-soluble methacrylate resin, Technovit 7100 (Kulzer Co., Ltd.), and polymerized at 45°C. The samples were sectioned at 5 μm longitudinally and stained with Delafield’s hematoxylin. Axial and radial length of cortex cells at the third layer from outside were measured under a light microscope.

To investigate the effects of mutant gene on continual production of new cells in the root apical meristem, we estimated the cortical cell flux in the seminal root of BRX180 and the wild type. Cell flux is the rate at which new cells are added to cell files or the rate of displacement of cells from the growth zone (Thomson and Atwell 1989; Fraser et al., 1990). We estimated the cell flux at the proximal end of the elongation zone as the number of mature cells displaced per hour (increase in seminal root length per hour was divided by the length of mature cortical cells). The mature cell length was determined at 10 mm behind the root tip where cell elongation has already stopped, and the increase in seminal root length per hour was calculated from the differences in root length between the 2nd and 4th day after germination.

6. Application of plant hormones and their inhibitors
Seeds of BRX180 and the wild type were surface-sterilized and germinated. The seedlings were grown in water culture without shaking for two days. Four seedlings of each type were transplanted to small containers with 500 ml distilled water containing indole-3-acetic acid (IAA), abscisic acid (ABA) or their inhibitors at various concentrations as follows or to 500 ml distilled water in an airtight container with ethylene at various concentrations.

(1) Application of plant hormones to the seedlings without mechanical stimulus
Ethylene was introduced through air to seedlings in airtight containers at the concentrations of 10^{-1}, 1, 10, 10^2, or 10^3 μL L^{-1}. IAA and ABA were applied to the water at the concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M. The water was not shaken throughout the growth period.

(2) Application of hormones to the seedlings under mechanical stress
Ethylene, IAA and ABA were applied at the same concentrations as mentioned above. Mechanical stress was given by shaking containers on reciprocal shaker operated at 40 rpm.

(3) Application of inhibitors to the seedlings under mechanical stress
Ethylene action inhibitor, AgNO₃, was applied to the water at the concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, or 10^{-7} M. Auxin action inhibitor, α-(p-chlorophenoxy)isobutyric acid (PCIB), was applied to the water at the concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3} M. ABA biosynthesis inhibitor, norflurazon (NF), was applied at the concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, or 10^{-1} M. Mechanical stimulus was given by shaking as mentioned above.

All the experiments were performed in a plant growth chamber maintained at 30±1°C under 12 h photoperiod (320 μmol photons m^{-2} s^{-1}). Seminal root length of these seedlings were measured before and after these treatments for 48 hours.

Results
1. Morphological characterization of mutant
In water culture without bubbling, the seminal root elongation of BRX180 did not differ from that of wild type (Fig. 2A). However, in water culture with bubbling, root elongation of the mutant was substantially reduced as compared with that of the wild type (Fig. 2B). Besides the defect in elongation of seminal root, the BRX180 seedlings showed many other abnormal root characteristics when grown in water with bubbling. Compared with the wild type, the mutant produced more crown roots, more lateral roots and shorter lateral roots, although the diameter of seminal root was not significantly different between BRX180 and wild type (Table 1). No significant difference was detected in either plant height and plant age in leaf number between BRX180 and wild type seedlings (Table 1).

2. Inheritance of mutant phenotype and allelism tests
Segregation ratio of BRX180 phenotype in M₃ progeny derived from selfed M₂ heterozygous plants fit the 3 (wild type):1 (mutant) (Table 2), indicating that the mutant phenotype is controlled by a single recessive gene. All the F₁ progenies from crosses of BRX180×m1/
Fig. 2. Ten-day-old seedlings of wild type and BRX180 grown in water culture conditions without (A) and with (B) bubbling. Bars = 50 mm.

Fig. 4. Longitudinal sections of the seminal roots in three-day-old seedlings of the wild type (A and C) and rrl3 mutant (B and D). A and B: median longitudinal sections of the root apices. C and D: median longitudinal sections of the mature regions. cc: central cylinder, e: cortex, ep: epidermis. Bars = 100 μm.

Table 1. Morphological characteristics of one-week-old seedlings of BRX180 and wild type.

| Character                        | Wild type         | BRX180          |
|---------------------------------|-------------------|-----------------|
| Plant height (mm)               | 57.3 ± 1.3        | 60.3 ± 1.3*     |
| Plant age in leaf number        | 2.1 ± 0.1         | 2.3 ± 0.1*      |
| Length of seminal root (mm)     | 153.0 ± 3.8       | 49.0 ± 1.1**    |
| Length of lateral root (mm)     | 7.8 ± 0.6         | 2.1 ± 0.3**     |
| Branching density of lateral root (cm⁻¹) | 13.1 ± 0.7       | 20.3 ± 0.9**    |
| Diameter of seminal root (μm)   | 418.4 ± 6.5       | 398.0 ± 6.3*    |

Data show means ± S.D. (n = 4).

1) Total length of the first order lateral roots developed from the basal 20 mm of a seminal root/number of the first order lateral roots on the same part.

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

3) This value was determined at 10.0 cm from the root tip.

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

Table 2. Segregation of BRX180 phenotype in M₄ progenies derived from selfed M₂ heterozygous plants for the BRX180 and in F₁ progenies of the crosses of BRX180 × nll and BRX180 × nll2-1.

| Phenotype in M₄ progenies | Normal | Mutant | χ²(3 : 1) | P.  |
|--------------------------|--------|--------|-----------|-----|
|                          | 177    | 67     | 0.19      | 0.50-0.75 |

Combination | Phenotype in F₁ progenies | Normal | Mutant |
|------------|--------------------------|--------|--------|
| BRX180 × nll | 18 | 0 |
| BRX180 × nll2-1 | 20 | 0 |

mutant and BRX180 × nll2-1 mutant showed normal phenotype (Table 2). These results indicate that the mutant gene of BRX180 was not the allele of nll and nll2 - 1, and thus was named the mutant gene RRL3.

3. Environmental factor regulating the expression of the short root trait of the mutant

The seminal root length of BRX180 seedlings was similar to that of the wild type seedlings throughout the culture period in HDO, MDO and LDO water (Figs. 3A, B, C), but was less than that of the wild type in HDO and LDO water, particularly in HDO water (Fig. 3D, E). The seminal root length of the mutant grown for seven days in HDO and LDO water was less
than one third and about two third of that of the wild type, respectively. These observations indicate that the environmental factor regulating the expression of short root trait of BRX180 is mechanical stimulus given by shaking the water, and the concentration of dissolved oxygen is, at least partially, involved in mechanism regulating abnormal root elongation of BRX180.

4. Histological observations and estimation of cell flux

To detect the cause of abnormal root elongation in m13 mutant histologically, we measured axial and radial length of the cortical cells at the third layer from the outermost layer on the seminal root of the mutant and wild type grown in water with bubbling. We also estimated the cortical cell flux. No significant difference was detected in either the mean axial or radial length of matured cortical cells between m13 mutant and wild type root (Figs. 4C, D, Table 3). However, the zone where cells had not yet begun elongation, i.e., the meristematic zone in this mutant root was smaller than that in the wild type root (Figs. 4A, B, 5). The cortical cell flux of this mutant was also significantly lower than that of the wild type (Table 3). These observations suggest that the short root trait observed in m13 mutant was caused not by a decrease in cell length but by a reduction of cell flux.

5. Effects of plant hormones and their inhibitors

Applying ethylene, IAA or ABA inhibited root elongation of m13 mutant and the wild type cultured in water without shaking (Figs. 6A, B, C). The rate of root elongation decreased as the concentration of these hormones increased, but the rate was not different between the mutant and wild type at any concentration. This indicates that the short root trait of m13 mutant is not caused by high sensitivities to these hormones.

In the water with shaking (under mechanical stress), the rate of root elongation in the wild type was higher than that in the mutant in the absence of exogenous plant hormones (Figs. 6D, E, F). The rate was decreased by applying these hormones dose dependently in both the mutant and wild type, but more severely in the wild type than in the mutant. The rate of root elongation in the mutant was similar to that in the wild type when the concentration of ethylene, IAA and ABA was higher than 10 μM−1, 10−6 M and 10−4 M, respectively. These results suggest that the root elongation of the mutant was suppressed, at least partly, by the action of these hormones. However, the short root trait of m13 mutant was not rescued by applying ethylene action inhibitor,
Table 3. Axial and radial length of mature cortical cells and estimated cortical cell flux in the growth zone of the rrl3 mutant and wild type roots grown in water with bubbling.

|                | Axial cell length (μm cell⁻¹) | Radial cell length (μm cell⁻¹) | Cell flux (no. cells h⁻¹) |
|----------------|-------------------------------|-------------------------------|--------------------------|
| Wild type      | 105.1 ± 7.8                   | 23.8 ± 4.6                    | 9.14 ± 0.28              |
| rrl3 mutant    | 104.0 ± 10.1*                 | 21.3 ± 4.3*                  | 3.20 ± 0.25**           |

Data show means ± S.D. (n = 4).

*: not significant and significant at 1% level from the wild type, respectively.

**AgNO₃, auxin action inhibitor, PCIB, or ABA biosynthesis inhibitor, NF.

Discussion

Our results showed that the environmental factor regulating the expression of short root trait of the rrl3 mutant was the mechanical stimulus. Many researchers have observed restricted root elongation and increased root diameter in compact soil or mechanically impeded growth media (Atwell, 1993; Bengough et al., 1997). The morphological changes result from a reduction in the rate of cell elongation accompanied by radial cell expansion (Peterson and Barber, 1981; Wilson et al., 1977) and by a decrease in the cell production rate (Croser et al., 1999). In rrl3 mutant, although the cortical cell flux was significantly lower than that in the wild type, neither the mean axial length nor radial length of mature cortical cells nor the diameter of seminal root showed a significant difference between the rrl3 mutant and wild type. These facts suggest that the RRL3 gene regulates specifically the cell production process in the root meristematic zone, but does not affect the axial and radial cell expansion processes in the elongation zone under a mechanically impeded condition.

In addition, compared with the wild type, the mutant developed more lateral roots on a unit length of seminal root axis (higher branching density) than the wild type although the mean length of lateral roots was shorter. Plants are known to have an ability to compensate for stress-induced suppression of root growth. For example, Goss (1977) showed that barley seminal roots growing in the ballotini bed, which subjected seminal roots but not lateral roots to impedance, had almost twice as long lateral roots and as high branching density of lateral roots than those in the non-stressed condition. Based on the idea of compensatory growth, it is considered that increase in branching density of the rrl3 mutant results from reduction in the length of the seminal roots. However, reduction in lateral root length of the mutant is a direct effect of the rrl3 gene rather than an indirect effect through the reduction of the length of the seminal root.

The rrl3 mutant was different from other stimulus-response mutants in the expression mode of developmental characteristics of roots. Arabidopsis mutants have been isolated, whose roots have altered tropic responses to such as gravity (Okada and Shimura, 1992; Hobbie and Estelle, 1995; Fukaki et al., 1997), light and physical obstacles (Okada and Shimura, 1990; 1992). These tropic responses resulted not from altered meristem development but from alterations in local rates of cell extension and a consequent differential elongation rate between the two sides of the roots (Pilet and Ney, 1981). This is also the case in rice mutant, LM 10, which showed increased root elongation when the root were exposed to light (Liang and Ichii, 1996), though light normally inhibits root growth. This trait of LM10 mutant also resulted from altered cell expansion process in the root elongation zone and not from reduction of cell production rate in the meristematic zone. Because of such novel characteristics of the rrl3, this mutant could be very useful in obtaining new information about the role of gene specifically involved in cell production process in roots under mechanical stress.

The rrl3 mutant and wild type did not differ in sensi-
Fig. 6. Effects of ethylene, IAA, ABA and their inhibitors at various concentrations on the elongation of the seminal roots of the rrl3 mutant and wild type grown in water without shaking (A-C) and with shaking (D-I). The error bars represent the standard deviations. Each value represents the mean measurement for 4 seedlings.

activities to ethylene, IAA and ABA indicating that the limited root growth did not result from higher sensitivities to these hormones. On the other hand, when these seedlings were grown in the water with shaking (under mechanical stress), the root elongation of the mutant was slower than that of the wild type in the absence of these plant hormones. The difference between the mutant and wild type in the rate of root elongation decreased as the concentration of these hormones increased, and the difference was not observed when the concentration of ethylene, IAA and ABA was higher than $10 \mu l^{-1}$, $10^{-6}$ M and $10^{-4}$ M, respectively. These results showed that the endogenous concentration or action of these hormones was increased by mechanical stimulus in the mutant. However, the short root trait of rrl3 mutant was not rescued by applying ethylene action inhibitor, AgNO3, auxin action inhibitor, PCIB, or ABA biosynthesis inhibitor, NF, suggesting that reduced root elongation of the mutant is not caused by higher endogenous ethylene, IAA nor ABA levels. It is necessary
to measure and compare the endogenous levels of these hormones between the wild type and the mutant under mechanical stress.

Many workers suggested that ethylene is involved in promoting the radial expansion and reducing elongation in mechanically impeded roots. This is because the mechanically impeded roots produce more ethylene than unimpeded roots, and the application of ethylene or ethylene–releasing compounds induces symptoms similar to those caused by mechanical stress (Kays et al., 1974; Wilkins et al., 1976; Sarquis et al., 1991). However, the radial expansion and reduced elongation of the roots treated with ethylene or ethylene–releasing compounds were induced only by a change in the direction of cell expansion, and it is not clear whether ethylene actually affects the meristematic activity in the roots. Moreover, endogenous levels of the other plant hormones have not been thoroughly measured in mechanically impeded roots. There has been only one report that showed an increased IAA level in mechanically impeded roots of maize so far (Lachno et al., 1982). Tardieu et al. (1992) and Mulholland et al. (1996) found that the ABA level in the roots of maize and barley was increased by mechanical stimulus, whereas Hartung et al. (1994) showed that the application of 1 μM ABA to unstressed maize seedlings promoted the growth of short thick roots like the mechanical impedance (Hartung et al., 1994). By contrast, Lachno et al. (1982) and Moss et al. (1988) failed to detect any increase in ABA level in the impeded roots of maize. As such, whether IAA and ABA are involved in the change in the root growth observed under mechanical impedance remains controversial.

Xu et al. (1995) isolated the Arabidopsis TCH4 gene, whose expression was strongly and rapidly induced in various tissues including primary root tips and lateral root primordia by mechanical stimuli such as touch and wind. In the paper, they also reported that the expression of the TCH4 gene can be induced by brassinosteroids. Therefore, hormones other than ethylene, IAA and ABA could be involved in the root growth observed under mechanical impedance and it may be important to examine whether the hormone such as brassinosteroids regulate short root trait expression of ntl3 mutant or not.

In summary, reduced elongation of seminal and lateral roots in ntl3 mutant was controlled by a single recessive gene. The environmental factor triggering the expression of the mutant phenotype was the mechanical stimulus. The concentration of dissolved oxygen was also involved in the mechanism of the expression of the mutant phenotype. RRL3 gene regulated specifically the cell production process in the meristematic zone but was not involved in axial and radial cell expansion processes in the elongation zone under mechanically impeded condition. The reduced root growth in the ntl3 mutant did not result from higher sensitivities to ethylene, IAA or ABA levels. This mutant could be very useful for obtaining new information about the role of the gene specifically involved in the cell production process in roots under mechanical stress.

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