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Interspecific hybridisation of cytoplasmic male-sterile rapeseed with *Ogura* cytoplasm and *Brassica rapa* var. *pekinensis* as a method to obtain male-sterile Chinese cabbage inbred lines

Piotr Kamiński · Małgorzata Podwyszyńska · Michał Starzycki · Elżbieta Starzycka-Korbas

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Abstract During 2011–2014, three self-compatible Chinese cabbage inbred lines (*Brassica rapa*, genome AA $2n = 20$) were sexually hybridised with cytoplasmic male-sterile (*CMS Ogu-INRA*) and fertile lines of rapeseed (*Brassica napus* var. *oleifera*, genome AACC $2n = 38$). Interspecific F_1 hybrids and their consecutive back-crosses (BCs) toward *B. rapa* were obtained, and their morphological and commercial characteristics, as well as their ability for generative propagation, were analysed. Cytometric and morphological analyses confirmed the interspecific character of the F_1 hybrids in comparison with *B. napus* and *B. rapa* parental lines. All interspecific F_1 hybrids had intermediate morphological characteristics typical of both parental components in the generative and vegetative stages. The F_1 hybrids of *CMS B. napus* × *B. rapa* were sterile, as were those of fertile *B. napus* × *B. rapa*. Good seed productivity from *CMS B. napus* × *B. rapa* and consecutive back-cross 1 (BC_1)–BC_3 generations with Chinese cabbage as the recurrent parent was obtained. Plants of the BC_1 generation of *CMS B. napus* × *B. rapa* were sterile and had highly diversified morphological characteristics, both in the vegetative and generative phases. The BC_3 generation of *CMS B. rapa* was also sterile and more uniform than BC_1, with good vigour and most of the typical Chinese cabbage agronomic traits. As a result, a collection of Chinese cabbage genotypes having the *CMS* trait, which is valuable for the breeding of commercial F_1 hybrids, was obtained for the first time in Poland.

Keywords *Brassica rapa* · *CMS* · Flow cytometry · Interspecific crosses · Morphological traits

Introduction

The species representing the tribe Brassicae of the Brassicaceae family are of great economic importance, as they are domesticated for edible oil, vegetables, spices, forage crops and ornamental plants (Pratap and Gupta 2009). Interspecific and intergeneric hybridisation between Brassicae species makes the transferring of valuable resistance and quality traits of commercial interest, as well as broadening the gene pool for genetic improvement, challenging. Hybridisation has been extensively performed to develop more potential varieties with desired morphological characteristics, resistance to

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P. Kamiński (✉) · M. Podwyszyńska
Research Institute of Horticulture, ul. Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland
e-mail: piotr.kaminski@inhort.pl

M. Starzycki · E. Starzycka-Korbas
Plant Breeding and Acclimatization Institute, National Research Institute, ul. Strzeszyńska 36, 60-479 Poznań, Poland

biotic and abiotic stresses, or male sterility suitable for breeding (Kaneko et al. 2009; Rahman 2001). The mechanism of chromosome elimination in interspecific *Brassica* species has not been completely described. However, the elimination of chromosomes that fall behind during mitosis (Sybenga 1992), abnormal spindle fibres that are formed during mitosis (Maiato et al. 2002), genetic controls that lead to chromosome elimination (Liu and Wendel 2000) and the responsibility of some genetic sequences for the non-random elimination of homologous sequences from different parental lines (Feldman et al. 1997) have been suggested. The hybridisation in *Brassica* proved to be very useful for the development of cytoplasmic male-sterile (*CMS*) crops by transferring the radish *Ogura* cytoplasm to *Brassica oleracea* and *Brassica napus* (Pelletier et al. 1988). The introgression of the alloplasmic *CMS* systems for the production of *Brassica* oilseeds allowed for the effective commercialisation, meeting plant breeding goals. With the development in 1994 of the improved *CMS Ogu-INRA* system in oilseed *Brassica*, seed companies have developed a number of hybrid varieties in Europe, Canada and Australia. This *CMS* sterility has also been widely used to produce commercial hybrids of *B. oleracea* vegetables (Prakash et al. 2009). Interspecific crosses attempted by plant breeders and geneticists between *B. napus* and *B. rapa* can occur readily owing to the high degree of relatedness, albeit with varying degrees of difficulty (Elling et al. 2010). Natural hybrids were found in fields where species are in physical proximity and have flowering synchrony (Anonymous 2002; Bing et al. 1996; Jorgensen and Andersen 1994).

Brassica rapa is a highly polymorphic species, represented by numerous important vegetables, and oilseed and fodder crops. Chinese cabbage (*B. rapa* ssp. *pekinensis* L.) is one of the most popular vegetables with a worldwide distribution, available for consumers year-round as a valuable source of carbohydrates, proteins, dietary fibre, vitamins A, C, K and B6, minerals, and glucosinolates (Dickson 2007). In Poland, the production area of Chinese cabbage ranges from 5000 to 7000 ha and depends entirely on F_1 cultivars supplied by foreign seed companies. For this reason, a breeding programme to obtain new sources of genetic diversity and develop domestic Chinese cabbage F_1 hybrids began at the Research Institute of Horticulture, Skierniewice, Poland in 2008. Production of commercial cabbage F_1 hybrids requires an effective, inexpensive and reliable system of generative propagation (Faulkner et al. 1977; Hodgkin 1980a, b; Ockendon 1973, 1975) and is currently based on either self-incompatibility or *CMS*. Maternally inherited *CMS* traits have been ascertained to be more successful than self-incompatibility in the ability to stably express pollen sterility without any evident changes in vegetative growth or female fertility (Kaneko and Bang 2014). Among several male sterility types of *Brassicae*, the *CMS* discovered by Ogura (1968) is used worldwide for the breeding of *B. napus*, *Brassica juncea*, *B. oleracea* and *Raphanus sativus* F_1 hybrids (Bartkowiak-Broda et al. 1979; Black et al. 2006). *Ogura-CMS* originated from a wild *Raphanus* that spontaneously hybridised with Japanese radish. An effective and reliable method for obtaining interspecific hybrids with the *CMS* trait is also important for the breeders of Chinese cabbage. Sterile and semi-fertile F_1 and back-cross 1 (BC_1) plants were obtained by interspecific sexual hybridisation between maternal *B. napus* var. *oleifera* and paternal *B. rapa* (Harsh et al. 2007). Interspecific F_1 hybrids consecutively back-crossed from a cross between male-sterile *B. napus* (*eru CMS*) and an inbreeding line of tsai–tsai (*B. rapa*) were successfully obtained by Wan et al. (2013). Rapeseed with *pol CMS* has also been used to produce *pol CMS* Chinese cabbage lines (Ke et al. 1992; Yang et al. 2006). The cold-tolerant *Ogura* cytoplasm was successfully incorporated from *B. napus* rapeseed into *B. rapa* (Heath et al. 1994); however, there are no further reports about the use of *CMS-Ogura* for the development of Chinese cabbage F_1 hybrids. The restorer fertility gene, which was introduced into *Brassica* from *R. sativus* by protoplast fusion (Sakai et al. 1996; Carlsson and Glimelius 2011), is also essential for the F_1 hybrid production of oilseed crops; however, the restoration of the fertility is not necessary for F_1 hybrids of *B. oleracea* vegetables with the *CMS* trait (Yamagishi and Bhat 2014).

Brassica species differ in their numbers of chromosomes (Nagaharu 1935; Johnston et al. 2005). This results in variations in nuclear DNA content (genome size) that were clearly confirmed using flow cytometry (FCM) by Sabharwal and Doležel (1993). The numbers of chromosomes and the nuclear DNA contents (2C) in *B. napus* are $2n = 38$ and a 2C of approximately 2.3 pg, respectively, while in *B. rapa* they are

2n = 20 and a 2C of approximately 1.1 pg, respectively (Bennett and Smith 1976; Johnston et al. 2005). Sabharwal and Doležel (1993) showed that F_1 hybrids between various pairs of *Brassica* species differing in genome sizes have intermediate nuclear DNA content values. The authors stated that the evaluation of the genome size by FCM can be a useful method to quickly identify *Brassica* interspecific hybrids. In this study, FCM was used as an additional tool to confirm the status of F_1 hybrids between *B. napus* and *B. rapa.*

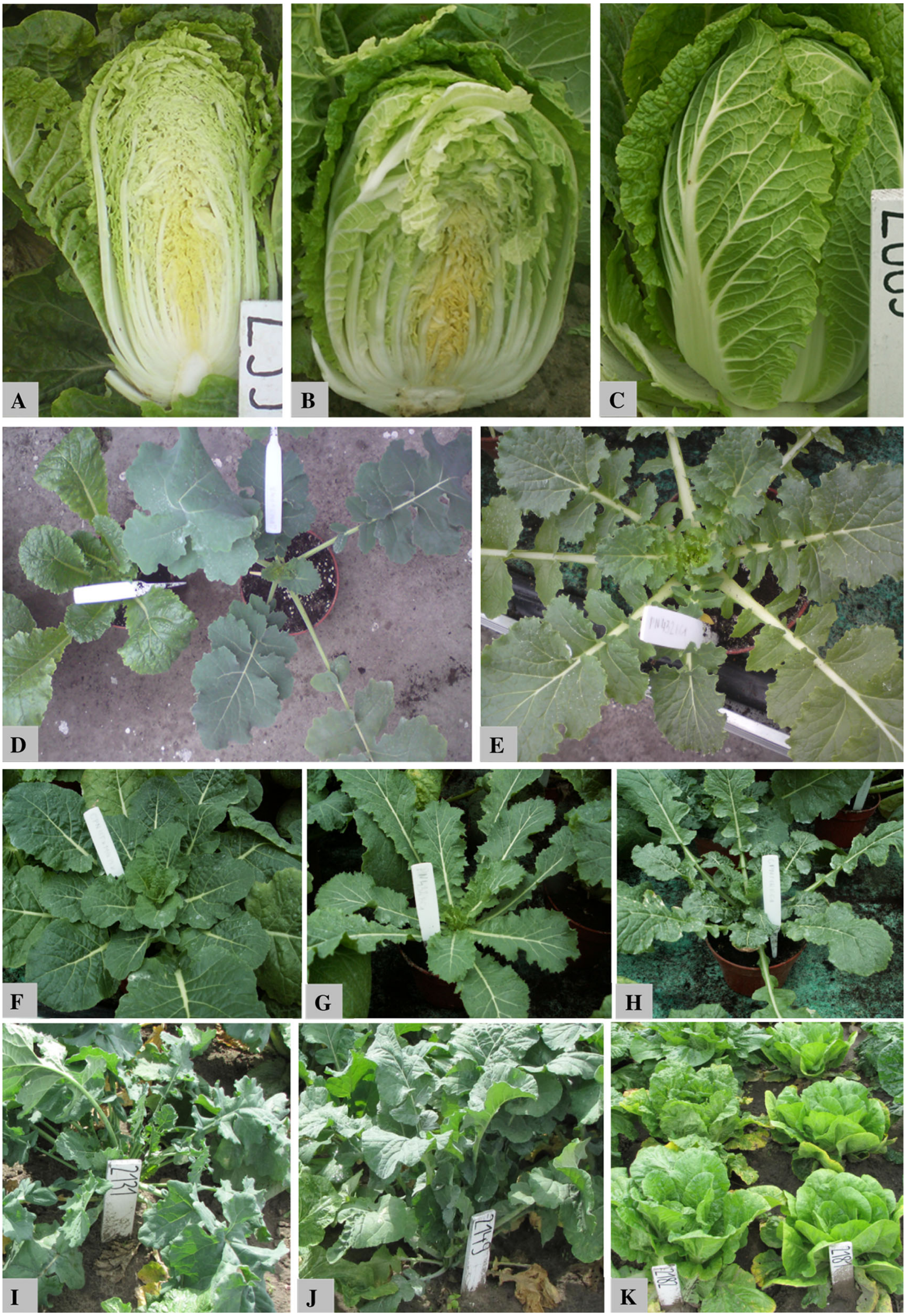
The aims of the study were to (1) introduce the *CMS Ogu-INRA* trait from rapeseed into Chinese cabbage inbred lines through sexual interspecific hybridisation, (2) evaluate the cytological and morphological characteristics, as well as the ability for generative propagation, of the interspecific F_1 hybrids and BC_1 generations, (3) obtain seeds of the consecutive BC_1–BC_3 generations of *CMS B. rapa* lines and (4) evaluate the agronomic traits of the new *CMS* Chinese cabbage lines of the BC_3 generation in comparison with those of the recurrent parents and interspecific F_1 hybrids in the field.

Materials and methods

Obtaining rapeseed × Chinese cabbage interspecific F_1 hybrids and their consecutive BC_1–BC_3 generations

The male-sterile C1162 rapeseed line (*B. napus*) with *Ogura* cytoplasm and an isogenic P1162 fertile line were obtained from the Plant Breeding and Acclimatization National Research Institute, Poznan, Poland in 2010. Three F_6 inbred lines of Chinese cabbage (*B. rapa*) (KW, HG and MR) were developed at the Research Institute of Horticulture, Department of Genetics, Breeding and Biotechnology of Vegetable Crops, Skierniewice, Poland. Selected Chinese cabbage lines were self-compatible, and they had the desired agronomic characteristics of a 71–77-days vegetation period from planting to harvest, good internal uniformity, good (HG) or moderate (KW and MR) vigour, and barrel (HG and KW) or cylindrical shaped heads (Fig. 1a–c). Seeds of the *B. napus* and *B. rapa* parental lines were sown at the beginning of September 2010 in a greenhouse. Three-week-old seedlings were transplanted into 10-cm plastic pots, fertilised and protected against pests and pathogens. Vernalisation started at the beginning of December when plants reached the 10–12-true-leaf stage, and it was conducted under a natural photoperiod at a temperature of 5–7 °C until the end of February 2011. At the beginning of March, three vernalised plants from each of the *B. napus* and *B. rapa* lines were selected, transplanted into 5-L plastic pots filled with substrate and placed on the ground in a greenhouse at a temperature of 16–20 °C. To avoid random and undesired cross-pollination, flower stacks were isolated using plastic and paper bags 2 days before anthesis. Pollen was collected individually from each of the Chinese cabbage inbred lines (KW, HG and MR) and transferred by hand to the stigmas of pistils of maternal rapeseed plants. The interspecific hybridisations KW × C1162, HG × C1162 and MR × C1162 were made on 7–10 freshly opened flowers of the *CMS* rapeseed C1162 line, while crosses KW × P1162, HG × P1162 and MR × P1162 were performed after emasculation at the green bud stage, 2 days before anthesis, of the fertile rapeseed P1162 line. From the second week of March until the end of April, three independent crosses were made for each of the combinations on consecutively developed flower stacks of maternal rapeseed plants. Mature siliques for all of the interspecific F_1 hybrids and inbred lines were harvested by hand in the middle of June. After the siliques were dried, seeds were extracted and counted for each combination.

In 2012, the consecutive BC_1 generation ((*B. napus*/*B. rapa*) × *B. rapa*) was obtained through the use of propagation and vernalisation, as described for the seed development of the interspecific F_1 hybrids. Three plants from each of the male-sterile interspecific F_1 hybrids (KW × C1162, HG × C1162 and MR × C1162) having flower structures typical of the *CMS Ogu-INRA*-type were selected for the BC pollination and crossed with superseding Chinese cabbage lines at the open flower stage. Plants of the KW × P1162, HG × P1162 and MR × P1162 F_1 hybrids were male fertile and, for this reason, were hybridised with superseding Chinese cabbage lines after emasculation at the green bud stage, 2 days before anthesis. In both experimental years, the self-pollination of the male-fertile parental lines (P1162, KW, HG and MR) and the sib-pollination of the sterile C1162 line with pollen of the complementary P1162 line were made at the open flower stage as controls. In contrast to the development of the F_1 and BC_1 interspecific crosses, the BC_1/BC_2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◀**Fig. 1** **a**–**c** Mature plants of the three Chinese cabbage inbred lines used as recurrent parents (from the *left*): lines MR, HG and KW, **d** morphological differences between KW (from the *left*) and rapeseed C1162 parental lines in the greenhouse, **e** interspecific F_1 hybrid of *Brassica rapa* × *Brassica napus*, **f**–**h** segregation of the back-cross 1 (BC_1) population of *B. rapa* × *B. napus*, **i** C1162 rapeseed line in the field, **j** interspecific C1162 × KW F_1 hybrid in the field, and **k** CKW line of the BC_3 generation (from the *left*) and KW *B. rapa* superseding line in the field

(year of propagation 2013) and BC_2/BC_3 (year of propagation 2014) generations were pollinized in the field by the red mason bee (*Osmia rufa* L.). Vernalised plants of the three superseding Chinese cabbage genotypes (KW, HG and MR) and *CMS* plants of the BC_1 generation, [(C1162 × KW) × KW] = CKW BC_1, [(C1162 × HG) × HG] = CHG BC_1 and [(C1162 × MR) × MR] = CMR BC_1, and BC_2 generation, [((C1162 × KW) × KW) × KW] = CKW BC_2, [((C1162 × HG) ≥ HG) × HG] = CHG BC_2 and [((C1162 × MR) × MR) × MR] = CMR BC_2, were cross-pollinated in three separate 9 m^2 growth cages, each covered by a nylon fibre grid. In the second week of April, for each pair of *CMS* and male-fertile superseding lines, five plants with developed generative stacks were transplanted to the open field into two rows, with 100 cm between rows and 50 cm between plants. In the first week of May, when plants started to bloom, 90 red mason bees were placed into each cage to ensure appropriate cross-pollination between male-sterile and fertile components. From the second week of July until the beginning of August, mature siliques were harvested from *CMS* and male-fertile lines separately from each plant. After siliques dried, seeds were extracted, cleaned and weighed. For the seed productivity of interspecific crosses and their parental components, the standard deviation (SD) and coefficient of variation (V%) were calculated.

FCM analysis

Since *B. rapa* and *B. napus* differ in their nuclear DNA contents, their F_1 hybrids should possess intermediate genomes of intermediate sizes compared with those of the two parental species. Therefore, FCM was used as an additional tool to confirm the status of *B. napus* × *B. rapa* F_1 hybrids. Samples were taken in February and March from the fully developed young leaves of plants grown during the winter in a cold greenhouse at a vernalisation temperature of 5–7 °C. Leaf tissue (0.5–1 cm^2) was chopped together with a piece (1 cm^2) of an internal plant standard in a Petri dish in 0.5 mL nuclei isolation Partec buffer supplemented with propidium iodide (50 µg/mL) and RNasa (50 µg/mL) (Śliwińska 2008). As an internal standard for the FCM analysis, the young leaves of *Zea mays* CE-777 (2C = 5.43 pg DNA) (Institute of Experimental Botany, Olomouc, Czech Republic) were used (Lysák and Doležel 1998). After adding 1.5 mL of the isolation buffer, the samples were passed through a 30-µm filter and incubated for 80 min at room temperature. The fluorescence of the nuclei was measured using a CyFlow ploidy analyser with CyView software (CyFlow PA, Partec, Germany) and an Nd–YAG green laser at 532 nm. Data were analysed by means of CyView software (Partec). The 2C DNA content of a sample was calculated as the sample peak's mean divided by the standard plant peak and multiplied by the amount of DNA of the standard plant. Five leaf samples from each plant having at least 5000 nuclei were measured, and each nucleic isolation extract was run twice. Data were subjected to a one-way analysis of variance (ANOVA) (STATISTICA package StatSoft v. 10). The means were compared by Tukey's test at $P = 0.05$. SDs (n = 5) were calculated.

Morphological characteristics of the interspecific *B. napus* × *B. rapa* F_1 hybrids and their BC_1 generation

In 2012, the morphological characteristics of the two rapeseed lines (C1162 and P1162), three Chinese cabbage lines (KW, HG and MR), six interspecific F_1 hybrids (P1162 × KW, P1162 × HG, P1162 × MR, C1162 × KW, C1162 × HG and C1162 × MR) and six BC_1 ((*B. napus*/*B. rapa*) × *B. rapa*) populations [((P1162 × KW) × KW), ((P1162 × MR) × MR), ((P1162 × HG) × HG), ((C1162 × KW) × KW), ((C1162 × HG) × HG) and ((C1162 × MR) × MR))] were analysed. Each line was represented by 10 plants. Seeds were sown at the beginning of September 2011 in the greenhouse, and 3-week-old seedlings were then transplanted and cultivated in 0.33-L plastic pots filled with substrate. In the second week of January 2012, the internal uniformity and morphological characteristics, such as diameter, height and type of plant, position of leaves, heights of stacks, size, type, colour, innervation, waxiness and shapes of leaves, width, and blistering and edges of leaf blades, were analysed in the parental lines and interspecific crosses of the F_1/BC_1 generations. At the beginning of March, for the development of the generative phase, five vernalised plants from each genotype were selected, transplanted into 5-L plastic pots filled with substrate and placed on the ground in a greenhouse at 20 °C. Average plant heights (cm), times of blooming, shapes and lengths of green buds, sizes of flowers (mm), shapes, positions and colours of petals, and the presence of pollen for Chinese cabbage, rapeseed and their F_1 and BC_1 generations in the generative phase were analysed during the blooming season. The coefficients of variance for the average plant heights and average bud lengths were calculated, and other traits were classified according to the multigrade International Union for the Protection of New Varieties of Plants (UPOV) scale.

Agronomic characteristics of the *CMS* Chinese cabbage BC_3 generation and interspecific (*B. napus* and *B. rapa*) F_1 hybrids in the field

In 2014, the agronomic characteristics of three Chinese cabbage *CMS* lines of the BC_3 generation (CKW, CHG and CMR) and three interspecific F_1 hybrids (C1162 × KW, C1162 × HG and C1162 × MR) were compared with those of their parents, *B. rapa* (KW, HG and MR) and *CMS B. napus* (C1162). The experiment was performed in the field at the Research Institute of Horticulture, Skierniewice, Poland. The soil type was a pseudopodsolic over loamy sand (1.5 % organic matter, pH 6.5). The tested plants were developed from seeds in the greenhouse in mid-June 2014. Seeds were sown at the beginning of July in the greenhouse. Three-week-old seedlings were planted 50 cm apart in rows with 60 cm between rows. The experiment was a randomised complete block design with three replications. Each plot consisted of 10 plants in one row. Fertilisation, irrigation, and pest and disease control followed the current recommendations for cabbage plants. Cabbage heads were harvested gradually from the second week of September until the end of October, when they reached maturity. Masses, and lengths and widths of heads were measured, and the head shape coefficient (length/width) was calculated. The results of the head mass and head shape coefficients for the parental lines, F_1 plants and BC_3 generation were elaborated statistically using an ANOVA with the STATISTICA package StatSoft v. 10. The significances of differences among the means were evaluated by Tukey's test at $P = 0.05$. Morphological characteristics of the Chinese cabbage population, such as lengths of vegetation periods from planting to harvest, intraline uniformity, plant vigour, head quality, and the colour and waxiness of external leaves, were classified separately for each plot according to the multigrade UPOV scale.

Results

Generative propagation

Rapeseed (*B. napus*) and Chinese cabbage (*B. rapa*) genotypes selected for interspecific hybridisation were characterised by a high self-compatibility and a good capability for generative propagation when pollinated at the open flower stage in 2011 (Online Resource, ESM_1.pdf). The total number of seeds (900) obtained for the C1162 male-sterile rapeseed line was higher than that for the P1162 fertile line (205). The average number of seeds/silique (18.0) for the sib pollination (C1162 × P1162) was fourfold higher than for self-pollination of the P1162 line (4.1 seeds/silique). Three *B. rapa* inbred lines developed 144 (MR) to 259 (KW) seeds after self-pollination in 2011 and 72 (HG) to 266 (KW) seeds in 2012. Line KW set also the highest average number of seeds/silique in both years of propagation (16.6 and 11.1 in 2011 and 2012, respectively). Cross-pollination between the male-sterile C1162 rapeseed line and three fertile Chinese cabbage lines resulted in the development of 373 (C1162 × KW) to 191 (C1162 × HG) seeds of interspecific F_1 hybrids. The highest average number of seeds/silique (16.3) was obtained for the (C1162 × KW) F_1 hybrid. Lower seed productivity was found in the interspecific crosses between fertile rapeseed line P1162 and Chinese cabbage superseding lines [12 seeds (P1162 × HG) to 95 seeds (P1162 × KW)].

In 2012, seeds of the BC_1 generation were obtained for each combination of interspecific F_1 hybrids and *B. rapa* superseding lines. The numbers of seeds of the BC_1 generation were diverse and ranged from 47 [((P1162 × HG) × HG)] to 281 [((C1162 × MR) × MR)]. The average number of seeds/silique for the BC_1 generation (1.9) was lower than that of the F_1 hybrids (6.4) and those of their parental rapeseed and Chinese cabbage lines (11.1 and 9.8, respectively) (Online Resource, ESM_1.pdf). The V% for the propagated population was diverse, ranging from 6.5 % (P1162) to 64.4 % (KW).

FCM analysis

The FCM analysis of young, fully expanded leaves revealed that all of the *Brassica* genotypes were characterised by a high degree endoreplication, which was illustrated on the histograms by at least three distinct peaks corresponding to 2C, 4C and 8C DNA nuclear contents. The FCM analysis carried out with *Z. mays* ($2C = 5.43$) as an internal standard revealed that the relative 2C nuclear DNA contents of *B. napus* lines P1162 and C1162 were similar, at approximately 2.5 pg. Also, *B. rapa* genotypes did not differ significantly in 2C genome sizes that were approximately 1.1 pg. The genome sizes of all of the F_1 hybrids (1.71–1.83 pg) demonstrated values approximately intermediate to those observed in the parental species (Online Resource, ESM_2.pdf).

Morphological characteristics for F_1 and BC_1 interspecific crosses of *B. napus* and *B. rapa*

Rapeseed and Chinese cabbage genotypes had morphological characteristics typical of their species and good intraline uniformity (Table 1). *B. napus* inbred lines (P1162 and C1162) were tall, with greater plant diameters in comparison with *B. rapa* lines (KW, MR and HG). Leaves of *B. napus* plants were large, lyrate-type, with a grey-green colour. Their leaf blades were wide with a strong waxiness, weak blistering and dentated or serrated leaf blade edges (Fig. 1d). All of the *B. rapa* lines had smaller plant diameters and were shorter in comparison with *B. napus* lines. They were also characterised by a semi-compact plant type with low (HG) or very low (KW and MR) stack heights. Leaves were typical for Chinese cabbage: small in size, whole with petioles, having green coloured blades and very weak, or absent, waxiness. The shape of the leaf blades for *B. rapa* lines was broad obovate (KW) or elliptical (MR and HG), with medium width, intermediate blistering and crenated leaf blade edges.

The interspecific F_1 hybrids of *B. napus* × *B. rapa* were internally uniform with intermediate morphological traits typical of their parental components (Fig. 1e). All of the F_1 generation plants were large in diameter, of medium height and had extensive leaf growth. Their stack heights, size, colour and waxiness of leaves were less than those of the rapeseed lines. The elliptical shaped leaves, with intermediate blistering, of the interspecific F_1 hybrids were more typical of the Chinese cabbage than the rapeseed parental line. However, the erect position and lyrate type of leaf with dentated edges were more typical for rapeseed than for *B. rapa* (Table 1).

The BC_1 generation ((*B. napus*/*B. rapa*) × *B. rapa*) was more diverse in the majority of the morphological characteristics. All of the BC_1 plants had very small stack heights, semi-compact plant types and horizontal or half-erect leaf positions, typical of Chinese cabbage recurrent parents. Leaves with absent or very light leaf waxiness with elliptical, medium-sized leaf blades, intermediate blistering and crenated leaf edges, typical of *B. rapa* plants, were also found. Plant diameters of the interspecific BC_1 population ranged from small to large, and plant heights were small or medium. The most evident diversity of the BC_1 population occurred in the leaf morphological characteristics, such as size, innervation, waxiness, shape, width, edge and blistering of leaf blades (Fig. 1f–h). Three types of leaves—whole without petiole, semi-lyrate and lyrate—were present. Most of the BC_1 plants had an intermediate level of leaf innervation; however, plants with weak or strong innervation, and leaf colours from light to dark green, were also observed. Parental lines evaluated for morphological characteristics in the greenhouse developed flower stacks typical for rapeseed and Chinese cabbage. The average size of blooming rapeseed plants was the highest of all of the tested genotypes and ranged from 180 to 190 cm (Table 2). Rapeseed lines started to bloom in the third week of March 2012, 2 weeks later than the three Chinese cabbage genotypes. Flowers of the P1162 rapeseed line were male fertile, with normally developed anthers and abundant pollen grains (Fig. 2a).The C1162 rapeseed line had flowers typical of *Ogura*-type *CMS*, having smaller anthers without pollen. The P1162 and C1162 lines developed bulgy-type buds 7.2–7.8 mm long and flowers in sizes of 19 and 17 mm, respectively. The petals of the rapeseed lines were close to one another, with round shapes and a yellow colour. The three Chinese cabbage parental lines were smaller (90–100 cm) compared with the rapeseed genotypes. They developed short (5 mm), cylindrical buds and male-fertile flowers that were

Table 1 Morphological characters of parental *B. napus* and *B. rapa* lines, their interspecific crosses of F_1 and back-cross 1 (BC_1) generations in the vegetative phase

Genotypes	Internal uniformity	Plant diameter	Plant height	Plant type	Position of leaves	Stack height	Leaf size	Leaf type	Leaf colour	Leaf innervation	Leaf wax	Shape of leaf blade	Width of leaf blade	Leaf blade blistering	Edge of leaf blade
Rapeseed (*B. napus*)															
P1162	1	9	7	7	7	5	7	7	4	3	5	2	7	3	9
C1162	1	9	7	7	7	5	7	7	4	3	5	2	7	3	7
Chinese cabbage (*B. rapa*)															
KW	1	5	3	5	5	1	1	3	2	3	1	5	5	5	5
HG	1	7	5	5	5	3	1	3	2	3	1	2	5	5	5
MR	1	5	3	5	5	1	1	3	2	3	1	2	5	5	5
F_1 hybrids (*B. napus* × *B. rapa*)															
P1162 × KW	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
P1162 × HG	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
P1162 × MR	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
C1162 × KW	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
C1162 × HG	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
C1162 × MR	1	7	5	7	7	3	5	7	3	3	5	2	5	5	7
BC_1 generation (*B. napus*/*B. rapa*) × *B. rapa*															
(P1162 × KW) × KW	3	3, 5	3, 5	5, 7	5	1	1, 5	3, 5, 7	1, 2, 3	5	1, 3	1, 2, 6	3, 5	3, 5, 7	5, 7, 9
(P1162 × MR) × MR	3	3, 5, 7	3, 5	5	3, 5	1	1, 5, 7	3, 5, 7	2	5	1	2, 4	3, 5	3, 5, 7	5, 7
(P1162 × HG) × HG	3	3, 5	3, 5	5	3, 5	1	1, 3, 5	3, 5, 7	1, 2, 3	5	1, 3	2	3, 5	3, 5, 7	5, 7
(C1162 × KW) × KW	3	3, 5, 7	3, 5	5	3, 5	1	1, 3, 5, 7	3, 5, 7	2, 3	3, 5	1, 3, 5	1, 2	5, 7	3, 7	5, 7, 9
(C1162 × HG) × HG	3	3, 5	3, 5	5	3, 5	1	1, 5	3, 5, 7	2, 3	3, 5	1, 3	2	3, 5	3, 5, 7	5, 7
(C1162 × MR) × MR	3	3, 5, 7	3	5	3, 5	1	1, 5	3, 5, 7	2, 3	5, 7	1, 3, 5	1, 2, 7	3, 5	3, 5, 7	5, 7, 9

Internal uniformity 1, uniform; 2, partially uniform; 3, lack of the uniformity, *plant diameter* 1, very small (<10 cm); 3, small (11–15 cm); 5, medium (16–25 cm); 7, large (26–30 cm); 9, very large (>30 cm), *plant height* 1, very small (<10 cm); 3, small (11–15 cm); 5, medium (16–20 cm); 7, high (21–25 cm); 9, very high (>25 cm), *plant type* 3, compact; 5, semi compact; 7, extensive, *position of leaves* 3, horizontal; 5, half-erect; 7, erect, *stack height* 1, very small (<10 cm); 3, small (11–15 cm); 5, medium (16–20 cm); 7, high (21–25 cm); 9, very high (>25 cm), *leaf size* 1, small (>10 cm); 5, medium (11–19 cm); 7, large (>20 cm), *leaf type* 1, whole without petiole; 3, whole with petiole; 5, semi-lyrate; 7, lyrate, *leaf colour* 1, light green; 2, green; 3, dark green; 4, grey green, *leaf innervation* 3, weak; 5, intermediate; 7, strong, *leaf wax* 1, absent; 3, weak; 5, medium; 7, strong; 9, very strong, *shape of leaf blade* 1, broad lanceolate; 2, elliptic; 5, broad obovate; 6, round; 7, ovate, *width of leaf blade* 1, very small (<7 cm); 3, small (8–12 cm); 5, medium (13–15 cm); 7, large (16–20 cm); 9, very large (>20 cm), *leaf blade blistering* 0, none; 3, weak; 5, intermediate; 7, strong, *edge of leaf blade* 3, entire; 5, crenate; 7, dentate; 9, serrate

Table 2 Morphological characters of parental *Brassica napus* and *Brassica rapa* lines and their interspecific crosses of F_1 and back-cross 1 (BC_1) generations in the generative phase

Genotypes	Average plant height (cm)	V (%)	Beginning of blooming	Bud shape	Average bud length (mm)	V (%)	Average size of flower (mm)	V (%)	Position of petals	Colour of petals	Shape of petals	Male fertility/ sterility
Rapeseed (*B. napus*)												
P1162	190	2.6	20.03	5	7.8	5.7	19.0	2.8	3	5	3	f
C1162	180	5.6	20.03	5	7.2	6.1	17.0	3.1	3	5	3	s
Chinese cabbage (*B. rapa*)												
KW	100	5.0	5.03	7	5.0	0.0	12.9	2.8	5	7	5	f
HG	90	16.7	5.03	7	5.0	0.0	12.1	2.9	5	7	5	f
MR	95	5.2	5.03	7	5.0	0.0	12.9	2.7	5	7	5	f
F_1 hybrids (*B. napus* × *B. rapa*)												
P1162 × KW	155	6.5	15.03	7	7.9	4.2	20.1	1.7	5	5	5	f
P1162 × HG	150	10.0	15.03	7	8.0	0.0	19.1	1.8	5	5	5	f
P1162 × MR	140	1.4	15.03	7	7.9	4.2	19.1	1.8	5	5	5	f
C1162 × KW	160	2.5	15.03	7	7.3	6.8	18.9	1.8	5	5	5	s
C1162 × HG	170	5.9	15.03	7	7.9	4.2	19.1	1.9	5	5	5	s
C1162 × MR	160	3.1	15.03	7	7.4	7.8	19.1	1.8	5	5	5	s
BC_1 generation (*B. napus*/*B. rapa*) × *B. rapa*												
(P1162 × KW) × KW	135	3.7	5.03	7	5.0	0.0	13.9	2.6	5	5	5	f/s
(P1162 × MR) × MR	125	2.4	5.03	7	5.3	9.4	14.9	2.4	5	5	5	f/s
(P1162 × HG) × HG	125	4.0	5.03	7	5.0	0.0	14.1	2.5	5	5	5	f/s
(C1162 × KW) × KW	140	7.1	5.03	7	5.1	6.5	13.9	2.6	5	5	5	s
(C1162 × HG) × HG	135	3.7	5.03	7	5.2	8.4	14.8	3.1	5	5	5	s
(C1162 × MR) × MR	135	3.0	5.03	7	5.6	9.5	14.3	3.2	5	5	5	s

Bud shape 5, bulgy; 7, cylindrical, *position of petals* 3, close; 5, intermediate; 7, broad, *colour of petals* 3, light yellow; 5, yellow; 7, dark yellow, *shape of petals* 3, round; 5, ovate

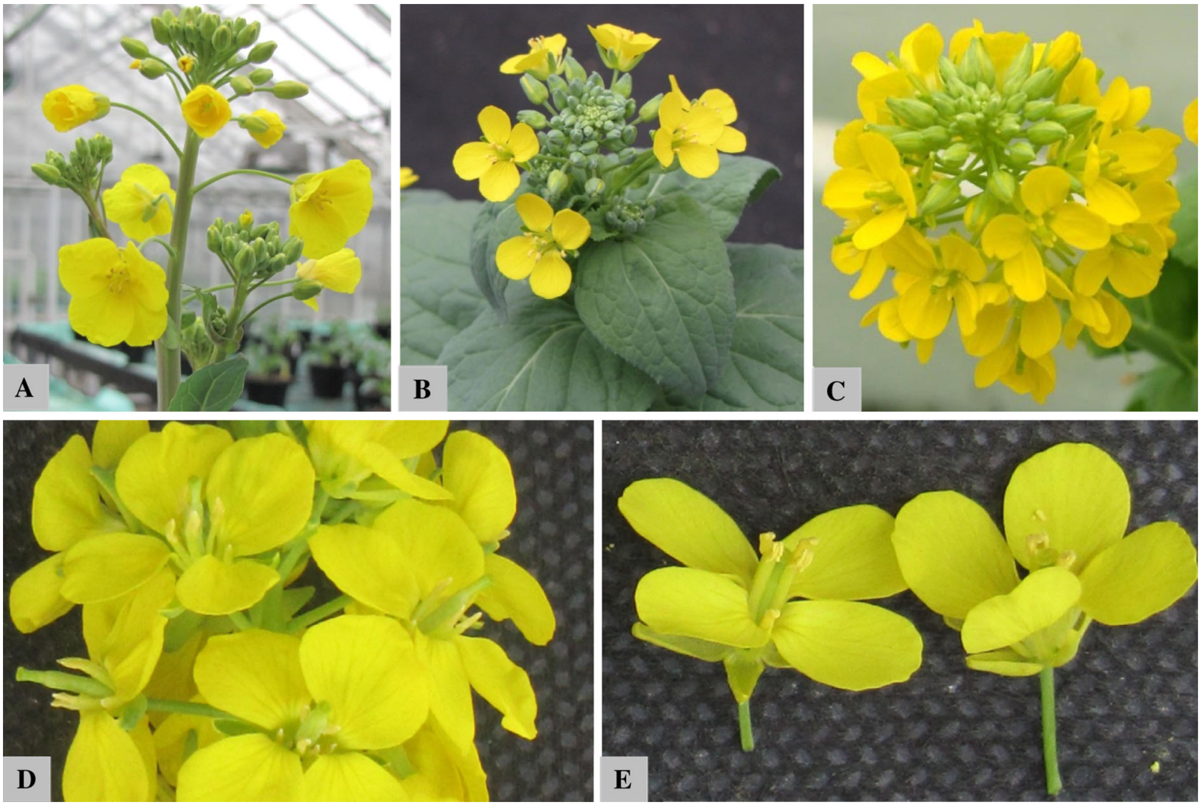

Fig. 2 Flower stacks of the **a** P1162 rapeseed inbred line, **b** Chinese cabbage KW inbred line, **c** *Ogu*-cytoplasmic male-sterile (CMS) interspecific F_1 hybrid *Brassica napus* × *Brassica rapa*, **d** flowers of the Ogu-*CMS* back-cross 3 (BC_3) Chinese cabbage line CKW, and **e** flowers of the male-fertile recurrent parental KW line

small in size (12.1–12.9 mm), with ovate petals and a dark yellow colour (Fig. 2b).

The interspecific F_1 hybrids of *B. napus* × *B. rapa* had intermediate plant heights (140–170 cm) in the generative stage. They started to bloom approximately 5 days earlier than the rapeseed and 10 days later than the Chinese cabbage lines (Table 2). The buds of F_1 hybrids were cylindrical, similar to Chinese cabbage but equal in size to the buds of rapeseed (7.3–8.0 mm). The flowers of F_1 hybrids (18.9–20.1 mm) were larger than those of both of their parental components. The shapes and positions of petals were similar to those of Chinese cabbage, but the yellow colour of the F_1 hybrids was more typical of rapeseed lines. The interspecific F_1 hybrids obtained from the crosses of emasculated fertile P1162 rapeseed × Chinese cabbage were also male fertile. All interspecific F_1 hybrids of male-sterile C1162 *B. napus* × *B. rapa* were male sterile (Fig. 2c).

The average height of plants for the BC_1 generation (125–140 cm) was less than that of the F_1 hybrids. The BC_1 genotypes started to bloom at the same time as their Chinese cabbage recurrent parents, in the first week of March. The sizes and shapes of buds of the BC_1 generation, as well as the characteristics of flowers, were more typical of Chinese cabbage than of the interspecific F_1 hybrids (Table 2). Three BC_1 lines, ((C1162 × KW) × KW), ((C1162 × HG) × HG) and ((C1162 × MR) × MR), were male sterile. Three other BC_1 lines, ((P1162 × KW) × KW), ((P1162 × HG) × HG) and ((P1162 × MR) × MR), were male fertile with lower pollen development capabilities than those of their parental components.

Seed effectiveness of the BC_2 and BC_3 generations of *CMS B. napus* × *B. rapa* propagated in the field

The BC_2 and BC_3 generations of the three Chinese cabbage lines (CKW, CHG and CMR) with *Ogura* cytoplasm were 100 % male sterile. Besides having pistils without pollen, male-sterile lines developed flowers (Fig. 2d) that did not differ from their fertile recurrent components (KW, HG and MR) (Fig. 2e). The use of the red mason bee as a pollinator insect enables the effective cross-pollination between male-sterile and male-fertile superseding lines and sib-pollination of the fertile components. The capability for seed development in male-sterile lines was similar in comparison with their fertile components (Table 3). In 2013, the average mass of seeds/plant for *B. rapa* fertile lines ranged from 13.6 g (MR) to 16.6 g (HG); the mass of seeds for the male-sterile BC_2 generation was slightly lower, from 9.2 g (CKW) to 14.0 g (CHG). In 2014, the average seed productivity for Chinese cabbage fertile lines (38.4 g) and male-sterile lines of the BC_3 generation (56.0 g) was higher than that in 2013. The two male-sterile lines (CKW and CHG) of the BC_3 generation developed a higher mass of seeds/plant (65.2 and 69.2 g) than did their recurrent fertile pollinators (41.6 and 39.1 g, respectively).

Agronomic characteristics of the *CMS* Chinese cabbage lines of the BC_3 generation

Rapeseed P1162 and C1162 inbred lines had the longest periods of vegetation (120 days from planting to the end of the growing season) when plants started their vernalisation (Table 4). Both *B. napus* lines were uniform with medium vigour, dark green coloured leaves and the medium waxiness typical of this species (Fig. 1i). Three Chinese cabbage parental lines had the shortest periods of vegetation, from 71 (HG) to 77 days (KW), good intraline uniformity, green coloured leaves and very weak, or absent, waxiness (Fig. 1k). Two lines (KW and MR) developed high-quality

Table 3 Seed effectiveness of cytoplasmic male-sterile (*CMS*) *Brassica napus* × *B. rapa* of back-cross 2 (BC_2)/BC_3 generations in comparison to *Brassica rapa* recurrent parental lines propagated in the field under isolated growth cages with the use of *Osmia rufa* as a pollinator

Year of propagation	Genotypes	Types of pollination	Average mass of seeds/plant (g)	Standard deviation (SD)	Coefficient of variation (V%)
2013	Chinese cabbage (*B. rapa*) lines				
	KW	Sib	13.6	3.7	27.5
	HG	Sib	16.6	6.4	38.7
	MR	Sib	13.4	3.9	29.4
	Average		14.5		
	B. napus CMS × *B. rapa* BC_2 generation				
	CKW BC_2	Back-cross	9.2	3.5	37.8
	CHG BC_2	Back-cross	14.0	3.8	27.2
	CMR BC_2	Back-cross	10.3	3.8	36.9
	Average		11.2		
2014	Chinese cabbage (*B. rapa*) lines				
	KW	Sib	41.6	9.7	23.3
	HG	Sib	39.1	16.3	41.7
	MR	Sib	34.3	14.0	40.7
	Average		38.4		
	B. napus CMS × *B. rapa* BC_3 generation				
	CKW BC_3	Back-cross	65.2	17.3	26.6
	CHG BC_3	Back-cross	69.2	19.8	28.6
	CMR BC_3	Back-cross	33.6	11.3	33.7
	Average		56.0		

Table 4 Agronomic characters of cytoplasmic male-sterile (*CMS*) Chinese cabbage lines of the back-cross 3 (BC_3) generation in comparison to F_1 *CMS Brassica napus* × *Brassica rapa* and their parental components

Genotypes	Length of vegetation from planting to harvest	Uniformity	Vigour of plants	Mass of head (kg)	Quality of head	Head shape coefficient	Colour of leaves	Waxiness
Rapeseed (*B. napus*)								
P1162	120	1	2	–	–	–	3	3
C1162	120	1	2	–	–	–	3	3
Chinese cabbage (*B. rapa*)								
KW	77	1	2	1.27 c	1	1.88 ab	2	1
HG	71	1	1	0.71 a	2	1.73 a	2	1
MR	74	1	2	1.36 c	1	2.09 abc	2	1
F_1 hybrids (*B. napus* × *B. rapa*)								
C1162 × KW	110	3	3	–	–	–	3	3
C1162 × HG	110	3	3	–	–	–	3	3
C1162 × MR	110	3	3	–	–	–	3	3
BC_3 generation (*B. napus*/*B. rapa*) × *B. rapa*								
CKW	86	2	1	1.70 d	1	2.36 c	2	2
CHG	86	2	1	1.00 b	2	2.14 bc	2	3
CMR	86	2	2	0.85 ab	2	3.00 d	3	1

Means followed by the same letter are not significantly different at $P = 0.05$

Uniformity 1, complete; 2, partial; 3, lack of uniformity, *vigour* 1, high; 2, medium; 3, low, *quality of head* 1, commercial quality; 2, slight defects; 3, non-commercial with major defects, *head shape coefficient* length/width, *colour of leaves* 1, light green; 2, green; 3, dark green; 5, blue green, *waxiness* 1, absent; 2, weak; 3, medium; 4, strong

commercial heads with masses greater than 1 kg, while the HG line had smaller heads (0.71 kg). Interspecific F_1 hybrids (*CMS B. napus* × *B. rapa*) developed plants with intermediate morphological characteristics of rapeseed and Chinese cabbage—without heads and with low vigour, dark green coloured leaves and strong waxiness (Fig. 1j). All of the BC_3 populations were more similar to their recurrent parental components with respect to their agronomic traits (Table 4). The vegetative length for the BC_3 generation was approximately 1 week longer (86 days from planting to harvest) than those of the fertile inbred lines of Chinese cabbage (71–77 days). The *CMS* lines of the BC_3 generation were characterised by the partial uniformity of morphological traits, and high or medium level of plant vigour. The CKW BC_1 line developed heads with significantly higher masses than those of the Chinese cabbage parental components, while the CHG and CMR lines were comparable with the fertile *B. rapa* inbred lines. The BC_3 generation produced longer, more elliptical heads than its recurrent parental components. CMR BC_3 lines developed green (CKW and CHG) or dark green (CMR) leaves. Line CMR had leaves without wax, but the CHG and CKW genotypes had strong or medium waxiness, which is atypical of parental Chinese cabbage lines.

Discussion

A method of developing novel and valuable breeding materials for Chinese cabbage with *Ogura-CMS* cytoplasm from *B. napus* (rapeseed) was presented in this study. We showed that interspecific F_1 hybrids and consecutive BC_1–BC_3 generations had 100 % male fertile flowers only when the male sterile CPN1162 rapeseed line was the source of the *Ogura-CMS* trait.

Interspecific F_1 hybrids (*B. napus* × *B. rapa*) and BC_1 generations ((*B. napus* × *B. rapa*) × *B. rapa*) obtained using the male-fertile maternal P1162 line (with *B. napus* cytoplasm) were male fertile. The C1162 and P1162 rapeseed lines were isogenic and differed only in respect to the cytoplasm type. For that reason the male fertility/sterility character of interspecific hybrids and their consecutive BCs was determined by the maternal component. The *CMS-Ogura* retained all of its characteristics in the consecutive F_1–BC_3 generations, irrespective of different environmental conditions and genetic backgrounds, with normal floral development in contrast with the *pol CMS* described by Fan et al. (1986). For plant breeders such stable expression of the maternally inherited male sterility through F_1–BC_3 generations should be sufficient proof for the presence of *Ogu-INRA* cytoplasm. The other factors, such as additional chromosomes from *B. napus*, probably had no impact on the expression of the CMS trait observed in consecutive BCs.

Our results have confirmed that evaluating genome sizes using FCM is a fast and reliable way to identify interspecific hybrids when parental genotypes differ considerably in genome size. The nuclear DNA contents of *B. rapa* and *B. napus* (approximately 1.1 and 2.5 pg, respectively) are comparable to the values reported by other authors (Arumuganathan and Earle 1991; Johnston et al. 2005), whereas the values of the F_1 hybrids revealed that they had intermediate DNA contents compared with those of the parental species. In addition, the values are similar to the theoretically expected (i.e., approximately 1.8 pg). Previously, FCM was used to detect interspecific hybrids obtained from reciprocal crosses between *B. napus* and *B. rapa*, as well as *B. napus* and *B. juncea* (Sabharwal and Doležel 1993). FCM was also useful for the very early detection of interspecific hybrids within the genera of *Lilium* (van Tuyl and Boon 1996), *Centaurium* (Banjanac et al. 2014) and *Hieracium* (Morgan-Richards et al. 2004). In our study, the FCM of young, fully expanded leaves revealed that all of the *Brassica* genotypes were characterised by high degrees of endoreplication. This phenomenon, also called endoreduplication, endoploidisation or polysomaty, refers to a well-known process in which multiple rounds of DNA replication occur without subsequent chromosome separation and cytokinesis (De Veylder et al. 2011). Such a high degree of endoreplication was also found in root samples of *B. oleracea* var. *capitata* 3–4-day-old seedlings, but this phenomenon was not observed in leaf samples (Šesek et al. 2005). Since the degree of endoreplication may be dependent on physiological or developmental phases, and may vary in different plant organs (Šesek et al. 2005; Rewers et al. 2009; De Veylder et al. 2011), we suppose that such endoreplication could be related to the physiological phase of *Brassica* plants undergoing vernalisation from which the samples were collected.

The results obtained in this study showed that interspecific F_1 hybrids of *B. rapa* and *B. napus* had intermediate traits of both parents; however, Wan et al. (2013) reported that F_1 hybrids of tsai–tsai (*B. rapa*) × *B. napus* were more similar to the female *B. napus* parent. The high level of diversity and evident segregation of morphological characteristics among the interspecific BC_1 population of *B. napus*/*B. rapa* were also reported for the genotypes obtained by Wan et al. (2013). The relatively good quality of the *CMS* genotypes of the BC_3 generation in comparison with their superseding Chinese cabbage lines reveals that most of the C chromosomes may have been eliminated in the very early BC stages of rapeseed × Chinese cabbage interspecific crosses. However, the lack of internal uniformity among all of the populations of the BC_3 generation showed that back-crossing should be continued for at least two more generations to obtain homozygous inbred lines suitable for the breeding of F_1 hybrids. Wan et al. (2013) also suggested that the recombination during back-crossing between A and C chromosomes may affect the higher-than-expected level of atypical traits among *CMS B. rapa* lines. This hypothesis was supported by the analysis of non-parental amplified fragments length polymorphism fragments, which were produced at much higher numbers than expected for the advanced BC populations of *B. rapa* and *CMS B. napus*.

The good seed productivity of BC_1–BC_3 generations of *CMS* Chinese cabbage lines obtained in this study showed that sexual hybridisation with *B. napus* can be effectively used for breeding (Kaneko and Bang 2014). The development of high volumes of seeds was possible because of the lack of the physiological barriers between *B. napus* and *B. rapa*, physical proximity and the flowering synchrony of both components (Anonymous 2002; Bing et al. 1996; Elling et al. 2010). The utilization of three highly self-compatible Chinese cabbage inbred lines (KW, HG and MR) for the interspecific hybridisation with rapeseed allowed for the efficient cross-pollination at the open flower stage in consecutive BCs. The use of the red mason bee to pollinate the BC_2 and BC_3 generations of *CMS B. rapa* lines in the field proved to be very effective and less time-consuming than pollinating by hand in the greenhouse, which is in agreement with the observation made by Steffan-Dewenter (2003). Greater masses of seeds for the *CMS* BC_3 generation in comparison with the self-pollinated inbred lines of fertile Chinese cabbage were probably caused by their higher vigour and the development of stronger blooming plants with abundant flowers, which is typical of heterosis. Relatively lower seed setting rates in 2013 compared with 2014 were probably affected by the low temperatures during the blooming season in April and May, as well as the extremely high volume of rainfall during seed development in June 2013. Weather conditions during hand-pollination, especially temperature and humidity, may be the reason for the high V% for seed development in the greenhouse. The lower seed effectiveness for the interspecific F_1 hybrids of fertile P1162 rapeseed crossed with *B. rapa* in comparison with interspecific F_1 hybrids of the *CMS* C1162 rapeseed line crossed with *B. rapa* was probably affected by the different pollination techniques (pollination at the green bud stage after emasculation and pollination at the open flower stage, respectively).

The method presented in this study can play an important role in the development of new and valuable sources of genetic diversity with stable pollen sterility and without any evident changes in vegetative growth or female fertility for Chinese cabbage. Our results showed, for the first time in the available literature, that the *CMS-Ogura* system could also be very effective, inexpensive and reliable for the breeding of Chinese cabbage male-sterile F_1 hybrids not only in Poland, but also in other regions where Chinese cabbage plays an important role as a vegetable crop.

Conclusions

Our paper presents the development of a novel and valuable Chinese cabbage breeding material with *Ogura* cytoplasm through the use of interspecific hybridization. The successful introgression of *Ogura-CMS* from *B. napus* (rapeseed) into *B. rapa* (Chinese cabbage), effectiveness of seed development and characteristics of interspecific F_1 hybrids and BC_1–BC_3 populations were described in this study for the first time. The interspecific F_1 hybrids had intermediate traits of both parents; however, the morphological characteristics segregated in the BC_1 generation, and, in general, the majority of plants were more similar to *B. rapa*. A high seed effectiveness for the interspecific F_1 hybrids and consecutive BCs was obtained both in the greenhouse and in the field. Plants of the *B. rapa* BC_3 generation had a high level of vigour and the good morphological characteristics typical of Chinese cabbage recurrent lines, but they need further BCs to improve the uniformity and stability. As a result of the study, a valuable collection of Chinese cabbage genotypes with the *CMS* trait, which could be very useful for producing domestic F_1 hybrids as well as for studies on their chromosomal and molecular characteristics, was obtained for the first time in Poland. The material presented in this paper will be supplied for research purposes upon request.

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