DNA Markers for the Cereal Cyst Nematode (*Heterodera avenae* Woll.) Resistance Gene in Barley

by

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Summary

The major aim of this study was finding DNA markers for the cereal cyst nematode (CCN) resistance gene in the barley cultivars Orge Martin and Chebec. To fulfil this aim, several sub-experiments were conducted, and the results are summarised.

To simplify the genetic study, and as a part of the barley breeding program, 79 doubled haploid (DH) lines were developed by anther culture of the F_1 plants of Clipper x Orge Martin. All DH lines were produced from spontaneous chromosome doubling.

The 79 DH lines developed were used to establish the genetic relationship between the *V/v* (2/6-row) character and the gene for resistance to the Australian CCN pathotype. The genetic distance between the CCN resistance gene in Orge Martin and the *V/v* character was estimated to be 20±4.6 cM. This CCN resistance gene was tentatively named $Ha_{(OM)}$, and was located on the long arm of barley chromosome 2.

Several RFLP probes were assayed to establish linkages to the $Ha_{(OM)}$ gene. For this experiment, two DH populations were used (Clipper x Orge Martin and Chebec x Harrington). Several RFLP markers (AWBMA21, BCD453, cMWG694, KsuD22, MWG503 and MWG865) established close linkages to the $Ha_{(OM)}$ gene. Three markers (AWBMA21, cMWG694 and MWG503) tightly flanked the $Ha_{(OM)}$ gene, and genetic distances were within 3 cM in the Chebec x Harrington DH population. Two other markers, BCD453-d and MWG865, were within 6 and 8 cM of the gene, respectively.

The use of RFLP markers gave clear explanations for some of the ambiguous CCN bioassay results previously obtained, which were difficult to explain by the conventional genetic experiments using few morphological markers. The CCN resistance gene(s) in Orge Martin and Chebec were determined to be a single gene.

A complementary pair of PCR markers for the $Ha_{(OM)}$ gene was developed utilising RFLP probe AWBMA21 and the Chebec x Harrington DH population. The genetic distance between the $Ha_{(OM)}$ gene and the PCR marker was 2.3±1.6 cM.

Genomic DNA subtraction was adopted to assist in determining genetic differences between barley NILs [Ingrid and BC^7 (Orge Martin x $Ingrid^7$)], and to develop new RFLP markers for the $Ha_{(OM)}$ gene. Genomic DNA subtraction produced a clone (172-2) which revealed a polymorphism between the NILs. The RFLP using the clone was loosely linked to the $Ha_{(OM)}$ gene (approximately 36 cM).

Declaration

I declare that this thesis is the results of my own work
except where due reference has been made.
None of data in this work has been submitted previously
for a degree at any University, or has been published by any other person.

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Y.W. Choe

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Chapter 1 Introduction and Literature Review

1.1 Introduction

CCN, cereal cyst nematode (*Heterodera avenae* Woll.), is a major cereal root pathogen widely distributed in the world, especially in important temperate cereal growing areas (Kort, 1972). Since CCN was first reported as a parasite of cereals in East Germany in 1874, at least 31 countries have reported yield losses in cereals due to CCN infection (Meagher, 1977). More than 20 pathotypes have been detected in the world (Brown, 1982).

In Australia, CCN is widespread in the south eastern region, infesting more than 2 million hectares of cereal cropping land (Brown, 1982; Brown, 1984). The main agent of dissemination of CCN in south eastern Australia is wind. During the dry season, wind causes soil erosion, and dust storms carry cysts of the nematode to adjacent areas. The habitation of CCN is not localised to particular soil types, for example this parasite has been found in heavy clay, fine sand and peat soil in England (Kort, 1972). In Australia, CCN is mainly distributed in the lighter soil (sands and loams) (Meagher, 1977; Southey, 1956).

CCN affects *Gramineae* family members such as barley, wheat, oats, rye, rye grass and barley grass (Meagher and Brown, 1974). The infected root systems are abnormal with stunted and knotted roots. Leaves of affected plants show discolouration that is similar to nitrogen and phosphorus deficiency (Kort, 1972), and these symptoms can cause difficulty and ambiguity in the identification of the cause of the disease.

CCN causes serious economic losses of up to 30% of yield in barley and 70% in some wheat and oat cultivars (Sparrow and Dubé, 1981). The estimated annual loss in

wheat production alone due to CCN infection in Australia is $US70 million (Brown, 1984).

Several agronomic strategies have been used to control CCN. One strategy is crop rotation including non-host crops such as legumes. This method decreases the nematode population, and greatly increased wheat yield. A low number of nematodes, however, still persists in the soil (Meagher and Rooney, 1966). The establishment of effective cropping sequences is an important factor for crop rotation, and it is useful in maintaining soil fertility (Corbin and Pratley, 1980). However, a long ley phase in cropping systems is not preferable for farmers, who want an intensified cereal cropping system (Brown, 1984). Other agronomic strategies are early sowing to reduce the damage of young seedling plants from the mass invasion of nematodes (Brown, 1984), and chemical treatment using nematicides (Brown and Pye, 1981; Gurner *et al.*, 1980; Rovira *et al.*, 1981). These strategies are effective for control of CCN (Brown, 1984; Rovira *et al.*, 1981). However, a number of problems exist, for example the cost of the nematicide. The environmental problems caused by the applying the nematicide strongly argue that more effective approaches to control CCN are required.

The use of resistant varieties is the most desirable strategy in the long term in Australia where only one CCN pathotype has been reported (Andersen and Andersen, 1982b; Brown, 1982; O'Brien and Fisher, 1979). Several CCN resistant cereal cultivars have been released in Australia. One of these, Galleon, is a feed barley released in South Australia in 1981; its use is an important strategy in the control of CCN (Sparrow and Dubé, 1981). Chebec, another CCN resistant cultivar, has been released in 1994 (Sparrow, 1994), and provides an additional strategy for using a resistant barley to control CCN. Also, the strategy using CCN resistant cultivars is now available in other cereals, such as wheat and oats. Resistance to CCN is

considered as a stable and long lasting character without frequent mutations of pathotypes to more virulent types (Gair *et al.*, 1987).

The selection of CCN resistant lines is an essential process. The current screening method, a CCN bioassay, is tedious and laborious, and needs a controlled environment which does not allow large scale screening in the given time during breeding programs (Fisher, 1982b). Also, this method is often ambiguous and ineffective in the determination of CCN resistance and susceptibility for heterozygous plants such as F_2 plants which require the progeny test. With the current bioassay, the progress in breeding programs often is delayed until a bioassay result is confirmed. For efficient incorporation of CCN resistance into breeding programs, advanced CCN screening methods are required (O'Brien and Fisher, 1974) which can evaluate resistance/susceptibility at any stage in breeding programs.

Closely linked molecular markers that flank a gene of interest have the potential to improve the efficiency of selection for the desired gene in breeding lines (Beckman and Soller, 1983). They offer more accurate information than morphological markers which have limited resolution in genetic analysis (Graner *et al.*, 1991). Also, the use of molecular markers can provide information about the diversity of the resistance genes that exist in several resistant barley varieties (Sparrow and Dubé, 1981). The use of molecular markers to integrate resistance genes into breeding programs may provide a more effective breeding process in the production of promising new cultivars.

1.2 Literature Review

1.2.1 The life cycle of CCN

The life cycles of all species of *Heterodera* follow a similar pattern, namely; dormancy of eggs, hatching, moulting and cysts (Brown, 1984; Jenkins and Taylor, 1967).

Dormant eggs in the cyst may survive for a long time; up to six years (Jenkins and Taylor, 1967). To overcome this dormancy, a low temperature of between 2°C and 20°C is needed, with an optimum temperature of 10°C (Fisher, 1981). However, hatching does not occur until there is sufficient moisture in the soil. In late autumn–winter of Southern Australia, low temperatures and rain offer a good environment for the breakage of dormancy, and cause mass hatchings in the field (Banyer and Fisher, 1971). This mass hatching causes maximum infestation and damage on plants from early June to mid-August. Approximately 50-75% of eggs hatch, and larvae are released into the soil . Other dormant eggs in the cyst remain in the soil and survive until next year (Brown, 1984).

Growing nematodes moult four times. During a moult, the old cuticle is shed, and a new cuticle surface is formed (Bird and Bird, 1991). The first moult occurs in the egg within the cyst (Fisher, 1981; Jenkins and Taylor, 1967). Recently hatched larvae are second stage larvae (0.4-0.6 mm in length), and these migrate through the soil. They usually penetrate roots behind the root tips of young seedlings (Jenkins and Taylor, 1967). Temperature affects the timing of the moult of CCN. The second moult occurs in 10 days at 15°C and in eight days at 30°C after penetration (Fisher, 1981). At the third stage, larvae are classified into male and female according to the shape of the genital primordia. The appearance of male and female larvae at the third stage is sausage-shaped (Jenkins and Taylor, 1967). The third moult begins at about 17 days (15°C)-14 days (30°C) after penetration (Fisher, 1981). At the fourth stage, the male larvae are still in the third cuticle and the female larvae are flask shaped. The male larvae leave the root and migrate into the soil (Jenkins and Taylor, 1967). It takes about 29 days to reach the fourth moult after penetration at 15°C and 18 days at 30°C (Fisher, 1981). Therefore, in the field, the relatively low temperatures during the Australian winter retard this moulting process. After the final moult, the posterior part of the female is exposed in the soil, and males are able to inseminate the females

(Jenkins and Taylor, 1967). After the female dies, the body is detached from the root and is the cyst (0.5-0.8 mm in length) containing up to 500 viable eggs (Jenkins and Taylor, 1967).

In spring, rapidly increasing temperatures induces dormancy of eggs in cysts, which prevents late hatching, and this helps the survival of eggs under the hostile environmental conditions of the hot and dry Australian summer (Banyer and Fisher, 1971).

1.2.2 Interaction between CCN and Host

The second stage larvae penetrate the roots, and root cells around the larvae are destroyed because of mechanical damage. The larva makes feeding sites in the vascular system of roots. The stylet of the nematode penetrates the cell wall, and a plug of material of unknown composition is formed around the stylet. The cell walls of neighbouring cells are degraded, and the cell contents merge to make a syncytium (elongated multi-nucleate cells around the lip region of nematodes in plant roots). As the fusion of neighbouring cells progresses, the syncytium grows along the root axis to reach 2-3 mm in length, and the syncytium contacts the vascular system (Jones, 1981). Generally, a larva forms a syncytium. As the syncytia develop in susceptible plant roots, high levels of enzyme activity are detected in the dense cytoplasm whilst in the syncytium of resistant plants only a slight increase in enzyme activity is observed followed by necrosis and deterioration of the syncytium (Endo and Veech, 1970). In wheat, the development of female juveniles has been observed (Williams and Fisher, 1993). In a susceptible cultivar, females increase in size during the later stages of their development, while the development of males fails beyond the fourth stage in resistant roots. The syncytia cell structure shows significant differences between resistant and susceptible wheat cultivars at about 14 days after inoculation. The syncytia of susceptible roots show a highly active and enlarged cytoplasm with

numerous mitochondria, free ribosomes, plastids and small vacuoles. In resistant roots, the syncytia are less active and highly vacuolated. The cross sectional diameter of juveniles increases rapidly at about 14-15 days after penetration (Fisher, 1981). In barley, the size of the syncytia and mitochondria in a resistant cultivar are small and a hypersensitive reaction is detected in feeding zones and surrounding cells (Subbotin *et al.*, 1991). The second stage juvenile successfully penetrates barley roots in a day (Fig. 1.1) (Choe, unpublished data). Within two days, juveniles reach the vascular system of barley roots (Fig. 1.2 and 1.3) (Choe, unpublished data). These juveniles are sedentary, and successfully develop syncytia. Developed syncytia have multi-nuclei, which means syncytia cells are alive, and represent a food reservoir for the developing nematode (Fig. 1.4) (Choe, unpublished data). Invaded root systems show extremely abnormal structures (Fig. 1.5) (Choe, unpublished data).

1.2.3 The genetics of the CCN resistance gene

In the study of CCN resistance genes, the definition of resistance differs from that of tolerance. Tolerance is defined as good growth and a high yield of host plants in spite of the invasion of nematodes, whilst resistance refers to a low number of cysts, indicating inefficient growth of the nematode in the host's roots (Sparrow and Dubé, 1981). In certain resistant genotypes of barley, the female CCN fails to reach maturity (Cotton and Hayes, 1969), and there are fewer cysts in the soil after growth of the resistant crop (Williams, 1970).

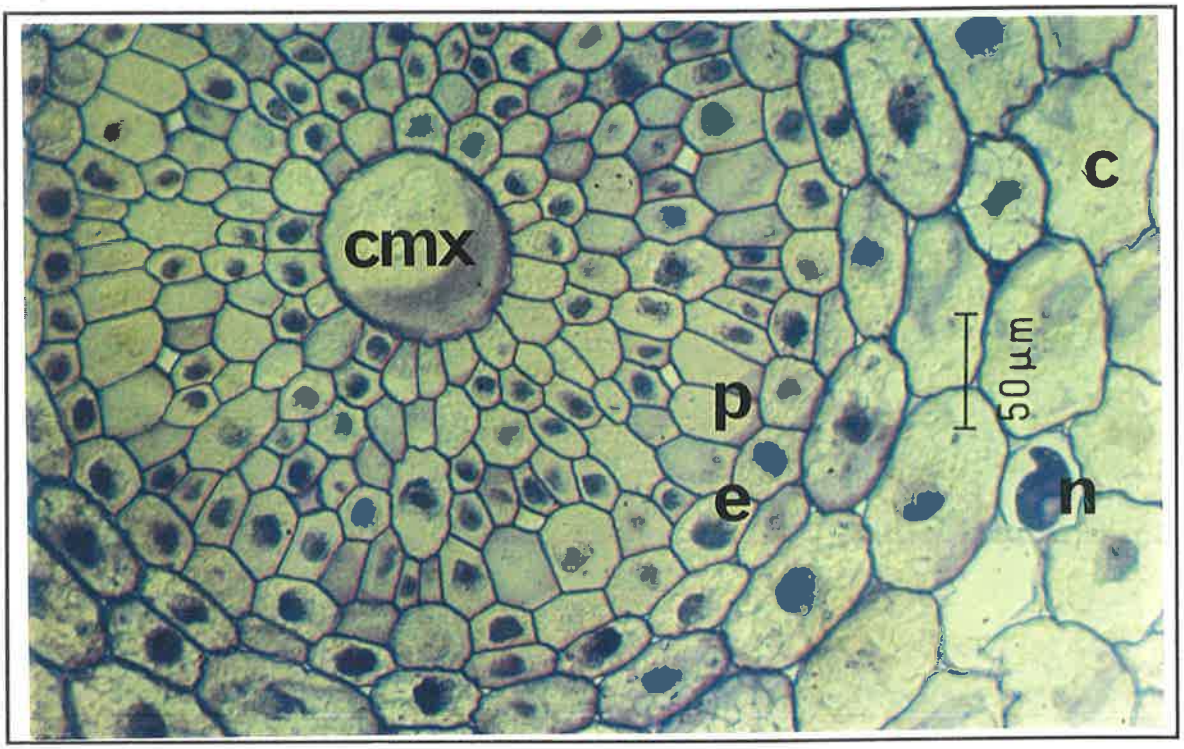


Fig. 1.1 The second stage larva penetrates the barley root within a day after inoculation (cross section of three day old barley root stained with 0.5% toluidine blue, x200). cmx, central meta xylem; c, cortex cells; e, endodermis; n, the second stage CCN larva; p, pericycle

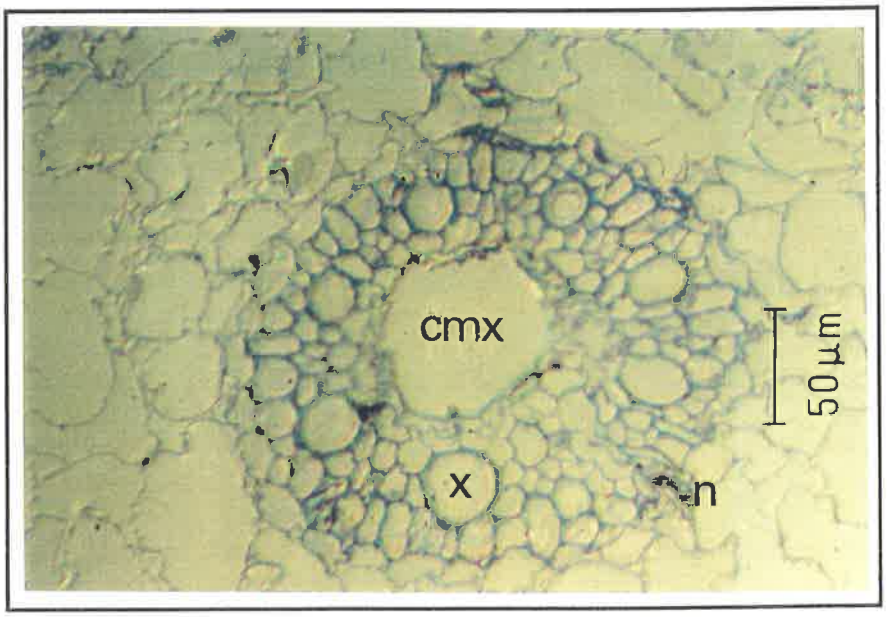


Fig. 1.2 The second stage larva reaches the vascular system of the barley root within two days after inoculation (cross section stained with 0.5% toluidine blue, x200). cmx, central meta xylem; n, nematode; x, xylem

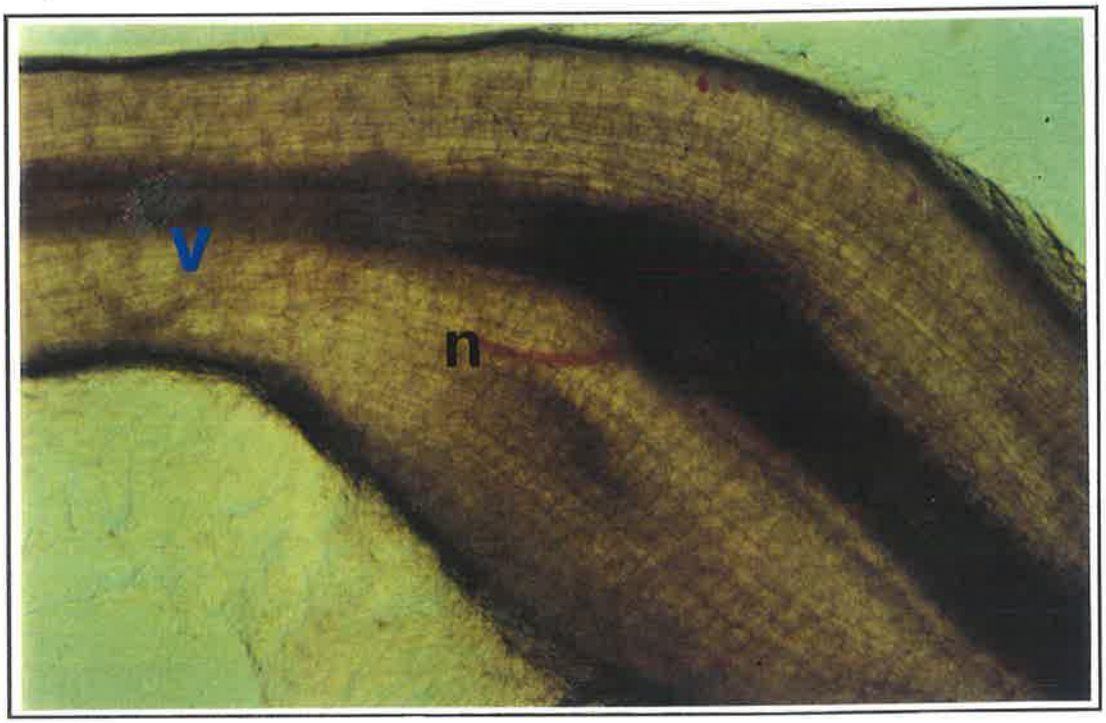


Fig. 1.3 The second stage CCN larva in a barley root at the second day after inoculation (a barley root pressed by a cover glass after stained with 0.05% acid fuchin). n, nematode; v, vascular system

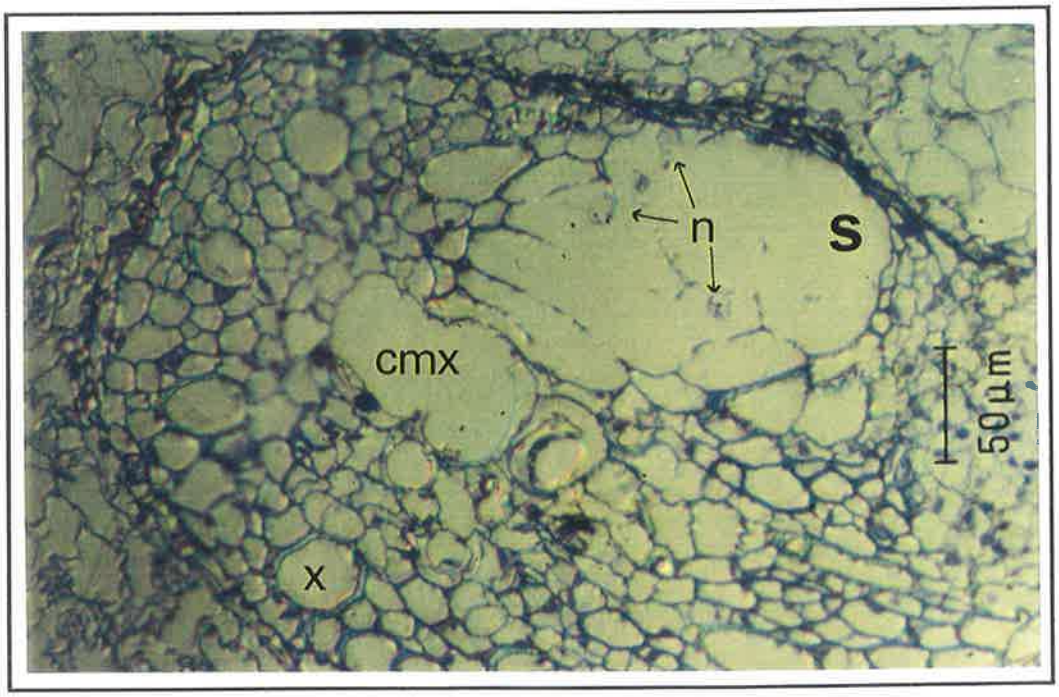


Fig. 1.4 The syncytium containing three nuclei in the susceptible barley root at the 29th day after inoculation (stained with 0.5% toluidine blue, x200). cmx, central meta xylem; n, nucleus; s, syncytium; x, xylem

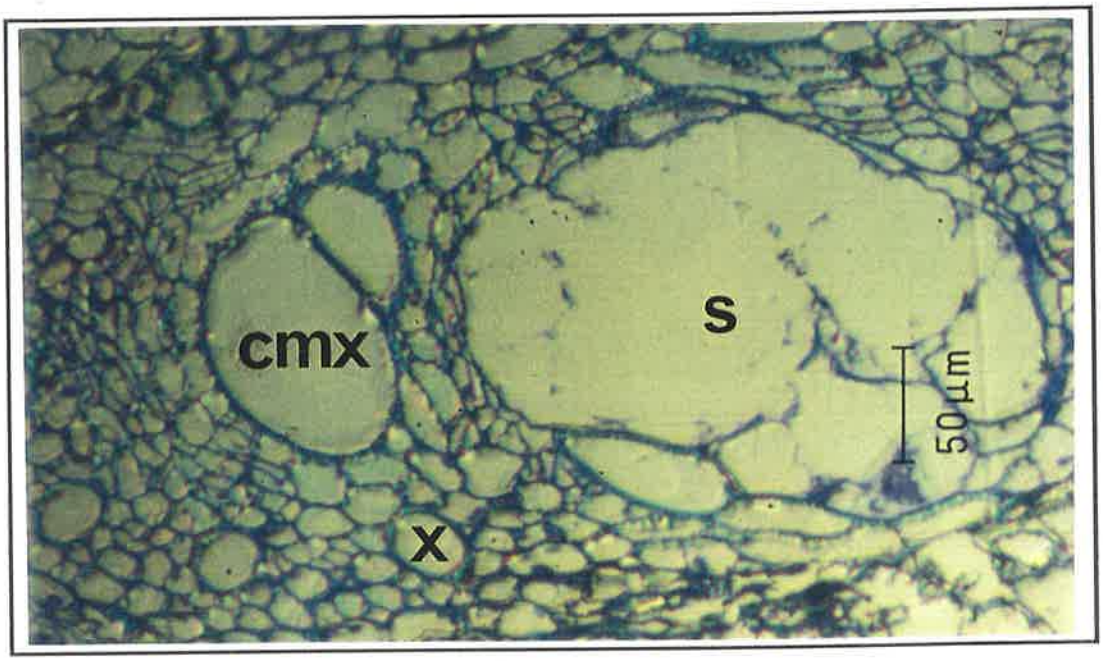


Fig. 1.5 Well developed syncytium, and abnormal shape of the vascular system of the barley root stained with 0.5% toluidine blue (x200). cmx, central meta xylem; s, syncytium; x, xylem.

According to Andersen (1959), resistance of barley cultivars to the cereal root eel worm was observed by Nilsson-Ehle as early as 1920. Since this report, much genetic research has been undertaken for various cereal varieties and at several locations to determine the number of pathotypes and the number of resistance genes being involved in the resistant reaction to CCN. Several pathotypes of CCN have now been classified in Europe. These can be divided into three groups; I, II and III. Group I contains six different pathotypes (Ha1I, Ha2I, Ha3I, Ha4I, Ha5I, Ha6I), Group II has one pathotype (Ha1II), and group III has three pathotypes (Ha1III, Ha2III, Ha3III) (Andersen and Andersen, 1982b). Pathotypes in Group I are avirulent on barley cultivars Drost and Ortolan, which have the resistance gene *Ha1*. Group II is virulent on cultivars containing *Ha1*, but avirulent on KVL 191 and Siri, which have the resistance gene *Ha2*. In group III, pathotypes are virulent on *Ha1* and *Ha2*, but avirulent on the *Ha3* gene found in the cultivar Morocco. However, it is not

clear whether *Ha3* is the only CCN resistance gene in Morocco. Resistance of *Ha2* is ambiguous. *Ha2* is resistant to pathotypes Ha1I, 2I, 3I, but not to pathotypes, Ha4I, 5I, 6I, and the reason for this split resistance was not clear (Andersen and Andersen, 1982b). There is sufficient ambiguity in the relationship between pathotypes and CCN resistance that further investigation of the various resistances and pathotypes is warranted. There is only one CCN pathotype in Australia, Ha1III, that is quite different from the European CCN pathotypes (Andersen and Andersen, 1982b; Brown, 1982; O'Brien and Fisher, 1979).

According to O'Brien *et al.* (1979), at least three and possibly five loci are involved in resistance to the Australian CCN pathotype in barley cultivars. They found that the resistance genes in cultivars Athenais and Marocaine are probably the same, and this gene is different from another resistance gene in the cultivar Nile. These two monogenic dominant genes differ from the third gene (*Ha3*) found in Morocco, which may have one or two resistance genes to Australian CCN pathotype. CI 8147 has a resistance gene which differs from that in Morocco (O'Brien *et al.*, 1979). It is not known whether the resistance in CI 8147 is the same as genes in Athenais, Marocaine and Nile. Soetopo (1986) reported that the CCN resistance gene in Athenais was different from Marocaine, but that they could be closely linked. Barley cultivars Athenais, Marocaine and Nile have a single resistance gene, and Morocco and Orge Martin have two independent resistance genes (Soetopo, 1986). The CCN resistance gene in Athenais and Nile was linked to the *V/v* gene that controls the barley 2/6-row head types, but the gene in Morocco and Orge Martin was not (Soetopo, 1986). The assumed relationships between these CCN resistance genes (or loci) are illustrated in Fig. 1.6. To date, the chromosomal location of the CCN resistance gene to the Australian CCN pathotype has not been determined rigorously.

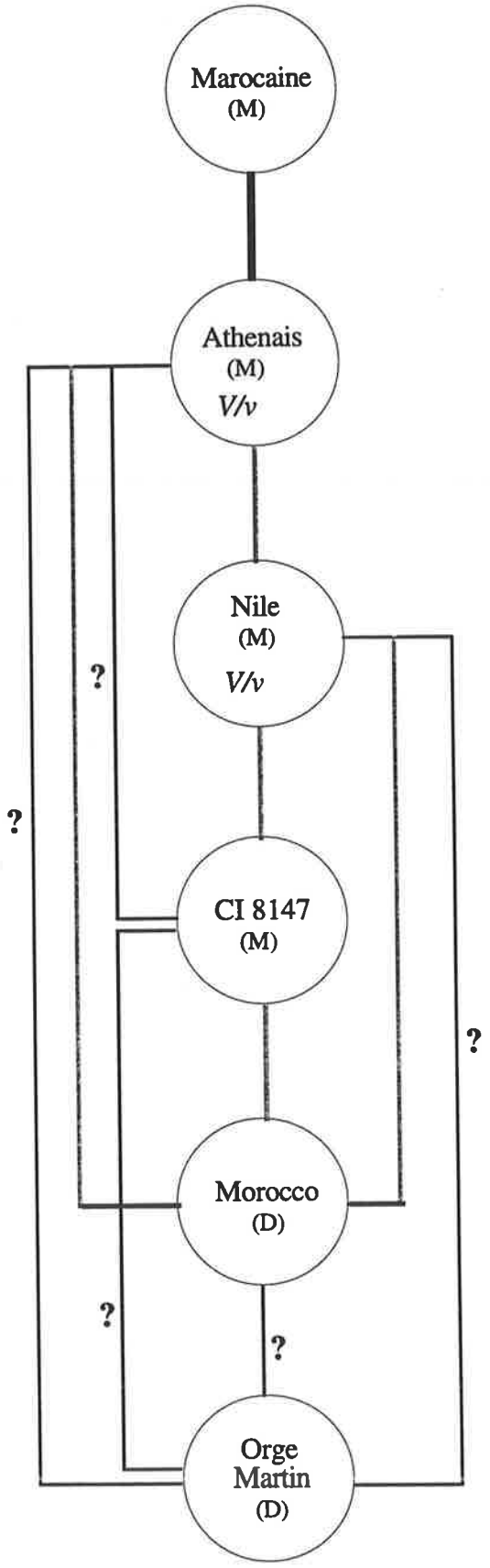


Fig. 1.6 Relationship between several CCN resistance genes (or loci) in barley to the Australian CCN pathotype. *V/v*, linked to the *V/v* gene; M, Monogenic; D, Digenic. (Soetopo, 1986)

Barley chromosome 2 has several CCN resistance genes against European CCN pathotypes. Two genes, including the single dominant gene *Ha1*, are probably located on the short arm of chromosome 2 (Cook and York, 1982; Nielson, 1982). *Ha2* , another dominant gene, has been located between two loci, *V/v* (2/6-row) and *Li* (liguless); 10.2±2.65 cM from *V/v* locus and 27.5±4.47 cM from *Li* locus on the long arm of chromosome 2 (Cotten and Hayes, 1969). Also *Ha2* is linked to several morphological genetic characters; *pau* (yellow and white green colour at the ligule, nodes of stem and the tip of awn), *Lk* (long awn), *e* or *log 1* (wider and long awns of outer glumes), *cer g10* (surface waxy), *Re2* (purple lemma, palea and pericarp) and *Vt* (very small lateral florets), and a translocation break point (T2-3f) (Andersen and Andersen, 1973) (Table 1.1). In addition, at least three different CCN resistance genes are linked to *Ha2* (Cotten and Hayes, 1969; Hayes and Cotten 1971). Therefore, it is assumed that barley chromosome 2 could have up to five different loci encoding resistance to CCN.

Table 1.1 The approximate recombination values between the *Ha2* gene and each of several morphological characters (Andersen and Andersen, 1973)

Gene or translocation point	Approximate recombination value
e (log1)	25
V/v, V^t, Lk	18
T2-3f	15
cer g^{10}	13
pau	3

Resistance to CCN has been reported in other cereals; wheat (O'Brien and Fisher, 1974), oats (Barr and Dubé, 1986) and rye (Asiedu *et al.*, 1990). In wheat, a CCN resistance gene, *Cre*, is located on the long arm of chromosome 2B, and this gene encoding moderate resistance to the Australian CCN pathotype is available in Loros

and a spring wheat Aus10894 (O'Brien and Fisher, 1974; O'Brien *et al.*, 1980). The gene, *Cre*, is flanked by two RFLP markers, Xglk605 and CDO588, with map distances of 7.3 cM and 8.4 cM, respectively (Williams *et al.*, 1994). Other resistance sources in wheat are Dural, Duramba, Festiguay and Psathias (Aus881). Cultivars Condor, Olympic and Halberd are susceptible to the Australian CCN pathotype (Fisher, 1982a). Williams (1994)suggested that the *Cre* gene could be linked to CCN resistance of barley.

Recently three CCN resistant oat cultivars; Wallaroo, Marloo and Potoroo, have been released in Australia (Barr *et al.*, 1988; Barr *et al.*, 1994). The major sources of CCN resistance in oats to the Australian CCN pathotype are Avon, New Zealand Cape and the *Avena sterilis* line Cc4658 (Barr *et al.*, 1988; Barr *et al.*, 1994). *Avena sterilis* line Cc4658 has resistance to all reported CCN pathotypes in Europe, and is an important resistance source in oat breeding programs (Barr and Dubé, 1986). A CCN resistance gene in *Avena sativa* CI3444 and *A. sterilis* line I. 376 was located on oat chromosome XV, and minor genes were located on chromosomes VIII and X (Chew *et al.*, 1981).

Rye cultivars, "South Australian", Rago and Weethalle have resistance to the Australian CCN pathotype, but little information is available (Brown, 1982; O'Brien and Fisher, 1979). The resistance in "South Australian" was more effective than the gene from Aus10894, and the gene has been located on chromosome 6R (Asiedu *et al.*, 1990). Further studies may demonstrate usefulness of transferring these alternative resistances between the cereal breeding programs.

1.2.4 Molecular markers in genetic studies and for plant breeding

The traditional barley genetic map consists of morphological markers that are derived mostly from mutated characters, and isozyme markers (Søgaard and Wettstein-

Knowles, 1987). Morphological markers and isozyme markers are ultimately due to differences between expressed functional genes, which represent a small part of the whole genome (Laurie *et al.*, 1992). Also, the classical genetic map is not well saturated, and has limited uses. However, molecular markers derived from the whole genome have greater potential to detect sequence polymorphisms with or without apparent differences in phenotypes (Laurie *et al.*, 1992).

1.2.4.1 RFLP markers

Sequence variation in genomic DNA can be detected by a combination of restriction endonuclease enzymes which recognise specific sequences in the DNA strands, and sequence specific DNA probes. Differences in the size of DNA fragments derived from restriction enzyme digestion, and visualised on X-ray films by radioactive or chemical labelling are referred to as restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980; Gusella, 1986). The choice of restriction enzymes depends on the cost of the enzyme and the degree of polymorphisms. DNA probes consisting of numerous single-copy or low-copy clones from genomic DNA and cDNA clones are used in the detection of polymorphisms. This advantage overcomes the limited availability of phenotype markers (Laurie *et al.*, 1992). The majority of markers in the published barley molecular maps are RFLP markers (Graner *et al.*, 1991; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993; Laurie *et al.*, 1992; Shin *et al.*, 1990). It is not necessary to isolate specific genes for probes in RFLP work (Beckman and Soller, 1983), but the work is costly and relies on highly accurate molecular techniques in the sequential process.

1.2.4.2 PCR based DNA markers

Despite several advantages, the RFLP marker system has some limitations; for example, costs and the low degree of polymorphisms between genetically closely

related sister lines (Whitkus *et al.*, 1994). An alternative or complementary marker system utilises PCR (Polymerase Chain Reaction) (Mullis, 1990; Mullis *et al.*, 1986) method. PCR using specific or random primers is widely used for efficient amplification of specific sequences of template DNA, and for the detection of polymorphisms (D'Ovidio *et al.*, 1990; Emanuel, 1991). Usually PCR methods use non-radioactive probes (primers), and are faster and cheaper than the RFLP marker system (Whitkus *et al.*, 1994).

Several PCR based methods have been developed in plant research; detection of size variations of simple sequence repeat (SSR) DNA (or micro satellite DNA) (Akkaya et al., 1992; Wu and Tanksley, 1993; Zhao and Kochert, 1993), Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990), Sequence Characterised Amplified Regions (SCARs) (Paran and Michelmore, 1993; Williamson *et al.*, 1994), and Sequence-Tagged-Site (STS) (Lee *et al.*, 1993; Talbert *et al.*, 1994; Tragoonrung *et al.*, 1992). These methods are described below.

One method involves finding polymorphisms based on the size variations of micro satellite or simple sequence repeats. Micro satellites are tandemly repeated DNA sequences, six base pairs or less, mainly di-nucleotide. In barley, the majority of micro satellites consists of $(AT)_n$ sequences that are spread randomly through the barley genome structure (Wang *et al* ., 1994). Variations in the size of micro satellites produce different sizes of amplified DNA fragments (polymorphisms) (Wu and Tanksley, 1993). These micro satellites are abundant, randomly distributed and highly informative. Tandem repeat sequences are thought to be formed by unequal crossovers, or by slippage during DNA replication. Polymorphisms derived from micro satellites are stable and co-dominant (Burr, 1994). Several micro satellite markers have been reported in rice and soybean (Akkaya *et al.*, 1992; Wu and Tanksley, 1993; Zhao and Kochert, 1993).

Another PCR based molecular marker system is random amplified polymorphic DNA (RAPD). The RAPD method uses random PCR primers (average 9-10 nucleotides) and less stringent reaction conditions (Caetano-Anollés, 1994). RAPD does not need any specific DNA sequences of primers, and the results are less reproducible. To increase the accuracy of RAPD, optimal reaction conditions mostly annealing temperature should be ascertained (Wolff *et al* ., 1993). Although this method is not often suitable for routine experimental approach in cereals (Kleinhofs *et al.*, 1993), it produces highly variable polymorphisms (Whitkus *et al.*, 1994).

RAPD markers, when found, can be converted to more reliable PCR markers through sequencing of RAPD products and developing new primers. This converted PCR oligomer method is called Sequence Characterised Amplified Regions (SCARs). The method has been used successfully in lettuce (Paran and Michelmore, 1993) and tomato (Williamson *et al* ., 1994).

Another related approach is the sequence-tagged-site (STS) PCR method. Restriction enzymes are often used to reveal polymorphisms in the products of the PCR reactions. For this method, PCR oligomers can be produced from the sequence of RFLP probes that have known location on chromosomes. This method is well used in barley (Tragoonrung *et al.*, 1992). The specificity of the PCR reaction can allow for allele specific amplification (allele specific PCR), which could separate single base differences of the specific sequence alleles (Huang *et al.*, 1992; Kwok *et al.*, 1990; Newton *et al.*, 1989; Wu *et al.*, 1989).

1.2.4.3 Genetic analysis with DNA markers

In genetic analysis of heterogeneous populations with RFLP markers, two co-dominant RFLP loci produce nine observable genotypes; AABB, AABb, AAbb, AaBB, AaBb, Aabb, aaBB, aaBb and aabb. When two loci show independent

assortment in the F_2 generation derived from selfing F_1 (AaBb), the expected segregation ratio of these genotypes is 1:2:1:2:4:2:1:2:1. When the result obtained deviates significantly from this expected segregation ratio, it suggests that there is linkage between the two loci (Laurie *et al.*, 1992). Established linkages permit the measurement of map distances among linked loci.

Map distance can be calculated in two ways, one of which involves no mapping functions, and one of which does (Crow and Dove, 1990; Laurie *et al.*, 1992; Mather, 1951). The genetic distance (originally "Morgan") can be measured by the percentage of recombinants under the assumption that there is only one crossover and no double crossover (complete interference) between adjacent loci (Crow and Dove, 1990). To efficiently use this mapping system, a large number of progeny containing numerous characters are required. The results can produce maps densely saturated with loci, so that no double crossovers are expected between adjacent loci (Crow and Dove, 1990; Manly and Cudmore, 1994). Alternative methods use mapping functions, and are based on the assumption of the presence of multiple crossovers between adjacent loci. Mapping functions are particularly useful when only a small number of loci are available (Crow and Dove, 1990). A small number of loci may produce long genetic distances, and may contain double or multi-crossovers that may not be detected by the first method (Haldane, 1919; Kosambi, 1944). Simply put, mapping functions are a correction for undetected double or multi-crossovers. When only one crossover is expected between two loci, the genetic distance calculated by the mapping function (estimated recombination frequency) is the same as that calculated as the percentage of recombinants (Laurie *et al.*, 1992). The use of the mapping function is based on the hypotheses that there are random crossovers over the whole genomes (Crow and Dove, 1990).

The major mapping functions being used in mapping work are Kosambi's and Haldane's (Crow and Dove, 1990). Haldane mapping function (Haldane, 1919) is based on the zero interference [coincidence (c) = observed double crossover/expected double crossover = 1]. The formula is;

$$m = -1/2 \log_e(1-2r)$$

(r, proportion of recombinants; m, map distance)

The Kosambi mapping function (Kosambi, 1944) hypotheseses that coincidence is a linear of proportion of recombinants [$c \propto r$, $c = 2r$ $(0 \leq r \leq 1)$]. The formula is;

$$m = 1/4 \log_e(1+2r/1-2r)$$

Generally, the Haldane mapping function produces a longer genetic map than that constructed with the Kosambi function, and using no mapping function gives the shortest genetic map (Nilsson *et al.*, 1994).

Once measured, map distances can be used for the construction of a map containing the best order of multi-loci. For the construction of the best order molecular map using multiple loci, computational analysis is essential and a number of computer programs are available such as; LINKAGE 1 (Suiter *et al.*, 1983), MAPMAKER (Lander *et al.*, 1987), GMENDEL (Liu and Knapp, 1990), JoinMap (Stam, 1993) and Map Manager (Manly and Cudmore, 1994).

The construction of the genetic map begins with the estimation of recombination frequencies, and with the test of the linkage between all possible pairs of loci for the determination of the linear order of markers in a linkage group (Ritter *et al.*, 1990). There are several methods for the linkage test, such as χ^2-test, LOD score ("log of the odds ratio") and Bayesian analysis (Manly and Cudmore, 1994). In the traditional genetic analysis using χ^2-test, the establishment of linkages can be achieved by rejecting null-hypotheses representing independent segregation of test alleles.

Usually, the significance values being used are 95% ($\alpha = 0.05$) or 99% ($\alpha = 0.01$). However, these levels only suggest possible linkages, since there are still possible errors of 5% or 1%. More stringent confidence levels are often required such as 99.9% and 99.99% (Manly and Cudmore, 1994). The LOD score is frequently used for the linkage tests (Gerber and Rodolphe, 1994). A LOD score of 3 is commonly used, and it means established linkages are 1000-fold more likely to be correct than not correct (Morton, 1955). Another method is Bayesian analysis (Neumann, 1990). This method is based on the hypotheses that new loci can be placed equally likely through the whole genome. It uses 95% and 99% of confidence levels, and the stringency of these levels are the same as LOD score 3.0 and 4.0, respectively (Manly and Cudmore, 1994). The conventional confidence level, 99.99% is equivalent to LOD score 2.6, approximately (Gerber and Rodolphe, 1994; Manly and Cudmore, 1994). Bayesian analysis may be better than conventional χ^2-test in terms of genetic analysis of mapping data containing distorted segregation ratios. When a genotype such as *aabb* is missing in progeny genotypes because of the distorted segregation, or because of using small number of test plants, χ^2-test is not suitable. In this case, Bayesian analysis is the preferred method (Lorieux *et al.*, 1995a). Simply put, the best order map has the least number of recombinants (Manly and Cudmore, 1994) and the highest log-likelihood value (Lander *et al.*, 1987; Laurie *et al.*, 1992; Manly and Cudmore, 1994).

An interesting mapping program is the "JOIN MAP" (Stam, 1993). This program contains a computerised statistical procedure to combine several different sources of linkage information produced from different mapping materials such as F_2, backcross and recombinant inbred lines. The key factor of combining several maps is based on the existence of common markers in the several maps being combined (Stam, 1993). Some difficult situations are expected in using this program, in case of chromosomal rearrangements and lack of common markers. However, the most likely integrated

map obtained may provide some information for the assumption of the most common types of linkages.

Various mapping results and orders of loci can be expected, if chromosomal rearrangements such as spontaneous reciprocal translocations, duplications, deletions and inversions occur in different mapping populations. Reciprocal translocations have a rearranged chromosomal constitutions, such as 2S + 4L (2S, the short arm of chromosome 2; 4L, the long arm of chromosome 4) resulting rearranged linkage groups Marthe and Künzel, 1994). Chromosomal inversion can produce ambiguous linear map orders because of different gene orders contained in the mapping population, and can reduce map distances because of the inversion loop (Powell and Nilan, 1968; Ramage and Suneson, 1961). These phenomena may cause difficulties in genetic mapping work. However, although difficult to use, translocation break points and deletions are valuable markers for physical mapping work (Heslop-Harrison, 1991; Marthe and Künzel, 1994; Sorokin *et al.*, 1994; Werner *et al.*, 1992).

1.2.4.4 Assignment of DNA markers to chromosomes

In wheat genetics, assignment of linkage groups to specific chromosomes is usually defined by using specially constructed genetic stocks where whole chromosome or chromosome arm(s) are added, substituted or recombined with other chromosomes (mostly belonging to the same homoeologous group). These genetic stocks have been used for assignment of linkage groups. Wheat-barley addition lines (Islam *et al.*, 1981) have full wheat chromosome (cv. Chinese Spring) plus a pair of barley chromosomes (cv. Betzes); except for chromosome five which was self-sterile. For example, when a clone has the same RFLP bands between a test barley cultivar and the 4H chromosome of the wheat-barley addition lines in RFLP analysis, this clone belongs to barley chromosome 4 (Laurie *et al.*, 1992). For the location of loci to specific chromosome arms and in relation to the centromere and for the determination

of the location of the centromere itself, wheat-barley di-telosomic addition lines are used where each addition line has a complete euploid set of wheat chromosome plus a pair of barley chromosome arms. (Graner *et al.*, 1991; Heun *et al.*, 1991; Islam *et al.*, 1981; Kleinhofs *et al.*, 1993).

1.2.4.5 Mapping populations

The choice of the mapping population is critically important. Usually F_2 plants, recombinant inbred (RI) lines and doubled haploid (DH) lines are used for mapping work. Although the F_2 population can be produced easily in a short period, this population is not suitable for the permanent mapping work because identical F_2 plants are not reproducible. In *Arabidopsis thaliana*, the bulk of F_3 plants produced from the selfing of individual F_2 plants are used instead of F_2 plants (Kochert, 1994). RI lines can be produced from each F_2 plant through the selfing and the single seed descent (SSD) method. After 5-6 generations, each line becomes largely homozygous. Mapping work with RI lines provides higher resolution than F_2 plants which are derived from only one round of meiosis (Burr *et al.*, 1988). The construction of RI lines needs continuous selection for several years. In maize, the level of heterozygosity of RI lines was about 1.6-2.7% after eight generations of selfing (Burr and Burr, 1991).

For practical breeding programs and stable mapping work, the use of homozygous mapping populations is recommended. In barley mapping, doubled haploid populations produced through anther culture or the *Hordeum bulbosum* technique provide reproducible homozygous mapping populations. Once established, DH mapping populations are permanent, and new markers can be placed on the current molecular map. The recombinant resolution of DH populations is the same as F_2 plants because of only one round of meiosis (Burr *et al.*, 1988).

1.2.5 Genomic DNA subtraction

One of the major contributions of molecular mapping work in the genetic analysis is flanking target genes with tightly linked DNA markers. However, finding polymorphisms between genetically close sister lines is one of the challenges in molecular marker work. Near iso-genic lines (NILs) may have almost the same genetic background except for the "dragged" donor genome containing the gene of interest. In the process of backcrossing, some of the donor chromosome segment containing the gene of interest is dragged (linkage drag) into a backcrossed line resembling the recurrent parent (Brown *et al.*, 1989; Stam and Zeven, 1981). If this dragged DNA region is isolated, it should be the DNA fragment closely linked to the target gene. One of the strategies for the isolation of these dragged chromosome segments or DNA fragments containing genetic differences between NILs is the genomic DNA subtraction method (Somerville, 1992). This method is based on the direct sequence comparison of the DNA of NILs. Some DNA fragments that are present in one of NILs, but not in another can be isolated after sequence comparison using the hybridisation process. These DNA fragments may have target sequences that can discriminate the genetic differences of NILs. This method is well demonstrated in the cloning of the differences between complex genomes (Lisitsyn, 1995; Lisitsyn *et al.* 1993; Wieland *et al.*, 1990; Yokota *et al.*, 1989; Yokota and Oishi, 1990), and for the isolation of addition or deletion sequences between mutant cereal lines (Clark *et al.*, 1992).

1.2.6 The use of doubled haploid plants for the genetic analysis and plant breeding

The development of both *in vitro* tissue culture and the chromosome doubling technique provides highly efficient approaches in plant research. The production of doubled haploid (DH) plants in particular has wide adaptability in plant breeding programs. For the successful use of the doubled haploid system in plant breeding, it

is important to produce easily a large number of doubled haploid plants in a limited time (Snape, 1981). To fulfil this goal, continuous developments have been made in the DH plant production systems, especially the anther culture method which has improved considerably the efficiency of green plant production (Finnie *et al.*, 1989; Kuhlman and Foroughi-Wehr, 1989).

The production of DH plants provides big advantages in the breeding program. In common cereal breeding systems, yield testing can begin at the F_5 generation or later, and at least 10 years are required to release a new variety. With the DH system, genetically fixed homozygous lines can be produced in a single generation, which may save at least four years in the conventional breeding program (Luckett and Darvey, 1992). Also, the fixation of genetic homozygosity is possible at any stage in the breeding program (Kasha, 1982). Homozygous lines provide easy and reliable screening for disease resistance. Contamination of seeds is easily detected in the field because each DH line has a uniform phenotype. Complete homozygosity of DH lines allows the yield and quality test of cereals in the early stages of testing. Genetically fixed DH lines show no significant variations under different environments (Kasha, 1982). In addition, relatively small numbers of DH lines provide more concentrated breeding schemes. When an agronomic character is controlled by two unlinked loci (A and B), 11 doubled haploid lines are the minimum number of DH plants that should be produced in order to obtain at least one specific homozygous plant (AABB) at 95% probability (Jansen, 1992; Mather, 1951). The equation for the population size is;

$$m = \log(1-p)/\log(1-a)$$

(m, population size; p, probability, ie. 95%; a, the frequency expected, ie. 0.25 in DH population) (Kerry and Lance, 1993; Mather, 1951)

If the two loci are linked, the suggested sample sizes at the 5% level are 298 DH lines to detect a 1% recombination value, compared to 150 and 9000 F_2 plants for a heterozygote and a homozygote, respectively (Allard, 1956).

The expected segregation ratio of DH lines is the same as that of backcross populations. However, the DH population more likely detects linkages among marker loci than backcross materials, especially for the quantitative characters (Carbonell *et al.*, 1993; Luo and Kearsey, 1991). This is due to a difference in the "scale" of characters between backcross and DH populations. A simplified example will be given to illustrate the point. A backcross population contains a homozygous group and a heterozygous group, and these can be stated as the Q_1Q_1 (or Q_2Q_2) and Q_1Q_2, respectively. An assignment of values for these genotypes in a statistic analysis could be +1 (or -1) and 0; ie. a scale of the value of 1. However, a doubled haploid population contains only homozygous groups such as Q_1Q_1 and Q_2Q_2 whose values could be assigned +1 and -1 (or *vice versa*); ie. a scale of the value of 2. This expanded scale may provide greater discrimination for quantitative traits and more clearly define genotypic classes for simply inherited characters, and therefore a more accurate estimation of linkages could be possible than that of a backcross population (Carbonell *et al.*, 1993).

1.2.7 Production of DH lines from different filial stages

In cereals, the most exploited doubled haploid system is based on F_1 plants. In some cases, however, one round of meiosis in F_1 may show high linkage disequilibrium because, there is a reduced chance of recombination (Powell *et al.*, 1990). If undesirable characters are closely linked to desirable agronomic characters, this linkage group has a greater chance of being separated. To achieve a higher level of variation in agronomic characters, the production of DH lines from F_2 plants has been recommended. DH populations produced from F_2 plants may contain up to 50%

more of the best recombinants than those produced from F_1 plants (Kasha and Reinbergs, 1981). DH plants from F_2 plants have two rounds of meiosis producing more frequent breakages of linkage groups. Frequent breakages of linkage groups, however, may also cause the loss of desirable linkage groups. DH lines produced from F_3 plants show only slightly higher variation than those produced from F_2 plants, and it takes time to establish F_3 plants (Snape and Simpson, 1981). However, the use of F_3 plant may be a useful strategy for the production of highly specific DH lines because only valuable F_3 plants selected from initial screening are used for the DH plant production (Simpson and Snape, 1981). In general, when target genes are in coupling phase, the use of DH system is recommended. In contrast, when two loci should be separated, traditional breeding methods or the application of DH system at more advanced filial stages, such as F_3 or F_4 should be used (Bjørnstad, 1987).

For critical genetic analysis, doubled haploid plants should be produced from a random sample of parental gametes. However, it has been reported that the segregation ratios of several molecular, biochemical and morphological markers in DH populations deviated from the expected segregation ratio (Heun *et al.*, 1991; Logue *et al.*, 1995; Powell *et al.*, 1986; Snape and Simpson, 1981; Thomson *et al.*, 1991; Zivy *et al.*, 1992). One of the reasons for the biased segregation is the differential transmission of alleles that may be due to different responses of genotypes in the anther culture, or different viabilities between gametes or zygotes because of one or more selected genes which is/are more viable (Thomson *et al.*, 1991; Zivy *et al.*, 1992). Distortion of segregation is not a unique phenomenon of DH lines. It has also been observed in backcross and F_2 populations (Lorieux *et al.*, 1995 a, b).

1.2.8 Summary and perspective for this study

Continuous monitoring of the target gene is essential in genetic studies and in breeding programs. To date, some genetic studies have been undertaken to understand the interaction between CCN and its hosts. However, reported results often have been complicated and ambiguous. The location of morphological markers in relation to the CCN resistance genes, *Ha1* and *Ha2* have been reported in some European studies.

The determination of the number of CCN resistance genes is important for efficient and effective use of resistance resources in breeding programs. It was reported that there could be up to five different loci expressing resistance in barley to the Australian CCN pathotype. It is not clear whether these loci originated from a single resistance gene, or are actually distinct and unique resistance genes. To obtain more accurate information, genetic studies involving the known sources of resistance to the Australian CCN pathotypes need to be conducted to establish the number of loci and allelic relationships of these genes. The use of DH lines for molecular mapping studies greatly simplifies the genetic analysis due to the homozygosity of the progenies in genetic studies.

Molecular genetic markers which are closely linked and flank the CCN resistance gene are desirable to maximise the efficiency and to minimise the complexity of genetic studies. Morphological and biochemical markers are rare and have limited application in such experiments. They also only represent small functional regions of DNA in the whole genome. Molecular markers, unlike morphological markers can be obtained from the whole genome and can provide unlimited and valuable research resources with the application of a large number of markers. A single DNA sequence variation can be used as a marker using allele specific PCR and this already has been demonstrated for CCN resistance in wheat (Williams, 1994). A molecular marker based screening method (marker assisted selection-MAS) for CCN resistance can

supplement the current CCN bioassay which has several limitations in terms of accuracy, speed and labour.

For the efficient management of the large amount of genetic information produced from using molecular markers, computational analysis is essential, and several mapping programs are available. These vary slightly, but the principles are the same; linkage establishment and the construction of best order maps.

Modern molecular biological techniques expands the range and precision of genetic experiments. Recently reported genomic DNA subtraction technology can reveal the different genetic constitutions between closely related sister lines such as NILs. The construction of DH lines for mapping populations, the finding of DNA markers for the CCN resistance gene(s) which can assist the monitoring of the gene(s) segregating in various populations, and the application of advanced molecular technologies are all essential for this study.

The aims of this study were

- To investigate and determine the number of the CCN resistance genes in the barley cultivars, Orge Martin and Chebec
- To establish the genetic relationship between a morphological character (*V/v* -2/6 row head type) and the CCN resistance gene(s) in Orge Martin
- To establish the chromosomal location of the CCN resistance gene(s) in Orge Martin and Chebec
- To find RFLP markers closely linked to the CCN resistance gene(s)
- To develop PCR markers for the CCN resistance gene(s)

- To investigate the genetic variation between barley NILs, and to develop new molecular markers for the CCN resistance gene with the use of barley genomic DNA subtraction

Chapter 2 Materials and Methods

Materials and methods described below were used throughout this study. The more specific materials and methods are described in each chapter.

2.1 Plant materials

2.1.1 Barley cultivars and lines

Orge Martin (OM), Ingrid (IN), BC^7 (OM x IN^7), Chebec (CB), Harrington (HA), Galleon (GA), Haruna nijo (HN) and Clipper (CL) were used. OM, IN and BC^7 were provided by Prof. James Mackey (Upsalla, Sweden). CB (WI 2737) has been recently released in Australia (Sparrow, 1994). GA is an Australian, OM is an Algerian, HN is a Japanese and HA is a Canadian barley. OM, CB, GA and BC^7 are CCN resistant, and IN, HA and HN are susceptible. CCN resistance in BC^7 and Chebec is from OM.

2.1.2 F_2 plants

60 F_2 plants were produced from IN x OM

2.1.3 Doubled haploid (DH) populations

1) 79 DH lines produced from F_1 plants of CL x OM (Section 3.2.2).
2) 88 DH lines of CB x HA were provided by Dr. S. Logue (Logue *et al.*, 1995).

2.2 CCN bioassay

2.2.1 Preparation of nematode farms

Field soil containing CCN cysts was provided by J. Lewis (SARDI-South Australian Research and Development Institute). Soil was washed on a set of sieves (sizes:

0.710 mm and 0.250 mm) under strong jet spray of tap water. Stones and organic matters were screened on the top sieve (size: 0.710 mm), and discarded. Washing was continued until clean water was released from the lower sieve (size: 0.250 mm). On the lower sieve, only a mixture of small particles of organic materials and cysts was collected. The jet spray of tap water was applied into a two litre container containing the mixture, and floating organic matter and cysts were collected quickly on another sieve (size: 0.250 mm). The collected mixture was placed in a cloth bag, and the bag was placed on the wire-grid with sufficient water supplied under the bag until the water level just touched the bag. A tray was kept at 10°C over one month to release the 2nd stage larvae from cysts.

2.2.2 CCN bioassay

The CCN bioassay procedure was described by Fisher (1982b). The CCN bioassay was carried out for 60 F_2 plants, 79 DH lines of CL x OM with 3 replications, and 88 DH lines of CB x HA with 3 replications. Dry seeds were treated at 36°C in an incubator for 24 hours to break dormancy. The seeds were then germinated in a 3 cm petri dish containing two layers of Whatman No.1 filter papers. Distilled water (1.3 ml) was added to each petri dish. The petri dish was sealed with Parafilm® and placed in a cold room (4°C) for 48 hours to allow imbibition of water, and was then kept at room temperature until 1-2 cm of roots had grown. Germinated seeds were planted in plastic tubes (inner diameter, 2.5 cm; length, 13 cm) filled with sterile sandy loam soil without organic matter. On the same day of every inoculation, CCN larvae were collected from the nematode farm. Water containing the released 2nd stage larvae in the tray (nematode farm) was poured on a sieve (size: 0.100 mm), and the larvae were kept in an appropriate volume of tap water, such that the number of larvae was approximately 100 per 1 ml of water. When the coleoptiles of test plants emerged above the soil surface in the tube, inoculation of the 2nd stage CCN larvae

(approximately 100 larvae at each time) was applied into the tube five times at three day intervals with a 1 ml pipette (inoculation volume, 1 ml). After the last inoculation, plants were grown under a 10 hour day length regime in a 15°C growth room for 10 weeks. The test tube was washed on a set of sieves (size: 0.710 mm and 0.250 mm) under jet-spray tap water. Plant debris was screened on a top sieve (size: 0.710 mm), and discarded except for roots. The number of white colour cysts on the root and lower sieve (size: 0.250 mm) was counted against a black colour plate.(Fisher, 1982b).

2.3 Abbreviations of chemicals and other units

A	adenosine
APS	ammonium per sulphate
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
CTP	cytosine triphosphate
dATP	2'-deoxy adenosine 5'-triphosphate
dCTP	2'-deoxy cytidine 5'-triphosphate
dGTP	2'-deoxy guanidine 5'-triphosphate
dTTP	2'-deoxy thymidine 5'-triphosphate
dNTPs	2'-deoxy ribonucleoside 5'-triphosphates
ddATP	2',3'-dideoxy adenosine 5'-triphosphate
ddCTP	2',3'-dideoxy cytidine 5'-triphosphate
ddGTP	2',3'-dideoxy guanidine 5'-triphosphate

ddTTP	2',3'-dideoxy thymidine 5'-triphosphate
ddNTPs	2',3'-dideoxy ribonucleoside triphosphates
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
8-Hydroxyquinoline	8-Hydroxy-1-azanaphthalene
G	guanine
GTP	guanosine triphosphate
HCl	hydrochloric acid
IPTG	isopropyl β-thiogalactopyranoside
kb	kilo base
KCl	potassium chloride
λ	lambda
LB	Luria-Bertaini
l	liter
M	molar
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
$MgCl_2$	magnesium chloride
MgOAc	magnesium acetate
µg	microgram
µl	microliter
µmole	micromolar
mRNA	messenger RNA

NaCl	sodium chloride
nmole	nanomole
pfu	plaque forming unit
pg	picogram
pmole	picomole
RNase	ribonuclease
SAM	S-adenosylmethionine
Sarkosyl	N-lauroylsarcosine, sodium salt
SDS	sodium dodecyl sulfate
T	thymidine
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TTP	thymidine triphosphate
Tris	tris[hydroxymethy]amino methane
Tween 20	polyoxyethylenesorbitan monolaurate
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
X-gal	bromo-(5)-4-chloro-3-indolyl-β-galactopyranoside
$ZnCl_2$	zinc chloride

2.4 Molecular techniques and solutions

2.4.1 Antibiotic, buffers and solutions

Ampicillin: 50 mg/ml in water

Buffers for restriction enzymes: buffers B, D, E, H (Promega, U.S.) are given in Table 2.1. 10X universal buffer (1 M potassium acetate/ 250 mM Tris-HCl, pH 7.6/ 100 mM MgOAc/ 5 mM β-mercaptoethanol/ BSA, 100 μg/ml) (Stratagene, U.S.).

Table 2.1 Restriction endonuclease buffers

Buffer	pH (at 37°C)	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	DTT (mM)
B	7.5	6	6	50	1
D	7.9	6	6	150	1
E	7.5	6	6	100	1
H	7.5	90	10	50	--

Denaturing solution: 1.5 M NaCl/ 0.5 M NaOH

DNA extraction buffer: 100 mM Tris-HCl, pH 8.5/ 100 mM NaCl/ 10 mM EDTA/ 1% sarkosyl

Ethidium bromide: 10 mg/ml

5X Sequenase buffer (Amersham, U.K.): 200 mM Tris-HCl, pH 7.5/ 100 mM $MgCl_2$/ 250 mM NaCl

Glycerol solution: 0.1 M $MgSO_4$/ 25 mM Tris-HCl/ 65% glycerol

Hybridisation solution: 1% BSA/ 1 mM EDTA/ 0.5 M $NaHPO_4$, pH 7.2/ 7% SDS (Church and Gilbert, 1984)

Labelling mix for sequencing (Amersham): 7.5 µM dGTP/ 7.5 µM dCTP/ 7.5 µM dTTP

LB medium (1l): bacto-tryptone 10g/ bacto-yeast extract 5g/ NaCl 5g, pH 7.5

LB plate (1l): LB medium/ bacto-agar 15g

Neutralising solution: 1.5 M NaCl/ 0.5 M Tris-HCl, pH 7.2/ 1 mM EDTA

Phage buffer: 20 mM Tris-HCl, pH 7.4/ 100 mM NaCl/ 10 mM $MgSO_4$

Sequenase dilution buffer (Amersham): 10 mM Tris-HCl, pH 7.5/ 5 mM DTT/ BSA (0.5 mg/ml)

Sequencing termination mix (Amersham): concentrations are in µM except for NaCl

	dATP	dCTP	dGTP	dTTP	ddNTPs	NaCl (mM)
ddA mix	80	80	80	80	8 µM of ddATP	50
ddC mix	80	80	80	80	8 µM of ddCTP	50
ddG mix	80	80	80	80	8 µM of ddGTP	50
ddT mix	80	80	80	80	8 µM of ddTTP	50

Solution I: 50 mM glucose/ 25 mM Tris-HCl, pH 8.0/ 10 mM EDTA

Solution II: 0.2M NaOH/ 1% SDS

TE-buffer: 10 mM Tris-HCl, pH 8.0/1 mM EDTA

10X CIAP buffer: 10 mM $ZnCl_2$/ 10 mM $MgCl_2$/ 100 mM Tris-HCl, pH 8.4

10X ligation buffer: 300 mM Tris-HCl, pH 7.8/ 100 mM $MgCl_2$/ 100 mM DTT/ 10 mM ATP

10X loading buffer: 100 mM Tris-HCl, pH 8.0/ 200 mM EDTA/ 0.25% bromophenol blue/ 0.25% xylene cyanol/ 30% ficoll, type 4000

10X methylase buffer: 1M Tris-HCl, pH 8.0/ 100 mM EDTA

10X TAE buffer: 400 mM Tris-HCl, pH 7.8/ 30 mM Na-acetate/ 10 mM EDTA

10X *Taq* polymerase reaction buffer: 200 mM $(NH_4)_2SO_4$/ 750 mM Tris-HCl, pH 9.0/ 0.1% Tween 20

10X TBE buffer: 1 M Tris-HCl, pH 8.3/ 0.863 M boric acid/ 10 mM EDTA

TM buffer: 50 mM Tris-HCl, pH 7.5/ 10 mM $MgSO_4$

Top agarose: 0.6% agarose in LB medium

Trituration buffer: 100 mM $CaCl_2$/ 70 mM $MgCl_2$/ 40 mM Na-acetate, pH 5.5

20X SSC: 3 M NaCl/ 0.3 M Trisodium citrate

2X Sequencing stop solution: 95% formamide/ 20 mM EDTA/ 0.05% bromophenol blue/ 0.05% xylene cyanol FF

2.4.2 Barley genomic DNA preparation ("minipreps")

This procedure followed the protocol described by Langridge *et al.* (1992). A piece of fresh barley leaf (approximately 10 cm) was placed in a 2 ml micro-centrifuge tube, and frozen in liquid N_2. A frozen leaf fragment was ground to a fine powder with a knitting needle. To the tube, 600 µl of DNA extraction buffer was added and homogenised by quick vortex. To the tube, 800 µl of phenol/chloroform/iso-amyl alcohol (25:24:1) was added and vigorously mixed by hand shaking for 5-10 min. on ice. The phases were separated by centrifuging (13,200 rpm) for 10 min., and the upper phase was transferred to a 2 ml micro-centrifuge tube. The phenol/chloroform/iso-amyl alcohol extraction was repeated, and 600 µl of the upper phase transferred to a fresh 1.5 ml micro-centrifuge tube. To the tube, 600 µl of chloroform/iso-amyl alcohol (24 : 1) was added, and vigorously mixed by hand shaking for 1-2 min. After centrifuging (13,200 rpm) for 10 min., the upper phase was transferred to a 1.5 ml micro-centrifuge tube, and barley genomic DNA was precipitated by adding 60 µl of 3M Na-acetate (pH 4.8) and 700 µl of iso-propanol. The tube was kept on ice for 10 min., and centrifuged (13,200 rpm) for 10 min. The supernatant was removed, and the white DNA pellet was washed with 70% ethanol. The air dried DNA pellet was resuspended in nanopure water containing *RNase* A (40 mg/ml). The final DNA concentration was approximately 3 µg/µl.

2.4.3 Hybridisation

The membrane (Hybond-N^+, Amersham) containing transferred DNA was pre-hybridised for 3-4 hours in a bottle (RATEK Instrument, Australia) at 65°C, which contained hybridisation solution (Church and Gilbert, 1984). The probe was denatured by boiling for 5 min. cooled down in ice water for 5 min., and added to the hybridisation bottle without changing the solution. Hybridisation was done overnight at 65°C in a rotating hybridisation oven (Hybaid). After hybridisation, the membrane was washed with the washing solutions series (2X SSC, 0.1% SDS/ 1X SSC, 0.1% SDS/ 0.5X SSC, 0.1% SDS/ 0.1X SSC, 0.1% SDS) for 20 min. each step at 65°C in a shaking water bath. After the last wash, excess washing solution was removed from the membrane, and the membrane was wrapped with Glad wrap®. Then, the membrane was exposed to Hyper film (Amersham, U.K.) for 4-5 days at -80°C.

2.4.4 Amplification of insert fragments by PCR, and purification of DNA fragments

Procedures described below followed the protocol of Langridge *et al.* (1992). Insert fragments (in the plasmid vector) were amplified by PCR with M13 primers. A PCR reaction was carried out in 50 µl reaction volume containing 0.1 volume of reaction buffer/ 1.5 mM $MgCl_2$/ 200 mM of each dNTPs/ 0.5 µg of each primers/ 1 unit of *Taq* polymerase/ 100 ng of plasmid DNA containing insert fragments. The program for PCR reaction on a MJ Research thermal cycler was 1) 94°C for 5 min.; 2) 94°C for 2 min.; 3) 55°C for 1 min.; 4) 72°C for 2 min.; 5) go to step 2), 35 more cycles; 6) 72°C for 5 min. *Taq* polymerase was purchased from Advanced Biotechnology (U.K.). PCR products were electrophoresed in a 1.5% agarose gel, and gel pieces containing amplified DNA fragments were excised. Insert DNA fragments were purified from the agarose gel piece using the "Gene clean" kit (Bio 101, U.S.). The gel piece was placed in a 1.5 ml micro-centrifuge tube containing 600-700 µl of 6M NaI. The tube was kept at 55°C until the gel piece was completely melted. Glass milk (5 µl) (Gene

clean kit) was added, and the tube was kept at room temperature for 5-10 min. A white pellet was formed by a quick centrifugation (5 seconds), and the pellet was washed 3 times with 500 µl of "wash solution" (Gene clean kit). The pellet was completely dried to remove "wash solution", and resuspended in 20 µl of nanopure water followed by centrifugation (13,200 rpm) for 2 min. The supernatant (20 µl) containing the insert DNA was transferred to a fresh 1.5 ml micro-centrifuge tube, and stored at -20°C.

2.4.5 Ligation

Dephosphorylated 100-150 ng of Bluescript vector (Stratagene, U.S.) digested with *Eco*RI, and 3-4 ng of insert fragments was ligated with 2.5 Weiss units of T_4 DNA ligase (Promega, U.S.) in a total volume of 20 µl containing 0.1 volume of 10X ligase buffer. The reaction was carried out at 12°C overnight, and ligated DNA was stored at -20°C.

2.4.6 Preparation of phenol

The procedure followed the protocol of Sambrook *et al.* (1989). Solid phenol was purchased from Sigma (U.S.). Phenol was melted at 68°C. 8-Hydroxyquinoline was mixed to phenol (final concentration 0.08-0.1%). An equal volume of 1M Tris-HCl (pH 8.0) was mixed with the melted phenol on a magnetic stirrer for 15-20 min. The upper phase was removed with a 50 ml glass pipette. To the phenol phase, 0.1M Tris-HCl (pH 8.0) was added, and stirred vigorously again. During this step, the pH of phenol was measured with a paper pH indicator. After the phenol was equilibrated to pH 8.0, the upper phase was removed, and 0.1 volume of 0.1M Tris-HCl (pH 8.0) was added. The phenol solution was kept in a dark bottle at 4°C.

2.4.7 Probe labelling

The labelling procedure followed the manufacturer's protocol for the "labelling kit" (Amersham, U.K.), which uses the principle of random oligonucleotides as primers (Feinberg and Vogelstein, 1983). Approximately 25 ng of insert fragments in 5 µl of nanopure water and 5 µl of random primer solution (labelling kit) were mixed in a 1.5 ml micro-centrifuge tube, and denatured by boiling for 5 min. The tube was briefly centrifuged and kept at room temperature for 3-4 min. After cooling, 10 µl of labelling buffer (labelling kit) (contained unlabelled dNTPs and reaction buffer), 25 µl of nanopure water, 3 µl of [α-^{32}P]dCTP (specific activity, ~4,000 Ci/mM) and 2 units of Klenow enzyme were added to the tube. The total reaction volume was 50 µl. The tube was incubated at 37°C in a water bath for 15 min. After incubation the tube was stored at -20°C for a few hours until it was used.

2.4.8 Purification of plasmid DNA containing insert fragments

The procedure followed was the "Alkaline lysis" method (Sambrook *et al.*, 1989). Overnight cultured bacterial cells (2 ml culture) were precipitated by centrifuging (3,000 rpm) for 10 min., and resuspended in 100 µl of Solution I. Resuspended bacterial cells were denatured by adding 200 µl of Solution II, and denatured bacterial proteins were precipitated by adding 150 µl of 3M Na-acetate (pH 4.8). After incubation on ice for 10 min., denatured bacterial debris was pelleted by centrifuging (13,200 rpm) for 10 min. in a 4°C cold room. The supernatant was transferred to a fresh tube, and the plasmid DNA precipitated by adding an equal volume of iso-propanol, followed by centrifuging (13,200 rpm) for 10 min. The white pellet was then washed with 70% ethanol. The air dried DNA pellet was resuspended in nanopure water. The final DNA concentration was 100 ng/µl.

2.4.9 Restriction enzyme digestion

Procedures described below followed the protocol of Langridge *et al.* (1992). Extracted barley DNA (approximately 20 µg) was digested independently with six restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RVand *Hin*dIII). All restriction enzymes were purchased from Promega (U.S.). Twenty units of each enzyme (high concentration) were used in total volume 15 µl containing 10X reaction buffer. Reaction was carried out at 37°C for 3-4 hours.

2.4.10 Gel blotting

Procedures described below followed the protocol of Langridge *et al.* (1992). Digested DNA was loaded onto a 1.5% agarose gel, and the gel electrophoresed in 1X TAE running buffer overnight at a current of 30 mA. The electrophoresed gel was soaked in denaturing solution for 20 min. and neutralised in neutralising solution for 20 min. DNA was transferred from the neutralised gel to Hybond N^+ membranes (Amersham, U.K.) through capillary blotting (Southern, 1975) with 20X SSC for 3-4 hours. The membrane containing transferred DNA was rinsed in 5X SSC for 2 min., and dried at 80°C in a drying oven for 1-2 hours. Once dried, the membrane was kept at 4°C until it was used.

Chapter 3 The chromosomal location and the number of the cereal cyst nematode (CCN) resistance gene(s) in barley cultivars, Orge Martin and Chebec

3.1 Introduction

Since studies of the genetics of resistance to CCN were initiated in 1960 (Hayes and Cotten, 1971), several different CCN pathotypes and CCN resistance genes have been reported (Andersen and Andersen, 1973; Andersen and Andersen, 1982a; Andersen and Andersen, 1982b). In Europe, at least 10 CCN pathotypes and at least five different CCN resistance genes in barley have been reported (Andersen and Andersen, 1982b; Hayes and Cotten, 1971) as described in Section 1.2.3.

In Australia, only one CCN pathotype (Ha1III) has been detected (Brown, 1982). A few genetic experiments have been undertaken to characterise the CCN resistance genes in Australian barley cultivars (Brown, 1982; Ellis and Brown, 1976; O'Brien *et al.*, 1979; O'Brien and Fisher, 1977; Soetopo, 1986; Sparrow and Dubé, 1981). Several morphological and isozyme markers have been reported for the resistance gene to the Australian CCN pathotype, for example *pau* (purple auricle), *ant-2* (anthocyamadin free), *cer* (surface wax) and *e* (elongated outer glume) (Ellis, 1987). Several genetic studies suggested that at least three, and possibly five unique CCN resistance genes have been involved in resistance to the Australian CCN pathotype (O'Brien *et al.*, 1979; Soetopo, 1986). One of several morphological markers, the *V/v* (2/6-row) locus has been linked to the gene for CCN resistance in barley cultivars, Athenais, Marocaine and Nile (Soetopo, 1986). However, the CCN resistance gene in Orge Martin was not reported to be linked to the *V/v* gene locus in a previous experiment (Soetopo, 1986).

Two independent genes, *V/v* and *I/i*, control the barley head types (Cattivelli *et al.*, 1994; Gymer, 1978; Leonard, 1942). The *V/v* gene on the long arm of the chromosome 2 controls the row number of barley head types. The dominant gene, *VV*, produces the 2-row head type, and the recessive gene, *vv* gives the 6-row head type. The *I/i* gene on the long arm of chromosome 4 controls the fertility of the lateral florets. Paired combinations of the alleles of these two independent genes (*V/v* and *I/i*) produce four distinct barley head types in the homologous status. When the *vv* allele co-operates with the *I/i* gene, two different 6-row barley head types are possible. The *vv/II* gives a 6-row head type without pedicels of lateral florets (the sessile type; commercial 6-row barley). The *vv/ii* combination produces a 6-row head type with pedicels (1-3 mm length) of lateral florets. Long pedicels may hamper the flow of nutrients from the internodes of spikes to the developing seeds. A negative correlation was reported between the pedicel length and seed weight (Cattivelli *et al.*, 1994; Gymer, 1978). When the *VV* allele co-operates with the *I/i* gene, two different 2-row head types are produced. The *VV/ii* forms a 2-row head type with complete sterile lateral florets (commercial 2-row barley). The *VV/II* gives a 2-row head type with inflated lateral florets featuring anthers, or sometimes with fertile lateral florets producing seeds (intermediate head type) (Gymer, 1978; Leonard, 1942).

It was reported that Orge Martin has *vv/II* head type (Soetopo, 1986). However, the genetic relationship between the CCN resistance gene and the *II/ii* gene, one of genes controlling barley head types, was not determined.

The aims of this chapter are to:

- determine the number of CCN resistance genes in the barley cultivars, Orge Martin and Chebec

- establish the linkage between the *V/v* gene and the CCN resistance gene(s) in Orge Martin
- establish the chromosomal location of the CCN resistance gene(s) from Orge Martin and Chebec.
- test the possible linkage between the CCN resistance gene(s) and the *II/ii* gene, one of genes controlling barley head types

3.2 Materials and methods

3.2.1 Plant materials

3.2.1.1 Barley cultivars

Orge Martin (OM), Chebec (CB), Clipper (CL), Harrington (HA) and Ingrid (IN) were used.

3.2.1.2 F_2 plants

60 F_2 plants were produced from IN x OM.

3.2.1.3 Doubled haploid (DH) populations

1) 79 DH lines from F_1 plants of CL x OM.
2) 88 DH lines from F_1 plants of CB x HA (Logue *et al.*, 1995).

3.2.2 Anther culture for the production of DH lines from F_1 plants of CL x OM

Procedure for production of anther culture derived doubled haploid mapping population

The procedure of anther culture followed the protocol described by Logue *et al.* (1993).

1) F_1 seeds were harvested from CL x OM. Three or four seeds were sown in eight inch pots. F_1 plants were grown in a growth room (temperature, 15°C; daylength, 10 hours; photon flux density, 850 µmole photons/m^2/s) until tillers were harvested (Logue *et al.*, 1993).

2) Tillers were regularly harvested when the flag leaf emerged approximately three cm from the penultimate leaf (the youngest leaf except for the flag leaf). Usually this growth stage indicated the mid-uninucleate stage of developing pollen. (Gaul *et al.*, 1976; Wheatley *et al.*, 1986).

3) Spikes were removed from tillers under sterile conditions in a laminar flow cabinet. Anthers were isolated from the most developed central florets, and were tapped out in aceto carmine (45% acetic acid; 2% carmine), and the pollen stages were checked under the microscope (x40).

4) Spikes containing the mid-uninucleate stage pollen were placed aseptically in a double compartment petri dish containing a moist Wettex® sponge, and kept in a cold room (darkness, 4°C) for four weeks (Huang and Sunderland, 1981).

5) After four weeks, anthers were removed from five or six central florets of each spike in a laminar flow cabinet. Removed anthers were plated aseptically on 30 mm petri dishes containing induction medium (Appendix 1-a).

6) Petri dishes sealed with Parafilm® were incubated in the growth cabinet (darkness, 25°C) for four weeks.

7) After incubation, calli were transferred to 55 mm petri dishes containing regeneration medium using sterile technique (Appendix 1-b).

8) Petri dishes containing calli were sealed with Parafilm®, and incubated in a growth cabinet (25°C, continuous lights) until green plantlets were generated.

9) Green plantlets were transferred into plastic bottles containing 10 ml of modified MS medium (Murashige and Skoog basal medium, 4.71g; sucrose, 30g; agar, 8g/L) (Murashige and Skoog, 1962).

10) Green plantlets were grown in a growth room (continuous light, 25°C) until shoots and roots were established. Plantlets were then transplanted into four inch pots containing sterile recycled soil, and grown in a glass house. Plants were covered with plastic cups for one week to maintain high humidity.

11) After four weeks, the lengths of stomatal guard cells were measured to estimate the ploidy level. A leaf segment (one to two cm) was removed from the tip of a young leaf of individual plants. These leaf segments were placed in tubes containing 70% ethanol in boiling water bath for 10 min. to remove chlorophyll. After chlorophyll was removed, leaf segments were fixed in fixation solution (3 parts of ethanol, 1 part of glacial acetic acid). Leaf fragments were mounted in a drop of distilled water, and covered with the cover glass on the microscope glass slide. The lengths of 10-12 stomatal guard cells were measured under the microscope (x400), and the eye piece unit (EPU; 1 EPU = 2.4 μm) was converted to the actual size (Borrino and Powell, 1988; Slayter and Slayter, 1992). The sizes, 40 μm and 58 μm were chosen as discrimination points for the separation of haploid/diploid and diploid/tetraploid, respectively (Borrino and Powell, 1988). Plants were grown without colchicine treatment until seed set, because the separation of haploid and diploid by the size of stomatal guard cells was sometimes ambiguous (Borrino and Powell, 1988). Plants setting no seed were considered sterile (haploid) and discarded.

3.2.3 CCN bioassay

The CCN bioassay procedure was described in Section 2.2

3.3 Results

3.3.1 Anther culture

3.3.1.1 Number of green and albino plants

A total of 8282 anthers (6150 anthers from F_1 plants, and 2132 anthers from parents plants as controls) were used for the anther culture (Table 3.1). The percentage of anthers which made calli and/or embryoids varied between parents (CL and OM) and F_1 plants. About 13% of the anthers of CL responded to make calli and/or embryoids. In contrast, anthers of OM responded poorly (1.5%). The anthers of the F_1 plants had an intermediate response (7%). This trend was repeated for the levels of green and albino plants produced (Table. 3.1). CL, OM and F_1 plants produced 3.3, 0.7 and 2.3 green plants per 100 anthers, respectively. A total of 142 green plants were regenerated from 6150 F_1 anthers.

Table 3.1 Number of anthers responsive to culture, and number of green/albino plants produced per 100 anthers

Plants	No. of anthers	Responding anthers	No. of green plants (per 100 anthers)	No. of albino plants (per 100 anthers)
CL	777	102 (13%)	26 (3.3%)	44 (5.9%)
OM	1335	20 (1.5%)	9 (0.7%)	19 (1.4%)
F_1	6150	447 (7%)	142 (2.3%)	365 (5.2%)
Total	8282	569 (6.9%)	177 (2.1%)	428 (5.2)

3.3.1.2 Establishment of doubled haploid (DH) lines

Among 142 green plants produced from F_1 anthers (Table 3.1); 101 plants survived to the heading stage. These remaining 101 plants were a mixture of haploids and diploids (Fig. 3.1). After setting seed, 22 sterile haploid plants were discarded. No tetraploid plants were found. Seventy nine fertile diploids were formed by spontaneous chromosome doubling without colchicine treatment. The yield level of spontaneously doubled haploid plants was 78%. One of 79 doubled haploid lines had an intermediate head type (plant number, 33B/2; see Appendix 2-a). The intermediate head contained several fertile lateral florets on the 2-row head, and was considered as the 2-row head type.

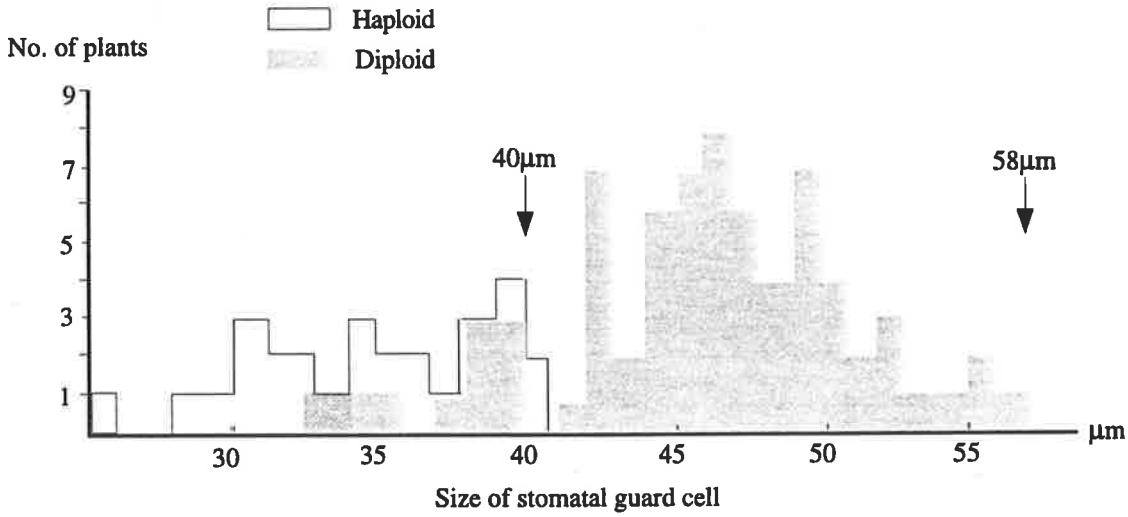


Fig. 3.1 The distribution of the size of stomatal guard cells. Some lines had the ambiguous size of stomatal guard cells. Arrows, discrimination points of haploid/diploid plants, and diploid/tetraploid plants

3.3.2 Segregation of the CCN resistance gene

The number of cysts produced on CCN resistant and susceptible cultivars was clearly delineated (Fig. 3.2). The average number of cysts produced from resistant cultivars, OM and CB, was zero. The average number of cysts in the susceptible cultivars were; 37, 19 and 19 for HA, CL and IN, respectively. The distributions for the number of cysts produced on the DH lines and the F_2 population was highly skewed toward the resistant parents, OM and CB (Fig. 3.2) (CCN bioassay results; see Appendix 2-a and -b).

The segregation ratios of CCN resistance:susceptibility were variable (Table 3.2). Previous observations have suggested that there were two independent CCN resistance genes in OM (Soetopo, 1986). The expected segregation ratios were therefore 3:1 for the CL x OM DH population and 15:1 for the F_2 population. The observed segregation ratios were 3:1 for DH lines and 3:1 for the F_2 population (Table 3.2). As the donor of CCN resistance in CB was OM (Sparrow, 1994), the genetic analysis for CB x HA DH lines was undertaken considering two models, for a single gene and two genes. The result obtained was 3:1, suggesting a two gene control (Table 3.2).

3.3.3 The genetic distance between the *V/v* gene and the CCN resistance gene in OM

To measure the genetic distance between the *V/v* gene and the CCN resistance gene in OM, the possibility of linkage between two characters was tested (Table 3.3). The genetic distance between two characters was approximately 20±4.6 cM, and the CCN resistance gene in OM is located on the chromosome 2. The *I/i* gene was independent from this CCN resistance gene (Table 3.4). Segregation ratios of the *VV*:*vv* and *II*:*ii* were both 1:1. The segregation ratios fitted well into the expected range (1:1) that meant unbiased segregation at the regions of the *V/v* gene and the *I/i* gene (Table 3.5).

Also, the *I/i* gene controlling the fertility of lateral florets was independent from the *V/v* gene (Table 3.5).

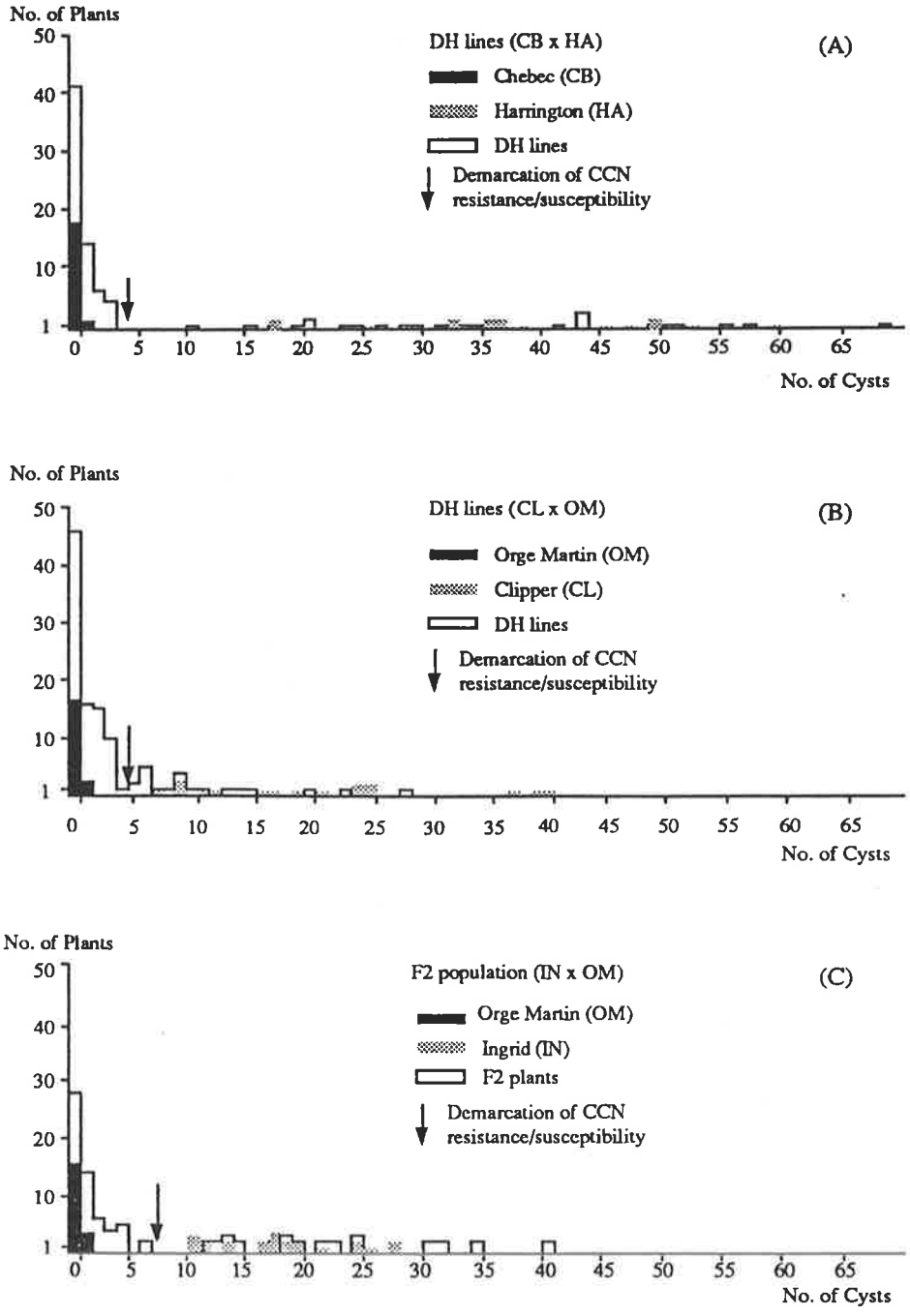


Fig. 3.2 The distribution of cysts produced from three different test populations

Table 3.2 Genetic segregation for the resistance gene(s) to CCN in three populations

Test populations	No. of plants tested	Expected segregation RR : rr	Observed segregation RR : rr	χ^2	(df=1)
CL/OM DH lines	76	19 : 57 (1 : 3)	26 : 50 (1 : 3)	3.43	0.1>P>0.05
		38 : 38 (1 : 1)	26 : 50 (1 : 3)	7.58	0.025>P>0.01
CB/HA DH lines	88	66 : 22 (3 : 1)	66 : 22 (3 : 1)	0	P=0.995
		44 : 44 (1 : 1)	--	22	P<0.005
IN/OM F_2 population	60	56.3 : 3.8 (15 : 1)	--	65	P<0.005
		45 : 15 (3 : 1)	41 : 19 (3 : 1)	1.43	0.5>P>0.1

RR, resistant; rr, susceptible

Table 3.3 Detection of the genetic linkage between the *V/v* gene for the barley head type and the $Ha_{(OM)}$ gene in the CL x OM DH population

No. of plant tested	Expected segregation ratio RR/VV : RR/vv : rr/VV : rr/vv	Observed segregation ratio	χ^2	(df=3)
76*	19:19:19:19 (1 : 1 : 1 : 1)	13 : 37 : 23 : 3	35.8	P<0.005

RR, CCN resistant; rr, susceptible; *VV*, 2-row head type; *vv*, 6-row head type
* CCN bioassay results for three DH lines were not determined.

Table 3.4 Test for the linkage between the *I/i* gene and the $Ha_{(OM)}$ gene in the CL x OM DH population

No. of plant tested	Expected segregation ratio RR/II : RR/ii : rr/II : rr/ii	Observed segregation ratio	χ^2	(df=3)
76*	19 : 19 : 19 : 19 (1 : 1 : 1 : 1)	27 : 22 : 13 : 14	7.03	0.1>P >0.05

* CCN bioassay results for three DH lines were not determined.
RR, CCN resistant; rr, susceptible

Table 3.5 Test for the linkage between the *V/v* gene and the *I/i* gene in the CL x OM DH population

No. of plant tested	Expected segregation ratio *VV/II : VV/ii : vv/II : vv/ii*	Observed segregation ratio	χ^2	(df=3)
79	19.7:19.7:19.7:19.7 (1 : 1 : 1 : 1)	22 : 17 : 20 : 20	0.16	0.995>P >0.975

3.4 Discussion

The barley anther culture method is well established at the Waite Agricultural Research Institute (Logue *et al.*, 1993). The anther culture procedure used in this study followed the established protocol without modification. Some gametoclonal variation was observed in the level of green plant production. CL produced more green plants than OM, 3.3% and 0.7%, respectively. These levels were lower than previously reported levels from this laboratory (Logue *et al.*, 1993). The reason for the observed variations could be genotype effects (Powell *et al.*, 1986).

The size of the stomatal guard cell was measured to evaluate the ploidy level (Borrino and Powell, 1988). Among 101 green plants, 25 lines had the size of stomatal guard cells (a size ≤ 40 μm) indicative of haploids. Nine of these were later verified as diploid lines since they produced seeds. Among the 76 putative diploid lines (40 μm ≤ the size of stomatal guard cell ≤ 58 μm), four lines failed in seed setting (haploids). No tetraploid plants were found. One of the advantages of the anther culture method in barley is that there is a large proportion of spontaneous chromosome doubling of haploid plants (Heberle-Bors, 1985; Logue *et al.*, 1993; Sunderland, 1974). It was reported that pre-meiotic colchicine treatments decreased the recombinations, but increased the frequency of double crossovers among recombinations in wheat (Curtis

and Lukaszewski, 1992), also it may cause heritable point mutations (Francis and Jones, 1989). Therefore, all regenerated plants were grown until the seed set; putative haploid plants were not treated with colchicine. After seed setting, 22 sterile plants were found and removed.

In summary, among the 101 green plants, about 78% were diploid, and these were produced from spontaneous chromosome doubling. One of the possible reasons for the spontaneous chromosome doubling is nuclear fusion in haploid pollen (Sunderland, 1974). Nuclear fusion was common for *Datura innoxia*, and this process was likely to be involved in the formation of diploid and tetraploid cereals (Clapham, 1977). Another theory for cereals is the formation of multicellular pollen grain through non-polarised mitosis forming equal size cells which can divide to form multicellular grain (Clapham, 1977; Sunderland, 1974).

As CL and OM had the 2-row and the 6-row head types respectively, a majority of 79 DH lines had the 2-row or the 6-row head types. One of the 79 DH lines had the intermediate head type containing several fertile lateral florets on the 2-row head. Spontaneous and induced mutant head types have been reported. Barley head types were often changed from 2-row to 6-row or intermediate head types (Lundqvist and Lundqvist, 1987 and 1988; Nilan, 1964). Also, a mutation of head types from the 6-row to the 2-row has been reported (Shebeski and Lawrence, 1954).

Several CCN bioassays were undertaken to determine the number of the CCN resistance genes in OM and CB. As previously reported results suggested that OM had two independent CCN resistance genes (Soetopo, 1986), the expected results of the CCN bioassay for the F_2 population of IN x OM was 15 : 1, but the result obtained in this study was 3 : 1, implying a single gene control which was different from Soetopo's report (1986). However, the result of the CCN bioassay for 79 doubled haploid lines of CL x OM was 3 : 1, representing two gene control. Another CCN

bioassay result from the doubled haploid population of CB x HA was 3 : 1, which is consistent with CB having two independent CCN resistance genes. As the CCN resistance in CB was from OM, this result suggested the possibility of the existence of two independent CCN resistance genes in OM and CB.

From the results of the CCN bioassay and the discrimination of barley head types, the linkage was established between the *V/v* gene and the CCN resistance gene in OM.

Soetopo (1986) concluded that the CCN resistance gene in OM was not linked to the *V/v* gene, but her data implied the linkage between two loci (Soetopo, 1986). In this study, the genetic distance between two characters was approximately 20 cM. This genetic distance is similar to the reported results for different barley cultivars and different CCN pathotypes in Europe (Andersen and Andersen, 1973).

As the Australian CCN pathotype is different from the European CCN pathotypes, and belongs to group III (Andersen and Andersen, 1982b), the CCN resistance gene in OM and CB may be *Ha3*. However, the relationship between the CCN resistance gene in OM and the reported *Ha3* gene in Morocco has not been fully investigated. The CCN resistance gene in OM and CB therefore has been tentatively named "$Ha_{(OM)}$".

Overall, it was difficult to determine the number of CCN resistance genes in OM and CB by CCN bioassay results obtained in this study. To determine whether there is a single locus or two CCN resistance gene loci in OM and CB, more precise experimental approaches are required using several markers, such as DNA markers.

Chapter 4 RFLP markers of the CCN resistance gene in barley

4.1 Introduction

One of the rapidly emerging modern technologies in plant genetic research is the use of molecular markers. As molecular markers are numerous, phenotypically neutral and free from allelic interactions (Laurie *et al.*, 1992), this technique has become an important strategy in the construction of genetic maps (Young, 1994). One of the important contributions of molecular marker based mapping work for plant breeding is tagging the gene of interest with tightly linked molecular markers. Molecular marker-facilitated research can monitor the manipulation of the target gene in crosses and in the resultant progenies (Stuber, 1994). Well characterised molecular markers for the gene of interest can be used for the selection of valuable breeding lines in any breeding system for the efficient incorporation of the target gene into other breeding lines. Closely linked flanking markers have the potential for improving the efficiency for the selection of disease resistant breeding lines, since the chance of double cross overs is rare between closely linked flanking markers (DeScenzo *et al.*, 1994).

To date, several morphological markers and a translocation break point [*V/v, Li, pau, Lk, e* or *(log1), cer g10, Re2, Vt* and T_{2-3}f] linked to the CCN resistance gene in barley have been reported in Europe as described in Section 1.2.3. To make use of these markers, barley cultivars containing them must be used for the crossing. This process is not practical in breeding programs, since many of these markers are undesirable traits. Another approach could be the use of backcross selection using repulsion relationships between marker genes and the CCN resistance gene. The marker genes can be transferred into several backgrounds with a donor CCN resistant barley, and backcross to the recurrent lines containing homozygous recessive markers.

The desirable CCN resistance lines can then be screened against the recessive marker genes. This approach would be cumbersome.

To achieve the efficient incorporation of CCN resistance into cereal breeding programs, a practical and accurate marker-assisted screening method is required. RFLP marker systems can fulfil these requirements (Botstein *et al.*, 1980; Gusella, 1986).

The aims of the research described in this chapter are:

- To determine the number of CCN resistance genes in barley cultivars Orge Martin and Chebec by using molecular genetic markers.
- To determine the chromosomal location of the $Ha_{(OM)}$ gene(s).
- To find tightly linked RFLP markers of the $Ha_{(OM)}$ gene(s).

4.2 Materials and methods

4.2.1 Plant materials

Plant materials were described in Section 2.1.3

4.2.2 CCN bioassay

The procedure for the CCN bioassay was described in Section 2.2, and the same results were used for mapping work.

4.2.3 Barley genomic DNA extraction

The procedure was described in Section 2.4.2.

4.2.4 RFLP clones

RFLP clones below were obtained through the Australian Triticeae Mapping Initiative (ATMI): ABC152, ABC451, ABG14, ABG19, BCD266, BCD355, BCD453, CDO680, cMWG694, KsuD22, KsuF15, MWG503, MWG699, MWG865 and MWG892. ABC, ABG, BCD and CDO clones are from the North American Barley Genome Mapping Project (NABGMP) (Kleinhofs and Kilian, 1994). cMWG and MWG clones belong to a German barley mapping team (Graner *et al.*, 1991). The clone AWBMA21 was originally isolated by P. Murphy from a Galleon root cDNA library at the Waite Agricultural Research Institute (WARI). Preliminary analysis by Dr. Langridge *et al.* (WARI) showed that AWBMA21 mapped to the region of the $Ha_{(OM)}$ gene(s).

Selected clones have been positioned on the centromere region and the long arm of barley chromosome 2 in current barley molecular maps (Graner *et al.*, 1991; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). Two RFLP clones, ABG19 and BCD355 have been located on the proximal region of barley chromosome 2 (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). ABC152, ABC451, ABG14, BCD266, BCD453, CDO680, KsuD22 and KsuF15 are distributed from the proximal region to the distal region of the long arm (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). Other clones, cMWG694, MWG503, MWG699, MWG865 and MWG892 are located near the *V/v* locus (Graner *et al.*, 1991).

4.2.5 Restriction enzymes

*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII were purchased from Promega (U.S.). Reaction conditions were described in Section 2.4.9.

4.2.6 Preparation of labelled probes and hybridisation

The following procedures were used for the preparation of labelled probes and hybridisation; restriction enzyme digestion of genomic DNA, gel blotting, purification of plasmid DNA, isolation of inserts, probe labelling, and hybridisation. Details of these procedures are described in Section 2.4.

4.2.7 RFLP analysis

After the development of X-ray films, each allele detected by the RFLP probes was scored and analysed with "Map Manager (Version 2.6)" mapping software (Manly and Cudmore, 1994). For utilising this mapping software, DNA fragments of Orge Martin (OM) and Chebec (CB) identified by each RFLP clone were scored as an "A", and those of Clipper (CL) and Harrington (HA) as "B". The confidence level used for the genetic analysis was 95%.

4.3 Results

4.3.1 Polymorphisms between parent cultivars of mapping populations

Pairwise combinations of 16 RFLP probes and six restriction enzymes were used to identify RFLPs between parent plants of each mapping population (CL x OM and CB x HA).

Among 16 probes, ABC152 produced multiple polymorphic bands between parent plants. Although most of the other clones hybridised to single copy DNAs, some clones produced two or three polymorphisms (low copy clone) with a single restriction enzyme. These multi-polymorphisms consisted of a major polymorphic band and one or two weakly hybridising polymorphic bands (Fig. 4.1). For each probe, the major polymorphism which had strong and distinct hybridisation to the RFLP probe was adopted for RFLP analysis of the mapping populations. The weak bands of minor

polymorphisms were often ambiguous, and were considered unsuitable for RFLP analysis. ABG19, CDO680, KsuF15, cMWG699 and MWG892 did not identify RFLPs between parent plants of mapping populations (CL x OM and CB x HA) with the restriction enzymes used. ABC152, the multi-copy probe, revealed many bands that were too difficult to resolve, and this caused ambiguity in mapping work. The probes which produced either no polymorphism or ambiguous RFLP bands were not used for further mapping work. A summary of the results of the parental screen is shown in Table 4.1.

Table 4.1 RFLP probes and restriction enzymes producing RFLPs between parent cultivars of two DH mapping populations

Probes	CL x OM	CB x HA
ABC152	BI, BII, DI, EI, EV	BI, BII, DI, EI, EV
ABC451	BI, BII, EI	BI, BII, EI
ABG14	DI	BI, DI, BII
ABG19	NP	NP
AWBMA21	EI	BI, HIII
BCD266	BI, EV	BI
BCD355	BI, BII, EV	BI, BII, EV
BCD453	BI, BII, DI, EI	BI, BII, DI, EI
CDO680	NP	NP
cMWG694	BI, EI, EV, HIII	BI, EI, EV, HIII
KsuD22	BI, BII, DI, EV	NP
KsuF15	NP	NP
MWG503	BI, EV	BI, EV
cMWG699	NP	NP
MWG865	BI	BI
MWG892	NP	NP

BI, *Bam*HI; BII, *Bgl*II; DI, *Dra*I; EI, *Eco*RI; EV, *Eco*RV
NP, no polymorphism

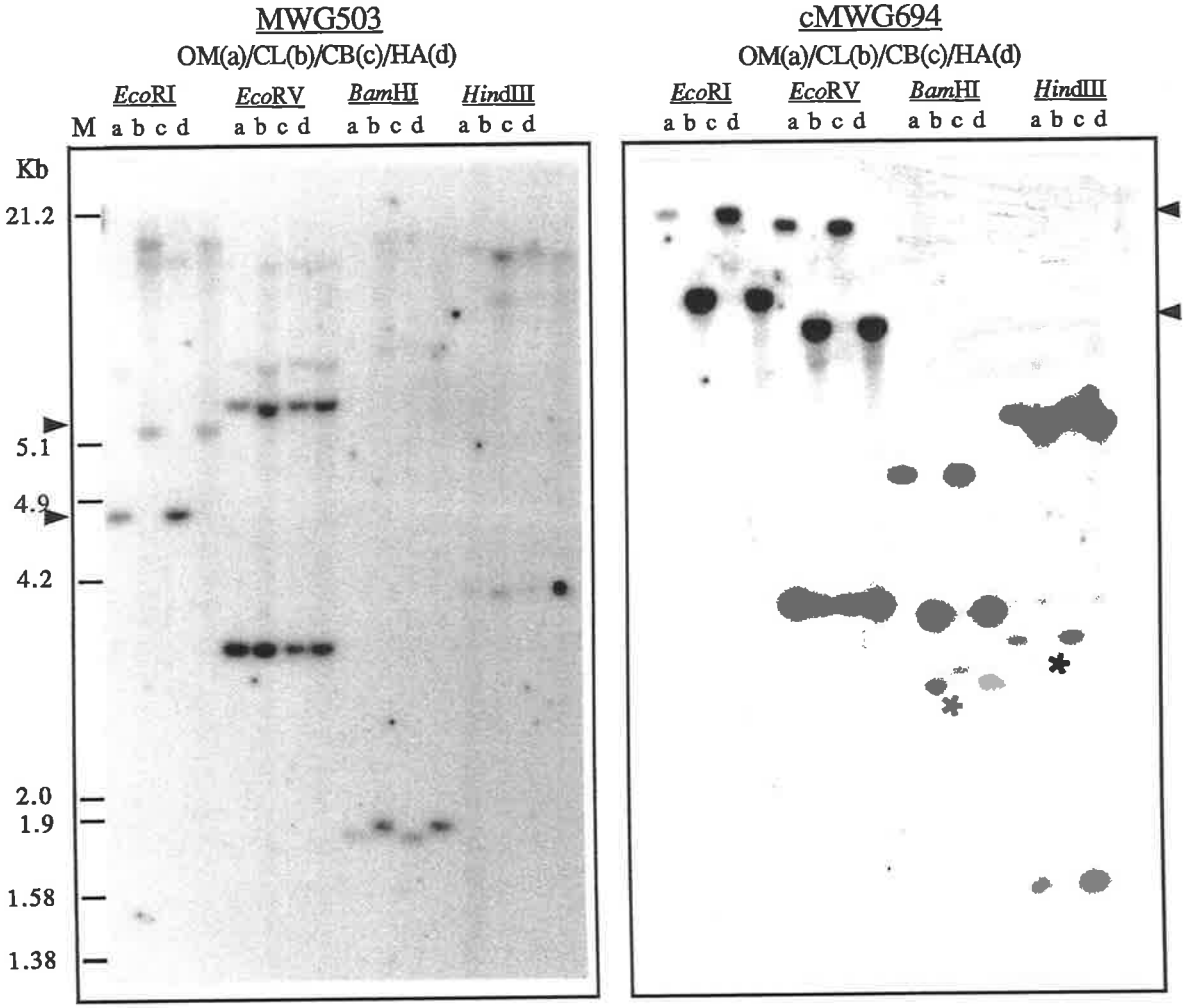


Fig. 4.1 Polymorphisms between parent cultivars of mapping populations. A probe, MWG503 revealed polymorphisms (left arrows) with *Eco*RI restriction enzyme, and cMWG694 produced various polymorphisms with four restriction enzymes used. Polymorphisms revealed by *Eco*RV enzyme (right arrows) were used for RFLP analysis. M, DNA size marker (lambda/*Hin*dIII/*Eco*RI); arrows, polymorphisms used for RFLP analysis. * marks, minor poly morphisms

Variation was found between the six different restriction enzymes for the percentage of RFLPs produced. *Bam*HI and *Hin*dIII were highly informative for the identification of genetic variation between the parental cultivars. *Bgl*II, *Dra*I, *Eco*RI and *Eco*RV were less informative for the four parental cultivars (CL, OM, CB and HA) in this study (Table 4.2).

Table 4.2 Restriction enzymes producing RFLPs between parent cultivars of mapping populations with the use of 16 probes

Restriction Enzymes	No. of uses	No. of RFLPs detected	Percentage of RFLPs
*Bam*HI	32	19	59%
*Bgl*II	32	10	31%
*Dra*I	26	7	30%
*Eco*RI	32	7	22%
*Eco*RV	32	12	38%
*Hin*dIII	7	3	43%

4.3.2 Mapping the CCN resistance gene relative to RFLP markers

4.3.2.1 Initial mapping work for tagging the $Ha_{(OM)}$ gene(s)

Initially, two RFLP clones (BCD453 and KsuD22) were selected. These clones previously have been located on the proximal region and the distal region of the long arm of barley chromosome 2, respectively (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). It was expected that a probe BCD453 may tag the $Ha_{(OM)}$ gene(s), if this gene was located on the proximal region, since the recombination is rare in this region producing short map distances (Graner *et al.*, 1991). The probe KsuD22 was expected to cover the distal region of the long arm of chromosome 2.

RFLP data of the two clones, CCN bioassay results and data of barley head types (2/6-row, from Section 3.3.3) were used for the genetic analysis. The probe BCD453 was a low copy probe, and produced common bands [the first arrow in Fig. 4.2 (A)] and an RFLP between CL and OM [the 2nd and 3rd arrows in Fig. 4.2 (A)]. Also, it produced a common band [the first arrow in Fig. 4.2 (B)] and an RFLP between CB and HA [the 2nd and 3rd arrows in Fig. 4.2 (B)]. The RFLP analysis using this clone produced unusual results for both mapping populations depending on how the RFLPs were scored and assigned to gene loci.

Thirteen and 31 DH lines from CL x OM and CB x HA mapping populations, respectively, had both parental RFLPs resembling duplicated bands ["D" marks in Fig. 4.2 (A) and (B)]. Also, 22 DH lines from the CL x OM population, and 9 DH lines from the CB x HA population had none of polymorphic parental bands resembling the absence of the polymorphic parental RFLP ["d" marks in Fig. 4.2 (A) and (B)]. These unusual RFLP patterns caused difficulties in mapping work. To reduce ambiguity, polymorphic bands were scored as BCD453-a [the 2nd arrow in Fig. 4.2 (A)], BCD453-b [the 3rd arrow in Fig. 4.2 (A)], BCD453-c [the second arrow in Fig. 4.2 (B)] and BCD453-d [the third arrow in Fig. 4.2 (B)]. Loci were then scored as being either present or absent and the appropriate allocation of "A" and "B" alleles ascribed to each locus. The loci BCD453-a and BCD453-c did not belong to the linkage group containing the $Ha_{(OM)}$ locus, and only BCD453-b and BCD453-d belonged to the linkage group. The number of DH lines were counted, which contain both parental RFLP bands ["D" marks in Fig. 4.2 (A) and (B)], or containing neither of parental RFLPs ["d" marks in Fig. 4.2 (A) and (B)]. The ratios between parental type DH lines ["P" marks in Fig. 4.2 (A) and (B)] containing one of parental alleles and other DH lines indicated by "D" and "d" marks were 41:35 (1:1) in the CL x OM population, and 47:40 (1:1) in the CB x HA population (Table 4.3). This result strongly suggests that the BCD453 probe is associated with two independent loci in

each population. To understand these results, several allelic hypotheses will be discussed.

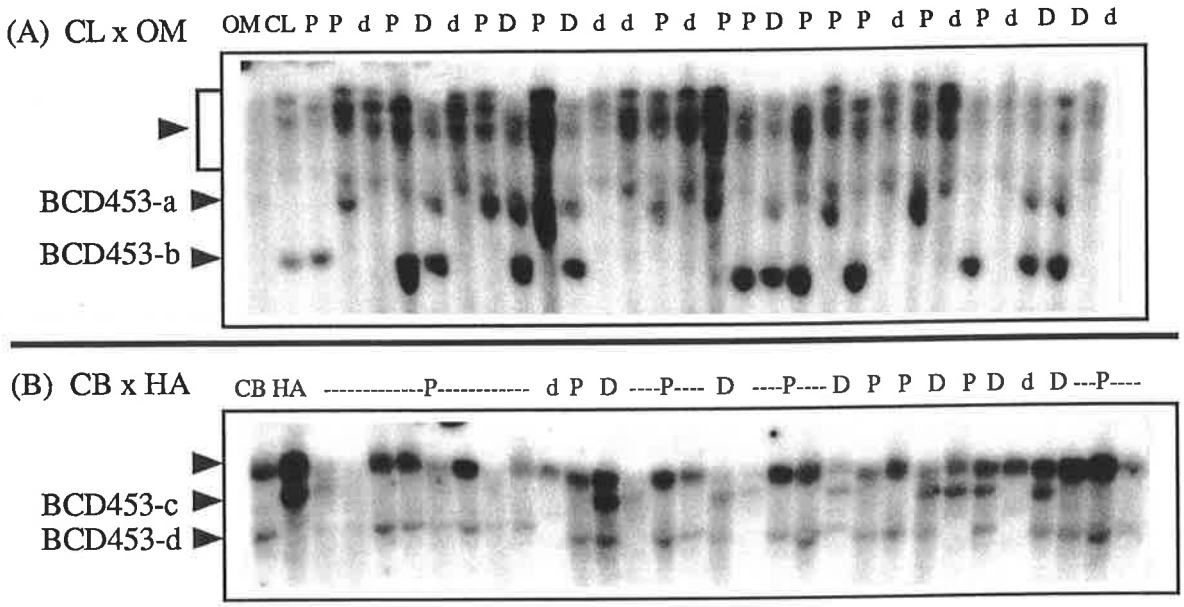


Fig. 4.2 RFLP analysis of two DH populations using BCD453 clone. About 50% of DH lines of both mapping populations had both parental alleles ("D" marks), or none of them ("d" marks). First arrows, common bands between parent cultivars in both mapping populations; BCD453-a, -b -c and-d; each RFLP was scored independently; D, duplication; d, deletion; P, parental type DH lines containing one of parental alleles

The loci of BCD453-b and KsuD22 were genetically linked to the $Ha_{(OM)}$ locus in the CL x OM population (Fig. 4.3). Of these, KsuD22 did not reveal RFLPs between CB and HA with several restriction enzymes used (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI and *Eco*RV). Map distances were variable; the genetic distance between the $Ha_{(OM)}$ locus and the BCD453-b locus was approximately 36 cM in the CL x OM, but was 4.7 cM in the CB x HA population. The $Ha_{(OM)}$ gene was located between the *V/v* and KsuD22 loci on the initial CL x OM map. The genetic distances were 20±4.6 and 29±5.3 cM, respectively (Fig. 4.3).

Table 4.3 The allele ratios of BCD453 probe producing one of parental RFLP bands, and producing duplicated or deleted parental RFLP bands

DH population	No. of plant tested	Number of DH lines	Ratio	χ^2	(df=1)
		P : D/d	P : D/d		
CL x OM	79	41 : 35	1 : 1	0.5	P>0.1
CB x HA	86	47 : 40	1 : 1	0.56	P>0.1

P, one of parental type RFLP bands; D/d, duplicated or deleted parental RFLP bands

4.3.2.2 Final mapping of the CCN resistance gene locus in Orge Martin

For the detailed mapping work with 79 DH lines (CL x OM), seven new RFLP probes (Table 4.4) were selected, that revealed distinct RFLPs between CL and OM. These RFLP probes belonged to a single linkage group, and three of them (MWG503, cMWG694 and AWBMA21) were located between the *V/v* and the $Ha_{(OM)}$ loci (Fig. 4.4). However, no RFLP locus was found between the $Ha_{(OM)}$ and KsuD22 loci. Pairwise genetic distances were measured for marker loci (Table 4.5). AWBMA21 was the closest to the $Ha_{(OM)}$ locus with a separation of 5.5±2.6 cM (Fig. 4.4 and Table 4.5) (mapping data, Appendix 3-a).

The segregation ratios of each allele of CL and OM produced from marker loci (from ABC451 to AWBMA21 in Fig. 4.4), including a morphological marker (*V/v*-2/6 row), gave the expected segregation ratio (1 : 1) of each allele.

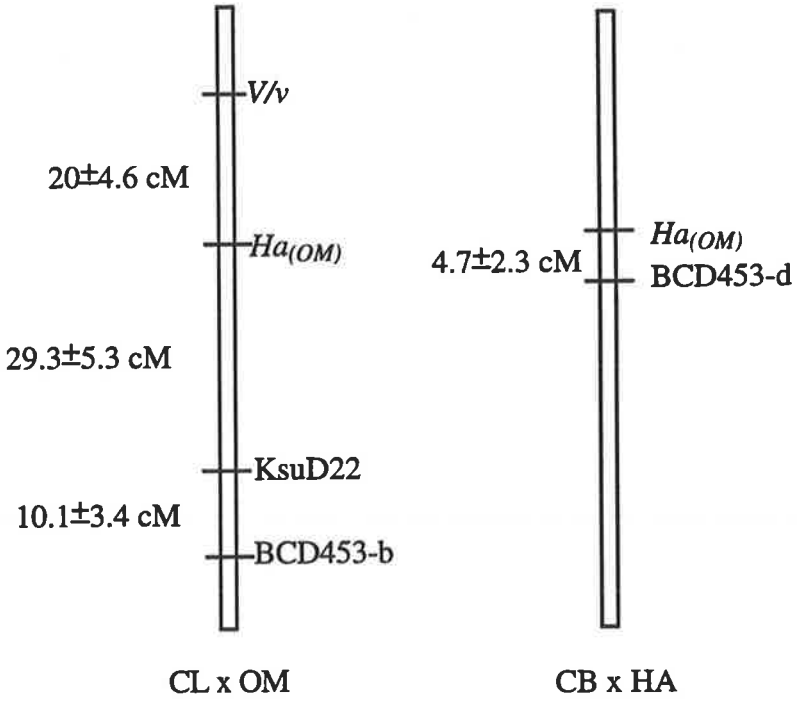


Fig. 4.3 Initial maps of two mapping populations (CL x OM and CB x HA). The $Ha_{(OM)}$ locus was located between the loci of the *V/v* and BCD453-b in a CL x OM map, and a BCD453-d locus closely linked to the $Ha_{(OM)}$ locus in a CB x HA map.

Table 4.4 Restriction enzymes used for the linkage analysis of the CL x OM DH population

RFLP probes	Restriction enzymes
ABC451	*Bam*HI
AWBMA21	*Eco*RI
BCD453-b	*Eco*RI
cMWG694	*Bam*HI
KsuD22	*Eco*RV
MWG503	*Eco*RI
MWG865	*Bam*HI

However, the segregation of both alleles of the *Ha(OM)* gene, KsuD22 and BCD453-b deviated significantly from a 1:1 ratio (Table 4.6). Whilst the distorted segregation ratio of the *Ha(OM)* locus gave an ambiguous result in determining the number of CCN resistance genes with the CCN bioassay (Section 3.3.2), the mapping result obtained showed, however, that there was a single locus for the *Ha(OM)* gene in Orge Martin. The CCN bioassay result from the F_2 population (IN x OM) supported this result, as a 3:1 segregation ratio was obtained (Section 3.3.2).

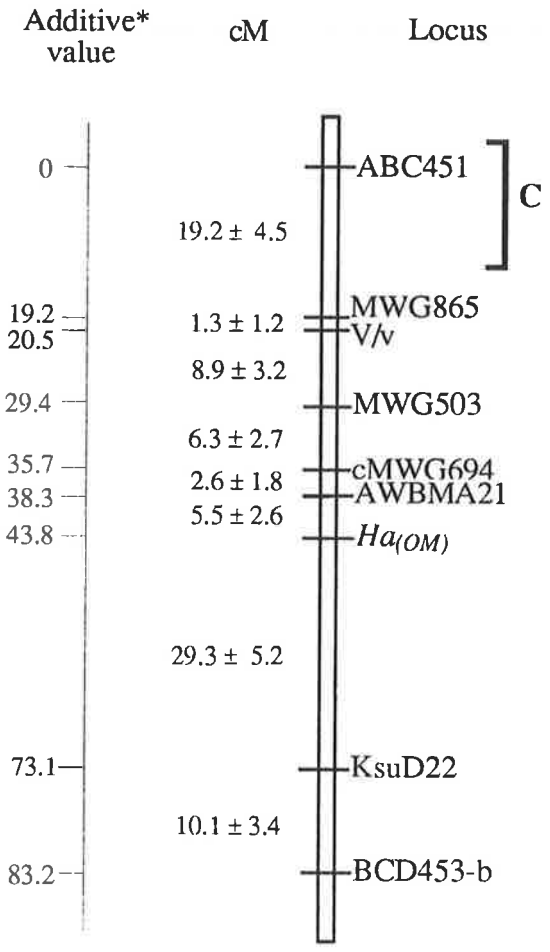


Fig. 4.4 The order of molecular markers around the CCN resistance gene, *Ha(OM)*, on the long arm of chromosome 2 on the CL x OM map. C, centromere region in the current barley molecular map (Kleinhofs *et al.*, 1993); cM, centi Morgan; *, without standard error value

Table 4.5 Approximate genetic distances (cM) between pairwise loci in the OM x CL population

Locus	ABC451	MWG865	*V/v*	MWG503	cMWG694	AWBMA21	*Ha(OM)*	KsuD22	BCD453-b
ABC451	--	19.2	20.5	29.5	33.3	34.2	39.2	42.3	50.0
MWG865		--	1.3	10.3	16.7	17.1	21.6	38.5	46.2
V/v			--	8.9	15.2	15.6	20.0	36.7	44.3
MWG503				--	6.3	6.5	10.7	32.9	43.0
cMWG694					--	2.6	9.3	26.6	36.7
AWBMA21						--	5.5	24.7	35.1
Ha(OM)							--	29.3	36.0
KsuD22								--	10.1
BCD453-b									--

Table 4.6 The segregation ratio of each marker locus around the *Ha(OM)* gene region on the long arm of the chromosome 2 on the CL x OM molecular map

Loci	N**	Expected (CL : OM)	Observed (CL : OM)	χ^2	(df=1)
ABC451	78	1 : 1 (39 : 39)	45:33	1.85	0.5>P>0.1
MWG865	78	1 : 1 (39 : 39)	38:40	0.05	P>0.5
V/v	79	1 : 1 (39.5 : 39.5)	39:40	0.01	P>0.5
MWG503	79	1 : 1 (39.5 : 39.5)	38:41	0.11	P>0.5
cMWG694	79	1 : 1 (39.5 : 39.5)	33:46	2.14	0.5>P>0.1
AWBMA21	77	1 : 1 (38.5 : 38.5)	32:45	2.20	0.5>P>0.1
Ha*	76	1 : 1 (38 : 38)	**26:50**	7.58	**0.005< P<0.01**
KsuD22*	79	1 : 1 (39.5 : 39.5)	**30:49**	4.60	**0.025< P<0.05**
BCD453*	79	1 : 1 (39.5 : 39.5)	**26:53**	9.23	**P<0.005**

* Loci in bold had biased segregation ratios
** Number of plants tested, few lines produced very weak RFLP bands which were difficult to read, or did not have the CCN bioassay result

4.3.2.3 Mapping the CCN resistance locus in Chebec

For the RFLP analysis of CCN resistance in CB, eight RFLP clones including AWBMA21 (described in Section 4.2.4) were selected whose map locations were close to the BCD453, KsuD22 and *V/v* loci on the current barley molecular maps (Graner *et al.*, 1991; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). They were low or single copy probes, and gave distinct single or low number of polymorphisms between CB and HA. The restriction enzymes used for each RFLP probe selected are given in Table 4.7.

Table 4.7 Restriction enzymes used for the linkage analysis of the CB x HA DH population

RFLP probes	Restriction enzymes
ABC451	*Eco*RV
ABG14	*Bam*HI
AWBMA21	*Eco*RV
BCD355	*Eco*RI
BCD453	*Bam*HI
cMWG694	*Eco*RV
MWG503	*Eco*RI
MWG865	*Bam*HI

The full set of RFLP data produced from each RFLP probe, and the results of the CCN bioassay were used for linkage analysis. It was found that all eight RFLP loci tested (ABC451, ABG14, AWBMA21, BCD355, BCD453-d, cMWG694, MWG503 and MWG865) and the CCN resistance gene locus belonged to a single linkage group (Fig. 4.5). Five of the RFLP probes (AWBMA21, BCD453-b, cMWG694, MWG503 and MWG865) were closely linked to the $Ha_{(OM)}$ locus (Fig. 4.5 and 4.6). Three RFLP markers (AWBMA21, cMWG694 and MWG503) were located within 3 cM of the $Ha_{(OM)}$ locus. BCD453-d and MWG865 were within 6 and 7 cM of the $Ha_{(OM)}$ locus, respectively in the CB x HA population (Table. 4.8) (mapping data, Appendix 3-b).

As the CB x HA mapping population was from doubled haploid lines, the expected segregation ratio of each locus was 1:1. However, the observed segregation ratios of all RFLP loci deviated significantly from expected segregation ratios (Table 4.9).

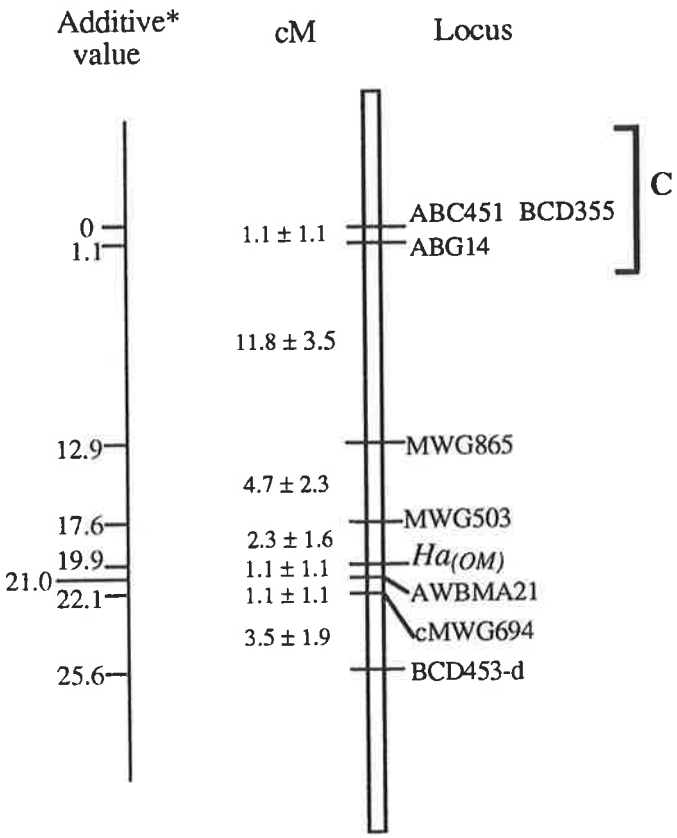


Fig. 4.5 The linear order of molecular markers and the CCN resistance gene, $Ha_{(OM)}$, on the long arm of the chromosome 2 in the CB x HA DH population. The $Ha_{(OM)}$ locus was flanked by three loci, MWG503, AWBMA21 and cMWG694.
C, centromere region; cM, centi Morgan; *, without standard error

Again the biased segregation of the chromosomal region of the $Ha_{(OM)}$ locus caused difficulties in determination of the number of $Ha_{(OM)}$ genes using bioassay data (Section 3.3.2). However, this molecular genetic mapping analysis indicates that there is a single locus in CB which should be the same as the $Ha_{(OM)}$ gene in OM, because the CCN resistance of CB was derived from OM (Sparrow, 1994).

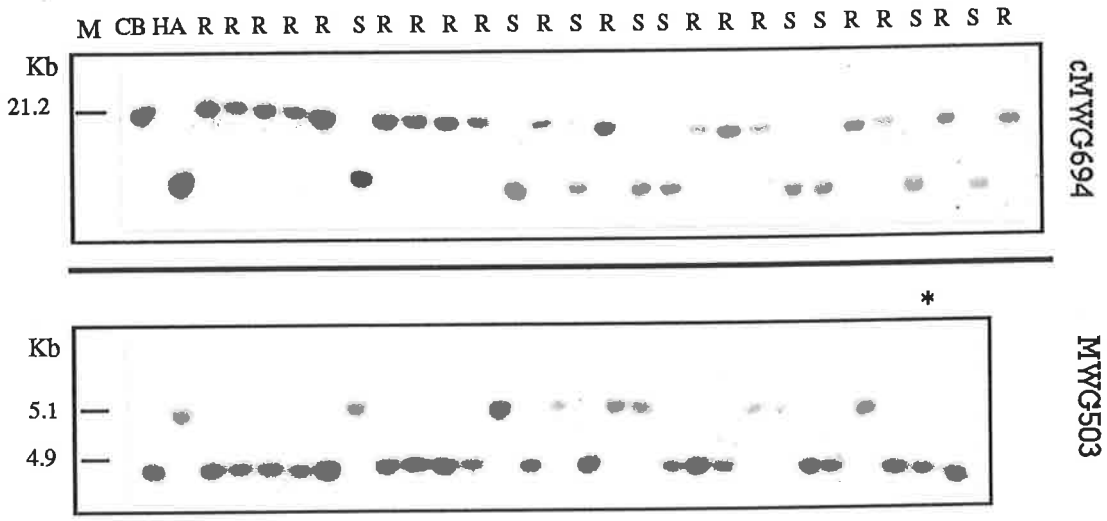


Fig. 4.6 RFLP analysis of DH lines (CB x HA) with clones cMWG694 and MWG503. R/S, CCN bioassay result; R, CCN resistant; S, susceptible; * , recombinant; M, DNA size marker (lambda/*Hin*dIII/*Eco*RI)

Table 4.8 Approximate genetic distances (cM) between pairwise loci in the CB x HA population

Locus	ABG14	MWG865	MWG503	*Ha(OM)*	AWBMA21	cMWG694	BCD453-b
ABG14	--	11.8	15.3	16.5	18.6	17.4	20.0
MWG865		--	4.7	7.1	5.9	8.1	11.8
MWG503			--	2.3	2.3	3.5	8.1
Ha(OM)				--	2.3	1.2	5.8
AWBMA21					--	1.2	5.8
cMWG694						--	4.6
BCD453-b							--

Table 4.9 Distorted segregation ratios of RFLP markers around the *Ha(OM)* gene region on the long arm of barley chromosome 2 in the CB x HA population

Loci	N**	Expected (CB : HA)	Observed (CB : HA)	χ^2	(df=1)
ABC451	86	1 : 1 (43 : 43)	**63 : 23**	18.6	P<0.005
ABG14	86	1 : 1 (43 : 43)	**64 : 22**	20.5	P<0.005
AWBMA21	87	1 : 1 (43.5: 43.5)	**66 : 21**	23.3	P<0.005
BCD355	88	1 : 1 (44 : 44)	**64 : 24**	18.2	P<0.005
cMWG694	88	1 : 1 (44 : 44)	**66 : 22**	22.0	P<0.005
Ha(OM)	87	1 : 1 (43.5: 43.5)	**65 : 22**	21.3	P<0.005
MWG503	87	1 : 1 (43.5: 43.5)	**66 : 21**	23.3	P<0.005
MWG865	86	1 : 1 (43 : 43)	**62 : 24**	16.8	P<0.005

* All loci in bold had biased segregation ratio.
** One or two lines among 88 DH lines produced very weak RFLP bands which were difficult to read, or did not have the CCN bioassay results

4.3.2.4 Variations of linear orders and map distances

The two genetic maps constructed were compared to each other. The linear order of marker loci in the CL x OM map was the same as that of the CB x HA map except for the location of the *Ha(OM)* locus (Fig. 4.7). The *Ha(OM)* locus in the CL x OM map was located between AWBMA21 and KsuD22 (Fig. 4.4 and 4.6). However, the locus in the CB x HA map was flanked by MWG503 and AWBMA21 (Fig. 4.7). Three closely linked loci, cMWG694, AWBMA21 and *Ha(OM)* showed a reversed order in the two best ordered molecular maps (Fig. 4.7).

Another difference was the map distances between marker loci. The CL x OM map had more expanded map distances between adjacent loci than did the CB x HA map. The map distance of the *Ha(OM)* locus from each of the three loci, AWBMA21, cMWG694 and MWG503 was 5.5, 9.3 and 10.7 cM, respectively on the CL x OM map (Table 4.5), but on the CB x HA map these were 2.1, 1.1 and 2.3 cM,

respectively (Table 4.8). The sum of map distances of the six common loci including the $Ha_{(OM)}$ locus was 43.7 cM on the CL x OM map, and 21.7 cM on the CB x HA map (Fig. 4.7).

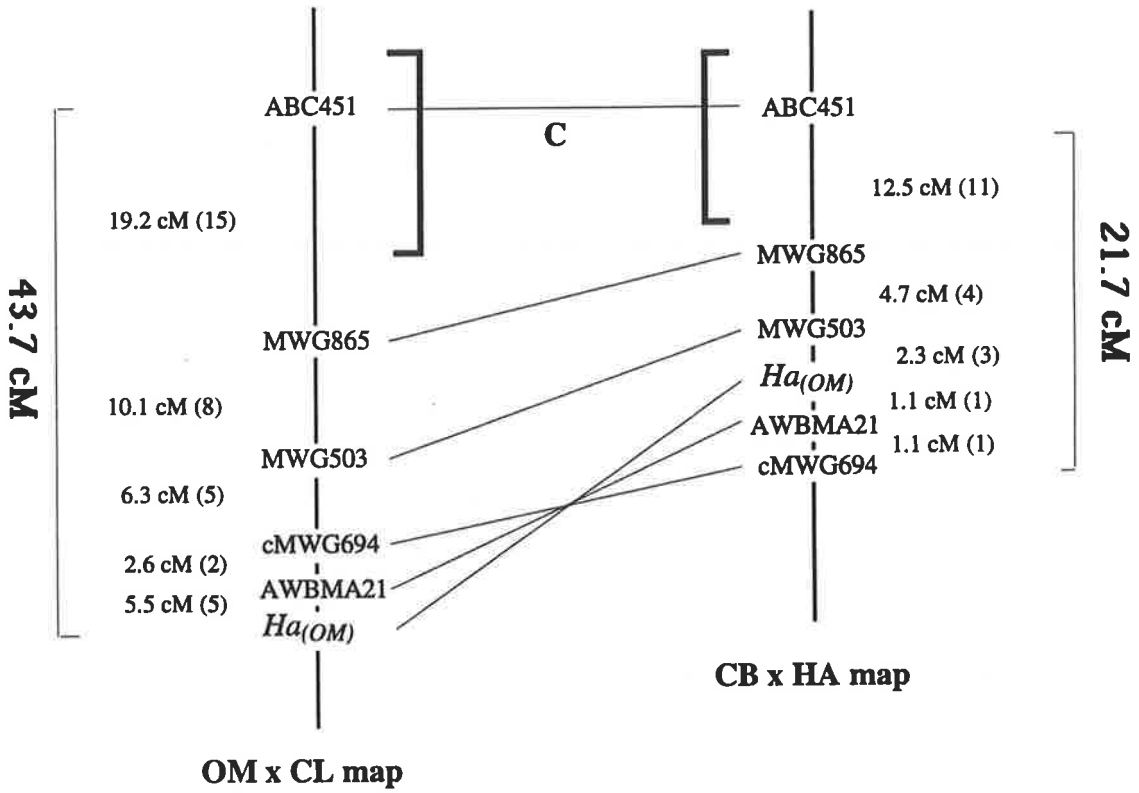


Fig. 4.7 Variations of genetic distances between marker loci. Two maps had reversed map locations of cMWG694 and $Ha_{(OM)}$ loci. The OM x CL map had more expanded map distances than that of the CB x HA map. (n), no. of recombinants between marker loci; C, centromere region

4.4 Discussion

The mapping analysis for tagging the CCN resistance gene was complicated. During the mapping analysis, several conflicting and ambiguous results were obtained including distorted segregation ratios, the determination of the number of the $Ha_{(OM)}$ genes, variation in the linear map order and variation in the map distances. Likely

alternate hypotheses are proposed for understanding results obtained, and the possible usefulness of RFLP markers investigated is discussed.

4.4.1 Selection of RFLP clones for tagging the $Ha_{(OM)}$ gene

A morphological character, *V/v* (2/6-row head types) in OM was established as having linkage to the $Ha_{(OM)}$ locus with the 79 DH lines (CL x OM) in the previous experiment (Chapter 3). This linkage provided the starting point for the RFLP mapping work. As the *V/v* gene has been located on the long arm of chromosome 2 at approximately 20 to 25 cM from the centromere (Kasha and Burnham, 1965), several RFLP clones (Table 4.1) were selected, which were distributed from the proximal region to the distal region of the long arm of chromosome 2.

The map distance between the two loci *Ha* and *V/v* (Section 3.3.3) was approximately 20 cM, which implied that the *Ha* locus could be located near the centromere or on the long arm. It was also possible that the *Ha* locus could also have been located on the short arm, as the centromere region has been shown to undergo few recombinations and therefore there are short map distances between loci in its vicinity (Graner *et al.*, 1991). To simplify the mapping work, an RFLP probe (BCD453) was selected from the proximal region, and another RFLP probe (KsuD22) was selected from the distal region of the long arm of barley chromosome 2. These probes established the initial linkages to the $Ha_{(OM)}$ gene, and provided the direction of further mapping work.

4.4.2 Variation in RFLP mapping work

Both molecular maps constructed had distorted segregation alleles for all or some of the loci assayed. The reason for the distorted segregation ratios could be the result of preferential survival of gametes during anther culture (Graner *et al.*, 1991; Logue *et al.*, 1995). Biased segregation ratios can be a difficulty for the determination of the number of target genes. The result of the CCN bioassay implied the presence of two

independent CCN resistance genes or two loci in CB. However the use of molecular markers provided accurate information for the determination of the number of the *Ha(OM)* loci. The result of this study strongly support the conclusion that both OM and CB have a single locus for resistance to the Australian CCN pathotype.

To determine the likely linkage orders, trial maps were tested without the *Ha(OM)* locus as the determination of it's map location was most critical. The trial linkage order of the *Ha(OM)* region in the CL x OM population was [- MWG503 - cMWG694 - AWBMA21 - KsuD22 - BCD453 -]. The likely position of the *Ha(OM)* locus was between AWBMA21 and KsuD22 loci, such as it presented in Fig. 4.4, which produced the least number of crossing-overs. The CB x HA trial linkage map had the order of [- MWG503 - AWBMA21 - cMWG694 - BCD453 -], that had the least number of recombinants. There were three possible positions of the *Ha(OM)* locus (A, B and C) (Fig. 4.8). All of these are equally likely and consistent with having the least number of crossovers. However, placing *Ha(OM)* at any of these loci (A, B and C), produced double or triple cross-overs in the *Ha(OM)* region for mapping lines #26 and #54 . To simplify ambiguous map orders, these two mapping lines were removed from the mapping population (CB x HA), and the likely map order was produced from RFLP data only. Then #26 and #54 were replaced and the final likely map order obtained was [- MWG503 - *Ha(OM)* - AWBMA21 - cMWG694 -], such as the map presented in Fig. 4.9. Determination of the map order of the three closely linked loci [AWBMA21, cMWG694 and *Ha(OM)*] was ambiguous. Although the suggested map (Fig. 4.5 and 4.9) had the likely map order, slight variation of the linear order will occur due to random chance, either by changing the number of test plants, or by using different mapping populations.

The two maps constructed had different map orders for three closely linked loci. The CL x OM map showed [- *Ha(OM)* - AWBMA21 - cMWG694 -] compared to [-

cMWG694 - AWBMA21 - $Ha_{(OM)}$ -] in the CB x HA map. One likely reason for these different map orders could be a paracentric inversion of three loci in the CB x HA population. If an inversion containing a crossover between paired homologs of a heterozygote containing inverted gene orders occurred, the linear map order [- $Ha_{(OM)}$ - AWBMA21 - cMWG694 -] can become [- cMWG694 - AWBMA21 - $Ha_{(OM)}$ -] (Suzuki *et al.*, 1989). The presence of inversions would cause ambiguous linkage orders during mapping work because of the presence of two different types of viable gametes carrying genes in reversed orders (Powell and Nilan, 1968; Suzuki *et al.*, 1989). It was also possible that the classification of CCN resistance/susceptibility was incorrect for one or a few lines, which might also cause the ambiguous mapping result in the $Ha_{(OM)}$ region of the CB x HA population.

Another distinct difference between the two molecular maps (CL x OM and CB x HA) was the map distances between adjacent loci. The CL x OM map had an expanded map distance compared to that of the CB x HA map. The sum of the map distance of common six loci located on both maps was about 43.7 cM [from ABC451 to $Ha_{(OM)}$] in the CL x OM map, and about 21.7 cM [from ABC451 to cMWG694] on the CB x HA map (Fig. 4.7). With markers separated by 1 or 2 cM, the population size of the DH population used was not sufficiently large. Two hundred DH lines at least would be required to get the resolution and the order of the markers used in this study. Some ambiguous results and different map orders between the two maps could be due to the use of the smaller population size.

The reason for different map distances could be explained by the presence of an inversion loop on the CB x HA map. As the inversion loop can restrict neighbour chiasmata (Lukaszewski and Curtis, 1993), the chromosomal region containing the inversion has fewer recombinations producing shorter map distances than expected (Powell and Nilan, 1968; Ramage and Suneson, 1961). This could be the reason

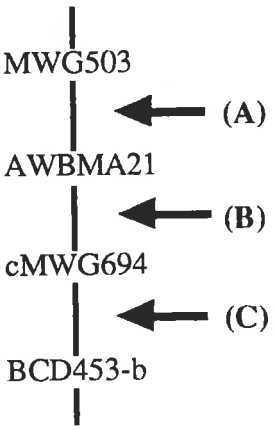


Fig. 4.8 Three possible map positions (A, B and C) of the $Ha_{(OM)}$ locus . These three possible map positions produced the least number of cross-overs. however, double or triple cross-overs were detected.

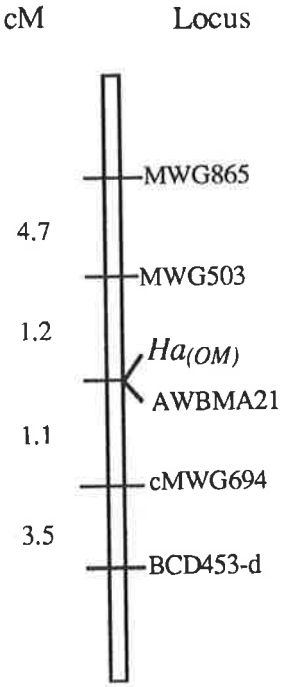


Fig. 4.9 The simplified linkage order in the CB x HA population. To determine the likely map position of the $Ha_{(OM)}$ locus, two mapping lines, #26 and #54 were removed, which caused ambiguous map orders.

why the CB x HA map had shorter map distances than those of the CL x OM map. Two loci, BCD453-b and BCD453-d may be the same locus, because the RFLP data of these two loci were produced from a single probe (BCD453), and the two loci were linked to the $Ha_{(OM)}$ gene. As for the other markers, the map distance between

BCD453-d and $Ha_{(OM)}$ in the CB x HA population is shorter than that between BCD453-b and $Ha_{(OM)}$ in the CL x OM population, because of the putative inversion in the CB x HA population. An inversion typically results ambiguous map orders and reduced map distances (Powell and Nilan, 1969; Suzuki *et al.*, 1989).

About 50% of DH lines in both mapping populations produced what could have been interpreted as duplicated parental alleles or none of the polymorphic parental alleles with the BCD453 clone (Fig. 4.2). This result could be explained by a non-reciprocal crossover (unequal crossover). When a non-reciprocal crossover has occurred between homologs during meiosis, four types of gametes can be produced, two of them are parental types, and the third one has duplicated alleles (duplication), and the fourth one has none of parental alleles (deletion). As a result of a non-reciprocal crossover, about 50% of progenies have one of parental alleles (parental type), and other 50% of progenies have duplicated or none of parental alleles (rearranged type) (Hagberg, 1986; Suzuki *et al.*, 1989). The result obtained in this study showed the 1:1 ratio between the number of parental type progenies and rearranged progenies (Section 4.3.2.1 and Table 4.3). Usually, deletions cause inviable gametes or sterile/non-viable plants (Hagberg, 1986), but both mapping populations had common alleles [the first arrow in Fig. 4.2 (A) and (B)], which may lie in a region that is essential for the viable plants. Usually duplications and deletions occur from the same event, and the duplication is an important chromosomal rearrangement in genome evolution (Suzuki *et al.*, 1989).

Another possible explanation was the independent chromosomal locations of both loci, BCD453-a and -b for the CL x OM population, and BCD453-c and -d for the CB x HA population. During the evolution of barley, chromosomal rearrangements such as translocations and duplications are common (Hagberg, 1986). If parental plants of both mapping populations had different loci of the BCD453 clone on different

chromosomes or arms because of a chromosomal rearrangement, RFLP analysis using this probe would produce independent map positions or different linkage groups of both loci, BCD453-a and -b for the CL x OM population, and BCD453-c and -d for the CB x HA population, as was obtained in this study. As this study was focused on the long arm of chromosome 2, the map location of BCD453-a and BCD453-c were not identified. RFLP bands of four loci (BCD453-a, -b, -c and -d) should be cloned and sequenced to know whether these loci are same or different. A similar result was reported in maize (Murigneux *et al.*, 1993). Two of 58 maize DH lines produced by anther culture had unexpected RFLP bands. One of two DH lines had both parental bands, and another had a non-parental band. Murigneux *et al.* (1993) suggested several hypothetic explanations, such as DH lines produced from diploid heterozygous cells, contamination of pollen, modification of DNA during anther culture and recombination between two restriction sites. In this study, however, the most likely hypotheses is a randomised chromosomal rearrangement resulting in the Mendelian segregation ratio such as described above for unequal crossover (Table 4.3).

The third possible explanation is a translocation on the long arm of chromosome 2. However, this possibility has little likelihood, because the distal and telomere region of the long arm of the chromosome 2 of the CB x HA map (constructed by Dr. P. Langridge *et al.*) include map locations of other RFLP clones whose molecular map locations are on the long arm of chromosome 2 in other current barley molecular maps (pers. commun. by Dr. P. Langridge *et al.*). The map position of BCD453 clone identified in a previous study is on the proximal region of chromosome 2 (Heun *et al.*, 1991), but it is far from the centromere region in this study.

4.4.3 The possible utility of RFLP markers identified in this study

Previous studies have suggested that barley has at least three, possibly five different loci encoding resistance to the Australian CCN pathotype. The use of closely linked

molecular markers for the *Ha(OM)* gene can be a useful tool for understanding the relationship of these different loci. It may be useful to apply all of the closely linked six RFLP markers (AWBMA21, BCD453, cMWG694, KsuD22, MWG503 and MWG865) for the screening of CCN resistance in the barley breeding programs using various test lines, since the map distances showed significant differences between crosses (Säll, 1990).

The genes for resistance to plant diseases are often located in the same chromosomal region rather than being randomly scattered on the whole genome (Jahoor *et al.*, 1993). The RFLP clone MWG503 was linked to the likely position of a CCN resistance gene, *Ha2* with the use of DH lines (Alf x Vogelsanger Gold) (Giese *et al.*, 1993). The *Ha(OM)* gene and the *Ha2* gene may be closely linked to each other. Also, a molecular marker MWG503 was linked to the powdery mildew resistance gene, *Ml(La)*, and linked to the gene for stripe mosaic virus (*Sm*) which are located on the long arm of barley chromosome 2 (Freialdenhoven *et al.*, 1994; Giese *et al.*, 1993). As the long arm of barley chromosome 2 has several disease resistance genes [*Ha(OM)*, *Ha2*, *Ml(La)*, and *Sm*], and as these genes have established chromosomal linkages to one another, the use of several molecular markers for the *Ha(OM)* gene obtained in this study can be useful for the barley breeding program in testing for incorporation of several disease resistance genes into the one genetic background.

In a barley molecular map (Graner *et al.*, 1991), an RFLP clone MWG87 is located between the two loci, MWG503 and cMWG694. In the map, the genetic distances of MWG87 from MWG503 and cMWG694 are 2.1 cM and 1.8 cM, respectively. These genetic distances are almost the same as those between MWG503, *Ha(OM)* and cMWG694 obtained in this study. Unfortunately, MWG87 was not available during this study, and therefore could not be tested. The establishment of the linkage of this clone to *Ha(OM)* is required.

Chapter 5 Allele specific PCR markers of the cereal cyst nematode (*Heterodera avenae* Woll.) resistance gene in barley

5.1 Introduction

For effective screening in breeding programs, marker assisted selection is highly desirable (Knapp, 1994). Recently developed molecular marker based screening methods using numerous markers derived from the whole genome are accurate and informative unlike those using phenotype markers representing only a small part of the genome (Laurie *et al.*, 1992). Two types of molecular markers have been used routinely, RFLPs and PCR based markers (Young, 1994).

The RFLP marker system requires precise technical skills in several sequential processes, and often involves the use of radioactive labelled probes (Ragot and Hoisington, 1993). As the RFLP method is based on the detection of single or low copy sequences from the whole genome, this method needs large amount of genomic DNA, a few hundred micrograms (Whitkus *et al.*, 1994). For this amount of genomic DNA, growing green plants is an essential step. This is a time consuming and costly process. These complexities could be the limiting factors for the general use of the RFLP technique in breeding programs. The use of a rapid and simple screening method is essential for handling thousands of test plants (Young, 1994).

As an alternative strategy, the PCR based method has recently been developed (Paran and Michelmore, 1993; Tragoonrung *et al.*, 1992; Williams *et al.*, 1990; Williamson *et al.*, 1994). In general, PCR based molecular markers require less labour, with, for example, no library construction necessary. They save time, and are easy to handle (Whitkus *et al.*, 1994). PCR can amplify specific target sequences from small amounts of template DNA (a few nanograms) (Caetano-Anollés, 1994) which makes

the PCR approach suitable for any stage of a breeding program, as small amounts of template DNA can be prepared from most mature plant tissues including seeds (Guillemaut and Maréchal-Drouard, 1992). The PCR method using DNA extracted from seeds (Kamalay *et al.*, 1990) is useful in reducing the time, labour and costs associated with the glass house work required for handling large number of samples (Young, 1994). Pre-screening with molecular markers for the target gene can reduce the number of test plants to be grown in breeding trials. Also, the immediate selection of homozygous alleles of the target gene from a heterogeneous population of heterozygous and homozygous individuals is possible.

The specificity of the PCR reaction allows allele specific amplification separating a single base difference between the sequence alleles (Huang *et al.*, 1992; Kwok *et al.*, 1990; Newton *et al.*, 1989; Wu *et al.*, 1989), and this specific discrimination can be used for the identification of genetic differences amongst genetically closely related plant materials which often contain few RFLPs. An allele specific PCR method of screening for a marker linked to CCN resistance has been demonstrated already for wheat (Williams, 1994).

As CCN causes serious economic losses in the southern Australian cereal growing areas (at least $US 70 million), developing CCN resistant cultivars is one of the major aims of cereal breeding programs (Sparrow and Dubé, 1981). The current screening method, the CCN bioassay, is effective for the screening of CCN resistant and susceptible lines. However, this bioassay is tedious (a few hundred test plants must be grown for 3-4 months) and laborious, and needs a controlled environment (Fisher, 1982b). This does not allow for mass screening in the limited time often required by breeding programs. However, a screening method using closely linked PCR markers will enable an efficient and rapid mass screen of the large numbers necessary for the selection of CCN resistant breeding lines in the time frame of a breeding program.

The aim of the research described in this chapter is to:

- develop a PCR based marker system for the CCN resistance gene, $Ha_{(OM)}$

5.2 Materials and methods

This experiment was conducted to convert an RFLP clone, AWBMA21 to a pair of allele specific PCR markers for the CCN resistance gene. In brief, the strategy used in this experiment was:

1) Sequencing of an RFLP clone; AWBMA21.

2) Design of PCR primers (BM21A and BM21B) from the sequence of the AWBMA21 clone to amplify specific DNA fragments (the 1st round PCR) from genomic DNA of Chebec (CB) and Harrington (HA), then subcloning amplified fragments.

3) Sequencing of subcloned fragments (CBM21, a clone containing the 1st round PCR fragment amplified from genomic DNA of CB; HAM21, a clone containing the 1st round PCR fragment amplified from genomic DNA of HA).

4) Finding a sequence difference between CBM21 and HAM21. In this experiment, a single base difference was found at the 14th nucleotide from the BM21A primer site.

5) Design of allele specific PCR (AS PCR) primers (BM21ICB and BM21IHA), which contain the single base difference between the CB and HA sequence at the 3' ends of each AS PCR primers.

6) The 1st round PCR; amplification of specific PCR fragments with BM21A and BM21B from genomic DNA of CB, HA and 88 DH lines (CB x HA), which will be used for the 2nd round PCR as template DNA.

7) Screening 88 DH lines with two AS PCR primer sets (BM21ICB/BM21B and BM21IHA/BM21B) using the first round PCR products as template DNA.

Detailed procedures are described in this Chapter, unless otherwise specified.

5.2.1 Plant materials

The barley cultivars, CB and HA, and 88 doubled haploid (DH) lines derived from the F_1 of the cross (CB x HA) (Section 2.1.3) were used in this study.

5.2.2 DNA extraction and CCN bioassay

The procedure for barley DNA extraction was described in Section 2.4.2. The procedure for the CCN bioassay was described in Section 2.2; the same results were used.

5.2.3 Sequencing, PCR reaction and molecular techniques

5.2.3.1 Sequencing of an RFLP clone AWBMA21

The RFLP clone AWBMA21 (barley cDNA clone from Galleon cDNA library) was sequenced with Version 2.0 T_7 DNA polymerase (DNA sequencing kit; USB, U.S.) using the principle of chain-terminating inhibitors (Sanger *et al.*, 1977). The sequencing reaction for double-stranded DNA templates followed the protocol of the "DNA sequencing kit". Five µg of plasmid DNA containing the insert was denatured by adding 0.1 volume of (2M NaOH/ 2mM EDTA), and incubated at 37°C in a water bath for 30 min. The denatured plasmid was neutralised by adding 0.1 volume of 3M Na-acetate (pH 4.8), and precipitated with two volumes of ethanol at -80°C for 20 min., followed by centrifuging (13,200 rpm) in a cold room for 10 min. The air dried DNA pellet was resuspended in 7 µl of nanopure water, and kept at -20°C until used. Two µl of 5X "Sequenase" buffer and 1 µl (1 µg) of one of the M13 primers were

added to the tube containing denatured plasmid DNA (total volume, 10 µl). A tube containing 10 µl of mixture was placed at 65°C in a water bath for 2 min., and slowly cooled to room temperature over 20 min. for the annealing between the denatured plasmid and the M13 primer. Annealed plasmid DNA was kept on ice until it was used for the labelling reaction. While the tube was cooling down, four micro-centrifuge tubes (0.5 ml) containing 2.5 µl of each ddA, ddC, ddG and ddT termination mixture (DNA sequencing kit) were prepared and kept at 37°C in a water bath for 5-10 min. To the annealed plasmid, 1 µl of 0.1 M DTT, 2 µl of labelling mix (DNA sequencing kit), 0.5 µl of [^{35}S]dATP and 2 µl of diluted Sequenase [1µl of Version 2.0 T_7 DNA polymerase in 7 µl of enzyme dilution buffer (DNA sequencing kit)] were mixed (total volume, 15.5 µl) and incubated at room temperature for 5 min.. An aliquot (3.5 µl) of labelling reaction solution was transferred to each of termination mixture, and the four tubes containing 3.5 µl of labelling reaction and 2.5 µl of termination mixture were incubated at 37°C in a water bath for 5 min., then 4 µl of stop solution (DNA sequencing kit) was added to each tube, and reactions were stored at -20°C.

5.2.3.2 Denaturing gel electrophoresis

Each sequencing reaction tube was placed in a water bath at 70°C for three min. to denature the products of the sequencing reaction, and then kept in iced water until it was loaded onto the vertical sequencing gel. The denaturing gel for the electrophoresis of the denatured sequencing product was prepared with the recipe as follows; 100 ml of 6% acrylamide gel solution was prepared (acrylamide, 5.7g/ bis-acrylamide, 0.3g/ urea, 42g/ 10X TBE, 10 ml/ formamide, 40 ml/ H_2O to make total volume, 100 ml). To this gel solution, 1 ml of 10% APS and 150 µl of TEMED were added, and this mixture was poured between gel forming glass plates. After the gel was set (thickness, 0.4 mm), the gel was pre-run for 45-60 min. at current of 27-30

mA to raise the temperature of the gel plate to 45-50°C. A volume of 0.5 µl of each of the denatured sequencing products was loaded onto the gel. The sample loading order was A, C, G and T. The gel was run at 27-30 mA (approximately 2,000 volt.) with 1X TBE running buffer until the green dye (xylene cyanol FF) was 4/5 of the way down of the gel. During the gel running, the temperature of the gel plate was 45-50°C. After the gel had run, one of the glass plates was removed, and the gel was placed in 5% acetic acid/15% methanol solution for 10 min. to remove urea, followed by drying. The gel was dried in a slab gel drier (Bio-RAD, U.S.) at 80°C for 45-60 min. An auto radiography film (Hyper film, Amersham, U.K.) was placed on the dried gel and left at room temperature overnight to expose.

5.2.3.3 Design of PCR primers (BM21A and BM21B)

After reading the complete sequence of AWBMA21 (BM21) clone, PCR primers, BM21A (5' TAAAAGTAGCAACAA-15 mer) and BM21B (5' ATCTTTAGCGTGGTCTT-17 mer) were selected with "Oligo (Version 4.0)" software (National Bioscience, U.S.) from both ends of the RFLP clone AWBMA21. These primers were synthesised by MACROMOLECULAR RESOURCES (Colorado State Uni., U.S.).

5.2.3.4 PCR reaction with BM21A and BM21B (the 1st round PCR)

Specific fragments were amplified with the PCR primers (BM21A and BM21B) from CB, HA and the 88 DH lines. The 1st round PCR was carried out with 0.1 volume of reaction buffer/ 1.5 mM $MgCl_2$/ 200 µM of each dNTPs/ 0.5 µg of each primers/ 1 unit of *Taq* polymerase/ 100 ng of template DNA in 25 µl reaction volume. The program for the amplification reaction was; [(1) 94°C for 5 min.; (2) 94°C for 2 min.; (3) 60°C for 1 min.; (4) 72°C for 30 seconds; (5) go to step (2), 35 more cycles; (6) 72°C for 1 min.] on a MJ Research thermal cycler. *Taq* DNA polymerase, 10X

reaction buffer and $MgCl_2$ (25 mM) were purchased from Advanced Biotechnologies (U.K.).

5.2.3.5 Subcloning of PCR products of Chebec and Harrington from the 1st round PCR

After electrophoresis of the 1st round PCR products of CB and HA, bands of interest from CB and HA were isolated from a 3% metaphor agarose gel (FMC, U.S.), and purified with the "Gene clean" kit (Bio 101, U.S.), as described in Section 2.4.4. As *Taq* polymerase showed a tendency to attach a single nucleotide "A" (adenosine) at the 3' end of amplified fragments (Clark 1988; Marchuk *et al.*, 1990), "T-vector" was prepared for subcloning of the "gene-cleaned" PCR products. Preparation of "T-vector" followed the protocol described by Marchuk *et al.* (1990). Five µg of Bluescript (Stratagene, U.S.) plasmid was digested with *Eco*RV restriction enzyme (Promega, U.S.) that produces blunt ends, and "T-tailed" with 5 units of *Taq* polymerase using 2 µl reaction buffer and including 2 mM dTTP (total reaction volume, 20 µl) at 72°C for 2 hours. T-tailed vector was purified by phenol extraction, precipitated, and air dried. The DNA pellet was resuspended in nanopure water to a final concentration of 200 µg/ml, and 1 µl was used for the ligation.

5.2.3.6 Ligation

T-vector (200 ng) and insert fragments (3-5 ng) were ligated. The procedure for ligation was described in Section 2.4.5.

5.2.3.7 Preparation of competent cells

The principle of transformation of *E. coli* strain DH5α followed the protocol described by Sambrook *et al.* (1989). A DH5α glycerol stock was streaked on an LB plate (90 mm), and incubated at 37°C overnight. A single colony was used to inoculate 25 ml

of LB medium, and incubated at 37°C overnight with shaking (200 rpm). Five ml of cultured cells were transferred to a 2L flask containing 500 ml of fresh LB media, and incubated with shaking (200 rpm) at 37°C until $O.D._{600}$ of 0.45-0.55 was reached. The cells were chilled on ice for 2 hours, then pelleted by centrifugation (2,500 g) for 20 min. in a cold room (4°C). The bacterial cell pellet was resuspended in 10 ml of trituration buffer, and diluted to 500 ml with trituration buffer. The diluted solution was kept on ice for 1 hour. Bacterial cells were collected by centrifugation (1,800 g) for 10 min. in a cold room, and resuspended in 50 ml of ice-cold trituration buffer. 80% glycerol was added to a final concentration of 15% (v/v). Aliquots (0.2 ml) were stored at -80°C.

5.2.3.8 Transformation and selection of transformed colonies

A tube containing competent cells was thawed on ice, then 3 µl of DMSO and 2 µl of ligation product were added. The tube was kept on ice for 30 min. and then subjected to heat shock at 42°C for 1 min. After heat shock, 1 ml of LB medium was added, and the bacterial cells were incubated with shaking (200 rpm) for 90 min. An aliquot (100 µl) was plated onto an LB plate (90 mm) containing ampicillin (50 mg/ml) overlaid with 40 µl of 2% X-gal, and 4 µl of 0.1M IPTG. After incubation at 37°C overnight, a white colony was selected and cultured in 5 ml of LB medium with shaking (200 rpm) at 37 °C overnight. An aliquot (0.5 ml) of the cultured cells was mixed with an equal volume of glycerol solution in a fresh 1.5 ml micro-centrifuge tube, and kept at -80°C.

5.2.3.9 Sequencing of subcloned PCR fragments and design of PCR primers (BM21ICB and BM21IHA) for the 2nd round PCR

Subcloned PCR fragments of CB (CBM21) and HA (HAM21) were sequenced with the "DNA sequencing kit" (USB). Sequencing of clones CBM21 and HAM21

revealed a single base difference between CB and HA (Fig. 5.1). Two AS PCR primers containing a single base difference at the 3' end (underlined) [BM21ICB (contained internal sequence of CBM21, 5'-ACCGAAGAAGAAGAA-15 mer); BM21IHA (contained internal sequence of HAM21), 5'-ACCGAAGAAGAAGAG-15 mer] were synthesised by MACROMOLECULAR RESOURCES (Colorado State Uni., U.S.). When two pairs of AS PCR primer sets (BM21ICB/BM21B and BM21IHA/BM21B) were used, the expected size of the PCR product was 169 bp.

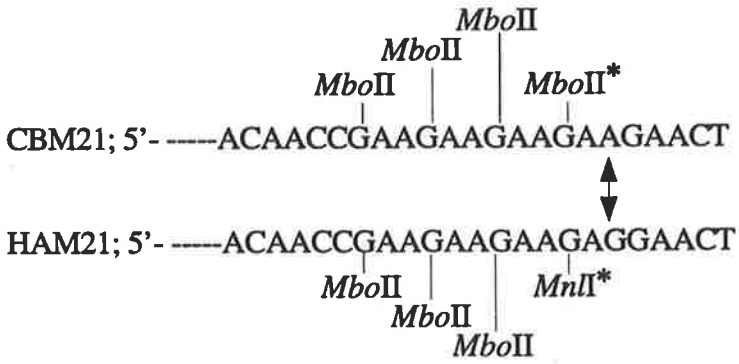


Fig. 5.1 A single base difference between CBM21 and HAM21 (double head arrow), and this difference produced a different restriction sites, *Mbo*II on the CBM21 and *Mnl*I on the HBM21 (asterisk marks). Three more *Mbo*II sites were recognised.

5.2.4 Screening of 88 DH lines

The 1st round PCR products of the 88 DH lines were diluted to 1/100, and 1 µl was used as the template DNA for the 2nd round PCR using two AS PCR primer sets (BM21ICB/BM21B and BM21IHA/BM21B). The reaction conditions for the 2nd round PCR were the same as for the 1st round PCR except for the template DNA.

5.2.5 Electrophoresis of PCR products

After the 2nd round PCR, 5-7 µl of PCR product was loaded onto a 5% non-denaturing polyacrylamide gel (Sambrook *et al.*, 1989) in a "Mighty Small II" gel electrophoresis unit (Hoefer Scientific, U.S.). 1X TBE running buffer was used with a current of 90 volts. for the one gel and 130 volts. for two gels. The gel was electrophoresed until the green dye was 2/3 of the way down the gel. The gel was stained with ethidium bromide solution (0.5 µg/ml) (Sambrook *et al.*, 1989).

5.2.6 Measurement of the genetic distance

The genetic distance between the PCR markers and the gene for CCN resistance was estimated using "Map Manager (Version 2.6)" mapping software (Manly and Cudmore, 1994), at a confidence level of 95%. For the use of this software, alleles of CB and HA produced from the second round PCR reaction were designated "A" and "B", respectively; and CCN resistant lines were "A" and susceptible lines were "B".

5.3 Results

An RFLP clone, AWBMA21 whose location was closest to the location of the CCN resistance gene on both molecular maps was sequenced to design PCR primers. The size of AWBMA21 was 272 bp (Fig. 5.2). A PCR primer (BM21A) was designed from the 53rd nucleotide of the 5' end of the upper strand, and another PCR primer (BM21B) was designed from the 38rd nucleotide of the 5' end of the lower strand (Fig. 5.2).

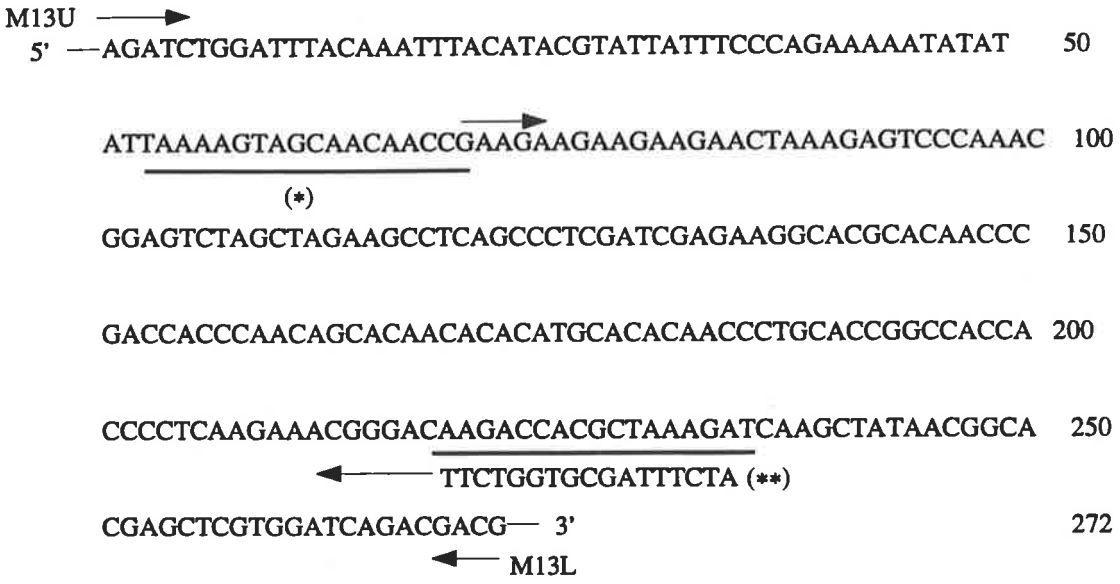


Fig. 5.2 The full sequence of a RFLP clone AWBMA21, and the sites of primers (BM21A and BM21B). BM21A is 15 nucleotides long, and BM21B is 17 nucleotides long. The expected size of the amplified fragment was 183bp. U, upper primer; L, lower primer; *, position of primer BM21A; **, primer BM21B

5.3.1 PCR with BM21A and BM21B

PCR primers, BM21A and BM21B were used to amplify specific fragments from CB, HA and 88 DH lines. Three PCR bands were produced from each cultivar (arrows in Fig. 5.3), and resulting products showed no polymorphism between cultivars (Fig. 5.3). The 3rd band was the expected one (183 bp) from sequence analysis. It was found by sequencing that the second PCR bands (the second arrow in Fig. 5.3) also had the perfect complementary sequences of the primers but unrelated internal sequences.

Fig. 5.3 PCR products of Chebec (CB) and Harrington (HA) amplified with BM21A and BM21B. Metaphor agarose gel, 3% (FMC, U.S.). M, DNA size marker (1k ladder, Gibco BRL, U.S.)

5.3.2 Sequence variations between CBM21 and HAM21

The 3rd PCR bands (183 bp) from CB and HA were purified, and subcloned into the T-vector. The clone containing the CB PCR fragment was named CBM21, and another clone containing the HA PCR fragment was named HAM21. Sequencing of these 2 clones revealed a single base difference at the 14th nucleotide from the primer BM21A. Clone CBM21 had an "A" (adenosine)as occurred in the original clone from Galleon, and HAM21 had a "G" (guanidine) (Fig. 5.4). Full sequence data are given in Appendix 4-a, b and c. This single base difference produced different restriction enzyme sites, *Mnl*I (HA sequence) and *Mbo*II (CB sequence) (Fig. 5.1). This difference could make it possible to use the "sequence-tagged-site" (STS) method (Tragoonrung *et al.*, 1992). CBM21 clone had three *Mnl*I restriction sites (at the 65th, 72nd and 151st nucleotide from 5' end of upper strand). HAM21 clone had four *Mnl*I restriction sites (at the 27th, 65th, 72nd and 151st nucleotide from 5' end of upper strand) (Appendix 3-b and 3-c). When the 183 bp "third band" from CB and HA were digested with *Mnl*I restriction enzyme (Stratagene, U.S.), the CB fragment was predicted to produce four bands (expected sizes of digested DNA were; 79, 65, 32 and 7 bp), and the HA fragment should produce five bands (expected sizes of digested DNA; 79, 38, 32, 27 and 7 bp). A polymorphism of the expected size was

found (Fig. 5.5), although small fragments (32, 27 and 6 bp) were not observed on the 7% polyacrylamide gel. Digestion with *Mbo*II restriction enzyme was also trialed, but the results obtained were difficult to resolve because of the three repeated restriction sites in the fragments (Fig. 5.1).

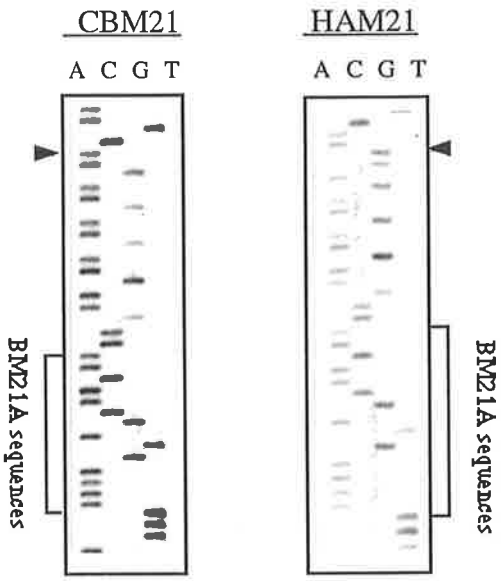


Fig. 5.3 A single base difference (arrow) between CBM21 and HAM21.CBM21 had "A" at the 14th nucleotide site from the end of BM21A primer site, and HAM21 had "G" at the same site.

5.3.3 AS PCR

During preliminary PCR tests, the two AS PCR primer sets (BM21ICB/BM21B and BM21IHA/BM21B) were able to distinguish a single base difference between the cloned template DNA, CBM21 and HAM21. The BM21ICB/BM21B primer set containing CB sequence at the 3' end of BM21ICB produced an allele specific PCR band on the CBM21 clone only. In contrast, the BM21IHA/BM21B primer set containing HA sequence at the 3' end of BM21IHA produced a complementary PCR

band on the HAM21 clone only using various annealing temperatures between 55°C and 60°C(Fig. 5.6).

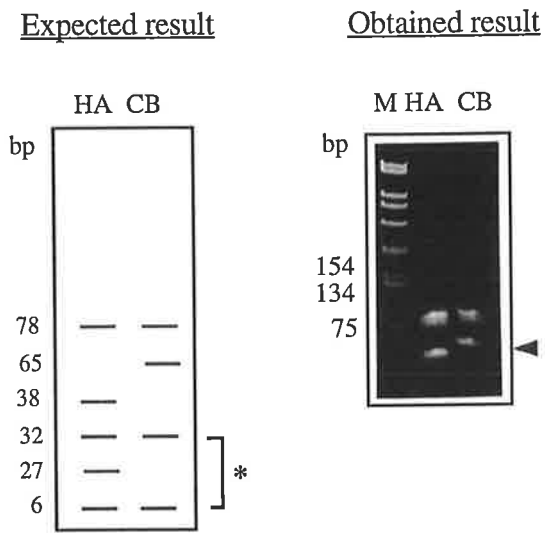


Fig. 5.5 A polymorphism (arrow) between CBM21 and HAM21 produced from "sequence-tagged-site" method using a *MnlI* restriction enzyme. Small fragments were not fully separated (asterisk mark) with 7% polyacryl amide gel

5.3.4 PCR markers of the CCN resistance gene in barley

The 1st round PCR products of the 88 DH lines (CB x HA) were screened with both AS PCR primer sets, and complementary results were obtained (Fig. 5.7). With the full set of complementary PCR results and the results of the CCN bioassay, the genetic distance was measured. The genetic distance between the two AS PCR markers and the CCN resistance gene was 2.3 cM (2 recombinants among 88 lines) (Fig. 5.8). One difference was found between the RFLP result using AWBMA21 probe and the AS PCR result. A DH line, 76B produced a CB allele on RFLP screening, but a HA allele was detected on PCR screening.

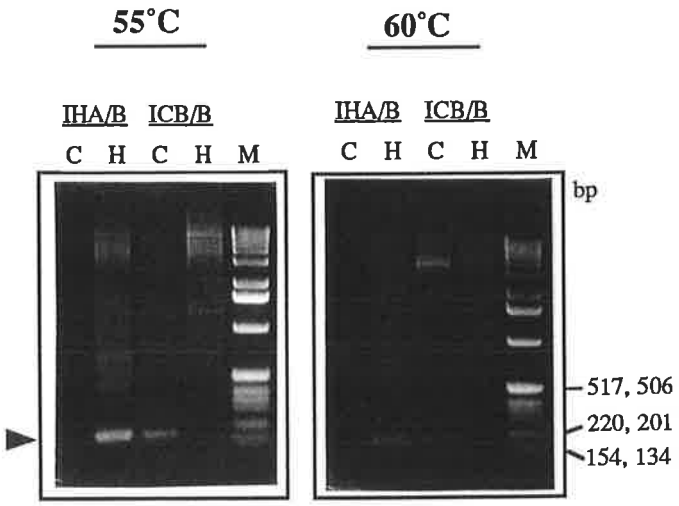


Fig. 5.6 Two allele specific PCR primer sets produced allele specific PCR products under different annealing temperatures (55°C and 60°C). C, CBM21; H, HAM21; IHA/B, BM21IHA/BM21B; ICB/B, BM21ICB/BM21B; M, 1kb DNA ladder (Gibco BRL, U.S.); 1.5% agarose gel

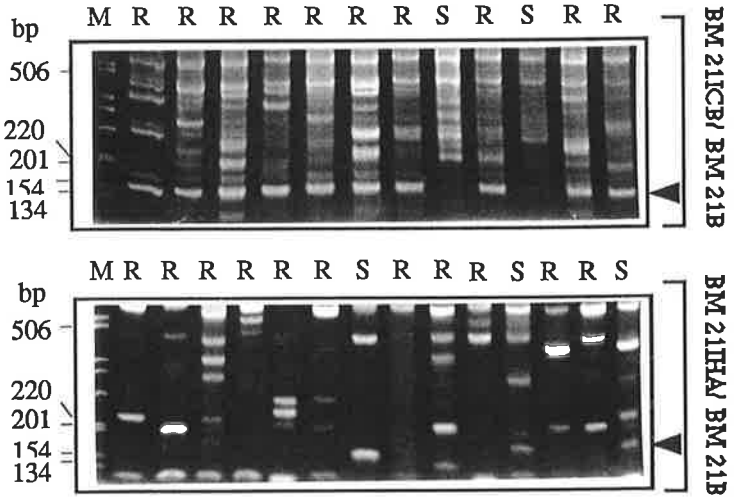


Fig. 5.7 Screening of barley DH lines with two allele specific PCR primer sets. BM21ICB/BM21B set detects only CCN resistant lines (top arrow), and BM21IHA/BM21B detects susceptible lines only (lower arrow). R, CCNresistant lines; S, susceptible lines; M, 1kb DNA ladder; 5% non-denaturing polyacrylamide gel

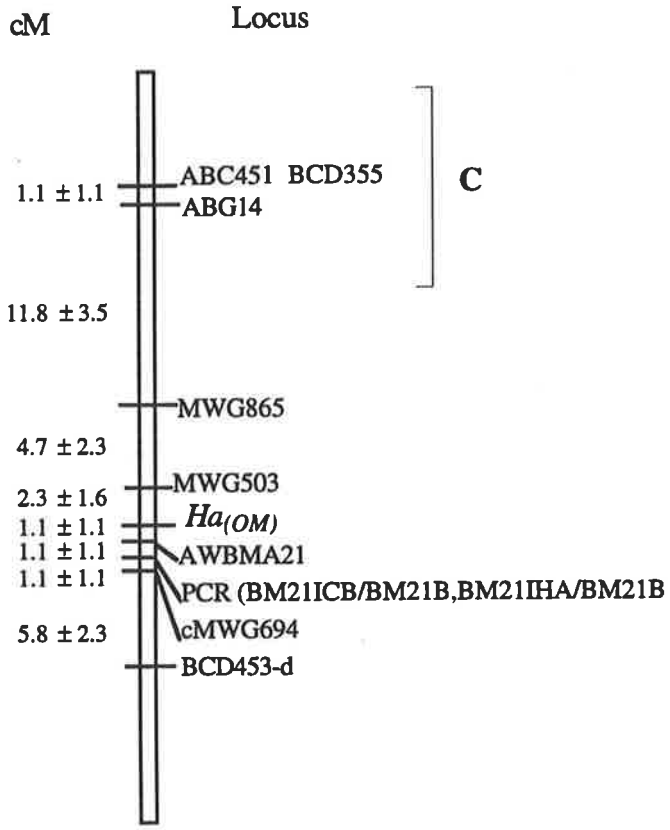


Fig. 5.8 The molecular map containing the location of allele specific PCR markers of the CCN resistance gene *Ha(OM)* on the long arm of the chromosome 2 in the CB x HA population. C, centromere; cM, centi Morgan

5.4 Discussion

The high specificity of PCR enabled discrimination of a single base mismatch between the primer and the template DNA (Huang *et al.*, 1992; Kwok *et al.*, 1990; Sommer *et al.*, 1992). To achieve the optimum results for the allele specific amplification, the character of the mismatched sequence and primer concentrations were considered to be

important conditions (Huang *et al.* 1992; Kwok *et al.* 1990). Although Huang *et al.* (1992) and Kwok *et al.* (1990) reported that the A:G mismatch was less stable than the C:T mismatch, in this study the A:G mismatch was clearly identified without reducing primer concentration. In addition, AS PCR was successful at annealing temperatures from 55°C-60°C with the use of cloned template DNA.

One of the advantages of the AS PCR was that two allele specific primer sets should produce complementary results on a test plant as shown in Fig. 5.6. When the PCR results produced from a test plant gave poor or ambiguous results, the PCR was repeated until complementary results were obtained. The complementary results provided the "control" for each other (Fig. 5.9). Eighty-eight barley DH lines were screened, representing a population of CCN resistant and susceptible lines. About 70% of the test lines produced clear and unambiguous complementary PCR results. The other 30% had to be repeated until satisfactory complementary results were obtained. These 30% DH lines often produced no PCR bands or non-complementary results. The reason for these unusual PCR results may be the purity of template DNA, and this could be a disadvantage of AS PCR.

	BM21ICB/BM21B	BM21IHA/BM21B
CB	+	–
HA	–	+

Fig. 5.9 Expecting complementary PCR results with allele specific PCR primer sets. A PCR primer set (BM21ICB/BM21B) containing CB sequence should make the target PCR band on CB DNA only, and another set (BM21IHA/BM21B) should make the band on HA DNA only. +, PCR band; –, no band

A single base difference between CB and HA produced different restriction sites on the target DNA fragments. This difference provided the possibility of the application of "sequence-tagged-site" (STS) method (Tragoonrung *et al.*, 1992). As AS PCR needs very specific discrimination, the use of the STS method might be an acceptable alternative approach, that could be more user-friendly than AS PCR which may need replicated PCR reactions to ensure PCR results obtained. However, some enzymes may not digest the 1st round PCR fragment for the STS method, unless it is repurified, which requires extra work (Tragoonrung *et al.*, 1992), and the cost of some restriction enzymes are prohibitive. Therefore AS PCR and STS methods should be considered supplementary to each other.

The genetic distance between the CCN resistance gene and the AS PCR markers (complementary markers) was 2.3 cM. The RFLP and the PCR results of DH lines were identical except for one DH line (76B). This result could be explained by a possible allele specific point mutation at the priming site of one of PCR primers, or by a mistake of *Taq* polymerase early in the first round of DNA amplification (Sambrook *et al.*, 1989). The sequence of the PCR fragment of 76B DH line was not checked.

It has been reported that the CCN resistance gene, *Cre*, in wheat and the CCN resistance gene, $Ha_{(OM)}$, in barley may be on the same homoeologous group, since both genes are loosely linked to the RFLP marker ABC451 (Williams, 1994). The homoeologous group 2 chromosomes of barley, wheat and rye have highly conserved gene orders (Devos *et al.*, 1993). This implies that as the PCR markers (BM21ICB/BM21B and BM21IHA/BM21B), and several RFLP markers (AWBMA21, BCD453, cMWG694, MWG503 and MWG865) are closely linked to the $Ha_{(OM)}$ gene in barley, they should also be tested to determine their linkage relationship to the *Cre* gene in wheat.

Unlike the RFLP method, which uses either presence or absence of restriction sites of particular restriction enzymes, AS PCR is based on a single base variation. Exploiting sequence variation can expand the utility of the PCR based molecular marker strategy in plant breeding, especially when the closely linked RFLP markers produce few or no polymorphisms between test plants (Graner *et al.*, 1991). Also, this method can be a useful approach for finger printing of genetically closely related sister lines.

For successful AS PCR, it should be considered that the quality of template DNA and the sequence specificity of primers can affect non-specific background bands. However, it was considered that non-specific bands are inevitable as far as the template DNA had priming sites of the PCR primers, especially with the relatively short primer sequences used when studying a complex genome like barley or wheat.. For example, the non-specific PCR fragment sequenced (the second PCR band ; Fig. 5.3) had perfect complementary sequences of the PCR primers in this study. Non-specific background could be reduced by the use of longer primers and high annealing temperatures, but increasing annealing temperatures should be avoided when sequence information about specific target DNA fragments are not available. Too many non-specific PCR products can be a disadvantage for utilising the STS method. Optimised PCR conditions and highly purified template DNA may allow direct allele specific amplifications without the 1st round PCR. Once well established PCR strategies have clear potential to be a handy tool for the efficient incorporation of a target gene into breeding programs.

Chapter 6 Finding polymorphisms between barley NILs using genomic DNA subtraction

6.1 Introduction

The molecular marker technique has become an important tool in genetic studies. One of the major contributions of the molecular map to plant breeding programs is flanking the target gene with tightly linked molecular markers, which can provide highly efficient selection. An efficient method for finding tightly linked molecular markers is screening near isogenic lines (NILs, Young *et al.*, 1988). However, fewer numbers of polymorphisms between NILs is one of the practical difficulties with this approach (Young *et al.*, 1988; Whitkus *et al.*, 1994).

In the process of producing NILs, some donor chromosome segments are dragged into the recipient line that otherwise resembles the recurrent parent. The reason for this introgression is the strong linkage between the gene of interest and the surrounding chromosomal region (Stam and Zeven, 1981). The theoretical size of this dragged donor genome is about 38 cM after six generations of backcrossing (BC^6), if the size of a chromosome is 200 cM (Stam and Zeven, 1981). If this dragged fragment is isolated, it should provide several markers closely linked to the gene of interest. The genomic DNA subtraction method is one strategy for the isolation of this dragged donor genome (Somerville, 1992).

Methods that are designed to specifically clone genetic differences that exist between two samples have been successfully used for finding DNA sequence variation between different tissues in a complex genome, for example monkey and rats (Yokota and Oishi, 1990; Yokota *et al.*, 1989). Such alteration of DNA sequences within an organism may be caused by somatic mutation during specific tissue development (Yokota *et al.*, 1989) or by infection with a DNA-based pathogen (Wieland *et al.*, 1990; Yokota *et al.*, 1989). This method, also, has been used to isolate cDNA clones

representing genes expressed or low levels in one tissue of a monkey, but not in another tissue (Travis and Sutcliffe, 1988), and has also been applied to clone plant developmental specific genes (Koltunow *et al.*, 1990). Generally, the difference cloning is suitable for the cloning of male-specific DNA sequences from the Y chromosome, and for the cloning of sequence variation of cancer cells (Lisitsyn, 1995). However, the genomic DNA subtraction may be inefficient for the higher eukaryotic because of high complexity of genomic DNA. For the higher eukaryotic DNA, the concentration of target DNA fragments should be increased by PCR amplification during the differential cloning process (Lisitsyn *et al.*, 1993; Lisitsyn, 1995).

Genomic DNA subtraction, first described by Straus and Ausubel (1990) for yeast, is a difference cloning method that is based on the direct DNA hybridisation between two independent DNA samples in order to clone of DNA sequences that differ between the two samples. This method was demonstrated to work with plant DNA by Sun *et al.* (1992) and shortly after, for wheat, using the addition or deletion lines for the homoeologous chromosome pairing locus (Clark *et al.*, 1992).

In this study, a modified genomic DNA subtraction method involving "in-gel reassociation" has been used for barley NILs (IN and BC^7) to isolate dragged donor DNA fragments from the recipient line (BC^7), which should be linked to the CCN resistance gene. These NILs have almost identical phenotypes except for the trait of CCN resistance/susceptibility.

6.2 Materials and methods

6.2.1 Plant materials

Plant materials are described in Section 2.1.1. For genomic DNA subtraction, IN and BC^7 were considered as "reference" and "target" DNA, respectively. The donor

cultivar of BC^7 was OM. For the test of selected clones from the genomic DNA subtraction library, 79 doubled haploid (DH) barley lines were used which were developed from F_1 plants of CL x OM through anther culture (Section 3.2.2.).

6.2.2 CCN bioassay

The CCN bioassay procedure was described in Section 2.2.

6.2.3 "In-gel reassociation"

6.2.3.1 Procedure

The process of "in-gel reassociation" followed the protocol described by Yokota and Oishi (1990) with minor modifications (Fig. 6.1). All enzymes and dephosphorylated *Eco*RI linkers were purchased from Promega (U.S.), and procedures of each reaction followed the protocol of the supplier, unless otherwise specified. Barley DNA was extracted from young leaves as described in Section 2.4.2. Approximately 20 µg each of "target" and "reference" DNA was digested independently with *Alu*I restriction enzyme in 20 µl of reaction volume. *Alu*I restriction enzyme digested most of barley "relic" DNA which is robust to be digested by six base-cutter restriction enzymes (Junghans and Metzlaff, 1988). Each digested DNA was subjected to methylation treatment with *Eco*RI *methylase*, and phenol extracted. After phenol extraction, each DNA was precipitated, followed by centrifugation (13,200 rpm) for 10 min. at 4°C. Air dried DNA pellets were resuspended in 10 µl of nanopure water. *Methylase*-treated "target" DNA (see method below) was kept at -20°C until it was used. *Methylase*-treated "reference" DNA was dephosphorylated with *Calf intestine alkaline phosphatase*, and phenol extracted. "Target" DNA was diluted to 1 x 10^{-2} in nanopure water, and 10 µl (approximately 50 ng) was used for the "in-gel reassociation". Diluted "target" DNA (10 µl) and 100-fold excess "reference" DNA

(10 µl) were mixed together, and loaded on a the 1.5% agarose gel. The gel size was 14.6 cm x 19 cm x 0.6 cm, and the size of each comb well was 7 mm x 1 mm.

The gel was electrophoresed in 1X TAE running buffer at a current of 25 mA for 12 hours. The electrophoresed gel was soaked in denaturing solution (0.5 M NaOH/ 0.6 M NaCl) for 20 min. at room temperature with gentle shaking. The denaturing step was repeated with fresh solution. The gel was gently rinsed with nanopure water two times, and incubated in reassociation solution [50% (v/v) formamide/ 25 mM sodium phosphate buffer, pH 6.8/ 1 M NaCl/ 5 mM EDTA/ 10% (w/v) PEG 8000] for 20 min. at room temperature. This incubation step was repeated two times, each time with fresh buffer. After the third incubation, the gel was soaked in fresh buffer in a plastic box at 45°C overnight in a very gently shaking water bath for the hybridisation between "reference" and "target" DNA in the gel to occur. A gel fragment containing 0.5-5.5 kb DNA fragments (Fig. 6.2) was cut out, and DNA fragments were purified using the "Gene clean" kit (Bio 101, U.S.). As the *Alu*I restriction enzyme produces blunt end DNA fragments, dephosphorylated *Eco*RI linkers (blunt ends) were attached to purified DNA fragments with T_4 DNA ligase. After ligation, *Eco*RI restriction enzyme was used to produce cohesive ends on the *Eco*RI linkers. As the insert DNA was treated already with *Eco*RI *methylase*, the insert DNA should have remained intact, and only the attached linkers should have been digested. The DNA fragments were then ligated with the vector λ gt10.

6.2.3.2 *Eco*RI methylation

The procedure of methylation followed the protocol of the *Eco*RI *methylase* supplier (Promega, U.S.). *Alu*I restriction enzyme-digested genomic DNA (approximately 20µg) was extracted with phenol, and precipitated. After precipitation, the air dried

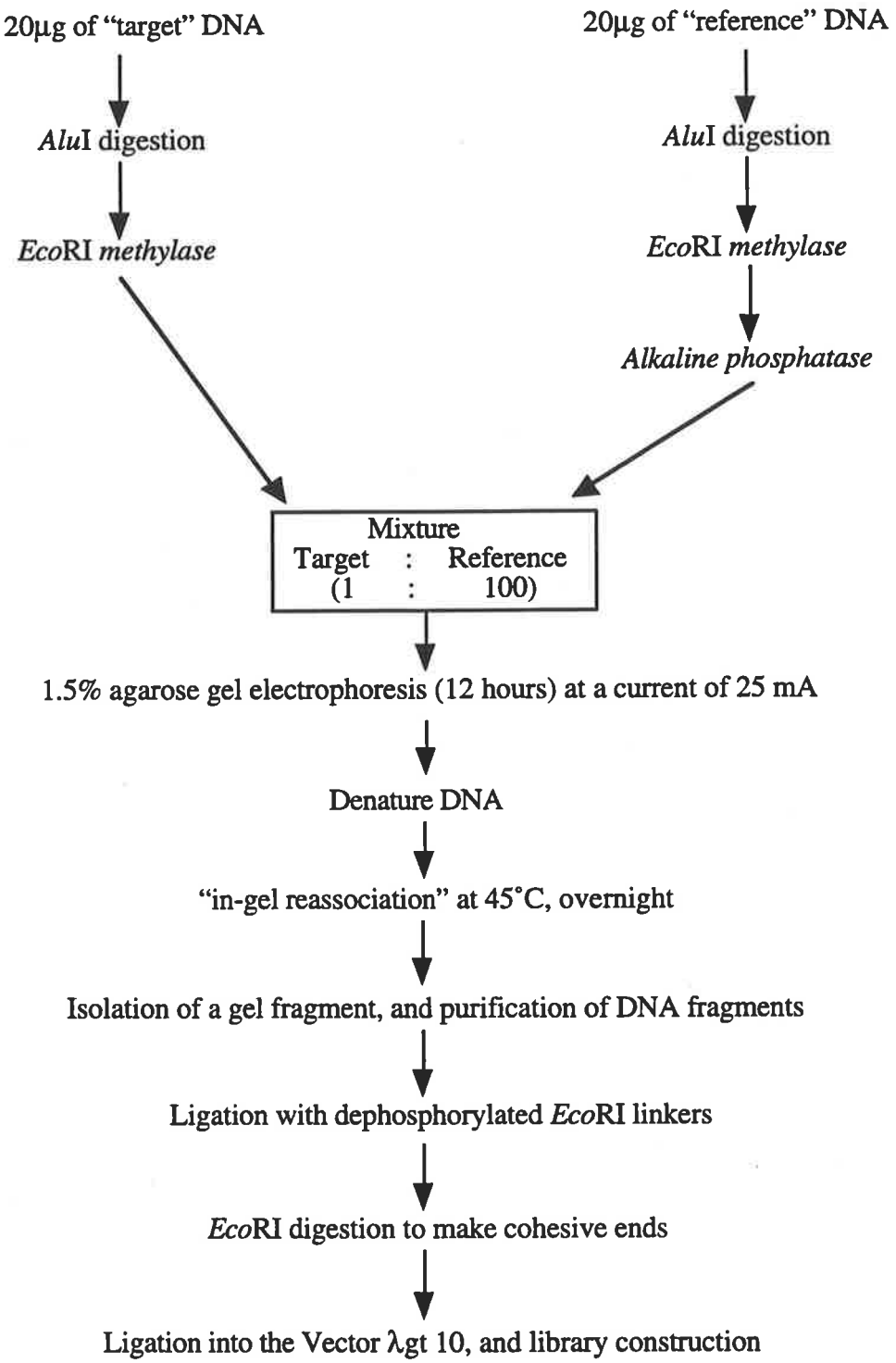


Fig. 6.1 Outline of genomic DNA subtraction used in this study. Detailed procedures were described in 'materials and methods'.

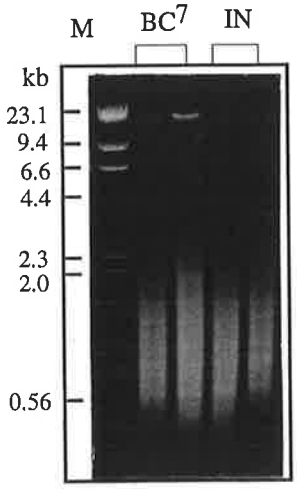


Fig. 6.2 *Alu*I restriction enzyme-treated barley genomic DNA. The sizes of digested DNA were mostly between 5.5 and 0.5 kb. IN,Ingrid;BC^7, NIL of IN (OM x IN^7); DNA size marker, (lambda/*Hin*dIII)

DNA pellet was resuspended in 10 µl of nanopure water. To a tube, one µl of 1 mM SAM, two µl of *Eco*RI *methylase* 10X buffer, one µl of BSA (100 µg/ml), 20 units of *Eco*RI *methylase* and water (to make total volume 20 µl) were added, and the tube was incubated at 37°C for 30 min. *Eco*RI *methylase* was inactivated by heating at 70°C for 10 min. and *Methylase*-treated DNA was extracted with phenol. Extracted DNA was precipitated, and the air dried DNA pellet was resuspended in 10 µl of nanopure water, and kept at -20°C until it was used.

6.2.3.3 Dephosphorylation

The process followed the protocol of the *calf intestinal alkaline phosphatase* supplier (Promega, U.S.). To a tube containing *Eco*RI *methylase*-treated DNA in 10 µl of water, 5 µl of 10X reaction buffer, 2 units of *calf intestinal alkaline phosphatase* (CIAP), and 33 µl of nanopure water were added (total volume, 50 µl), and the tube incubated at 37°C for 60 min. After incubation, CIAP was inactivated by phenol extraction, and the DNA was precipitated. The air dried DNA pellet was resuspended in 5 µl of nanopure water, and stored at -20°C.

6.2.4 Library construction

The process of the ligation of the insert and λ gt10 vector and the library construction followed the procedure described by the supplier of the λ gt10 vector system (Promega, U.S.).

6.2.4.1 Ligation of inserts into the vector λ gt10

To a tube containing purified DNA (mixture of target DNA, reference DNA and hybrids of "target/reference" DNA fragments in 5 µl of nanopure water), 0.1 µg of dephosphorylated lambda vector DNA, 1 µl of 10X reaction buffer, 2.5 Weiss units of T_4 DNA ligase and nanopure water were added to make a total volume of 10 µl. This mixture was incubated at room temperature for 3 hours, and kept at -20°C. For the positive control, 10 ng of insert control DNA was used. Also, a negative control was prepared without inserts.

6.2.4.2 Packaging of ligated DNA

For the packaging of ligated DNA, Packagene® system (Promega, U.S.) was used. Packagene® extract (50 µl) was kept on ice to thaw, and the ligation product was immediately added to Packagene® extract (total volume, 60 µl), and mixed well by tapping the bottom of the tube. After incubation at room temperature for 2 hours, 25 µl of chloroform and 415 µl of phage buffer were added and mixed by gentle inversion, and this mixture was stored at 4°C until it was used for titration.

6.2.4.3 Preparation of host bacteria

Host bacteria, C600*hfl* strain was grown to an $O.D._{600}$ of 0.6-0.7 in 5 ml of LB medium with moderate shaking (200 rpm).

6.2.4.4 Titration of packaged phage

For the titration, 100 µl (1/5 of total) of packaged phage was mixed (without dilution) into 300 µl of host bacterial solution. The mixture was kept at 37°C for 30 min. for infection. A total of five batches were prepared using whole packaged phages without dilution, since a 10^{-2} dilution of phage produced four plaques. During infection, three ml of top agarose was kept at 47°C. To pre-warmed top agarose, infected host cells (200 µl) were gently added then mixed by swirling, and immediately plated on an LB plate (150 mm). After the top agarose had hardened, each plate was inverted and kept at 37°C overnight. The average size of plaques was less than 2 mm (diameter). The negative and positive controls were also plated out.

6.2.5 Library screening

6.2.5.1 The number of plaques

After incubation at 37°C overnight, 213 plaques were formed from the product of genomic DNA subtraction. The plaque forming unit (pfu) of λ gt10 arms positive control, background and packagene extract positive control were $4.8x10^4$ pfu/µg, $6.4x10^3$ pfu/µg and $9.9x10^5$ pfu/µg, respectively. Of the 213 plaques, 32 plaques were randomly selected for screening.

6.2.5.2 Diffusion of phage

A single plaque was removed using a wide bore one ml pipette tip prepared with the end cut off. The agar plug was placed into 100 µl of phage buffer, and the tube was kept at 4°C overnight to allow phage diffusion.

6.2.5.3 λ DNA preparation using liquid culture ("minipreps")

This procedure followed the protocol of Sambrook *et al.* (1989) with minor modifications. Host bacteria (C600*hfl*) were grown in five ml of LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ with shaking (200 rpm) until $O.D._{600}$ was 0.6-0.7. An aliquot of 20 µl of diffused phage solution was added to 500 µl of host cells in a 1.5 ml micro-centrifuge tube, and the tube was kept at 37°C for 20-25 min. for infection. Infected cells were transferred to 10 ml of LB medium supplemented with one ml of 1M $MgSO_4$ in a 100 ml flask, and incubated with vigorous shaking (250 rpm) until lysis occurred (5-6 hours). After cell lysis, bacterial debris was removed by centrifugation (8,000 g) for 10 min. at 4°C. The supernatant (approximately 10 ml) was transferred to a 50 ml centrifuge tube, and 1 µl each of *RNase* A (1 mg/ml) and *DNase* I (1 mg/ml) was added. The mixture was incubated at 37°C for 1 hour. To each tube, PEG 8000 and NaCl were added to final concentrations of 20% and 2 M, respectively. Tubes were gently shaken by inversion until PEG 8000 and NaCl had completely dissolved, and then were kept on ice for at least 1 hour. The bacteriophage was collected by centrifugation (10,000 g for 15 min. at 4°C), and the fluid was removed from the phage pellet by inversion of tubes on paper towel. Phage were resuspended in 0.5 ml of TM buffer, and transferred to a 2 ml micro-centrifuge tube. To the tube, EDTA (final conc. 20 mM), Proteinase K (final conc. 50 µg/ml) and SDS (final conc. 0.5%) were added, and incubated at 57°C for one hour. After incubation, 10 µl of 5 M NaCl was added to each tube. Phage DNA was extracted once with an equal volume of Tris-buffered phenol (pH 8.0), once or twice with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1, v/v) and once or twice with an equal volume of chloroform/iso-amyl alcohol (24:1, v/v), each extraction lasting at least 5 min. on ice. After extraction with chloroform/iso-amyl alcohol, the upper phase containing phage DNA was transferred to a 2 ml micro-centrifuge tube, and 0.6-1 volume of isopropanol was added to precipitate phage

DNA. The phage DNA precipitated was removed from the tube with a 1 ml pipette tip, and transferred into a 1.5 ml micro-centrifuge tube containing 700 µl of 70% ethanol. The DNA was pelleted by centrifugation (13,200 rpm) for 2-3 min. at room temperature. Air dried DNA pellets were resuspended in 500 µl of nanopure water containing *RNase A* (20 µg/ml). These samples were incubated at 37°C overnight, and the DNA was purified again; once with phenol/chloroform/iso-amyl alcohol and once or twice with chloroform/iso-amyl alcohol followed by precipitation and pelleting. The air dried DNA pellet was resuspended in nanopure water, and kept at 4°C. The final phage DNA concentration was approximately 500 µg/ml.

6.2.5.4 Isolation of inserts, and subcloning

After λ DNA purification, 11 insert fragments were isolated with *Eco*RI restriction enzyme (Section 2.4.9), and subcloned into the Bluescript vector (Section 2.4.5).

6.2.6 RFLP analysis

Each subcloned insert was used as a probe to determine whether it identified RFLPs between barley NILs. Restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, *Dra*I, *Bgl*II, *Alu*I) were purchased from Promega (U.S.), and membranes and labelling kit were from Amersham (U.K.). The procedures for DNA gel electrophoresis, "southern blotting" and hybridisation are described in Section 2.4.3 and 2.4.10.

6.2.7 Construction of a consensus map

A computer software program, "JOIN MAP", was used for the construction of a consensus map containing mapping data of CB x HA map and CL x OM map, and the map position determined for one RFLP clone produced from the genomic DNA subtraction. The LOD score adopted for the consensus map was 2.

6.3 Results

The genomic DNA subtraction method was used for the isolation of the dragged donor genome from the recipient line (BC^7).

6.3.1 The principle of agarose gel limiting genomic DNA subtraction

The principle of genomic DNA subtraction using barley NILs (IN and BC^7) in this study was based on the direct comparison of DNA sequences between NILs (Fig. 6.1). The general procedure is the same as that described by Somerville (1992), and Yokota and Oishi (1990). Excess amount of the "reference" DNA hybridised the "target" DNA if there were complementary sequences between both DNA fragments during "in-gel reassociation". As the *Alu*I restriction enzyme produces blunt end DNA fragments, dephosphorylated blunt end *Eco*RI linkers were used for the isolation of "target" DNA fragments that could not find complementary "reference" DNA fragments during "in-gel reassociation". Dephosphorylated "reference" DNA, and the hybrid of "target/reference" DNA could not ligate with dephosphorylated blunt end *Eco*RI linkers. Ideally, only the "target" DNA whose sequences were not homologous to "reference" DNA sequences, and which had blunt ends can ligate with the dephosphorylated blunt end *Eco*RI linker. After the use of the *Eco*RI restriction enzyme, only the "target" DNA fragment containing the cohesive *Eco*RI linker should be incorporated into the vector λ gt10.

6.3.2 A polymorphism between barley NILs

The genomic DNA subtraction library produced 213 plaques, and 32 plaques were randomly selected. After the λ DNA purification, 11 insert fragments were isolated from eight plaques with *Eco*RI restriction enzyme (another 24 plaques representing background), and subcloned into the Bluescript vector. The sizes of the insert fragments varied from 0.6 to 5 kb (Fig. 6.3). One of them, the clone number 172-2

(0.9 Kb) revealed a polymorphism between barley NILs that were digested with *Alu*I restriction enzyme (Fig. 6.4). The donor plant (OM) and BC^7 had the same size bands, and IN had a smaller band. No polymorphisms were found with the other clones as probes.

6.3.3 The genetic distance between clone 172-2 and the CCN resistance gene

The 172-2 clone identified a polymorphism between OM and CL (parent cultivars of DH lines) with *Bgl*II restriction enzyme (Fig. 6.5). This polymorphism was used for RFLP analysis of 79 DH lines. Among 79 DH lines, 29 recombinants were found (Fig. 6.6), and the genetic distance was estimated to be 36±5.5 cM.

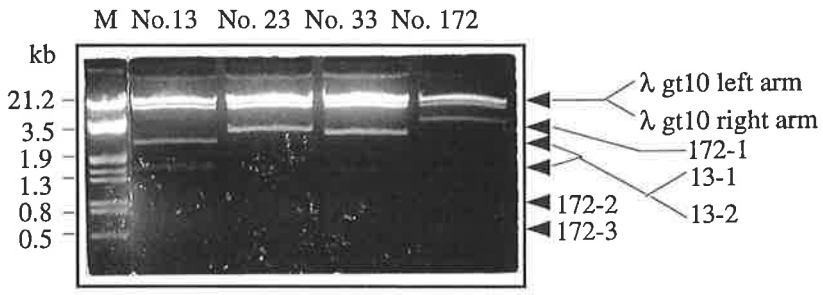


Fig. 6.3 The insert fragment isolated from λ gt10 vectors. No. 13 clone had two inserts (13-1 and -2), and No. 172 clone had three inserts (arrows) (172-1, -2 and -3); M, DNA size marker (lambda/*Hin*dIII/*Eco*RI)

6.3.4 Map location of a clone, 172-2, and a consensus map

The complete mapping data for the CB x HA map and the CL x OM map, and the RFLP data for clone 172-2 were used for the construction of a consensus map shown in Fig. 6.8. As the two molecular maps had different segregation ratios for each locus

and different map distances among loci, the map order of the consensus map showed slight variations from both independent maps (Fig. 4.4 and 4.5 in Chapter 4).

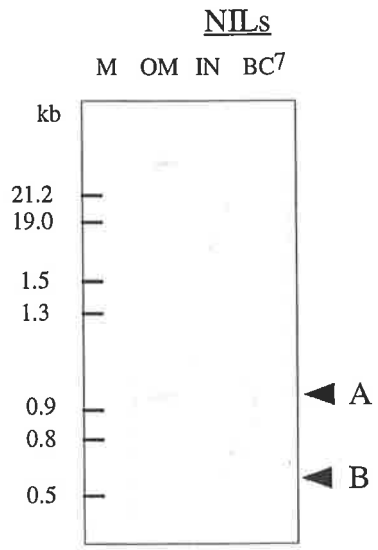


Fig. 6.4 Detection of polymorphisms (arrows) between barley NILs with clone 172-2. OM and BC^7 had the same size band (A), and IN had a smaller size band (B). Restriction enzyme was *AluI*. M, DNA size marker (lambda/*Hin*dIII/*Eco*RI)

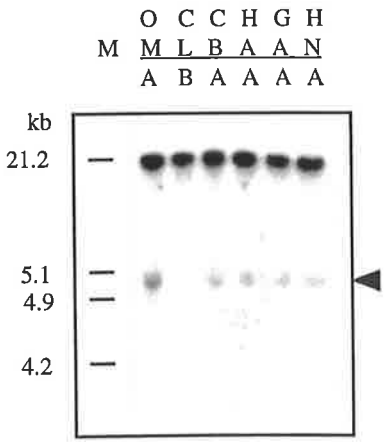


Fig. 6.5 DNA gel blot analysis of several barley cultivars hybridised with clone 172-2. Restriction enzyme was *Bgl*II. A polymorphism between OM and CL (arrow) was found, which was used for the RFLP analysis in Fig. 6.6. M, DNA size marker (lambda/*Hin*dIII/*Eco*RI); A/B, presence/absence of a band indicated by an arrow

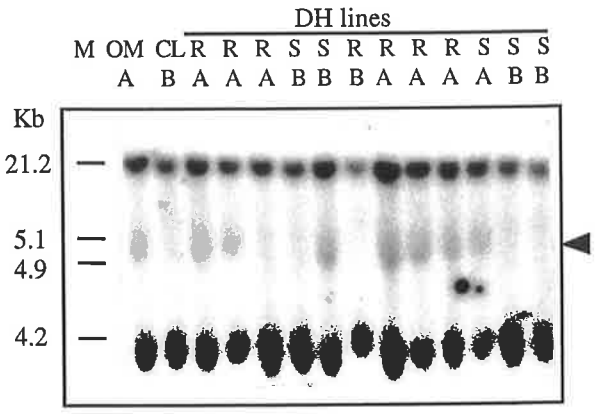
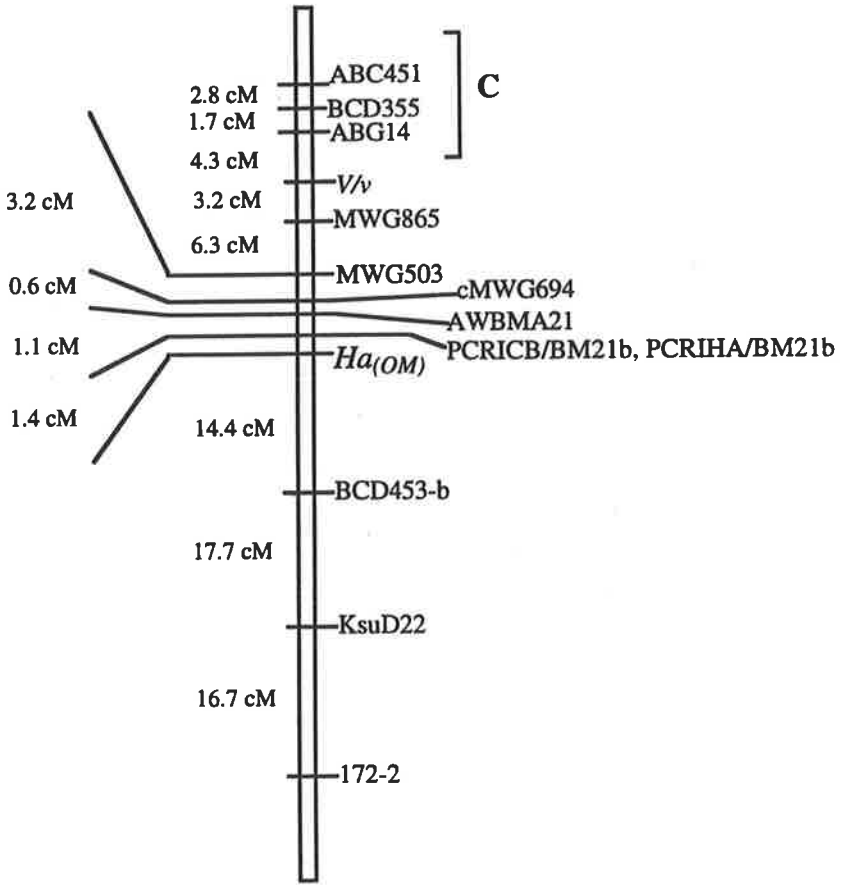


Fig. 6.6 The RFLP analysis of clone 172-2 with DH lines (CL x OM). Restriction enzyme was *Bgl*II. OM (CCN resistance); CL (CCN susceptible); R, resistant lines; S, susceptible lines; M, DNA size marker (lambda/*Hin*dIII/ *Eco*RI); A/B, presence/absence of the band of interest (arrow)

Overall, the map order of the consensus map resembled that of the CL x OM map. However, two loci, BCD453-b and KsuD22 had reversed locations in the consensus map (Fig. 6.7). The genomic subtraction clone, 172-2, belonged to the $Ha_{(OM)}$ linkage group, and the genetic distance was about 16.7 cM from the KsuD22 locus (Fig. 6.7).

6.4 Discussion

The genomic DNA subtraction method is a useful approach for finding polymorphisms between genetically close breeding lines. As the NILs have small differences representing absence or presence of the donor genome, the products of genomic DNA subtraction should include the introgressed donor genome that is linked to the gene of interest.



Barley chromosome 2, long arm

Fig. 6.7 A consensus map produced from combination of the CL x OM map and the CB x HA map. The genomic subtraction clone 172-2 belongs to the $Ha_{(OM)}$ linkage group. Overall, the linear map order was the same as that of the CL x OM map except for the loci of BCD453-b and KsuD22. These two loci had the reversed locations on the CL x OM map. C, centromere region on the current barley molecular map.

*Alu*I restriction enzyme-treated barley genomic DNA resulted in relatively small sized DNA fragments mostly between 0.5-5.5 kb (Fig. 6.2), and these DNA fragments were purified after "in-gel reassociation" for the library construction. The amount of intact "target" DNA purified after "in-gel reassociation" was unknown, because the product of "in-gel reassociation" was the mixture of intact "target", "reference", and

the hybrid of "target/reference" DNA. It was therefore difficult to decide on the amount of vector DNA to use. Approximately 100 ng of vector DNA was used in this study, and several plaques containing two or three inserts were found (Fig. 6.3), suggesting a larger amount of vector would have given clearer cloning results.

Screening the library was a time consuming process, since each plaque was screened to determine whether it had an insert or not. Of the 213 plaques, 32 plaques were randomly selected, and the presence/absence of inserts was checked by lambda DNA purification. This process was tedious and restricted the number of plaques tested. Library screening using PCR amplification was not suitable because of the likely presence of two or three inserts in the clones.

With the library screening, an interesting clone (172-2) was obtained, and it revealed the DNA polymorphisms between NILs. The genetic distance between the loci of this clone and the *Ha*$_{(OM)}$ gene in the CL x OM DH population was 36±5.5 cM. Although the recombination value did not result from the progenies of BC^7 x IN, it is evidence for the presence of dragged donor background in the BC^7 used in this study. If the average size of barley chromosome 2 was 220 cM (Kleinhofs and Kilian, 1994), the theoretical size of the introgressed donor chromosome segment is approximately 35 cM in BC^7 (Stam and Zeven, 1981). The obtained genetic distance; 36 cM was almost the same as the theoretical size of the introgressed donor genome size in BC^7. A similar result was reported in barley using three backcross generations (Brown *et al.*, 1989). The sizes of dragged segments after three backcross generation were measured by the use of several isozyme markers on the barley chromosome 5. The recombination values between isozyme markers were between 31 and 55 cM, and these values were close to the estimated recombination values in the three backcross generation calculated by Stam and Zeven (1981) (Brown *et al.*, 1989).

The result obtained should be considered carefully. It is possible that the polymorphism between NILs may be produced from a single base difference caused by sequence mutations or sequence methylation resulting in absence or presence of the specific *Alu*I restriction sites producing RFLPs between NILs obtained in this study. However, a more likely explanation for the RFLPs obtained is the presence or absence of a donor chromosomal segment between NILs. The restriction site of *Alu*I enzyme in BC^7 producing a polymorphism between NILs originated from the donor plant, OM as OM and BC^7 produced no polymorphism. There could be several sequence variations between NILs, which were not detected by the restriction enzymes used.

The other 10 insert fragments tested did not show RFLPs between barley NILs with several restriction enzymes used. These could be "background" inserts which ligated into the vector because of the low efficiency of "in-gel reassociation" using barley genomic DNA. To improve the efficiency of genomic DNA subtraction, the use of at least two cycles of "in-gel reassociation" was recommended (Yokota and Oishi, 1990).

It may be possible to find clones more closely linked to the $Ha_{(OM)}$ gene by the further screening of other clones from the library, and by using other restriction enzymes. The sequence of "target" DNA may be very similar to that of "reference" DNA. To clone the $Ha_{(OM)}$ gene, other supplementary experiments such as cDNA library screening could also be undertaken.

The size of introgressed donor genome is a particularly interesting point in genetic studies and breeding (Bjørnstad, 1987). Genomic DNA subtraction is expected to be used effectively for studying different genetic constitutions of closely related sister lines. If there was some alien chromosomal addition or deletion, such as wheat-barley addition lines (Islam *et al.*, 1981) the genomic DNA subtraction method can be used to isolate the alien DNA fragments. This study has demonstrated the feasibility of using this approach for such theoretical studies.

Chapter 7 General discussion

The major purpose of this study was finding molecular markers (RFLP and PCR markers) for the CCN resistance gene found in Orge Martin and Chebec. Initially, some efforts focused on the histology work to understand the mechanism of resistance to CCN. During this work, the process of a developing syncytium was observed. However, no distinctly different cell structures were found between resistant and susceptible syncytium cells. Embedding with Spurrs resin and sectioning of the intact syncytium cell of resistant barley was difficult, as this syncytium was extremely fragile. Some photographs representing the developing process of the syncytium cell are illustrated as unpublished results in Section 1.2.2.

For the efficient incorporation of CCN resistance into the barley breeding programs, the determination of the number of CCN resistance genes and elucidation of their chromosomal location of these genes are essential. To determine the CCN resistance gene was located on the long arm of barley chromosome 2, the genetic linkage was examined between a morphological marker, *V/v* and the CCN resistance gene in OM. The genetic distance between the two loci was approximately 20 cM. This CCN resistance gene was tentatively named $Ha_{(OM)}$, since it is unknown whether this gene is *Ha3*. The determination of the number of CCN resistance genes using the CCN bioassay method was ambiguous, and it was often difficult to separate resistant and susceptible lines by counting the number of cysts during CCN bioassay. When a monogenic result was expected, the CCN bioassay result was digenic, or the result deviated significantly from expectations. These complicated results were explained well by using molecular markers that provided a higher resolution for the genetic analysis.

Several RFLP markers (AWBMA21, BCD453, cMWG694, KsuD22, MWG503 and MWG865) established close linkages to the $Ha_{(OM)}$ gene, and the use of these RFLP

markers and two DH populations gave clear explanations for some of the genetic variation that was otherwise difficult to explain by classical genetic analysis using few morphological markers using segregating populations, such as F_2 plants.

The chromosomal region containing the *Ha(OM)* gene had strongly biased segregation ratios for all closely linked loci tested of the CB x HA anther culture derived doubled haploid mapping population, and for several loci of the CL x OM anther culture derived doubled haploid mapping population. More frequent CB and OM alleles were observed for most loci tested. The locus of *Ha(OM)* gene also showed biased assortments for both resistant parental alleles. This distorted segregation caused complicated results in the determination of the number of the *Ha(OM)* genes by CCN bioassay. In fact, the results of this study gives strong and direct evidence that OM and CB have a single CCN resistance gene. Using densely located markers surely improves the resolution in the genetic analysis, and provides clues for understanding complicated genetic results. The use of DNA markers disclosed several interesting genetic phenomena. The CL x OM map contained more frequent recombinations between marker loci than that of the CB x HA map. Frequent recombinations expanded the map length. Three loci, cMWG694, AWBMA21 and *Ha(OM)* had reversed map locations between both genetic maps. The expanded map distances on the CL x OM map (or condensed map distances on the CB x HA map) and reversed map positions of three loci could be explained by an inversion in the CB x HA population, as the inversion loop reduces the number of recombinations in the vicinity of the inversion resulting in short genetic distances. However, it should be considered that inversions are rare in barley. Further, more intensive genetic investigation will need to be undertaken to either confirm or negate this suggestion. Another possible reason for the reversed map positions could be that the CCN bioassay results may have some variations, although the CCN bioassay was repeated to be sure the results obtained. However, this reason can not explain the expanded

map distances among marker loci in the CL x OM population, as map distances among marker loci were obtained from RFLP analysis, and as RFLP results were acceptable. Overall the two molecular maps had well conserved order of loci tested except for the locus of the $Ha_{(OM)}$ gene.

The use of molecular markers produced highly accurate information for the genetic analysis. Also, it raised several questions; the structure of the chromosomal region of the $Ha_{(OM)}$ gene and the possibility of chromosome walking toward the $Ha_{(OM)}$ gene with the use of closely linked flanking RFLP markers (AWBMA21, cMWG694 and MWG503). The application of map based chromosome walking is one of the possibilities to characterise the structure of the $Ha_{(OM)}$ gene. However, it should be considered that the physical map distance is not linear with the genetic map distance. This is one of the major difficulties in the chromosome walking (Heslop-Harrison, 1991; Sorokin *et al.*, 1994).

The RFLP clone ABC451 which was linked to the *Cre* gene in wheat (Williams *et al.*, 1994) has linkage to the $Ha_{(OM)}$ gene in barley (Fig. 4.7 in this study) (Williams *et al.*, 1994). The use of several RFLP markers that were closely linked to the $Ha_{(OM)}$ gene in barley should be considered as invaluable research tools for further genetic studies of CCN resistance in wheat and other cereals, as the gene order of homoeologous group 2 chromosomes of barley and wheat has been well conserved (Devos, *et al.*, 1993).

The long arm of chromosome 2 has several disease resistance genes, such as $Ha_{(OM)}$, *Ha2, Ml(La)* and *Sm* which may have genetic linkages among them (Giese *et al.*, 1993). Exploiting the chromosomal region of the $Ha_{(OM)}$ gene can provide valuable information for understanding disease resistance mechanisms and for the disease resistance breeding.

One of the RFLP markers of the $Ha_{(OM)}$ gene, AWBMA21 was converted to a two allele specific PCR (AS PCR) marker system closely linked to the $Ha_{(OM)}$ locus by utilising sequence characterisation of the DNA fragments of CB and HA. As the AS PCR system is based on the sequence variation on the double stranded template DNA, two allele specific PCR primers can be constructed which have complementary sequence at the 3'-end of each primer. AS PCR results obtained from such primers can be used as positive controls for each other, and hence as a standard to know that AS PCR reactions were correct or not. The sequence characterisation has expanded the utility of PCR base molecular markers for genetic studies and for use in breeding programs. Sequence characterisation can discriminate very specific variation of the DNA sequences. This discrimination of even a single base difference can greatly increase the level of polymorphisms that is otherwise often difficult to detect by the RFLP method which is based on absence/presence of restriction sites. The increased number of polymorphisms are obviously beneficial for the genetic studies and for "finger printing" of closely related genetic stocks. However, AS PCR required the extremely accurate DNA polymerization process, and it could be a negative point for the AS PCR, since establishing the very same PCR conditions every experiment is often difficult, although commercialized PCR components were used. For the optimized AS PCR system, several methods can be applied, such as restriction enzyme digestion of template DNA, increasing primer size, high annealing temperature and improved purity of template DNA.

The genomic DNA subtraction method was used for developing new molecular markers for the $Ha_{(OM)}$ gene, and for adding to our understanding of the genome structure of barley NILs. In the process of developing NILs, the donor genome is introgressed into recipient lines. Ideally, this "dragged" donor genome should be linked to the gene of interest. An interesting clone was found from the constructed genomic DNA subtraction library using barley NILs (Ingrid, CCN susceptible; BC^7,

CCN resistance). This clone (172-2) produced a polymorphism between barley NILs, that was linked to the $Ha_{(OM)}$ gene (36 cM) in the CL x OM DH population. This genetic distance is similar to the theoretical donor genome size in the BC^7 computed by Stam and Zeven (1981). This polymorphism and genetic distance obtained may represent the size of introgressed donor genome in BC^7 of NILs. However, the polymorphism may be produced from a point mutation resulting the presence/absence of restriction sites of *Alu*I enzyme, or may be produced from the variation of methylation at the *Alu*I restriction sites.

There could be some of the donor genome in the recipient lines, which are not linked to the gene of interest, since some chromosomal regions are very resistant and appear to suppress cross overs, especially in the proximal regions of the chromosomes. For the isolation of these donor genome fragments from the recipient line and for the isolation of alien genome, the genomic DNA subtraction method can be an effective alternative strategy. A number of successful results were reported using subtraction method in plant and animal experiments. Large amounts of repeated sequences in cereals could reduce the efficiency of subtraction techniques. To improve the efficiency and accuracy, the number of subtraction cycles using PCR should be increased, which can enrich the target DNA fragments.

At the beginning of this study, several questions were asked; the chromosomal location of the CCN resistance gene, how many CCN resistance genes in Orge Martin and Chebec, finding closely linked RFLP markers for the CCN resistance gene, developing a PCR-based method for screening of CCN resistant lines in barley and exploring genetic variation between barley NILs to develop new RFLP markers for the CCN resistance gene. As results, some answers were obtained as described above. However, more intensive experiments will be required to develop a practical PCR-based screening method for the CCN resistance gene.

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Appendix 1-a

The ingredients of induction medium for barley anther culture

Ingredients	Concentration
Stock A(1)	50 ml of stock A used/l; Stock A: KNO_3, 38g; NH_4NO_3, 3.3g; $CaCl_2 \cdot 2H_2O$, 8.8g; $MgSO_4 \cdot 7H_2O$, 7.4g; KH_2PO_4, 3.4g; NaFeEDTA, 0.74g/l
Stock B(1)	5 ml of Stock B used/l; Stock B: H_3BO_3, 1.24g; $MnSO_4 \cdot 4H_2O$, 4.46g; $ZnSO_4 \cdot 7H_2O$, 1.72g; $Na_2Mo_4 \cdot 2H_2O$, 50 mg; KI, 166 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; $CoCl_2 \cdot 6H_2O$, 5 mg/l
Myo-inositol	100 mg/l
Thiamine HCl(2)	0.4 ml of Stock used (Stock: 1g/l)
Glutamine	750 mg/l
Indole-3-acetic acid (IAA)	1 ml of Stock used Stock: 1 g/l
Benzyl-6-amino-purine (BAP)	1 ml of Stock used Stock: 1 g/l
Maltose	63 g/l
Filter sterilisation	The above ingredients were disolved in 500 ml nanopure water, and filter sterilised (size:0.45 μm) (Nalge)
Agarose	8 g of Agarose (Sigma type VII) was melted in 500 ml of nanopure water, cooled until hand-hot, and mixed with filter sterilised solution (500 ml) to make 1litre

(1), Stock A and Stock B should be replaced every 6 weeks.
(2), Solution should be replaced every month.

Appendix 1-b

The ingredients of regeneration medium (1litre) for barley anther culture

Ingredients	Concentration
Stock A*	50 ml of stock A used
Stock B*	5 ml of stock B used
Myo-inositol*	100 mg/l
Thiamine HCl*	0.4 ml of stock used
Glutamine*	750 mg/l
Indole-3-acetic acid (IAA)	0.4 ml of stock used
Benzyl-6-amino-purine (BAP)	0.4 ml of stock used
Maltose	15.75 g/l
Filter sterilisation	The above ingredients were disolved in 500 ml nanopure water, and filter sterilised (size: 0.45 µm) (Nalge)
Agarose	8g of Agarose (Sea Kem, Le Agarose) was melted in 500 ml of nanopure water, cooled to hand-hot, and mixed with filter sterilised solution to make 1litre

*, Solutions are the same as the induction medium.

Appendix 2-a

CCN bioassay results of 79 DH lines (CL x OM) and head types

Mapping number	Plant number	Head types	CCN bioassay	Mapping number	Plant number	Head types	CCN bioassay
1	193B/1	2	A	40	165B/4	6	B
2	220A/1-1	2	B	41	37B/1	2	A
3	183B/1	6	A	42	181A/3	2	B
4	175A/1	2	B	43	181A/5	6	A
5	22B/2	2	A	44	130A/9	2	B
6	179B/2	6	A	45	185B/1	2	B
7	219B/1	2	B	46	193B/3	6	A
8	220B/3	6	A	47	164A/4	2	B
9	22B/1	6	A	48	169B/1	2	B
10	30B/2	2	U	49	11A/1	2	B
11	30B/4	6	A	50	175B/2	6	A
12	37A/1	6	A	51	16A/1	6	A
13	220B/2	6	A	52	16A/2	2	B
14	100A/2	6	A	53	16A/6	2	B
15	179B/1	2	A	54	183A/3	6	A
16	177A/3	6	A	55	181A/4	2	B
17	30B/1	2	B	56	100A/1	6	A
18	30A/1	6	A	57	16A/3	6	A
19	216A/1	2	B	58	130A/10	2	B
20	181B/3	6	A	59	179B/6	2	B
21	37A/2	6	A	60	52A/1	6	A
22	177A/2	6	B	61	179B/3	6	A
23	7B/1	6	A	62	130A/1	2	A
24	33B/2	2	B	63	202A/1	6	A
25	130A/7	6	A	64	164A/3	2	A
26	183A/1	6	A	65	33B/1	6	A
27	181B/4	6	A	66	177A/6	2	A
28	177B/4-1	2	B	67	30B/2	2	B
29	37A/3	2	B	68	164A/1	2	A
30	177B/4	2	A	69	258B	6	A
31	175A/2	2	U	70	9A/1	6	A
32	179A/1	2	B	71	9A/12	6	A
33	220A/2	2	A	72	175B/1	2	B
34	130A/11	2	A	73	193B/4	6	A
35	11B/1	6	A	74	16A/3	6	A
36	177B/6	2	B	75	9A/1-1	6	A
37	219B/2-1	6	B	76	130A/8	6	A
38	177A/4	6	A	77*	33B/2	2	U
39	259B/1	6	A	78	181A/1	2	B
				79	165B/1	2	A

A, CCN resistance; B, CCN susceptible; U, unidentified
*, intermediate head type

Appendix 2-b

CCN bioassay results of 88 DH lines (CB x HA)

Mapping number	DNA code	CCN bioassay	Mapping number	DNA code	CCN bioassay
1	1B	A	48	61B	A
2	2B	A	49	62B	A
3	3B	A	50	63B	A
4	4B	A	51	64B	B
5	5B	A		65B	B
6	6B	B	52	66B	A
7	7B	A	53	67B	B
8	8B	A		68B	
9	9B	A	54	69B	B
10	10B	A	55	70B	A
11	11B	B	56	71B	A
12	12B	A		72B	
	13B		57	73B	A
13	14B	B	58	74B	A
14	15B	A	59	75B	A
15	16B	B	60	76B	U
16	17B	B		77B	A
17	18B	A		78B	B
18	19B	A	61	79B	A
19	20B	A		80B	A
20	21B	B	62	81B	A
21	22B	B	63	82B	A
22	23B	A		83B	A
	24B	A		84B	A
	25B		64	85B	B
	26B	A		86B	
23	27B	A	65	87B	A
24	28B	B	66	88B	A
25	29B	A	67	89B	A
26	30B	B	68	90B	B
27	31B	A	69	91B	A
28	32B	A	70	92B	B
29	33B	A	71	93B	B
	34B	A		94B	B
30	35B	A		95B	A
	36B	A	72	96B	A
	37B		73	97B	A

31	38B	A	74	98B	B
	39B	A	75	99B	A
32	40B	A	76	100B	B
33	41B	A	77	101B	A
	42B	B		102B	A
34	43B	A		103B	
35	44B	A		104B	A
36	45B	A		105B	A
37	46B	B		106B	A
38	47B	A		107B	
39	48B	A	78	108B	A
	49B		79	109B	A
40	50B	B	80	110B	A
41	51B	A	81	111B	A
	52B		82	112B	A
42	53B	A	83	113B	A
43	54B	A	84	114B	A
	55B	A	85	115B	A
44	56B	B	86	116B	A
	57B	A	87	117B	A
45	58B	A		118B	
46	59B	A		119B	
47	60B	B	88	120B	A

A, CCN resistance; B, CCN susceptible; U, unidentified

Mapping data of 79 DH lines (CL x OM)

CL x OM

Chr	Two	Backcross Stats, 95% Limit															
		1	11111	11112	22222	22223	33333	33334	44444	44445	55555	55556	66666	66667	77777	7777
		12345	67890	12345	67890	12345	67890	12345	67890	12345	67890	12345	67890	12345	67890	12345	6789
ABC451		BBBBB	ABAUB	ABBAB	ABBBA	AAABA	ABBAB	BABBA	BAABA	BBBBB	BBBBA	ABBAA	ABABA	ABABA	BBBAA	ABBBA	ABBA
		X	?	XX	X		X	X	X	X	X	X	XX			XX	X
MWG865		BBABB	ABAUB	AAAAB	ABABA	AAABA	AABAB	BBBBA	BAAAA	BBABB	ABBBA	ABBAB	AABBA	ABABA	BBBAA	ABAAA	ABBB
			?				X										
V/v		BBABB	ABAAB	AAAAB	ABABA	AAABA	AABBB	BBBBA	BAAAA	BBABB	ABBBA	ABBAB	AABBA	ABABA	BBBAA	ABAAA	ABBB
		X				X	X		X	X	X				X		
MWG503		ABABB	ABAAB	AAAAB	ABABA	ABABA	AABBA	BBBBA	BBAAB	ABABB	ABBBA	ABBAB	AABBA	ABABA	ABBAA	ABAAA	ABBB
		X		X								X				X	X
cMWG694		ABABA	ABAAB	AAAAA	ABABA	ABABA	AABBA	BBBBA	BBAAB	ABABB	ABBBA	ABAAB	AABBA	ABABA	ABBAA	AAAAA	ABBA
														X	?	X	
BM21		ABABA	ABAAB	AAAAA	UBABA	ABABA	AABBA	BBBBA	BBAAB	ABABB	ABBBA	ABAAB	AABBA	ABAAA	ABUAA	ABAAA	ABBA
								XX				X		X	?		
Ma		ABABA	ABAAU	AAAAA	ABABA	ABABA	AAUBA	UBAAA	BBAAB	ABABB	ABBBA	ABBAB	AABBA	AAAAA	ABAAA	ABAAA	AUBA
		X	X	X	XXX	X	X	X	X	X	X	XX	X	XX	X	X	XX	X
KsuD22		AAABA	ABBAB	ABAAA	BABBA	AAABB	AABBA	BBBAA	BBBAA	AAABB	BABAA	ABABB	AAABA	ABAAA	ABAAA	AABAA	ABAA
					X		X	X	X				X		X		XX
BCD453-b		AAABA	ABBAB	ABAAA	BABBB	AAABB	AAABA	ABBAA	BABAA	AAABB	BABAA	ABABB	AAAAA	ABAAA	AAAAA	AABAA	BAAA

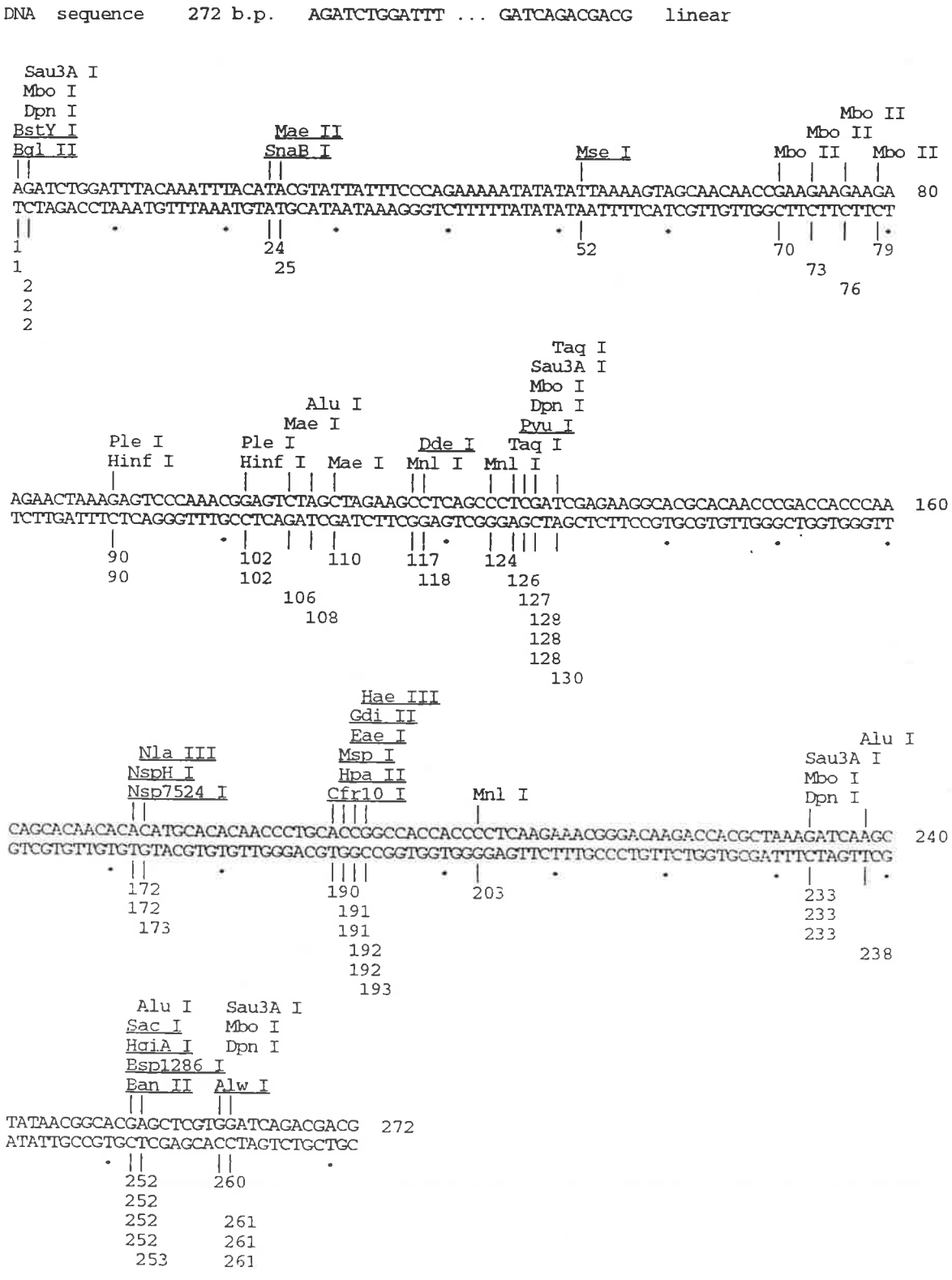
Mapping data of 88 DH lines (CB x HA)

CB X HA

Chr Two	Backcross Stats, 95% Limit																	
	1 11111 11112 22222 22223 33333 33334 44444 44445 55555 55556 66666 66667 77777 77778 88888 888																	
	12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 678																	
BCD355	AAAAA	BAAAA	AABAB	BBBAA	BAABA	AAAAA	AAAAA	BABAB	AAABA	ABAAA	BAABA	BABAA	AAAAA	BABAA	BAABA	BAAAA	AAABA	BAA
ABC451	AAAAA	BAAAA	AABAB	BBUAA	BAABA	AAAAA	AAAAA	BABAB	AAABA	ABAAA	BAABA	BABAA	AAAAA	BABAA	BAABA	BAAAA	AAABA	BAU
ABG14	AAAAA	BAAAA	AABAB	BBUAA	BAABA	AAAAA	AAAAA	BABAB	AAABA	ABAAA	BAABA	BABAA	AAAAA	BABAA	BAABA	BAAAA	AAABA	AAU
MWG865	AAAAA	BAAAA	BABAU	BBBAB	BAABA	AAAAA	AAAAA	BBAAB	AAABA	ABAAA	BAABA	AAAAB	AAABA	AABAB	BAABA	BAAAA	AAABA	AAU
MWG503	AAAAA	BAAAA	BABAB	BAAAB	BAABA	AAUAA	AAAAA	ABAAB	AAABA	ABAAA	BAABA	AAAAB	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
Ha	AAAAA	BAAAA	BABAB	BAAAB	BAABA	BAAAA	AAAAA	ABAAB	AAABA	ABAAA	BABBA	AAAAU	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
BM21	AAAAA	BAAAA	BABAB	BAUAB	BAABA	AAAAA	AAAAA	ABAAB	AAABA	ABAAA	BABAA	AAAAB	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
PCRICB/B	AAAAA	BAAAA	BABAB	BAAAB	BAABA	AAAAA	AAAAA	ABAAB	AAABA	ABAAA	BABAA	AAAAA	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
PCRIHA/B	AAAAA	BAAAA	BABAB	BAAAB	BAABA	AAAAA	AAAAA	ABAAB	AAABA	ABAAA	BABAA	AAAAA	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
cMWG694	AAAAA	BAAAA	BABAB	BAAAB	BAABA	BAAAA	AAAAA	ABAAB	AAABA	ABAAA	BABAA	AAAAB	AAABA	AABAB	BAABA	BAAAA	AAAAA	AAA
BCD453	AAAAA	BAAAA	BABAB	BAAAB	BAABA	BAUAA	AAAAA	ABAAB	AAABA	AAAAA	BABAA	AAAAB	AAAAA	AABAB	BAAAA	BAAAA	AAAAA	AAU

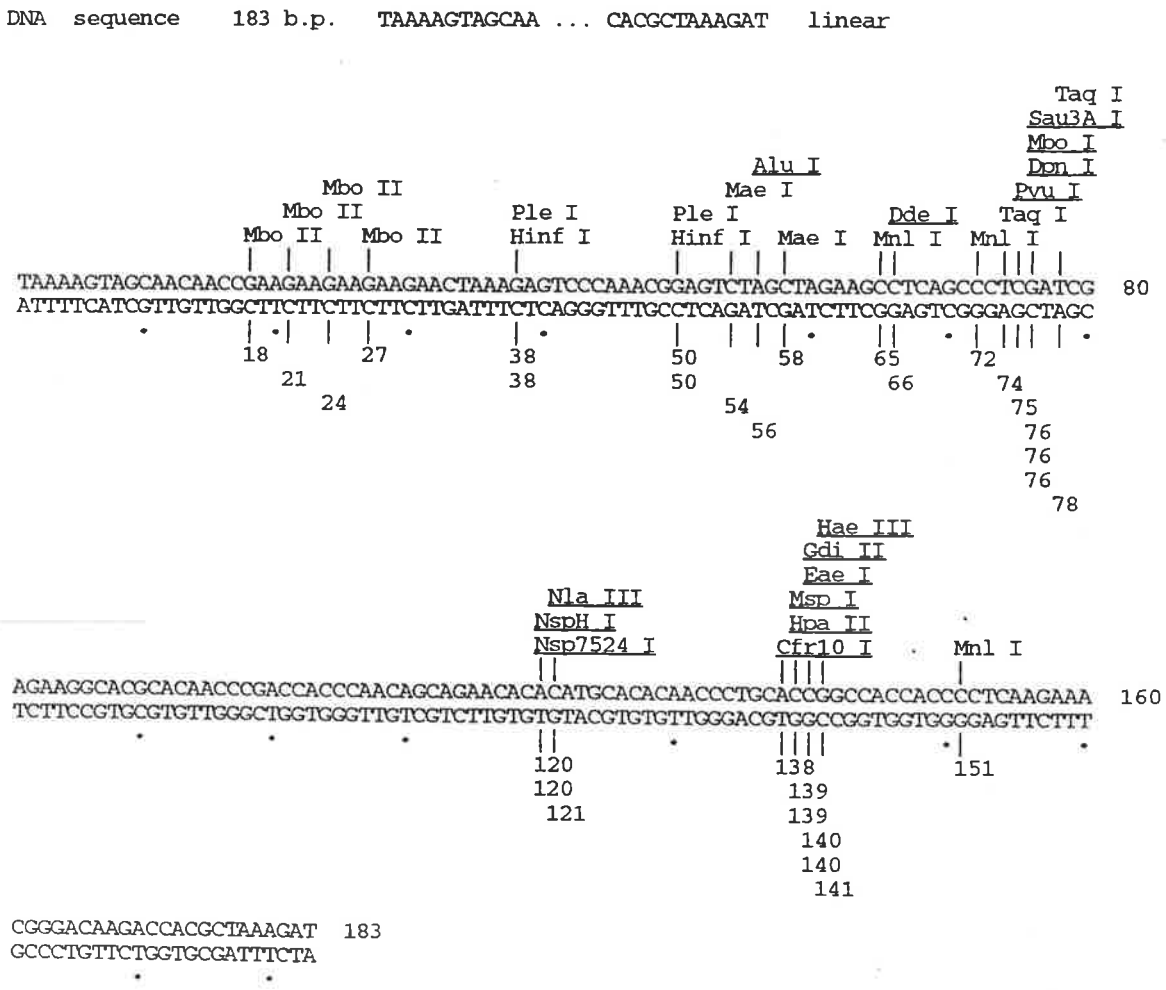
Appendix 4-a

AWBMA21 full sequence (restriction map)



Appendix 4-b

CBM21 full sequence (restriction map)



Appendix 4-c

HAM21 full sequence (restriction map)