Breeding for Disease Resistance in Brassica Vegetables Using DNA Marker Selection

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Abstract

The Brassica genus comprises of agro-economically important vegetables. Disease causes great yield loss of Brassica vegetables worldwide. Different traditional methods such as crop rotation and chemical control have limited effect on different diseases of Brassica vegetables and cannot completely eradicate the pathogens by these methods. Development of disease resistant cultivars is one of the most effective, ecofriendly, and cheapest measure to control Brassica diseases. With the development of genomics, molecular biology techniques, and biological methods, it is possible to discover and introduce resistance ($R$) genes to efficiently control the plant diseases caused by pathogens. Some $R$ genes of major diseases such as Fusarium wilt and clubroot in Brassica vegetables have been already identified. Therefore, we will focus to review the Fusarium wilt and clubroot resistance in Brassica vegetables and the methodologies for identification, mapping, and pyramiding of $R$ genes/quantitative trait loci (QTLs) to develop disease resistant cultivars. These techniques will be helpful for sustainable crop production and to maintain global food security and contribute to ensure protection of food supply in the Asian country as well as throughout the world.

Keywords: $R$ gene, marker assisted selection, Fusarium wilt, clubroot, Brassica

1. Introduction

Brassica is a commercially important genus that contains vegetables, oilseeds, condiments, and fodder crops, and they provide nutrition and health-promoting substances to humans worldwide [1]. The commercially important vegetables such as Chinese cabbage (var. *pekinensis*), pak choi (var. *chinensis*), and turnip (var. *rapa*) are involved in *Brassica rapa* L., and cabbage (var. *capitata*), broccoli (var. *italica*), and cauliflower (var. *botrytis*) are involved in *Brassica oleracea* L. [1].

Production of Brassica vegetables constantly threatened by emerging viral, bacterial, and fungal diseases, whose incidence has increased in recent years [2, 3]. The major diseases of Brassica vegetables are Black rot, clubroot, Downy mildew, Fusarium wilt, soft rot, and Turnip mosaic virus [2]. Cultural, physical, biological, or chemical controls, or a combination of these controls, integrated
pest management, are used for disease control [2, 3]. However, soil-borne phytopathogens such as Fusarium wilt or clubroot are hard to control by physical and chemical methods, and they can survive in the soil for many years in dormant conditions and become devastating when they find suitable host [2–4]. Thus, breeding the disease resistant cultivars of Brassica vegetables, especially against soil-borne phytopathogens, is the best way for effective disease control. Recently, some disease resistance genes (R genes) have been isolated in Brassica vegetables, and DNA marker assisted selection is applicable in some diseases [2, 3].

In this chapter, we focus on the Fusarium wilt and clubroot and present the breeding for these disease resistances in Brassica vegetables using DNA marker selection.

2. DNA marker selection for breeding Fusarium wilt disease resistant cultivars in Brassica vegetables

Fusarium species are highly host specific and comprise more than 120 formae speciales (f. sp.) further sub grouped into races [5, 6]. Fusarium oxysporum is considered as one of the top ten most devastating plant pathogens throughout the world and can infect approximately 150 of independent host plants or over, including economically important agricultural crops such as cabbage, tomato, onion, pepper, cucumbers, bananas, melons, cotton, etc. [5, 7–12]. Two formae speciales of F. oxysporum (f. sp. conglutinans and f. sp. rapae) mainly invade in Brassica vegetables [3, 13].

Fusarium wilt was first identified in the United States by Smith in the 1890s, and in the following decades it was subsequently found in Japan and several other countries [14, 15]. In recent years, Fusarium wilt has been overspread in China [16]. F. oxysporum f. sp. conglutinans infects Brassica vegetable roots (young roots are more vulnerable), and thereafter, it colonizes and blocks the xylem vessels by their growth leading to blockage of the water transport inside the plant. Finally, it leads to show the disease symptoms such as dull green to yellow green color of the leaves initially, yellowing, wilting, necrosis of leaf, defoliation, stunting, and death of seedling [17].

2.1 Traditional management

A number of traditional techniques have been adopted to manage Fusarium wilt disease. Crop rotation is effective to control Fusarium wilt disease [18], and soil solarization [19] and soil steam sterilization [20, 21] can suppress significantly the F. oxysporum f. sp. conglutinans population. Application of chemical fungicides such as prochloraz, carbendazim, and Bavistin is also used [22], but it is not strongly recommended to control Fusarium wilt [23, 24]. Chemical fumigants such as sodium azide, chloropicrin, and methyl bromide etc. are environmentally hazardous and most of them are not available nowadays. A few chemical fumigants may be available in commercial market in some countries, but it needs to be applied according to the sustainable regulations [25–27]. An alternative environment friendly method is biological control, but there are not any registered biological control agents for the Fusarium wilt in Brassica vegetables [27]. Combining different independent strategies are used for the more efficient control of Fusarium wilt.

For example, combining the organic soil amendment (Brassica carinata defatted seed meals and compost) with a short period of soil solarization can significantly reduce both F. oxysporum f. sp. conglutinans and F. oxysporum f. sp. raphani [28].
2.2 Isolation of resistance genes

Most R genes encode proteins with leucine-rich repeats (LRR), a central nucleotide binding site (NBS) domain, and in the N-terminus a domain that contains homology to cytosolic domains of the Drosophila Toll or animal interleukin-1 receptors (TIR) (termed TIR–NBS–LRR) or a potential coiled coil (CC) domain (termed CC–NBS–LRR) [29–31]. R gene of F. oxysporum f. sp. conglutinans has been identified in B. rapa using transcriptome analysis focusing on differentially expressed putative R genes that have NBS, LRR, TIR, or CC motifs between Fusarium wilt resistant and susceptible lines [32]. Two TIR-NBS-LRR genes (Bra012688 and Bra012689), which located next to each other in the same transcriptional direction, have been identified as candidates of R gene (FocBr1), and presence and absence of these two genes were identical to the resistant and susceptible phenotypes, respectively, by inoculation test using F₂ population derived from crossing between susceptible and resistant lines [32, 33]. However, it has not been clarified which gene is FocBr1 [32].

R gene of F. oxysporum f. sp. conglutinans (FocBo1) has also been identified in B. oleracea by genetic approach [34, 35], and candidate R gene in B. oleracea is ortholog of Bra012688 [35]. It suggests that Bra012688 could be FocBr1. The susceptible B. oleracea lines have mutations causing frame shift and there are several susceptible alleles [34–36], suggesting that mutations leading to susceptibility have occurred multiple times independently. In contrast, no mutations other than deletion leading to loss of function of FocBr1 have been found in B. rapa [32, 33].

2.3 DNA marker selection system

Selection by inoculation test is labor-intensive and highly influenced by the environmental factors, and selection of suitable plants highly depends on the experience of breeders. In contrast, DNA marker selection is rarely affected by the environmental conditions. DNA marker selection also has merits that it can be performed at early developmental stages, can handle many samples, and can test multiple traits in a sample [37]. Identification of R gene or locus linked to R gene enables us to develop DNA marker for disease resistance [2, 3, 38].

As the susceptible allele (focbr1–1) of Fusarium wilt in B. rapa is due to deletion of FocBr1, a dominant DNA marker (Bra012688m), which confirms amplification of FocBr1, has been developed. This dominant DNA marker cannot distinguish the homozygous (FocBr1/FocBr1) and heterozygous (FocBr1/focbr1–1) alleles (Table 1). The SSR marker (SSR687int), which locates close to FocBr1, was identified, and we have confirmed this DNA marker can identify the heterozygous alleles in some lines (Figure 1). However, as there were several lines showing not identical to genotype information with resistance phenotypes by inoculation test, the genotypes determined by this DNA marker (SSR687int) and disease resistance by inoculation test must be confirmed before applying the DNA marker selection. To shorten the time required for PCR and to allow simultaneous determination of two dominant DNA markers of Bra012688 and Bra012689 (multiplex), the new DNA marker sets (YR688s and YR689s) were developed (Table 1).

In B. oleracea, three different susceptible alleles (focbo1–1, focbo1–2, and focbo1–3) were found [36]. As DNA marker sets covering these three susceptible alleles have been developed [36], it is necessary to select a DNA marker suitable for lines and will be available for breeder to use these primer sets (Table 2).
| Primer sequence          | PCR condition                                                                 | R  | S  | Ref.       |
|-------------------------|-------------------------------------------------------------------------------|----|----|------------|
| Bra012688 (FocBr1)      |                                                                               |    |    |            |
| Bra012688m F AGTCGCTTGGAGTAGCTCGAGG | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min. | A  | NA | [32, 33]  |
| R GAGCTAACAACATAACTGAGACC |                                                                               |    |    |            |
| YR688s F CTCACATCTGAGGATGGAAGTTGATACAAGCTCGGA | 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30s and 68 °C for 30s, and final extension at 68 °C for 2 min. This primer set is used with YR689s for multiplex PCR. | A  | NA |            |
| R GCTCCGAATTCGAATTTGGTAATATCGCATACGAG |                                                                               |    |    |            |
| SSR687/SS687F CGTCAAACCCTTTTTGCTTA | 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 30s, and final extension at 72 °C for 2 min. This marker is co-dominant DNA marker | LB | SB |            |
| R CAAACGCTGGCTCTGCAAAT |                                                                               |    |    |            |
| Bra012689m F GCAATCAAAGCAAATAATCG | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min. | A  | NA | [32, 33]  |
| R CATTATAGTAGAACCAGTTGTGCACCC |                                                                               |    |    |            |
| YR689s F CCACCTGAGTGTGTTGAGAGTCTGATACCATCG | 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30s and 68 °C for 30s, and final extension at 68 °C for 2 min. This primer set is used with YR688s for multiplex PCR. | A  | NA |            |
| R AGAGAGGAGCTTGATCCACAAAGTAGTGATTC |                                                                               |    |    |            |

R, resistance; S, susceptible; A, amplification of PCR product; NA, no amplification of PCR product; LB, large sized band; SB, small sized band.

Table 1.
DNA marker for predicting fusarium wilt resistance in Brassica rapa.
3. DNA marker selection for breeding clubroot disease resistant cultivars in Brassica vegetables

Clubroot is also one of the most devastating diseases in Brassica vegetables and spreads almost all over the world [39]. Clubroot disease is caused by an obligate plant pathogen *Plasmodiophora brassicae*, which has distinct pathotypes or physiological races over the world [40, 41]. Plants infected by *P. brassicae* form clubs on roots, which interfere with the host plant’s water and nutrients uptake. This interference leads to leaf yellowing, wilting, stunted growth, and death of the host plants.

3.1 Traditional management

Clubroot is quite difficult to control completely by the traditional methods due to the long survival spores of the *P. brassicae* in soil, their pattern of life cycle, and their pathotype specific infection, so that *P. brassicae* ultimately causes a broad diversity of virulence [3]. However, some traditional management systems can control clubroot disease in some extent. Crop rotation with non-cruciferous plants can reduce the infestation of *P. brassicae* [18, 42], but cannot eliminate the *P. brassicae* completely [43, 44]. It is recommended not to grow any cruciferous plants on the infested site at least five to seven years.

Some biocontrol agents against *P. brassicae* such as *Bacillus subtilis*, *Streptomyces griseorube*, etc. are able to reduce the severity of clubroot infection [45, 46]. Soil sterilants like chloropicrin, diazomet, methyl, or ethylene dibromide etc. are effective to control clubroot [47]. Application of fungicides fluazinam and cyazo-famid can effectively reduce the viability of resting *P. brassicae* spores and prevent infection [47]. However, the real fact is that these chemicals are not commercially approved for clubroot management since a long ago [47]. Integrated application of cultural or physical, chemical, and biocontrol agents can also be practiced for the more efficient management of *P. brassicae* [47, 48].

3.2 Isolation of resistance genes

In *B. rapa*, clubroot resistance is controlled by major dominant genes and pathotypes specific [3]. About 20 clubroot resistance loci have been identified, and highest numbers of clubroot resistance genes were found in chromosome A03 [3]. Two clubroot resistance genes (*CRA/b* and *Crr1a*) have been cloned and both genes encode a TIR-NB-LRR class R protein [49–51]. Recently, new clubroot resistance loci were found in a locus close to *Crr1a* in chromosome A08. A clubroot resistance locus, covering *CRs* gene, has been identified, and Bra020876 and Bra020918 have been identified as candidates of the *R* gene. Another clubroot resistance locus, covering
| Primer sequence | PCR condition | R   | S   | Ref. |
|-----------------|---------------|-----|-----|------|
| *Fusa*-6        | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min | D   | ND  | [61] |
|                 | *Hind* III digestion |     |     |      |
|                 | *FocBo1* vs. *focbo1*-1 |     |     |      |
| R               | CAATCGCTTCTTGCTTCTCC |     |     |      |
| *Fusa*-4        | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min | ND  | D   | [61] |
|                 | *Eco* RI digestion |     |     |      |
|                 | *FocBo1* vs. *focbo1*-1 |     |     |      |
| R               | TAGCTTCATGCCATAGTCGTCCTGG |     |     |      |
| #1              | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min | ND  | D   | [36] |
|                 | *Eco* RI digestion |     |     |      |
|                 | *FocBo1* vs. *focbo1*-1 |     |     |      |
| R               | AGATGGGTAATTTGCTGGTGCG |     |     |      |
| #2              | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min | SB  | LB  | [36] |
|                 | *Eco* RI d-caps marker |     |     |      |
|                 | *FocBo1* vs. *focbo1*-2 |     |     |      |
| R               | ATCCCAAGTTGATATCAGTAGGAAGAG |     |     |      |
| #3              | 1 cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, and final extension at 72 °C for 3 min | ND  | D   | [36] |
|                 | *Eco* RI d-caps marker |     |     |      |
|                 | *FocBo1* vs. *focbo1*-3 |     |     |      |
| R               | GCCTCTGAAAGATCTGGAAAAGAA |     |     |      |

*R*, resistance; *S*, susceptible; *D*, PCR products are digested; *ND*, PCR products are not digested; *LB*, large sized band; *SB*, small sized band.

Table 2.
DNA marker for predicting *fusarium* wilt resistance in *Brassica oleracea*.
Rcr9 gene, was also identified in chromosome A08, and Bra020936 has been identified as a candidate gene [52]. Two clubroot resistance loci, Rcr3 and Rcr9, have been mapped in chromosome A08 being 1.17 Mb apart each other. Three genes, Bra020951, Bra020974, and Bra020979, have been identified as candidates of the Rcr3 gene, and three genes related to immune-system-process (Bra020827, Bra020828, Bra020814) have been identified as candidates of the Rcr9 gene [53]. An clubroot resistance locus, covering PbBrA08hangim gene, was also detected on chromosome A08, where is near to Crr1, CRs, and Rcr9 [54]. These reports suggest that they are not allelic, thus chromosome A08 covering these genes has an R gene cluster.

In B. oleracea, clubroot resistance is quantitative, and QTLs have been identified. Effect of each QTL is weak, and little progress in isolation of R gene or fine mapping of R gene in B. oleracea [3]. It clarifies resistant mechanism of clubroot in B. oleracea is polygenic nature where multiple clubroot loci combinedly responsible to the clubroot resistance [55].

3.3 DNA marker selection system

A breeding for clubroot resistance is much more complex compared with Fusarium wilt resistance due to the complexity of plant–pathogen interactions. A number of clubroot resistance locus has been identified by the different research groups in B. rapa, and this variation is due to the pathotype specific pathogenicity of P. brassicae [2, 3]. As CRa/CRh and Crr1a have been isolated, DNA markers in these genes have been developed. A dominant resistance (CRAim-T) and susceptible (Craim-Q) DNA marker set of CRa has been developed [49], and co-dominant indel marker (mCrr1a) of Crr1a has been developed (Table 3) [33, 50]. In B. rapa, as the other clubroot resistance genes have not been isolated, linkage DNA markers are developed (Table 3). Some clubroot resistant cultivars in Chinese cabbage have been produced by introducing a single gene for clubroot resistance from European turnip. However, there is a problem that the loss of resistance by the presences of multiple pathotypes of P. brassicae or arising new pathotypes has been found [56, 57]. Thus, the accumulation of multiple genes of clubroot resistance could make small risks to the breakdown of resistance [58, 59]. The introduction of DNA marker selection is essential for the simultaneous selection of multiple clubroot resistance genes. Furthermore, high-throughput genotyping system such as multiplex PCR could be useful. We have developed the multiplex DNA marker selection system (Figure 2). Indeed, the accumulation of three major clubroot resistance genes (Cra/CRh, CRk, and CRc) by DNA marker selection in Chinese cabbage represented the highly resistance against six isolates of P. brassicae [60]. A high clubroot resistant Chinese cabbage cultivar, ‘Akimeki’, was also developed by the accumulation of Crr1a, Crr2, and CRA/CRh genes by DNA marker selection [3].

In B. oleracea, it has also been compared by the independent and cumulative incorporation of the clubroot resistance locus to combat various isolates, where one major clubroot resistance gene (PbBo(Anju)1) accumulated independently, as well as combined with four minor clubroot resistance genes (PbBo(Anju)2, PbBo(Anju)3, PbBo(Anju)4, PbBo(GC)1). Accumulation of five clubroot resistance genes (one major and four minor clubroot resistance genes) represented the highest resistance against six P. brassicae isolates. Here, the major QTL, PbBo(Anju)1, is a main player for the resistance mechanism against P. brassicae and the introgression of other four minor clubroot resistance QTLs boosted up the resistance [55]. As this major clubroot resistance gene acts as repressive, heterozygous of this gene shows susceptibility to P. brassicae. Previously, we have tested using linkage DNA marker of PbBo(Anju)1 in 35 cabbage F1, cultivars in Japan, and 12 cultivars (34%) have homozygous of PbBo(Anju)1 allele [61], suggesting that about 60%
| Primer sequence   | PCR condition                                                                 | R     | S    | Ref. |
|------------------|-------------------------------------------------------------------------------|-------|------|------|
| **CRa/CRb**      |                                                                               |       |      |      |
| CRaim-T          | F TATATTAATGATAAAGCAGAAGAAGAA                                               |       |      | [33, 49] |
|                  | R AATGCGACTGAGAAGTTTGTAG                                                     |       |      |      |
| CRaim-Q          | F TGAAGAATGCGGGCTACGTCCTGAAATC                                               |       |      |      |
|                  | R GAAATGATGAGGGTTTATTGTAGGG                                                   |       |      |      |
| **CRb**          |                                                                               |       |      |      |
| TCR108           | F CGGATATTCGATGTCGTTCA                                                        | A     | NA   |      |
|                  | R AAAATGTATGTGTTATGTGTTTATG                                                  |       |      | [33, 62] |
| mCrr1a           | F CGATGAGATGCTGCTTCTTCT                                                     | SB    | LB   |      |
|                  | R TCTGAGATCAACGGCTTCA                                                        |       |      | [33]  |
| **CRc**          |                                                                               |       |      |      |
| B50-C9           | F GATTCAATGCATTTCTCTGAT                                                       | A     | NA   | [60]  |
|                  | R CGTATTAATATCTCTCTGTCATCC                                                   |       |      |      |
| B50-6R           | F AATGCAATTTCGCTCAACC                                                        | NA    | A    |      |
|                  | R CGTATTATAATCTCTCTCATCC                                                     |       |      |      |
| **CRk**          |                                                                               |       |      |      |
| HC688–4          | F TCTCTGATTCGTTGACTG                                                        | A     | NA   | [60]  |
|                  | R ATATGTTGAGCCTATGTCT                                                        |       |      |      |
| HC688–6          | F TCTCTGATTCGTTGACTG                                                        | NA    | A    |      |
|                  | R AAATATATGTGAACTTTATGATC                                                    |       |      |      |
| **Crr2**         |                                                                               |       |      |      |
| Primer sequence | PCR condition | R  | S  | Ref. |
|-----------------|---------------|----|----|------|
| BRMS-096        | 1 cycle of 94 °C for 1 min, 35 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min, and final extension at 72 °C for 4 min. | LB  | SB | [63] |
| OPC11–2S        | 1 cycle of 94 °C for 30s, 45 cycles of 94 °C for 30s, 40 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 7 min. | LB  | SB | [64, 65] |

R, resistance; S, susceptible; A, amplification of PCR product; NA, no amplification of PCR product; LB, large sized band; SB, small sized band.

Table 3.
DNA marker for predicting clubroot resistance in Brassica rapa.
of cabbage cultivars could be susceptible to *P. brassicae*. One reason for clubroot resistance breeding in *B. oleracea* being behind in *B. rapa* is due to the theoretically impossible to introduce recessive resistance gene by backcrossing with inoculation test. However, DNA marker selection can overcome this problem as co-dominant DNA marker can distinguish the heterozygosity and homozygosity of \( PbBo(Anju)1 \) allele, suggesting that introduction of DNA marker selection is indispensable in *B. oleracea*. However, the current DNA markers are linkage markers, making it difficult to use them universally. Thus, it will be necessary to develop gene markers based on mutations that cause susceptibility, and this will require the isolation of clubroot resistance genes in *B. oleracea*.

### 4. Perspective

Both Fusarium wilt and clubroot are the serious disease for Brassica vegetables. Breeders are trying to develop the resistant lines for the both diseases by DNA marker assisted breeding. It has already been successfully developed Fusarium wilt and clubroot resistant lines. However, a Fusarium wilt resistant line can be infested by the clubroot or vice versa, while the clubroot has the virulence complexity. It is quite difficult to inoculate the multiple pathogens/races in an individual plant, while resistant breeding independently for each disease will make a further issue. DNA marker-based selection will enables us to overcome the mentioned issue. It has already found an association between a Fusarium wilt resistance allele and clubroot susceptible allele in *B. napus*, but their recombination was also reported [66]. It is necessary to identify the possible linkage between the genes responsible for the Fusarium wilt and clubroot diseases in Brassica vegetables. A Fusarium wilt resistance gene (*FocBr1*) is located on the region covering *CR* genes (*CRa/CRb, Rcr1, Crr3, and CRk*) with a physical distance approximately 2 Mb in chromosome A03 [3]. Recombination of two genes has been found [33], thus we can accumulate Fusarium and clubroot resistant alleles. In *B. oleracea*, *FocBo1* is close to a minor clubroot QTL in chromosome C06, but they are not closely linked each other [35, 55, 61, 67]. A linkage between dissimilar resistance loci can allow to inherit the resistance genes both for Fusarium wilt and clubroot, which can lead us for the development of resistant cultivars for both diseases.

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Conflict of interest

The authors declare no conflict of interest.

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