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ORIGINAL ARTICLE

Identification and characterization of *dwarf 62*, a loss-of-function mutation in *DLT/OsGRAS-32* affecting gibberellin metabolism in rice

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Abstract A dwarf mutant, *dwarf 62* (*d62*), was isolated from rice cultivar 93-11 by mutagenesis with γ-rays. Under normal growth conditions, the mutant had multiple abnormal phenotypes, such as dwarfism, wide and dark-green leaf blades, reduced tiller numbers, late and asynchronous heading, short roots, partial male sterility, etc. Genetic analysis indicated that the abnormal phenotypes were controlled by the recessive mutation of a single nuclear gene. Using molecular markers, the *D62* gene was fine mapped in 131-kb region at the short arm of chromosome 6. Positional cloning of *D62* gene revealed that it was the same locus as *DLT/OsGRAS-32*, which encodes a member of the GRAS family. In previous studies, the *DLT/OsGRAS-32* is confirmed to play positive roles in brassinosteroid (BR) signaling. Sequence analysis showed that the *d62* carried a 2-bp deletion in ORF region of *D62* gene which led to a loss-of-function mutation. The function of *D62* gene was confirmed by complementation experiment. RT-PCR analysis and promoter activity analysis showed that the *D62* gene expressed in all tested tissues including roots, stems, leaves and panicles of rice plant. The *d62* mutant exhibited decreased activity of α-amylase in endosperm and reduced content of endogenous GA_1. The expression levels of gibberellin (GA) biosynthetic genes including *OsCPS1*, *OsKS1*, *OsKO1*, *OsKAO*, *OsGA20ox2/SD1* and *OsGA2ox3* were significantly increased in *d62* mutant. Briefly, these results demonstrated that the *D62* (*DLT/OsGRAS-32*) not only participated in the regulation of BR signaling, but also influenced GA metabolism in rice.

Keywords *DLT/OsGRAS-32* · *Dwarf 62* (*d62*) · Gibberellin (GA) · Positional cloning · Rice (*Oryza sativa* L.)

Abbreviations

bp	Base pair
BR	Brassinosteroid
GA	Gibberellin
GRAS	GAI-RGA-SCR
GUS	β-Glucuronidase
InDel	Insertion/Deletion
ORF	Open reading frame
SSR	Simple sequence repeat
UTR	Untranslated region

Introduction

Dwarfism is one of the most important agronomic traits in crop breeding programs because dwarf cultivars are more resistant to lodging. During the famous "green revolution", dwarfism was adopted to improve crop architecture which significantly increased the grain yield of cereal crops in the world (Sasaki et al. 2002; Hedden 2003). In rice, more than 60 dwarf mutants have been reported so far and several of them have been characterized thoroughly (http://www.gramene.org/rice_mutant/).

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There are various factors responsible for dwarfism in plants, but gibberellin (GA) and brassinosteroid (BR) are the most intensely studied factors in determining plant height (Fujioka and Yokota 2003; Yamaguchi 2008). The GAs which control diverse biological processes including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation, induction of flowering, fruit development, etc., are considered the most important bioactive growth regulators in plants (Olszewski et al. 2002). In recent years, numerous GA-related genes have been isolated from various plants (Hedden and Phillips 2000; Sun and Gubler 2004; Itoh et al. 2008) and the "green revolution" genes, such as the wheat *Rht* and rice *sd1*, are the most notable examples (Peng et al. 1999; Sasaki et al. 2002).

GA-related mutants are categorized into GA-deficient mutants and GA-insensitive mutants according to their responses to exogenous GAs (Mitsunaga et al. 1994). In rice, GA-deficient or -insensitive dwarf mutants display typical dwarfism with wide leaf blades and dark-green leaves (Ueguchi-Tanaka et al. 2000, 2005; Itoh et al. 2001; Sasaki et al. 2003). In GA-deficient dwarfs, the mutations are usually ascribed to the deficiency in GA metabolic pathway, where the GA metabolism is blocked or weakened (Hedden and Phillips 2000). In recent years, most of the genes encoding enzymes of GA metabolism have been identified in *Arabidopsis*, rice and other model plants (Sakamoto et al. 2004; Grennan 2006), and the complex pathways of GA metabolism have been elucidated in higher plants (Hedden and Phillips 2000; Sun and Gubler 2004; Yamaguchi 2008). In GA-insensitive dwarfs, the mutants are deficient in GA signaling and exhibit altered GA responses (Gomi and Matsuoka 2003). Also, there are slender-type mutants, such as *slender rice* (Ikeda et al. 2001) and *slender barley* (Lanahan and Ho 1988), which exhibit constitutive activation of GA responses. In recent years, the power of molecular genetics has dramatically facilitated our understanding of all aspects of GA signaling, especially the principal steps associated with GA perception and signal transduction in *Arabidopsis* and rice (Ueguchi-Tanaka et al. 2007).

The identification and characterization of a dwarf mutant, designated as *dwarf 62* (*d62*), are reported in the present study. The *D62* gene was isolated via positional cloning approach and was shown to be the same locus as *DLT/OsGRAS-32*. In previous studies, the *DLT/OsGRAS-32* has been confirmed to play positive roles in BR signaling (Tong et al. 2009). However, the *d62* mutant exhibited decreased activity of α-amylase in endosperm and reduced content of endogenous GA_1. The expression levels of GA biosynthetic genes including *OsCPS1*, *OsKS1*, *OsKO1*, *OsKAO*, *OsGA20ox2/SD1* and *OsGA2ox3* were up-regulated in the *d62* mutant. These results demonstrated that the *D62* (*DLT/OsGRAS-32*) also influenced GA metabolism in rice.

Materials and methods

Plant materials

The *d62* mutant was isolated from the M_2 population of rice cultivar 93-11 (*Oryza sativa* L. ssp. *indica*; supplied by the Beijing Genomics Institute, Beijing, China) by mutagenesis with γ-rays. The mapping population consisted of field grown F_2 individuals derived from a cross between *d62* mutant and normal rice cultivar 02428 (*Oryza sativa* L. ssp. *japonica*; supplied by the Jiangsu Academy of Agricultural Sciences, Jiangsu Province, China). A total of 546 mutant individuals were identified from the F_2 mapping population. In addition, eight F_1 hybrids from the cross between *d62* mutant and 93-11 were obtained. Cultivar 93-11 was used as the wild-type line in the present study. All plants were grown in the paddy field of Zhejiang University.

GA-induction in shoot elongation

Elongation of shoots was measured according to the method of Ueguchi-Tanaka et al. (2000) with minor modifications. Rice seeds were divested of glumes and surface sterilized, washed three times with sterile distilled water, then imbibed at 30°C for 24 h. The seeds were placed on 1/2 MS medium containing various concentrations of GA_3 and grown at 30°C under a 16-h light/8-h dark period. After an interval of 7 days, the length of second leaf sheath and the seedling height were measured.

Assay of α-amylase activity

The α-amylase assay was performed as described by Lanahan and Ho (1988). Seeds were cut transversely, and the half-seeds containing embryos were grown to identify their phenotype. The embryoless half-seeds were sterilized with 2% NaClO for 15 min and washed with sterile distilled water five times. These half-seeds were then placed on 2% agar plates containing 0.2% soluble potato starch, 10 mM sodium acetate and 2 mM $CaCl_2$ at pH 5.3. GA plates were made by adding 1 μM GA_3 to the cooled agar after autoclaving. Agar plates were incubated in the dark for 3 days at 30°C and then developed by flooding the plates with a solution of I_2–KI (0.72 g/L I_2 + 7.2 g/L KI) in 0.2 N HCl. Half-seeds which synthesized and secreted α-amylase had transparent halos around the seed resulting from the digestion of the starch by α-amylase.

GAs purification and ELISA analysis

The procedure used to purify GAs from rice plants was as described previously (Yang et al. 2001; Zhu et al. 2005). Leaf tissues from each plant were homogenized in liquid nitrogen and then extracted in 4 mL of 80% (v/v) ice-cold aqueous methanol containing butylated hydroxytoluene (1 mM) and polyvinylpyrrolidone (60 mg/g fresh weight). The samples were incubated overnight at 4°C and centrifuged at 10,000*g* for 10 min. The resulting supernatants were collected individually and filtered through C_{18} Sep-Pak cartridges (Waters, Milford, MA, USA). Efflux of each sample was collected, dried by evaporation with N_2 and measured for GA_1 content with enzyme-linked immunosorbent assay (ELISA) kit according to the protocols recommended by the manufacturer.

Mapping and cloning of the *D62* gene

Rice genomic DNA was extracted from fresh leaves of rice plants according to the method of Murray and Thompson (1980) with minor modifications. The *D62* gene was mapped primarily with SSR markers (http://www.gramene.org/), by using 136 mutant individuals of F_2 mapping population. To fine map the *D62* gene, an InDel marker M14 (forward: 5′-CTCAGGAGCAAGAAGAGGAATA-3′; reverse: 5′-CACTAATGTTGTAGCAAACTGAT-3′) was newly developed based on the sequence difference between Nipponbare (spp. *japonica*) and 93-11 (spp. *indica*). A SSR marker MM0124 (forward: 5′-ATCAAGGAGGAGAAGG AGCC-3′; reverse: 5′-CGCACCTAGAGGAGATGAGG-3′) from Zhang et al. (2007) was also used in fine mapping. Thus, the *D62* gene was further narrowed to a region between the markers MM0124 and RM19320 by using 546 mutant individuals of the F_2 mapping population. This region of 131-kb in length was present in three BAC contigs, AP001168, AP002838 and AP000391. To identify the mutation site of the *d62* mutant, the candidate *D62* gene was amplified using genomic DNA extracted from *d62* mutant and wild-type pants by using five pairs of primers, P1, P2, P3, P4 and P5 (Table 1). The amplified DNA fragments were cloned into pMD19-T vector (TaKaRa, Dalian, China) and sequenced.

RNA isolation and RT-PCR analysis

Total RNA was extracted from various rice tissues of wild type and *d62* mutant by using RNAiso^{TM} Plus (TaKaRa). The extracted RNA was treated with RNase-free DNaseI (Fermentas, Beijing, China) to eliminate genomic DNA contamination according to the protocols recommended by the manufacturer. The first strand of cDNA was synthesized from 4 μg of total RNA using oligo$(dT)_{18}$ (TaKaRa) as primers. The first-strand cDNA products equivalent to 320 ng of total RNA were used as templates in a 20 μl PCR reaction system. To amplify the ORF region of *D62* gene, the primers of DORF (forward: 5′-GAGGAGGTCT CTTCTTGGCACG-3′; reverse: 5′-CACCATTCATCGTC AGCTTAGCT-3′) were used in RT-PCR. The amplified cDNA fragments were sequenced as described above.

To investigate the effects of GA_3 on the expression of *D62* and GA biosynthetic genes, the seedlings at 5-leaf stage were incubated in 100 μM GA_3 solution or control solution (distilled water) for 4 days and sprayed with the solution per day. After 4 days of incubation, total RNA was isolated from leaves as described above. For expression analysis of *D62*, RT-PCR was performed using the primers RT-D62 (Table 1). For expression analysis of the GA biosynthetic genes including *OsCPS1*, *OsKS1*, *OsKO1*, *OsKAO*, *OsGA20ox2/SD1*, *OsGA3ox2/D18* and *OsGA2ox3*, the primers used in RT-PCR analysis are listed in Table 1. The rice *Actin1* gene was used as an internal control in RT-PCR analysis.

Real-time qPCR analysis

Real-time qPCR was performed with SYBR Premix Ex Taq^{TM} HS (Takara) on the Applied Biosystems 7500 Real-Time PCR System. The relative expression levels of each transcript were obtained by normalization to rice *Actin1* gene. PCR was carried out with the two-step protocol as follows: activation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 62°C for 34 s. For real-time qPCR analysis of the GA biosynthetic genes *OsCPS1*, *OsKS1*, *OsKAO, OsGA20ox2/SD1* and *OsGA3ox2/D18*, the primers for each gene are listed in Table 1. The rice *Actin1* gene was used as an internal control in real-time qPCR analysis.

Complementation experiment

A 6.1-kb DNA fragment containing the entire *D62* coding region, 3,093 bp of the sequence upstream of the start codon and 1,153-bp downstream of the stop codon, was amplified from wild-type genomic DNA by HiFi DNA polymerase with the primers CT6.1 (forward: 5′-CTGG TGGTTTGGGGATTGGAGTTG-3′; reverse: 5′-CTGGA GATTTGGAGTGCGTGATGG-3′). The amplified fragment was cloned into the pMD19-T vector and sequenced, then digested with *Eco*RI and *Hin*dIII, and inserted into the binary vector pCAMBIA1301 to generate a transformation plasmid pCT6.1. The binary plasmid was introduced into *Agrobacterium tumefaciens* EHA105, and then transformed into *d62* callus as described by Hiei et al. (1994). The empty vector pCAMBIA1301 was introduced into *d62* callus as a control.

Table 1 List of primers used in this study

Primer name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Trials
P1	AACGGCTATTGCTTTGGACCT	TTGCCTATCTATGAACCACTCG	Sequencing
P2	GCAAATCAACCATTACAGGCGT	TTGCCTCACCATTACCACTCCT	Sequencing
P3	TGGTAATGGTGAGGCAACAAGA	TGGATTGATGGAACCATACCTC	Sequencing
P4	GACGGCGATGAGGTATGGTT	GAACATCTCCTCCGCCTTGG	Sequencing
P5	CTCAACTCGGGGAGGTGGGA	TAGAGATGTGGGTGGGCTGGAT	Sequencing
RT-D62	CAACCACGAGGCCGCCAACTACTAC	GTGAAGTGCAGGAACCTCG GGATGG	RT-PCR
OsCPS1	ACGAATTGAGGAGGCAGCATCTATG	GAGCAAGTTCTTGCATACCCAACTC	RT-PCR
OsKS1	GACAAGGGACCAGCTCCAGACATTGGAG	CAGGAGCAGCAATCTGCTCATCCATGGC	RT-PCR
OsKO1	AAGCATTGTCTGTGATAAGC	CCTGTTTGTCAGGGTTCTTG	RT-PCR
OsKAO	GAGATCGTCGACGTCCTCATCATGTACC	AGATGTTGACGCAGCGAAGTGTCTCGTC	RT-PCR
OsGA20ox2	CCCTCACCATCCTCCTCCAG	GGCAGCTCTTATACCTCCCGT	RT-PCR
OsGA3ox2	TCTCCAAGCTCATGTGGTCCGAGGGCTA	TGGAGCACGAAGGTGAAGAAGCCCGAGT	RT-PCR
OsGA2ox3	TTCTTCGTCAACGTCGGCGACTCGTTGC	TCTCAAACTGGGCCAGCCTGTTGTCTCC	RT-PCR
OsActin1	CGTCAGCAACTGGGATGATATG	GTGTGGCTGACACCATCACCAG	RT-PCR
qOsCPS1	CCTCTATGAAGAAAATGGCAATG	ATCATCCATTCAATCCAAGCAC	Real-time qPCR
qOsKS1	CCCGACTCTGTATTTCGTAGGAC	CCACCACTGTGATGAACAAGCA	Real-time qPCR
qOsKAO	GCAGAACGGATTAACCCTCAG	GGGGATAAGATAACCGTTCACA	Real-time qPCR
qOsGA20ox2	CCCTCACCATCCTCCTCCAG	GGCAGCTCTTATACCTCCCGT	Real-time qPCR
qOsGA3ox2	CCACATCCTCACCAACGGC	GAGGAAGTAGCCGAGCGAGAC	Real-time qPCR
qOsActin1	GTGGTCGCCCCTCCTGAAAG	GGCTTAGCATTCTTGGGTCCG	Real-time qPCR

Promoter activity analysis

For the construction of the fusion between the *D62* promoter and *GUS* coding sequence (*D62* promoter::*GUS*), the 1.6-kb upstream of *D62* gene was amplified from wild-type genomic DNA and inserted into the 5′-end of the *GUS* gene (*gusA*) in pCAMBIA1301 at the *Eco*RI and *Nco*I sites to create a transformation plasmid of *GUS* driven by *D62* promoter. The primers used in PCR amplification were DP (forward: 5′-AT*GAATTC*CTACTCATCACCGTCGCATT TCTT-3′; reverse: 5′-AAT*CCATGG*GACGTGCCAAGA AGAGACCTCCT-3′; *Eco*RI and *Nco*I sites are italicized, respectively). The plasmid was introduced into *Agrobacterium tumefaciens* EHA105, and then transformed into Nipponbare (*Oryza sativa* L. ssp. *japonica*; supplied by the College of Life Sciences, Zhejiang University) callus as described by Hiei et al. (1994). Transgenic plants harvested at different developmental stages were incubated with X-gluc buffer overnight at 37°C to ensure that they develop blue color (Jefferson et al. 1987). Green tissues were incubated with 70–100% ethanol at room temperature to remove chlorophyll before their observation under a dissecting microscope.

Phylogenetic analysis

Protein BLAST search was performed with the BLASTP program (http://blast.ncbi.nlm.nih.gov/). The deduced amino acid sequence was aligned using the Clustal X program (Thompson et al. 1997) with the default parameters. A phylogenetic tree was generated using MEGA4 (Tamura et al. 2007) with the neighbor-joining method. GenBank/EMBL/DDBJ and Swiss-Prot accession numbers for the genes mentioned in present study are as follows: D62 (BAC24836 or BAA90816), At RGA (CAA72177), At GAI (CAA75492), At RGL1 (AAL05911), At RGL2 (NP_186995), At RGL3 (NP_197251), At SCR (AAB06318), At SCL28 (AAG51600), Br RGA1 (AAX33297), Br RGA2 (AAX33298), Cs SCR (CAI30892), Hv SLN1 (AAL66734), La SCR1 (ACQ84011), La SCR2 (ACR48080), Ls DELLA1 (BAG71200), Ls DELLA2 (BAG71201), Os SCR (BAD22576), Os SLR1 (BAE96289), Os SLRL1 (AAR31213), Os SLRL2 (AAT69589), Pn SCR (BAE48702), Pp DELLAL1 (ABX10764), Pp DELLAL2 (ABX10765), Ps SCR (ABH85406), Pt GRAS18 (EEE84364), Pt GRAS19 (EEE78544), Rc GAI1 (EEF49399), Rc RGL1 (EEF51101), RHT-D1A (CAB51555), Sm DELLA1 (ABX10758), Vv GAI1 (AAM19210), Zm DWARF8 (CAB51557), Zm DWARF9 (ABI84226) and Zm SCR (AAG13663).

Results

Characterization of the *d62* mutant

Before the 3-leaf stage, no obvious morphological differences could be observed between the *d62* mutant and wild

type. Beginning from the 4-leaf stage, the *d62* mutant showed some characteristic phenotypes, such as dwarfism, abnormal leaf blade morphology, dark-green leaves, etc. (Fig. 1). Figure 1a–c displayed the gross morphology of *d62* mutant at seedling stage (Fig. 1a), tillering stage (Fig. 1b) and grain-filling stage (Fig. 1c). After the heading stage, the mutant line reached ~66% of the height of wild type (Fig. 1c, Table 2). All internodes of *d62* mutant were shorter than that of the wild type, but the first (uppermost) and second internodes of the mutant were less affected (Table 2). Compared with wild type, the *d62* mutant showed reduced tiller numbers, late and asynchronous heading and partial male sterility. The leaf blades of *d62* mutant were shorter and wider (Fig. 1d), and edges of leaf tips were more rounded than that of the wild type (Fig. 1e). In *d62* mutant, crinkly leaf phenotype was observed after heading stage (Fig. 1f). The *d62* mutant also showed smaller panicles and shorter rachis-branches (Fig. 1g). The grains of *d62* became shorter and wider than those of the wild type (Fig. 1h), and increased with larger chalkiness was observed in its brown rice (Fig. 1i). In addition, the *d62* mutant had short roots (Fig. 1j). These observations indicated that the *d62* mutant had multiple morphological defects.

Positional cloning of the *D62* gene

To determine whether *d62* was controlled by a single gene or multiple genes, in 576 F_2 individuals from the cross between *d62* mutant and *japonica* cultivar 02428, 440 wild-type individuals and 136 mutants were identified, respectively. The segregation of normal plants and mutants was fitted to a 3:1 segregation ratio ($\chi^2 = 0.59 < \chi^2_{0.05} = 3.84$). Also, all F_1 hybrids derived from the crosses between *d62* mutant and 93-11, or *d62* mutant and 02428, showed a normal phenotype. So these results indicated that the *d62* mutation was controlled by a single recessive nuclear gene.

To elucidate the molecular function of *D62*, the gene was isolated by positional cloning. The *D62* gene was primarily mapped at the short arm of chromosome 6 by SSR markers RM19289 and RM19320 (Fig. 2a), and was subsequently fine mapped at an interval of 131 kb bracketed by SSR markers MM0124 and RM19320 (Fig. 2b). Using the rice Gramene database (http://www.gramene.org/) and Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp/viewer/gbrowse/build4/), 20 putative genes were annotated in this region (Fig. 2c, Suppl. Table 1). Among them, the gene (IRGSP locus: Os06g0127800; TIGR locus:

Fig. 1 Morphological characterization of the wild type (*left* image of each part) and *d62* mutant (*right*). **a–c** Gross morphology of the *d62* mutant at seedling stage (**a**); tillering stage (**b**); grain-filling stage (**c**); **d** leaf blades; **e** leaf tips; **f** partial enlarged morphology of leaf blades; **g** panicles; **h** grains; **i** the brown rice observed under a X-ray viewer. *Bars* 2 cm (**e**, **f**), 5 cm (**g**), 10 cm (**a**, **d**, **h**, **i**, **j**), 15 cm (**b**), 20 cm (**c**)

Table 2 The plant height, panicles and internodes length of *d62* mutant

Type	Plant height (cm)	Length of panicles (cm)	Length of internodes (cm)					
			First	Second	Third	Fourth	Fifth	Sixth
WT	100.3 ± 4.9	23.7 ± 1.0	31.4 ± 1.9	14.5 ± 1.0	15.4 ± 2.4	8.5 ± 2.0	4.5 ± 1.0	2.0 ± 0.9
d62	66.4 ± 3.6	18.3 ± 0.7	22.8 ± 1.0	11.4 ± 0.7	7.1 ± 0.8	4.1 ± 0.6	2.7 ± 0.5	0.4 ± 0.4

Values are mean ± SD ($n = 10$)

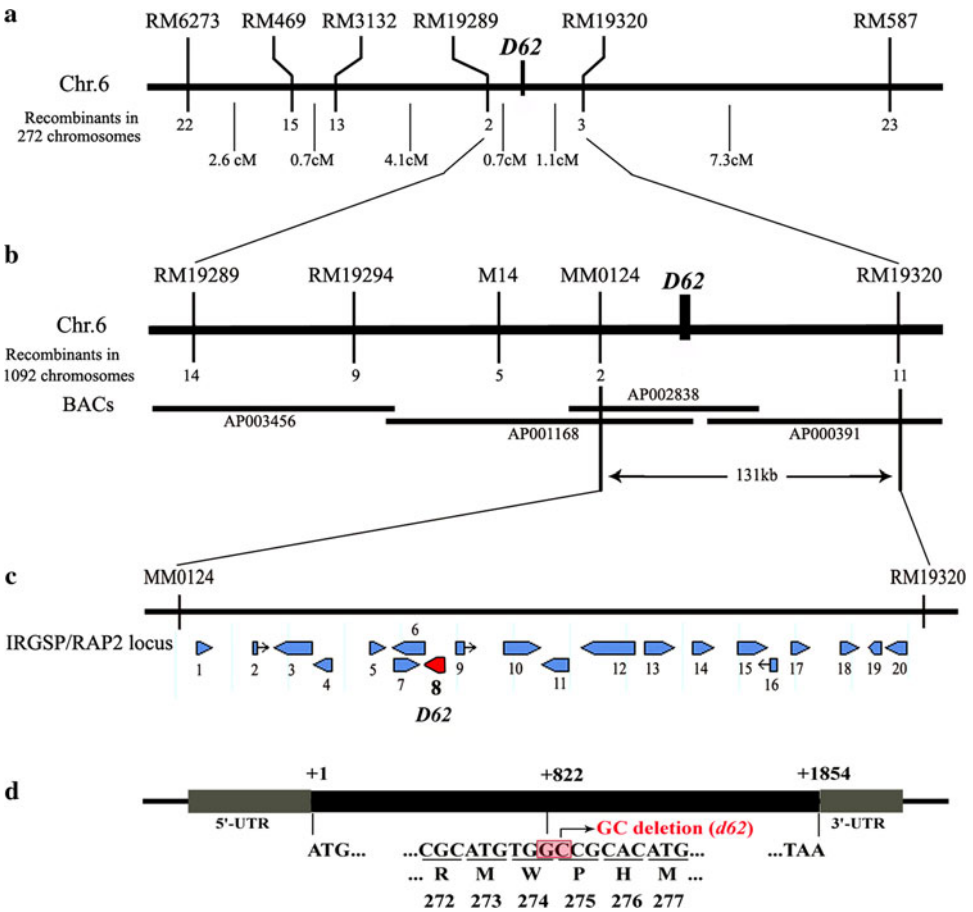

Fig. 2 Isolation of *D62* gene by positional cloning. **a** The *D62* gene was primarily mapped on chromosome 6 based on the genotyping of 136 F_2 mutant individuals. Marker names are indicated above the *vertical lines* and the numbers of recombinants are shown below the corresponding markers. The genetic distances (cent Morgan [cM]) between adjacent markers are indicated. **b** The *D62* gene was fine mapped to an interval of 131-kb by genotyping of 546 F_2 mutant individuals. Numbers of recombinants are shown below the corresponding markers. BAC contigs covering the *D62* gene are shown below the linkage map. The physical distance of 131 kb was calculated from the map-based sequence published by IRGSP (2005). **c** In the 131 kb region, 20 putative genes were annotated in RAP-DB database. The candidate gene 8 (IRGSP locus: Os06g01 27800; TIGR locus: Os06g03710) that encodes a putative gibberellin response modulator was suspected to be the *D62* gene. **d** The structure of *D62* gene and the mutation site. The start codon (ATG) and stop codon (TAA) are indicated. *Black* and *gray boxes* indicate the ORF region and untranslated region (UTR) of *D62* gene, respectively. The *d62* had a 2-bp deletion at the ORF region

Os06g03710) which encodes a putative GA response modulator of GRAS transcription factor was specially focused in present experiment. The results of BLASTP search indicated that Os06g0127800 was homologous to DELLA and DELLA-like proteins of the GRAS family. In rice, the overexpression of DELLA-like protein SLRL1 induced a dwarf phenotype with increased levels of *OsGA20ox2/SD1* gene expression (Itoh et al. 2005). These results elicited the possibility that *Os06g0127800* was the *D62* gene. In fact, recent findings indicate that *Os06g01 27800* is *DLT/OsGRAS-32*, which plays positive roles in BR signaling (Tian et al. 2004; Tong et al. 2009). The *dlt*

mutant produces dwarf and low-tillering phenotypes and exhibits insensitivity or less response to BR application (Tong et al. 2009).

The locus *Os06g0127800* was therefore amplified from *d62* mutant and wild-type plants by PCR and then sequenced. The results of DNA sequencing revealed that the *d62* mutant had a 2-bp deletion of GC at the ORF region of locus *Os06g0127800* (Fig. 2d). To confirm the mutation site, the ORF region of locus *Os06g0127800* was amplified by RT-PCR. Sequencing of the cDNA fragment further confirmed the 2-bp deletion in the *d62* mutant. These results indicated that the locus *Os06g0127800* was the *D62* gene.

The full-length cDNA of *D62* (Genbank Accession: AK106449) was 3,084-bp in length containing an ORF of 1,854-bp; the 5′-UTR was 770-bp long, and the 3′-UTR was 460-bp long; the ORF was predicted to encode a polypeptide of 617-amino acids with calculated molecular mass of 65.85 kD and a pI of 6.02 (Suppl. Fig. 1). There was no intron in *D62* gene, as is the case of most members of the GRAS family in rice and *Arabidopsis* (Tian et al. 2004). In the *d62* mutant, a 2-bp deletion at the position +822–823 (start codon is position +1) of the ORF caused frame shift mutation (Suppl. Fig. 1).

Confirmation of the 2-bp deleted ORF as the *d62* was achieved by complementation experiment. A genomic DNA fragment of 6.1-kb in length, including the entire sequence of the *D62* gene, was introduced into *d62* mutant with *Agrobacterium tumefaciens*-mediated transformation method (Hiei et al. 1994). Six independent transgenic lines were obtained by identifying the expression of GUS reporter gene (Suppl. Fig. 2). The mutative phenotypes of *d62* were rescued in these transgenic plants (Fig. 3), which indicated that the locus *Os06g0127800* was indeed the *D62* gene and the *d62* was a loss-of-function mutation in this locus. Based on the fact that *Os06g0127800* was *DLT/OsGRAS-32*, these results also indicated that the *D62* gene was the same locus as *DLT/OsGRAS-32* and the *d62* was allelic to the *dlt* mutant reported by Tong et al. (2009).

Phylogenetic analysis of D62 and its homologous proteins

The results of BLAST search indicated that the D62 protein shared homology (33–37% identity and 49–53% similarity) with DELLA or DELLA-like proteins, the repressors of GA signaling pathway. The D62 protein also showed homology (34–37% identity and 51–53% similarity) to SCR (SCARECROW) proteins, another GRAS subfamily involved in asymmetric cell divisions in plants (Di Laurenzio et al. 1996; Lim et al. 2000; Kamiya et al. 2003). Sequence analysis found that the N-terminal region of D62 protein contained no DELLA or TVHYNP motifs which were conserved in DELLA proteins, but the D62 protein shared GRAS domains with DELLA proteins, such as LHRI, VHIID, LHR μ, PFYRE and SAW motifs, with high similarity (Fig. 4a). The sequence alignment of D62 protein with DELLA-like and SCR proteins also showed that the N-terminal of D62 protein was highly variable, but the C-terminal of D62 protein was highly similar to those of DELLA-like or SCR proteins (Suppl. Figs. 3, 4). However, phylogenetic analysis demonstrated that the D62 protein could be categorized into a novel group distinct from DELLA, DELLA-like or SCR groups (Fig. 4b).

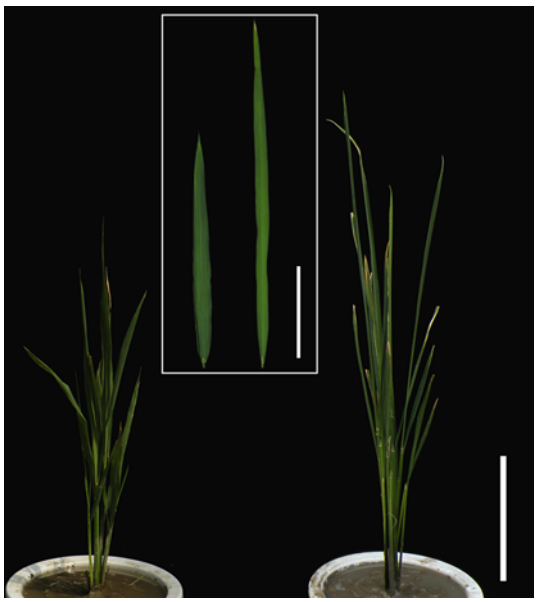

Fig. 3 Phenotypic complementation by the introduction of *D62* gene. Regenerated *d62* mutant plants containing the empty vector pCAMBIA1301 (*left*) and pCT6.1 vector encompassing the entire *D62* gene (*right*) are shown. *Bar* 20 cm. The image superimposed at the *top middle* is a close-up view of the leaf blades. *Bar* 10 cm

Expression patterns of the *D62* gene

To study the functions of *D62*, expression patterns of *D62* gene were analyzed by using semi-quantitative RT-PCR. Total RNA was extracted from leaves, stems and roots at 5-leaf stage and young panicles of wild-type plants. The results showed that the *D62* gene expressed in leaves, stems, roots and panicles (Fig. 5a). The expression levels in roots were lower than those in other tissues. For reconfirmation of the expression patterns, a vector of *GUS* driven by *D62* promoter was constructed and introduced into rice plants. The GUS staining showed that *Gus* gene expressed in roots, stems, leaves and panicles of the transgenic lines (Fig. 5b–e). These results indicated that the *D62* gene expressed in all tested tissues including roots, stems, leaves and panicles of rice plant.

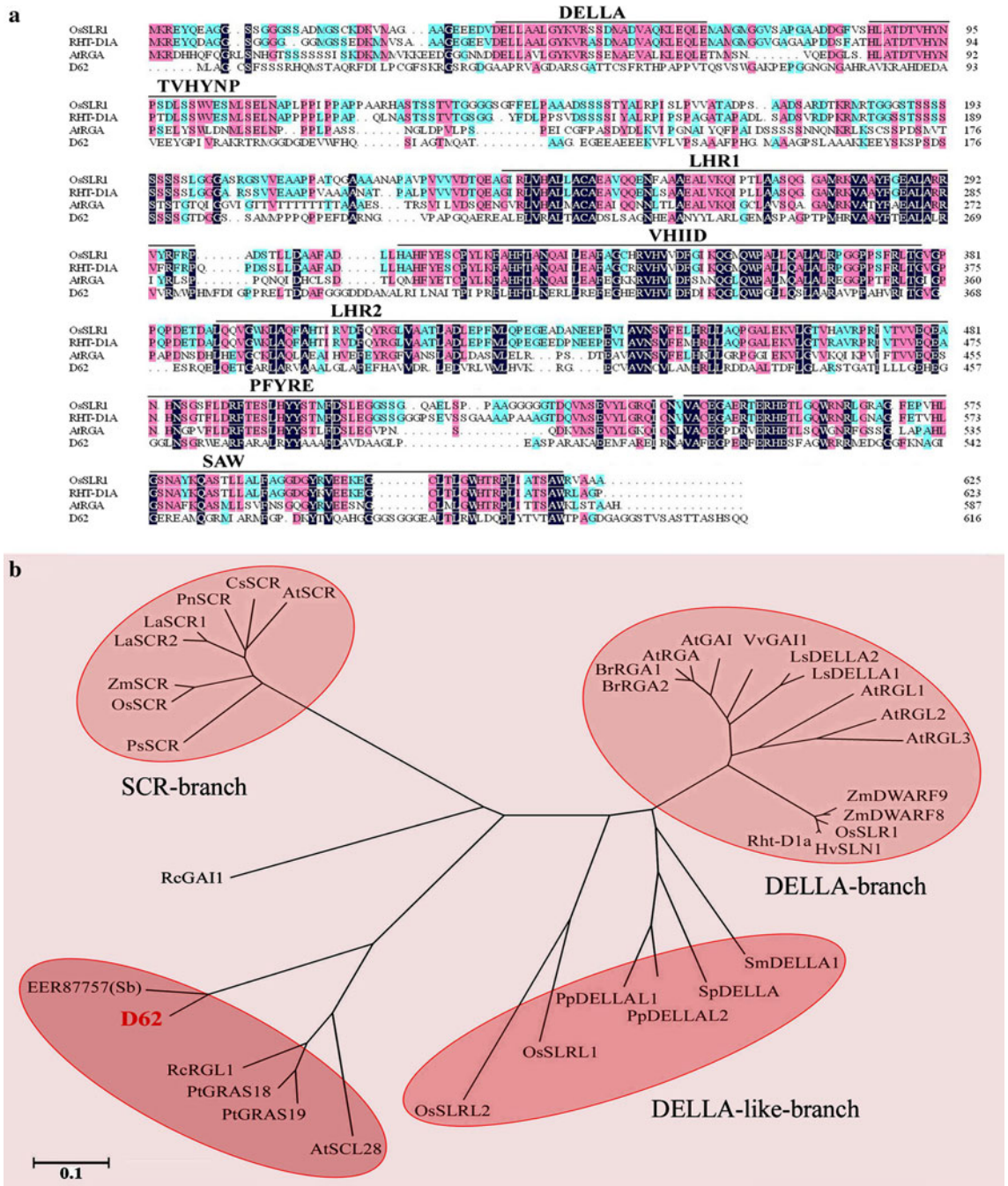

Fig. 4 Sequence and phylogenic analysis of D62 protein. **a** Alignment of D62 and DELLA proteins. Full-length amino acid sequences of D62 and DELLA proteins from rice, *Arabidopsis* and wheat were aligned using the Clustal X program. *Black* and *gray boxes* indicate identical and similar amino acids, respectively. The *lines* above the alignment indicate the locations of the conserved regions in GRAS proteins as defined by Pysh et al. (1999). **b** Phylogenetic tree of GRAS proteins. The phylogenetic analysis was performed with the Clustal X program and phylogenetic tree was constructed using the MEGA4 program with the neighbor-joining method by 1,000 bootstrap replicates. *Scale* 0.1 nucleotide substitutions per site

Reduced α-amylase activity and GA_1 contents in the *d62* mutant

To investigate the possible relationship between *D62* and the GA-related pathway, the shoot elongation and α-amylase activity induction by GA application, both of which are GA-mediated physiological processes, were examined in this experiment. To examine the GA-induction of α-amylase activity, embryoless half-seeds were placed on the starch plate with or without GA_3 for 3 days, and the

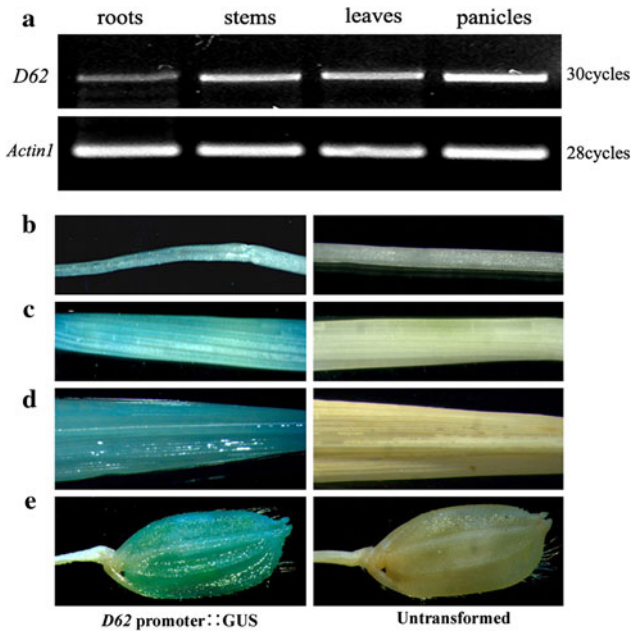

Fig. 5 Expression patterns of *D62* gene. **a** Detection of *D62* transcript by RT-PCR. Total RNA was isolated from roots, stems and leaves at 5-leaf stage, and panicles at heading stage of wild type. The rice *Actin1* gene was used as a control. **b**–**e** GUS activity was detected in *D62* promoter::*GUS* transgenic plants: lateral root (**b**), stem (**c**) and leaf blade (**d**) at tillering stage, and young spikelet (**e**)

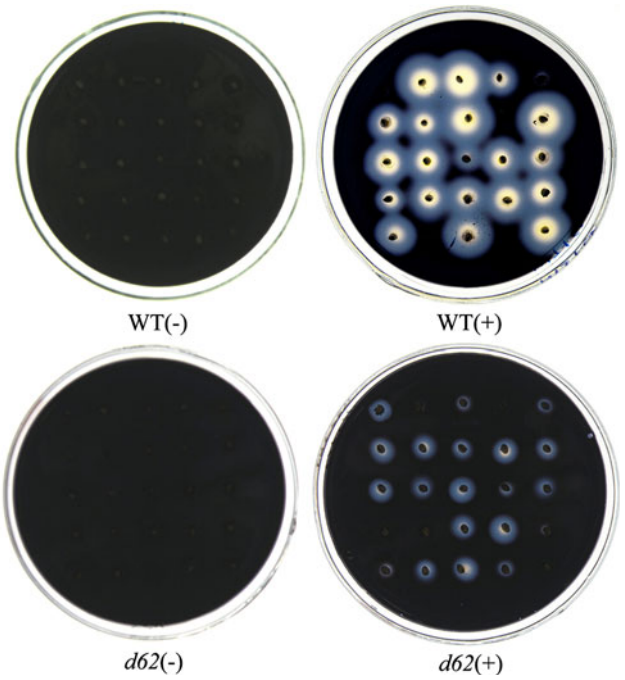

Fig. 6 α-Amylase production from embryoless half-seeds of the wild type (WT) and *d62* mutant. The half-seeds were placed on starch plates containing 1 μM GA_3 (+) or no GA_3 (−) for 3 days, and the starch plates were detected by staining with iodine

starch plate was stained with iodine. Production of α-amylase was observed as plaques both in wild-type and in *d62* mutant on the plate containing GA_3, but the activity of α-amylase was significantly decreased in the *d62* mutant (Fig. 6). This indicated that the *d62* mutant was related to the GA pathway.

GA-induction in shoot elongation was also analyzed in the *d62* mutant. When the second leaf sheaths of *d62* and wild-type plants were compared, the *d62* seedlings were found to be normally responsive to exogenous GA_3 treatment (Suppl. Fig. 5). The shoot elongation was triggered both in the *d62* mutant and in the wild type by treatment with GA_3 concentrations higher than 10^{-8} M. The elongation ratios were similar between *d62* and wild-type plants, and the response curve in *d62* mutant was also more or less parallel with that of wild-type plants. This indicated that the dwarf phenotype of *d62* mutant was limited by factors independent of GA signaling.

For further characterization of *d62*, levels of endogenous GA_1 (a major active GA in rice vegetative tissues) were measured in the *d62* mutant and wild type. GAs were extracted from fresh leaf tissues at 4-leaf stage and measured for GA_1 content with ELISA kit. The result showed that the levels of endogenous GA_1 in *d62* mutant were lower than those in wild-type plants (Fig. 7). This further confirmed that the *d62* mutant was related to the GA pathway.

Increased expression of GA biosynthetic genes in the *d62* mutant

To further investigate the relationship between the *D62* gene and GA metabolic pathway, the expression of GA biosynthetic genes was examined by using semi-quantitative RT-PCR. First, two important genes involved in GA biosynthesis, the *OsGA20ox2/SD1* and *OsGA3ox2/D18*, were analyzed and the expression of which was negatively regulated by the levels of GA in a feedback manner (Itoh et al. 2001; Sasaki et al. 2003). As expected, the expression levels of the two genes were decreased by GA_3 treatment (Suppl. Fig. 6). Compared with the wild-type, the expression of *OsGA3ox2/D18* was not affected in *d62* mutant, but the expression level of *OsGA20ox2/SD1* significantly increased in *d62* mutant (Suppl. Fig. 6). The expression levels of other GA biosynthetic genes, including *OsCPS1*, *OsKS1*, *OsKO1* and *OsKAO* were also examined by using semi-quantitative RT-PCR. The results showed that the expression levels of *OsCPS1*, *OsKS1*, *OsKO1* and *OsKAO* significantly enhanced in the *d62* mutant (Suppl. Fig. 6). The expression level of *OsGA2ox3* which encodes GA 2-oxidase (GA2ox) was also analyzed. GA2ox proteins function in the control of GA levels by inactivating the bioactive GAs. The results from semi-quantitative RT-PCR showed that the expression level of *OsGA2ox3* increased in *d62* mutant (Suppl. Fig. 6). Furthermore, the expression

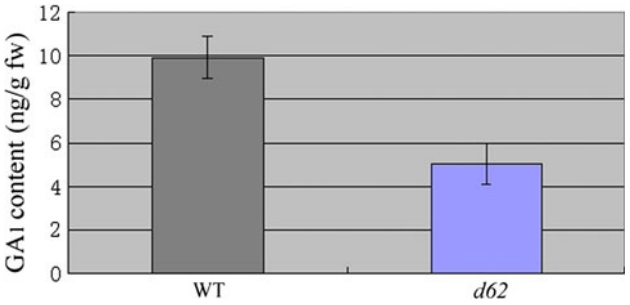

Fig. 7 Levels of endogenous GA_1 in the wild type (WT) and *d62* mutant. The content represents the mean ± SD of measurement from three plants. Extract residues were dissolved in PBS buffer to measure GA_1 content by the indirect ELISA technique using anti-GA_3 antibodies. *gfw* gram fresh weight

levels of *OsCPS1*, *OsKS1*, *OsKAO*, *OsGA20ox2/SD1* and *OsGA3ox2/D18* were analyzed by real-time qPCR (Fig. 8, Suppl. Fig. 7), and the result showed that the mRNA level of *OsGA3ox2/D18* in *d62* mutant is slightly higher than that in wild-type plants (Suppl. Fig. 7). However, the mRNA levels of *OsCPS1*, *OsKS1*, *OsKAO* and *OsGA20ox2/SD1* in *d62* mutant were 4–8 times higher than those in wild-type plants (Fig. 8). These results indicated that *D62* gene influenced the expression of GA biosynthetic genes.

Discussion

This investigation was carried out for the characterization of the rice mutant *dwarf62* (*d62*) and the isolation of *D62* gene. Under normal growth conditions, the *d62* mutant produced many abnormal phenotypes throughout development, such as dwarfism, wide and short leaf blades, dark green and crinkly leaves, round leaf tips, reduced tiller numbers, short roots, late and asynchronous heading, partial male sterility, smaller panicles, shorter rachis-branches, etc. Genetic analysis indicated that the *d62* mutation was controlled by a single recessive nuclear gene. The *D62* gene was primarily mapped to the short arm of chromosome 6 by SSR markers RM19289 and RM19320, and was subsequently bracketed in the 131-kb region by newly developed molecular markers. Positional cloning of the *D62* gene had shown that it encodes a GRAS protein homologous to DELLA, DELLA-like and SCR proteins of the GRAS (GAI-RGA-SCR) family. The *D62* gene is the same locus as *DLT/OsGRAS-32*, which has been shown to act down-stream of the BR signaling pathway by Tong et al. (2009). However, several findings led toward the conclusion that the *D62* gene was related to the pathway of GA metabolism in rice. First, the *d62* mutant showed reduced activity of α-amylase in endosperm induction by GA application. Second, the *d62* mutant had decreased levels of endogenous bioactive GA. Third, the expression levels of GA biosynthetic genes, including *OsCPS1*, *OsKS1*, *OsKO1*, *OsKAO*, *OsGA20ox2/SD1* and *OsGA2ox3* were up-regulated in the *d62* mutant.

Positional cloning of the target gene revealed that *D62* encodes a member of the GRAS family. Plant-specific GRAS proteins are suggested to play important roles in all

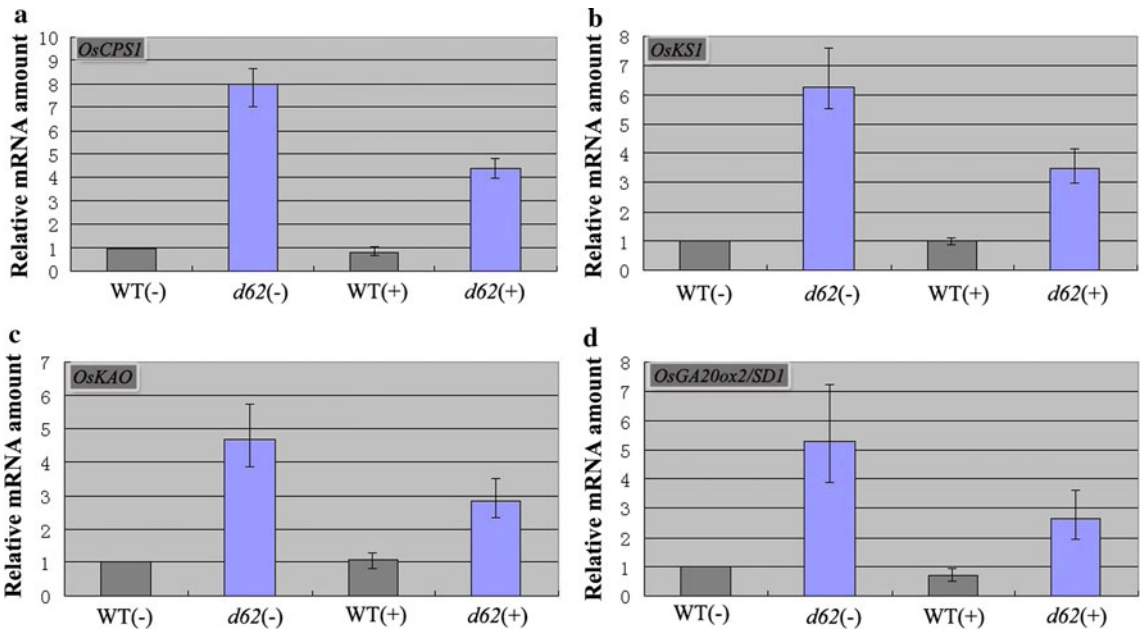

Fig. 8 Expression analysis of the GA biosynthetic genes by real-time qPCR. Total RNA was isolated from the wild-type (WT) and *d62* plants treated with 10^{-4} M GA_3 solution (+) or control solution (−). The mRNA levels of *OsCPS1* (**a**), *OsKS1* (**b**), *OsKAO* (**c**) and *OsGA20ox2/SD1* (**d**) were measured by real-time qPCR

aspects of plant growth and development (Pysh et al. 1999; Bolle 2004; Tian et al. 2004). As one of the most important subfamilies of the GRAS family, DELLA proteins are considered as the repressors in GA-related biological processes. In *Arabidopsis*, there are five distinct DELLA proteins (RGA, GAI, RGL1, RGL2 and RGL3), which have overlapping functions as repressors of GA signaling during GA-regulated plant growth processes, such as stem elongation, flower development and seed germination (Peng et al. 1997; Dill and Sun 2001; King et al. 2001; Lee et al. 2002; Cheng et al. 2004). Loss-of-function in any of these five genes shows a reduction in GA responsiveness (Silverstone et al. 1998; Wen and Chang 2002). In rice, SLR1 is considered as the sole DELLA protein suppressing GA signals and controls almost all GA-regulated events because *SLR1* loss-of-function mutant shows the typical GA constitutive responses of slender phenotype (Ikeda et al. 2001). In addition, Itoh et al. (2005) identified two DELLA-like proteins (SLRL1 and -L2), which contain regions highly similar to the C-terminal GRAS domains of SLR1, but lacking the N-terminal DELLA domain that is unique to DELLA proteins. The overexpression of *SLRL1* in wild-type rice plants produces a dwarf phenotype with increased expression levels of *OsGA20ox2* and diminished the GA-induced shoot elongation (Itoh et al. 2005). It is suggested that SLRL1 functions as a repressor of GA signaling and prevents an excessive response to GA (Itoh et al. 2005). Another representative of GRAS family that has been well-characterized is SCR proteins, the members of which function as specifying asymmetric cell divisions throughout development and are especially crucial for root and shoot cell radial patterning (Di Laurenzio et al. 1996; Dolan 1997; Lim et al. 2000; Kamiya et al. 2003). Molecular analyses of *D62* gene demonstrated that it encodes a GRAS domain protein homologous to DELLA, DELLA-like or SCR proteins. The D62 (OsGRAS-32) shared C-terminal GRAS domains with DELLA, DELLA-like and SCR proteins, with high similarity, but in fact the N-terminal of D62 protein was highly variable. However, phylogenetic analysis showed that D62 could be categorized into a novel group distinct from DELLA, DELLA-like or SCR groups. These results suggested that the D62 might play different roles in the control of development of rice plant.

Tian et al. (2004) performed a genome-wide analysis of the GRAS gene family in rice and *Arabidopsis* and identified a total of 57 GRAS genes from rice. Recently, Tong et al. (2009) reported the cloning of *DWARF AND LOW-TILLERING* (*DLT*), which is shown to play positive roles in BR signaling in rice. The *dlt* mutant is characterized by the production of dwarf and low-tillering phenotypes and insensitivity or less response to BR application (Tong et al. 2009). It is also shown that the transcription of BR biosynthetic genes and BR response genes is altered in *dlt* mutant (Tong et al. 2009). The *DLT* gene is mapped within the 1,310-kb region of chromosome 6 and confirmed to be the locus *OsGRAS-32,* which encodes a new member of the GRAS family (Tian et al. 2004; Tong et al. 2009). In the present study, positional cloning of *D62* gene revealed that the *D62* was the same locus as *DLT/OsGRAS-32* and the *d62* was allelic to the *dlt* mutant reported by Tong et al. (2009). However, the characterization of *d62* mutant indicated that the *D62* (*DLT/OsGRAS-32*) was related to the GA pathway. In the *d62* mutant, reduced activity of α-amylase was observed in endosperm induction by GA application and decreased level of endogenous bioactive GA_1 was also measured in leaves. Furthermore, the expression levels of GA biosynthetic genes increased in the *d62* mutant. These observations suggested that the *D62* influenced GA metabolism besides BR signaling. In fact, recent molecular studies have provided much evidence for interactions of GA and BR. Microarray analysis of cDNA library in rice treated with GA and BR has demonstrated some specific genes coordinately regulated by GA and BR (Yang et al. 2004). Recently, Wang et al. (2009) reported that *OsGSR1*, a member of the GAST (GA-stimulated transcript) gene family, plays important roles in both BR and GA pathways, and also mediates an interaction between the two signaling pathways in rice. The *OsGSR1* is induced by GA and repressed by BR (Wang et al. 2009). The *OsGSR1* RNAi transgenic rice exhibits a reduced sensitivity to GA treatment, an increased expression of the GA biosynthetic gene *OsGA20ox2*, and an elevated level of endogenous GA (Wang et al. 2009). Furthermore, the *OsGSR1* RNAi transgenic plants also show a reduced level of endogenous BR and dwarf phenotypes similar to BR-deficient plants (Wang et al. 2009). Altered GA metabolism also exists in some BR-related mutants. For example, the expression levels of GA biosynthetic genes including the *OsKAO*, *OsGA20ox1*, *OsGA20ox2/SD1* and *OsGA3ox2/D18* increased in the rice BR-deficient *brd1*, and the high expression levels of the GA biosynthetic genes were suppressed by BR treatment (Mori et al. 2002; Komorisono et al. 2005). Komorisono et al. (2005) also characterized a dwarf mutant, *dwarf and gladius leaf 1* (*dgl1*), which exhibits increased expression of the GA biosynthetic genes and only minimal response to GA or BR treatment. Bouquin et al. (2001) reported that, in *Arabidopsis*, the *bri1-201* (BR-insensitive mutant) and *cpd* (BR-deficient mutant) displayed decreased expression levels of *GA5* (*GA20ox1*). RNA-blot analysis revealed that BR and GA antagonistically regulate the accumulation of mRNAs of the GA-responsive *GASA1* gene and the GA-repressible *GA5* gene (Bouquin et al. 2001). In other words, BR may act as a positive regulator of GA 20-oxidation, a key step in GA biosynthesis. These results suggest that cross talk

exists between these two important hormones, and that GA and BR modulate the expression of *GA5* in *Arabidopsis* (Bouquin et al. 2001). In contrast, this may not be the case in pea as the GA_{20} levels are elevated in BR-deficient *lkb* mutant and application of BR to *lkb* plants reduced GA_{20} levels (Jager et al. 2005). These indicate that BR actually negatively regulates GA_{20} levels in pea (Jager et al. 2005). Even though a clear interaction exists between BR levels and the level of GA_{20}, it is suggested that this interaction is not biologically significant in pea (Jager et al. 2005). Based on the evidence reported by Tong et al. (2009), it indicated that the *d62* was a BR-insensitive dwarf mutant. However, we found that the expression levels of several GA biosynthetic genes, including *OsGA20ox2/SD1*, were elevated in the *d62* mutant. This demonstrated that the BR-responsive *D62* (*DLT/OsGRAS-32*) gene also influenced GA metabolism. In this respect, we propose that the *D62* may mediate a cross talk between GA and BR in rice.

The inductions of α-amylase and shoot elongation by GA, both of which are GA-mediated control of physiological processes, are classical model systems for studying how GA acts (Lanahan et al. 1992; Matsukura et al. 1998). Although the *d62* mutant exhibited reduced activity of α-amylase in endosperm induction by GA_3 application, it was normally responsive to GA_3 treatment for shoot elongation. These observations suggested that the dwarf phenotype of *d62* mutant was caused by some factors independent of GA signaling. It was possible that the reduced α-amylase activity resulted from low background levels of endogenous GA in the *d62* mutant. This conclusion was consistent with the result that *d62* mutant had low level of endogenous bioactive GA_1. Furthermore, the expression level of *OsGA2ox3* was up-regulation in the *d62* mutant. In rice, the *OsGA2ox3* encodes GA2ox protein which functions in the control of GA levels by inactivating the bioactive GA, such as GA_1 (Sakamoto et al. 2004). Thus, it was possible that the decreased level of GA_1 was caused by the increased expression of *OsGA2ox3* in the *d62* mutant.

In higher plants, the final stage of bioactive GA synthesis, from GA_{53}/GA_{12} to GA_1/GA_4, is catalyzed by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) (Hedden and Phillips 2000). For the GA biosynthetic genes, *OsGA20ox2/SD1* and *OsGA3ox2/D18*, the expression of which was negatively regulated by the levels of GA in a feedback manner (Itoh et al. 2001; Sasaki et al. 2003). As expected, the expression levels of the two genes were decreased by GA_3 treatment. It was noteworthy that the expression level of *OsGA20ox2/SD1* was significantly increased in *d62* mutant. Although semi-quantitative RT-PCR analysis showed that the expression level of *OsGA3ox2/D18* was not significantly altered in *d62* mutant (Suppl. Fig. 6), the results of real-time qPCR analysis indicated that the expression level of *OsGA3ox2/D18* in *d62* mutant was slightly higher than those in wild-type plants (Suppl. Fig. 7). It was possible that the slight elevation of *OsGA3ox2/D18* expression was caused by feedback regulation of low GA_1 level in *d62* mutant. Furthermore, the GA biosynthetic genes, *OsCPS1*, *OsKS1*, *OsKO1* and *OsKAO*, the expression of which was independent of the levels of GA, were also significantly increased in *d62* mutant. These observations indicated that *D62* affected the expression of the GA biosynthetic genes. Based on these results, it was suggested that the *D62* influenced GA metabolism in rice. The effect could be considerably down-stream of *D62* (*DLT/OsGRAS-32*) and may be related to developmental changes.

In addition, RT-PCR analysis and promoter activity analysis showed that the *D62* gene expressed in all tested tissues including roots, stems, leaves and panicles of rice plant. Consistent with the expression patterns of *D62* gene, the phenotypic analysis also showed that the *d62* mutant had many morphological disorders, such as dwarfism, abnormal leaves and roots, smaller panicles, reduced tiller numbers, late and asynchronous heading, partial male sterility, etc. These results indicated that the *D62* gene was related to multiple processes of morphological development in rice.

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