Genetic Interaction between 2 Tillering Genes, Reduced Culm Number 1 (rcn1) and Tillering Dwarf Gene d3, in Rice

NAOKO YASUNO, YUTA YASUI, ITUSURO TAKAMURE, AND KIYOAKI KATO

From the Department of Crop Science Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-5 Inada-cho, Obihiro, Hokkaido, 080-8555 Japan (Yasuno, Yasui, and Kato); and the Graduate School of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido, 060-8589 Japan (Takamure).

Address correspondence to K. Kato at the address above, or e-mail: kiyoa@obihiro.ac.jp.

Mutant genes, reduced culm number 1 (rcn1) and bunketsu-waiko tillering dwarf (d3), affect tiller number in rice (Oryza sativa L.) in opposite directions. The d3 mutant was reported to increase tiller number and reduce plant stature. Our objective was to compare the phenotype of the d3rcn1 double mutant with each single mutant and parental rice cultivar “Shiokari” and to clarify whether the Rcni gene interacted with the D3 gene. We recovered a new rcn1 mutant from Shiokari and developed d3rcn1 double mutant with Shiokari genetic background. A new rcn1 mutant, designated as “S-97-61”, exhibited a reduction in tiller number and plant stature to about the same level as the previously reported original rcn1 mutant. Three near-isogenic lines, rcn1 mutant, d3 mutant, and d3rcn1 double mutant, were grown together with the parental Shiokari. The reduction in tillering by the rcn1 mutation was independent of the d3 genotype, and tillering number of d3rcn1 double mutant was between those of the d3 and rcn1 mutants. These results demonstrated that the Rcni gene was not involved in the D3-associated pathway in tillering control.

Shoot branching is important for the establishment of plant architecture and productivity. Shoot branching is regulated by 2 developmental steps, the formation of axillary meristems and the outgrowth of axillary buds. The axillary meristems are formed as lateral organs from shoot apical meristems on every leaf axil and differentiate into axillary buds. The outgrowth activity of axillary buds is controlled by multiple genetic, developmental, and environmental signals (Shimizu-Sato and Mori 2001; Beveridge et al. 2003; Leyser 2003). Suppression for the outgrowth of axillary buds by the primary shoot apex is well known as apical dominance (Thimann and Skoog 1933). Apical dominance is mediated by plant hormone signals. The plant hormone auxin (Indole-3-acetic acid) transported from shoot apical meristems inhibits the outgrowth of axillary meristems. Apically derived auxin inhibited indirectly axillary bud growth showing the possibility for the existence of a second signal for auxin (Morris 1977). Many potential second messengers for auxin action have been suggested (for review, see Cline 1991; Napoli et al. 1999). The plant hormone cytokinin is one good candidate. From a series of genetic studies using bushy mutants Arabidopsis, pea, and petunia, an additional undefined graft transmissible signal was proved as a second messenger for auxin action (Beveridge et al. 1994; Napoli 1996; Stirnberg et al. 2002). Molecular characterization of Arabidopsis bushy mutants, more axillary branching 1 (max1) to max4 mutants, demonstrated that the branching is regulated by at least one carotenoid-derived hormone (Booker et al. 2005). MAX1, MAX3, and MAX4 acting in hormone synthesis and MAX2 acting in perception (Booker et al. 2005).

In rice, 2 bushy mutant genes were molecularly identified. The tillering dwarf mutant gene D3 is orthologous to Arabidopsis MAX2/ORE9 (Ishikawa et al. 2005). In addition, the high tillering dwarf mutant gene, Htd1, is orthologous to Arabidopsis MAX3/CCD7 (Zou et al. 2005). These comparative genomics suggested that the mechanisms controlling shoot branching are conserved between monocots and eudicots.

Alternatively, a series of reduced culm number (rn) rice mutants, rn1 to rn9, were reported (Takamure and Kinoshita 1985, 1993; Takamure 1994; Jiang et al. 2006). Among them, rn1 mutant exhibited a severe reduction in tillering together with reduced culm length and panicle length (Takamure and Kinoshita 1985). To understand the mechanism of rice tillering, it is important to elucidate interactions among these bushy mutants and rn1 mutants.

In the present study, our objective was to compare the phenotype of d3rn1 double mutant with each single mutant and parental rice cultivar “Shiokari” and to clarify whether the Rn1 gene interacted with the D3 gene.

Materials and Methods

Mutant Screening

Screening for the rn1-like mutant in the rice cultivar Shiokari genetic background was performed by using M2 progeny
derived from 220 Gy gamma-ray-irradiated Shiokari. The M₂ progeny was grown in a paddy field at Hokkaido University, and plants were screened for the \textit{nn} phenotype. Self-pollinated M₃ seeds for each putative mutant were planted to confirm the reduced culm phenotype in the next generation.

Inheritance of Mutant Characters and Allelism Test

To examine the inheritance mode of the \textit{nn} mutant, segregation of the phenotype in F₂ progeny derived from a cross between Shiokari and the \textit{nn} mutant was investigated. Next, to determine whether the putative \textit{nn} mutation is allelic to the \textit{nn} locus, the \textit{nn} mutant was cross-pollinated with “\textit{N}-133,” the original \textit{nn} line (Takamura and Kinoshita 1985). Self-pollinated F₂ progenies were examined in a paddy field. The segregation of 2 genes derived from N-133, \(R_c\) brown pericarp and seed coat gene and \(R_d\) red pericarp and seed coat gene, was checked.

Development of Double Mutant

To clarify the genetic interaction between \(D_3\) and \(R_n1\), we developed \(d3r_n1\) double mutant in Shiokari genetic background. Near-isogenic line “\textit{ID3}” carrying \(d3\) mutant gene originated from “bunetsuwaito,” backcrossed with Shiokari 5 times, was cross-pollinated with the new \(nn\) mutant derived from Shiokari. F₁ plants carrying \(d3d3 r_n1r_n1\) derived from self-pollinated \(d3\) \((d3d3 + nn)\) plants were screened. F₂ seeds derived from self-pollinated F₁ plants carrying \(d3d3 r_n1r_n1\) were used as \(d3r_n1\) double mutant in the present study.

Phenotypic Evaluation

From 15–20 plants per each genotype, Shiokari, \textit{ID3}, \textit{nn} mutant, and \(d3r_n1\) double mutant, were grown under natural conditions where there was no control for temperature and day length, at Obishi University of Agriculture and Veterinary Medicine from May to September in 2006. Each plant was planted in a 3-L pot filled with soil compost with 1.2 g of each N, P₀₅, and K₂O per pot. Plants were arranged at random and rerandomized daily to minimize positional effects. Between the second and the 10th leaf stages, the number of tillers and plant height at each leaf stage were recorded. At heading time, active tiller number and culm length of each plant were recorded. Tukey’s Honesty Significantly Different (HSD) test (Tukey 1977) was performed in the statistical analysis for active tiller number and culm length.

Results

A New \textit{nn} Mutant

A putative \textit{nn} mutant, designated as “S-97-61,” was screened from the M₂ progeny of rice cultivar Shiokari irradiated with gamma rays. The reduced culm number and length phenotypes were stable in the next generation. F₁ plants derived from a cross between Shiokari and S-97-61 showed wild-type phenotype. In the F₂ progeny derived from a self-pollinated F₁ plant, we observed 127 wild-type and 45 mutants and that fitted a 3:1 ratio (\(\chi^2 = 0.12, P = 0.72\)).

To confirm whether the putative \textit{nn} mutation was allelic to the \textit{nn} locus, the original \textit{nn} line, N-133, was cross-pollinated with S-97-61. F₁ plants showed a typical \textit{nn} phenotype. In F₂ progeny derived from self-pollinated F₁ plants, all 50 plants showed the \textit{nn} phenotype. In F₂ plants, we checked the segregation of 2 morphological genetic markers, \(R_c\) and \(R_d\). A new \textit{nn} mutant, designated as S-97-61, was recovered in the Shiokari genetic background.

Development of Double Mutant

To develop a double mutant, \textit{ID3} was cross-pollinated with S-97-61. The F₁ plants were self-pollinated. Forty-four F₂ plants were grown in a paddy field. After the filling stage, F₂ plants were classified into 4 phenotype groups; ++, +\textit{nn}, +\textit{nn}, and +\textit{nn}, respectively, which fitted a 9:3:3:1 \((\chi^2 = 0.28, P = 0.96)\) (Table 1). Three F₂ plants suspected as double mutant genotypes were too short to emerge the spikes from water level and fill the grains. Thus, no F₃ seeds were derived from these 3 F₂ plants. On the basis of F₃ progeny test, 7 \(d3\) F₃ plants were classified into 2 groups, 3 \(d3d3 + nn\) and 4 \(d3d3 + nn\). We obtained 19 F₃ plants estimated as \(d3d3 r_n1r_n1\) genotype derived from \(d3d3 + nn\) F₂ plants. In the present study, we used F₃ seeds derived from self-pollinated \(d3d3 r_n1r_n1\) F₃ plants as double mutant genotype.

Genetic Interaction between \textit{Rn1} and \textit{D3}

Developmental growth of tillering and plant height at the early stage between second and 10th leaf stages are shown in Figure 1. The active tiller emerged at the seventh leaf stage in Shiokari, and the eighth leaf stage in S-97-61, respectively. In S-97-61, however, the rate of tiller increase was much lower. These data showed that \textit{nn} inhibited tillering after the seventh leaf stage. In \textit{ID3}, the active tiller emerged at the fourth leaf stage showing that \textit{d3} promoted tillering after the fifth leaf stage. In the \textit{d3r_n1} double mutant, the active tiller emerged from the fourth leaf stage, and similar tillering development with \textit{ID3} between the fourth and the seventh leaf stages were observed. After the eighth leaf stage, the rate of tiller increase of the \textit{d3r_n1} double mutant was suppressed in comparison with that of \textit{ID3}. This showed that \textit{nn} inhibited tillering in the \textit{d3} genetic background that was the same stage in the Shiokari genetic background.

Between the second and the fifth leaf stages, the 4 genotypes were similar in plant height development. After the sixth leaf stage, the elongation rate depended on the

| Table 1. Segregation of wild-type, \textit{nn} plants, \textit{d3} plants, and \textit{d3r_n1} double mutant in an F₂ population of a cross between \textit{ID3} and S-97-61 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| \textit{Observed number} | ++ | +\textit{nn} | \textit{d3} | +\textit{nn} | \textit{d3r_n1} | \textit{Total} |
| \textit{Fitted number} | 25 | 9 | 7 | 3 | 44 | \(\chi^2 = 0.28\) | 0.96 |

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genotype. Among 4 genotypes, Shiokari was the longest after the sixth leaf stage. S-97-61 and ID3 were significantly shorter than Shiokari after the seventh leaf stage. The \textit{d3rcn1} double mutant was the shortest among 4 genotypes after the sixth leaf stages.

Phenotypes of the 4 genotypes at heading time are shown in Figure 2 and Table 2. The S-97-61 developed only 5.3 tillers, which was 15.5\% that of Shiokari, whereas ID3 developed 195.1 tillers, which was more than 5 times that of Shiokari. The \textit{d3rcn1} double mutant had 74.6 tillers, which was significantly different from ID3 and S-97-61 at the 0.01\% level. In culm length, S-97-61 was 71.1\% that of Shiokari and ID3 was 39.8\% that of Shiokari. The \textit{d3rcn1} double mutant was the shortest among the 4 genotypes and was significantly different from the other genotypes at the 0.01\% level.

\textbf{Discussion}

We recovered a new \textit{rcn1} mutant, designated as S-97-61 from Shiokari in the present study. S-97-61 developed 1 or 2 tillers in a paddy field, which suppressed tillering to about the same level as the original \textit{rcn1} mutant line, N-133 (Takamure and Kinoshita 1985). In the present environmental condition, S-97-61 developed 5 active tillers at heading time, which was 15.5\% of the original wild-type Shiokari (Table 2). This reduction was similar to 18.2\%, which was the reduction of N-133 in comparison with wild type, “A-5” in a paddy field experiment (Takamure and Kinoshita 1985). An original \textit{rcn1} is reported as a low temperature–sensitive \textit{rn} mutant gene (Takamure and Kinoshita 1985). Takamure and Kinoshita (1985) reported that tillering of N-133 increased in response with nitrogen to the same level as wild-type A-5 in a plastic house. Whereas, tillering of N-133 was strongly suppressed in any nitrogen, potassium, and phosphate contents in a paddy field experiment. In future studies, we need to elucidate whether both \textit{rcn1} mutant alleles are sensitive to low temperature in a various genetic backgrounds under controlled environmental conditions.

In \textit{d3} genetic background of Shiokari, \textit{rcn1} started the inhibition of tillering from the eighth leaf stage. At heading

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
\textbf{Strain} & \textbf{Culm number} & \textbf{Culm length (cm)} \\
\hline
Shiokari & 34.3 ± 4.8\* & 71.3 ± 5.3\* \\
S-97-61 & 5.3 ± 1.4\* & 50.7 ± 4.6\* \\
ID3 & 195.1 ± 25.4\* & 28.4 ± 2.1\* \\
DM & 74.6 ± 33.4\* & 13.5 ± 2.0\* \\
\hline
\end{tabular}
\caption{Culm number and length (mean ± standard deviation) of Shiokari, a new \textit{rcn1} mutant S-97-61, \textit{d3} mutant of near-isogenic line ID3, and \textit{d3rcn1} double mutant (DM) at heading time}
\end{table}

Means followed by the same letter are not significantly different at the 1\% level according to Tukey’s HSD test (Tukey 1977).
time, tillering of the \( d3rnl \) double mutant exhibited 38.2% that of ID3, which was between ID3 and S-97-61. These results proved that promotion of \( Rnl1 \) in tillering is independent of \( D3 \) inhibition in the tillering pathway. In eudicots MAX pathway, shoot branching is regulated by modulating auxin transport capacity through the control of \( PIN \) transcript levels (Bennett et al. 2006). The family of PIN auxin transport facilitator proteins mediates the amount and direction of polar auxin transport (Chen et al. 1998; Galweiler et al. 1998; Frielm, Benkova, et al. 2002; Frielm, Wisniewska, et al. 2002). We need to clarify whether there is a possibility that \( Rnl1 \) is integrated in promotion of auxin transport via PIN family protein by further studies.

Few tillers in rice is one of the main target traits for “super-rice ideotype” (Khush 2000). Many breeders and scientists are focusing to control rice tillering. To clarify the molecular basis of tillering, it is important to clone new genes from rice using different mutants. The present new \( rnl1 \) mutant will facilitate the map-based cloning of the \( rnl1 \) gene in future studies. In addition, \( rnl2 \) mutants \( rnl2 \) to \( rnl9 \) were reported (Takamure and Kinoshita 1993; Takamure 1994; Jiang et al. 2006). No tillering mutant was also reported as monoculm mutant, \( MOC1 \), with defects in formation of tiller buds (Li et al. 2003). As we understand the molecular basis of rice tillering using these mutants, we will combine these tillering genes in rice and breed high-yielding super-rice ideotypes by conventional and molecular breeding procedures.

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