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ORIGINAL PAPER

A missense mutation in the VHYNP motif of a DELLA protein causes a semi-dwarf mutant phenotype in *Brassica napus*

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Abstract Although dwarf genes have been widely used to improve lodging resistance and enhance harvest index in cereal crops, lodging is still a serious problem in rapeseed (*Brassica napus*) production. A semi-dwarf *B. napus* mutant, *ds-1*, was identified through EMS mutagenesis of a microspore-cultured DH line. The mutant had a significant reduction in height due to a lower first branch position and shorter internodes when compared with wild-type cultivars. This dwarfism was inherited as a single semi-dominant gene, *ds-1*. *DS-1* locus was mapped to chromosome A6, and co-segregated with a microsatellite marker BnEMS1125 derived from the gene *BnRGA*. *BnRGA* encodes a DELLA protein that functions as a GA signaling repressor. The expression of a mutant *BnRGA* allele from *ds-1*, *Bnrga-ds,* caused dwarf phenotypes in *Arabidopsis*. Comparative sequencing of *RGA* open-reading frames (ORFs) of *ds-1* and wild-type cultivars revealed a single proline (P)-to-leucine (L) substitution that may lead to a gain-of-function mutation in GA signaling. The expression of the *Arabidopsis* homolog, *Atrga-ds*, bearing this site-directed mutation also rendered dwarf phenotypes in *Arabidopsis*, which demonstrated that the P-to-L mutation in the VHYNP motif of Bnrga-ds is responsible for the dwarfism. A yeast two-hybrid assay confirmed that this mutation inhibited the interaction between Bnrga-ds/Atrga-ds and the GA receptor, AtGID1A, in the presence of GA_3, suggesting that the conserved proline residue in the VHYNP motif of DELLA protein directly participates in DELLA-GID1 interaction. Identification and characterization of the dwarf gene *ds-1* will facilitate its utilization in improving lodging resistance in *Brassica* breeding.

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Introduction

Lodging is an important problem in rapeseed (*Brassica napus*) production, which can lead to severe yield loss (Islam and Evans 1994) and difficulty harvesting. The extensive planting of hybrid cultivars exacerbates lodging due to increased plant height (Foisset et al. 1995). The introduction of semi-dwarf genes into rice (*Oryza sativa*) and wheat (*Triticum aestivum*) cultivars effectively overcame lodging and increased harvest index, which was a critical factor for the success of the Green Revolution.

Gibberellins (GAs) are essential phytohormones that regulate many aspects of plant growth and development, including seed germination, leaf expansion, stem and root extension, flower induction and development, seed development, and fruit expansion (Fleet and Sun 2005; Swain and Singh 2005). The dwarf genes, *sd1* in rice and *Rht-B1b* and *Rht-D1b* in wheat, used in the Green Revolution are involved in the GA biosynthesis and signaling pathways, respectively. 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. The 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, wheat mutants containing dwarf genes *Rht-B1b*

and *Rht-D1b* could not be rescued with exogenous GA treatment. The wild-type *Rht* alleles, *Rht-B1a* and *Rht-D1a*, encode DELLA proteins that serve as GA signaling repressors. GA-induced degradation of DELLA proteins is required for normal GA signaling. *Rht-B1b* and *Rht-D1b* encode mutant DELLA proteins that are resistant to GA-induced degradation and GA signaling is constitutively blocked (Peng et al. 1999; Silverstone et al. 2001).

DELLA proteins belong to the GRAS protein family that regulates diverse aspects of plant growth and have been identified from several plant species. In *Arabidopsis*, the DELLA subfamily has five members, RGA, GAI, RGL1, RGL2 and RGL3. Except for RGL3, the other four members have partially overlapping functions in repressing GA-regulated plant growth and development (Lee et al. 2002; Peng et al. 1997; Silverstone et al. 1998; Wen and Chang 2002). Their orthologs, rice SLR1 (Ikeda et al. 2001; Itoh et al. 2002), maize (*Zea mays*) d8 (Peng et al. 1999), barley (*Hordeum vulgare*) SLN1 (Chandler et al. 2002; Fu et al. 2002; Gubler et al. 2002), grape (*Vitis vinifera*) VvRGA (Boss and Thomas 2002), *B. rapa* BrRGA1 (Muangprom et al. 2005) and tomato (*Solanum lycopersicum*) LeGAI (Bassel et al. 2004), have conserved functions as GA signaling repressors. Although the biochemical function of DELLA proteins are not well defined, recent results have shown that RGA interacts with the promoters of its response genes and activates their expression (Zentella et al. 2007). In addition, DELLA proteins can also interact with Phytochrome Interacting Factor 3/4 (PIF3/4), a bHLH transcription factor, to prevent PIF3/4 from promoter binding and restrain transcriptional activation (de Lucas et al. 2008). DELLA proteins have a conserved DELLA domain at their N-terminus, which contains a DELLA motif, a VHYNP motif and a poly S/T region. Most gain-of-function mutations in DELLA proteins are present in the DELLA domain. Deletions in the DELLA motif and/or VHYNP motif (*Arabidopsis* gai-Δ17; wheat Rht-B1b and Rht-D1b; maize D8-1, D8-MP1, D8-2023) or a point mutation in the DELLA motif (grape Vvgai1; barley Sln1d) rendered DELLA proteins resistant to GA-induced degradation and resulted in dwarf phenotypes. Therefore, the DELLA domain is important for the degradation of DELLA proteins (Thomas and Sun 2004). DELLA proteins also have a conserved GRAS domain at their C-terminus, which includes a leucine heptad repeat I (LHR I) motif, a VHIID motif, a leucine heptad repeat II (LHR II) motif, a PFYRE motif, and a SAW motif (Pysh et al. 1999). All loss-of-function mutations in rice and barely were found to occur in this domain and cause a slender phenotype (Chandler et al. 2002; Fu et al. 2002; Ikeda et al. 2001; Itoh et al. 2002). Thus, the GRAS domain is also required for DELLA proteins to function as GA signaling repressors.

DELLA protein degredation is a critical event in the GA signaling pathway. In addition to GA, the soluble GA receptor GID1 (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2005, 2007; Willige et al. 2007), and E3 ubiquitin ligase $SCF^{SLY1/GID2}$ (Dill et al. 2004; Gomi et al. 2004; McGinnis et al. 2003) are involved in this process. When a bioactive GA molecule binds to GID1 to form a GA-GID1 complex, GID1 can interact with DELLA protein at the DELLA domain to form a GA-GID1–DELLA complex, which induces a conformational change of the GRAS domain of DELLA protein into the form that can be recognized by the F-box protein SLY1/GID2. SLY1/GID2-DELLA interaction leads to the polyubiquitylation of DELLA protein, which are then degraded by the 26S proteasome. Yeast two-hybrid assays showed that the DELLA domain is the DELLA-GID1 interacting region (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2007; Willige et al. 2007).

In this study, we identified and characterized a semi-dwarf mutant *ds-1* in *B. napus*, and demonstrated that a gain-of-function point mutation in the VHYNP motif of a DELLA protein rendered its dwarfism. The effect of this mutation on the interaction between DELLA and GA receptor was also investigated.

Materials and methods

Plant materials

The dwarf mutant *ds-1* was a double haploid (DH) line obtained from EMS-treated microspore cultures of a breeding line, 92-B10. Double-low (low erucic acid and low glucosinolate) cultivar Zhong Shuang 4 (ZS4) was used as a wild-type control for trait evaluation and mapping population.

Field trial and trait measurements

One field trial was carried out in Changyang county, Hubei province, in the summer of 2000. Four rows of *ds-1* and ZS4 plants (10 plant/row) were grown side-by-side in the field. At maturity, 19 *ds-1* and 11 ZS4 plants in the middle rows were phenotyped for plant height-related traits in the field. Plant height was measured from the ground to the tip of the main inflorescence. First primary branch height was measured from the ground to the base of first primary branch. Main inflorescence length was measured from the base of the last primary branch to the highest point of the inflorescence. The number of total nodes was counted for all nodes of the main stem. Average internode length was measured as last primary branch height divided by the number of total nodes. The analysis of variation was performed on these experimental data to test trait difference between *ds-1* and ZS4.

To map the *ds-1* gene, an F_2 population containing 486 plants was derived from the cross between *ds-1* and ZS4. In the growing season of 2005–2006, F_2 plants along with parental lines *ds-1* and ZS4 and F_1 plants were grown at the experimental station of Huazhong Agricultural University, Wuhan, Hubei province. Young leaves were collected from each F_2 plant for molecular marker analysis. At maturity, plant height of each individual was measured in the field. Each F_2 plant was self-pollinated to obtain F_3 seeds. In addition, 10–20 plants for each F_3 family were grown to investigate the segregation of plant heights to confirm the genotype of corresponding F_2 plant in Wuhan in the growing season of 2006–2007.

DNA extraction and marker analysis

Genomic DNA was extracted from young leaves using the CTAB method (Doyle and Doyle 1987). DNA concentration was measured by a Beckman spectrophotometer (Beckman, Fullerton, USA) and adjusted to a final concentration of 25 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Equal amounts of genomic DNA from ten extremely short plants and ten extremely tall plants from the F_2 mapping population were pooled separately to construct the short (SB) and tall bulks (TB), respectively. The dwarf and wild-type parental DNAs and the two bulks were subject to bulked segregant analysis (BSA) (Michelmore et al. 1991) with SSR markers.

Marker analysis was performed as described previously (Zhang et al. 2007). SSRs prefixed by Na, Ra, Ol, Ni, BN, MB, BRMS and MR were obtained from http://www.brassica.info/ssr/SSRinfo.htm. The markers prefixed by sN, sR and sO are developed by Agriculture and Agri-Food Canada (http://brassica.agr.gc.ca/index_e.shtml), the FITO markers were obtained at http://www.osbornlab.agronomy.wisc.edu/research/maps/ssrs.html and BRAS and CB were from Piquemal et al. (2005). Primers prefixed "BnGMS" (Cheng et al. 2009), "BnEMS", "BrGMS" and "BoGMS" (unpublished data) were developed by us. The PCR procedure and primer sequences for SCAR marker SCM07 were previously described (Barret et al. 1998). Linkage analysis was performed using JoinMap3.0 (Van and Voorrips 2001). The threshold for goodness-of-fit was set to $\leq$5.0, a recombination frequency of <0.4, and minimum logarithm of odds scores of 2.0. All genetic distances were expressed in centimorgans as derived by the Kosambi (1944) function.

RNA extraction and RT-PCR analysis

Both ZS4 and *ds-1* plants were grown in the greenhouse under conditions of 25°C and 16 h/day light. Leaves from 4-week-old seedlings were harvested for RNA extraction with the Trizol reagent (Invitrogen, Carlsbad, USA) to compare the transcriptional levels of *BnRGA*. Equal amount of total RNA was reverse-transcribed with M-MLV reverse-transcriptase (Fermentas, Vilnius, Lithuania). Primers BnRGA-1F (5′-TCTAATCAGTTCGCGTTTGC-3′) and BnRGA-1R (5′-ATCTGCTTAACGAGAGCCTC-3′) were used to amplify a 260-bp fragment of *BnRGA*. The *Actin* gene was amplified using primers Actin-F (5′-TGCG ACAATGGAACTGGAATG-3′) and Actin-R (5′-CAAGA CGTAGGATAGCATGTG-3′) as the equal loading control of RNA samples between the two genotypes.

Amplification and sequencing of the *RGA* coding regions

The 2.1kb-*RGA* fragments were PCR amplified from *ds-1* and ZS4, respectively, using primers BnRGA-F (5′-GATC AGAAATGAAGAGGGATC-3′) and BnRGA-R (5′-GAT TTACAGTTCACACATTGTGAAG-3′) designed from the *BrRGA1* sequence (GenBank accession number AY928549) of *B. rapa*, and cloned into the pCR2.1 T-vector (Invitrogen). Ten positive clones were sequenced to obtain the consensus sequences for *ds-1* and ZS4, respectively. The *RGA* fragments were also amplified from other 12 wild-type cultivars (Rainbow, Oscar, Monty, Glacier, Surpass 600, Quantum 1, Quantum 2, Cao 221167, Sc-ug 3, Sc-ug 4, Sc-ug 8, and Pinnacle). The PCR products were sequenced directly, and aligned with the sequences from ZS4 and *ds-1* to identify single-nucleotide polymorphisms (SNPs).

Transformation assay

To generate plasmids for *Arabidopsis* transformation, a 3.3-kb DNA fragment including the *BnRGA* promoter region (1.2-kb upstream of the start codon), the entire ORF (1.7 kb) and the 3′-terminator region (0.4-kb sequence downstream of the stop codon) was amplified from ZS4 and *ds-1*, respectively, with primers BnRGA-PF (5′-CAC*CTG CAG*AGGTCTGATGACTTTCCAAAACCC-3′) and RGA-ER (5′-CGC*GAATTC*GATTTACAGTTCACACAT TGTGAAG-3′). The PCR reaction mixture (50 μl) contained 1× Phusion polymerase buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 1 U of Phusion DNA polymerase (New England Biolabs, Beijing, China) and 50 ng of genomic DNA. PCR amplification was conducted with a first denaturation step of 96°C/30 s, 30 cycles of 96°C/10 s, 60°C/20 s, 68°C/1 min 30 s, and a final extension step of 68°C/5 min. The PCR products from ZS4 and *ds-1* were digested with *Pst*I and *Eco*RI, then ligated into the binary vector pCAMBIA2300 to produce construct pBnRGA:BnRGA and pBnRGA:Bnrga-ds, respectively. The two constructs were transformed into wild-type *Arabidopsis* (Columbia ecotype) using an *Agrobacterium*-mediated floral-dip method (Clough and Bent 1998). Transgenic lines that exhibited a 3:1 ratio for kanamycin resistant: sensitive

in the T_2 generation was selected to obtain homozygous transgenic plants for further analysis.

Site-directed mutagenesis

A 4.44-kb genomic DNA fragment including the 1.69-kb upstream region, 1.76-kb ORF and 0.99-kb 3′ UTR of *AtRGA* gene was amplified from the wild-type *Arabidopsis* (ecotype Columbia) using primers AtRGA-F (5′-AGA*CTGCAG*GCGTTAAGCAATGTAACGGGAATCTC-3′) and AtRGA-R (5′-AGA*CTGCAG*CTGAGCTTCTACTAATTTCTGGAACC-3′), then digested with *Pst*I and cloned into pBluescript KS (−) (Stratagene, La Jolla, USA) to generate the pKS-AtRGA construct. Site-directed mutagenesis of *AtRGA* was performed using the Phusion site-directed mutagenesis kit (New England Biolabs, Beijing, China). A pair of 5′-end phosphorylated primers VHYNL-F (5′-GTTCATTATAATCTGTCGGAGCTTTA-3′) and VHYNL-R (5′-AGTATCCGTCGCGAGATGAGATAAAC-3′) was used to introduce a P-to-L mutation into the VHYNP motif of AtRGA. After amplification with primers VHYNL-F and VHYNL-R, the 7.4-kb PCR product was circularized through self-ligation by T4 DNA ligase to generate the pKS-Atrga-ds construct. After sequencing, the mutated *Atrga-ds* gene was inserted into pCAMBIA2300 to form plasmid pAtRGA:Atrga-ds, which was transformed into wild-type *Arabidopsis*. The wild-type *AtRGA* gene was also cloned into pCAMBIA2300 to form the plasmid pAtRGA:AtRGA to produce transgenic plants and used as control.

Yeast two-hybrid assay

The ORFs of *BnRGA* and *Bnrga-ds* were amplified from ZS4 and *ds-1* using primers AD-BnRGA-F (5′-GTC*GAATTC*ATGAAGAGGGATCTTCATCAGTTC-3′) and AD-BnRGA-R (5′-ATA*GGATCC*GTGCACCGCCGAGAGCTTC-3′), respectively. The ORFs of *AtRGA* and *Atrga-ds* were amplified from pKS-AtRGA and pKS-Atrga-ds using primers AD-AtRGA-F (5′-ATA*CCATGG*AGATGAAGAGAGATCATCACCAAT-3′) and AD-AtRGA-R (5′-ATA*GGATCC*GTACGCCGCCGTCGAGAG-3′), respectively. The ORF of *AtGID1A* was generated by RT-PCR using primers DB-AtGID1A-F (5′-AGT*GAATTC*ATGGCTGCGAGCGATGAAG-3′) and DB-AtGID1A-R (5′-ATA*GGATCC*ACATTCCGCGTTTACAAACGC-3′) from *Arabidopsis* Columbia ecotype mRNA. The ORFs of *BnRGA, Bnrga-ds, AtRGA* and *Atrga-ds* were fused to the activation domain (AD) of pGADT7 (Clontech, Palo Alto, USA) to generate constructs pAD-BnRGA, pAD-Bnrga-ds, pAD-AtRGA and pAD-Atrga-ds, respectively. The ORF of *AtGID1A* was fused to the BD domain of pGBKT7 (Clontech) to form the pBD-GID1A plasmid. The AD and BD fusion constructs were co-transformed into *Saccharomyces cerevisiae* strain AH109 and the transformants were screened on SD/Leu–Trp media. Positive transformants were further streaked on SD/-Leu-Trp-His-Ade media containing X-α-gal to test the interaction between AD and BD fusion proteins in the absence and presence of 100 μM GA_3.

Results

Morphological traits of *ds-1* mutant

The *ds-1* mutant was isolated from DH plants regenerated from EMS-treated somatic embryos of microspore cultures. At the seedling stage, *ds-1* showed prostrate growth with dark-green leaves. At maturity, the height of *ds-1* was significantly reduced as compared to the wild-type cultivar ZS4; *ds-1* grew to only 58% of the height of ZS4. *ds-1* had a similar main inflorescence length and number of total nodes to ZS4. However, *ds-1* had a first primary branch at a significantly lower position on the main stems than ZS4. The average internode length of *ds-1* was also significantly shorter (Table 1). Therefore, the reduced height of *ds-1* was primarily due to shorter internodes and the lower position of the first primary branch.

Genetic analysis of *ds-1* mutant

The inheritance of the dwarf phenotype in *ds-1* was analyzed using a cross between *ds-1* and ZS4. F_1 plants had intermediate plant heights compared to the two parents (Fig. 1a). Plant heights in the F_2 population showed a

Table 1 Comparison of plant height and its component traits between ZS4 and *ds-1*

Trait	ZS4	*Ds-1*	*F* value	*P* value
Plant height (cm)	119.5 ± 11.5[a]	69.3 ± 7.7	206.14	0.0000
First branch height (cm)	53.5 ± 11.2	31.5 ± 8.1	107.28	0.0000
Main inflorescence length (cm)	42.9 ± 5.1	37.8 ± .6.2	5.24	0.0298
Number of total nodes	26.1 ± 2.3	27.3 ± 1.9	2.22	0.1472
Average internode length (cm)	2.86 ± 0.2	1.16 ± 0.2	695.22	0.0000

[a] Mean ± standard deviation

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

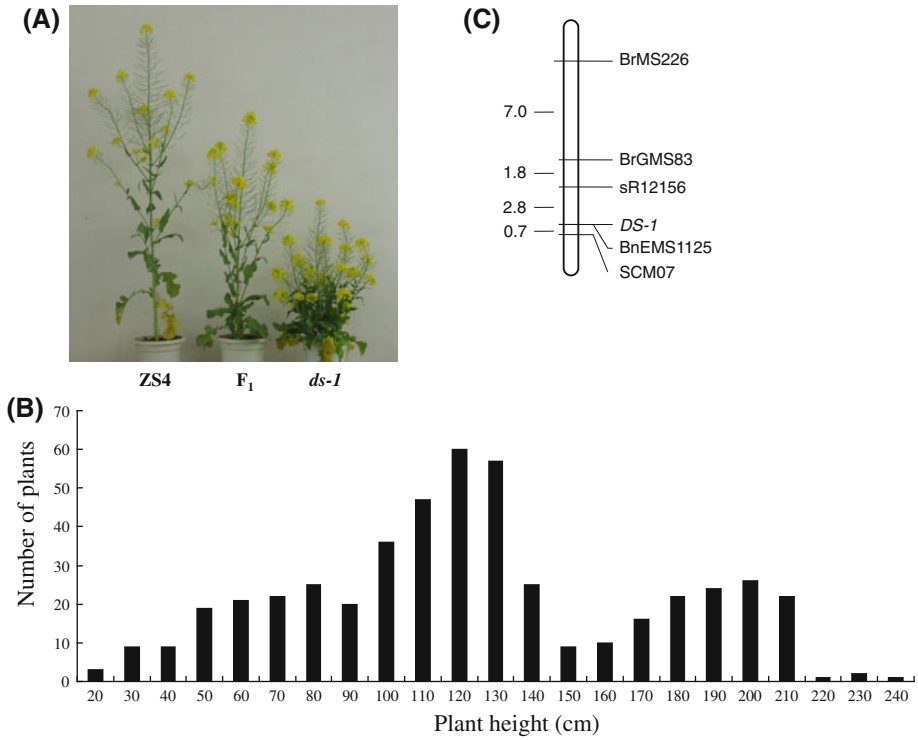

Fig. 1 Genetic mapping of the *DS-1* gene. **a** Phenotypes of ZS4 (*left*), *ds-1* (*right*) and their F_1 hybrid (*center*). **b** Plant height distribution of F_2 population derived from the cross of *ds-1* and ZS4. **c** Map location of *DS-1* locus on chromosome A6 in *B. napus*. The *numbers* at *left* are the distance between the markers (in centimorgans)

trimodal distribution from 25 to 240 cm, with three peaks at about 80, 120 and 200 cm. All F_2 individuals could be roughly classified into three groups corresponding to putative homozygous dwarf, heterozygous, and homozygous wild-type genotypes (Fig. 1b). All F_2 plants were self-pollinated to harvest F_3 seeds; 10 to 20 F_3 progeny were grown from each F_2 individual to confirm its corresponding F_2 genotype. Progeny testing showed that the F_2 population contained 118 homozygous dwarf, 245 heterozygous intermediate and 123 homozygous tall plants, and the segregation fit an expected Mendelian inheritance ratio of 1:2:1 ($\chi^2 = 0.136$, $0.90 < P < 0.95$). These data indicated that the dwarf phenotype of *ds-1* was controlled by a single semi-dominant allele. This interpretation was supported by the lack of segregation of progeny of the dwarf and tall plants, whereas the progeny derived from intermediate plants segregated for plant height.

Genetic mapping of the *DS-1* gene

The BSA strategy was used to identify molecular markers linked to *DS-1*. Ten extremely short and tall homozygous plants in the F_2 population were separately selected and pooled to make a short (SB) and a tall (TB) bulk, respectively. A total of 640 publicly available SSR markers were used to survey polymorphisms between the dwarf and tall parents and bulks. Only sR12156 detected polymorphisms between the dwarf and tall parents and bulks. sR12156 detects two loci, one of which was previously mapped to chromosome A6 of *B. napus* (Chen et al. 2007).

Comparative mapping revealed that chromosome A6 in the A-genome is homologous to chromosome C7 in the C-genome of *B. napus* (Parkin et al. 2005). To determine the chromosomal location of the *DS-1* gene, we further selected 121 newly developed SSR markers from chromosomes A6 and C7 from a high-density SSR linkage map constructed using a DH population (Cheng et al. 2009; Xu and Liu unpublished data). Among them, three markers, BrGMS83, BrMS226 and BnEMS1125 on chromosome A6 detected polymorphisms between the short and tall parents and the contrasting bulks, and were used to genotype the F_2 individuals. BnEMS1125, a microsatellite marker derived from an expressed sequence tag (EST) of *B. napus*, completely co-segregated with the *DS-1* locus (Fig. 1c). BLAST analysis indicated that the source cDNA sequence (GenBank accession number CX189060) of BnEMS1125 encodes a DELLA protein that is a component of the GA signaling pathway. Previously, Muangprom et al (2005) identified a dwarf gene, *dwf2*, on chromosome R6 of *B. rapa* genome, which corresponds to chromosome A6 of *B. napus*. The gain-of-function mutant allele *Brrga1-d* (*dwf2*) similarly caused a dwarf phenotype in *B. rapa*. These information prompted us to consider the *BnRGA* gene as an established candidate for further characterization.

A dwarf mutant *bzh* was previously identified in *B. napus* and its dwarfism was also controlled by a single

semi-dominant gene. One RAPD marker OPMO7-730, which was sequentially converted into a SCAR marker SCM07, was closely linked to the dwarf locus at 0.8 ± 0.7 cM (Barret et al. 1998; Foisset et al. 1995). To test allelism between *DS-1* and *Bzh*, SCM07 was used to genotype the F_2 population used in this study. SCM07 also showed close linkage to the *DS-1* gene at a distance of 0.7 cM in our population (Fig. 1c), indicating that the *Bzh* could be allelic to *DS-1*.

Identification of *RGA* as a candidate gene for *DS-1*

We first checked the expression level of the *BnRGA* gene in both ZS4 and *ds-1*. Semi-quantitative RT-PCR analysis demonstrated that the *BnRGA* genes had similar transcript levels in ZS4 and *ds-1* (Fig. 2a), indicating that the dominant dwarf phenotype of *ds-1* was not caused by an increase of *BnRGA* transcription. Subsequently, we checked the coding region of *BnRGA* to identify putative mutation in *ds-1*. Because the wild-type progenitor line 92-B10 was not kept, we were unable to obtain the original wild-type *RGA* allele of *ds-1* for sequence analysis. Thus, we amplified and sequenced the full-length coding sequences of *RGA* from *ds-1* and ZS4 to identify polymorphisms between the *BnRGA* alleles. The ORFs of *BnRGA* from ZS4 and *Bnrga-ds* from *ds-1* are 1,719 and 1,734-bp in length, and encode proteins having 572 and 577 amino acids, respectively. A comparison of the ORFs identified 20 SNPs and two indels. Sixteen of the SNPs in *Bnrga-ds* did not result in any amino acid changes; the other four SNPs resulted in four single-residue variations (M1, M3, M4 and M6). The two indels, insertions of a 6 bp and a 9 bp, resulted in 2 (M2) and 3 amino acid insertions (M5) in the gene product of *Bnrga-ds*, respectively (Fig. 2b). The 6-bp insertion caused the polymorphism detected between *ds-1* and ZS4 by the BnEMS1125 marker in the mapping analysis. M2, M3, M4 and M6 are located in non-conserved regions of the DELLA protein, whereas M5 is located in the poly S/T region. The SNP in M1 resulted in a proline (P)-to-leucine (L) change in the N-terminal VHYNP motif, which belongs to the DELLA domain. Amino acid sequence alignment of the VHYNP regions of Bnrga-ds and BnRGA with those of

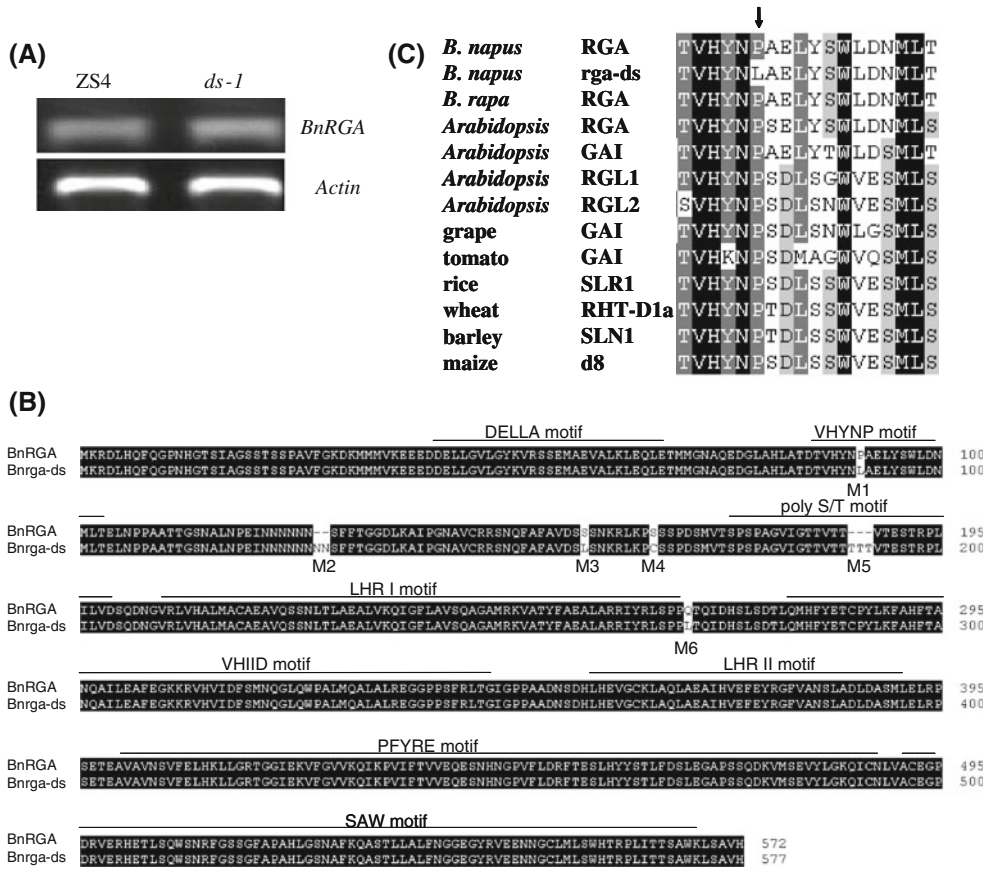

Fig. 2 Mutation of the conserved P residue in the VHYNP motif of BnRGA causes dwarfism. **a** Comparison of transcriptional level of *BnRGA* in cultivar ZS4 and *Bnrga-ds* in *ds-1* mutant. *Actin* was used as a control. **b** Comparison of the amino acid sequence between Bnrga-ds in *ds-1* and BnRGA in ZS4 (wild type) revealed six mutations (M1–M6). The conserved regions are indicated *above* the sequences. *White* on *black letters* represent identical amino acid. **c** Amino acid sequence alignment compared the VHYNP motifs of Bnrga-ds and BnRGA with those of other DELLA proteins from different plant species. The *arrow* indicates the position of the mutated amino acid in Bnrga-ds

Table 2 Amino acid sequence of 14 *B. napus* lines or cultivars at the six mutation sites in BnRGA

Accession	M1	M2	M3	M4	M5	M6	Phenotype
ds-1	L	8Ns	L	C	5Ts	L	Dwarf
ZS4	P	6Ns	S	S	2Ts	Q	Wild type
Rainbow	P	8Ns	L	C	5Ts	L	Wild type
Oscar	P	7Ns	S	S	2Ts	Q	Wild type
Monty	P	7Ns	S	S	2Ts	Q	Wild type
Glacier	P	6Ns	S	S	2Ts	Q	Wild type
Surpass 600	P	8Ns	S	S	2Ts	Q	Wild type
Quantum 1	P	5Ns	S	C	2Ts	Q	Wild type
Quantum 2	P	5Ns	S	C	2Ts	Q	Wild type
Cao 221167	P	6Ns	S	S	2Ts	Q	Wild type
Sc-ug 3	P	5Ns	S	C	2Ts	Q	Wild type
Sc-ug 4	P	6Ns	S	S	2Ts	Q	Wild type
Sc-ug 8	P	6Ns	S	S	2Ts	Q	Wild type
Pinnacle	P	5Ns	S	C	2Ts	Q	Wild type

other DELLA proteins from different plant species indicated that this proline (P) is absolutely conserved (Fig. 2c).

To determine which mutation caused the dwarf phenotype in *ds-1*, we sequenced the *BnRGA* coding region of other 12 wild-type *B. napus* cultivars (Table 2). Mutations M2, M3, M4, M5 and M6 were found in at least one of these cultivars, suggesting that these five mutations did not change the GA resistance of these DELLA proteins. M1 was not found in these wild-type cultivars, but was specific to the *ds-1* mutant. These results suggested that the P-to-L substitution in the VHYNP motif of *Bnrga-ds* might render the dwarfism in *ds-1*.

Arabidopsis transformed with the *Bnrga-ds* allele and *Atrga-ds* gained dwarf phenotype

Mutations in the DELLA domain of RGA usually caused gain of function (Boss and Thomas 2002; Chandler et al. 2002; Peng et al. 1997, 1999). Therefore, we attempted to transform *Bnrga-ds* into wild-type *Arabidopsis* to gain the dwarf phenotype. The 3.3-kb DNA fragments of *BnRGA* and *Bnrga-ds* containing their native promoter, coding sequence and terminator were, respectively, introduced into wild-type Columbia ecotype. Eighteen pBnRGA:Bnrga-ds transgenic lines and 10 pBnRGA:BnRGA lines were obtained. The homozygous transgenic pBnRGA:Bnrga-ds progenies displayed dwarf phenotype, whereas the homozygous pBnRGA:BnRGA progenies did not show obvious phenotype (Fig. 3a), suggesting that *Bnrga-ds* is responsible for the dwarfism of *ds-1* mutant.

To investigate the effect of the conserved proline in the DELLA proteins of other species, we performed site-directed mutagenesis on the wild-type *AtRGA* sequence of *Arabidopsis* (Columbia ecotype) to obtain *Atrga-ds* encoding an identical mutation as in *Bnrga-ds*. Fourteen pAtRGA:Atrga-ds and 10 pAtRGA:AtRGA *Arabidopsis* transformants were obtained. The homozygous pAtRGA:Atrga-ds plants were dwarfed compared with homozygous pAtRGA:AtRGA and wild-type plants (Fig. 3b). These results demonstrated that the effect of the P residue in the VHYNP motif of DELLA on GA signaling is conserved in different plant species, and further confirmed that the P-to-L substitution in the VHYNP motif of Bnrga-ds cause the dwarfism in *ds-1*.

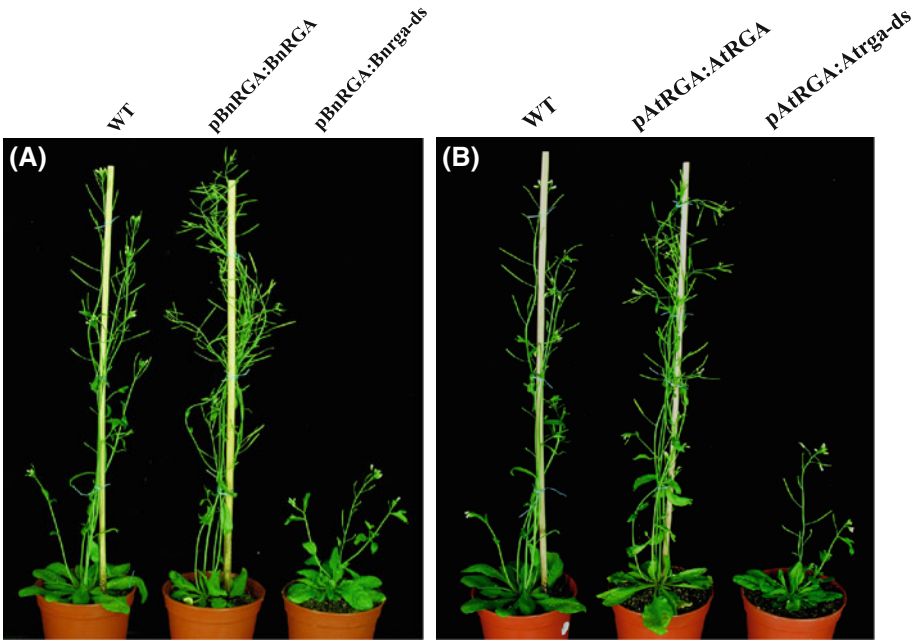

Fig. 3 Expression of the *Bnrga-ds* and *Atrga-ds* transgene rendered dwarf phenotypes in *Arabidopsis* ecotype Columbia. **a** Phenotypes of wild type (*left*), transgenic *BnRGA* (*center*) and *Bnrga-ds* (*right*) plants at 7 weeks. **b** Phenotypes of wild type (*left*), transgenic *AtRGA* (*center*) and *Atrga-ds* (*right*) plants at 7 weeks

Bnrga-ds and Atrga-ds protein cannot interact with AtGID1A

GID1s, the soluble GA receptors, can interact with DELLA proteins in a GA-dependent manner and the DELLA domain is involved in their interaction (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2005; Ueguchi-Tanaka et al. 2007; Willige et al. 2007). There are three GID1s (AtGID1A, AtGID1B, AtGID1C) in *Arabidopsis* and they have redundant function acting as GA receptors (Nakajima et al. 2006). We speculated that the P residues in the VHYNP motif of BnRGA and AtRGA might directly participate in the interaction between DELLA and GID1. To test this hypothesis, we examined the interactions between AtGID1A and BnRGA, Bnrga-ds, AtRGA and Atrga-ds using yeast two-hybrid assay. In the absence of GA_3, AtGID1A cannot interact with BnRGA and AtRGA (Fig. 4c), while it can interact with BnRGA and AtRGA in the presence of GA_3 (Fig. 4d). However, AtGID1A cannot interact with the mutated Bnrga-ds and Atrga-ds in the presence or absence of GA_3 (Fig. 4c, d). These results indicated that BnRGA and AtRGA can interact with AtGID1A in a GA-dependent manner. The P-to-L mutation in the VHYNP motif abolished these interactions, suggesting that this conserved P residue in the VHYNP motif plays an important role in RGA-GID1 interaction.

Discussion

In this study, we mapped the dwarf gene *ds-1* to chromosome A6 in *B. napus*, which is possibly allelic to the previously mapped dwarf gene *Bzh*. *BnRGA*, an ortholog of *B. rapa RGA,* was identified as *DS-1*. We found that a P-to-L substitution in the VHYNP motif of Bnrga-ds caused the resistance to GA-induced degradation of this DELLA protein and resulted in the GA-insensitive dwarf phenotype (Fig. 2). This constitutes the first point mutation that has been identified in the VHYNP motif. Most gain-of-function mutations identified previously contained deletions in DELLA domain, which include *Arabidopsis* gai-Δ17 (Peng et al. 1997), wheat RHT-B1b and RHT-D1b, maize D8-1, D8-MP1 and D8-2023 (Peng et al. 1999), except for the L-to-H substitution in the DELLA motif of Vvgai1 (Boss and Thomas 2002) and the G-to-E substitution in the DELLA motif of Sln1d (Chandler et al. 2002) and the Q-to-R substitution in the VHIID motif of GRAS domain of Brrga1-d (Muangprom et al. 2005). Alignment of the VHYNP regions of different DELLA proteins indicated that this P residue is absolutely conserved in plant species. Expression of a mutated *Atrga-ds* that contained the P-to-L mutation found in Bnrga-ds also conferred a dwarf phenotype in *Arabidopsis*, suggesting that this conserved P residue has a similar role in DELLA proteins of different plant species.

Soluble GA receptors (GID1s) play important roles in GA-induced degradation of DELLA protein. It was revealed that GID1s interact with DELLA proteins in a GA-dependent manner (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2005; Ueguchi-Tanaka et al. 2007; Willige et al. 2007). Deletions of DELLA and/or VHYNP motifs abolish the GID1-DELLA interaction, suggesting that both motifs are interacting regions for DELLA with GID1 (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2007). Crystallographic analysis of the GA-GID1–DELLA complex further revealed that the VHYNP motif is located in loop C–D and helix αD of the DELLA domain, and the V and P residues in the VHYNP motif

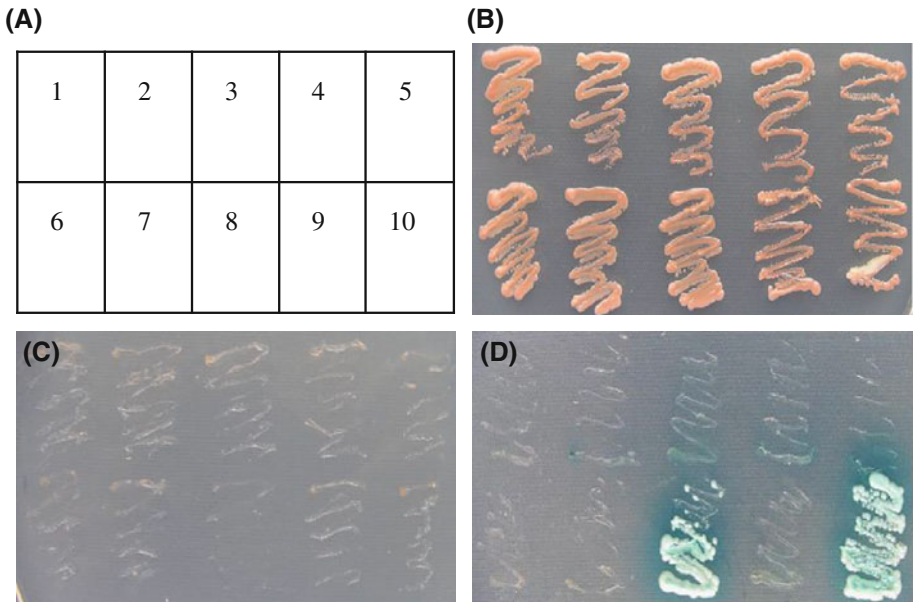

Fig. 4 The conserved P in VHYNP motif is essential for the DELLA–GID1 interaction. **a** The chart indicated the AD and BD constructs co-transformed into the yeast strain AH109. 1, AD/BD; 2, AD-AtGID1A/BD; 3, AD/BD-Bnrga-ds; 4, AD/BD-BnRGA; 5, AD/BD-Atrga-ds; 6, AD/BD-AtRGA; 7, AD-AtGID1A/BD-Bnrga-ds; 8, AD-AtGID1A/BD-BnRGA; 9, AD-AtGID1A/BD-Atrga-ds; 10, AD-AtGID1A/BD-AtRGA. Growth test of transformants streaked onto selection media. **b** SD/-Leu-Trp (control). **c** SD/-Leu-Trp-His-Ade/X-α-Gal. **d** SD/-Leu-Trp-His-Ade/X-α-Gal/GA_3

sandwich the aromatic ring of Tyr 48 of the GID1A to form a hydrogen bond with the phenolic hydroxyl group (Murase et al. 2008), indicating that the conserved P residue in the VHYNP motif is in direct contact with GID1. The GA-GID1–DELLA interaction promotes the DELLA protein to change its conformation and be recruited by SLY1 and subsequently be proteolyzed by the 26S proteosome (Ueguchi-Tanaka et al. 2007). Our yeast two-hybrid assay demonstrated that the P-to-L mutation in the VHYNP motif could block the DELLA–GID1 interaction in the presence of GA_3 (Fig. 4), suggesting that the conserved P residue is essential for this interaction and important for the degradation of the DELLA protein.

Dwarf genes have been extensively used to improve lodging resistance and to enhance harvest index in cereal crops. However, lodging is still one of the key factors related to yield loss in *B. napus*. The *ds-1* mutant and hybrids carrying the *ds-1* allele showed significant height reduction as compared to wild-type cultivars (Fig. 1a). This height reduction was due to shorter internodes and a lower position of the first primary branch. Because the height of *ds-1* is approximately half that of ZS4, it is considered a semi-dwarf phenotype, and thus would be suitable for height reduction in current cultivars (Hedden 2003) and the lower position of the first branch of *ds-1* increases lodging resistance. Introduction of the dwarf genes *dwf2* and *Bzh* into oilseed rape inbred lines and hybrids significantly decreased plant heights and improved lodging resistance (Foisset et al. 1995; Muangprom et al. 2006). Therefore, the *ds-1* gene, together with the *dwf2* and *Bzh* genes would be useful for height reduction in *Brassica* breeding, and thus could lead to yield improvement due to lodging resistance in *Brassica* crops. In addition, the PCR markers linked to *DS-1* locus identified in this study will be useful for selection of *ds-1*, *dwf2* and *Bzh* by different breeders in different breeding programs.

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