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Identification and evaluation of intervarietal substitution lines of rapeseed (*Brassica napus* L.) with donor segments affecting the diploidization rate of isolated microspores

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Abstract In *Brassica* species microspore derived doubled haploid lines are an important tool in breeding and research. A limiting step in the production of doubled haploid lines is the diploidization of the microspores. Strong differences have been observed in diploidization rates between different genotypes but little is know about the genetic control of these differences. To identify genomic regions that carry genetic factors controlling the diploidization rate of isolated microspores of rapeseed, marker segregations were compared between segregating populations of diploid and haploid microspore derived embryos and a BC_1 from a cross between 'Express 617' and 'RS239'. After map construction ten intervarietal substitution lines from the same cross were selected with donor segments covering five genomic regions that showed a pattern of skewed marker segregations across the three populations indicative of the segregation of genes controlling the diploidization rates. The diploidization rates of microspores of the ten lines ranged from 23.9 to 58.7 % while the recurrent parent 'Express 617' showed a rate of 52.5 %. For three lines the diploidization rates were significantly lower (P = 0.05) than the rate of 'Express 617'. By comparing donor segments between the significant and the non-significant lines, seven genomic regions that cover just between 4.17 and 6.16 % of the rapeseed genome were identified that may contain genetic factors controlling diploidization rates in rapeseed. In addition, one marker was found that has a high probability to be linked to such a factor. The significant lines represent an ideal material for further in depth studies of this trait.

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Introduction

Microspores or immature pollen grains normal developmental pathway in vivo is to differentiate into male gametophytes, but in vitro they can be diverted from this pathway to a sporophytic development by a stress treatment, usually a mild heat stress. Cultured in a liquid medium isolated microspores start to divide repeatedly and develop into embryo like structures, so

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called "microspore derived embryos" (MDEs; Lichter 1982; Swanson et al. 1987). MDEs can develop into fully-grown plants after transfer to a solid medium (Baillie et al. 1992; Burnett et al. 1992). Since microspores are the products of meiosis, microspore derived plants would be haploids, although some spontaneous diploidization usually occurs by duplication of the original chromosome set, leading to so called doubled haploid (DH) plants or lines, which are entirely homozygous. In rapeseed the spontaneous diploidization rates are low, in the range of 10–30 %, which is too low for practical applications (Möllers and Iqbal 2009). During the development of DH lines in rapeseed, diploization rates are increased by a treatment with colchicine, a natural alkaloid extracted mainly from the plant *Colchicum autumnale*, which depolymerises microtubules and disrupts regular mitotic cell division. (Chen et al. 1994; Möllers et al. 1994; Zhao et al. 1996a, 1996b; Hansen and Andersen 1996; Zhou et al. 2002a, 2002b; Weber et al. 2005; Seguí-Simarro and Nuez 2008). As discussed by Möllers and Iqbal (2009), the most efficient protocol for the colchicine treatment is a treatment of the freshly isolated microspores during the first days of cultivation, which will lead to entirely diploid plants with full fertility and diploidization rates of up to 90 %.

In rapeseed and related *Brassica* crops doubled haploid (DH) plants are a valuable material in modern breeding programs. Traits are fixed and homozygous true breeding lines are produced within one generation, which can help reduce the time needed to develop new varieties (Thomas et al. 2003; Ferrie and Caswell 2011; Ferrie and Möllers 2011). For the same reasons, in vitro microspore culture has become an important tool for mutagenesis (Barro et al. 2001, 2002; Sonntag and Rudloff 2004; Liu et al. 2005; Ferrie et al. 2008) and in vitro selection (Albrecht et al. 1995; Möllers et al. 2000; Liu et al. 2005). In addition, microspores, MDEs and DH plants are being used for transformation studies. Different approaches have been reported, using *Agrobacterium tumefaciens* (Dormann et al. 2001; Cegielska-Taras et al. 2008), particle gun bombardment (Fukuoka et al. 1998; Chen and Beversdorf 1994; Nehlin et al. 2000), or microinjection (Guerche et al. 1987; Jardinaud et al. 1993; Huang 1992).

One major application of DH lines is in genetic mapping. In rapeseed, F_1 derived segregating DH populations have been extensively used for map construction and the genetic analysis of important traits (Ferreira et al. 1994; Parkin et al. 1995; Sharpe et al. 1995; Uzunova et al. 1995; Zhao et al. 2005; Delourme et al. 2006; Qiu et al. 2006; Radoev et al. 2008). In these mapping studies distorted marker segregations have been observed regularly (summarized in Ferrie and Möllers 2011). This is often explained by the assumption that the selection of favourable alleles at loci controlling microspore embryogenesis leads to skewed segregations at linked marker loci (Foisset et al. 1993; Cloutier et al. 1995; Foisset and Delourme 1996). On the other hand, the production of doubled haploid lines in *Brassica* encounters three limiting factors: (1) the embryogenic potential of the microspores, (2) the diploidization capacity of the microspores after colchizine treatment, and (3) the direct embryo to plant conversion (DEPC) rate (Möllers and Iqbal 2009). For all three of these traits large variations have been observed between different genotypes of rapeseed, clearly indicating that genetic factors play a major role in the control of the three traits (Ferrie and Keller 2007; Chen et al. 1994; Möllers et al. 1994; Klutschewski 2013). Accordingly, skewed segregations in F_1 derived DH populations may not only result from selection of favourable alleles at loci controlling the embryogenic potential of microspores, but may also be due to selection at loci controlling the diploidization capacity of microspores or the DEPC rate of MDEs. In addition, skewed segregations may also result from earlier selection events during microspore formation. When analysing a DH population that consists of fully developed plants, it is not possible to distinguish between these possibilities. To assign a specific cause to skewed segregations in a specific region of the genome, it will be necessary to analyse and compare skewed segregations in different populations that have experienced only one or different subsets of the selective factors described above. Furthermore, since real segregating populations always represent samples, skewed segregations may occur just by chance, necessitating an independent verification of effects assigned to a genomic region.

While there has been a small number of studies on the genetics of the embryogenic potential of isolated microspores in rapeseed and *B. rapa*, either by comparing skewed segregations in F_1 derived populations of non-colchicinized MDEs and F_2 or BC_1

populations (Foisset et al. 1993; Ecke et al. 2015) or by determining the embryogenic potentials of microspores from all individuals of an F_2 mapping population (Zhang et al. 2003), to our knowledge there have been no studies until now about the genetic control of the diploidization rates of isolated microspores.

In this study we compared skewed segregations between F_1 derived populations of diploid and haploid MDEs and a corresponding BC_1 population of rapeseed to identify genomic regions putatively affecting the diploidization rates of isolated, colchicine treated microspores in rapeseed. The putative effects of selected regions were verified using intervarietal substitution lines (ISLs; Howell et al. 1996) with donor segments covering these regions. By comparing the donor segments in ISLs with diploidization rates significantly lower than the recurrent parent of the ISLs with the donor segments in ISLs that did not show significant differences in their diploidization rates, a number of genomic regions corresponding to only a few per cent of the rapeseed genome were identified that should carry genes that control the diploidization rate of isolated, colchicine treated microspores in this crop species.

Materials and methods

Plant materials and genetic maps

Isolated colchicinized microspores from one clonally propagated F_1 plant of a cross between 'Express 617', an inbred line of the winter rapeseed variety 'Express', and 'RS239', a resynthesized rapeseed line, were used to develop a segregating mapping population of 197 diploid MDEs for analysing skewed marker segregations. In addition, a population of 323 doubled haploid ISLs, derived from microspores of BC_4 plants of the same cross with 'Express 617' as recurrent parent, was available to verify effects of specific genomic regions on the diploidization rate of microspores. The genetic characterization of the ISL population with 484 AFLP markers from a map constructed in a corresponding F_1DH population (DH map) has been summarized in Ecke et al. (2015); full details can be found in Nurhasanah (2010). Shortly, the ISLs carry an average of three donor segments, with a minimum of one and a maximum of nine. In total, the minimum and maximum donor segment coverage on the DH map is

951 cM and 1587 cM, respectively, out of 2003 cM. Furthermore, information on marker segregations in a haploid MDE and a BC_1 population with 191 and 185 genotypes, respectively, derived from the same F_1 plant as the diploid MDE population (Ecke et al. 2015) and the corresponding genetic maps (haploid MDE and BC_1 map) was available for comparison with the diploid MDE population.

Donor plant growth

Microspore donor plants were grown in the greenhouse until they reached the four to six leaf stage after which they were vernalized in a controlled climate chamber at 4 °C under a photoperiod of 8 h for 8 weeks. Afterwards the plants were transferred to 13 cm plastic pots with compost soil and grown in the greenhouse. When the first flower buds appeared, plants were moved to an environmentally controlled growth chamber with a photoperiod of 16 h with day/night temperatures of 12°/6 °C, a light intensity of 400 µmol/m²s (Li-1400 Data Logger, Licor Biosciences, Nebraska, Canada) and a relative humidity of 65 %.

Bud harvest, microspore isolation and cultivation

Flower buds, 2–3 mm in length, were harvested and placed on ice. The harvested flower buds were surface sterilized under moderate agitation for 5 min in 1 % calcium hypochloride laced with some drops of tween 20 and rinsed three times with cold sterile water.

For microspore isolation, 16 buds were transferred to a sterile nylon sieve with a diameter of 5 cm and a pore size of 40–50 µm (Wilson Sieves, Nottingham, UK). The sieve was placed in a 9 cm sterile petri dish (92 × 16 mm, Sarstedt AG and Co., Nürnbrecht, Germany) filled with 5 ml cold sterile NLN medium [0.039 % (w/v) NLN medium basal salt mixture, 0.104 % (w/v) NLN medium vitamin mixture (Duchefa Biochemie B.V., Haarlem, The Netherlands), 0.05 % (w/v) Ca(NO$_3$)$_2$·4H$_2$O (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), 13 % (w/v sucrose)] (Lichter 1982). The buds were crushed with a sterile pestle and the microspores were released into the sieve. The pestle and the sieve were rinsed with 7 ml cold NLN medium and the medium containing the microspores was transferred to a sterile glass centrifuge tube and centrifuged for 5 min at 1000 rpm.

The pellet was resuspended in 12 ml cold NLN medium and centrifuged again. The washing step with 12 ml cold NLN medium was repeated one more time. The final pellet was resuspended in 5 ml cold NLN medium and transferred to a 9 cm petri dish already containing 6.4 ml cold NLN medium and 60 µl of a stock solution of colchicine (2 mg/ml). The petri dish was sealed with two layers of Parafilm and incubated in the dark at 32 °C for three days. After the three days the suspension with the microspores was centrifuged again for 5 min at 1000 rpm and the microspores were washed twice with 12 ml cold NLN medium. The final pellet was resuspended in 5 ml cold NLN medium and transferred to a 9 cm petri dish already containing 7 ml cold NLN medium. The petri dish was sealed with two layers of Parafilm and incubated in the dark at 28 °C for 7 days. Afterwards, the microspores were transferred to a shaker set to 80 rpm and incubated at a continuous temperature of 22 °C and a photoperiod of 12 h for 3 weeks.

Analysis of ploidy level

The ploidy level of MDEs was determined by flow cytometry from 0.5 cm^2 cotyledonary samples using a Partec Ploidy Analyser PA-I (Partec GmbH, Görlitz, Germany) according to the manufacturer's protocol: The samples were chopped up with a razor blade in 0.5 ml of CyStain UV Ploidy (Partec GmbH), a DAPI staining solution. After filtering through 30 µm partec CellTrics filters into sample tubes and waiting for 2–5 min at room temparature, 2 ml CyStain were added to the samples. Afterwards the samples were loaded onto the ploidy analyser and measured for about 20 s. Before MDE samples were measured, the ploidy analyser was calibrated using samples from a haploid and a diploid genotype of the rapeseed variety 'Drakkar' as standards. After measurements, the MDEs were frozen in liquid nitrogen and stored at −20 °C. Based on the analysis of ploidy levels, a population of diploid MDEs was selected.

DNA preparation, AFLP analysis and map construction

Total genomic DNA was extracted from frozen MDEs using the innuSPEED Plant DNA kit (Analytik Jena AG, Konrad-Zuse-Straße 1, Jena, Germany) according to the manufacturer's instructions. AFLP analyses were carried out following the multiplexing protocol described by Ecke et al. (2010). The same five primer sets (20 primer combinations) used in the development of the DH, haploid MDE and BC$_1$ maps and for the characterization of the ISLs were again used for the analysis of skewed segregations and for map construction in the diploid MDE population.

χ^2 tests were used to test the fit of marker segregations to the expected 1:1 segregation ratio. Significant deviations were considered at a level of P = 0.05. The χ^2 values were also used as a measure for the strength of the deviation of marker segregations from the expected segregation ratio.

For the construction of the genetic map in the diploid MDE population MAPMAKER/EXP 3.0b and a Perl script were used. The script automates the mapping process and distributes time critical processes to multiple copies of MAPMAKER in order to speed up the analysis. The mapping approach that was followed, which results in a high fidelity (HF) map of a subset of well ordered markers and a full map with all markers, was described in detail by Ecke et al. (2015).

Statistical analysis

The diploidization rates of isolated microspores of the ISLs and the recurrent parent 'Express 617' were determined from 10 to 20 repeated microspore cultures per genotype. For 'Express 617' the cultures were treated as replicates of one experiment. The mean diploidization rate and its 99 % confidence interval were calculated according to Gardner and Altman (1998). For the ISLs the diploidization rate was taken from the total of all MDEs that developed in the repeated microspore cultures and determined as the fraction of diploid MDEs. To identify ISLs with diploidization rates significantly different from the mean diploidization rate of 'Express 617' the diploidization rates of the ISLs were compared with the boundaries of the 99 % confidence interval of the rate of 'Express 617'. For all ISLs with diploidization rates between these boundaries the hypothesis of a significant difference to 'Express 617' was rejected. For ISLs with diploidization rates outside the boundaries a χ^2 test was applied with the calculated fractions of diploid and non-diploid MDEs at the closer boundary as expected frequencies. A significant difference to 'Express 617' was declared when the χ^2 test yielded a P $\leq$ 0.05.

Results

Genetic mapping in the diploid MDE population

To localize genetic factors that have an effect on the diploidization rate of isolated microspores of rapeseed, 526 AFLP markers were scored in the diploid MDE population. Using these markers, a genetic map of 470 markers could be constructed (Table S1), which consists of 19 linkage groups that could be assigned to the 19 chromosomes of rapeseed based on markers shared with the DH map and a map from the cross 'Express 617' × 'R53' (Ecke et al. 2010). Of linkage group C4 only as a short fragment of five markers was present. The full map had a total length of 2094.9 cM. A summary of the diploid MDE map and a comparison with the corresponding maps of the haploid MDE and the BC$_1$ populations is shown in Table 1.

Analysis of skewed segregations in the diploid MDE population

From the 470 AFLP markers on the diploid MDE map 186 (39.6 %) showed a significant deviation from the expected 1:1 segregation ratio in the diploid MDE population (Tables 2, S1). These skewed markers were distributed across all linkage groups except C3. Of the 186 skewed markers, 177 clustered in 23 regions with some regions also containing up to two markers with regular segregations. The number of markers in these regions ranged from 2 to 26, covering from 1.6 to 115.9 cM, in total 645.7 cM (30.8 %), of the map. Moreover, another nine skewed markers were found dispersed across the map, suggesting additional small regions with disturbed segregations. In total, 32 genomic regions showed skewed segregations in the diploid MDE population, with fifteen regions displaying an excess of 'Express 617' alleles and seventeen regions an excess of 'RS239' alleles. Peak χ^2 values in these regions ranged from 3.94 to 71.41. The nine single marker regions (nos. 5, 8, 9, 19, 25, 26, 27, 29, 31 in Table 2) were excluded from further analysis. From the 23 extended regions six regions showed regular segregations in the corresponding regions on the haploid MDE and BC$_1$ maps (nos. 2, 6, 12, 13, 15, 30), the pattern expected for the segregation of a genetic factor controlling diploidization efficiency. Two additional regions (nos. 21, 24) showed a comparable pattern only that here no information

was available for the BC$_1$ map since all markers in the two regions could not be mapped on this map. Together, these eight regions constitute the group of regions where skewed segregations were only observed in the diploid MDE population. Of the remaining fifteen regions, one region was not covered by donor segments in the ISLs (no. 28), five regions showed similarly skewed segregations in the diploid MDE map as in the haploid MDE map (nos. 1, 10, 18, 22, 23), four regions showed segregations less skewed in the diploid MDE map than in the haploid MDE map (nos. 3, 11, 16, 17), and five regions showed different segregation patterns between the diploid and haploid MDE maps across the region (nos. 4, 7, 14, 20, 32). A closer investigation of these five regions revealed that all contained subsections where the χ^2 values in the diploid MDE population greatly exceeded the values in the haploid MDE population and the BC$_1$ population (Table S1), which was considered to also indicate the segregation of a genetic factor controlling diploidization efficiency in these subsections. This notion was reinforced in some of these regions by the specific pattern of change in the skewed segregations across the regions. For example in region no. 14 on A7, skewed segregations in the haploid MDE population rise to a peak at 53.8 cM after which the χ^2 values fall again. On the other hand, in the diploid MDE population χ^2 values rise more slowly, but strongly exeed the values in the haploid MDE population after 60.4 cM, reaching a peak at 71.3 cM. This pattern was interpreted as the segregation of two loosely linked loci, one around 71.3 cM that affected the diploidization efficiency in the diploid MDE population, the other around 53.8 cM, which affected the segregation ratio in the haploid MDE population.

Selection of regions with skewed segregations and of ISLs with donor segments covering these regions

The selection of regions with skewed segregations in the diploid MDE population for further analysis was based on peak χ^2 values, the distribution of skewed segregations across the regions, and the availability of ISLs carrying donor segments covering these regions. Three regions on A3, A8 and C4 (nos. 6, 15, 24 in Table 2; Fig. 1) were chosen from the group of regions where skewed segregations were only observed in the diploid MDE population. The

Table 1 Comparison of linkage groups, number of markers and length of linkage groups of the high fidelity (HF) and full maps of the diploid MDE, the haploid MDE and the BC$_1$ populations

| LG | Diploid MDE population | | | | Haploid MDE population | | | | BC$_1$ population | | | |
| | HF map | | Full map | | HF map | | Full map | | HF map | | Full map | |
	No. markers	Length (cM)	No. markers	Length (cM)	No. markers	Length (cM)	No. markers	Length (cM)	No. markers	Length (cM)	No. markers	Length (cM)
A01	13	114.2	20	114.2	16	91.7	20	91.7	11	76.8	16	76.8
A02[a]	24	133.9	32	133.9	16	104.2	23	104.2	8	55.5	12	55.5
					5	22.0	11	22.0				
A03	31	126.3	46	129.9	41	125.0	46	125.0	14	134.5	21	152.5
A04	14	117.1	17	118.2	14	108.4	16	108.4	3	9.5	5	28.3
A05	23	108.0	30	108.0	24	60.7	29	60.7	11	65.0	17	65.0
A06	19	98.5	24	98.5	24	112.8	25	112.8	7	39.6	9	40.2
A07	14	84.2	20	87.0	16	88.9	20	88.9	11	88.7	11	88.7
A08	15	71.1	19	72.3	17	62.4	19	62.4	8	61.3	9	62.2
A09	21	106.4	30	106.4	26	114.8	28	114.8	10	86.5	13	86.5
A10	14	87.8	17	87.8	14	74.2	16	74.2	8	79.7	9	79.7
C01	18	189.2	24	189.2	17	108.9	23	134.8	10	60.4	11	60.4
C02	16	110.2	22	110.2	16	85.8	23	85.8	6	72.9	7	72.9
C03	27	130.7	29	130.7	28	149.1	30	149.1	12	122.1	17	122.1
C04	5	15.5	5	15.5	4	17.1	4	17.1	–	–	–	–
C05	24	86.7	32	86.7	26	83.5	32	83.5	12	58.2	18	58.2
C06	18	114.9	21	115.9	20	94.6	22	94.7	9	81.2	11	90.5
C07[a]	22	141.1	25	141.1	23	126.0	25	126.0	5	16.1	7	18.4
									2	2.2	2	2.2
									2	3.4	2	3.4
C08	16	110.0	20	112.2	20	114.2	20	114.2	8	70	9	70
C09	26	137.2	37	137.2	32	127.0	38	127.0	13	91.1	13	91.1
Naa	–	–	–	–	2	12.3	3	14.5	–	–	–	–
Nbb	–	–	–	–	2	1.6	3	20.4	2	2.8	2	2.8
Ncc	–	–	–	–	2	8.3	2	8.3	–	–	–	–
Ndd	–	–	–	–	3	2.7	3	2.7	–	–	–	–
Sum	360	2083.0	470	2094.9	408	1896.2	481	1943.2	172	1277.5	221	1327.4

[a] A2 is represented by two disjointed parts, A2a and A2b, in the haploid MDE map and C7 is represented by three disjointed parts, C7a, C7b and C7c in the BC$_1$ map (Ecke et al. 2015)

remaining regions in this group either showed only low χ^2 values (<7.5) or were rather long (≥40 cM), which made it difficult to cover them by donor segments. In addition, two regions on A2 and A7 (nos. 4 and 14) were chosen from the regions with subsections where χ^2 values in the diploid MDE population strongly exceeded the values in the other populations. These regions were chosen based on higher peak χ^2 values (≥12), the pattern of skewed segregations along the region and a good coverage by donor segments (Fig. 1). On A2 a subsection from 54.2 to 64.4 cM showed highly skewed segregations in the diploid MDE map while, with one exception, in the haploid MDE population the χ^2 values of the markers only just exceeded the significance threshold. Only one marker in this subsection showed a high χ^2 value of 8.13 in the haploid MDE population, which, nevertheless, was still less than the lowest value in the

Table 2 Regions with skewed segregations on the diploid MDE map and comparison with the corresponding regions on the haploid MDE and BC₁ maps

| No. | LG | Diploid MDE map | | | | | | Haploid MDE map | | | | | | BC₁ map | | | | | |
		Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2	Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2	Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2
1	A01	21.8	23.4	1.6	3/3	R	6.84	66.2	67.8	1.6	3/2	R	5.08	5.7	8.0	2.3	3/0	–	0.45
2	A01	88.3	114.2	25.9	3/3	E	6.95	0.0	8.3	8.3	2/0	–	2.17	48.4	76.8	28.8	3/0	–	1.78
3	A02	0.0	12.5	12.5	3/3	R	21.60	0.0	18.2	18.2	5/5	R	36.45	41.7	50.5	8.8	5/1	R	4.65
4	A02	54.2	77.3	23.1	8/8	R	16.49	48.8	104.2	55.4	18/9	R	8.13	23.2	24.9	1.7	2/0	–	1.42
5	A03	0.0	0.0	0.0	1/1	R	10.80	–	–	–	–	–	–	134.5	134.5	0.0	1/0	–	0.09
6	A03	123.0	129.9	6.9	8/7	E	14.88	0.0	3.8	3.8	8/0	–	2.66	46.3	49.7	3.4	5/0	–	0.79
7	A04	–1.1	61.2	62.3	12/11	R	45.55	0.0	95.4	95.4	12/11	R	28.81	0.0	28.3	28.3	5/5	R	87.23
8	A04	117.1	117.7	0.0	1/1	R	22.84	–	–	–	–	–	–	26.3	26.3	0.0	1/1	R	20.45
9	A05	0.0	0.0	0.0	1/1	R	13.41	–	–	–	–	–	–	–	–	–	–	–	–
10	A05	33.6	96.2	62.6	26/26	E	28.81	17.8	58.0	40.2	26/26	E	32.40	13.6	50.2	36.6	14/2	R	6.92
11	A06	17.0	33.4	16.4	4/4	E	6.22	25.5	42.2	16.7	4/4	E	9.38	–	–	–	–	–	–
12	A06	58.7	98.5	39.8	6/4	E	16.18	70.2	106.3	36.1	6/1	E	3.86	12.7	39.6	26.9	5/0	–	0.57
13	A07	–2.8	6.0	8.8	4/4	E	6.35	79.7	88.9	9.2	4/0	–	0.28	0.0	6.0	6.0	2/0	–	3.76
14	A07	29.8	79.8	50.0	13/13	E	11.94	5.6	54.7	49.1	13/10	E	20.45	33.1	88.7	55.6	8/0	–	2.22
15	A08	–1.2	10.4	11.6	9/8	E	11.00	0.0	8.3	8.3	9/0	–	3.38	51.8	62.2	10.4	4/0	–	0.56
16	A08	62.7	71.1	8.4	2/2	E	10.83	56.5	62.4	5.9	2/2	E	26.34	0.0	0.0	0.0	1/0	–	1.41
17	A09	0.0	24.6	24.6	18/17	R	12.39	90.4	114.3	23.9	17/17	R	23.01	62.8	86.5	23.7	7/6	E	8.50
18	A09	61.4	71.9	10.5	5/3	E	8.99	40.7	52.4	11.7	5/1	E	7.04	22.8	28.6	5.8	2/0	–	2.30
19	A10	0.0	0.0	0.0	1/1	E	9.80	–	–	–	–	–	–	–	–	–	–	–	–
20	C01	69.6	94.0	24.4	7/7	R	12.80	42.0	61.3	19.3	7/6	R	9.28	–	–	–	–	–	–
21	C01	141.6	189.2	47.6	4/4	R	17.42	69.4	108.9	39.5	5/1	R	2.86	–	–	–	–	–	–
22	C02	0.0	24.9	24.9	9/9	E	71.41	0.0	23.0	23.0	11/10	E	51.86	–	–	–	–	–	–
23	C02	45.8	52.3	6.5	3/3	E	41.63	35.3	38.2	2.9	2/2	E	16.18	–	–	–	–	–	–
24	C04	0.0	15.5	15.5	5/5	E	20.11	0.0	17.1	17.1	4/0	–	0.90	–	–	–	–	–	–
25	C05	5.6	5.6	0.0	1/1	R	3.94	78.5	78.5	0.0	1/0	–	0.05	–	–	–	–	–	–
26	C05	39.3	39.3	0.0	1/1	R	4.95	40.9	40.9	0.0	1/0	–	3.06	–	–	–	–	–	–
27	C05	86.7	86.7	0.0	1/1	R	5.37	0.0	0.0	0.0	1/0	–	2.63	–	–	–	–	–	–

Table 2 continued

| No. | LG | Diploid MDE map | | | | | | Haploid MDE map | | | | | | BC$_1$ map | | | | | |
		Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2	Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2	Start (cM)	End (cM)	Length (cM)	tm/sm[a]	Favored allele[b]	Peak χ^2
28	C06	0.0	115.9	115.9	21/21	E	20.11	0.0	94.7	94.7	22/22	E	48.02	0.0	90.5	90.5	11/1	E	4.65
29	C07	0.0	0.0	0.0	1/1	R	5.63	13.4	13.4	0.0	1/1	R	8.51	–	–	–	–	–	–
30	C08	50.9	77.3	26.4	5/5	R	7.40	24.4	46.0	21.6	5/0	–	1.20	28.8	50.1	21.3	4/0	–	1.84
31	C09	0.0	0.0	0.0	1/1	R	17.34	127.0	127.0	0.0	1/0	–	0.78	–	–	–	–	–	–
32	C09	117.7	137.2	19.5	7/7	R	14.53	0.0	24.1	24.1	8/6	R	9.78	84.4	91.1	6.7	2/0	–	1.41
Sum				645.7	194/186					626	203/136					356.8	85/16		

[a] tm: total number of markers in the region; sm: number of markers with significantly skewed segregations (P = 0.05) in the region

[b] R = 'RS239' allele, E = 'Express 617' allele

diploid MDE population. In the BC$_1$ map the markers in this region could not be mapped. As already stated above, on A7 the pattern of skewed segregations across the populations indicated the segregation of two loosely linked factors, one of which affects diploidization efficiency. Here marker segregations were strongly skewed in the diploid MDE population from 66.4 to 79.8 cM, while the same markers showed regular or less skewed segregations in the haploid MDE population. In the BC$_1$ population the marker segregations in this region were regular. Among the chosen regions an excess of 'RS239' alleles was observed on A2, while an excess of 'Express 617' alleles occurred on A3, A7, A8, and C4.

In total 10 ISLs were selected with donor segments covering the selected regions with skewed segregations (Fig. 1). On A2, the donor segment in ER1284, with a minimal extent from 12.5 to 64.4 cM, covers the whole subsection from 54.2 to 64.4 cM where the χ^2 values in the diploid MDE population exceed the values in the haploid MDE population. In its maximal extent it also covers the region from 64.4 to 77.3 cM, part of a region where a transition from highly skewed segregations in the diploid MDE population to highly skewed segregations in the haploid MDE population occurs. The remainder of this transitory region is covered by the donor segments in two additional ISLs, ER137 and ER199. On A3 the minimal extent of the donor segments in the two selected ISLs, ER984 and ER1433, covers 87.5 % of the selected region with skewed segregations. Taking into account the maximal extent of the donor segments the whole region, which extents from 123.0 to 129.9 cM, is covered. On A7 the selected ISL, ER1437, carries a donor segment that covers the entire subsection where skewed segregations are more pronounced in the diploid MDE population than in the haploid population. On A8 the minimal extent of the donor segments of the two selected ISLs, ER444 and ER807, covers 71 % of the region with skewed segregations, while, taking into account the maximal extent of the donor segments, the whole region is covered. The peak χ^2 value of this region falls within the minimal extent of the donor segments. On C4 the two selected ISLs, ER281 and ER964, carry donor segments that cover the entire region.

In the original characterization of the donor segments in the ISL population based on the DH map, the donor segments of ER984 on A3 is a

Fig. 1 Distribution of marker segregation patterns in the diploid and haploid MDE and the BC₁ populations and donor segments of selected ISLs on linkage groups A2, A3, A7, A8 and C4 of the diploid MDE map. Values from a χ^2 test for 1:1 segregation are displayed as a measure for the degree to which marker segregations deviate from the expected segregation ratio. The horizontal *purple line* indicates the significance threshold at $P = 0.05$. Each *blue*, *red* and *green mark* represents a marker on the diploid MDE, the haploid MDE and the BC₁ map, respectively. Positions refer to the positions of the markers on the diploid MDE map. The *black* and *yellow bars* represent minimal and maximal extents, respectively, of the donor segments of selected ISL on the five linkage groups. (Color figure online)

subsegment of the corresponding segment in ER1433, distiguished by a pair of cosegregating markers at 7.9 cM both of which show donor alleles in ER1433, but only one in ER984 (Fig. S1). In the diploid MDE map a marker, E44M50-147E, has been mapped at the end of A3 at 129.9 cM (Table S1), which corresponds to the beginning of A3 in the DH map since A3 has an inverted orientation in the diploid MDE map with respect to the orientation in the DH map. This marker had not been mapped in the DH map, but had been scored in the ISL population. E44M50-147E shows a donor allele in ER984 but a recurrent parent allel in ER1433 and therefore also distiguishes the two lines as shown in Fig. 1. Since in the upper respectively lower part of A3 the alignement between the DH and the diploid MDE map was poor, it was not possible to extrapolate a position for E44M50-147E on the diploid MDE map. The marker is therefore not represented in Fig. S1 and was entered in Table 3, which shows an overview of all donor segments in the selected ISLs, as a separate one-marker segment with a position based on the diploid MDE map. In addition to ER984, E44M50-147E also shows donor alleles in ER281 and ER964.

Determination of the diploidization rates
of microspores of 'Express 617' and the selected
ISLs

To determine the diploidization rate of isolated microspores of 'Express 617', 23 independent microspore isolations were cultured (Table 4) and the ploidy level of the resulting MDEs was analysed. Of the 23 cultures, 12 yielded ≥10 MDEs. For each of these cultures an independent estimation of the diploidization rate was determined. The remaining cultivations yielded less than ten MDEs each and a 13th estimation of the diploidization rate was determined from the total of MDEs from these cultivations. Based on these 13 independent estimates a mean diploidization rate of 'Express 617' of 52.5 % was calculated, with a 99 % confidence interval ranging from 34.8 to 70.2 %.

The diploidization rates of the ten selected ISLs were calculated from the total number of MDEs derived from 10 to 20 microspore preparations (Table 4). The number of MDEs analysed for ploidy level ranged from 107 to 395 and the diploidization rates of the lines from 23.9 % for ER281 to 58.7 % for ER444. These rates cover a range of 0.46–1.12 times

the mean rate of the recurrent parent 'Express 617'. A comparison of the diploidization rates of the ISLs with the boundaries of the 99 % confidence interval of the mean rate of 'Express 617' identified three ISLs, ER281, ER964, and ER984, with diploidization rates significantly lower than 'Express 617', indicating that the donor segments in these lines carry alleles that reduce the diploidization efficiency of isolated microspores. No lines were identified with significantly enhanced diploidization efficiencies.

Donor segments involved in the control
of the diploidization rate of microspores

The available marker data showed that the number of donor segments carried by the selected ISLs range from one to six (Table 3, Fig. S1). In individual ISLs the sum of the minimal extents of the donor segments ranges from 3.2 to 66.9 cM, covering between 0.16 and 3.34 % of the total length of the DH map. Considering the maximal extent, the coverage by the donor segments ranges from 7.7 to 76.2 cM, corresponding to 0.38 and 3.80 % of the DH map, respectively (Table 5).

To identify genomic regions that may carry genetic factors involved in the control of the diploidization rate of microspores, the distribution and overlap of donor segments in the ISLs with significantly lower diploidization rates were compared with the donor segments in the non-significant ISLs (Fig. S1, Table 3). Seven regions on five linkage groups were identified where donor segments only occurred in the significant lines, which, accordingly, may carry genetic factors controlling the diploidization rate of microspores (Table 6). The seven regions are on chromosomes A03, A08, C03, C04 and C05. The minimal extent of these regions ranges from 0.0 to 56.8 cM and covers 83.6 cM (4.17 %) of the total length of the DH map. Taking into account the maximal extent of these regions the range is from 3.3 to 57.6 cM, covering 123.4 cM (6.16 %) of the total length of the DH map.

Discussion

In order to identify genomic regions that carry genetic factors affecting the diploidization rate of isolated, colchicine treated microspores in rapeseed, skewed

Table 3 Genomic regions with skewed segregations in the diploid MDE population and the donor segments of intervarietal substitution lines selected for coverage of these regions

LG	A02	A03	A07	A08	C04
Extent[a]	54.4 - 64.2 cM	123 - 129.9 cM	66.4 - 79.8 cM	-1.2 - 8 cM	0 - 17 cM
Extent[b]	58.8 - 65.3 cM	0 - 11.1 cM	74.4 - 90.2 cM	0 - 5.5 cM	46.9 - 94.5 cM
Favoured allele[c]	R	E	E	E	E
Selected ISLs	ER137 ER199 ER1284	ER984 ER1433	ER1437	ER444 ER807	ER281 ER964
No. of segments[d]	2 1 2	6 1	3	1 1	3 3

Donor segments of the selected ISLs

LG	Segments (ER137)	(ER199)	(ER1284)	(ER984)	(ER1433)	(ER1437)	(ER444)	(ER807)	(ER281)	(ER964)
A02	0.0 - 42.8[e]	27.0 - 42.8	**54.5 - 78.3**	54.5 - 58.8; 62		0.0 - 12.4				
A03				**7.9 - 11.1**; (129.9)[f]	7.9 - 11.1	7.9 - 11.1			(129.9)[f]; 136.8	(129.9)[f]; 136.8
A07						**71.2 - 91.7**				
A08/lg22				0.0 - 4.6[g]			**0.0 - 5.5**	**0.0 - 5.5**		
C01			44.3							
C02	0.0									
C03				77.1 - 79.3						
C04									**46.9 - 80.7; 84.4 - 94.5**	**23.9 - 80.7; 84.4 - 94.5**
C05				34.9 - 44.8						

Donor segments that cover the selected genomic regions with skewed segregations are in bold

Segments with bold box borders cover or overlap genomic regions that may be involved in the decreased diploidization rates of the significant ISLs

Positions are in cM and refer to the DH map if not otherwise indicated

[a] Positions of genomic regions with skewed segregations in the diploid MDE population with respect to the diploid MDE map

[b] Positions of the regions with respect to the DH map based on an alignment with the diploid MDE map

[c] R, E: excess of 'RS239' or 'Express 617' alleles in the respective region

[d] Number of donor segments in the selected ISLs according to the DH map

[e] Minimal extent of donor segments

[f] Segment marked by E44M50-147E, position according to diploid MDE map

[g] Posititions are from a pair of markers that constitute lg22 in the DH map that were shown to belong to chromosome A8 in the diploid MDE map

marker segregations were analysed in a segregating diploid MDE population and were compared to skewed segregations found in corresponding haploid MDE and BC$_1$ populations. In a second step, the effects of selected regions on the diploidization rate were verified using ISLs carrying donor segments covering these regions.

For the analysis of skewed segregations in the diploid MDE population a genetic map was constructed. With 470 markers and a total length of 2094.9 cM this map is similar to the map constructed in the haploid MDE population, which has 481 markers and a length of 1943.2 cM. In contrast, with only 221 markers and 1327.4 cM, the BC$_1$ map has both, a lower marker density and lower genome coverage than the diploid and haploid MDE maps, although the same primer sets were used for marker analysis in all three populations. The lower marker number on the BC$_1$ map is a consequence of the dominant inheritance of the AFLP markers used for marker analysis, which allowed only the mapping of markers with the

Table 4 Diploidization rate of microspores of the 10 selected ISLs compared to the diplodization rate of microspores of the recurrent parent 'Express 617'

ISL	n^a	N^b	Mean[c]	Diploid MDEs (%)[d]	Ratio to express	χ^{2e}
ER137	16	110	6.9	43.6	0.83	$3.79^{n.s.}$
ER199	10	107	10.7	36.4	0.69	$0.13^{n.s.}$
ER281	19	142	7.5	23.9	0.46	7.38^{***}
ER444	10	143	14.3	58.7	1.12	$8.91^{n.s.}$
ER807	13	395	30.4	45.6	0.87	$20.19^{n.s.}$
ER964	20	207	10.4	27.1	0.52	5.48^{***}
ER984	11	168	15.3	25.6	0.49	6.27^{***}
ER1284	13	119	9.2	32.8	0.63	$0.22^{n.s.}$
ER1433	17	166	9.8	45.2	0.86	$7.88^{n.s.}$
ER1437	16	123	7.7	56.1	1.07	$11.62^{n.s.}$
Express 617	23	276	11.5	52.5	1	

[a] Number of microspore preparations

[b] Number of individual MDEs analysed by flow cytometry

[c] Mean number of embryos per microspore preparation

[d] Percentage of diploid MDEs

[e] χ^2 test using the calculated ratio of diploid and non-diploid MDEs of 'Express 617' at the closer border of the 99 % confidence interval ($34.8 \leq 52.48 \leq 70.2$) as the expected ratio

*, **, *** diploidization rate is significantly different from the mean diploidization rate of 'Express 617' at $P = 0.05$ ($\chi^2 = 3.84$), $P = 0.01$ ($\chi^2 = 6.64$) and $P = 0.001$ ($\chi^2 = 10.83$), respectively, n.s.: not significant ($P > 0.05$ or the diploidization rate is between the boundaries of the confidence interval of the mean of 'Express 617')

Table 5 Minimal and maximal genome coverage by the donor segments in the selected ISLs according to the DH map

ISL	Minimal coverage		Maximal coverage	
	Absolute coverage (cM)	Percentage of map (%)	Absolute coverage (cM)	Percentage of map (%)
ER137	42.8	2.14	62.4	3.12
ER199	15.8	0.79	32.2	1.61
ER281	43.9	2.19	74.1	3.70
ER444	5.5	0.27	7.7	0.38
ER807	5.5	0.27	7.7	0.38
ER964	66.9	3.34	76.2	3.80
ER984	24.2	1.21	75.1	3.75
ER1284	23.8	1.19	46.2	2.31
ER1433	3.2	0.16	10.8	0.54
ER1437	36.1	1.80	62.6	3.13
Mean	26.8	1.34	45.5	2.27

dominant (visible) allele coming from the donor parent 'RS239' in the BC_1 population.

The diploid MDE map consists of nineteen linkage groups corresponding to the nineteen chromosomes of *B. napus*. With about one marker every 4.5 cM the marker density of this map can already be considered high. Nevertheless, one linkage group, C4, consisted of only five markers covering just 15.5 cM, although

Table 6 Genomic regions that may control the diploidization rate of microspores

LG	Minimal extent			Maximal extent			No. Markers	ISLs
	Start (cM)[a]	End (cM)	Length (cM)	Start (cM)	End (cM)	Length (cM)		
A03[b]	129.9	129.9	0	127.4	129.9	2.5	1	ER281, ER964, ER984
A03	136.8	136.8	0.0	134.4	137.7	3.3	1	ER281, ER964
A08/lg22[c]	0.0	4.6	4.6	0.0	4.6	4.6	2	ER984
C03	77.1	79.3	2.2	74.4	83.7	9.3	2	ER984
C04	23.9	80.7	56.8	23.9	81.5	57.6	10	ER281, ER964
C04	84.4	94.5	10.1	81.5	96.8	15.3	7	ER281, ER964
C05	34.9	44.8	9.9	34.9	65.7	30.8	7	ER984
Sum			83.6			123.4		
Map coverage			4.17 %			6.16 %		

[a] Positions refer to the DH map if not indicated differently

[b] Marker position is referring to the diploid MDE map

[c] Two markers constituting lg22 in the DH map that belong to A08 according to the diploid MDE map

in other maps, for example the map published by Ecke et al. (2010), C4 (N14) was much larger. In addition, in the haploid MDE map and the BC$_1$ map—both maps developed in mapping populations derived from the same F$_1$ plant as the diploid MDE population—C4 was also only present as a small fragment or missing altogether. This indicates that, even though the marker density was high, there are some parts of the genome, at least on C4, which are not covered by the AFLP markers of these maps. A possible explanation is that in this part of the genome extensive deletions or homoeologous exchanges between the two genomes of rapeseed have occurred. As a result marker loci may be deleted or, in the case of non-reciprocal homoeologous exchanges, duplicated on different chromosomes, which would render the markers unmappable. It has actually been shown that homoeologous exchanges occur with higher frequency in resynthesized rapeseed genotypes or in crosses between such genotypes and natural rapeseed than in natural rapeseed itself (Parkin et al. 1995; Song et al. 1995; Udall et al. 2005; Szadkowski et al. 2010; Chalhoub et al. 2014). Furthermore Ecke et al. (2015) found some evidence that such occurrences have happened during the development of the ISLs or already in the donor parent 'RS239'.

About 40 % of the markers (186 out of 470 markers) on the diploid MDE map showed skewed segregations in the diploid MDE population. This is less than the 48 % that had been observed in the haploid MDE population (Ecke et al. 2015), a somewhat unexpected result since the diploid MDE population has experienced an extra limiting step, the diploidization, which should have caused additional skewed segregations. This contradiction may indicate that sample effects, that is chance, may have contributed to the occurrence of skewed segregations in both populations.

On the diploid MDE map, the skewed markers cluster in 32 regions, including nine regions marked by only one marker. Overall, both parental alleles were favoured in a similar number of regions. Contrary to this, among the eight regions where skewed segregations were only observed in the diploid MDE population—the pattern expected when the skewed segregations are due to the segregation of alleles at a locus controlling the diploidization efficiency—all but two regions showed an excess of 'Express 617' alleles. On the other hand, among the five regions with changing patterns of skewed segregations, where only part of the region showed the expected pattern, five showed an excess of 'RS239' alleles. In addition, 'RS239' alleles were predominantly favoured among the single marker regions where eight out of nine showed an excess of this allele. This distribution of the favoured allele across different types of regions with skewed segregations may indicate that 'Express 617' contributed more alleles or more strongly acting alleles favourable for the diploidization efficiency of isolated microspores of rapeseed than 'RS239'.

Single marker regions were excluded from the selection of regions with skewed segregations for verification. This exclusion was based on two considerations: (1) when a region with skewed segregations is only indicated by just one marker there is an increased probability that the underlying genetic factor has only a minor effect, and (2) with individual markers there is always the possibility that skewed segregations are due to a bias in allele scoring. If one of the alleles is more difficult to score, more failed scorings may occur for this allele, leading to a deviation from the expected allele ratio among the scored genotypes.

To verify possible effects of the five genomic regions chosen because they showed the pattern of skewed segregations across the populations expected from the segregation of a locus controlling the diploidization capacity of isolated microspores, ten ISLs were selected with donor segments covering the chosen regions. The comparison of the diploidization rates of isolated microspores of the ISLs with the rate of 'Express 617' was hampered by the fact that microspores of 'Express 617' and most of the ISLs have a very low embryogenic potential (Ecke et al. 2015). As a consequence, many of the repeated microspore cultivations yielded less than ten MDEs, a number considered too small a sample size for a proper analysis of variance. Therefore, diploidization rates derived from the total number of MDEs of an ISL were compared to the rate of 'Express 617' by χ^2 tests. To at least take into account the statistical error in the determination of the diploidization rate of 'Express 617' in these comparisons, a larger number of microspore cultivations was carried out for this genotype until 12 cultures were available that had produced at least ten MDEs from which 12 estimates of the diploidization rate could be calculated. To not loose the information from the additional MDEs produced in the remaining cultures completely, these were combined and a 13th estimate was calculated. Based on these estimates a mean and a 99 % confidence interval for the diploidization rate of microspores of 'Express 617' could be calculated, with the boundaries of the latter providing the expected frequencies for the χ^2 tests.

The mean diploidization rate of 'Express 617' was 52.5 % and the ISLs showed a range from 23.9 to 58.7 %. These values fall into the lower to middle range of the diploidization rates of 37–94 % reported

in the literature for isolated microspores after colchicine treatment (Chen et al. 1994; Möllers et al. 1994; Iqbal et al. 1994; Hansen and Andersen 1996; Klutschewski 2013). Three ISLs had significantly reduced diploidization rates of 23.9, 25.6 and 27.1 % respectively. No lines with significantly increased diploidization rates were found. This is perhaps not surprising since four of the five regions with skewed segregations chosen for verification showed an excess of 'Express 617' alleles, which would indicate that the donor alleles should decrease diploidization rates. This bias in the selection of regions for verification was due to the fact that three regions were chosen from the regions that showed the expected pattern of skewed segregations along their whole length, which, with two exceptions, all showed an excess of 'Express 617' alleles. The other two regions were chosen from the regions with changing patterns, but here the one region showing an excess of 'Express 617' alleles was included in the selection. This region was chosen because of a subsection showing a clear pattern of skewed segregations with the χ^2 values in the haploid MDE population continuously falling along the subsection and the values in the diploid MDE population rising to a peak in the middle of the subsection before falling again. The latter pattern is exactly the pattern expected when the skewed segregations are caused by a selective factor segregating at the peak position.

The comparison of the donor segments of the lines with significantly reduced diploidization rates with the segments of the non-significant lines identified seven genomic regions that were only covered by donor segments in the significant lines. Therefore, these regions, which cover only between 4.17 and 6.16 % of the rapeseed genome, are candidate regions for carrying the genetic factor(s) that reduced the diploidization rates in the significant lines. On the other hand, the seven regions may not represent all differences between the significant and non-significant lines. Three of the lines, ER137, ER1284, and ER1437, had been included in the high-throughput SNP analysis published by Ecke et al. (2015). In this analysis additional, mostly small donor segments were detected, which could not be taken into account in the current analysis because it was not possible to include all of the ISLs analysed here in the SNP analysis. The detection of additional donor segments in the ISLs not discovered in the original AFLP analysis reflects on a basic problem inherent in using ISLs for genetic

analysis. ISLs are produced by several rounds of backcrossing, with one round of meiotic recombination in each generation. This increases the probability that small donor segments are produced that may not be detected in the marker analysis used to characterize the lines. Furthermore, in rapeseed, especially in crosses with resynthesized rapeseed, further genetic changes may be present in ISLs due to homoeologous exchanges whose detection may depend on the marker type used (Ecke et al. 2015).

Only based on the comparison of donor segments between significant and non-significant lines it was not possible to assign a genetic factor to a specific genomic region because all three of the significant lines carried several segments that were not present in the non-significant lines. On the other hand, the 10 ISLs had been selected because they carry donor segments that cover or overlap regions with strongly skewed segregations in the diploid MDE population and regular segregations in the haploid MDE and BC_1 populations. For the region on A3, two ISL, ER984 and ER1433, had been selected. While ER984 showed a significantly reduced diploidization rate of 25.6 %, the diploidization rate of ER1433, 45.2 %, was much higher and not significantly different from the rate of 'Express 617'. According to the genetic characterization of the ISLs based on the DH map (Fig. S1) the donor segment in ER984 on A3 is a subsegment of the segment in ER1433 and ER1437, another non-significant line. On the other hand, ER984 and ER1433 are distiguished by E44M50-147E from the diploid MDE map, wich shows a donor allele in ER984 but not in ER1433 or ER1437. Interestingly, this marker also shows donor alleles in ER281 and ER641, the two other significant lines, but in none of the non-significant lines. This finding provides a strong argument that the AFLP marker E44M50-147E on A3 is indeed linked to a gene controlling the diploidization rate of isolated microspores. The reduced diploidization rates of the significant ISLs would indicate that 'Express 617' contributes the favourable allele, which is in agreement with the excess of 'Express 617' alleles observed at the marker loci in the respective region of A3 in the diploid MDE map.

The other two lines with significantly reduced diploidization rates, ER281 and ER964, had been selected because they carry donor segments that cover the region with skewed segregations on C4. Since these donor segments do not occur in any of the non-significant lines, the significantly reduced diploidization rates of ER281 and ER964 support the idea that the skewed segregations on C4 are due to the segregation of alleles at a locus controlling diploidization rates in isolated microspores of rapeseed. Again, 'Express 617' would have contributed the favourable allele. On the other hand, ER281 and ER964 show donor alleles at the marker locus E44M50-147E, too, which is strongly implicated to be linked to a factor controlling diploidization rates. In addition, the diploidization rates of the two lines are quite similar to one another and to the rate of ER984, raising the possibility that just one factor, a gene linked to E44M50-147E, may be responsible for the reduced diploidization rates of the three lines. Then again, the broad range of diploidization rates from 37 to 94 % described in the literature (Chen et al. 1994; Möllers et al. 1994; Iqbal et al. 1994; Hansen and Andersen 1996; Klutschewski 2013) would argue for a polygenic control of this trait. Taking into account the large genetic distance between the parents of the mapping populations, 'Express 617' being a natural biannual rapeseed type and 'RS239' an annual resynthesized rapeseed, it is quite likely that more than one gene controlling diploidization rates in rapeseed is segregating in our populations. Accordingly, the possibility that the reduced diploidization rates of the three significant ISLs are due to more than one genetic factor residing on different donor segments cannot be discarded just based on the current results.

The ISLs selected for donor segments covering the regions with skewed segregations on A2, A7 and A8 did not show significant differences in their diploidization rates from the rate of 'Express 617'. Accordingly, the hypothesis that the skewed segregations in these regions are caused by the segregation of different alleles at a locus controlling diploidization rates could not be verified in this study. On the other hand, the results do not actually disprove this hypothesis. There are two reasons why the verification could have failed although the hypothesis is correct: (1) the non-significant ISLs still show a broad range of diploidization rates, from 32.8 to 58.7 %. There may be true effects that just could not be detected due to limits to the statistical power of the experiments conducted. (2) On A8, the region with the expected pattern of skewed segregations is not completely covered by the minimal extent of the donor segments, raising the possibility

that the gene causing the skewed segregations is not located on the donor segments.

As far as we are aware the work presented here is the first study into the genetics of the diploidization capacity of isolated microspores in rapeseed. In this study it was possible to identify seven genomic regions, which may carry genetic factors that affect the diploidization rate of isolated microspores of *B. napus* and to identify one marker that has a high probability to be actually linked to one of these factors. It was not possible to assign one of the genetic factors responsible for the reduced diploidization rates of the significant ISLs unequivocally to a specific region of the genome or to determine their exact number, but the seven genomic regions that should carry these factors cover only about 4 % of the rapeseed genome in their minimal extent. Furthermore, three ISLs with significantly reduced diploidization rates compared to the recurrent parent 'Express 617' have been identified. These lines are distinguished from 'Express 617' by only a few donor segments that cover in their minimal extent just between 1.21 and 3.34 % of the genome according to the genetic characterization of the lines based on the DH map. This makes these lines together with 'Express 617' an ideal material for further genetic studies on diploidization efficiency, for example by using transcriptomic, proteomic or metabolomic approaches. They also represent an ideal starting material for a fine mapping of the genes controlling the diploidization rate. By again backcrossing the lines with 'Express 617' new ISLs with a smaller set of donor segments and with donor segments representing subsegments of the segments in the significant lines could be produced, which could be used to further delimit the position of the genes controlling the diploidization rate. The three ISLs and the recurrent parent 'Express 617' are availabel upon request from the corresponding author.

Compliance with ethical standards

References

Albrecht S, Möllers C, Röbbelen G (1995) Selection in vitro for erucic acid content in segregating populations of microspore-derived embryoids of *Brassica napus*. Plant Breed 114:210–214

Baillie AMR, Epp DJ, Hutcheson D, Keller WA (1992) In vitro culture of isolated microspores and regeneration of plants in *Brassica campestris*. Plant Cell Rep 11:234–237

Barro F, Fernández-Escobar J, De la Vega M, Martin A (2001) Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. Plant Breed 120:262–264

Barro F, Fernández-Escobar J, De la Vega M, Martin A (2002) Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by UV treatment of isolated microspores. Euphytica 129:1–6

Burnett L, Yarrow S, Huang B (1992) Embryogenesis and plant regeneration from isolated microspores of *Brassica rapa* L. ssp. *oleifera*. Plant Cell Rep 11:215–218

Cegielska-Taras T, Pniewski T, Szala L (2008) Transformation of microspore-derived embryos of winter oilseed rape (*Brassica napus* L.) by using *Agrobacterium tumefaciens*. J Appl Genet 49:343–347

Chalhoub B, Denoeud F, Liu S, Parkin IAP et al (2014) Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science 345:950–953

Chen JL, Beversdorf WD (1994) A combined use of micro-projectile bombardment and DNA imbibition enhances transformation frequency of canola (*Brassica napus* L.). Theor Appl Genet 88:187–192

Chen ZZ, Snyder S, Fan ZG, Loh WH (1994) Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breed 113:217–221

Cloutier S, Cappadocia M, Landry BS (1995) Study of microspore-culture responsiveness in oilseed rape (*Brassica napus* L.) by comparative mapping of a F_2 population and two microspore-derived populations. Theor Appl Genet 91:841–847

Delourme R, Falentin C, Huteau V, Clouet V, Horvais R, Gandon B, Specel S, Hanneton L, Dheu JE, Deschamps M, Margale E, Vincourt P, Renard M (2006) Genetic control of oil content in oilseed rape (*Brassica napus* L.). Theor Appl Genet 113:1331–1345

Dormann M, Wang H-M, Oelck M (2001) Transformed embryogenic microspores for the generation of fertile homozygous plants. USA Pat 6(316):694

Ecke W, Clemens R, Honsdorf N, Becker HC (2010) Extent and structure of linkage disequilibri-um in canola quality winter rapeseed (*Brassica napus* L.). Theor Appl Genet 120:921–931

Ecke W, Kampouridis A, Ziese-Kubon K, Hirsch AC (2015) Identification and genetic characterization by high-throughput SNP analysis of intervarietal substitution lines of rapeseed (*Brassica napus L.*) with enhanced embryogenic potential. Theor Appl Genet 128:587–603

Ferreira ME, Williams PH, Osborn TC (1994) RFLP mapping of *Brassica napus* using doubled haploid lines. Theor Appl Genet 89:615–621

Ferrie AMR, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104:301–309

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the *Brassicaceae*. In Vitro Cell Dev Biol Plant 43:348–355

Ferrie AMR, Möllers C (2011) Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss Organ Cult 104:375–386

Ferrie AMR, Taylor DC, MacKenzie SL, Rakow G, Raney JP, Keller WA (2008) Microspore mutagenesis of *Brassica* species for fatty acid modifications: a preliminary evaluation. Plant Breed 127:501–506

Foisset N, Delourme R (1996) Segregation distortion in androgenic plants. In: Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants. Kluwer Academic Publisher, Dordrecht, pp 189–201

Foisset N, Delourme R, Lucas MO, Renard M (1993) Segregation analysis of isozyme markers on isolated microspore-derived embryos in *Brassica napus* l. Plant Breed 110:315–322

Fukuoka H, Ogawa T, Matsuoka M, Ohkawa Y, Yano H (1998) Direct gene delivery into isolated microspores of rapeseed (*Brassica napus L.*) and the production of fertile transgenic plants. Plant Cell Rep 17:323–328

Gardner MJ, Altman DG (1989) Statistics with confidence: confidence intervals and statistical guidelines. British Medical Journal, London

Guerche P, Charbonnier M, Jouanin L, Tourneur C, Paszkowski J, Pelletier G (1987) Direct gene transfer by electroporation in *Brassica napus*. Plant Sci 52:111–116

Hansen NJP, Andersen SB (1996) In vitro chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. Euphytica 88:159–164

Howell PM, Marshall DF, Lydiate DJ (1996) Towards developing intervarietal substitution lines in *Brassica napus* using marker-assisted selection. Genome 39:348–358

Huang B (1992) Genetic manipulation of microspores and microspore-derived embryos. In Vitro Cell Dev Biol Plant 28:53–58

Iqbal MCM, Möllers C, Röbbelen G (1994) Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus l*. J Plant Physiol 143:222–226

Jardinaud M-F, Souvre A, Alibert G (1993) Transient GUS gene expression in *Brassica napus* electroporated microspores. Plant Sci 93:177–184

Klutschewski S (2013) Methodical improvements in microspore culture of *Brassica napus* L. Dissertation, Georg-August-Universität Göttingen. http://hdl.handle.net/11858/00-1735-0000-0001-BC20-2. Accessed 15 July 2015

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Zeitschrift für Pflanzenphysiologie 105:427–434

Liu S, Wang H, Zhang J, Fitt BDL, Xu Z, Evans N, Liu Y, Yang W, Guo X (2005) In vitro mutation and selection of doubled-haploid *Brassica napus* lines with improved resistance to *Sclerotinia sclerotiorum*. Plant Cell Rep 24:133–144

Möllers C, Iqbal MCM (2009) Doubled haploids in breeding winter oilseed rape. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, Heidelberg, pp 161–170

Möllers C, Iqbal MCM, Röbbelen G (1994) Efficient diploidization of *Brassica napus* by colchicine treatment of microspores and regeneration of doubled haploid plants. Euphytica 75:95–104

Möllers C, Rücker B, Stelling D, Schierholt A (2000) In vitro selection for oleic and linoleic acid content in segregating populations of microspore derived embryos of *Brassica napus*. Euphytica 112:195–201

Nehlin L, Möllers C, Bergman P, Glimelius K (2000) Transient ß-gus and GFP gene expression and viability analysis of microprojectile bombarded microspores of *Brassica napus*. J Plant Physiol 156:175–183

Nurhasanah (2010) QTL mapping using intervarietal substitution lines in rapeseed (*Brassica napus* L.). Dissertation, Georg-August-Universität Göttingen. Cuvillier Verlag, Göttingen, Germany

Parkin IAP, Sharpe AG, Keith DJ, Lydiate DJ (1995) Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). Genome 38:1122–1131

Qiu D, Morgan C, Shi J, Long Y, Liu J, Li R, Zhuang X, Wang Y, Tan X, Dietrich E, Weihmann T, Everett C, Vanstraelen S, Beckett P, Fraser F, Trick M, Barnes S, Wilmer J, Schmidt R, Li J, Li D, Meng J, Bancroft I (2006) A comparative linkage map of oilseed rape and its use for QTL analysis of seed oil and erucic acid content. Theor Appl Genet 114:67–80

Radoev M, Becker HC, Ecke W (2008) Genetic analysis of heterosis for yield and yield components in rapeseed (*Brassica napus* L.) by QTL mapping. Genetics 179:1547–1558

Seguí-Simarro JM, Nuez F (2008) Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet Genome Res 120:358–369

Sharpe AG, Parkin IAP, Keith DJ, Lydiate DJ (1995) Frequent nonreciprocal translocations in the amphidiploid genome of oilseed rape (*Brassica napus*). Genome 38:1112–1121

Song K, Lu P, Tang K, Osborn TC (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proc Natl Acad Sci USA 92:7719–7723

Sonntag K, Rudloff E (2004) Microspore mutagenesis in transgenic oilseed rape for the modification of fatty-acid composition. Acta Universitatis Latviensis Biol 676:227–230

Swanson EB, Coumans MP, Wu SC, Barsby TL, Beversdorf WD (1987) Efficient isolation of microspores and the production of microspore-derived embryos from *Brassica napus*. Plant Cell Rep 6:94–97

Szadkowski E, Eber F, Huteau V, Lodé M, Huneau C, Belcram H, Coriton O, Manzanares-Dauleux MJ, Delourme R, King GJ, Chalhoub B, Jenczewski E, Chèvre A-M (2010) The first meiosis of resynthesized *Brassica napus*, a genome blender. New Phytol 186:102–112

Thomas WTB, Forster BP, Gertsson B (2003) Doubled haploids in breeding. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, pp 337–349

Udall JA, Quijada PA, Osborn TC (2005) Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* l. Genetics 169:967–979

Uzunova M, Ecke W, Weißleder K, Röbbelen G (1995) Mapping the genome of rape-seed (*Brassica napus* L.). I. Construction of an RFLP linkage map and localization of QTLs for seed glucosinolate content. Theor Appl Genet 90:194–204
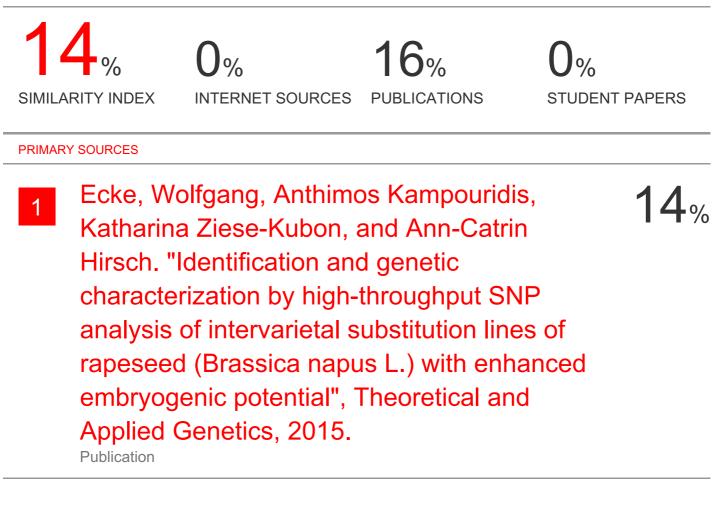
Weber S, Unker F, Friedt W (2005) Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment in vitro and ploidy determination by flow cytometry. Plant Breed J 124:511–513

Zhang FL, Aoki S, Takahata Y (2003) RAPD markers linked to microspore embryogenic ability in *Brassica* crops. Euphytica 131:207–213

Zhao JP, Simmonds DH, Newcomb W (1996a) High frequency production of doubled haploid plants of *Brassica napus* cv. Topas derived from colchicine-induced microspores embryogenesis without heat shock. Plant Cell Rep 15:671–688

Zhao JP, Simmonds DH, Newcomb W (1996b) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas Planta 198:433–439

Zhao J, Becker HC, Zhang D, Zhang X, Ecke W (2005) Oil content in a European x Chinese rapeseed population: QTL with additive and epistatic effects and their genotype–environment interactions. Crop Sci 45:51–59

Zhou WJ, Hagberg P, Tang GX (2002a) Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. Euphytica 128:27–34

Zhou WJ, Tang GX, Hagberg P (2002b) Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul 37:185–192

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PRIMARY SOURCES

1 Ecke, Wolfgang, Anthimos Kampouridis, Katharina Ziese-Kubon, and Ann-Catrin Hirsch. "Identification and genetic characterization by high-throughput SNP analysis of intervarietal substitution lines of rapeseed (Brassica napus L.) with enhanced embryogenic potential", Theoretical and Applied Genetics, 2015.
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