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ORIGINAL ARTICLE

Brassica napus DS-3, encoding a DELLA protein, negatively regulates stem elongation through gibberellin signaling pathway

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Abstract

Key message **Identification and characterization of a semi-dwarfing gene *ds-3* encoding a mutant DELLA protein regulating plant height through gibberellin signaling pathway.**

Abstract Lodging is one of the most important factors causing severe yield loss in oilseed rape. Utilization of semi-dwarf varieties has been proved the most effective way to increase lodging resistance and yield in many crops. To develop semi-dwarf germplasm in oilseed rape, we identified a semi-dwarf mutant *ds-3* which showed a reduced response to phytohormones gibberellins (GAs). Genetic analysis indicated the dwarfism was controlled by a single semi-dominant gene, *ds-3*. The *DS-3* gene was mapped to a genomic region on chromosome C07, which is syntenic to the region of a previously identified semi-dwarf gene *ds-1* (*BnaA06.RGA*). In this region, *DS-3* (*BnaC07.RGA*) gene was identified to encode a DELLA protein that functions as a repressor in GA signaling pathway. A substitution of proline to leucine was identified in *ds-3* in the conserved VHYNP motif, which is essential for GA-dependent interaction between gibberellin receptor GID1 and DELLA proteins. Segregation analysis in the F_2 population derived from the cross between *ds-1* and *ds-3* demonstrated that *BnaA06.RGA* displayed a stronger effect on plant height than *BnaC07.RGA*, indicating that different *RGA* genes may play different roles in stem elongation. In addition to *BnaA06.RGA* and *BnaC07.RGA*, two more *RGA* genes (*BnaA09.RGA* and *BnaC09.RGA*) were identified in the *Brassica napus* (*B. napus*) genome. Reverse-transcription polymerase chain reaction (RT-PCR) and yeast two-hybrid (Y2H) assays suggest that both *BnaA09.RGA* and *BnaC09.RGA* are transcribed in leaves and stems and can mediate GA signaling in vivo. These genes represent potential targets for screening ideal semi-dwarfing alleles for oilseed rape breeding.

Introduction

The introduction of the *semi-dwarf1* (*sd1*) and the *Reduced height* (*Rht*)-*B1b* and *Rht-D1b* semi-dwarfing genes into rice and wheat made a major contribution to impressive higher yields during the Green Revolution (Hedden 2003). The higher yields were associated with improved lodging resistance and increased harvest index (Youssefian et al. 1992). The rice and wheat semi-dwarf mutants demonstrated altered response to plant hormone gibberellin. The rice semi-dwarf gene, *sd1*, encodes a defective GA biosynthetic enzyme, GA20 oxidase (GA20ox), which causes a deficiency of bioactive GA in the *sd1* mutant. Application of exogenous GA to *sd1* plants was sufficient to restore normal plant height (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). In contrast, the wheat semi-dwarf mutants could not be rescued by application of exogenous GA. This abnormal response to GA is conferred by dwarfing alleles at one of the two *Rht* loci that encode truncated DELLA proteins, orthologues of the *Arabidopsis*

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Gibberellin-Insensitive (*GAI*) gene (Peng et al. 1997) and *Repressors of ga1-3* (*RGA*) (Silverstone et al. 1997, 1998).

Gibberellins are a class of well-characterized plant hormones that regulate plant growth and influence various developmental processes, including stem elongation, seed dormancy and germination, flowering and fruit development (Cheng et al. 2004; Davière and Achard 2016; Tyler et al. 2004). DELLA proteins act as negative regulators that repress GA signaling and restrict plant growth. They modulate gene expression by interacting with transcription factors to regulate plant growth (Marín-de la Rosa et al. 2015; Yoshida et al. 2014). GA-induced degradation of DELLA proteins is required for normal GA signaling. Binding of GA to its soluble receptor, GIBBERELLIN-INSENSITIVE DWARF1 (GID1), triggers the formation of GID1–GA–DELLA complex (Sun 2010). DELLA protein in the complex is then recruited to the $SCF^{SLY1/GID2}$ ubiquitin E3 ligase complex for poly-ubiquitination and subsequent degradation by the 26 S proteasome (Ariizumi et al. 2011; Hirano et al. 2010). DELLA proteins from different plant species share a highly conserved N-terminal DELLA domain, which is essential for GA-induced degradation (Davière and Achard 2013; Peng et al. 1999). GID1 directly binds to the conserved DELLA, LEXLE and VHYNP motifs within the N-terminal domain of DELLA proteins in a GA-dependent manner (Murase et al. 2008). Mutations in the DELLA and VHYNP motifs destroy the binding between DELLA and GID1, and thus block the GA-induced degradation of bound DELLA protein (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2007; Willige et al. 2007). Therefore, mutants bearing mutations in DELLA and VHYNP motifs are insensitive to exogenous GA and display dwarfish growth.

Oilseed rape (*Brassica napus* L.) is one of the major winter crops in provinces along the Yangtze River. Lodging is an important problem in rapeseed production in this region, which can lead to severe yield loss and difficulty harvesting. It is urgent to breed semi-dwarf varieties with improved resistance to lodging. In oilseed rape, several dwarfing genes have been described and their application in hybrid production has been explored. Liu et al. (2010) described a dwarf mutant *ds-1* with a height of about 70 cm. *DS-1* encodes a DELLA protein and is a homolog of the *RGA* gene in *Arabidopsis thaliana, Rht* in wheat and *dwarf-8* (*d8*) gene in maize. Another dwarfing gene *bzh* on *B. napus* chromosome A06 (DY6) additively controls plant height (Foisset et al. 1995, 1996). A semi-dwarf hybrid cultivar with better lodging resistance and harvest ability was released in France using the dwarfing *bzh* gene (Miersch et al. 2016). The dwarf gene *Brrga1-d* in *Brassica rapa* (*B. rapa*) was transferred to *B. napus* through interspecific hybridization of *B. rapa* and *Brassica oleracea* (*B. oleracea*). Yields of hybrids containing *Brrga1-d* were similar to wild-type hybrids (Muangprom et al. 2006). Dwarf mutant *ndf-1* is also GA insensitive. The dwarf phenotype is controlled by a single semidominant gene which possibly encodes the GA receptor GID1 (Li et al. 2011b). *bnaC.dwf* is also a GA-insensitive mutant, but its dwarfism is controlled by a single recessive gene (Zeng et al. 2011). *BnDWF1*, a major dominant locus controlling the dwarfism of *Bndwf1*, was mapped onto a 152-kb interval of *B. napus* chromosome A09 (Wang et al. 2016). These dwarf and/or semi-dwarf mutants provide opportunities to study the genetic basis of plant architecture in oilseed rape.

In this study, we reported the identification of a new semi-dwarf mutant *ds-3* and its inheritance. We showed that a single nucleotide substitution in the N-terminal VHYNP domain of an *RGA* homolog confers the dwarfism of *ds-3*. In addition, we identified other *RGA* homologues in *B. napus* and characterized their expression patterns and activity in GA signaling pathway. The identification of the *DS-3* gene and characterization of these *RGA* genes will facilitate the improvement of lodging resistance in oilseed rape.

Materials and methods

Plant materials and field experiment

To create mutants in oilseed rape, 1000 grams of dry seeds of Huashuang 5 (HS5), an elite cultivar with double-low quality (low erucic acid and glucosinolate), were soaked in water for 2 h and then mutagenized with freshly made 0.3% EMS solution (W/V, Catalog M-0880, Sigma) for 18 h. The mutagenized seeds (M_1 generation) were directly sown in the field. M_1 plants were self-pollinated to harvest M_2 seeds. A total of 5,200 M_2 lines were sown in the field with one row for each line. Mutants with phenotypic alterations were screened and confirmed in M_3 generation. One dwarf mutant named *ds-3* was obtained and confirmed to be inheritable. Besides, one inbred line, G127 with normal plant height, was used to develop mapping population with *ds-3*, and seven cultivars or inbred lines (Yunyou-8, Zhongyou 821, Zhongza-H8002, Qingyou 331, Rainbow, Pinnade(TT) and Alto(oo)) were used for comparative sequencing of the causal mutation. All the materials were grown in the field of Huazhong Agricultural University in Wuhan (114.31_E, 30.52_N), China, during normal rapeseed growing season for phenotype observation. The plant heights were measured and calculated from the ground to the top of main inflorescence when the oilseed had ripened.

Measurement of hypocotyl elongation

Hypocotyl elongation was quantified as previously described (King et al. 2001). Seeds from HS5 and ds-3 plants were surface sterilized for 20 min with a 3% NaClO solution, washed five times with sterile distilled water, soaked in the distilled water for 3 days at 4 °C. To investigate the role of GA_3 on the elongation of the hypocotyl, seeds of HS5 and ds-3 mutant plants were germinated on Murashige and Skoog (MS) media supplemented with various concentrations of GA_3. All plants were grown for 10 days in a growth room at 23 °C under long-day conditions (16-h-light/8-h-dark cycle). For each treatment, twelve seedlings were randomly selected for hypocotyl length measurement in each experiment. All experiments were carried out in duplicate. Difference between each group was analyzed based on a two-tailed Student's *t* test.

Genetic analysis

To reveal the inheritance of the dwarfism, crosses were made between *ds-3* and its original parent HS5. For evaluating the number of genes controlling the dwarfism of *ds-3*, the resultant F_1 hybrids were self-pollinated and backcrossed to HS5 to obtain F_2 and backcross (BC_1) populations, respectively. The F_2 and BC_1 populations and their parental lines were grown in the field and plant heights of all the F_2 and BC_1 individuals were measured from the ground to the top of main inflorescence at the maturity. The segregation of each population was tested by a Chi-squared (χ^2) goodness-of-fit test. In addition, the dwarf mutant *ds-1* (Liu et al. 2010) was crossed to *ds-3* for allelism test and interaction analysis using the F_1 and F_2 generations.

Genetic mapping of *ds-3*

To map the *ds-3* gene, an F_2 population containing 461 plants was developed from the cross between *ds-3* and a wild-type variety G127 with normal height. F_3 families obtained from self-pollinated F_2 plants were grown in two rows (ten plants/row) to validate the phenotype of corresponding F_2 individuals. Genomic DNA was extracted from young leaves using cetyl-trimethylammonium bromide (CTAB) method (Doyle 1987). Ten extremely dwarf plants and ten extremely tall plants were selected from the F_2 mapping population. Equal amounts of DNA from the dwarf and tall extremes were pooled to form the dwarf bulk (DB) and tall bulk (TB), respectively. Bulked segregant analysis (BSA) (Michelmore et al. 1991) was performed to identify molecular markers linked to the dwarf gene *ds-3*. To identify molecular makers linked to the dwarf gene, 292 evenly distributed simple sequence repeat (SSR) markers were selected from the previous genetic linkage maps constructed in our laboratory (Cheng et al. 2009; Li et al. 2011a, 2013; Xu et al. 2010) and subjected to polymorphism screening. Linked SSR markers were used to genotype all F_2 individuals. Linkage analysis was performed using JoinMap 4.0 software (Van Ooijen 2006). The threshold for goodness-of-fit was set to ≤ 5.0, with a recombination frequency of < 0.4 and minimum logarithm of odds (LOD) scores of 2.0. Map distances in centiMorgans (cM) were calculated using the Kosambi mapping function (Kosambi 1943). The sequences of SSR primers used for mapping are listed in Supplemental Table S1.

Comparative sequencing of the candidate *RGA* gene

A gene-specific primer pair, BnaC07.RGA_orf-F and BnaC07.RGA_orf-R (Supplemental Table S2), was designed based on the complementary DNA (cDNA) sequences of *Bol004667* and *BnaA06.RGA* (Liu et al. 2010) and used to amplify the open-reading frames (ORF) of *BnaC07.RGA* from *ds-3* and the wild-type HS5. PCR was performed in 50-μL reaction volumes containing 100 ng of DNA template, 1 × KOD Buffer, 0.2 mM of each dNTP, 1.0 U KOD-plus (Catalog KOD-201, TOYOBO), 0.3 μM forward and reverse primers under the following conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 58 °C for 30 s, and 68 °C for 2 min; a final extension was performed at 68 °C for 5 min. PCR products were cloned into pGEM®-T Easy vector (Catalog A1360, Promega) and then transformed into DH5α cells. Three positive clones were sequenced for each PCR product. The ORF of *BnaC07.RGA* was also amplified from other eight wide-type cultivars and sequenced, and aligned with the *BnaC07.RGA* sequences from HS5 and *ds-3* to identify the causal mutations in VHYNP motif. Multiple alignments of nucleotide and deduced amino acid of *RGA* homologs were performed using the online software Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignment results were edited and labeled by GeneDoc software (http://www.nrbsc.org/gfx/genedoc/).

Arabidopsis transformation

To validate the effect of mutant allele *BnaC07.rga-ds* on dwarf phenotype, the *BnaC07.rga-ds* gene was expressed under the control of its native promoter. A 4.131-kb genomic fragment including the entire 1.731-kb coding region, 2.0 kb upstream sequence from the translation start codon and 0.4 kb downstream sequence from the stop codon was amplified from *ds-3* and HS5, respectively, using KOD-plus DNA polymerase with primers BnaC07.RGA_g-F and BnaC07.RGA_g-R (Supplemental Table S2). PCR products from *ds-3* and HS5 were digested by FastDigest® *Eco*RI and *Kpn*I (Catalog

FD0274 and FD0524, Thermo Fisher Scientific), then ligated into the binary vector pCAMBIA2301 to produce construct pBnaC07.RGA:BnaC07.rga-ds and pBnaC07.RGA:BnaC07.RGA, respectively. These two plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Arabidopsis (Columbia ecotype) was transformed using the *Agrobacterium*-mediated floral-dip method (Clough and Bent 1998). Transgenic T_1 generation was selected on MS media containing 50 mg L^{-1} kanamycin (Catalog GR0408-5G, Generay, China). Two positive lines in the T_2 generation that exhibited a 3:1 segregation ratio for kanamycin resistant:sensitive were selected to generate homozygous transgenic plants (T_3) for further analysis. Plant heights of the T_3 transgenic lines and wild-type Columbia plants were measured at maturation.

RNA extraction and RT-PCR analysis

Total RNA was extracted from leaves and stems collected from *ds-3* and HS5 plants in initial stage of bolting with the Trizol reagent (Invitrogen, http://www.invitrogen.com/). HS5 seedlings at 7 days after germination were treated with 100 μM GA_3 solution for 0.5, 1, 3, 6 and 8h and sampled for RNA extraction at each time point. Equal amount of total RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Catalog K1622, Thermo Fisher Scientific). Gene-specific primer pairs were used to amplify individual mRNA transcripts of the four *RGA* homologs *BnaC07.RGA, BnaA06.RGA, BnaC09.RGA* and *BnaA09.RGA* in *B. napus*. The *BnaActin1* gene was amplified to indicate the equal amount of input RNA with a primer pair, BnaActin1-F and BnaActin1-R (Zhang et al. 2015). Quantitative reverse-transcribed PCR (qRT-PCR) was performed to measure transcript levels of the four *BnaRGA* genes in three replications using the CFX96 Real-time system (Bio-Rad). Gene-specific primers, BnaC07.RGA_qRT-F/R, BnaA06.RGA_qRT-F/R, BnaC09.RGA_qRT-F/R and BnaA09.RGA_qRT-F/R, were used for the *BnaRGA* genes expression analysis, respectively. Expression of the *BnaENTH* gene (Yang et al. 2014) was used as an internal control. The corresponding primers sequences are listed in Supplemental Table S2.

The mRNA transcripts of *BnaC07.RGA* in transgenic *Arabidopsis* plants were amplified using BnaC07.RGA_dCAPS primers to verify the expression of the transgenes. The expression of *AtACTIN2* (*AT3G18780*) was used as the indicator of the equal amount of input RNA.

Development of allele-specific marker and polymorphism detection

The 268–273th nucleotides from the translation start site in the wild-type allele of *BnaC07.RGA* in HS5 are AACCCT, which was mutated to AACC*T*T in *ds-3* (the italic and underlined letter indicates the mutation). To detect the causal point mutation in *BnaC07.RGA*, a locus-specific derived cleaved amplified polymorphic sequence (dCAPS) marker was developed by introducing a mismatch into the forward primer (BnaC07.RGA_dCAPS-F, Supplemental Table S2) to create a *Hind*III recognition site (AAGCTT) in the mutant allele *BnaC07.rga-ds* (Neff et al. 2002; http://helix.wustl.edu/dcaps/). PCR products amplified from both wild-type and mutant alleles are expected to be 353 bp in length. PCR products were digested with *Hind*III (Catalog FD0504, Thermo Fisher Scientific) and separated on 3% agarose gel. Additionally, an INDEL marker was developed based on sequence difference between *BnaA06.rga-ds* and *BnaA06.RGA* (Liu et al. 2010) to detect the *BnaA06.rga-ds* allele using a primer pair, BnaA06.RGA_InDel-F and BnaA06.RGA_InDel-R (Supplementary Table S2).

Phylogenetic analysis of *RGA* genes in *B. napus*

The amino acid sequence of *Arabidopsis RGA* (*At2g01570*) was used as query sequence to search against the *B. rapa, B. oleracea* (Wang et al. 2011b; Liu et al. 2014; http://brassicadb.org/brad/) and *B. napus* genomes (Chalhoub et al. 2014; http://www.genoscope.cns.fr/brassicanapus/). Subject sequences with *E* value = 0 and identity > 80% were defined as target genes. Putative *B. rapa, B. oleracea* and *B. napus RGA* sequences were downloaded from these *Brassica* databases. Gene structures were predicted using FGENESH (http://linux1.softberry.com/berry.phtml). Homology and similarity of nucleotide and the deduced amino acids sequences among *RGA* genes were analyzed using the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was obtained by analyzing the nucleotide sequence divergence of *Brassica RGA* genes using the neighbor-joining method based on *p* distance model of nucleotide substitutions type. A non-parametric bootstrap method was implemented in MEGA5.1 software (Tamura et al. 2011) and the number of bootstrap replication was 1000.

Yeast two-hybrid assay

The ORFs of four *BnaRGA* homologs and *AtGID1a* were amplified from genomic DNA of *ds-3* and mRNA of *Arabidopsis* (Columbia ecotype), respectively, and cloned into the pGEM®-T Easy vector (Catalog A1360, Promega). The ORFs of the four *BnaRGA* homologs were further fused to the activation domain (AD) of pGADT7 (Clontech, Palo Alto, USA) to generate constructs pAD-BnaA06.RGA, pAD-BnaC07.RGA, pAD-BnaC09.RGA and pAD-BnaA09.RGA, respectively. The ORF of *AtGID1a* was fused to the binding domain (BD) of pGBKT7 (Clontech,

Palo Alto, USA) to form the pBD-GID1a plasmid. The AD and BD fusion constructs were co-transformed into *Saccharomyces cerevisiae* strain AH109 and transformants were screened on Synthetic Dropout (SD)/-Leu-Trp media. Positive transformants were further streaked on SD/-Leu-Trp-His-Ade media containing 20 μg/mL X-α-gal (NALCO, USA) to test interactions between the AD and BD fusion proteins in the absence and presence of 100 μM GA_3. The corresponding primers are listed in Supplemental Table S2.

Results

Identification of a GA-sensitiveness-reduced dwarf mutant *ds-3*

To identify genes regulating plant architecture in oilseed rape, we screened our EMS mutagenized HS5 collection for mutants exhibiting reduced plant height compared to the wild-type HS5 plants. A mutant with significantly reduced plant height and dark green leaves was identified and named as *ds-3*. At maturity, the height of *ds-3* was 69.7 ± 4.5 cm ($n = 30$), which is only 40% of height of wild-type HS5 (177.7 ± 7.2 cm, $n = 20$) (Fig. 1a). Mutant *ds-3* had less number of total nodes, shorter internodes and main inflorescences and lower positions of the first primary branches than HS5 (Table 1), which together resulted in the reduced height of *ds-3*.

To test if GA_3 has effects on the *ds-3* mutant, we sow the seeds of both *ds-3* and HS5 on MS medium supplied with different concentrations of GA_3. Hypocotyl length was measured at 10-day after germination. Under all concentrations of exogenous GA_3, the relative hypocotyl elongation rates of *ds-3* were significantly lower than that of the wild-type HS5 counterpart (Fig. 1b). In addition, as the concentration of exogenous GA_3 increases,

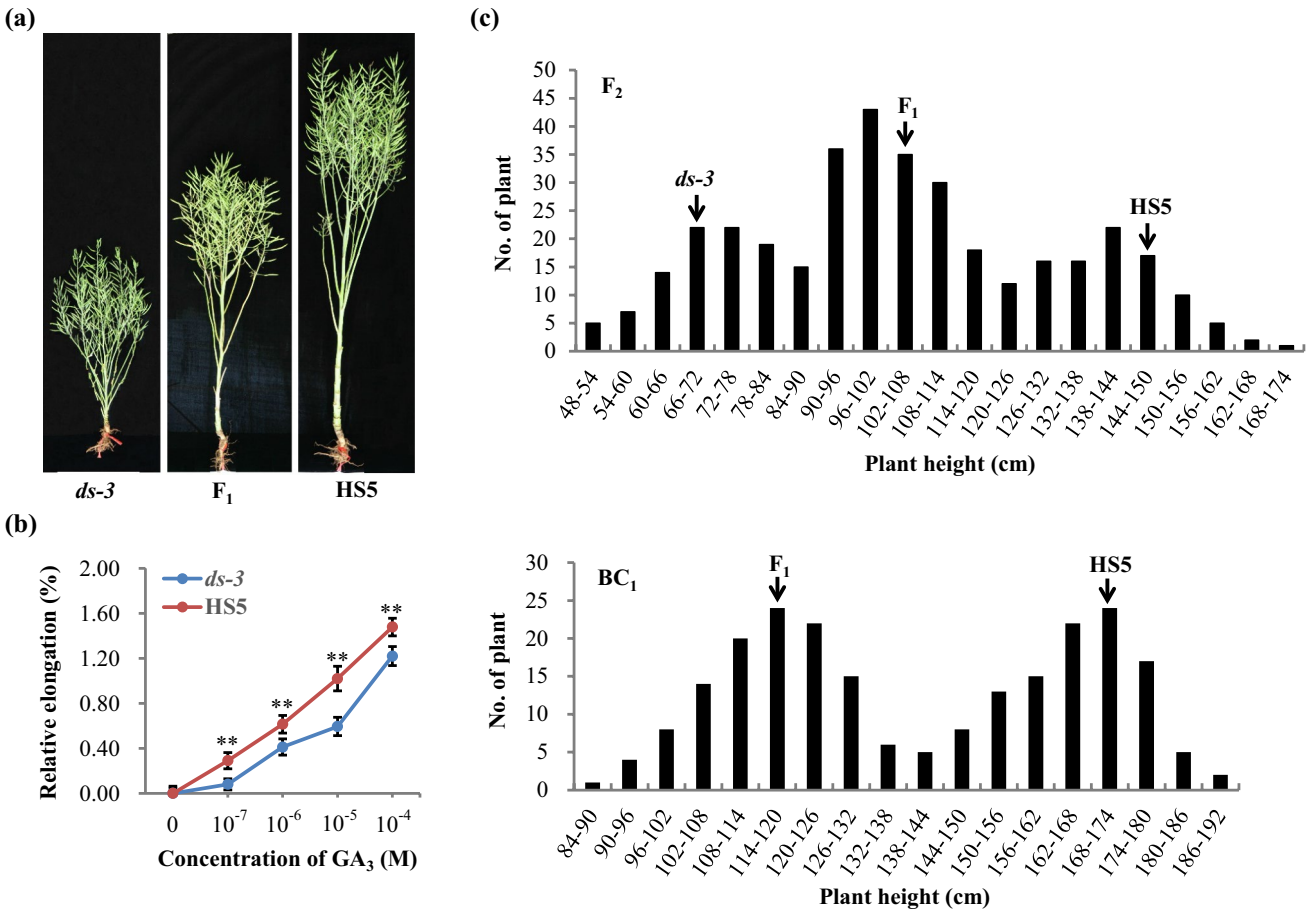


Fig. 1 Phenotype and trait inheritance of *ds-3*. **a** Phenotype of *ds-3* (*left*), HS5 (*right*) and their F_1 hybrid (*middle*) at maturity. **b** Effects of different GA_3 concentrations on hypocotyl elongation. The *x*-axis indicates concentration of GA_3 in the MS medium and the *y*-axis represents relative hypocotyl elongation compared to control. Values are mean ± SD ($n = 12$). **Denotes significant differences at 1% probability level based on a two-tailed Student's *t* test. **c** Distribution of plant height in the F_2 population (*top*) derived from the *cross* of *ds-3* and HS5 and BC_1 population (*bottom*) derived from the *backcross* of F_1 and HS5. *Black arrows* indicate average heights of HS5, *ds-3*, and their F_1 hybrids

Table 1 Comparison of plant height and its component traits between *ds-3* and HS5 at mature stage

Trait	*ds-3*	HS5	*F* value	*P* value
Plant height (cm)	69.7 ± 4.5[a]	177.7 ± 7.2	1574.8	0.0000
First branch height (cm)	2.5 ± 1.6	34.6 ± 3.8	734.7	0.0000
Main inflorescence length (cm)	37.1 ± 4.2	55.9 ± 5.9	80.5	0.0000
Number of total notes	15.5 ± 2.7	21.9 ± 3.6	24.7	0.0000
Average internode length (cm)	2.1 ± 0.5	5.5 ± 0.9	142.7	0.0000

F and *P* values were obtained through one-way ANOVA for each trait

[a]Mean ± standard deviation

the hypocotyl length of *ds-3* did not restore to that of the HS5 counterpart (Supplemental Fig. S1), suggesting that *ds-3* mutant has reduced response to exogenous GA_3.

Inheritance of *ds-3*

To explore the inheritance of dwarfism in *ds-3*, crosses were made between *ds-3* and its original parent HS5. F_1 plants from the crosses displayed intermediate plant heights compared to *ds-3* and HS5 (Fig. 1a). The F_2 individuals from the cross of *ds-3* × HS5 could be roughly classified into dwarf, intermediate and tall types when compared to the heights of *ds-3*, HS5 and their F_1 hybrids. Plant height of the F_2 population displayed a trimodal distribution (Fig. 1c). $F_{2:3}$ progeny testing showed that the F_2 population had 90 homozygous dwarfs, 195 heterozygous intermediates and 82 homozygous tall plants. Plant heights of the BC_1 individuals displayed a bimodal distribution (Fig. 1c). The segregation of plant heights fit an expected Mendelian ratio of 1:2:1 (χ^2 = 1.79, $P = 0.41$) for the F_2 population and 1:1 for the BC_1 population (χ^2 = 1.28, $P = 0.26$). These results indicated that the dwarf phenotype of *ds-3* was controlled by a single semi-dominant gene, which was named *DS-3*.

The dwarfism and altered response to GA of *ds-3* are very similar to that of *ds-1*, a previously reported dwarf mutant in *B. napus* (Liu et al. 2010). To test if *ds-3* is allelic to *ds-1*, allelism test was conducted by crossing these two dwarf mutants. The F_1 hybrids were 68.4 ± 5.2 cm in height, which is similar to the *ds-3* plants, while F_2 individuals segregated for plant height (Supplemental Fig. S2). In 316 F_2 plants, 17 plants (5.4%) showed similar height to wild-type controls and 105 plants (33.2%) showed similar or shorter height than *ds-1* and *ds-3*, which suggest that *ds-3* is non-allelic to *ds-1* but a novel mutant.

Genetic mapping of the *DS-3* gene

To map the *DS-3* gene, an F_2 population with 657 plants was derived from the cross between *ds-3* and a wild-type inbred line G127 with normal height. Based on the distribution of plant heights in the F_2 population (Supplemental Fig. S3), 10 extremely dwarf and 10 extremely tall plants were selected from the F_2 population and equal amount of DNA from these plants was pooled to make a dwarf and a tall bulk, respectively. Bulked segregant analysis (BSA) was performed with a total of 292 evenly distributed SSR markers from previous genetic linkage maps constructed in our laboratory (Cheng et al. 2009; Li et al. 2011a, 2013; Xu et al. 2010). Two SSR markers, BoGMS2499 and cnu_ssr223b on chromosome C07 showed polymorphisms between the dwarf and tall parents and between the dwarf and tall bulks. Using the sequences of the two SSRs as queries, BLAST search against the *B. oleracea* reference genome sequence (Liu et al. 2014) identified two scaffolds, Scaffold00153 and Scaffold00309. To further map the *DS-3* gene, 25 SSR markers were developed from these two scaffolds and their flanking scaffolds. Four markers, BoGMS4029, BoGMS4033, BoGMS4044, and BoGMS4051, detected polymorphisms between the dwarf and tall parents and bulks. These linked SSR markers were used to genotype extremely tall recessive individuals selected from the F_2 mapping population. Of the six linked SSR markers, BoGMS4033 and BoGMS4044 are the two closest linked markers with distances of 4.9 and 1.1 cM to the *DS-3* locus on both sides, respectively (Fig. 2a).

In *B. napus*, chromosome C07 in the C subgenome is homeologous to A06 in the A subgenome (Parkin et al. 2005). Two previously identified semi-dwarf genes, *Brrga1-d* on *B. rapa* A06 (Muangprom et al. 2005) and *DS-1* (*BnRGA*) (here renamed as *BnaA06.RGA* following the nomenclature rules in *Brassica* spp. (Østergaard and King 2008)) on *B. napus* A06 chromosomes (Liu et al. 2010), encode DELLA proteins that have conserved function as repressors in GA signaling. To determine whether the *DS-3* region on C07 is collinear with the *DS-1* region on A06, markers linked to the *DS-3* locus were aligned to the *B. napus* reference genome sequence (Chalhoub et al. 2014). Four consecutive markers on one side of *DS-3* showed good collinearity to a DNA region of 3035-kb on A06 (Fig. 2a). The most closely linked marker BoGMS4044 was 1.1 cM apart from *DS-3*, its homologous sequence on A06 being 215-kb apart from *DS-1*. In general, the genetic distance of 1.0 cM corresponds to a physical distance of about 500 kb in the *B. napus* genome (Raman et al. 2013; Wang et al. 2011a). But in some regions, the physical distance is only 40 kb/cM (Yi et al. 2010). The mapping result together with the similar phenotype to *ds-1* prompted us to consider an orthologous gene of *BnaA06.RGA* as the candidate gene

of *DS-3*. As the C subgenome in allotetraploid *B. napus* originates from diploid *B. oleracea* (Chalhoub et al. 2014), we searched the *B. oleracea* database using the coding sequence of *BnaA06.RGA* as a query and found that a gene model *Bol004667* is annotated to encode a DELLA protein in the target region of *DS-3* on *B. oleracea* chromosome C07 (Fig. 2b). We thus took *BnaC07.RGA*, the orthologous gene of *Bol004667* in *B. napus*, as the candidate gene of *DS-3* for further characterization.

Identification of causal mutation in *ds-3*

To identify the causal mutation in *ds-3*, we first amplified the open reading frame of *BnaC07.RGA* from the *ds-3* mutant and its original genotype HS5. Comparative sequencing identified two variations in *ds-3*, an insertion of 3 nucleotides (+118 GAA + 120 respect to the start codon) and a C-to-T transition at the +272 nucleotide from the translation start point. The 3-bp insertion is a repeat unit of a simple sequence repeat and resulted in an addition of glutamic acid (Glu) residue. The C-to-T transition caused a substitution of proline (Pro) to leucine (Leu) at the 91st amino acid (designated as P91L) located in the N-terminal VHYNP motif (Fig. 2c). Alignment of amino acid sequences of BnaC07.RGA from eight rapeseed cultivars and inbred lines indicated that the proline at the 91st position is conserved, whilst the number of glutamic acid residues is variable (Table 2). In addition, the proline residue in the VHYNP motif is absolutely conserved in all known DELLA proteins from different plant species (Fig. 2d), which indicated that this missense mutation may cause the dwarf phenotype of *ds-3*.

To determine whether the C-to-T transition is directly associated with the dwarf phenotype of *ds-3*, co-segregation analysis was conducted with the locus-specific BnaC07.RGA_dCAPS marker developed from this single nucleotide polymorphism (SNP). A recognition site of *Hind*III (AAGCTT) was introduced into the PCR product amplified from mutant *ds-3*. Digestion of the PCR product with *Hind*III generated a fragment of 312 bp, which was referred as the dwarf (D) allele. The corresponding site from the tall (T) allele had the sequence AAGCCT, which lead to an uncleavable product of 353 bp. BnaC07.RGA_dCAPS co-segregated with plant heights of individuals in the F_2 and BC_1 populations derived from the cross between *ds-3* and HS5 (Supplemental Fig. S4).

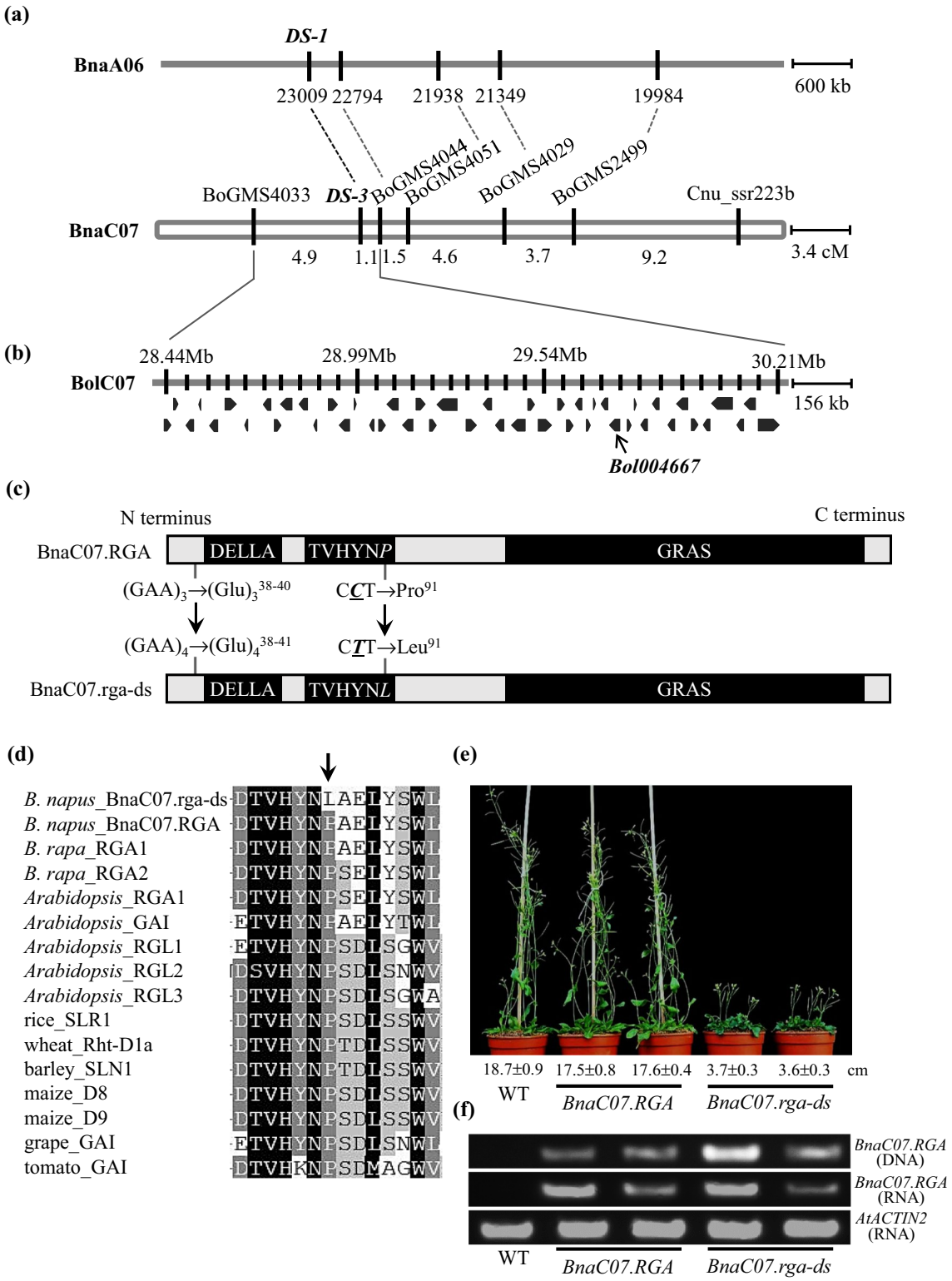
The P91L mutation in BnaC07.rga-ds leads to the dwarf phenotype

To further confirm the C-to-T transition in *BnaC07.RGA* is the causal mutation, we transformed the mutant and wild-type *BnaC07.RGA* genes driven by their native promoter into wide-type *Arabidopsis* Columbia ecotype. The integration and expression of the transgenes in transgenic *Arabidopsis* were confirmed by amplifying the genomic DNA and RNA using BnaC07.RGA_dCAPS primers (Fig. 2f). The average height of the transgenic *Arabidopsis* lines expressing the wild-type allele *BnaC07.RGA* (*pBnaRGA:BnaC07.RGA*) was 17.6 ± 0.6 cm, which is similar to that of wild-type *Arabidopsis* plants (18.7 ± 0.9 cm), whereas transgenic lines expressing the mutant allele *BnaC07.rga-ds* (*pBnaRGA:BnaC07.rga-ds*) were 3.6 ± 0.3 cm, showing obviously dwarfed stature (Fig. 2e). In addition, the height of T_3 transgenic plants co-segregated with BnaC07.RGA_dCAPS (Supplemental Fig. S5). These results implied that *BnaC07.rga-ds* is responsible for the dwarfism of the *ds-3* mutant and the C-to-T transition which caused the P91L substitution in *BnaC07.rga-ds* is the causal mutation.

The DELLA and VHYNP motifs in DELLA proteins are important for the formation of GA-GID1-DELLA complex upon GA induction (Murase et al. 2008; Ueguchi-Tanaka et al. 2007; Willige et al. 2007). We assumed that the P91L substitution in the VHYNP motif may prevent the interaction between DELLA protein and GID1 in the presence of GA. To test this hypothesis, we examined the interaction between AtGID1a and BnaC07.rga-ds through yeast two-hybrid assays. In the absence of GA_3, BnaC07.RGA cannot interact with AtGID1a, while it can interact with AtGID1a in the presence of GA_3. However, BnaC07.rga-ds cannot interact with AtGID1a with or without GA_3 (Fig. 3). These results indicated that the P91L mutation in the VHYNP motif indeed abolished the interaction between BnaC07.rga-ds and AtGID1a, and thus rendered the dwarfism of *ds-3*.

BnaC07.RGA exerts a weaker effect on plant height than *BnaA06.RGA*

Previous studies indicated that different DELLA isoforms have different effects on stem growth (Asano et al. 2009; Pearce et al. 2011). To test the effects of *BnaC07.RGA* and *BnaA06.RGA* on plant height, we crossed *ds-3* to *ds-1* and measured the stem heights of their F_2 progenies at maturity stage. We also genotyped all F_2 individuals using two gene-specific markers, the BnaC07.RGA_dCAPS marker for *DS-3* (*BnaC07.RGA*) and an INDEL marker for *DS-1* (*BnaA06.RGA*). The F_2 individuals were classified into nine genotypes and plant heights between different genotypes had significant differences. The *ds-1ds-1/ds-3ds-3* genotypes have an average height of 38.2 ± 3.9 cm, which is significantly shorter than either *ds-1ds-1/DS-3DS-3* (47.0 ± 8.9 cm) or *DS-1DS-1/ds-3ds-3* (62.2 ± 8.8 cm) (Fig. 4), suggesting that *DS-1* and *DS-3* redundantly and additively regulate plant heights in oilseed rape. The



ds-1ds-1/DS-3DS-3 individuals are significantly shorter than *DS-1DS-1/ds-3ds-3* individuals, and *DS-1ds-1/DS-3DS-3* (72.1 ± 10.6 cm) individuals are significantly shorter than *DS-1DS-1/DS-3ds-3* (92.8 ± 12.1 cm) individuals (Fig. 4). These results indicated that *BnaA06.RGA* has a

◂**Fig. 2** Comparative mapping and identification of *DS-3*. **a** Genetic and comparative mapping of *DS-3*. The *horizontal solid line* represents the pseudomolecule of *B. napus* chromosome A06. The *horizontal empty box* represents the genetic linkage map of the *DS-3* region on *B. napus* chromosome C07. The *dash lines* indicate local synteny between the *DS-1* (*BnaA06.RGA*) region on chromosome BnaA06 and the *DS-3* region on BnaC07. The *short vertical solid lines* represent SSR markers and their homologous sequences on the linkage map and pseudomolecule. **b** The *broad arrows* represent predicted genes in the 1.71 Mb genomic region on chromosome BolC07 of *B. oleracea* delimited by SSR markers BoGMS4033 and BoGMS4044. *Bol004667* is indicated as the candidate gene. **c** Deduced amino acid sequences of DELLA proteins encoded by BnaC07.RGA from wild-type HS5 (*top*) and *ds-3* (*bottom*). *Solid boxes with texts* represent the conserved motifs in DELLA proteins. The two nucleotide mutations and their corresponding amino acid mutations are indicated. **d** Alignment of partial amino acid sequence surrounding the 91th amino acid of 16 DELLA proteins from different plant species. The *arrow* indicates the conserved amino acid residue proline (P) in the VHYNP motif. The accession numbers of the DELLA proteins are as follows: *B. rapa*_RGA1 (*Brassica rapa*, Q5BN23), *B. rapa*_RGA2 (*Brassica rapa*, Q5BN22), *Arabidopsis*_RGA1 (*Arabidopsis thaliana*, NP_178266), *Arabidopsis*_GAI (*Arabidopsis thaliana*, NP_172945), *Arabidopsis*_RGL1 (*Arabidopsis thaliana*, NP_176809), *Arabidopsis*_RGL2 (*Arabidopsis thaliana*, NP_186995), *Arabidopsis*_RGL3 (*Arabidopsis thaliana*, NP_197251), rice_SLR1 *(Oryza sativa*, NP_001051032), wheat_Rht-D1a (*Triticum aestivum*, AGQ43580), barley_SLN1 (*Hordeum vulgare*, Q8W127), maize_D8 (*Zea mays*, NP_001130629), maize_D9 (*Zea mays*, ABI84225), grape_GAI (*Vitis vinifera*, XP_002284648), tomato_GAI (*Solanum lycopersicum*, NP_001234365). Accession numbers for the amino acid sequences of DELLA in *B. napus, B. rapa* and *B. oleracea* are described in article. **e** Confirmation of the causal mutation of *ds-3* in *Arabidopsis* ecotype Columbia. WT, wild-type Arabidopsis ecotype Columbia; *BnaC07.RGA*, transgenic *Arabidopsis* lines expressing the wild-type allele *BnaC07.RGA; BnaC07.rga-ds*, transgenic *Arabidopsis* lines expressing the mutant allele *BnaC07.rga-ds*. **f** Genome integration and expression analysis of the transgenes in transgenic *Arabidopsis* lines. The genomic DNA and RNA were amplified using BnaC07.RGA_dCAPS. *AtACTIN2* were used as the indicator of the equal amount of input RNA in RT-PCR analysis

Table 2 Amino acid sequences of 10 *B. napus* lines or cultivars at the two mutation sites in BnaC07.RGA

Accession	$(Glu)_3^{38-40}$	Pro^{91}	Phenotype
HuangShuang 5	3Glus	Pro	Wild type
ds-3	4Glus	Leu	Dwarf
Yunyou-8	3Glus	Pro	Wild type
G127	4Glus	Pro	Wild type
Zhongyou 821	4Glus	Pro	Wild type
Zhongza-H8002	4Glus	Pro	Wild type
Qingyou 331	3Glus	Pro	Wild type
Rainbow	4Glus	Pro	Wild type
Pinnade (TT)	3Glus	Pro	Wild type
Alto(oo)	4Glus	Pro	Wild type

relatively stronger effect than *BnaC07.RGA* on regulating stem elongation.

Identification and characterization of *RGA* homologs in *B. rapa, B. oleracea* and *B. napus*

B. napus is an allotetraploid species originated from natural hybridization between two diploid progenitors, *B. rapa* and *B. oleracea*. Before the hybridization, both progenitors had experienced whole genome triplication events in the process of speciation (Liu et al. 2014; Wang et al. 2011b). Therefore many genes contain four to six copies in the *B. napus* genome (Cheung et al. 2009; Yang et al. 2006). To identify the number of *RGA* genes in the genomes of *B. rapa, B. oleracea* and *B. napus*, reciprocal BLASTp searches were conducted using the *Arabidopsis RGA* (*At2g01570*) amino acid sequence as a query to identify homologous genes in *B. rapa, B. oleracea* and *B. napus*. Two *RGA* gene models, *Bra024875* on A06 and *Bra017443* on A09 (*BraA09.RGA*), were identified in the *B. rapa* genome and renamed as *BraA06.RGA* and *BraA09.RGA*, respectively, following the nomenclature rules in *Brassica* spp. (Østergaard and King 2008). The two *RGA* gene models, *Bol004667* on C07 (*BolC07.RGA*) and *Bol018868* on C09 (*BolC09.RGA*), were identified in the *B. oleracea* genome. The two *B. rapa* gene models, *BraA06.RGA* (*Bra024875*) and *BraA09.RGA* (*Bra017443*), correspond to previously identified genes *BrRGA1* and *BrRGA2* (Muangprom et al. 2005), respectively. Four *RGA* gene models, *BnaA06g34810D, BnaC07g20900D, BnaA09g18700D* and *BnaC09g52270D*, were identified in the *B. napus* genome. *BnaA06g34810D* and *BnaC07g20900D* correspond to the *BnaA06.RGA* gene identified in previous study (Liu et al. 2010) and the *BnaC07.RGA* gene identified in this study, respectively. The other two gene models, *BnaA09g18700D* and *BnaC09g52270D*, are named as *BnaA09.RGA* and *BnaC09.RGA*, respectively. These two genes have not been identified in previous studies. All these genes or gene models have only one exon and encode intact DELLA proteins containing all N-terminal and C-terminal conserved domains (Supplemental Fig. S6).

Phylogenetic tree was constructed based on the nucleotide sequences of these *RGA* genes using *AtRGA* as the out-group. As shown in Figure 5a, the *Brassica RGA* genes are classified into four groups. Group I contains *BraA06.RGA* and its orthologous gene *BnaA06.RGA*. Group II contains *BolC07.RGA* and its orthologous gene *BnaC07.RGA*. In Group III, *BraA09.RGA* and *BnaA09.RGA* are orthologs. In Group IV, *BnaC09.RGA* and *BolC09.RGA* are orthologs. Additionally, Group I and III showed closer relationship with Group II and IV, respectively, suggesting that Group I and Group II are orthologs and Group III and Group IV are orthologs. The

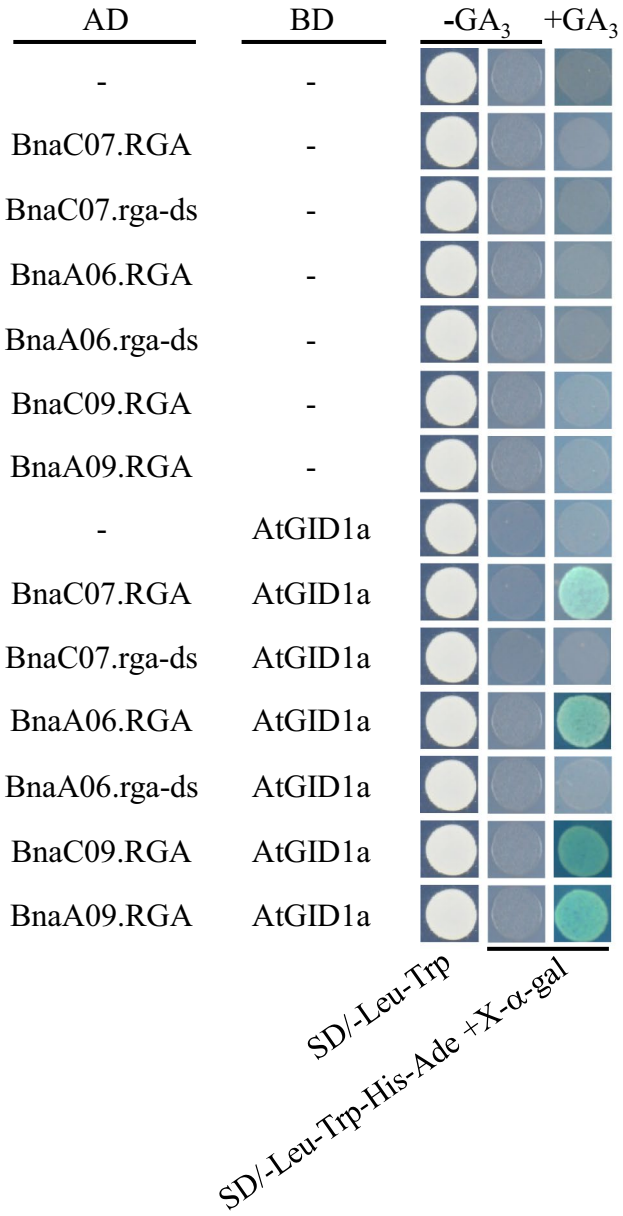


Fig. 3 Yeast-two-hybrid assay for the interaction between DELLA proteins and GID1 mediated by gibberellin. The *AD* panel indicates the DELLA protein in the activation domain. The *BD* panel indicates the GA receptor in the binding domain. Yeast transformants were spotted on control medium (SD/-Leu-Trp) and selective medium (SD/-Leu-Trp-His-Ade) containing 20 μg/mL X-a-gal with (the $-GA_3$ panel) or without 100 μM GA_3 (the $+GA_3$ panel)

homologies between different groups are consistent with the synteny between the A and C genomes or subgenomes (Chalhoub et al. 2014; Parkin et al. 2005). BnaA06.RGA and BnaA09.RGA have the highest amino acid sequence identity with BraA06.RGA (99%) and BraA09.RGA (97%), and BnaC07.RGA and BnaC09.RGA show the highest identity with BolC07.RGA (99%) and BolC09. RGA (99%), respectively (Table 3).

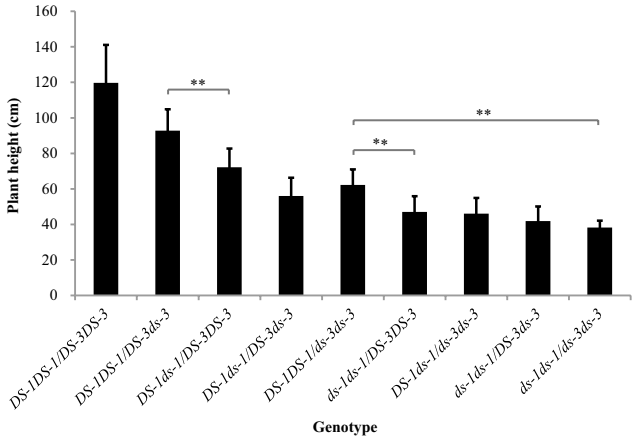


Fig. 4 Allelism and effect test between *DS-1* and *DS-3*. Individuals in F_2 population derived from *ds-1*×*ds-3* were classified into nine genotypes based on two locus-specific markers. *x*-axis represents nine genotypes. *y*-axis represents plant heights of each genotype. The *t* test significance level was set to $P < 0.01$

Of the four *B. napus RGA* genes, the function of *BnaA06.RGA* and *BnaC07.RGA* in regulating stem length had been identified through dwarf mutants in this and previous studies, while the functions of *BnaA09.RGA* and *BnaC09.RGA* are unknown. RT-PCR analysis indicated that each *BnaRGA* gene has similar levels of mRNA transcripts in leaves and stems (Fig. 5b). To address the GA-induced expression patterns of the four *BnaRGAs*, we analyzed the GA-induced kinetics of the relative expression level at different time point (0, 0.5, 1, 3, 6, 8 h) in 7-day-old seedlings treated with 100 μM GA_3 (Fig. 5c). qRT-PCR indicated that *BnaA09.RGA* responds much quicker but its expression is kept at a much lower level than the other *BnaRGA*s. *BnaC07.RGA* and *BnaC09.RGA* have similar expression patterns upon GA_3 induction, their highest levels of expression appeared at 1 h after GA_3 treatment. *BnaA06.RGA* responses much slower and the expression peak appeared after 3-h GA_3 treatment. Its expression level is two or more times higher than that of the other *BnaRGA* genes after GA_3 treatment (Fig. 5c).

To verify if BnaA09.RGA and BnaC09.RGA function as repressors in a GA-dependent manner, we assayed their interaction with the soluble GA receptor AtGID1a using yeast two-hybrid assay. In the absence of GA_3, all four RGA proteins cannot interact with AtGID1a, while they can interact with AtGID1a in the presence of GA_3 (Fig. 3). These results demonstrated that, in addition to *BnaA06.RGA* and *BnaC07.RGA, BnaA09.RGA* and *BnaC09.RGA* also encode functional DELLA proteins and they are likely to play a redundancy function in regulating the growth and development of *B. napus*.

Table 3 Amino acid homology among *Brassica* DELLA homologs

Gene	Identity								
	BolC09.RGA	BnaC09.RGA	BraA09.RGA	BnaA09.RGA	BolC07.RGA	BnaC07.RGA	BraA06.RGA	BnaA06.RGA	AtGAI
AtRGA	82	81	82	83	82	83	80	80	74
AtGAI	81	80	78	79	76	76	76	76	
BnaA06.RGA	82	82	80	82	94	94	99		
BraA06.RGA	82	82	80	82	94	94			
BnaC07.RGA	82	82	80	82	99				
BolC07.RGA	82	82	80	82					
BnaA09.RGA	97	97	97						
BraA09.RGA	97	95							
BnaC09.RGA	99								

Discussion

Lodging can cause severe yield loss in oilseed rape and thus is one of the major restraints for yield stability. Breeding practices in many crops have demonstrated that application of semi-dwarf genes could effectively improve lodging resistance and reduce yield loss (Hedden 2003). In this study, we identified a semi-dwarf mutant *ds-3* in oilseed rape. *ds-3* had a height of about 70 cm at maturity and displayed a reduced response to exogenous GA, which are similar to previously reported dwarf mutants *bzh* (Foisset et al. 1995), *ds-1* (Liu et al. 2010) and *ndf-1* (Wang et al. 2004). The F_1 hybrid between *ds-3* and wild-type HS5 showed a semi-dwarf stature with a mean height of 120 cm, an ideal height for rapeseed production (Zhao and Tian 2009). The height of *ds-3* hybrid is similar to the released hybrid cultivars derived from dwarf genes *Brrga1-d* and *bzh* (Miersch et al. 2016; Muangprom et al. 2006), suggesting that *ds-3* is also a useful gene resource for improvement of lodging resistance in oilseed rape hybrid breeding program.

The molecular mechanism of GA-regulated stem elongation has been revealed in many plant species. In this study, we identified the *DS-3* gene that encodes a DELLA protein acting as a repressor in GA signaling pathway (Davière and Achard 2013). A single nucleotide mutation was identified in *ds-3* (*BnaC07.rga-ds*), which led to a Pro-to-Leu substitution in the VHYNP motif at the N terminus (Fig. 2c). Alignment of DELLA proteins from many plants indicated that the proline residue in this motif is absolutely conserved (Fig. 2d). An identical substitution was also identified in a previously reported dwarf mutant *ds-1* with similar phenotype. Interaction assays between GID1 and DELLA and protein crystallographic analysis had demonstrated that the VHYNP motif and the proline residue are essential for the interaction between GID1 and DELLA (Griffiths et al. 2006; Murase et al. 2008; Ueguchi-Tanaka et al. 2007). Variations that cause truncations or missense mutations in the conserved DELLA and VHYNP motifs in the DELLA domain always render dwarfism to plants including *Arabidopsis*, wheat, maize, barley and oilseed rape (Chandler et al. 2002; Liu et al. 2010; Peng et al. 1997, 1999). Mutations in these motifs damage the binding between DELLA and the GA receptor GID1, which finally blocks the degradation of DELLA protein upon GA induction (Asano et al. 2009; Liu et al. 2010; Wu et al. 2011).

Different plant species have different number of DELLA proteins. The Arabidopsis thaliana genome contains five genes encoding highly homologous DELLA proteins, GAI and RGA, RGA-LIKE1 (RGL1), RGL2 and RGL3. These DELLAs display overlapping but also distinct functions in repressing GA responses (Gallego-Bartolomé et al. 2010; King et al. 2001; Tyler et al. 2004; Wen and Chang 2002). The hexaploid wheat genome has three homologous *RGA* genes (*Rht-A1, Rht-B1* and *Rht-D1*) encoding DELLA proteins. *Rht-B1* and *Rht-D1* redundantly repress GA-responsive growth, and their mutant alleles *Rht-B1b* and *Rht-D1b* show similar semi-dwarf stature (Pearce et al. 2011; Peng et al. 1999). The function of *Rht-A1* is elusive, although it is expressed at comparable levels to the other homologs, because no semi-dwarf alleles of *Rht-A1* have been identified (Pearce et al. 2011). In the *B. napus* genome, four *RGA* orthologs (*BnaA06.RGA, BnaC07.RGA, BnaA09.RGA* and *BnaC09.RGA*) were identified (Fig. 5a, Supplemental Fig. S6). Y2H assays and qRT-PCR indicated that they are functional in GA signaling (Figs. 3, 5c). The two orthologs, *BnaA06.RGA* and *BnaC07.RGA* located in homeologous chromosome regions, have been found to redundantly

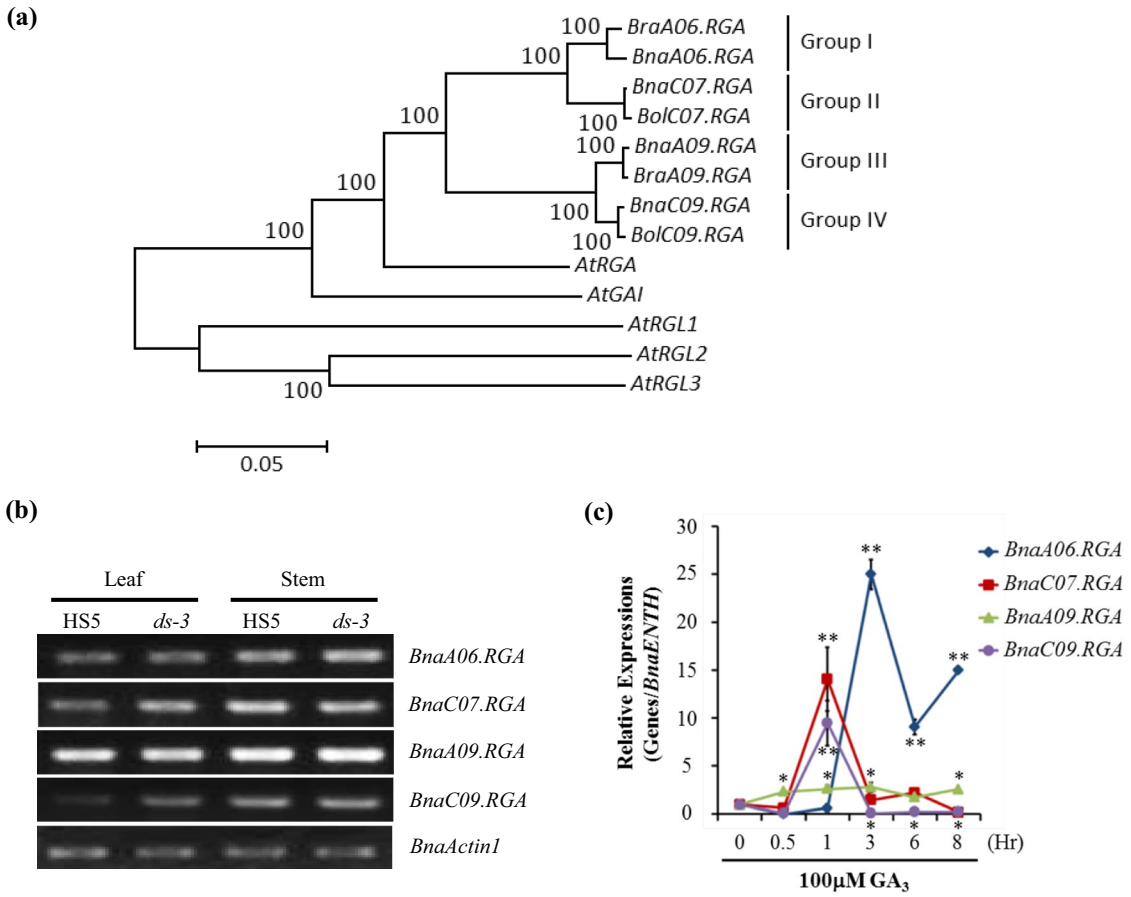


Fig. 5 Phylogenetic relationship and expression analysis of *RGA* homologs in *Brassica napus*. **a** Phylogenetic tree of the *Brassica RGA* and *Arabidopsis* DELLA-encoding genes. The bootstrap neighbor-joining phylogenetic tree was constructed based on nucleotide sequence using MEGA 5.1. The lengths of the branches refer to the nucleotide variation rates. **b** Expression pattern of the four *BnaRGAs* detected by RT-PCR in leaves and stems from cultivar HS5 and mutant *ds-3*. The *BnaActin1* gene was amplified as the equal loading control of RNA samples. **c** The dynamic expression patterns of *BnaRGAs* at different time point. The expression levels were detected by qRT-PCR using 7-day seedlings treated with 100 μM GA_3. The expression level was normalized by *BnaENTH* gene. * and ** denote significant differences at 5 and 1% probability levels, respectively, based on a two-tailed Student's *t* test. *Error bars* represent SDs from three biological replicates

regulate elongation growth in a GA-dependent manner by surveying respective dwarf mutant (Figs. 2a, 3, 4). However, the functions of the other pair of orthologs, *BnaA09.RGA* and *BnaC09.RGA*, are unknown currently because no such dwarf mutants have been identified so far. They may redundantly repress stem elongation with *BnaA06.RGA* and *BnaC07.RGA* because they share over 80% sequence identity (Table 3) and similar expression pattern in leaf and stem as *BnaA06.RGA* and *BnaC07.RGA* (Fig. 5a). Creation of mutants in the *BnaA09.RGA* and *BnaC09.RGA* genes via EMS mutagenesis or CRISPR/Cas9 gene editing is necessary for further characterizing their functions in regulating stem elongation. These two genes represent potential targets for producing novel dwarfing alleles.

In the wheat Green Revolution genes *Rht-B1* and *Rht-D1*, a series of dwarfing alleles were identified conferring different N-terminal mutant forms in terms of the same DELLA give plant distinguishing dwarf stature (Pearce et al. 2011; Peng et al. 1999). Y2H and α-amylase assays indicated that the extent of affinity between DELLAs (Rht-B1c, Rht-B1b and Rht-B1a) and GID1 corresponded to the dwarf severity in *Rht-B1c, Rht-B1b*, and *Rht-B1a* (Wu et al. 2011). Three mutant forms of the *d8* locus, *D8-1, D8-2030* and *D8-mp1* encoding defective N-terminal DELLAs displayed distinguishing dwarf stature (Harberd and Freeling 1989; Peng et al. 1999; Winkler and Freeling 1994). These studies demonstrated that different mutations of the same *RGA* gene or mutations in different *RGA* genes may display different dwarf severity. In this study, the same mutations of *DS-1* (*BnaA06.RGA*) and *DS-3* (*BnaC07.RGA*) displayed different effects on stem elongation. Based on these observations, it is possible to screen for more semi-dwarf

mutants with ideal plant heights to breed for rapeseed cultivars with strong lodging resistance and high yields.

Author contribution statement BZ, HTL, JJL, and CD performed the experiments. BZ, JW and KDL wrote the manuscript. HTL and BW help analyze the data. KDL conceived and supervised the study. All the authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

References

Ariizumi T, Lawrence PK, Steber CM (2011) The role of two F-box proteins, SLEEPY1 and SNEEZY, in *Arabidopsis* gibberellin signaling. Plant Physiol 155:765–775

Asano K, Hirano K, Ueguchi-Tanaka M, Angeles-Shim RB, Komura T, Satoh H, Kitano H, Matsuoka M, Ashikari M (2009) Isolation and characterization of dominant dwarf mutants, *Slr1-d*, in rice. Mol Gen Genet 281:223–231

Chalhoub B, Denoeud F, Liu S, Parki IAP, Tang H, Wang X, Chique J, Belcram H, Tong C, Samans B, Corréa M, Silva CD, Just J, Falentin C, Koh CS, Clainche IL, Bernard M, Bento P, Noel B, Labadie K, Alberti A, Charles M, Arnaud D, Guo H, Daviaud C, Alamery S, Jabbari K, Zhao M, Edger PP, Chelaifa H, Tack D, Lassalle G, Mestiri I, Schnel N, Paslier M-CL, Fan G, Renault V, Bayer PE, Golicz AA, Manoli S, Lee T-H, Thi VHD, Chalabi S, Hu Q, Fan C, Tollenaere R, Lu Y, Battail C, Shen J, Sidebottom CHD, Wang X, Canaguier A, Chauveau A, Bérard A, Deniot G, Guan M, Liu Z, Sun F, Lim YP, Lyons E, Town CD, Bancroft I, Wang X, Meng J, Ma J, Pires JC, King GJ, Brunel D, Delourme R, Renard M, Aury J-M, Adams KL, Batley J, Snowdon RJ, Tost J, Edwards D, Zhou Y, Hua W, Sharpe AG, Paterson AH, Guan C, Wincker P (2014) Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science 345:950–953

Chandler PM, Marion-Poll A, Ellis M, Gubler F (2002) Mutants at the *Slender1* locus of barley cv Himalaya. Molecular and physiological characterization. Plant Physiol 129:181–190

Cheng H, Qin L, Lee S, Fu X, Richards D, Cao D, Luo D, Harberd N, Peng J (2004) Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. Development 131:1055–1064

Cheng X, Xu J, Xia S, Gu J, Yang Y, Fu J, Qian X, Zhang S, Wu J, Liu K (2009) Development and genetic mapping of microsatellite markers from genome survey sequences in *Brassica napus*. Theor Appl Genet 118:1121–1131

Cheng F, Liu S, Wu J, Fang L, Sun S, Liu B, Li P, Hua W, Wang X (2011) BRAD, the genetics and genomics database for *Brassica* plants. BMC Plant Biol 11:136

Cheung F, Trick M, Drou N, Lim YP, Park JY, Kwon SJ, Kim JA, Scott R, Pires JC, Paterson AH, Town C, Bancroft I (2009) Comparative analysis between homoeologous genome segments of *Brassica napus* and its progenitor species reveals extensive sequence-level divergence. Plant Cell 21:1912–1928

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Davière J-M, Achard P (2013) Gibberellin signaling in plants. Development 140:1147–1151

Davière J-M, Achard P (2016) A pivotal role of DELLAs in regulating multiple hormone signals. Mol Plant 9:10–20

Doyle JJ (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Foisset N, Delourme R, Barret P, Renard M (1995) Molecular tagging of the dwarf *BREIZH* (*Bzh*) gene in *Brassica napus*. Theor Appl Genet 91:756–761

Foisset N, Delourme R, Barret P, Hubert N, Landry B, Renard M (1996) Molecular-mapping analysis in *Brassica napus* using isozyme, RAPD and RFLP markers on a doubled-haploid progeny. Theor Appl Genet 93:1017–1025

Gallego-Bartolomé J, Minguet EG, Marín JA, Prat S, Blázquez MA, Alabadí D (2010) Transcriptional diversification and functional conservation between DELLA proteins in *Arabidopsis*. Mol Biol Evol 27:1247–1256

Griffiths J, Murase K, Rieu I, Zentella R, Zhang Z-L, Powers SJ, Gong F, Phillips AL, Hedden P, Sun TP, Thomas SG (2006) Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. Plant Cell 18:3399–3414

Harberd NP, Freeling M (1989) Genetics of dominant gibberellin-insensitive dwarfism in maize. Genetics 121:827–838

Hedden P (2003) The genes of the Green Revolution. Trends Genet 19:5–9

Hirano K, Asano K, Tsuji H, Kawamura M, Mori H, Kitano H, Ueguchi-Tanaka M, Matsuoka M (2010) Characterization of the molecular mechanism underlying gibberellin perception complex formation in rice. Plant Cell 22:2680–2696

King KE, Moritz T, Harberd NP (2001) Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. Genetics 159:767–776

Kosambi DD (1943) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Li H, Chen X, Yang Y, Xu J, Gu J, Fu J, Qian X, Zhang S, Wu J, Liu K (2011a) Development and genetic mapping of microsatellite markers from whole genome shotgun sequences in *Brassica oleracea*. Mol Breed 28:585–596

Li H, Wang Y, Li X, Gao Y, Wang Z, Zhao Y, Wang M (2011b) A GA-insensitive dwarf mutant of *Brassica napus* L. correlated with mutation in pyrimidine box in the promoter of *GID1*. Mol Biol Rep 38:191–197

Li H, Younas M, Wang X, Li X, Chen L, Zhao B, Chen X, Xu J, Hou F, Hong B (2013) Development of a core set of single-locus SSR markers for allotetraploid rapeseed (*Brassica napus* L.). Theor Appl Genet 126:937–947

Liu C, Wang J, Huang T, Wang F, Yuan F, Cheng X, Zhang Y, Shi S, Wu J, Liu K (2010) A missense mutation in the VHYNP motif of a DELLA protein causes a semi-dwarf mutant phenotype in *Brassica napus*. Theor Appl Genet 121:249–258

Liu S, Liu Y, Yang X, Tong C, Edwards D, Parkin IAP, Zhao M, Ma J, Yu J, Huang S, Wang X, Wang J, Lu K, Fang Z, Bancroft I, Yang T-J, Hu Q, Wang X, Yue Z, Li H, Yang L, Wu J, Zhou Q, Wang W, King GJ, Pires JC, Lu C, Wu Z, Sampath P, Wang Z, Guo H, Pan S, Yang L, Min J, Zhang D, Jin D, Li W, Belcram H, Tu i, Guan M, Qi C, Du D, Li J, Jiang L, Batley J, Sharpe AG, Park B-S, Ruperao P, Cheng F, Waminal NE, Huang Y, Dong C, Wang L, Li J, Hu Z, Zhuang M, Huang Y,

Huang J, Shi J, Mei D, Liu J, Lee T-H, Wang J, Jin H, Li Z, Li X, Zhang J, Xiao L, Zhou Y, Liu Z, Liu X, Qin R, Tang X, Liu W, Wang Y, Zhang Y, Lee J, Kim HH, Denoeud F, Xu X, Liang X, Hua W, Wang X, Wang J, Chalhoub B, Paterson AH (2014) The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploid genomes. Nat Commun 5:3930

Michelmore RW, Paran I, Kesseli R (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Miersch S, Gertz A, Breuer F, Schierholt A, Becker HC (2016) Influence of the semi-dwarf growth type on seed yield and agronomic parameters at low and high nitrogen fertilization in winter oilseed rape. Crop Sci 56:1573–1585

Monna L, Kitazawa N, Yoshino R, Suzuki J, Masuda H, Maehara Y, Tanji M, Sato M, Nasu S, Minobe Y (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

Muangprom A, Thomas SG, Sun TP, Osborn TC (2005) A novel dwarfing mutation in a green revolution gene from *Brassica rapa*. Plant Physiol 137:931–938

Muangprom A, Mauriera I, Osborn TC (2006) Transfer of a dwarf gene from *Brassica rapa* to oilseed *B. napus*, effects on agronomic traits, and development of a 'perfect'marker for selection. Mol Breed 17:101–110

Murase K, Hirano Y, Sun TP, Hakoshima T (2008) Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. Nature 456:459–463

Neff MM, Turk E, Kalishman M (2002) Web-based primer design for single nucleotide polymorphism analysis. Trends Genet 18:613–615

Østergaard L, King GJ (2008) Standardized gene nomenclature for the *Brassica* genus. Plant Methods 4:10

Parkin IA, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics 171:765–781

Pearce S, Saville R, Vaughan SP, Chandler PM, Wilhelm EP, Sparks CA, Al-Kaff N, Korolev A, Boulton MI, Phillips AL, Hedden P, Nicholson P, Thomas SG (2011) Molecular characterization of *Rht-1* dwarfing genes in hexaploid wheat. Plant Physiol 157:1820–1831

Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The Arabidopsis *GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Gene Dev 11:3194–3205

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) 'Green revolution' genes encode mutant gibberellin response modulators. Nature 400:256–261

Raman H, Raman R, Kilian A, Detering F, Long Y, Edwards D, Parkin IA, Sharpe AG, Nelson MN, Larkan N, Zou J, Meng J, Aslam MN, Batley J, Cowling WA, Lydiate D (2013) A consensus map of rapeseed (*Brassica napus* L.) based on diversity array technology markers: applications in genetic dissection of qualitative and quantitative traits. BMC Genom 14:277

Rosa NM-dl, Pfeiffer A, Hill K, Locascio A, Bhalerao RP, Miskolczi P, Grønlund AL, Wanchoo-Kohli A, Thomas SG, Bennett MJ, Lohmann JU, Blázquez MA, Alabadí D (2015) Genome wide binding site analysis reveals transcriptional coactivation of cytokinin-responsive genes by DELLA proteins. PLoS Genet 11:e1005337

Sasaki A, Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Kobayashi M, Khush GS, Kitano H, Matsuoka M (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. Nature 416:701–702

Silverstone AL, Mak PYA, Martinez EC, Sun TP (1997) The new *RGA* locus encodes a negative regulator of gibberellin response in *Arabidopsis thaliana*. Genetics 146:1087–1099

Silverstone AL, Ciampaglio CN, Sun TP (1998) The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell 10:155–169

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

Sun TP (2010) Gibberellin-GID1-DELLA: a pivotal regulatory module for plant growth and development. Plant Physiol 154:567–570

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP (2004) DELLA proteins and gibberellin-regulated seed germination and floral development in *Arabidopsis*. Plant Physiol 135:1008–1019

Ueguchi-Tanaka M, Nakajima M, Katoh E, Ohmiya H, Asano K, Saji S, Hongyu X, Ashikari M, Kitano H, Yamaguchi I, Matsuoka M (2007) Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. Plant Cell 19:2140–2155

Van Ooijen J (2006) JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma BV, Wageningen 33:10–1371

Wang M, Zhao Y, Chen F, Yin X (2004) Inheritance and potentials of a mutated dwarfing gene *ndf1* in *Brassica napus*. Plant Breed 123:449–453

Wang J, Lydiate DJ, Parkin IA, Falentin C, Delourme R, Carion PW, King GJ (2011a) Integration of linkage maps for the amphidiploid *Brassica napus* and comparative mapping with *Arabidopsis* and *Brassica rapa*. BMC Genom 12:101

Wang X, Wang H, Wang J, Sun R, Wu J, Liu S, Bai Y, Mun J-H, Bancroft I, Cheng F, Huang S, Li X, Hua W, Wang J, Wang X, Freeling M, Pires JC, Paterson AH, Chalhoub B, Wang B, Hayward A, Sharpe AG, Park B-S, Weisshaar B, Liu B, Li B, Liu B, Tong C, Song C, Duran C, Peng C, Geng C, Koh C, Lin C, Edwards D, Mu D, Shen D, Soumpourou E, Li F, Fraser F, Conant G, Lassalle G, King GJ, Bonnema G, Tang H, Wang H, Belcram H, Zhou H, Hirakawa H, Abe H, Guo H, Wang H, Jin H, Parkin IAP, Batley J, Kim J-S, Just J, Li J, Xu J, Deng J, Kim JA, Li J, Yu J, Meng J, Wang J, Min J, Poulain J, Wang J, Hatakeyama K, Wu K, Wang L, Fang L, Trick M, Links MG, Zhao M, Jin M, Ramchiary N, Drou N, Berkman PJ, Cai Q, Huang Q, Li R, Tabata S, Cheng S, Zhang S, Zhang S, Huang S, Sato S, Sun S, Kwon S-J, Choi S-R, Lee T-H, Fan W, Zhao X, Tan X, Xu X, Wang Y, Qiu Y, Yin Y, Li Y, Du Y, Liao Y, Lim Y, Narusaka Y, Wang Y, Wang Z, Li Z, Wang Z, Xiong Z, Zhang Z (2011b) The genome of the mesopolyploid crop species *Brassica rapa*. Nat Genet 43:1035–1039

Wang Y, He J, Yang L, Wang Y, Chen W, Wan S, Chu P, Guan R (2016) Fine mapping of a major locus controlling plant height using a high-density single-nucleotide polymorphism map in *Brassica napus*. Theor Appl Genet 129:1479–1491

Wen C-K, Chang C (2002) *Arabidopsis RGL1* encodes a negative regulator of gibberellin responses. Plant Cell 14:87–100

Willige BC, Ghosh S, Nill C, Zourelidou M, Dohmann EM, Maier A, Schwechheimer C (2007) The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE

DWARF1A gibberellin receptor of *Arabidopsis*. Plant Cell 19:1209–1220

Winkler RG, Freeling M (1994) Physiological genetics of the dominant gibberellin-nonresponsive maize dwarfs, *Dwarf8* and *Dwarf9*. Planta 193:341–348

Wu J, Kong X, Wan J, Liu X, Zhang X, Guo X, Zhou R, Zhao G, Jing R, Fu X, Jia J (2011) Dominant and pleiotropic effects of a *GAI* gene in wheat results from a lack of interaction between DELLA and GID1. Plant Physiol 157:2120–2130

Xu J, Qian X, Wang X, Li R, Cheng X, Yang Y, Fu J, Zhang S, King GJ, Wu J, Liu K (2010) Construction of an integrated genetic linkage map for the A genome of *Brassica napus* using SSR markers derived from sequenced BACs in *B. rapa*. BMC Genom 11:594

Yang T-J, Kim JS, Kwon S-J, Lim K-B, Choi B-S, Kim J-A, Jin M, Park JY, Lim M-H, Kim H-I, Lim YP, Kang JJ, Hong J-H, Kim C-B, Bhak J, Bancroft I, Park B-S (2006) Sequence-level analysis of the diploidization process in the triplicated *FLOWERING LOCUS C* region of *Brassica rapa*. Plant Cell 18:1339–1347

Yang H, Liu J, Huang S, Guo T, Deng L, Hua W (2014) Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in *Brassica napus* L. Gene 538:113–122

Yi B, Zeng F, Lei S, Chen Y, Yao X, Zhu Y, Wen J, Shen J, Ma C, Tu J, Fu T (2010) Two duplicate *CYP704B1*-homologous genes *BnMs1* and *BnMs2* are required for pollen exine formation and tapetal development in *Brassica napus*. Plant J 63:925–938

Yoshida H, Hirano K, Sato T, Mitsuda N, Nomoto M, Maeo K, Koketsu E, Mitani R, Kawamura M, Ishiguro S, Tada Y, Ohme-Takagi M, Matsuoka M, Ueguchi-Tanaka M (2014) DELLA protein functions as a transcriptional activator through the DNA binding of the indeterminate domain family proteins. Proc Natl Acad Sci USA 111:7861–7866

Youssefian S, Kirby E, Gale M (1992) Pleiotropic effects of the GA-insensitive *Rht* dwarfing genes in wheat. 2. Effects on leaf, stem, ear and floret growth. Field Crop Res 28:191–210

Zeng X, Zhu L, Chen Y, Qi L, Pu Y, Wen J, Yi B, Shen J, Ma C, Tu J, Fu T (2011) Identification, fine mapping and characterisation of a dwarf mutant (*bnaC.dwf*) in *Brassica napus*. Theor Appl Genet 122:421–428

Zhang B, Liu C, Wang Y, Yao X, Wang F, Wu J, King GJ, Liu K (2015) Disruption of a *CAROTENOID CLEAVAGE DIOXYGENASE 4* gene converts flower colour from white to yellow in *Brassica* species. New Phytol 206:1513–1526

Zhao F, Tian Z (2009) Research and utilization of dwarf or semi dwarf gene resources in rapeseed. Crop Res 23:157–160 (**in Chinese**)