Predicting Cloned Disease Resistance Gene Homologs (CDRHs) in Radish, Underutilised Oilseeds, and Wild Brassicaceae Species

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Abstract: Brassicaceae crops, including Brassica, Camelina and Raphanus species, are among the most economically important crops globally; however, their production is affected by several diseases. To predict cloned disease resistance (R) gene homologs (CDRHs), we used the protein sequences of 49 cloned R genes against fungal and bacterial diseases in Brassicaceae species. In this study, using 20 Brassicaceae genomes (17 wild and 3 domesticated species), 3172 resistance gene analogs (RGAs) (2062 nucleotide binding-site leucine-rich repeats (NLRs), 497 receptor-like protein kinases (RLKs) and 613 receptor-like proteins (RLPs)) were identified. CDRH clusters were also observed in Arabis alpina, Camelina sativa and Cardamine hirsuta with assigned chromosomes, consisting of 62 homogeneous (38 NLR, 17 RLK and 7 RLP clusters) and 10 heterogeneous RGA clusters. This study highlights the prevalence of CDRHs in the wild relatives of the Brassicaceae family, which may lay the foundation for rapid identification of functional genes and genomics-assisted breeding to develop improved disease-resistant Brassicaceae crop cultivars.

Keywords: Brassicaceae cultivated and weedy species; resistance gene analogs and homologs

1. Introduction

The Brassicaceae family, also known as Cruciferae due to its cross-shape four-petal flower [1], is one of the most diverse and agronomically important plant families, consisting of 44 tribes, 372 genera and 4060 species [2,3]. The Brassica species (B. rapa, B. nigra, B. oleracea, B. juncea, B. napus and B. carinata), Camelina sativa, Raphanus sativus and Sinapis alba are crop members, which are produced for vegetables, edible oil, herbs, spices, condiments and fodder. The Brassicaceae also contains many model species that are used in various areas of research, including Arabidopsis thaliana for genetic studies [4], Arabidopsis thaliana for heavy metal (e.g., cadmium and zinc) accumulation and tolerance [5], Arabis alpina in ecological studies [6], Barbarea vulgaris for insect resistance [7], Boechera species in apomixis research [8], Brassica species in crop evolution [9], C. sativa in metabolic oils [10], Cardamine hirsuta in leaf structure and morphology [11], Eutrema salsugineum in salinity stress [12] and Lepidium meyenii in floral structure [13]. In addition, species, such as Amoracia rusticana, Cheiranthes cheiri, Isatis tinctoria, Matthiola incana and Raphanus raphanistrum, have industrial uses (biofuels, dyes, etc.) [14–18], while species in the genera Aethionema, Cheiranthis, Erysimum, Hesperis, Iberis, Lobularia, Lunaria, Malcolmia and Matthiola are cultivated as ornamentals [19,20].

The production of Brassicaceae species, especially the crop members, is limited by various pathogenic species, such as Lepidium species (L. maculans, L. biglobosa), Sclerotinia sclerotiorum, Albugo candida, Hyaloperonospora species (H. parasitica, H. arabidopsidis), Pseudomonas syringae, Plasmidiphora brassicae, Xanthomonas spp., Fusarium oxysporum mattioli, Botrytis cinerea, Erysiphe cichoreacrum and Alternaria species (A. brassicicola, A. brassicicola), which cause blackleg, Sclerotinia stem rot, white rust, downy mildew, bacterial leaf spot, clubroot, black rot, Fusarium wilt, grey mould, powdery mildew and Alternaria black spot diseases,
respectively [21–25]. Crops have qualitative and quantitative disease resistance to overcome pathogens. The quantitative resistance, governed by many minor genes, is a partial resistance manifesting at later stages of the crop, while qualitative resistance, governed by major genes or resistance genes \( R \) genes, is largely manifested from the early stages up to the maturity stage of the crop. Among the types of resistance in Brassicaceae crops, qualitative resistance is commonly used to screen lines in early stages of the genotypes for disease resistance breeding and development. For instance, a set of pathogen isolates containing avirulence \( (Avr) \) genes is used to screen white rust resistance in \( B. \) juncea genotypes [26] and blackleg resistance in \( B. \) napus genotypes [27,28] by assessing a hypersensitive response observed in the cotyledons. Clubroot resistance is also screened either in the cotyledon and roots of the seedlings in Brassicaceae species [29–32].

The crop wild relatives (CWRs) of the cultivated Brassicaceae species can be used to improve disease resistance by integrating favourable alleles harboured by the CWRs into the crop members. For example, \( B. \) fruticulosa and \( Erucastrum \) cardaminoides were introgressed, via wide hybridization, including chromosome doubling and bridging species, to \( B. \) juncea with disease \( R \) genes against Sclerotinia stem rot [33,34]. In addition, \( B. \) juncea-\( S. \) alba hybrids were developed through somatic hybridization, which leads to the transfer of Alternaria black spot disease resistance to \( B. \) juncea [35]. The wild \( C \) genome of \( B. \) incana was also introduced to \( B. \) napus through interspecific hybridization and pyramiding for Sclerotinia stem rot resistance [36], while Alternaria black spot and white rust resistance from the wild crucifers \( Diploptaxis \) erucoides and \( B. \) maurorum were introduced into \( B. \) rapa with the aid of sequential ovary-ovule culture [37]. Lastly, \( A. \) thaliana, \( B. \) insularis, \( B. \) atlantica, \( B. \) macrocarpa, \( D. \) muralis, \( Eruca \) pinnatifia, \( E. \) gallicum, \( R. \) raphanistrum, \( S. \) arvensis, \( S. \) loeselii and \( T. \) arvense have been found with proteins/compounds that may enhance blackleg resistance in \( B. \) napus [38–45].

Plant disease \( R \) genes, also called resistance-gene analogs (RGAs), play a significant role in triggering the genetic resistance-defence response in crops [46] and are grouped into three main classes: nucleotide-binding site (NBS)-leucine rich repeats (LRR) (NLRs), receptor-like protein kinases (RLKs) and receptor-like proteins (RLPs). NLRs, with the subclasses coiled-coil (CC)-NBS (CN), CNL, NBS, NBS-LRR (NL), Toll/Interleukin-1 receptor (TIR)-NBS-LRR (TNL), TIR-NBS (TN), TIR with unknown domains (TX), NLR with other domains (Other-NLR), are generally involved in effector-triggered plant immunity (ETI) and plant defence [47–50]. On the other hand, RLKs, with the subclasses, including LRR-RLK, Lysin motif (LsyM) (LysM-RLK) and other receptor (Other-RLK) [51] and RLPs, with the subclasses, including LRR-RLP and LysM (LysM-RLP), are not only involved in the first line of defence by recognising pathogen elicitors [52,53], but also in plant development [54,55].

This study aimed to determine what RGAs are homologous to cloned fungal and bacterial \( R \) genes across 20 Brassicaceae genomes and to assess the retention and diversification of RGA domains in the homologs and their physical clustering patterns.

2. Results

2.1. Prediction of RGAs in \( B. \) cretica, \( C. \) bursa-pastoris and \( S. \) alba

RGAugury predicted a combined total of 3738 RGAs in \( B. \) cretica (982 RGAs; with 230 NLRs, 614 RLKs and 138 RLPs), \( C. \) bursa-pastoris (1474 RGAs; with 353 NLRs, 925 RLKs and 196 RLPs) and \( S. \) alba (1282 RGAs; with 208 NLRs, 943 RLKs and 131 RLPs) genomes (Figure 1, Table S1). Of these RGAs, 791 were NLRs (195 TNL, 161 NL, 161 TX, 110 CNL, 53 TN, 51 NBS, 26 CN and 34 Other-NLR), 2482 were RLKs (1486 Other-RLK, 982 LRR-RLK and 14 LysM-RLK) and 465 were RLPs (457 LRR-RLP and 8 LysM-RLP) (Figure 1).
Figure 1. The number and distribution of resistance-gene analog (RGA) subclass nucleotide-binding site (NBS), coiled-coil (CC)-NBS or CN, CN-leucine rich repeats (LRR) or CNL, NBS-LRR or NL, Toll/Interleukin-1 receptor (TIR)-NBS-LRR or TNL, TIR-NBS or TN, TIR with unknown domains or TX, NBS-LRR with other domains or Other-NLR, LRR- receptor like kinase (RLK) or LRR-RLK, Lysin motif (LsyM)-RLK or LysM-RLK, RLK with other receptor or Other-RLK, LRR- receptor-like protein (RLP) or LRR-RLP and LysM-RLP in Brassica cretica (Bcr), Capsella bursa-pastoris (Cbp) and Sinapis alba (Sal) genomes.

2.2. Identification of CDRHs across the Study Species and Diseases

The 3172 cloned disease R gene homologs (CDRHs) identified were all RGAs: 2062 NLRs, 497 RLKs and 613 RLPs, with an average of 159 CDRHs (RGAs) in each of the 20 studied genomes/species (Figure 2, Table S2). C. sativa contained the highest number of CDRHs: 307, followed by Boechera stricta (296), C. hirsuta (240), A. alpina (226), C. bursa-pastoris (197), B. vulgaris (171) and Arabidopsis lyrata (162) (Figure 2). The rest of the studied Brassicaceae contained less than the average CDRHs per species, with the lowest in Schrenkiella parvula (62), Leavenworthia alabamica (91), Capsella rubella (94) and R. raphanistrum (99) (Figure 2). It should also be noted that A. lyrata, C. bursa-pastoris and R. sativus (135 CDRHs) had the highest number of CDRHs in their respective subfamilies (Figure 2).
Figure 2. The number and distribution of cloned disease resistance gene homologs containing resistance domains including nucleotide-binding site (NBS), coiled-coil (CC)-NBS or CN, CN-leucine rich repeats (LRR) or CNL, NBS-LRR or NL, Toll/Interleukin-1 receptor (TIR)-NBS-LRR or TNL, TIR-NBS or TN, TIR with unknown domains or TX, NBS-LRR with other domains or Other-NLR, LRR- receptor like kinase (RLK) or LRR-RLK, Lysin motif (LsyM)-RLK or LysM-RLK, RLK with other receptor or Other-RLK, LRR- receptor like protein (RLP) or LRR-RLP and LysM-RLP in Arabidopsis halleri (Aha), Arabidopsis lyrata (Aly), Arabis alpina (Aal), Barbarea vulgaris (Bvu), Boechera stricta (Bst), Brassica cretica (Bcr), Camelina sativa (Csa), Capsella grandiflora (Cgr), Capsella bursa-pastoris (Cbp), Capsella rubella (Cru), Cardamine hirsuta (Chi), Eutrema salsugineum (Esa), Leavenworthia alabamica (Lal), Lepidium meyenii (Lme), Raphanus raphanistrum (Rra), Raphanus sativus (Rsa), Sinapis alba (Sal), Sisymbrium irio (Sir), Schrenkiella parvula (Spa) and Thlaspi arvense (Tar) genomes.

The cloned R genes against bacterial leaf spot (At_ADR1, At_BAK1, At_FLS2, At_NDR1, At_NRG1a, At_NRG1b, At_PBS1, At_RLP30, At_RLP32, At_RPM1, At_RPS2, At_RPS4, At_RPS5, At_RRS1 and At_SOBIR1) had a total of 752 CDRHs (Figure 3). C. sativa had the highest number of CDRHs, 85, followed by 59 and 58 in C. hirsuta and L. meyenii, respectively (Figure 3). For the gene conferring resistance to another bacterial disease (black rot), At_RLP1, a total of 36 CDRHs were identified, with the highest numbers found in C. hirsuta and C. bursa-pastoris with 6 and 5, respectively (Figure 3).
Figure 3. The number and distribution of cloned disease resistance gene homologs associated to Alternaria black spot (ABS), blackleg (BL), black rot (BR), bacterial leaf spot (BLS), clubroot (CR), downy mildew (DM), Fusarium wilt (FW), grey mould (GM), powdery mildew (PM), Sclerotinia stem rot (SSR) and white rust (WR) resistance in *Arabidopsis halleri* (Aha), *Arabidopsis lyrata* (Aly), *Arabis alpina* (Aal), *Barbarea vulgaris* (Bvu), *Boechera stricta* (Bst), *Brassica cretica* (Bcr), *Camelina sativa* (Csa), *Capsella grandiflora* (Cgr), *Capsella bursa-pastoris* (Cbp), *Capsella rubella* (Cru), *Cardamine hirsuta* (Chi), *Eutrema salsugineum* (Esa), *Leavenworthia alabamica* (Lal), *Lepidium meyenii* (Lme), *Raphanus raphanistrum* (Rra), *Raphanus sativus* (Rsa), *Sinapis alba* (Sal), *Sisymbrium irio* (Sir), *Schrenkiella parvula* (Spa) and *Thlaspi arvense* (Tar) genomes.

In total, 921 CDRHs associated with cloned *R* genes against the fungal disease downy mildew (*At_ADR1*, *At_NRG1a*, *At_NRG1b*, *At_RLP42*, *At_RPP1*, *At_RPP2a*, *At_RPP2b*, *At_RPP4*, *At_RPP5*, *At_RPP7*, *At_RPP8*, *At_RPP13* and *At_RPP39*) were identified (Figure 3). Of these, 89 and 86 CDRHs were the highest numbers obtained in *C. sativa* and *B. stricta*, respectively. The cloned *R* genes against white rust (*Bju_WRR1*, *At_RAC1*, *At_WRR4a*, *At_WRR4b*, *At_WRR8*, *At_WRR9* and *At_WRR12*) (Table 1) were recorded having a total of 544 CDRHs (Figure 3). The highest count was found in *B. stricta*: 106 CDRHs, followed by *A. alpina* (52 CDRHs). For blackleg, the cloned *R* genes (*Bna_MAPk*, *Bna_LepR3/Rlm2*, *Bna_Rlm9/4/7*, *At_RLM1a*, *At_RLM1b* and *At_RLM3*) had a total of 509 CDRHs (Figure 3). Both *A. alpina* and *B. stricta* had the most CDRHs, with 49 each, followed by *C. hirsuta* with
44 and *C. sativa* with 40. For Sclerotinia stem rot, the cloned R genes (*At_BAK1, At_RLP23, At_RLP30* and *At_SOBIR1*) had a total of 310 CDRHs with the highest count observed in *C. sativa* with 48 (Figure 3).

Table 1. Cloned genes with resistance against Brassicaceae diseases and their corresponding homologs (similar by sequence identity) along the homolog types across the 20 studied genomes.

| Cloned Gene (RGA Subclass) | Same RGA Domain | Different RGA Domain (Total) | Total |
|----------------------------|-----------------|------------------------------|-------|
| *At_ADR1* (NL)            | 33 NL           | 5 CNL, 6 NBS, 1 TNL (12)     | 45    |
| *At_BAK1* (LRR-RLK)       | 117 LRR-RLK     | 2 Other-RLK (2)              | 119   |
| *At_FLS2* (LRR-RLK)       | 24 LRR-RLK      | 0                            | 24    |
| *At_NDR1* (TM)            | 0               | 0                            | 0     |
| *At_NRG1a* (RNL)          | 0               | 31 CNL, 28 NL, 1 LRR-RLP, 3 CN, 3 NBS (66) | 66    |
| *At_NRG1b* (RNL)          | 0               | 31 CNL, 26 NL, 1 LRR-RLP, 3 CN, 3 NBS (64) | 64    |
| *At_PBS1* (Other-RLK)     | 20 Other-RLK    | 0                            | 20    |
| *At_RAC1* (TNL)           | 48 TNL          | 10 NL, 3 NBS, 13 TN, 6 TX, 1 Other-NLR (33) | 81    |
| *At_RFO1* (Other-RLK)     | 119 Other-RLK   | 0                            | 119   |
| *At_RFO2* (LRR-RLP)       | 31 LRR-RLP      | 28 LRR-RLP (28)              | 59    |
| *At_RFO3* (Other-RLK)     | 50 Other-RLK    | 0                            | 50    |
| *At_RIN1* (CC)            | 0               | 0                            | 0     |
| *At_RLM1a* (TNL)          | 61 TNL          | 5 NBS, 12 NL, 9 Other-NLR, 16 TN, 38 TX (80) | 141   |
| *At_RLM1b* (TNL)          | 81 TNL          | 4 NBS, 23 NL, 8 Other-NLR, 16 TN, 31 TX, 1 LRR-RLP (83) | 164   |
| *At_RLM3* (TN)            | 5 TNL           | 3 NL, 2 NBS, 1 Other-NLR, 7 TNL, 4 TX (17) | 22    |
| *At_RLP1* (LRR-RLP)       | 36 LRR-RLP      | 0                            | 36    |
| *At_RLP2* (LRR-RLP)       | 117 LRR-RLP     | 0                            | 117   |
| *At_RLP30* (LRR-RLP)      | 47 LRR-RLP      | 0                            | 47    |
| *At_RLP32* (LRR-RLP)      | 159 LRR-RLP     | 1 LRR-RLK (1)                | 160   |
| *At_RLP42* (LRR-RLP)      | 112 LRR-RLP     | 0                            | 112   |
| *At_RPM1* (NL)            | 14 NL           | 1 LRR-RLP, 1 NBS (2)         | 16    |
| *At_RPP1* (TNL)           | 26 TNL          | 1 CNL, 22 Other-NLR, 2 NBS, 6 NL, 15 TN, 30 TX (76) | 102   |
| *At_RPP13* (CNL)          | 14 CNL          | 4 NBS, 1 CN, 16 NL (21)      | 35    |
| *At_RPP2a* (TNL)          | 56 TNL          | 19 NL, 9 Other-NLR, 7 TN, 7 TX (42) | 98    |
| *At_RPP28* (TNL)          | 20 TNL          | 1 CNL, 2 NBS, 3 NL, 4 Other-NLR (10) | 30    |
| *At_RPP39* (CNL)          | 71 CNL          | 11 CN, 3 NBS, 26 NL, 3 LRR-RLP (43) | 114   |
| *At_RPP4* (TNL)           | 8 TNL           | 3 NL, 2 Other-NLR, 5 TN, 5 TX (15) | 23    |
| *At_RPP5* (TNL)           | 8 TNL           | 2 NL, 3 Other-NLR, 6 TN, 11 TX (22) | 30    |
| *At_RPP7* (NL)            | 56 NL           | 1 CN, 12 CNL, 1 LRR-RLP, 10 NBS (24) | 80    |
| *At_RPP8* (CNL)           | 80 CNL          | 12 CN, 6 NBS, 24 NL (42)     | 122   |
| *At_RPS2* (NL)            | 6 NL            | 18 CNL, 3 NBS (21)           | 27    |
| *At_RPS4* (TNL)           | 32 TNL          | 1 NBS, 6 NL, 7 Other-NLR (14) | 46    |
| *At_RPS5* (TNL)           | 0               | 58 CNL, 6 CN, 7 NBS, 22 NL (93) | 93    |
| *At_Rps8.1* (RNL)         | 0               | 0                            | 0     |
| *At_Rps8.2* (RNL)         | 0               | 0                            | 0     |
| *At_RS1* (TNL)            | 26 TNL          | 0 (15)                       | 41    |
| *At_SOBIR1* (LRR-RLK)     | 26 LRR-RLK      | 1 Other-RLK (1)              | 27    |
| *At_WRR12* (TNL)          | 29 TNL          | 5 NL, 2 TX, 4 LRR-RLP (11)   | 40    |
| *At_WRRd* (TNL)           | 37 TNL          | 4 NL, 4 Other-NLR, 6 TN, 33 TX (47) | 84    |
Table 1. Cont.

| Cloned Gene (RGA Subclass)          | Same RGA Domain | Different RGA Domain (Total) | Total |
|------------------------------------|-----------------|-----------------------------|-------|
| At_WRAR6 (TNL)                    | 51 TNL          | 2 LRR-RLP, 5 NL, 6 Other-NLR, 17 TN, 38 TX (68) | 119   |
| At_WRAR8 (TNL)                    | 56 TNL          | 12 TN, 4 NBS, 11 NL, 2 Other-NLR, 6 TX (35)    | 91    |
| At_WRAR9 (NL)                     | 6 NL            | 1 NBS, 1 Other-NLR, 9 TN, 35 TNL, 16 TX (62)  | 68    |
| Bju_WRRA1 (CNL)                   | 39 CNL          | 10 NL, 9 CN, 3 NBS (22)     | 61    |
| Bna_LepR3/Rlm2 (LRR-RLP)          | 97 LRR-RLP      | 0                           | 97    |
| Bna_MAPk (Other-RLK)              | 8 Other-RLK     | 0                           | 8     |
| Bna_Rlm9/4/7 (Other-RLK)          | 101 Other-RLK   | 0                           | 101   |
| Bol_FocBo1 (TNL)                  | 23 TNL          | 3 Other-NLR, 7 TN, 14 TX, 8 NL (32)            | 55    |
| Bra_cRa/cRb (TNL)                 | 14 TNL          | 1 Other-NLR, 5 TN, 1 NBS, 7 TX (14)            | 28    |
| Bra_Crr1a (TNL)                   | 28 TNL          | 7 NL, 6 Other-NLR, 28 TN, 19 TX, 2 NBS (62)   | 90    |
| Total                             | 1992            | 1181                         | 3172  |

At = Arabidopsis thaliana, Bju = Brassica juncea, Bol = Brassica oleracea, Bra = Brassica rapa, Bna = Brassica napus, Resistance-gene analogs (RGA) domain in comparison to the cloned gene. CN = coiled-coil (CC)-nucleotide-binding site (NBS), CNL = CC-NBS-leucine rice repeats (LRR), NL = NBS-LRR, TN = Toll/Interleukin-1 receptor (TIR)-LRR, TNL = Toll/Interleukin-1 receptor (TIR)-NBS-LRR, TX = Toll/Interleukin-1 receptor (TIR) with other domains, Other-NLR = NBS-LRR with other domains, RNL = resistance to powdery mildew 8 (Rpw8)-NBS-LRR, LRR-RLK = LRR-receptor-like kinase proteins (RLK), Other-RLK= RLK with other domains, LRR-RLP = LRR-receptor-like proteins, TM = transmembrane.

The cloned R genes (Bol_FocBo1, At_RFO1, At_RFO2 and At_RFO3) against Fusarium wilt had 283 CDRHs in total with the highest numbers being 38 (C. sativa) and 23 CDRHs (C. hirsuta and A. alpina) (Figure 3). The cloned R genes against grey mould (At_RLP42 and At_RLM3) had a total of 134 CDRHs with the highest CDRHs obtained in C. sativa (21 CDRHs), S. alba (13 CDRHs) and C. bursa-pastoris (13 CDRHs) (Figure 3). The cloned R genes (Bra_Crr1a and cRa/cRb) against clubroot had a total of 117 CDRHs with A. alpina and S. alba containing the highest counts with 17 and 12 CDRHs, respectively (Figure 3). At_ADR1 against powdery mildew had 45 CDRHs, with 7 CDRHs in C. sativa as the highest count. At_RLM3 conferring resistance to Alternaria black spot had 22 CDRHs with 2 CDRHs as the highest in each of eight species (A. alpina, B. stricta, B. vulgaris, C. bursa-pastoris, Capsella grandiflora, E. salsugineum, R. sativus and T. arvense) (Figure 3).

2.3. Retention and Diversification of RGA Domains in CDRHs

In terms of RGA subclasses, CDRHs were composed of 647 TNL, 613 LRR-RLP, 402 NL, 361 CNL, 301 Other-RLK, 271 TX, 196 LRR-RLK, 168 TN, 89 Other-NLR, 78 NBS and 46 CN (Figure 2), which shows the variation in CDRHs throughout the Brassicaceae family.

The RGA domain retention in the CDRHs (same RGA domain compared to its reference cloned R gene) and diversification (different RGA domain compared to its reference cloned R gene) were also noted in this study (Table 1). In total, 1992 (63%) and 1180 (37%) out of the 3172 CDRHs had retained and diversified their RGA domain compared to their reference cloned R gene, respectively (Table 1). It can be noted that the cloned R genes classed as Other-RLK had their corresponding CDRHs also classified as Other-RLK (100%, 298 out of 298 CDRHs). The next highest numbers retaining the same RGA domain were 98%, 95% and 61% in CDRHs from the LRR-RLK (167 out of 170 CDRHs), LRR-RLP (599 out of 628 CDRHs) and CNL (204 out of 332 CDRHs) cloned R genes, respectively. The remaining CDRHs from the NL, TNL and TN cloned R genes had 49% (115 out of 236 CDRHs), 45% (604 out of 1356 CDRHs) and 38% (5 out of 13 CDRHs) RGA domain retention, respectively.

The gene diversification could either be through truncation (one or two domains omitted), addition (one or two domains were added) or the combination of truncation and addition of RGA domains. Of the diversification results in CDRHs, 100% (130 CDRHs) of the CDRHs from RNL cloned R genes did not have an RNL domain. Diversification was also observed in CDRHs from cloned R genes that were TN (62% or 8 out of 13 CDRHs),
TNL (55% or 752 out of 1356 CDRHs), NL (51% or 121 out of 236 CDRHs), CNL (49% or 128 out of 332 CDRHs), LRR-RLP (5% or 29 out of 628 CDRHs) and LRR-RLK (2% or 3 out of 170 CDRHs). Of the cloned R genes, which were NLs, all the CDRHs (29) had additional RGA domains, while for the LRR-RLP cloned R genes 59% (71 out of 121 diversified CDRHs) had an additional one or two RGA domains. On the other hand, the combination of truncation and addition of RGA domains was observed in CDRHs from cloned R genes TN (63% or 5 out 8 diversified CDRHs), TNL (55% or 411 out of 752 diversified CDRHs) and RNL (54% or 70 out of 130 diversified CDRHs).

2.4. Identification of CDRH Clusters in Arabis alpina, Camelina sativa and Cardamine hirsuta

The organisation of CDRHs with RGA domains across chromosomes of A. alpina, C. sativa and C. hirsuta was studied to investigate the gene clustering of CDRHs in Brassica crop relatives. We identified a total of 72 gene clusters, consisting of 62 homogeneous RGA clusters (38 NLR, 17 RLK and 7 RLP clusters) and 10 heterogeneous RGA clusters (Figures 4–6). C. sativa contained the highest number of gene clusters with 28 (Figure 5), followed by C. hirsuta with 24 gene clusters (Figure 6) and A. alpina with 20 gene clusters (Figure 4).

Figure 4. Distribution of cloned disease resistance gene homologs in Arabis alpina (1st inner layer in their corresponding position in A. alpina genome). The tracks in the circos plot, from outer to inner, show chromosome (Chr) number and types of gene cluster. GC_NLR = gene cluster (GC) with all nucleotide-binding site leucine rice repeats (NLR) members, GC_RLP = GC with all receptor-like proteins (RLP) members, GC_RLK = GC with all receptor-like kinase proteins (RLK) members, GC_H = GC with members are a mixture of NLR, RLK and/or RLP, Chr = chromosome and M = position in million base pairs.
Figure 5. Distribution of cloned disease resistance gene homologs in *Camelina sativa* (1st inner layer in their corresponding position in *C. sativa* genome). The tracks in the circos plot, from outer to inner, show chromosome (Chr) number and types of gene cluster. GC_NLR = gene cluster (GC) with all nucleotide-binding site leucine rice repeats (NLR) members, GC_RLP = GC with all receptor-like proteins (RLP) members, GC_RLK = GC with all receptor-like kinase proteins (RLK) members, GC_H = GC with members are a mixture of NLR, RLK and/or RLP, Chr = chromosome and M = position in million base pairs.

Figure 6. Distribution of cloned disease resistance gene homologs in *Cardamine hirsuta* (1st inner layer in their corresponding position in *C. hirsuta* genome). The tracks in the circos plot, from outer to inner, show chromosome (Chr) number and types of gene cluster. GC_NLR = gene cluster (GC) with all nucleotide-binding site leucine rice repeats (NLR) members, GC_RLP = GC with all receptor-like proteins (RLP) members, GC_RLK = GC with all receptor-like kinase proteins (RLK) members, GC_H = GC with members are a mixture of NLR, RLK and/or RLP, Chr = chromosome and M = position in million base pairs.
3. Discussion

By aligning the 49 cloned R genes from 11 diseases, across 20 Brassicaceae genomes (crop species C. sativa, R. sativus and S. alba and wild species A. halleri, A. lyrata, A. alpina, B. vulgaris, B. stricta, B. cretica, C. grandiflora, C. bursa-pastoris, C. rubella, C. hirsuta, E. salsugineum, L. alabanica, L. meyenii, R. raphanistrum, Sisymbrium irio, S. parvula and T. arvense), an inventory of specific RGAs associated with cloned R genes was found. This provides an opportunity to search for novel CDRHs, which may confer disease resistance (especially the CDRHs in wild species), which can be used for future crop improvement once function is established in the crop species. Once cloned, molecular markers can be developed as a diagnostic tool in screening additional germplasm to characterise further lines for resistance.

The RGAs in B. cretica, C. bursa-pastoris and S. alba genomes and specific RGAs (CDRHs) obtained here are additional gene resources to the previously identified Brassicaceae RGA repertoire [51,56,57]. The number of S. alba RGAs in this study was higher than the RGAs obtained in the 18 species: Aethionema arabicum, A. halleri, A. lyrata, A. thaliana, A. alpina, B. vulgaris, B. stricta, B. rapa, C. grandiflora, C. rubella, C. hirsuta, E. salsugineum, L. alabanica, R. raphanistrum, R. sativus, S. irio, S. parvula and T. arvense genomes; the number of C. bursa-pastoris RGAs identified in this study was higher than the number of RGAs in the 21 species: Aethionema arabicum, A. halleri, A. lyrata, A. thaliana, A. alpina, B. vulgaris, B. stricta, B. rapa, B. nigra, B. oleracea, C. grandiflora, C. rubella, C. hirsuta, E. salsugineum, L. alabanica, R. raphanistrum, R. sativus, S. irio, S. parvula and T. arvense genomes [51,58]. Only the tetraploid Brassica crops (B. juncea, B. napus and B. carinata), C. sativa (hexaploid) and the wild species L. meyenii (octaploid) had greater numbers of RGAs than C. bursa-pastoris (tetraploid) [51,56], indicating that polyploidisation is a factor leading to more RGAs in species in the Brassicaceae family. Polyploid plants also have a greater number of transposable elements, an evolution driver of genome expansion [59,60], compared to its progenitors [61,62].

*Brassica* crops have experienced extensive breeding and development to improve disease resistance due to their long history of domestication that may have been a factor for RGA number expansion [63]. A previous study showed an average of 1563 RGAs in 11 genomes of the domesticated species compared to the average of 863 RGAs in 19 genomes of the wild species [51]; a similar trend was observed in this study between the domesticated and wild species. The number of RGAs in B. cretica (wild species) in this study was lower compared to the number of RGAs found in domesticated Brassica crops. This was also the case with the specific RGAs for R. sativus and R. raphanistrum (CDRHs in this study) and the RGAs obtained in a previous study [51], where domesticated radish had more RGAs compared to wild radish. However, this is not always the case as B. macrocarpa (wild cabbage species) had more RGAs compared to 10 domesticated cabbage species in pangenoome analysis [58]. Here, the lesser RGAs in B. cretica and R. raphanistrum than their domesticated counterpart species may also be due to the quality of genomes, as domesticated crops often have better genome qualities.

The domesticated Brassicaceae members (used in this study) have also been reported as excellent sources of disease resistance. For instance, C. sativa has been reported to have R genes providing resistance against Alternaria black spot, blackleg, downey mildew and Sclerotinia stem rot [40,64,65]. R. sativus has resistance against black rot [66], clubroot [67,68], downey mildew [69,70], Fusarium wilt [71], white rust [72] and Turnip mosaic virus [73,74] and S. alba has resistance to blackleg [39,75], Turnip mosaic virus [76] and Sclerotinia stem rot [77,78]. However, further investigation is needed as to whether the RGAs we identified in these three species are associated with the resistant phenotype. Nevertheless, our study supports the previous findings and the RGAs we identified are a valuable reference for future studies.

Unlike the cultivated crops, information towards genetic disease resistance in Brassicaceae wild species is limited. Of the wild Brassicaceae species we included, a few of them have been reported previously as potential R gene source against a particular disease,
for instance, *B. vulgaris* against Alternaria black spot and black rot [79], *B. cretica* against Verticillium wilt disease [80], *C. bursa-pastoris* against clubroot [81], Sclerotinia stem rot [82] and Alternaria black spot [83]. *R. raphanistrum* against blackleg [38], clubroot [84], downy mildew [85] and Sclerotinia stem rot [86] and *T. arvense* against blackleg [42]. However, the association between the reported phenotypic disease resistance in these species and the identified RGAs here needs further research.

The retention and diversification of RGA domains in the Brassicaceae family are a result of evolutionary events, such as whole-genome triplication/duplication [87–91]. Homologs may confer similar or dissimilar function to the reference gene [92,93]. A functional study revealed the *A. lyrata* homologs AL.MTP11A and AL.MTP11B are redundant to AT.MTP11 in *A. thaliana* [94], a gene involved in Mn$^{2+}$ transport and tolerance [95]. Similarly, AL.TSO2A and AL.TSO2B in *A. lyrata* are homologous to AT.TSO2 in *A. thaliana*, a gene functionally related to ribonucleotide reductase [96]. On the other hand, diversification in domains may indicate a different function of the original gene. For instance, the *At_RPP1* homolog *At_RPP1*$_{ND}$ (Nd accession) recognises a single allele of *Avr* gene *ATR1*$_{NdWsB}$, while *At_RPP1*$_{WsB}$ (WsB accession) also detects *ATR1*$_{NdWsB}$ plus three additional alleles with divergent sequences to confer resistance against downy mildew [97].

RGAs have also been reported to be prone to alteration, such as truncation or even loss of function, as they respond to selection pressure (e.g., presence of virulent pathogens) [98,99]. Truncated R genes encoding two-part proteins, such as CN, TN and NL, are evolutionary gene reservoirs and they readily allow for the formation of new genes through duplications, translocation and fusions [100–102]. In an RGA, added LRR domains can indicate pathogen specificity. For instance, the LRR domain in *At_RPP1* directly interacts with *Avr ATR1* [103], much like the L6 recognition of *Avrl*567 and the L11 recognition of *AvrL11* [104,105]. The LRR domain is also important for gene/protein stability [106]. Solo RGA domains could also confer resistance, as reports showed that the overexpression of NBS domains in a potato *R* gene *Rx* (CNL) resulted in an HR [107]. However, the case is different to the CC domain overexpression in *At_RPP5*, as it did not yield a hypersensitive response, but when both CC and NBS were overexpressed, it resulted in a hypersensitive response [108].

In gene clustering, *C. sativa* contained the highest total number of CDRHs clusters due to its higher number of chromosomes, 20, compared to 8 chromosomes of *A. alpina* and *C. hirsuta*. The RGA clusters are more prone to evolutionary processes, such as sequence exchanges, insertion or duplication, followed by neofunctionalisation [109–112]. The NLRs in a gene cluster can undergo mono or polymerisation, which results in massive expansions of pathogen recognition [111]. For instance, an NLR cluster with eight members contained two functionally characterised *R* genes, *At_RPP4* and *At_RPP5*, recognizing the *Avr* genes *ATR4* and *ATR5* in the downy mildew resistance response, respectively [113]. Furthermore, it has been shown that RLPs in a gene cluster are most likely pathogen responsive [114]. Two cloned RLP genes, *At_RLP30* and *At_RLP32*, which are involved in bacterial leaf spot resistance, form a gene cluster on At03 in *A. thaliana* [56,115,116], while a gene cluster on A10 in *B. napus* consists of *LepR3/Rlm2*, two alleles of a cloned RLP gene that confers blackleg resistance [117,118] and a homolog of *At_PBS1* [56]. On the other hand, 16 RLP clusters associated with disease resistance were found in *A. thaliana* and *Brassica* crops [56]. Heterogeneous gene clusters with members having RGA domains and including secreted peptides associated to blackleg and clubroot were also observed in *B. napus* [119]. Thus, the CDRHs obtained here, especially those that were clustered, are putative *R* genes that may confer disease resistance.

4. Materials and Methods

4.1. Mining the Protein Sequences of the Cloned Genes

In total, 49 cloned *R* genes identified in *Brassica* crop species and *A. thaliana* that confer resistance against fungal and bacterial diseases that affect Brassicaceae species (Table 2) were selected based on the following criteria set in a previous study [56]: (1) the *R* gene
pairs to an effector or Avr gene in a gene-for-gene resistance or (2) confers resistance in the form of a hypersensitive response (usually observed early stage), indicating its involvement in a gene-for-gene interaction or (3) acts as a helper or accessory gene pairing to the existing R–Avr interaction. The protein sequences of the 49 cloned R genes were retrieved from the UniProtKb [https://www.uniprot.org/uniprot/, verified and accessed on 8 August 2022] [120] or NCBI [https://www.ncbi.nlm.nih.gov/, verified and accessed on 8 August 2022] website.

### Table 2. The 49 cloned R genes from Arabidopsis thaliana (At), Brassica juncea (Bju), Brassica napus (Bna) and Brassica rapa (bra) used for homology searches.

| Gene (Accession ID/Reference) | Pathogen |
|-------------------------------|----------|
| At_ADR1 (Q9FW44 U) [121–123]  | Hyaloperonospora arabidopsidis F, Erysiphe cichoracearum F and Pseudomonas syringae B |
| At_BAK1 (Q9F4F2 U) and At_SOB1R1 (Q9SK82 U) [124,125] and At_RLP30 (Q9MAA8 U) [115,126] | P. syringae and Sclerotinia sclerotiorum F |
| At_RPS2 (Q42484 U) [127], At_RPS4 (Q9XGM3 U) [128] and At_RPS5 (Q64973 U) [129], At.Flush2 (Q9FL28 U) [130,131], At_NDR1 (O48915 U) [132], At_RSBI (Q9FE20 U) [133], At_RLP32 (Q69M9O U) [116], At_RPM1 (Q93214 U) [134,135], At_RIN4 (Q8GYN5 U) [136–140] and At_RRS1 (P0DKH5 U) [141,142] | P. syringae |
| At_NGR1a (Q9FKZ1 U) and At_NGR1b (Q9FKZ0 U) [122,123] | Albugo candida F, H. arabidopsidis, and P. syringae |
| At_RFO1 (Q8RY17 U) [143], At_RFO2 (Q9SH14 U) [144] and At_RFO3 (Q9LW83 U) [145] | Fusarium oxysporum matthioli F |
| At_RLM1a (F4I594 U) and At_RLM1h (Q9CAK1 U) [146], Bna_MPK9 (A0A0781FE9 U) [147], Bna_LepR3/Rlm2 (I7C3X3 U/A0A05B5618 U) [118,148], Bna_Rlm9/4/7 (CDX67982.1 N) [119,150] | Leptosphaeria maculans F |
| At_RLM3 (Q9FT77 U) [151] | L. maculans, Botrytis cinerea F, Alternaria brassicicola F and A. brassicicola F |
| At_RLP1 (Q9FLNV9 U) [152,153] | Xanthomonas spp. B |
| At_RLP23 (O48849 U) [125,154] | S. sclerotiorum |
| At_RLP42 (Q9LJ30 U) [155] | B. cinerea and H. arabidopsidis |
| At_RPP1 (F4J339 U) [156], At_RPP2a (F4J778 U) and At_RPP2b (F4T80 U) [157], At_RPP4 (F4JNA9 U) [158], At_RPP5 (F4JNB7 U) [159], At_RPP7 (Q8W3K0 U) [160,161], At_RPP8 (Q8W4J9 U) [162], At_RPP13 (Q8M667 U) [163] and At_RPP39 (I9BP9R U) [164] | H. arabidopsidis |
| At_Rps8.1 (Q9C5Z7 U) and At_Rps8.2 (Q9C5Z6 U) [165] | E. cichoracearum |
| At_RAC1 (Q6X558 U) [166], At_WRR4a (Q9C7X0 U) and At_WRR4b (MK034466 N) [167], At_WRR8 (MK034464 N), At_WRR9 (MK034464 N) and At_WRR12 (MK034462 N) [168] and Bju_WRR1 (A0A0B5L618 U) [169] | A. candida |
| Bra_cra/cRb (M5A83 U) [170,171] and Bra_Crr1a (AB605024.1 N) [172] | Plasmodiophora brassicae F |
| Bol_FecB1 (BAQ21734.1 N) [173] | F. oxysporum f. sp. Conglutinans F |

F = fungus, B = bacteria, RGA = resistance-gene analog, U = https://www.uniprot.org/uniprot/, accessed on 10 October 2020 website, N = https://www.ncbi.nlm.nih.gov/ (accessed on 10 October 2020).

### 4.2. Mining of Resistance Gene Analogs

The list of predicted RGAs and their subclasses (CN, CNL, NBS, NL, TNL, TX, CNL, TN, Other-NLR, Other-RLK, LRR-RLK, Lysm-RLK, LRR-RLP and Lysm-RLP) derived from the RGAugury pipeline [174] in A. alpina, A. halleri, A. lyrata, B. vulgaris, B. stricta, C. sativa, C. grandiflora, C. rubella, C. hirsuta, E. salsugineum, L. alabamica, L. meyenii, R. raphanistrum, R. sativus, S. irio, S. parvula and T. arvense genomes were taken from a previous study [51], available at https://research-repository.uwa.edu.au/en/datasets/brassicaceae-
rga-candidate-protein-sequences, accessed on 23 November 2020. The RGAugury pipeline was also used in this study to perform in silico prediction of RGAs and their subclasses in the genomes of *B. cretica* [175], *C. bursa-pastoris* [176] and *S. alba* [177].

4.3. Identification of Homologs

The RGAs from the 20 Brassicaceae genomes and the 49 cloned *R* genes were aligned using Protein Basic Local Alignment Search Tool (BLASTp) [178]. From the BLASTp results, the criteria of the previous studies in identifying homologous genes in plants were applied by removing hits with greater than E-45 [56,179–181] and less than 148 amino acid or aa (coverage) [56] from further analyses. We applied an additional criterion by removing any BLASTp results lower than 60% similarity from further analyses as the homology search was conducted between crop *R* genes and several wild species. Further classification of RGAs was undertaken, according to whether they had the similar resistance domain to their homologous cloned *R* gene counterpart or whether it was different [56].

4.4. Gene Cluster Analysis

Among the 20 Brassicaceae species used in this study, only three genomes, *A. alpina* [182], *C. hirsuta* [11] and *C. sativa* [183], were used for gene cluster analysis, due to the accessibility of their pseudo-chromosomes (assigned chromosomes), from which gene clusters were derived. Two types of gene clusters were then identified, with the first defined as a homogenous RGA cluster (having at least 2–8 RGAs of the same class either NLR, RLK or RLP) situated within a 200 kb region on the same chromosome [184,185]. The second was defined as a heterogeneous cluster, containing different classes of RGAs [184,185].

5. Conclusions

CWRs with exotic genetic libraries provide rare RGAs, which could be a GMO alternative in improving disease resistance in Brassicaceae crops. This study suggests several domesticated and wild species could be a potential *R* gene source for a particular disease resistance. Based on their CDRHs having RGA domains, *A. alpina* and *B. stricta*, *C. hirsuta* and *C. bursa-pastoris* and *C. sativa* are good sources of resistance against white rust, black rot and Sclerotinia stem rot, respectively. Though the challenge remains in the gene transfer, several methodologies, such as bridging crosses, chromosome doubling after hybrid crossing and somatic hybridization, have found success in Brassicaceae crop breeding. Several CDRHs have also been found in less-explored disease resistance, such as Alternaria black spot, bacterial leaf spot, black rot, grey mould and powdery mildew in Brassicaceae crops, and the RGAs obtained are a valuable starting reference for future studies. Lastly, the current findings of CDRHs in crops *C. sativa*, *R. sativus* and *S. alba* and the 17 wild Brassicaceae species and the previous findings of CDRHs in *A. thaliana* and *Brassica* crops [56] provide an opportunity to study the evolutionary differences in 49 cloned *R* genes (reference in this study) and their homologs throughout the Brassicaceae family.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11223010/s1, Table S1: List of resistance-gene analogs (RGAs) in *Brassica cretica*, *Capsella bursa-pastoris* and *Sinapis alba* using RGAugury pipeline. Table S2: List of resistance-gene analogs (RGAs) homologous to cloned disease resistance genes (*R* genes) and the E-value and similarity basis.

Author Contributions: A.Y.C. and J.B. conceptualized the paper; A.Y.C. wrote the original draft along with formal analyses; W.J.W.T. helped improve the paper by suggesting additional ideas and by thorough revision/editing; P.E.B. analysed the *Brassica cretica*, *Capsella bursa-pastoris* and *Sinapis alba* genes using the RGAugury pipeline; D.E. and J.B. supervised, reviewed and suggested revisions to the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This study is funded by the Australian Research Council projects (DP200100762 and DP210100296) and Grains Research and Development Corporation UWA1905-006RTX.

Institutional Review Board Statement: Not applicable.
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