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Review

Understanding *R* Gene Evolution in *Brassica*

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Abstract: *Brassica* crop diseases caused by various pathogens, including viruses, bacteria, fungi and oomycetes, have devastating effects on the plants, leading to significant yield loss. This effect is worsened by the impact of climate change and the pressure to increase cultivation worldwide to feed the burgeoning population. As such, managing *Brassica* diseases has become a challenge demanding a rapid solution. In this review, we provide a detailed introduction of the plant immune system, discuss the evolutionary pattern of both dominant and recessive disease resistance (*R*) genes in *Brassica* and discuss the role of epigenetics in *R* gene evolution. Reviewing the current findings of how *R* genes evolve in *Brassica* spp. provides further insight for the development of creative ideas for crop improvement in relation to breeding sustainable, high quality, disease-resistant *Brassica* crops.

Keywords: *R* genes; evolution; *Brassica*; advanced technology

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1. Introduction

The *Brassica* genus (family Brassicaceae) contains economically important crop species that are widely grown throughout the world in the Americas, Asia, Europe and Oceania [1].There are six *Brassica* species that form the main vegetable and oilseed food crops, including three diploid members, namely *B. rapa* (rapeseed, AA genome, *n* = 10), *B. nigra* (black mustard, BB genome, *n* = 8) and *B. oleracea* (cole crops, CC genome, *n* = 9); there are also three allotetraploid members which were formed from pairwise hybridisation of those three diploid members, namely *B. juncea* (Indian mustard, AABB genome, *n* = 18), *B. napus* (canola, AACC genome, *n* = 19) and *B. carinata* (Ethiopian mustard, BBCC genome, *n* = 17), of which the relationship can be described as the triangle of U [2].

Brassicas, which are closely related to *Arabidopsis* (tribe Arabideae), are a member of the tribe Brassiceae within the family Brassicaceae, constituting nearly 50% of the entire 3740 species in the family [3], and are represented by large morphological diversity [4]. After the split of the Arabideae and Brassiceae tribes 5–9 million years ago, whole-genome triplication (WGT) of the hexaploid *Brassica* ancestor occurred, leading to massive chromosomal rearrangements, with re-construction to a more stabilised diploid *Brassica* species belonging to the Brassiceae tribe through many rounds of polyploidisation [5–7]. The benefit of the WGT event unique to the *Brassica* lineage is the expansion of genes related to abiotic and biotic stress adaptability along with plant hormonal networks [5].

One of the challenges in sustaining *Brassica* crop yields is biotic stress. Various pathogens, including viruses, bacteria, fungi and oomycetes, can infect *Brassica* crops, causing major economic losses. The main diseases of *Brassica* are caused by the bacterium *Xanthomonas campestris* (black rot), turnip mosaic potyvirus (TuMV), the oomycetes *Albugo candida* (white rust) and *Hyaloperonospora parasitica* (downy mildew), the protist *Plasmodiophora brassicae* (clubroot) and the fungi *Alternaria brassicae* (Alternaria blight), *Erysiphe*

cruciferarum (powdery mildew), *Fusarium oxysporum* (Fusarium wilt), *Leptosphaeria maculans* (blackleg), *Neopseudocercosporella capsellae* (white leaf spot), *Sclerotinia sclerotiorum* (Sclerotinia stem rot) and *Verticillium longisporum* (Verticillium wilt), amongst others.

To survive and multiply in the natural ecosystem, plants have developed an innate immune system to defend themselves against pathogen invasion. Substantial research has shown that plants are equipped with a two-tiered innate immune system; pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), in terms of the types of pathogen molecules interacting with the host receptors that activate the immune system [8]. Responsible for non-host resistance, PTI is triggered when cell surface-localised or transmembrane pattern recognition receptors (PRRs) recognise extracellular pathogen/microbial-associated molecular patterns (PAMPs/MAMPs). During the co-evolution of the host–pathogen process, pathogens have developed pathways to evade PTI and secrete effectors that enter plant cytoplasm. ETI is then activated by the recognition of these specific pathogen effectors, or avirulence (Avr) proteins, via corresponding intracellular receptors, leading to a complete resistance. The receptors are mainly encoded by a large resistance gene family featuring nucleotide-binding site leucine-rich repeats (NLRs). Due to the specific recognition involved, this host-mediated resistance is also known as gene-for-gene resistance [9–12]. Proteins encoded by the resistance (*R*) genes are termed R proteins, referring to PRRs and NLRs. PRRs are mainly from the receptor-like kinase (RLK) and receptor-like protein (RLP) gene families [13]. NLRs can be further divided into two subtypes according to domains at their N-terminus. With the presence of a Toll/interleukin-1 receptor (TIR) domain, they are classified as TNLs, while with the presence of a coiled coil (CC) domain, or in a rather rare situation, with the appearance of resistance to the Powdery Mildew 8 (RPW8) domain, they are classified as nTNLs, termed as CNLs or RNLs, respectively [14–17].

Though PTI- and ETI-triggering signals lead to several shared cellular responses, how PTI and ETI interplay spatially to arrest pathogens is poorly understood [18]. Recent groundbreaking studies in the model plant *Arabidopsis* have revealed a revised immunity model in which PTI and ETI undertake their roles with mutual potentiation [18–20]. According to two independent studies in *Arabidopsis*, PTI is required for the effective complete resistance of ETI; in other words, the activation of surface receptors can effectively enhance the intracellular-receptor-dependent hypersensitive cell death response (HR) and impede pathogens from further propagation [19,20]. Furthermore, the activation of NLRs in ETI, such as TIR signalling, augments the accumulation of PRR signalling components and boosts plant defence during PTI by upregulating the transcripts and protein levels in *Arabidopsis* lines induced by the PTI elicitor flg22/nlp20 [18]. The mutual enhancement suggests that breeding for crops with accumulated *R* genes not only strengthens host-mediated resistance but also supports non-host resistance. This synergistic work between PTI and ETI demonstrates the complexity of the plant immune system, which necessitates taking this synergy into consideration in the study of plant resistance gene evolution (Figure 1).

In this review, we expound the sources and variations of *Brassica* resistance genes, highlight forces of evolution on disease resistance genes in *Brassica* species and discuss how these forces shape immunity in the plant, describe the cutting-edge and less studied perspectives related to the study of *R* gene evolution and review the latest technologies applied in this field.

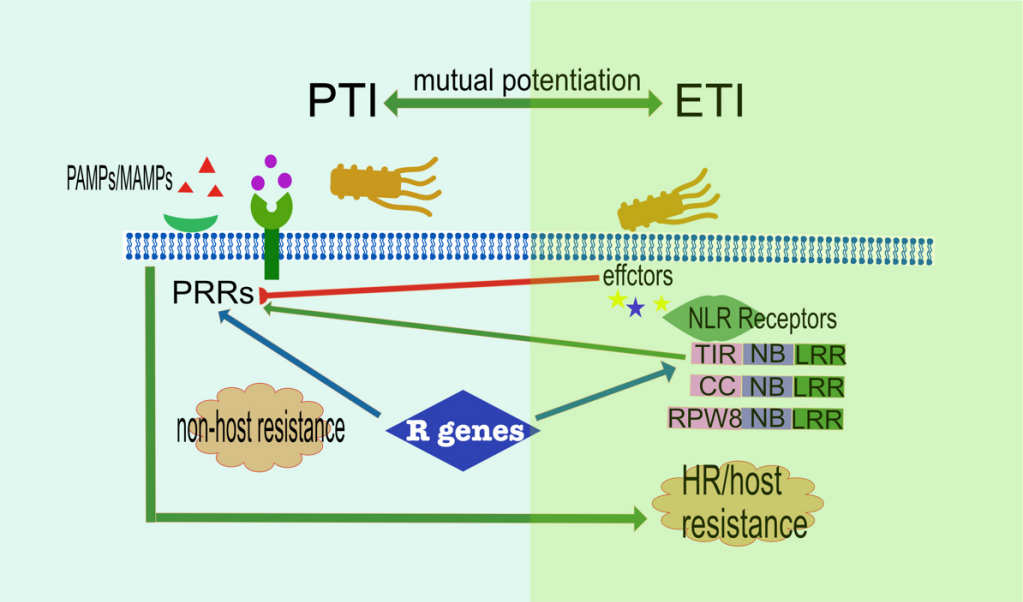

Figure 1. Schematic view of two-tiered immune system of plants. PTI is triggered when surface-localised and transmembrane receptors (PRRs) recognise PAMPs/MAMPs. ETI is triggered when intracellular receptors (NLR receptors) recognise the specific effectors released by pathogens. Effectors hinder the PRRs' recognition to evade PTI (indicated by red flat arrow). PTI and ETI present mutual potentiation (indicated by green arrows), with the activation of surface receptors in PTI enhancing hypersensitive response (HR) in ETI and the signalling of TNL (TIR-NB-LRR) receptor augmenting PTI through upregulated response induced by PTI elicitor such as flg20/nlp20. *R* genes encode receptors important in the immune system (indicated by blue arrows).

2. Evolutionary Origin of *R* Genes

2.1. Polyploid Ancestry

On the evolutionary origin of the *Brassica* species, it has been suggested that ancient *B. rapa* and *B. oleracea* were domesticated separately in China and Europe until both species were introduced and crossed with each other during the opening of Silk Road that links China and the Mediterranean, with further backcrossing creating the diverse morphotypes that we see today [21]. More recently, a genotyping-by-sequencing (GBS) study on the origin of *B. rapa*, the largest diversity study to date on representatives of domesticated and weedy *B. rapa*, pointed towards a true wild relative lineage or highly admixed feral lineage from the Caucasus mountains situated at the intersection between Europe and Asia, with a domestication event of turnips and/or oilseed types in Central Asia happening 3000–5000 years ago, followed by distribution across Europe and East Asia [22]. In *B. oleracea*, an updated RNA-seq study proposed the origin of *B. oleracea* being a monophyletic ancestor coming from the Eastern Mediterranean and the likely progenitors being *B. cretica* and *B. hilarionis*, with events of gene flow happening between these wild species (and other wild relatives such as *B. incana*, *B. montana*, etc.) and cultivated species throughout the domestication process [23]. The origin of *B. juncea* was reported to be in West Asia 8000 to 14,000 years ago via natural interspecific hybridisation with subsequent independent domestication events happening near Central Asia, the Indian subcontinent and East Asian regions, giving rise to different morphotypes of mustards [24].

When investigating the relationship between the *Brassica* species, comparative genomics studies, using mitochondrial and chloroplast DNA, revealed that *B. napus* (AACC) is genetically closer to *B. rapa* (AA), *B. juncea* (AABB) and *B. oleracea* (CC), while *B. nigra* (BB) and *B. carinata* (BBCC) are more diverged from these *Brassica* species but show a closer relationship with *Sinapsis arvensis*, a relative of the *Brassica* species [25]. Whole-genome re-sequencing revealed that the A subgenome of *B. napus* originated from European turnip *B. rapa* and the C subgenome from the ancestor of *B. oleracea*, but it is not known which

B. rapa type—the wild or feral type—and the form of the *B. oleracea* ancestor remains unknown [26]. Nevertheless, gathering all of this information on the historical origin of the *Brassica* genomes provides context on how disease resistance genes evolve across time through polyploidisation and crop-domestication events.

2.2. Disease Resistance Genes from Introgression Lines

Due to the high compatibility of genomes between members of the *Brassica* species, *R* genes can be introgressed through interspecific hybridisation, thereby creating new polyploids with enhanced disease resistance. Aside from producing introgression lines of oilseed *B. napus* using closely related species or wild relatives to obtain novel disease resistance genes, for instance against blackleg [27–29], clubroot [30,31] and Sclerotinia stem rot [32,33], stable allohexaploids with rich sources of *R* genes have now been created using improved cytogenetics techniques. For instance, interspecific triploid hybrids (ABC genomes) resulting from a cross between *B. nigra* (B genome) and *B. napus* (AC genomes) show promising introgression of blackleg resistance genes from the B genome into the A or C genome [34–36]. The application of *Brassica* genus-wide pangenomics (ABC genomes) including related genomes has enabled us to distinguish the donor from the recipient genomes in introgression lines and study the signatures of these hybridised genome patterns [37].

2.3. Studying Disease Resistance Genes from Close Relatives of Brassica

The close genomic relationship between *Arabidopsis* and *Brassica* species allows us to discover important *Brassica* disease resistance genes using the *Arabidopsis* plant system to look for *R* gene homologs. This was recently accomplished for Alternaria blight disease: the disease resistance genes were identified in *A. thaliana* using publicly available microarray datasets and mapped onto the *B. rapa* genome to identify the homologous genes in *B. rapa* [38]. The *Arabidopsis-Pseudomonas syringae* pathosystem shows that RLKs and RLPs are involved in ETI immunity [20], while the *Arabidopsis-Albugo candida* (white rust) pathosystem revealed the involvement of NLRs [39]. Homologous genes of Fusarium wilt resistance (caused by the fungal pathogen *Fusarium oxysporum*) in radish, *Raphanus sativus,* were identified in *A. thaliana*, *B. rapa* and *B. oleracea* genomes [40]. The updated BRAD database, with the expansion of the collection of Brassicaceae genomes, has greatly enhanced our interrogation of *R* gene homologs [41]. Although many *R* gene homologs can be inferred in *Arabidopsis* for many of the *Brassica* diseases, the predicted race-specific candidate *R* genes must still be validated through rigorous gene functionality testing to ensure durable resistance in *Brassica*. With the pan-NLRome available for *A. thaliana* and the proposed pan-RGAome in the *Brassica* genus, species-wide *R* gene homologs can be identified [42,43].

2.4. Structural Variation of Brassica Resistance Genes

A large number of NLR genes against the three major diseases of *Brassica*—blackleg, clubroot and Sclerotinia stem rot—were found in a selective sweep region, implying strong selection pressure associated with the domestication process and promoting quick diversification of the *R* genes in this region [44]. The location of *R* genes in these highly variable regions in *Brassica* has warranted a search for *R* gene candidates using a pangenome approach, revealing signification patterns of SNPs and PAVs (presence/absence variation) influencing the *R* gene diversity in *B. napus* [45,46] and *B. oleracea* [47]. A pangenome is developed from a compilation of genome assemblies coming from various *Brassica* ecotype representatives, and by using sophisticated algorithms, the number and type of core vs. dispensable genes can be uncovered. For example, through the study of the pangenomes of *B. napus* and its progenitors *B. oleracea* and *B. rapa*, it was discovered that gene loss events following polyploidisation in *B. napus* were linked with homoeologous recombination, differentiating them from gene loss events in *B. rapa* and *B. oleracea* linked with transposable elements [48]. Supporting these findings is the *in-silico* sequence comparison of the NBS-

encoding genes in these three *Brassica* genomes, showing gene expansion and loss through non-homologous recombination [49].

The implementation of genomics and bioinformatics tools in the study of *R* genes in *Brassica* species, including members within the Brassicaceae, has greatly increased our understanding about how these genes evolved [17]. Common QTL co-located with NBS genes against blackleg, clubroot and Sclerotinia stem rot have been found in *B. napus* [49], suggesting multiple disease-resistance effects. Tandem and segmental duplications of the NBS gene family are commonly found in polyploids and diploids such as radish (*Raphanus sativus*) [40]. Examples of tandemly duplicated TNL genes responsible for *Plasmodiophora brassicae,* or clubroot, resistance are *Crr3T*^{sc} in *B. napus* and the *Cra*/*Crb*/*CRb*^{kato} locus in *B. rapa* [50,51]. Genomic regions associated with quantitative resistance to blackleg were found to be duplicated in *B. napus* [52], with their density bias being towards the A subgenome, with higher collinearity at the homoeologous loci than paralogous loci [53]. The recently cloned qualitative blackleg *R* genes in *B. napus*—*Rlm4*, *Rlm7* and *Rlm9*—were identified as allelic variants on chromosome A07 [54,55], with a large insertion of about 6 kb found within the coding sequence of these allelic variants [56]. Another blackleg *R* gene, *Rlm13*, was found to be located in a genomic region that has high numbers of structural variants such as SNPs, indels and PAVs on chromosome C03 [57]. On the other hand, a 700 bp deletion on *B. napus* chromosome C05 within a major QTL for *V. longisporum* resistance was identified through long-read sequencing [58], with gene PAV also found to be involved based on Illumina resequencing and *Brassica* 60K SNP analyses [59]. All of these evidences show that *R* genes in *Brassica* species evolve through highly dynamic processes of gene expansion and loss resulting in structural variations (SVs).

2.5. Complex Host-Pathogen Interaction

The level of a host's susceptibility is often affected by its interaction with the pathogen, and the disease outcome could potentially rely on a complex interplay between the developmental stage of the host (cotyledon vs. adult stage) and environmental conditions, as shown in the glasshouse study of the *AvrLmS-Lep2 Brassica* blackleg pathosystem [60]. It has been shown that climate changes, such as variation in the CO_2 concentration, temperature fluctuations and water availability, play a role in influencing disease outcomes in host plants [61]. Disease severity is also impacted by the type of disease resistance of the host, whether it is qualitative or quantitative; in *Brassica* blackleg disease [62–68], or in the qualitative type, it depends on the specific pathotype that infects the host. For example, in the *B. napus–Pyrenopeziza brassicae* (light leaf spot) interaction, the host resistance is specific to the isolated pathotypes [69]. In the blackleg and clubroot pathosystems, the resistance genes of the host exert direct selection pressure on *L. maculans* and *P. brassicae*, thus increasing the adaptability of the pathogens and causing higher severity of disease in the host due to resistance breakdown, particularly when cultivars of major *R* genes are being deployed on large scales in fields [70,71]. To minimise sudden losses of *R* gene efficacy leading to major yield losses from *L. maculans* infection, growing *Brassica* varieties with different *R* genes on a rotational basis has been shown to be an effective strategy as a means to select against the corresponding virulence allele [72]. The tight interaction in the host–pathogen relationship, with the influence of physiological and environmental conditions, forces the *Avr* genes to evolve rapidly, thus leading to susceptibility of the host.

2.6. Complex Signalling Network Influencing Plant Immunity

Plant growth and defence is controlled by complex signalling pathways involving interplays of hormones, for instance, auxin, abscisic acid, brassinosteroids, cytokinin, ethylene, gibberellin, jasmonate and salicylic acid [73]. The *B. napus* valine-glutamine (VQ) genes, implicated in *B. napus* growth and development, were found to enhance resistance towards blackleg at the adult stage by interacting with the transcription factor WRKY, invoking the SA and JA signalling pathways, especially at the necrotrophic stage of *L. maculans* infection [74]. Abiotic stress hormones may also affect the biotic response

pathways. In a study exploring the relationship between long-term (seven years) exposure to drought stress in *B. rapa* and the level of susceptibility of the plant towards *Alternaria brassicae* (Alternaria blackspot), a positive correlation of both variables was found with the involvement of the JA signalling pathway [75]. These examples demonstrate a complex signalling network in *Brassica* that influences *R* gene evolution and the extent of plant resistance developed over time. By taking advantage of some common genes along the signalling pathways, such as WRKY transcription factors, which have been found to play a role in many plant physiological systems—e.g., vernalisation in Chinese cabbage, or *B. rapa* [76], and Sclerotinia stem rot resistance in *B. napus* [77]—we increase the pool of candidate genes for gene pyramiding and stacking strategy, thus achieving the breeding of durable resistance in *Brassica* crops.

2.7. Epigenetics and R Gene Evolution

Since the beginning of this century, there has been an awareness that epigenetics can revolutionise medicine and agriculture [78]. Widely studied epigenetic modifications, such as DNA (de)methylation, histone post-translational acetylation, methylation or ubiquitination, chromatin assembly and RNA methylation, can regulate genomic activities such as chromatin density and gene expression/silencing, thus controlling varied phenotypes [79,80]. Experiments have revealed that epigenetic modifications play a role in transcriptional regulation of plant immunity against pathogens. Pathogen-infection-induced DNA hypomethylation results in elevated pathogen resistance, which is manifested by the interactions between *Arabidopsis* and bacteria, soybean and nematode, tobacco and virus, and *Aegilops auschii* and fungus [81–84]. In *Arabidopsis* resistance against the bacteria *Pst* DC300 and fungus *Verticillium dahlia*, histone H2B mono-ubiquitination (H2Bub1) plays a positive role [85,86]. Moreover, during ubiquitination in *Arabidopsis*, two novel ubiquitin E3 ligases, SNIPER1 and its homolog SNIPER2, were found to globally control the protein levels of sensor NLRs (sNLRs) reversely to maintain homeostasis and immune output [87]. All of these epigenetic modifications are important sources of genome evolution, participating in eukaryote genome regulation.

Among the studied epigenetic modifications, RNA N^6-methyladosine (m^6A) modification is the subject of the most up-to-date research, and it is the most prevalent internal post-transcriptional modification of mRNA. In experiments looking into the plant–pathogen arms race, results suggested that m^6A modification is activated in pathogen-infected plants, which leads to different effects on the m^6A and mRNA levels of genes related to plant–pathogen interaction, indicating a variety-specific m^6A modification [88,89]. m^6A has been shown to evolve synchronously with genome evolution and mRNA abundance [90]. According to Miao's latest research on evolutionary analysis of m^6A methylomes of 13 plant species representing evolution spanning over half a billion years, including *A. thaliana* in the family Brassicaceae, for plant *R* gene families, the m^6A methylation ratio is negatively correlated with the number of family members. Furthermore, for all genes studied in the research and previous studies, the m^6A methylation ratio is negatively correlated to genome size and gene members. Thus, the more expansion a genome experienced during its evolution, such as local gene duplication, the more m^6A elimination it might induce. In addition, the earlier the orthologous genes split, the less diverse the m^6A modification presents. Thus, the abundance of m^6A can be used as a reference to determine the chronological order of gene evolution and isolation. This finding exposes new perspectives in the analysis of plant *R* gene evolution [91]. Although limited epigenetic studies of *Brassica* have been published, the intriguing correlation between m^6A level and *R* gene evolution among *Brassica* and wild relatives suggests that *R* genes could be introduced into *Brassica* from such relatives with novel evolution.

2.8. Recessive Resistance Genes

In addition to the dominant *R* genes, recessive resistance genes also play an important role in plant host resistance [92]. Currently, there are two main hypotheses for the

mechanism of these genes: according the first hypothesis, the dominant allele of a recessive resistance gene (also known as a susceptibility gene) might encode a specific host factor that is essential for a pathogen to complete its life cycle in plants. If the plant has the recessive resistance gene, it will lack or present a mutated version of the host factor, which makes the plant resistant to the pathogen [93]. The second hypothesis proposes that the recessive resistance gene might encode an inhibitor which interferes with some stage of the infection cycle [93].

Recessive resistance accounts for 50% of the 200 virus-resistance genes in crops [94], and to date, all the studied virus recessive resistances are governed by the abovementioned first hypothesis. The second hypothesis, on the other hand, can explain fungal recessive resistance [95]. Recessive resistance traits can be introduced into crop species by crossing, random mutagenesis, selection and genome editing [96,97]. It is proposed to be more durable than dominant resistance. The eukaryotic translation initiation factors (eIF) 4E and eIF4G and their isoforms (hereafter eIF4Es) are the most common recessive resistance genes identified to date. They are essential protein complexes involved in the translation of mRNA into proteins. They have been found in a range of plants to confer resistance to viruses, employing the first resistance hypothesis mentioned above: loss of susceptibility due to a deficiency of the *eIF4Es* gene. For example: *eIF4Es*-mediated resistance against viruses has been identified in *A. thaliana* [98–100], wild tomato (*Solanum habrochaites*) [101], lettuce (*Lactuca sativa)* [102], melon (*Cucumis melo*) [103], barley [104,105] and rice (*Oryza sativa*) [106], as well as in *Brassica*. In *Brassica rapa*, the recessive resistance gene *retr01/retr02* was identified, which is an eIF4E-encoding gene associated with broad-spectrum resistance to turnip mosaic virus (TuMV) [107–109]. The resistance occurs due to a mis-splicing of the *eIF4E* allele [110].

In addition, another recessive resistance gene, *retr03*, encoding eIF2B was cloned in *B. rapa* [111]. In 2020, Shopan et al. reported that a total of 190 *eIFs* were detected in the *B. juncea* genome, 99 and 91 from the A and B subgenomes, respectively [112]. They were further clustered phylogenetically into 40 distinct subfamilies. Gene duplication plays an important role in the evolution and expansion of this *eIF* gene family. They identified a total of 33 duplicate gene pairs in the A subgenome and 35 pairs of duplicate/triplicate genes in the B subgenome of *B. juncea* [112]. After a duplication event, some gene copies were retained, owing to their critical function, while some genes were lost due to functional redundancy. The *eIFs* are highly conserved in Brassicaceae, with nearly 60% identity in *A. thalian* and *B. juncea* orthologs [112]. The orthologs did not diverge significantly between the A and B subgenomes of *B. juncea* [112].

MLO (mildew resistance locus O) is another class of recessive resistance gene. It also employs the first mechanism by encoding a negative regulator of plant immunity to achieve infection. Conversely, its mutation leads to broad-spectrum, high-efficiency and lasting resistance to disease in plants. It was first discovered in barley (*Hordeum vulgare* L.) with resistance to powdery mildew [113]. In *B. nigra*, a recessive resistance gene *lm1* conferring resistance to blackleg (*L. maculans*) was classified as an *MLO* gene [114]. Yan et al. [115] explored the *MLO* gene families in *A. thaliana*, *B. rapa*, *B. oleracea* and *B. napus*. A total of 123 *MLO* genes were identified, which included 15 in *A. thaliana*, 23 in *B. rapa*, 28 in *B. oleracea* and 57 in *B. napus* [115]. Evolutionary analysis found that these 123 *MLO* genes were clustered into three different subgroups. Through comparative genome analysis, they found only 2 out of 15 *A. thaliana MLO* genes—*MLO3* and *MLO9*—had no homologous genes in *Brassica*. Further comparison of *MLO* genes in *A. thaliana* and *Brassica* species identified that most of the *MLO* genes in *A. thaliana* had expanded in *Brassica* species, except *MLO3* and *MLO9*. Among the transmembrane motifs of the 123 *MLO* genes, 71 genes have more than seven common transmembrane motifs. Conserved domains of these 71 *MLO* genes had one conserved amino acid sequence (462 aa long), which might be the main functional domain [115]. In addition to dominant resistance, recessive resistance genes also account for a great amount of disease resistance in crops. In *Brassica*, they mainly evolve through gene duplication.

The mechanisms governing *R* gene evolution in *Brassica* as discussed in this section have shed light on managing various diseases to increase crop production (Table 1).

Table 1. Summary of the eight mechanisms of *R* gene evolution and their impact on disease management and crop production.

Mechanism of *R* Gene Evolution	Main Findings	Impact on Disease Management and Crop Production
1. Polyploid ancestry	• Wild, weedy and domesticated types of *Brassica* species proposed and identified • Comparative genomics of *Brassica* genomes revealed the genome relatedness between each species and their close relatives	• Novel *R* genes sources can be identified from different varieties of *Brassica* species, especially the wild type • Acceleration of interspecific hybridisation process in disease-resistant *Brassica* breeding
2. Disease resistance genes from introgression lines	• Genome compatibility is established, and genome patterns after hybridisation can be studied using a pangenome approach	• Produce varieties with hybrid vigour that can better resist various *Brassica* pathogens
3. Studying disease resistance genes from close relatives of *Brassica*	• *R* gene homologs explored • Immune response in *Arabidopsis* and other close relatives observed and compared with *Brassica*	• Increase the gene pool of *R* genes for breeding purposes • Speculate host–pathogen interaction and immunity response in different pathosystems
4. Structural variation of *Brassica* resistance genes	• *R* gene behaviour studied, e.g., tandem and segmental duplications, allelic variants, SNPs, Indels, PAVs, etc.	• Facilitate cloning of *R* genes using pangenome approach
5. Complex host–pathogen interaction	• Factors affecting disease severity determined • Examples of several pathosystems showed tight "arms- race" interaction influencing *R* gene evolution (Section 3)	• Assist in pathotype and host resistance screening • Develop better strategy for quality resistance crop breeding
6. Complex signalling network influencing plant immunity	• Other genes such as those involving in growth and development are associated with immunity.	• Enhance potential gene pyramiding strategy for more durable resistance
7. Epigenetics and *R* gene evolution	• The m^6A methylation ratio is negatively correlated with the number of *R* gene family members. • The abundance of m^6A can be used as a reference to determine the chronological order of *R* gene evolution and isolation.	• Expose new perspectives in the analysis of plant *R* gene evolution
8. Recessive resistance genes	• The mutation of recessive resistance genes plays important role in plant immunity. • Recessive resistance genes might evolve through gene duplication.	• Provide sustainable wide-spectrum resistance in crops

3. *R* Gene Evolution in the Blackleg *B. napus–L. maculans* Pathosystem and the Impact on Disease Management

For blackleg resistance, 19 *R* genes/alleles have been identified in *Brassica* species, and 5 of them have been cloned [116]. In the tight arms race of the *Brassica–L. maculans* pathosystem, the host *R* genes are often located in recombination hotspots within the genome and within regions of complex structural variation to allow more flexibility of genetic changes in response to the selection of highly evolved virulent pathogen genotypes [117,118]. When the *R* gene fails to evolve in advance of the virulent pathogen, the efficacy of the *R* gene fails [119,120] as soon as within three years [121,122]. More often than not, the resistance breakdown is not necessarily due to the direct effect of *R–Avr* interaction

but due to dual specificity of the avirulence gene [116], showing that close monitoring of *L. maculans* pathotype screening and disease incidence in the field are equally important in managing blackleg disease.

Identification of *Brassica* blackleg *R* genes from different varieties such as breeding lines, cultivars, wild relatives, landraces, feral species and possibly ancient forms obtained from germplasm sources could enrich the gene pool of *R* genes. With advanced biotechnologies and the integration of genomics, phenomics and machine learning [123], supported by revolutionary CRISPR/Cas9 and other modern cloning and genetic transformation techniques [124–126], breeding disease-resistant *Brassica* varieties at accelerated speed is no longer a major constraint. The main challenge, however, is the implementation of the *R* genes in each cultivar and the deployment strategy of these cultivars in the field, taking into consideration the evolutionary mechanisms between the two players of the blackleg pathosystem, to ensure durability of *R* genes.

It was recently shown through a simulation study that rotating *B. napus* cultivars containing a single *R* gene with different *R* genes every five years and pyramiding two genes in a cultivar, with two different pyramided genes changing every year, in two-year rotations are effective strategies to extend the durability of *R* genes [72]. This indicates that the greater the *R* gene resources, the higher the chance of successful implementation of crop rotation and stacking. In addition, the *B. napus* resistance outcome is dependent on the genetic background of the host, as it was seen that within the same *R* gene–*L. maculans* effector interaction in different genotypes (*R* gene introgressed lines), the dynamics of the gene expression of defence-related genes was different; hence, the intensity of immunity was also different [127]. This has practical implications for *Brassica* disease management, where these defence-related genes that are differentially expressed in genotypes with different genetic background can be used as markers to determine which parental genotype will be most suitable when breeding *R*-gene-resistant *B. napus*. Another effective strategy to deploy *R* genes in the field is to breed *B. napus* cultivars that have qualitative *R* gene resistance in combination with quantitative resistance [128]. The biology behind this mixture of resistance types is that there would be higher genetic variation in the host population, thus disrupting the selection pressure on *L. maculans* and decreasing the evolution frequency of virulent pathotypes [117,129]. Some other ways to control the pathogen population include agricultural practices such as applying fungicide, crop isolation and treating the infected cultivar residue by burning, tillage or burial [130].

4. Technologies to Study Evolutionary Origins of *Brassica*

4.1. Genome Level

Structural variation (SV) plays an important role in the evolution of plant genomes. Large-scale structural variants can be detected using advanced third-generation sequencing methods and read mapping strategies such as high throughput chromosome conformation capture (Hi-C) and BioNano optical mapping technologies, as well as third-generation whole-genome sequencing strategies such as PacBio single-molecule real-time sequencing (SMRT) and Oxford Nanopore (ONT), which produce long reads and have been shown to overcome limitations on short reads to detect various SVs [131,132]. For example, in *B. oleracea*, large (100 kb or greater) SVs were detected using a combination of PacBio SMRT (DNA sequencing), PacBio Iso-Seq (RNA sequencing), BioNano optical mapping and Hi-C technologies [133], with genes related to stress response found to be related to these SVs. In *B. napus*, a 700 bp deletion of the *V. longisporum* resistance gene was identified using PacBio ONT sequencing [58].

Whole-genome sequencing, RNA sequencing, flow cytometry and some cytogenetic technologies such as fluorescence in situ hybridisation/genomic in situ hybridisation (FISH/GISH), depending on hybridisation between fluorescent probe DNA and introduced target DNA, help to reveal the process of allopolyploid formation and alien chromosome fragment introgression, like the artificial creation of hexaploid/octoploid *Brassica* and natural development of other allopolyploid species like tetraploid cotton [134–136]. Using

advanced sequencing and mapping tools on the complex polyploid *Brassica* genomes formed through interspecific hybridisation, either through artificial or spontaneous means, will allow us to understand plant genome evolution at greater depth and identify clearly the valuable genes underlying the genomes in each of the *Brassica* species [137].

At the gene level, candidate gene identification through bulked segregant analysis (BSA), whole-genome resequencing (WGRS) and QTL-seq methods have been shown to be very effective, delimiting the QTL interval of the candidate region in *Brassica* plants—for example, in flower colour candidate gene determination in *B. juncea* [138], as well as flowering and trichome formation and shoot branching in *B. rapa* [139,140]. The BSA-RNA sequencing approach was undertaken to identify resistance genes for clubroot *P. brassicae* in *B. oleracea* [141]. Some of the main advantages of using the BSA-QTL-seq strategy for candidate gene identification include being highly robust, as it can be applied not only on *Brassica* plants but fungal isolates such as *L. maculans*, and only a limited number of representatives (10 samples) from the segregating population are required instead of hundreds of individuals for genotyping and phenotyping work [60]. The *Brassica* 60K Illumina Infinium array and WGRS approaches using high-quality *Brassica* genome assemblies are useful tools to explore SNPs at the whole-genome level. For example, WGRS on 991 *B. napus* accessions representing various ecotypes identified more than 50 candidate NLR genes located within highly evolved genomic regions of *B. napus* [44]. A more targeted approach towards candidate *R* gene identification using the "bait capturing" method to capture all RGA-like sequences within the genome, called RenSeq (*R* gene enrichment sequencing) and RLP/KSeq (receptor-like protein/kinase enrichment sequencing) [142,143], collectively referred to as RGASeq, can be useful in large, complex genomes like *Brassica* [42]. All of these advanced NGS tools offer high-resolution mapping and quick discovery of novel *R* genes for the study of the evolutionary history of *R* genes for resistance breeding.

4.2. Pangenome Level

With more sequenced *Brassica* genomes available, the pangenome approach has become more attractive, considering the rich evolutionary information that can be deciphered at the genome and gene levels. It is currently feasible to use pangenomes with the implementation of state-of-the-art machine learning, not only to discover all the genes present in different *Brassica* species and explore the genome changes that happen in each of these polyploid species during the evolutionary pathway, but also to use this wealth of information in genomic selection, which offers a very promising future for *Brassica* crop breeding [144]. By developing pangenomes of *B. napus* and its progenitors, *B. oleracea* and *B. rapa*, it was revealed that defence- and stress-related genes are common dispensable genes and that gene loss events observed in specific *Brassica* plants are attributed to various evolutionary mechanisms [48]. A *B. napus* pangenome database was recently built, called BnPIR, which is a one-stop platform for users to look for pangenome resources using a built-in browser, making possible the comprehensive study of evolution-related variations in *B. napus* [145].

4.3. Epigenetic Level

There has been rapid development in high-throughput epigenetic and epitranscriptomic sequencing in recent years. Whole-genome bisulfite sequencing (WGBS), targeted methylome sequencing (TMS), such as shotgun bisulfite sequencing, and post-bisulfite adaptor tagging (PBAT)-assisted TMS are used in DNA methylome analysis [146,147]. Enriching abundant m^6A-modified RNA fragments through immunoprecipitation with m^6A-specific antibodies and technology like methylated RNA immunoprecipitation with next-generation sequencing (MeRIP-seq) has been widely applied to enable transcriptome-wide profiling of RNA m^6A modification related to pathogen resistance or genome evolution [91,148]. The data analysis is carried out using bioinformatic technologies to reveal the epigenetic and epitranscriptomic changes formed during adaption to environmental changes like biotic and abiotic stress.

4.4. High-Throughput Phenotyping

Association analysis between plant genotypes and phenotypes is an important method to understand the differences in crucial traits regulated by genetic variations formed during plant genome evolution and development. As we reviewed above, there has already been major development in technologies to acquire abundant genetic and genomic data. However, the traditional labour- and time-consuming phenotyping methods remain a bottleneck in association analysis to understand plant genome development [149].

Nowadays, there are some high-throughput phenotyping technologies that have been introduced into plant improvement which allow data acquisition in a rapid and non-invasive way. These high-throughput phenotyping methods always rely on dynamic optical imaging equipment and machine learning [150]. Take its application in pathogen resistance, for instance: hyperspectral imaging was used to assess disease severity, such as cellular level changes in barley leaves infected with powdery mildew [151,152]; an automated imaging scanner was used to assess resistance against Septoria tritici blotch (STB, caused by fungus *Zymoseptoria tritici*) in wheat in a field experiment, with 26 chromosomal intervals harbouring several novel loci identified, showing quantitative resistance emerging from co-evolution with the pathogen [153]. High-throughput digital imaging and R scripts were conducted in the genome-wide association study (GWAS) of *Arabidopsis* against *Botrytis cinerea*, identifying 23 candidate genes [154]. All of these high-throughput phenotyping technologies present potential to help us further understand the coevolution between plants and pathogens.

5. Conclusions

The *Brassica R* genes have evolved through many evolutionary forces. With such a deep history of hybridisation and polyploidy formation in *Brassica* species, we believe many more novel evolutionary forces and novel *R* genes have yet to be uncovered (Figure 2). With currently available high-quality *Brassica* genome assemblies and pangenome resources and many more adaptations of NGS in DNA, RNA or methylation sequencing using sophisticated bioinformatics algorithm, coupled with the application of advanced flow cytometry and cytogenetical technologies such as FISH and GISH, *R* gene evolution and discovery in *Brassica* will be accelerated in the near future. High-throughput phenotyping approaches exploited across all *Brassica* crops, along with continuous efforts to characterise pathogen isolates, will further enhance our knowledge of host–pathogen interaction for breeding high-quality *Brassica* crops.

Furthermore, the newly confirmed synergy of PTI and ETI in *Arabidopsis* might show a novel point in the study of plant *R* gene evolution. The heredity and epigenetic memory might show new perspectives in crop resistance breeding, with a possibility that the m^6A modification ratio reflects *R* gene evolution conservation. Understanding *R* gene evolution and the genetic diversity across crops' wild relatives may also help in the introgression of novel *R* genes for sustainable breeding of high-quality *Brassica* crops.

Compared with major *R* genes, which evolve quickly to win the race against pathogens, the resistance guaranteed by recessive resistance genes is rather conserved. This conservation provides a sustainable, wide-spectrum resistance. The mutation of recessive *R* genes can be considered as a method in engineering broad-spectrum resistance in crops, together with the utilisation of E3 ligases controlling global sNLRs in plants.

There are still many pieces of puzzles that need to be researched and understood to achieve a complete picture of the plant immune system and its evolution. With the development of high-throughput genotyping and other advanced gene validation tools such as CRISPR/Cas9, achieving a near-complete understanding of *R* gene evolution and precision breeding is very promising. In this manner, it is favourable to achieve higher accuracy in the study of plant *R* gene evolution and of breeding for broad-spectrum resistance in crops through advanced methods, causing fewer public concerns.

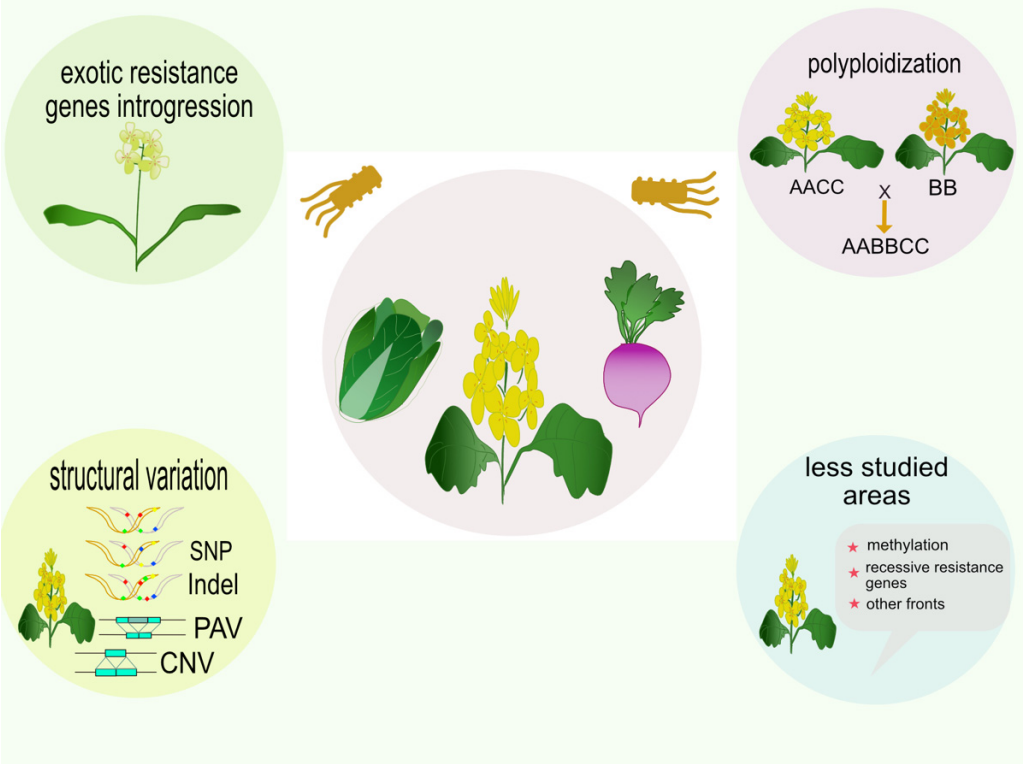

Figure 2. Major forces related to study of *R* gene evolution in *Brassica*.

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