Recent amplification of Osr4 LTR-retrotransposon caused rice D1 gene mutation and dwarf phenotype

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A novel rice d1 mutant was identified using map-based cloning and comparative analysis of known d1 mutants. The mutant (d1-a) shows a mild dwarf trait, which differs only slightly from the wildtype in plant height at the tilling stage. The d1-a mutant is different from other d1 mutants. We found that it was interrupted by an Osr4 long terminal repeat (LTR)-retrotransposon, which resulted in the loss of exon 7 in the mutant D1 mRNA. A paralog of the D1 gene, D1-like, was revealed. D1-like is a truncated gene that might have resulted from recombination between retrotransposons. We identified 65 Osr4 LTR-retrotransposons in Nipponbare, and found more LTR variants in contrast to coding DNA sequence (CDS) in the retrotransposons. We also identified five possible regulatory motifs inLTRs which may control the expression of the retrotransposons. In addition, we predicted six putative functional Osr4 retrotransposons that contain complete CDSs and all important elements. Osr4 retrotransposons were classified into 4 groups, and this type of retrotransposon only appears to be present in monocots.

Members of group I-1, which included all putative functional retrotransposons, showed a high similarity with each other. The retrotransposons were expressed in all tissues, at especially higher levels in some leaves and seeds. These findings imply that transpositions of group I-1 members might have occurred frequently and recently.

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1. Introduction

The dwarf phenotype in plants provides an opportunity for studying regulatory mechanisms of plant growth and development. This character is also favored in breeding. Dwarf mutants have been discovered in many species, and the regulatory mechanisms underlying this trait have been partially revealed (Daviere and Achard, 2013; Waldie et al., 2014; Zhang et al., 2014). Various factors can result in dwarf phenotypes associated with the gibberellin (Daviere and Achard, 2013), brassinosteroid (Zhang et al., 2014) and/or strigolactone pathways (Waldie et al., 2014).

In rice, at least 20 dwarf genes have been cloned, and most of them are involved in the synthesis and signal transduction of gibberellin, brassinosteroid and/or strigolactone (http://www.ricedata.cn/gene/gene_sd.htm). Rice dwarf mutant Daikoku, which carries the d1 gene, was first isolated as a spontaneous mutant (Ashikari et al., 1999; Fujisawa et al., 1999). The d1 mutant shows abnormal gross morphologies, such as dwarfism, shortened internodes, small-rounded seeds and erect leaves (Ashikari et al., 1999; Fujisawa et al., 1999). The D1 gene encodes a subunit of a G-protein, the GTP-hydrolysing Gα, the mutation of which alters responses to both gibberellin and brassinosteroid (Oki et al., 2009c; Ueguchi-Tanaka et al., 2000). G-proteins are membrane-associated heterotrimer consisting of Gα, Gβ and Gγ subunits. The Gα subunit binds tightly to GDP-bound Gα, enhancing the coupling of Gα to the G-protein-coupled receptor. Upon receptor stimulation by a signal molecule, the state of the receptor changes and Gα dissociates from the receptor and Gβγ, while GTP is exchanged for the bound GDP, which leads to Gα activation. Then, Gα goes on to activate other molecules in the cell (Urano and Jones, 2014).
Long terminal repeat (LTR) retrotransposons are class I transposable elements and are the most common type. They are ubiquitous in the plant kingdom, and are the main constituents of large plant genomes (Kumar and Bennetzen, 1999; Ma et al., 2004). For example, LTR-retrotransposons comprise more than 50% of the maize genome (Kumar and Bennetzen, 1999) and more than 22% of rice genome (Ma et al., 2004). About 41 families, including 13,000 elements, have been revealed in rice (Vitte et al., 2007). However, only Tos elements have been studied extensively, and Tos17 was used for use as molecular markers. BSA (Michelmore et al., 1991) was used to perform to determine the insert using Wang’s method (Wang et al., 2011). All primers are shown in Table 2.

2. Plant materials and methods

2.1. Plant materials and genetic mapping

The dwarf mutant was found in the Min Nong 1 rice paddy field. To identify the dwarf gene, two F2 populations from crosses between the mutant and Nipponbare or LPBG08 plants were produced for genetic mapping. DNA was extracted from fresh leaves from each of the F2 plants using the SDS method (Zheng et al., 1995) with a few modifications. SSRs were amplified for use as molecular markers. BSA (Michelmore et al., 1991) was used to find the markers that linked to the target gene.

2.2. Identification of the mutant gene and the insert

Total RNA was extracted from rice leaves and flowers following Bilgin’s method (Bilgin et al., 2009). Total RNA (1 μg) was reverse-transcribed using M-MLV reverse transcriptase in a final volume of 20 μl to obtain cDNA. RT-PCR was used to detect gene expression and to find the deletion of the mutant gene.

A set of PCR was done with the primers on D1 gene. Tail-PCR was performed to determine the insert using Wang’s method (Wang et al., 2011). All primers are shown in Table 2.

2.3. Analysis of the Osr4 LTR-retrotransposons

We used Chr4-1 as a query to search the rice genome sequence (IRGSO-1.0) with Blastn (http://rapdb.dna.affrc.go.jp/tools/blast) to identify the Osr4 LTR-retrotransposons in Nipponbare. The High-scoring Segment Pairs in the Blast result, whose sequence identities were more than 80% and lengths more than 350bp, were selected. The homologous retrotransposon sequences, LTR mutations, CDS mutations and directed repeats were manually analyzed and revealed. The alignment and phylogenetic tree were constructed with MEGA6 (http://megasoftware.net/). To find the Osr4 LTR-retrotransposons in other species, we used Chr4-1 as query to search a nr/nt database, and the High-scoring Segment Pairs whose sequence identities are more than 80% and lengths are more than 4000bp were manually analyzed, and their CDSs were used to construct the phylogenetic tree.

3. Results

3.1. Mapping the dwarf gene

A spontaneous dwarf mutant which shows broad, dark green leaves, compact panicles, and short, round grains was found in Min Nong 1 paddy field (Fig. 1). Each internode of the mutant is shorter than in the wildtype, and the second internode is extremely short (Fig. 1C and D). However, the plant heights of the wildtype and the mutant are not obviously different at the tillering stage (Fig. 1E).

The segregation ratio of dwarf plants to wildtype plants fits to 1:3 in the single Mendelian factor model, which indicates that one recessive gene controls the dwarf phenotype. We made crosses between Nipponbare and the mutant, and between the mutant and LPBG08. We tested the segregation ratios, and both crosses segregated according to the Mendelian factor model.

We developed two DNA pools: wildtype pool and dwarf pool. For the dwarf pool, nine dwarf plants were selected from the F2 mutant; their DNA was extracted and pooled. For the wildtype pool, individuals of the cross between Nipponbare and the dwarf mutant are not obviously different at the tillering stage (Fig. 1). Each internode of the mutant is shorter than in the wildtype, and the second internode is extremely short (Fig. 1C and D). However, the plant heights of the wildtype and the mutant are not obviously different at the tillering stage (Fig. 1E).

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We developed two DNA pools: wildtype pool and dwarf pool. For the dwarf pool, nine dwarf plants were selected from the F2 individuals of the cross between Nipponbare and the dwarf mutant; their DNA was extracted and pooled. For the wildtype pool, the DNAs from nine F2 wildtype individuals were mixed. We detected their polymorphisms identify the Osr4 LTR-retrotransposons in Nipponbare. The High-scoring Segment Pairs in the Blast result, whose sequence identities were more than 80% and lengths more than 350bp, were selected. The homologous retrotransposon sequences, LTR mutations, CDS mutations and directed repeats were manually analyzed and revealed. The alignment and phylogenetic tree were constructed with MEGA6 (http://megasoftware.net/). To find the Osr4 LTR-retrotransposons in other species, we used Chr4-1 as query to search a nr/nt database, and the High-scoring Segment Pairs whose sequence identities are more than 80% and lengths are more than 4000bp were manually analyzed, and their CDSs were used to construct the phylogenetic tree.

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| Table 1 |
| --- |
| **Number of different LTR retrotransposons.** |
| Structure | No. of elements |
| Intact elements with TSDs | 33 |
| Intact elements without TSDs | 7 |
| One LTR partially deleted | 8 |
| Both LTR partially deleted | 17 |

| Table 2 |
| --- |
| **Primer sequences used in this study.** |
| Name | Primer sequence | Purpose |
| P1 | TTACCTTGTCTCCGTTCTT | Determine insert position |
| P2 | CTCGAACTTCATGATTGCTC | Determine insert position |
| P3 | GCACATGACGAGCTTGCTCG | Determine insert position |
| P4 | CATGACGAGGACACCTTGTCT | Determine insert position |
| P5 | AGCATGTTGACCCCTATGT | Determine insert position |
| P6 | CATGTTGAGCACTCTCTG | Determine insert position |
| D1-R2-2 | CCACCTTCCAACAGAAAAC | Tail-PCR |
| D1-R2 | ACCATGTGACACCTATGAC | Tail-PCR |
| D1-EXON-R1 | CCTTCGTATCAGAGCTCTG | Tail-PCR |
| CT-R2 | AACAGACGACGACCTTGTCT | Osr4 expression |
| T-F2 | GCCAGGGAGATCACTAAGGCA | Osr4 expression |
| CT-R3 | TCCTTCACTGGGCTGATCA | Osr4 expression |
| T-R1 | AACGGCTGCTCCTCCGAAAGA | Osr4 expression |
| D1-cDNA-R1 | AGCTCTCTCGTAAAGACAGCA | D1 cDNA |
| D1-cDNA-R2 | AGCGTCTGAAAGCCCAAGCT | D1 cDNA |
| RT-F | GTCTGACATTGCCGATATA | D1 gene expression |
| RT-R | GCTCTGCTGCTCTGGAAGA | D1 gene expression |
among the two DNA pools and the parents. The SSR markers RM289, RM509, RM430 and RM163 on chromosome 5 exhibited polymorphisms between the two DNA pools, and these results indicated that the gene controlling the dwarf trait was most likely linked to the four markers. Further analysis showed that the dwarf gene is located between RM289 and RM430 and co-segregates with RM509 (Fig. 2).

3.2. The dwarf gene

The phenotype of our mutant is very similar to the dwarf 1 mutant (Ashikari et al., 1999; Fujisawa et al., 1999, 2001; Miura et al., 2009; Oki et al., 2009b; Ueguchi-Tanaka et al., 2000), which is located on chromosome 5 near SSR marker RM509. We speculated that our dwarf mutant was controlled by an allele of the D1 gene. To confirm this, we detected the D1 cDNA with PCR, and found that the PCR product from the dwarf mutant is smaller than that from the wildtype (Fig. 3A). We sequenced the PCR products, and discovered a 102-bp deletion in our dwarf mutant gene, covering all of exon 7 (Fig. 2), but didn’t reveal an expression difference between the wildtype and the mutant (Fig. 3B). We termed this mutant gene d1-a.

To analyze the exact length of the deletion in the genome, we amplified genomic DNA that included exon 7, but we did not obtain PCR products in the mutant (Fig. 3D). We hypothesized that there was an insert fragment in the D1 gene of the mutant. To validate this hypothesis, we performed several PCRs, and found that the insert should be in exon 7 (Fig. 3C–E). However, to our surprise, we obtained double PCR product bands in the wildtype, and one smaller band in the mutant, and one bigger band in Nipponbare with primer P1 and P4 (Fig. 3E). The D1 gene was previously thought to be a single copy gene in the rice genome (Ishikawa et al., 1995). However, our results did not support this assumption and implied that there may be other D1 variants in rice plants.

In order to identify the variants, we performed PCR with primer P1 and P3, which were on the same side of the insert (Fig. 3C). The results exhibited two bands in the wildtype and the mutant, but one bigger band in Nipponbare (Fig. 3F). It is difficult to explain these results. Fortunately, using Blastn, we identified a truncated D1 gene which was also on chromosome 5 and about 394.7 kb distant from the D1 gene in Nipponbare (Fig. 4A). This truncated gene, which we termed D1-like, contained seven exons of the D1 gene (Fig. 4B). We checked Min Nong 1 and RP Bio-226, and found that both of them contained the D1-like gene. However, the D1-like gene was longer in Nipponbare than in Min Nong 1 and RP Bio-226 (indica). In addition, the P1–P3 and P1–P4 regions of the D1-like gene were almost identical to the same regions in the D1 gene in Nipponbare, RP Bio-226 and the wildtype (Fig. 4C). The differences led to the results shown in Fig. 3E and F. The P1–P4 region included an insert in d1-a, so only the shorter band could be obtained (Fig. 3E). In addition, the D1-like gene contained many point mutations compared with the D1 gene.

The D1-like gene was a section of Os05t0341300 gene (http://rapdb.dna.affrc.go.jp/viewer/gene_detail/irgsp1?name=Os05t0341300), and exons 1, 2, 3, 4, 5, 6 and 7 of D1-like were also exons of Os05t0341300. The gene prediction of Os05t0341300 was supported by the transcript AK241716, but no start codon was indicated in the prediction. We used the DNA sequence of AK241716 as query to perform a Blastx search, and only D1 (Gα) proteins in different species were hit. The homologous regions covered the partial region of AK241716, and contained a stop codon. These results suggest that Os05t0341300 may be a pseudogene. Moreover, we also found at least two remains of LTRs in intron 8 of Os05t0341300 (Fig. 4B).

3.3. An active LTR-retrotransposon inserted rice D1 gene

We used a tail PCR to reveal the insert sequence. The results showed that the sequence of the tail-PCR product was about the...
same as the partial sequence of an LTR-retrotransposon. We designed primers according to the LTR-retrotransposon sequence (ref NC_008397.2: 31604515 to 31610215), and performed PCR with one primer on the LTR-retrotransposon and one primer on D1 gene, and then sequenced the PCR products. The results showed that the sequences of both sides of the insert were nearly identical to that of the sides of an LTR-retrotransposon (ref NC_008397.2: 31604515 to 31610215). The insert position is shown in Fig. 5A. However, it is difficult to determine whether the insert contains a complete LTR-retrotransposon. Further analysis showed that the LTR-retrotransposon (ref NC_008397.2: 31604515 to 31610215) belongs to the Osr4 group (McCarthy et al., 2002; Vitte et al., 2007). We checked the expression of the LTR-retrotransposon, and found that the expression in leaves was much higher than in flower tissues (Fig. 5C). To further investigate the expression of the LTR-retrotransposon in other tissues, we used the sequence of the LTR-retrotransposon as the query to search PLEXdb (http://www. plexdb.org/modules/tools/plexdb_blast.php), and obtained a probe set (ID-Os.45957.3.S1_x_at), which matches the LTR-region. The expression profile of Os.45957.3.S1_x_at is shown in Fig. 5A, which partially reflects the expression of the LTR-retrotransposon. The LTR-retrotransposon is expressed in all tissues, with higher expression levels in some seeds and leaves. These results suggested that at least one of the Osr4 members should be active.

3.4. Members of Osr4 LTR-retrotransposons in Nipponbare and other species

We found 65 members of the Osr4 LTR-retrotransposons in the Nipponbare genome, and revealed 11 retrotransposons with a complete CDSs (Coding DNA sequence) in addition to 54 with mutant CDSs (Table 1 and S2). We also found some point mutations in the 11 retrotransposons, but did not determine whether the mutant sites affect their function. Comparing the CDSs of retrotransposons, we revealed many more changes in the LTR region (data not shown) thought to regulate expression (Grandbastien, 2015). Short directed repeats were detected at the insert sites in 35 LTR-retrotransposons, while they were absent in another 30 LTR-retrotransposons (Table S2).

We classified the 65 homologs into 4 groups according to a phylogenetic tree. Fifteen members in group I-1 showed high similarity with each other, including 10 LTR-retrotransposons with a complete CDS within the group (Table S2 and Fig. 5B). The insert of d1 gene in the mutant also exhibited high similarity with Chr4-1 in group I-1. These results implied that the members of group I-1, which showed higher activity, may have undergone transpositions frequently and recently.

The Osr4 members were distributed over all chromosomes (Fig. S1), and most of them were located in introns (33) and intergenic regions (29), while only 3 members were found in exons.
Fig. 5. (A) The exact position of the insert and a typical Osr4 LTR retrotransposon. LTR (Long terminal repeat) with TG at the 5' extremity and CA at the 3' extremity; TSR (target site repeat) is a 5bp short direct repeat string flanking the 5' and 3' extremities of an element; PBS, a sequence complemented to the 3' tail of some tRNA; PPT (polypurine tract) is a short rich purine segment. Protein domains: The gag gene encodes proteins involved in maturation, packaging of retrotransposon RNA and proteins into a form suitable for integration into the genome. Int, integrase; RT, reverse transcriptase; RH, RNase H. (B) The Neighbor-joining tree of 65 LTR-retrotransposon homologs with their DNA sequences. The scale bar indicates the simple matching distance. *Indicate the retrotransposon with a complete CDS. **Indicate the possible functional retrotransposons. (C) The expression of the retrotransposon was detected with RT-PCR. Three different pairs of primers were used for retrotransposon. (D) The Neighbor-joining tree of LTR-retrotransposons in different species with their coding region sequences. *Indicates retrotransposons with a complete CDS.
(Table S2). Some Osr4 members were located in promoters (Table S2), and might affect downstream gene expression.

The cis-regulation of LTR-retrotransposon gene expression mainly located in the LTR region, and some regulatory motifs in LTR play a key role in the expression (Dhadi et al., 2015; Grandbastien, 2015; Takeda et al., 1999). We checked the left LTR region, and revealed two new types of putative regulatory motifs: Box-A and Box-B (Fig. 6A). There were two Box-A motifs and three Box-B motifs in the LTR region. Only eight out of 65 Osr4 members contained all five motifs. Six of these are predicted to be functional retrotransposons containing the five motifs, a TATA box, PBS (a ~18bp sequence complemented to the 3’ tail of some tRNA, which is very important because tRNA binding process is the first step of initiating reverse transcription.), a complete CDS and polyuridine tract (Fig. 6B). In addition, some functional retrotransposons lacked the short directed repeats at the insert sites (Table S2 and Fig. 6B).

We searched the NCBI nr/nt database with the Chr4-1 DNA sequence, and only discovered the Osr4 members in monocots. However, most of them contained frameshift mutations or stop codons in the CDS, while some of them lost short directed repeats. We found only one LTR-retrotransposon in some species and more in other species. It is not certain whether there were active LTR-retrotransposons in the species. The phylogenetic tree of the LTR-retrotransposons in different species is shown in Fig. 5D.

4. Discussion

In rice plants, d1 mutants were originally identified in 1999 (Ashikari et al., 1999; Fujisawa et al., 1999), and have been used in subsequent G-protein studies (Oki et al., 2009a, 2009c; Suharsono et al., 2002; Ueguchi-Tanaka et al., 2000). Thereafter, many other d1 mutants which contained base substitutions, insertions or deletions in the d1 gene have been identified and show very similar phenotypes (Ashikari et al., 1999; Fujisawa et al., 1999; Oki et al., 2009b). This implies there may be several mutation hotspots in the D1 gene region. The d1-a gene lost its exon 7 of mRNA, and exon 7 encodes 34 amino acid residues (161–194), which includes one GoLoco binding site. GoLoco binding sites of Ga protein are responsible for binding with regulatory proteins possessing GoLoco motifs, which maintain G-protein subunit dissociation in the absence of Ga activation (Kimple et al., 2002). This implied the Ga protein in the d1-a mutant lost its activity completely or partially. Our mutant showed a similar phenotype as other d1 mutants, and the mutant gene was located at the same position as the D1 gene. These results suggested that the dwarf mutant phenotype in our study was caused by a mutation in the d1-a gene.

Previous studies showed that the d1-4 mutant exhibited a mild phenotype, characterized by bigger seeds and higher plants in contrast to other mutants (Oki et al., 2009b). Most d1 mutants...
contain deletions (Ashikari et al., 1999; Chen et al., 2011; Fujisawa et al., 1999; Oki et al., 2009b), and three d1 mutants contained stop codons (Oki et al., 2009b). We found a novel d1 mutant, which contained an Ors4 copia retrotransposon in exon 7. This insert resulted in loss of exon 7 in d1 mRNA of the d1-a mutant. Compared with some severe d1 mutants (d1-1, -2, -3, -5 etc.) (Oki et al., 2009b), the d1-a mutant is predicted to be a mild mutant, and we found no obvious difference in plant height between d1-1 mutant and wild-type plants at the tillering stage (Fig. 1E). Our results revealed a D1-like gene followed by two LTR-retrotransposon remnants (Fig. 4B), which implied that the D1-like gene may have been truncated by recombination between retrotransposons after D1 gene duplication. The D1-like gene may exist in all cultivated rice.

Os4 LTR-retrotransposon was firstly identified in 2002 (McCarthy et al., 2002). The length of the LTR retrotransposon reference copy is 5665bp, and its LTR length is 350bp (McCarthy et al., 2002; Vitte et al., 2007). However, up to this date, the Os4 copies have not been characterized. We carefully analyzed Os4 retrotransposons in Nipponbare, and revealed 65 homologs. We predicted six possible active retrotransposons, which contained all motifs in LTR, a complete CDS, PBS and PPT (Table S2 and Fig. 6B). The members of group I-1 could transpose recently, and included all possible active retrotransposons. One of Os4 retrotransposons inserted into D1 gene in the d1-a mutant, and resulted in loss of gene function. The Os4 retrotransposon is expressed in all tissues. These findings suggested that at least one Os4 retrotransposon was activated. Os4 retrotransposon expression in some seeds and leaves was higher than in other tissues (Fig. S2), but the transposition that occurred in somatic cells may have produced plant mosaics, and only the transposition which occurred in sexual cells may have produced plant phenotypes.

Author’s contributions

XS identified the gene and the insert, JC and HZ identified the data, KL and YG completed the field experiments. XS drafted the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pld.2017.01.003.

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