Development and Characteristics of Interspecific Hybrids between *Brassica oleracea* L. and *B. napus* L.

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Abstract: Interspecific hybridization between *B. oleracea* inbred lines of head cabbage, Brussels sprouts, kale and *B. taurica* and inbred lines of rapeseed (*B. napus* L.) were performed aiming at the development of the new sources of genetic variability of vegetable Brassicas. Using conventional crosses and the embryo-rescue techniques the following interspecific hybrids were developed: 11 genotypes of *F* 1 generation, 18 genotypes of *F* 2 and *F* 1 × *F* 2 generations (produced after self- and cross-pollination of interspecific *F* 1 hybrids), 10 plants of the BC 1 generation (resulted from crossing head cabbage cytoplasmic male-sterile lines with interspecific hybrids of the *F* 2 and *F* 1 generations) and 8 plants of BC 1 × ( *F* 1 × *F* 2). No viable seeds of the BC 2 generation (*B. oleracea*) were obtained due to the strong incompatibility and high mortality of embryos. The morphological characteristics during the vegetative and generative stages, pollen characteristics, seed development and propagation, nuclear DNA contents and genome compositions of interspecific hybrids were analyzed. All the interspecific *F* 1 hybrids were male-fertile with a majority of undeveloped and malformed pollen grains. They showed intermediate values for morphological traits and nuclear DNA contents and had nearly triploid chromosomal numbers (27 to 29) compared with parental lines. The *F* 2 generation had a doubled nuclear DNA content, with 52 and 56 chromosomes, indicating their allohexaploid nature. *F* 2 hybrids were characterized by a high heterosis of morphological characteristics, viable pollen and good seed development. *F* 1 × *F* 2 hybrids were male-fertile with a diversified DNA content and intermediate pollen viability. BC 1 plants were male-sterile with an intermediate nuclear DNA content between the *F* 2 and head cabbage, having 28 to 38 chromosomes. Plants of the BC 1 × ( *F* 1 × *F* 2) generation were in majority male-fertile with 38–46 chromosomes, high seed set, high heterosis and intermediate values for morphological traits. The obtained interspecific hybrids are valuable as new germplasm for improving *Brassica*-breeding programs.

Keywords: *B. oleracea* × *B. napus*; embryo-rescue; flow cytometry; fluorescence in situ hybridization; interspecific crosses; morphological traits

1. Introduction

The genus *Brassica* includes a large number of widely distributed and economically important species that were domesticated to produce edible oil, vegetables, spices, forage crops and ornamental plants [1,2]. Three of these species are diploid (*B. rapa*, AA 2n = 20; *B. nigra*, BB 2n = 16; *B. oleracea*,
CC 2n = 18), while three others are allotetraploids (B. juncea, AABB 2n = 36; B. napus, AACC 2n = 38; B. carinata, BBCC 2n = 34) [3]. B. oleracea and B. rapa are two important vegetable crops, both composed of dozens of subpecies encompassing hundreds of varieties and cultivars [4]. Closely related Brassica species from the triangle of U [5] can hybridize with each other more or less efficiently owing to their genomic similarities and common ancestors. The phylogeny of the tribe Brassicaceae (Brassicaceae) is still under reconstruction and remain unresolved hampering comparative research. Hybridization across the species, gene or even distant lineages and polyplody resulted in the lack of phylogenetically informative sequence variation [6,7]. Collinearity between B. rapa and B. oleracea genomes has been confirmed based on genomic syntenic analyses [4,8–11]. Spontaneous and induced interspecific hybridization between Brassica species can allow the transfer of desired traits, including increased biomass accumulation, and can broaden the genetic diversity and variation. Thus, hybridization can be used for the development of varieties and cultivars with desired morphological characteristics, such as resistance to biotic and abiotic stresses or the male-sterility trait [9,11–13]. Many studies were reported on resynthesis of B. napus with the use of B. oleracea and B. rapa parents to expand the available gene pool [14]. On the other hand, only a few studies reported improvement of B. oleracea through interspecific hybridization [15–17].

Brassica polyploids can be obtained spontaneously or induced by the union of unreduced gametes and through the somatic doubling of F1 interspecific hybrids [18]. Polyploidy induced during the process of resynthesis can overcome crossing barriers due to endosperm failure in interploidy crosses [19]. Interspecific hybridization and genome duplication allow the accumulation of diverse alleles from diploid donors, and they can assist in the development of de novo variation and the creation of synthetic species [14]. The progeny often revealed excellent variations in morphological traits, which resulted in phenotypical divergence and increased breeding values for the new germplasm [20]. Hybridization between allotetraploid B. napus and diploid B. oleracea is difficult, producing progeny that are highly sterile. The sterility of crosses of B. oleracea and B. napus lines could be caused by interspecific incompatibility and differences in genome sizes between parental components [9,11]. Most interspecific crosses do not produce mature seeds owing to the failure of endosperm development. Fertilization may take place but abortions occur during early development [21,22]. The instability and infertility in subsequent generations of resynthesized Brassica allopolyploids described by Mason et al. [23] probably resulted from a high rate of non-homologous chromosomal interactions between the closely related A and C genomes.

Sexual incompatibility barriers that prevent cross-pollination between distant Brassicas may be overcome by pollination at the very young green flower-bud stage and the use of subsequent embryo-rescue techniques [24,25]. Embryo-rescue techniques have been successfully used for the development of interspecific and intergeneric hybrids of Brassica [11,15,26,27].

Brassica species differ in their numbers of chromosomes [3,5], which leads to variation in the nuclear DNA content (genome size) as confirmed by Sabharwal and Doležel [28] using flow cytometry (FCM). The numbers of chromosomes and nuclear DNA contents (2C DNA) in B. napus are 2n = 38 and 2C DNA approximately 2.3 pg, respectively, while in B. rapa, they are 2n = 20 and 2C DNA approximately 1.1 pg, respectively [5,29]. Sabharwal and Doležel [28] showed that the F1 hybrids between various Brassica species that differed in genome size have intermediate nuclear DNA contents. That was confirmed in our earlier studies [12]. The evaluation of the genome size by FCM is a useful method for the fast identification of Brassica interspecific hybrids.

Brassica chromosomes, apart from being numerous and small, are characterized by limited morphological diversity [30]. Since the identification of chromosomes during karyotyping is the starting point in cytogenetic studies, it is necessary to identify appropriate markers that distinguish individual chromosomes and homologous chromosomal pairs. For Brassica chromosomes differentiation, several staining methods including C banding, silver staining, CMA3/DAPI fluorescence staining, fluorescence in situ hybridization (FISH) with chromosome-specific DNA probe as well as BAC-FISH were used [31]. The use of the rDNA sequences has allowed the identification of significant numbers of chromosomes in
complements of diploid [30,32,33] as well as allopolyploid Brassica species [30,34,35]. Combining FISH with respective DNA sequences and BAC-FISH enabled to identify each chromosome of both A and C genomes in B. napus (2n = 38; AACC) [32]. Similarly, Xu et al. [36] characterized all of the chromosomes of allopolyploid B. juncea using multicolor FISH with six repeat sequences. Using FISH with rDNA probes has become increasingly important in Brassica breeding because it provides information on chromosomal rearrangements and the origins of particular chromosomes in hybrids.

In this study, we aimed to develop new sources of genetic variability for the breeding of vegetable brassicas using the interspecific hybridization of B. oleracea inbred lines with rapeseed (B. napus). The specific objectives were: (i) morphological characteristics of the offspring of F1, F2, F1 × F2, BC1 (B. oleracea) and BC1 × (F1 × F2) generations in the vegetative and generative stages; (ii) the assessment of the interspecific hybrids in respect to nuclear DNA contents and genome compositions using the rDNA sequences.

2. Materials and Methods

2.1. Interspecific Hybridization B. oleracea × B. napus

Two Brussels sprouts lines (B. oleracea L. var. gemmifera) (P18 and J13), two male-sterile head cabbage lines (B. oleracea L. var. capitata) with Ogus-INRA cytoplasm (CKA251 and CIW1018) and their fertile complementary lines (KA251 and IW1018, respectively) were developed at the Research Institute of Horticulture, Department of Genetics and Breeding, Skierniewice, Poland. Two kale lines (B. oleracea L. var. acephala) (ZGH02 and ZGH08) and the B. taurica T02009 line were obtained from the germplasm collection of the Research Institute of Horticulture. The rapeseed lines (B. napus) a male-sterile C1162 having Ogus-INRA cytoplasm and an isogenic P1162 fertile line were obtained from the Plant Breeding and Acclimatization National Research Institute, Poznań, Poland. Most of parental B. oleracea and B. taurica genotypes were partially self-compatible, with the exception of P18 Brussels sprout line that was highly self-incompatible.

Seeds of the B. oleracea and B. napus parental lines were sown at the beginning of September 2013 in a greenhouse. Three-week-old seedlings were transplanted into 10-cm plastic pots, fertilized and protected against pests and pathogens and grown at 20–22 °C in the greenhouse at 12 h day length. Vernalization in a growth chamber at 4–7 °C started at the beginning of December when plants reached the 14–16-true-leaf stage and was conducted under 12/12 day/night photoperiod until the end of February. At the beginning of March 2014, five vernalized plants of each parental lines were transplanted into 5 L plastic pots filled with Kronen substrate and placed on the ground in a greenhouse at 16–20 °C. From the second week of March until the end of April, flower stacks were isolated using bags made from plastic wrap and paper to avoid random cross-pollination. Pollen was collected individually from each of the fertile lines and transferred by hand to the stigmas of the pistils of maternal genotypes. Interspecific hybridization between B. oleracea and B. napus lines was performed for 10–30 opened flowers and green buds of maternal lines using the pollen of fertile paternal genotypes. Fertile maternal lines were emasculated before cross-pollination at the green-bud stage. Three replications were performed for each cross at consecutively developed flower stacks. The self-pollination of the male-fertile parental lines and sibling-pollination of the CMS lines with the pollen of their fertile isogenic lines were performed as controls. Matured siliques were harvested individually by hand in the middle of June for each combination, and after siliques were dried, seeds were then extracted and counted.

In 2015 and 2016, self- and cross-pollinations between 11 interspecific F1 hybrids (B. oleracea × B. napus) were performed in the greenhouse. In 2017, 11 interspecific F1 and F2 hybrids of kale, head cabbage, Brussels sprout and rapeseed were crossed as males with 7 head cabbage lines having Ogus-INRA cytoplasm (KLG, KLJ, TKL, CKA251, CIW1018, CPS8 and KLH), with 2 male-fertile head cabbage lines (IW1018, KA251) and with B. taurica (T02009). Head cabbage lines selected for the cross pollinations with interspecific F1/F2 hybrids were developed at the Research Institute of Horticulture.
and have valuable morphological characters. In 2018 ten interspecific hybrids of the BC \textsubscript{1} generation were backcrossed with two head cabbage sterile inbred lines. The hybridization between three interspecific hybrids of the BC \textsubscript{1} generation (\textit{B. oleracea}) and four plants of the F\textsubscript{1}, F\textsubscript{2} and F\textsubscript{1} × F\textsubscript{2} generations was also performed. The numbers of cross-pollinated buds and flowers, crossing directions and choices of parental components depended on the synchronization of the blooming and availability of flower stacks from parental lines. In 2019 five hybrids of the F\textsubscript{1} generation, six hybrids of the F\textsubscript{2} and F\textsubscript{1} × F\textsubscript{2} generations, ten hybrids of the BC \textsubscript{1} generation and eight hybrids of BC \textsubscript{1} × F\textsubscript{1}/F\textsubscript{2} were open pollinized between all lines by bees in the greenhouse to evaluate their seed set.

2.2. Embryo Rescue

The embryo-rescue technique was applied simultaneously with conventional cross-pollination to obtain \textit{B. oleracea} × \textit{B. napus} interspecific F\textsubscript{1} hybrids and the BC \textsubscript{1} generation. At 3–4 weeks after cross-pollination, siliques that developed to the globular or heart-shaped embryo stages were dehisced and transferred at the Plant Breeding and Acclimatization National Research Institute, Poznań. The obtained embryos were placed in Gamborg’s B-5 media containing 1 mg·mL\textsuperscript{-1} of 6-benzyloaminopurine and 0.01 mg·mL\textsuperscript{-1} of the auxin indole-3-acetic acid). The embryos were then transferred to controlled conditions with a 12 h photoperiod and 15 °C day/10 °C night thermocycle. After 4–6 weeks, the obtained plants were cloned in vitro using hydroponics \cite{25,37}. The best developed plants were transferred for further research at the Research Institute of Horticulture, Skiermiewice.

2.3. Analysis of the Morphological Characteristics

From 2015 to 2019 parental genotypes and interspecific hybrids of \textit{B. oleracea} × \textit{B. napus} were propagated vegetatively and maintained in the greenhouse. The morphological characteristics during the vegetative and reproductive stages of 11 F\textsubscript{1} hybrids, 2 F\textsubscript{2} hybrids, 8 F\textsubscript{1} × F\textsubscript{2} hybrids, 10 hybrids of the BC \textsubscript{1} and 8 hybrids of BC \textsubscript{1} × F\textsubscript{1}/F\textsubscript{2} generations were evaluated. The morphological characters of the interspecific hybrids and their parental components were classified using the multigrade International Union for the Protection of New Varieties of Plants descriptors for \textit{Brassica}, which included plant diameter and type, leaf position, stack height, leaf size, type, color, innervation and waxiness, shape, blistering, and leaf blade edge and width during the vegetative phase. At the beginning of March, the average plant height, bloom start time, bud shape and length, flower size, position and color, petal shape and male fertility/sterility of the vernalized hybrids and their parental components were analyzed during the generative phase.

2.4. Nuclear DNA Content

The nuclear DNA contents (genome sizes) were evaluated using FCM. Leaf tissue (0.5 cm\textsuperscript{2}) was chopped along with a piece (1 cm\textsuperscript{2}) of a plant internal standard in a Petri dish containing 0.5 mL Partec nuclei isolation buffer to which propidium iodide (50 mg·mL\textsuperscript{-1}) and RNase (50 mg·mL\textsuperscript{-1}) had been added \cite{38}. As an internal standard, the young leaves of \textit{Zea mays} CE-777 (2C = 5.43 pg DNA) were used \cite{39}. After adding 1.5 mL of the isolation buffer, the samples were passed through a 30-µm filter and incubated for 50–60 min at room temperature. The fluorescence of the nuclear DNA was measured using a CyFlow Ploidy Analyser with CyView software (CyFlow PA, Partec nuclei extraction buffer) with an Nd-YAG green laser at 532 nm. Data were analyzed using CyView software (Partec, Germany). The 2C DNA content of each sample was calculated as the sample peak mean divided by the standard plant peak and multiplied by the amount of DNA in the standard plant. Samples with at least 5000 nuclei were measured from five leaves of each plant, with two runs per nuclei isolation extract.

2.5. Pollen Grain Length and Viability

In total, the pollen grain length, minimal and maximal pollen grain sizes and the percentage of deformed and undeveloped grains from 8 male-fertile parental lines and 15 male-fertile interspecific hybrids were evaluated. A mixed sample of pollen from 2–6 anthers of each genotype was stained with
Alexander’s solution [40]. The measurements were determined for 100 pollen grains using a Nikon Eclipse 80i microscope at 400× magnification and the program NIS-Elements BR 2.30 (Nikon, Japan).

### 2.6. Chromosome Preparation

For FISH, ~2-cm-long root tips were harvested from a rapeseed line (P1162), an inbred line of *B. taurica* (T02009), two kale lines, two head cabbage lines, Brussels sprouts line, seven *B. oleracea* × *B. napus* interspecific F1 hybrids, two F2 hybrids, five BC1 hybrids toward head cabbage and five hybrids of BC1 × F1/F2. Roots were pre-treated with 2 mM 8-hydroxyquinoline for 4 h, fixed in 3:1 ethanol:glacial acetic acid solution for at least 12 h and then stored at −20 °C until use. The roots were subjected to enzymatic digestion in a mixture comprising 20% pectinase (Sigma), 1% cellulase (Calbiochem) and 1% cellulose ‘Onozuka R-10’ (Serva) at 37 °C for ~1 h. Meristems were squashed in a drop of 45% acetic acid. After freezing in liquid nitrogen, cover slips were removed using a razor blade, and the preparations were dehydrated in absolute ethanol, air dried and stored at −20 °C.

### 2.7. Fluorescence In Situ Hybridization

The following ribosomal DNA sequences were used as probes: 5S rDNA (the wheat clone pTa794) [41] and a 2.3-kb Clai subclone of the 25S rDNA coding region of *Arabidopsis thaliana* [42]. The latter probe was used to determine the chromosomal localization of 18S–5.8S–25S rRNA genes (35S rDNA). The 5S and 35S rDNAs were labeled with tetramethyl-rhodamine-5-dUTP and digoxigenin-11-dUTP (Roche), respectively, using nick translation according to the manufacturer’s instructions (Roche).

Methods for in situ hybridization essentially followed Mizuochi et al. [43]. The hybridization mixture consisted of 50% deionized formamide, 20% dextran sulphate, 2x saline sodium citrate (SSC) and salmon sperm blocking DNA in 50–100× excess of labelled probes. The ribosomal DNA probes were mixed to a final concentration of ~2.5 ng/mL and pre-denatured at 80 °C for 10 min. The slides with chromosomal materials were denatured in the hybridization mixture at 70 °C for 4.5 min and allowed to hybridize for 12–18 h in a humid chamber at 37 °C. The post-hybridization washes were carried out for 15 min in 2× SSC at room temperature, followed by washes in 0.1× SSC at 42 °C for 30 min (73% stringency) and 2× SSC for 15 min at room temperature. The immunodetection of digoxigenated probes was performed using fluorescein isothiocyanate-conjugated anti-digoxigenin antibodies (Roche). The preparations were mounted and counterstained in Vectashield (Vector Laboratories, Burlingame, U.S.A.) containing 2.5 g/mL 4’,6-diamidino-2-phenylindole (DAPI) (Serva). Microscopic slides were analyzed using an epifluorescent microscope Optiphot-2 (Nikon, Japan) with an image analysis system NIS-Elements Basic Research ver. 4.00 (Nikon Instruments Inc., Tokyo, Japan) and an appropriate filter block at the following excitation wavelengths: 350 nm for 4’,6-diamidino-2-phenylindole, 495 nm for fluorescein isothiocyanate and 550 nm for rhodamine. Microscopic images were captured using a digital CCD camera PS-Fi1 (Nikon, Tokyo, Japan).

### 2.8. Statistical Analyses

The nuclear DNA contents (genome sizes) and pollen lengths were subjected to a one-way analysis of variance (STATISTICA package StatSoft v. 10). The means were compared using Tukey’s test at *p* = 0.05. Standard deviations (*n* = 5) were calculated.

### 3. Results

#### 3.1. Interspecific Hybridization

Parental lines of rapeseed, Brussels sprouts, kale, *B. taurica* and head cabbage, showed generally good generative propagative capabilities after self/sibling pollination at the open-flower and at the green-bud stages (Table S1).
In 2014, 913 green buds and 634 flowers were cross-pollinated to form nine combinations between *B. oleracea* and *B. napus* inbred lines. Siliques after cross-pollination had slower growth rates in comparison to self- or sibling-pollinated genotypes of the same species. The majority of cross-pollinated siliques were retarded at early developmental stages and dried and dropped at 2–4 weeks after pollination (Figure S1a,b). A single seed was obtained from the cross of male-sterile C1162 rapeseed with the pollen of the male-fertile head cabbage KA251 (X20) (Table S1). In total, 23 embryos were successfully extracted from the 3–4 week-old siliques of eight interspecific crosses. Twelve embryos with chlorosis did not develop into plants, dying during the first steps of in vitro culture. Ten interspecific hybrids of *B. oleracea × B. napus* obtained by embryo rescue were regenerated using hydroponics and adapted to cultivation in the substrate in the greenhouse (Figure S1c–g; Table S1).

In 2015 and 2016, 2210 green buds and flowers of 11 interspecific F$_1$ hybrids (*B. oleracea × B. napus*) were self-pollinated. Self-pollinated siliques of F$_1$ hybrids were usually empty and dried 2–3 weeks after flower development. An exception was the S14 F$_1$ hybrid (Brussels sprout × rapeseed) in which two viable seeds of the F$_2$ generation were found. The single interspecific S14 F$_1$ × SI4 F$_1$ hybrid was self-pollinated at 80 green buds and flowers and developed 45 true seeds of the S14 F$_3$ generation. In total, 23 crosses between interspecific hybrids of the F$_1$ and F$_2$ generations were performed using 807 green buds and flowers. Eight crosses resulted in the formation of 18 true seeds. (Table S2).

Nine interspecific F$_1$ hybrids and two hybrids of the F$_2$ generation were back-crossed with *B. oleracea* fertile and sterile inbred lines at 1551 buds and flowers. Despite repeated cross-polinations from the beginning of April to the end of June in 2015 and 2016, no fertile seeds were obtained by conventional methods (Table S2). For the 14 backcrosses, 38 embryos were obtained, and 18 back-crosses did not result in any viable embryos. Ten embryos of the BC$_1$ generation that developed without a chlorophyll deficiency were regenerated using hydroponics and adapted to greenhouse conditions (Table S2). Three interspecific hybrids of the BC$_1$ generation were successfully hybridized with interspecific plants of F$_1$, F$_2$ and F$_1$ × F$_2$ generations resulting in fourteen true seeds for four different combinations. Ten interspecific hybrids of the BC$_1$ generation were back-crossed with two *B. oleracea* sterile inbred lines at 626 green buds and flowers. In total, 16 embryos were successfully extracted from siliques, however all of them died because of chlorosis during in vitro culture. No viable seeds of the BC$_2$ generation were obtained due to the strong incompatibility between parental genotypes (Table S2).

The ability for the seed development after open pollination of *Brassica* interspecific hybrids was much higher for the F$_2$ and F$_1$ × F$_2$ and BC$_1$ × F$_1$/F$_2$ generations (aver. seed number 232 and 134 respectively) than for the F$_1$ and BC$_1$ generations (aver. seed number 9.8 and 3.6 respectively). The average seed number per siliqua for F$_2$, and F$_1$ × F$_2$ generations (3.22) was fifteen fold higher than that of the F$_1$ generation (0.21). The average number of seeds per siliqua (1.92) for interspecific hybrids of the BC$_1$ × F$_1$/F$_2$ generation was thirty fold higher than that of the BC$_1$ (0.06) (Table S3).

### 3.2. Morphological Characteristics

*B. oleracea* and *B. napus* parental lines had morphological characteristics that were typical of their species in the vegetative and generative stages (Figure 1a–j; Table S4.). Rapeseed P1162 and C1162 inbred lines had larger plant diameters in comparison with *B. oleracea* genotypes resulting in extensive plant types and high stacks. Leaf blades were large, lyrate-type, with a grey-green color, strong waxiness, weak blistering and dentated or serrated edges (Figure 1a,b). *B. oleracea* plants were diversified in their morphological characteristics during the vegetative stage. The two Brussels sprouts lines (P18 and J13) were compact with horizontal leaf positions. Their leaf blades were whole with petioles, darker than those of head cabbage with a stronger waxiness and round shape (Figure 1c). The two kale lines (ZGH02 and ZGH08) were semi-compact with horizontal, small leaves without petioles. Their leaf blades were grey-green with intermediate blistering and crenated (ZGH08) or serrated (ZGH02) edges (Figure 1d,e). The *B. taurica* line (T02009) had medium-diameter plants with half-erect leaf positions and a semi-compact plant type. The leaf type of *B. taurica* was semi-lyrate and more typical of rapeseed than other *Brassica* species (Figure 1f). The four head cabbage lines were semi-lyrate and strong in waxiness, weak blistering and dentated or serrated edges (Figure 1a,b).
compact with medium diameters and half-erect leaves. Their leaves were whole without petioles but small (IW1018 and CIW1018) or medium (KA251 and CKA251) in size, with entire, broad, obovate leaf blades (Figure 1g–j). Rapeseed P1162 and C1162 inbred lines developed flower stacks during the first and second weeks of March, which was earlier than B. oleracea genotypes (Table S5). Flowers of the male fertile parental lines developed anthers and abundant pollen. The C1162 rapeseed and CKA251 and CIW1018 head cabbage lines with flowers typical of Ogura-type CMS did not develop pollen grains. Two isogenic head cabbage lines (KA251 and CKA251) had bulgy-type buds, while the other parental lines were cylindrical. All the parental lines developed medium long (5–7 mm) buds with diversified flower sizes. The B. taurica (T02009) line had the largest flowers (25–30 mm) in comparison with the other parental forms. Flowers of other male-fertile lines (P1162, KA251, IW1018, ZHG08 and ZGH02) were larger (23–25 mm) than those of the sterile CKA251 and CIW1018 lines as well as the Brussels sprouts (P18 and J13) lines (19–22 mm). Petals of rapeseed lines were close to one another, with round shapes and a yellow color, while petals of the B. oleracea genotypes were intermediate-broad and ovate.

Eleven interspecific F$_1$ hybrids of B. oleracea × B. napus were characterized by good vigor and intermediate morphological characteristics typical of their parental components (Figure S2a–f). The majority of F$_1$ hybrids were medium in diameter with high stacks and half-erect leaf positions, with the exception of the S20 and S15 genotypes (kale × rapeseed), which were smaller in size than the other interspecific F$_1$ hybrids during the vegetative phase. The lyrate or semi-lyrate leaves were half-erect and of medium or small size. Interspecific F$_1$ hybrids were diversified in leaf color, waxiness, innervation and leaf-blade shape.

Four hybrids were grey-green with strong wax on the leaf surfaces. Seven F$_1$ hybrids obtained with kale and B. taurica parental lines had stronger leaf blistering and crenated or dentated leaf blade edges in comparison with F$_1$ hybrids obtained with head cabbage and Brussels sprouts parental lines. The S14 F$_1$ × S14 F$_1$ and S15 F$_1$ × S2 F$_1$ hybrids differed from interspecific hybrids of the F$_1$ generation by having larger plant diameters and leaf sizes, wider leaf blades, broad obovate shapes and intermediate leaf-blade blistering (Table S4). Ten interspecific hybrids of the BC$_1$ generation [B. oleracea × (B. oleracea × B. napus)] were characterized by medium diameters and semi-compact plant types during the vegetative stage (Figure 2d–f). Their leaves were half-erect, semi-lyrated, medium or small, with weak blistering and crenated leaf blades. Eight interspecific hybrids of the BC$_1$ × F$_1$/F$_2$ generation had intermediate morphological characteristics typical for BC$_1$ and F$_1$/F$_2$ parental components but were more diversified in respect to the plant diameter, position, innervation and waxiness of leaves and edge of leaf blade (Figure 2, Table S4).

Most of the interspecific F$_1$ hybrids started to bloom during mid-March, with the exception of the S1, S2 and S14 lines, which developed flowers at the beginning of March, and the S15 line, which started to bloom in April (Table S5). Interspecific F$_1$ hybrids developed medium or tall plants during the generative stage, similar to both B. napus and B. oleracea. Their buds were intermediate in shape and longer than those of their parental lines, with the exception of S9 and S7 hybrids, which had shorter buds. Flowers were medium with intermediate positions and shaped petals. Ten interspecific hybrids of the F$_1$ generation developed male-fertile flowers. The X20 F$_1$ interspecific hybrid obtained by the cross of the CMS Ogu INRA C1162 rapeseed line with male fertile head cabbage (KA251) was male-sterile (Table S5).

In contrast to the interspecific F$_1$ hybrids, genotypes of the F$_2$ generation started to bloom 2 weeks later and developed larger buds and flowers with an abundance of pollen. Both siliques and seeds from F$_2$ hybrids were larger than those of the F$_1$ generation and B. oleracea and B. napus inbred lines. Eight genotypes obtained by the cross-pollination between F$_1$ and F$_2$ interspecific hybrids had smaller diameters, with lower stacks, erect leaf positions and dentated or serrated leaf-blade edges (Table S5). Buds of the F$_1$ × F$_2$ generation were larger than those of the F$_1$ hybrids and the parental B. napus and B. oleracea lines. All the flowers of the F$_1$, F$_2$ and F$_1$ × F$_2$ generations were male fertile. BC$_1$ genotypes started to bloom at the beginning of April, which was later than the majority of other hybrids and parental lines. All the genotypes of the BC$_1$ generation were male-sterile with typical flowers for
Ogu-INRA cabbage inbred lines, which were used as their maternal components for the back-cross pollination. In contrast to BC$_1$ generation, the majority of BC$_1$ $\times$ F$_1$/F$_2$ plants developed male fertile flowers (Figure 2, Table S5).

**Figure 1.** Parental lines of *B. oleracea* $\times$ *B. napus* interspecific hybrids. (a) rapeseed P1162 line, (b) rapeseed C1162 line, (c) Brussels sprouts P18 line, (d) kale ZGH02 line, (e) kale ZGH 08 line, (f) *B. taurica* T02009 line, (g) head cabbage IW1018 line, (h) head cabbage CIW1018 line, (i) head cabbage KA251 line, (j) head cabbage CKA251 line.
3.3. Nuclear DNA Contents

Parental lines of *B. napus* and *B. oleracea* had DNA volumes typical for their species. The DNA volume for the rapeseed PN1162 and CPN116 lines (2.51 to 2.47 pg) was significantly higher than for the *B. oleracea* genotypes, which ranged from 1.40 to 1.57 pg (Table S5). The 11 interspecific *F*₁ hybrids obtained by embryo rescue did not differ significantly from one another in nuclear DNA contents, which ranged from 1.97 (S15 *F*₁) to 2.08 pg (S9 *F*₁). These values were intermediate between *B. napus* and *B. oleracea* and *B. taurica*. A single *F*₁ hybrid (X20 *F*₁) obtained from a true seed by the cross pollination of CMS *B. napus* (CPN1162) with a head cabbage fertile line (KA251) had a significantly higher volume of nuclear DNA (2.73 pg) than the other interspecific *F*₁ hybrids. S14 *F*₁ × S14 *F*₁ and S15 *F*₁ × S2 *F*₁ hybrids were characterized by doubled nuclear DNA contents (3.96 and 3.94 pg, respectively) in comparison with the interspecific hybrids of the *F*₁ generation. The nuclear DNA
contents for the $F_1 \times F_2$ interspecific hybrids were diversified and ranged from 3.06 pg to 4.75 pg (Table S5). Two $F_1 \times F_2$ hybrids were characterized as having nuclear DNA contents similar to those of the $F_2$ generation: 3.83 pg and 4.01 pg.

Eight genotypes of the $BC_1$ generation obtained after the backcrossing of fertile $F_2$ interspecific hybrids with CMS head cabbage lines had intermediate nuclear DNA contents between the parental lines that ranged from 2.57 pg to 2.87 pg. The $KLJ \times (S15F_1 \times S2F_1)$ and $KLG \times S7 F_1$ hybrids had a significantly smaller DNA content (1.89 and 2.0 pg respectively) in comparison with the other back crossed hybrids. Six plants of the $BC_1 \times F_1/F_2$ generation had significantly higher volume of nuclear DNA (from 3.16 to 3.54 pg) than $BC_1$ generation. Two genotypes of $(KLG \times S7 F_1) \times S18 F_1$ had smaller nuclear DNA contents (2.32 and 2.58 pg respectively) (Table S5).

### 3.4. Pollen Characteristics

The average pollen lengths for the seven fertile lines of *B. oleracea* ranged from 21.33 µm to 27.01 µm, and they were significantly shorter in comparison with the interspecific hybrids of the $F_1$, $F_2$ and $F_1 \times F_2$ generations (Table 1; Figure 3a–d).

| Genotype                  | Avg. Length of Pollen (µm) | Std. Dev | Malformed and Undeveloped Grains (%) |
|---------------------------|-----------------------------|----------|-------------------------------------|
| **Parental lines**        |                             |          |                                     |
| P1162 ♀rapeseed           | 26.63 f                     | 1.90     | 0.00                                |
| P18 ♀Brussels sprout      | 25.49 f                     | 0.83     | 10.99                               |
| J13 ♀Brussels sprout      | 21.33 g                     | 2.40     | 5.26                                |
| ZHG02 ♀kale               | 24.79 g-f                   | 1.12     | 4.34                                |
| ZGH08 ♀kale               | 25.51 g-f                   | 2.01     | 2.73                                |
| T02009 ♀*B. taurica*      | 27.01 f                     | 2.87     | 7.24                                |
| KA251 ♀head cabbage       | 24.94 g-f                   | 1.23     | 3.00                                |
| IW1018 ♀head cabbage      | 24.90 g-f                   | 2.89     | 11.50                               |
| **Interspecific hybrids of the $F_1$ generation** |                             |          |                                     |
| S1 $F_1$ kale × rapeseed  | 38.06 a                     | 3.15     | 79.32                               |
| S2 $F_1$ kale × rapeseed  | 36.13 a–d                   | 4.53     | 84.72                               |
| S15 $F_1$ kale × rapeseed | 36.43 a–d                   | 4.44     | 88.39                               |
| S20 $F_1$ kale × rapeseed | 36.60 a–c                   | 4.43     | 86.00                               |
| X22 $F_1$ kale × rapeseed | 37.71 a                     | 4.44     | 90.10                               |
| S9 $F_1$ *B. taurica* × rapeseed | 35.73 a–d | 1.70 | 81.71                               |
| S7 $F_1$ head cabbage × rapeseed | 37.50 a | 3.93 | 93.12                               |
| S14 $F_1$ Brussels sprout × rapeseed | 39.64 a | 4.01 | 77.49                               |
| S18 $F_1$ Brussels sprout × rapeseed | 38.10 a | 4.78 | 90.40                               |
| **Interspecific hybrids of the $F_2$ generation** |                             |          |                                     |
| S14 $F_1$ × S14 $F_1$     | 34.72 a–c                   | 3.13     | 7.21                                |
| S15 $F_1$ × S2 $F_1$      | 33.20 b–c                   | 1.35     | 11.36                               |
| **Interspecific hybrids of the $F_1 \times F_2$ generation** |                             |          |                                     |
| S1F1 × (S15 $F_1$ × S2 $F_1$) | 34.64 a–e  | 3.40     | 35.79                               |
| S7 $F_1$ × (S14 $F_1$ × S14 $F_1$) | 37.60 a  | 3.15     | 45.30                               |
| S7 $F_1$ × (S15 $F_1$ ×S2 $F_1$) | 37.74 b–e  | 3.20     | 48.59                               |
| S20 $F_1$ × (S14 $F_1$ × S14 $F_1$) | 32.93 d–e  | 3.56     | 42.50                               |

* Average pollen lengths in the column having the same lowercase letters are not significantly different according to Tukey’s test ($p = 0.05$).

The pollen length of the fertile rapeseed P1162 line (26.63 µm) did not differ significantly from the majority of *B. oleracea* genotypes, with the exception of the J13 line (Figure 3a,b) (Table 1). The average pollen-grain lengths for interspecific $F_1$ hybrids ranged from 31.32 µm ($S7$) to 38.10 µm ($S18$) and were similar for those of the $F_2$ and $F_1 \times F_2$ generations. The majority of pollen grains for the interspecific
F1 hybrids were undeveloped, malformed and unstained (Figure 3c and Figure S3a–i). In contrast to the F1 hybrids, two genotypes of the F2 generation had 88.60% and 98.80% respectively, of the pollen stained (Figure S3j,k). Four fertile F1 × F2 hybrids had intermediate numbers of undeveloped, malformed and unstained pollen grains, ranging from 35.79% to 48.59% of the total pollen (Figure 3d and Figure S3l–o).

3.5. Cytology and Physical Mapping of 5S and 35S rDNA Loci in Parental Genotypes and Interspecific Hybrids of B. oleracea × B. napus Using FISH

The FISH technique with 5S and 35S rDNA probes was used to assess chromosome numbers and the genome compositions of parental genotypes and selected interspecific hybrids of B. oleracea × B. napus obtained by embryo rescue and classical breeding methods (Table 2). The varieties of B. oleracea (B. taurica (T02009), kale lines (ZGH02 and ZGH08), head cabbage lines (IW1018 and KS251) and Brussels sprouts line (P18) had 2n = 18 chromosomes and in all the varieties, except for B. taurica, two 5S rDNA loci and four 35S rDNA loci were found (Figure 4a). In the latter genome an additional two loci of 35S rDNA were observed. The paternal genotype B. napus (P1162) had 2n = 38 chromosomes bearing 8 loci of 5S and 12 loci of 35S rDNA (Figure 4b).

In hybrids high variability in respect to chromosome number and the number of ribosomal DNA loci was observed (Figure 4c–f). Chromosome number differed from 27 in the F1 generation to 56 in F2 hybrids (Table 2). In the F1 hybrids, from 4 to 8 signals of 5S rDNA and from 6 to 9 loci of 35S rDNA were observed. In two genotypes representing the F2 generation, 9 and 13 loci of 5S rDNA and 13 loci of 35S rDNA were determined. Similarly, the number of rDNA loci differed among hybrids of the BC1 and BC1 × (F1 × F2) generation. In the former from 3 to 5 loci of 5S rDNA and from 6 to 9 loci of 35S rDNA were observed, while in the later from 4 to 8 loci of 5S rDNA and from 11 to 14 loci of 35S rDNA were determined.
Table 2. Cytogenetic analysis of parental genotypes and their progeny as assessed by rDNA fluorescence in situ hybridization (FISH).

| Genotype       | Chromosome Number | 5S rDNA | 35S rDNA | Marker Chromosomes | Genome C | Genome A | A1 | A3 | A10 | A5/A6/A9 |
|----------------|-------------------|---------|----------|-------------------|----------|----------|----|----|-----|---------|
|                |                   |         |          | C4 8 C8 C7        |          |          |    |    |     |         |
| **Parental lines** |                  |         |          |                   |          |          |    |    |     |         |
| PN1162 rapeseed  | 38                | 8       | 12       | 2 2 2 2 2 2 2 2 2 | 4        |          |    |    |     |         |
| T0209 B. taurica | 18                | 2       | 6        | 2 2 2             |          |          |    |    |     |         |
| ZHG08 kale      | 18                | 2       | 4        | 2 2 2             |          |          |    |    |     |         |
| ZGH02 kale      | 18                | 2       | 4        | 2 2 2             |          |          |    |    |     |         |
| IW1018 head cabbage | 18               | 2       | 4        | 2 2 2             |          |          |    |    |     |         |
| KA251 head cabbage | 18              | 2       | 4        | 2 2 2             |          |          |    |    |     |         |
| P18 Brussels sprout | 18           | 2       | 4        | 2 2 2             |          |          |    |    |     |         |
|                |                   |         |          |                   |          |          |    |    |     |         |
| **Interspecific hybrids of the F1 generation** |                  |         |          |                   |          |          |    |    |     |         |
| S1 F1 kale × rapeseed | 27          | 4–5     | 8        | 2 2 3 1 1           | 1        |          |    |    |     |         |
| S2 F1 kale × rapeseed | 28          | 5       | 8        | 2 2 2 1 1           | 1        |          |    |    |     |         |
| S7 F1 head cabbage × rapeseed | 28    | 6       | 8        | 2 2 2 1 1           | 1        |          |    |    |     |         |
| S14 F1 Brussels sprout × rapeseed | 28 | 6       | 8        | 2 2 2 1 1           | 1        |          |    |    |     |         |
| S15 F1 kale × rapeseed | 29       | 7       | 8        | 2 2 2 1 1           | 1        |          |    |    |     |         |
| S18 F1 Brussels sprout × rapeseed | 29      | 8       | 9        | 3 2 2 2 2           | 1        |          |    |    |     |         |
| S20 F1 kale × rapeseed | 27       | 5       | 6        | 3 2 2 1 1           | 1        |          |    |    |     |         |
|                |                   |         |          |                   |          |          |    |    |     |         |
| **Interspecific hybrids of the F2 generations** |                  |         |          |                   |          |          |    |    |     |         |
| S14 F1 × S14 F1 | 52                | 8       | 13       | 2 2 1 1 1           | 1        |          |    |    |     |         |
| S15 F1 × S2 F1 | 56                | 13      | 13       | 2 2 2 5 2           | 4        |          |    |    |     |         |
|                |                   |         |          |                   |          |          |    |    |     |         |
| **Interspecific hybrids of the BC1 generation (head cabbage × F1/F2)** |                  |         |          |                   |          |          |    |    |     |         |
| CPS8 × (S15 F1 × S2 F1) | 37,38          | 4–5     | 6        | 2 2 2 1 1           | 1        |          |    |    |     |         |
| KLG × S7 F1 | 28,                | 3       | 8        | 2 3 3 1           | 1        |          |    |    |     |         |
| KLG × (S14 F1 × S14 F1) | 34,36          | 5       | 9        | 3 2 3 1           | 1        |          |    |    |     |         |
| TKL × (S15 F1 × S2 F1)/1 | 38           | 4       | 9        | 1 1 3 1           | 1        |          |    |    |     |         |
| TKL × (S15 F1 × S2 F1)/2 | 28           | 5       | 8        | 2 2 1 1           | 1        |          |    |    |     |         |
|                |                   |         |          |                   |          |          |    |    |     |         |
| **Interspecific hybrids of the BC1 × (F1 × F2) generation** |                  |         |          |                   |          |          |    |    |     |         |
| (KLG × S71) × (S1 × S14)/1 | 39,40       | 7       | 14       | 3 6 4 1           | 2        | 4        |    |    |     |         |
| [(KLG × S7) × (S1 × S14)/2] | 46          | 6       | 13       | 2 3 3 1           | 1        | 2        |    |    |     |         |
| [CPS8 × (S15 ×S2)] × S14/1 | 43           | 4–5     | 12–14    | 1–2 4 4           | 2        | 1        |    |    |     |         |
| [CPS8 × (S15 ×S2)] × S14/2 | 38,39       | 8       | 12       | 2 4 3 1           | 2        | 1        |    |    |     |         |
| [(CPS8 ×(S15×S2)] × S1 × (S15×S2)/1 | 39         | 5       | 11       | 1–2 2 2           | 1        | 1        |    |    |     |         |

* Marker chromosome nomenclature according to Xiong and Pires [31].

Based on the number and distribution of the in situ hybridization signals on chromosomes, selected chromosome marker pairs were identified. The identification of marker chromosomes, as well as their nomenclature (A1, A3, A10, A5/A6/A9, C4, C7 and C8) for genomes A and C, were according to Xiong and Pires [29]. In genome A, chromosomes A1 and A3 have both 5S and 35S rDNA signals that occupy different chromosomal positions. A5/A6/A9 chromosomes are characterized by the presence of 35S rDNA loci, while A10 chromosomes have 5S rDNA loci. In the C genome, the 5S rDNA loci are located on the long arm of C4 chromosomes, while the chromosome C7 and C8 pairs had 35S rDNA loci on their short arms. The centromeric location of the 35S rRNA genes is characteristic of genome A. In B. napus, chromosomes that have 35S rDNA genes located in the terminal position on chromosomes represent the C genome. In the B. rapa A genome, rDNA sequences allow the identification of A1 and A3 chromosomal pairs (carrying 5S and 35S rDNAs), A10 pairs (carrying 5S rDNA) and A5/A6/A9 pairs (carrying 35S rDNAs), while in the B. oleracea C genome, this approach allows C4 chromosomal pairs (carrying 5S rDNAs) and C7 and C8 pairs (carrying 35S rDNAs) to be distinguished.
**Figure 4.** Simultaneous FISH of 5S rDNA (red fluorescence) and 35SrDNA (green fluorescence) probes to metaphase chromosomes of parental genotypes and interspecific hybrids of *Brassica oleracea* × *Brassica napus*. (a) KA251 (head cabbage) (b) PN1162 (rapeseed). (c) S1 F$_1$ (kale × rapeseed). (d) S18 F$_1$ (Brussels sprout × rapeseed). (e) S15 F$_1$ × S2 F$_1$ [(kale × rapeseed) × (kale × rapeseed)]. (f) KLG × (S14 F$_1$ × S14 F$_1$) [head cabbage × (Brussels sprout × rapeseed) × (Brussels sprout × rapeseed)]. Letters A indicates marker chromosomes for genome *B. rapa* and C represents marker chromosomes for *B. oleracea*, respectively, nomenclature according to Xiong and Pires [31]. Chromosomes are stained with 4′,6-diamidino-2-phenylindole (DAPI). Bars represent 5 µm.
An analysis of individual chromosomal pairs showed that the number of chromosome markers is generally stable for the analyzed F1 Brassica hybrids. In the hybrids of BC1 and BC1 × (F1 × F2) generations, a high variability in the number of marker chromosomes was observed. A large change in the number of chromosomes was observed for A10 pairs, A5/A6/A9 and C4 pairs, and C8 and C7 pairs. Chromosome A3 (one chromosome) was the most stable in the analyzed Brassica hybrids.

4. Discussion

Distant hybridization plays an important role in Brassica crops germplasm innovation, desired traits introduction and crop improvement [14]. Our work aimed to improve the genetic diversity of vegetable Brassica oleracea genotypes by hybridization with rapeseed. Novel and valuable interspecific hybrids between B. oleracea genotypes and rapeseed were presented in this study. B. oleracea and B. napus belong to different species of Brassica genus and for this reason it is very difficult to obtain hybrids by hand pollination. Embryo rescue has been proved to be an effective method to obtain interspecific hybrids between B. oleracea and B. napus [44,45]. In our work embryo rescue in combination with repeated cross-pollination at the open flower/bud stage improved the efficiency of interspecific hybrid development of F1, F2, F1 × F2, BC1 (B. oleracea) and BC1 × F1/F2 generations. Interspecific F1 hybrids obtained in this study were similar to both parents, however we observed diversity of morphological characters in vegetative and generative stage between plants obtained from different B. oleracea. Intermediate morphological characters between parents and hybrid vigor were described by Yu et al. [45] for the crosses of Chinese kale (B. oleracea) with rapeseed. Our F1 genotypes had moderate vigor but interspecific plants F2 and F1×F2 generations had larger leaves, buds and flowers in comparison to F1 and parental genotypes. Positive correlation between the introgressed B. oleracea genomic components and heterosis was observed in hybrids made with rapeseed lines by Li et al. [44].

Low seed set under natural pollination for F1 and BC1 generations confirmed that hybridization between allotetraploid (B. napus) and diploid (B. oleracea) species is difficult, producing progeny that are highly sterile. The difficulty in seed development after the conventional crossing of B. oleracea and B. napus lines could be caused by interspecific incompatibility and differences in genome sizes between parental components [9,11]. According to Karim et al. [21] and Weerakoon et al. [22] most interspecific crosses do not produce mature seeds owing to the failure of endosperm development. Fertilization may take place but abortions occur during early development. Poor seed set of interspecific B. oleracea × B. napus F1 hybrids were also described by Yu et al. [45], but further generations had better ability for the seed development [46]. The ability for the seed development after open pollination of Brassica interspecific hybrids was much higher for the F2, F1 × F2 and BC1 × F1/F2 generations than for the F1 and BC1 generations. Our results confirm that in consecutive generations seed sets become significantly improved and stable and seeds can be produced without embryo rescue.

Interspecific and intergeneric hybrids usually showed abnormal or disordered chromosome segregation and combination due to their differences between parents. Backcrossing is a common method to eliminate alien chromosome fragments [45]. In our study we recorded numerous changes in the numbers of 5S and 35S rDNA loci and the number of marker chromosomes. The polymorphism in the numbers of rDNA loci was also visible in the genotypes with the same numbers of chromosomes. In BC1 and BC1 × F1/F2 generations the variation in the number of rDNA sites was increased as compared to the F1 and F2 generations. The polymorphism concerned both 5S and 35S rDNA loci. In hybrids the alterations in rDNA sites can be caused by chromosomal rearrangements, unequal crossing-over, gene conversion and transpositional events [47,48]. Among our hybrids we observed instability in the number of marker chromosomes especially in BC1 and BC1 × F1/F2 generations. The changes affected both genomes A and C. Within the genome C, the most variable was chromosome C8 whose number ranged from two to six. Among the F1 and BC1 generations, the trisomy of C chromosomes C4 were observed in S18 F1 (Brussels sprout × rapeseed) and S20 F1 hybrids (kale × rapeseed). In respect to genome A the elimination of chromosome A1 (bearing 5S and 35S rDNA) and A10 (bearing 5S rDNA) was recorded in BC1 and BC1 × F1/F2 generations. The changes in the number
of chromosome A1 have already been reported in the A genome of species and hybrids of the genus *Brassica* [31,49,50].

Obtained results showed that backcrossing of interspecific *B. oleracea* × *B. napus* hybrids toward *B. oleracea* is much more difficult than that reported toward *B. napus* to obtain allosynthetic rapeseed [12]. We faced difficulties in the creation of BC_2_ generation due to the chlorosis and high mortality of embryos after crosses of the BC_1_ generation with head cabbage inbred lines. No viable seeds of the BC_2_ generation were obtained due to the strong incompatibility between parental genotypes. Severe chlorosis for sesquidiploid hybrids between *Brassica* crop species and their wild relatives was also reported by Kaneko and Bang [11]. Yu et al. [46] suggests that it is necessary to enlarge the population of backcrossing in the distant hybridization, especially at the early generations. In our study, we used a wide range of *B. oleracea* genotypes to maximize the crossing’ success rate. Selfing and crossing may be a substitution method to introduce the alien target fragments for distant hybridization of *Brassicaceae* such as resistance to clubroot or other important characters, although it needs more generations to eliminate the alien genetic background [51]. We observed that plants of BC_1_ × F_1_ and BC_2_ generations had high vigor and good ability for the seed development after pollination by the mixture of pollen. Analysis of the consecutive generations obtained from the open pollination of interspecific hybrids in respect to their morphology, genome structure, fertility and agronomical characters will show their usefulness for breeding in the future.

We demonstrated that interspecific F_1_ hybrids (S7 and S19) obtained with the use of the male-sterile *Ogu-INRA* *B. oleracea* maternal lines (CIW1018 and CKA257) crossed with *B. napus* P1162 line with Rfo gene developed male-fertile flowers. The interspecific hybrids of the F_1_ × F_2_, S7 F_1_ × (S14 F_1_ × S14 F_1_), and S7 F_1_ × (S15 F_1_ × S2 F_1_) generations were also male-fertile as well as the majority of interspecific hybrids obtained by the cross of the sterile BC_1_ generation with fertile F_1_/F_2_ hybrids. The expression of the male fertility among F_1_, F_1_ × F_2_ and BC_1_ × F_1_/F_2_ interspecific hybrids with CMS *Ogu-INRA* cytoplasm was caused by nuclear fertility restorer (*Rfo*) genes from rapeseed, allowing the plants to produce functional pollen [52]. The restoration of fertility of interspecific F_1_ hybrids between Chinese kale (*B. oleracea* var. *albogabra*) and rapeseed with *Rfo* gene was previously described by Yu et al. [45,46]. In contrast to the interspecific F_1_, F_2_ and F_1_ × F_2_ hybrids, ten genotypes of the BC_1_ generation did not recover male-fertility and had male-sterile flowers typical of *Ogu-INRA* cabbage inbred lines used for backcrossing. These results are different from those described by Yu et al. [45,46] who obtained BC_1_ offsprings with the *Rfo* gene and restored male-fertility. We hypothesize that the lack of male fertility in the BC_1_ (*B. oleracea*) generation was associated with these particular cross-combinations. However, most plants obtained from crosses of BC_1_ male-sterile plants with male fertile F_2_ and F_1_ × F_2_ paternal lines had restored fertility.

The novel combination of techniques used in this study, such as embryo rescue, conventional crossing, morphological characteristic analysis, nuclear DNA content, pollen grain length, viability analyses, and fluorescence in situ hybridization allowed a complex investigation of the interspecific *B. oleracea* × *B. napus* hybrids using F_1_, F_2_, F_1_ × F_2_, BC_1_ (*B. oleracea*) and BC_1_ × F_1_/F_2_ generations. The interspecific states of the F_1_ hybrids analyzed using FISH and FCM methods showed intermediate chromosomes numbers and nuclear DNA contents between those of the parental *B. oleracea* and *B. napus* lines, as well as the presence of chromosome markers characteristic of C and A genomes. Interspecific hybrids between *B. oleracea* (CC) and *B. napus* (AACC) had allotriploid ACC genomes, intermediate morphological characteristics between *B. napus* and *B. oleracea*, lower pollen vitality and lower seed set. We demonstrated that most of the pollen grains of the interspecific F_1_ hybrids were unstained, malformed and undeveloped, and only a small percentage of *B. oleracea* × *B. napus* pollen grains were developed normally and were larger in size in comparison with their parental genotypes. The greater pollen size of the *Brassica* amphidiploid interspecific hybrids has been observed previously by Hossain et al. [33] and Mason et al. [54] and it is explained by the pre-meiotic doubling of chromosomal numbers, the phenotypic expression of the hybrid genome and a greater ploidy effect. Meiotic configurations in pollen mother cells were not analyzed in our study; however, the low viability
and high variation level in the pollen lengths of the interspecific F1 hybrids suggests high rate of abnormal meiotic events during pollen development. The low number of normally developed pollen grains in the interspecific F1 hybrids probably resulted from non-homologous interactions between the closely related A and C genomes during meiosis, resulting in a loss of chromosomes, unstable gametoclonal inheritance and low fertility rates in subsequent generations [4,6,8,9,21]. We showed that two interspecific hybrids of the F2 generation, (S14 F1 × S14 F1) and (S15 F1 × S2 F1), had 2n = 52 and 2n = 56 chromosomes, respectively, increased numbers of 5S and 35S signals, doubled nuclear DNA contents, higher vigor levels, larger generative and vegetative organs and greater generative propagation capabilities in comparison with their parental F1 hybrids. The development of two true seeds in the F2 generation could have occurred after the spontaneous internal genome duplication of gametic cells, or genome duplication after fertilization of unpaired ACC genomes of parental plants, which would allow the recovery of fertility and normal meiosis. The obtained interspecific F2 hybrids could be described as allohexaploids (A ACCCC) and near hexaploids, possessing doubled copy of parental ACC genome typical for F1 hybrids. Our results are in accordance with those of Mason et al. [54] and Szadkowski et al. [18] in which "Brassicaceae" allopolyploids were produced spontaneously by the union of unreduced gametes, especially under natural conditions, after the self- or cross-pollination of F1 interspecific hybrids. Newly formed allohexaploids provide the unique opportunity to investigate the immediate genetic and genomic consequences of Brassica alleles.

5. Conclusions

We developed the novel interspecific B. oleracea × B. napus hybrids of the F1, F2, F1 × F2, BC1 and BC1 × F1/F2 generations with the aid of embryo rescue. The hybrids displayed a number of significant differences in vegetative and reproductive traits as compared to parent genotypes. The interspecific F1, F2, F1 × F2 and BC1 × F1/F2 hybrids had intermediate characteristics of both parents; however, the morphological characteristics of the BC1 generation were more similar to those of head cabbage (B. oleracea). The majority of interspecific hybrids of the F1 × F2 and BC1 × F1/F2 generations had good seed set and were also male-fertile. The obtained germplasm of F2, F1 × F2 generations can be used as a genetic resource for the development of the new leafy vegetables, fodder crops or seed crops and to cross with high-performance cultivars.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/9/1339/s1, Table S1: Self/sibling pollination and interspecific hybridization of Brassica oleracea and Brassica napus genotypes in Skierniewice during 2014. Figure S1: Development of interspecific hybrids of B. oleracea × B. napus using embryo-rescue: (a) pods, 2 weeks after pollination, (b) embryos development – torpedo stage, (c) calluses obtained from globular embryos, (d) in vitro regenerants of interspecific hybrids, (e) hypodromonic culture, an explant before planting in soil, (f) transfer of the interspecific hybrids from in vitro to in vivo cultures. (g) Seeds of interspecific hybrids of F2 generation (bottom) in comparison to B. oleracea (middle) and B. napus (top). (h) Pods of ZGH08 × PI1662 hybrid (kale × rapeseed) after cross-pollination. Table S2: Seed and embryo development of interspecific hybrids (B. oleracea × B. napus) after self- and cross-pollination. Figure S2: Morphological characteristics of interspecific F1 hybrids: (a) leaf and flower stack of S1 F1 kale × rapeseed, (b) leaf and flower stack of S15 F1 kale × rapeseed, (c) S7 F1 plant head cabbage × rapeseed, (d) S14 F 1 plant Brussels sprout × rapeseed, (e) S19 F1 plant head cabbage × rapeseed, (f) X20 F1 plant head cabbage × rapeseed. Table S3: Seed set of interspecific hybrids (B. oleracea × B. napus) after open-pollination by mixture of pollen in Skierniewice 2019. Figure S3: Pollen staining with Alexander’s solution. (a–i) Interspecific hybrids of F1 generation. (j–k) Interspecific hybrids of F2 generation. (l–o) Interspecific hybrids of F1 × F2 generation. (a) S1 F1 kale × rapeseed, (b) S2 F1 kale × rapeseed, (c) S7 F1 head cabbage × rapeseed, (d) S9 F1 B. taurica × rapeseed (e) S14 F1 B. napus × rapeseed (f) S15 F1 kale × rapeseed, (g) S16 F1 Brussels sprout × rapeseed, (h) S20 F1 kale × rapeseed, (i) X22 F1 kale × rapeseed, (j) S14 F1 × S14 F1, (k) S15 F1 × S2 F1, (l) S1F1 × (S15 F1 × S2 F1), (m) S7 F1 × (S14 F1 × S14 F1). (n) S7 F1 × (S15 F1 × S2 F1). Table S4: Morphological characteristics of parental B. napus and B. oleracea lines and the interspecific hybrids of the F1, F2, F1 × F2 and BC1 generations during the vegetative phase. Table S5: Morphological characteristics and nuclear DNA contents of parental B. napus and B. oleracea lines and the interspecific hybrids of the F1, F2, F1 × F2 and BC1 generations during the reproductive phase.

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