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GISH analysis of disomic *Brassica napus-Crambe abyssinica* chromosome addition lines produced by microspore culture from monosomic addition lines

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Abstract Two *Brassica napus–Crambe abyssinica* monosomic addition lines (2*n*=39, AACC plus a single chromosome from *C. abyssinca*) were obtained from the F_2 progeny of the asymmetric somatic hybrid. The alien chromosome from *C. abyssinca* in the addition line was clearly distinguished by genomic in situ hybridization (GISH). Twenty-seven microspore-derived plants from the addition lines were obtained. Fourteen seedlings were determined to be diploid plants (2*n*=38) arising from spontaneous chromosome doubling, while 13 seedlings were confirmed as haploid plants. Doubled haploid plants produced after treatment with colchicine and two disomic chromosome addition lines (2*n*=40, AACC plus a single pair of homologous chromosomes from *C. abyssinca*) could again be identified by GISH analysis. The lines are potentially useful for molecular genetic analysis of novel *C. abyssinica* genes or alleles contributing to traits relevant for oilseed rape (*B. napus*) breeding.

Keywords *Brassica napus* · *Crambe abyssinica* · Addition lines · Microspore culture · GISH

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Introduction

Crambe abyssinica Hochst. ex RE Fr., belonging to the tribe *Brassiceae*, is an annual herb with a high content of erucic acid (55–60%) in the seed oil. Moreover, it possesses further valuable characteristics, such as a short growing period, wide adaptation and insect resistance, that make it a potential gene donor for the modification of oilseed rape (Wang et al. 2000). In an attempt to increase the erucic-acid content in the seed of *B. napus* by combining different genetic systems involved in erucic-acid biosynthesis, asymmetric somatic hybridization was performed between *B. napus* and *C. abyssinica*, and 20 asymmetric somatic hybrids were produced (Wang et al. 2003). Among progeny plants of F_2 and BC_1, aneuploids with 1–30 additional chromosomes from *C. abyssinica* were characterized in a previous study (Wang et al. 2004). Plants with 38 chromosomes were also produced after elimination of *C. abyssinica* chromosomes following backcrossing with *B. napus*. Analyses via genomic in situ hybridization (GISH) and cleaved amplified polymorphic sequences (CAPS) of the *fae1* gene confirmed intergenomic recombination between *B. napus* and *C. abyssinica*. In order to recover and maintain intergenomic recombinations and to achieve homozygous lines, microspore culture was performed using two plants ($S_{3/1}$, $S_{4/1}$) that contained 39 chromosomes (*B. napus* genome plus a single *Crambe* addition chromosome) as microspore donor.

The development of haploids is recognized as the most rapid route to the achievement of homozygosity and production of pure lines in a number of plant species. In *Brassica* species, an isolated microspore culture protocol was first reported by Lichter (1982). To date, it is possible to recover haploid plants in *B. napus* and *B. carinata* (Chuong and Beversdorf 1985), *B. nigra* (Leelavathi et al. 1987), *B. rapa, B. oleracea* (Lichter 1989) and *B. juncea* (Thiagarajah and Stringham 1993). Leino et al. (2004) obtained 90 haploid plants from a fertile BC_5 plant produced by somatic *B. napus* (+) *Arabidopsis thaliana* hybrids; however to our knowledge, there are few further reports on the production of haploid plants of progeny

from interspecific *B. napus* lines containing an addition chromosome from a donor species Monosomic alien addition lines (MAALs) that carry only one chromosome from a wild species are potentially interesting for plant breeders as a bridge to transfer genes of interest via recombination or translocation events, in an effort to introduce novel germplasm into elite lines (Chen et al. 2004). Gene introgressions through MAALs have been accomplished in a number of important crops, such as wheat (Kishii et al. 2004), rice (Jena and Khush 1989), sugar beet (Gao et al. 2001; Gao and Jung 2002), and cucumber (Chen et al. 2004). In *B. napus*, intergeneric MAALs have been described from *B. napus* + *Diplotaxis erucoides* (Chévre et al. 1994), *B. napus* + *Sinapis arvensis* (Snowdon et al. 2000), and *B. napus* + *R. sativus* (Peterka et al. 2004). In order to produce a stable, non-segregating disomic addition line from an MAAL, either self-pollination or colchicine treatment may be applied. Use of chromosome doubling by colchicines treatment was first used by Islam and Shepherd (1981) to produce disomic wheat–barley addition lines from monosomic additions, and Linc et al. (2003) demonstrated in wheat–maize addition lines that chromosome doubling via colchicines treatment can be more efficient than self-pollination for production of fertile disomic addition lines. The *B. napus* + *C. abyssinica* addition lines we described previously (Wang et al. 2004) showed a low fertility and regeneration frequency after selfing, hence the objectives of the current study were (1) to produce haploid plants comprising the *B. napus* genome plus a single chromosome from *C. abyssinca* by microspore culture, and (2) to recover homozygous disomic chromosome addition lines by colchicine treatment. The resulting monosomic and disomic plants were characterised cytologically by GISH analysis.

Materials and methods

Plant materials

Asymmetric somatic hybrids obtained by fusion of *B. napus* protoplasts with UV-irradiated *C. abyssinica* protoplasts have been described previously (Wang et al. 2003). Sexual progenies of the hybrids were obtained by self-pollination (F_2) or backcrosses (BC_1) with *B. napus* cv. 'Maplus'. Among 24 progenies investigated, two plants ($S_{3/1}$ and $S_{4/1}$) were confirmed to have 39 chromosomes (Wang et al. 2004) and were used as materials for microspore culture.

Microspore isolation, embryo culture and plant regeneration

Flower buds (3–4.5 mm long) were harvested from the progeny plants with 39 chromosomes. The buds were surface-sterilized in 96% ethanol for 15 s, then in a 4% (v/v) commercial bleach for 15 min, followed by three rinses with sterile distilled water. Fifteen buds for each sample were blended in 25 ml NLN medium (Lichter 1982) containing 13% (w/v) sucrose and then lightly pressed with a pestle. The material was filtered through a 45 μm sieve and collected in 10 ml centrifuge tubes, then centrifuged at 1000 rpm for 5 min and washed once with NLN medium containing 13% sucrose. The pellet was diluted to a concentration of 6×10^4 microspores per milliliter with NLN medium containing 13% sucrose, and then dispensed into 9 cm petri dishes. The isolated microspores were incubated in the dark for 5 days at 33°C, then for a further 2 weeks at 25°C. Subsequently the dishes were transferred to a rotary shaker (90 rpm) under dim light for 1 week and propogated under normal light until well-developed cotyledons were formed. Cotyledonary embryos about 4–5 mm in length were transferred into a solid medium [MS medium (Murashige and Skoog 1962) +20 g/l of sucrose +7.5 g/l of agarose +0.2 mg/l of NAA (naphthaleneacetic acid) +0.2 mg/l of 6-BA (6-benzylaminopurine)] and cultured at 24°C under cool-white fluorescent light with a 16 h (light) photoperiod. Shoots emerged after 3–4 weeks and were transferred to MS medium without hormones for plantlet development.

Determination of ploidy level, colchicine doubling and seed set

The ploidy level of the microspore-derived plants was detected by flow cytometry with a 'Partec Cell Analyzer CA-II' (Partec GmbH, Germany) using a previously described method (Wang et al. 2003). Plants displaying haploidy were treated with colchicine as follows: Shortly before floral emergence the roots of the plants were washed with running water and immersed in a 3.4 g/l of colchicine solution for 90 min (Lionneton et al. 2001). The treated roots were rinsed with running water for 2 h and then the plants were planted in soil. For each plant, flowers were self-pollinated, and fertility (seed set) was measured by the number of seeds obtained per pod.

Genomic in situ hybridization (GISH)

Root tips and young flower buds from the microspore-derived plants were used as experimental material for GISH analysis. Cytological preparations and fluorescence in situ hybridization were performed according to previously-described methods (Wang et al. 2004; Snowdon et al. 1997). Total genomic DNA was extracted from young leaves of *B. napus* and *C. abyssinica* plants using a DNeasy Plant Maxi Kit (Qiagen, Germany). Genomic DNA of *C. abyssinica* was fluorescently labeled with tetramethyl-rhodamine-5-dUTP or fluorescein-12-dUTP using a nick-translation kit (Catalog No. 976776, Roche, Germany) according to the manufacturer's instructions. To prevent non-specific, intergenomic cross-hybridization, a 30-fold excess of sheared genomic DNA from *B. napus* was added to the hybridization solution. The DNA was sheared by autoclaving (5 min,

1 bar), yielding fragments of around 300–500 bp in size. Labeled probe and chromosomes were denatured simultaneously on cleaned microscope slides at 80°C for 4 min and hybridized overnight at 37°C. After hybridization, slides were washed at 42°C for 5 min each in 2 × SSC and 0.4 × SSC, respectively. Chromosomes were counterstained with DAPI (4,6-Diamidino-2-phenylindole) for rhodamine-labeled probe, or propidium iodide for FITC-labeled probe, and fluorescence was visualized using an Olympus BX40 microscope. At least five cells were observed for each plant. Images of the single fluorophores were obtained using a computer-assisted, cooled charge-coupled device (CCD) camera, and composite GISH images were generated by merging using Adobe Photoshop version 5.5 software.

Fatty-acid analysis

Seeds from the microspore-derived plants were subjected to analysis of fatty acid composition. For every plant, 30 single seeds (3 replications of 10 seeds) harvested in the same greenhouse were analyzed. The chemical analysis of the fatty-acid composition was carried out by capillary-gas chromatography of fatty-acid methyl ethers (FAME) using a gas chromatograph HP 5890 with an HP 7673 auto-sampler (Hewlett Packard), as described previously (Wang et al. 2004). Fatty acids with chain lengths between C_{14} and C_{24} were determined. The erucic-acid content was expressed as the percentage of the total fatty acids. Statistical analyses of erucic-acid variation were performed with SAS/STAT software release 6.12 (SAS Institute Inc.).

Results

Production of microspore-derived plants and examination of the ploidy level

The first division of the microspores was observed about 1 week after isolation from the monosomic addition plants (Fig. 1A). Proembryos were formed after 2 weeks (Fig. 1B). Globular embryos were visible after 3 weeks of culture, and were then transferred onto a shaker under light. Well-developed cotyledonary embryos were formed after 6 weeks (Fig. 1C) and transferred to solid medium for plant regeneration. In total, 405 well-developed cotyledonary embryos were obtained. From these, 27 plants (15 from $S_{3/1}$, 12 from $S_{4/1}$) were regenerated (Table 1). Before transferring the plants to the greenhouse, the ploidy level was determined by flow cytometry. From the 27 regenerated plants, 14 (51.9%) were found to be spontaneous diploids. The remaining 13 plants were confirmed as haploids (Fig. 1D) and these were treated with colchicine immediately prior to flowering.

Table 1 Seed set and erucic acid content of plants derived from microspore culture compared with *B. napus* cv. 'Maplus' as control

Origin	Ploidy	Seed set (seeds/pod)	Mean erucic acid (%) ± SD
S3/1	S[a]	7.5	53.1±0.1
	D1	10.1	50.3±0.1*
	D2	19.1	50.6±0.4*
	D3	13.8	48.7±0.1
	D4	11.4	51.3±0.1*
	D5	16.3	52.3±0.2*
	D6	8.2	52.6±0.2*
	D7	13.7	51.5±0.2*
	D8	8.2	52.8±0.8*
	D9	13.3	52.6±0.3*
	DH1	5.3	50.2±0.1*
	DH2	3.1	50.5±0.4*
	DH3	4.2	48.9±0.8
	DH4	6.9	51.3±0.1*
	DH5	6.2	52.0±0.3*
	DH6	5.3	51.9±0.1*
S4/1	S[a]	4.8	53.0±0.3
	D10	6.2	54.3±0.7*
	D11	5.3	51.2±0.3*
	D12	5.4	48.6±0.7
	D13	5.5	51.1±0.4*
	D14	5.1	51.6±0.3*
	DH7	3.2	54.0±0.6*
	DH8	4.7	51.7±0.7*
	DH9	2.5	54.8±0.4*
	DH10	5.5	51.5±0.2*
	DH11	4.3	54.2±0.4*
	DM1	3.7	52.6±0.1*
B. napus *cv.* *'Maplus'*	DM2	2.4	53.0±0.4*
		16.9	48.1±0.4

Note. D: spontaneous diploid; DH: doubled haploid; DM: disomic alien addition line. Significant difference compared with *B. napus* cv. 'Maplus' ($P<0.05$) is shown by an asterisk

[a]Selfing progenies from $S_{3/1}$ and $S_{4/1}$ plants were harvested via self-pollination, but not grown in the same season as the other plants

Morphological and cytological characterization of the monosomic addition line and microspore-derived plants

The progeny plants with 39 chromosomes exhibited minor morphological differences from the control plant (*B. napus* cv. 'Maplus'), for example in the presence of numerous trichomes on the stems and waxy leaves in the young plant (plant $S_{3/1}$, Fig. 1E), or very dark green leaves (plant $S_{4/1}$, Fig. 1F); both of these characteristics are typical for the chromosome donor *C. abyssinica*. GISH analysis of these two plants indicated that each of them contained 39 chromosomes comprising the complete diploid genome of *B. napus* ($2n=38$) and a monosomic addition chromosome from *C. abyssinca* (Fig. 1G). The 14 spontaneous diploid plants derived from microspore culture all possessed a normal *B. napus* karyotype with $2n=38$ and no

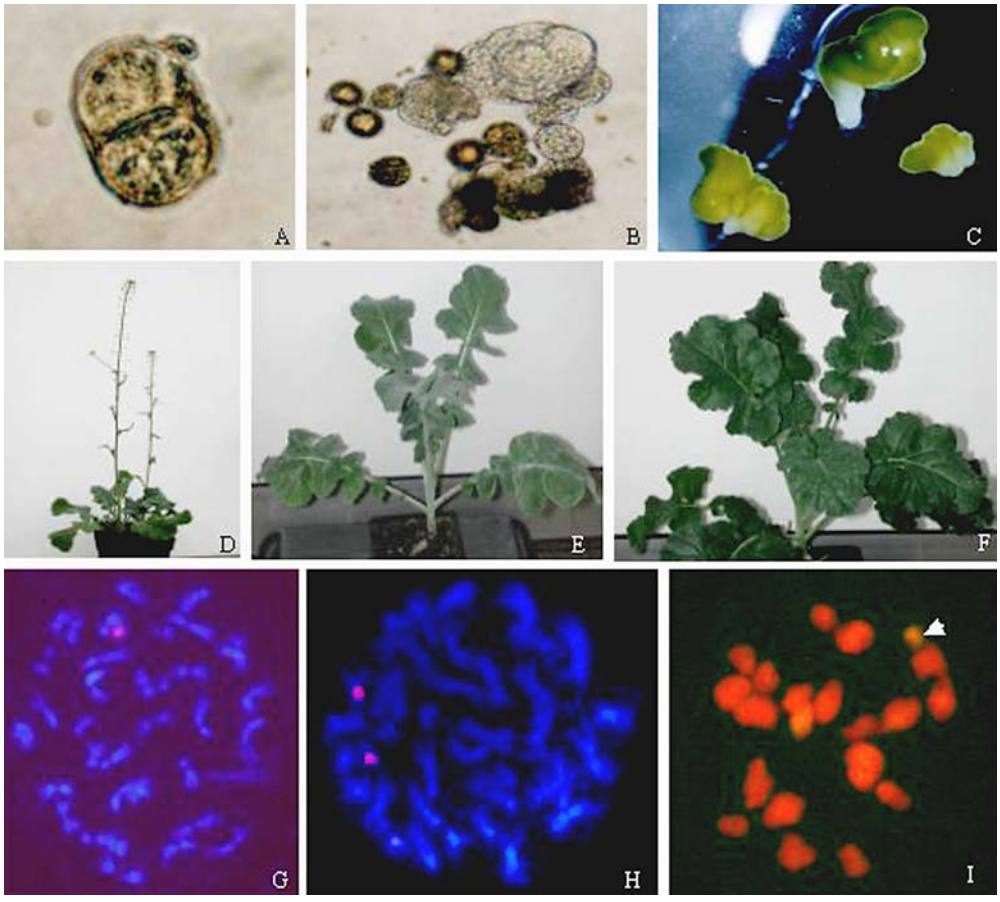

Fig. 1 **A–C** Microspore culture in a *Brassica napus* plant containing a monosomic addition chromosome from *Crambe abyssinica* introduced by asymmetric hybridization. **(A)** First microspore division. **(B)** Proembryo formation. **(C)** Well-developed embryos. **(D)** Haploid plant. **(E, F)** Monosomic plants (2*n*=39). **(G, H)** GISH analysis of mitotic cells from the alien addition chromosome plants. *Crambe abyssinica* chromosomes are labeled red with rhodomine, whereas non-labeled *B. napus* chromosomes are stained blue with DAPI. **(G)** Original monosomic addition plant with 2*n*=38 chromosomes from *B. napus* in blue and one single chromosome from *C. abyssinica* labelled red. **(H)** Disomic addition plant obtained by microspore culture and chromosome doubling, containing 2*n*=38 chromosomes from *B. napus* and two homologous chromosomes from *C. abyssinca*. **(I)** GISH analysis of meiotic cells from the disomic addition plants, showing 19 II from *B. napus* stained with PI in red, and 1 II from *C. abyssinca* stained with fluorescein-12-dUTP in yellow (indicated by arrow)

complete *C. abyssinica* addition chromosomes. These plants exhibited a large range of seed set, with 5.1 to 19.1 seeds per pod (Table 1). The seeds contained 51.4% erucic acid, ranging from 48.6% to 54.3%, which is significantly higher than that of the control (*B. napus* cv. 'Maplus', 48.1%). After colchicine doubling of 13 haploids, two offspring from plant $S_{4/1}$ were confirmed to possess 40 chromosomes, representing 2*n*=38 *B. napus* chromosomes and two homologous *C. abyssinica* chromosomes confirmed by GISH (disomic addition line) (Fig. 1H). These plants had a seed set ranging from 2.4 to 3.7 and an erucic acid level between 52.6 and 53.0%. The meiosis of these plants showed 20 normal bivalents (II) at diakinesis, which were shown by GISH to comprise 19 (II) from *B. napus* (no GISH signals) and 1 (II) from *C. abyssinca* (strong GISH signals, Fig. 1I). The remaining 11 seedlings exhibited 38 chromosomes and showed no GISH signals with labelled *C. abyssinica* genomic DNA. Most of cells (86%) exhibited normal pairing at diakinesis/metaphase of meiosis, whereby the remaining 14% of the cells presented laggards indicating irregular segregation at anaphase. These doubled haploids had a low seed set, from 2.5 to 6.9, and compared to the control (*B. napus* cv. 'Maplus') they contained a higher level of erucic acid ranging from 48.9% to 54.8% (Table 1).

Discussion

In the *Brassicaceae*, MAALs are generally generated from natural or synthetic amphidiploid lines by the following steps: 1) production of an amphidiploid through distant hybridization; 2) production of the sesquidiploid by backcrossing the amphidiploid to the diploid species; and 3) production of the MAALs through repeated backcrossing to the diploid species followed by selection (Chen et al. 2004). *Brassica napus* and *C. abyssinca* belong to different genera, but the same tribe (*Brasicacaeae*). The former has 38 chromosomes, whereas the latter is a hexaploid with 90 chromosomes, making it virtually impossible to generate a

full set of *C. abyssinca* MAALs. Nevertheless, individual MAALs containing enhanced traits from the donor species provide us with a potentially powerful tool to transfer genes of interest from *C. abyssinica* to *B. napus* and to enhance our understanding of the relationships and the genomic structure among the two genera. In this work, two MAALs were obtained in the F_2 progeny of asymmetric somatic hybrids between *B. napus* and *C. abyssinica*. Because of the very high chromosome number of *C. abyssinica* (2*n*=90), the reduction of chromosomes was accelerated by low-level UV-irradiation of the donor protoplasts (Wang et al. 2003). In this manner, it was possible to eliminate unwanted *C. abyssinica* chromosomes in only a few number of backcrossing steps in order to obtain useful breeding lines with limited linkage drag. The success of this strategy confirms that asymmetric somatic hybridization via UV-irradiation is a powerful tool to produce highly asymmetric somatic hybrids. The two monosomic addition lines generated (Wang et al. 2004) were distinguishable from *B. napus* by morphological traits.

MAALs might be used as a bridge to transfer genes of interest from one species to another via recombination or translocation events. Successful examples of gene introgressions by MAALs include the transfer of leaf spot resistance from *Beta corolliflora* into sugar beet, *B. vulgaris* (Gao and Jung 2002), and the transfer of brown rust resistance from *Oryza officinalis* into rice (Jena and Khush 1989). MAALs of *B. napus* with alien chromosomes from *S. arvensis* were shown to exhibit resistance to *Leptosphaeria maculans* (Snowdon et al. 1997). More recently, five different multiple *B. napus–R. sativus* (oil radish) addition chromosomes (a–i) were generated by Peterka et al. (2004) and used to identify a chromosome containing genes for beet cyst nematode resistance. The potential use of MAALs also includes chromosomal assignment of molecular markers or dominant plant traits (Chen et al. 1992; Peffley et al. 1985; Suen et al. 1997), molecular mapping of alien genes (Jung et al. 1992), the construction of chromosome-specific libraries (Schmidt et al. 1990), and production of disomic addition lines (Jahier et al. 1989).

In some cases, the generation of cytologically stable, non-segregating, fertile disomic addition lines via selfing of an MAAL can be difficult, possibly due to restricted competitiveness of gametes carrying an alien chromosome (Leino et al. 2004). In the present study, we were unable to generate stable disomic addition lines by selfing, hence as an alternative strategy dihaploidization was performed to recover homozygous lines. The recovery rate of disomic additions after microspore culture and colchicines treatment was low, with only 2 disomic addition lines being produced out of 27 regenerated plants, indicating that the microspores carrying an alien chromosome may have been subject to a selective disadvantage in the haploid plants. However, meiotic GISH showed that the two disomics derived from microspore culture exhibited normal pairing at diakinesis. Most of the plants with 38 chromosomes contained a higher level of erucic acid as compared to the control, with some individual plants, attaining more than 54.0% erucic acid from the individual $S_{4/1}$, which showed among the highest eruci acid levels, exhibited *C. abyssinica*-specific bands after CAPS analysis of *fae1* gene (Wang et al. 2004), indicating that one or more alleles governing erucic-acid biosynthesis from *C. abyssinca* may have been transferred to the recipient genome. In these plants no *C. abyssinica* signals were observed by GISH analysis, although laggards and irregular segregation were observed at anaphase of meiosis in some cells. Hence it appears that an intergenomic recombination has been achieved, possibly involving genome segments containing the *fae1* gene copies from *B. napus* and/or *C. abyssinica*. The inability to detect what are presumably *C. abyssinica* translocations to *B. napus* chromosomes is probably due to fact that the chromosome arms in *Brassica* and related genera contain unusually low copy numbers of dispersed repeat sequences. Because such sequences generally form the basis of chromosome "painting" and GISH signals, strong GISH signals are normally only observed at centromeric regions in *Brassica* (Heslop-Harrison and Schwarzacher 1996; Snowdon et al. 1997; Wang et al. 2004). This means that small recombinations or translocations in non-heterochromatic regions from interspecific *Brassica* hybrids can generally not be detected using GISH analysis (Snowdon et al. 1997, 2000). In the present case, the clear increase of erucic-acid content in some individuals suggests that alleles governing erucic-acid synthesis have been transferred from *C. abyssinica* to *B. napus*, although further molecular genetic analyses are necessary to confirm this hypothesis.

Microspore-derived haploids in *Brassica* can double spontaneously. Many studies show that the frequency of spontaneous doubling depends on the genotype used, the microspore stage and the culture conditions (Möllers et al. 1994). In microspore cultures of *B. juncea*, 22% spontaneous diploids were found (Lionneton et al. 2001), whereas different genotypes of *B. oleracea* showed different degrees of spontaneous doubling ranging from 21% to 67% (Rudolf et al. 1999). In the present study, about 51.9% of microspore-derived plants were diploids without any chromosome doubling treatment. The high regeneration rate may result from the use of aneuploid plants (2*n*=38+1) for microspore isolation, which could lead to endoreduplication or meiotic disturbances in the microspores. Such spontaneous diploids are very useful to breeders for rapid production of homologous lines. Further experiments are in progress to observe the agronomical characteristics and breeding behavior of the materials generated in the present study.

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