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ORIGINAL PAPER

Identification, fine mapping and characterisation of a dwarf mutant (*bnaC.dwf*) in *Brassica napus*

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Abstract In the present study, we have obtained one dwarf mutant (*bnaC.dwf*) from the *Brassica napus* inbred line T6 through chemical mutagen ethyl methanesulfonate (EMS). We have determined the phenotypic effects and genetic characteristics of dwarf mutant (*bnaC.dwf*). The dwarf mutant was insensitive to exogenous GA_3 for plant height, suggesting that it is significantly playing a crucial role in the gibberellins response pathway. Genetic analysis revealed that one recessive gene is responsible for controlling the phenotypic expression of dwarf mutant. Amplified Fragment Length Polymorphism (AFLP) technique was applied for selecting markers linked to the *BnaC.DWF* gene which assisted in screening of dwarf and normal individuals in the BC_4 population. We have screened 1,024 primer combinations and then identified nine AFLP markers linked to the *BnaC.DWF* gene. Identification and linkage of the markers were carried out by analysing 2,000 individuals from a larger population of the BC_4. Two markers EA10MC09 and EA12MC02 were located on the flanking region of the *BnaC.DWF* gene at a distance of 0.2 and 0.05 cM, respectively. Four AFLP markers EA09MG05, EA02MC07, EA01MC01 and EC04MC07 were successfully converted into Sequence Characterised Amplified Region markers namely SCA9G5, SCA2C7, SCA1C1 and SCC4C7. We further integrated *BnaC.DWF* linked Simple Sequence Repeat markers into two populations (Piquemal et al. Theor Appl Genet 111:1514–1523, 2005; Cheng et al. Theor Appl Genet 118:1121–1131, 2009). *BnaC.DWF* was mapped to the linkage region N18. The molecular markers developed from these investigations will greatly accelerate the selection process for developing dwarf varieties *in B. napus* by Marker Assisted Selection and genetic engineering.

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Introduction

Lodging is severely impacting the crop productivity. It leads to an unprecedented decrease in crop yield. It was during the era of green revolution, when we came to understand the potential use of dwarf varieties in the breeding programmes. The "Green Revolution" led by Norman Borlaug, Monkombu Swaminathan and Gurudev Khush enabled the world's food supply to be tripled during the last three decades of the 20th century by using dwarf and semidwarf high yielding varieties (HYVs) of crops (Elliott 2008). The introduction of the rice semi-dwarf variety "*IR8*" (Khush 1999) and a dominant wheat semi-dwarf cultivar "*Rht*" was crucial to this revolution (Evans 1998). These dwarf varieties were associated with increased yields, higher fertility, early maturity and high tillering capacity (Hedden 2003; Khush 2001).

The dwarf genes used in the "Green Revolution" (*sd1* in rice and *Rht-B1b* and *Rht-D1b* in wheat) were involved in the GAs biosynthesis and signalling pathways. The previous studies on rice mentioned about a semidwarf1 (*sd1*) gene, encoding for a defective GAs biosynthetic enzyme, GA 20 Oxidase (GA 20-ox), involved in the gibberellic acid biosynthetic pathway, but it was observed that the application of exogenous GAs could restore *sd1* plants similar to the wild types (Monna et al. 2002; Sasaki et al. 2002;

Spielmeyer et al. 2002). However, the application of exogenous GAs could not rescue wheat mutants containing dwarf genes *Rht-B1b* and *Rht-D1b*. The *Rht-B1b* or *Rht-D1b* gene encodes a mutant form of DELLA protein that are resistant to GAs-induced degradation and constitutively blocks GAs signalling (Peng et al. 1999). Recently, the DELLA proteins encoded by the *GAI* (Peng et al. 1997), *RGA* (Silverstone et al. 1998) in *Arabidopsis*; *d8* in maize (*Zea mays*, Winkler and Freeling 1994); *VvGAI* in grape (V*itis vinifera*, Boss and Thomas 2002); *SLN1* in barley (*Hordeum vulgar*, Chandler et al. 2002) and *SLR1* in rice (*Oryza sativa*, Ikeda et al. 2001) have been isolated (Sun and Gubler 2004).

Lodging is also a serious problem in *B. napus*, resulting a significant decrease in crop yield and causing difficulty in harvesting (Islam and Evans 1994). Identification and characterisation of the dwarf genes could be helpful for improving lodging resistance in oilseeds (Liu and Tang 1990). Compared to studies of dwarfing genes in cereal crops, fewer studies have been carried out in *Brassica* species. In previous studies, some dwarf mutants have been identified in *B. napus* such as *Bzh* (Foisset et al. 1995), *ndf-1* (Wang et al. 2004) controlled by *BnGID1a*, a homolog of *A. thaliana* (Li et al. 2010) and *99CDAM* (Mei et al. 2006). *Brassica* species and *A. thaliana* belong to the same family *Brassicaceae* (*Cruciferae*) (Lagercrantz 1998), so the studies carried out in *A. thaliana* can be a valuable piece of information for correlating the GAs studies in *Brassica* species. Based on the informations of dwarf genes in *Arabidopsis*, the semi-dwarf mutant *dwf2* (*Brrga1-d*) was identified, mapped and characterised in *B. rapa* (Muangprom and Osborn 2004; Muangprom et al. 2005). The *Brrga1-d* gene was transferred from *B. rapa* to the cultivated *B. napus* by hybridizing *B. rapa* × *Brassica oleracea* and doubling the chromosomes of the hybrids (Muangprom et al. 2006). The dwarf gene *Bnrga* is a homolog of *Brrga1-d* and was cloned in *B. napus*, which encodes for a DELLA protein causing missense mutation in the VHYNP motif (Liu et al. 2010).

In this paper, we report on the identification, mapping and characterisation of the dwarf mutants (*bnaC.dwf*) in *B. napus*. We determined the effects of exogenous GAs on the plant height of the mutants and also carried out the genetic analysis of the dwarf phenotype.

Materials and methods

Plant materials

The dwarf mutant (*bnaC.dwf*) was obtained from the *B. napus* inbred line T6 (+/+) through chemical mutagen ethyl methanesulfonate (EMS). The F_1 plants were obtained by crossing the *BnaC.dwf* line with Zhong shuang 9 (ZS9, a semi-winter cultivar of *B. napus* in China).

Gibberellic acid (GA) treatment

The dwarf mutants (*bnaC.dwf*) and T6 (+/+) seeds were planted in a plastic plate (10 × 15 × 10 cm) in 2009, October. The plates were kept in a growth chamber under normal conditions and watered daily to soil saturation. After squaring 0, 0.1, 10 or 100 mg GA_3 (Sigma) in 20 ml of 50:50 (v/v) ethanol:water were applied to the shoot tips of 10 seedlings for each treatment once a week till the inception of flowering. The plant heights were measured from the cotyledon to the tip of the main inflorescence just after flowering over. Analysis of variance was performed on these experimental data using the SAS system (SAS 8.1).

Endogenous GAs determination

The functional leaves (the 3rd or 4th leaf from the top) and stem tips of the dwarf mutants (*bnaC.dwf*) and T6 (+/+) lines were sampled at the elongation stage. The samples were frozen in liquid nitrogen immediately, and the fresh weight was determined. Subsequently, the samples were grinded and extracted overnight at 4°C in cold 80% methanol. The mixture was centrifuged at 5,000 rpm for 5 min, and the supernatant was collected. The colour of one millilitre supernatant was removed by Sep-Pak C18. The endogenous GAs was determined by Enzyme Linked Immunosorbent Assay (ELISA) (Wu et al. 1988), and three time replications were carried out in each sample.

Field trial and trait measurements

The F_2 population containing 258 plants were derived from the cross between ZS9 and dwarf mutants (*bnaC.dwf*). In 2008, the F_2 plants were grown at the Rapeseed Research Base of Huazhong Agricultural University in Northwest of China at Hezheng, Gansu Province. Each F_2 plants were self-pollinated to obtain F_3 seeds, and each F_3 family was grown in two rows (10 plants/row), the segregation of plant heights were used to confirm the genotype of the corresponding F_2 plants at Huazhong Agricultural University, Wuhan, Hubei Province in 2008–2009. At maturity, five plants of each F_3 family in the middle rows were used for measuring the plant height from the ground to the tip of the main inflorescence.

The mapping population (BC_4) was derived by subsequent four backcrosses of F_1 to the dwarf mutant (*bnaC.dwf*). BC_4 population was grown at Huazhong

Agricultural University, Wuhan, Hubei Province. BC_4 seeds were planted at early October 2009 in about 300 rows having 1.5 m width with 0.20 m distance between each row. The dwarf mutants (*bnaC.dwf*) and T6 (+/+) plants (as controls) were grown alternatively in the field at every ten rows. The plants were spaced sufficiently far apart to avoid any interplant competition (10 plants/row). Before squaring, every plant of BC_4 was ranked based on the growth feature. At maturity, 146 plants ranked with the same grade were selected to study the plant height. Plant heights were measured from the cotyledon to the tip of the main inflorescence just after the flowering over.

Data from both experiments were analysed using the SAS system (SAS 8.1). All the measurements in the experiments were analysed as completely random design.

DNA extraction, BSA and AFLP analysis

Bulk segregant analysis (BSA; Michelmore et al. 1991) combined with the AFLP technique were used to identify molecular markers linked to the dwarf gene (*bnaC.dwf*). Ten plants of each phenotypic class (extremely short plants and extremely tall plants) from the BC_4 mapping population were randomly selected for constructing the dwarf bulk (DB) and tall bulk (TB).

The DB and TB DNA samples (50 ng/μl) were treated with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 12.5 μl digested solution (*Eco*RI and *Mse*I) were used for digesting the DNA samples. Specific double-stranded adapters were subsequently ligated to the restriction fragment ends by T4 DNA ligase. After fivefold dilutions, the adapter-ligated DNA was pre-amplified with AFLP primers (EA/MC, EA/MG, EC/MC, EC/MG) in 25 μl solution. Subsequent analyses were done in a 1.0% agarose gel electrophoresis. The pre-amplified products were diluted 10- to 30-folds for selective amplification (Negi et al. 2000). Selective amplification products were separated on a 6% polyacrylamide denaturing sequencing gel and visualised by a silver staining system (Lu et al. 2001, 2004) with some modifications.

Converting AFLP markers into SCAR markers

The AFLP fragments exhibiting polymorphism were cloned and sequenced (Ke et al. 2004; Yi et al. 2006). Based on these sequences the specific primers were designed by the software Primer3 (Rozen and Skaletsky 1999). These primers were used to amplify genomic DNA from dwarf and tall individuals. Finally, the PCR products were detected on a 6% polyacrylamide denaturing sequencing gel for testing the phenotypic polymorphism.

Genetic map of the *BnaC.DWF* gene

For constructing a rough flanking map linked to the *BnaC.DWF* gene, we used the BC_4 population consists of 96 individuals. Subsequently, a larger population of the BC_4 with 2,000 individuals were selected and analysed to fine map the *BnaC.DWF* gene. The DH mapping populations derived from the cross Tapidor × Ningyou7 (Qiu et al. 2006) were utilised to locate the SSR6 markers (derived from our laboratory) to a specific linkage group. The SSR6 was mapped to linkage group N18, and then 17 SSR markers in the linkage group were selected for a polymorphism survey. The data of these markers and individual phenotypes were analysed with the MAPMAKER/EXP 3.0 program (Lander et al. 1987; Lincoln et al. 1992). Map order was estimated by maximum-likelihood.

Results

Response to exogenous and endogenous levels of gibberellins (GAs) in the wild type (T6) and dwarf mutants (*bnaC.dwf*)

In our experiments, we observed that the plant height of dwarf mutants (*bnaC.dwf*) was insensitive ($P > 0.05$) to all the concentrations of exogenous GAs, but the wild type T6 (+/+) lines showed a significant subsequent increase in plant heights with the increasing exogenous concentrations of GA_3 (Fig. 1). The ELISA results showed that the levels of endogenous GAs in the leaf and the stem of the dwarf mutants (*bnaC.dwf*) line were 74% ($P < 0.01$) and 45% ($P < 0.01$), respectively, which was relatively higher than

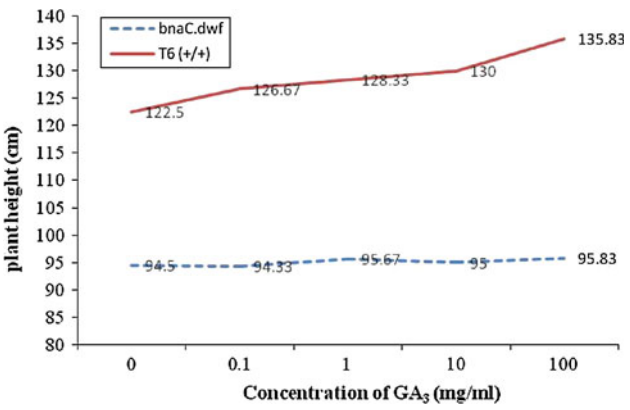

Fig. 1 Effects of gibberellin (GA_3) on plant height of *B. napus* T6 (+/+) and *bnaC.dwf*. After squaring, 0–200 mg of GA3 in 20 ml of 50:50 (v/v) ethanol:water were applied once a week until flowering. Plant heights were measured from the cotyledon to the tip of the main inflorescence just after the flowering overs

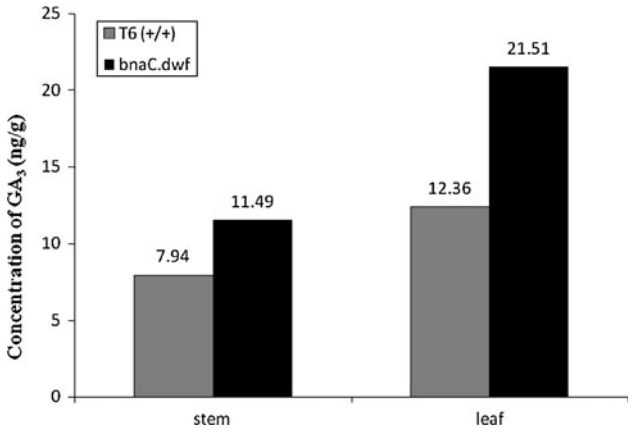

Fig. 2 The levels of endogenous GAs (leaves and stems) in T6 (+/+) and *bnaC.dwf*. The endogenous levels of GAs in functional leaves (the 3rd or 4th leaf from the top) and stem tips of T6 (+/+) and *BnaC.dwf* were determined by ELISA

the average levels of GAs present in the T6 (+/+) line (Fig. 2).

Genetic analysis of the dwarf mutants (*bnaC.dwf*)

The phenotypic expression of the filial generations obtained by crossing dwarf mutant (*bnaC.dwf*) and normal parent (ZS9) were thoroughly investigated. The height of the F_1 plants were close to the mean height of the normal parent (ZS9) (Fig. 3). Plant heights in the $F_{2:3}$ families showed a bimodal distribution from 90 to 190 cm, with two peaks at approximately 130 cm. The $F_{2:3}$ families could be roughly classified into two groups of dwarf and tall phenotypes, comparing their heights with the parental phenotypes (Fig. 4a). This analysis showed an expected Mendelian ratio of segregation 3:1 ($\chi^2 = 0$, $P > 0.95$). Plant heights in the BC_4 population showed a bimodal distribution from 60 to 140 cm, with two peaks at approximately 105 cm (Fig. 4b). All BC_4 individuals segregated into two groups namely heterozygous and the putative homozygous dwarf genotypes. The segregation showed an expected Mendelian inheritance ratio 1:1 ($\chi^2 = 0.62$, $P > 0.95$). These data indicated that the phenotype of the dwarf mutants (*bnaC.dwf*) was controlled by one single recessive gene.

Genetic mapping of the *BnaC.DWF* gene

We have developed nine linked AFLP markers to the *BnaC.DWF* gene by using AFLP assay combined with the BSA (Table 1). In the rough mapping experiment, all AFLP markers were used to identify the genotype of 96 plants, and all the markers were mapped to a region around the *BnaC.DWF* gene. In our study, we have converted four of nine AFLP markers into the SCAR markers (Table 1).

Fig. 3 Phenotypes of dwarf mutant (*bnaC.dwf*) (A); wild type T6 (+/+) (B); the F1 hybrid derived from ZS9 × *bnaC.dwf* (C) and ZS9 (D)

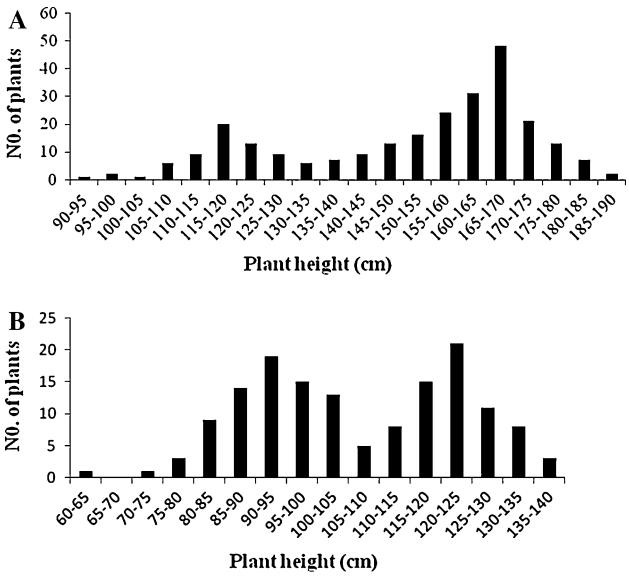

Fig. 4 Plant height distribution of $F_{2:3}$ (**a**) and BC_4 (**b**) generations derived from ZS9 × *bnaC.dwf* and backcrossed to *bnaC.dwf*. Plant height was measured from the ground to the tip of the main inflorescence just after the flowering over

Two SCAR markers SCC4C7 and SCA9G5 were detected on flanking region of the *BnaC.DWF* gene (Fig. 5a). All individuals from the BC_4 populations were tested by these

two SCAR markers. Eighty recombination events were detected between the *BnaC.DWF* gene and SCC4C7, and 40 recombinants of the *BnaC.DWF* gene and SCA9G5 were identified. We restrict our study only to these recombinants and used them to identify other molecular markers. We have successfully mapped four AFLP, two SSR and five SCAR markers to the region of 19 cM in the franking region of the *BnaC.DWF* gene. The two AFLP markers EA10MC09 and EA12MC02 were closely linked to the *BnaC.DWF* gene at a distance of 0.2 and 0.05 cM, respectively (Fig. 5a).

For determining the location of the *BnaC.DWF* gene in the *B. napus*, all the SCAR markers linked to the *BnaC.DWF* gene were used for screening two parents of TN DH populations derived from Tapidor × Ningyou7. Only the SSR6 marker showed polymorphism in the TN DH populations. As a result, the SSR6 was mapped to linkage group N18 of TN DH populations. We further confirmed the location of the *BnaC.DWF* gene by using two reference maps (Piquemal et al. 2005; Cheng et al. 2009) for selecting SSR markers from N18. These SSR markers from N18 were used to screen 96 plants derived from the BC_4 population. One SSR marker CB10092 in the reference map (Piquemal et al. 2005) showed polymorphism and it is located at a distance of 11.0 cM to the *BnaC.DWF* gene (Fig. 5b). The other SSR marker BnGMS340 in the reference map (Cheng et al. 2009) also showed polymorphism and it is located at a distance of 8.0 cM to the *BnaC.DWF* gene (Fig. 5c). These results showed that the *BnaC.DWF* gene is located on linkage group N18.

Discussion

Gibberellins are an essential endogenous plant growth regulator (Brian 1959). In the previous studies, the dwarf mutants were specifically used for analysing GAs functions. These dwarf mutants with aberrant GAs responsiveness can be divided into two classes: mutants that are defective in the GAs biosynthesis, and mutants that are defective in the GAs response pathway (Silverstone et al. 1998). The two classes of dwarf mutants mentioned above display a similar phenotype. In the previous studies it was emphasised that the mutants that were defective in the GAs biosynthesis pathway were also sensitive to exogenous GAs application and could be rescued with exogenous GAs treatment, whereas the mutants that are defective in the GAs response pathway were insensitive to exogenous GAs treatment (Sun 2000). In accordance with the previous studies used in elucidating GAs pathway, in our present studies, we have also used dwarf mutants to understand the functions of GAs. Our results indicated that the *bnaC.dwf* mutants were insensitive to exogenous GAs application in plant height (Fig. 1), and the levels of endogenous gibberellins (GAs) present in leaves and stems of these mutants were significantly higher than that of the T6 (+/+) plants (Fig. 2).

In our present investigation, we have also studied the effect of brassinosteriod (BR) on the *bnaC.dwf* mutants. Previous studies have established that the mutants which were defective in the biosynthesis of BR or in the BR response pathway showed similarity to the mutants with severe GAs-deficiency. Intensive studies carried out on the model plant *Arabidopsis* showed that the GAs mutant seedlings were morphologically similar to that of the wild type when germinated in the dark, whereas the BR mutants displayed a de-etiolated growth habit with short hypocotyls, open cotyledon and no hook (Fridborg et al. 1999). In our experiment, we observed that the *bnaC.dwf* seedlings grown in the dark were morphologically similar to the wild type (data not shown), suggesting that the *bnaC.dwf* seedlings were not involved in BR pathway. The above-mentioned studies have clearly indicated that *bnaC.dwf*

Table 1 Description of AFLP markers that are tightly linked to the *BnaC.DWF* gene

AFLP marker	Primer combinations	Size of markers (bp)	Extended for SCARs	SCAR name derived from AFLP	Map distance (cM)
Am01	E-AAA/M-CAA	92	Yes	SCA1CI	3.00
Am02	E-AAT/M-CTC	445	Yes	SCA2C7	2.00
Am03	E-ATC/M-CAA	200	No		3.00
Am04	E-ACT/M-CCA	450	No		0.20
Am05	E-ACG/M-CAT	280	No		0.05
Am06	E-ACA/M-GTA	162	Yes	SCA9G5	2.00
Am07	E-ACA/M-GGG	680	No		0.40
Am08	E-AGC/M-GGT	650	No		0.45
Am09	E-CAG/M-CTC	446	Yes	SCC4C7	4.00

E = *Eco*RI primer, 5′-GACTGCGTACCAATTC-3′; M = *Mse*I primer, 5′-GATGAGTCCTGAGTAA-3′

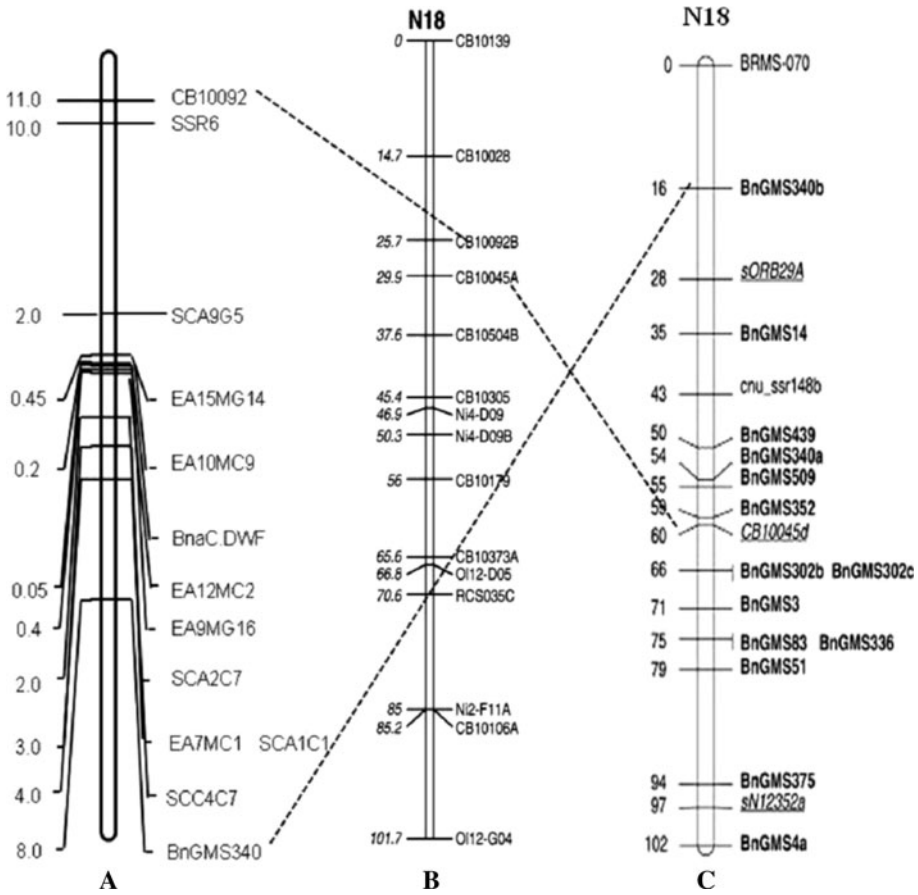

Fig. 5 Partial linkage maps of *B. napus* indicated the relative location of the *BnaC.DWF* gene on linkage group N18 of the two reference maps. **a** Linkage map of the region surrounding the *BnaC.DWF* gene from the BC_4 population. **b** The linkage group N18 (Piquemal et al. 2005) consisted of SSR markers. **c** The linkage group N18 (Cheng et al. 2009). *Dotted lines* indicated the relationship of three genetic maps

mutants were defective in the GAs response pathway, and GAs signal transduction pathway was constitutively blocked.

The previous studies on the crop species like *Rht-B1b* or *Rht-D1b* in wheat (*Triticum* spp., Peng et al. 1999); *d8* in maize (*Zea mays*, Winkler and Freeling 1994); *VvGAI* in grape (V*itis vinifera*, Boss and Thomas 2002); *SLN1* in barley (*Hordeum vulgar*, Chandler et al. 2002); *SLR1* in rice (*Oryza sativa*, Ikeda et al. 2001); *Brrga1-d* in *B. rapa* (Muangprom et al. 2005) and *Bnrga* in *B. napus* (Liu et al. 2010) have showed that these mutants of GAs responsive pathways were controlled by semi-dominant genes. To our knowledge, the previous studies have never mentioned about a recessive gene controlling the dwarfness in crop plants; however, our present study in this paper mentions that one single recessive gene is responsible for controlling the GAs responsive pathways in *B. napus*, an economically important oil crop.

We strongly believe that mapping of this dwarf gene will be helpful in map-based cloning and marker-assisted selection breeding. The mapping of EA10MC09 and EA12MC02 in the flanking region to the *BnaC.DWF* gene with distances of 0.2 and 0.05 cM, respectively (Fig. 5a) can be helpful to isolate the *BnaC.DWF* gene by the map-based cloning strategy because the distance of 0.1 cM would correspond to <50 kb in *B. napus* (Foisset et al. 1996). The height of the *bnaC.dwf* mutants may be affected by the genotype of the material selected and the environmental condition. Therefore, it was difficult to select precisely the homozygotic *bnaC.dwf* genotype from the segregating population. The molecular markers linked to the *BnaC.DWF* locus can be used for selecting the homozygotic *bnaC.dwf* line because these markers are tightly linked to the *BnaC.DWF* gene. This could greatly help to shorten the breeding time and save space.

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