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RECENT PROGRESS OF CLUBROOT RESISTANCE BREEDING THROUGH MARKER-ASSISTED SELECTION IN *Brassica rapa* AND *Brassica oleracea*

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Abstract

Clubroot disease *(Plasmodiophora brassicae*) is one of the most damaging diseases of vegetable *Brassica* in the world. Different research groups from various countries of the world have identified eight clubroot resistance (*CR*) loci; *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRa*, *CRb*, *CRc* and *CRk* in *Brassica rapa* and twenty seven genetic loci encoding *CR* in *B. oleracea* by Quantitative Trait Loci (QTLs) analysis till now. Some *CR* loci are located in the near position of the same chromosome and others are distributed in different chromosomes. The current status of knowledge of clubroot resistance genes considering the future prospects for the development of CR resistant cultivars only are reviewed. The article provides relevant reviews on the clubroot resistant genes and their accumulation through Marker-assisted selection (MAS) in both *B. oleracea* and *B. rapa*.

Keywords: Brassica crops, clubroot disease, *Plasmodiophora brassicae*, resistance gene, MAS

Introduction

Clubroot disease is one of the most damaging diseases of vegetable crops belonging to the family Brassicaceae worldwide. The pathogen *Plasmodiophora brassicae*, an obligate biotroph has ability to survive as resting spores for a long time in soil. The nutrient absorption and growth of infected plants is inhibited due to club formation clubs on root and finally leads to a substantial reduction of crop quality and yield. The eukaryote pathogen, *P. brassicae* is a member of the group Plasmodiophorids. Previously, this group was classified as fungi but the recent phylogenic studies based on small sub-units ribosomal RNA, actin and the ubiquitin genes have classified Plasmodiophorids into Protozoa or Protoctista (Castlebury and Domier 1998; Van *et al*. 2000; Braselton, 2001; Down *et al*. 2002).

The infection occurs through two phases. The resting spores in soil germinate and then the resultant zoospores attack root hairs, and grow into the multi-nucleate plasmodia (primary plasmodia) in the root hairs. The plasmodia cleave and then form secondary zoospores. The zoospores migrate to root cortical tissue, and induce abnormal growth of the root tissue forming a distorted massive gall called club (Hirai, 2006). Secondary plasmodia are formed in the clubs, then after meiosis, numerous resting spores are formed. Upon the decay of the clubs, resting spores are released into soil, where they can survive for many years (Wallenhammar, 1996). The spores spread to production areas via drainage water and infected root debris. The disease is difficult to prevent by agricultural practices such as liming and drainage (Dixon, 2007 and 2009). Thus, breeding of clubroot resistant (CR) cultivars is one of the most effective and sustainable approaches to the prevention of clubroot disease for minimizing crop loss from infection of the pathogen. Recently, there have been dramatic advances in the understanding of the molecular nature and mechanisms associated with natural *CR* resistant genes. Dominant and recessive resistance genes have been characterized at molecular level and we are beginning to understand some new principles of innate immunity to pathogens associated with gene silencing.

This article depicts an overview about the molecular and genetic character of *CR* genes in *Brassica rapa* and *B. oleracea* crops and their accumulation towards CR resistance. Breeding for resistance need not to be dependent upon a full molecular characterization of the resistance gene alleles and the corresponding pathogen avirulence (avr) determinants. In a practical sense, the successful deployment of a novel resistance gene into a crop depends upon the identification of a positive phenotype, identification of genetic markers for marker-assisted selective breeding (MAS) and understanding of how the novel resistance will behave under different genetic backgrounds and pathogenic pressures in the field. Several CR cultivars with higher resistance have recently been developed but these

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are still insufficient to protect. In the following paragraphs, this article has provided recent information about *CR* resistance genes which are indispensible for CR breeding because it allows the acquisition of higher resistance by combining different resistance genes.

Breeding and identification of *CR* loci in *Brassica rapa*

Clubroot resistant traits have been found in European turnips (*B. rapa* ssp. *rapifera*) such as Gelria R, Siloga, Debra and Milan White. Yoshikawa (1981) successfully has introduced clubroot resistance (*CR*) genes from the European fodder turnips into the Chinese cabbage (*B. rapa* spp. *pekinensis*). Subsequently a number of resistant F_1 hybrid cultivars were released by using the *CR* parental lines. Recent molecular genetics analyses have identified eight *CR* loci, namely *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRa*, *CRb*, *CRc* and *CRk* in *Brassica rapa* (Table 1). All eight *CR* genes present in *B. rapa* have been identified through QTL mapping using a range of resistant sources and molecular markers. The DNA markers linked to *CR* locus have been developed for marker-assisted selection in *B. rapa* (Matsumoto *et al*. 2012; Sakamoto *et al.* 2008; Piao *et al.* 2009; Suwabe *et al*. 2006; Hirai *et al*. 2004).

Table 1. *CR* loci reported on *Brassica rapa*

***CR* locus**	**Found as**	**Map position**	**Linked marker**	**Gene source**	**Reference**
CRa	Major gene	R3	HC352b-SCAR *CRa* gene	T136-8(DH line)	Matsumto *et al*. 1998, Hayashida *et al*. 2008, Ueno et al. 2012
Crr1	Major gene	R8	BRMS-088 BSA7	Siloga	Kuginuki *et al*. 1997, Suwabe *et al.* 2003, Suwabe *et al* . 2012
Crr2	Major gene	R1	BRMS-096	Siloga	Suwabe *et al*. 2003
Crr3	Major gene	R3	OPC11-2S	Milan White	Hirai *et al*. 2004
Crr4	QTL	R6	WE24-1	Siloga	Suwabe *et al.* 2006
CRb	Major gene	R3	TCR05 KBrH059N21F B0902	Gelria	Piao *et al.* 2004, Kato *et al.* 2012, Kato *et al.* 2013
CRc	Major gene	R2	m6R	C9 (DH line of Debra)	Sakamoto *et al.* 2008
CRk	Major gene	R3	OPC11-2S	K10(DH line of CR Kanko)	Sakamoto *et al.* 2008

Breeding and identification of *CR* loci in *Brassica oleracea*

In *B. oleracea*, different research groups from various regions of the world have identified several sources of clubroot resistance through the screening of germplasm (Crute *et al.* 1980; Dixon and Robinson 1986; Dixon *et al*. 1986; Dixon 1988; Crisp *et al.* 1989; Dias *et al.* 1993; Voorrips and Kanne 1997; Manzanares-Dauleux *et al.* 2000; Carlsson *et al.* 2004). Only few completely resistant accessions have been identified in *B. oleracea* in contrast to *B. rapa*. Genetic analyses of clubroot resistance were studied in *B. oleracea* using diallel crossing methods or segregating population. Most of the classical genetic studies without molecular markers revealed that inheritance of this trait is polygenic in nature (Hirai 2006; Piao *et al.* 2009). A total of twenty seven QTLs were identified till now (Table 2); two QTLs, *CR2a* and *CR2b* in rutabaga cv Wilhelmsburger (Landy *et al.* 1992), three QTLs in broccoli (Figdore *et al.* 1993), two QTLs in kale (C10) (Grandclement *et al.* 1996), two major QTLs, *pb-3* and *pb-4*, and a minor QTL in cv Bindsachsener (Voorrips *et al.* 1997), one QTL in kale (K269) (Moriguchi *et al.* 1999), three QTLs in kale (K269) (Nomura *et al.* 2005), nine QTLs in kale (C10) (Rocherieus *et al*. 2004) and five QTLs in cabbage cv. Anju (Nagaoka *et al.* 2010). The identification of several *CR*-QTLs indicates that the clubroot resistance in *B. oleracea* is controlled by polygenic manner, confirming the complex genetic basis of clubroot resistance in *B. oleracea*. The comparison of these QTLs is impossible due to lacking of common molecular markers among different researchers and use of the different CR sources and pathogen isolates (Piao *et al.* 2009). For better

understanding the genetics and genomics of *CR* loci in *B. oleracea*, QTL identification based on common PCR-based markers is required.

Table 2. Mapping of *CR* loci reported on *Brassica oleracea*

***CR* locus**	**Map position**	**Linked marker**	**Gene source**	**Reference**
CR2a	LG6	2NF11, 2ND3	Swede cv Wilhelmsburger	Landy *et al.* 1992
CR2b	LG1	3NE4a, 3ND3		
3	LG1	14a	Broccoli (OSU[a] CR-7)	Figdore *et al.* 1993
	LG4	48		
	LG9	177b		
2	-	OPL6-780, OPB11-740, OPA18-14900, OPA4-700, OPE20-1250, OPA1-1880, OPA16-510	Kale (C10)	Grandclement *et al.* 1996
pb-3	LG3	4NE11a	Bindsachsener	Voorrips *et al.* 1997
pb-4	LG1	2NA8c		
1	LG3	WG6A1, WG1G5	Kale (K269)	Moriguchi *et al.* 1999
QTL1	LG1	SCA02a2	Kale (K269)	Nomura *et al*. 2005
QTL3	LG3	SCB50b, SCB74c		
QTL9	LG9	SOPT15a, SCA25		
Pb-Bo1	LG1	Ae05.8800, T2	Kale (C10)	Rocherieus *et al.* 2004
Pb-Bo2	LG2	PBB38a, r10.1200		
Pb-Bo3	LG3	ae15.100, RGA8.450		
Pb-Bo4	LG4	ELI3.983,aa9.983		
Pb-Bo5a	LG5	PBB7b, ae05.135		
Pb-Bo5b	LG5	ELI3.115, a18.1400		
Pb-Bo8	LG8	c01.980, t16.500		
Pb-Bo9a	LG9	aj16.570, W22B.400		
Pb-Bo9b	LG9	a04.1900, ae03.136		
Pb-Bo(Anju)1	LG2	KBrH059L13	Cabbage cv Anju	Nagaoka *et al*. 2010, Tomita *et al.* 2013
Pb-Bo(Anju)2	LG2	CB10026		
Pb-Bo(Anju)3	LG3	KBrB068C04		
Pb-Bo(Anju)4	LG7	KBrB089H07		
Pb-Bo(GC)1	LG5	CB10065		

Accumulation of *CR* genes in *Brassica rapa* crops

Though CR cultivars have been being used widely for major production areas, these are still insufficient to protect from the attack of the clubroot disease. This is due to appearance of multiple races of *P. brassicae* (Matsumoto *et al*. 2012). In addition, complexity of plant-pathogen interaction prevents the breeding of CR cultivars in Brassica crops. For example, *CR*-genes show different reactions against variable virulence of *P. brassicae* (Suwabe *et al.* 2003 and Nomura *et al*. 2005). Suwabe *et al.* (2006) found that *Crr1, Crr2* and *Crr4* were effective in two different *P. brassicae* isolates i.e. Wakayama-01 and Ano-01. The simultaneous involvement of *Crr1* and *Crr2* was specifically effective against the most virulent Wakayama-01 isolate, whereas the expression of resistance to the Ano-01 isolate was effective without *Crr2*. It explained that *Crr2* is an enhancer for the resistance expressed by *Crr1* and /or *Crr4*, rather than a determinant for recognition specificity. The inheritance of resistance in kale (*B. oleracea*) was controlled by some dominant alleles with a predominance of additive effects with incomplete dominance (Laurens and Thomas, 1993). Yoshikawa (1993) demonstrated that clubroot resistance of the European fodder turnips, including cv Siloga, was controlled mainly by a major gene and a few minor genes. James *et al.* (1978) identified three independent dominant genes that conferred resistance in three *B. rapa* genotypes to race 6 of *P. brassicae*. Heterozygotes in *CR* locus are less resistant than homozygotes (Hirai, 2006).

Although complexity of plant-pathogen interaction is problematic for clubroot resistance breeding of Brassica crops, previous reports have indicated that combining different *CR*-genes exhibited higher resistance to the disease (Nomura *et al*. 2005; Matsumoto *et al*. 2012). Matsumoto *et al*. (2012) attempted to accumulate 3 *CR*-genes, *CRa, CRk* and *CRc* in Chinese cabbage (*B. rapa* spp. *pekinensis*) through marker-assisted selection and found homozygous lines for 3 *CR*-genes, whether selfed or crossed, exhibited exceedingly high resistance against all of 6

field isolates of *P. brassicae*. Similarly NARO Institute of Vegetable and Tea Science (NIVTS) has developed a highly *CR*-resistant Chinese cabbage cultivar by accumulating *Crr1, Crr2* and *CRb* genes. It is proven that accumulation of *CR*-genes through MAS strengthened resistance and consequently it can be resistant to the multiple races of *P. brassicae* in *B. rapa*. Hatakeyama *et al.* (2013) have reported that the CR level dose-dependence increased according to the expression level of the *Crr1*a gene in the transformed *B. rapa* plants, suggesting possibility in latter one. The effect of accumulation of different *CR* genes could be controlled by dose-dependent accumulation of CR proteins (Kou and Wang, 2010; Liu *et al*. 2004).

Accumulation *CR* genes in *B. oleracea* crops

Brassica rapa possesses several major *CR* loci, which confer differential (pathotype-specific) resistance to particular isolates of *Plasmodiophor brassicae* , and are *CR* loci such as *Crr1, CRa*, and *CRk* have a large effect on resistance (Kuginuki *et al*. 1999; Suwabe *et al.* 2003, 2006; Matsumoto *et al.* 2012). That's why CR cultivars in which the three *CR*- genes accumulated have been readily developed (Matsumoto *et al.* 2012). In *B. oleracea*, resistance of genotypes has generally been identified less frequently than in the genotypes of *B. rapa* and *B. napus*, and the level of resistance is lower (Crisp *et al.* 1989). This might be polygenic nature of resistance in *B. oleracea* (Tomita *et al.* 2013).

Nagaoka *et al.* (2010) developed the progeny of *B. oleracea* derived from the crossing between 'GC' and 'Anju' to evaluate effectiveness of accumulating the major and minor QTLs. In their study, a single F_1 plant was self-pollinated (bud pollination technique) to produce F_2 seeds. Afterwards, selected F_2 plants were self-pollinated to obtain F_3 seeds. Heterozygous condition of *CR* locus in F_2 plants was confirmed by closest marker (Table 3). The F_3 lines were inoculated by resting spore of *P. brassicae* race 4 at 10^6 spores ml^{-1} (Fig. 1).

Nomura *et al.* (2005) have identified three CR-QTL in the F_2/ F_3 population from the cross between cabbage and kale line K269, and demonstrated that the accumulation of those three *CR*-genes showed broad resistance to three isolates.

Manzanares *et al.* (1996) identified the isolate-specific reaction between *P. brassicae* and *B. oleracea* genotypes in inoculation tests using isolates derived from a single spore. Rocherious *et al.* (2004) identified nine *CR-QTLs* which differentially act on five different isolates of *P. brassicae.* Similarly, Werner *et al.* (2008) mapped *CR-QTL* from B. *rapa* and *B. oleracea* after inoculation into *B. napus*, and identified eight *CR-QTLs* in the *B. oleracea* C genome, all showing pathotype specificity.

Table 3. Position of *CR-QTLs* and linked markers in *B. oleracea* (Nagaoka *et al.* 2010; Doullah *et al.* 2012)

QTLs	Linked markers	Linkage group	Marker position (cM)	QTL peak (cM)	LOD score
PbBo(Anju)1	BRMS228		16.13		
	KBrH059L13	O2	18.83	18.7	11.7
	IGF5224g		27.41		
PbBo(Anju)2	BoGMS0985		41.29		
	CB10026	O2	48.36	49.1	4.8
	BoGMS1394		55.06		
PbBo(Anju)3	KBrB068C04	O3	119.36	125.3	2.7
	CB10021		126.75		
PbBo(Anju)4	CB10634		20.31		
	KBrB089H	O7	23.01	20.5	4.2
	BoGMS0537		24.19		
PbBo(GC)1	CB10435		19.24		
	CB10065	O5	20.37	20.4	3.9
	BoGMS1330		31.55		

Nagaoka *et al.* (2010) found that the resistant parent (Anju) showed an ID of 0.2 and susceptible parent (GC) had an ID of about 5.0 (Fig. 1 & 2). The *CR* test of F_3 progenies revealed that the line having only major QTL *PbBo(Anju)1* showed moderate resistance (ID=3.0).The lines involving minor *CR*-genes without *PbBo(Anju)1* showed distinct susceptibility (ID= 4.1-4.6), whereas *PbBo(Anju)1* plus one or some minor *CR*-genes brought out moderate resistance (ID= 1.5-3.0). Particularly, *PbBo(Anju)1* plus combination of less

than that of 3 minor QTLs exhibited no strong resistance. Doullah *et al.* (2012) also revealed that *PbBo(Anju)1* and three minor QTLs (*PbBo(Anju)2*, *PbBo(Anju)4* and *PbBo(GC)1* play a critical role in the acquisition of resistance to clubroot (Fig. 2). They concluded that *PbBo(Anju)1* plays a crucial role in the expression of clubroot resistance and also pyramiding minor *CR*-gene is essential for getting higher resistance. Later, Tomita *et al.* (2013) clarified their effectiveness for controlling disease involving various isolates. In exploring the overall disease incidence, a single involvement of the major *CR*- gene located in the *PbBo(Anju)1* locus, or accumulation of *CR*-genes in the minor *CR-QTL*, is not enough to confer sufficient resistance. One major *CR*-gene in the QTL *PbBo(Anju)1* locus plus two to three minor *CR*- genes conferred moderate resistance. They found that the genotype in which all of the *CR*-genes locating in the five QTLs including *PbBo(Anju)1* were accumulated showing the highest resistance, and it was broadly resistant against six *P. brassicae* isolates. This is in agreement with previous papers that reported the importance of pyramiding *CR*- genes (Nomura *et al.* 2005; Matsumoto *et al.* 2012).

The results from Tomita *et al.* (2013) indicated that the developed DNA markers can select the genes that are required for the acquisition of resistance efficiently and these markers could be powerful tool for CR breeding in *B. oleracea*. The novel breeding method developed by CR research groups that clubroot resistance can be reinforced by pyramiding minor *CR*-genes through MAS which is available in both *B. oleracea* and *B. rapa*.

Fig 1. Disease severity index; 0= no clubs; 1 = a few small clubs usually confined to lateral roots; 2 = moderate clubbing on lateral roots; 3 = large clubs on lateral roots and slight swelling of main roots; 4 = large clubs in mainroots and 5 = severe clubbing. Arrows indicate the club(S) on roots.

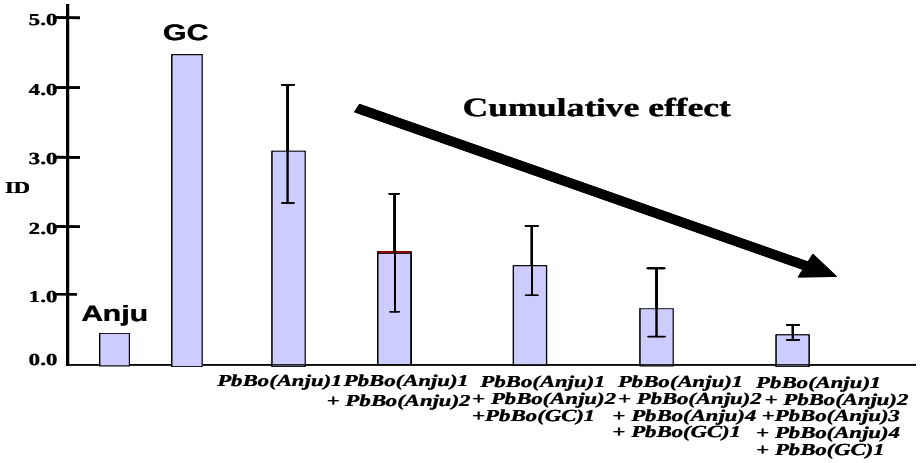

Fig. 2. Cumulative effect of five QTLs for resistance to clubroot disease in *B. oleracea*

Marker-assisted Selection (MAS) in clubroot disease

For genetic accumulation of *CR*-genes corresponding to wide pathogenicity of clubroot fungus, MAS is indispensable because it allows precise identification of how many *CR*-genes involves in cultivars and monitoring accumulation of *CR*-genes in the progeny in the breeding process. On the other hand, it is impossible to

accumulate *CR*-genes by the ordinary inoculation method, which isn't capable of distinguishing homo/heterozygote alleles of *CR*-genes, and furthermore time-consuming and imprecise for identification of disease incidence. To increase the durability of clubroot resistant cultivars to a broader spectrum of physiological race, the combination of different *CR*-genes into a single line will be an indispensable means. To do this, MAS is essential for CR breeding in Brassica crops.

Nagaoka *et al.* (2010) analyzed *CR*-QTLs using the mean phenotypes of F_3 progenies from the cross of a susceptible broccoli double haploid (DH) line 'GC' and resistant parent cabbage DH line 'Anju'. As a result, one major QTL *PbBo(Anju)1* and four minor QTLs namely, *PbBo(Anju)2*, *PbBo(Anju)3*, *PbBo(Anju)4* and *PbBo(GC)1* were identified against *P. brassicae* race 4 in the present findings. Subsequently Tomita *et al.* (2013) successfully developed MAS system by designing the specific primers of those QTLs (Table 3).

Conclusion and Perspectives

In the area of clubroot disease resistance, Quantitative Trait Loci (QTLs) analysis has been identified clubroot resistance (*CR*) loci; *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRa*, *CRb*, *CRc* and *CRk* in *Brassica rapa*. Also more than twenty genetic loci encoding *CR* in *B. oleracea* were extensively screened and identified. Some *CR* loci are located in near position of the same chromosome and others are distributed in different chromosomes.

B. rapa and *B. oleracea* possesses several major *CR* loci, which confer differential (pathotype-specific) resistance to particular isolates of *Plasmodiophora brassicae*. This article has reviewed that several research groups have tried to accumulate several *CR*- genes. In *CR* breeding program of *B. rapa*, NARO Institute of Vegetable and Tea Science (NIVTS) has developed a highly CR-resistant Chinese cabbage cultivar by accumulating *Crr1*, *Crr2* and *CRb* genes. Due to the lack of common molecular markers among different studies and limited information of specific primer sequences linked to the *CR*- genes, it is very difficult to know *CR* locus identified by different researchers corresponds to which *CR* locus. So, additional studies that can disclose specific primer sequences linked to the *CR*- genes are needed to develop marker-assisted selection for clubroot resistance in *Brassica* species. Accumulation of several *CR*- genes by MAS is necessary to conduct *CR* breeding in *B. oleracea* and *B. rapa*. Different CR research groups developed DNA markers that can select required genes for the acquisition of resistance efficiently and these markers could be powerful tool for CR breeding in *B. rapa* and *B. oleracea*.

The best strategy to prevent or limit the extent of the infection is the development of genetic resistance. MAS could assist the incorporation of durable clubroot resistance (CR) into cultivars. The effectiveness for controlling disease, combinations of various *CR*-genes which were selected by using the DNA markers closely associated with each *CR*-*QTL* are very much useful. DNA markers detecting *CR* loci will be very useful for pyramiding *CR*-genes in Brassica with polygenic in nature. Uses of these markers developed by different CR researchers are good achievement for CR breeding. Accumulations of *CR*-genes through MAS strengthen resistance and consequently, confer resistance to the multiple pathotypes of *Plasmodiophora brassicae* in Brassica crops. It is likely that increased durability will be conferred by the pyramiding of the resistance genes. Specific primer sequences of a number of DNA markers linked to *CR*-genes are now available for clubroot resistance.

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