Brassica oleracea

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Brassica oleracea L. is an important vegetable, fodder, and ornamental diploid (2n = 18) species which belongs to the genus *Brassica* and mustard family Brassicaceae Burnett. *B. oleracea* probably originates from the Western Mediterranean region, Great Britain and Northern-Central China.

Keywords: Brassica oleracea ; heterosis ; male sterility ; plant reproduction

1. Introduction

According to the habit of the plant and its edible parts, *B. oleracea* can be divided into seven different varieties or cultivars: Capitata Group (cabbage, savoy cabbage, red cabbage; *B. oleracea* var. *capitata*), Acephala Group (kale, borecole, collards; *B. oleracea* var. *acephala*) and Tronchuda Group (Portuguese cabbage, seakale cabbage), Italica Group (purple sprouting, sprouting broccoli; *B. oleracea* var. *italica*), Botrytis Group (broccoli, cauliflower, broccoflower, calabrese; *B. oleracea* var. *botrytis*), Gongylodes Group (kohlrabi, knol-kohl; *B. oleracea* var. *gongylodes*), Gemmifera Group (sprouts, Brussels sprouts; *B. oleracea* var. *gemmifera*), and Alboglabra Group (Chinese kale, Chinese broccoli, gai lan, kai lan; *B. oleracea* var. *alboglabra*) [1][2]. *B. oleracea* vegetables are rich in nutrients [3], have strong adaptability and resistance to stress environments, and are widely cultivated all over the world. According to the Food and Agriculture Organization (FAO) statistics, the global cabbage, and other brassicas harvest area in 2018 was 2.41 million hm^2 (http://faostat.fao.org/), and cabbage is one of the main vegetables consumed in Europe, North and South America, Asia, and Oceania.

B. oleracea vegetables are typically cross-pollinated crops. Across *B. oleracea* strong heterosis is displayed on yield, quality, disease resistance, and stress tolerance [4]. Heterosis breeding is the main way of improving *B. oleracea* varieties. At present, most *B. oleracea* varieties used in agriculture are hybrids. In heterosis breeding, self-incompatible and male sterile lines can be used to produce *B. oleracea* hybrid seeds. Self-incompatibility genes are ubiquitous across *B. oleracea*. When using self-incompatible lines to produce hybrids, the hybrid seeds can be obtained from both parents, so seed yield is high [5]. Before the 21st century, *B. oleracea* hybrids were mainly produced using self-incompatible lines, such as Jingfeng No.1, the first cabbage hybrid in China. However, this method has some shortcomings. For example, it is difficult to attain a 100% hybridization rate. Inbred lines will degenerate after multiple generations of selfing events and propagating inbred lines through artificial pollination during the bud stage is costly [5].

Male sterility refers to the degeneration or loss of function of male organs in bisexual plants. When male sterile lines are used to produce hybrids, the hybridization rate can reach 100%. Moreover, the maintenance of male sterile lines can be self-compatible lines that are propagated by bees, which could save labor and reduce the overall production cost [5]. Due to these reasons, scientists have always placed great importance on male sterility in *B. oleracea*.

2. Types and Genetic Characteristics of Male Sterility in *B. oleracea*

Ever since the German botanist Joseph Gottlieb Kolreuter first reported the male sterility in plants back in 1763, the male-sterile phenomenon has been found across 43 families, 162 genera, and 320 species of plants [6][7]. According to the genetic characteristics of male sterile genes, male sterility can be divided into genic male sterility (GMS) and cytoplasmic male sterility (CMS). GMS is controlled by nuclear genes and CMS is controlled by mitochondrial genes and a smaller subset of nuclear genes [8].

2.1. Genic Male Sterility (GMS)

GMS in *B. oleracea* includes mainly dominant genic male sterility (DGMS) and recessive genic male sterility (RGMS). At present, most GMS in *B. oleracea* crops were found to be controlled by recessive nuclear genes. For example, 83121A is a spontaneous male sterile mutant in cabbage. Genetic analysis showed that male sterility was controlled by a recessive

gene in cabbage. The 83121A line exhibits normal vegetative development, has fully open flowers, well-developed nectaries, and normal pistils. However, the anthers of 83121A are severely degraded and have no pollen grains [9][10]. RGMS does not have a typical maintenance line, and at most, only 50% of male sterile plants can be obtained from test cross progeny.

The cabbage breeding group of the Chinese Academy of Agricultural Sciences (CAAS) discovered the dominant male sterile plant DGMS79-399-3 in the 1970s. Genetic analysis showed that male sterility of DGMS79-399-3 was controlled by a dominant gene and was affected by other small-effect genes [11]. A few sensitive DGMS plants produced traces of pollen induced at low temperatures. After selfing with the trace pollen, the ratio of male sterile plants to fertile plants in the progeny was 3:1, from which dominant homozygous sterile plants could be obtained. DGMS lines have beneficial economic characteristics, large flowers, and well-developed nectaries [5]. The DGMS has been successfully applied in the production of cabbage hybrids.

2.2. Cytoplasmic Male Sterility (CMS)

Most CMS in *B. oleracea* was transferred from other cruciferous crops (e.g., *Raphanus sativus* L. and *B. napus* L.). The main types of CMS include Ogura (Ogu) CMS, Polima (Pol) CMS, and Nigra (Nig) CMS.

2.2.1. Ogu CMS

Ogu CMS is a completely infertile, naturally mutated type of CMS found in radish (*Raphanus raphanistrum* subsp. *sativus* L.) [12]. So far, Ogu CMS is the most widely studied and widely used type of male sterility in cruciferous vegetable breeding. This sterility type is induced by the interaction of a homozygous nuclear gene $rf_{og}rf_{og}$ and a sterile Ogu cytoplasm. Ogu CMS in radish was originally transferred to cabbage by distant hybridization and embryo rescue in order to obtain Ogu CMS R1 [13]. Because the nucleus and cytoplasm are not coordinated, the nectaries and pistils of Ogu CMS R1 do not develop normally, and the leaves of Ogu CMS R1 are yellow at low temperatures. Using asymmetric protoplast fusion technology, radish chloroplasts in Ogu CMS R1 were successfully replaced with broccoli chloroplasts to obtain Ogu CMS R2, which do not turn yellow at low temperatures, but its siliques are deformed and its nectaries degenerate after multiple generations of backcrossing [14]. Following Ogu CMS R2, the company U.S. Asgrow reorganized the mitochondria of Ogu CMS R2, again, through asymmetric protoplast fusion technology and obtained Ogu CMS R3, which has stable sterility, does not turn yellow at low temperatures, and has well-developed siliques [15]. Similarly, the well-known *Brassica* Ogu-INRA CMS was also obtained by the plant somatic fusion method [16]. These male sterile lines derived from Ogu CMS R3 or Ogu-INRA CMS have been widely used for the production of cabbage hybrids.

2.2.2. Pol CMS

Pol CMS was discovered in a homonymous rapeseed (*B. napus* L.) variety bred in Poland [17]. Its male sterility was controlled by both cytoplasmic and nuclear genes. Concerning the temperature dependence of the male sterility, Pol CMS lines can be divided into three types: low-temperature, high-temperature, and stable CMS lines [18][19][20][21]. It is easy to find Pol CMS fertility-restored materials in *B. napus*, *B. campestris*, and *B. juncea*. Both two-line and three-line schemes are used to produce the F_1 hybrids based on the Pol CMS [21]. The Pol CMS was transferred from *B. napus* to *B. oleracea* by using the protoplast fusion method [20]. However, the obtained male sterile plants showed abnormal development of flowers and siliques, as well as incomplete pollen abortion, which meant that the male sterile type could not be used for breeding.

2.2.3. Nig CMS

The F_1 of *B. nigra* (wild mustard) × *B. oleracea* (broccoli) was treated with colchicine to double the number of chromosomes and was then repeatedly backcrossed with cabbage to obtain Nig CMS [22]. The Nig CMS fertility-restored materials could be found in cabbage and kale. However, most Nig CMS flowers do not open normally and its nectaries degenerate markedly. Moreover, the proportion of male sterile plants in the test cross progeny was only 33.7–60.0%, which means that the male sterile type could not be used for breeding.

3. Cytological Study of Male Sterility in *B. oleracea*

The main goal of plant male sterility cytology research is to determine the abortion period and abortion mode of microspores, as well as to explore the factors leading to microspore abortion from a cell morphology perspective. A large number of studies have shown that microspore abortion can occur at any stage of microspore development, including from the archesporial cell stage to the mature pollen stage [6]. The peak period of microspore abortion occurs from the tetrad stage to the unicellular stage [6].

The main male sterile stages and characteristics for the four types of male sterility in *B. oleracea* were analyzed in detail by paraffin section, scanning electron microscopy, and transmission electron microscopy techniques. Microspore abortion of RGMS 83121A occurred at the early unicellular stage [10]. The tapetum of 83121A was strikingly degraded at the uninucleate stage. The development of microspores in 83121A stopped at the uninucleate stage and was followed by breakdown. Moreover, microspores of 83121A did not form pollen exine after being released from the tetrad [10]. DGMS abortion occurred at the late tetrad stage. The important characteristic of DGMS was the abnormal development of the tapetum. Moreover, the pollen mother cell primary wall surrounding the developing microspores in DGMS remained intact until the very late pollen stage [23][24]. Nigra CMS abortion occurred at the sporogenesis cell stage with abnormal tapetal cell differentiation and development [24]. Ogu CMS abortion often occurred at the early tetrad stage, and its abnormal activities of tapetal cells were observed after meiosis. Most of the Ogu CMS microspores were released from tetrads and then were aborted after being squashed by hypertrophic tapetum cells [24].

In conclusion, although the abortion period and abortion characteristics of the various male sterility types in *B. oleracea* are different, almost all of them show abnormal development of the tapetum. The tapetum is the innermost cell of the anther wall. It transports various nutrients to the pollen mother cell and plays a key role in the development of the pollen mother cells and microspores [25][26]. Many studies have shown that abnormalities in the differentiation and development of tapetum cells can directly or indirectly lead to pollen abortion and male sterility [27].

4. Molecular Biological Study of Male Sterility in *B. oleracea*

4.1. Expression Analysis of Male Sterile Related Genes

The expression of DGMS related genes in cabbage was studied at the transcriptional level using the cDNA-AFLP differential display method. The results showed that the expression of a dominant male sterile gene (*Ms-cd1*) may hinder the normal release of microspores in tetrads and inhibit the expression of genes encoding pectin methylesterase, pectase lyase, thioredoxin, rapid alkalinization factor, and proline-rich protein [23]. Using an *Arabidopsis* whole-genome microarray, the genome-wide gene expression profiles during anther abortion of four types of *B. oleracea* male sterility (Nig CMS, RGMS, Ogu CMS, and DGMS) were comprehensively analyzed. In total, 105 candidate genes specifically expressed in the tapetum were identified [24]. Moreover, it was shown that the main reason for the designation of four types of male sterility was the disturbance of the abnormal tapetum during normal development of the microspores. Label-free quantitative mass spectrometry was used to analyze the differential protein levels of RGMS 83121A and its wild-type buds before the microspore binuclei stage. A total of 1245 protein types were identified to have significant differential abundances [28]. The identified proteins were mainly involved in pollen wall synthesis, fatty acid metabolism, amino acid synthesis, and protein processing modification, suggesting that these metabolic pathways play an important role in cabbage reproductive development.

4.2. Molecular Markers Associated with Male Sterility

Many studies have reported the molecular markers associated with dominant genic male sterile gene *Ms-cd1* in cabbage [29][30]. For example, the SSR (Simple Sequence Repeats) and SRAP (Sequence-related Amplified Polymorphism) markers linked to *Ms-cd1* were obtained by bulk segregant analysis. The genetic distance between SSR marker 8C0909 and *Ms-cd1* was found to be 2.06 cM [30]. Three SRAP markers, ENA14F-CoEm7RSC, ENA20R-rem2SC, and CoEm17RE37SC, were converted into SCAR (Sequence Characterized Amplified Region) markers, and the genetic distances between the SCAR markers and *Ms-cd1* were 0.18, 0.39, and 4.23 cM, respectively [30]. A KASP (Kompetitive Allele Specific PCR) molecular marker closely linked to DGMS was developed from resequencing data and *Ms-cd1* gene mapping [31]. This marker can be used for rapid identification of the dominant male sterile gene locus.

Based on comparative genomic and transcriptomic analysis, *BoCYP704B1* was identified as an important candidate gene linked to RGMS in the 83121A line [10]. Cloning and sequencing showed that a 5424-bp Ty3-gypsy type retrotransposon was inserted in the first exon of *BoCYP704B1* in 83121A. The retrotransposon insertion in *BoCYP704B1* not only blocked gene expression, but also changed the structure of the encoded protein. Molecular markers completely linked to the male sterile gene in 83121A were developed from the mutation of *BoCYP704B1* [10]. Using map-based cloning technology, the RGMS gene *ms3* of cabbage line 51S was fine mapped in a 187.5 kb region on chromosome C01, and *BoTPD1* was identified as a candidate gene for male sterility [32]. It was found that a 182 bp fragment was inserted in the *BoTPD1* gene of the male sterile mutant. The molecular marker designed according to this variant site is closely linked to male sterility and can be used for assisted screening of male sterile plants [32].

CMS in *B. oleracea* is typically regulated by mitochondrial-specific genes. For example, the male sterility of Ogu CMS and Pol CMS is controlled by the mitochondrial specific genes *orf138* and *orf224*, respectively [33][34][35]. According to the sequence of *orf138*, several specific molecular markers were designed, which can be used for the identification of Ogu CMS plants [36][37][38][39].

4.3. The Mechanism of Ogu CMS

Ogu CMS has been widely used in the breeding of *B. oleracea*. Several studies have shown that the Ogu CMS was controlled by *orf138* gene, which was generated by the rearrangement of the mitochondrial genome [40][41]. Previous studies of *orf138* revealed that at least nine variants of *orf138* designated as A, B, …, I were identified; these variants included the F type characterized by a 39-bp deletion [42]. This type was also called *Kosena* according to the name of a radish variety from which the Kos CMS line was obtained [43]. It is well known that the F type variant of *orf138* was also discovered in white-headed cabbage. Studies have shown that the protein encoded by the *orf138* gene would accumulate on the mitochondrial membrane, which may interfere with the expression of some key genes, such as *atp6*, *atp8*, and *cox I*, in the electron-transport chain and inhibit the normal development of anthers [44][45][46][47][48]. The whole mitochondrial genome sequencing showed that the mitochondrial genome of Ogura CMS type was highly rearranged compared with the normal-type genome [49]. Four unique regions were generated from the rearrangement in Ogu CMS mitochondrial genome, and most of the unique regions are composed of known Brassicaceae mitochondrial sequences [49]. The results suggested that the unique regions of Ogu CMS mitochondrial genome were produced by integration and shuffling of pre-existing mitochondrial sequences during the evolution of Brassicaceae, and novel genes such as *orf138* may have been generated from the shuffling process of the mitochondrial genome [49]. The conjoint analysis of transcriptome and proteome suggested that the tapetum programmed cell death was disturbed and the synthesis of sporopollenin was inhibited in Ogu CMS cabbage [50].

4.4. The Fertility-Restored Gene Rfo of Ogu CMS

Studies have shown that the Ogu CMS fertility-restored materials only exist in R. sativus [51][52][53]. At present, the Ogu restorer materials have been found in European radish, Japanese radish, and Chinese radish. The restorer nuclear gene in the Ogu CMS restorer line can disturb the stability of the protein ORF138, which would reduce the accumulation of ORF138, leading to the restoration of fertility [47][47,54]. The restorer locus Rfo has been obtained by map-based cloning [55][56]. Studies showed that the Rfo locus contains three genes (PPR-A, PPR-B, and PPR-C) organized in tandem, which are predicted to encode highly similar proteins [57]. PPR-B was genetically defined as the restorer gene and is predicted to encode a pentatricopeptide repeat (PPR) protein comprising 17 PPR repeats. Compared with PPR-B, the protein encoded by PPR-A has a longer C-terminal tail and a deletion of four amino acids in the third PPR repeat. The PPR-C gene contains a 17-bp deletion compared with PPR-A and PPR-B, which leads to a frameshift and a premature stop codon about in the middle of the gene. Genetic transformation experiments further showed that only PPR-B instead of PPR-A and PPR-C can restore the fertility of Ogu CMS [57]. Koizuka et al. (2003) also cloned the fertility-restored gene orf687 (Rfk) of radish Kos CMS, which was identified as the same gene as Rfo [58]. In addition to Rfo, some new restorer genes have been found in R. sativus, and most of them are homologous genes of Rfo. For example, a novel fertility-restored gene Rft controlling fertility restoration of Ogu CMS was identified in Japanese wild radish [59]. In addition, a number of new homologous genes of Rfo were found in Chinese radish materials. These genes, including Rfob, Rfoc, RsRf3-1/RsRf3-2, RsRf3-4, and RsRf3-5, were mainly produced by recombination during hybridization [60][61][62][63][64].

4.5. Other Male Sterile Related Genes in B. oleracea

Male sterile plants can be used as a useful tool to study the expression patterns and biological functions of anther development-related genes [65]. Many genes associated with male sterility have been cloned, such as *BoBHLH1*, *BoMF1*, *BoMF2*, and *BoMYB1*. The *BoBHLH1* gene is downregulated in Ogu CMS and encodes the bHLH transcription factor, which is homologous to *Arabidopsis* AtBHLH151 and is induced by jasmonic acid signaling. *BoBHLH1* is preferentially expressed in cabbage anthers and has two expression peaks in the early and late stages of anther development [66]. The promoter of Ogu CMS related gene *BoMF1* from *B. oleracea* was cloned by genomic walking. The promoter was able to drive the GUS gene that is exclusively expressed in anther and pollen of *Arabidopsis thaliana* [67]. *BoMF2* encodes the AT-hook DNA binding protein and was found to be up-regulated in Ogu CMS. *BoMF2* was mainly expressed in wild-type cabbage stamens during the tetrad stage. However, *BoMF2* expression continued into the mature pollen stage of Ogu CMS flowers [68]. *Arabidopsis* with overexpression of *BoMF2* showed significantly shorter siliques than the wild type, as well as a decrease in pollen viability [68]. *BoMYB1* encodes a MYB transcription factor and was downregulated in Ogu CMS. This gene was preferentially expressed in cabbage anthers and reached its expression peak in the late stage of anther development. The expression of *BoMYB1* was induced by the plant hormones salicylic acid and methyl jasmonate and regulated the expression of anther development genes [69][70].

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