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ORIGINAL PAPER

Candidacy of a chitin-inducible gibberellin-responsive gene for a major locus affecting plant height in rice that is closely linked to Green Revolution gene *sd1*

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Abstract Appropriate plant height is crucial for lodging resistance to improve the rice crop yield. The application of *semi-dwarf 1* led to the green revolution in the 1960s, by predominantly increasing the rice yield. However, the frequent use of single *sd1* gene sources may cause genetic vulnerability to pests and diseases. Identifying useful novel semi-dwarf genes is important for the genetic manipulation of plant architecture in practical rice breeding. In this study, introgression lines derived from two parents contrasting in plant height, Zhenshan 97 and Pokkali were employed to locate a gene with a large effect on plant height by the bulk segregant analysis method. A major gene, *ph1*, was mapped to a region closely linked to *sd1* on chromosome 1; the additive effects of *ph1* were more than 50 cm on the plant height and 2 days on the heading date in a BC_4F_2 population and its progeny. *ph1* was then fine mapped to BAC AP003227. Gene annotation indicated that LOC_OS01g65990 encoding a chitin-inducible gibberellin-responsive protein (CIGR), which belongs to the GRAS family, might be the right candidate gene of *ph1*. Co-segregation analysis of the candidate gene-derived marker finally confirmed its identity as the candidate gene. A higher expression level of the CIGR was detected in all the tested tissues in tall plants compared to those of short plants, especially in the young leaf sheath containing elongating tissues, which indicated its importance role in regulating plant height. *ph1* showed a tremendous genetic effect on plant height, which is distinct from *sd1* and could be a new resource for breeding semi-dwarf varieties.

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Introduction

Rice is the main food crop in many parts of the world, but predominantly in Asia and Africa. The rice yield is determined either directly or indirectly by many traits. Among them, plant height plays a pivotal role, and dwarfism is a valuable trait in crop breeding, because it increases lodging resistance and decreases crop damage due to wind and rain, thereby increasing the crop yield. The second half of the 20th century is very prominent in crop science, because of the famous 'green revolution', where the semi-dwarf variety IR8, also known as 'miracle rice', enabled dramatic yield increases and helped to avert predicted food shortages in Asia during that period (Khush 1999). During the same period in wheat, another dominant semi-dwarf cultivar, *Rht*, facilitated a considerable increase in productivity and led to the 'green revolution' in wheat (Evans 1998). The short stature of IR8 is due to a mutation in the plant's *sd1* gene, which was later identified as encoding an oxidase enzyme (*GA20ox-2*) involved in the biosynthesis of gibberellin, a plant growth hormone (Ashikari et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). Gibberellin is also implicated in green revolution varieties of wheat, but the reduced height of those crops is conferred by defects in the hormone's signaling pathway (Peng et al. 1999).

Moreover with the availability of the complete rice sequence and advancement in molecular marker technology, conventional breeding was almost replaced by

molecular breeding (Moose and Mumm 2008). Different types of mapping populations such as double haploids, F_2, F_3, recombinant inbred lines (RILs), backcross inbred lines (BILs) and introgression lines (ILs) were developed by molecular marker technology for quantitative trait locus (QTL) identification (Fan et al. 2006; Nair et al. 1995; Pooni and Jinks 1978; Xing et al. 2001). A limited number of near isogenic lines (NILs), backcross inbred lines (BILs), advanced backcross (AB) lines or introgression lines (ILs) can be used to precisely identify QTLs instead of a large F_2 or recombinant inbred line (RIL) population (Keurentjes et al. 2007; Tanksley and Nelson 1996; Zamir 2001). Since such lines are homozygous, numerous genetically identical plants can be evaluated, thus increasing the accuracy of phenotyping without increasing the efforts of genotyping. Moreover, ILs are effective as a tool for the genetic analysis of QTLs. Any differences between ILs and their parents must be due to a QTL located in the introgressed region. These IL series help the fine mapping of QTLs as well as the precise estimation of the effect of each QTL (Yano and Sasaki 1997). Several research groups have reported QTLs that control plant height in rice (Ishimaru et al. 2001, 2004; Xiao et al. 1996; Zhuang et al. 1997). Among all the genes identified, *sd1* is the dominant one widely used in breeding to develop semi-dwarf varieties.

It was reported that the *sd1* carriers were more susceptible to the bacterial leaf blight (*Xanthomonas oryzae*) than their tall parents (Hu 1973). The frequent use of single *sd1* gene sources may cause genetic vulnerability to pests and diseases (Chang et al. 1985; Reddy et al. 1983; Reddy and Ram Rao 1997). Therefore, it is necessary to develop alternate or new sources of dwarfs for broadening the genetic base of dwarfism. Undoubtedly, the identification of more plant height-related genes/QTLs will provide us with more opportunities to breed diverse semi-dwarf varieties that can resist lodging. In addition, the functional dissection of more gene-regulated plant height traits will be helpful to further understand the molecular mechanism involved in semi-dwarfism. Identifying useful novel semi-dwarf genes is important for the genetic manipulation of plant architecture in practical rice breeding.

In this study, we identified a major QTL on chromosome 1, *ph1*, which controls plant height and heading date from introgression lines derived from two parents contrasting in plant height, Zhenshan 97 and Pokkali. Further, we report here the fine mapping of *ph1* and validation of its candidate gene by comparative sequencing and expression analysis between its parents.

Materials and methods

Plant materials and development of mapping population

A set of introgression lines (BC_4F_2) was developed from a cross between two *Oryza sativa* L. *indica* varieties contrasting in plant height, namely Zhenshan 97, a Chinese native, and Pokkali, an Indian native (Fig. 1a); Zhenshan 97 was used as the recurrent parent. One introgression line (BC_4F_2 family) showed a clear segregation in plant height in 2003. The family, which consisted of 172 plants, and its progeny (BC_4F_3) were grown in summer 2004 and 2005 in a bird-net-equipped experimental farm of Huazhong Agricultural University, Wuhan, China (29°58′N, 113°41′E). Field experiments were carried out in a randomized complete block design.

For the progeny test, 24 plants of each BC_4F_3 family were grown in a two-row plot in Wuhan in 2006. The 20 plants growing in the middle were investigated for plant height and heading date. Field management, including irrigation, fertilizer application and pest control, followed essentially the normal agricultural practices.

Fine mapping population

A large BC_4F_2 population of 6,400 plants was grown for plant height in 2005 in Wuhan. The progenies of 12 recombinants between the gene *Ph1* and two closely linked flanking markers were sowed in summer 2007 in the same field where the progeny of BC_4F_2 population was grown.

DNA extraction and marker selection

DNA was extracted from fresh leaf samples collected at the seedling stage using a modified CTAB protocol (Murray and Thompson 1980). PCR was performed using a hot start Taq polymerase (TaKaRa) under the following conditions: 95°C for 4 min, 35 cycles of 95°C for 40 s, 55–58°C for 40 s and 72°C for 1 min, followed by 72°C for 6 min. SSR markers were used for developing a genetic linkage map. In addition to SSR markers, three newly developed InDel markers for fine mapping are listed in Table S1, according to the publicly available rice genome sequences (http://www.rgp.dna.affrc.go.jp). Markers of the Monsanto rice genome (MRG) series were designed according to the rice genome sequences of the Monsanto Company (McCouch et al. 2002) and those of the rice microsatellite (RM) series according to Temnykh et al. (2000, 2001). The SSR assay was conducted by polyacrylamide gel electrophoresis, as described by Wu and Tanksley (1993).

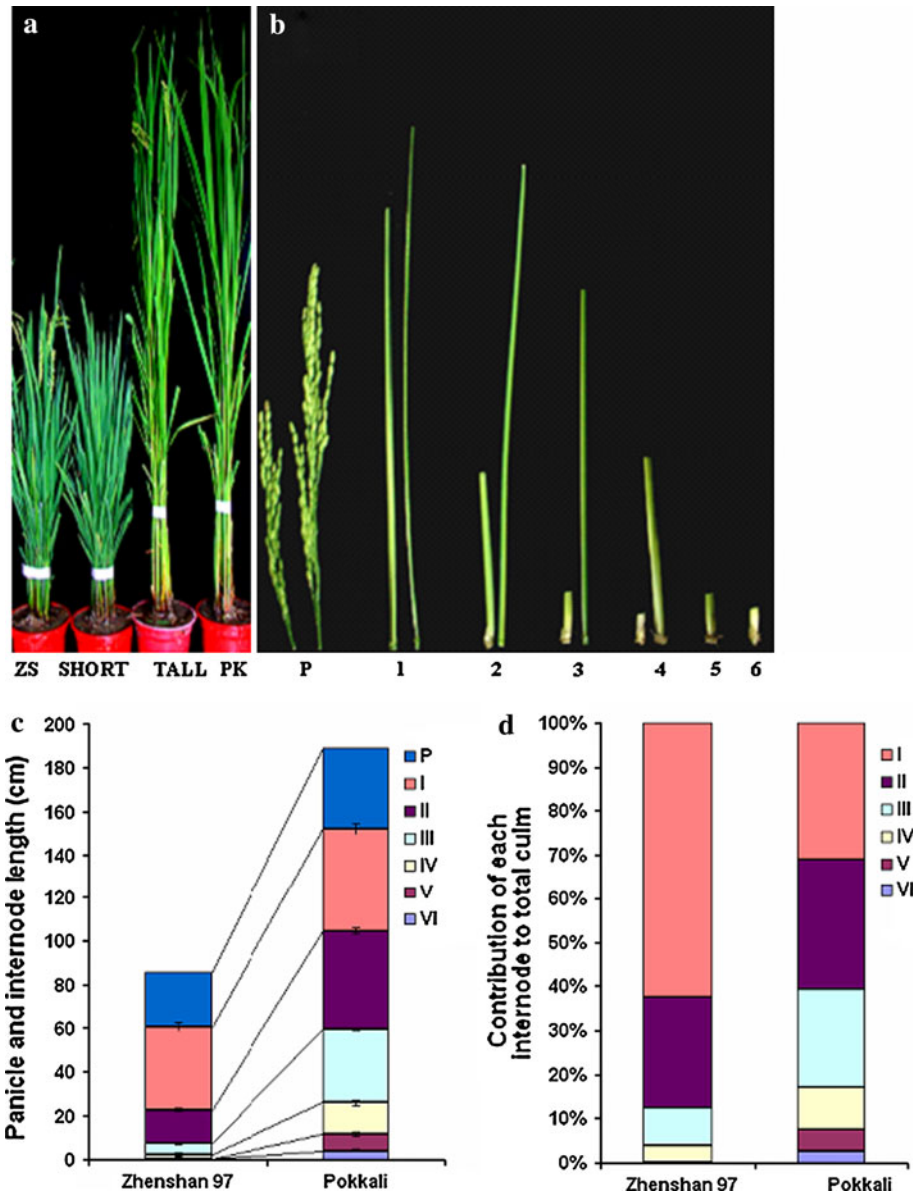

Fig. 1 Morphology and characterization of plants in the BC_4F_2 population. **a** Zhenshan 97 (left) and Pokkali (right). In the middle, the short and tall plants are NILs. **b** Panicles and internodes of the short (left) and tall (right) plants. **c** Comparison of the length of the panicles and internodes of tall (right) and short (left) plants. Data are the averaged lengths of the panicles and internodes of the 14 main culms. In Pokkali, there were no significant differences in first two internodes; *P*, panicle. The number of each internode is labeled (*1*, *2*, *3*, *4*, *5* and *6*). **d** Schematic representation of the internode elongation patterns of the short and tall plants

Plant height and heading date measurement

Plant height was recorded from the field surface to the top of the highest panicle of each plant for BC_4F_2 and its progeny BC_4F_3. At the seed maturation stage, the average panicle length, number of internodes and their lengths were recorded for both Zhenshan 97 and Pokkali. The heading date was recorded as the days from sowing to the first panicle appearance for each plant in BC_4F_3.

Bulk segregant analysis for primary mapping

Five extremely tall individuals with a plant height greater than 200 cm and five extreme short individuals with plant height less than 90 cm were selected from the BC_4F_2 population for developing two DNA bulks. Equal quantities of leaf tissue from each individual were bulked, and DNA was extracted from the bulk. To identify the polymorphic markers, the two parents Zhenshan 97 and Pokkali were genotyped with 440 SSR markers covering 12 rice chromosomes. Markers showing polymorphism in the form of a clearly visible difference in band intensity were selected for genotyping these two tail bulks.

Genetic linkage map and genetic effect analysis

The molecular linkage map was constructed using Mapmaker/EXP 3.0 (Lincoln et al. 1993). The Kosambi function was used to calculate the genetic distance. Interval mapping was conducted using Mapmaker/QTL (Lander

and Botstein 1989). The LOD threshold was of 3.0, determined by 1,000 random permutations at a false-positive rate of 0.05 for the trait.

Sequencing analysis of *sd1* gene and probable candidate gene

Based on the BAC clones AP003561 and AP003227 sequences (http://www.ncbi.nlm.nih.gov), eight and five specific primer pairs were designed to sequence the promoter and coding regions of the *sd1* gene and the probable candidate gene, respectively (Table S1). The two genes were amplified using LA Taq (Takara) from genomic DNA of Zhenshan 97 and Pokkali, and the PCR products were purified. These purified PCR fragments were sequenced using the big dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Data were collected using the ABI Prism 3730 DNA Analyzer (Applied Biosystems) and interpreted using SEQUENCHER 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequence alignment was performed with the BLAST network service (http://www.ncbi.nlm.nih.gov), National Center for Biotechnology Information, (NCBI) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), European bioinformatics institute, EBI).

RNA extraction and quantitative real-time PCR analysis (qRT-PCR)

At the eight-leaf stage, 100 mg of the youngest leaf sheath, which contained young elongating tissues, leaf blade and root were separately pooled from ten plants of Zhenshan 97 and Pokkali. In total, 30 plants of each genotype were sampled as three biological repeats for each tissue. Total RNA was extracted from duplicate biological samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality of the RNA samples was evaluated by absorbance measurements using a Nanodrop Spectrophotometer ND-1000 (Nanodrop Technologies, USA). All the RNA samples used in the qRT-PCR reactions showed a 260/280 nm absorbance ratio of 1.9–2.2. Prior to qRT-PCR, the total RNA samples were pretreated with RNase-free DNase I to eliminate any contaminating genomic DNA.

First-strand cDNAs were synthesized from the DNaseI-treated total RNA samples using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a reaction volume of 40 μl, according to the manufacturer's instructions. qRT-PCR was performed in an optical 96-well plate with an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed for the *sd1* gene and the candidate gene (*CIGR*) (Table S1). Each reaction contained 12.5 μL of SYBR Premix Ex Taq (TAKARA), 0.5 μL of ROX Reference Dye (TAKARA), 5.0 μL of cDNA samples and 10 μM gene-specific primers in a final volume of 25 μL. The rice actin1 gene was used as the endogenous control; it was amplified with the primers 5′-TGGCATCTCTCAGCACATTCC-3′ and 5′-TGCACAATGGATGGGTCAGA-3′. The thermal cycle used was as follows: 95°C for 10 s, 45 cycles of 95°C for 5 s and 60°C for 35 s. All qRT-PCR reactions were carried out in triplicate. The relative expression levels were analyzed as described by Livak and Schmittgen (2001).

Results

Phenotypic variations of plant height in BC_4F_2 population and its progeny

The two parents, Zhenshan 97 and Pokkali, showed highly significant differences in plant height. Zhenshan 97 was of short stature with an average height of 88 cm and ranging from 85.0 to 89.5 cm, while Pokkali was tall with an average height of 196 cm and ranging from 195.0 to 218 cm (Table 1). In the BC_4F_2 population, the difference in average plant height between short and tall plants (carrying homozygous Zhenshan 97 alleles and Pokkali alleles at the targeted gene, respectively) was large, reaching up to

Table 1 Descriptive statistics of plant height in BC_4F_2 and BC_4F_3 populations and parents

Genotype	Number of plants	BC_4F_2		BC_4F_3	
		Range (cm)	Mean (cm)	Range (cm)	Mean (cm)
ZZ	40	70.0–118.6	90.2 ± 15.6	85.2–127.1	113.9 ± 8.5
ZP	88	142.0–203.0	177.9 ± 13.1	151.6–188.6	166.5 ± 6.5
PP	44	161.0–233.0	204.4 ± 14.2	187.3–218.5	203.3 ± 7.3
Zhenshan 97	14	83.0–89.5	88.7 ± 2.2	82.0–88.7	85.5 ± 1.3
Pokkali	14	195.0–218.0	196.2 ± 8.5	191.7–216.6	195.9 ± 7.3

Genotype is inferred by progeny test

ZZ, PP and ZP indicate mean Zhenshan 97 homozygote, Pokkali homozygote and heterozygotes at *Ph1*, respectively

100 cm (Fig. 1a). The major differences were caused by both the length of internodes and the number of internodes (Fig. 1b). The short plants had an average of four elongated internodes, while tall plants had six. All the other internodes, except the first, of the tall plants were significantly longer than the counterparts of the short plants. Meanwhile, the tall plants had a longer panicle compared to the short plants (Fig. 1c). In tall plants, the first and second internodes were of similar length and together contributed approximately 60% to the total culm, while in short plants, the first internode alone contributed more than 60% to the total culm (Fig. 1d).

In BC_4F_2, plant height varied widely and ranged from 70 to 233 cm. Among the 172 BC_4F_2 plants, there were 40 short and 132 tall plants, which was in agreement with the expected segregation ratio (1:3) of a single Mendelian gene ($\chi^2 = 0.22$, $P = 0.639$). Further progeny tests (BC_4F_3) confirmed that 40 and 44 plants were homozygous alleles for Zhenshan 97 and Pokkali at the locus of the target plant height gene, whose progeny expressed identical short and tall plants, respectively. However, the progenies of 88 plants showed varied plant height, indicating that they were heterozygous. The mean heights for short, heterozygote and tall plants were 90.2, 177.9 and 204.4 cm, respectively (Fig. 2). The frequencies of the three genotypes matched the expected Mendelian ratio (1:2:1) for single locus segregation ($\chi^2 = 0.28$, $P = 0.869$). This analysis suggested that one gene (termed *ph1*) controlled the variation of plant height in the BC_4F_2 population.

Primary mapping of *ph1*

Bulk segregant analysis was used to detect the major plant height gene. A total of 150 polymorphic simple sequence repeat (SSR) markers distributed evenly on all the 12 chromosomes between the parents were selected and screened for two bulks, the tall bulk and the short bulk. Among the 150 SSR markers, there was no polymorphism between the two bulks at the loci of 143 markers; the bulks were identical to Zhenshan 97 genotype at 135 marker loci, while the bulks were heterozygotes at eight markers. Polymorphisms were identified between the tall and short bulks at the loci of seven markers (RM3304, RM3825, RM6439, RM472, RM5382, RM1339 and RM1387) located on chromosome 1 (Figure S1). Consequently, these seven markers were regarded as being linked to the plant height gene (termed *ph1*) and were used to genotype the 172 BC_4F_2 plants. A local linkage map covering 35.8 cM was constructed (Fig. 3a). The individual BC_4F_2 genotypes at *ph1* were determined by progeny (BC_4F_3) tests. Then, *ph1* treated as a marker was directly localized into the linkage group, where it was located in a 0.6-cM region flanked by markers RM5382 and RM1339 and co-segregated with RM1339 (Fig. 3a). It is closely linked to *sd1*.

The interval mapping method was conducted to estimate its genetic effects by using the phenotypic data from BC_4F_2 and its progeny. A major plant height QTL (*ph1*) was detected in the 0.6-cM region between the markers, RM5382 and RM1339 (Fig. 4a). *ph1* explained 90.3% of the phenotypic variance with additive and dominant effects of 57.1 and 30.6 cm, respectively. In the progeny test (BC_4F_3), *ph1* explained 92.7% of plant height variation, but the additive effect was less than that of the F_2 population. *ph1* acted as partially dominant in both generations; the Pokkali allele increased plant height (Table 2). Variations in heading date were also observed in the BC_4F_3 population (Figure S2). Tall plants always flowered 3–4 days earlier than short ones, indicating the pleiotropic effect of *ph1* on heading date. One QTL for heading date was also detected in the same interval where *ph1* was located. This QTL could explain 31.2% of the total phenotypic variance with LOD scores of 13.7. The additive effect was 1.5 days. Pokkali allele promoted heading date (Table 2).

Fine mapping of *ph1*

The revolutionary gene *sd1* is located on the right side of the marker RM1339. RM1339 is more than 516.13 kb from *sd1*. Therefore, to provide evidence that the large effect of

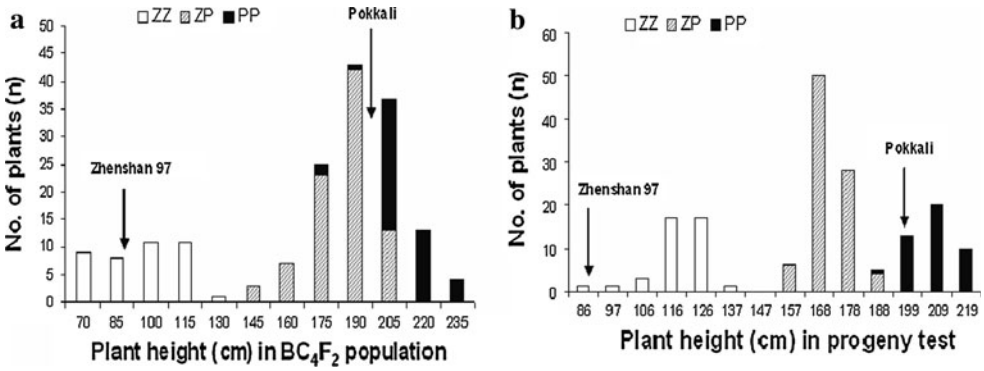

Fig. 2 Frequency distribution of plant height in the BC_4F_2 and BC_4F_3 populations. ZZ, PP and ZP refer to Zhenshan 97 homozygote, Pokkali homozygote and heterozygotes, respectively

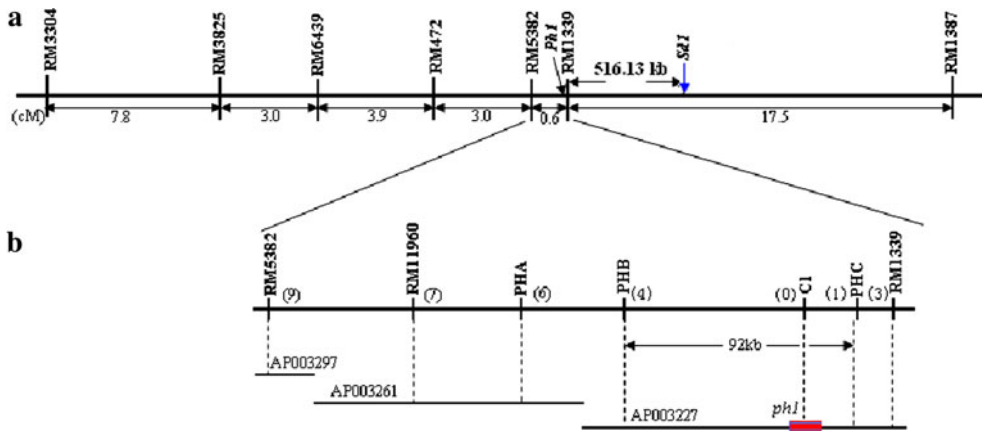

Fig. 3 Genetic and physical maps of the *ph1* gene. **a** Linkage map of chromosome 1 constructed using 172 BC_4F_2 individuals by bulk segregant analysis. The *ph1* gene was mapped to the region between markers, RM5382 and RM1339. The *numbers* show the genetic distance between the adjacent markers. The *arrow* under *sd1* represents the position of *sd1* on the map, which is 516.13 kb (http://www.gramene.org) from the nearest marker RM1339. **b** Fine mapping of the *ph1* gene. The *ph1* gene was narrowed down to a 92-kb region in the BAC contig AP003227 between markers PHB and PHC. The number of recombinants between the markers and *ph1* is indicated under the linkage map. The *thick bar* represents the candidate gene LOC_Os01g75900 encoding a gibberellin-responsive protein, which might be the right candidate and which segregated with the *ph1* specific marker (*C1*)

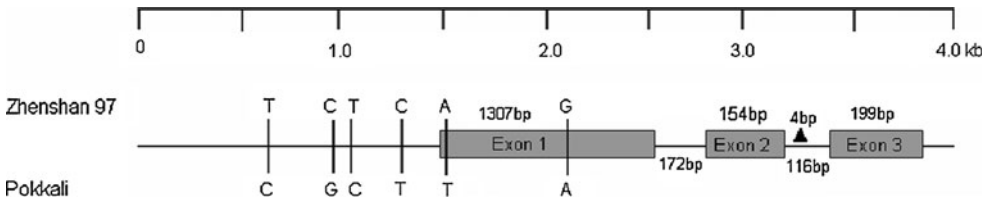

Fig. 4 Polymorphism of the *CIGR* between Zhenshan 97and Pokkali. The *vertical bars* represent SNPs. The *numbers* indicate the lengths of each exon and intron region. In Zhenshan 97, two SNPs were found at 1,151 and 1,377 bp in the promoter region, and one SNP was located 650 bp away from ATG in exon 1. In Pokkali, two SNPs were found at 706 and 1,048 bp in the promoter region, and one SNP was located in exon 1 at 13 bp away from ATG. The *triangle* in the intron region indicates a 4-bp deletion in Zhenshan 97

Table 2 Genetic effects of *Ph1* estimated in BC_4F_2 and its progeny BC_4F_3 population

Population		Flanking makers	LOD	Add[a] (cm)	D[b] (cm)	Var[c] (%)
BC_4F_2	*Ph1*	MRG4382-MRG0339	87.3	57.1	30.6	90.3
BC_4F_3	*Ph1*	MRG4382-MRG0339	106.2	51.0	26.6	92.7

Positive additive effect indicates Pokkali allele increased the plant height

[a] Additive effect

[b] Dominant effect

[c] Variance explained by QTL

ph1 on plant height is not the allelism of *sd1*, we performed sequencing analysis of Zhenshan 97 and Pokkali with *sd1*. The sequencing analysis of *sd1* showed no sequence difference in the three exons and two introns (Figure S3). However, we identified one SNP in the promoter region of *sd1*. To further investigate the role of *sd1*, we performed expression analysis. The quantitative real-time polymerase chain reaction (qRT-PCR) results showed that there was no difference in the expression levels of *sd1* in root, leaf sheath and leaf blade tissues in both Zhenshan 97 and Pokkali (Figure S4). Thus, clear molecular-level evidence determined that *ph1* was distinct from *sd1*.

It is worth isolating *ph1* because it is a novel plant height gene. In total, 1,250 extremely short plants with a plant height of less than 100 cm, which were assumed to be Zhenshan 97 homozygotes at *Ph1*, were selected from 6,400 plants of the BC_4F_2 population to screen recombinants between *Ph1* and markers RM5382 and RM1339.

Nine and three recombinants were identified between RM5382 and *ph1*, and RM1339 and *ph1*, respectively. In case the trait measurement yielded any false positives in the Zhenshan 97 homozygous plants, progeny tests of the 12 recombinants between RM5382 and RM1339 were conducted. Each recombinant progeny showed an identical short plant height, which was highly significantly shorter than the control Pokkali homozygotes, but showed no difference with the control Zhenshan 97 homozygotes (Table 3). This result confirmed the homozygous identity of the 12 Zhenshan 97 recombinants at *ph1*. To enhance the resolution of the *ph1* local linkage map, we used one SSR (RM11960) and four InDel markers (Table S1) to screen the 12 recombinants; 7 recombinants were identified between RM11960 and *ph1*, 6 between PHA and *ph1*, 4 between PHB and *ph1* and 1 between PHC and *ph1* (Fig. 3b). Thus, *ph1* was narrowed down to the region of approximately 92 kb bounded by markers PHB and PHC (Fig. 3b). One Nipponbare BAC (AP003227) spanned the region exactly.

The candidate gene in the 92-kb target region

There are 17 predicted genes in the 92-kb region according to the rice genome automated annotation database (http://www.rice.plantbiology.msu.edu/) (Table S2). Of these, 12 genes have homology with rice full-length cDNAs. Among these 17 genes, 5 are of unknown function; the functional annotations of the 12 remaining genes are given in Table S2. Gibberellin-responsive genes were reported to be associated with plant height (Itoh et al. 2001; Kobayashi et al. 1989; Murakami 1972; Sasaki et al. 2002; Sakamoto et al. 2004). Among all the 12 putative genes, LOC_Os01g65900, which encodes a chitin-induced gibberellin-responsive protein (CIGR, (http://www.rice.plantbiology.msu.edu/), was gibberellin responsive; thus, *CIGR* was regarded as the *ph1* candidate.

Sequencing analysis of the 3,640-bp *CIGR* gene showed single-base substitutions in the promoter region and exon 1 of both Zhenshan 97 and Pokkali, and also detected a 4-bp deletion in the intron region of Zhenshan 97 (Fig. 4). Between the parents, four SNPs were found at 706, 1,048, 1,151 and 1,377 bp upstream of ATG, and two SNPs were located in exon 1. *CIGR* contains three exons; it encodes a predicted protein with 553 amino acids, but modifications in two amino acids were observed between the parents (Figure S6).

Co-segregation of the candidate

To confirm whether the candidate gene (*CIGR*) co-segregated with plant height, we developed a gene-specific marker (C1) covering the SNP region in the first exon of *ph1* and screened all five recombinants (32, 58, 67, 83 and 121). The genotype data showed that all the five recombinants are homozygous to Zhenashan 97 at C1 (Figure S5) and confirmed that *CIGR* co-segregated with plant height. Thus, *CIGR* might be the real candidate for *ph1*. In addition, our qRT-PCR results showed that Pokkali had significantly higher expression levels of *CIGR* in root, leaf sheath and leaf blade tissues than Zhenshan 97 (Fig. 5).

Discussion

Comparison of the genetic effects of *ph1* to *sd1*

In this study, it was clearly confirmed that *ph1* is distinct from *sd1* by fine mapping with a large BC_4F_2 population.

Table 3 Plant height performance in the progenies of recombinants

Recombinants	Plant height (cm)		Marker				
	F_2	Progenies	RM11960	PHA	RM11961	PHB	PHC
32	82.9	82.5 ± 2.7[c]	PZ	PZ	PZ	PZ	ZZ
58	81.7	82.1 ± 2.1[c]	PZ	PZ	PZ	PZ	ZZ
67	84.3	84.1 ± 2.3[c]	PZ	PZ	PZ	PZ	ZZ
83	84.8	83.4 ± 2.1[c]	PZ	PZ	PZ	PZ	ZZ
121	83.1	82.1 ± 2.4[c]	ZZ	ZZ	ZZ	ZZ	PZ
CK-Z[a]	85.5	84.8 ± 2.2	ZZ	ZZ	ZZ	ZZ	ZZ
CK-P[b]	139.2	135.4 ± 4.2[d]	PP	PP	PP	PP	PP

ZZ, PZ and PP indicate Zhenshan 97 homozygotes, heterozygotes and Pokkali homozygotes at the locus of the marker, respectively

[a] The control near isogenic line with Zhenshan 97 allele of *Ph1*

[b] The control near isogenic line with Pokkali allele of *Ph1*

[c] No significant difference between the recombinant and CK-Z at $P = 0.05$

[d] Highly significant difference between the two controls CK-Z and CK-P, and between CK-P and recombinants at $P = 0.001$

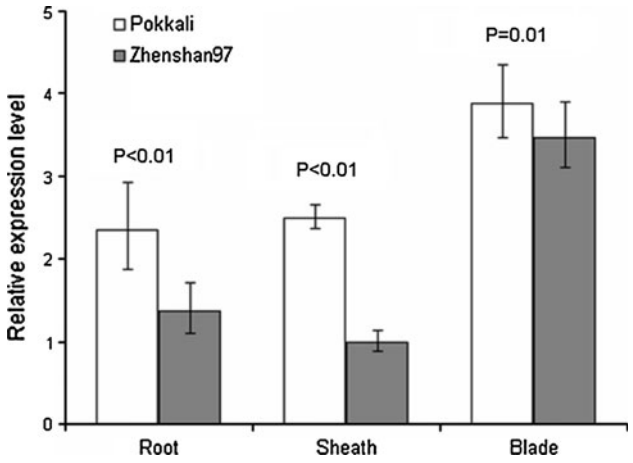

Fig. 5 Expression analysis of the candidate gene *CIGR* in different tissues of Zhenshan 97 and Pokkali. The qRT-PCR results showed significant difference of *CIGR* expression levels in root and leaf sheath tissues compared to leaf blade in Zhenshan 97 and Pokkali. The ratio of the transcript level relative to actin is defined as "relative expression". *Error bars* represents the standard deviation of relative expression level

Comparative sequencing between the parents further confirmed that *sd1* did not contribute to plant height variation. We detected no difference in the expression level of *sd1* between ZS97 and Pokkali, and this was in agreement with the result that *ph1* was a novel gene distinct from *sd1*. Although *ph1* was not the allele of *sd1*, it showed similar genetic characters on plant height but with larger effects. *ph1* had a large genetic effect; two homozygotes of *ph1* showed a big difference of 110 cm in plant height.

The variation in the final plant height in the BC_4F_2 population between tall and short plants is mainly contributed by the difference in the length of the panicle, and the second, third and fourth uppermost stem internodes. In particular, the second and third showed differences of about 25 cm, but the uppermost stem length showed no significant difference (Fig. 1b), while variation in the final plant height by *sd1* results from differences in stem length of the first (subtending panicle) and second (subtending flag leaf) stem internodes (Shi and Shen 1996; Spielmeyer et al. 2002). Meanwhile, the average number of elongated internodes of tall and short lines is different (Fig. 1c). That is to say, *ph1* mainly controls the number of internodes and the length of uppermost internodes except the first one. In addition, *ph1* has a pleiotropic effect on the heading date. Pokkali alleles increase the plant height and decrease the days to heading, which is in agreement with their negative correlation ($r = -0.55$, $P < 0.01$). Taken together, these results confirm that *ph1* is a novel gene based on phenotypic variation.

Chitin-inducible gibberellin-responsive gene might be the probable candidate gene

First, we fine mapped *ph1* to the BAC clone AP003227 (Fig. 3b) containing 12 putative known functional genes. Then, with the help of bioinformatics analyses, we inferred the *CIGR* gene (LOC_OS01g65900) encoding a chitin-induced gibberellins-responsive protein as the candidate of *ph1*. Finally, the identity of the candidate gene was validated by co-segregation analysis.

It is a well-known fact that gibberellin plays a key role in the plant height. Many important genes like *sd1* and *d18* are influenced by gibberellins (Itoh et al. 2002; Spielmeyer et al. 2002). *CIGR* genes are gibberellin-response genes and were reported to play key roles in defense and plant development (Bolle 2004; Day et al. 2004; Itoh et al. 2005; Richards et al. 2000; Tian et al. 2004). Moreover, the *CIGR* gene belongs to the GRAS family. Comparative analysis showed important genes like *SCARECROW LIKE 1* (*SCL1*) in *Arabidopsis*, *Dwarf8* in maize and *MOC1* (*MONOCULM 1*) in rice belong to this family and are related to plant growth and development. *CIGR* is 85% similar to *SCL* in *Aeluropus littoralis* and 77% to the *SCL* in *Arabidopsis*. Recently, Wang et al. (2010) reported that *SCL6* is involved in shoot branching and plant height by testing double and triple mutants of *SCL6-III*, *SCL6-IV* and *SCL6-V* in Arabidopsis.

In addition, it was reported that the induction of *CIGR1* and *CIGR2* both located on chromosome 7 was observed only upon application of phytoactive GA, and the accumulation of mRNAs for *CIGR1* and *CIGR2* is correlated with the bioactivities of GA in suspension-cultured cells of rice and also in the leaves (Day et al. 2004). CIGR genes may be involved in developmental GA-mediated events in rice. *CIGR1* and *CIGR2* are excellent markers of GA signal transduction (Day et al. 2004). In fact, high levels of *CIGR1* and *CIGR2* mRNAs accompany a high concentration of phytoactive GA, which promotes plant height. In this study, different expression levels of the *CIGR* (LOC_OS01g65900) between Zhenshan 97 and Pokkali were detected in all the three tested tissues. The difference was the smallest in leaf blade and largest in young leaf sheath containing young elongating tissues; this finding suggests that tall plants contain a higher concentration of phytoactive GA that resulted in increased plant height. Transformation of the candidate gene will ultimately reveal its biological functions on plant height.

Plant height gene cluster on chromosome 1

In rice, disease-resistant (*R*) gene clusters contributing significantly to plant defense were observed and studied extensively to elucidate their mechanisms (Michelmore

and Meyers 1998; Richter and Ronald 2000). The characteristic clustering of *R* genes has been proposed to facilitate the evolution of novel resistance specificities via recombination or gene conversion (Hulbert 1997). Therefore, the organization of functionally related genes in clusters is expected to have an evolutionary advantage to the organism. Besides, many genes controlling the same agronomic traits were located in a very small region, making it difficult to determine whether they are one or two genes in a primary mapping population. For example, *Gn1*, which is regarded as one gene controlling rice grains per panicle, is located on the nearest marker BB-85. However, it was dissected into the two closely linked genes, *Gn1a* and *Gn1b*, which both control the trait in advanced populations (Ashikari et al. 2005). Later, *SPP1*, which controls the number of spikelets per panicle, was also detected in the nearby region (Liu et al. 2009). A similar situation was observed for the genes controlling plant height. *qCL1* is located 1.4 and 2.6 cM away from two plant height genes, *d18* and *d2*, which are located at 1.2 cM distance on chromosome 1 (Hori et al. 2009). *QTLph1* and *sd1* are linked on chromosome 1 with a 1.7-Mb physical distance (Ishimaru et al. 2004; Monna et al. 2002). The important plant height genes, *sd1*, *QTLph1*, *d2*, *d18*, *qCL1* and *ph1*, from this study are all located on chromosome 1. Many of these genes are located at the distal end, indicating a hot spot region for plant height genes. However, the mechanism or functions of plant growth or development gene clusters are not clear. Activating these clusters will uncap a vast resource of novel enzymes, compounds, pathways and diverse chemistries, which can be exploited for a wide range of applications in plant height adaptation.

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References

Ashikari M, Sasaki A, Ueguchi-Tanaka M, Itoh H, Nishimura A et al (2002) Loss-of-function of a rice gibberellin biosynthetic gene, *GA20 oxidase* (*GA20ox-2*), led to the rice green revolution. Breed Sci 52:143–150

Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T et al (2005) Cytokinin oxidase regulates rice grain production. Science 309:741–745

Bolle C (2004) The role of GRAS proteins in plant signal transduction and development. Planta 218(5):683–692

Chang TT, Zuno C, Marciano Ramena A, Loresto GC (1985) Semidwarfs in rice germplasm collections and their potentials in rice improvement. Phytobreedon 1(1):1–9

Day RB, Tanabe S, Koshioka M, Mitsui T, Itoh H et al (2004) Two rice GRAS family genes responsive to *N*-acetylchitooligosaccharide elicitor are induced by phytoactive gibberellins: evidence for cross-talk between elicitor and gibberellin signaling in rice cells. Plant Mol Biol 54(2):261–272

Evans LT (1998) Feeding the ten billion. Plant and population growth. Cambridge University, Cambridge

Fan CC, Xing YZ, Mao HL, Lu TT, Han B et al (2006) *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112:1164–1171

Hori K, Yamamoto T, Ebana K, Takeuchi Y, Yano M (2009) A novel quantitative trait locus, qCL1, involved in semi-dwarfism derived from Japanese rice cultivar Nipponbare. Breed Sci 59:285–295

Hu CH (1973) Evaluation of breeding semidwarf rice by induced mutation and hybridization. Euphytica 22:562–574

Hulbert SH (1997) Structure and evolution of the *Rp1* complex conferring rust resistance in maize. Annu Rev Phytopathol 35:293–310

Ishimaru K, Yano M, Aoki N, Ono K, Hirose T et al (2001) Toward the mapping of physiological and agronomic characters on a rice function map: QTL analysis and comparison between QTLs and expressed sequence tags. Theor Appl Genet 102:793–800

Ishimaru K, Ono K, Kashiwagi T (2004) Identification of a new gene controlling plant height in rice using the candidate-gene strategy. Planta 218:388–395

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M et al (2001) Cloning and functional analysis of gibberellin 3b-hydroxylase genes that are differently expressed during the growth of rice. Proc Natl Acad Sci USA 98:8909–8914

Itoh H, Ueguchi-Tanaka M, Sakamoto T, Kayano T, Tanaka H et al (2002) Modification of rice plant height by suppressing the height-controlling gene, D18, in rice. Breed Sci 52:215–218

Itoh H, Shimada A, Ueguchi-Tanaka M, Kamiya N, Hasegawa Y et al (2005) Overexpression of a GRAS protein lacking the DELLA domain confers altered gibberellin responses in rice. Plant J 44(4):669–679

Keurentjes JJB, Bentsink L, Alonso-Blanco C, Hanhart CJ, Blankestijn-De Vries H et al (2007) Development of a near-isogenic line population of *Arabidopsis thaliana* and comparison of mapping power with a recombinant inbred line population. Genetics 175:891–905

Khush GS (1999) Green revolution: preparing for the 21st century. Genome 42:646–655

Kobayashi M, Sakurai A, Saka H, Takahashi N (1989) Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. Plant Cell Physiol 30:963–969

Lander E, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199

Lincoln S, Daly M, Lander E (1993) Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1: a tutorial and reference manual, 2nd edn. Whitehead Institute Tech Rep, Cambridge, Massachusetts

Liu T, Mao DH, Zhang YS, Xu CG, Xing YZ (2009) Fine mapping *SPP1*, a QTL controlling the number of spikelets per panicle, to a BAC clone in rice (*Oryza sativa*). Theor Appl Genet 118:1509–1517

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{[-\Delta\Delta CT\ C\ (T)]}$ method. Methods 25(4):402–408

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K et al (2002) Development and mapping of 2,240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9:199–207

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Monna L, Kitazawa N, Yoshino R, Suzuki J, Masuda H et al (2002) Positional cloning of rice semidwarfing gene, *sd-1*: rice "green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. DNA Res 9:11–17

Moose SP, Mumm RH (2008) Molecular plant breeding as the foundation for 21st century crop improvement. Plant Phys 147:969–977

Murakami Y (1972) Dwarfing genes in rice and their relation to gibberellin biosynthesis. In: Carr DJ (ed) Plant growth substances. Springer, Berlin, pp 166–174

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325

Nair S, Rao UP, Bennett J, Mohan M (1995) Detection of a highly heterozygous locus in recombinant inbred lines of rice and its possible involvement in heterosis. Theor Appl Genet 91:978–986

Peng J, Richards DE, Hartley N, Murphy GP, Devos KM et al (1999) "Green Revolution" genes encode mutant gibberellin response modulators. Nature 400:256–261

Pooni HS, Jinks JL (1978) Predicting the properties of recombinant inbred lines derived by single seed descent for two or more characters simultaneously. Heredity 40:349–361

Reddy TP, Ram Rao DM (1997) Genetic analysis of semidwarf mutants induced in indica rice (*Oryza sativa* L.). Euphytica 95:45–48

Reddy TP, Vaidyanath K, Reddy GM (1983) Investigation on semidwarf mutants in Indica rice. In: First research coordination meeting on semidwarf mutants for rice improvement in Asia and the Pacific, Los Banos. IRRI, Phillippines, pp 24–28

Richards DE, Peng J, Harberd NP (2000) Plant GRAS and metazoan STATs: one family? Bioessays 22(6):573–577

Richter TE, Ronald PC (2000) The evolution of disease resistance genes. Plant Mol Biol 42:195–204

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M et al (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol 134:1642–1653

Sasaki A, Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A et al (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. Nature 416(6882):701–702

Shi CH, Shen ZT (1996) Effects of semidwarf gene *sd1* on agronomic traits in rice (*Oryza sativa* subsp. *indica*). Chin J Rice Sci 10(1):13–18 (In Chinese with English abstract)

Spielmeyer W, Ellis MH, Chandler PM (2002) Semidwarf (*sd-1*), green revolution rice, contains a defective *gibberellin 20-oxidase* gene. Proc Nat Acad Sci USA 99(13):9043–9048

Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203

Temnykh S, Park WD, Ayres N, Cartihour S, Hauck N et al (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100:697–712

Temnykh S, Declerck G, Luashova A, Lipovich L, Cartinhour S et al (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Res 11:1441–1452

Tian C, Wan P, Sun S, Li J, Chen M (2004) Genome-wide analysis of the GRAS gene family in rice and Arabidopsis. Plant Mol Biol 54(4):519–532

Wang L, Mai XY, Zhang YC, Luo Q, Yang HQ (2010) MicroRNA171c-targeted *SCL6-II*, *SCL6-III*, and *SCL6-IV* genes regulate shoot branching in *Arabidopsis*. Mol Plant 3(5):794–806

Wu KS, Tanksley SD (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241:225–235

Xiao J, Li J, Yuan L, Tanksley SD (1996) Identification of QTLs affecting traits of agronomic importance in a recombinant inbred population derived from a subspecific rice cross. Theor Appl Genet 92:230–244

Xing YZ, Tan YF, Xu CG, Hua JP, Sun XL (2001) Mapping quantitative trait loci for grain appearance traits of rice using a recombinant inbred line population. Acta Bot Sin 43(8):840–845

Yano M, Sasaki T (1997) Genetic and molecular dissection of quantitative traits in rice. Plant Mol Biol 35:145–153

Zamir D (2001) Improving plant breeding with exotic genetic libraries. Nat Rev Genet 2:983–989

Zhuang JY, Lin HX, Lu J, Qian HR, Hittalmani S et al (1997) Analysis of QTL environment interaction for yield components and plant height in rice. Theor Appl Genet 95:799–808