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Review

Advances in Genetics and Molecular Breeding of Broccoli

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Abstract: Broccoli (*Brassica oleracea* L. var. *italica*) is one of the most important vegetable crops cultivated worldwide. The market demand for broccoli is still increasing due to its richness in vitamins, anthocyanins, mineral substances, fiber, secondary metabolites and other nutrients. The famous secondary metabolites, glucosinolates, sulforaphane and selenium have protective effects against cancer. Significant progress has been made in fine-mapping and cloning genes that are responsible for important traits; this progress provides a foundation for marker-assisted selection (MAS) in broccoli breeding. Genetic engineering by the well-developed *Agrobacterium tumefaciens*-mediated transformation in broccoli has contributed to the improvement of quality; postharvest life; glucosinolate and sulforaphane content; and resistance to insects, pathogens and abiotic stresses. Here, we review recent progress in the genetics and molecular breeding of broccoli. Future perspectives for improving broccoli are also briefly discussed.

Keywords: broccoli; progress; genetic researches; molecular breeding

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1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is a member of the Brassicaceae family and is widely cultivated as an important vegetable crop worldwide [1,2]. It produces edible hypertrophic reproductive organs (floral head and stalk), with rich health benefits and nutritious properties, such as vitamin A, vitamin K, calcium, magnesium and anticancer bioactive compounds, including glucosinolates, sulforaphane, selenium and flavonoids [3–5].

The *italica* group arises from the cultivation and domestication of *Brassica oleracea* (CC genome; 2n = 18) in the Mediterranean region. Accurate knowledge about the cultivation of *B. oleracea* mustard plants can be traced to the Hellenic culture, starting in approximately the 6th century BC [6]. By distinguishing the *B. oleracea* cultivars, 'Broccoli' is probably a colloquial Latin word for any projecting shoots of the cabbage family [6]. Broccoli-like varieties were developed from selections of desirable *B. oleracea* types during the past 2000 years and formed various broccoli landraces mainly in Italy [6–9]. The broccoli variety 'Vrocculi o Sparaceddi' is considered the first domesticated form of wild brassica from which broccoli originated [10]. During the past 300 years, the heading broccoli has greatly improved, largely attributed to selection by Danish and English horticulturists [6]. For a long time, the consumption of broccoli as a vegetable was confined to the Italian peninsula and it was grown mainly as sprouting broccoli cultivars [10]. With the breeding and improvement of calabrese broccoli varieties, a particular type producing large and compact heads more similar to cauliflower, broccoli spread and gained popularity worldwide [10,11]. Various broccoli landraces were introduced to the United Kingdom in the 1700s and to the United States in the 1800s and became popular after World War II [11]. Broccoli was initially introduced into several southern provinces of China in the 1980s and has been a popular vegetable widely grown in China. In recent years, China, with a cultivation area

of over 80,000 ha, has become the largest producer of broccoli in the world [12]. Driven by scientific evidence that broccoli is beneficial to human health, the market demand is still increasing in the main broccoli-producing countries, including China, the US and India [12–14].

With the development of molecular biology technology and functional genomics, a large number of studies on broccoli have been performed. Marker-assisted selection (MAS) and genetic transformation were combined with conventional breeding to improve broccoli for high yield, quality, resistance to biotic and abiotic stresses, etc. We review the recent progress on the genetics and molecular breeding of broccoli, focusing on desirable agronomic traits, male sterility, abiotic stress resistance, disease resistance, secondary metabolites and genetic transformation. Postharvest yellowing (or prolonging shelf life) of broccoli is also a research hotspot that has been reviewed recently and thus is not included in this review [2]. Broccoli improvement by genetic engineering was reviewed in 2016 [1], so relevant advances in recent years from 2016 to 2021 are included in this review.

2. Genetics and Molecular Breeding of Broccoli

2.1. Abiotic Stress Resistance

2.1.1. Heat Stress

Broccoli production faces challenges of demand to extend plant areas and maintain production security under extreme weather brought by climate change [13,15]. Broccoli is suitable for growth in cool weather with optimal temperatures ranging from 15 to 23 °C during the early stages of floral development [16]. High temperatures above 25 severely reduce broccoli quality because (1) most broccoli germplasms require vernalization at temperatures below 23 °C and superoptimal temperatures would even result in no head formation; (2) some broccoli germplasms do not require vernalization, but floral development under high temperatures (e.g., above 30 °C) results in undesirable traits, such as bracting, uneven head surface and sizes of buds, discoloration or even brown bead, making the broccoli products unmarketable; and (3) high temperatures during the head maturity stage decrease broccoli yield [15,17,18]. In recent years, substantial progress has been made in creating heat-tolerant breeding lines and genetically controlling heat tolerance in broccoli. In the USA, researchers have made efforts to achieve sustainable broccoli production under heat tolerance in the main production area on the east coast, supported by projects (National Institute of Food and Agriculture (NIFA) Project No. 2010-51181-21062 and the USDA Vegetable Brassica Research Project (CRIS No. 6080-21000-019-00D)) [16,19,20]. In Asia, researchers are trying to introduce broccoli to subtropical and tropical regions, such as in Taiwan, China and Indonesia [21,22].

The ability to produce high-quality heads by several broccoli germplasms under heat stress is considered a quantitative trait controlling multiple positive loci [13,15]. Lin et al. identified 31 QTLs for head size and weight phenotypes of broccoli grown in high-temperature seasons (average 36.4 °C day/25.9 °C) [23]. Branham et al. constructed a high-density genetic map by genotyping-by-sequencing of a DH broccoli segregating population for heat tolerance and identified five QTLs and one positive epistatic interaction between *QHT_C03* and *QHT_C05*, explaining 62.1% of phenotypic variation [15]. Using a new DH population of broccoli, Branham et al. performed whole-genome resequencing of bulked segregants and identified two novel heat tolerance QTLs, of which *QHT_C09.2* may explain the negative correlation between maturity and heat tolerance [13].

Using reversed genetic approaches, a heat-stress-related broccoli catalase gene was cloned, and ectopic expression of this gene in *Arabidopsis* can enhance heat tolerance, but whether it plays a role in maintaining a high-quality head under high temperatures is still unknown [24,25]. In addition, benefiting from improved sequencing techniques and the release of reference genomes, some researchers performed omics-related studies and identified differentially expressed microRNAs/genes and potential pathways involved in heat tolerance [26,27].

2.1.2. Other Abiotic Stresses

Several studies have focused on broccoli resistance to other stresses, such as proteomic analysis for waterlogging stresses [28], microRNA analysis for salt stress [29] and transcriptome and metabolomics for wounding stress [30]; differentially expressed proteins/microRNAs/genes were identified as possibly related to resistance to these stresses [28–30].

In addition, cuticular waxes on the plant surface contribute to resistance to many environmental stresses, such as drought, UV light, high radiation and both bacterial and fungal pathogens [31]. Some loci and linked markers for this trait have been obtained. Using a natural glossy (cuticular wax defective) mutant, Branham and Farnham identified three candidates, *Bo3g001070*, *Bo3g122030* and *Bo3g008780*, for this trait on C03 [32]. In the broccoli × Chinese kale-derived BolTBDH population, leaf color was segregated, which resulted from the differences in cuticular waxes between broccoli and Chinese kale; a locus for this trait, *LC_C09@15.1*, was identified on C09, explaining 45.64% of the phenotypic variation [33].

2.2. Desirable Agronomic Traits

2.2.1. Heading

Broccoli produces edible reproductive organs characterized by proliferation and developmental arrest of floral buds [17]. Floral head quality is the most important agronomic trait selected by breeders. With forward and reversed genetic approaches, some genes/loci related to head formation have been identified, but the genetic basis remains elusive [34].

Some works tried to identify homologs of the Arabidopsis floral meristem identity genes *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) and implied that *BoCAL* and *BoAP1* are involved in curding in cauliflower, a subspecies similar to broccoli but different in the developmental stage of the reproductive meristem at harvest [35–37]. Subsequent studies suggest that heading is quite complex in both cauliflower and broccoli, which seems not to be controlled solely by these floral genes [34].

In the 1990s, researchers started to construct genetic maps by crossing broccoli cultivars/inbred lines with various materials, including broccoli cultivar/landrace, cabbage, cauliflower, kale and Chinese kale, to detect loci of important traits, such as disease resistance, head morphology, nutritional quality and flowering/maturation time [38–42]. Several quantitative trait loci influencing head traits, including head weight, head height/width and floret height/width, have been identified, but the early constructed genetic maps are hard to unify [23,42,43] due to the differences in plant germplasm, marker types and linkage group nomenclature and the lack of *B. oleracea* reference genomes before 2014. Using a double-haploid BolTBDH mapping population derived from Early Big (broccoli DH line) and TO1000DH3 (nonhead Chinese kale), Stansell et al. identified heading-quality QTLs, including *BU_C04@51.5*, *BR_C09@49.5*, *HC_C09@48.8*, *HU_C09@48.8*, *HE_C09@47.7* and *OQ_C09@49.5* (Table S1), and found genomic regions of approximately 49 Mb on C09 harboring *FLOWERING LOCUS C* (*FLC*) homologs *Bo9g173400* and *Bo9g173370*, as hotspots contributing largely to over 40% phenotypic variance of the heading phenotype [33]. In another study, three head quality QTLs, *qCQ-2*, *qCQ-3* and *qCQ-6*, associated with subtropical adaptation were identified [21]; and specific haplotype combinations of candidates *BoFLC3* in the interval containing *qCQ-3* and *PERIANTHIA* (*PAN*, a bZIP-transcription factor required for *AGAMOUS* activation) in the interval containing *qCQ-6*, were supposed to adapt broccoli to high ambient temperature and short daylength. Along with these key head-related traits, QTL mapping for bud morphology was also reported by Stansell et al. and Lin et al. [21,33]. These studies provide genetic information and breeding materials for improving broccoli varieties.

2.2.2. Flowering Time

Flowering is an important agronomic trait of broccoli, as it influences maturity, head quality, hybrid seed production and geographical region adaptation. Flowering time is con-

sidered to be controlled by multiple QTLs. To detect QTLs/genes associated with this trait, segregation populations were generated by crossing broccoli with different germplasms, such as broccoli × cabbage, broccoli × Chinese kale and broccoli × broccoli. Different QTLs were detected in these studies, even using similar populations, such as broccoli × cabbage and broccoli × Chinese kale [21,33,44–47]. Most studies have implied that flowering is largely controlled by one or a few major QTLs [21,33,44–47]. As early as the late 1990s, using populations of broccoli (nonvernalization type) × cabbage (vernalization type), broccoli (late flowering type) × Chinese kale (early flowering type), several QTLs for flowering time were mapped [44–47]. Two subsequent studies used a similar population derived from broccoli × cabbage but obtained different results [48,49], possibly due to the differences of the specific germplasms used and the planting environmental conditions. Okazaki et al. detected six QTLs controlling flowering time (from February to July, 2001, Niigata, Niigata Prefecture, Japan), among which the major QTL in the interval BRMS215–F2-R4b, accounting for 36.8% of the phenotypic variance and *BoFLC2* in the interval is thought to be the candidate control of flowering time [48]. Similarly, using a broccoli × cabbage population, Shu et al. combined QTL-seq and a traditional linkage map to detect flowering time loci (from the spring of 2013 to the winter of 2014, Beijing China). A major QTL *Ef2.1* is located on C02 2.65–2.68 Mb, responsible for early flowering and explaining 51.5% of the phenotypic variation, and a homolog of *GROWTH-REGULATING FACTOR 6* (*BolGRF6*) is a possible candidate [49]. Using DH populations of broccoli × Chinese kale, Stansell et al. (2019) identified two QTLs *DM_C03@6.4* and *DM_C09@50.0* for days to maturity, two QTLs *DF_C03@6.4* and *DF_C09@50.0* for days to flowering, and the major QTLs *DM_C09@50.0* and *DF_C09@50.0* on C09 at approximately 50 Mb, explaining approximately 50% of the phenotypic variation [33].

Broccoli is usually sensitive and not feasible in high-temperature areas/seasons, which are thought to impede vernalization, resulting in defects in floral meristem development. Using tropical accessions in Taiwan, China, Lin et al. 2018 identified nonvernalization-responsive QTLs that contribute to subtropical adaptation (high ambient temperature and short day length) [21]. The candidate gene *BoFLC3* identified in the major QTL *qDCI-3* may function as an alternative pathway for the control of flowering in temperate and tropical environments [21].

2.2.3. Plant Architecture

Plant architecture is a complex trait attributed to stem and leaf morphologies, including plant height, leaf size, leaf shape, leaf angle, petiole length and lateral shoot growth. It affects the planting density, yield and quality of broccoli [50]. Several QTLs for plant architecture-relevant stem and leaf traits have been reported [23,27,32,51,52]. Before the release of reference genomes, researchers mapped QTLs associated with leaf lamina width on linkage groups C01 and C07 [51], stem width on LG5 [23], leaf apex on linkage groups C06 and C07 [43], leaf shape on linkage group C3 [43], leaf length on linkage group C7 [43], wing petiole length on linkage group C7 [43] and lobe number, wing number, leaf shape and lamina petiole length on linkage group C3 [43]. In recent years, in addition to focusing on heading traits, Stansell et al. mapped several QTLs for leaf morphology and lateral shoots. Four QTLs for leaf apex, two QTLs for leaf margin and leaf-associated hotspot genomic regions, *Lea3* on C03 0.7–1.7 Mb and *Lea7* on C07 37.0–39.5 Mb were identified. A *GRF1-INTERACTING FACTOR 1* (*GIF1*) homolog (*Bo7g093130*) within major QTL *LA_C07@36.6* may be responsible for the narrow leaf phenotype, and a *LATE MERISTEM IDENTITY1* ortholog (*BoLMI1, Bo3g002560*) near the major *LM_C03@0.7*, explaining over 40% phenotype variation, may be responsible for leaf margin phenotype [33]. Three lateral shoot growth-associated QTLs, *LT_C03@5.9*, *LT_C04@15.0* and *LT_C09@9.0*, are located on C03, C04 and C09, although no likely candidates were predicted [33]. Huang et al. constructed a genetic linkage map using a broccoli DH population and identified QTLs for plant height (PH), maximum outer petiole length (PL) and leaf width (LW), including

major QTLs *phc1* for PH on chromosome 1, *plc6−2* for PL on chromosome 6 and *lwc3−1* for LW on chromosome 3 [52].

2.2.4. Stem Development

Broccoli hollow stem is an undesirable phenotypic disorder showing symptoms of cracks in the internal stem tissue [53,54]. It reduces the quality of broccoli products because hollow stems can result in (1) yield reduction, as harvested broccoli comprise partially edible stalks; (2) secondary pathogen infection and rotting of stems and florets [38,39]. The incidence of hollow stems increases when plants grow rapidly, triggered by, for example, high levels of nitrogenous and warm weather but also varies in different broccoli accessions, indicating that this trait is largely genetically determined and can be controlled by breeding resistant varieties [53,54]. However, relevant studies on this trait are very limited. Yu et al. constructed a genetic map using specific locus-amplified fragment (SLAF) sequencing in a double-haploid segregation population of broccoli and defined nine QTLs on C02, C03, C05, C06 and C09 for hollow stems, among which *QHS. C09-2* could explain 14.1% of the phenotypic variation [55].

2.2.5. Head Color

Broccoli is rich in anthocyanin, an important nutritional value with antioxidant activity, can improve health, increase life expectancy and prevent diseases [56]. Anthocyanin accumulation in broccoli inflorescences, especially in septals, makes the appearance range from green/blue to purple. Some cultivars, such as 'Purple Sprouting Early', are selected for rich anthocyanin contents, producing obvious purple heads [56]. Purple traits in *B. oleracea* are attributed to the independent activation of *Brassica oleracea MYB DOMAIN PROTEIN 2* (*BoMYB2*) in subspecies of cabbage, cauliflower, kohlrabi and possibly broccoli [57].

On the other hand, broccoli cultivars producing heads with green-purple color are considered not beautiful and would be less attractive to consumers than the completely green type, especially in the market of China [58]. This green-purple type is sensitive to temperature, and cool weather would induce and deepen the purple degree. Yu et al. mapped this purple sepal trait using a DH population and SLAF sequencing; three QTLs were detected, with a major locus, *qPH. C01–2*, located on linkage group (LG)1, and two loci, *qPH. C01–4* and *qPH. C01–5*, located near *qPH. C01–2* [59].

2.3. Male Sterility and Fertility Restoration

Broccoli displays obvious heterosis and most commercial broccoli varieties are F1 hybrids. The production of broccoli F1 hybrids depends on self-incompatibility before the early 21st century and now nearly completely depends on male sterility-based breeding systems [11,12,59]. Male sterility comprises cytoplasmic male sterility (CMS) and genic male sterility (GMS) [60]. Among them, Ogura CMS, with the advantages of complete male gamete abortion, maternal inheritance and easy transfer, is now the most widely studied and applied male sterility source in broccoli seed production [60,61]. Ogura CMS is a natural mutation found in radish populations [62], which is caused by a mitochondrial gene named *orf138*, and can be fully restored by the nuclear gene *RFO* (*PPR-B*) [63,64].

Researchers have made efforts to introduce the CMS source to *B. oleracea* by distant hybridization and/or protoplast fusion, but the initially created CMSR1 and CMSR2 contain too much radish cytoplasm, displaying undesirable characteristics, including yellowing at low temperature, deformed flower shape and poor seed setting, which cannot be used in seed production [65–67]. Until the late 1990s, the American Asgrow company applied the method of asymmetric protoplast fusion to reduce the proportion of radish mitochondria, creating CMSR3 with normal fertility and pistil structure; this CMS has been transferred to many elite parent lines, playing a dominant role in the seed production of *B. oleracea* crops [67,68]. During the creation and transfer processes of Ogura CMS, specific *orf138* PCR markers were developed for MAS [69]. Additional mitochondrial markers were developed to distinguish the CMS types; detected by these six *orf138*-related and two simple sequence

repeat markers in 2016, Shu et al. divided 39 CMS broccoli accessions into five groups, and observed that CMSR3 constituted 79.49% of the CMS accessions from China [67].

In addition to the Ogura CMS, GMS resources and GMS-based seed production systems were reported as promising alternatives [60,70–73]. A special dominant genic male sterility (DGMS) resource, 79–399–3, which arose in cabbage populations in China, has been successfully and widely applied in cabbage hybrid seed production [61,72]. The DGMS-based breeding system has been established in *B. oleracea* crops, including cabbage, broccoli and kohlrabi [61,72]. Compared with the Ogura CMS, the DGMS-based breeding system displayed advantages of much higher seed quality and yield [61]. However, its utilization is limited in broccoli, largely because homozygous DGMS plants must be preserved and reproduced by tissue culture, which is not effective for large-scale hybrid seed production [61]. Despite these disadvantages, this DGMS-based system has been preserved as an alternative for broccoli hybrid seed production. In recent decades, dozens of broccoli DGMS lines have been created, and several markers have been developed for MAS for the rapid creation of DGMS lines [61,74]. Shu et al. developed generic SSR markers linked to the male-sterile gene, with the marker scaffold10312a showed the highest accuracy of $\geq$96.43% [74]. By distinguishing the amplified products polyacrylamide gel, these markers were successfully used for identification of male and sterile plants in broccoli breeding lines DGMs8554, DGMs93219 and DGMs94174; enabled DGMS plants selection in the seedling stage. Han et al. developed a high-throughput kompetitive allele specific PCR (KASP) marker K6 with high accuracy and no genetic background bias applicable to all *B. oleracea* crops, including broccoli [61]. This marker was based on allele specific fluorescence on an Applied Biosystems Viia 7 real-time PCR system for high-throughput detection. In the DGMS-based breeding system, this marker was used for identifying homozygous DGMS plants from selfing progenies of heterozygous plants as an alternative to test crossing, which requires at least two years and additional labor in tissue culture [61]. These DGMS-specific markers enable effective selection in breeding programs.

On the other hand, there is increasing demand for the reutilization of CMS resources in *B. oleracea* crops. The Ogura CMS restorer gene *RFO* (*PPR-B*) was introduced from radish to rapeseed and recently to *B. oleracea* crops [75,76]. Liu et al. applied strategies of interspecific hybridization and backcrossing and introduced the *RFO* gene from rapeseed to broccoli. The foreground Rfo-specific markers BnRFO-AS2F/BnRFO-AS2F and BnRFO-AS2F/BnRFO-NEW-R, were used for detecting *Rfo*-positive interspecific hybrids; and 28 background SSR markers were used for detecting true intergeneric hybrids and assessing the genetic backgrounds of *Rfo*-positive interspecific hybrids. By evaluating polymorphism loci of the 28 background markers, the BC2 *Rfo*-positive individuals were found closer to the broccoli's genetic background [76].

2.4. Disease Resistance

2.4.1. Downy Mildew

Downy mildew, caused by the obligate fungus *Hyaloperonospora parasitica* (Pers. Fr.), is a destructive disease that affects brassica crops, including broccoli [77,78]. Broccoli plants are often stunned or killed when infected with downy mildew at the young seedling stage or infection can result in quality reduction and yield loss at the adult stage [79,80]. The disease is prevalent in cool weather, with initial symptoms of light green-yellow lesions on the upper leaf surface and later on the undersurface; the spot enlarges and turns yellow; white fungi are visible on the undersurface of leaves under high humidity conditions [79,80]. High resistance to downy mildew both at the young and adult stages is present in some broccoli germplasms and is controlled by a single dominant locus [79,81–84]. Resistance loci were mapped and linkage markers were developed for MAS, but the gene has not been cloned [79,82,83]. Giovannelli et al. identified 8 RAPD (random amplification of polymorphic DNA) markers linked to downy mildew resistance in broccoli (cotyledon and true leaf stage), among which two, $UBC3596_{620}$ and $OPM16_{750}$, were converted to SCAR (sequence characterized amplified regions) markers linked to the locus with 6.7 and

3.3 cM [82]. Farinhó et al. mapped the locus *Pp523* for downy mildew resistance to adult plants of broccoli and developed flanking RAPD markers OPK17_980 and AFLP marker AT. CTA_133/134, with genetic distances of 3.1 cM and 3.6 cM, respectively [83]; in a later study, new AFLP markers were developed and some of them were more user-friendly SCAR and CAPS (cleaved amplified polymorphic sequence) markers; sequencing indicated that *Pp523* is syntenic to the top arm end of *Arabidopsis thaliana* chromosome 1 [79]. We aligned the marker sequences to the broccoli HDEM reference genome [85] and found that the target *Pp523* region is 49.29–50.68 Mb on C8.

2.4.2. Clubroot

Clubroot, caused by the soil-borne pathogen *Plasmodiophora brassicae*, is one of the most devastating diseases of Brassica crops, including broccoli [86–88]. Plants infected by the pathogen form galls on roots, which prevent plant uptake of nutrients and water and become stunted and wilt under warm weather [89]. *B. olearcea* lacks germplasm highly resistant to clubroot, although it has been identified and studied for mining resistance loci/genes in its close relatives, such as turnip, radish and rapeseed [90–93]. The resistance gene *CRa* has been introduced from *B. rapa* to *B. olearcea* by distant hybridization and MAS [94]; in this process *CRa*-specific markers SC2930-Q-FW/SC2930-RV were applied for detection of *CRa* gene in the F1 and each backcross plants, enabled successful introgression of the *CRa* gene into the cabbage inbred lines. In recent years, commercial broccoli varieties with the *CRa* resistance gene, bred by the Syngenta Corporation, are available on the market of China, but the MAS process is not available.

While highly clubroot-resistant germplasms are lacking, some moderate clubroot resistance has been identified in *B. oleracea* [95–97]. There are two studies on genetic mapping for resistance loci related to broccoli, although both of them used broccoli as susceptible parents. These studies are useful for the rapid introduction of clubroot resistance from other subspecies/related species to broccoli with MAS [95,96]. Rocherieux et al. generated F2:3 segregation populations by crossing clubroot-resistant kale and clubroot-susceptible broccoli and constructed a restriction fragment length polymorphism (RFLP) based genetic map. The populations were infected by five isolates and two to five QTLs were identified depending on the isolates; one of these QTLs, *Pb-Bo1*, showed broad-spectrum resistance detected in all isolates [95]. Using populations of crossing resistant double-haploid line (Anju) with a susceptible double-haploid line (GC), Nagaoka et al. identified five CR-QTLs, *pb-Bo(Anju)1*, *PbBo(Anju)2*, *PbBo(Anju)3* and *PbBo(Anju)4* derived from Anju and *pb-Bo(GC)1* from the susceptible parent GC; this study also provided specific primer sequences linked to CR loci and a comparison with known *B. rapa* CR genes [96].

2.4.3. Black Rot

Black rot, caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Pam.) Dowson (*Xcc*), is also one of the most destructive diseases of brassica crops in the world [98,99]. The pathogen often invades plants through hydathodes and spreads through vascular tissue, forming V-shaped lesions at the leaf margins, causing systemic infection and great loss of quality and yield [98–100]. While some resistant plant resources have been reported in *B. oleracea*, few loci/genes have been identified [101–103]. Camargo et al. identified genomic regions associated with young and adult plant resistance to black rot in linkage groups 1, 2 and 9 using a population of black rot-resistant cabbage line BI-16 and susceptible inbred broccoli line OSU Cr-7 [38]. Doullah et al. identified two genomic regions on LG 2 and LG 9 significantly associated with resistance to black rot, with a disease rating of populations from susceptible broccoli green commet P09 and resistant Reiho P01 [104]. In a later study using the same plant materials, Tonu et al. improved the previous genetic map and identified three QTLs, *XccBo(Reiho)1*, *XccBo(Reiho)2 and XccBo(Reiho)1,* for resistance to black rot, and the major QTL, *XccBo(Reiho)2*, was from parent Reiho [105]; comparison using common markers of the previous study by Camargo et al. revealed that *XccBo(Reiho)1* and *XccBo(GC)1* may be identical to the previously reported QTLs [104,105].

Iglesias-Bernabé et al. performed QTL analysis of black rot resistance (*Xcc* race 1) in the BolTBDH mapping population and identified four QTLs, including *Xcc1.1* showing overlap with the previously reported cabbage resistance locus *BRQTL-C1_1*, *BRQTL-C1_2* [106], *Xcc6.1* showing overlap with *BRQTL-C6*, *Xcc8.1* showing overlap with *XccBo(Reiho)2* [105] and a novel locus, *Xcc9.1* [107]; in addition, this study indicated that resistance might be related to the synthesis of secondary metabolites [107].

2.5. Secondary Metabolites

Broccoli contains a number of beneficial secondary metabolites, including glucosinolates/sulforaphane, carotenoids, phenolic acids and flavonoids. Several loci/genes regulating the accumulation of these compounds in broccoli have been identified. Genetic models of secondary metabolite biosynthesis in *Arabidopsis* provide a convenient tool for homologous studies in broccoli [108,109]. Via a homologous cloning strategy, some broccoli genes are isolated directly, including *cytochrome P450 79F1* (*CYP79F1*), *cytochrome P450 83A1* (*CYP83A1*), *UDP-glucosyltransferase 74B1* (*UGT74B1*), *sulfotransferase 18* (*ST5b*) and flavin-containing monooxygenase *GS-OX1* (*FMOGS-OX1*), *cytochrome P45083B1* (*BoCYP83B1*), *BoMYB51*, *GSL-PRO*, *GSL-ELONG*, *GSL-ALK*, *GSL-OH*, *Myb28* and *BoMYB51* for glucosinolate biosynthesis [109–113], and *BoPAL*, *BoDFR*, *BoTT8* and *BoTTG1* for anthocyanin biosynthesis [114]. Genetic loci determining the variation in these secondary metabolites were also detected by genetic mapping. Sotelo et al. performed genetic analysis to identify the genome regions regulating glucosinolate biosynthesis in the DH mapping population BolTBDH and detected eighty-two significant QTLs for individual and total glucosinolate synthesis in leaves, seeds and flower buds, and *QTL9.2* (proposed candidate as *GSL-ALK*) plays a central role in determining glucosinolate variation, showing epistatic interactions with other loci [115]. Brown et al. constructed a genetic linkage map with a broccoli mapping population, identified 14 QTLs associated with the accumulation of aliphatic, indolic or aromatic glucosinolates in florets, and a locus *GSL12* on C09 explains approximately 40% of the phenotypic variability of progoitrin [116]. Li et al. performed genetic mapping for sulforaphane metabolism with a DH population; 18 QTLs for sulforaphane metabolism in broccoli florets were identified, and six QTLs among them were detected in more than one environment [117]. Using the same population previously reported [116], Brown et al. constructed a genetic linkage map with an SNP array and identified three QTLs for carotenoid variation in broccoli florets [118]. Gardner et al. performed QTL analysis saturated with SNP markers in an Illumina 60 K array for total phenolic concentration and its individual components in the population previously reported by Brown et al. [118] and obtained twenty-three loci identified in at least two analyses [119]. In the BolTBDH mapping population, 33 QTLs were identified controlling phenolic concentrations in leaves, flower buds and seeds [120]. In addition, transcriptome analyses were performed to identify differentially expressed genes related to glucosinolate metabolism in broccoli seeds, sprouts and byproducts [121–123].

2.6. Development of Omics Research

Advances in techniques and reduced costs of high-throughput next- and third-generation sequencing have brought high-throughput tools for genomic-related studies and the improvement of broccoli. In 2014, *B. oleracea* draft genome-based short reads of the next generation were released [124,125]; in 2018, the first broccoli (HDEM) reference genome, a high-quality daft genome based on third-generation nanopore long reads and optical maps, was accessible [85]. These studies provided information on genome duplication and gene divergence and the direct prediction of genes related to phytochemicals and morphological variations and, as mentioned above, provided a reference for high density marker development [97,116,118]. Bulked-segregant analysis combined with whole genome resequencing (BSA-seq) for rapid gene/QTL mapping and candidate searching [13,32] and omics-related studies exploring differentially expressed genes/miRNAs related to important traits [26–30,121–123]. In addition, high-throughput strategies promote KASP marker-based fingerprinting for the essential

broccoli germplasm [126], genetic diversity and population structure analysis for broccoli cultivars [11,127], and the genomic and morphological domestication syndrome of broccoli calabrese landraces, hybrids and sprouting broccoli [11].

2.7. Genome Editing

Genome editing is a powerful tool for efficient and targeted genome manipulations in living organisms. Depending on the genome editing tools, four engineered nucleases were developed: Meganucleases [128], zinc finger nucleases (ZFNs) [129], transcription activator-like effector-based nucleases (TALENs) [130] and short palindromic repeat (CRISPR)-associated protein (Cas9) systems [131,132]. CRISPR/Cas9 has proven to be a cost-effective and versatile tool for precise and efficient genome editing and in recent years, it has been extensively studied and applied to manipulate desired genes in plants [133]. While it has been realized in some *B. oleracea* crops [134,135], genome editing by CRISPR/Cas9 has not succeeded in complete broccoli background plants. Only one study applied this tool to broccoli-related plant material DH1012, a doubled haploid genotype from the crossing of *B. oleracea alboglabra* (A12DHd) with *B. oleracea italica* (Green Duke GDDH33), targeting *BolC.GA4.a* (*Bol038154*), resulting in dwarf stature [136,137].

2.8. Genetic Transformation

Agrobacterium-mediated transformation in broccoli was first reported by Metz et al. [138]. In the last decade, this genetic engineering tool has been applied for improving broccoli regarding (1) insect resistance by the genes *cry1A(c)*, *cry1C* and *cryIA(b)*; (2) fungal resistance by the Trichoderma harzianum endochitinase gene, *PR-1* and *PR-2*; (3) abiotic stress resistance by *AtHSP101*; (4) herbicide resistance by *Bar* gene; (5) prolonged shelf-life/delayed postharvest yellowing by *ipt* (isopentenyl transferase) gene, *ACC synthase 1*, *BoCLH1* and *ACC oxidase* gene; and (6) flowering control by *CYP86MF*, *SLG*, *FCA* and *CONSTANS*, which has been reviewed by Kumar and Srivastava in 2016 [1]. Thus, we review the advances of broccoli transgenic improvement in recent years (Table 1).

Table 1. Broccoli improvement by genetic transformation in recent years.

Gene Transferred	Origin	Recipient Plant	Performance	References
BoAPX	broccoli	broccoli	enhanced resistance to downy mildew enhanced tolerance to heat stress	[139]
BoWRKY6	broccoli	broccoli	enhanced resistance to downy mildew	[140]
BoiCesA (RNAi)	broccoli	broccoli	enhanced salt tolerance; dwarf and smaller leaves	[141]
BoC3H	broccoli	broccoli	enhanced salt stress tolerance	[142]
BoC3H4	broccoli	broccoli	enhanced salt stress tolerance; more susceptible to *S. sclerotiorum*	[143]
BoERF1	broccoli	broccoli	enhanced salt stress tolerance; enhanced resistance to Sclerotinia stem rot	[144]
cryIAa	Bacillus thuringiensis	broccoli	resistance to diamondback moth	[145]
BoMYB29	wild *B. oleracea*	DH line AG1012, (partial broccoli background)	increased glucosinolate content	[146]
BoTSB1, BoTSB2	broccoli	Arabidopsis	increased glucosinolate content	[147]
BroMYB28 (transient overexpression)	broccoli	broccoli	increased glucoraphanin content	[148]

Table 1. *Cont.*

Gene Transferred	Origin	Recipient Plant	Performance	References
MAM1	broccoli	broccoli	increased sulforaphane content	[149]
FMOGS–OX2	broccoli	broccoli	increased sulforaphane content	[149]
Myrosinase	broccoli	broccoli	increased sulforaphane content	[149]
BoiDAD1F (RNAi)	broccoli	broccoli	recoverable male sterility	[150]
bol-miR171b	broccoli	broccoli	nearly completely male sterile and increased the chlorophyll content	[151]

2.8.1. Transgenic Breeding for Fungal Resistance

In two independent studies, Jiang et al. generated transgenic broccoli plants overexpressing the cytosolic ascorbate peroxidase gene *BoAPX* and the WRKY transcription factor gene *BoWRKY6*; both of them obtained enhanced resistance to downy mildew [139,140]. *BoAPX*-overexpressing broccoli, with a lower level of electrical conductivity and a higher level of APX enzyme activity, exhibited significantly higher resistance to *Hyaloperonospora parasitica* infection, as well as to heat stress, than wild-type plants [139]. *BoWRKY6*-overexpressing broccoli exhibited significantly increased resistance to downy mildew but varied from low to very high [140]; two of them, lines BWK14 and BWK31, exhibited very high resistance to downy mildew [140].

2.8.2. Transgenic Breeding for Abiotic Stress Resistance

Li et al. generated RNAi transgenic broccoli lines targeting the cellulose synthase gene *BoiCesA*; the *BoiCesA* knockdown plants showed a loss of cellulose content and significantly enhanced salt tolerance, and the expression of related genes (*BoiProH*, *BoiPIP2;2*, *BoiPIP2;3*) was significantly changed but also displayed phenotypic defects characterized by dwarfs and smaller leaves [141].

In three independent studies, Jiang et al. reported that the overexpression of the C3H-type zinc finger genes *BoC3H* and *BoC3H4* and the ethylene response transcription factor gene *BoERF1* enhanced salt stress tolerance [142–144]. The BoC3H-overexpression lines exhibited higher germination rates, dry weight and chlorophyll content under salt stress and less cell death in the leaves due to the decreased hydrogen peroxide level, relative electrical conductivity and malondialdehyde contents but increased free proline content and catalase, peroxidase and superoxide dismutase enzyme activities [142]. The *BoC3H4*-overexpression lines exhibited increased salinity stress tolerance, with an increase in proline and H_2O_2 and a decrease in chlorophyll loss, MDA and REC compared with WT plants; however, the lines were more susceptible to *S. sclerotiorum*, possibly due to the inhibited expression of the *BoPDF1.2* gene [143]. The *BoERF1*-overexpression lines exhibited a higher seed germination rate and less chlorophyll loss under salt stress, with less cell death in the leaves similar to the *BoC3H*-overexpression lines; in addition, the transgenic lines showed enhanced resistance to *Sclerotinia* stem rot [144].

2.8.3. Transgenic Breeding for Insect Resistance

Transgenic broccoli for insect resistance was extensively studied in the late 1990s and the beginning of the 21st century [1], but in recent years there have been few related studies. Kumar et al. generated transgenic broccoli overexpressing *cryIAa*, which showed effective resistance to infestation by diamondback moth (*Plutella xylostella*) larvae [145].

2.8.4. Transgenic Breeding for Enriched Glucosinolate/Sulforaphane Content

In recent years, improving the anticancer metabolite glucosinolate/sulforaphane content in broccoli by the genetic engineering of biosynthesis-/regulation-related genes has increased [146–149,152]. Zuluaga et al. reported that the overexpression of *BoMYB29* in DH line AG1012 resulted in the upregulation of the aliphatic glucosinolate pathway and

higher production of methylsulphinylalkyl glucosinolates, including glucoraphanin [146]. Li et al. isolated two tryptophan synthase beta subunit (TSB) genes from broccoli and generated overexpression lines of *BoTSB1* or *BoTSB2* in Arabidopsis, which showed accumulation of tryptophan, indole-3-acetic acid (IAA) and indole glucosinolates; this study provides a target for improving glucosinolates, but no broccoli transgenic plants were generated [147]. Studies on *BroMYB28* revealed its possible role in the biosynthesis of glucoraphanin [152], but its function was not proven in broccoli until 2019 [148]. *Agrobacterium*-mediated transient overexpression of *BroMYB28* in broccoli results in the accumulation of glucoraphanin [148]. Cao et al. generated transgenic broccoli by overexpressing *MAM1*, FMO_{GS-OX2} and *Myrosinase* independently or in triple [149]. Compared with wild-type plants, independent transgenes of *MAM1* FMO_{GS-OX2} and *Myrosinase* enhanced sulforaphane content by 1.7–3.4-, 1.6–2.7- and 3.7-fold, while transgenic plants with the triple gene enhanced sulforaphane content by 1.86–5.5-fold [149].

2.8.5. Transgenic Breeding for Manipulating Male Fertility

Creation of a new male-sterile type by genetic engineering strategies can rapidly provide alternative resources for hybrid seed production. Male-sterile transgenic broccoli was reported by Chen et al. via RNAi of the jasmonic acid pathway gene *BoiDAD1F* [150]. These transgenic plants showed male sterility under normal conditions but recovered to fertility when treated with exogenous JA and were thus suitable for utilization in a two-line seed production system [150]. Li et al. reported that the overexpression of a microRNA *bol-miR171b* in broccoli resulted in nearly complete male sterility and increased the chlorophyll content [151].

3. Conclusions and Future Perspectives

In recent years, progress has been made in the molecular breeding of broccoli for agronomic traits, secondary metabolites, male sterility, abiotic stress resistance, disease resistance and insect resistance. MAS facilitates the breeding of heat-stress-resistant varieties and clubroot-resistant varieties. However, the molecular breeding of broccoli is still restrained by a lack of basic research and an unknown genetic basis of most desirable traits. Future research on the molecular breeding of broccoli may pay attention to the following aspects.

3.1. Mining Functional Loci/Genes

Some linked markers and mapped genes/QTLs for desirable traits have been reported, and with the development of sequencing technology in recent years, candidates have been predicted for mapped genes/QTLs. Omics technologies, such as transcriptomics, proteomics and metabolomics, have been employed to understand the mechanism of desirable traits. Despite efforts, quite a few genes in broccoli have been cloned and functionally verified. Further research should focus on mining and functionally verifying more genes/QTLs for guiding and promoting the breeding work of broccoli: (1) As the most desirable traits in broccoli are controlled by complex QTLs, secondary mapping populations, including near-isogenic lines, introgression lines and chromosome segment substitution lines, should be developed for fine mapping and isolation of these genes; (2) by reverse genetics approaches, released databases and advanced sequencing technology can be used to identify more functional genes; and (3) the obtained target genes, neither from fine mapping nor homology cloning, should be verified by transient expression or genetic transformation.

3.2. Improving Broccoli by Landraces or Other B. oleracea Subspecies

Modern broccoli has very narrow genetic diversity, which may cause undesirable quality, yield and resistance. Broccoli landraces (especially from Italy) and other subspecies provide diverse genetic resources with promising traits, such as differential heading type, differential flowering/maturation time, high glucosinolate content and strong disease

resistance. Desirable genes can be introduced by MAS from landraces/different subspecies to breeding materials to improve the quality and extend the genetic diversity of broccoli. A particular case is the head compactness of calabrese broccoli, the most popular broccoli type; in recent decades, head compactness of this broccoli has been significantly enhanced for easy transport and storage, which may be improved via genomic fragment introgression from cauliflower (no published literature).

3.3. Introducing Disease Resistance Genes from Related Species

Broccoli lacks resistance to some devastating diseases, such as clubroot and black rot. To guard broccoli genotypes against these diseases, distant hybridization and MAS can be used to introduce pyramid resistance genes/loci from related species. The clubroot pathogen *P. brassicae* evolved many physiological races showing different infection responses on host plants. Only one resistant locus, *CRa* for race 4, has been introduced from *B. rapa*, which is not enough for sustainable production of broccoli under the threat of other *P. brassicae* races. More resistance genes/loci should be introduced from related species, such as turnip, radish and rapeseed, and pyramided in broccoli. For black rot disease, strong resistance sources have been reported in the A and B genomes of Brassica species, and moderate clubroot resistance has been reported in the C genome of cabbage. These resistance genes/loci can be introduced from *Brassica carinata* and cabbage to broccoli.

3.4. Improving the CRISPR/Cas9 Genome Editing System

The CRISPR/Cas9 system has been proven to be a highly efficient genome editing method in plants. In recent years, this genome editing system has been successfully applied in many crops, including rice, maize, soybean and tomato, for gene function studies and crop improvement, such as high yield, disease resistance, herbicide resistance, ideal plant architecture and other desirable traits. However, the CRISPR/Cas9 system has not been established in broccoli; thus, future studies should pay more attention to improving and employing the CRISPR/Cas9 system for broccoli improvement.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7090280/s1, Table S1: Genetic mapping of genes/QTLs in broccoli.

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