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Identification, genetic characterization, GA response and molecular mapping of *Sdt97*: a dominant mutant gene conferring semi-dwarfism in rice (*Oryza sativa* L.)

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Summary

Semi-dwarfism is an important agronomic trait in rice breeding programmes. *sd-1*, termed the 'Green Revolution gene', confers semi-dwarf stature, increases harvest index, improves lodging resistance, and is associated with increased responsiveness to nitrogen fertilizer. It has contributed substantially to the significant increase in rice production. In this paper, a novel semi-dwarf mutant in rice is reported. Genetic analysis revealed that only a single dominant gene locus non-allelic to *sd-1*, temporarily designated *Sdt97*, is involved in the control of semi-dwarfism of the mutant. The semi-dwarfism of the mutant could be partly restored to the tall wild-type by application of exogenous GA_3, suggesting that the mutant gene *Sdt97* may be involved in the gibberellin (GA) synthesis pathway and not the GA response pathway in rice. A residual heterozygous line (RHL) population derived from a recombinant inbred line (RIL) was developed. Simple sequence repeat (SSR) and bulked segregation analysis (BSA) combined with recessive class analysis (RCA) techniques were used to map *Sdt97* to the long arm of chromosome 6 at the interval between two STS markers, N6 and TX5, with a genetic distance of 0·2 cM and 0·8 cM, respectively. A contig map was constructed based on the reference sequence aligned by the *Sdt97* linked markers. The physical map of the *Sdt97* locus was defined to a 118 kb interval, and 19 candidate genes were detected in the target region. This is the first time that a dominant semi-dwarf gene has been reported in rice. Cloning and functional analysis of gene *Sdt97* will help us to learn more about molecular mechanism of rice semi-dwarfism.

1. Introduction

Dwarf genes have been utilized extensively in plant breeding to improve lodging resistance. Their applications have been associated with increased yields, higher fertility, early maturity and high tillering capacity. The popularization of dwarf cultivars was a major factor in the success of the 'Green Revolution' in rice and wheat (Khush, 2001; Hedden, 2003; Muangprom & Osborn, 2004). There are various reasons for the dwarf phenotype in plants, but defects in biosynthesis and the perception of gibberellin (GA) is the important determinant of plant height (Sasaki *et al.*, 2002). GAs are essential endogenous regulators of plant growth and development that affect many aspects of a plant's life cycle, including seed germination, leaf expansion, stem elongation, flower initiation, and flower and fruit development (Harberd *et al.*, 1998). The important dwarf genes used in agriculture are mutations of genes in the GA biosynthesis or response pathways (Sakamoto *et al.*, 2004). Mutations of genes in the biosynthesis pathway cause GA deficiency and dwarf phenotypes, and exogenous GA application can restore the wild-type phenotype

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in these mutants (Phillips, 1998). The predominant dwarf gene in rice cultivars, semi-dwarf1 (*sd1*), affects the GA biosynthesis pathway (Monna *et al*., 2002; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002; Muangprom & Osborn, 2004).The semi-dwarf stature of $d35^{Tan\text{-}Ginbozu}$ is caused by a defective early step of GA biosynthesis, which is catalysed by ent-kaurene oxidase (KO) (Itoh *et al*., 2004). Two GA 3β-hydroxylase genes, *OsGA3oxl* and *OsGA3ox2*, corresponding to the *d18* locus, encoded proteins showing 3β-hydroxylase activity for the steps GA_{20} to GA_1, GA_5 to GA_3, GA_{44} to GA_{38}, and GA_9 to GA_4 (Itoh *et al*., 2001).

Dwarf mutants in the GA response pathway display a similar phenotype to the GA biosynthesis mutants, although they fail to respond to exogenous GA treatment (Sun, 2000). GA-insensitive dwarf1 gene *GID1* encodes a soluble receptor mediating GA signalling in rice (Ueguchi-Tanaka *et al*., 2005; Hartweck *et al*., 2006), *GID2* encodes a rice F-box protein, which is essential for GA-mediated DELLA protein degradation (Gomi *et al*., 2004). Rice GA-insensitive dwarf mutant gene *Dwarf 1* encodes the subunit of GTP-binding protein; dwarf mutant *d1*, which is defective in a subunit of the heterotrimeric G protein, affects GA signal transduction (Ueguchi-Tanaka *et al*., 2000; Ashikari *et al*., 1999). *SLR1* is an intermediate of the GA signal transduction pathway; *slender* mutant (*slr1-1*) results in a constitutive GA response phenotype (Ikeda *et al*., 2001). Most modern commercial wheat cultivars contain Rht mutant alleles; *Rht-B1b* and *Rht-D1b* are mutations in the GA response pathway (Silverstone & Sun, 2000; Muangprom & Osborn, 2004).

So far, five genes (*ga1*, *ga2*, *ga3*, *ga4* and *ga5*) encoding key enzymes in the GA biosynthesis pathway and one gene in the GA response pathway (*GAI*) have been cloned in *Arabidopsis* (Sun *et al*., 1992; Yamaguchi *et al*., 1998; Helliwell *et al*., 1998; Chang *et al*., 1995; Xu *et al*., 1995; Peng *et al*., 1997). The GA biosynthesis orthologous genes have been cloned in several plant species, such as *ls* (Ait-Ali *et al*., 1997) and *le* (Martin *et al*., 1997) in pea, *D8* in maize and *Rht1* in wheat (Peng *et al*., 1999).

More dwarf mutant genes related to GA in rice have also been cloned, such as *d18* (Itoh *et al*., 2001), *D35* (Itoh *et al*., 2004) and *sd-1* (Monna *et al*., 2002; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002) with a deficiency in the GA biosynthesis pathway, and *D1* (Ueguchi-Tanaka *et al*., 2000; Ashikari *et al*., 1999) and *Gid2* (Gomi *et al*., 2004) with a deficiency in the GA response pathway.

In 1997, a novel semi-dwarf mutant rice was isolated in our research (Tong *et al*., 2001). In this paper, the genetic characterization, GA response and molecular mapping of the semi-dwarf mutant gene are reported.

2. Materials and methods

(i) *Plant materials and field planting*

A semi-dwarf mutant was found in the F_6 generation of a medium *japonica* rice cross between M9056 and R8018 XUAN in 1997. In 1998, the seeds harvested from this mutant were planted in the field and the plant heights of their progenies were recorded and analysed. To study the genetic character and map the mutant gene, plant materials used in these researches were as follows: the semi-dwarf mutant, tall wild-type, and the F_1, F_2 progenies derived from reciprocal crosses between the semi-dwarf mutant and wild-type; Hua-jing-xian74 (an elite *indica* rice cultivar); a residual heterozygous line (RHL) population derived from RHL63–146; and a recombinant inbred line (RIL) (F_6) originated from an inter-subspecific cross between the semi-dwarf mutant (*japonica* cv.) and Hua-jing-xian74 (*indica* cv.).

These plant materials were planted in the field during the rice-growing seasons from 2000 to 2004 at the experiment stations at Hefei (31°N, 117°E), Anhui province, and Sanya (18°N, 109°E), Hainan province, China. The planting density was 13·3 cm between plants in a row, and 16·7 cm between rows, with 11 plants per row. Field management followed normal agricultural practices. Irrigation of the field was maintained to avoid drought stress. Plant heights were recorded at maturity.

(ii) *GA response experiment design*

In agriculture, exogenous GA_3 treatment has usually been used to stimulate panicle emergence in male sterile (MS) lines to gain greater yield in hybrid rice seed production in China. In order to study the response of the semi-dwarf mutant to exogenous GA, GA_3 solution was sprayed on both the semi-dwarf mutant and the tall wild-type at a dosage of $5{\cdot}6 \times 10^{-4}$ g per plant at different rice developmental periods including the seedling stage, tillering stage, heading stage and milking ripe stage. From the jointing stage to the heading stage in 2002, the same dosage of GA_3 solution was sprayed on the semi-dwarf mutant and the tall wild-type on 17 July, 25 July and 2 August, respectively. The plant height, panicle and elongation internode lengths were recorded at maturity.

(iii) *Mapping population development*

To dissect the genetic factors underlying the semi-dwarf mutant, temporarily designated *Sdt97*, and to construct a population for mutant gene *Sdt97* mapping, an inter-subspecific cross was carried out

between the mutant, a semi-dwarf *japonica* rice line, and Hua-jing-xian74, a semi-dwarf *indica* rice cultivar.

A line with a heterozygous segment surrounding a gene is denoted as a residual heterozygous line (RHL), and can be used for gene mapping in map-based cloning (Yamanaka *et al*., 2005). In the *Sdt97* mapping, a segregated $F_{6:7}$ progeny derived from a single line (named RHL63-146), which derived from the 186 RILs (F_6) and was identified to be heterozygous around locus *Sdt97* (Yamanaka *et al*., 2005; Tuinstra *et al*., 1997), was selected for *Sdt97* mapping (see Section 3 below).

(iv) *DNA extraction, BSA and RCA*

Genomic DNA was extracted individually from fresh leaves of the parental $F_{6:7}$ plants, using the modified CTAB method (Doyle & Doyle, 1987; Ragers & Bendich, 1998). PCR amplification was performed with the Programmable Thermal Controller PTC 100 (MJ Research, Watertown, MA). PCR reactions consisted of 2·5 μl of 10× reaction buffer (with $(NH_4)_2SO_4$, 100 mM), 2·0 μl $MgCl_2$ (25 mM), 1·0 μl dNTPs (10·0 μM), 1·0 unit *Taq* DNA polymerase, 100 ng template DNA and 1·5 μl of primer (10 μM), made up to 25·0 μl with distilled water, and then covered with a drop of mineral oil. Amplifications were performed using the following profile: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Amplification products were analysed on 4% agarose gels stained with ethidium bromide, and photographed using the Gel Doc 2000 system. When necessary, the amplification products were further analysed on 6% polyacrylamide gel stained with 0·1% silver nitrate.

Bulked segregant analysis (BSA) (Michelmore *et al*., 1991) combined with recessive class analysis (RCA) (Chen *et al*., 2005; Pan *et al*., 2003; Zhu *et al*., 2004; Zhang *et al*., 1994) was used to identify molecular markers linked to the mutant gene *Sdt97* in this study. Genomic DNA from 30 semi-dwarf individuals and 30 dwarf individuals in the $F_{6:7}$ segregated progenies was pooled to create the semi-dwarf and dwarf DNA bulks, respectively. The parental DNA and the two bulks were used for BSA. Markers were examined for polymorphism between the mutant and Hua-jing-xian74.

Polymorphic markers from the two parents were screened against the two DNA bulks, and polymorphic markers between the two DNA bulks were screened against the recessive individuals. The marker that was linked to the target gene *Sdt97* was screened against the entire $F_{6:7}$ segregated population. Polymorphic markers were used for co-segregation analysis with the plant height genotype. The formula for recombination fraction calculation is:

$$r = \frac{\text{the recombination gamete}}{\text{total gamete}},$$

where r is recombination fraction. The plant height genotype of the individuals in the $F_{6:7}$ populations was validated by progeny testing of $F_{7:8}$.

(v) *Marker development, linkage analysis and fine-mapping of Sdt97*

Only PCR-based SSR markers were used in the primary map study. A set of 832 SSR markers (data not shown) evenly distributed throughout the 12 chromosomes was used. Their map locations, primer sequences and other details are available online at http://gramene.org.ricemicrosat.html. The primer sets were adopted from the International Rice Microsatellite Initiative (IRMI, http://www.gramene.org; McCouch *et al*., 2002), and the detection procedures are as described by Zhu *et al*. (2004). SSR primers were synthesized by Shanghai CASarray Co. Ltd.

For further linkage analysis, position-specific microsatellite (PSM) and sequence-tagged site (STS) markers were developed in the target region defined by the SSR markers through bioinformatics analysis (BIA) using the publicly available reference sequences of the entire rice genome of two subspecies: *japonica* (cv. Nipponbare; http://rgp.dna.affrc.go.jp) and *indica* (cv. 93–11; http://www.genomics.org.cn). In particular, primer sets for PSM markers were designed based on the reference sequence of cv. Nipponbare using the software tools SSRIT (http://www.gramene.org/microsat/ssrtool) and Primer Premier 5.0 (PREMIER Biosoft International, http://www.premierbiosoft.com). Primer sets for STS markers were designed according to the sequence comparison between the two subspecies in the target region using a software tool, Pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). When a large deletion existed between the two subspecies, the sequence was considered a candidate from a putative STS marker.

To determine the linkage relationship between the *Sdt97* locus and molecular markers, the genotype of plant height was combined with DNA marker data for linkage analysis. Linkage analysis was conducted using the Mapmaker/Exp 3.0 program (Lincoln *et al*., 1992) at a LOD threshold of 3·0 to construct a local genetic map for the *Sdt97* genomic region. Map distance between marker and semi-dwarf gene was estimated by the Kosambi mapping function:

$$x = \frac{1}{4}\ln\frac{1+2r}{1-2r},$$

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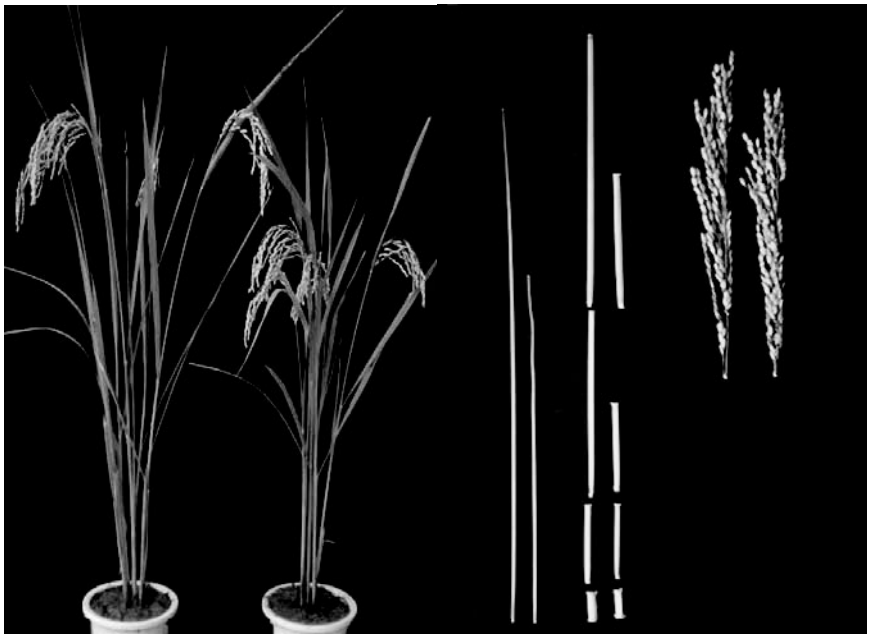

Fig. 1. Plant height, panicle and elongation internode length of the tall wild-type (left) and semi-dwarf mutant (right).

where x is the map distance and r is the recombination fraction (Kosambi, 1944).

3. Results

(i) *Identification and genetic analysis of the semi-dwarf mutant*

A semi-dwarf mutant was isolated from the tall F_6 progenies which derived from the cross between M9056 and R8018 XUAN in 1997 (Fig. 1). In 1998, seeds harvested from this mutant were planted in the field. Individuals in this population could be divided into two groups: semi-dwarf and tall. All the individuals in this population were self-bred and field-planted in 1999. Among 38 progenies, 9 exhibited semi-dwarf non-segregation, 21 exhibited continued segregation and 8 exhibited tall non-segregation. The ratio of non-segregation semi-dwarf progenies to segregation progenies to non-segregating tall progenies was 1 : 2·333 : 0·889 ($\chi^2 = 0{\cdot}4737$, $P > 0{\cdot}05$). Among 21 segregating progenies, there were 532 semi-dwarf and 172 tall individuals; the ratio of semi-dwarf to tall individuals equalled 2·993 ($\chi^2 = 0{\cdot}0473$, $P > 0{\cdot}05$). These results revealed that there was only one dominant gene locus involved in the control of the semi-dwarfism of the semi-dwarf mutant.

In order to analyse the genetic basis of semi-dwarfism of the mutant further, the plants derived from the non-segregating semi-dwarf progeny (Y98149) and the non-segregating tall progenies (Y98148) were selected as parents, and reciprocal crosses between them were made. In 2000, Y98149, Y98148 and the reciprocal F_1 were field-planted.

The plant height of Y98149 was $65{\cdot}8 \pm 4{\cdot}613$ cm (mean $\pm$ SD; 20 plants were measured, for these and subsequent measurements), the plant height of Y98148 was $84{\cdot}3 \pm 4{\cdot}814$ cm, that of Y98149 × Y98148/F_1 was $65{\cdot}0 \pm 6{\cdot}486$ cm, and that of Y98148 × Y98149/F_1 was $68{\cdot}3 \pm 2{\cdot}517$ cm. It was clear that the reciprocal F_1 exhibited the same plant height as that of the semi-dwarf mutant (Y98149), and they showed a significant difference compared with the height of the tall wild-type (Y98148).

The reciprocal F_2 were planted in 2001, and obvious segregation in plant heights occurred. In the Y98148 × Y98149/F_2 population, distribution of plant heights appeared to be definitely bimodal, breaking at the point in the apparent valley at 99·5 cm: of all 552 individuals, 427 were semi-dwarf and 124 were tall. The ratio of semi-dwarf individuals to tall individuals was 3·4436 ($\chi^2 = 1{\cdot}6993$, $P > 0{\cdot}05$). In the Y98149 × Y98148/F_2 population, the distribution of plant height appeared to be definitely bimodal also, breaking at the point in the apparent valley at 101·5 cm; of all 544 individuals, 412 were semi-dwarf and 132 were tall. The ratio of semi-dwarf individuals to tall individuals was 3·1212 ($\chi^2 = 1{\cdot}2010 \times 10^{-1}$, $P > 0{\cdot}05$) (Fig. 2). These results illustrate that a single dominant nuclear gene locus is involved in the control of semi-dwarfism of the mutant, and that the semi-dwarfism expression of the mutant is not affected by its cytoplasm. Similar results were also obtained in 2002 and 2003 (data not shown). It was deduced that the semi-dwarfism of the mutant did not result

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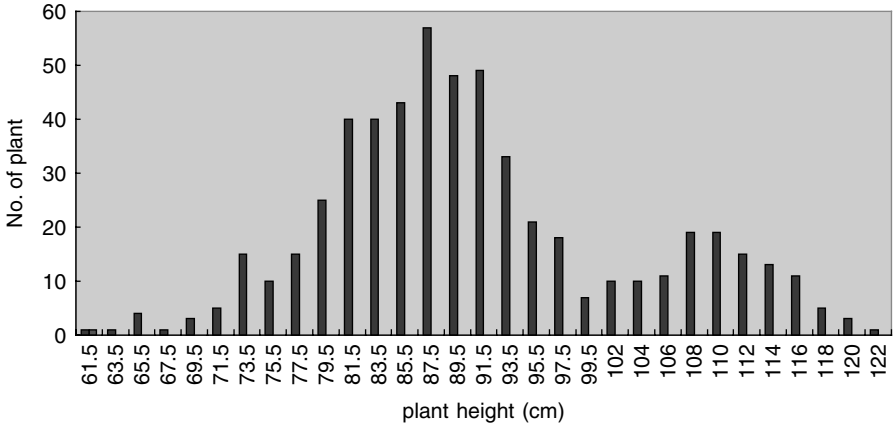

Fig. 2. Plant height distribution of the 544 plants in the mutant × wild-type/F_2 population.

from the cross-fertilization of the tall wild-type to other dwarf or semi-dwarf rice cultivars, but that a spontaneous mutation had occurred in the tall wild-type gene locus.

(ii) *Responses of the mutant to exogenous GA*

In agriculture, exogenous GA_3 treatment has usually been used to stimulate panicle emergence in MS lines to gain greater yield in hybrid rice seed production in China. To research the responses of the semi-dwarf mutant to exogenous GA, exogenous GA_3 solution was sprayed on the semi-dwarf mutant at different developmental periods. The results showed that the semi-dwarf mutant was sensitive to exogenous GA_3 only during the period from the stem elongation stage to the heading stage. During this time, the semi-dwarfism of the mutant could be partly restored to normal phenotype by exogenous GA_3.

At the seedling stage, the plant heights of semi-dwarf mutants treated with GA_3 and those treated with water were $69{\cdot}7 \pm 7{\cdot}5$ cm and $68{\cdot}2 \pm 2{\cdot}7$ cm, respectively; at the milking ripe stage they were $69{\cdot}4 \pm 3{\cdot}5$ cm and $67{\cdot}6 \pm 3{\cdot}0$ cm, respectively. At the seedling stage and at the milking grain stages, the semi-dwarf mutants treated with GA_3 showed the same plant height as those treated with water. But at the stem elongation stage and heading stages, the plant height of mutants treated with GA_3 was significantly different from that of the plants treated with water. At the stem elongation stage, the heights of GA-treated and untreated plants were $80{\cdot}9 \pm 8{\cdot}2$ cm and $72{\cdot}0 \pm 4{\cdot}3$ cm, respectively, and at the heading stage they were $82{\cdot}9 \pm 5{\cdot}0$ cm and $71{\cdot}6 \pm 3{\cdot}5$ cm, respectively.

The above results for the semi-dwarf mutant imply that the mutant gene *Sdt97* might be involved in the GA synthesis pathway and not the GA response pathway in rice.

(iii) *Mapping population development*

To map the semi-dwarf mutant gene *Sdt97*, a cross between the semi-dwarf mutant and the tall wild-type was made. A total of 680 SSR markers distributed evenly throughout 12 chromosomes were selected for polymorphism scanning between the semi-dwarf mutant and the wild-type; however, no polymorphism was detected. This result implied that the F_2 population derived from the cross between the semi-dwarf mutant and tall wild-type is difficult to use for the mapping of *Sdt97*.

To dissect the genetic factors underlying the semi-dwarf mutant, and to construct a population for *Sdt97* mapping, an inter-subspecific cross was carried out between the mutant, a semi-dwarf *japonica* rice line with the genotype *Sdt97Sdt97 Sd-1Sd-1*, and Hua-jing-xian74, a semi-dwarf *indica* rice cultivar with the genotype *sdt97sdt97sd-1sd-1*. There is a two-locus difference relating to dwarfism between these two varieties. However, the plant height of the F_2 population was more continuously distributed, Plant height phenotypes of the individuals in this F_2 population cannot easily be separated into discrete classes. This being the case, we attempted to develop another mapping population.

In the segregated F_2 progeny, extreme dwarf individuals with a genotype of *Sdt97_sd-1sd-1* were selected. In the segregated $F_{2:3}$ (hereafter $F_{2:3}$ means the F_3 population derived from one of the F_2 individuals) progenies, dwarf individuals with the same genotype were selected. Among the segregated $F_{3:4}$ progenies, one of the $F_{3:4}$ progeny populations had two discrete classes of plant height: semi-dwarf individuals and dwarf individuals. The ratio of dwarf individuals to semi-dwarf individuals fitted the expected Mendelian segregated ratio of 3 : 1. This suggested that only one dominant Mendelian factor was involved in controlling the segregation of plant height in the population. Because no tall individuals

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Fig. 3. Semi-dwarf plant (left) and dwarf plant (right) in RHL63-146, the mapping population of segregating $F_{6:7}$ progenies.

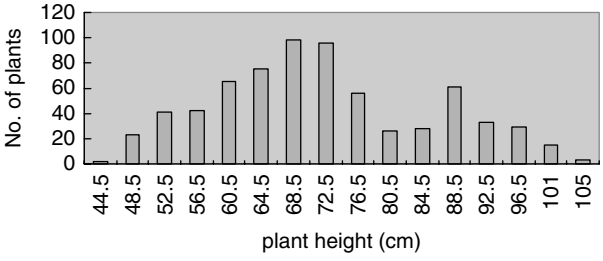

Fig. 4. Plant height distribution of the 693 plants in RHL63-94, a segregating $F_{6:7}$ progeny population.

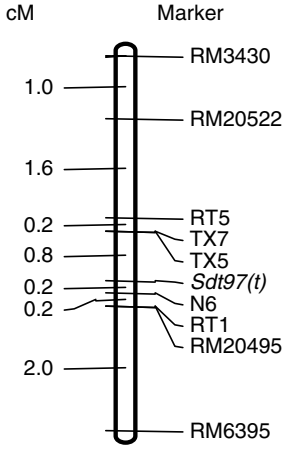

Fig. 5. Fine mapping of *Sdt97*.

occurred, the impact of *sd-1* can be eliminated and the genetic effects of *Sdt97* can be investigated.

Dwarf individuals with genotype *Sdt97_sd-1sd-1* were selected in the segregated $F_{3:4}$ progeny. A similar process was used to select dwarf individuals in segregated $F_{4:5}$ and segregated $F_{5:6}$ progenies. A segregated $F_{6:7}$ progeny derived from a single line (named RHL63-146), which derived from the 186 RILs (F_6) and was identified to be heterozygous around the locus *Sdt97* (Yamanaka *et al*., 2005; Tuinstra *et al*., 1997), was selected for *Sdt97* mapping; it comprised 257 individuals.

(iv) *Identification of Sdt97 by RHL population*

In the $F_{6:7}$ progenies, populations were categorized into two groups according to plant height. Group 1, derived from homozygous dwarf F_6 individuals, exhibited dwarf non-segregation; 44 $F_{6:7}$ progenies were categorized in this group. Group 2, derived from heterozygote dwarf F_6 individuals, showed continued plant height segregation and resulted in both semi-dwarf and dwarf individuals; 82 $F_{6:7}$ progenies were categorized in this group (Fig. 3). The ratio of group 2 to group 1 progenies was 1·86. The ratio of the segregating $F_{6:7}$ progenies to the non-segregating dwarf $F_{6:7}$ progenies did not differ significantly from 2 : 1 ($\chi^2 = 0{\cdot}081$, $P > 0{\cdot}05$). The results confirmed there was only one dominant gene controlling the segregation of plant height in the RHL population and the gene was designated *Sdt97*.

One segregating $F_{6:7}$ progeny population, RHL63-94, was investigated further. Distribution of plant height in this population appeared to be bimodal. Of the 693 individuals, 524 were dwarf and 169 were semi-dwarf giving a ratio of dwarf to semi-dwarf plants of 3·10 ($\chi^2 = 0{\cdot}1082$, $P > 0{\cdot}05$) (Fig. 4). It was suggested that only one single dominant gene locus was involved in the segregation of plant height in this population. Similar results were obtained in 2004 (data not shown).

The parents of the segregating $F_{6:7}$ progenies were a residual heterozygous line (RHL) (Yamanaka *et al*., 2005; Tuinstra *et al*., 1997), and the two chromosomes in their nuclear genome showed close chromosomal similarity to each other along the entire genome length with the only heterozygous sequences covering

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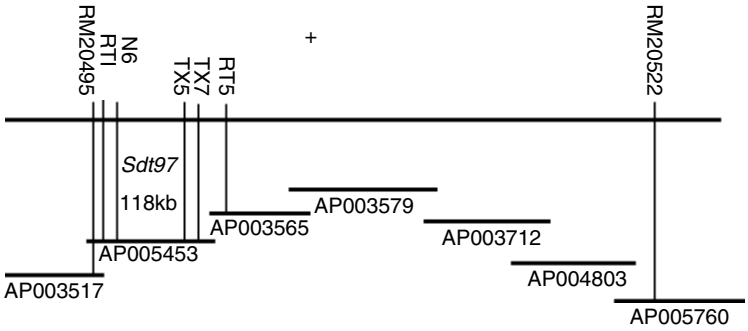

Fig. 6. A contig map covering the *Sdt97* allele region. RM20495 and RM20522 are SSR markers, RT1 and RT5 are PSM markers, and N6, TX5, and TX7 are STS markers. The long horizontal line indicates the genomic region encompassing the *Sdt97* locus. The short horizontal line represents BAC/PAC clones of cv. Nipponbare with the accession numbers indicated. The vertical lines indicate the relative position of the corresponding marker on BAC/PAC clones

the *Sdt97* locus. The heterozygous chromosomal region (approximately 25·5 cM, 6646 kb, starting at RM3430 and ending at RM6395; data not shown) initiated from different parents, one carrying the *Sdt97* gene derived from the mutant, and the other carrying the *sdt97* gene derived from Hua-jing-xian74. The DNA marker polymorphism was easily detected in this chromosome region.

(v) *Molecular mapping of Sdt97*

One segregating $F_{6:7}$ progeny, RHL63-146, comprising 257 individuals, was selected for *Sdt97* gene mapping in this study. Six hundred and eighty known SSR markers selected from 12 rice chromosomes with intervals of 2·7 cM were tested in the segregated $F_{6:7}$ populations, using the BSA approach. One SSR marker (RM340), located on the long arm of chromosome 6, showed positive polymorphisms for the mutant gene in the mapping populations. The RCA approach was then employed to determine the linkage relationships between the semi-dwarf mutant gene and marker RM340. A total of 72 extremely semi-dwarf individuals were subjected to linkage analysis and 16 distinct recombinants were identified. Polymorphic markers were confirmed by testing the plants in the populations individually. The results showed that marker RM340 co-segregated with the mutant gene.

To confirm this result, an additional 77 SSR markers located on chromosome 6 were tested. Results showed that markers RM3138, RM5509, RM3430, RM6395, RM5371, RM5314 and RM5957 co-segregated with the semi-dwarf gene locus in the mapping population. Markers RM3430 and RM6395 were closer to the semi-dwarf locus, and the *Sdt97* locus was flanked by RM3430 on the telomeric side and RM6395 on the centromeric side at a distance of 3·6 cM and 2·4 cM, respectively, indicating that the gene involved in the mutation is located on the long arm of chromosome 6.

To find additional markers flanking the *Sdt97* locus, 40 new SSR markers located in the target region were adopted from IRMI and the Rice Genome Sequence Program, Japan (RGP; web site: http://rgp.dna.affrc.go.jp). Among them, the two markers RM20495 and RM20522, where 2 and 11 recombinants from those identified at RM6395 and RM3430 loci, respectively, were detected, indicating that the *Sdt97* locus was further defined by the markers on both sides at 0·4 and 2·6 cM, respectively.

For further fine-mapping of the *Sdt97* locus, 14 PSM markers and 74 STS markers were developed in the smaller region based on the reference sequences of cv. Nipponbare by BIA. Among these new PCR-based markers, two PSM markers (RT1, RT5) and three STS markers (TX5, TX7, N6) showed polymorphism to the parents. The five polymorphic markers were further tested and the two markers RT1 and RT5, where 2 and 5 recombinants from those identified at RM20495 and RM20522 loci, respectively, were detected. The three STS markers, TX5, TX7 and N6, where 4, 4 and 1 recombinants from those identified at RT5 and RT1 loci, respectively, were detected, and no recombinant was detected at the other loci. These revealed that a total of 15 markers (RM340, RM3138, RM5509, RM3430, RM20522, RT5, TX7, TX5, N6, RM20495, RT1, RM6395, RM5371, RM5314 and RM5957) co-segregated with the *Sdt97* locus. The genetic region spanning the *Sdt97* locus between TX5 and N6 was estimated to be 1·0 cM in length (Fig. 5).

A contig map covering the *Sdt97* locus through Pairwise BLAST analysis was constructed. The physical distance between markers N6 and RT5 is 118 kb on the RGP BAC/PAC contig (Fig. 6). Based on the available sequence annotation database (http://www.rgp.dna.affrc.go.jp; http://www.tigr.org), there are 19 predicted genes in the 118 kb target region of the cultivated rice genome. Of these, 10 protein genes, 7 putative genes and 2 hypothetical protein genes were identified (see Supplementary Table 1).

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Identification of the candidate gene of *Sdt97* is still in progress.

4. Discussion

One technique that has been widely used to isolate the genes identified as quantitative trait loci (QTLs) is map-based cloning. To clone genes by this method, investigators adopt a fine-mapping strategy using a series of near-isogenic lines (NILs), introgression lines or chromosome-substitution lines. For the fine-mapping of QTLs, it is necessary to carry out high-resolution linkage analysis using a large number of plants that segregate only around the QTL being investigated. However, The RHL strategy has two main advantages over using an NIL developed by backcrossing. The first is that in developing the population for fine-mapping, only one line from an RIL population need be selected on the basis of its genotype; repeated backcrossings and selections based on DNA markers or phenotypes are not required. The second advantage is that the genomic composition of a RHL can be determined merely by checking the genotype data of the linkage map; it is not necessary to analyse the genotypes of any of the markers (Yamanaka *et al*., 2005). Using an RHL derived from an RIL, Yamanaka *et al*. (2005) succeeded in fine-mapping the soybean flowering-time QTL, and using the RHL population, we succeeded in mapping the semi-dwarf mutant gene *Sdt97* to the long arm of chromosome 6 in rice.

sd-1, termed the 'Green Revolution gene', was first identified in the Chinese variety Dee-geo-woo-gen (DGWG), and developed in the semi-dwarf cultivar IR8, which produced record yields throughout Asia and formed the basis for the development of new high-yielding, semi-dwarf plant types (International Rice Research Institute, 1967). It has contributed substantially to the significant increase in rice production and averted a chronic food shortage, an issue of great concern after the rapid expansion of the world population since the 1960s (Jennings, 1964; Spielmeyer *et al*., 2002).

Since the 1960s, more than 60 dwarf genes have been identified in rice; most of these are recessive genes, and only one dominant gene, *D53*, was reported. These genes are associated with traits such as severe dwarfism, floret sterility, or abnormal plant and grain development; therefore, most of the dwarf mutants identified in rice (*d1* to *d60*) have not been used in crop improvement (Aquino & Jennings, 1966; Kinoshita, 1995). In recent years, new semi-dwarf genes non-allelic to *sd-1* have been identified in rice (Liang *et al*., 1994, 2004; Li *et al*., 2001, 2003; Jiang *et al*., 2002; Zhao *et al*., 2005), but *sd-1* is still the primary semi-dwarf gene used in rice breeding (Kinoshita, 1995). About one-half of the stock from the International Rice Research Institute collection is allelic to Dgwg. In southern China, the semi-dwarf gene found in varieties of economic importance is found at the same locus as *sd-1* (Gu & Zhu, 1979; Xiong *et al*., 1989).

Frequent usage of the *sd-1* gene may reduce genetic diversity and bring about genetic vulnerability to pests and diseases. It is of great significance to develop a new source for broadening the genetic basis of semi-dwarfism. Given that the dwarf or semi-dwarf germ plasm in rice reported previously results mostly from recessive genes, and can not be directly utilized in heterosis, it is obvious that the discovery and utilization of a dominant semi-dwarf gene may be of great significance, not only in genetic theory but also in rice breeding practice (Tong *et al*., 2001, 2003).

The dominant mutant gene *Sdt97* reported here is not allelic to the *sd-1* gene on chromosome 1 (Monna *et al*., 2002; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002), *sd-t* (Li *et al*., 2000; Jiang *et al*., 2002), *sd-t2* (Zhao *et al*., 2005) on chromosome 4, *sd-g* (Liang *et al*.,1994, 2004) and *sd-n* (Li *et al*., 2003) on chromosome 5, or dwarf gene *D53* on chromosome 11 (Wei *et al*., 2006). It is a new kind of semi-dwarf gene reported in rice.

The semi-dwarf mutant was deduced to result from a spontaneous mutation, and possessed a number of desirable characteristics. In conventional rice breeding programmes the semi-dwarf mutant could be used as an elite parent. If used in inter-subspecific heterosis utilization, it could provide a power genetic tool to resolve the problem of excessive height in the inter-subspecific F_1 hybrid. In our research, *Sdt97* and *pms3* have been recombined together, a series of semi-dwarf PSGMS rice has been bred successfully, and they are now available for two-line hybrid rice breeding programmes.

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