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Identification of loci associated with embryo yield in microspore culture of *Brassica rapa* by segregation distortion analysis

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Abstract

Key message **We identified three physical positions associated with embryo yield in microspore culture of *Brassica rapa* by segregation distortion analysis. We also confirmed their genetic effects on the embryo yield.**

Abstract Isolated microspore culture is well utilized for the production of haploid or doubled-haploid plants in *Brassica* crops. *Brassica rapa* cv. 'Ho Mei' is one of the most excellent cultivars in embryo yield of microspore culture. To identify the loci associated with microspore embryogenesis, segregation analysis of 154 DNA markers anchored to *B. rapa* chromosomes (A01–A10) was performed using a population of microspore-derived embryos obtained from an F_1 hybrid between 'CR-Seiga', a low yield cultivar in microspore-derived embryos, and 'Ho Mei'. Three regions showing significant segregation distortion with increasing 'Ho Mei' alleles were detected on A05, A08 and A09, although these regions showed the expected Mendelian segregation ratio in an F_2 population. The additive effect of alleles in these regions on embryo yield was confirmed in a BC_3F_1 population. One region on A08 containing Br071-5c had a higher effect than the other regions. Polymorphism of nucleotide sequences around the Br071-5c locus was investigated to find the gene possibly responsible for efficient embryogenesis from microspores.

Introduction

Haploid and doubled-haploid lines are useful materials in breeding programs to produce homozygous lines at every locus. Anther and isolated microspore cultures have been developed to produce haploid and doubled-haploid plants (Germanà 2011; Ferrie and Caswell 2011). In anther culture of genus *Brassica*, the first successful production of a haploid plant was reported in cabbage, *Brassica oleracea*, by Kameya and Hinata (1970), followed by several reports in *Brassia napus* and *Brassica rapa* (Thomas and Wenzel 1975; Keller et al. 1975). Additionally, Keller and Armstrong (1979) improved the efficiency of anther culture by heat-shock treatment just after in vitro culture. Following this improvement of anther-culture techniques, Lichter (1982) developed a microspore-culture technique in *B. napus*. This technique has been further improved and applied to other *Brassica* crops such as Chinese cabbage (Sato et al. 1989), cabbage (Lichter 1989), broccoli (Takahata and Keller 1991) and radish (Takahata et al. 1996). The efficiency of embryogenesis derived from microspores has been reported to depend on genotypes in *B. napus* (Ohkawa et al. 1987; Gland-Zwerger et al. 1988), *B. rapa* (Burnett et al. 1992; Baillie et al. 1992), *B. oleracea* (Takahata and Keller 1991) and *B. juncea* (Hisamatsu et al. 1994).

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To identify genetic factors for the efficiency of embryogenesis derived from microspores, several forward and reverse molecular genetic studies have been conducted. A large number of ESTs (expressed sequence tags) in microspores just after heat-shock treatment and/or microspore-derived embryos have been identified in *B. napus* (Tsuwamoto et al. 2007; Malik et al. 2007). In a forward genetic study by diallel analysis, Zhang and Takahata (2001) have revealed that the microspore embryogenesis is controlled by two loci in *B. napus*. Additionally, several loci showing deviations from an expected 1:1 Mendelian ratio in a population of microspore-derived embryos from an F_1 individual have been found in *B. rapa* and *B. napus* (Cloutier et al. 1995; Zhang et al. 2003; Ajisaka et al. 1999), suggesting that the distortion results from the difference of embryogenic ability during in vitro androgenesis. However, loci and chromosomes harboring the genes responsible for embryogenic ability have not yet been identified, although whole genome sequences of those *Brassica* species have been published (Wang et al. 2011; Chalhoub et al. 2014). In this study, using DNA markers anchored to the physical positions of *B. rapa* chromosomes, we identified the number and the positions of loci showing segregation distortion in a microspore-derived embryo population from an F_1 individual, which was produced by crossing a high embryo yield cultivar, 'Ho Mei', and a very low embryo yield cultivar, 'CR-Seiga'. Additionally, the genetic effect of the candidate loci on the embryo yields was confirmed using another microspore-derived embryo population and a backcross population. Surveying the candidate genomic regions, the coding sequences of several genes were investigated to infer the gene responsible for the difference of embryo yields.

Materials and methods

Plant materials

Two *Brassica rapa* lines, a doubled-haploid (DH) line of 'Ho Mei' ('HM-DH1', hereafter) (Zhang and Takahata 2001) and an inbred line of a self-compatible mutant of 'CR-Seiga 65' (Fujimoto et al. 2006) ('CRS1', hereafter), were used as plant materials. 'HM-DH1' was crossed with 'CRS1' to produce F_1 progenies, and then an F_1 individual was used for production of an embryo population by microspore culture. An F_2 population was also produced by self-pollination of the F_1 individual. The F_1 individual was backcrossed with 'CRS1' to produce BC_1F_1 plants. After marker-assisted selection with Br071-5c, which showed segregation distortion in this study, a heterozygous progeny at the Br071-5c locus was backcrossed with 'CRS1' to

produce BC_2F_1 plants. The backcrossing was repeated to produce BC_3F_1 progenies.

Microspore culture and DNA preparation

Microspore culture was carried out by an improved method enabling the handling of multiple samples according to Takahashi et al. (2011). The microspores were suspended at a density of 1.0×10^5 mL^{-1} in 1/2 NLN-11 medium. Two microliters of the microspore suspension was cultured in 60×15 mm plastic Petri dishes, followed by heat-shock treatment at 33 °C for 1 day. After the treatment, the dishes were placed in the conditions at 25 °C for 3 weeks, and then the developed embryos were counted. Cotyledonary embryos were cultured in B5 medium with 2 % sucrose and 0.8 % agar at 25 °C with a 16-h photoperiod. Total DNAs from the cotyledonary embryos and leaves of 'HM-DH1' and 'CRS1' were extracted by a modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1990).

DNA marker production and genotyping

Based on the whole-genome sequences of *B. rapa* published in *B. rapa* database version 1.5 (http://www.brassica.info/) by Wang et al. (2011), primer sets were designed for SCAR (sequence characterized amplified region) analysis, PCR–RFLP (PCR-restriction fragment length polymorphism) analysis and dot-blot-SNP (single nucleotide polymorphism) analysis (Shiokai et al. 2010). PCR was performed in a 10-μl reaction mixture containing 10 ng of genomic DNA, forward and reverse primers of 1 μM, $1 \times$ Ex *Taq* buffer (TaKaRa Biomed., Japan), 0.2 mM dNTP (TaKaRa Biomed., Japan) and 0.25 U Ex *Taq* (TaKaRa Biomed., Japan). A thermal cycle was carried out at 94 °C for 1 min (one cycle), and then at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 1 min (40 cycles). Amplified products were electrophoresed in 1 % agarose gel, followed by staining with ethidium bromide. For PCR–RFLP analysis, the PCR product was digested by a restriction enzyme, *Acc*II, *Alu*I, *Eco*O109I, *Hae*III, *Mbo*I, *Msp*I, *Mva*I or *Spe*I, and then electrophoresed in 6 % polyacrylamide gel. The DNA fragments were stained with ethidium bromide. For production of dot-blot-SNP markers, amplified PCR products from 'HM-DH1' and 'CRS1' were sequenced by the Sanger method using a DNA analyzer (CEQ 2000, Beckman Coulter) and SNPs were identified by alignment of the sequences using SEQUENCER^{TM} version 4.8 (Gene Code Corporation, USA). Bridge probes were designed and dot-blot analyses were performed according to Shiokai et al. (2010). Additionally, out of the dot-blot-SNP markers developed by Li et al. (2009) and Tonosaki et al. (2013), 269 markers were

also examined for the genotyping analysis according to Shiokai et al. (2010).

Results

Segregation distortion of microspore embryogenesis in F_1

'Ho Mei' has been reported to be a cultivar with high embryo yield in microspore culture (Zhang and Takahata 2001). High embryo yield of a doubled-haploid (DH) line of 'Ho Mei', 'HM-DH1', was confirmed in this study (Table 1). On the other hand, a self-compatible mutant of 'CR-Seiga 65' ('CRS1') showed low embryo yield in microspore culture (Table 1). To produce DNA markers showing polymorphism between these lines, we first examined dot-blot-SNP markers produced by Li et al. (2009) and Tonosaki et al. (2013). Out of 269 dot-blot-SNP markers, 26 markers exhibited polymorphism between these lines (Supplementary Table 1). Second, based on the *B. rapa* whole genome sequence version 1.5 (Wang et al. 2011), PCR primer sets were designed. Of these sets, DNA fragments amplified by 14 primer sets exhibited polymorphisms in size, i.e., co-dominant SCAR markers (Supplementary Table 2). PCR products amplified by 61 designed primer sets showed polymorphisms by digestion with *Acc*II, *Alu*I, *Eco*O109I, *Hae*III, *Mbo*I, *Msp*I, *Mva*I or *Spe*I (PCR–RFLP marker, Supplementary Table 3). The other DNA fragments amplified by 145 primer sets were sequenced to discover SNPs and the polymorphisms were used to produce dot-blot-SNP markers. Forty-seven designed dot-blot-SNP markers clearly detected polymorphisms between these lines (Supplementary Table 1). Additionally, four dot-blot-SNP markers for embryogenesis-related genes participating in seed formation, i.e., *BABY BOOM 1* (*BBM1*, Boutilier et al. 2002), *LEAFY COTYLEDON 1* (*LEC1*, Meinke et al. 1994), *ABSCISIC ACID INSENSITIVE 3* (*ABI3*, Parcy et al. 1994), and *CYP81F* (cytochrome P450 family 81 subfamily F:Malik et al. 2007), were also designed (Supplementary Table 1). Polymorphisms of three genes for a *CLE* (*CLAVATA/ESR-RELATED*)-like gene (Miwa et al. 2009), *HSP70B* (70 kDa class heat-shock protein) (Cordewener et al. 1995) and *Bnms4D-84* (Tsuwamoto et al. 2007) were also used for

producing dot-blot-SNP markers (Supplementary Table 1) and a PCR–RFLP marker (Supplementary Table 3). The total number of produced markers was 154, i.e., 20 in A01, 12 in A02, 17 in A03, 12 in A04, 13 in A05, 19 in A06, 10 in A07, 23 in A08, 17 in A09 and 11 in A10, and physical positions of these markers are shown in Fig. 1 and Supplementary Table 4.

The segregation ratio of the co-dominant DNA markers in a population of microspore-derived embryos is expected to be a 1:1 Mendelian ratio. However, since alleles of 'HM-DH1' are assumed to be associated with high embryogenic ability, distortions of the segregation ratio of the alleles can be expected. To identify the loci showing segregation distortion resulting in an increase of 'HM-DH1' alleles, one hundred and thirty-six embryos from microspores of an F_1 plant produced by crossing 'HM-DH1' and 'CRS1' were genotyped by the DNA markers. Distorted loci toward 'HM-DH1' and toward 'CRS1' were observed to be significant in A03, A04, A05, A06, A08, and A09, and in A02 and A07, respectively, at the 1 % level by χ^2 test (Fig. 1; Supplementary Table 4). Especially, the region containing the Br071-5c locus (named region A) on A08 showed the most prominent distortion, more than 3.0 in relative values of the number of the 'HM-DH1' type and the number of the 'CRS1' type (Table 2; Supplementary Table 4). Other highly distorted regions showing distortion of more than 3.0 were also observed around the Br110p marker (region B) on A05 and a region including Br103p and Br082p markers (region C) on A09 (Table 2). In a microspore-embryo population from another F_1 plant, the three regions, i.e., regions A, B and C, showed distorted segregation ratios reproducibly at the 1 % level (Supplementary Table 5).

Segregation of the markers located in regions A, B and C in an F_2 population developed by crossing 'HM-DH1' and 'CRS1' was also investigated. All investigated markers showed expected ratios of segregation, i.e., 'HM-DH1' homozygote:heterozygote:'CRS1' homozygote = 1:2:1, 'HM-DH1' homozygote:heterozygote and 'CRS1' homozygote = 1:3, or 'HM-DH1' homozygote and heterozygote:'CRS1' homozygote = 3:1 (Supplementary Table 6).

Additive effect of three loci showing segregation distortion on microspore embryogenesis in BC_3F_1

The effects of regions A, B and C on microspore-derived embryo yields were investigated using 37 BC_3F_1 plants, which were obtained by three-time backcrossing of an F_1 plant with 'CRS1'. A significant difference at the 1 % level in the efficiency of microspore embryogenesis was observed between heterozygotes and homozygotes of the 'CRS1' allele of the Br071-5c locus in region A (Table 3). Similarly, a significant difference was also observed

Table 1 Average embryo yield of the parents

	Embryo yield	SE	N
HM-DH1	187	31	7
CRS1	0.7	0.3	11

Average number of embryos per Petri dish. Microspores of 1.0×10^5 were cultured in a Petri dish

Fig. 1 Physical positions of the
DNA markers used in this study.
Physical positions were
determined based on the
Brassica Database version 1.5
(Wang et al. 2011). # and ##
represent distorted marker
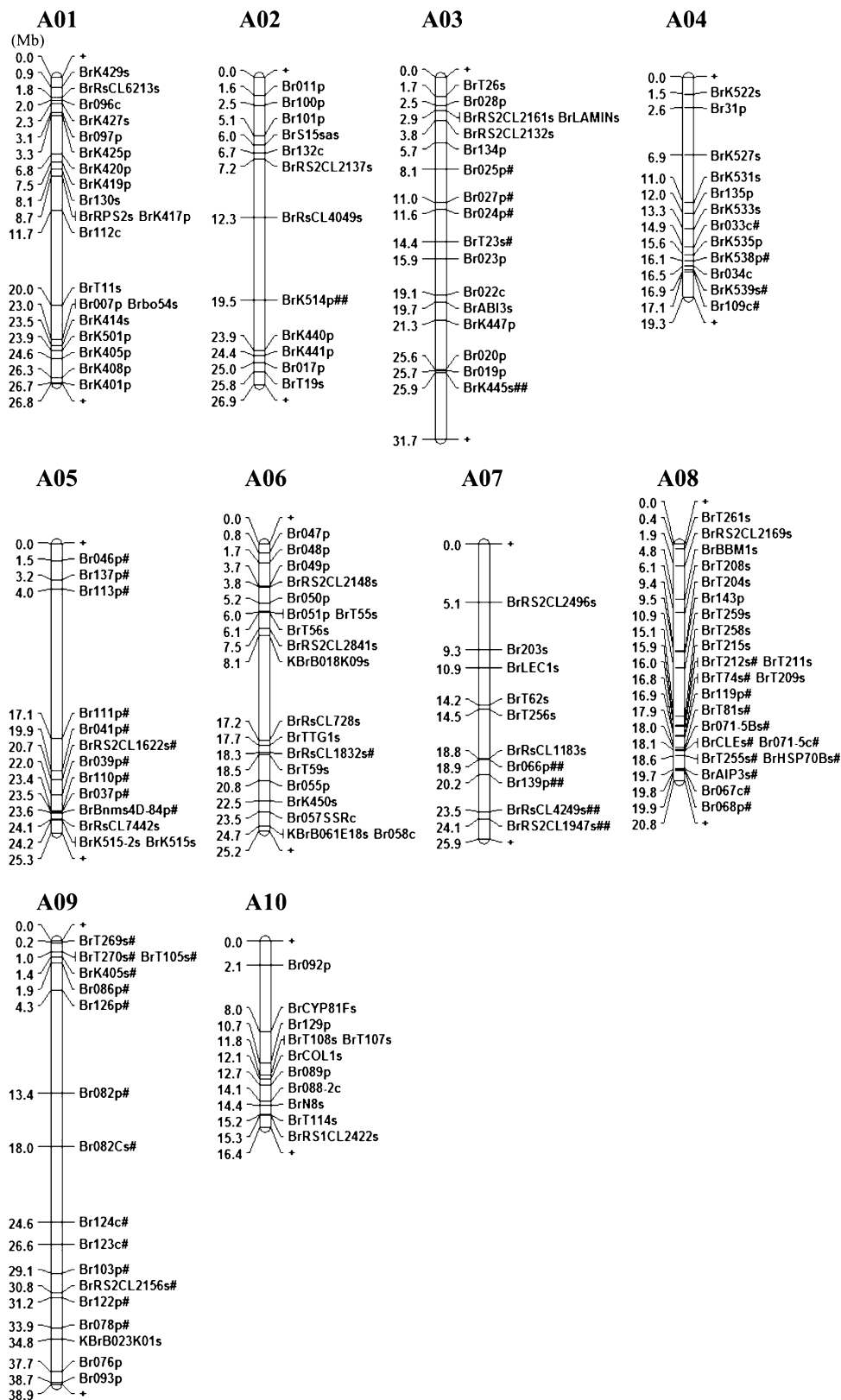toward 'HM-DH1' and 'CRS1',
respectively

Table 2 DNA markers showing distorted segregation in microspore embryo population derived from an F_1 plant of 'HM-DH1' × 'CRS1'

Chromosome	Position (Mb)	Marker	Ratio of segration HM-DH1' allele /'CRS1' allele	Note
A05	1.5	Br046p	2.05 **	
	3.2	Br137p	2.08 **	
	4.0	Br113p	1.61 **	
	17.1	Br111p	2.18 **	
	19.9	Br041p	2.87 **	
	20.7	BrRS2CL1622s	2.32 **	
	22.0	Br039p	1.95 **	
	23.4	Br110p	3.48 **	Region B
	23.5	Br037p	1.89 **	
	23.6	BrBnms4D-84p	1.90 **	
	24.1	BrRsCL7442s	1.52	
	24.2	BrK515-2s	1.58	
	24.2	BrK515s	1.52	
A08	0.4	BrT261s	1.46	
	1.9	BrRS2CL2169s	1.31	
	4.8	BrBBM1s	0.69	
	6.1	BrT208s	1.44	
	9.4	BrT204s	1.36	
	9.5	Br143p	1.49	
	10.9	BrT259s	1.53	
	15.1	BrT258s	1.42	
	15.9	BrT215s	1.57	
	16.0	BrT212s	1.63 **	
	16.0	BrT211s	1.56	
	16.8	BrT74s	1.73 **	
	16.8	BrT209s	1.15	
	16.9	Br119p	2.19 **	
	17.9	BrT81s	3.45 **	Region A
	18.0	Br071-5Bs	3.06 **	
	18.1	BrCLEs	3.86 **	
	18.1	Br071-5c	3.96 **	
	18.6	BrT255s	3.03 **	
	18.6	BrHSP70Bs	3.14 **	
	19.7	BrAIP3s	2.2 **	
	19.8	Br067c	1.91 **	
	19.9	Br068p	1.82 **	
A09	0.2	BrT269s	1.65 **	
	1.0	BrT270s	1.84 **	
	1.0	BrT105s	1.75 **	
	1.4	BrK405s	1.58 **	
	1.9	Br086p	2.38 **	
	4.3	Br126p	1.98 **	
	13.4	Br082p	3.29 **	Region C
	18.0	Br082Cs	2.55 **	
	24.6	Br124c	2.45 **	
	26.6	Br123c	3.19 **	
	29.1	Br103p	2.54 **	
	30.8	BrRS2CL2156s	1.96 **	
	31.2	Br122p	2.18 **	
	33.9	Br078p	1.77 **	
	34.8	KBrB023K01s	1.39	
	37.7	Br076p	1.24	
	38.7	Br093p	1.15	

Double asterisks show significant difference from the expected ratio (1.0) at 1% level.

between genotypes of the Br110p locus in region B at 5 % level. However, the Br082p locus in region C did not show a significant difference (Table 3). Cumulative effects of these three loci were investigated. Plants having 'HM-DH1' alleles in all three loci showed the highest embryo yield (Table 4), followed by plants having the 'HM-DH1' allele of Br071-5c and the 'HM-DH1' allele of either Br110p or Br082p. In contrast, plants having the 'CRS1' allele of Br071-5c with the 'HM-DH1' allele of Br110p or Br082p did not show a significant difference in the embryo yield compared with plants with the 'CRS1' alleles in all three loci (Table 4).

Discussion

Loci showing distorted segregation

In segregation analysis of microspore-derived embryos from an F_1 plant produced by crossing 'HM-DH1' and 'CRS1', three regions were found to have high segregation distortion resulting in an increase in 'HM-DH1' alleles. The most highly distorted region was around the Br071-5c locus on A08 (region A), and the second and third regions were around the Br110p locus on A05 (region B) and the Br082p locus on A09 (region C), respectively. However, these regions were segregated in Mendelian manner in the F_2 population derived from the same parents. These results suggest that the distorted segregations are caused not by a difference in male gametogenesis but by a difference of ability in microspore embryogenesis. Analysis using the BC_3F_1 population revealed that both region A and region B had predominant cumulative effects on the embryo yield from microspores (Table 4). Especially, the embryo yield of plants without the 'HM-DH1' allele in region A showed no significant difference from that of homozygotes of the 'CRS1' alleles in all three regions (Table 4). These results suggest that a 'HM-DH1' allele in region A containing the

Table 3 Effect of alleles on embryo yield in a BC_3F_1 population

Locus	Genotype	Embryo yield	SE
Br110p (A05)	*H/S* ($n = 13$)	10.2*	2.0
	S/S ($n = 23$)	5.2	1.0
Br071-5c (A08)	*H/S* ($n = 22$)	9.6**	1.4
	S/S ($n = 15$)	3.0	0.8
Br082p (A09)	*H/S* ($n = 17$)	7.8	1.8
	S/S ($n = 20$)	6.2	1.2

Efficiency is calculated per 1.0×10^5 microspores

**, * Show significant differences between *H* ('HM-DH1' allele)/*S* ('CRS1' allele) and *S/S* at each locus at 1 and 5 % level by *t* test, respectively

Br071-5c locus plays a significant role for enhancement of microspore embryogenesis.

Ajisaka et al. (1999) and Zhang et al. (2003) studied segregation distortions of RAPD (random amplified polymorphic DNA) markers in microspore-embryo populations developed from F_1 plants, and found two and seven distorted loci significantly associated with embryo yield from microspores, respectively. Together with the findings of this study, it is suggested that more than two genes are responsible for embryo yield from microspores. However, because RAPD markers are generally anonymous, identification of correspondence between the RAPD markers of Ajisaka et al. (1999), those of Zhang et al. (2003) and the markers developed in this study is difficult. Furthermore, the positions of the markers reported by Ajisaka et al. (1999) and Zhang et al. (2003) on *B. rapa* chromosomes were unclear. In this study, we identified the physical positions of the distorted loci based on the international standard *B. rapa* map (Wang et al. 2011). Choi et al. (2007) reported loci showing distorted segregation in a population of regenerated plants by anther culture of F_1. To most of chromosomes, except A01, the loci showing distorted segregation were distributed in clusters. By consideration with the genetic maps constructed by Choi et al. (2007) and Kim et al. (2009), a region containing an mSNRC73b marker on A05 and a region from a nia_m050a marker to an mEJU3a marker on A08 were found to be located on loci near region B and region A in this study, respectively. Although degrees of segregation distortion were not shown in Choi et al. (2007), regions around both region A and region B are considered to be regions reproducibly distorted in segregation of microspore-derived embryo populations and, therefore, those participating in microspore-derived embryogenesis.

Zhang and Takahata (2001) have revealed that dominant effects largely contributed to the microspore embryogenesis in *B. rapa*, suggesting that the responsible loci for dominant effects work sporophytically on microspore embryogenesis, although the loci have not been identified. To the contrary, as well as the loci reported by Ajisaka et al. (1999), Zhang et al. (2003), and Choi et al. (2007), the distorted region A, B, and C identified in this study have gametophytic effects on microspore embryogenesis. Therefore, these loci with the gametophytic effects might be different from those reported by Zhang and Takahata (2001).

Candidate genes associated with microspore-derived embryogenesis

BBM1 (Boutilier et al. 2002), *LEC1* (Meinke et al. 1994), *ABI3* (Parcy et al. 1994) and *CYP81F* (Malik et al. 2007), which are genes responsible for embryogenesis in seed

Table 4 Cumulative effect of 'HM-DH1' alleles on the Br071-5c locus and the two other loci on embryo yield in a BC$_3$F$_1$ population

Genotype[a]	Number of plants	Embryo yield	SE
H/S//H/S//H/S	5	15.4**	3.3
H/S//H/S//S/S, S/S//H/S//H/S	17	7.8*	1.2
H/S//S/S//H/S, H/S//S/S//S/S, S/S//S/S//H/S	9	3.6	1.3
S/S//S/S//S/S	6	2.2	1.0

**, * Show significant differences at 1 and 5 % level by *t* test, respectively, compared with value of an *S/S//S/S//S/S* genotype

[a] Genotype is represented by Br110p locus//Br071-5c locus//Br082p locus. *H* and *S* indicate the 'HM-DH1' and 'CRS1' allele, respectively

formation, have been located on A10, A07, A03 and A10, respectively. However, these loci did not exhibit significantly distorted segregation ratios in the microspore embryo population. From this result, it can be considered that these genes might not be key genes responsible for embryogenesis from microspores or that mutations causing change of function might not exist in these genes between 'HM-DH1' and 'CRS1'.

Based on the whole-genome sequence information of *B. rapa* (Wang et al. 2011), we attempted to find a candidate gene associated with embryo yield from microspores in regions A, B and C. Tsuwamoto et al. (2007) identified several ESTs, which are expressed during microspore embryogenesis in *B. napus*. One of them, which is a gene homologous to *Bnm4D-84* (Bra029631 in BRAD), was found to be located near the Br110 locus on A05 at a distance of 110 kb. The amino acid sequence encoded in the 7th exon of *Bnm4D-84* exhibits high homology with the zinc-finger domain of RING zinc-finger protein (Pfam algorism, http://pfam.xfam.org) and *Bnm4D-84* is expressed highly in early to middle stages of embryogenesis just after heat treatment to induce embryogenesis from microspores (Tsuwamoto et al. 2007). In comparison of coding sequences of *Bnm4D-84* between 'HM-DH1' and 'CRS1', seven polymorphisms were observed. Of these, one in the 5th exon and three in the 6th exon are non-synonymous substitutions (Supplementary Fig. 1a). However, no polymorphisms were observed in the Zinc-finger motif, which is a key motif for the function for DNA binding. Several candidate genes, i.e., *CLE* (CLAVATA/ESR-RELATED)–like gene (Bra016537), *MEE67* (MATERNAL EFFECT EMBRYO ARREST 67) (Bra016560) and HSP70B (70 kDa class heat-shock protein) (Bra016644), were found to be located adjacent to the Br071-5c locus on A08 at a distance of 11, 148, and 486 kb, respectively. *CLE* encodes a secreted small molecule with a signal peptide at the N-terminal and the CLE domain, and CLE protein may function as a signal molecule to regulate plant morphogenesis at the shoot apical meristem (Miwa et al. 2009). Comparison between two alleles in the coding sequence of Bra016537 (a *CLE-like* gene) detected two non-synonymous substitutions in the signal peptide region

(Supplementary Fig. 1b). However, SOSUI analysis (http://bp.nuap.nagoya-u.ac.jp/sosui/) indicated that these changes cause little difference in the degree of hydrophobicity responsible for secretion. Bra016644 (a *HSP70B* gene) is reported to be induced by heat shock and has no intron in its structure (Cordewener et al. 1995). PROSITE (http://prosite.expansy.org) and InterPro (www.ebi.ac.uk/interpro) predicted three conserved domains and a peptide-biding domain in the coded protein, respectively (Supplementary Fig. 1c). In comparison between both alleles in the coding sequences, 31 polymorphisms were observed. Only one was non-synonymous substitution causing a change of Arg251 in 'HM-DH1' to Lys251 in 'CRS1' (Supplementary Fig. 1c). However, because both amino acids are basic and the change is not in the conserved regions, this change may not cause functional differences as a HSP70B protein. Bra016560 (a *MEE67* gene) has four exons and is reported to be involved in embryo development, especially at the two-cell embryo stage (Pagnussat et al. 2005). In the coding region, there were no polymorphisms between the two lines. Investigating polymorphisms of these four genes, we were unable to infer a gene participating in the difference of microspore-derived embryogenesis. Further analysis is required for identification of the gene responsible for microspore embryogenesis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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