Genetic Analysis of Resistance to *Xanthomonas campestris* pv.*campestris* in *Brassica oleracea*

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Abbreviations

BLAST	Basic Local Alignment Search Tool
Bolbase	*Brassica oleracea* Genome Database
CAPS	Cleaved Amplified Polymorphic Sequences
CFU	Colony forming unit
CIM	Composite Interval Mapping
cM	CentiMorgans
CTAB	Cetyltrimethylammonium bromide
DH	Double Haploid
DLA	Diseased Leaf Area
DNA	Deoxyribonucleic acid
GC	Green comet
LG	Linkage Group
LOD	Logarithm (base 10) of odds
MAS	Marker assisted selection
ml	Milliliter
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
QTL	Quantitative trait loci
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single-stand Conformation Polymorphism
SSR	Simple Sequence Repeats
VE	Variance
Xcc	*Xanthomonas campestris* pv. *campestris*
YDC	Yeast Extract Dextrose Calcium Carbonate

Summary

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*), is possibly the most important disease of Brassica worldwide. Control of the disease is difficult and is usually attempted through the use of healthy planting materials and the elimination of other potential inoculum sources. An alternative approach is use of *Xcc*-resistant cultivars, but in practice has had only limited success. In order to produce *Xcc*-resistant cultivars, disclosure of mechanism of *Xcc*-resistance is needed. Firstly, as a preliminary to genetic analysis of resistance, an attempt was taken to screening the current cultivated local varieties of *Brassica oleracea* for identifying sources of resistance to *Xcc* race 1.

I conducted artificial leaf inoculation test where the resistance level was evaluated with infected symptom area (cm^2). Screening test resulted the most susceptible variety was Green comet (GCP09) (0.98 cm^2) followed by Savoyace (0.94cm^2) and Irodori (0.93cm^2). Among the resistant variety, lowest disease severity was recorded, ReihoP01 (0.13cm^2) followed by Beru fore (0.17cm^2) and Akazukin (0.19cm^2).

Based on this result, we selected GC(P09) as a susceptible parent and Reiho(P01) as a resistant parent in order to carry out QTL analysis in the subsequent F2 generation. We constructed a genetic map from the F2 population derived from GC(P09) × Reiho(P01). As a result, 181 markers of SSR and CAPS were distributed in 9 Linkage groups covering 1099.4cM. QTL analysis for *Xcc*-resistance detected 3 QTLs in Chromosome 5, 8 and 9, respectively where the major QTL, *XccBo(Reiho)2*, was derived from Reiho with a maximum LOD score (7.7) in C8. The QTL *XccBo(GC)1* (LOD 4.4) located in C9, was derived from the susceptible GC. The other QTL *XccBo(Reiho)1* (LOD 4.4), was found in C5.

To compare chromosomal positions of *Xcc*-resistance QTL in *B. oleracea* between the present and published studies, anchor markers that are common among the different maps were mapped to our map. In the 9 linkage groups obtained (C1-C9), based on common anchor markers, it was possible to compare our finding *Xcc*-resistance QTLs with the *B. oleracea Xcc* loci reported by previous authors; *XccBo(Reiho)2* and *XccBo(GC)1* may be identical to the Xcc resistance QTLs reported previously or a different member contained in the same resistance gene cluster. Our map includes public SSR markers linked to *Xcc*-resistance genes that will promote pyramiding *Xcc*-resistance genes in *B. oleracea*. The present study will also contribute to a better understanding of genetic control of *Xcc*-resistance.

General introduction

Xanthomonas campestris pv. *campestris* (*Xcc*) (Pammel) Dowson is a Gram-negative bacterium that causes black rot, the most important disease of vegetable brassica crops worldwide. The genus *Xanthomonas* includes economically important pathogenic bacteria that are generally associated with plants (Hayward, 1993; Vauterin *et. al*., 1990). The taxonomy of this genus was initially determined according to host preference and, consequently, a large number of species and pathovars have been defined (Burkholder, 1957). Morphological and other physiological and biochemical characters were subsequently used to classify the *Xanthomonas* isolates into eight phenotypic groups (Van Den Mooter and Swings, 1990). The *Xanthomonas* species were later reclassified on the basis of DNA–DNA hybridization, leading to *X. campestris* being restricted to comprise only the vascular pathogen *X. campestris* pv. *campestris* (Pammel) Dowson (*Xcc*), which causes black rot of Brassica species, and additional pathovars that cause vascular or leaf spot diseases in cruciferous hosts, including *X. campestris* pv. *aberrans* (Knösel) Dye, *armoraciae* (McCullock) Dye, *barbareae* (Burkholder) Dye, *incanae* (Kendrick & Baker) Dye and *raphani* (White) Dye (Vauterin *et. al*., 1995).

The bacterium *X. campestris* pv. *campestris* (Pammel) Dowson is a Gram-negative rod, that occurs mostly alone or in pairs and is usually motile by means of a single polar flagellum. Most strains form yellow, mucoid, glistening colonies. The yellow pigments, xanthomonadins (mono- or dibromo-arylpolyene structures), and the exopolysaccharide xanthan, responsible for the mucoid or viscous cultures, are typical of the genus (Vauterin *et. al*., 1995), although the existence of atypical pigmented isolates has been reported (Poplawsky and Chun, 1995). The taxonomy of the genus was mainly based on the hosts of origin and the phenotypic characteristics until the early 1990s. A detailed study of the phenotypic characteristics of the genus was conducted by Van den Mooter and Swings (1990). Vauterin *et. al*. (1995) later reclassified the genus on the basis of DNA–DNA hybridization studies. In the new classification, the species *X. campestris* was restricted to strains that cause disease in Brassicaceae (Cruciferae) plants (including *X. campestris* pv. *aberrans*, *armoraciae*, *barbarea*, *campestris*, *incanae*, *raphani* and, possibly, *plantaginis*). The reclassification is mainly supported by data obtained through other molecular techniques, including amplified fragment length polymorphism (AFLP) and polymerase chain reaction (PCR) fingerprinting (Rademaker *et. al*., 2000), but there has been some discussion on the shifts in the classification of some groups of isolates (Schaad *et. al*., 2000; Vauterin *et. al*., 2000).

There are many views regarding to what constitutes different pathovars. For example, some authors, such as Alvarez *et. al*. (1994), have considered that *X. campestris* pv. *raphani*, a pathovar originally described by White (1930), which has a broad range of hosts within the Brassicaceae and Solanaceae, and *X. campestris* pv. *armoraciae*, described one year earlier by McCulloch (1929) as a leaf spot disease of horse radish, are synonymous. Other authors, such as Tamura *et. al*. (1994) and Vicente *et. al*. (2006), have considered them to be distinct pathovars with a different host range.

Other *X. campestris* pathovars have received less attention. Some of these pathovars, such as *X. campestris* pv. *aberrans*, may not be distinct from *Xcc* (Fargier and Manceau, 2007; Fargier *et. al*., 2011; Vicente *et. al*., 2001). Fargier and Manceau (2007) considered that the species can be restricted to three pathovars (*campestris*, *raphani* and *incanae*), but some isolates from ornamental crucifers, which are currently identified as pv. *campestris* or *incanae*, may still belong to distinct pathovars (Vicente *et. al*., 2006).

Garman (1894) first described Black rot as a disease of cabbage in Kentucky, USA. He isolated two types of bacteria from diseased plants, but could not determine which type of bacterium was causing the disease. In Iowa, USA, Pammel (1895a, b) observed a similar disease in rutabaga and turnip, and showed that the disease was caused by a bacterium (named *Bacillus campestris*) with yellow pigmented colonies in culture. Reports from Wisconsin also attributed the disease of turnips and cabbage to the yellow bacterium (Russell, 1898; Smith, 1898). Since then, the disease has been identified in all continents wherever Brassicaceae crops are grown (Bradbury, 1986), and is considered to be the most important disease of vegetable Brassica crops worldwide (Williams, 1980). *Brassica oleracea* (including cabbage, cauliflower, broccoli, Brussels sprouts and kale) is economically most important host of *Xcc*. However, the disease also occurs in other Brassica crops, radish, ornamental crucifers and related weed species (Bradbury, 1986). Some accessions of *Arabidopsis thaliana*, the model plant for molecular plant research, are also susceptible when inoculated with *Xcc.*

Cook *et. al*., (1952), described Black rot as a seed-borne disease primarily. However, the disease can also be transmitted in infected transplants, infested soil, crop residues and carry-over in related weed species (Schaad and Alvarez, 1993; Walker, 1953). Schaad and White (1974) and Dane and Shaw (1996) showed that *Xcc* can survive in the soil, independent from the host, for approximately 40 days in winter and 20 days in summer. The results of Arias *et. al*. (2000) showed that high soil matric potential (saturated soils) can reduce the survival of the pathogen. The pathogen can survive longer in soil within plant

tissues than as free living cells. Kocks and Zadoks (1996) showed that crop residues in fresh (2 weeks) refuse piles are more effective in spreading the disease than older (4 months) piles. In some conditions, cruciferous weeds can survive all year round and can provide potential carry-over inoculums for the crops (Schaad and Dianese, 1981). Arias *et. al*. (2000) showed that epiphytic survival of the bacteria on the phylloplane is dependent on the plant species, as bacteria survived for 48 days on cabbage, mustard and lettuce, but only for 9 days on rice. In some cases, infected crops have also been shown to provide inoculum for the weeds (Dane and Shaw, 1996), and one study has indicated that weeds do not play an important role in the dissemination of black rot (Schaad and Thaveechai, 1983).

The bacterium can disperse over short distances via wind, insects, aerosols, irrigation water, rain, farm equipment and workers. Commercial vegetable brassica crops are raised from transplants. In plant nurseries that produce module-raised transplants, the overhead irrigation system can increase significantly the dissemination of the bacteria, and can subsequently lead to a high level of disease in the field; changing the irrigation method can therefore limit the spread of the disease (Roberts *et. al*., 2007).

Generally bacteria enter the plant through hydathodes on the leaf margins, when droplets of guttation contaminated with bacteria are reabsorbed into the leaf (Russell, 1898). This mode of entry is dependent on a combination of environmental, biological and mechanical factors (Meier, 1934). In contrast, stomata generally do not appear to be important for *Xcc* infection, because the disease generally does not spread into surrounding tissues, even though the bacteria can enter the plant through the stomata and produce small dark spots (Cook *et. al*., 1952). This suggests that vascular movement of bacteria is essential for disease development. The bacteria can also enter the plant through wounds caused by machinery, insects, animals, rain, irrigation and wind. The typical symptom of black rot is the formation of V-shaped, chlorotic yellow lesions with vertices towards the middle vein of the leaves and darkened veins that result from bacterial movement in the vascular system. The affected tissues can become necrotic, and leaves can fall prematurely; systemic infections can cause stunted growth and the death of young plants. Secondary infection by other bacterial species can also contribute to further development of severe rotting of vegetable tissue. The infection is often latent when temperatures are low, as the bacteria can persist in the vascular system without producing symptoms and, when the temperature rises, the typical symptoms become evident (Cook *et. al*., 1952; Schaad, 1982; Walker, 1953).

Xcc considered as a severe disease agent in warm, humid climates and, consequently, is most serious in tropical, subtropical and humid continental regions

(Williams, 1980). Given the global distribution of *Xcc*, black rot will become an increasingly important disease constraint favored by climate change in more northern latitudes of vegetable production, including the warmer regions of Europe.

Control of black rot disease is difficult and usually attempted through the use of disease free planting materials (seeds or transplants) and the elimination of other potential inoculam sources such as infected crop debris and cruciferous weeds (Taylor *et. al*. 2002). Sanitation and management practices, including crop rotation, weed control and the use of assayed clean seed, can provide significant control of the disease (Schaad and Alvarez, 1993). Black rot was a minor disease in the most important production areas in the USA during certain decades, probably because growers followed the recommended practices, including the use of tested, disinfected seed and rotation of seedbeds. However, there was a resurgence of the disease during the 1970s, probably associated with the use of F1 hybrid seed produced in areas in which the disease was endemic (Williams, 1980). Standard seed testing methods have been developed (Roberts and Koenraadt, 2006). The tolerance for reliable disease control through seed testing needs to be adjusted according to the system of production, e.g. the number of seeds tested should be higher for transplants raised with overhead irrigation than for direct-drilled crops (Roberts *et. al*., 2007). Seed treatments, including hot water, antibiotics, and sodium hypochlorite, hydrogen peroxide and hot acidified cupric acetate or zinc sulphate, are available, but no treatment is totally effective. Several methods can be used to reduce the spread of the disease during transplant rising, including the use of web and flow irrigation systems instead of overhead irrigation and of chlorine dioxide in the irrigation water (Krauthausen *et. al*., 2011).

The development and use of black rot-resistant cultivars have long been recognized as important methods of control, but, in practice, have had only limited success (Taylor *et. al*., 2002). Natural variation and the inheritance of black rot resistance have been studied in several Brassica species and, so far, no disease resistance gene has been cloned. Most studies have focused on *B. oleracea* (representing the C genome of Brassicas), and a limited number of sources of resistance have been identified, including the cabbage cultivar Early Fuji and the cabbage accession PI 436606 (cv. Heh Yeh da Ping Tou) (Camargo *et. al*., 1995; Dickson and Hunter, 1987; Hunter *et. al*., 1987; Taylor *et. al*., 2002; Vicente *et. al*., 2002; Williams *et. al*., 1972). Badger Inbred-16, a line derived from Early Fuji, contains quantitative trait loci (QTLs) for black rot resistance which have been genetically mapped (Camargo *et. al*., 1995).

The most common and potentially useful sources of black rot resistance occur in

the A and B genomes of brassica species, and a number of sources of resistance have been identified in the different species containing these genomes (Bain, 1952; Taylor *et. al*., 2002; Westman *et. al*., 1999). Inheritance of major gene resistance has been studied in the diploid *B. rapa* (A genome) and in the tetraploids *B. carinata* (BC genome) and *B. napus* (AC genome) (Guo *et. al*., 1991; Ignatov *et. al*., 2000; Vicente *et. al*., 2002). A single dominant race-specific gene has been mapped to the A genome in *B. napus* (Vicente *et. al*., 2002), and QTLs that control resistance to at least two of the most prevalent races of *Xcc* have been mapped in a Chinese cabbage accession of *B. rapa* (Soengas *et. al*., 2007). Genes present in the brassica A and B genomes could potentially provide durable black rot control, especially if strong race specific genes (matching the most prevalent races) could be combined in a genetic background of race-nonspecific genes (e.g. providing quantitative resistance). To achieve this aim, genes from the wild relative *A. thaliana* could potentially be easier and quicker to characterize molecularly, and either be used directly in transgenic brassica crops, or facilitate the identification and interspecific transfer of homologous black rot resistance genes from A or B genome sources into vegetable crops. Interestingly, most *A. thaliana* accessions are resistant to one or more races of *Xcc*, and more than half exhibit broad-spectrum resistance to all major races of the pathogen (described below), suggesting that this wild relative of brassica crops could indeed provide useful sources of durable black rot resistance (Holub, 2007). Tsuji *et. al*. (1991) showed that the resistance to an *Xcc* isolate in the accession Columbia is controlled by a single dominant gene/locus. In addition, Buell and Somerville (1997) described a monogenic and a digenic resistance mechanism in this accession, and mapped the three genes involved. Plant mutants impaired in resistance to *Xcc* have been isolated and a gene involved in the establishment of the hypersensitive response (HR) and defense response has been identified and mapped (Lummerzheim *et. al*., 2004). However, although *A. thaliana* and *Xcc* provided one of the earliest experimental models for the investigation of the interactions of *A. thaliana* with a major crop pathogen (Simpson and Johnson, 1990), the molecular basis of natural variation in black rot resistance is largely unexplored in this pathosystem.

The DNA–DNA hybridization technique is not suitable for the routine identification of new pathogen isolates, and so other molecular methods have been developed. Simões *et. al*. (2007) differentiated species of *Xanthomonas* by PCR - restriction fragment length polymorphism of the genes *rpfB* and *atpD* involved in the regulation of pathogenicity factors and the synthesis of ATP. Methods based on DNA sequencing have become more popular as the cost of sequencing has decreased. The sequencing of genes that encode conserved proteins involved in essential cell processes and collectively constitute the ‘core genome’ has been developed for the identification of pathogens. Parkinson *et. al*. (2007,

2009) have shown that sequences of DNA gyrase subunit B (*gyrB*) can be used as an identification tool at the genus, species and, possibly, pathovar level of *Xanthomonas*; this method does not have sufficient resolution to differentiate isolates within each pathovar.

The identification of *Xcc* at the pathovar level is generally based on the isolation of the pathogen using semi-selective media. The currently used protocol for the detection of the pathogen in seeds uses Fieldhouse-Sasser and mCS20ABN media (Koenraadt *et. al*., 2005; Roberts and Koenraadt, 2006). The morphology of the cultures is generally then checked in subcultures on media such as Yeast Dextrose Calcium Carbonate. Classic bacteriological tests, carbon source metabolic fingerprinting (Biolog, Hayward, CA, *Xanthomonas campestris* pv. *campestris* USA) (Poplawsky and Chun, 1995), fatty acid analysis (MIDI, Newark, DE, USA) (Massomo *et. al*., 2003) and serological tests using polyclonal or monoclonal antibodies (Alvarez *et. al*., 1994; Franken, 1992) have been used to speed up the identification of the organisms. All of these methods rely on the availability of databases with the results obtained with representative isolates of different species and pathovars, but frequently problems with the standard isolates used (e.g. misidentification) can complicate the interpretation of new results. The inoculation of susceptible brassica seedlings is still the most reliable method, as it provides the ultimate confirmation of the identification of the pathovar (Roberts and Koenraadt, 2006). However, all of these methods are time consuming and inadequate for high-throughput screening. Several molecular methods have been used for the identification and characterization of the molecular diversity of *Xcc* and related pathovars. Rademaker *et. al*. (2005) used PCR primers that amplified repetitive sequences dispersed across bacterial genomes to generate a method to distinguish DNA 'fingerprinting' of isolates. Several studies have demonstrated that rep-PCR (using REP, ERIC and BOX primers) can differentiate isolates at the species, pathovar and intrapathovar level of *X. campestris* (Rademaker *et. al*., 2005; Vicente *et. al*., 2006). Nevertheless, the comparison of gel profiles and the standardization of the method between laboratories are still difficult to achieve (Parkinson *et. al*., 2007). A DNA probe was developed for the detection of *Xcc*, but, although the method worked for infected leaves, it was generally not sufficiently sensitive to detect the pathogen in seeds (Shih *et. al*., 2000).

This gene cluster is involved in plant–pathogen interactions, the growth and development of symptoms in plants and is largely conserved; therefore, these genes are good candidates for molecular diagnostics of different species or pathovars. Berg *et. al*. (2006) and Zaccardelli *et. al*. (2007) developed PCR methods using primers that amplify part of the *hrpF* gene and the *hrcC* secretinlike gene, respectively. These methods allowed the identification of a range of *Xcc* isolates, but were also positive for isolates of the closely related pathovars *aberrans*, *armoraciae*, *raphani*, *barbarea* and *incanae*. In the near future,

the comparison of whole genome sequences might constitute the basis for the classification and identification of *X. campestris,* and PCR methods with primers related to pathogenicity genes might become part of the routine protocol for the identification of *Xcc*.

A race structure for *Xcc* was first proposed by Kamoun *et. al*. (1992). The authors described five races (numbered 0–4) based on the reaction of different brassica species. Vicente *et. al*. (1998) and Ignatov *et. al*. (1998b) have subsequently shown that race 1 can be subdivided into two or three races on the basis of their reaction on several accessions of *B. oleracea* and *B. carinata*. A revised race classification was proposed by Vicente *et. al*. (2001) based on a much larger collection of isolates. Three races (1, 2 and 4) were retained from Kamoun *et. al*. (1992); however, no isolate was found that matched race 3, and so this race was dropped from the new race classification. Three variant classes were identified amongst the previous race 1 isolates based on the reactions of two *B. oleracea* accessions and an accession of *B. carinata*: a new race 1 that refers to the most commonly found variant, a new race 3 to accommodate a rare variant represented by the type strain of *Xcc* (ATCC33913; NCPPB 528) and an additional race 5 for three non-UK isolates, including an isolate previously included in *X. campestris* pv. *aberrans* (Vicente *et. al*., 2001). It was proposed that race 0 should be reassigned to a new race 6 to avoid the implication that these isolates lacked avirulence genes; although these isolates are pathogenic in all the differentials currently used, partial resistance to this race has been observed in brassica accessions (J. D. Taylor *et. al*., unpublished data; Horticulture Research International,Warwick, UK). Race 2 is only represented by a single isolate (HRI 3849A), which was used in the earliest molecular investigations of black rot resistance in *A. thaliana* (Buell and Somerville, 1997; Kamoun and Kado, 1990; Tsuji *et. al*., 1991). More recently, race 7 has been added by Jensen *et. al*. (2007, 2010) and Fargier and Manceau (2007). In addition, Fargier and Manceau (2007) included races 8 and 9 for the classification of isolates that have a narrow host range in the differential cultivars. Doubled haploids from several accessions of *B. oleracea*, *B. napus*, *B. carinata* and *B. juncea* were produced at the University of Warwick,Warwick HRI (now part of the School of Life Sciences), to replace the previous differential lines described by Vicente *et. al*. (2001). These include doubled haploid lines that replace Cobra, PI199947, Florida Broad Leaf Mustard and Miracle F1.

Gene-for-gene interactions can be used to explain the relationship between bacterial isolates and differential lines. The genes that confer resistance to the most important races (1 and 4) are designated *R1* and *R4*. The model allows for the possible inclusion of additional gene pairs if new races and differentials are identified. In general, the model was constructed in a manner that reflects the origin of the allotetraploid brassica

species (Nagaharu, 1935): *R1* originates from the B genome, *R3* from the C genome and *R4* from the A genome. The proposed model needs to be supported by genetic and molecular data from both the host and the pathogen to be fully validated. In the case of the host, results of crosses made to establish the inheritance of resistance to some of the races indicate that *R1*, *R3* and *R4* are single dominant genes (Vicente *et. al*., 2002).

A simpler gene-for-gene model has been proposed by He *et. al*. (2007) based on the interactions between *Xcc* isolates and cultivars of *Brassica* (*B. juncea*, *B. oleracea*, *B. rapa*), radish (*Raphanus sativus*) and pepper (*Capsicum annuum*). Races 1 and 4 are predominant worldwide, but their relative frequencies in *B. oleracea* crops appear to vary with geographical region. For example, race 1 appears to be more common than race 4 in the UK, whereas race 4 has been shown to be the predominant race in Portugal (Vicente, 2004), northwestern Spain (Lema *et. al*., 2012) and some East African countries, such as Tanzania and Uganda (Mulema *et. al*., 2012). Other races are generally rare, but may be more common in other host species that are less frequently surveyed. Races 2 and 6 were absent in a collection of isolates from Japan and Russia (Ignatov *et. al*., 1998a). Nepal and northwest Spain seem to have diverse populations of *Xcc*, with five different races identified in *B. oleracea* crop plants (Jensen *et. al*., 2010; Lema *et. al*., 2012). The low frequency of race 3 worldwide may be a result of the extensive use of cultivars that are resistant to this race.

The gene-for-gene model and the availability of defined ‘race type strains’ should assist in the selection and evaluation of plant material for breeding programmes and may be the basis for molecular studies. Disease resistance screening should be performed with isolates that represent the pathogenic variation of *Xcc*, and therefore should at least include the major races 1 and 4. In addition, isolates of race 6 should be useful to detect potential race-nonspecific resistance. The monitoring of the frequency and distribution of races worldwide is essential to the development of effective strategies for the breeding of black rot-resistant cultivars. Future brassica crops will benefit from the combination of major genes that confer strong resistance to the most common races of the pathogen (*R1* and *R4*) and, if possible, race-nonspecific genes that could confer quantitative resistance to all known races.

Many phytopathogenic bacteria produce a large number of factors that might be essential or contribute to cause disease. The bacteria from the genus *Xanthomonas* typically produce yellow, membrane-bound pigments, called xanthomonadins. These pigments have a role in the maintenance of the ecological fitness of the bacteria, protecting the cells against photooxidative stress. *Xcc* produces a range of extracellular enzymes (including proteases,

pectinases and endoglucanase). The extracellular enzymes are capable of degrading the plant cell components and may be required to overcome plant defense responses, to allow bacteria to move into uncolonized plant tissues and to mobilize plant polymers for nutritional purposes (Torres *et. al*., 2007).

Research into *Xcc* and closely related pathovars has now reached the genomic age, although it still lags behind the progress made from the investigation of *Pseudomonas* pathogens, such as *P. syringae* pv. *tomato* and *maculicola*. Our understanding of *Xcc* is increasing rapidly through functional and comparative genomic studies, and we are starting to understand the role of some of the key genes involved in pathogenicity. Nevertheless, there are still many areas that require further work, including the study of the mode of entry of the pathogen, such as comparisons between the vascular pathogen *Xcc*, which generally penetrates the host via the hydathodes, and the nonvascular pathogen *X. campestris* pv. *raphani*, which generally penetrates the host through stomata. The effect of the environment and genetic factors in determining the preferred mode of entry of these pathogens is still under-studied. The application of functional genomics and proteomics to bacteria *in planta* to identify virulence factors, and the application of functional genomics and proteomics to both resistant and susceptible host plants inoculated with *Xcc*, will provide key information on the interaction between the bacteria and the hosts. Research on the diversity of *Xcc*, pathogenicity factors and evolution, together with host–pathogen interaction studies, should lead to improvements in the prevention and control of the black rot of crucifers.

Therefore, my present studies were taken to find out resistance source to *Xcc* in the cultivated local varieties against the specific race (Chapter 1). By using *Xcc* resistance source found by my extensive screening, I identified *Xcc* resistance QTLs by using F2 populations developed from *Xcc* susceptible Green comet × resistant Reiho of *B. oleracea* plants. Simultaneously, we did a comparative analysis between our mapped QTL positions and the positions of QTLs on the previously published maps by incorporating common markers in our developed map (Chapter 2).

Chapter 1

Identification of race and screening of resistance to black rot (*Xanthomonas campestris* pv. *campestris*) in *Brassica oleracea*

1.1 Abstract

A screening test was under taken in order to selection resistant variety of black rot in different Brassica crops (cabbage, cauliflower, broccoli, flowering kale, Chinese cabbage). *Xanthomonas campestris* pv. *campestris*, isolate no. *Xcc-03-01967* was used as the inoculam source. For inoculation, 48h new bacteria grown on YDC media were used. Leaves were inoculated by cutting 1.0 cm with mid vein near the margins using nail cutter. After 15 days of inoculation, lesions enlarged as they progressed towards the midrib resulting in typical chlorotic, V-shaped lesions on the susceptible plants and on resistant plants, lesions were restricted in size and were often associated with a small necrotic area surrounding the cut portion. A total of thirty (30) commercial cultivars including two (2) double hybrid lines (DH lines), Reiho and Green commet were used. Screening test resulted the most susceptible variety, was Green commet-GC (0.98 cm^2) followed by Savoyace (0.94cm^2) and Irodori (0.93cm^2). Among the resistant variety, lowest disease severity was recorded in Reiho (0.13cm^2) followed by Berufore (0.17cm^2) and Akazukin (0.19cm^2). We used differential cultivars for identifying race of the isolate *Xcc-03-01967* in this study, was found to be race 1.

1.2 Introduction:

Black rot of crucifers, caused by *Xanthomonas campestris*, is considered the most destructive disease of crucifers worldwide (Williams, 1980). The disease has been a problem for many years but has become progressively more common and therefore more economically important in the last few years because of it's seed borne nature (Cook *et. al*., 1952, Monteith, 1921, Walker and Tisdale, 1920) and frequently transmit to descendents by direct infection of developing seeds. Most efforts of controlling the disease have been taken by eradicating the pathogen from the seeds. The disease has a wide geographical distribution and is particularly destructive to *Brassica oleracea* L. vegetables causing reduction in yield and quality (Williams, 1980), but it can also attack other *Brassica* spp., cruciferous weeds and ornamentals. In *B. rapa*, the disease can be serious in turnip and turnip greens (Pammel, 1985; Vicente, 2004) and it has also been reported in Chinese cabbage crops (Schaad and Thaveeschai, 1983; Ignatov *et. al*. 2000).

Since 1963, black rot disease of cauliflower, *Xanthomonas campestris* (Pam.) Dowson, has been very prevalent in India where cultivar 'Snow ball' cultivars are highly susceptible, and often the seed crop is seriously damaged by the disease (Sharma *et. al.* 1977). Three genotypes derived from Indian cultivars, possessed a high degree of resistance rather than other genotypes including 'Snow ball' and their relative cultivars. Russell (1898) reported the reaction of cabbage varieties and related plants to black rot disease. In cabbage relative, there were little differences in susceptibility where all varieties readily yielding to the disease, if the causal organism is once present. However, among closely related plants cauliflower rated as the most susceptible where cabbage as readily affected by the disease, and broccoli, Kohl rabi and Brussels sprouts as quite susceptible. Bain (1952) observed the reaction of seedlings of a number of varieties and strains of Brassica to black rot from inoculated seeds. Turnip, mustard and other brassica like *B. nigra*, *B. napus* were in the low percentage black rot group. On the other hand cauliflower, broccoli, rutabaga, collard, kale, brussels sprouts and kohl rabi were in the high percentage black rot group while cabbage was intermediate. Varieties and stains within these groups varied from high to low percentage.

Till now nine races of *Xcc* have been identified where race 1 and 4 are predominant worldwide and other races, 2, 3, 5 and 6, were rare (Kamoun *et. al*., 1992, Ignatov *et. al*., 1998, Vicente *et. al*., 2001, Taylor *et. al*., 2002, Fargier *et. al*., 2007). Race 1 and 4 are the most important races in *B. oleracea* crops. Therefore, resistance to both of these two races is a minimum requirement to be of value in controlling black rot (Soengas *et. al*., 2007).

In cabbage, resistance is to be governed by a single dominant gene (Bain, 1955). The pattern of inheritance of resistance was investigated in cauliflower (Sharma *et. al*. 1972) and found resistance is dominant and governed by polygenic. The dominance component of variation was greater than the additive in almost all the crosses. It was suggested that Snow ball cultivar is to be adopted in breeding methods by incorporating resistant genes.

Taylor *et. al*, (2002) screened two hundred and seventy-six accessions of Brassica for resistance to different races of *Xcc*. In *B. oleracea* (C genome), the majority of accessions were susceptible to all races, but 43% showed resistance to one or more of the rare races 2, 3, 5, and 6 and a single accession showed partial resistance to races 1, 3, 5, and 6. Strong resistance to race 4 was frequent in *B. rapa* (A genome) and *B. napus* (AC genome), indicating that A genome is the origin of resistance to the race 4. Moreover, resistance to races 1 and 4 was present in *B. nigra* (B genome) and *B. carinata* (BC genome) accessions, indicating that B genome also important to races 1 and 4. On the other hand, *B. juncea* (AB genome) is the most resistant species, showing either strong resistance to races 1 and 4 or quantitative resistance to all races. Potentially race-nonspecific resistance was also found but in lower frequency in *B. rapa*, *B. nigra*, and *B. carinata*. The combination of race specific and race-nonspecific resistance could provide durable control of black rot of crucifers.

The control measure of the disease is difficult and limited. Usually attempts are made through the use of disease-free planting materials and the elimination of other potential inoculums sources such as infected crop debris and cruciferous weeds (Taylor *et. al*, 2002). An alternative approach through the development and use of resistant cultivars has long been recognized, but in practice has had only limited success. Early studies of resistance to *Xcc*, identified cabbage cultivars with varying levels of field resistance which were mainly concerned with *B. oleracea* (Alvarez,*et. al*.,1994, Dane and Shaw, 1996). Bain (1952) made resistant selections from cabbage cvs. Huguenot and Early Fuji. Resistance to *Xcc* was much more common in other *Brassica* spp. like *B. nigra, B. napus, B. juncea*, and *B. rapa* than in *B. oleracea*.

Hunter *et. al*. (1987) found the cabbage accession PI 436606 expressed resistance in both seedlings and adult plants. Resistance at the seedling stage was attributed to a single recessive gene (Chen *et. al*., 1994). Both Early Fuji and PI 436606 have been used in *B. oleracea* breeding programs. Several other studies have identified sources of resistance in *B. oleracea* (Ignatov, *et al*., 1998, McCulloch, 1929).

A high level of resistance in the accessions PI 199947 and PI 199949 (*B. napus*)

and a moderate level of resistance were found in two Chinese cabbage accessions *(B. rapa*) (Guo *et. al*. 1991). Beside that a number of accessions of *B. nigra* and *B. juncea* with a high proportion of resistant plants were also identified (Westman *et. al*. 1999). The limitation of the studies was that they failed to recognize the existence of pathogenic variants (races) of *Xcc.* Although screening and identification of black rot resistance in *B. rapa* and *B. napus* were done by various researchers but very few information are available in *B. oleracea* (Soengas, *et. al*. 2007). However, despite the number of resistance studies in *B. oleracea*, available sources with useful levels of resistance are very limited and scarce. Therefore our present studies were taken to find out resistance source to *Xcc* in the current cultivated local varieties and to identify race used in the study.

1.3 Materials and methods:

Plant Materials :

Thirty cultivars of *Brassica oleracea* used for screening of resistance to black rot disease, were collected from different seed companies of Japan (Table 1). All the plants were grown on blotter paper in 9 cm plastic petridish from seeds for 2-3 days and seedlings were transferred to 42 celled plastic trays (cell size: 3.5 cm × 3.0 cm) using soil (Honen Agri., Japan) in the green house (25°C/15℃ day/night cycle) up to 20 days and then transferred to 12 cm plastic pot containing soil Honen Agri., Japan.

Bacterial culture and preparation of inoculam:

Xanthomonas campestris pv. *campestris* (isolate no. *Xcc-03-01967*) used in this study was obtained from Gene bank of National Institute of Agrobiological Resources, Japan and was maintained on Yeast Dextrose Calcium Carbonate Yeast Dextrose Calcium Carbonate (YDC) agar slant at 4°C. Before inoculation, a 48h new culture grown on YDC medium at 28°C was used for prepare the suspension. Bacteria were grown on YDC medium at 28°C for 48h before inoculation. Bacterial cells were scraped from the plates and suspended in saline solution (0.85% NaCl) and adjusted to 108 CFU/ml (0.2OD A600 nm by Gene Quant (1300) Spectrophotometer).

Inoculation Procedure:

For disease development, leaves of approximately 50-day old plants were inoculated by cutting 1.0 cm with mid vein near the margins using nail cutter with *X.*

campestris pv. *campestris* (Kau-chi *et. al*., 1982). Three youngest leaves of each plant and nine plants of each cultivar were inoculated. For every inoculation, nail cutter was dipped into the bacterial solution. After inoculation, plants were moved to artificial plastic chamber for maintaining 100% moisture at 28℃ until the disease development. V-shaped symptom (Figure 1) area (cm2) was measured 14 days after inoculation following the equation of (width × length) ×1/2. Plants with a mean disease area <0.50 cm2 were classified as resistant, those having 0.51-0.90 cm2 were border line resistant and those having of >0.90 cm2 were classified as susceptible to the disease. The experiment was arranged in a completely randomized block design with three replicates (three leaves per plant and three plants per replication) maintaining appropriate controls.

Identification of the race of *Xcc*:

For identification race, seedlings and plants were grown in a greenhouse at Niigata University. Approximately 50-day old plants were used for the inoculation test. Leaves were inoculated by cutting the mid vein near the leaf margins, 1.0 cm in width using a nail cutter that had been dipped in the bacterial suspension (Ohata *et. al*. 1982). For every inoculation, the nail cutter was dipped into the bacterial suspension.

Identification of race was according to the following criteria; race 0 infected all of four differential cultivars, race 1 infects Marathon F1, Tokyo Cross Hybrid Turnip, and Seven Top Turnip but not Florid Broad Leaf, race 2 infects Marathon F1, Tokyo Cross Hybrid Turnip, and Florid Broad Leaf but not Seven Top Turnip, race 3 infects Marathon F1 and Seven Top Turnip but not Tokyo Cross Hybrid Turnip and Florid Broad Leaf, race 4 infects only Marathon F1 (Kamoun *et. al*. 1992, Ignatov *et. al*. 1998 and Vicente *et. al*. 2001).

Statistical analysis:

Data of the experiments were subjected to analysis of variance (ANOVA), and inter-mean differences between treatments were determined by Turkey test and standard deviation (SD) was calculated by Microsoft Excel (2007).

Results

Inoculation of plants by leaf cut method for developing of black rot disease was highly effective in establishing the disease judged by symptom expression on the susceptible

parent and controls. Symptom began appearing 5 days after inoculation as a water-soaked lesion around the cut site. Lesions enlarged as they progressed towards the midrib resulting in typical chlorotic, V-shaped lesions on the susceptible plants, (Figure 1.1 a, d). On resistant plants, lesions were restricted in size and were often associated with a small necrotic area surrounding the cut portion after 15 days of inoculation (Figure 1.1 b, e). The V-shaped lesion was regarded as a triangle shape to calculate disease leaf area (DLA).

A total of 30 cultivars of cabbage, cauliflower and broccoli were used for disease screening for the *Xcc* resistance (Table 1.2). Significant differences in resistance were found among the cultivars used. The cabbage cultivars showed variable susceptibility compared to cauliflower, broccoli and others *B. oleracea* cultivars. The highly susceptible cultivars was found in broccoli cultivar Green comet with a mean DLA 0.98 cm2, followed by Savoyace (0.95 cm2), Fujiwase (0.93 cm2) and Irodori (0.93 cm2), respectively (Table 1.2, Figure 1.2) where Reiho showed highly resistant with mean DLA 0.13 cm2 followed by Berufore (0.16 cm2) and Akazukin (0.19 cm2), respectively. Among the 15 cabbage cultivars Savoyace (0.95 cm2) showed the highly susceptible disease interaction where Reiho was most resistance (0.13 cm2). Similarly, Cauliflower 60 exhibited the highest susceptible reaction (0.81 cm2) where none showed resistance. On the other hand in broccoli, Green comment showed highly susceptibility (0.98 cm2) where cultivar Berufore was tolerant (0.18 cm2) to the disease. No tolerant cultivar was found in other *B. oleracea* (Table 1.2, Figure 1.2).

For race identification of the isolate no. *Xcc-03-01967*, we used four differential cultivars namely Marathon (*B. oleracea*), Tokyo Cross Hybrid Turnip (*B. rapa*), Seven Top Turnip (*B. rapa*) and Florida Broad Leaf (*B. juncea*) (Table 1.3) including resistant and susceptible cultivars Reiho P01 and GC P09, respectively. The lesion on the susceptible differential cultivars and GC P09, enlarged towards the midrib, resulting in typical chlorotic V-shaped lesions (Figure 1.3). The V-shaped lesion was regarded as a triangle shape to calculate disease leaf area (DLA). The lesion on resistant cultivars and the Reiho P01 was restricted in the portion of leaves that was inoculated. Among the differential cultivars, only Florida broad leaf (mustard) was found to be resistant to the isolate no. *Xcc-03-01967*. This type of reaction between the differential cultivars and the isolates revealed that the race used in this study was race 1 (Table 1.3).

Discussion:

Black rot (*X. campestris* pv. *campestris*) is the most important bacterial disease of crucifers (Camargo *et. al*., 1995; Westman, 1998; Vicente *et. al*., 2001). Different inoculation

methods are developed and applied by several authors, like spraying method (Camargo *et. al*., 1995), pinning method (Hansen and Earle, 1995) and clipping method (Camargo *et. al*., 1995; Ignatov *et. al*., 1998; Vicente *et. al*., 2001). Doullah *et. al*., (2011) found the inoculation of leaves by cutting 1.0 cm with mid vein near the margins was highly effective in establishing the disease. Regarding the results among the currently cultivated *B. oleracea* cultivars Green commet was the most susceptible and Reiho was the highly resistant.

Nine races of *Xcc* have been identified to date from pathogenicity tests based on the interaction between differential cultivars and races (Kamoun *et. al*. 1992, Ignatov *et. al*., 1998, Vicente *et. al*., 2001, Taylor *et. al*., 2002, Fargier *et. al*., 2007). Vicente 2001, reported that the appearance of race 1 and 4 was predominant worldwide and other races, 2, 3, 5 and 6, were rare. Race 1 and 4 are the most important races in *B. oleracea* crops. Therefore, resistance to both of these two races is a minimum requirement to be of value in controlling black rot (Soengas *et. al*., 2007).

Screening for *Xcc* resistance was performed in *Brassica* species and related species (Bain, 1952, Sharma *et. al*., 1977, Ferreira, 1993, Westman *et. al*., 1999) and extensive screening using more than 100 genotypes was done by (Taylor *et. al*., 2002). As a result, resistance to *Xcc* has been identified in genotypes of *B. rapa* (A genome), *B. nigra* (B), *B. oleracea* (C), *B. carinata* (BC), *B. juncea* (AB), and *B. napus* (AC). Some genotypes of *B. nigra*, *B. carinata* and *B. juncea* with B genome revealed the highest level of resistance to races 1, 3 and 4, indicating the existence of R1, R3 and R4 resistance genes that were postulated based on the gene-for gene model (Taylor, 2002). In addition, the high level resistance of race 4, conferred by the R4 gene, was found in *B. rapa* and *B. napus* with A genome. In *B. oleracea*, resistance to race 3 and race 5 is common, but resistance to race 1 is very rare. Overall single R genes corresponding to AVR genes (avirulence gene) in each race are considered to confer the high level of qualitative resistance in *Brassica* species, and those R genes are dominant. Inheritance of such race-specific resistance genes was confirmed by the phenotypic segregations in the F2 progenies derived from crossing between susceptible and resistant genotypes; the observed ratio (Resistance: Susceptibility) fit to the 3:1 ratio expected in segregation of a single dominant gene (Vicente *et. al*., 2002).

We identified isolate no. *Xcc-03-01967* as race 1 in this study. By using race 1 for our study, we successfully identified highly resistance cultivars to *Xcc*. Therefore, our screening of resistance to black rot of *B. oleracea* will be helpful for incorporating resistance into cultivated species.

Table 1.1 List of different Brassica Oleracea cultivar used for screening of Black rot Disease

Sl no.	Name of Cultivar	Group	Distributer
1	Reiho	Cabbage	Ishi Seed Co. LTD.
2	KEX708	Cabbage	Kaneko Seed Co. LTD.
3	Matsunami	Cabbage	Takii Seed Co. LTD.
4	Shiramoni	Cabbage	Takii Seed Co. LTD.
5	Anju	Cabbage	Takii Seed Co. LTD.
6	Nanpou	Cabbage	Takii Seed Co. LTD.
7	Okina	Cabbage	Takii Seed Co. LTD.
8	Ayahikari	Cabbage	Takii Seed Co. LTD.
9	MiniX 40	Cabbage	Muratane Seed Co. LTD.
10	Hatsudayori	Cabbage	Nozaki Seed Co. LTD
11	KEX716	Cabbage	Kaneko Seed Co. LTD.
12	KEX713	Cabbage	Kaneko Seed Co. LTD.
13	Fujiwase	Cabbage	Muratane Seed Co. LTD.
14	Irodori	Cabbage	Nozaki Seed Co. LTD
15	Savoyace	Cabbage	Takii Seed Co. LTD.
16	Minicauliflower	Cauliflower	Kaneko Seed Co. LTD.
17	Cauliflower40	Cauliflower	Kaneko Seed Co. LTD.
18	Newball	Cauliflower	Takii Seed Co. LTD.
19	Snow new dia	Cauliflower	Takii Seed Co. LTD.
20	Yukimatsuri	Cauliflower	Watanabe noji Seed Co. LTD.
21	Cauliflower60	Cauliflower	Kaneko Seed Co. LTD.
22	Berufore	Broccoli	Watanabe noji Seed Co. LTD.
23	Indevar	Broccoli	Takii Seed Co. LTD.
24	Wineberu	Broccoli	Watanabe noji Seed Co. LTD.
25	Salinas early	Broccoli	Kaneko Seed Co. LTD.
26	Green commet	Broccoli	Takii Seed Co. LTD.
27	Akazukin	Flowering Kale	Muratane Seed Co. LTD.
28	YR 50	Chinese cabbage	Nozaki Seed Co. LTD.
29	Shiun	Red cauliflower	Nozaki Seed Co. LTD.
30	Newrubi	Red cabbage	Musashino Seed Co. LTD

Table 1.2 Mean Diseased leaf area (cm^2) and the standard deviations (SD) of the screening for the black rot caused by *Xanthomonas campestris* pv. *campestris* in 30 cultivars of *Brassica oleracea*

Cultivar	Mean (cm^2) ±SD	Cultivar	Mean (cm^2) ±SD
Cabbage		**Caulilfower**	
Reiho	0.13±0.05	Minicauliflower	0.38±0.07
KEX 708	0.22±0.09	Cauliflower 40	0.41±0.10
Matsunami	0.22±0.06	Newball	0.57±0.05
Shiramoni	0.26±0.06	Snow new dia	0.57±0.06
Anju	0.33±0.22	Yukimatsuri	0.62±0.07
Nanpou	0.49±0.04	Cauliflower 60	0.81±0.12
Okina	0.62±0.07	**Broccoli**	
Ayahikari	0.62±0.06	Berufore	0.18±0.07
MiniX 40	0.71±0.08	Indevar	0.35±0.07
Hatsudayori	0.72±0.19	Wineberu	0.73±0.11
KEX 716	0.76±0.09	Salinus early	0.87±0.12
KEX 713	0.90±0.21	Green Commet	0.98±0.09
Fujiwase	0.93±0.13	**Other**	
Irodori	0.93±0.08	Akazukin	0.20±0.07
Savoyace	0.95±0.33	YR 50	0.36±0.07
		Shiun	0.45±0.13
		Newrubi	0.51±0.11

Table 1.3. Race identification by using differential cultivars.

Differential cultivars [a]	DLA	Susceptibility (+) /resistance (-)
Marathon (*B. oleracea*)	3.1	+
Tokyo Cross Hybrid Turnip (*B. rapa)*	6.7	+
Seven Top Turnip (*B. rapa*)	5.8	+
Florida Broad Leaf (*B. juncea*	0.02	-

[a] Differential cultivars were chosen from (kamuon *et al*. 1992) and (Vicente *et al*. 2001)

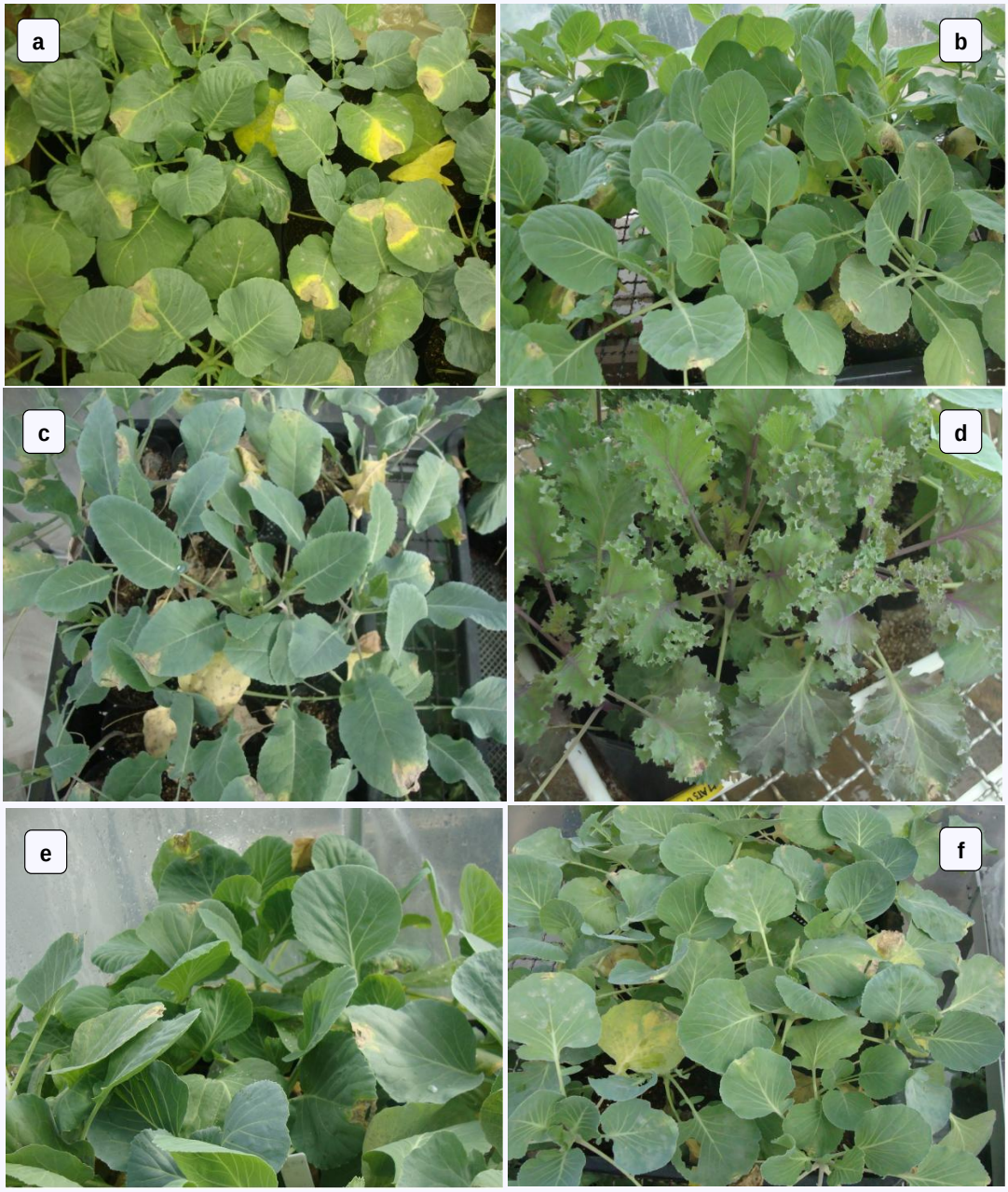

Figure 1.1 Plants showing Disease interactions 14 DAI with *Xanthomonas campestris* pv. *campestris* through leaf cut method in Green house. Figure shows (a) Green commet (b) Reiho, (c) Cauliflower 60, (d) Matsunami, (e) KEX 713, (f) Savoyace after 14 DAI respectively. Green commet (a) showing typical `V`- shaped symptom and Reiho (b) showing necrosis are resticted around the cut portion.

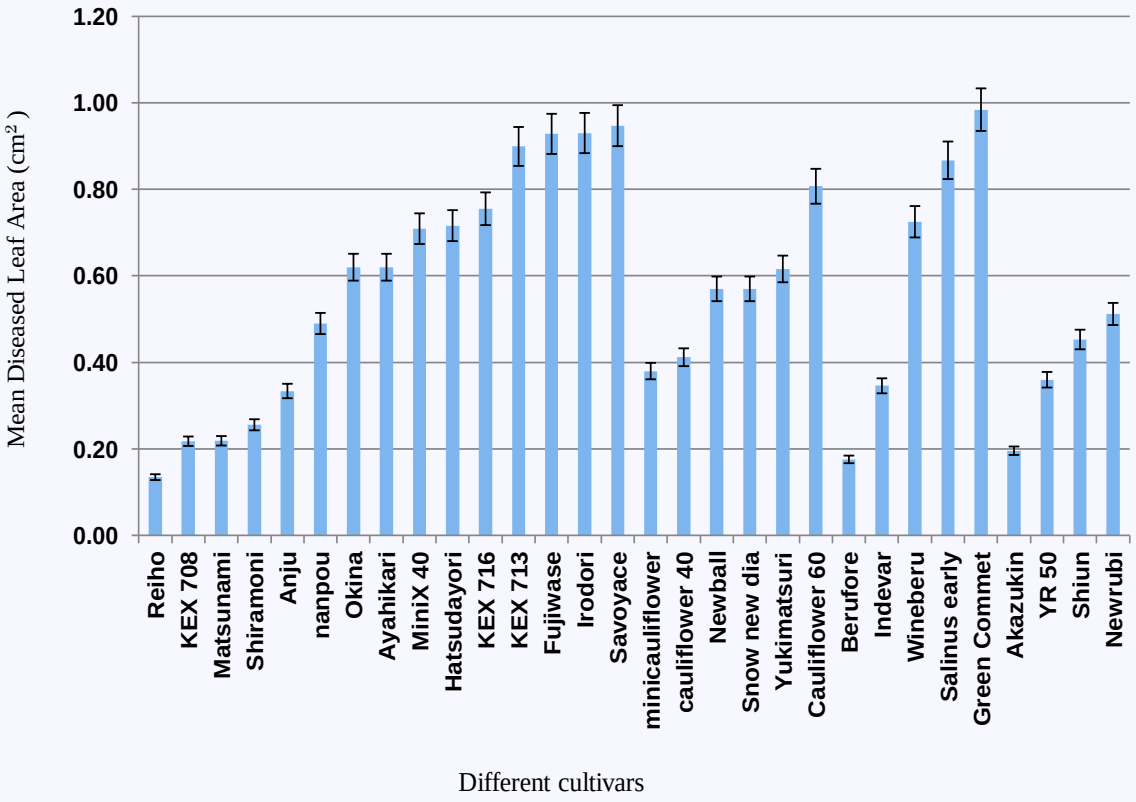

Figure 1.2 Frequency distribution of mean disease area (cm^2) of black rot (*Xanthomonas campestris* pv. *campestris*) disease in 30 cultivated variety of *Brassica oleracea.* Plants with diseased area 0-0.6 cm^2 considered as resistant, 0.7-0.9 cm^2 are border line resistant and those having more than 0.9 cm^2 are considered as susceptible to the disease. Error bars indicate standard errors of the mean value.

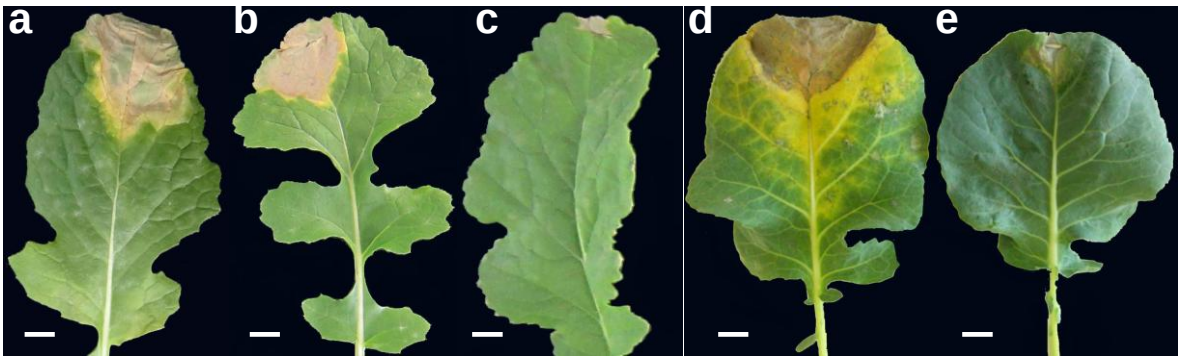

Figure 1. 2. Black rot disease symptoms produced on differential cultivars and the parent used in QTL analysis. a: Tokyo Cross Hybrid Turnip, b: Seven Top Turnip, c: Florida Broad Leaf, d: GCP09, e: Reiho P01. Bar = 1cm.

Chapter 2

Comparison of positions of QTLs conferring resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica oleracea*

2.1 Abstract

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) is possibly the most important disease of *Brassica* worldwide. To compare chromosomal positions of *Xcc* resistance loci in *Brassica oleracea* between the present and published studies and to develop marker assisted selection (MAS) to resistance against *Xcc* race 1, we constructed a *B. oleracea* map, including pW, pX and BoCL markers that were closely linked to previously reported *Xcc* resistance QTLs. We also analyzed *Xcc* resistance QTLs by improving our previously reported map derived from the cross of a susceptible double-haploid line (GC P09) with a resistant double-haploid line (Reiho P01). In the nine linkage groups obtained (C1-C9), the major QTL, *XccBo(Reiho)2*, was derived from Reiho with a maximum LOD score (7.7) in C8. The QTL (LOD 4.4) located in C9, *XccBo(GC)1*, was derived from the susceptible GC. The other QTL (LOD 4.4), *XccBo(Reiho)1*, was found in C5. Based on common markers, it was possible to compare our finding *Xcc* resistance QTLs with the *B. oleracea Xcc* loci reported by previous authors; *XccBo(Reiho)2* and *XccBo(GC)1* may be identical to the *Xcc* resistance QTLs reported previously or a different member contained in the same resistance gene cluster. Our map includes public SSR markers linked to *Xcc* resistance genes that will promote pyramiding *Xcc* resistance genes in *B. oleracea*. The present study will also contribute to a better understanding of genetic control of *Xcc* resistance.

2.2 Introduction

Black rot, caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Pam.) Dawson (*Xcc*), is the most destructive disease in crucifer crops (Williams, P.H. 1980). *Xcc* enters leaves not only through insect or mechanically wounded tissue (Shelton and Hunter, 1985) but also through hydathodes at leaf margins and spreads through vascular tissue, clogging vessels and producing V-shaped chlorotic lesions (Cook *et. al*. 1952). Such symptoms lead to a systemic infection in susceptible plants so that crop quality and yield substantially decrease. Crop debris and cruciferous weed are potential inoculum sources in field (Schaad and Dianese, 1981). The pathogen can be retained in seeds via vessels and causes severe incidence in descent seedlings; consequently, *Xcc* is difficult to prevent by agricultural practices such as seed treatment, crop rotation and use of agrochemicals. Thus, utilization of *Xcc* resistant cultivars is one of the most effective approaches to minimize crop loss from infection of the pathogen.

Till now nine races of *Xcc* have been identified (Kamoun *et. al*. 1992, Ignatov *et. al*., 1998, Vicente *et. al*., 2001, Taylor *et. al*., 2002, Fargier *et. al*., 2007). Race 1 and 4 was found to be predominant (Vicente 2001) and the most important races in *B. oleracea* crops where other races, 2, 3, 5 and 6, were rare. Therefore, resistance to both of these two races is a minimum requirement to be of value in controlling black rot (Soengas *et. al*., 2007).

Overall single R genes corresponding to AVR genes (avirulence gene) in each race are considered to confer the high level of qualitative resistance in *Brassica* species, and those R genes are dominant. Inheritance of such race-specific resistance genes was confirmed by the phenotypic segregations in the F2 progenies derived from crossing between susceptible and resistant genotypes; the observed ratio (Resistance: Susceptibility) fit to the 3:1 ratio expected in segregation of a single dominant gene (Vicente *et. al*., 2002).

On the other hand, (Bain, 1952) found that the Japanese cabbage cultivar, Early Fuji, had a high level of resistance to *Xcc*. and he showed that this resistance was controlled by one or two dominant genes. Williams *et. al*., 1972, found that resistance of cabbage cultivars BI-16, derived from Early Fuji, was quantitative under oligogenic control; they postulated one major recessive resistance gene, *f*, the expression of which in heterozygous conditions was influenced by one recessive and one dominant modifier genes. Vicente *et. al*., 2002, also reported that the resistance of BI-16 to race 1 was quantitative and recessive. Camargo *et. al*., 1995, Identified the two *Xcc* resistance QTLs on LG2 and another two QTLs on LG1 and LG9, respectively, in the mapping population derived from the cross of

the resistant cabbage and the susceptible broccoli. Kifuji *et. al*., 2013, also identified QTLs on C2, C4, and C5. Doullah *et. al*. 2011, detected the two significant QTLs controlling resistance to *Xcc* on LG2 and LG9. Those results indicate that resistance to *Xcc* was under oligogenic control. Comparison of the QTLs identified by previous authors is, however, quite difficult because no anchor markers can align the linkage maps contracted by different authors, and furthermore, some of the linkage maps do not follow the international nomenclature established for the C genome of *B. oleracea*.

In our previous study (Doullah *et. al*., 2011), the total length of the linkage map constructed was 320 cM, which is not enough long to analyze locations of QTLs at a genome-wide level. The objectives of this study, therefore, were (1) to analyze *Xcc* resistance QTLs by using an improved F2 population map of *B. oleracea* plants, and (2) to do a comparative analysis between our mapped QTL positions and the positions of QTLs on the previously published maps by incorporating common markers in our developed map.

2.3 Materials and Methods

Plant Materials:

A doubled hybrid (DH) broccoli line (P09) of *B. oleracea* subsp. *Italica* cv. Green Comet (GC) (Takii Seed Co. Ltd.,Japan) was crossed as the female parent to a DH line P01 of *B. oleracea* subsp. *capitata* cv. Reiho (Ishii seed company, Japan). The 'GC P09' was susceptible to *X. campestris* pv. *campestris* diseases, whereas the Reiho P01 was tolerant. We used the F2 population produced in the study of Doullah *et. al*. 2011, to construct our linkage map. In summary, seeds of F2 were produced by bud-selfing of a F1 plant and F2 plants selected for QTL analysis were self-pollinated to produce F3 lines. Marathon F1 (*B. oleracea*), Tokyo Cross Hybrid Turnip (*B. rapa*), Seven Top Turnip (*B. rapa*), and Florid Broad Leaf (*B. juncea*) were collected from Twilley Seed Co., Inc., SC, US) and used for identification of the race of *Xcc*. Marathon F1 was used as a susceptible check.

Preparation of inoculum and Inoculation test :

The strain confirmed as race 1 that was used for this study, *Xanthomonas. campestris* pv. *campestris* strain (isolate no. 03-01967), was the same as previous study. Inoculums of the bacterium was cultured in Yeast Dextrose Calcium Carbonate (YDC) agar plate for 48 h at 28°C, and then bacterial cells were scraped from plates and adjusted to a concentration of 108 CFU/ml (0.2OD A600 nm) with 0.85% NaCl solution. V-shaped lesion

area (cm2) was measured two weeks after inoculation according to the equation of (lesion width × length) ×1/2.

The seedlings and plants were grown in a greenhouse at the agricultural field of Niigata University. Approximately 50-day old plants were used for the inoculation test. Leaves were inoculated according to Ohata *et. al*. 1982 as described previously. Twelve plants from each F3 line were tested. The three youngest fully expanded leaves were inoculated per plant. The mean DLA of the 12 seedlings per F3 line was used as representative of DLA for each F2 plant.

DNA polymorphism and QTL analysis

The sample set of Genomic DNA of the parents and 94 F2 individuals used in the study of Doullah *et. al*. 2011was also used for the linkage construction. DNA of each sample was amplified by the GenomiPhi V2 DNA Amplification kit (GE Healthcare). Ten µl PCR cocktail containing 10 ng genomic DNA, 0.2µM each primer and 1× EmeraldAmp Max PCR Master Mix (Takara Bio. Inc., Japan) were used for CAPS (Cleaved Amplified Polymorphic Sequences) and SSR (Simple Sequence Repeat) analyses. Standard three step PCR was performed. Annealing temperature and extension time for PCR were set according to the primer sequence and gene size. The primer sequences were taken from various reports listed in Table 2.1. For CAPS analysis, the amplicons were digested with one of four restriction enzymes (*Alu*I, *Msp*I, *Hin*fI or *Mbo*I). DNA fragments obtained from restriction enzyme digestion and PCR were separated on 8-15% polyacrylamide gel according to fragment size (Kikuchi *et. al*., 2004). The gel was subsequently stained with a Gelstar solution (0.1µl/10ml) (Takara Bio. Inc., Japan).

Linkage analysis was performed using Ant Map programe, version 1.2(Iwata and Ninomiya, 2006). The QTL detection for *X. campestris* pv. *campestris* resistance was analyzed using a QTL Cartographer software version 2.5 (Basten *et. al*. 2005) in which composite interval mapping (CIM). CIMs were performed at LOD threshold values which were estimated by means of a permutation test with 1000 permutations with QTL Cartographer.

Alignment of different maps

Previous studies identified four *Xcc* resistance QTLs (Camargo el. Al. 1995) and self-incompatibility locus (Camargo *et. al*. 1997) in the cabbage BI-16× broccoli OSU Cr-7

mapping population, of which linkage map was constructed using WG, TG, and EC RFLP markers. Those markers were renamed as pW and pX according to the NCBI DNA data base. In order to correlate all the linkage groups of the BI-16×OSU Cr-7 map to the international nomenclature established for the C genome, we aligned the BI-16×OSU Cr-7 map to the consensus map constructed by (Udall *et. al*. 2005). After that, common pW and pX markers were used as anchor markers for map position comparisons of *Xcc* resistance QTLs. DNA sequences of the pW/pX RFLP markers were collected from NCBI DNA data base, and then, by using those DNA sequences, we did BLAST search at the *Brassica oleracea* Genomics Project web site, *Bol*base, to obtain coding sequences or genomic sequences corresponding to the RFLP markers. From the identified DNA sequences, we designed primer sets by which the chromosomal regions specifically associated with the pW/pX markers were detected (Table 2.2). For comparison of positions of QTLs identified by (Kifuji *et. al*., 2013) and our map, the primer sets were designed based on the sequence of the EST-SNP markers that Kifuji *et. al*. 2013 described. By using the primer set, we amplified the chromosomal region identified by EST-SNP markers and, thereafter, detected the polymorphism by CAPS or PCR-SSCP analysis.

2.4 Results

In the present study, the inoculation data was cited from Doullah *et. al*. (2011), who reported that DLA of the F3 lines showed a continuous distribution pattern, with some F3 lines showing lower DLA values than the resistant parent (Figure 2.1). This time the genotyping of each F2 plant at the *Xcc*-resistance QTL (*XccBo(Reiho)2*) was newly conducted to analyze association between phenotypic and genotypic data of F2 plants (Details given later). In this study, 94 F2 individuals were used for the linkage construction. A total 181 markers were distributed in 9 linkage groups covering 1099.4cM, and the average interval between markers was 6.1cM. The linkage map included 155 SSR and 26 CAPS markers (Table 2.3). To align our map to the internationally accepted *Brassica* map, we used pW, pX, CB, BRMS, BoGMS and BoSF, markers reported by (Udall *et. al*.2005, Piquemal *et. al*., 2005, Suwabe, *et. al*., 2006, Chen, *et. al*.,2011, Wang, *et. al*. 2011 and Wang *et. al*. 2012).

QTL analysis was performed using the appropriate significance threshold calculated in the permutation test (1000 iterations) and we detected three significant QTLs (Table 2.4, Figure 2.2). These results indicated that *Xcc* resistance was controlled by an oligogenic system. Three QTLs for *Xcc* resistance were detected in C5, C8, and C9. The largest QTL effect (LOD of 7.7) for *Xcc* resistance was detected between the loci EMS1010

and CB10419 on C8 and was closely linked to marker BoGMS0971. This QTL, which explained 34% of the total phenotypic variation, was named *XccBo(Reiho)2*. The QTL located in C9 came from the susceptible broccoli parent (Table5), and therefore this *Xcc* locus was named *XccBo(GC)1*. Despite high susceptibility of the susceptible parent GC P09 to *Xcc*, this *Xcc* locus accounted for 17.9% of the variation, suggesting that there may be epistatic genes that interact with *XccBo(GC)1* in other regions of the genome. The other minor QTLs found in C5, which came from the resistant parent, accounted for 6.6% of the variation, named *XccBo(Reiho)1*. Genotyping at the BoGMS0971 marker that was closely linked to the major QTL indicated that higher resistance was associated with the homozygous Reiho genotype versus the homozygous GC genotype, with the heterozygotes having varying resistance levels (Figure 2.1).

For comparison of the positions of *Xcc* resistance QTLs identified by different authors (Camargo *et. al*. 1995, Kifuji *et. al*. 2013), we first compared the linkage map (I) of Camargo *et. al*. 1995, with the linkage map (II) of (Camargo *et. al*. 1997) who mapped self-incompatibility locus. Although they used the same mapping population (BI-16×OSU Cr-7), the constructed linkage group numbering differed; the LG 2 in linkage map (I) corresponded to LG2 and LG9 in linkage map (II), and LG 9 in linkage map (I) to the upper portion of LG1 in linkage map (II) (Table 2.5). Next, for assigning positions of *Xcc* resistance QTLs identified by (Camargo *et. al*. 1995) to the consensus map constructed by (Udall *et. al*. 2005), common pW and pX markers in the two linkage maps were used as anchor markers (Figure 2.3). As a result, LG1 and LG 9, that had the major QTLs in BI-16×OSU Cr-7 mapping population, were assigned to the lower portion of C3 and the bottom distal end of C9, respectively. The two minor *Xcc* resistance QTLs on LG 2 corresponded to C5 and C6, respectively. The markers, pW164 (WG3C5) and pW114 (TG4D2), that were closely linked to one of the *Xcc* resistance QTLs identified in LG2 by (Camargo *et. al*. 1995) were mapped to the central portion of C5 in our map, where *XccBo(Reiho)1* located. Similarly, the markers, pW143 (WG8A9) and pX117 (EC2D9), that were closely linked to the QTL identified in LG9 were mapped to the distal end of C9 in our map where *XccBo(GC)1*was located. The BoCL6244s marker closely linked to the QTL-3 mapped by (Kifuji *et. al*. 2013) was mapped in the central region of C5 in our map. The two *Xcc* resistance QTLs on C3 and C9 were detected by (Doullah *et. al*. 2011) who used the same mapping population used for our study. In the present study, we did not detect significant QTLs on the bottom distal end of C3, although there was a LOD peak (LOD=2.1) in the same region of C3 where (Doullah *et. al* 2011) found the *Xcc* resistance QTL.

2.5 Discussion

Previous studies reported that resistance to *Xcc* occurs with race-specific manner in cruciferous plants including common *Brassica* species and such interaction between *Xcc* and its host was controlled by a gene-for-gene relationship (Kamoun *et. al*, 1992; Vicente *et. al*. 2002). Single dominant genes that are highly resistant against *Xcc* races have been commonly found in *B. nigra*, *B. rapa* and their amphidiploid species, whereas a few sources of race-specific resistance have been identified in *B. oleracea*. On the other hand, non-differential resistance has been found in *B. oleracea* (Taylor *et. al*, 2002); for instance, Vicente *et. al*. 2002, reported that F1 plants obtained from the cross of resistant cabbage BI-16 × A12DHd were susceptible and the subsequent F2 plants showed quantitative resistance to *Xcc* races 1 and 3, indicating that resistance was mainly controlled by one recessive gene (*xca6*) or by linked genes. Williams *et. al*. 1972, found that resistance of BI-16 was quantitative under oligogenic control. In the GC P09 × Reiho P01 mapping population, appearance of susceptible phenotype in F1 plants and detection of multiple QTLs controlling resistance to *Xcc* (Doullah *et. al*. 2011, this study) showed that the inheritance of resistance to *Xcc* was recessive and controlled in a quantitative manner. Taken together, both single (qualitative) and multiple (quantitative) resistance genes must control resistance to *Xcc* in *B. oleracea*.

Our linkage map was constructed from the 94 F2 plants derived from the cross of GCP09 × Reiho P01 and comprises nine linkage groups, corresponding to the nine chromosomes of *B. oleracea*. The map length of 1099.4cM was similar to the map length, 1112, 1048, and 1197.9 cM of (Parkin *et. al*. 2005 Nagaoka *et. al*. 2010 and Wang *et. al*. 2012), respectively, and longer than the map lengths, 891.4, 320.5, 928.7 cM of (Iniguez-Luy *et. al*.2009, Doullah *et. al*. 2011 and Kifuji *et. al*. 2013), respectively. In our map, the average interval between markers was 6.1cM, indicating that the length and marker distribution of our map was suitable for QTL analysis of *Xcc* resistance. In our study, the QTL analysis that was performed using the appropriate significance threshold successfully detected several significant QTLs, indicating that *Xcc* resistance was controlled by an oligogenic system. We detected one major locus *XccBo(Reiho)2* on C8 that accounted for 34% of the variation. Alleles from Reiho at the *XccBo(Reiho)2* locus act additively and contribute to resistance, as indicated by the negative value of the additive gene action. Genotyping at the BoGMS0971 marker that was closely linked to *XccBo(Reiho)2* showed that the homozygous Reiho genotypes were resistant, whereas the homozygous GC genotypes tended to be susceptible, and the resistance level of the heterozygotes fluctuated (Figure 2.1). This result suggests that the *Xcc* resistance gene at the *XccBo(Reiho)2* locus

established stable expression in homozygous plants. We identified two smaller QTLs, *XccBo(Reiho)1* and *XccBo(GC)1*, on C5 and C9, respectively. The resistance allele at *XccBo(GC)1* locus on C9 come from the susceptible parent. This secret gene effect could explain the fact that some plants exhibited transgressive segregation beyond the level of the resistant parent. In fact, the five most resistant F3 lines were derived from F2 plants which were either homozygous for broccoli alleles or heterozygous at this locus on C9. The disease resistance alleles coming from susceptible parents have been reported in QTL analyses of black rot (Camargo *et. al*. 1995) and clubroot (Nagaoka *et. al*. 2010) in *B. oleracea* and leaf blight in *Zea mays* (Schechert *et. al*. 1999).

Camargo *et. al*. (1995) identified the four *Xcc* resistance QTLs in the BI-16×OSU Cr-7 map in which two QTLs on LG1 (abbreviation, QTL-LG1) and LG9 (QTL-LG9) were associated with both young and adult plant resistance and the two additional QTLs on LG2 (QTL-LG2a, QTL-LG2b) were associated only with young plant resistance. Kifuji *et. al*., 2013, detected the three *Xcc* resistance QTLs on C2, C4 and C5. Comparative map data with the common pW, pX and BoCL markers revealed that the central portion of C5 harboring *XccBo(Reiho)1* corresponded to that of QTL-LG2a identified by Camargo, *et. al*., (1995) as well as to that of QTL-3 identified by Kifuji *et. al*. 2013. Furthermore, the bottom distal end of C9 harboring *XccBo(GC)1* corresponded to that of QTL-LG9 identified at the interval pX117 (EC2D9) - pW143 (WG8A9). The assignment of different linkage maps with the common markers suggests that our identified QTL, *XccBo(Reiho)1*, may be equivalent to QTL-LG2a and QTL-3 identified by (Camargo *et. al*., 1995 and Kifuji *et. al*., 2013) respectively and furthermore, the *XccBo(GC)1* may correlate to QTL-LG9 identified by (Camargo *et. al*., 1995). The QTL-LG1 in the BI-16×OSU Cr-7 map corresponded to the bottom distal end of C3 based on the location of pW125, pW181, pW245 and pW188 markers (data not shown). We also mapped pW188 on the distal end of C3 in our linkage map where one candidate QTL (LOD=2.1) was detected. However, it is difficult to conclude whether the QTLs that are linked to the same molecular markers involve just one gene or are family members of clustered *Xcc* resistance genes. Microsynteny analysis in these regions in *B. oleracea* is needed to identify the relationship between these resistance loci.

Although we identified the *XccBo(Reiho)2* on C8, previous authors did not find any *Xcc* resistance QTLs on C8 (Camargo *et. al*., 1995, Doullah *et. al*., 2011 and Kifuji *et. al*., 2013). The discrepancy in positions of the detected QTLs might be due to differences of races used, inoculation methods, and plant materials. In addition, the magnitude of QTL effects could change in response to different environmental conditions. The upper part of C5, one of middle part of C8 and the distal end of C8 shared conserved regions with *Arabidipsis*

thaliana chromosome 1(Lukens *et. al*., 2003 and Parkin *et. al*., 2005). In addition, it is known that the large conserved regions are duplicated in C5 and C8 (Parkin *et. al*., 2005 and Carlier *et. al*.; 2011). This indicates that *XccBo(Reiho)1*on C5 and *XccBo(Reiho)2* on C8 might be homologous loci. The diploid *Brassica* genome was formed by the whole-genome triplication followed by multiple chromosome rearrangements via insertions, deletions, and translocations. Through this process, disease resistance genes were located on various chromosomal regions and furthermore, clustering of disease resistance genes occurred as a result of long-term host–parasite co-evolution (Holub, 1997). The QTL regions conferring *Xcc* resistance to *B. oleracea* plants might have originated from common chromosomal regions existing in the progenitor diploid species.

Screening genetic resources of *B. oleracea* revealed that resistance to race 3 and race 5 is common, but resistance to race 1 is very rare (Taylor *et. al*., 2002). Therefore, our finding three QTLs that conferred resistance to race 1 is important for resistant breeding in *B. oleracea*. Pyramiding those QTLs, one novel major QTL on C8 and the other two QTLs that might coincide with previously mapped QTLs, will promote *Xcc* resistance breeding in *B. oleracea*, and the markers closely linked to the QTLs will be useful in MAS for improving resistance to black rot across environments.

Table 2.1. List of DNA markers used in this study.

Marker symbols	Type of markers	Origin	Reference	No. of marker used in the linkage map
BnGMS	SSR	*B. napus*	Cheng *et al.* (2009)	1
BoCL	SNP	*B. oleracea*	Kifuji *et al.* (2012)	5
BoE	SSR	*B. oleracea*	Wanxing Wang *et al*. (2012)	10
BoGMS	SSR	*B. oleracea*	Li *et al*. (2011)	43
BrSF	SSR	*B. oleracea*	Wanxing Wang *et al*. (2012)	1
BoSF	SSR	*B. oleracea*	Wanxing Wang *et al*. (2012)	21 [a]
BRAS	SSR	*B. napus*	Piquemal *et al*. (2005)Radoev *et al*. (2008)	3
BRMS	SSR	*B. rapa*	Suwabe *et al*. (2006)	7 [a]
BSA	SSR	*B. rapa*	Suwabe *et al*. (2006)	2
CB	SSR	*B. napus*	Piquemal *et al*. (2005)Radoev *et al*. (2008)	34
EMS	SSR	*B. oleracea*	Wanxing Wang *et al*. (2012)	3
FITO	SSR	*B. oleracea*	Iniguez-Luy et al. (2008)	1
KBr	SSR	*B. rapa*	Nagaoka *et al*. (2010)	12
MR	SSR	*B. napus*	Radoev et al. (2008	1
Na	SSR	*B. napus*	Piquemal *et al*. (2005)	5
Ni	SSR	*B. nigra*	Piquemal *et al*. (2005)	1
Ol	SSR	*B. oleracea*	Piquemal *et al*. (2005)	2
pW, pX	CAPS	*B. napus*	Udall *et al*. (2005)	5
	CAPS	*B. oleracea*	Udall *et al*. (2005)	21 [b]
Total markers				178

[a]Markers mapping more than one position
[b]CAPS markers mapped by using the genotyping data of Doullah et al. (2011).

Table 2.2 List of the primer sequences by which the chromosomal regions mapped by pW, PX and BoCL markers were detected

Name	Forward (5'—3')	Reverse (5'—3')	Chr. identified
BoCL6200s	GGTTGGAAAGCAATTGGTGAAC	GGTTCGACACACAAAGAAACCA	C2
BoCL5584	CAAGAGCACAATCTCGGTCCTA	ATGACACGCGTTTACACTCTGC	C2
pW188	GATGTGATCACCTCTTATCGA	ACAATGCCCCCAACAAAGCG	C3
BoCL5860	AGATGCTACAGCAACAGCTCTC	GAGGAGCTGAGTTGAGAAGCTCA	C5
BoCL1135	TACAAGTACCGGCCATAGGTGA	GCATGCTGAAAGATTCTCTGTG	C5
pW114	TTCCCAATGTTGGAGGCAGT	TATATATCGCTCAAGCTCAATC	C5
pW164	CAGCAGCACGATAACGAGGTGCA	CGTGTGATCGTAACGAGCAATTGG	C5
pX117	CGTCCCTTACCTTCCTCCG	TCCTCCGTAGATAACGGTCG	C9
pW143	ATGAGCAGAGCACAAGATCCACCGA	ACAACGGCTTCTCAGAGACCG	C9

Table 2.3. Characteristics of *B. oleracea* linkage map.

Linkage	Length	Number of markers		
Group	（cM）	SSR	CAPS	Total
1	113.7	15	0	15
2	106.8	16	7	23
3	138.5	12	6	18
4	163.6	22	4	26
5	170.1	18	2	21
6	90.0	17	2	19
7	88.2	19	0	19
8	114.2	19	3	22
9	114.3	16	2	18
Total	1099.4	155	26	181

Table 2.4. Summary of QTLs significantly detected for black rot disease against *Xcc* race1 using F2 populations derived from GC P09 × Reiho P01.

QTL name	Chr.	Closest marker	Position (cM)	LOD[a]	Additive effect[b]	Dominance effect[b]	R^2 (%)[c]
XccBo(Reiho)1	C5	BoGMS1330	90.7	4.4	- 0.3	0.4	6.6
XccBo(Reiho)2	C8	BoGMS0971	58.0	7.7	- 0.9	0.1	34.0
XccBo(GC)1	C9	CB10459	81.0	4.4	0.5	0.0	17.9

[a]Peak LOD score of the QTL
[b] Additive and dominant effect of resistant parent allele in DLA
[c] Percentage of variance explained by quantitative trait loci.

Table 2.5. Alignment of linkage maps for assigning positions of Xcc-resistance QTLs identified by Camargo et al. (1995) on the consensus map.

Consensus map (Udall et al.2005)	Linkage map (II) (Camargo et al. 1997)	Linkage map (I) (Camargo et al. 1995)	Marker interval in which Xcc-resistance QTL detected in the linkage map (I)	Abbreviation of the QTLs
N11(C1)	LG8	-		
N12(C2)	LG7	-		
Top of N13(C3)	LG5	-		
Bottom of N13(C3)	Bottom of LG1	LG1	WG2G11(pW245)-WG6G5(pW224)[a] WG1E3(pW188)-WG6G5(pW224)[b]	OTL-LG1
N14(C4)	LG4	-		
N15(C5)	LG9	LG2	WG6H1(pW245)-TG4D2(pW114)[a]	OTL-LG2a
N16(C6)	LG2	LG2	EC5E12(pX130)-EC2H2(pX144)[a]	OTL-LG2b
N17(C7)	LG3	-		
N18(C8)	LG6	-		
N19(C9)	Top of LG1	LG9	WG8A9(pW147)-WG4D7[a] WG8A9(pW147)-EC2D9(pX117)[b]	OTL-LG9

[a] The marker intervals were identified in the greenhouse trial (young plant); [b] in field trial (adult plant).

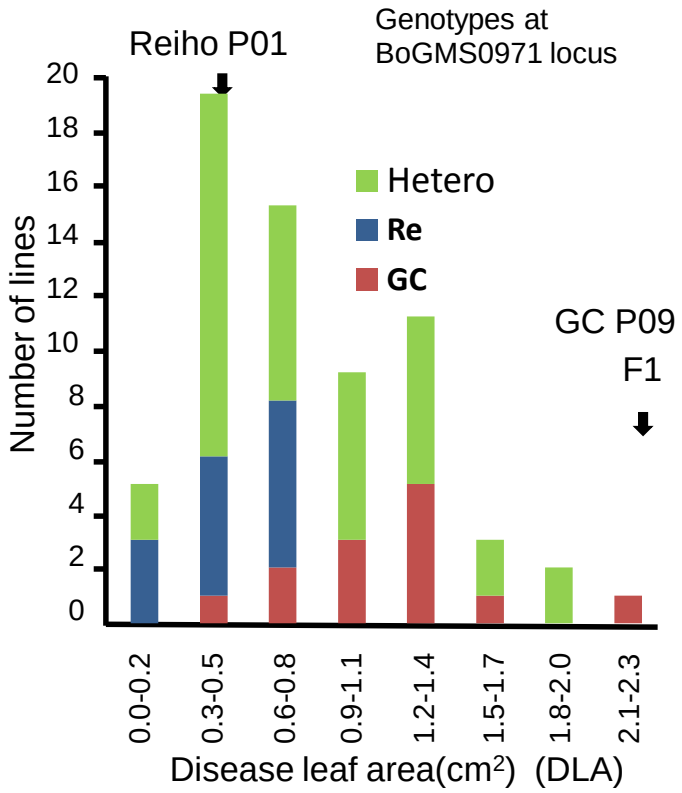

Figure 2.1. Genotyping data at BoGMS0971 locus in the mapping population that Doullah et al. (2010) described previously. Frequency distribution data of mean diseased leaf area (DLA) of black rot disease in the F3 lines. Arrows indicate values obtained for parental (Reiho P01 and GC P09) and F1 plants. The mapping population were genotyped at the BoGMS0971 locus. F2 plants homozygous for the 'Reiho' BoGMS0971 locus, homozygous for the 'GC' BoGMS0971 locus, or heterozygous at the BoGMS0971 locus are indicated by blue, red, or green bars, respectively.

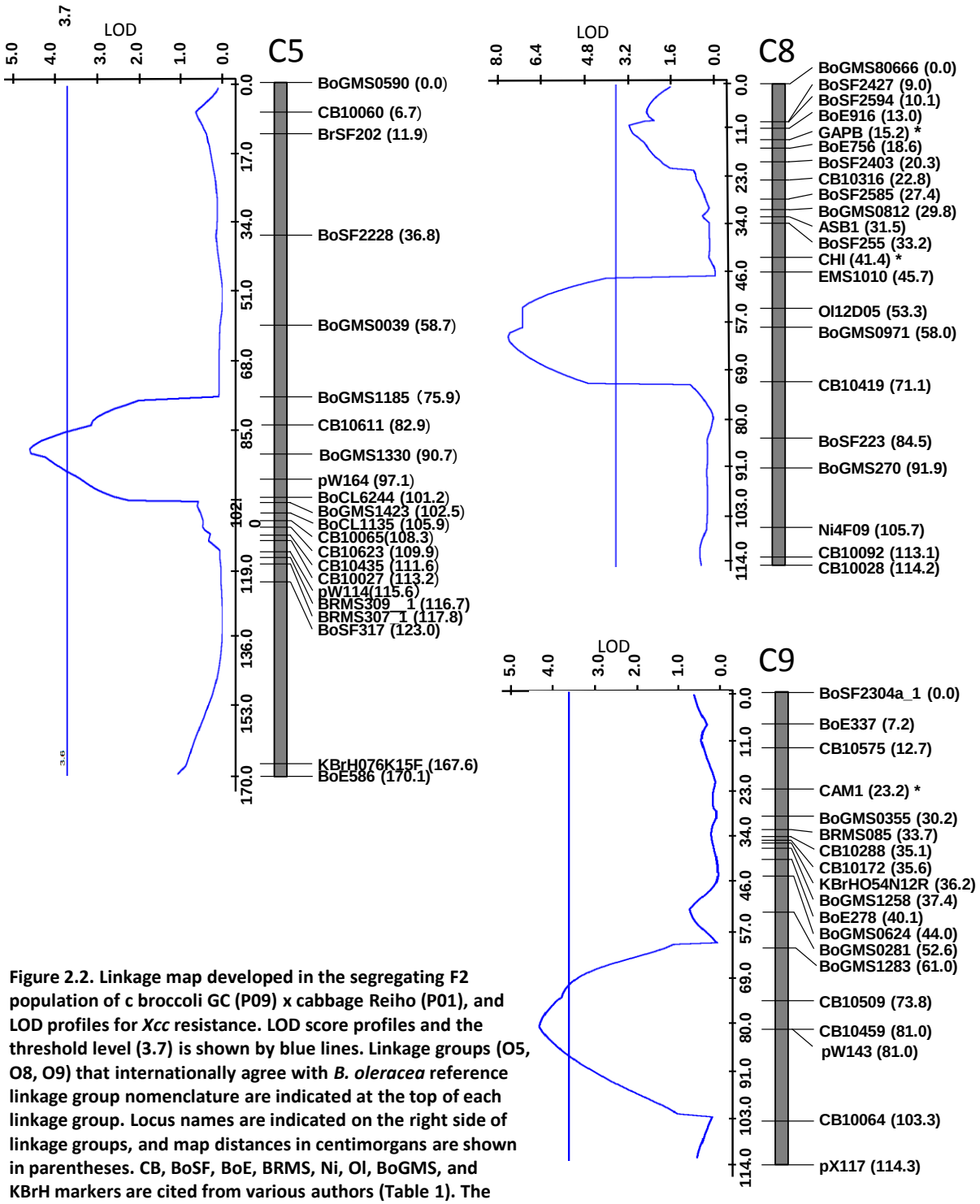

Figure 2.2. Linkage map developed in the segregating F2 population of c broccoli GC (P09) x cabbage Reiho (P01), and LOD profiles for *Xcc* resistance. LOD score profiles and the threshold level (3.7) is shown by blue lines. Linkage groups (O5, O8, O9) that internationally agree with *B. oleracea* reference linkage group nomenclature are indicated at the top of each linkage group. Locus names are indicated on the right side of linkage groups, and map distances in centimorgans are shown in parentheses. CB, BoSF, BoE, BRMS, Ni, Ol, BoGMS, and KBrH markers are cited from various authors (Table 1). The CAPS markers shown by asterisks were mapped by using the data of Doullah et al. (2011).

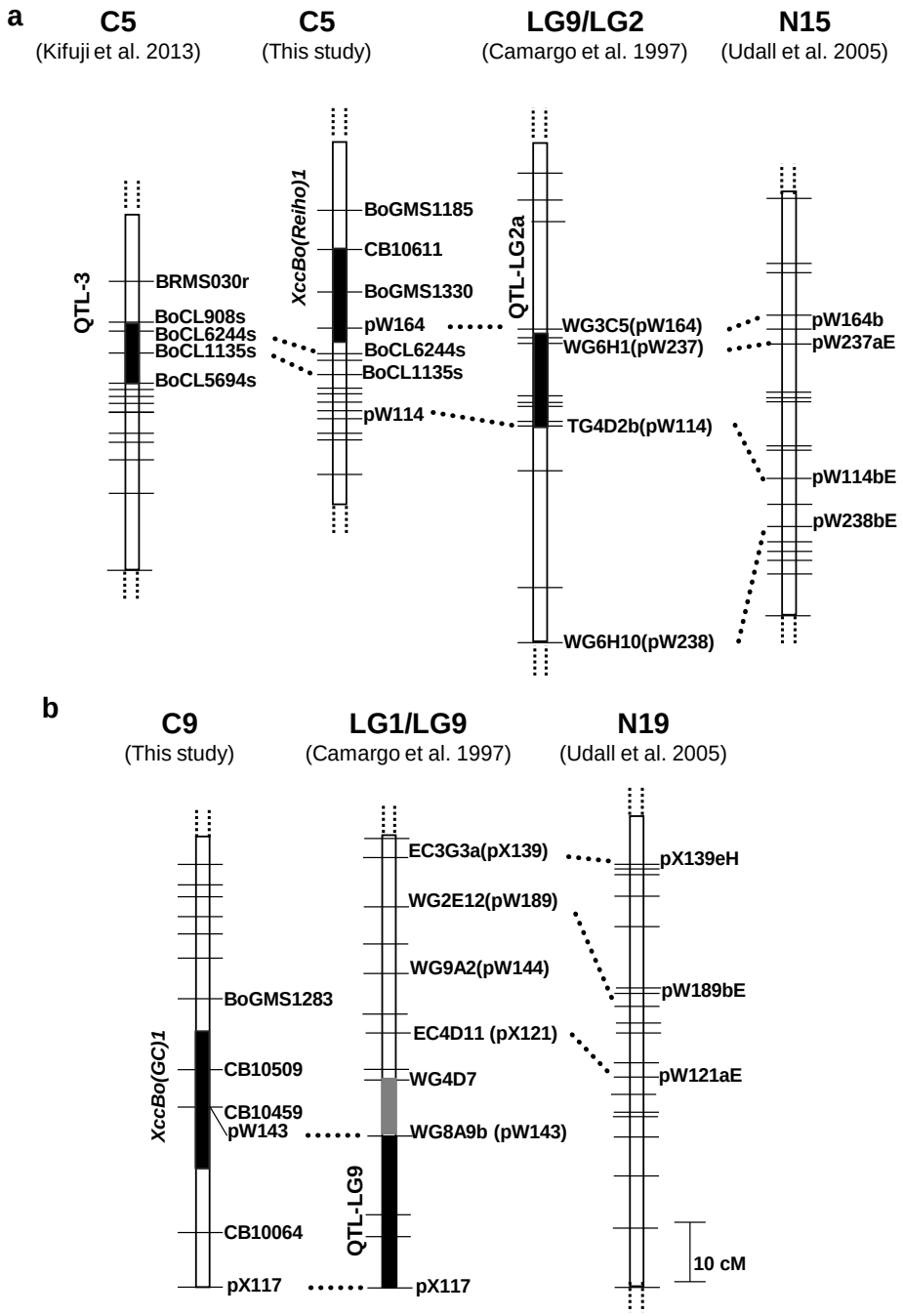

Figure 2.3. Comparison of chromosomal positions of *Xcc* resistance QTLs on C5 (a) and C9 (b). The filled vertical bars indicate the marker intervals where the *Xcc* resistance QTLs identified by Camargo et al. (1995), Kibushi et al. (2013) and this study. The gray and black vertical bars on LG1/LG9 represent QTLs identified by Camargo et al. (1995) in the greenhouse and the field trials, respectively. The numbers at the top indicate *B. oleracea* reference linkage groups. Consensus map constructed by Udall et al. (2005) (right) was aligned to identify linkage groups constructed by Camargo et al. (1995). Horizontal bars indicate the positions of markers included in each map and the marker names were omitted but the marker intervals were the same as described by the authors.

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Appendix 1

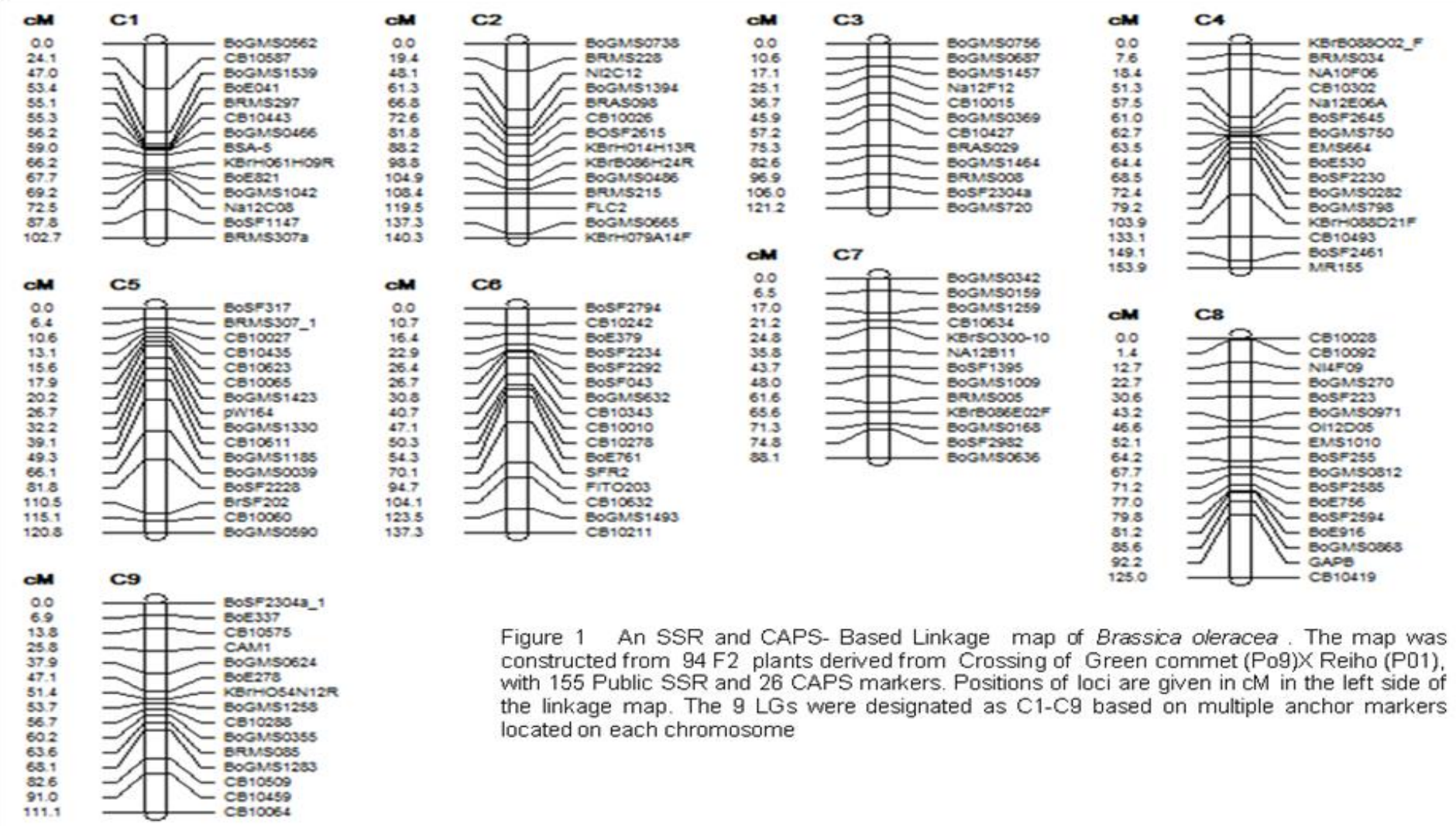

Figure 1 An SSR and CAPS- Based Linkage map of *Brassica oleracea* . The map was constructed from 94 F2 plants derived from Crossing of Green commet (Po9)X Reiho (P01), with 155 Public SSR and 26 CAPS markers. Positions of loci are given in cM in the left side of the linkage map. The 9 LGs were designated as C1-C9 based on multiple anchor markers located on each chromosome

Appendix 2

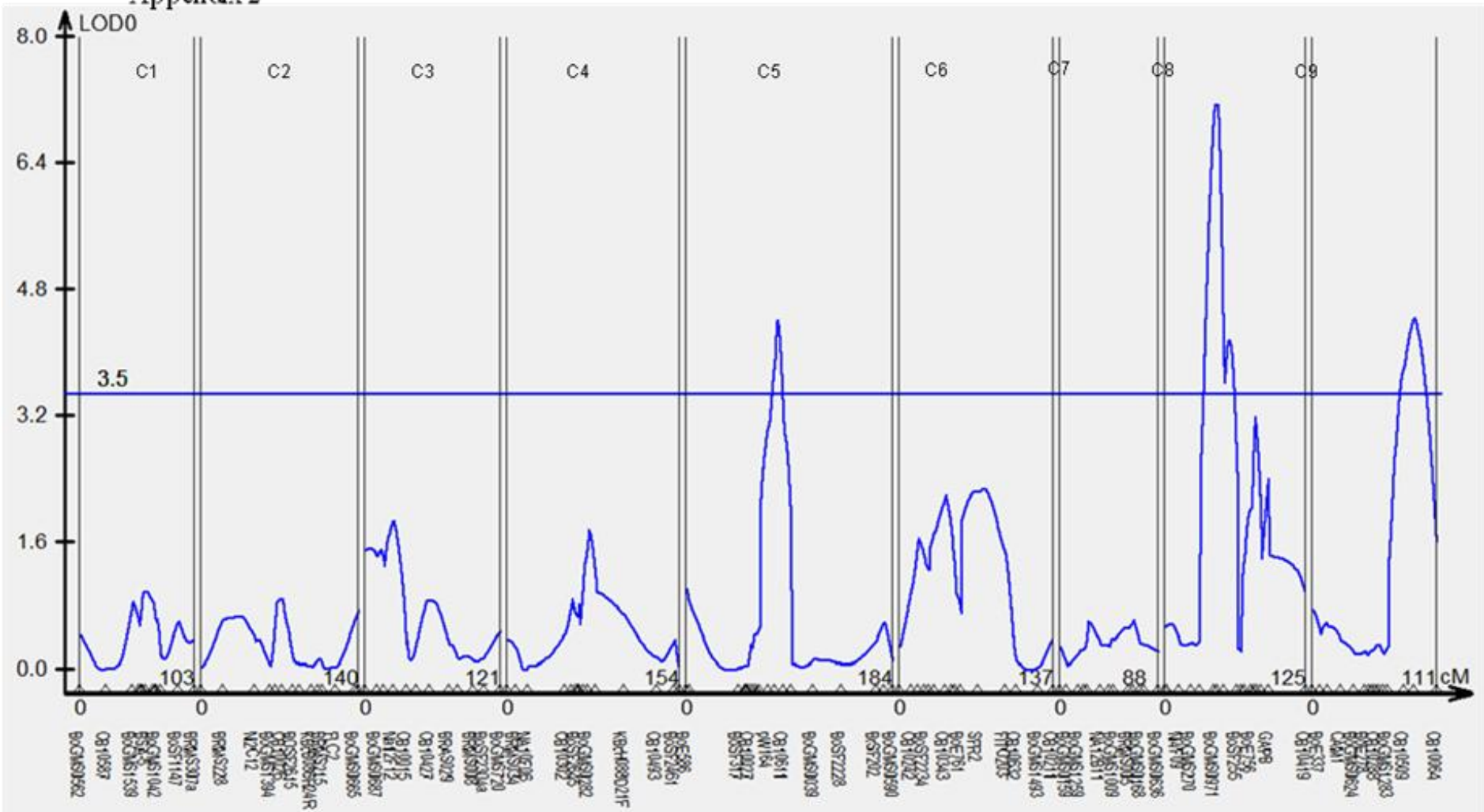

Figure 2 QTL profile developed in a segregating F2 Population of broccoli 'GC' (P09) X cabbage 'Reiho' (P01), and LOD profiles for Black rot resistance. Linkage groups (C1 – C9) that internationally agree with B. oleracea reference linkage group nomenclature are indicated at the top of each linkage group. The threshold value (3.5) is shown as a continuous line. The positions of the markers are shown at the bottom

Appendix 3 List of markers used in the linkage map of *Brassica oleracea* in this study

	Marker Type	Marker Name	Forward Primer	Reverse Primer	Linkage Group	Source
1	SNP	BoCL6200	GGTTGGAAAGCAATTGGTGAAC	GGTTCGACACACAAAGAAACCA	C2	Kifuji *et al.* (2012)
2	SNP	BoCL5584	CAAGAGCACAATCTCGGTCCTA	ATGACACGCGTTTACACTCTGC	C2	Kifuji *et al.* (2012)
3	SNP	BoCL2635	AAAGGATGAGGACCATGCAACT	CTTTACCCACACGTGCATCATT	C4	Kifuji *et al.* (2012)
4	SNP	BoCL5860	AGATGCTACAGCAACAGCTCTCT	GAGGAGCTGAGTTGAGAAGCTCA	C5	Kifuji *et al.* (2012)
5	SSR	BoE041	CTTGGCTAGGGTTTTGGTAT	ACTAGTCCCGAGGTTATTGTTT	C1	Wanxing Wang *et al.* (2012)
6	SSR	BoE278	GGGGAAGGGACGAAATACAGGAGT	CTTTAAGGGCTTCGATGGAGTTGG	C9	Wanxing Wang *et al.* (2012)
7	SSR	BoE337	AACTGAGACTGAAAAAGACAAACA	TTCAGCATCAATCAACTTATCAAT	C9	Wanxing Wang *et al.* (2012)
8	SSR	BoE379	GCGGGGACTCTACCTCTA	AGCAGCTCAGCATACAAG	C6	Wanxing Wang *et al.* (2012)
9	SSR	BoE530	TGGCTCCACCGGTCACTATCAGA	TCATGGACTTGGGAGGGTTTTG	C4	Wanxing Wang *et al.* (2012)
10	SSR	BoE756	GATTGCTCGGCGACGGTAGAT	CTGGCTTAATGTTGGAGATGAAAA	C8	Wanxing Wang *et al.* (2012)
11	SSR	BoE761	CATTCAGCGACTTCCTTCAAACTT	GGCGCACTTCTTCCCCTGTA	C6	Wanxing Wang *et al.* (2012)
12	SSR	BoE821	ACAATCACAATGGGTTACG	CTTCGGCTTGGAGTGTC	C1	Wanxing Wang *et al.* (2012)
13	SSR	BoE916	CCTGTCTCGGGGATGATGCTAT	GGCGCCGAACCCGAATGTAT	C8	Wanxing Wang *et al.* (2012)
14	SSR	BoGMS0039	TGGACATTGGACATAAGGTAA	AAGAAGTGTAAATAGGCACAAGA	C5	Li *et al*. (2011)
15	SSR	BoGMS0092	AAACATAAATCTGAAAGTGAAA	AATGGTGAATGCCCGTCT	C4	Li *et al*. (2011)
16	SSR	BoGMS0118	ACGAGAGGAACACGATGA	GTGGAGTCAATCAGAAGAGG	C7	Li *et al*. (2011)
17	SSR	BoGMS0159	TCAAGAAAGAGAGAACTAAGCC	GTGCGGTGTTGTGAGTCC	C7	Li *et al*. (2011)
18	SSR	BoGMS0168	GTCTTGTATGAAGCCCAGTAG	AGAGGAAGTGTCGGGAAG	C7	Li *et al*. (2011)
19	SSR	BoGMS0270	GCAATGAAATCCCTACCTG	GAATCTGCCTTGAATCTGAC	C8	Li *et al*. (2011)
20	SSR	BoGMS0281	ATCCTTCTGCCTTCTCTG	TGACATCCATCCACACATT	C9	Li *et al*. (2011)

21	SSR	BoGMS0282	CCCTTGTAGAGAGAGAGAGGA	AAACGAAATAAGATGACGAGA	C4	Li	*et al*. (2011)
22	SSR	BoGMS0342	ATGAATAACCAAGCGACAAG	TAAGCCAGAAGGCACTGTT	C7	Li	*et al*. (2011)
23	SSR	BoGMS0355	GTGAAAGCACGCCAAGAAC	TCTCAACCTAATCAAACACAAA	C9	Li	*et al*. (2011)
24	SSR	BoGMS0369	AGGACAGCATCGGTATGA	CTGGATAGTTTCTCTTTCTTGG	C3	Li	*et al*. (2011)
25	SSR	BoGMS0466	TCTCTCATCCTCTTCTTCTTC	GTGATTCTCCACCAGATACAG	C1	Li	*et al*. (2011)
26	SSR	BoGMS0486	AAGGAGGAACCAAATGCC	TGATAATGCCACTGATAGGAC	C2	Li	*et al*. (2011)
27	SSR	BoGMS0562	CCTAACGAAGAAGACGAATAA	GTCGGAGAATCGGTGGTG	C1	Li	*et al*. (2011)
28	SSR	BoGMS0590	TGGTTTATCTTCATTCTTTGG	TATTGAGTTGTCGCACTTGA	C5	Li	*et al*. (2011)
29	SSR	BoGMS0624	AAGACGAAGTCAAGTCAAGGT	CGTATCATCCAGAGTATCCAG	C9	Li	*et al*. (2011)
30	SSR	BoGMS0632	ATCATCGTCCTCTTCTTCTTC	TATCATCCTTATTGGGTCTC	C6	Li	*et al*. (2011)
31	SSR	BoGMS0636	ACCTCTGCTCCTCATTCAC	GTGTAGCCCATCTCATCTTT	C7	Li	*et al*. (2011)
32	SSR	BoGMS0665	TCAGAGATGAACAAGAAGCAC	GCGAACCTTTCCCAACCT	C2	Li	*et al*. (2011)
33	SSR	BoGMS0687	GACAACACAACAGACGCA	GCATTTCCCATTACTTCCA	C3	Li	*et al*. (2011)
34	SSR	BoGMS0720	GCCATCTCTCTCTCTCTCTCT	AAGCACAATCTTCCATTCAC	C3	Li	*et al*. (2011)
35	SSR	BoGMS0738	TTGAGGAAGGAACACGAA	GTGGGAGAGTGAGGGTAGTAA	C2	Li	*et al*. (2011)
36	SSR	BoGMS0750	CTGACTTGGCGTTGATGT	CGGAAATAGGATAATAGAGGATT	C4	Li	*et al*. (2011)
37	SSR	BoGMS0756	AACAAGAAGAAGAAAGGAGGA	GTGGGAGACAGCGAGGAC	C3	Li	*et al*. (2011)
38	SSR	BoGMS0798	AATGAAACTGCTTGAGGAAA	CTGTTGCGGTATGTTGTTG	C4	Li	*et al*. (2011)
39	SSR	BoGMS0812	GCTGGCACATAGTTGTAATG	CTCATCTCCTCTGCTGGA	C8	Li	*et al*. (2011)
40	SSR	BoGMS0868	AAATCCCAACGAGATAGGTAG	AGAAAGAAAGGAAGAAAGTGG	C8	Li	*et al*. (2011)
41	SSR	BoGMS0971	TAATCCGAACAACACGAA	CACCCAATAAGCGATGAG	C8	Li	*et al*. (2011)
42	SSR	BoGMS1009	CGAAACCAGGATAAGTCA	CAATGCTTCTGTATGCGTC	C7	Li	*et al*. (2011)
43	SSR	BoGMS1042	ATAGTGAATAATGGAAGGCTG	GAGAGAGGAGAGAACAGAGGA	C1	Li	*et al*. (2011)
44	SSR	BoGMS1065	GGGTTGATTGGGAAGTGT	CTTAGCACCATTTGTTTGTATT	C7	Li	*et al*. (2011)

45	SSR	BoGMS1185	ATACAAGATGCGAAGGAGAA	CGGCAAGAGAATAATAGACAG	C5	Li *et al*. (2011)
46	SSR	BoGMS1258	TTATTATTCTCCTGCTCCTTG	ACACACAAACGATGCTCAC	C9	Li *et al*. (2011)
47	SSR	BoGMS1259	ACAAGGAGAAGGAGAAGACAC	TGAGGAAGATGAAGTTGAAGA	C7	Li *et al*. (2011)
48	SSR	BoGMS1283	TTGTCATCATCCTCTTCACTC	TGCTATCCACTCTTCTTCTCA	C9	Li *et al*. (2011)
49	SSR	BoGMS1330	AGGAGAAGAAGGAAGATACCA	AGAAAGGAAAGAAAGACCAGA	C5	Li *et al*. (2011)
50	SSR	BoGMS1394	ATTGTGTCAGAGAAGGGTT	GGTGGAAGTAGAGGAAGAAGA	C2	Li *et al*. (2011)
51	SSR	BoGMS1423	AACTAAACCAAGCAAAGGAAA	AACCCTCTCACCTACTTCAAC	C5	Li *et al*. (2011)
52	SSR	BoGMS1457	AACAGACAACTAAATCAACGG	TGGAAGAGGAGGTCACTAACT	C3	Wanxing Wang *et al*. (2012)
53	SSR	BoGMS1464	CTGATGAACGGAGACACAG	AAGCAAAGCAGAGCATAAAC	C3	Li *et al*. (2011)
54	SSR	BoGMS1493	CGTAGAGAGTATTGGAAGCA	GTCCTCCTCGTAATGGTGT	C6	Li *et al*. (2011)
55	SSR	BoGMS1539	GTGCTTCACTTGTATGATGACT	ATAATGGGCTGCTCTTCTTC	C1	Li *et al*. (2011)
56	SSR	BrSF202	GCGATCAGATCCAAACGAAT	AAGACGATCTCTTTCGCTGC	C5	Wanxing Wang *et al*. (2012)
57	SSR	BoSF043	TTTTGGATGAAGCCCAAGAC,	GCTAATCAGATCTCTCGCGG	C6	Wanxing Wang *et al*. (2012)
58	SSR	BoSF223	AGCTCACGAATCTGCTCCAT	CCGAAACCAAGATAGGTGGA	C8	Wanxing Wang *et al*. (2012)
59	SSR	BoSF255	AGTGGGTGAACCAAGGACAA	AAGCAAGTTTCAGCCAAAGC	C8	Wanxing Wang *et al*. (2012)
60	SSR	BoSF317	CCAACTCCGGTCAATCATCT	GCCCCTTTCTGTGTGACATT	C5	Wanxing Wang *et al*. (2012)
61	SSR	BoSF1147	CCCCATCGTGTTTACGATTC	GCGTGGGTTCAATTACCACT	C1	Wanxing Wang *et al*. (2012)
62	SSR	BoSF1269	AATCAAATAGCCGCAGCGT	TCAAAAACCGTGACATCGAA	C4	Wanxing Wang *et al*. (2012)
63	SSR	BoSF1395	TCACGATGTCCCTTGTGATG	TCTGGCGATGATGAGAAGAA	C7	Wanxing Wang *et al*. (2012)
64	SSR	BoSF2212	GAACCCAAGGAAACATGCAC	TCAGAGTGTCCTCTGCCTCA	C4	Wanxing Wang *et al*. (2012)
65	SSR	BoSF2228	TTTTCGTGAAGCTGATGCTG	CCAGCTTTCTTTAAAATCCCAA	C5	Wanxing Wang *et al*. (2012)
66	SSR	BoSF2230	ATCGGAAAGACTTCACCCCT	CAATCATGTCCCCAAAGGAC	C4	Wanxing Wang *et al*. (2012)
67	SSR	BoSF2234	CCCCCACCTAGTGAAACTCA	CCTCTTCCTCACCAACCAAC	C6	Wanxing Wang *et al*. (2012)
68	SSR	BoSF2292	GGGCGTATCAGGAAGAGTGA	ATGTGTGCCTGGGAGAAAAT	C6	Wanxing Wang *et al*. (2012)

69	SSR	BoSF2304a	AGAAACGCAAATGGTCTTCG	CTGTATGAGCATGGCTTCCA	C3,C9	Wanxing Wang *et al*. (2012)
70	SSR	BoSF2403	AGGTGATCTCACCAACAGGC	GGGGTGGGTAGGTTGACTTT	C8	Wanxing Wang *et al*. (2012)
71	SSR	BoSF2427	CATCAACGATGGTGATGCTC	AAACCAAGTACGATCGGCAG	C8	Wanxing Wang *et al*. (2012)
72	SSR	BoSF2461	CTGATTTGGAGGGAAAAGCA	CACTCTTCCGACAACAGCAA	C4	Wanxing Wang *et al*. (2012)
73	SSR	BoSF2585	AGCTCGTCGGAGTCTGATGT	TCATTCATCTTTCTTCCCCG	C8	Wanxing Wang *et al*. (2012)
74	SSR	BoSF2594	ATGGCCATCGTACTTTGAGG	CAAAGCCACGATTGCATTTA	C8	Wanxing Wang *et al*. (2012)
75	SSR	BoSF2615	CGTTGTCTCAAATCAATGGC	TCATCCATTATCATCGGGGT	C2	Wanxing Wang *et al*. (2012)
76	SSR	BoSF2645	GGTTTCTCAGTTCGACGGAA	GCGCAAAAGCCATATAATCC	C4	Wanxing Wang *et al*. (2012)
77	SSR	BoSF2794	TGGTACCTACCACCGTCTCC	CCGGTTTGGTTTCAGTGAGT	C6	Wanxing Wang *et al*. (2012)
78	SSR	BoSF2982	TTCTCCTTCTTTCTTTACTCCCATTCT	TGGACCCAAACTCAAATAAAAATCA	C7	Wanxing Wang *et al*. (2012)
86	SSR	BRAS029	GTTCAACCTCCCTCGTCTCT	AGGTGCCAACTCATTTCTCAA	C3	Piquemal *et al*. (2005)Radoev *et al*. (2008)
85	SSR	BRAS098	GAA AGA AAG CGG ATA GG	CAA AAC CCA CAC GAA AGC	C2	Piquemal *et al*. (2005)Radoev *et al*. (2008)
87	SSR	BRAS119	ATG AAA ATA TAA ACG CTG CT	TAC CTT GAG GAC CTG CGA CT	C7	Piquemal *et al*. (2005)Radoev *et al*. (2008)
88	SSR	BRMS005	ACCTCCTGCAGATTCGTGTC	GCTGACCTTTCTTACCGCTC	C7	Suwabe *et al*. (2006)
90	SSR	BRMS008	AGGACACCAGGCACCATATA	CATTGTTGTCTTGGGAGAGC	C3	Suwabe *et al*. (2006)
92	SSR	BRMS034	GATCAAATAACGAACGGAGAGA	GAGCCAAGAAAGGACCTAAGAT	C9	Suwabe *et al*. (2006)
89	SSR	BRMS085	ACTCCACACTCTCACTTCCTCTATT	TTACGCTTGTTTCTGTTTTTGAATA	C9	Suwabe *et al*. (2006)
94	SSR	BRMS215	TATCGTCCACATGAACAATTCATAC	GAAGGTTTTCCTAATATGTCGGATT	C2	Suwabe *et al*. (2006)
93	SSR	BRMS228	GTGGGGAGAAGAGAGAATTCAAA	AGCTAGCTTTCAAATCAAAACGAAT	C2	Suwabe *et al*. (2006)
91	SSR	BRMS297	AAACTCAAAAACCTCCACTTTCTCG	ATGTGGAGGTGGGACCCATTA	C1	Suwabe *et al*. (2006)
95	SSR	BRMS307_1	GTGACATCTATCCCCAAACGTACAG	ATAAACGAAGCGAGTGTGATGGTTA	C1,C5	Suwabe *et al*. (2006)
96	SSR	BRMS309_1	TGGTGGCTTGAGATTAGTTC	ACTCGAAGCCTAATGAAAAG	C5	Suwabe *et al*. (2006)
97	SSR	BSA-5	ATTCTTTGATATTGAGCCATGTGGT	TCCAGAGAGATACACAGTTCTCATCA	C1	Suwabe *et al*. (2006)
98	SSR	CB10010	TTATCTTTGAATGAGCATCT	ACCCTGTTCCTTCTACTAT	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)

99	SSR	CB10021	ACGGAGACGGGGACGG	CTTCTTCGCCTTTAGTCCTT	C3	Piquemal *et al*. (2005), Radoev *et al*. (2008)
100	SSR	CB10026	TCGTTCTGACCTGTCGTTAT	GGAAATGGCTGCTCATGTT	C2	Piquemal *et al*. (2005), Radoev *et al*. (2008)
101	SSR	CB10027	CGGCTTGTAAACCTTG	GACTCGAAAATCACTAACAC	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
102	SSR	CB10028	GGAAATGGCTGCTCATGTT	AAATCAACGCTTACCCACT	C8	Piquemal *et al*. (2005), Radoev *et al*. (2008)
103	SSR	CB10060	CTGTACGGCTTCATCTCATAC	AGAATGTTATCTTGCCTTCAC	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
104	SSR	CB10064	CTCTCTCATCATATTCGGTG	TAGCAGAAAGAGTAAGAGGG	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)
105	SSR	CB10065	CGGCAATAATGGACCACTGG	CGGCTTTCACGCAGACTTCG	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
106	SSR	CB10092	TTGATCCGAAATTCTCTGG	AGGCAAGCAATAGATAAAGG	C8	Piquemal *et al*. (2005), Radoev *et al*. (2008)
107	SSR	CB10172	ATTGGTCTCTTAACCCGC	TTCTCGAATCCCTCGAA	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)
108	SSR	CB10211	CAGCAGAGATCGATGGAG	ATAGAAGGCTGCCCCTC	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
109	SSR	CB10213	CCTACCTTCCTTTACCAACC	GGTGATGATGATGGGAGA	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
110	SSR	CB10242	TTCCTTTCACGGTTTTCA	TTCCACAGGCACTTTCTC	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
111	SSR	CB10278	TGAAGAAGCTGGGACAAG	CAATGCAATACAGCACCA	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
112	SSR	CB10288	GCAATGCATATCGACCTT	AACCGCGCTATCAAGAAT	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)
113	SSR	CB10302	CGATACTTGGAGCGTGTC	CTGGTGTCTTAACCACGC	C4	Piquemal *et al*. (2005), Radoev *et al*. (2008)
114	SSR	CB10316	TGGTGTATATGGGATCGG	GTTTGCAGACCATTCTCG	C2,C8	Piquemal *et al*. (2005), Radoev *et al*. (2008)
115	SSR	CB10343	ATGCACGTCTCACAGACC	AGCCCATTGGAGCTAGAG	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
116	SSR	CB10416	GCTGTTGCTGTAGGTTTGA	GAGCCAGCGTTGATAAGA	C2	Piquemal *et al*. (2005), Radoev *et al*. (2008)
117	SSR	CB10419	CTGGAGTCGATGGAACTG	GCCAACATAAGCCAAAGA	C8	Piquemal *et al*. (2005), Radoev *et al*. (2008)
118	SSR	CB10427	TCCCAACAAAAGAGTCCA	CAGCGAACCGAGTCTAAA	C3	Piquemal *et al*. (2005), Radoev *et al*. (2008)
119	SSR	CB10433	CTGTGACTGCATTGCTGA	ACACAATAAAGTGCGGCT	C7	Piquemal *et al*. (2005), Radoev *et al*. (2008)
120	SSR	CB10435	GGTCCTGTCAAGCGTCTA	TTCAATCCCACCTGTGC	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
121	SSR	CB10443	CAGAAACCATCATAGCCG	TGATTTGGGAGACGAAGA	C1	Piquemal *et al*. (2005), Radoev *et al*. (2008)
122	SSR	CB10459	CCTGCTTTTGCTCTGTTC	GCGATGAAACCAAAGCTA	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)

123	SSR	CB10493	TGACGTGTGAGCAACAGA	CTGAGTCACAAGCCGAGT	C4	Piquemal *et al*. (2005), Radoev *et al*. (2008)
124	SSR	CB10509	TAGTCCCGATCCCTTTTC	TCACTTTGTTGTGGCTGA	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)
125	SSR	CB10575	TGGTGGTTGAGTTCGTCT	CACAGACCCCGTAAAGGT	C9	Piquemal *et al*. (2005), Radoev *et al*. (2008)
126	SSR	CB10587	TTGTGTTTTGCCTTCTGA	TTTGCGCACAAACAATAA	C1	Piquemal *et al*. (2005), Radoev *et al*. (2008)
127	SSR	CB10611	GTATCTGCGACAGTGGGA	AGCTTGGCTGTAATGACG	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
128	SSR	CB10623	GAGATCGAAGGTCTCGGT	GAGTCGAAACAGTGGTGG	C5	Piquemal *et al*. (2005), Radoev *et al*. (2008)
129	SSR	CB10632	CGAGGGCGAATTGGA	CCATCAACAGCCATCTTC	C6	Piquemal *et al*. (2005), Radoev *et al*. (2008)
130	SSR	CB10634	CCCATTGCTTTCACTCTG	GCAATGATGAGATCCGAG	C7	Piquemal *et al*. (2005), Radoev *et al*. (2008)
131	SSR	EMS664	CATCTCGATTACCTTGAAGC	CGCTCTTTCTTAGCAATGAT	C4	Wanxing Wang *et al*. (2012)
132	SSR	EMS748	TGCTTCTCTACCTTCTTTCCT	AGATGTTGCTTTCGTTCACT	C4	Wanxing Wang *et al*. (2012)
133	SSR	EMS1010	AAGACAAAGTCGGTGAAGAA	GCTACGAATCTCATCCTCTG	C8	Wanxing Wang *et al*. (2012)
134	SSR	FITO203			C6	
135	SSR	KBrB086E02F-F	AGT ATG GAA TTG ATC ACC ATC TTC	AGT ATG GAA TTG ATC ACC ATC TTC	C7	Nagaoka *et al*. 2010
136	SSR	KBrB086H24R-F	AGT AGA CTC CCA GAG GCA AAT TCC	GAA AGC AGA GTT GCG ATG ACA AGA	C2	Nagaoka *et al*. 2010
137	SSR	KBrB001K15F	ATC GTC AAT GAT CAT CGG TTA CCT	TCC AAA TAG AAA GCG ACC ACC ACT	C4	Nagaoka *et al*. 2010
138	SSR	KBrB027J02	CTCCCAAATGGAAAGAGACGTG	CATATGGCTCCTTTTCCTCAGCT	C7	Nagaoka *et al*. 2010
139	SSR	KBrB088O02F-F	ACC TTT GAG TAT AGG CAG ATG GAT	GAA GAG TTC GAA GTC GAG TGG CAT	C4	Nagaoka *et al*. 2010
140	SSR	KBrH079A14F-F	AGC TTT CCT ACC TTT TTC CCC TTC	GAG TGC GAC TTG GAA TTT CTC CAT	C2	Nagaoka *et al*. 2010
141	SSR	KBrB088D21F-F	ACT TGT GAC GGA GGC ATA GAC TTT	GTC CCT GGA ATG TCA AAG GTT	C4	Nagaoka *et al*. 2010
142	SSR	KBrH061H09R-F	ATT CAA CTC CCA AGC GAA AAT CGT	GAA GTC ACA GTA TGT GCA ATG GGC	C1	Nagaoka *et al*. 2010
143	SSR	KBrH014H13R-F	ATG TTT CCG AGG GAG AAC CTC TTT	CCA TAA ATA GAG GGA CGC GAA TCA	C2	Nagaoka *et al*. 2010
144	SSR	KBrH054N12R	ACC ATG GTA ACA TCT GGA AGG TGA	GTG CAG GCA CTT CTA CAC CAG	C9	Nagaoka *et al*. 2010
145	SSR	KBrSO300-10	TCACTCCTCTCGCAGATTCA	TGGAATCGCTTTAAGCAGATGC	C7	Nagaoka *et al*. 2010
146	SSR	MR155	CAACTCCAGCACCAATAC	AGAGCTGGATATTACGACATT	C4	Nagaoka *et al*. 2010

147	SSR	NA10F06	CTCTTCGGTTCGATCCTCG	TTTTTAACAGGAACGGTGGC	C4	Piquemal et al. (2005), Radoev et al. (2008)
148	SSR	NA12B11	AAGCTTCCTCGTTCTCCTCC	TTGTCTTCACTCGTTTTGCG	C7	Piquemal et al. (2005), Radoev et al. (2008)
149	SSR	Na12C08	GCAAACGATTTGTTTACCCG	CGTGTAGGGTGATCTAGATGGG	C1	Piquemal et al. (2005), Radoev et al. (2008)
150	SSR	Na12E06A	TTGGGTTGACTACTCGGTCC	CCGTTGATTTGGCTAAGACC	C4	Piquemal et al. (2005), Radoev et al. (2008)
151	SSR	Na12F12	CGTTCTCACCTCCGATAAGC	TCCGATGTAGAATCAGCAGC	C3	Piquemal et al. (2005), Radoev et al. (2008)
152	SSR	Ni4F09	CTGTTATGCAAGGTCATCGC	TGTTCCAGGTGAAGAAACCG	C8	Piquemal et al. (2005), Radoev et al. (2008)
153	SSR	Ol10C01	ATGACTGCTTAAACAGCGCC	CTTCTCCAACAAAAGCTCGG	C4	Piquemal et al. (2005), Radoev et al. (2008)
154	SSR	Ol12D05	TCCATGACCAACGACAAGGTC	AAGAGGCGACTTCTATTGCG	C8	Piquemal et al. (2005), Radoev et al. (2008)
158	CAPS	pW114	TTCCCAATGTTGGAGGCAGT	TATATATCGCTCAAGCTCAATC	C5	Udall *et al*. (2005)
157	CAPS	pW164	CAGCAGCACGATAACGAGGTGCA	CGTGTGATCGTAACGAGCAATTGG	C5	Udall *et al*. (2005)
155	CAPS	pW188	GATGTGATCACCTCTTATCGA	ACAATGCCCCCAACAAAGCG	C3	Udall *et al*. (2005)
156	CAPS	pX117	CGTCCCTTACCTTCCTCCG	TCCTCCGTAGATAACGGTCG	C9	Udall *et al*. (2005)
159	CAPS	Px144	AGGCTAGGTTCTGGGTTGAT	AGACCGATGTCCACATACCCAA	C6	Udall *et al*. (2005)
160	CAPS	AC002333-1	AATGGTTTATTCAAGAARGCNCA	CGAACGAGTTTGAAAGTRTTYT	C4	Doullah *et al. (*2011)
161	CAPS	ACS2	AGCTACATGCAACAGCCATG	AGTCGTTGTCTTCTTCCTCG	C4	Doullah *et al. (*2011)
162	CAPS	ASB1	CGCAACCCAAGAATGCAATC	ACCAGAACATTCCATCCACT	C8	Doullah *et al. (*2011)
163	CAPS	BoHM13	TATGCACTTCCGGTCAGACC	CAGCTTATCTCTCAACTCTG	C3	Okazaki et al. (2007)
164	CAPS	BORED	GTGGCCAGGCTATCACNTTYGG	AGTCGCTGTGTAGTTTGCYTGNCC	C3	Okazaki et al. (2007)
165	CAPS	CAM2	TGACCGATGACCAGATCTCA	GTCGCAACCGAATCAAGTTC	C2	Okazaki et al. (2007)
166	CAPS	CHI	GTGGAAGGGAAAAACTACGGAGGAG	CCGGTTTCAGGGATACTATCATCTT	C8	Kuittinen et al.2002
167	CAPS	FLC1	GAGGAATCAAATGTCGATAA	ATGAGCCACGGCTAAGTCAA	C2	Okazaki et al. (2007)
168	CAPS	FLC2	CCATGAGCTACTAGAACTTG	ATGAGCCACGGCTAAGTCAA	C2	Okazaki et al. (2007)
169	CAPS	FLC3	GTGGAATCAAATGTCGGTGG	ATGAGCCACGGCTAAGTCAA	C3	Okazaki et al. (2007)
170	CAPS	GAPB	GGCTAGAAGTCGCTGAATTC	TGGTAGAGACATCAGAGCAC	C8	Nagaoka *et al.* (2010)

171	CAPS	GSL-ELONG	TGGCATCGTCACTTCTGACA	CTAATGCTACTCGCGACCAT	C2	Nagaoka *et al.* (2010)
172	CAPS	IPI	ATGCTGTTCAAAGACGCCTC	TACAGCTTCACCGAGAGTTC	C3	Okazaki et al. (2007)
173	CAPS	myro	CATAAAGCTTCTTCATGGAC	TCATGCATCAGCGAGCTTCT	C2	Doullah *et al. (*2011)
174	CAPS	MSP1	GAGATCGAGGAGCGACTAAT	TGTTGTCCTCAGCAACACTG	C2	Doullah *et al. (*2011)
175	CAPS	Nit2	ACATCTCTGGAACGTTGCAT	CCTTGAGTAATGTCCGACC	C4	Nagaoka *et al.* (2010)
176	CAPS	SFR2	TCGGCTACAGAATCTCTCAC	TCTAGATCAGCAGCTGCTAG	C6	Okazaki et al. (2007)
177	CAPS	TFL1	GGTTTCACGAGTGGCTTATTCC	CCGTCGTCATCCTCACCTTC	C2	Kuittinen et al.2002
178	CAPS	tmt1	TCCTGCCTGAAACTGTTGAG	ACCTCCCAAGCTTCTCTTTG	C4	Okazaki et al. (2007)
179	CAPS	VIN3			C3	