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Identification and evaluation of intervarietal substitution lines of rapeseed (*Brassica napus* L.) with donor segments affecting the direct embryo to plant conversion rate of microspore-derived embryos

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Abstract Microspore culture has become an important tool in many species, including *Brassicas*, for the production of entirely homozygous lines, so called double haploid (DH) lines. The primary products of microspore culture are embryo-like structures, called microspore-derived embryos (MDEs). A major problem in the development of DH lines is the often low efficiency of Direct Embryo to Plant Conversion (DEPC). During the development of DH populations, favourable alleles of genes affecting the DEPC rate will be under selection. This selection should lead to skewed segregations at markers linked to these genes. By comparing skewed marker segregations in four populations, a population of doubled haploid plantlets, a haploid and a doubled haploid MDE population, and a BC$_1$ population, 20 genomic regions were identified, which showed patterns of skewed segregations across the populations, indicative of the segregation of genetic factors controlling DEPC rates. Four regions and eight intervarietal substitution lines (ISLs) with donor segments overlapping these regions were selected for further studies. Three ISLs, ER654, ER661 and ER653 with DEPC rates of 49.1, 54.5 and 57.2 %, showed significantly reduced DEPC rates compared to the rate of the recurrent parent of 76.5 %. By comparing donor segments between the significant and the non-significant lines, eight genomic regions were identified that may contain genetic factors controlling the DEPC rate in rapeseed. These regions range in size from 0 (represented by just one marker) to 16.5 cM and cover together just 1.33 % of the genetic map used to characterize the donor segments in the ISLs.

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Introduction

Microspores or immature pollen grains of many plant species can be diverted from the normal gametophytic to a sporophytic developmental pathway by a stress treatment, for example a mild heat stress. When cultured in a nutrient-rich medium, they start to divide repeatedly and develop into embryo like structures, so called "microspore derived embryos" (MDEs), which

can be grown into fully developed plants after transfer to solid medium (Touraev et al. 2001; Forster et al. 2007; Ferrie and Caswell 2011). This phenomenon is known as microspore embryogenesis or androgenesis. By including a treatment with a mitosis inhibitor, usually colchicine, during the development of the plants, the haploid chromosome complement of the original microspore can be doubled, leading to diploid, entirely homozygous plants (Chen et al. 1994; Möllers et al. 1994) that can be propagated as true breeding lines, so called doubled haploid lines (DH lines).

In *Brassica* and specifically in rapeseed, androgenesis and DH lines are now used routinely for breeding and research activities. Many rapeseed varieties currently grown are hybrids of DH lines. Also, microspores and MDEs can be used for mutagenesis and in vitro selection (Albrecht et al. 1995; Barro et al. 2001; Ferrie et al. 2008; McClinchey and Kott 2008) and it is possible to utilize microspores for genetic transformation (Guerche et al. 1987; Fukuoka et al. 1998; Nehlin et al. 2000; Dormann et al. 2001). The main advantage of androgenesis is, that traits can be fixed in one generation rather than after multiple self-pollinated generations (Dunwell 2010; Ferrie and Caswell 2011; Germanà 2011).

When MDEs are transferred to solid medium, only part of the MDEs develop directly into plants by producing roots and shoots (Direct Embryo to Plant Conversion, DEPC). Many MDEs produce calli or undergo secondary embryogenesis, which will eventually also lead to plants, but only after several rounds of sub-culturing, which is labour intensive and time consuming (Huang et al. 1991). DEPC rates are dependent on the age of the MDEs and on culture conditions (Kott and Beversdorf 1990; Huang et al. 1991). Rates can be significantly increased by a partial dessication or a cold pretreatment of the MDEs (Kott and Beversdorf 1990; Huang et al. 1991; Klutschewski 2013), but large differences in DEPC rates have been observed between different genotypes (Chuong et al. 1988). In a number of experiments studying DEPC rates under different culture conditions and pretreatments using a range of genotypes, Klutschewski (2013) found a strong, in many experiments a predominant, effect of the genotype on DEPC rates. These results clearly indicate that the DEPC rate of MDEs of rapeseed is under genetic control. Nevertheless, to our knowledge there have been no studies until now to elucidate the genetics of this trait.

In an F_1 derived population of DH lines developed from MDEs that underwent DEPC, the segregation of different alleles at loci controlling the DEPC rate should lead to a selection of alleles favourable for DEPC. This in turn should lead to a deviation from the expected 1:1 segregation ratio at linked marker loci. Skewed marker segregations, therefore, could provide a way to localize genes controlling DEPC rates on a genetic map, although in this approach it has to be taken into account that DEPC is not the only selective step in the development of a DH population. In addition to the DEPC rate, the embryogenic potential and the diploidization capacity of isolated microspores are also traits where large differences between geno-types have been observed, indicating a strong genetic contribution to the variation in these traits (Ferrie and Keller 2007; Chen et al. 1994; Möllers et al. 1994; Klutschewski 2013). Ecke et al. (2015) and Kampouridis et al. (2016) actually have identified a number of genomic regions that may carry genes controlling embryogenic potential and diploidization capacity of isolated microspores. In addition, skewed marker segregations may also result from earlier selection events during microspore formation. When using skewed marker segregations to localize genes controlling DEPC rates of MDEs, it will therefore be necessary to compare marker segregation ratios in different populations that have experienced only one or different subsets of the above mentioned selective steps. Furthermore, since real populations always represent samples, skewed segregations may also occur just by chance. This necessitates an independent verification of effects of genomic regions implicated by skewed segregations to be involved in the genetic control of the DEPC rate.

In this study, skewed marker segregations were compared between an F_1 derived population of plantlets derived by DEPC from MDEs of a rapeseed cross and corresponding populations of diploid and haploid MDEs and a BC_1 population to identify genomic regions putatively involved in the genetic control of the DEPC rate of MDEs. To verify the putative effects of selected regions, the DEPC rates of intervarietal substitution lines (ISLs; Howell et al. 1996) with donor segments covering the regions were determined. By comparing the donor segments of ISLs with DEPC rates significantly different from the DEPC rate of the recurrent parent of the ISL population with the donor segments of non-significant lines, a

number of genomic regions could be identified that should carry genes affecting DEPC rates in rapeseed.

Materials and methods

Plant materials, genetic maps

For analysing skewed marker segregations, a population of 214 double haploid (DH) plantlets were developed (DEPC population) that were derived from MDEs that had directly converted to plants after transfer to solid medium. The MDEs were derived from isolated, colchicine treated 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. For the verification of effects of specific genomic regions on the DEPC rate of MDEs, a population of 323 doubled haploid ISLs, derived from microspores of BC_4 plants of the same cross with 'Express 617' as the recurrent parent, was available. 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 and 1587 cM, respectively, out of 2003 cM. In addition, marker information and corresponding genetic maps were available for three additional populations derived from the same F_1 plant as the DEPC population: a population of diploid MDEs (Kampouridis et al. 2016) and a population of haploid MDEs as well as a BC_1 population with 'Express 617' as recurrent parent (Ecke et al. 2015).

Donor plant growth

Seed-derived and in vitro propagated plants used as microspore donors 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/m^2 s (Li-1400 Data Logger, Li-Cor Biosciences, Nebraska, USA) and a relative humidity of 65 %.

Bud harvest, microspore isolation and cultivation

Flower buds, 2–3 mm in length, were harvested and placed in flasks on ice. The 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 & 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(NO3)2·4H2O (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 100 g. The supernatant was discarded and the pellet was resuspended with 12 ml cold NLN medium and centrifuged again. The washing step was repeated once and the final pellet resuspended in 5 ml cold NLN medium and transferred to a 9 cm petri dish containing 6.4 ml cold NLN medium and 600 µl of a 100 mM stock solution of colchicine. The petri dish was sealed with two layers of Parafilm and incubated in the dark at 32 °C for 3 days. The suspension with the microspores was transferred again into a glass centrifuge tube and washed twice with 12 ml cold NLN medium. The final pellet was suspended in 5 ml cold NLN medium and transferred to a 9 cm petri dish 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.

MDEs that reached a length of 0.7–1.0 cm were transferred to solidified Gamborg B5 medium (Gamborg et al. 1968; Supplier: Duchefa, Haarlem, The Netherlands), supplemented with 0.1 mg/L gibberellic acid (GA3, Duchefa, Haarlem, The Netherlands), in sterile plastic boxes (10.5 × 8 × 5 cm, vol. 0.24 L, Huhtamaki GmbH & Co. KG, Alf, Germany). Eight MDEs were transferred into each box. The boxes with the MDEs were incubated for a period of 2 weeks in a light thermostat at 1.5 °C in continuous darkness. After two weeks, the MDEs were transferred to a light room for further incubation of 4 weeks at a continuous temperature of 22 °C and a photoperiod of 12 h. At the end of this period the number of MDEs that had developed shoots was counted.

Ploidy analysis, DNA preparation, AFLP analysis, and map construction

The DH plantlets of the DEPC population were selected based on a ploidy analysis by flow cytometry using a Partec Ploidy Analyser PA-I (Partec GmbH, Görlitz, Germany) according to the manufacturer's protocol: Leaf samples of about 1 cm^2 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 DH plantlets were frozen in liquid nitrogen and stored at −20 °C. DNA was extracted from the frozen plantlets 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 map, the maps of the two MDE and the BC$_1$ populations, as well as in the characterization of the ISLs were again used for AFLP analysis in the DEPC 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 degree to which marker segregations deviated from the expected segregation ratio.

For the construction of the genetic map of the DEPC 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). Regions with skewed segregations were defined as clusters of markers with skewed segregations where none of the skewed markers was further apart from the next skewed marker than 15 cM.

Statistical analysis

To determine the DEPC rates of selected ISLs and the recurrent parent 'Express 617', between 10 and 14 microspore cultures were carried out per genotype. Per culture 24 to 72 MDEs were transferred to solid medium and an estimate of the DEPC rate of the respective genotype was determined as the percentage of MDEs that produced shoots. For the statistical analysis, which was performed with the program Statistica v.10.0 (StatSoft, Inc 2300, East 14th Street Tulsa, OK 74104, USA), the repeated microspore cultures were treated as replicates of one experiment. Dunnett's test was used to test for significant differences between ISLs and 'Express 617' at P = 0.05. The statistical analysis was based on the immediate phenotypic data without transformation since statistical tests had shown no significant deviation from normal distribution or variance homogeneity. To test for normal distribution within groups the Shapiro-Wilks-Test was used and Levenés test was applied to test for variance homogeneity.

Results

Genetic mapping in the DEPC population

To localize genetic factors affecting the DEPC rate of rapeseed, 608 AFLP markers were scored in the DEPC population. A genetic map with 534 AFLP markers could be constructed (Table S1). The DEPC map

consists of 20 linkage groups that could be assigned to the 19 chromosomes of rapeseed based on markers shared with the DH map and the maps of the diploid and haploid MDE populations and the BC$_1$ population. Linkage groups A1 and A4 were both present in two separate disjointed parts and linkage group C4 was not found in the DEPC map. The total length of the DEPC map was 1915.2 cM. A summary of the DEPC map and a comparison with the corresponding maps of the diploid and haploid MDE and the BC$_1$ populations is shown in Table 1.

Analysis of skewed segregations in the DEPC population

From the 534 AFLP markers that were mapped in the DEPC population, 320 (59.9 %) showed a significant deviation from the expected 1:1 segregation ratio (Table S1). Skewed marker segregations were distributed on all linkage groups of the DEPC map. Of the 320 skewed markers, 314 clustered in 26 extended regions (Table 2), with some regions also containing up to four markers with regular segregations (nos. 1, 21, 32, Table 2). The number of markers in these regions ranged from 2 to 45, covering between 3.2 cM (no. 19) to 93.7 cM (no. 7), in total 824.0 cM (43.0 %) of the DEPC map. Moreover, another six skewed markers were found across the map, suggesting small regions with disturbed segregations. In total, 32 genomic regions showed skewed segregations in the DEPC population, with 16 regions each displaying an excess of 'Express 617' alleles and 'RS239' alleles, respectively. Peak χ^2 values in these regions ranged from 6.28 to 101.30. The six single marker regions (nos. 2, 4, 6, 15, 18, and 23) were excluded from further analysis. In addition, two markers on C1 and C2 in the regions 20 and 21 were not taken into account in the further analysis because they showed improbably high χ^2 values in the DEPC and diploid MDE populations, respectively. On C1, the marker E32M49-266R at 53.5 cM showed a χ^2 value of 48.09 in the DEPC population, while the closely linked marker E41M50-288E at 53.8 cM showed only a value of 7.56. On the other side, the marker E39M50-183R at 48.4 cM also showed only a value of 18.15 (Table S1). On C2 the marker E44M50-141E at 54.2 cM showed a χ^2 value of 41.63 in the diploid MDE population although the cosegrating marker E34M49-268-2E showed only a value of 0.67, while

the flanking markers on both sides showed values of 0.9 and 1.74. These discrepancies in χ^2 values between closely linked markers make it highly likely that the high χ^2 values of E32M49-266R and E44M50-141E in the DEPC respectively the diploid MDE population are due to a strong bias in allele scoring (see "Discussion" section).

Among the 26 extended regions with skewed segregations in the DEPC population were four regions (nos. 26, 28, 30, and 31) where skewed segregations were only observed in the DEPC population (Table S1), a pattern expected if only a factor controlling DEPC frequencies is segregating in these regions. In addition, there were four regions (nos. 7, 11, 16, and 20) where this pattern was observed across substantial subsections of the regions, ranging in size from 8.2 to 80.9 cM. Furthermore, there were twelve regions where markers showed skewed segregations in at least one of the other populations, but the χ^2 values were significantly higher in the DEPC population, either across the whole length of the region (nos. 1, 3, 8, 17, 24, 25, and 29) or across large subsections (nos. 5, 9, 10, 12, and 32). This was interpreted as the joint segregation of (a) factor(s) leading to skewed marker segregations in the other population(s) and a factor controlling DEPC frequency, which would increase skewed segregations in the DEPC population above that observed in the other populations.

Selection of ISLs with donor segments covering selected regions with skewed segregations in the DEPC population

From the pool of 20 regions, where the pattern of skewed segregations across populations indicated the possible presence of a genetic factor controlling DEPC frequency, four regions or subsections on A2, C1, C3, and C9 (regions 5, 18, 21, 27, and Fig. 1) were chosen for further analysis. The selection of these regions was based on peak χ^2 values, the distribution of skewed segregations across the region in the different populations, and the availability of ISLs carrying donor segments covering the respective region. Three of the selected regions showed an excess of 'RS239' alleles and one—on C9—an excess of 'Express 617' alleles.

A subsection of region five was selected in the upper half of A2, ranging from 32.1 to 63.7 cM. The markers in this subsection showed highly skewed segregations in the DEPC population with χ^2 values up

Table 1 Comparison of linkage groups (LG), number of markers and length of linkage groups of the high fidelity (HF) and full maps of the DEPC, diploid MDE, haploid MDE and BC_1 populations

LG	DEPC population				Diploid MDE population				Haploid MDE population				BC_1 population			
	HF map		Full map		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]	No. markers	Length [cM]	No. markers	Length [cM]
A01a[1]	13	66.8	20	66.8	13	114.2	20	114.2	16	91.7	20	91.7	11	76.8	16	76.8
A01b[1]	6	25.3	7	25.3												
A02[2]	21	122.8	34	122.8	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	38	115.8	47	115.8	31	126.3	46	129.9	41	125.0	46	125.0	14	134.5	21	152.5
A04a[1]	5	58.2	10	59.3	14	117.1	17	118.2	14	108.4	16	108.4	3	9.5	5	28.3
A04b[1]	8	40.7	14	49.6												
A05	14	86.5	31	86.5	23	108.0	30	108.0	24	60.7	29	60.7	11	65.0	17	65.0
A06	26	105.4	31	105.4	19	98.5	24	98.5	24	112.8	25	112.8	7	39.6	9	40.2
A07	23	93.7	26	93.7	14	84.2	20	87.0	16	88.9	20	88.9	11	88.7	11	88.7
A08	19	68	21	68	15	71.1	19	72.3	17	62.4	19	62.4	8	61.3	9	62.2
A09	32	108	34	108.0	21	106.4	30	106.4	26	114.8	28	114.8	10	86.5	13	86.5
A10	19	102.2	23	102.2	14	87.8	17	87.8	14	74.2	16	74.2	8	79.7	9	79.7
C01	13	78.5	22	83.6	18	189.2	24	189.2	17	108.9	23	134.8	10	60.4	11	60.4
C02	20	108.9	23	133.8	16	110.2	22	110.2	16	85.8	23	85.8	6	72.9	7	72.9
C03	27	129.8	31	129.8	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	30	86.7	36	87.7	24	86.7	32	86.7	26	83.5	32	83.5	12	58.2	18	58.2
C06	26	126.2	28	127.3	18	114.9	21	115.9	20	94.6	22	94.7	9	81.2	11	90.5
C07[3]	24	125.6	29	126.7	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	20	105.6	23	106.1	16	110.0	20	112.2	20	114.2	20	114.2	8	70	9	70
C09	30	114.8	44	116.8	26	137.2	37	137.2	32	127.0	38	127.0	13	91.1	13	91.1
Naa[4]	–	–	–	–	–	–	–	–	2	12.3	3	14.5	2	2.8	2	2.8
Nbb									2	1.6	3	20.4				
Ncc									2	8.3	2	8.3				

Table 1 continued

LG	DEPC population				Diploid MDE population				Haploid MDE population				BC₁ population			
	HF map		Full map		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]	No. markers	Length [cM]	No. markers	Length [cM]
Ndd					–	–	–	–	3	2.7	3	2.7				
Sum	414	1869.5	534	1915.2	360	2083	470	2094.9	408	1896.2	481	1943.2	172	1277.5	221	1327.4

[1] Linkage groups A1 and A4 were each present in two disjointed parts in the DEPC map but were continuous in the other maps

[2] Linkage group A2 was present in two disjointed parts, A2a and A2b, in the haploid MDE map

[3] Linkage group C7 was present in three disjointed parts, C7a, C7b and C7c in the BC₁ map

[4] Naa–Ndd: three unjoined triplets and one pair that could not be assigned to the 19 chromosomes of *Brassica napus* in the haploid MDE map

to 96.90, while in the diploid and haploid MDE populations all χ^2 values were much lower, with the highest χ^2 value reaching just 12.98. In the BC₁ map, this region was not covered by markers (Fig. 1). On C1, a subsection of region 20 in the lower part of the linkage group ranging from 59.1 to 67.3 cM was selected, which showed regular segregations in the diploid and haploid MDE populations and in the BC₁ population (Fig. 1). On C3, region 24 at the beginning of the linkage group, ranging from 0.0 to 29.8 cM, was selected. This region showed high χ^2 values of up to 51.09 in the DEPC population, while the highest value in the other populations was only 11.07. On C9, a subsection of region 32, ranging from 62.7 to 116.3 cM with a peak χ^2 value of 18.13 in the DEPC population, was selected. The subsection showed regular segregations in the diploid MDE and the BC₁ population and mostly regular segregations in the haploid MDE population, with a few markers showing significant χ^2 values of up to 7.32.

Eight ISLs were selected with donor segments covering the selected regions. On A2, the donor segment of the selected ISL, ER1284, covers 59 % of the selected region in its minimal extent, including the two peaks in the distribution of χ^2 values in the DEPC population. Taking into account the maximal extent of the donor segment of ER1284, the coverage is increased to 87 %, extending from 32.1 to 59.5 cM. On C1, the marker E44M50-197R at 61.4 cM on the DEPC map indicated the presence of a donor segment in ISL ER240 within the selected subsection with skewed segregations on this linkage group. In its maximal extent this donor segment covers the full subsection from 59.1 to 67.3 cM. ER240 carries a second donor segment on C1, outside the selected region. To account for this segment in a comparison of DEPC rates, ER89 was also selected, an ISL that carries a donor segment covering the same region as the second segment in ER240 (Fig. 1). On C3, the donor segments in ISLs ER653 and ER654 cover 33 % of the selected region with skewed segregations, from 20.0 to 29.3 cM (Fig. 1). In addition, the first marker on C3 in the DEPC map, E35M47-174R, which showed the highest χ^2 value, indicates a second donor segment in these lines at the beginning of the linkage group. The second segment is also present in the ISL ER661, which is lacking the first segment. In their maximal extend, the segments in the three lines cover 82 % of the selected region with skewed segregations.

Table 2 Regions with skewed segregations on the DEPC map and comparison with the corresponding regions on the diploid and haploid MDE and BC$_1$ map

No.	LG	DEPC map					Diploid MDE map					Haploid MDE map					BC$_1$ map				
		Start [cM]	Length [cM]	Tm/sm[a]	FA[b]	Peak χ^2	Start [cM]	Length [cM]	Tm/sm[a]	FA[b]	Peak χ^2	Start [cM]	Length [cM]	Tm/sm[a]	FA[b]	Peak χ^2	Start [cM]	Length [cM]	Tm/sm[a]	FA[b]	Peak χ^2
1	A01a	14.9	30.4	14/12	E	49.55	21.8	36.4	12/3	R	6.84	35.5	32.3	12/5	R	5.14	5.7	21.1	10/0	–	2.02
2	A01a	66.8	0.0	1/1	R	8.70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	A01b/Ncc	0.0	25.3	7/7	E	29.45	73.8	40.4	5/3	E	6.95	0.0	0.5	2/2	R	12.13	41.0	35.8	5/0	–	1.78
												0.0	8.3	2/0	–	2.17					
4	A02/A2b	0.0	0.0	1/1	R	17.04	0.0	0.0	1/1	R	21.60	0.0	0.0	1/1	R	36.45	–	–	–	–	–
5	A02/A2a	32.1	39.2	21/21	R	96.90	35.8	68.4	18/6	R	12.98	30.5	50.2	17/7	R	12.66	–	–	–	–	–
6	A03	0.0	0.0	1/1	R	27.55	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	A03	22.1	93.7	45/45	R	55.37	0.0	106.3	37/1	R	10.80	15.5	83.8	36/0	–	2.99	57.6	76.9	13/0	–	1.99
8	A04a	–1.1	6.6	3/3	R	24.53	60.1	6.4	3/1	R	5.63	94.3	8.7	3/3	R	9.78	–	–	–	–	–
9	A04b	–8.9	49.6	14/14	R	71.85	–1.1	36.1	10/10	R	45.55	0.0	63.7	9/8	R	28.81	0.0	28.3	5/5	R	87.30
10	A05	0.0	77.0	30/30	E	76.56	26.9	81.1	28/25	E	28.81	0.0	60.7	28/27	E	32.40	13.6	51.4	14/2	R	6.92
11	A06	21.1	27.1	7/7	R	10.10	58.2	23.6	6/3	E	10.95	68.4	25.0	6/0	–	3.63	12.7	19.5	4/0	–	0.28
12	A07	37.4	56.3	12/12	R	29.13	–2.8	56.6	10/8	E	6.35	33.1	55.8	10/5	E	20.45	0.0	51.8	6/0	–	3.76
13	A08	0.0	4.8	6/6	E	8.31	–1.2	5.5	6/6	E	11.00	0.0	5.0	6/0	–	3.38	61.3	0.9	3/0	–	0.56
14	A08	60.9	7.1	2/2	E	8.70	62.7	8.4	2/2	E	10.83	56.5	5.9	2/2	E	26.34	0.0	0.0	1/0	–	1.41
15	A09	8.1	0.0	1/1	E	7.85	84.1	0.0	1/0	–	2.89	24.2	0.0	1/1	E	10.71	6.4	0.0	1/0	–	1.24
16	A09	25.2	20.8	7/7	E	12.64	48.2	23.7	7/3	E	8.99	40.7	21.9	7/1	E	7.04	22.8	5.8	2/0	–	2.30
17	A10	13.1	69.8	20/20	R	30.72	22.0	51.5	15/0	–	3.70	0.0	53.6	15/9	R	8.50	0.0	59.1	8/0	–	2.02
18	A10	102.2	0.0	1/1	E	6.28	0.0	0.0	1/1	E	9.80	–	–	1/1	E	–	–	–	–	–	–
19	C01/Naa	7.0	3.2	2/2	R	13.63	11.2	2.9	2/0	–	2.86	12.1	2.4	2/2	R	11.26	15.2	7.1	2/0	R	0.46
20	C01	32.0	40.6	15/15	R	48.09	43.6	73.1	14/5	R	11.19	2.2	51.7	14/4	R	9.28	30.2	30.2	8/0	–	0.97
21	C02	41.0	24.9	13/9	E	20.02	45.8	29.0	8/3	E	41.63	35.3	26.7	7/5	E	16.18	–	–	–	–	–
22	C02	99.1	9.8	3/3	R	15.72	–	–	–	–	–	–	–	–	–	–	54.6	1.2	2/0	–	0.67
23	C02	133.8	0.0	1/1	R	22.59	110.2	0.0	1/0	–	2.13	85.8	0.0	1/1	R	5.06	72.9	0.0	1/0	–	0.46
24	C03	0.0	29.8	9/9	R	51.09	102.9	27.8	9/0	–	2.22	0.0	39.4	9/2	R	11.07	14.5	28.4	6/0	–	2.66
25	C05	24.3	33.7	23/23	E	23.11	24.3	37.9	19/1	R	4.95	16.5	39.2	19/7	E	4.74	0.0	37.0	12/0	–	1.60
26	C05	74.0	5.5	4/4	E	6.55	10.8	3.5	4/0	–	1.56	68.6	6.3	4/0	–	1.20	51.6	0.0	1/0	–	0.27
27	C06	–1.1	47.6	12/12	E	101.30	71.9	44.0	10/10	E	19.67	58.4	36.3	10/10	E	48.02	44.5	46.0	6/0	–	1.27

Table 2 continued

No.	LG	DEPC map					Diploid MDE map					Haploid MDE map					BC$_1$ map				
		Start [cM]	Length [cM]	Tm/sm^a	FA^b	Peak χ^2	Start [cM]	Length [cM]	Tm/sm^a	FA^b	Peak χ^2	Start [cM]	Length [cM]	Tm/sm^a	FA^b	Peak χ^2	Start [cM]	Length [cM]	Tm/sm^a	FA^b	Peak χ^2
28	C07/C07a	52.1	17.3	5/5	E	27.87	65.3	15.9	3/0	–	1.47	52.5	17.1	3/0	–	1.93	–	–	–	–	–
29	C07/C07a	98.2	27.4	16/16	E	15.85	0.0	38.7	15/1	–	5.63	0.0	30.9	15/9	R	8.51	-2.3	18.4	7/4	E	6.35
30	C08	8.4	12.8	2/2	E	9.69	9.7	7.6	2/0	–	2.38	71.3	10.7	2/0	–	0.89	0.0	0.0	1/0	–	1.26
31	C08	98.0	4.3	7/7	R	7.20	98.9	11.1	5/0	–	1.23	0.0	5.9	5/0	–	2.83	70.0	0.0	1/0	–	0.05
32	C09	56.9	59.4	24/21	E	18.13	28.1	57.6	21/0	–	1.39	50.6	51.7	21/9	E	7.32	0.0	50.0	9/1	E	7.93
Sum			824.0	329/320				893.5	275/93				793.7	269/120				568.9	128/12		

^{a} tm: total number of markers in the region; sm: number of markers with significantly skewed segregation (P = 0.05) in the region

^{b} Favored allele: R = 'RS239' allele, E = 'Express 617' allele

On C9, the donor segments of the two selected ISLs, ER644 and ER1298, cover in their minimal extent all of the selected subsection with skewed segregations.
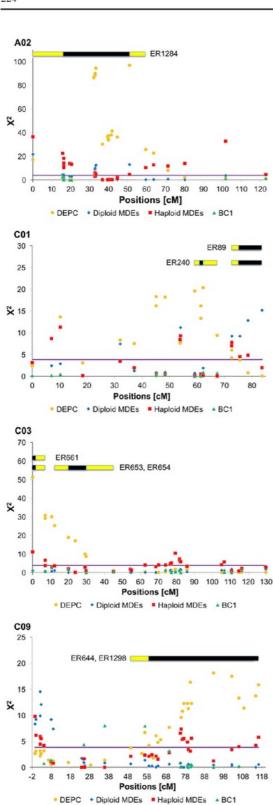
Figure 1 shows the donor segments of the selected ISLs on the respective linkage groups according to an analysis based on the DEPC map to better display the coverage of the selected regions with skewed segregations. Fig. S1, on the other hand, shows the donor segments in the selected ISLs according to an analysis based on the DH map. This map is the reference map for characterizing and comparing donor segments in the lines of the ISL population, basically because the analysis based on the DH map has been applied to all lines of the population. The DEPC map includes a number of markers, including E44M50-197R on C1 and E35M47-174R on C3, which had been scored in the ISL population but could not be included in the DH map. Accordingly, the donor segments defined by these markers as shown in Fig. 1 are not represented in Fig. S1.

Determination of the DEPC rate of the selected ISLs

The DEPC rates of seven of the eight selected ISLs were determined and compared with the DEPC rate of the recurrent parent 'Express 617' (Table 3). The rate of ER1284 could not be determined since it was not possible to obtain a sufficiently large number of MDEs from this line. ER1284 was, accordingly, omitted from all further analyses. The mean value of the DEPC rate of 'Express 617' was 76.5 %, while the mean rates of the ISLs ranged from 49.1 to 79.1 %, corresponding to ratios of 0.65–1.05 times the DEPC rate of 'Express 617'. Based on a Dunnett's multiple comparison test, two ISLs, ER654 and ER661, showed significantly reduced DEPC rates of 49.1 % (P = 0.002) and 54.5 % (P = 0.018). A third ISL, ER653, was not significant at a threshold of P = 0.05, but with a DEPC rate of 57.2 %, and a P value of just 0.051 was also considered to have a significantly reduced DEPC rate compared to 'Express 617'.

Donor segments involved in the control of the DEPC rate

The number of donor segments carried by the selected ISLs ranged from 1 to 6 (Table 4) according to the available marker data. In individual ISLs, the minimal

Fig. 1 Distribution of marker segregation patterns in the DEPC, diploid and haploid MDE and the BC_1 populations and donor segments of selected ISLs on linkage groups A2, C1, C3 and C9 of the DEPC 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 *orange* (*circle*), *blue* (*diamond*), *red* (*square*) and *green* (*triangle*) mark represents the χ^2 values of the respective marker on the DEPC map in the DEPC, diploid MDE, haploid MDE and BC_1 populations, respectively. The *black* and *yellow bars* represent minimal and maximal extents, respectively, of the donor segments of selected ISL on the four linkage groups

extent of the donor segments ranged from 3.7 to 30.6 cM, with the segments covering 0.18–1.53 % of the total length of the DH map (Table 5). Taking into account the maximal extent, the coverage of the donor segments as detected by the DH map ranges from 26.1 to 54.6 cM, corresponding to 1.3 to 2.73 % of the DH map, respectively.

To identify genomic regions that may carry genetic factors involved in the control of the DEPC rate in rapeseed, the distribution and overlap of donor segments in the ISLs with significantly reduced DEPC rates were compared with the donor segments in the non-significant ISLs. Eight regions on six linkage groups were identified where donor segments only occurred in the significant lines and which, accordingly, may carry genetic factors controlling the DEPC rate (Table 6). The eight regions are on chromosomes A2, A4, A5, A7, A9 and C3. The minimal extent of these regions ranges from 0.0 to 16.5 cM and covers 26.7 cM (1.33 %) of the total length of the DH map. Taking into account the maximal extent of these regions, the range is increased to 9.6 to 28.0 cM, covering 115.5 cM of the rapeseed genome, corresponding to 5.77 % of the DH map.

Discussion

In the development of doubled haploid lines in *Brassica* three liming factors are encountered: the embryogenic potential and the diploidization capacity of microspores as well as the DEPC rate of MDEs (Möllers and Iqbal 2009). Selection at loci controlling these factors should lead to skewed segregations at linked marker loci in segregating DH populations. To identify regions carrying factors affecting the DEPC

Table 3 Comparison of the DEPC rates of seven selected ISLs and the recurrent parent 'Express 617'

ISL	N[1]	N[2]	Mean[3]	Stdev[4]	Ratio[5]	Minimum	Maximum	P[6]
ER89	14	472	65.6	18.28	0.81	31.3	97.2	0.429[n.s]
ER240	10	264	65.4	12.64	0.94	50	87.5	0.506[n.s]
ER644	10	320	79.1	17.27	1.05	41.1	100.0	1.000[n.s]
ER653	10	256	57.2	18.06	0.76	31.3	79.2	0.051[+]
ER654	10	336	49.1	16.94	0.65	23.4	70.8	0.002[***]
ER661	10	384	54.5	18.58	0.72	17.5	79.2	0.018[*]
ER1298	10	296	71.2	23.70	0.94	28.1	100.0	0.971[n.s]
Express 617	13	496	76.5	9.01	1	62.5	89.6	

[1] Number of microspore cultures

[2] Number of MDEs that were transferred to solid B5 medium

[3] Mean DEPC rate

[4] Standard deviation

[5] Ratio of DEPC rates to the rate of 'Express 617'

[6] P values, +, *, **, *** significant at $P = 0.1, 0.05. 0.02$ and 0.01, respectively, *n.s* not significant

rate of microspore-derived embryos, skewed marker segregations were analysed and compared between a segregating population of doubled haploid plantlets that had developed directly from MDEs and diploid and haploid MDE populations. In addition, a BC_1 population was included in the comparison since there may be additional selective steps acting during microspore formation.

With about 60 %, the DEPC population had the highest fraction of markers with skewed segregations from all four populations, while the BC_1 population with just 12 % showed the lowest number (Ecke et al. 2015). This conforms to expectations since the DEPC population had passed the largest number of selective steps, while the BC_1 population had passed the least number of all four populations. On the other hand, this expectation did not hold for the diploid and haploid MDE populations, which showed 40 and 48 % of markers with skewed segregations, respectively. As discussed by Kampouridis et al. (2016), this may indicate that sample effects played a larger role in determining the number of regions with skewed segregations.

Among the 32 regions with skewed segregations observed in the DEPC population, 20 showed a pattern of skewed segregations across populations indicative of the segregation of a genetic factor controlling the DEPC rate. Of these regions, ten favoured the 'RS239' allele and ten the 'Express 617' alleles. These results

indicate that the DEPC frequency in the cross 'Express 617' × 'RS239' is controlled by a large number of genes and that both parents contributed about equal numbers of favourable alleles.

In total, eight ISLs with donor segments covering or overlapping four selected regions with skewed segregations were chosen to evaluate possible effects of these region on the DEPC rate in the cross 'Express 617' × 'RS239'. 'Express 617', the recurrent parent of the ISL population, has a very low embryogenic potential and some of the ISLs, among them ER1284, an even lower potential (Ecke et al. 2015), a fact that was not yet known when the line was selected. Due to the very low number of MDEs obtained from ER1284, the DEPC rate of this genotype could not be determined and, accordingly, the possible effect of the region with skewed segregations on A2 not be evaluated. The remaining seven ISLs and 'Express 617' showed DEPC rates ranging from 49.1 to 79.1 %. DEPC rates have been reported in the literature (Chuong et al. 1988; Mathias 1988; Kott and Beversdorf 1990; Zhang et al. 2006), but these rates cannot readily be compared because different cultivation protocols were used and, in addition to genotypes, DEPC rates are also dependent on the cultivation protocol. The protocol for cultivating MDEs used in this study was developed by Klutschewski (2013) as an optimized protocol to induce DEPC in MDEs of rapeseed. Klutschewski used this protocol to

Table 4 Genomic regions with skewed segregations in the DEPC population and the donor segments of intervarietal substitution lines selected for coverage of these regions

LG	ER89	ER240	ER653	ER654	ER661	ER644	ER1298
LG	C01		C03			C09	
Extent[a]	32.0 - 72.6 cM		0 - 29.8 cM			56.9 - 116.3 cM	
Extent[b]	44.3 - 68.0 cM		-1.4 - 2.2 cM			62.4 - 93.0 cM	
Favoured allele[c]	R		R			E	
Selected ISL	ER89	ER240	ER653	ER654	ER661	ER644	ER1298
No. of segments[d]	1	4	6	6	4	1	1
LG	*Donor segments of the selected ISLs*						
A02			0.0[ef]	0.0	0.0		
A03		136.8					
A04			0.0 - 0.0	0.0 - 0.0			
A05			86.0 - 86.0	86.0 - 86.0			
A07					13.9 - 20.4		
A09					35.2 - 51.7		
C01		**61.4**[g]					
	71.5 - 94.1	71.5 - 78.4					
C02		0.0					
C03			**0 .0 cM**[g]	**0 .0 cM**[g]	**0 .0 cM**[g]		
			-5.0 - -1.4	**-5.0 - -1.4**			
			2.2 - 2.3	**2.2 - 2.3**			
C09						62.4 - 93.0	62.4 - 93.0

[a] Genomic regions with skewed segregations in the DEPC population. The positions refer to the DEPC map

[b] The positions of the regions with skewed segregations on the DH map according to an alignment with the DEPC map

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

[d] Number of donor segments in the selected ISLs

[e] Single figures indicate donor segments detected by only one marker

[f] Minimal extent of donor segments. Positions are in cM and refer to the DH map if not otherwise indicated. Donor segments that cover the selected regions with skewed segregations are in bold. Segments within bold boxes represent genomic regions that may be involved in the decreased DEPC rates of the significant ISLs

[g] Positions according to the DEPC map since the markers detecting these segments were not mapped in the DH map

determine DEPC rates of 13 different rapeseed genotypes, which showed a range from 33 to 100 %, with a mean of 71 %. The range observed with the seven ISLs covers about the middle third of this range, indicating that a significant part of the genetic variability for the DEPC rate observed by Klutschewski between the 13 genotypes is present in the ISL population. The seven ISLs and 'Express 617', as well as the 13 genotypes analysed by Klutschewski, show a continuous distribution of DEPC rates, indicating that this trait is controlled by many genes, of which a larger number should be segregating in the cross 'Express 617' × 'RS239'. This assumption is supported by the rather large number of 20 genomic regions where skewed markers segregations show a pattern indicative of the segregation of such genes.

The seven ISLs whose DEPC rates were determined had been selected because their donor segments cover or overlap regions with skewed segregations on linkage groups C1, C3 and C9. The regions on C1 and C3 showed an excess of 'RS239' alleles. Accordingly, donor alleles in the ISLs should increase DEPC rates, but only one line, ER644, which does not carry a donor segment within the regions on C1 and C3, showed a DEPC rate slightly higher than 'Express 617'. This line, and the ISL ER1298, had actually been selected because of donor segments partially covering a region with skewed segregations on C9, which showed an

Table 5 Minimal and maximal genome coverage of the donor segments in the respective ISLs according to the AFLP analysis of the DH map

ISL	Donor segments[a]	Minimal coverage		Maximal coverage	
		Absolute coverage [cM]	Percentage of map [%]	Absolute coverage [cM][b]	Percentage of map [%][c]
ER89	1	22.6	1.13	26.1	1.30
ER240	4	6.9	0.34	47.2 (55.4)	2.36
ER653	6	3.7	0.18	54.6 (61.6)	2.73
ER654	6	3.7	0.18	54.6 (61.6)	2.73
ER661	4	23.0	1.15	52.4 (59.4)	2.62
ER644	1	30.6	1.53	36.0	1.80
ER1298	1	30.6	1.53	36.0	1.80
Mean		18.1	0.90	44.1	2.20

[a] Number of donor segments in the selected ISLs

[b] Coverage according to the DH map, in parentheses: coverage when taking into account the maximal extend of the segments only discovered by markers mapped on the DEPC map

[c] refers to coverage according to DH map

Table 6 Genomic regions that may control the DEPC rate of MDEs

LG	Minimal extent			Maximal extent			No. markers	ISLs
	Start [cM][1]	End [cM]	Length [cM]	Start [cM]	End [cM]	Length [cM]		
A02	0.0	0.0	0.0	0.0	12.4	12.4	1	ER653, ER654, ER661
A04	0.0	0.0	0.0	0.0	9.6	9.6	2	ER653, ER654
A05	86.0	86.0	0.0	76.7	91.4	14.7	3	ER653, ER654
C03	−5.0	−1.4	3.6	−5.0	12.9	17.9	2	ER653, ER654
C03	2.2	2.3	0.1				2	ER653, ER654
C03[2]	0.0	0.0	0.0	0.0	7.0	7.0	1	ER653, ER654, ER661
Sum			26.7			115.5		
Map coverage			1.33 %			5.77 %		

[1] Positions refer to the DH map if not otherwise indicated

[2] Positions refer to the DEPC map

excess of 'Express 617' alleles. Here, donor alleles should decrease DEPC rates. While ER644 shows a higher DEPC rate than 'Express 617', the DEPC rate of ER1298 is lower, but the rate is the second highest among the seven ISLs and quite close to the rate of 'Express 617'. In conclusion, it has not been possible to confirm effects of the regions with skewed segregations on C1, C3 and C9 on the DEPC rate of MDEs using the seven selected ISLs. This failure may be due to the fact that no ISLs could be found whose donor segments would fully cover the regions with skewed segregations. On all three linkage groups, the minimal extent of the donor segments of the chosen ISLs cover only part of the regions. On C1 and C9 the maximal extents of the donor segments cover the full regions, but the maximal extent is only the maximal possible extent; the true extent will be smaller in most cases. This leaves the possibility that the genes causing the

skewed segregations do not reside in the regions actually covered by the donor segments. On the other hand, it is also possible that the skewed segregations in these regions in the DEPC population are not due or not directly due to the segregation of genetic factors controlling the DEPC rate. The DEPC population has passed all three selective steps encountered during the production of doubled haploids. Accordingly, the skewed segregations observed in this population are a complex overlay of segregation patterns caused by the different selective steps, which may make it difficult to localize signals caused by an individual step. Also, sample effects, that is chance, may also have a significant effect on the observed segregation patterns.

Nevertheless, it has been possible in this study to identify three ISLs, ER653, ER654 and ER661, with DEPC rates significantly lower than the DEPC rate of the recurrent parent of the ISL population, indicating that the donor segments in these lines carry genes controlling the DEPC rate of MDEs of rapeseed. The lines ER653 and ER654 carry six donor segments and ER661 four. None of these segments occur in a non-significant line or overlap with donor segments of such a line. Accordingly, it was not possible to further narrow down the position of the genetic factors causing the reduced DEPC rates of the significant lines by a comparison with the non-significant lines. On the other hand, the donor segments in the three significant lines cover just 1.33 or 5.77 % of the DH map in their minimal or maximal extent, respectively, which means that it was possible to assign at least one genetic factor controlling DEPC rates to a rather small fraction of the rapeseed genome. The lines ER653 and ER654 share two small segments on A2 and C3 with ER661, but the larger segments are different, indicating the possibility that the reduced DEPC rate of ER661 is caused by a different genetic factor or a different set of factors than the reduced rates of ER653 and ER654. Although ER653 and ER654 share all donor segments, ER653 has a DEPC rate more than 8 % higher than ER654. While this difference is not large, it nevertheless may indicate a genetic difference between these lines. This difference may result from differences in the true extent of the donor segments, which cannot be known due to limits in the marker density of the maps used to characterize the ISLs, or due to the presence of undetected donor segments. As discussed by Kampouridis et al. (2016), there is

always the possibility with ISLs that small donor segments have escaped detection, like the segments on C1 and C3 that were only detected by an analysis using the DEPC map but had been missed by the original analysis with the DH map.

Although it was not possible to assign genetic factors controlling DEPC rates of MDEs in rapeseed to specific locations in the rapeseed genome or to determine the number of factors involved in the reduced DEPC rates of the significant ISLs, the identification of these ISLs is an important result. These ISLs represent an ideal starting point for a further in depth analysis of the genetics of the DEPC rate of MDEs. By backcrossing the lines again with 'Express 617' and developing new ISL with only single donor segments of subsegments of larger segments, it should be possible to fine map the genes in these lines that control the DEPC rate. Furthermore, since the significant lines are distinguished from 'Express 617' in only a small fraction of their genome, they are ideal for a molecular analysis of the trait DEPC rate by transcriptomic, proteomic or metabolomic approaches.

Compliance with ethical standards

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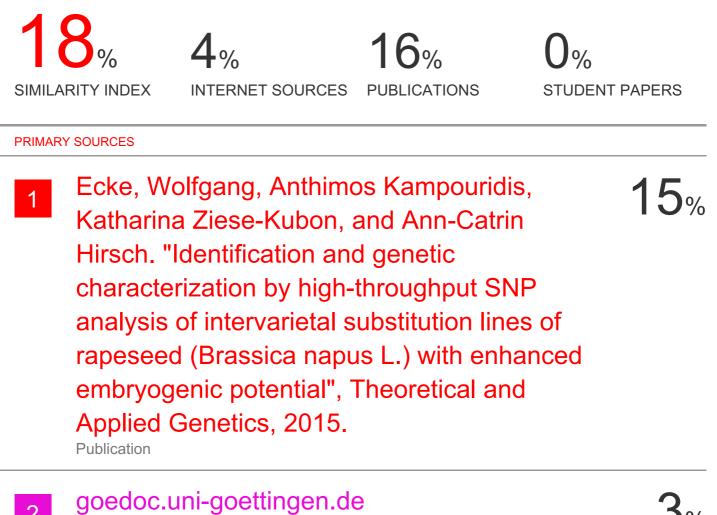
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