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Effect of Meiotic Polyploidisation on Selected Morphological and Anatomical Traits in Interspecific Hybrids of *Brassica oleracea* × *B. napus*

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Abstract: In *Brassica*, interspecific hybridisation plays an important role in the formation of allopolyploid cultivars. In this study, the ploidy of F_1 and F_2 generations resulting from interspecific hybridisation between *B. oleracea* inbred lines of head cabbage (*B. oleracea* L. var. *capitata*) (2n = 18) and kale (*B. oleracea* L. var. *acephala*) (2n = 18) with inbred lines of rapeseed (*B. napus* L.) (2n = 38) was examined by flow cytometry analysis and chromosome observation. Furthermore, the effect of meiotic polyploidisation on selected phenotypic and anatomical traits was assessed. The F_1 hybrids of head cabbage × rapeseed (S3) and kale × rapeseed crosses (S20) were allotriploids with 2n = 28 chromosomes, and nuclear DNA amounts of 1.97 (S3) and 1.99 pg (S20). These values were intermediate between *B. oleracea* and *B. napus*. In interspecific hybrids of the F_2 generation, which were derived after self-pollination of F_1 hybrids (FS3, FS20) or by open crosses between F_1 generation hybrids (FC320, FC230), the chromosome numbers were similar 2n = 56 or 2n = 55, whereas the genome sizes varied between 3.81 (FS20) and 3.95 pg 2C (FC230). Allohexaploid F_2 hybrids had many superior agronomic traits compared to parental *B. napus* and *B. oleracea* lines and triploid F_1 hybrids. In the generative stage, they were characterised by larger flowers and flower elements, such as anthers and lateral nectaries. F_2 hybrids were male and female fertile. The pollen viability of F_2 hybrids was comparable to parental genotypes and varied from 75.38% (FS3) to 88.24% (FC320), whereas in triploids of F_1 hybrids only 6.76% (S3) and 13.46% (S20) of pollen grains were fertile. Interspecific hybrids of the F_2 generation derived by open crosses between plants of the F_1 generation (FC320, FC230) had a better ability to set seed than F_2 hybrids generated from the self-pollination of F_1 hybrids. In the vegetative stage, F_2 plants had bigger and thicker leaves, larger stomata, and significantly thicker layers of palisade and spongy mesophyll than triploids of the F_1 generation and parental lines of *B. oleracea* and *B. napus*. The allohexaploid F_2 hybrids analysed in this study can be used as innovative germplasm resources for further breeding new vegetable *Brassica* crops at the hexaploid level.

Keywords: *B. oleracea* × *B. napus*; anatomy; flow cytometry; interspecific crosses; phenotype evaluation; polyploidy breeding; phenotypic variation

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1. Introduction

Interspecific hybridisation plays an important role in the origins of polyploid cultivars, and it is acknowledged as the main source of genetic variation in cultivated plants. Approximately 70% of angiosperms have undergone polyploidisation, including whole genome duplication (WGD), during species development [1,2]. Polyploidy may result

from the functioning of *2n* gametes (meiotic doubling), distant hybridisation followed by chromosome doubling, or induced through artificial chromosome doubling (mitotic polyploidisation) [3,4]. The rate of *2n* gamete production in plants is low. Most of the species of Brassicacea produce less than 2% of *2n* male gametes, whereas a small number has more than 5% (up to 85%) production [5]. According to Ramsey and Schemske [6], distant hybridisation increase the frequency of unreduced gamete production. Meiotic polyploidisation via crossing with *2n* gamete producing genotypes is one of the main methods currently used to obtain polyploidy tulip [4]. Similarly, in *Lilium* many triploid hybrids have resulted from the use of unreduced gametes [4]. Polyploid plants originating from the process of meiotic polyploidisation are superior to those produced from somatic doubling due to enhanced meiotic recombination, which leads to the rapid creation of genetic diversity [7]. Polyploidisation usually has a positive effect on growth habits, enhanced vigour, the size of generative and vegetative organs, and yield [8]. Good examples are triploid cultivars of apples, most of which are characterised by immunity to scab and high fruit marketable quality [9]. Similarly, allohexaploid wheat has a higher yield potential and is more vigorous than its diploid and tetraploid progenitors [10]. Allopolyploid crops that contain two or more genomes derived from distant species usually benefit from combining their traits and exhibit improved agronomic characteristics, better generative reproduction, and are useful as material for the breeding of new polyploid crop species [11].

Brassica is the most distinguished genus in the family Brassicaceae, which includes agriculturally important food crops domesticated for edible oil, vegetables, spices, forage crops, and ornamental plants [12,13]. Their genomes show high flexibility with respect to the ploidy modification, which allows them to easily adapt to various environmental conditions. The allotetraploid species produced by spontaneous interspecific polyploidisation include *Brassica juncea* (Indian mustard, AABB genome) formed by the hybridisation of diploid *B. rapa* (turnip rape, AA genome) and *B. nigra* (black mustard, BB genome), *B. carinata* (Ethiopian mustard, BBCC genome) formed from *B. nigra* and *B. oleracea* (cabbage, kale, CC genome), and *B. napus* (oilseed rape, rapeseed, swede, AACC genome) [14]. *B. napus* is a more recent allopolyploid species that formed about 7500 years ago by hybridisation between *B. rapa* and *B. oleracea* [15], which are mainly used as oil vegetables. These allopolyploids are morphologically diverse, including leaf type and shape, wax thickness, position of flowers, inflorescence types, and stack height and thickness [13]. Moreover, they are characterised by several desirable traits. For instance, *B. juncea* (AABB genome) has non-shattering siliques and disease resistance that are not present in the A or C genomes of *B. napus* [16,17]. *B. carinata* (BBCC genome) is resistant to diseases that commonly affect other oilseed *Brassica* spp., such as black rot disease and powdery mildew [18,19], and is resistant to various abiotic stressors, such as salt and heat [20,21]. Similarly, allotetraploid *B. napus* gives a higher yield and has increased resistance to biotic and abiotic stress compared to diploid *B. rapa* [22].

Allopolyploids have been subjected to extensive interspecific hybridisation with diverse parents, and there are examples of the introgression of various desirable characteristics into *Brassica* crops. For instance, high levels of resistance to black rot disease were found in somatic hybrids between *B. oleracea* var. *botrytis* and *B. nigra* and in all BC_1 progeny [23], whereas triploid hybrids (2n = 3x = ABC) produced from the cross *B. napus* (rapeseed, AACC) × *B. nigra* (black mustard, BB) and their allohexaploid hybrids (AABBCC) had resistance to blackleg disease inherited from *B. nigra* [24]. Allotetraploid hybrids of the F_2 generation between *Brassica rapa* var. *parachinensis* (Chinese cabbage) and *Brassica oleracea* var. *italica* (broccoli) showed new traits and a high level of nutritional elements [25]. In South Korea, one new leafy vegetable crop was bred from intergeneric the allopolyploid *Brassicoraphanus* between *B. rapa* and *Raphanus sativus* [26].

B. oleracea is mainly used as an edible vegetable. The most important *B. oleracea* crops are kale (var. *viridis*, var. *costata*, var. *medullosa*, and var. *sabellica*), cabbage (var. *capitata* and var. *sabauda*), branching bush kale (var. *ramosa*) cultivated for edible foliage, cauliflower and broccoli (var. *botrytis* and var. *italica*) cultivated for their thickened edible

inflorescences, Brussels sprouts (var. *gemmifera*) grown for its edible axillary bud leaves, kohlrabi (var. *gongyloides*) cultivated for its above ground thickened stem, and Chinese kale (var. *alboglabra*), a leaf vegetable often treated as *Brassica alboglabra* (Figure 1) [27]. Several genotypes were selected for interspecific crosses. The rapeseed line RAP62, head cabbage IW1234, and kale IW08 inbred lines had valuable agronomical characteristics, including high tolerance to biotic stress, such as resistance to clubroot and *Alternaria* leaf spot, desired agronomic characteristics, internal uniformity, and high combining ability. We expected that this breeding program could potentially yield new, improved allopolyploid leafy vegetables exhibiting heterosis in respect to their morphology, genome structure, fertility, and improved agronomic characteristics. The neopolyploids could also serve as innovative germplasm resources for further genomic and genetic research. In this study, we aimed to assess the effect of interploidy hybridisation between *B. oleracea* and *B. napus* on the ploidy level of the progeny and the variability of selected morphological and anatomical traits in F_1 and F_2 generation hybrids.

Figure 1. The diversity of *Brassica oleracea* vegetable crops. (**a**) Head cabbage (**b**) Leafy cabbage plants (**c**) Ornamental leafy cabbage; (**d**) Brussels sprouts; (**e**) Kale; (**f**) Cauliflower; (**g**) Purple cauliflower.

2. Materials and Methods

2.1. Plant Materials

The parental genotype of head cabbage IW1234 (*B. oleracea* L. var. *capitata*) was developed at the National Institute of Horticultural Research, Department of Genetics and Breeding, Skierniewice, Poland, as a valuable inbred line, which is characterised by a round head shape, good firmness with a 71–77-d vegetation period from planting to harvest, internal uniformity, and good vigour. Kale line (*B. oleracea* L. var. *acephala*) IW08 was obtained from the germplasm collection of the National Institute of Horticultural Research. The kale IW08 line had a semi-compact shape with grey-green horizontal, small leaves without petioles with intermediate blistering and crenated edges and was characterised by low susceptibility to clubroot and bacterial diseases in the field. The rapeseed (*B. napus*) RAP62 line was obtained from the Plant Breeding and Acclimatization National Research Institute, Poznań, Poland. This line had a high seed yield and lower susceptibility to clubroot and *Alternaria* leaf spot.

Interspecific F_1 hybrids (S3, S20) between *B. oleracea* IW1234 and IW08 and *B. napus* RAP62 lines were obtained via embryo rescue according to Starzycki et al. [28]. FS3 and FS20 interspecific hybrids of the F_2 generation were derived after self-pollination of the F_1 hybrids. Hybrids FC320 and FC230 were obtained by open crosses between plants of the F_1 generation. Interspecific hybrids were pollinated following the method described by Kamiński et al. [29,30].

Vernalised plants of parental lines and F_1 and F_2 interspecific hybrids were grown in a greenhouse in 5 L plastic pots filled with Kronen substrate placed on the ground and grown

at 16–20 °C with a 12 h day length. Each genotype was represented by four plants. Matured siliques were harvested individually by hand in the middle of June for each combination, and after siliques were dried, seeds were extracted and counted.

2.2. Assessment of the Ploidy Level

2.2.1. Flow Cytometry Analysis (FCM)

The nuclear DNA content was evaluated for parental lines and hybrids using FCM according to the method reported previously [31]. Leaf fragments (0.5 cm^2) were cut with a razor blade in a Petri dish that contained 0.5 mL nuclei isolation Partec buffer supplemented with propidium iodide (50 $mg \cdot mL^{-1}$) and RNase (50 $mg \cdot mL^{-1}$). The young leaves of *Zea mays* CE-777 (2C = 5.43 pg DNA) were used as an internal standard. After chopping, 1.5 mL of the isolation buffer was added, and the samples were filtered through a 30 µm filter and incubated at room temperature for 15–30 min in the dark. Nuclei fluorescence was measured using a CyFlow Ploidy Analyser with CyView software (CyFlow PA, Partec, Germany) with an Nd-YAG green laser at 532 nm. Data were analysed using CyView software (CyFlow PA, Partec, Germany). Samples with at least 5000 nuclei were measured from five leaves of each plant, with two runs per nuclei isolation extract. The 2C DNA content of each sample was calculated as the sample peak mean divided by the standard plant peak and multiplied by the value of the nuclear DNA content of the standard plant.

2.2.2. Chromosome Count

Cytogenetic observation was performed according to Marasek-Ciolakowska et al. [32]. Root tips were 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 digested in a mixture of enzymes comprised of 20% pectinase (Sigma-Aldrich, St. Louis, MO, USA), 1% cellulase (Calbiochem, San Diego, CA, USA), and 1% cellulose 'Onozuka R-10' (Duchefa, Haarlem, The Netherlands) at 37 °C for 1 h. Root meristems were squashed in a drop of 45% (*v*/*v*) 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. The preparations were stained with 2.5 g/mL 4′,6-diamidino-2-phenylindole (DAPI) (Serva, Heidelberg, Germany) and closed in glycerol. For each genotype, at least 5 instances of metaphase were photographed with a digital CCD camera PS-Fi1 (Nikon, Tokyo, Japan) attached to an epifluorescent microscope Optiphot-2 (Nikon, Jappan) using UV excitation for DAPI visualisation.

2.3. Analysis of the Morphological Characteristics

The morphological characteristics during the vegetative and reproductive stages of 2 F_1 hybrids, 4 F_2 hybrids, and their parental components were evaluated. At the beginning of April, measurements of the leaf surface were made using a surface scanner AM350 (Geomor Technik, Szczecin, Poland). Bud length, flower size, size of median and lateral nectaries, fertility/sterility, pod length, and number of seeds per pod of the vernalised hybrids and their parents were analysed during the generative phase. Measurements of flower diameter and size of flower components (nectary, anther, pistil, and stamen) were determined for 5 flowers under a stereo microscope SZX16 (Olympus, Tokyo, Japan) with imaging software Cell (Olympus, Münster, Germany).

2.4. Pollen Grain Diameter and Viability

A mixed sample of pollen from 3–6 mature anthers were stained according to Alexander [33]. The pollen grain diameter was evaluated for 100 grains with three repetitions. Pollen viability was determined based on pollen stainability with Alexander's stain in 5 fields of view at 100× magnification. Pollen grain diameter and viability were assessed using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan) with imaging software NIS-Elements Br ver. 2.30 (Nikon Instruments Inc., Tokyo, Japan) for photo documentation and measurements.

2.5. Leaf Anatomy

In a histological study, 10 × 5 mm fragments of the third, fully developed leaves were sampled. Five samples were collected for each genotype. The material was fixed in a chromic acid, acetic acid, and formalin (CrAF) mixture for 48 h at room temperature, dehydrated in graded series of ethanol (70, 80, 90, and 100%), and embedded in paraffin according to a previously reported method [34]. Cross sections (10 µm thick) were cut with a rotary microtome (Leica, Wetzlar, Germany) and stained with safranin (1% prepared in ultrapure water) and fast green (1% prepared in 95% ethanol). The sections were observed under a light microscope as described for pollen analysis (see above). For each sample, the thickness of the lamina, abaxial and adaxial epidermal layer, and spongy and palisade mesophyll were determined. For statistical analysis, three replicates were used for each genotype, and each replicate consisted of 20 measurements.

2.6. Stomata Length and Density

Samples of the abaxial epidermis from the middle part of the third fully developed leaf were isolated and stained with toluidine blue according to Dyki and Habdas [35]. For each genotype, the number of stomata per 1 mm^2 (n = 3 replication/genotype) and length of stomata (n = 3 replications × 100 stomata/genotype) were determined at 100 and 400× magnification, respectively, using a Nikon Eclipse 80i microscope (Eclipse 80i, Nikon, Tokyo, Japan) with the program NIS-Elements BR ver. 2.30 (Nikon Instruments Inc., Tokyo, Japan).

2.7. Statistical Analyses

The nuclear DNA content, pollen grain diameter and viability, leaf surface, bud length, flower diameter, pistil and stamen size, size of median and lateral nectary, pod length, stomata length and density, thickness of the lamina, thickness of the abaxial and adaxial epidermal layer, and thickness of the spongy and palisade mesophyll were subjected to a one-way analysis of variance (STATISTICA package StatSoft v. 10, Dell Inc. Round Rock, TX, USA). The means were compared using Tukey's test at $p = 0.05$. Standard deviations for nuclear DNA content, leaf surface, bud length, pistil and stamen size, nectaries and pod length were calculated in 5 replications (n = 5), whereas standard deviations for pollen grain diameter, thickness of the lamina and epidermal layers, and thickness of the spongy and palisade mesophyll, stomata length and density were calculated in 3 replications (n = 3).

3. Results

3.1. Genome Size Determination by FCM and Somatic Chromosome Count

The ploidy levels of parental lines and hybrids were determined by flow cytometry (FCM) analysis and chromosome count (Figures 2 and 3, Table 1). The amount of nuclear DNA and the number of chromosomes in parental lines of *B. napus* and *B. oleracea* agreed with data found in the literature. Head cabbage IW1234 (*B. oleracea* L. var. *capitata*) and kale IW08 (*B. oleracea* L. var. *acephala*) are diploids with 2n = 18 chromosomes and a DNA volume of 1.48 and 1.51 pg 2C, respectively. Allotetraploid rapeseed RAP62 (*B. napus* L.) had 38 chromosomes and a significantly higher amount of DNA (2.49 pg) than *B. oleracea* genotypes. The F_1 hybrids of head cabbage × rapeseed (S3) and kale × rapeseed crosses (S20) were allotriploids with 2n = 28 chromosomes; the nuclear DNA content was intermediate to the genome size of progenitors (1.97 and 1.99 pg, respectively). In interspecific hybrids of the F_2 generation, which were derived after self-pollination of F_1 hybrids (FS3, FS20) or by open crosses between plants of the F_1 generation (FC320, FC230), chromosome numbers were similar (2n = 56 or 2n = 55), whereas genome sizes varied between 3.81 ± 0.10 pg 2C for FS20 and 3.95 ± 0.091 pg 2C for FC230.

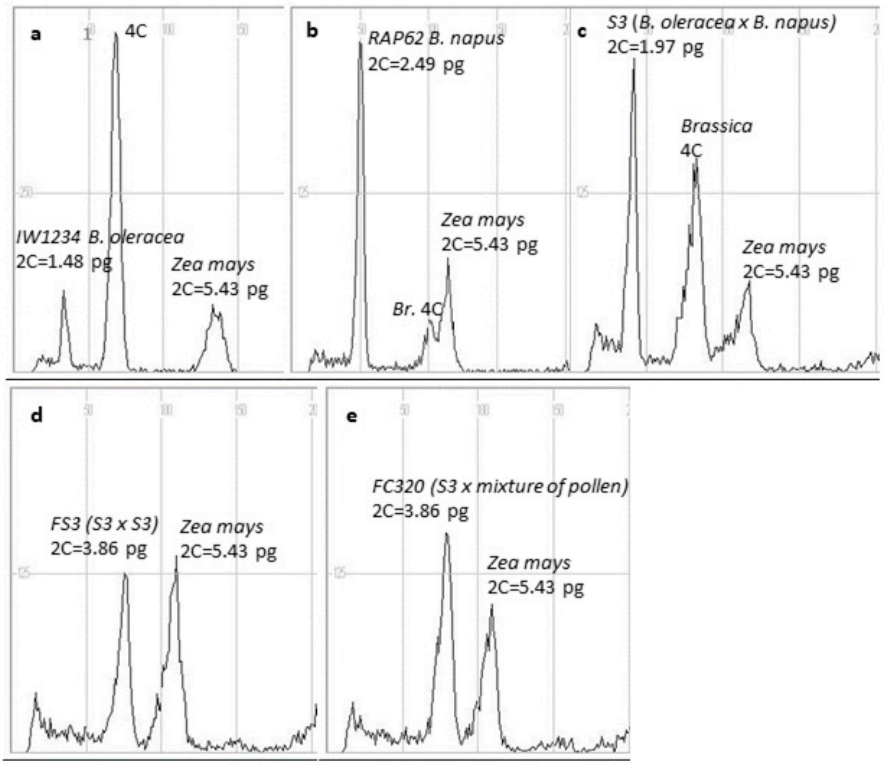

Figure 2. Histograms of nuclear DNA estimation using flow cytometry (FCM) for *Brassica oleracea* and hybrids with internal standard *Zea mays* (2C DNA = 5.43 pg). (**a**) IW1234 *B. oleracea* L. var. *capitata* (head cabbage); (**b**) RAP62 *B. napus* (rapeseed); (**c**) S3 F_1 (head cabbage × rapeseed); (**d**) FS3 F_2 (S3 × S3); (**e**) FC320 F_2 (S3 × mixture of pollen).

Table 1. Nuclear DNA content and chromosome number of parental *Brassica napus* and *B. oleracea* lines and their interspecific hybrids in the F_1 and F_2 generation.

Genotype	Genome	DNA Content (pg)	Std. dev.	Chromosome Number
IW1234 ♀ head cabbage	CC	1.48 d *	0.021	18
IW08 ♀ kale	CC	1.51 d	0.022	18
RAP62 ♂ rapeseed	AACC	2.49 b	0.036	38
Interspecific hybrids of the F_1 generation				
S3 (head cabbage × rapeseed)	ACC	1.97 c	0.030	28
S20 (kale × rapeseed)	ACC	1.99 c	0.017	28
Interspecific hybrids of the F_2 generation				
FS3 (S3 × S3)	AACCCC	3.86 a	0.034	56
FS20 (S20 × S20)	AACCCC	3.81 a	0.102	55
FC320 (S3 × mixture of pollen)	AACCCC	3.86 a	0.141	56
FC230 (S20 × mixture of pollen	AACCCC	3.95 a	0.091	55

* Means in the columns followed by the same lowercase letters are not significantly different according to Tukey's test at $p = 0.05$. ♀ maternal line; ♂ pollen donor.

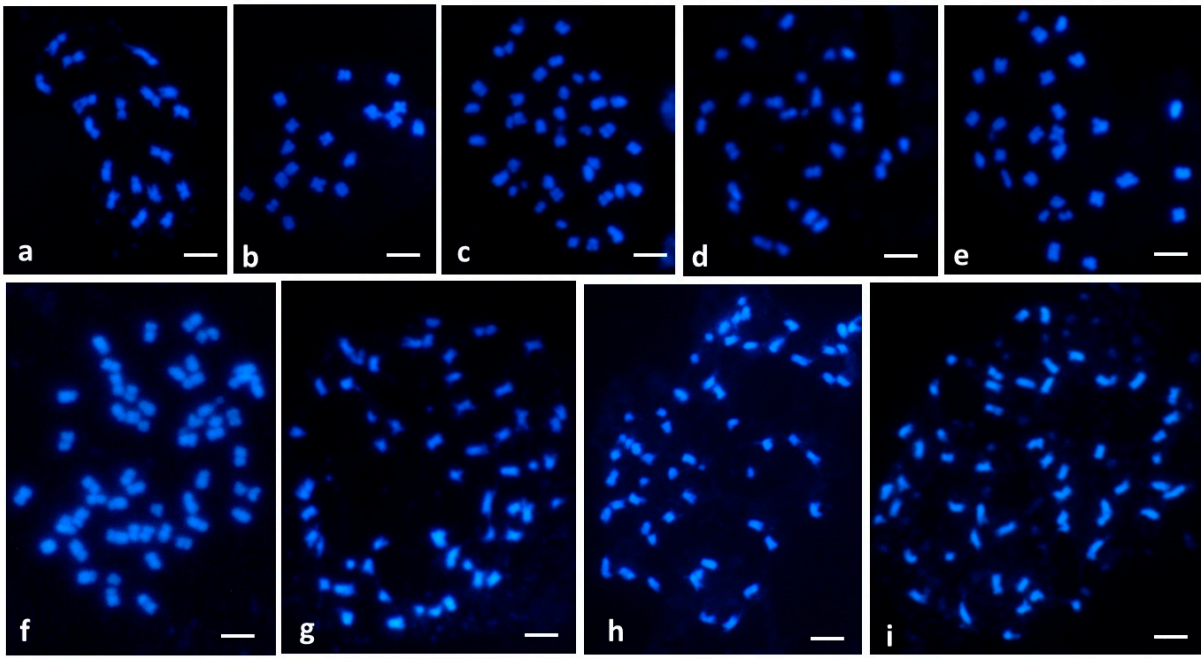

Figure 3. Chromosome counts of *Brassica* genotypes. (**a**) IW1234 *B. oleracea* L. var. *capitata* (head cabbage), 2n = 18; (**b**) IW08 *B. oleracea* L. var. *acephala* (kale), 2n = 18; (**c**) RAP62 *B. napus* (rapeseed), 2n = 38; (**d**) S3 F_1 (head cabbage × rapeseed), 2n = 28; (**e**) S20 F_1 (kale × rapeseed), 2n = 28; (**f**) FS3 F_2 (S3 × S3), 2n = 56; (**g**) FS20 F_2 (S20 × S20), 2n = 55; (**h**) FC320 F_2 (S3 × mixture of pollen), 2n = 55; (**i**) FC230 F_2 (S20 × mixture of pollen), 2n = 55. Scale bars represent 5 µm.

3.2. Characteristics of Parental Genotypes and Interspecific Hybrids of the F_1 and F_2 Generations in the Generative Stage

In the generative stage, interspecific polyploids of the F_1 and F_2 generation displayed significant heterosis with respect to flower morphology, nectary size, fertility, and ability to set seeds compared to the parent genotypes (Figure 4 and Figure S1). Allohexaploids representing F_2 hybrids had significantly longer buds, flower diameters, and longer anthers than those of parental *B. napus* and *B. oleracea* lines and triploid F_1 hybrids (Table 2). There were no significant differences in the length of the pistils. Each *Brassica* flower had two pairs of nectaries, the lateral (inner) and median (outer) pairs, that differed in morphology, size, and nectar productivity (Table 2, Figure 4c and Figure S1). The lateral nectaries were of equal size in *B. oleracea* L. var. *capitata* and *B. napus*, and they were shorter by approximately 20% compared to *B. oleracea* L. var. *acephala*. In contrast, there was no significant difference in the length of median nectaries among parental lines and interspecific hybrids. Lateral nectaries of the F_1 generation had a similar size to parental lines, but they were shorter by 30% than lateral nectaries of the F_2 generation (Table 2). Pollen viability was high in parental lines and varied from 85% in kale to 99% in rapeseed, and their average pollen size was 24.54 and 29.86 µm, respectively. All interspecific hybrids of the F_1 and F_2 generations developed male-fertile flowers. In triploid F_1 hybrids, pollen viability was low, and only 6.76 (S3) and 13.46% (S20) of pollen grains were stained by Alexander's solution (Figures 4e and S1, Table 2). In interspecific hybrids of the F_2 generation, pollen viability was comparable to the parental genotypes and varied from 75.38 (FS3) to 88.24% (FC320). The average pollen lengths for the interspecific hybrids of the F_1 and F_2 generations were significantly larger compared to the parental lines (Table 2, Figure S1). The distribution of pollen size was similar for the hybrids of head cabbage (Figure 5a) and kale (Figure 5b). A high variation in the size of viable pollen was recorded for F_1 hybrids, whose length differed from 19.40 to 36.10 µm in S3 (head cabbage × rapeseed) and from 20.85 to 32.40 µm in S20 (kale × rapeseed) hybrids (Figure 5). Among parental genotypes, differences in seed set and pod length were observed, where rapeseed produced longer pods by approximately

10% and more seeds than parental lines of *B. oleracea*. The negative effect on the length of pods and seed set was observed especially in the interspecific hybrid of the F_1 generation, in which pods were approximately 70% shorter compared to parental lines, and they were usually empty (Table 2, Figure 4h). Hybrids of the F_2 generation produced longer pods, and most of them had more seeds per pod than hybrids of the F_1 generation. However, among the F_2 hybrids, differences in the number of seeds were observed depending on the pollinator. Interspecific hybrids of the F_2 generation derived by open crosses between plants of the F_1 generation (FC320, FC230) had better seed set compared to F_2 hybrids derived from the self-pollination of F_1 hybrids (FS3, FS20) (Table 2).

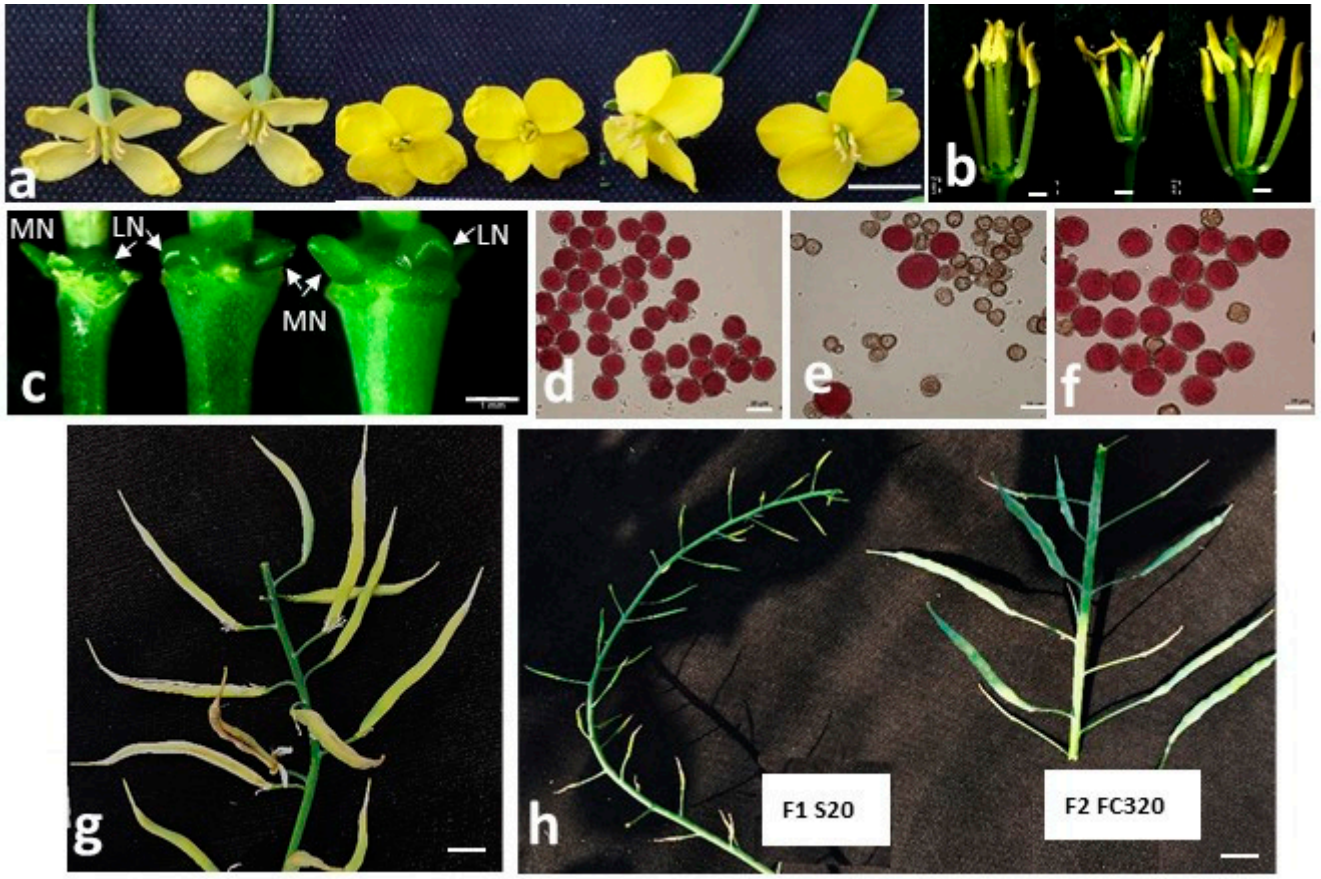

Figure 4. Phenotypic characteristics of maternal line IW1234 and the interspecific hybrids of head cabbage × rapeseed in the F_1 and F_2 generations in the generative stage. (**a**) Flowers of head cabbage IW1234 (left), S3 F_1 (head cabbage × rapeseed) (middle) and FC320 F_2 (S3 × mixture of pollen) (right, Bar = 1 cm; (**b**) Flowers with removed sepals and petals of head cabbage IW1234 (left), S3 F_1 (middle) and FC320 F_2 (right), Bar = 2 mm; (**c**) Receptacles with lateral and median nectaries, head cabbage IW1234 (left), S3 F_1 (middle) and FC320 F_2 (right), MN—median nectary, LN—lateral nectary, Bar = 1 mm; (**d**–**f**) Pollen staining with Alexander's solution, Bar = 25 µm; (**d**) IW1234; (**e**) S3 F_1 (**f**) FC320 F_2; (**g**) Pod of head cabbage IW1234; (**h**) Pod of S20 F_1 and FC320 F_2 generations. Scale bars represent 1 cm (**g**,**h**).

Table 2. Comparison of phenotypic characteristics of parental genotypes and interspecific hybrids of the F_1 and F_2 generations at the generative stage.

Genotype	Bud Length (mm)	Flower Diameter (mm)	Pistil Length (mm)	Stamen Length (µm)	Anther Length (mm)	Median Nectary Length (µm)	Lateral Nectary Length (µm)	Pollen Diameter (µm)	Pollen Viability %	Pod Length (cm)	Seed Number/pod
IW1234 ♀ head cabbage	8.00 bc * ±0.10	24.72 c ±0.17	11.83 b ±0.43	11.82 bc ±0.67	3.35 bc ±0.20	738.638 b ±86.02	517.64 e ±73.76	25.79 c ±1.86	89.50 a ±2.93	7.34 ab ±0.44	2.66 c ±0.331
IW08 ♀ kale	8.22 bc ±0.13	24.60 c ±0.21	15.65 a ±0.75	16.40 a ±0.40	3.17 bc ±0.17	1095.78 ab ±113.40	651.31 cd ±67.70	24.54 c ±1.34	85.82 a ±8.12	7.50 ab ±0.75	5.18 ab ±1.01
RAP62 ♂ rapeseed	8.56 bc ±0.14	23.40 d ±0.26	9.37 d ±0.47	10.97 bc ±0.45	3.51 bc ±0.13	978.29 ab ±44.35	578.51 e ±63.93	29.86 b ±1.50	99.37 a ±1.09	8.20 a ±0.47	6.91 a ±0.62
Interspecific hybrids of the F_1 generation											
S3 (head cabbage × rapeseed)	7.41 c ±0.11	22.04 e ±0.36	9.92 d ±0.15	10.06 b ±0.43	2.71 b ±0.11	932.03 ab ±40.28	624.40 cd ±35.35	33.74 ab ±4.16	6.765 b ±2.77	2.46 de ±0.15	0.00 d ±0.00
S20 (kale × rapeseed)	9.22 ab ±0.06	23.00 de ±0.07	11.66 bc ±0.13	11.64 bc ±0.39	3.11 bc ±0.046	1154.26 a ±52.06	688.85 b–d ±50.53	33.04 ab ±3.78	13.46 b ±9.29	2.22 e ±0.13	0.03 d ±0.07
Interspecific hybrids of the F_2 generation											
FS3 (S3 × S3)	9.46 ab ±0.23	24.60 c ±0.17	11.53 bc ±0.50	11.51 bc ±0.34	4.03 a,c ±0.15	1014.29 ab ±79.48	717.99 a–d ±39.95	33.63 ab ±1.95	75.38 a ±4.96	4.46 ce ±0.50	0.07 d ±0.15
FS20 (S20 × S20)	10.42 a ±0.13	27.80 a ±0.22	11.66 bc ±0.65	13.30 a,c ±0.32	4.24 a,c ±0.15	1076.63 ab ±60.01	729.12 a–d ±81.35	34.04 a ±2.06	85.79 a ±3.09	4.96 cd ±0.65	0.47 cd ±0.15
FC320 (S3 × mixture of pollen)	10.19 a ±0.19	26.60 b ±0.20	11.72 bc ±0.49	13.39 a,c ±1.09	4.86 a ±0.25	1102.10 ab ±63.37	839.01 a ±47.96	33.81 a ±2.24	88.24 a ±4.03	6.26 ac ±0.49	1.67 cd ±0.49
FC230 (S20 × mixture of pollen)	10.57 a ±0.13	25.20 c ±0.42	10.47 cd ±0.59	12.69 bc ±1.15	4.71 a ±0.47	1120.84 a ±123.23	803.28 ab ±85.51	32.71 ab ±2.20	78.71 a ±2.52	4.28 ce ±0.59	2.76 bc ±0.38

* Means in the columns followed by the same lower case letters are not significantly different according to Tukey's test at $p = 0.05$. ♀ maternal line; ♂ pollen donor.

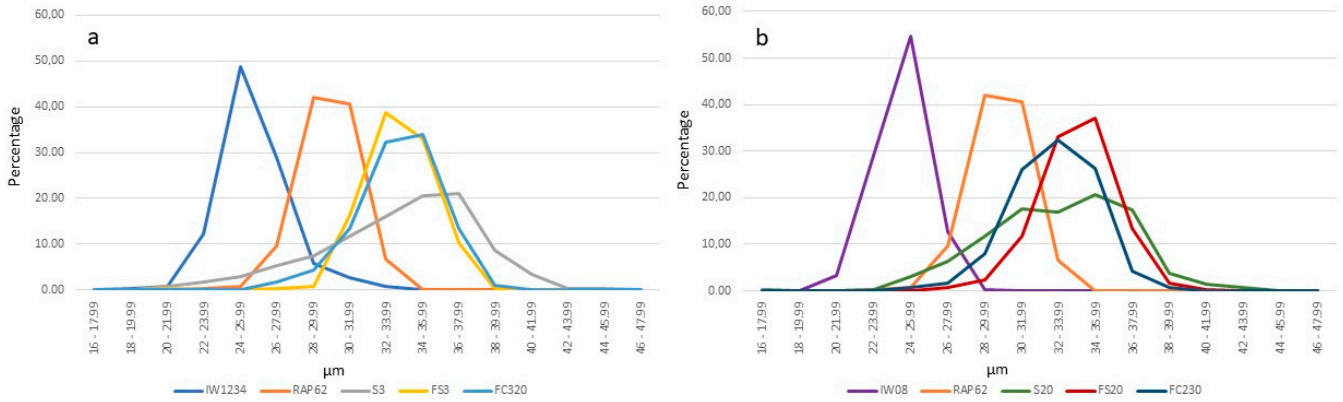

Figure 5. Distribution of pollen grain sizes (%) in parental genotypes and interspecific hybrids. (**a**) Head cabbage IW1234, rapeseed RAP62, S3F1 (head cabbage × rapeseed), FS3 F2 (S3 × self), and FS320 F2 (S3 × mixture of pollen). (**b**) Kale IW08, rapeseed RAP62, S20 F1 (kale × rapeseed), FS20 (S20 × self), and FS230F2 (S20 × mixture of pollen).

3.3. Characteristics of Morphological and Anatomical Traits of Leaves of Parental Genotypes and the Interspecific Hybrids of the F_1 and F_2 Generations

The morphological and anatomical characteristics of the leaves of F_1 and F_2 hybrids were compared with those of parental genotypes (Table 3). Allohexaploid hybrids of the F_2 generation had bigger and thicker leaves than triploids of the F_1 generation and parental lines of *B. oleracea* and *B. napus* (Figure 6a and Figure S2). The largest leaf surface and thickest leaves were observed in FC320, which resulted from open pollination of F_1 S3 hybrids; the leaves were larger than leaves of head cabbage and F_1 S3 hybrids by approximately 250% and thicker by 50–60%. Similarly, the comparison of the abaxial epidermis (Figure 6b–d and Figure S2b–j) showed that hexaploid F_2 hybrids had larger stomata than triploids of the F_1 generation and parental lines of *B. oleracea* and *B. napus*, but their density per mm^2 was the lowest. Figure 7 shows that in the diploid parental lines of *B. oleracea*, approximately 42% of head cabbage stomata and kale stomata had sizes of 24–26 and 22–24 µm in length, respectively. In tetraploid *B. napus*, there was significant variation in stomata length, which differed from 7.07 to 32.76 µm, and approximately 20% of stomata had a length of 24–26 µm. In the F_1 generation, 28% of stomata of head cabbage and 37% of kale stomata had a length of 26–28 µm. F_2 hybrids of kale (FS20, FC230) had a similar stomata size distribution, and about 30% of them had lengths from 30 to 36 µm. F_2 hybrids of head cabbage showed high variation in stomata sizes. Approximately 26% of FS3 hybrids had stomata 32–34 µm in length, and 28% of FC320 hybrids had stomata 36–38 µm in length. Histological analysis of leaf fragment cross sections revealed high variation among genotypes with respect to the internal structure of leaves (Figures 6e–g and S2k–s). In general, the thickness of the abaxial and adaxial epidermis was higher in interspecific hybrids of the F_2 generation derived after self-pollination of F_1 hybrids (F_2 FS3, FS20) compared to F_2 hybrids obtained by open crosses between plants of the F_1 generation (FC320, FC230). The thickness of the adaxial epidermis was similar in the F_1 generation and the parental lines; similarly, there was no relationship between ploidy level and the thickness of the abaxial epidermis among parental lines and F_1 hybrids (Table 3). The cells of the epidermis differed in shape and size, which were tightly arranged into a single layer. The characteristic feature of both head cabbage and kale hybrids was the presence of large, strongly vacuolated cells among smaller ones in the epidermal layer. Layers of both the palisade and spongy mesophyll were significantly thicker in hexaploids of the F_2 generation. In general, the examined genotypes differed in the thickness of particular cell layers in leaves, which was related to the thickness of the lamina. Leaves of FC320 F_2 hybrids were characterised by a more compact mesophyll structure with smaller intercellular spaces than other genotypes (Figure 6e–g and Figure S1k–s).

Table 3. Comparison of morphological and anatomical characteristics of leaves of parental genotypes and the interspecific hybrids of the F_1 and F_2 generations.

Genotype	Leaf Surface (cm^2)	Leaf Thickness (μm)	Palisade Mesophyll Thickness (μm)	Spongy Mesophyll Thickness (μm)	Abaxial Epidermis (μm)	Adaxial Epidermis (μm)	Stomata Length (μm)	Stomata Density (No./mm^2)
IW1234 ♀ head cabbage	164.50 c * ±9.43	264.60 cd ±19.63	103.3 bc ±15.56	113.9 c–e ±16.48	20.42 bc ±0.65	15.53 bd ±2.65	24.38 b ±0.07	114.11 ce ±6.26
IW08 ♀ kale	85.24 c ±8.61	234.30 d ±14.33	93.6 c ±13.61	103.5 c–f ±15.93	15.43 d ±1.47	13.24 d ±1.68	22.34 b ±0.54	271.22 a ±13.80
RAP62 ♂ rapeseed	137.54 c ±12.76	232.32 d ±21.08	91.96 c ±12.30	92.6 f ±15.68	20.05 c ±2.90	15.86 bd ±1.73	23.54 b ±4.12	181.33 b ±18.28
Interspecific hybrids of the F_1 generation								
S3 (head cabbage × rapeseed)	165.58 c ±18.91	249.14 cd ±17.17	114.1 bc ±15.65	94 ef ±16.28	21.26 bc ±2.05	15.16 cd ±1.82	26.89 b ±1.28	140.78 bc ±10.58
S20 (kale × rapeseed)	133.20 c ±26.13	294.83 bc ±16.88	129.6 bc ±15.31	123.6 bd ±15.54	19.23 cd ±1.38	14.99 cd ±1.65	26.87 b ±0.66	120.89 cd ±5.83
Interspecific hybrids of the F_2 generation								
FS3 (S3 × S3)	346.50 b ±9.24	327.91 b ±12.73	138.3 b ±18.66	138 b ±14.38	26.05 a ±4.08	22.47 a ±2.82	32.66 a ±0.39	87.88 ce ±1.39
FS20 (S20 × S20)	364.06 b ±12.09	291. 76 bd ±18.53	120.4 bc ±15.62	120.3 b–d ±15.20	24.41 ab ±2.55	19.74 ab ±2.74	32.45 a ±0.97	67.00 de ±7.36
FC320 (S3 × mixture of pollen)	603.72 a ±13.03	409.60 a ±28.72	183 a ±26.25	180.1 a ±24.76	17.84 cd ±2.40	17.50 bd ±2.80	32.50 a ±1.33	104.00 ce ±3.34
FC230 (S20 × mixture of pollen)	372.34 b ±20.35	294.76 bc ±14.07	119.6 bc ±10.91	129.9 bc ±14.33	24.24 ab ±1.31	19.32 ac ±2.98	37.40 a ±1.29	55.13 e ±3.29

* Means in the columns followed by the same lowercase letters are not significantly different according to Tukey's test at $p = 0.05$. ♀ maternal line; ♂ pollen donor.

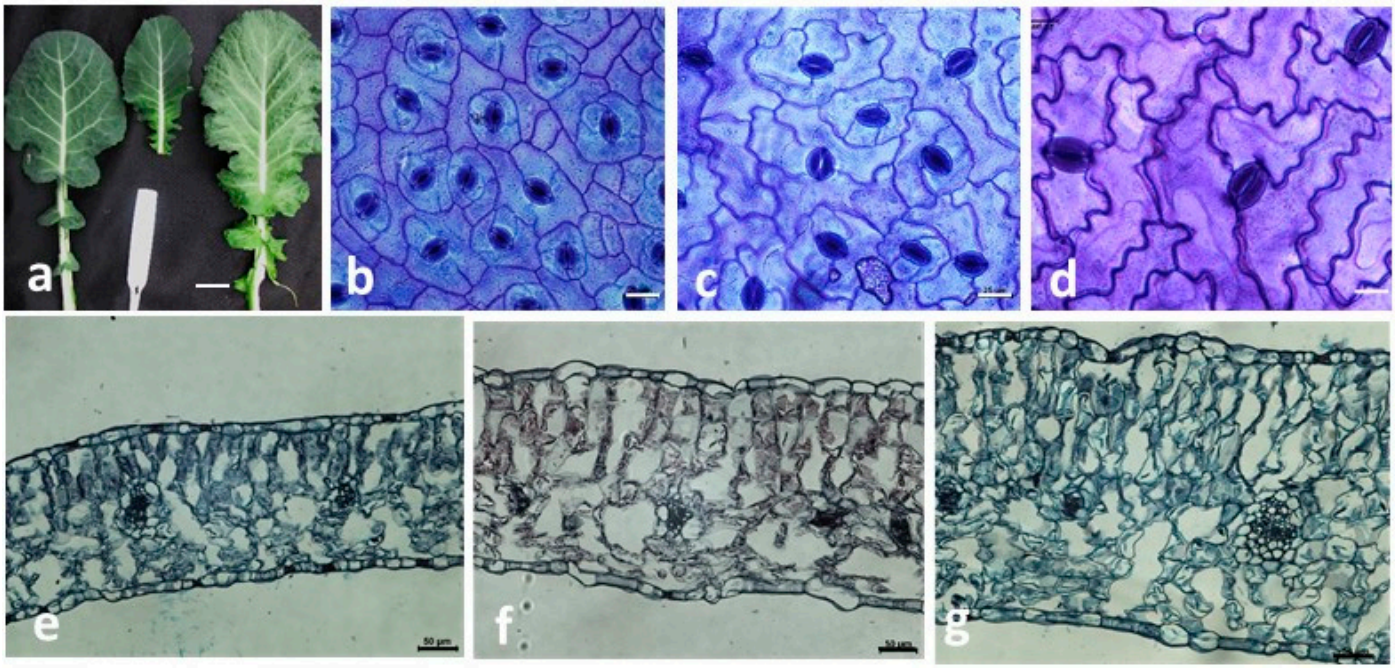

Figure 6. Characteristics of morphological and anatomical traits of leaves of maternal line IW1234 and the interspecific hybrids of head cabbage × rapeseed in the F_1 and F_2 generations. (**a**) Leaf of maternal genotype IW1234 (middle) and F_2 generation FS3 (left), FC320 F_2 (right), Bar = 2 cm; (**b**–**d**) Stomata on the abaxial leaf surface, Bars = 25 µm; (**b**) IW1234; (**c**) F_1 S3; (**d**) FC320 F_2; (**e**–**g**) Leaf cross-section, Bars = 50 µm; (**e**) IW1234; (**f**) F_1 S3; (**g**) FC320 F_2.

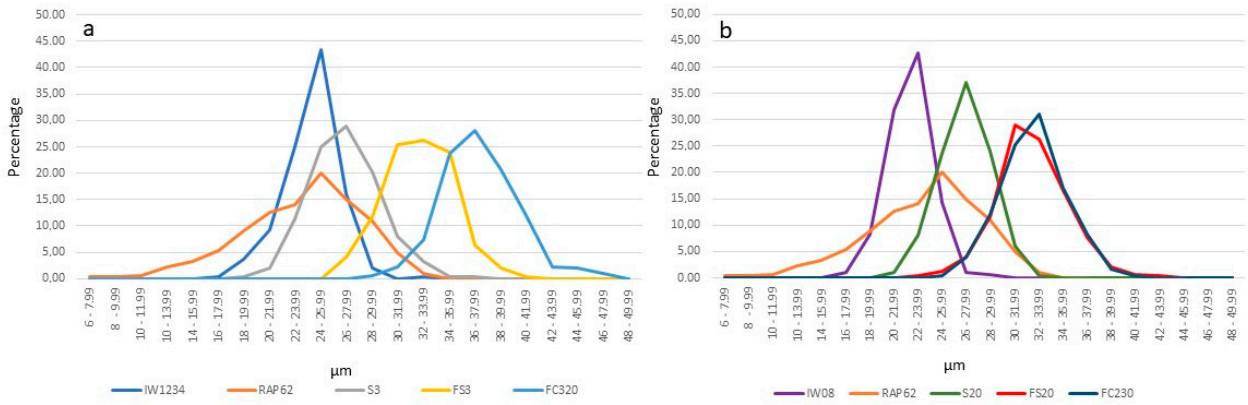

Figure 7. Distribution of stomata length in percentage in parental genotypes and interspecific hybrids. (**a**) Head cabbage IW1234, rapeseed RAP62, S3F1 (head cabbage IW1234 × rapeseed RAP62), FS3 F2 (S3 × self), and FS320 F2 (S3 × mixture of pollen). (**b**) Kale IW08, rapeseed RAP62, S20 F1 (IW08 kale × RAP62 rapeseed), FS20 (S20 × self), and FS230 F2 (S20 × mixture of pollen).

4. Discussion

In the genus *Brassica*, hexaploid species do not exist in nature, as tetraploidy (4x) is the highest naturally occurring ploidy [36]. There are different approaches to hexaploid induction. Trigenomic hexaploids (AABBCC) with 54 chromosomes were produced in *Brassica* through interspecific hybridisation of *B. napus* (AACC genome) and *B. nigra* (BB genome), followed by colchicine treatment [36]. In a similar manner, trigenomic hexaploids (AABBCC) were obtained from *B. campestris sp. chinensis* (AA) and *B. carinata* (BBCC) crosses after artificial chromosome doubling of F_1 hybrids [37]. Treatment of triploid ACC hybrids with colchicine led to hexaploid AACCCC genotype development; these hybrids were

largely used as bridge plants for the introgression of genomic components of *B. rapa* into *B. napus* [38]. Mason et al. [39] showed the possibility of producing trigenomic allohexaploid *Brassica* through unreduced gametes. The allohexaploid (6x) and aneuploid AACCCC *Brassica* hybrids analysed in this study were created by interploid crosses between diploids of kale (CC) and head cabbage (CC), with tetraploid *B. napus* (AACC), followed by self-pollination and open-cross-pollination of triploid F_1 hybrids (ACC). These results indicate that ploidy manipulation in *Brassica* is possible via euploid gametes produced by hybrids. The distribution of pollen sizes showed great variation in grain sizes, both in $S3F_1$ (head cabbage × rapeseed) and $S20F_1$ (head cabbage × rapeseed) hybrids (Figures4e, 5 and S1q,r). There was considerable evidence in the literature that in *Brassica*, interspecific triploid hybrids can produce euploid (x, 2x, 3x) and aneuploid gametes [22,40–42], and the success in ploidy manipulation depends on the lines selected for hybridisation. According to He et al. [43], due to abnormal meiotic processes and cytogenetic instability, aneuploids can be expected when using triploids or tetraploids in crosses with other genotypes, regardless of their ploidy levels. Li et al. [22] reported a high frequency of allotetraploid-like gametes in the interspecific hybrids between *B. napus* and *B. oleracea*.

The total nuclear DNA content per cell significantly influenced the phenotypic traits (nucleotypic effects). Cell size, pollen grain diameter, stomata length, and stomatal plastid number are positively correlated with genome size, and they are often used as morphological markers for identifying the ploidy level of plants [44–46]. Furthermore, seed size, stomatal density, and pollen fertility are usually related to the ploidy level [46]. For instance, a positive relationship was found between the ploidy level and stomatal guard cell length and width, and a negative correlation was observed between ploidy level and stomatal density for diploid and triploid *Citrus clementina* [47] and stomata length and pollen size in diploid and tetraploid tulips [31] and apples [48]. In *Brassica*, a high correlation between ploidy and stomata lengths was reported for trigenomic hexaploid hybrids (AABBCC) [36]. Similarly, in our study, both stomata length and pollen grain diameters were significantly higher as ploidy increases, whereas the density of stomata on the abaxial epidermis decreased. The number and distribution of stomata per unit leaf area may have consequential roles in moisture exchange between the leaves and atmosphere [49]. According to Padoan et al. [47], leaf water loss is higher in diploid plants than in triploids, which are characterised by decreased stomatal density. Thus, the size and stomatal density influence the adaptation to various environmental conditions and productivity of the plants.

The doubling of the cell genome usually leads to increased organ size. In our study, the vegetative and reproductive organs of hexaploids were larger than those of the diploid and tetraploid parents and triploid F_1 hybrids (Figure 4, Figure S1 and S2). Larger vegetative and reproductive organs were also observed in hexaploids derived from *B. rapa* and *B. carinata* [50]. Trigenomic hexaploid *Brassica* hybrids (AABBCC) derived from *B. napus* L. and *B. nigra* were more vigorous, had broader and thicker leaves, and the hairs on leaves and stems were more abundant compared to triploid (ABC) hybrids and their parents [36]. Some of the changes observed in polyploids are of high agronomical value. Plants of the F_2 generation had bigger and thicker leaves and significantly thicker layers of palisade and spongy mesophyll than triploids of the F_1 generation and parental lines of *B. oleracea* and *B. napus*, whereas FC320 F_2 had a compact mesophyll structure with small intercellular spaces. The leaf surface and leaf morphological characteristics may affect their resistance to infestation by insect herbivores [51–53]. Marasek-Ciolakowska et al. [53] showed that reduced infestation by the cabbage whitefly (*Aleyrodes proletella*) on leaves of resistant cultivars of kale and savoy cabbage was related to the structure of the epidermis, thickness of the lamina, and compactness of the mesophyll.

The morphological appearance of flowers in F_1 (ACC) and F_2 (AACCCC) hybrids was similar to *B. napus*, except that the organs were larger. Hexaploids had significantly larger flower buds, flowers, and anthers compared to triploids. Larger flowers, petals, and stamens were also observed in hexaploid *Brassica* (AABBCC) hybrids derived from a colchicine treatment of triploid (ABC) hybrids of *B. napus* (AACC) × *B. nigra* (BB) [36]

and *B. carinata* (BBCC) × *B. rapa* (AA) [54]. Flowers and flower elements of 4x plants of *B. rapa* were larger than diploid and haploid plants [55]. We observed a negative effect of polyploidy on the length of pods and seed set of the allopolyploid hybrids when compared to diploid and tetraploid progenitors. However, pod length and seed number per pod in the hexaploid plants increased significantly compared to triploids, and seeds were produced without embryo rescue. The ability for seed development was better after open pollination compared to self-pollination of F_1 hybrids. Similarly, the seed development in plants of $F_1 \times F_2$ and $BC_1 \times F_1/F_2$ generations of *B. oleracea* × *B. napus* after pollination by the mixture of pollen was higher than that of F_1 and BC_1 (*B. oleracea*) generations [30].

Another feature of agronomic importance is the size of nectaries. Nectaries secrete floral nectar, which attracts flower pollinators. Flowers of *Brassica* spp. have two pairs, which differ in structure and location [55]. Lateral nectaries are found above the base of shorter stamens, whereas median nectaries are located external to the base of the long stamen. In our study, the lateral nectaries of hexaploid plants were significantly larger than their triploid F_1 progenitors (Table 2, Figure S1). This characteristic suggests that flowers of allohexaploid *Brassica* hybrids may be more attractive to pollinators.

Our observations agree with previous studies showing that the *Brassica* genome is characterised by high plasticity with respect to the level of ploidy. Besides the described triploids, tetraploids, and hexaploids, there are also successful examples of octoploid formation with a significantly changed appearance [11,56]. For example, autoalloctoploids *B. napus* (AAAACCCC; 2n = 8x = 76) were obtained by doubling the genome of allotetraploid *B. napus*. Compared to tetraploids, octoploid *B. napus* showed significant differences in phenotype, such as decreased vegetative organ size, increased number of pollen apertures, moderate reproductive ability, high pollen variability, and a relatively stable meiotic process [11].

In our study, we confirmed that meiotic polyploidisation can be a promising method to obtain new germplasm showing the traits of both *B. napus* and *B. oleracea* that can be used as initial material for the development of new types of leafy vegetables.

5. Conclusions

Allohexaploid AACCCC *Brassica* hybrids of the F_2 generation were created by crossing diploid kale and head cabbage with tetraploid *B. napus*, followed by self-pollination. A positive correlation between genome size and chromosome number was found in F_1 and F_2 interspecific hybrids of *B. oleracea* × *B. napus*. Allohexaploid F_2 hybrids showed abundant phenotypic and anatomical variations, including larger and thicker leaves and larger flowers than the diploid and tetraploid parents and triploid F_1 hybrids. Stomata size was generally positively correlated with ploidy level. Interspecific hybrids of the F_2 generation derived by open crosses between plants of the F_1 generation (FC320, FC230) had a good seed set ability. Moreover, a comparative anatomical analysis showed that leaves of FC320 F_2 hybrids were characterised by a thicker and more compact mesophyll structure than other genotypes.

All F_2 hybrids were also male fertile, and pollen fertility of the F_2 population was significantly increased compared to F1 hybrids and even reached normal levels of the diploid and tetraploid parents. Analysed hybrids can be used for breeding *Brassica* at the hexaploid level, potentially leading to the development of new polyploid leafy vegetables.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12010026/s1, Figure S1: Phenotypic characteristic of flowers and pollen viability of parental genotypes and the interspecific hybrids of *B. oleracea* × *B. napus* of the F_1 and F_2 generations; Figure S2: Morphological and anatomical characteristics of leaves and flower stacks.

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A.M.-C.; writing—original draft, A.M.-C. and P.K.; writing—review and editing, A.M.-C., P.K. and M.P. All authors have read and agreed to the published version of the manuscript.

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References

1. Masterson, J. Stomatal size in fossil plants: Evidence for polyploidy in majority of Angiosperms. *Science* **1994**, *264*, 421–424. [CrossRef]
2. Casacuberta, J.M.; Jackson, S.; Panaud, O.; Purugganan, M.; Wendel, J. Evolution of plant phenotypes, from genomes to traits. *G3 Genes Genomes Genet.* **2016**, *6*, 775–778. [CrossRef]
3. Sattler, M.C.; Carvalho, C.R.; Clarindo, W.R. The ploidy and its key role in plant breeding. *Planta* **2016**, *243*, 281–296. [CrossRef]
4. Marasek-Ciolakowska, A.; Sochacki, D.; Marciniak, P. Breeding aspects of selected ornamental bulbous crops. *Agronomy* **2021**, *11*, 1709. [CrossRef]
5. Kreiner, J.M.; Kron, P.; Husband, B.C. Frequency and maintenance of unreduced gametes in natural plant populations: Associations with reproductive mode, life history and genome size. *New Phytol.* **2017**, *214*, 879–889. [CrossRef]
6. Ramsey, J.; Schemske, D.W. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annu. Rev. Ecol. Syst.* **1998**, *29*, 467–501. [CrossRef]
7. Pecinka, A.; Fang, W.; Rehmsmeier, M.; Levy, A.A.; Scheid, O.M. Polyploidization increases meiotic recombination frequency in Arabidopsis. *BMC Biol.* **2011**, *9*, 24. [CrossRef] [PubMed]
8. Dar, J.A.; Beigh, Z.A.; Wani, A.A. Polyploidy: Evolution and crop improvement. In *Chromosome Structure and Aberration*; Chapter 10; Bhat, T.A., Wani, A.A., Eds.; Springer: New Delhi, India, 2017; pp. 201–217.
9. Podwyszyńska, M.; Marasek-Ciolakowska, A. Ploidy, Genome Size, and Cytogenetics of Apple. In *The Apple Genome, Compendium of Plant Genomes*; Korban, S.S., Ed.; Springer: Cham, Switzerland, 2021. [CrossRef]
10. Lage, J.; Trethowan, R.M. CIMMYT's use of synthetic hexaploid wheat in breeding for adaptation to rainfed environments globally. *Aus. J. Agri. Res.* **2008**, *59*, 461–469. [CrossRef]
11. Yin, L.; Zhu, Z.; Luo, X.; Huang, L.; Li, Y.; Mason, A.S.; Yang, J.; Ge, X.; Long, Y.; Wang, J.; et al. Genome-Wide duplication of allotetraploid *Brassica napus* produces novel characteristics and extensive ploidy variation in self-pollinated progeny. *G3 Genes Genomes Genet.* **2020**, *10*, 3687–3699. [CrossRef]
12. Cheng, F.; Wu, J.; Wang, X. Genome triplication drove the diversification of *Brassica* plants. *Hortic. Res.* **2014**, *1*, 14024. [CrossRef]
13. Katche, E.; Quezada-Martinez, D.; Katche, E.I.; Vasquez-Teuber, P.; Mason, A.S. Interspecific hybridization for *Brassica* crop improvement. *Crop Breed. Genet. Genom.* **2019**, *1*, e190007. [CrossRef]
14. Nagaharu, U. Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. *Jap. J. Bot.* **1935**, *7*, 389–453.
15. Chalhoub, B.; Denoeud, F.; Liu, S.; Parkin, I.A.; Tang, H.; Wang, X.; Chiquet, J.; Belcram, H.; Tong, C.; Samans, B.; et al. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science* **2014**, *345*, 950–953. [CrossRef]
16. Roy, N.N. Interspecific tranfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus*. *Euphytica* **1984**, *33*, 295–303. [CrossRef]
17. Gerdemann-Knorck, M.; Nielen, S.; Tzscheetzsch, C.; Iglisch, J.; Schieder, O. Transfer of disease resistance within the genus *Brassica* through asymmetric somatic hybridization. *Euphytica* **1995**, *85*, 247–253. [CrossRef]
18. Sharma, B.B.; Kalia, P.; Singh, D.; Sharma, T.R. Introgression of Black Rot Resistance from *Brassica carinata* to Cauliflower (*Brassica oleracea botrytis* Group) through Embryo Rescue. *Front. Plant Sci.* **2017**, *8*, 1255. [CrossRef]
19. Tonguc, M.; Griffiths, P.D. Transfer of powdery mildew resistance from *Brassica carinata* to *Brassica oleracea* through embryo rescue. *Plant Breed.* **2008**, *123*, 587–589. [CrossRef]
20. Mafakheri, M.; Kordrostami, M. Biotechnological Approach for Enhancing Capability of *Brassica oleracea* var. *italica* Against Stresses Under Changing Climate. In *The Plant Family Brassicaceae*; Hasanuzzaman, M., Ed.; Springer: Singapore, 2020. [CrossRef]
21. Seepaul, R.; Kumar, S.; Iboyi, J.E.; Bashyal, M.; Stansly, T.L.; Bennett, R.; Boote, K.J.; Mulvaney, M.J.; Small, I.M.; George, S.; et al. Brassica carinata: Biology and agronomy as a biofuel crop. *GCB Bioenergy* **2021**, *13*, 582–599. [CrossRef]
22. Li, Z.; Wang, Y. Cytogenetics and germplasm enrichment in Brassica allopolyploids in China. *J. Integr. Agric.* **2017**, *16*, 2698–2708. [CrossRef]
23. Wang, G.-X.; Tang, Y.; Yan, H.; Sheng, X.-G.; Hao, W.-W.; Zhang, L.; Lu, K.; Liu, F. Production and characterization of interspecific somatic hybrids between *Brassica oleracea* var. *botrytis* and *B. nigra* and their progenies for the selection of advanced pre-breeding materials. *Plant Cell Rep.* **2011**, *30*, 1811–1821. [CrossRef]

24. Gaebelein, R.; Alnajar, D.; Koopmann, B.; Mason, A.S. Hybrids between *Brassica napus* and *B. nigra* show frequent pairing between the B and A/C genomes and resistance to blackleg. *Chromosome Res.* **2019**, *27*, 221–236. [CrossRef]
25. Wei, Y.; Zhu, M.; Qiao, H.; Li, F.; Zhang, S.; Zhang, S.; Zhang, H. Characterization of interspecific hybrid between flowering Chinese cabbage and broccoli. *Scientia Hortic.* **2018**, *240*, 552–557. [CrossRef]
26. Lee, S.S.; Lee, S.A.; Yang, J.; Kim, J. Developing stable progenies of ×*Brassicoraphanus*, an intergeneric allopolyploid between *Brassica rapa* and *Raphanus sativus*, through induced mutation using microspore culture. *Theor. Appl. Genet.* **2011**, *122*, 885–891. [CrossRef]
27. Dixon, G.R. *Vegetable Brassicas and Related Crucifers*; CAB International: Wallingford, UK, 2007; 327p.
28. Starzycki, M.; Starzycka, E.; Pszczoła, J. Development of Alloplasmic Rape. *Adv. Bot. Res.* **2007**, *45*, 313–335.
29. Kamiński, P.; Podwyszyńska, M.; Starzycki, M.; Starzycka-Korbas, E. 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. *Euphytica* **2016**, *208*, 519–534. [CrossRef]
30. Kamiński, P.; Marasek-Ciolakowska, A.; Podwyszyńska, M.; Starzycki, M.; Starszycka-Korbas, E.; Nowak, K. Development and characteristics of interspecific hybrids between *Brassica oleracea* L. and *B. napus* L. *Agronomy* **2020**, *10*, 1339. [CrossRef]
31. Podwyszyńska, M.; Trzewik, A.; Marasek-Ciołakowska, A. In vitro polyploidisation of tulips (*Tulipa gesneriana* L.)—Phenotype assessment of tetraploids. *Sci. Hortic.* **2018**, *242*, 155–163. [CrossRef]
32. Marasek-Ciolakowska, A.; He, H.; Bijman, P.; Ramanna, M.S.; Arens, P.; van Tuyl, J.M. Assessment of intergenomic recombination through GISH analysis of F1, BC1 and BC2 progenies of *Tulipa gesneriana* and *T. fosteriana*. *Plant Syst. Evol.* **2012**, *298*, 887–899. [CrossRef]
33. Alexander, M.P. Differential staining of aborted and nonaborted pollen. *Stain. Technol.* **1969**, *44*, 117–122. [CrossRef]
34. Marasek-Ciolakowska, A.; Saniewski, M.; Dziurka, M.; Kowalska, U.; Góraj-Koniarska, J.; Ueda, J.; Miyamota, K. Formation of the secondary abscission zone Induced by the interaction of methyl jasmonate and auxin in *Bryophyllum calycinum*: Relevance to auxin status and histology. *Int. J. Mol. Sci.* **2020**, *21*, 2784. [CrossRef] [PubMed]
35. Dyki, B.; Habdas, H. Metoda izolowania epidermy liści pomidora i ogórka dla mikroskopowej oceny rozwoju grzybów patogenicznych. (The method of isolation of epidermis of tomato and cucumber leaves for microscopic investigation of pathogenic fungus development). *Acta Agrobot.* **1996**, *49*, 123–129. (In Polish) [CrossRef]
36. Pradhan, A.; Plummer, J.A.; Neslon, M.N.; Cowling, W.A.; Yan, G. Successful induction of trigenomic hexaploid *Brassica* from a triploid hybrid of *B. napus* L. and *B. nigra* (L.) Koch. *Euphytica* **2010**, *176*, 87–98. [CrossRef]
37. Meng, J.L.; Shi, S.; Li, G.; Li, Z.; Qu, X. The production of yellow-seeded *Brassica napus* (AACC) through crossing interspecific hybrids of *B. campestris* (AA) and *B. carianata* (BBCC) with *B. napus*. *Euphytica* **1998**, *103*, 329–333. [CrossRef]
38. Zhang, K.; Mason, A.S.; Fariiq, M.A.; Islam, F.; Quezada-Martinez, D.; Hu, D.; Yang, S.; Zou, J.; Zhou, W. Challenges and prospects for a potential allohexaploid *Brassica* crop. *Theor. App. Genet.* **2021**, *134*, 2711–2726. [CrossRef] [PubMed]
39. Mason, A.S.; Yan, G.; Cowling, W.A.; Nelson, M.N. A new method for producing allohexaploid *Brassica* through unreduced gametes. *Euphytica* **2012**, *186*, 277–287. [CrossRef]
40. Silkova, O.G.; Shchapova, A.I.; Shumny, V.K. Patterns of meiosis in ABDR amphihaploids depend on the specific type of univalent chromosome division. *Euphytica* **2011**, *178*, 415–426. [CrossRef]
41. Xiong, Z.Y.; Gaeta, R.T.; Pires, J.C. Homoeologous shufing and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 7908–7913. [CrossRef]
42. Bhatia, R.; Sharma, K.; Parkash, C.; Pramanik, A.; Singh, D.; Singh, S.; Kumar, R.; Dey, S.S. Microspore derived population developed from an inter-specific hybrid (*Brassica oleracea* × *B. carinata*) through a modifed protocol provides insight into B genome derived black rot resistance and inter-genomic interaction. *Plant Cell Tissue Organ Cult. (PCTOC)* **2021**, *145*, 417–434. [CrossRef]
43. He, D.; Jing, J.; Snowdon, R.J.; Mason, A.S.; Shen, J.; Meng, J.; Zou, J. Exploring the gene pool of *Brassica napus* by genomics-based approaches. *Plant Biotech. J.* **2021**, *19*, 1693–1712.
44. Yuan, S.-x.; Liu, Y.-m.; Fang, Z.-y.; Yang, L.-m.; Zhuang, M.; Zhang, Y.-y.; Sun, P.-t. Study on the relationship between the ploidy level of microspore-derived plants and the number of chloroplasts in stomatal guard cells in *Brassica oleracea*. *Agr. Sci. China* **2009**, *8*, 939–946. [CrossRef]
45. Sohn, S.-I.; Oh, Y.-J.; Lee, K.-R.; Ko, H.-C.; Cho, H.-S.; Lee, Y.-H.; Chang, A. Characteristics analysis of F1 hybrids between genetically modified *Brassica napus* and *B. rapa*. *PLoS ONE* **2016**, *11*, e0162103. [CrossRef] [PubMed]
46. Dewitte, A.; Van Laere, K.; Van Huylenbroeck, J. Use of 2n gametes in plant breeding. In *Plant Breeding*; Abdurakhmonov, I.Y., Ed.; Intech.: Rijeka, Croatia, 2011; pp. 59–86.
47. Padoan, D.; Mossad, A.; Chiancone, B.; Germana, M.A.; Kjan, P.S.S.V. Ploidy levels in Citrus clementine affects leaf morphology, stomatal density and water content. *Theor. Exp. Plant. Physiol.* **2013**, *25*, 283–290. [CrossRef]
48. Podwyszyńska, M.; Markiewicz, M.; Broniarek-Niemiec, A.; Matysiak, B.; Marasek-Ciolakowska, A. Apple autotetraploids with enhanced resistance to apple scab (*Venturia inaequalis*) due to genome duplication-phenotypic and genetic evaluation. *Int. J. Mol. Sci.* **2021**, *22*, 527. [CrossRef]
49. Brownlee, C. The long and short of stomata density signals. *Trends Plant Sci.* **2001**, *6*, 441–442. [CrossRef]
50. Li, M.; Qian, W.; Meng, J.; Li, Z. Construction of novel *Brassica napus* genotypes through chromosomal substitution and elimination using interploid species hybridization. *Chromosome Res.* **2004**, *12*, 417–426. [CrossRef]

51. Seki, K. Leaf-morphology-assisted selection for resistance to two-spotted spider mite *Tetranychus uriticae* Koch (Acari: Tetranychidae) in carnations (*Dianthus caryophyllus* L.). *Pest Manag. Sci.* **2016**, *72*, 1926–1933. [CrossRef]
52. Goiana, E.S.S.; Dias-Pini, N.S.; Muniz, C.R.; Soares, A.A.; Alves, J.C.; Vidal-Neto, F.C.; Da Silva, C.S.B. Dwarf-cashew resistance to whitefly (*Aleurodicus cocois*) linked to morphological and histochemical characteristics of leaves. *Pest Manag. Sci.* **2020**, *76*, 464–471. [CrossRef] [PubMed]
53. Marasek-Ciolakowska, A.; Soika, G.; Warabieda, W.; Kowalska, U.; Rybczyński, D. Investigation on the relationship between morphological and anatomical characteristic of savoy cabbage and kale leaves and infestation by cabbage whitefly (*Aleyrodes proletella* L.). *Agronomy* **2021**, *11*, 275. [CrossRef]
54. Malek, M.A.; Rahman, L.; Das, M.L.; Hassan, L.; Rafii, M.Y.; Ismail, M.R. Development of hexaploid *Brassica* (AABBCC) from hybrids (ABC) of *Brassica carinata* (BBCC) × *B. rapa* (AA). *Aust. J. Crop Sci.* **2013**, *7*, 1375–1382.
55. Davis, A.R.; Fowke, L.C.; Sawhney, V.K.; Low, N.H. Floral nectar secretion and ploidy in *Brassica rapa* and *B. napus* (Brassicaceae). II. Quantified variability of nectary structure and function in rapid-cycling lines. *Ann. Bot.* **1996**, *77*, 223–234. [CrossRef]
56. Liu, S.; Liu, Y.; Yang, X.; Tong, C.; Edwards, D.; Parkin, I.A.; Zhao, M.; Ma, J.; Yu, J.; Huang, S.; et al. The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploid genomes. *Nat. Commun.* **2014**, *5*, 3930. [CrossRef] [PubMed]