BIOLOGICAL AND GENETIC STUDIES OF WHEAT RESISTANCE TO *HETERODERA AVENAE*

by

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Contents

Summary

The cereal cyst nematode (CCN) *Heterodera avenae* (Woll.) is an economically damaging root pathogen of cereals worldwide. This thesis presents the results of studies into the biology and genetics of resistance to CCN in wheat conferred by the gene *Cre*.

Following the development of a synchronised infection system, growth of *H.avenae* juveniles within the roots of resistant and susceptible near-isogenic lines (NILs) was investigated for evidence of resistance gene expression. Female nematodes developed similarly in both cultivars up till 15 days, when development was halted in the resistant line. This correlated with the appearance of the nematode-induced feeding site, a syncytium, in the resistant line. It became vacuolate at about this time, eventually degenerating totally while the syncytia of the susceptible line appeared to have high metabolic activity. This study indicates that the CCN resistance response occurs late in the infection process, and is not a hypersensitive-type reaction.

Protein profiles of syncytium-enriched root sections from the NILs were examined for resistance-related differences. Micro-dissection of roots was used to obtain stele sections containing syncytia. No specifically resistance-related proteins were observed. A major 15 kD protein accumulated in sections from 27-35 day old roots of susceptible lines. This may be a syncytium-specific protein, as syncytia in resistant roots are generally degenerate by this time. Protein differences were observed between sections from syncytium-containing roots and those from uninfected roots, indicating nematode-directed alteration of host gene transcription and/or translation.

A directed search was made for *Cre*-linked RFLP markers using the NILs. Two out of fifty-eight probes produced a RFLP between the lines. These were mapped using aneuploid stocks of "Chinese Spring" and two F2 populations segregating for CCN

resistance. *Cre* was mapped to the proximal region of the long arm of chromosome 2B in the line "AUS10894". A linkage group containing *Cre* and four RFLP loci was identified.

Molecular techniques were employed to develop the RFLP marker closest to *Cre* into a PCR-based assay. The clone was sequenced and PCR primers designed. A dominant PCR marker was produced when amplification products from resistant and susceptible lines were digested with a restriction enzyme. These PCR products were cloned and a single base substitution was found in the resistant line. Allele-specific primers were designed with respect to the mutation, and a co-dominant *Cre*-linked PCR marker was produced using a dual-PCR strategy.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university. To the best of my knowledge and belief, it contains no material previously written by any other person, except where due reference is made in the text.

I give consent for this thesis, when deposited in the University library, to be made available for loan or photocopying provided due acknowledgment is made.

Kevin Williams

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Chapter 1 Introduction and Literature Review

Introduction

The cereal cyst nematode (CCN) *Heterodera avenae* (Wollenweber, 1924; Filipjev, 1934) is an obligate parasite of cereals. It has a wide range of gramineous hosts, including the economically important cereals. *H. avenae* is distributed throughout the worlds grain growing areas, having been identified in over 30 countries (Meagher, 1977).

H. avenae was probably introduced from Europe in the late nineteenth century (Meagher, 1977). It was first reported in South Australia by Davidson (1930). It was later found on the roots of herbarium wheat specimens collected in 1904 (Meagher, 1972). Known hosts in Australia are species of the introduced genera *Avena*, *Hordeum*, *Lolium*, *Phalaris*, *Secale*, *Triticum* (McLeod 1992), while the nematode has not been found on any native plants (Fisher, 1987).

The detrimental effects of CCN on cereal growth in Australia are found in early reports by Hickinbotham (1930), and Millikan (1938). Control of damage by the use of rotations with non-hosts was first proposed at this time (Garret, 1934; Millikan, 1938). More recent research has confirmed its status as the most damaging pathogen of wheat in southern Australia (Brown, 1984), where it is found on all soil types. Annual crop losses caused by CCN are very high. In the 1986-1987 growing season, they were estimated at $54 million (Brennan and Murray, 1988).

Literature review

Biology of the Nematode

At least 20 pathotypes of *H. avenae* have been identified worldwide (Andersen and Andersen, 1982), although there is thought to be only one pathotype in Australia

Figure 1.1 Severe chlorotic symptoms in wheat crop caused by *H. avenae*.

Figure 1.2 Mature white females of *H. avenae* attached to wheat roots.

(O'Brien and Fisher, 1979). Cereals infected with *H. avenae* have chlorotic leaves and stunted growth habits. Seminal root growth is reduced, with many lateral roots arising from galls formed at the nematode feeding site (Volkmar, 1989). Plants suffer reduced tillering (Davies, 1961), and lower kernel weight (Zancada and Althofer, 1994). In combination, these symptoms result in serious yield depression under Australian conditions (Stanton and Fisher, 1988; Fisher and Hancock, 1991). Cereal crops susceptible to CCN may suffer losses of the magnitude of 20-50 % (Gurner *et al.*, 1980; Rovira and Simon, 1982).

H. avenae eggs become hatchable as the temperature falls in Autumn with optimum rate of hatching at about 10°C, depending on the availability of water in the soil (Banyer and Fisher, 1971, 1972). This soil temperature occurs at the optimum time for sowing cereals in southern Australia (Georg *et al.*, 1989), leading to massive infection rates in susceptible cultivars. Juveniles penetrate at the root tip and migrate intracellularly through the root cortex until they settle permanently at their feeding site, a syncytium induced by the nematode (Dropkin, 1969). Once a feeding site has been established, the nematode enlarges and completes its final molts. The life cycle is dependent on temperature (Fisher, 1981), with females maturing in 6-9 weeks and males in about 3 weeks. The immobile adult females are fertilised by migrating males and the eggs are contained in cysts formed from the tanned female body. The eggs may survive for many years within the cyst (Thorne, 1961).

Ultrastructure of Syncytium

The syncytium induced by *H. avenae* in wheat roots is evidence of the intimate interaction between this sedentary obligate parasite and its host. A nematode-induced syncytia is one of the most complex responses initiated in a host plant by a pathogen (Opperman and Conkling, 1994). Syncytial ultrastructure has therefore been extensively studied in some interactions. It is a prerequisite to determining how the nematode alters cell development to its own advantage, and it may also be an appropriate point to

engineer control strategies because of the complete dependence of the sedentary nematode on its syncytium.

Syncytia resemble the transfer cells of plants described by Pate and Gunning (1972), so named because of their characteristic wall membrane apparatus. The cells of the syncytium are modified to sequester nutrients from the host plant for ingestion by the nematode (Jones, 1981). Observations of *H. schachtii* in roots of *Arabidopsis* indicated that adult females may remove nutrients equivalent to four times the syncytial volume per day (Sijmons *et al.*, 1991). The transport of solutes is facilitated by cell wall ingrowths which form extensively adjacent to xylem vessels (Jones and Northcote, 1972a,b), increasing membrane surface area by 10 - 15 times (Jones and Dropkin, 1976).

Cyst nematodes initiate syncytia by perforating the wall of the initial syncytial cell with their stylet, followed by a resting period prior to the start of feeding (Wyss, 1992). The walls of neighbouring cells are dissolved and their protoplasts fuse. The syncytium expands acropetally and basipetally by incorporating xylem parenchyma and stellar parenchyma cells and may be up to 2 mm long (Magnusson and Golinowski, 1991). In contrast, root knot nematodes (*Meloidogyne* spp.) induce the formation of a single giant cell. This forms by repeated mitosis without cytokinesis (Huang and Maggenti, 1969). Morphological features of syncytia and giant cells induced by both cyst and root-knot nematodes include cell and nuclear hypertrophy, main vacuole reduction, and cytoplasmic organelle proliferation (Jones and Northcote, 1972a,b).

The ultrastructure of interactions between various sedentary nematode species and their compatible and incompatible hosts has been studied in an attempt to observe cellular changes that may be associated with resistance. Several different resistant host responses have been reported. The root-knot nematode *M. incognita* fails to develop a giant cell in resistant cultivars of tomato due to a localised hypersensitive reaction (Paulson and Webster, 1970; Bleve-Zacheo *et al.*, 1982). Resistance to the potato cyst nematode *Globodera rostochiensis* also appears to be due to a hypersensitive reaction around the developing syncytium (Rice *et al.*, 1987). In contrast, the soybean cyst nematode *H.*

schachtii initiates a syncytium in resistant cultivars, but it breaks down after 4 days with nuclear and cytoplasmic degeneration observed in various resistant cultivars (Endo, 1965; Riggs *et al.*, 1973; Wyss *et al.*, 1984).

The ultrastructure of syncytia induced in resistant and susceptible wheat cultivars were investigated in two studies by Grymaszewska and Golinowsky (1987, 1991). Each study proposed mechanisms for resistance which appear to be contradictory; the early degeneration of syncytia in the first study, and the late development of syncytia in the latter. These apparently confounding results may have been due to the inoculation system, which did not provide reliable synchronised infection.

Syncytial proteins

Syncytial biochemistry has received relatively little attention. The ultrastructural evidence indicates that cells incorporated into syncytia have a very different metabolism to surrounding unaffected cells, but researchers have not even begun to characterise the differences at the metabolic level. Biochemical studies are complicated by the limited amount of tissue available, and the necessity to obtain syncytial material free of associated nematodes. The first study of proteins from syncytia induced in roots of balsam by *M. incognita* concluded that syncytial metabolism was the same as that of root meristematic cells rather than being the same as that of transfer cells of leaf glands, whose appearance is similar to syncytial cells (Jones, 1980). In the only other study to use only syncytium-containing root sections, Grundler *et al.*, (1991) and Betka *et al.*, (1991) found that changes in some amino acids may have affected the sex determination of *H. schachtii* juveniles in rape, while changes in the total amount of protein or amino acids had no affect.

Hammond-Kosack *et al.*, (1989) investigated the accumulation of leaf apoplast proteins in potato plants infected with *G. rostochiensis*. The leaf proteins expressed were probably pathogenesis-related (PR), as their expression could be mimicked by applications of aspirin, which is known to be an elicitor of defence-related compounds

(Williams and Leung, 1993). Later studies from the same group also found massive changes in translatable mRNA in leaves of *G. rostochiensis* infected potato plants, with small qualitative changes in root mRNA that may have been associated with a resistant or susceptible response (Hammond-Kosack *et al.*, 1990). The massive rates of inoculation used in these experiments may have caused the systemic PR protein response observed, masking other more specific nematode-related protein or mRNA differences.

CCN control strategies

Chemical control of CCN has been shown to be effective under experimental conditions (Brown, 1973; Rovira, 1990). However, large scale pesticide use is now considered to be environmentally and socially unacceptable and uneconomic in low value crops. Biological control of CCN has been investigated under English conditions with some success (Kerry *et al.*, 1980) but under Australian conditions it was not effective enough to reduce numbers of *H. avenae* eggs in the soil to below damaging thresholds (Stirling and Kerry, 1983).

The use of rotations with non-hosts can reduce the damage to susceptible cereals by CCN. This strategy depends on the availability of a resistant rotational crop. Although rotations reduce the population of eggs in the soil, the use of non-income producing crops may not be economic. This option is also not available in some agricultural systems where only monocultures or limited numbers of crops can be grown due to climatic or soil limitations. The development of resistant cultivars is, therefore, seen as the most cost effective and environmentally sustainable method of reducing nematode damage (Cook and Evans, 1987). Income-producing nematode-resistant crops can then be used in a farming strategy with those more susceptible, maintaining returns to the grower. The benefits of developing crops resistant to nematodes can be great. For example, in North America the one million dollar cost of developing a soybean cultivar resistant to *H. glycines* was more than recouped by saving $400 million worth of damage over six years (Bradley and Duffy, 1982).

Resistance to CCN in wheat

It is probable that the highly specialised plant parasitic nematodes coevolved with their hosts to overcome the original resistance condition (Krall and Krall, 1978; Stone, 1986,1987). Resistance genes are relics from the ancient resistance genotype. Nematode pathotypes result from host evolution towards susceptibility. This hypothesis suggests that host resistance and nematode virulence genes will interact according to Flor's gene-for-gene model (Flor, 1956). Host plant resistance has often been found to be mediated by single genes (Parrott, 1982; Sidhu and Webster, 1981a,b).

Resistance to CCN has been described in various members of the Gramineae. Resistance genes have been identified in barley (Hayes and Cotten, 1970), oats (Cotten and Hayes, 1972), cultivated rye (Asiedu *et al.*, 1990) and wheat (Nielsen, 1966; O'Brien and Fisher, 1974). Sources of resistance amongst the wild grasses include *Triticum tauschii* (Eastwood *et al.*, 1991) and *Aegilops* spp. (Dosba *et al.*, 1978). Therefore, in most cereals there is a pool of genetic material available for breeders to use in their programs to develop CCN resistant commercial cereal cultivars. Single resistance genes are the most useful, as plant breeders have difficulty working with multiple gene resistance.

To date there has only been one gene reported for CCN resistance in wheat. Resistance in the variety Loros was discovered by Andersen (1961) and was determined to be monogenic by Nielsen (1966). The resistance gene locus was located on chromosome 2B by monosomic analysis using the variety Redman (Slootmaker *et al.*, 1974). The spring wheat variety AUS10894 also carries a single dominant CCN resistance gene but it has been shown to be the same as, or allelic to, that of Loros (O'Brien *et al.*, 1980; Nielsen, 1982).

Molecular markers

When genetic markers are linked to genes of interest, they can be used as tools in breeding programs to produce improved crop species (Stuber and Edwards, 1986). Selection of progeny can be based on the presence of a linked marker rather than having

to assay for the phenotype of the gene of interest. Multiple characters can potentially be scored at the same time. Morphological and cytological characteristics can be used as genetic markers, although they are of little use in breeding programs as they are difficult to score in many instances (Tanksley, 1989). Isozyme markers have been used in genetic studies (McIntyre, 1988) but their use is limited by low frequencies of polymorphic loci in many crops (Dudley, 1989).

RFLP markers

Molecular markers based on DNA polymorphisms such as restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980) have been used to produce genetic maps of many crop species. RFLP maps have been constructed for cereals, including barley (Heun *et al.*, 1991; Graner *et al.*, 1991; Kleinhofs *et al.*, 1993), rice (Nagamura, 1993) and hexaploid wheat (Liu and Tsunewaki, 1991; Anderson *et al.*, 1992).

The potential of marker-assisted selection using molecular markers has given impetus to the mapping of agronomically important genes. In cereals, RFLP markers that are linked to several disease resistance genes have been identified. Examples include the *Mlg* and *Mla* loci for powdery mildew resistance in barley (Schuller *et al.*, 1992; Gorg *et al.*, 1993), the *Rpg1* locus for stem rust resistance in barley (Kilian *et al.*, 1994) and the *Pm3* locus for powdery mildew resistance in wheat (Hartl *et al.*, 1993).

PCR markers

Markers based on the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) have been proposed as a quicker and technically simpler alternative to RFLPs (Ragot and Hoisington, 1993). In particular, randomly amplified polymorphic DNAs (RAPDs) can be used as genetic markers in mapping and genomic fingerprinting (Williams *et al.*, 1990, Welsh and McClelland, 1990, Rafalski *et al.*, 1991) and to find markers linked to agronomically important genes. RAPDs have been used with tomato near-isogenic lines to find markers linked to the *Pto* gene for resistance to *Pseudomonas syringae* (Martin *et*

al., 1991). They have also been used with bulk segregant analysis to quickly find markers in designated areas of the lettuce genome (Michelmore *et al.*, 1991).

Several groups have investigated using RAPDs as genetic markers in wheat. Weining and Langridge (1991) used a combination of RAPDs and semi-random primers to generate mappable polymorphisms between wheat, rye and barley. However, more detailed studies with wheat showed that the non-homologous, non-dose responsive and dominant nature of RAPDs reduced their value for genetic mapping in this species (Devos and Gale, 1992). He *et al.*, (1992) found that RAPDs revealed more polymorphisms in wheat when analysed with a denaturing gradient gel electrophoresis (DGGE) system.

Microsatellites are another type of PCR-based genetic marker. Site-specific length polymorphism of tandemly repeated nucleotide motifs can be detected using PCR (Litt and Luty, 1989). Morgante and Olivieri (1993) found microsatellites to be ideal genetic markers in soybeans. The only cereal crop in which microsatellites have been investigated is rice. They were found to overcome the problems of low heterozygosity found with RFLPs, and could be easily mapped to rice chromosomes (Wu and Tanksley, 1993).

Mapping in cereals

To date, the cereal crop which has been mapped with the highest degree of marker saturation is rice. The considerable efforts of the Rice Genome Research Program have put 1000 markers on the rice map, with a goal of 2000, or one every centimorgan. The prospects for genetic and physical mapping in rice are much better than in wheat. This is because of the complexity (2n=6x=42) and size (16,000 Mb / haploid genome) of the wheat genome, compared with the diploid (2n=2x=24) rice genome with only 400 Mb per haploid genome (Arumuganathan and Earle, 1991). The large difference in the genome sizes is caused by the large amount of repetitive DNA (75 %) in the wheat genome (Rimpau *et al.*, 1978). Notwithstanding the difference in genome sizes,

considerable gene order synteny between rice and wheat has recently been discovered (Ahn *et al.*, 1993).

Mapping of nematode resistance genes

Nematode resistance genes have been mapped in a number of diploid crop species. The *Mi* gene of tomato which confers resistance to *M. incognita* has been linked to, and may lie between, the isozyme locus *Aps-1* and the RFLP marker *GP79* on chromosome six (Klein-Lankhorst *et al.*, 1991; Messeguer *et al.*, 1991). Physical mapping of these markers indicated that they were approximately 550 kb apart (van Daelen *et al.*, 1993).

Two genes for resistance to the potato cyst nematode have been mapped. The gene *Gro1* (derived from *Solanum spegazzinii*) has been mapped to potato chromosome VII (Barone *et al.*, 1990). The *H1* gene (derived from *S. tuberosum* ssp. andigena) has been mapped to chromosome V using RFLP markers (Gebhardt *et al.*, 1993; Pineda *et al.*, 1993).

Molecular markers have also been identified that are linked to genes for resistance to *H. schachtii* in cultivated beet (Salentijn *et al.*, 1992), sugarbeet (Jung *et al.*, 1992; Uphoff and Wricke, 1992) and soybean (Weisemann *et al.*, 1992).

Research Objectives

This research project had two major gaols: to increase our understanding of wheat resistance to CCN; and to develop molecular markers linked to the CCN resistance gene *Cre*.

The first goal required fundamental studies on the biology of the interaction between *H. avenae* and susceptible and resistant wheat lines. The specific aims of this research were:

- To investigate the cellular structure of nematode-induced syncytia in near-isogenic lines of wheat, one of which carries a CCN resistance gene from the cultivar AUS 10894, to gather information on how and when the resistance mechanism may be operating.

- To investigate proteins of syncytia from the wheat near-isogenic lines, to search for differential accumulation of proteins associated with the compatible or incompatible interaction.

The second and more applied goal of the project was to use genetic analysis and molecular biology to:

- Identify *Cre*-linked molecular markers using near-isogenic lines and construct a linkage map containing the resistance gene and flanking markers.
- Develop a PCR-based assay for CCN resistance from any markers linked to *Cre*.

Chapter 2 Materials and methods

Plant materials

The CCN-resistant near-isogenic line (NIL) AUS10894 x Prins (AP) was developed by Prof. James MacKey (Upsalla, Sweden) after seven backcross generations with "Prins" as the recurrent parent and "AUS10894" as the donor line. Prins is a susceptible Swedish wheat line, while AUS10894 carries the only known wheat CCN resistance gene, *Cre* (O'Brien and Fisher, 1974; Nielsen, 1982). Other susceptible control varieties used were Schomburgk, Olympic, Spear, Warrigal, Condor and Egret. Breeders lines resistant to CCN and carrying the *Cre* gene were supplied by Dr A. Rathjen. "Chinese Spring" nulli-tetrasomic (Sears, 1966) and ditelosomic lines (Sears and Sears, 1978) were used to localise RFLPs to chromosomes and chromosome arms. For linkage analysis, F2 progeny of crosses between "AUS10894" x "Spear", and "AP" x "Prins", were kindly supplied by F. Green.

Molecular techniques

The experimental protocols described below are modified versions of those described by Langridge, Appels and Sharp (1992).

DNA isolation (miniprep)

A small piece of healthy leaf tissue was snap frozen in a 2 ml eppendorf tube in liquid N2 and ground to a powder with forceps. The powder was homogenised with 600 µl of extraction buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 1% Sarkosyl, pH 8.5). To the tubes 600 µl phenol/chloroform/iso-amyl-alcohol (25:24:1 (V/V)) was added and the tubes shaken vigorously for 1 min. The phases were separated at 12,000 g for 5 min and the upper aqueous phase transferred to a fresh tube. DNA was precipitated by adding 75 µl 3M Na-acetate pH 5.1 and 600 µl isopropanol, mixing and standing at

room temperature for 1 min. The DNA was pelleted at 12,000 g for 10 min. The supernatant was discarded and the pellet washed with 1 ml 70% ethanol. The pellet was dried and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 40 µg/ml RNaseA.

DNA ligation

Vector preparation: The vector (1µg) was digested with *Sma*1. Digested DNA was precipitated with 2 µl Na-acetate and 40 µl ethanol. Following pelleting by centrifugation the pellet was washed twice with 70% ethanol. The pellet was resuspended in 18 µl water and 2 µl 10x phosphatase buffer. To the tubes 1 µl calf intestinal phosphatase (CIP) (Boehringer) was added and the tubes incubated for 30 min at 37°C, followed by 10 min at 65°C. Further CIP (0.5 µl) was added and the tubes incubated again at 37°C for 30 min. The reactions were then extracted twice with phenol/chloroform and ethanol precipitated, before being resuspended in 5 µl TE.

Insert preparation: PCR was performed using kinased primers [10 µl primers were incubated with 1.3 µl 10x kinase buffer, 1 µl 10 mM ATP, 10 units T4 polynucleotide kinase (Promega)]. PCR products were purified using Geneclean according to manufacturers instructions (Bio101).

For ligation, 200 ng vector and 3 ng insert were incubated with 1 µl 10x ligase buffer, 1 µl 10 mM ATP, 1 µl T4 DNA (Promega) ligase in a total volume of 10 µl at 12°C overnight. The ligated DNA was ethanol precipitated, and resuspended in 40 µl TE.

Transformation

Transformation of the *Escherichia coli* strain DH5α used a protocol based on that of Hanahan (1985). Fifty ml of DH5α cells were grown to O.D. 600 of 0.45-0.55 in SOB (1 litre contained 20 g bacto tryptone, 5 g yeast extract, 0.6 g NaCl, 0.19 g KCl, 10 mM MgSO, 10 mM MgCl). Cells were placed on ice for 10-15 min. Cells were pelleted at 2500 g for 12 min at 4°C and resuspended in 8.5 ml TFB (10 mM MES, 45 mM MnCl,

100 mM RbCl, 10 mM CaCl, 3mM hexamine cobalt chloride). They were then placed on ice for 10 to 15 min then pelleted as above. Cells were resuspended in 2 ml TFB, 70 µl dimethyl sulphonide (DMSO), and left on ice for 5 min. Cells had 157 µl 1M dithiothreitol (DTT) added, followed by 10 min on ice, after which 75 µl DMSO was added, followed by 10 min on ice.

The ligation mix was added to 210 µl cells, and left for 30 min on ice before being heat shocked for 2 min at 42°C. SOC (800 µl) (SOB supplemented with 7 µl/ml 50% glucose) was added prior to incubation for 50-60 min at 37°C. Aliquots (100 µl) were plated onto LB plates containing ampicillin (50 µg/ml) overlaid with 20 µl Bromo-(5)-4-chloro-3-indolyl-ß-galactopyranoside (Xgal) (2% w/v in dimethylformamide) and 10 µl Isopropyl ß-thiogalactopyranoside (IPTG) (0.1 M).

Southern hybridisation

Plant DNA was transferred to Hybond N+ membrane (Amersham) according to the manufacturers instructions based on the method developed by Southern (1975). Restricted genomic DNA was electrophoresed overnight in 1% (W/V) agarose gels. The DNA was transferred via capillary blotting to Hybond N+ membranes. After staining with ethidium bromide, gels were soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min and were then neutralised in 1.5 M NaCl, 0.5 M Tris-HCl, 0.001 M EDTA (pH 7.2) for 30 min. The Hybond N+ membrane was placed on the inverted gel, and sandwiched between several layers of Whatman 3MM filter paper on a sponge soaked in 20x SSC (3 M NaCl, 0.3 M trisodium citrate). A 4 cm stack of paper towels was placed on top of the sandwich. After the transfer the membrane was rinsed in 5x SSC and dried under vacuum at 80°C for 20 min. The DNA was fixed to the membrane in 0.4 M NaOH for 20 min, followed by 5 min in neutralising solution and 2 min in 2x SSC.

Probe labelling

Approximately 50 ng of probe DNA and 3 µl oligolabelling mix (0.05 µg random primers, 60 mM dATP, dTTP, dGTP, 150 mM Tris-HCl pH 7.6, 150 mM NaCl, 30 mM $MgCl_2$, 0.9 µg BSA) in 8 µl total volume were boiled for 5 min and then chilled on ice. To the mix 12.5 µl of labelling buffer, 3 µl [alpha-^{32}P]dCTP and 1 µl Klenow enzyme was added, followed by incubation at 42°C for 60 min. The labelled DNA was separated from the unincorporated nucleotides on a Sephadex G-100 column.

Hybridisation

The membranes were pre-hybridised overnight at 65°C in 3 ml water, 3 ml 5x HSB (3 M NaCl, 100 mM PIPES, 25 mM Na_2EDTA; pH 6.8), 3 ml 25% (W/V) Dextran sulphate, 1 ml Denhardts III (2% (W/V) BSA, 2% (W/V) Ficoll 400, 2% PVP 360, 10% (W/V) SDS) and 1 µg boiled salmon sperm DNA. The labelled probe was then boiled with a further 1 µg salmon sperm DNA, added to the membrane and hybridised overnight. The membrane was washed at 65°C in 2x SSC, 0.1% SDS for 20 min and 0.2x SSC, 0.1% SDS for 20 min, before being exposed to x-ray film for 4-6 days at -80°C.

Sequencing

Sequences were determined using an Applied Biosystems Inc.(Foster City, California, U.S.A.) automated sequencer. DNA was prepared according to the manufacturers instructions.

Insert amplification

Clone inserts were PCR amplified using M13 forward and reverse primers. PCR reactions contained 20 mM NH_4SO_4, 75 mM Tris-HCl pH 9, 0.01 % (w/v) Tween, 50 mM each dNTP, 1.5 mM $MgCl_2$, 150 ng each primer, 100 ng template DNA and 0.2 units *Taq* polymerase in 50 µl total volume. Reactions were carried out in a MJ Research thermal cycler. Temperature conditions were 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min, followed by 1 cycle of 72 °C for 5

min. The products were separated on an agarose gel and the insert band excised and purified using Geneclean.

Plasmid DNA Preparation

Cells were pelleted and resuspended in 100 µl of buffer 1 (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, 0.4 µg lysozyme), and left on ice for 10 min. To the mixture 200 µl of freshly prepared buffer 2 (0.2 M NaOH, 0.1% SDS) was added prior to incubation on ice for a further 10 min, before 150 µl of 3M Na-acetate (pH 4.8) was added and the mixture incubated at -20°C for 10 min. After centrifugation at 12,000 g for 15 min, the supernatant was transferred to a fresh tube and 1 ml cold ethanol added. After incubation for 10 min on ice, the DNA was pelleted at 12,000 g and the pellet washed with 70% ethanol. The pellet was air-dried and resuspended in 30 µl TE buffer.

Chapter 3 Morphology of Nematode-induced Syncytia

Introduction

Little is known about the development of *Heterodera avenae* within host roots. It has been observed that the juveniles of other cyst nematode genera invade the tips of host roots and migrate through the cortex, settling permanently at their feeding site. The cells of the feeding site, or syncytium, are modified to sequester nutrients from the host plant vascular system for ingestion by the nematode.

The ultrastructure of interactions between various cyst nematode species and their compatible and resistant hosts has been investigated. Several different resistant host responses have been observed: the deterioration of the syncytium following its successful establishment (Gipson *et al.*, 1971; Riggs *et al.*, 1973; Grymaszewska and Golinowski, 1987), the resistance of cells to syncytial incorporation (Magnusson and Golinowsky, 1991) and the late development of syncytia in resistant varieties (Grymaszewska and Golinowski, 1991).

Resistance to CCN in lines of wheat, barley and oats has been observed (O'Brien and Fisher, 1974) but the mechanisms of resistance have not been conclusively determined. The aim of the research described in this chapter is to investigate the cellular structure of nematode-induced syncytia in near-isogenic cultivars of wheat, one of which carries the CCN resistance gene *Cre* from the line AUS 10894, to gather information on how and when the resistance mechanism may be operating.

Materials and Methods

Roots of the near-isogenic line AP (seven backcross generations) and the susceptible wheat cultivar Prins were examined. Surface-sterilised grains were germinated on 1 % agar in the dark at 20 °C for 48 h. The emerging roots were then inoculated with approximately 50 juvenile cereal cyst nematodes (*H. avenae*) of the Australian pathotype (Andersen and Andersen, 1982). Second stage juveniles were obtained from cysts extracted from soil and hatched at 10 °C. The juveniles were surface sterilised in 0.125 % (W/V) penicillin-G, 0.125 % (W/V) streptomycin sulphate and 0.0125 % (W/V) tetracycline hydrochloride overnight and re-suspended in sterile distilled water at a concentration of 2500 J2 per ml.

The inoculum (20 µl) was pipetted onto the surface of the agar immediately adjacent to the root tips. After two days the seedlings were removed from the inoculation plates to prevent further penetration and to ensure synchronised infection, and were transferred to 100 ml of B and D medium (Kondorosi *et al.*, 1984) in 500 ml containers at 15 °C with a 12 h day:night photoperiod.

Root samples were taken at various times from two to 36 days after inoculation for light and transmission electron microscopy. Sections of primary root (approx. 3 mm) containing a nematode-induced gall and associated lateral roots were pre-fixed overnight in 3 % (V/V) gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7). Tissues were post-fixed for 3 h in 1 % (W/V) osmium tetroxide and for 30 min in 0.5 % (W/V) uranyl acetate, prior to dehydration in acetone and embedding in Spurrs resin.

For light microscopy on a Zeiss axiophot microscope, thick (3 µm) transverse and longitudinal sections were cut on a LKB microtome, stained with 0.1 % (W/V) methylene blue and 0.1 % (W/V) azure II in 1 % (W/V) borax solution (Richardson *et al.*, 1960), and mounted in DePeX mounting medium (BDH, U.K.). For transmission electron microscopy on a Philips TEM, 70 nm sections cut on a LKB ultramicrotome were picked up on Formvar-coated grids and stained for 12 min in 0.4 % (W/V) uranyl

acetate and for 8 min in Reynolds lead citrate (Reynolds, 1963). For nematode counting and size measurement, roots were stained with 0.05 % (W/V) acid fuchsin in lactoglycerol (Bridge *et al.*, 1982). Body sizes were determined by measuring the plan area of drawings made with a camera lucida with an planimeter (OTT, Leonberg, Germany). Approximately 20 individuals were measured at each time point.

Results

Aseptic inoculation

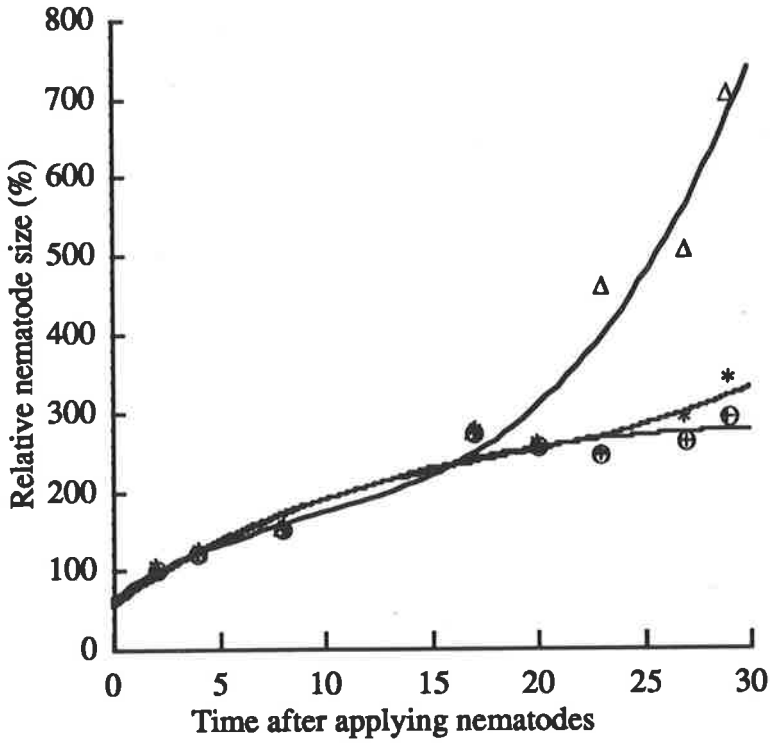
During all experiments, the susceptible and resistant lines were treated similarly. Nematode development on the susceptible cultivar demonstrated that the axenic culture system did not affect the ability of juveniles to penetrate roots and develop fully. Most nematodes had made their way onto the surface of the root tip by 24 hr post inoculation and had penetrated the root tip within 48 hours, at which stage the seedlings were transferred to fresh media.

Following inoculation, the region of the root immediately behind the root apical meristem became densely populated by root hairs resulting from the inhibition of root elongation by the nematodes. This reaction signalled syncytial initiation and these regions swelled into galls within four days and produced lateral roots within 14 days. When few juveniles penetrated the roots, gall formation was slower or failed to occur, so that the time of appearance and the size of galls indicated the efficiency of inoculation. The synchronised infection system and gall size selection were, therefore, used to examine root sections containing nematodes and root tissues of similar age and density.

Nematode growth rates

Figure 3.1 shows the increase in nematode body size for juveniles in the resistant and susceptible cultivars. Juveniles in both cultivars increased in size at the same rate until their third moult at seventeen days. At this stage males had ceased feeding. Females on

Figure 3.1 The development of *H. avenae* in susceptible and resistant wheat cultivars. Development is expressed as a percentage of body size relative to the size at two days post-inoculation. Δ - females and + - males in the susceptible cultivar, * - females and O - males in the resistant cultivar.


800
700
600
500
400
300
200
100
0
Relative nematode size (%)
0
5
10
15
20
25
30
Time after applying nematodes

the susceptible cultivar increased in size rapidly after 20 days, while growth of those in the resistant cultivar was greatly reduced, so that they failed to develop to maturity, giving an unequal final sex ratio.

Syncytia in susceptible wheat roots

Figure 3.2 presents cellular responses to invasion of roots of the susceptible wheat cultivar Prins by second stage juveniles of *H. avenae*. By day four, the juveniles had migrated towards the centre of the root, settled against the endodermis and initiated their permanent feeding site in the stele. The feeding site, a syncytium, was formed by cell wall dissolution of pericycle and vascular parenchyma cells and in some cases it incorporated immature metaxylem (Fig. 3.2a). Necrotic areas appeared at an early stage between the nematode body and the syncytium as endodermal cells collapsed. This necrosis sometimes extended in an arc around the vascular cylinder but was not observed between the syncytium and vascular cells (Fig. 3.2b).

At day eight, the syncytium was still characterised by extensive vacuolation (Fig 3.2c). By day fifteen, a major change occurred in the appearance of the syncytium in the susceptible cultivar, as large vacuoles decreased in number, with a corresponding increase in cytoplasmic volume. The cytoplasm stained intensely and the large vacuoles were clear or contained an amorphous material (Fig 3.2d). Lateral roots were initiated, and vascular elements were forced into irregular arrangements to avoid these and the large syncytia.(Fig. 3.2d) Osmiophilic granules appeared in the cytoplasm of very active syncytia (Fig. 3.2e). By day seventeen, syncytia were highly active, with hypertrophied nuclei and few large vacuoles, even when the nematode head was surrounded by necrotic cells (Fig. 3.2f).

Syncytia in resistant wheat roots

Figure 3.3 shows root cell responses to juvenile invasion in the resistant wheat cultivar "AP". By day four, syncytia were established. They had large vacuoles and a small amount of cytoplasm, differing in appearance from other vascular parenchyma cells (Fig.

Figure 3.2 Structure of syncytia induced in the roots of the susceptible wheat cultivar Prins by *H. avenae*.

a. Transverse section (TS) of day four syncytium incorporating central metaxylem element (Bar = 50 μm).

b. TS of day eight stele containing large syncytium, with extensive arc of necrosis between the nematode and syncytium (Bar = 100 μm).

c. TS of day eight syncytium showing large vacuoles and close contact with xylem and phloem (Bar = 20 μm).

d. Longitudinal section (LS) of two fifteen-day-old syncytia, showing few large vacuoles, multiple lateral meristems, and disorganised vascular tissue (Bar = 200 μm).

e. TS of day nineteen syncytium. Cytoplasm stains intensely, and contains osmiophilic granules (Bar = 25 μm).

f. TS of two day 21 syncytia. Few large vacuoles remain, nuclei are hypertrophied, and cytoplasm stains intensely. Necrosis is evident around nematode head (Bar = 50 μm).

S, S1, S2 - syncytia, N - nematode, nec - necrosis, LV - large vacuole, SV - small vacuole, x - xylem, p - phloem, LR - lateral root.

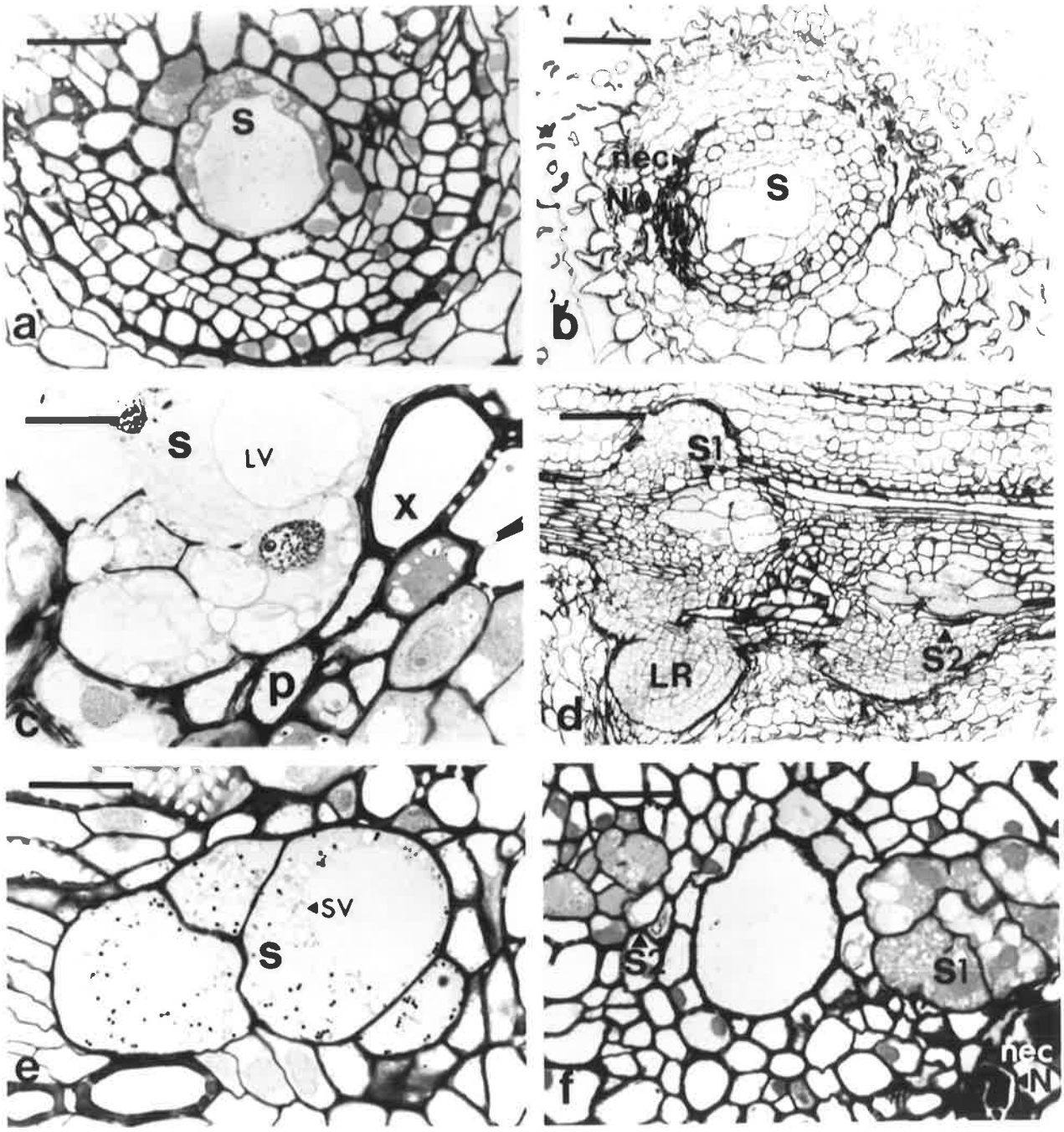

a
S
b
nec
N
S
c
S
LV
X
P
d
S1
LR
S2
e
SV
S
f
S2
S1
nec
N

Figure 3.3 Structure of syncytia induced in the roots of the resistant wheat cultivar AUS 10894 x Prins by *H. avenae*.

a. TS of day four syncytium, and vascular parenchyma cells (Bar =25 μm).

b. LS of day four syncytium, showing hypertrophied cells, cell wall breakdown, and large vacuoles. Endodermal cells adjacent to syncytium are necrotic (Bar = 50 μm).

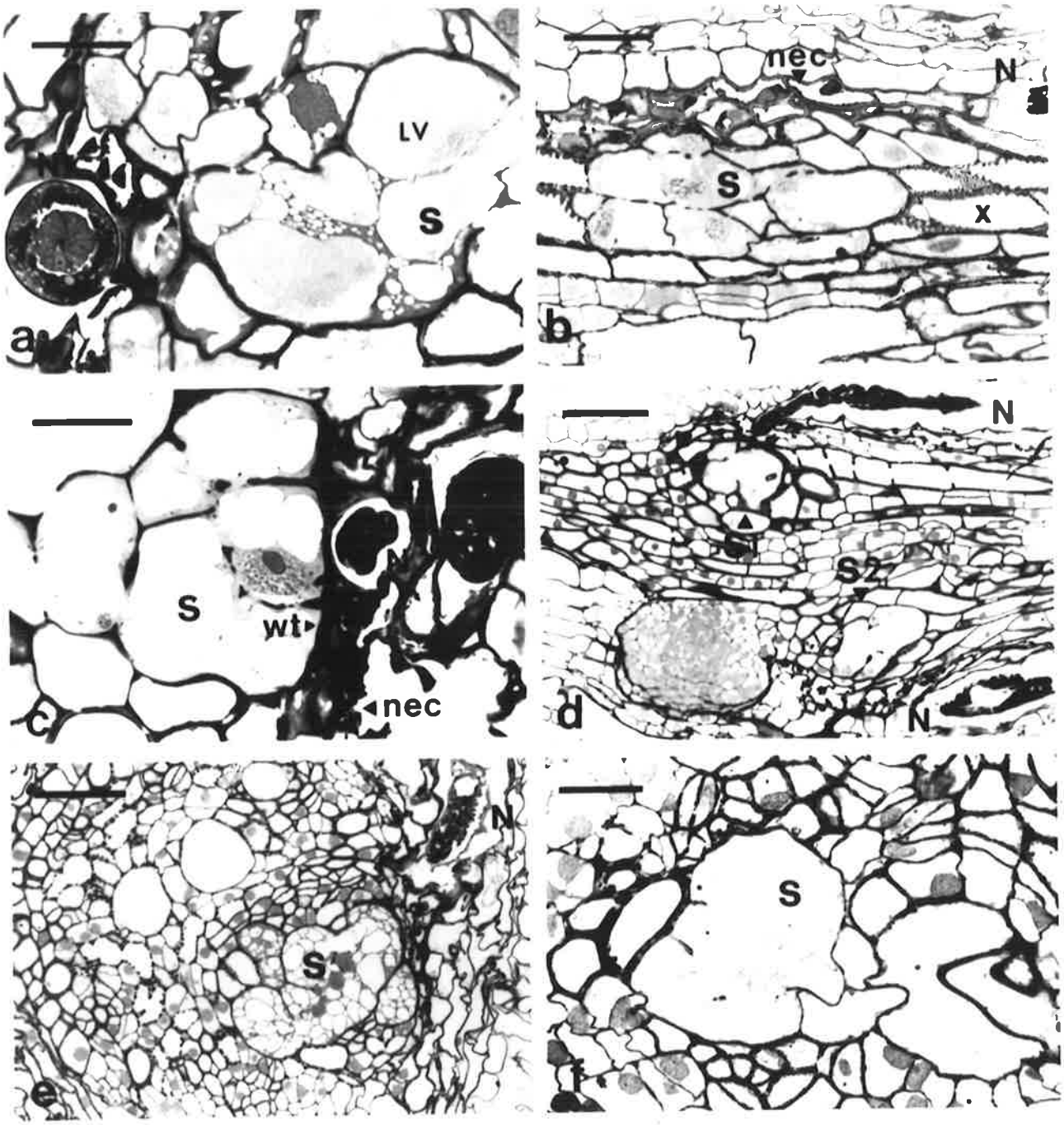
c. TS of day eight syncytium with large vacuoles and wall thickening adjacent to nematode (Bar = 25 μm).

d. LS of two day fifteen syncytia and associated juveniles. Syncytial cytoplasm stains weakly (Bar = 100 μm).

e. TS of day seventeen syncytium, showing predominance of large coalescing vacuoles (Bar = 200 μm).

f. TS of day 33 syncytium, showing complete cytoplasmic degeneration.(Bar = 35 μm)

S, S1, S2 - syncytia, N - nematode, nec - necrosis, LV - large vacuole, wt - wall thickening, x - xylem


LV
S
a
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S
x
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wt
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S1
S2
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d
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S
e
S
f

3.3a). As in the susceptible cultivar, the endodermis adjacent to the syncytium became necrotic (Figs. 3.3a,b). Cells incorporated into the syncytium were hypertrophied, their walls partially digested, leaving wall fragments which indicated their original shape (Fig. 3.3b).

The syncytium was multinucleate, nuclei were hypertrophied with scattered chromatin. The syncytium extended longitudinally along the axis of the root and had extensive contact with vascular elements (Fig. 3.3b). At eight days post-inoculation, the syncytium was still similar in appearance to earlier stages (Fig. 3.3c). Wall dissolution continued as the syncytium integrated more cells. However, the outer wall of the syncytium was unaffected and was thickened adjacent to the nematode (Figs. 3.3c,d). By fifteen days, syncytia in the resistant cultivar were still mainly dominated by large vacuoles, with weakly staining cytoplasm, indicating low activity (Figs. 3.3d). Proliferation of coalescing vacuoles in the syncytium of the resistant cultivar by nineteen days greatly reduced the volume of cytoplasm (Fig. 3.3e). At thirty-three days, syncytia may be totally nonfunctional, with complete tonoplast breakdown (Fig. 3.3f).

Syncytial ultrastructure

Figure 3.4 shows the ultrastructure of syncytia in resistant and susceptible wheat cultivars. Syncytia in the susceptible cultivar had dense cytoplasm, with numerous mitochondria, plastids, endoplasmic reticulum, free ribosomes and small vacuoles (Fig. 3.4a). Membrane-bound wall ingrowths were found adjacent to the nematode feeding site and vascular elements in mature syncytia (Fig. 3.4b). In contrast, syncytia in the resistant cultivar had cytoplasm reduced to a thin layer around the cell walls and wall fragments (Fig. 3.4c). Cell organelles were compressed into this thin layer of cytoplasm (Fig. 3.4c). In the later stages of syncytial breakdown, cellular membranes lost their integrity, with cytoplasm and chromatin dispersing (Fig. 3.4d).

Figure 3.4 Ultrastructure of syncytium in susceptible and resistant wheat cultivars. Susceptible cultivar:

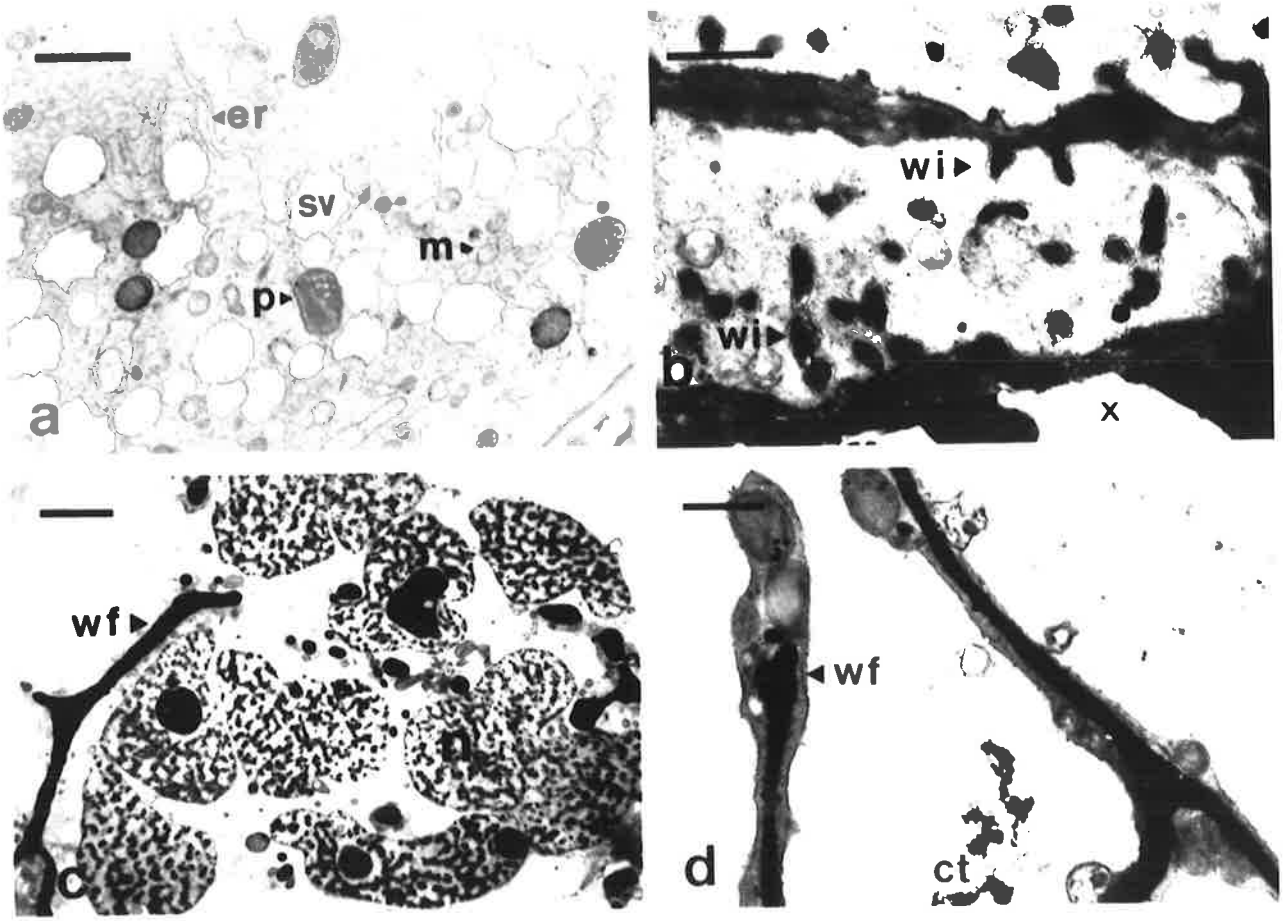
a. Cytoplasm of day fifteen syncytium with numerous organelles, small vacuoles and abundant endoplasmic reticulum (Bar = 2 μm).

b. Membrane-bound wall-ingrowths adjacent to xylem in a day nineteen syncytium(Bar = 2 μm). Resistant cultivar:

c. Cluster of hypertrophied amoeboid nuclei with prominent nucleoli and scattered chromatin in day seventeen syncytium, surrounded by small quantity of cytoplasm with few organelles (Bar = 5 μm).

d. Wall fragments with thin layer of cytoplasm containing plastids and mitochondria. Free chromatin released from degenerated nucleus (Bar = 2 μm).

er - endoplasmic reticulum, sv - small vacuole, p - plastid, m - mitochondria, wi - wall ingrowth, wf - wall fragment, ct - chromatin, x - xylem.


er
sv
m
p
a
wi
wi
b
x
wf
c
wf
d
ct

Discussion

This study has shown that juveniles of *H. avenae* successfully initiate feeding sites in resistant and susceptible wheat cultivars. Potential females in the susceptible cultivar undergo a massive increase in size in the later stages of their development, while in the resistant cultivar they fail to develop beyond the fourth stage. The most significant cellular difference observed between the cultivars was seen at about fifteen days, when the syncytia of the susceptible cultivar became highly active with increased cytoplasm volume. In contrast, the syncytium in the resistant cultivar was less active and highly vacuolated. Up till this time, syncytial development appeared to be the same in both cultivars.

Within four days of penetrating the primary roots, juveniles had initiated a feeding site or syncytium, which may be induced by the nematode stylet injecting secretions into an initially modified pericycle cell (Rice *et al.*, 1987). The cytoplasm of this cell then became confluent with vascular parenchyma cells as they were incorporated by wall degradation. The syncytium extended longitudinally along the root axis and had good contact with vascular elements along its length in both cultivars. This contrasts with *H. schachtii* induced syncytia in *Sinapsis alba*, where in the resistant cultivar contact with the xylem was prevented by necrosis of xylem parenchyma (Magnusson and Golinowski, 1991). The syncytium developed into a shape that gave it extensive contact with vascular elements, maximising its function as a conduit for the removal of nutrients from the plant by the nematode.

In wheat roots, a zone of necrotic cells was often observed between the nematode and its syncytium, where cells of the endodermis had broken down and become electron dense. This response appears to be similar to the necrosis noted in other nematode interactions (Endo, 1991). However, it occurs in both the resistant and susceptible cultivars and does not completely isolate the syncytium, nor does it appear to hinder syncytial development and nematode feeding. The necrosis may, therefore, be part of a non-specific endodermal

response to a pathogen being detected near the stele and, in this case, is ineffective against the nematode.

Syncytia in the susceptible cultivar at later stages are characterised by reduced vacuole volume, active cytoplasm with numerous organelles and wall ingrowths. The wall ingrowths and wall thickenings are found where the syncytium abuts vascular elements and the nematode head, and provide an enlarged membrane surface area for enhanced transport into and out of the syncytium. They are formed as a result of nematode demand for nutrients (Jones and Northcote, 1972b) and are characteristic of transfer cells (Pate and Gunning, 1972).

The nuclei of syncytia were hypertrophied and often amoeboid in shape. Nuclear enlargement is probably the result of endomitosis, the total or partial replication of the genome within one nucleus (Jones, 1981a). Endomitosis may provide for increased levels of RNA synthesis, possibly resulting in greater protein synthesis within the syncytium. The enlarged nucleoli may also be indicative of an increase in RNA synthesis. The amoeboid shape of the nuclei increases its capacity for nucleocytoplasmic exchange of RNA (Jones, 1981a).

The resistant response mediated by the AUS10894 CCN resistance gene is evidenced by differences in syncytia of the resistant cultivar at about fifteen days. Syncytia became largely vacuolate, cytoplasm was very reduced and membrane degeneration was observed. Nutrient flow to the nematode may therefore be reduced, at a stage when the female demand for nutrients is high. This type of resistant response, where syncytia are initiated but break down at some later stage, is common with *Heterodera* species and with some other nematode genera. Syncytia induced on resistant *Rhaphanus* by *H. schachtii* showed an increase in vacuolation three days after formation and cytoplasm was very compressed, with tonoplast breakdown after five days (Wyss *et al.*, 1984). A similar response occurred in potato cultivars resistant to *Globodera rostochiensis* (Rice *et al.*, 1985), although different resistance genes produce varying responses (Rice *et al.*, 1987).

In contrast, the resistant response to *Meloidogyne* spp. is usually a quick hypersensitive reaction of necrosis surrounding the syncytium (Paulson and Webster, 1970).

The only previous reports on the structure of syncytia in the resistant wheat cultivar, AUS10894, found that late initiation of syncytia (Grymaszewska and Golinowski, 1991), increased necrosis and early syncytial degeneration (Grymaszewska and Golinowski, 1987) were signs of a host resistance reaction. The reason for these contrasting findings may be that cysts were used as inoculum, rather than the quantitative synchronous inoculation system reported here. O'Brien and Fisher (1974) showed that when AUS10894 is challenged with the Australian pathotype of *H. avenae*, an equal number of juveniles penetrates the roots and initiates galls in both the resistant cultivar and a susceptible cultivar. This report finds the same for the AUS10894 x Prins cross, with syncytia being established by four days. The necrotic reaction was similar in both the susceptible and resistant cultivars and syncytial differences became apparent just prior to the third moult of the juvenile, which occurs at seventeen days when plants are grown at 15 ^{o}C (Fisher, 1981).

A major feature of the syncytial breakdown of the resistant response noted in this study and by others, is that male development is unaffected, either because of their lower nutrient requirement or because they finish feeding before the syncytium degenerates. While the timing of the breakdown differs between species, its occurrence at a stage which does not affect males may indicate that it is the female that triggers the response at a particular point in its development. Juveniles of *H. avenae* initially increase body size slowly at 15 ^{o}C, then female body size increases rapidly after males have stopped feeding. A gene product produced by the female at this stage may induce the host resistance response. This hypothesis fits with the accepted model of gene-for-gene interactions for the induction of host resistance reactions, and would explain the observed delay in cellular responses to the pathogen.

Chapter 4 Protein profiles of syncytia

Introduction

As few naturally occurring CCN resistance genes are available, it may be necessary to develop synthetic resistance. A prerequisite to such manipulation is an understanding of the biochemical and molecular events during the plant-nematode interaction. These events have not been investigated for any *H. avenae*-host interaction.

There have been several studies on nematode-induced proteins in other interactions. Most have found that the greatest differences are observed in leaf tissue extracts (Hammond-Kosack 1989,1990; Rahimi, 1993). This unexpected result is possibly due to the induction of PR-proteins as a result of the high level of root infection. The only study on proteins extracted from individual syncytia free from nematode contamination showed that syncytial metabolism is similar to that of root meristems (Jones, 1980).

This study examines the separation profiles of proteins from syncytia induced in the roots of resistant and susceptible near-isogenic lines of wheat by *H. avenae*. The differences observed are discussed with respect to the recent molecular evidence of syncytial gene regulation.

Materials and methods

Protein Extraction

Nematode-induced galls from roots of the resistant near-isogenic line (NIL) AP and its susceptible recurrent parent Prins were excised at approximately two-day intervals and carefully dissected under a microscope. Root cortical parenchyma cells were peeled away, exposing the syncytium-containing stele. Developing nematodes were removed. The 2 mm section of stele was snap frozen in liquid nitrogen and then homogenised with

fine forceps in a 5 µl droplet of 10 mM phosphate buffer (pH 6.8). The homogenate was either used immediately or stored at -80°C.

SDS-PAGE

The proteins in the homogenates were analysed by discontinuous SDS-PAGE (Hames, 1990) using pre-cast Mini-Protean 4-20 % gradient polyacrylamide gels (Bio-rad). Homogenates were boiled in sample buffer (0.0625 M Tris-HCl pH 6.8, 2% (W/V) SDS, 5% (V/V) 2-mercaptoethanol, 10% (W/V) sucrose, 0.002% (W/V) bromophenol blue) and centrifuged for 5 min at 12,000 g prior to loading. Two extracts were loaded per lane for NIL comparison, while one extract was loaded per lane for infected/control experiment. Gels were run at 150V for 1 hr.

Silver staining

After electrophoresis gels were stained with silver-nitrate (Hames, 1990). Gels were fixed overnight in 25% (V/V) ethanol plus 7.5% (V/V) acetic acid, followed by fixing for 1 h in 12.5% (V/V) ethanol plus 3.75% (V/V) acetic acid. They were rinsed in water for 15 min, then in 0.0005% DTT for 30 min. After two 10 min washes in water they were stained in 0.1% (W/V) AgNO for 30 min. They were then rinsed in water for 1 min, and in 3% (W/V) NaCO for 1 min, before being developed in 3% (W/V) NaCO containing 0.0185% (V/V) formaldehyde. The reaction was stopped with 2.3 M citric acid.

Results

The very small amount of homogenised syncytium-containing tissue led to the extraction of minute quantities of protein, estimated from staining intensity of gel banding to be about 1-5 µg per section. Small, 8 x 10 cm 1 mm thick 4-20% gradient gels were therefore used to separate the proteins, which were visualised with an ultra sensitive silver stain capable of detecting proteins in sharp bands above 0.4 $ngmm^{-2}$. The tissues were homogenised first in phosphate buffer, and then extracted with sample buffer containing SDS to obtain membrane-bound proteins.

Figure 4.1 Separation of proteins from syncytium-rich root samples. Samples were taken from the near-isogenic lines AUS10894 x Prins^7 (AP) (CCN resistant) and Prins (P) (CCN susceptible). Proteins were extracted from 2 mm syncytium-containing stele sections from roots at **(a)** 3, 5, 7, 9 and **(b)** 11, 13, 15, 17 days post-inoculation (DPI), and separated on 4-20% gradient SDS-polyacrylamide gels. Bands were visualised by silver staining. N - proteins extracted from 35 day old female nematode, M - size markers, kD - kilodaltons.

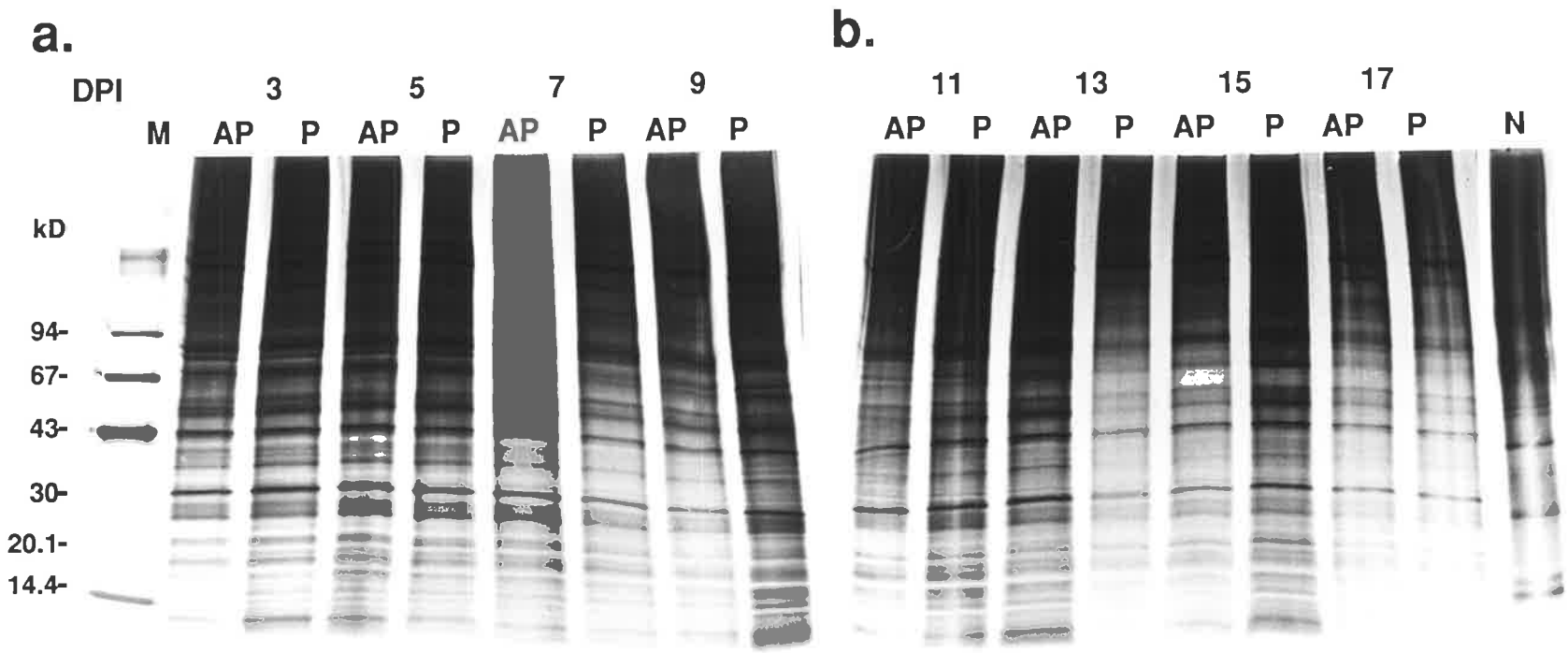

a.
DPI
3
5
7
9
M
AP
P
AP
P
AP
P
AP
P
kD
94-
67-
43-
30-
20.1-
14.4-
b.
11
13
15
17
AP
P
AP
P
AP
P
AP
P
N

Figure 4.2 Separation of proteins from syncytium-rich root samples. Samples were taken from the near-isogenic lines AUS10894 x Prins^{7} (AP) (CCN resistant) and Prins (P) (CCN susceptible). Proteins were extracted from 2 mm syncytium-containing stele sections from roots at **(a)** 19, 21, 23, 27 and **(b)** 33, 35, 38 days post-inoculation (DPI), and separated on 4-20% gradient SDS-polyacrylamide gels. Bands were visualised by silver staining. N - proteins extracted from 35 day old female nematode, M - size markers, kD - kilodaltons.

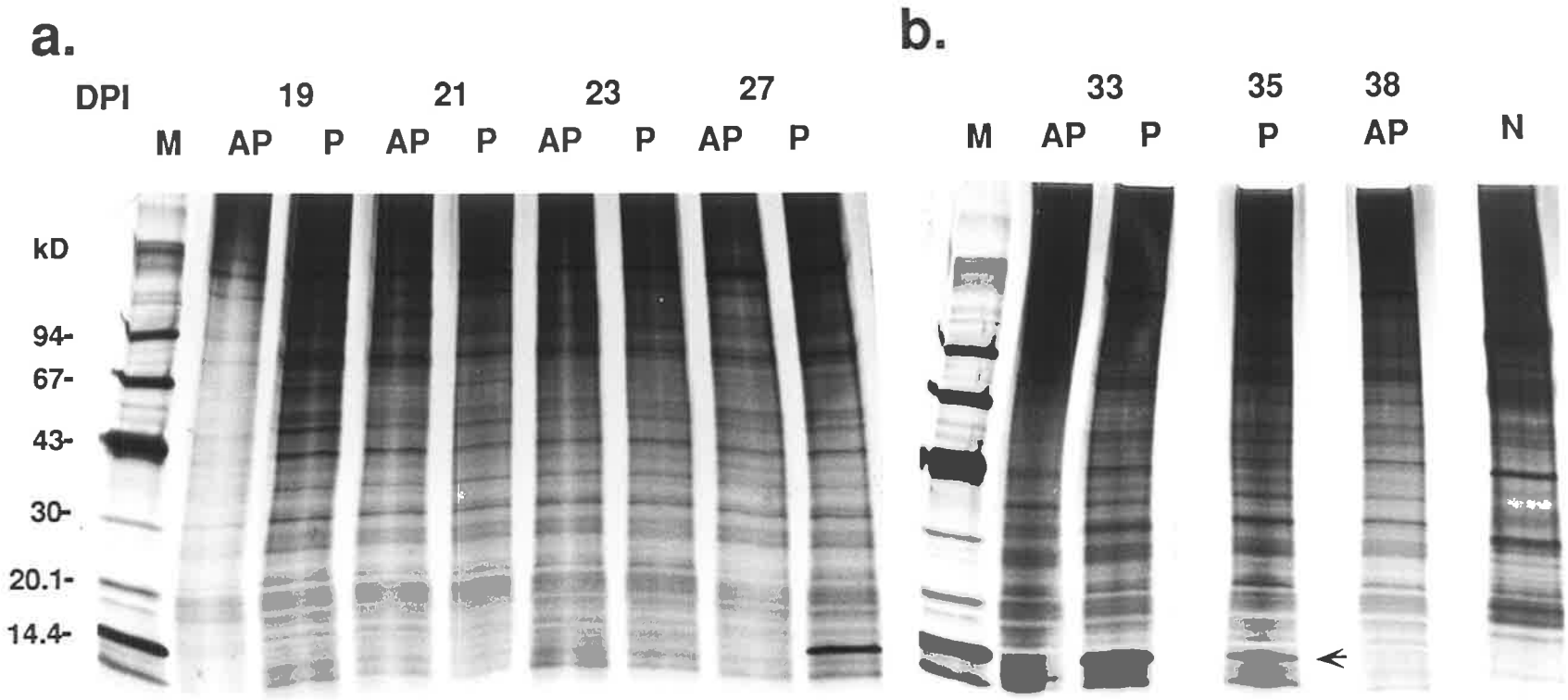
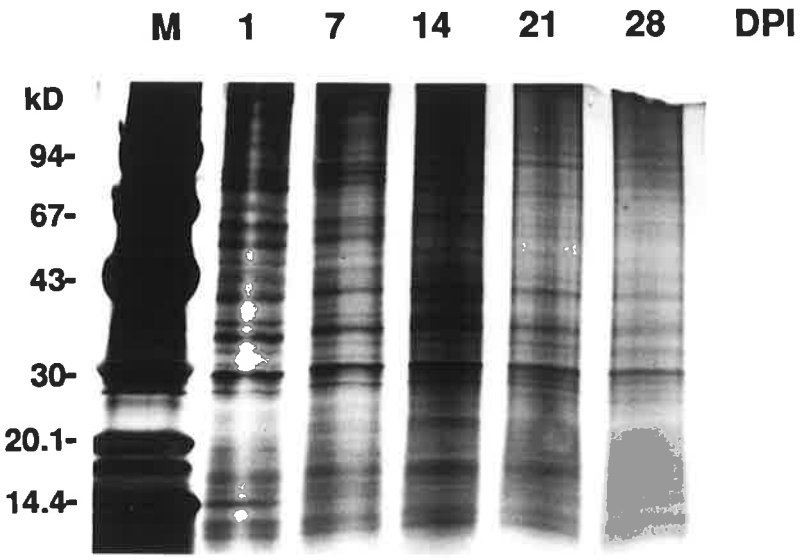

a.
DPI
19
21
23
27
M
AP
P
AP
P
AP
P
AP
P
kD
94-
67-
43-
30-
20.1-
14.4-
b.
33
35
38
M
AP
P
P
AP
N

Figure 4.3

(a) Separation of proteins from uninoculated stele sections. Proteins were extracted from 2 mm stele sections from Prins roots at 1, 7, 14, 21 and 28 days.

(b) Separation of proteins from nematode-inoculated and water-inoculated stele sections. Proteins were extracted from 2 mm stele sections from Prins roots at 1, 7, 14 and 21 days post inoculation (DPI). Proteins were separated on 4-20% gradient SDS-polyacrylamide gels and visualised by silver staining. N - proteins extracted from 35 day old female nematode. -N - water-inoculated root stele section, +N - syncytium-containing root stele section.

a.


M
1
7
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21
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DPI
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43-
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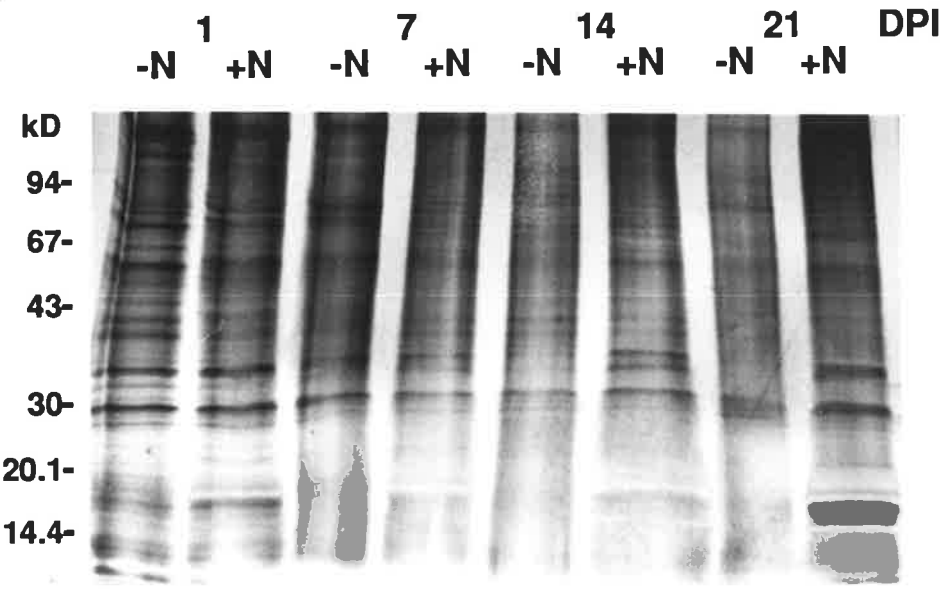
14.4-

b.


1
7
14
21
DPI
-N
+N
-N
+N
-N
+N
-N
+N
kD
94-
67-
43-
30-
20.1-
14.4-

The protein profiles of syncytium-enriched root sections were compared at approximately two day intervals to detect any differences between the resistant and susceptible varieties. Up to 23 days, no consistent differences in banding pattern could be observed between the NILs (Figs. 4.1a,b). From 27-35 days, a major new 14 kD band appeared in extracts from the susceptible line Prins (Figs. 4.2a,b). This band appeared to be most intense at 33 days. Nematode extracts from 30 and 35 day old females did not appear to contain this band.

The protein profiles of sections of root from nematode-inoculated and control water-inoculated susceptible plants were also compared to determine if infection alters normal root stele protein levels. Proteins from the control group of uninfected root sections taken at 1, 7, 14 and 21 days post-inoculation were separated (Figure 4.3a). Several differences were observed between 1 and 7 and 14 and 21 days. The total protein content also appeared to reduce with time, as only half of the day 1 and 7 extracts was loaded. This reduction was more noticeable in Fig 4.3b, where equal amounts of uninfected and infected tissue were loaded in adjacent lanes. While the uninfected tissue protein content reduces to an almost undetectable level, the infected tissue continued to have a relatively high protein content at 21 days. The most noticeable individual protein difference was the high expression of a 18 kD band in the infected tissues at all ages, and a 16 kD band at 14-21 days. There were also several differences between the profiles of infected and uninfected tissue in the higher molecular weight range.

Discussion

Plant protein separation has been investigated as a means of identifying polypeptides linked to resistance to pathogens with mixed success. Two-dimensional electrophoresis was used to identify putative protein changes associated with barley resistance to powdery mildew (Manners *et al.*, 1985). However, other extensive studies have failed to identify resistance-related proteins. Gabriel and Ellingboe (1982) failed to find a resistance-linked protein in congenic wheat lines, and a CCN resistance-linked protein difference was not found in an extensive study of barley near-isogenic lines at this

institute. Therefore, it was decided to conduct a more directed search for protein differences at the site of the plant-nematode interaction rather than looking for constitutively expressed resistance-related proteins.

The separation of proteins in one-dimensional gels gives only a limited qualitative indication of gene expression of plant cells. However, by separating proteins from excised syncytium-enriched tissue from near-isogenic lines over the initial stages of infection with one-dimensional electrophoresis, it was hoped that some of the ultrastructural findings of this study could be related to biochemical events.

The reason that one-dimensional PAGE was used is that the amount of protein required for separation and visualisation with silver nitrate on a two-dimensional gel is approximately 20 µg (Rickwood, 1990), while the syncytium-containing sections obtained by dissection in this study may have contained less than 1 µg total protein. To use two-dimensional electrophoresis a large number of sections would need to be taken or, alternatively, proteins could be labelled in-planta with a radioactive substrate to increase the sensitivity of detection.

This type of study has not previously been conducted for any nematode interaction. The difficulty may have been the location of the nematode within the host root during the early stages of infection. This problem has been addressed in this study by removing the root cortex so that a section of stele containing the syncytium could be obtained without associated nematodes. These sections were also free of the numerous lateral root meristems that are induced by nematode infection and would have had a high protein content. The disadvantage of dissection is that it may have resulted in the accumulation of wound-induced proteins, although all samples, including controls, had identical manipulations.

The protein content of section extracts varied considerably, and made comparison difficult. Although it may have masked individual syncytium differences, two syncytium-containing section extracts were loaded onto each gel lane to improve band

visualisation after staining, and to equalise protein loading to some extent. Ideally, the proteins from only one syncytium would be analysed.

Overall this study revealed little difference in pairwise comparison of proteins from syncytium-containing sections from the susceptible and resistant NILs. There were no resistance-related protein differences observed. The only major protein difference seen was the accumulation of a 14 kD protein in syncytial sections from the susceptible line. This had a specific temporal expression, appearing at 27 days, with a possible peak of expression at 33 days. Examination of nematode protein extracts indicates that there was no nematode contamination of these samples, although it is still possible that the protein was of nematode origin.

It is interesting to note that the female undergoes its fourth moult at 28 days at the temperature used for this study (Fisher, 1981). It could be speculated that the protein seen in extracts from the susceptible plant after 27 days is essential for this stage of the nematodes development and its absence from resistant plant extracts is directly related to resistance. Alternatively, it could be absent because nematode development has been affected by host resistance at an earlier stage, altering normal syncytial function so that it no longer resembles that of syncytia in compatible interactions.

Comparing protein profiles from infected and uninfected stele sections revealed some individual protein differences, with a few proteins accumulating to high levels in extracts from infected tissue. These proteins could be associated with unique characteristics of the syncytium, such as wall degradation and deposition, nuclear hypertrophy and increased transcription, or new organelle synthesis.

The overall level of protein in infected tissues appeared to be maintained at the level found in young roots, compared to the concentration decrease over time which was observed in uninfected roots as they pass through normal cell development and ageing. This corresponds with what is observed microscopically. Over time, normal stellar cells

become vacuolate with very little cytoplasm. In infected sections, the syncytium makes up a large proportion of the stele, and appears to be very active metabolically.

The only comparisons available for this study are those with several recent investigations into the molecular biology of syncytia. Differential screening of syncytial-enriched and uninfected tissue has been used to isolate syncytium specific cDNAs. This has been achieved in interactions between *G. rostochiensis* and potato (Gurr *et al.*, 1991) and also in *H. schachtii* induced syncytia in *Arabidopsis* (Burrows, 1992).

Altered gene expression in syncytia has also been investigated using the technique of promoter tagging. In a study of the *H. schachtii/Arabidopsis* interaction, gene regulation was downregulated in syncytia to such an extent that it did not match that of uninfected cells (Goddijn *et al.*, 1993). In other studies, it was observed that some genes were up-regulated in syncytia (Sijmons *et al.*, 1992; Cramer, 1992; Taylor *et al.*, 1992).

These studies confirm to some extent that the developing nematode is able to massively redirect gene expression in syncytia compared with normal tissues. Protein studies may allow us to visualise some of these changes, although more informative investigations will have to be carried out at the mRNA and DNA level. If it was considered desirable to isolate syncytium-specific genes via their polypeptide products, the protein differences observed in this study indicate that more intensive investigations may facilitate this goal. Bulked tissue samples could be used on two-dimensional gels to identify syncytium-specific proteins, which could be isolated and used to raise antibodies for in-situ or expression-library studies.

Chapter 5 Identification and Mapping of RFLP markers

Introduction

Molecular markers can be used to tag genes of agronomic importance. Once a gene has been mapped relative to one or more markers, these markers can be used to indirectly select progeny with the required genotype. To date there has only been one resistance gene for CCN reported in hexaploid wheat (Nielsen, 1966; O'Brien and Fisher, 1974). The development of CCN resistant cultivars is a high priority of breeders, although the current bioassay is limiting the rate at which this is achieved. If a molecular marker was identified that was closely linked to the resistance locus, rapid marker-assisted selection would be a possible alternative to the current 12-week assay.

RFLP markers linked to genes conferring nematode resistance have previously been identified in potato (Gebhardt *et al.*, 1993), sugarbeet (Jung *et al.*, 1990) and tomato (Messeguer *et al.*, 1991).

In wheat, the dominant CCN resistance allele at the locus *Cre* is located on chromosome 2B in the lines "Loros" (Slootmaker, 1974) and "AUS10894" (Nielsen, 1982; O'Brien *et al.*, 1980). This chapter describes the search for *Cre*-linked molecular markers using near-isogenic lines.

Materials and methods

Plant Material

The near-isogenic lines "AP" and "Prins" were used to screen for probes that revealed RFLPs. "Chinese Spring" nulli-tetrasomic (Sears, 1966) and ditelosomic lines (Sears and Sears, 1978) were used to localise RFLPs to chromosomes and chromosome arms.

F2 progeny of crosses between "AUS10894" x "Spear", and "AP" x "Prins" were used for linkage analysis.

Nematode assay

F2 progeny were tested for their resistance to *H. avenae* using the bioassay developed by Fisher (1982). Seedlings were planted in 3 cm tubes filled with sterile soil, and inoculated five times at 3 day intervals with 100 second stage juveniles. After 12 weeks at 15°C, the number of cysts formed on roots were counted. To confirm the resistance score, six to eight F3 progeny of each F2 individual from the "AUS10894" x "Spear" cross were also assayed by the same procedure.

DNA clones

Clones were obtained through the Australian Triticeae Mapping Initiative. Group 2 cDNA and genomic clones were selected from maps of barley (Heun *et al.*, 1991) and wheat (Liu and Tsunewaki, 1991).

Cot fractionation

Cot fractionation was carried out as described by Eastwood *et al.* (1994). DNA from AUS10894, AP and Prins was sonicated for 6 sec to produce 2-3 kb fragments. The DNA was boiled for 5 min, and renatured at 60°C to a Cot [=moles nucleotide/litre x incubation time [sec] (Smith and Flavell, 1975)] value of 130. The samples were then loaded onto a 5 cm^3 Hydroxyapatite (Biorad) column. The column was washed with 0.01 M phosphate buffer (pH 6.8) three times, before the single stranded DNA was eluted with 0.15 M phosphate buffer (pH 6.8) (60°C). New columns were packed for each sample. The eluted DNA was ethanol precipitated and diluted to 50 ng μl^{-1} for use in PCR.

RAPD analysis

RAPD reactions were similar to those described by Williams *et al.* (1990). PCR reactions contained 20 mM NH_4SO_4, 75 mM Tris-HCl pH 9, 0.01 % (w/v) Tween, 50 mM each dNTP, 1.5 mM $MgCl_2$, 200 nM 10mer primer, 50 ng template DNA and 0.5 units *Taq* polymerase in 25 µl total volume. Reactions were carried out in a MJ Research thermal cycler. Temperature conditions were 5 cycles of: 92°C for 2 min, 35°C for 2 min, 72°C for 90 sec; followed by 35 cycles of 92°C for 30 sec, 40°C for 60 sec, 72°C for 90 sec; followed by 1 cycle of 72°C for 5 min.

RFLP analysis

DNA extraction, restriction digestion, Southern blotting, and hybridisation were carried out as described in chapter two. NIL leaf DNA was digested with *Hin*DIII, *Eco*RI, *Eco*RV, *Dra*I, *Bam*HI and *Xba*I. DNA membranes of F2 progeny were screened with RFLP probes and the results analysed with Mapmaker (Lander *et al.*, 1987) and Joinmap (Stam, 1993) software using the Kosambi map unit function (Kosambi, 1944) with the critical LOD score at 3.

Results

Segregation of the nematode resistance gene

A total of ninety-two F2 progeny were tested for resistance to *H. avenae*. The segregation of individuals from the cross "AUS10894" x "Spear" was 42 resistant : 13 susceptible ($\chi^2 = 0.09$) and that of "AP x Prins" was 29 resistant : 8 susceptible ($\chi^2 = 0.14$). The χ^2 values indicate that the segregation's fit the expected 3:1 ratio for a single dominant gene for resistance to CCN.

Progeny testing was used to confirm the resistance status of 55 F2 individuals (Appendix I). For eleven F2 individuals the data from F3 testing resulted in the reclassification of the F2 plants reaction to CCN. In nine out of eleven instances, a resistant classification was changed to a susceptible one. This may be the result of the F2 seedling "escaping"

infection, or failing to support nematodes due to poor growth as a result of environmental conditions.

RFLP Analysis

Fifty-eight group 2 probes were tested (Appendix II), using six restriction enzymes, on the near-isogenic lines and the donor parent "AUS10894" to identify those showing RFLPs. Both cDNA and genomic probes from barley, oats and wheat showed RFLPs between the wheat parental lines at similar frequencies (Table 5.1). The restriction enzymes used also produced polymorphisms at about the same rate, although *Hin*DIII was the most effective (Table 5.2).

Table 5.1 RFLPs detected between AUS10894 and Prins by various clone types.

Probes	Number tested	RFLPs detected	% detecting RFLPs
ABC (Barley cDNA)	8	6	75
BCD (Barley cDNA)	11	7	64
BG (Barley genomic)	1	1	
CDO (Oat cDNA)	11	6	55
IPSR (Wheat cDNA)	1	0	
KSU (*T.tauschii* genomic)	4	1	
Tag (Wheat genomic)	19	14	74
WG (Wheat genomic)	3	3	
TOTALS	58	38	65

Table 5.2 RFLPs detected between AUS10894 and Prins by various restriction enzymes.

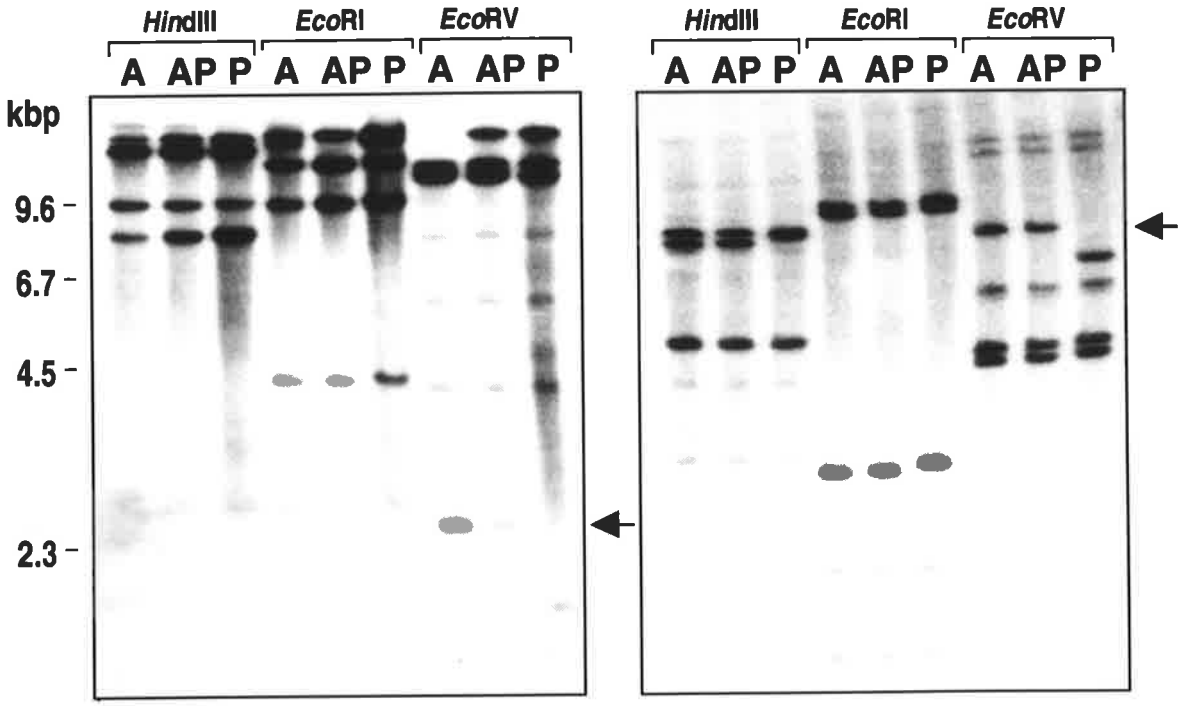
Restriction Enzyme	No. Probes tested	No. probes detecting RFLP	% Probes detecting RFLP
*Hin*dIII	58	16	27.6
*Eco*RI	58	9	15.5
*Eco*RV	58	13	22.4
*Dra*I	58	9	15.5
*Bam*HI	58	11	19.0
*Xba*I	58	11	19.0
Average	58	11.5	19.8

Membranes were made of DNA from near-isogenic lines digested with six restriction enzymes. From the 58 clones tested only two, CDO588 and Tag605, displayed RFLPs between the NIL "AP" and its recurrent parent "Prins" (Fig. 5.1). When probed with CDO588, the *Hin*DIII digest of AUS10894 and AUS10894xPrins^{7} shows a band of about 7.7 kbp which is missing in the Prins parent. The *Eco*RV digest of these lines also revealed a polymorphism when probed with CDO588. In this case a band of about 8 kbp in AUS10894 and AUS10894xPrins^{7} was replaced by one of about 6.8 kbp in Prins. When probed with Tag605, the *Hin*DIII digest of AUS10894 and AUS10894xPrins^{7} shows a band of about 2.4 kbp which is missing in the Prins parent.

A third clone, ABC451 (barley cDNA), gave an identical restriction pattern to CDO588 (oat cDNA). Partial sequencing of these clones showed 88% homology at the 3' end (Appendix V), indicating that they may be products of identical loci.

It was observed that the *Eco*RV digests of Chinese Spring and AUS10894 lines gave the same hybridisation patterns when probed with Tag605 and CDO588. Chinese Spring

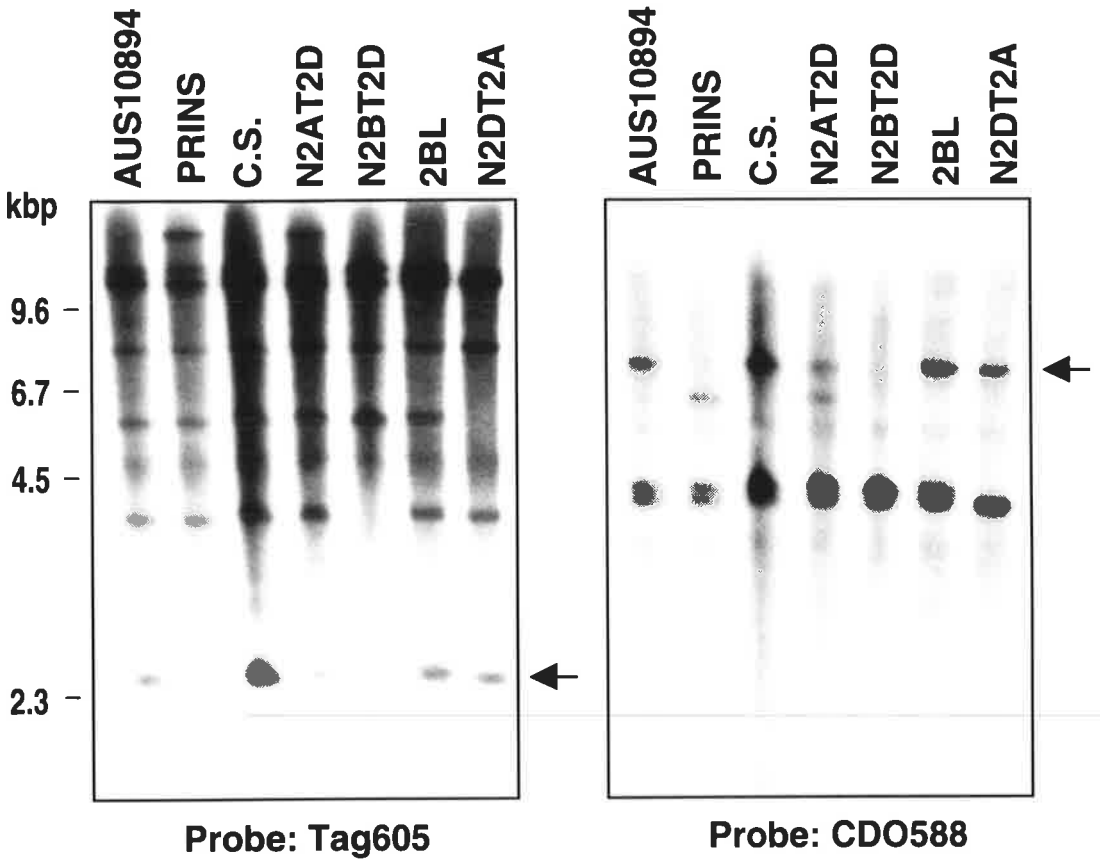
Figure 5.1 Southern analysis of near-isogenic lines. Plant DNA was digested with three restriction enzymes, and probed with Tag605 or CDO588. The arrow indicates the polymorphic band. A - AUS10894, AP - AUS10894 x Prins^{7}, P - Prins.



Probe: Tag605

Probe: CDO588

Figure 5.2 Chromosomal localisation of RFLPs. Southern blot of nulli-tetrasomic (N2AT2D - nulli 2A tetra 2D) and ditelosomic ($2B_L$) lines digested with *Eco*RV and probed with Tag605 and CDO588. C.S. - Chinese Spring. Absence of polymorphic bands (arrow) in N2BT2D and presence in $2B_L$ indicates that RFLP is derived from $2B_L$.


AUS10894
PRINS
C.S.
N2AT2D
N2BT2D
2BL
N2DT2A
kbp
9.6
6.7
4.5
2.3
Probe: Tag605
AUS10894
PRINS
C.S.
N2AT2D
N2BT2D
2BL
N2DT2A
Probe: CDO588

aneuploid lines could, therefore, be used to verify the chromosomal binding locations of these probes. The clones were hybridised to DNA membranes of nulli-tetrasomic and ditelosomic lines of Chinese Spring wheat digested with *Eco*RV (Fig. 5.2). When the membranes were probed with Tag605, the polymorphic 2.4 kbp band was missing from the nullisomic 2B-tetrasomic 2D line and was present in the ditelosomic $2B_L$ line. A similar pattern was observed with CDO588 probing of the membranes. The polymorphic 8 kbp band was missing from the nullisomic 2B-tetrasomic 2D line and was present in the ditelosomic $2B_L$ line. Both probes also produced bands that were missing from the nullisomic 2D-tetrasomic 2A lines (Fig 5.2). The restriction patterns indicated that these probes identify loci on chromosomes 2B and 2D and that the RFLPs observed were derived from the long arm of chromosome 2B.

Linkage analysis of the RFLP loci relative to the *Cre* locus was conducted on 55 F2 individuals of the cross "AUS10894" x "Spear". CDO588 linkage was also measured on 37 F2 progeny of a "AP" x "Prins" cross. Examples of RFLP analysis of "AUS10894" x "Spear" F2 progeny scoring for CDO588 and Tag605 are shown in Figs. 5.3 and 5.4. In both cases EcoRV digests of parental and F2 DNA were probed, and the presence or absence of each allele scored.

Linkage analysis with Mapmaker software (LOD > 3) produced a linkage map for the AUS10894 x Spear F2 population (Fig. 5.5a), which positioned the *Cre* gene between *Xcdo588*/*Xabc451* and *Xglkg605* with the additional two distal flanking markers *Xwg996* and *Xglk632*. The AP^7 x Prins F2 population was also used to determine *Xcdo588* linkage to *Cre* (Fig. 5.5b). The recombination between Cre and *Xcdo588* appears to be considerably different in the two populations. This may be the result of mis-scoring in the non-progeny tested small AP x Prins F2 population, as just one alteration changes the recombination frequency by 5 %.

A consensus map was produced by combining the data from both populations using Joinmap software with a critical LOD score of three. The consensus map positions the

Figure 5.3 RFLP analysis of F2 population. AUS10894 x Spear F2 DNA was digested with *Eco*RV and probed with CDO588. → AUS10894 allele, → Spear allele.

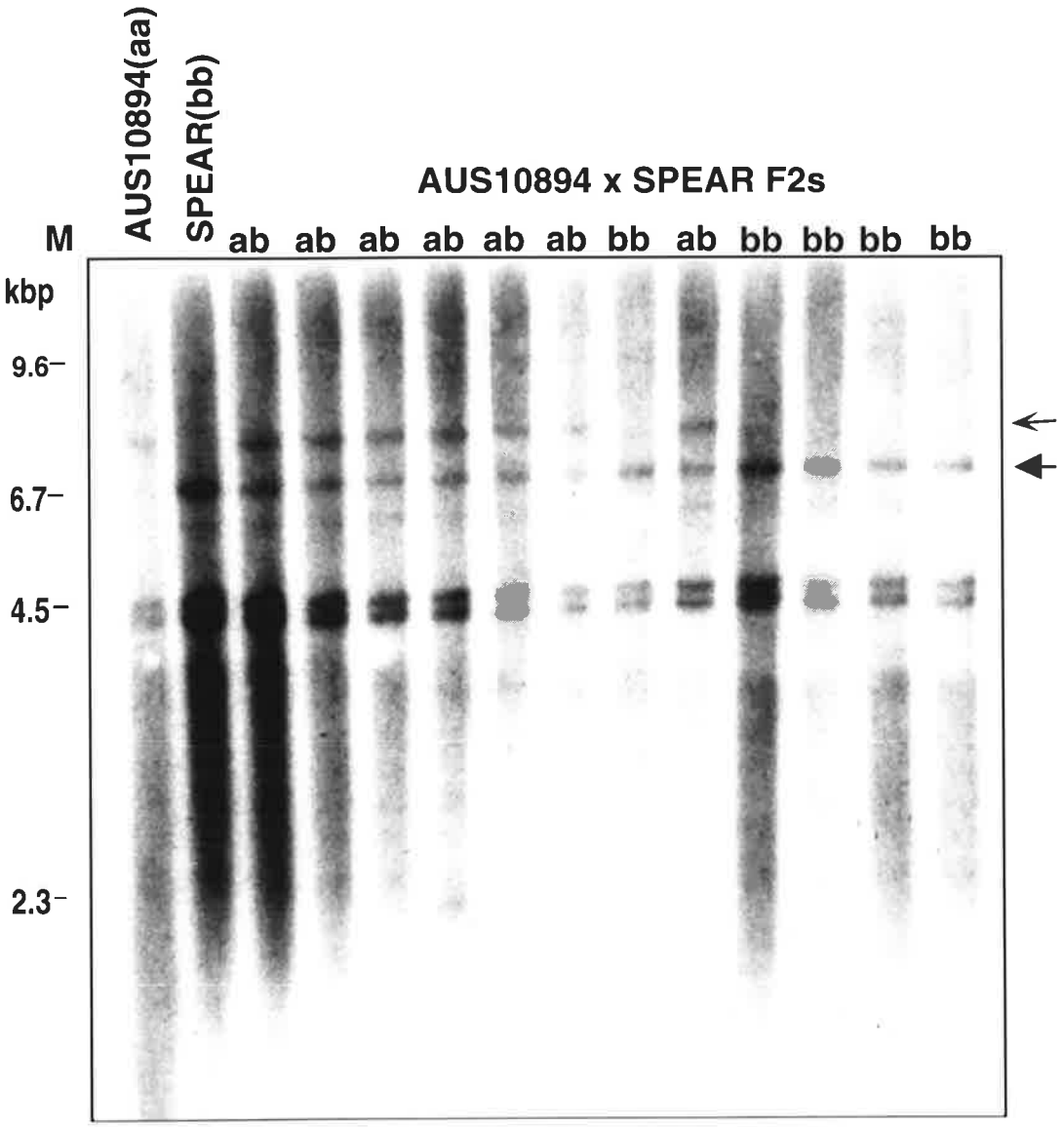

AUS10894(aa)
SPEAR(bb)
AUS10894 x SPEAR F2s
M
ab ab ab ab ab ab bb ab bb bb bb bb
kbp
9.6
6.7
4.5
2.3

Figure 5.4 RFLP analysis of F2 population. AUS10894 x Spear F2 DNA was digested with *Eco*RV and probed with Tag605. → AUS10894 allele, → Spear allele.

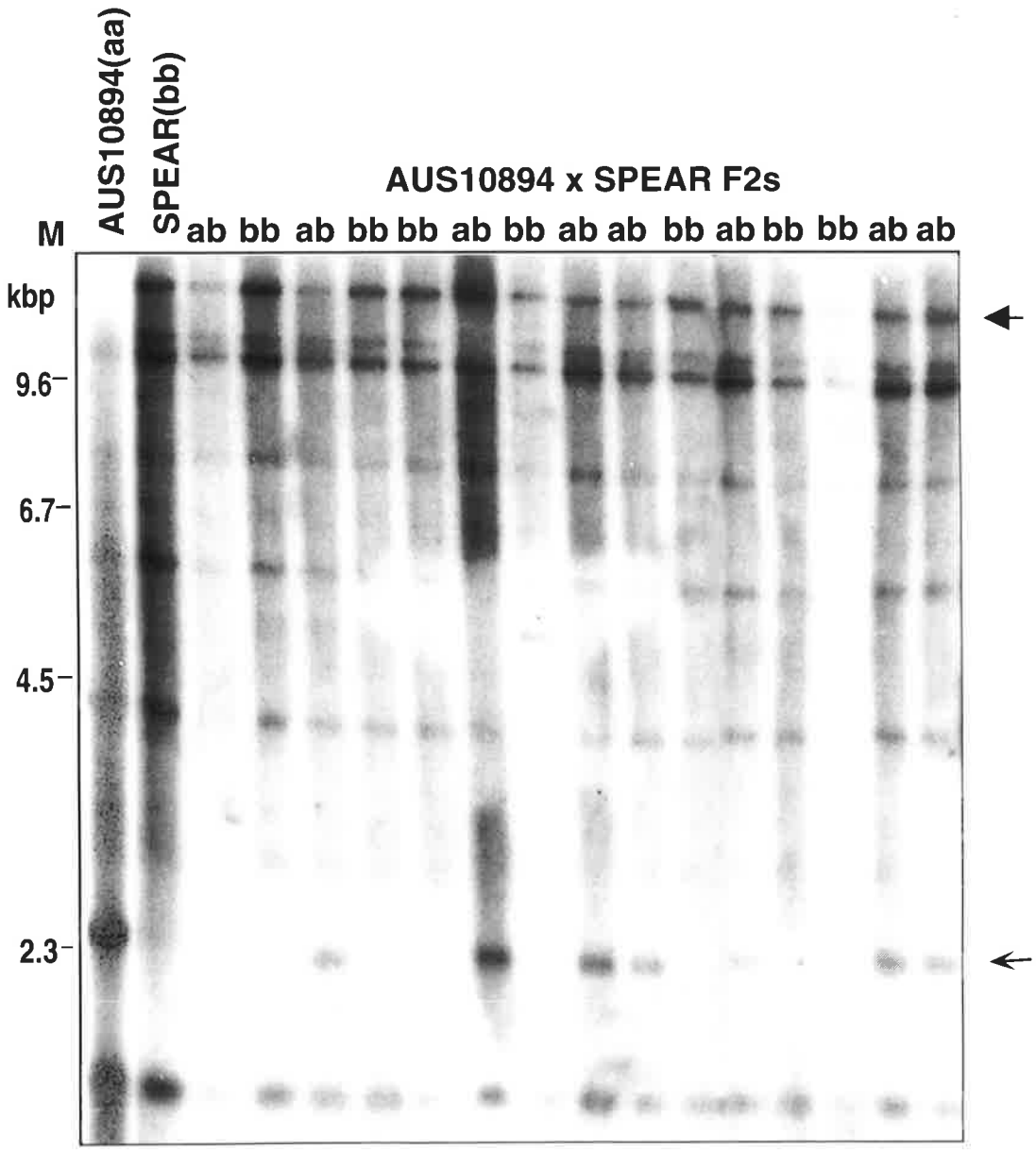
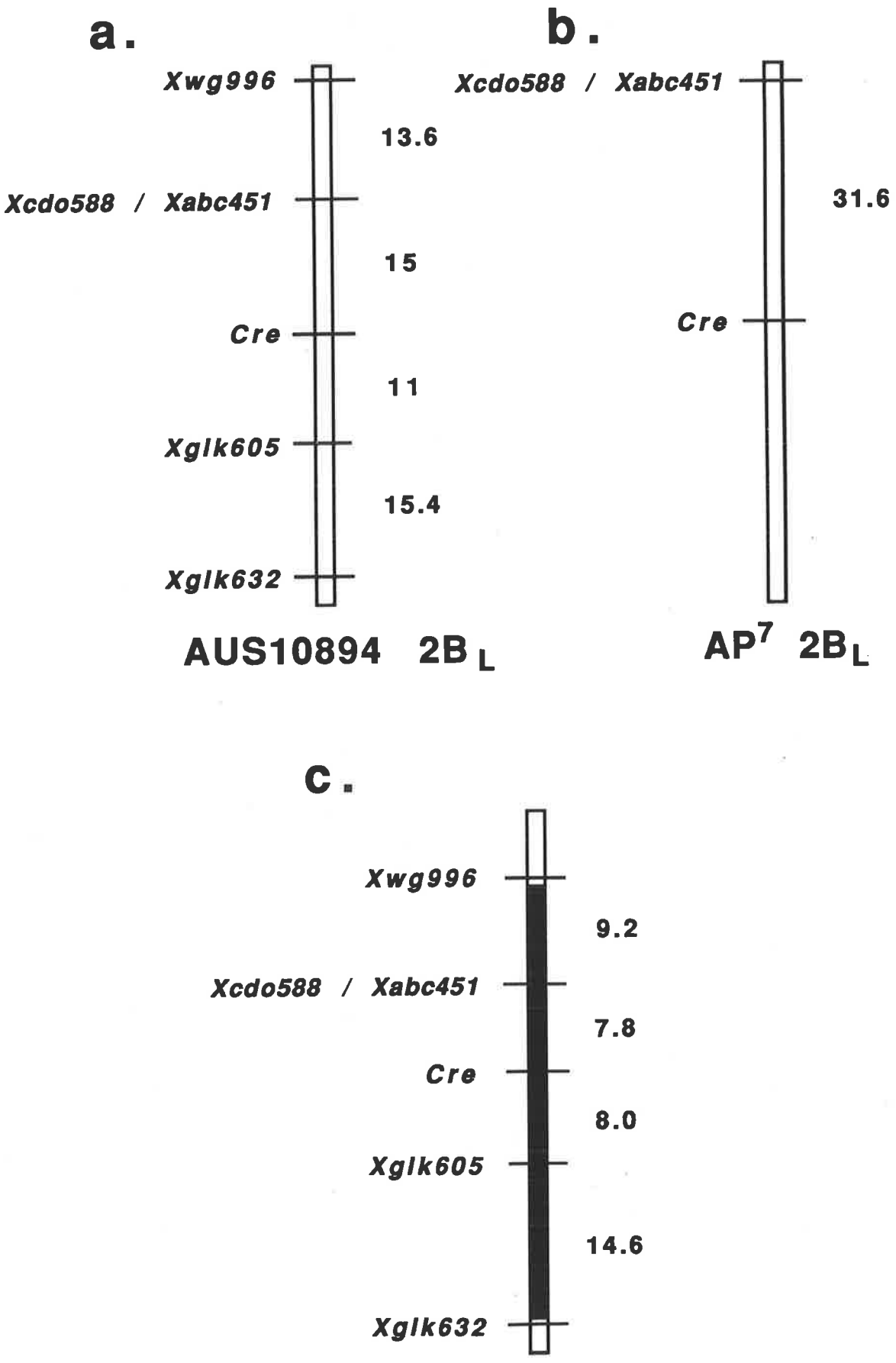

AUS10894(aa)
SPEAR(bb)
AUS10894 x SPEAR F2s
M
ab bb ab bb bb ab bb ab ab bb ab bb bb ab ab
kbp
9.6
6.7
4.5
2.3

Figure 5.5 Partial linkage maps of the long arm of wheat chromosome 2B derived from linkage analyses of progeny from crosses between (**a.**) "AUS10894" x "Spear", and (**b.**) "AP" x "Prins". Map distances, in centimorgans (Kosambi 1944) were determined using Mapmaker software with LOD > 3. (**c.**) Consensus map produced with data from both populations analysed with Joinmap (LOD > 3). The shaded area represents the estimated maximum size of the "AUS10894" chromosome segment that was introgressed into the "AP" backcross line.


a.
Xwg996
13.6
Xcdo588 / Xabc451
15
Cre
11
Xglk605
15.4
Xglk632
AUS10894 2B L
b.
Xcdo588 / Xabc451
31.6
Cre
AP7 2B L
c.
Xwg996
9.2
Xcdo588 / Xabc451
7.8
Cre
8.0
Xglk605
14.6
Xglk632
AUS10894 2B L

markers *Xcdo588/Xabc451* and *Xglkg605* 7.8 and 8.0 cM, respectively, either side of *Cre*.(Fig. 5.5c).

The markers *Xwg996* and *Xglk632* were also contained in the linkage group but were not polymorphic between the NIL's (Figs. 5.6 and 5.7). Digests of NILs with six restriction enzymes were probed with WG996, revealing five RFLPs between the parents, but none between the NILs AP and Prins (Fig. 5.6). Similarly, probing of NIL DNA with Tag632 revealed nine RFLPs between the parents without showing any between the NILs. These probes revealed high levels of polymorphism between the parents, but not the NILs, indicating that the distance between *Xwg996* and *Xglk632* represents the maximum size of this DNA segment introgressed from "AUS10894" into "AP" (Fig. 5.5c).

As an alternative verification of *Xglk605* linkage to Cre, breeders lines carrying *Cre* were tested for the presence of the marker (Fig. 5.8). Four lines of known pedigrees were probed with Tag605: ED135 [AUS15567/Cocamba A/Cocamba (approx. 6% AUS10894)]; ED086 [Cook/Millewa/TM56 (approx. 6% AUS10894)]; ED089 [TM56*2/Ausen 4-21/3 Ag 3/4* Condor (approx. 1% AUS10894)]; EL240 [Takari/TM56/Cocamba (approx. 6% AUS10894)]. Three of the lines scored positive for the marker *Xglk605* (Fig. 5.8).

Chinese Spring CCN resistance

During RFLP probing with group two clones it was noted that Chinese Spring consistently produced RFLP patterns which matched those from the CCN resistant variety AUS10894 and were different to all susceptible lines tested. Chinese Spring is thought to have some resistance to CCN (Asiedu *et al.*, 1990). A small experiment was conducted to investigate whether the RFLP homology meant that there was also a CCN resistance gene on chromosome 2B.

Figure 5.6 Southern analysis of near-isogenic lines. Plant DNA was digested with six restriction enzymes, and probed with WG996. A - AUS10894, AP - AUS10894 x Prins^{7}, P - Prins.

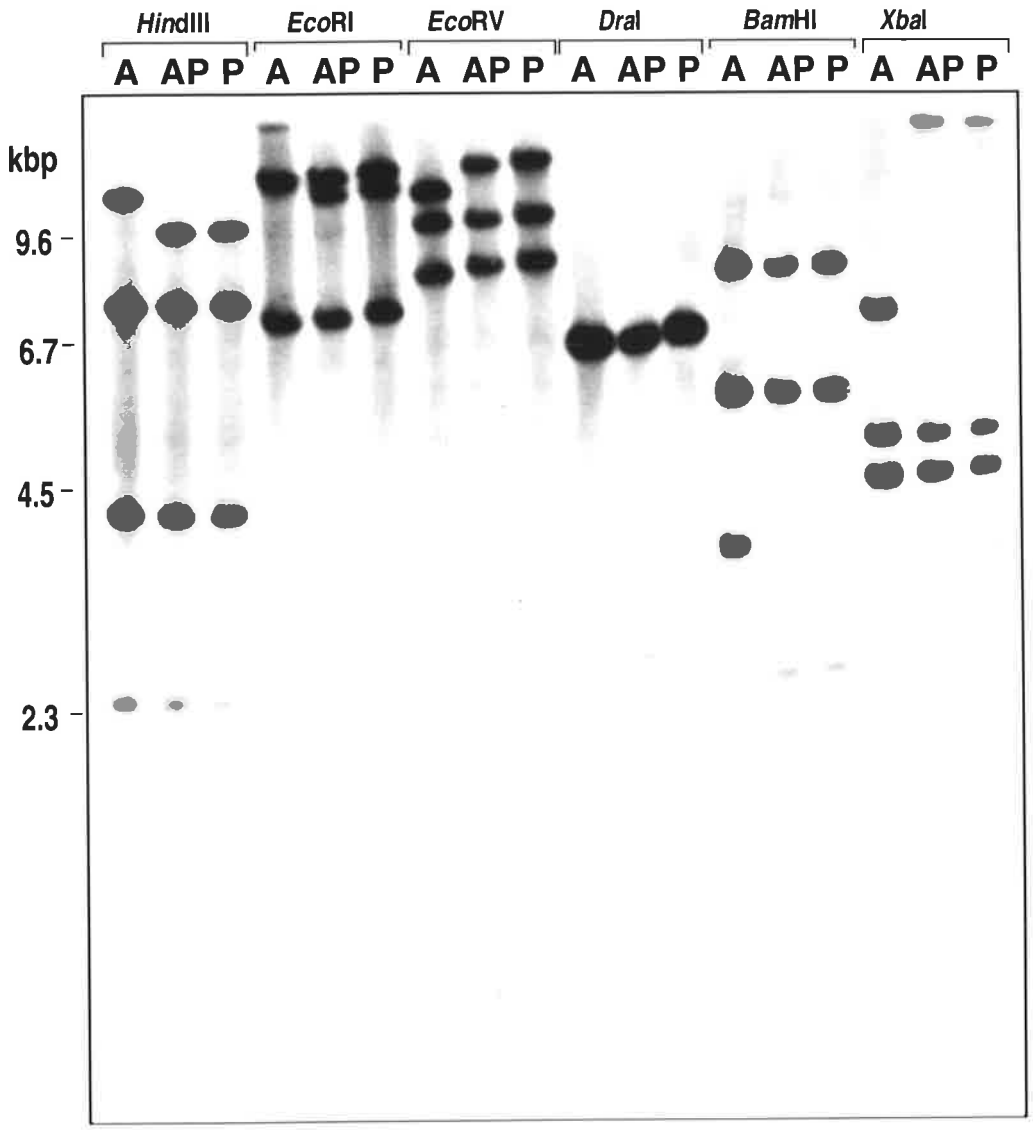

HindIII
EcoRI
EcoRV
DraI
BamHI
XbaI
A AP P A AP P A AP P A AP P A AP P A AP P
kbp
9.6
6.7
4.5
2.3

Figure 5.7 Southern analysis of near-isogenic lines. Plant DNA was digested with six restriction enzymes, and probed with Tag632. A - AUS10894, AP - AUS10894 x $Prins^7$, P - Prins.

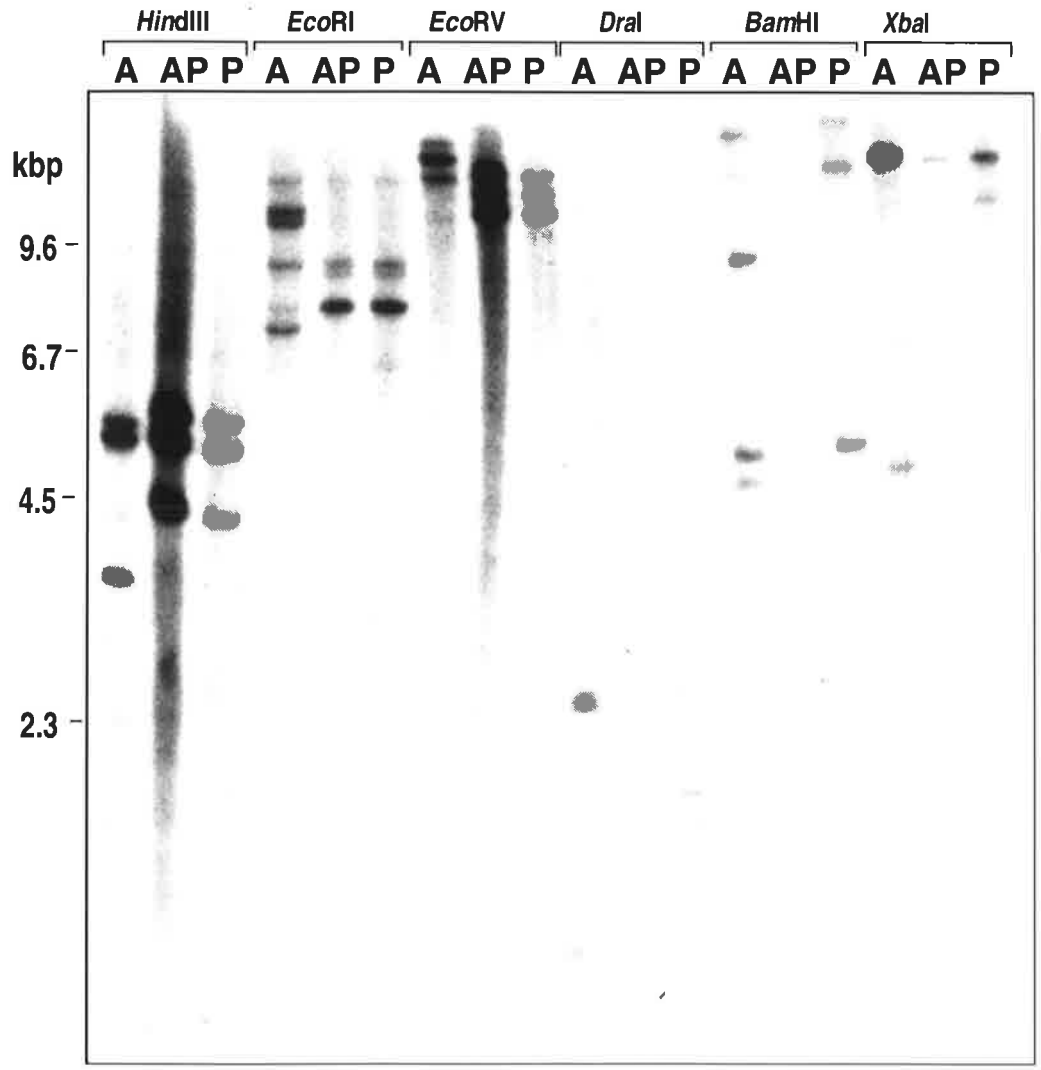

HindIII
EcoRI
EcoRV
DraI
BamHI
XbaI
A AP P A AP P A AP P A AP P A AP P A AP P
kbp
9.6
6.7
4.5
2.3

Figure 5.8 Southern analysis of breeders lines. Plant DNA was digested with *Eco*RV, and probed with Tag605. ED135, EL240 and ED089 are lines with complex pedigrees containing approximately 1-6% AUS10894 DNA. R - resistant to CCN, S - susceptible to CCN. The arrow indicates the position of the band cosegregating with resistance to CCN.

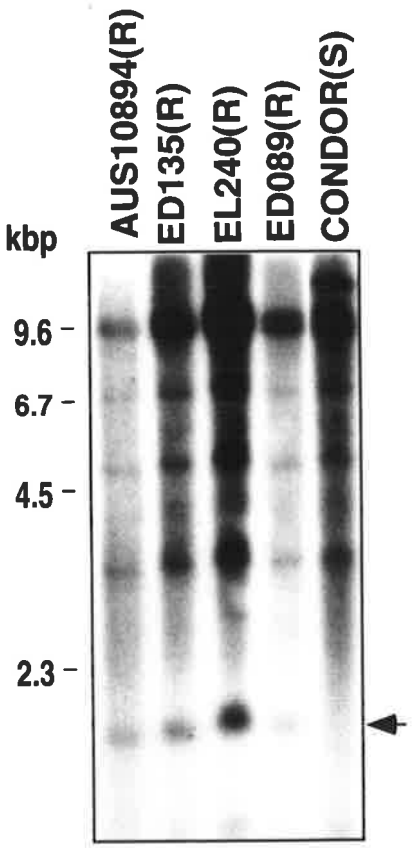

AUS10894(R)
ED135(R)
EL240(R)
ED089(R)
CONDOR(S)
kbp
9.6
6.7
4.5
2.3
Probe: TAG 605

Table 5.3 Numbers of females on roots of Chinese Spring aneuploids.

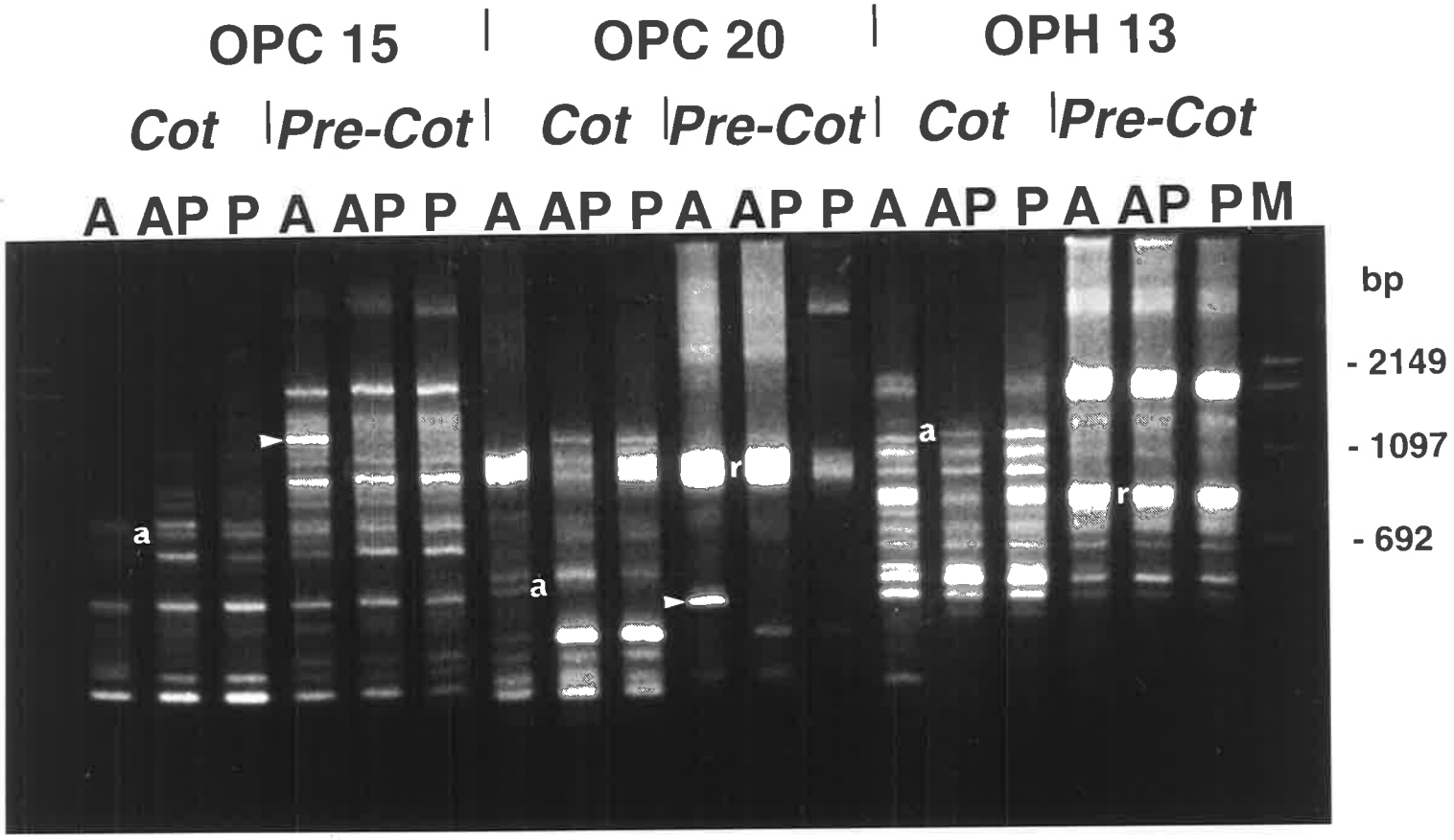
Plant Number	Chinese Spring	N2B-T2A	N2B-T2D	Ditelo 2B$_L$	AUS10894	Egret
1	2	8	3	0	1	10
2	5	3	0	0	1	15
3	1	1	3	0	0	9
4	3	4	6	0	0	11
5	1	3	3	2	0	10
6	3	3	8	0	1	32
7	0	6	6		1	24
8	2	0	3		0	40
9					0	17
10					1	
	2.1 ±1.4	3.5 ± 2.4	4.0 ± 2.3	0.3 ± 0.7	0.5 ± 0.7	18.7 ± 10.4

The results of the bioassays on this small sample show that Chinese Spring does have some resistance to CCN compared with the susceptible control Egret (Table 5.3). The high standard errors observed in this small experiment prevent any conclusions being made about the extra susceptibility of the nulli-2B lines. The only clear result was the extremely high resistance of the 2B$_L$ ditelosomic line, which is equivalent to that of the CCN resistant wheat AUS10894.

RAPD analysis

Using the approach of Eastwood *et al.* (1994), denatured sonicated genomic DNA from AUS10894, AP and Prins was renatured to a Cot value of approximately 130 at 60°C. The partially renatured DNA was run through a hydroxyapatite column to remove some of the repetitive sequences. Approximately 5-10 % of the original DNA was eluted from the column. This should have been enriched for low copy sequences. One hundred and

Figure 5.9 RAPD analysis of near-isogenic lines. Low-copy and total DNA from near-isogenic lines was amplified with the 10 base primers OPC 15, OPC 20, OPH 13. Amplification products were separated on a 2% agarose gel. **a** - Bands that were polymorphic only with low-copy template. ► Bands that were polymorphic only with total genomic template. **r** - bands thought to be derived from repetitive DNA. A - AUS10894, AP - AUS10894 x Prins[7], P - Prins, Cot - low copy DNA, Pre-cot - total genomic DNA.


OPC 15
OPC 20
OPH 13
Cot
Pre-Cot
Cot
Pre-Cot
Cot
Pre-Cot
A AP P A AP P A AP P A AP P A AP P A AP P M
a
a
a
r
r
bp
- 2149
- 1097
- 692

sixty-five random primers were used in PCR reactions with Cot-fractionated DNA from AUS10894, AP and Prins. Of these, sixty-nine revealed polymorphisms between the parental lines. However, no polymorphisms were detected between the NILs. A gel of products from representative reactions with three random primers is shown in Figure 5.9. The PCR products amplified from Cot-fractionated DNA were smaller than those amplified from total genomic DNA (Fig 5.9). This was expected because of the shearing caused by sonication. Reactions with the treated DNA had more distinct banding patterns and scorable polymorphisms than those with untreated DNA. However, in some cases polymorphisms that were observed between parental total genomic DNA were not observed in the reactions using the low-copy DNA fraction. Strong bands that were probably derived from repetitive DNA were missing or less intense in the low-copy DNA reactions (Fig 5.9).

Discussion

Molecular markers can be powerful tools for indirect selection of an agronomically important gene in a breeding program (Tanksley, 1983; Beckman and Soller, 1983).

This study used near-isogenic lines to identify RFLP markers linked to the cereal cyst nematode resistance gene *Cre*. Near-isogenic lines have been used previously to facilitate the identification of RFLP markers linked to disease resistance genes, as reported by Schuller *et al.* (1992) for the *Mla* locus in barley, Klein-Lankhorst *et al.* (1991) for the *Mi* locus in tomato, and Hartl *et al.* (1993) for the *Pm3* locus in wheat. As near-isogenic lines should be identical apart from a segment of DNA containing the gene selected for, any RFLPs between the NIL's should be, and are, closely linked to *Cre*. *Cre* and its closest linked markers reside on a small segment of "AUS10894" that has been integrated into "AP" chromosome 2B.

The location of *Cre* determined in this study can be compared with those of other known cereal CCN resistance genes. The genes with known locations have been located on either homeologous group two or six chromosomes. The species for which evidence has

been presented localising CCN resistance on homeologous group two chromosomes are barley (*Ha*) (Giese et al., 1993; this study-see chapter six), *T. tauschii* (*Ccn-D2*) (Eastwood *et al.*, 1994) and wheat (*Cre*) (Slootmaker *et al.*, 1974; this study). The species which have CCN resistance genes on homeologous group six chromosomes are triticale (rye genome) (Asiedu *et al.*, 1990), *T. tauschii* (*Ccn-D1*) (Eastwood *et al.*, 1994) and *Aegilops ventricosa* (*Cre2*) (Rivoal *et al.*, 1986).

The CCN resistance gene Ccn-D2 was mapped to the proximal region of chromosome 2D in *T. tauschii* (Eastwood *et al.*, 1994). However, only loose linkage was obtained with flanking RFLP markers, possibly due to inconclusive resistance scoring. It could be speculated that the weak *Ccn-D2* is a homeolocus of the wheat CCN resistance gene *Cre*. The much stronger *T. tauschii* resistance gene *Ccn-D1* was mapped to the distal end of the long arm of chromosome 6D (Eastwood *et al.*, 1994). The strong rye CCN resistance gene maps to 6R, making it a possible homologue of *Ccn-D1*.

While this study identified molecular markers with linkage to *Cre*, it would be desirable to find others with closer linkage. Finer mapping around *Cre* may be affected by the nature of the proximal region of chromosome 2B and by the strength of the resistance mediated by *Cre*.

The region of chromosome 2B mapped in this study is one of the most variable in the wheat genome (Liu and Tsunewaki, 1991). There also appears to be extremely localised crossing over in the proximal region of wheat group two chromosomes, leading to the cluster of RFLP loci around the centromeres (Devos *et al.*, 1993). Any recombinational hot-spots may affect the accuracy with which genetic markers can tag genes. This could be one explanation for the different recombination frequencies observed between two loci in the different crosses used in this study.

The resistance mediated by the *Cre* gene is not strong, causing some uncertainty when using the bioassay to determine the resistance phenotype. This has been reported previously by those studying the *Cre* gene (O'Brien et al., 1980). For this reason as

many F3 families as possible were tested to confirm the F2 resistance score in this study. An essential prerequisite for gene mapping is a reliable assay of genotype, as mis-scores can have a large effect on linkage analysis (Nilsson *et al.*, 1993). For instance, Eastwood *et al.* (1994) were able to find much closer marker linkage to the *T. tauschii* CCN resistance gene *Ccn-D1*, which gave strong resistance and clear F2 segregation, than to the weaker gene *Ccn-D2* which produced a less clear segregation.

In addition to RFLP mapping, an attempt was made to identify a *Cre*-linked molecular marker using RAPD analysis on the near-isogenic lines. RAPDs are not often used in genetic analysis of wheat, as its high amount of repetitive DNA and the complexity of its genome produces low levels of polymorphism and multiple similar-size products from homologous sequences (Devos and Gale, 1992). In an attempt to reduce the problems caused by repetitive DNA, the technique of Cot-fractionation of DNA described by Eastwood *et al.* (1994) was used. This should produce DNA templates enriched for low-copy sequences and has been used to identify a RAPD linked to a CCN resistance gene in *T. tauschii* (Eastwood *et al.*, 1994). However testing with 156 random primers using Cot-fractionated DNA did not reveal a RAPD between the near-isogenic lines. RAPDs were revealed between the parental lines, but at a much lower frequency than RFLPs. The search for RAPDs was not extended beyond 150 primers, as the more directed search using RFLPs produced *Cre*-linked markers.

While using the Chinese Spring aneuploid stocks to map the chromosomal location of markers, it was noted that their RFLP patterns matched those of the resistant line AUS10894. As there has been some suggestion of resistance to CCN in Chinese Spring (Asiedu *et al.*, 1990), a small study was conducted to determine whether this line had a CCN resistance gene on chromosome 2B. The presence of a CCN resistance gene on 2B was not determined. The most interesting result was the extremely high resistance of the ditelosomic $2B_L$ line. This may indicate that there are genes on the short arm that are important for nematode survival, or it may carry genes that have a negative interaction with a CCN resistance gene.

Chapter 6 Development of PCR markers

Introduction

Most genetic mapping in cereals has used RFLPs because of their sensitivity and reliability. Although these features give RFLP analysis an advantage in genetic research in the laboratory, the technique has limited potential in marker-assisted selection. It requires a high level of technical expertise and considerable equipment investment. The cost of each assay is also high, due to the expensive materials used and labour requirement (Ragot and Hoisington, 1993). In contrast, molecular markers based on the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) allow high throughput of material and have low startup and running costs.

Random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990) are one type of PCR marker that can be used to map resistance genes (Martin *et al.*, 1991; Michelmore *et al.*, 1991). Another type of PCR marker is the "sequence-tagged-site" (STS) marker (Olson *et al.*, 1989), or "sequence-characterised-amplified-region" (SCAR) marker (Paran and Michelmore, 1993). STS and SCAR markers are genomic sequences amplified by PCR using specific oligonucleotide primers.

This chapter describes attempts to develop PCR-based STS markers from RFLP clones.

Materials and methods

The plant materials and DNA extractions used are as described in Chapter 5.

Primer design

The ends of the RFLP clones Tag605, Tag699, ABC451, CDO588 and WG996 were sequenced using M13 forward and reverse primers. PCR primer sets were designed from the sequence of each of the clones. Approximately 400 bp of sequence from the 5' end of

the Tag605 insert was used to design an 18mer PCR primer set, designated AWP1. Cloned AWP1 products were sequenced and allele-specific PCR primer sets synthesised, designated AWP2 and AWP3. Sequencing was done on an ABI automated sequencer and primers were chosen using "Oligo" software (National Biosciences, Plymouth, MN, USA). Primers were synthesised by DNAExpress (Boulder, CO, USA).

PCR

PCR reactions contained 20 mM NH_4SO_4, 75 mM Tris-HCl pH 9, 0.01 % (w/v) Tween, 50 mM each dNTP, 1.5 mM $MgCl_2$, 200 nM each primer, 100 ng template DNA and 0.2 units *Taq* polymerase in 50 µl total volume. Reactions were carried out in a MJ Research thermal cycler. Temperature conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min, followed by 1 cycle of 72 °C for 5 min. PCR products were digested with restriction enzymes where necessary. Ethanol precipitation of DNA was required before digestion with *Bfa*1. Restricted products were separated on 3 % agarose gels and stained with ethidium bromide or on 10 % polyacrylamide gels in TBE buffer and stained with silver (Bassam *et al.*, 1991). For dual PCR, AWP1 products were diluted 1:100 and 1 µl used as template for the second amplification, in which the AWP2 or AWP3 primers were annealed at 52 °C. Products were separated on 2.5 % agarose gels and stained with ethidium bromide.

Cloning of AWP1 fragment

PCR was carried out using kinased AWP1 primers, with "AUS 10894" and "Spear" DNA as templates. The band of interest was excised from an agarose gel, purified using Geneclean and cloned into the dephosphorylated vector pTZ19u. Putative transformants were checked using colony swabs as templates for PCR with M13 forward and reverse primers.

Results

RFLP clones that mapped close to *Cre* were partially sequenced to enable PCR primers to be designed (Table 6.1). Partial clone sequences for CDO588, Tag699 and WG996 and primer sequences are given in Appendices III and IV. As the sequence of only one DNA strand was determined, ABC451 and CDO588 insert ends were sequenced twice and those of Tag605 and Tag699 sequenced three times to give accurate consensus sequences. Attempts were made to develop a PCR marker from the probe Tag699 although it was not able to be mapped in this study because no RFLP was found. According to the map of Liu and Tsunewaki (1991) it is close to Tag605 and could be tested for linkage to *Cre* if a PCR-based marker could be developed.

For the clones ABC451, CDO588 and Tag605 upper and lower primers were designed at each end of the insert in an attempt to amplify the full sequence. These primer sets were 451T, 588T and 605T (Table 6.1). This strategy failed to produce products of the expected size, so primer sets were designed from either end of the inserts to amplify approximately 300 bp fragments as the PCR appears to preferentially amplify smaller fragments. The primer sets designed from M13R sequence were 451R, 588R, 605R and 699R, while those designed from M13U sequence were 605F and 996F. Of the primers designed and tested with the NILs and other controls, only the sets from the M13R ends of the ABC451 and Tag605 inserts produced a clear banding pattern with a predominant product of the expected size (Table 6.1, Figs. 6.2 & 6.4).

Table 6.1 RFLP clones sequenced for primer design

CLONE	Cre Linkage (cM)	Vector	Insert size (kb)	M13U sequence (bp)	M13R sequence (bp)	Primers designed and tested	Correct size product
ABC451	7.8	pBS-SK	1.3	438	490	451T, 451R	451R
CDO588	7.8	pBS-SK	1.6	360	317	588T, 588R	
Tag605	8.0	pUC119	3.0	446	385	605T, 605F, 605R(2)	605R
Tag699	?	pUC119	3.0	511	402	699R	
WG996	17	pGEM 4	1.0	332		996F	

Characterisation of ABC451 product

The 451R primer set was predicted to produce a PCR product of 300 bp based on the ABC451 sequence (Fig 6.1). Amplification of resistant and susceptible varieties produced 300 and 700 bp bands. However, the 300 bp product showed no size polymorphism between varieties (Fig 6.2). The genomic origin of the product was tested using group two nulli-tetrasomic stocks. The 300 bp product was produced from all lines, although its intensity may have been lower from the nullisomic 2B-tetrasomic 2D line (Fig 6.2). The intensity of the 700 bp product appeared to be reduced in the nullisomic 2D-tetrasomic 2A line (Fig 6.2).

As an alternative method of verifying the identity of the 300 bp 451R PCR product, the amplified fragment was used to probe membranes which displayed the RFLP with the original ABC451 clone. The 451R primers were used to amplify DNA from AUS10894 and Spear. The 300 bp product from these reactions was cloned. The 451R-Spear clone was used to probe a membrane containing DNA from near-isogenic lines. The cloned PCR product produced almost exactly the same RFLP pattern as the original full-length clone, indicating that the 451R primer set is amplifying a fragment of DNA with considerable homology to part of the ABC451 clone (Fig. 6.3a). Further confirmation of the products identity was obtained by probing nulli-tetrasomic lines. The resulting

Figure 6.1 Partial sequence of the clone ABC451 generated by duplicate single-strand sequencing using M13 forward and reverse primers. Positions of 451T and 451R primer sets are shown. U - upper primer, L - lower primer

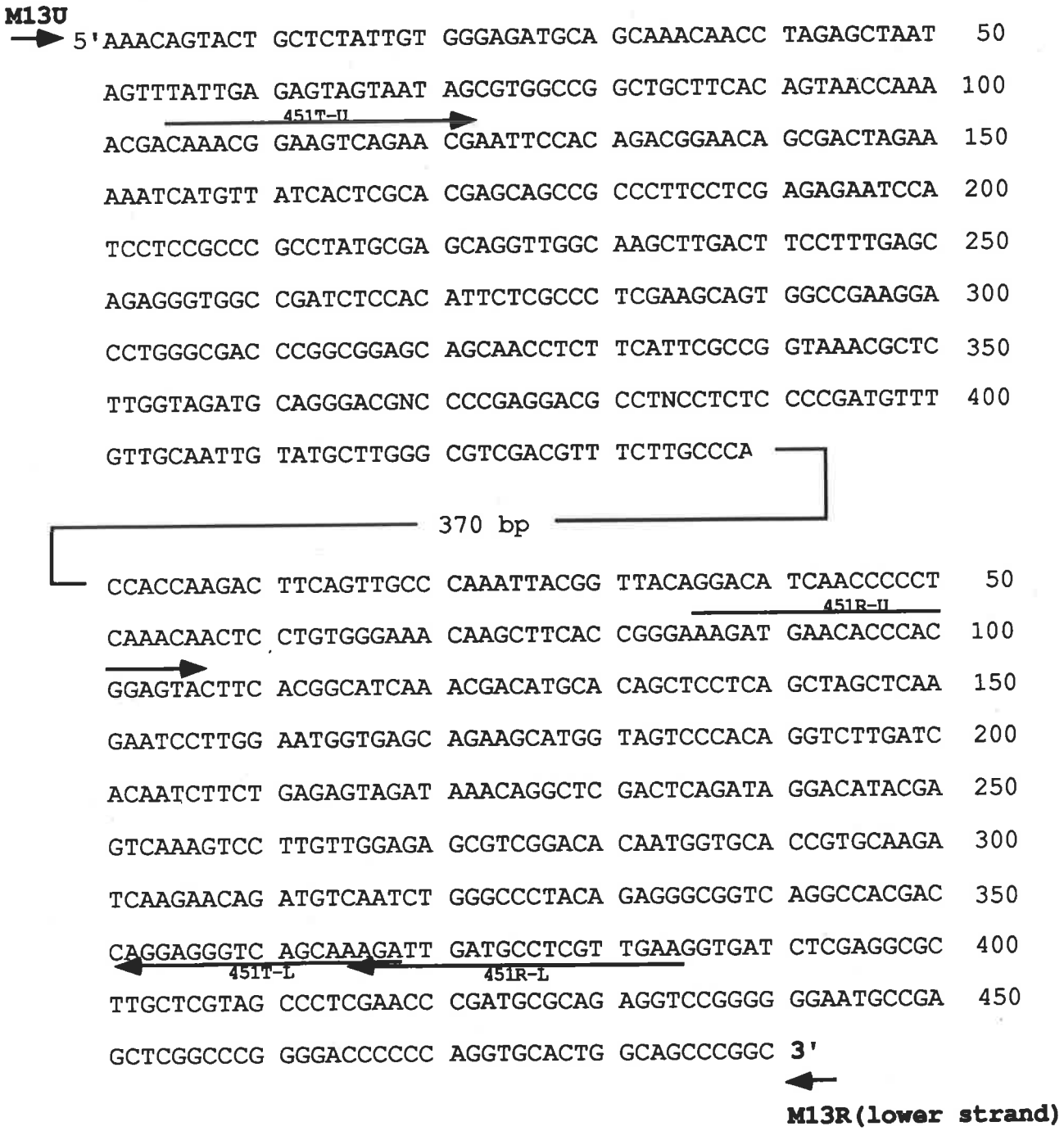

M13U
5'AAACAGTACT GCTCTATTGT GGGAGATGCA GCAAACAACC TAGAGCTAAT 50
AGTTTATTGA GAGTAGTAAT AGCGTGGCCG GCTGCTTCAC AGTAACCAAA 100
451T-U
ACGACAAACG GAAGTCAGAA CGAATTCCAC AGACGGAACA GCGACTAGAA 150
AAATCATGTT ATCACTCGCA CGAGCAGCCG CCCTTCCTCG AGAGAATCCA 200
TCCTCCGCCC GCCTATGCGA GCAGGTTGGC AAGCTTGACT TCCTTTGAGC 250
AGAGGGTGGC CGATCTCCAC ATTCTCGCCC TCGAAGCAGT GGCCGAAGGA 300
CCTGGGCGAC CCGGCGGAGC AGCAACCTCT TCATTCGCCG GTAAACGCTC 350
TTGGTAGATG CAGGGACGNC CCCGAGGACG CCTNCCTCTC CCCGATGTTT 400
GTTGCAATTG TATGCTTGGG CGTCGACGTT TCTTGCCCA
370 bp
CCACCAAGAC TTCAGTTGCC CAAATTACGG TTACAGGACA TCAACCCCCT 50
451R-U
CAAACAACTC CTGTGGGAAA CAAGCTTCAC CGGGAAAGAT GAACACCCAC 100
GGAGTACTTC ACGGCATCAA ACGACATGCA CAGCTCCTCA GCTAGCTCAA 150
GAATCCTTGG AATGGTGAGC AGAAGCATGG TAGTCCCACA GGTCTTGATC 200
ACAATCTTCT GAGAGTAGAT AAACAGGCTC GACTCAGATA GGACATACGA 250
GTCAAAGTCC TTGTTGGAGA GCGTCGGACA CAATGGTGCA CCGTGCAAGA 300
TCAAGAACAG ATGTCAATCT GGGCCCTACA GAGGGCGGTC AGGCCACGAC 350
CAGGAGGGTC AGCAAAGATT GATGCCTCGT TGAAGGTGAT CTCGAGGCGC 400
451T-L
451R-L
TTGCTCGTAG CCCTCGAACC CGATGCGCAG AGGTCCGGGG GGAATGCCGA 450
GCTCGGCCCG GGGACCCCCC AGGTGCACTG GCAGCCCGGC 3'
M13R(lower strand)

Figure 6.2 PCR products generated with primers targeted to the *abc451* locus. The arrow indicates the expected product at 300 bp. No size polymorphism was seen between resistant and susceptible varieties, or between nulli-tetrasomic lines (N2AT2D - nullisomic 2A-tetrasomic 2D). C.S. - Chinese Spring. R - resistant to CCN, S - susceptible to CCN. The PCR products were fractionated on a 2.5 % agarose gel and stained with ethidium bromide.

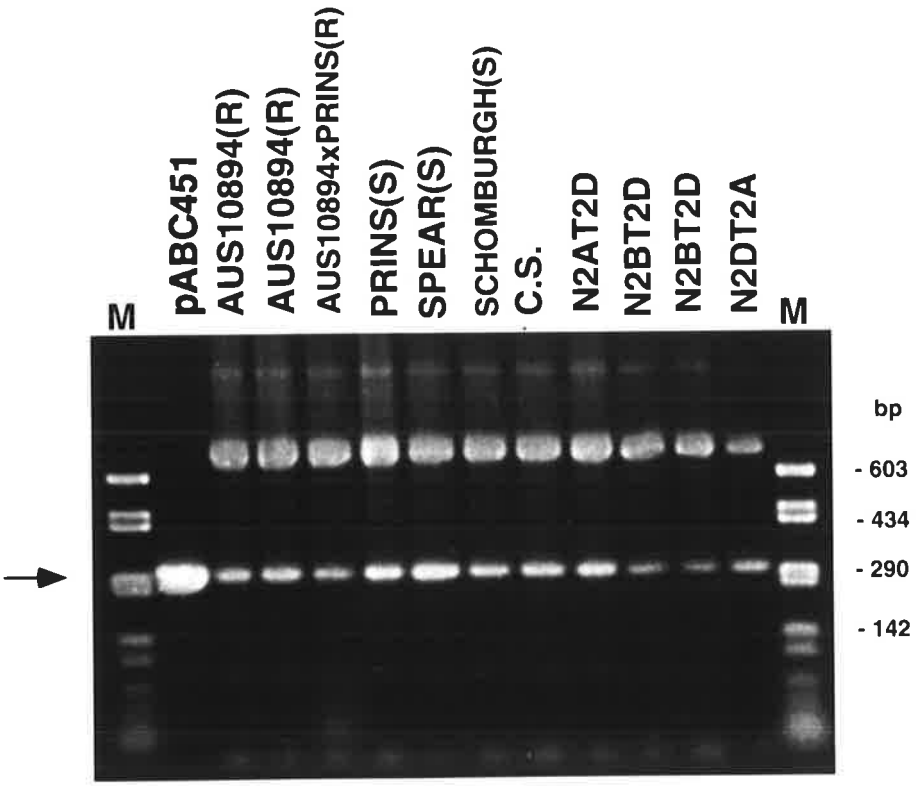
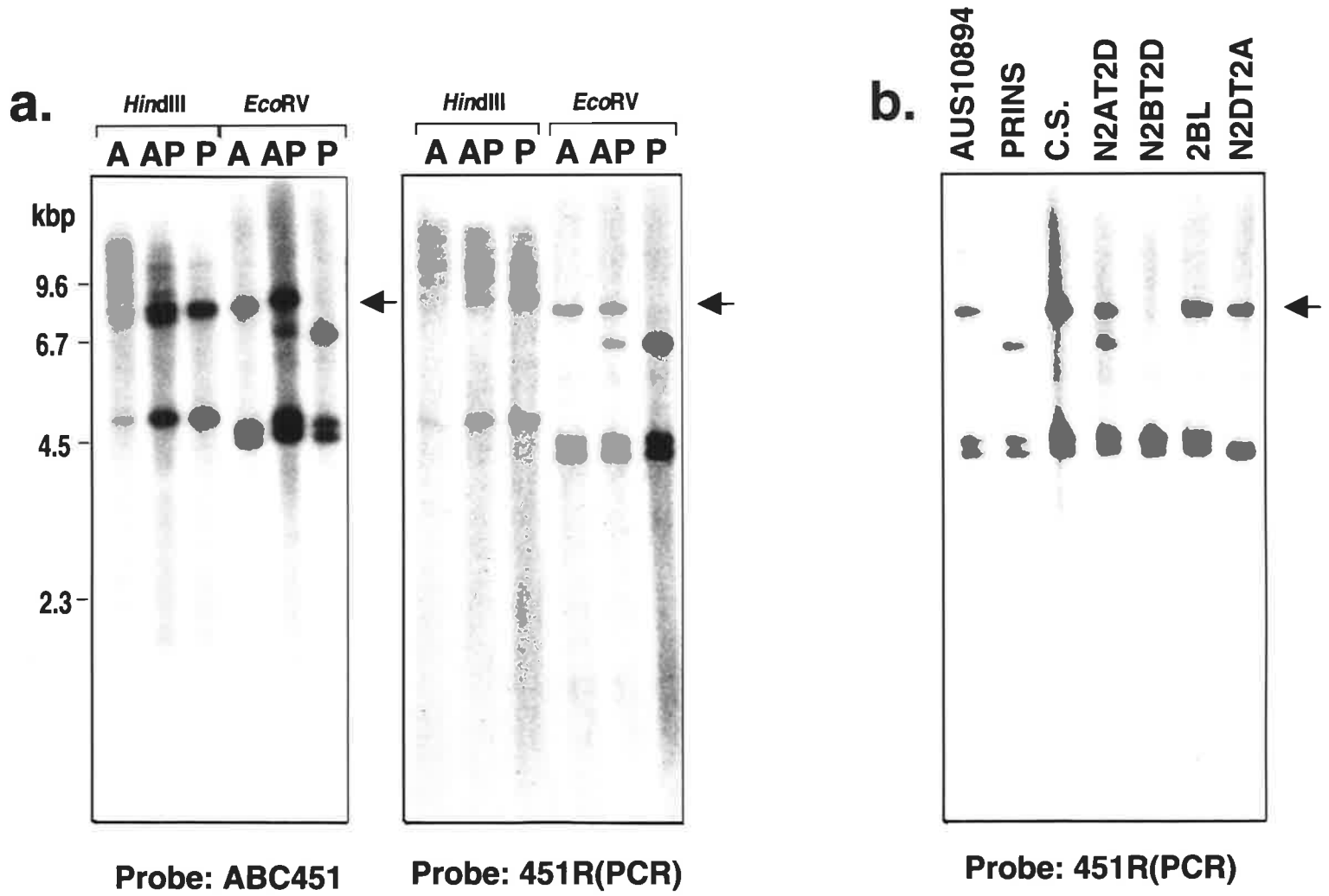

M
pABC451
AUS10894(R)
AUS10894(R)
AUS10894xPRINS(R)
PRINS(S)
SPEAR(S)
SCHOMBURGH(S)
C.S.
N2AT2D
N2BT2D
N2BT2D
N2DT2A
M
bp
- 603
- 434
- 290
- 142

Figure 6.3

(a) Southern analysis of near-isogenic lines. Plant DNA was digested with *HinDIII* or *Eco*RV and probed with either the original ABC451 probe or the cloned 451R PCR product. Both probes gave the same RFLP pattern. The arrow indicates the polymorphic band linked to the resistance allele. A - AUS10894, AP - AUS10894 x Prins[7], P - Prins. **(b)** Chromosomal localisation of RFLPs. Southern blot of nulli-tetrasomic (N2AT2D - nulli 2A tetra 2D) and ditelosomic ($2B_L$) lines digested with *Eco*RV and probed with the cloned 451R PCR product. C.S. - Chinese Spring. Absence of polymorphic band (arrow) in N2BT2D and presence in $2B_L$ indicates that RFLP is derived from $2B_L$.


a.
HindIII
EcoRV
A AP P A AP P
kbp
9.6
6.7
4.5
2.3
HindIII
EcoRV
A AP P A AP P
Probe: ABC451
Probe: 451R(PCR)
b.
AUS10894
PRINS
C.S.
N2AT2D
N2BT2D
2BL
N2DT2A
Probe: 451R(PCR)

hybridisation pattern indicated that the PCR product hybridised as expected to the long arm of chromosome 2B and also to chromosome 2D (Fig. 6.3b).

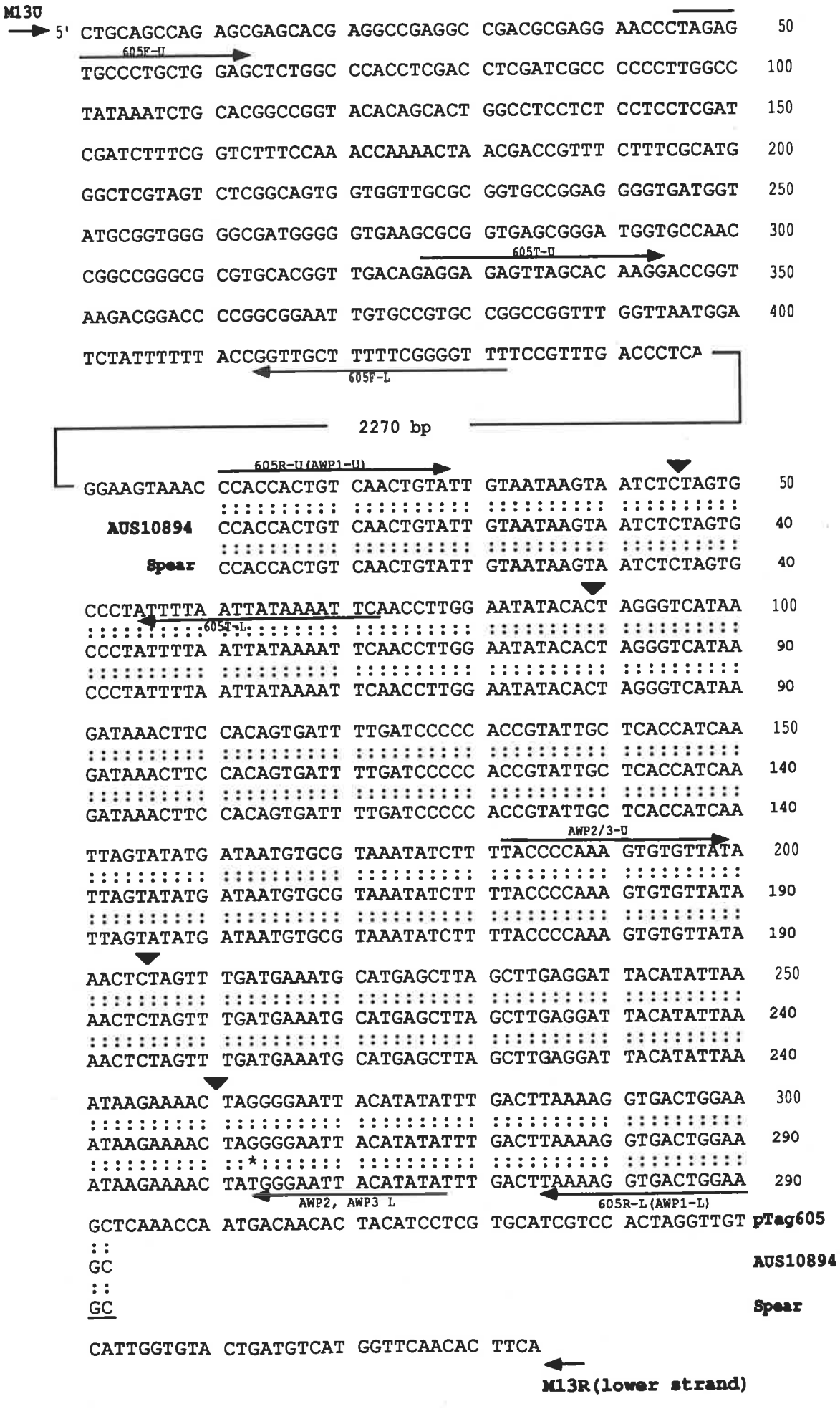
To try to generate a PCR marker based on the ABC451 clone, the 451R product was tested for DNA polymorphism between resistant and susceptible varieties. Products from PCR reactions with DNA from resistant and susceptible lines as template were digested with restriction enzymes chosen from the sequence of the ABC451 clone (Figure 6.1) and various frequent cutters. The products were restricted with the following enzymes: *Alu*I, *Dde*I, *Hae*III, *Hinf*1, *Hpa*II, *Mnl*I, *Msp*I, *Rsa*I, *Sac*I, *Sau*3AI, *Sau*96I, *Sca*I, *Sty*I. The recognition sequences for these enzymes cover about 50 bases of the 451R product sequence and will be searching at least 15% of the sequence for mutations. No polymorphisms were observed between any of the lines with the enzymes tested.

It was noticed during probe screening for RFLPs that ABC451 and CDO588 gave the same hybridisation pattern (Figs 5.1 and 6.3). The probes also mapped to the same position. When the clones were partially sequenced for primer design, considerable sequence homology was also observed (Appendix V). When all sequences were compared with the major genomic databases, ABC451 and CDO588 were the only clones to produce a close match with other sequences. Their 3' termini had amino acid homology with the potato tuberisation-related cDNA TUB13 (Taylor *et al.*, 1992). CDO588 had the closest homology, with 63% identity over 44 amino acids. TUB13 is thought to be a plant S-adenosylmethionine decarboxylase gene, and CDO588 does in fact have 46% identity with a yeast S-adenosylmethionine decarboxylase proenzyme. Not enough sequence information is available to establish the functions of ABC451 and CDO588, but they do share homology with domains of this enzyme involved in polyamine biosynthesis (Taylor *et al.*, 1992).

Characterisation of AWP1 PCR marker

PCR primers were designed from partial sequence of the 3' end of the wheat genomic clone Tag605 (Fig 6.4). The primer set (designated AWP1) produced a major PCR

Figure 6.4 Partial sequence of Tag605 clone generated by triplicate single-strand sequencing using M13 forward and reverse primers. Position of 605T, 605F and 605R primer sets are shown. The sequences of cloned AWP1 products amplified from AUS10894 and Spear are aligned with the Tag605 clone sequence. Conserved bases are indicated (:) and the single base difference is shown (*). Allele-specific lower primers (AWP2 and AWP3) are shown with their common upper primer. ▼ - indicates the positions of *Bfa*1 restriction enzyme recognition sites.


M13U
5' CTGCAGCCAG AGCGAGCACG AGGCCGAGGC CGACGCGAGG AACCCTAGAG 50
605F-U
TGCCCTGCTG GAGCTCTGGC CCACCTCGAC CTCGATCGCC CCCCTTGGCC 100
TATAAATCTG CACGGCCGGT ACACAGCACT GGCCTCCTCT CCTCCTCGAT 150
CGATCTTTCG GTCTTTCCAA ACCAAAACTA ACGACCGTTT CTTTCGCATG 200
GGCTCGTAGT CTCGGCAGTG GTGGTTGCGC GGTGCCGGAG GGGTGATGGT 250
ATGCGGTGGG GGCGATGGGG GTGAAGCGCG GTGAGCGGGA TGGTGCCAAC 300
605T-U
CGGCCGGGCG CGTGCACGGT TGACAGAGGA GAGTTAGCAC AAGGACCGGT 350
AAGACGGACC CCGGCGGAAT TGTGCCGTGC CGGCCGGTTT GGTTAATGGA 400
TCTATTTTTT ACCGGTTGCT TTTTCGGGGT TTTCCGTTTG ACCCTCA
605F-L
2270 bp
605R-U(AWP1-U)
GGAAGTAAAC CCACCACTGT CAACTGTATT GTAATAAGTA ATCTCTAGTG 50
AUS10894 CCACCACTGT CAACTGTATT GTAATAAGTA ATCTCTAGTG 40
Spear CCACCACTGT CAACTGTATT GTAATAAGTA ATCTCTAGTG 40
CCCTATTTTA ATTATAAAAT TCAACCTTGG AATATACACT AGGGTCATAA 100
605T-L
CCCTATTTTA ATTATAAAAT TCAACCTTGG AATATACACT AGGGTCATAA 90
CCCTATTTTA ATTATAAAAT TCAACCTTGG AATATACACT AGGGTCATAA 90
GATAAACTTC CACAGTGATT TTGATCCCCC ACCGTATTGC TCACCATCAA 150
GATAAACTTC CACAGTGATT TTGATCCCCC ACCGTATTGC TCACCATCAA 140
GATAAACTTC CACAGTGATT TTGATCCCCC ACCGTATTGC TCACCATCAA 140
AWP2/3-U
TTAGTATATG ATAATGTGCG TAAATATCTT TTACCCCAAA GTGTGTTATA 200
TTAGTATATG ATAATGTGCG TAAATATCTT TTACCCCAAA GTGTGTTATA 190
TTAGTATATG ATAATGTGCG TAAATATCTT TTACCCCAAA GTGTGTTATA 190
AACTCTAGTT TGATGAAATG CATGAGCTTA GCTTGAGGAT TACATATTAA 250
AACTCTAGTT TGATGAAATG CATGAGCTTA GCTTGAGGAT TACATATTAA 240
AACTCTAGTT TGATGAAATG CATGAGCTTA GCTTGAGGAT TACATATTAA 240
ATAAGAAAAC TAGGGGAATT ACATATATTT GACTTAAAAG GTGACTGGAA 300
ATAAGAAAAC TAGGGGAATT ACATATATTT GACTTAAAAG GTGACTGGAA 290
ATAAGAAAAC TATGGGAATT ACATATATTT GACTTAAAAG GTGACTGGAA 290
AWP2, AWP3 L
605R-L(AWP1-L)
GCTCAAACCA ATGACAACAC TACATCCTCG TGCATCGTCC ACTAGGTTGT pTag605
GC AUS10894
GC Spear
CATTGGTGTA CTGATGTCAT GGTTCAACAC TTCA
M13R(lower strand)

product of the expected size of 292 bp with no size polymorphism when DNA from resistant and susceptible varieties was used a the PCR template (Fig 6.5). The origin of the amplified product was tested using DNA from "Chinese Spring" nulli-tetrasomic and ditelosomic stocks as PCR templates. The pattern of amplification of the 292 bp fragment confirms that it is derived from the long arm of chromosome 2B while the 700 bp minor product is derived from 2D (Fig 6.6). This corresponds with the previous chromosomal assignments of the RFLP clone from which the AWP1 primer set was designed (Fig 5.2).

To search for sequence differences between varieties, AWP1 PCR products amplified from CCN-resistant and susceptible lines were digested with a panel of restriction enzymes chosen from the sequence of the Tag605 clone (Fig. 6.4). The enzymes tested were *Alu*I, *Dde*I, *Hinf*I, *Mae*I(*Bfa*I), *Mnl*I, *Mse*I, *Nla*III, *Nsi*I, *Rsa*I, *Sau*3AI, *Sau*96I, *Sty*I, *Taq*I. Of these, only *Bfa*1 (isoschizomer of *Mae*1) generated a polymorphic banding pattern between lines with or without the *Cre* gene (Fig 6.7). Based on the pTag605 sequence, a *Bfa*1 digest of the 300 bp AWP1 product should produce restriction fragments of 116, 55, 44, 43 and 34 base pairs in length. These bands are most clearly seen in the lane where the pTag605 clone was used as the PCR template, but are also present in digests of AWP1 products from reactions with AUS10894 and Chinese Spring templates (Fig. 6.7) In digests of products from the CCN-susceptible cultivar Schomburgk, the 55 bp fragment is replaced by one of 97 bp (Fig 6.7).

The identity of the AWP1 fragment could be further verified by analysis of F2 individuals from a population segregating for CCN resistance with known scores for the RFLP marker Tag605 (Fig. 6.8). The *Aawp1-Bfa1* marker co-segregated with the RFLP marker from which it was derived. However, for practical purposes, only the 97 bp fragment can be reliable scored (Fig. 6.8). The 97 bp fragment identifies the allele *Aawp1-2B-Bfa1b*. As this assay is based on the presence or absence of a single band, it is a dominant marker and as such cannot discriminate between individuals homozygous

Figure 6.5 AWP1 PCR products from resistant and susceptible varieties. Arrow indicates expected product at 292 bp. The pTag605 lane shows the product obtained when the original probe was used as template. No size polymorphism was seen between resistant and susceptible varieties. The products were fractionated on a 15% polyacrylamide gel and stained with ethidium bromide. R - resistant to CCN, S - susceptible to CCN.

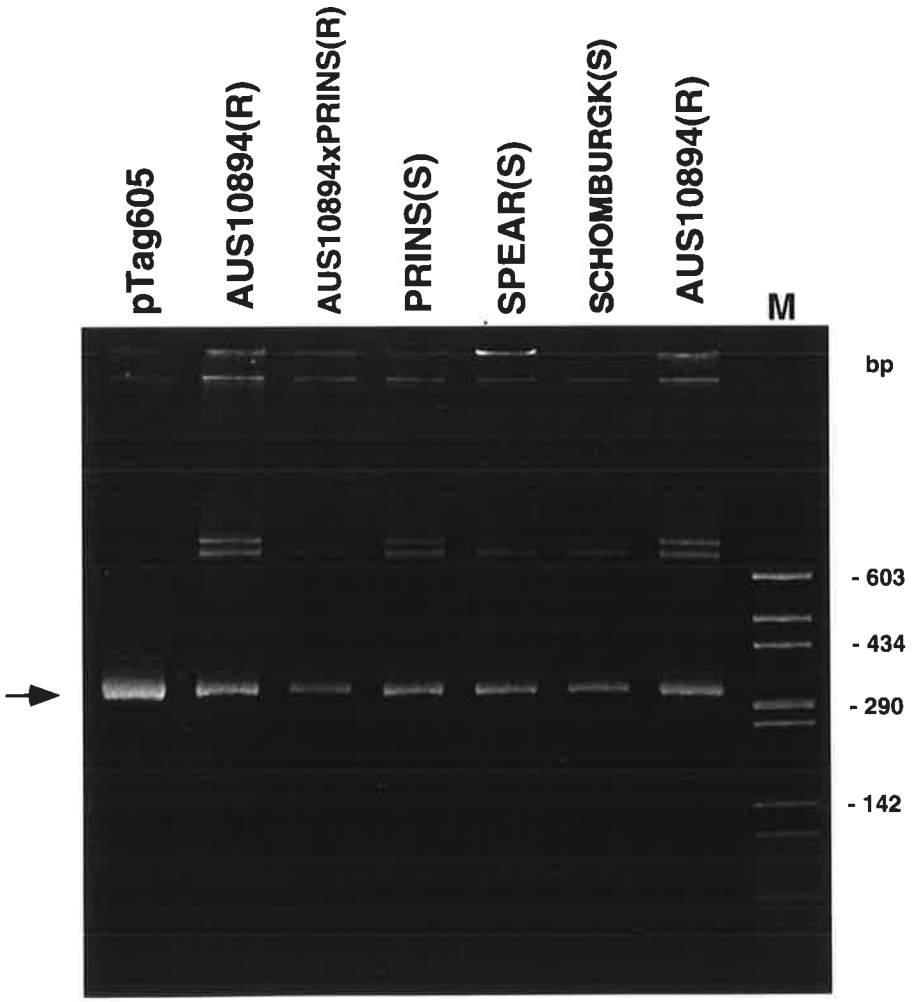

pTag605
AUS10894(R)
AUS10894xPRINS(R)
PRINS(S)
SPEAR(S)
SCHOMBURGK(S)
AUS10894(R)
M
bp
- 603
- 434
- 290
- 142

Figure 6.6 AWP1 PCR products from resistant and susceptible varieties and from nulli-tetrasomic and ditelosomic lines. **(a)** Arrow indicates expected product at 292 bp. No size polymorphism was seen between resistant and susceptible varieties. **(b)** Major 292 bp product is amplified from all lines except nullisomic 2B-tetrasomic 2D line. Minor 700 bp product is missing from nullisomic 2D-tetrasomic 2A line. The results indicate that major product is from chromosome 2B long arm, minor product is from chromosome 2D. R=resistant, S=susceptible, AP=AUS10894xPrins NIL, CS=Chinese spring, DT2BL= ditelosomic $2B_L$, N2AT2D= nullisomic 2A-tetrasomic 2D, etc. The products were fractionated on a 2.5 % agarose gel and stained with ethidium bromide.

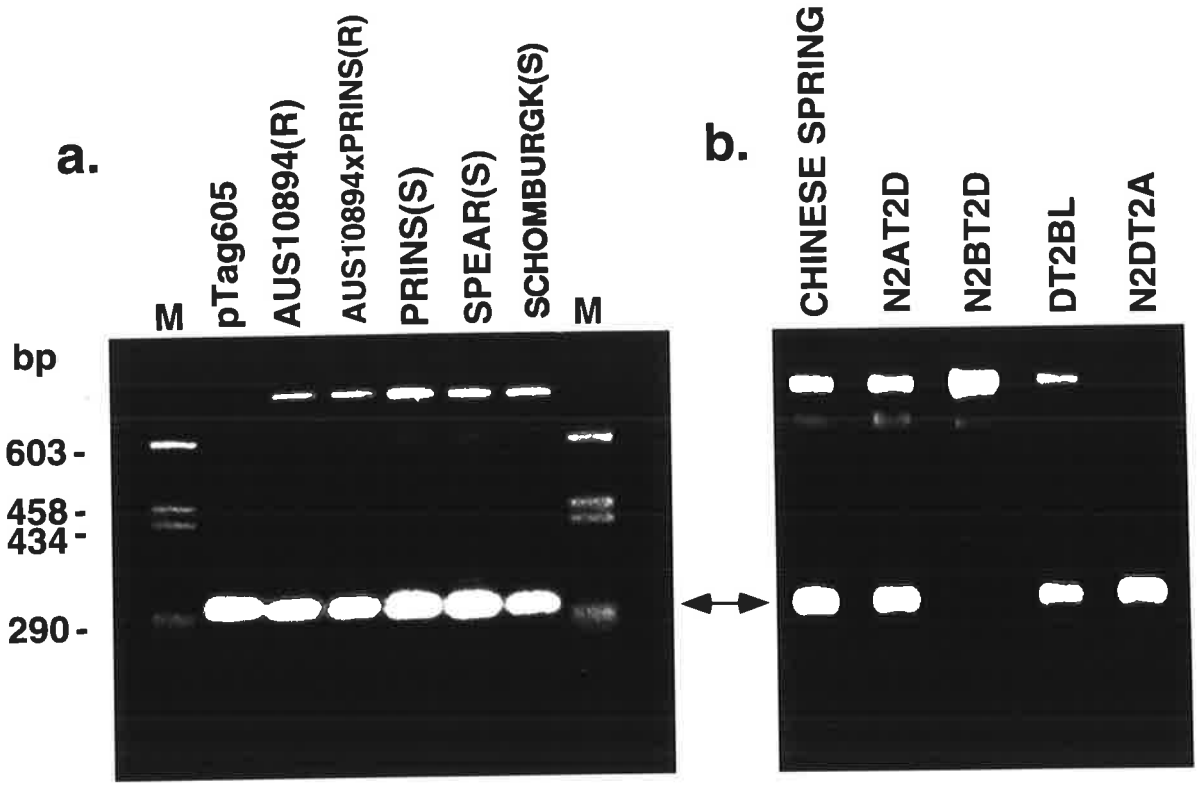

a.
M
pTag605
AUS10894(R)
AUS10894xPRINS(R)
PRINS(S)
SPEAR(S)
SCHOMBURGK(S)
M
bp
603 -
458 -
434 -
290 -
b.
CHINESE SPRING
N2AT2D
N2BT2D
DT2BL
N2DT2A

Figure 6.7 AWP1 PCR products digested with *Bfa*1. DNA templates for PCR were the Tag605 plasmid, Chinese Spring, AUS10894 (CCN Resistant) and Schomburgk (CCN Susceptible) → 97 bp Schomburgk allele band, → 55 bp AUS10894 allele band. The digested PCR products were fractionated on a 10 % polyacrylamide gel and stained with silver nitrate.

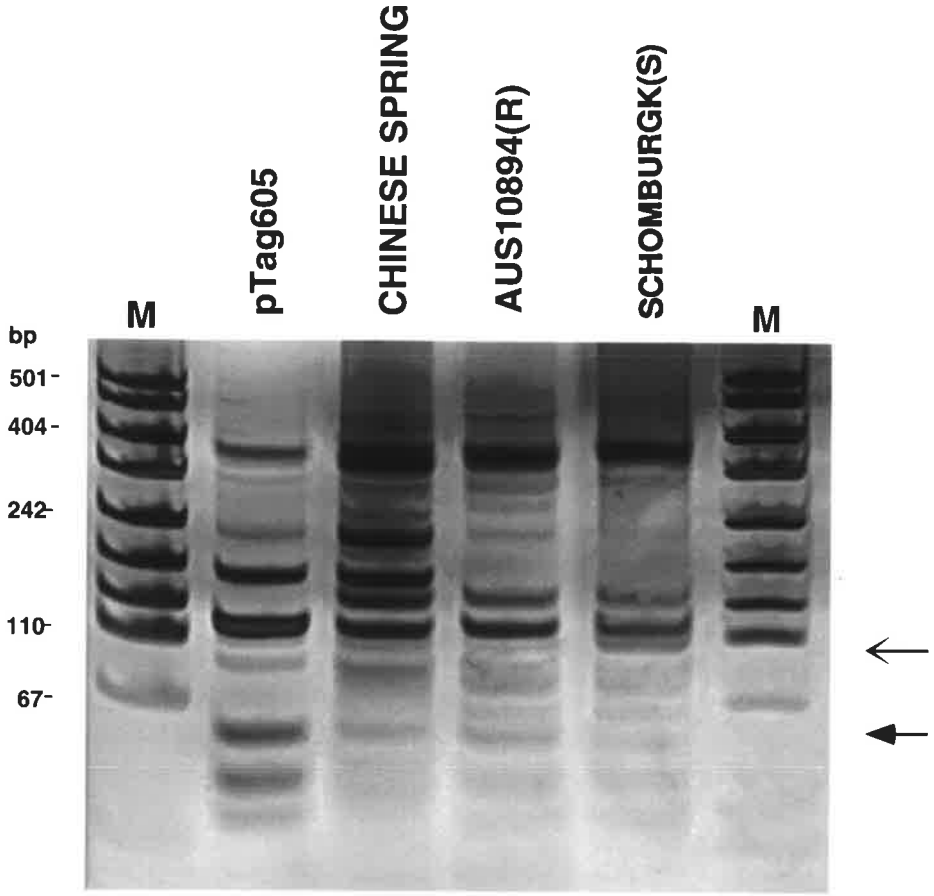
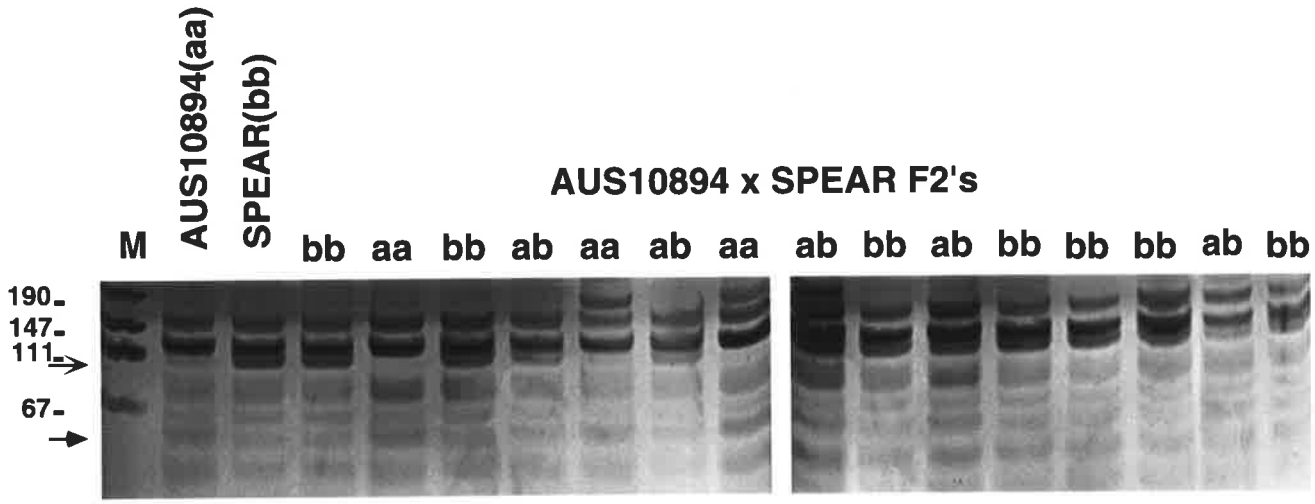

M
pTag605
CHINESE SPRING
AUS10894(R)
SCHOMBURGK(S)
M
bp
501
404
242
110
67

Figure 6.8 AWP1 PCR products digested with *Bfa*1. Template DNA for PCR was extracted from AUS10894 x Spear F2s segregating for CCN resistance and Tag605 (RFLP score above lanes). → 97 bp Spear allele band, → 55 bp AUS10894 allele band. The digested PCR products were fractionated on a 10 % polyacrylamide gel and stained with silver nitrate.


AUS10894(aa)
SPEAR(bb)
AUS10894 x SPEAR F2's
M
bb aa bb ab aa ab aa ab bb ab bb bb bb ab bb
190
147
111
67

or heterozygous for this allele. This is a major deficiency in terms of using this assay as a marker for the *Cre* gene.

Allele-specific PCR

In order to design a more informative PCR assay based on the sequence polymorphism that produced the AWP1-*Bfa*1 restriction pattern, the 292 bp AWP1 PCR product was sequenced. The AWP1 primer set was used to amplify products from the resistant line "AUS 10894" and the susceptible cultivar "Spear". The 292 bp AWP1 product was blunt-end cloned into pTZ19U. Putatively transformed colonies were checked using colony swabs as templates for templates for PCR with M13 forward and reverse primers. The identity of the correctly sized products was confirmed with *Bfa*I digestion (Fig 6.9). Putative clones and genomic DNA were used as a template for PCR with AWP1 primers, followed by digestion with *Bfa*1. The restriction pattern of the clone products matched those of the amplification products from genomic DNA (Fig. 6.9).

Double-stranded sequencing of the AWP1 clones derived from amplification of AUS10894 and Spear with the AWP1 primers revealed a single base difference between the two lines at position 263, where a G in the resistant line was replaced by an T in the susceptible line (Fig. 6.4). This base substitution destroys the fourth *Bfa*1 restriction site and explains the restriction fragment pattern difference observed between *Bfa*1 digests of AWP1 products from the two lines (Fig 6.8). PCR primers were designed with 3' termini that corresponded with those of the resistant or susceptible alleles to determine whether the 3' mismatch would result in the preferential amplification of the respective alleles. Resistance-allele specific lower primers from 14 to 18 bases long (designated Cre1,4,5,6)were tested with a common upper primer at increasing annealing temperatures to determine if they would discriminate between the resistant and susceptible alleles (Table 6.2). Similarly, two susceptible-allele specific primers 16 and 18 bases long (designated Susc2,3) were tested (Table 6.2). The results of this experiment were not conclusive. Although there were temperatures at which the some of the primers were able to preferentially amplify their respective alleles, the results were not

Figure 6.9 AWP1 PCR products from AUS10894 and Spear digested with *Bfa*1. Templates for PCR were genomic AUS10894 and Spear, and cloned AWP1 products from AUS10894 and Spear (AWP1-AUS and AWP1-SPEAR). → 97 bp Spear allele band, → 55 bp AUS10894 allele band. The digested PCR products were fractionated on a 3 % agarose gel and stained with ethidium bromide.

bp
M
AUS10894
AWP1-AUS
AWP1-AUS
AWP1-SPEAR
AWP1-SPEAR
SPEAR
603 -
458 -
290 -

reliable when F2 progeny were tested, possibly because of variation in the quality and concentration of the template DNA.

Table 6.2 Single PCR allele-specific amplification at increasing annealing temperatures. The PCR reaction was carried out with the upper primer AWP2/3 (5' TAC CCC AAA GTG TGT TAT 3') and the lower primers described below which had 3' bases corresponding to either the resistant allele sequence (designated *Cre 1,4,5,6*) or the susceptible allele sequence (designated *Susc 2,3*). The templates for the reaction were AUS10894 (Resistant) and Prins (Susceptible). Shaded areas represent primer-temperature combinations that preferentially amplified the correct allele.

Allele-specific primers	Resistant template sequence *Primer Sequence* (5'-3') Susceptible template sequence	Template	Allele-specific PCR (↔ = correct product amplified) (— = no amplification) Annealing Temperature (°C)						
			44	46	47	48	49	50	51
	CAAATATATGTAATTCCCCTAG	Resistant		↔	↔	—		—	
Cre 6	*ATATGTAATTCCCC* ->								
	CAAATATATGTAATTCCCATAG	Susceptible		↔	↔	—		—	
	CAAATATATGTAATTCCCCTAG	Resistant	↔	↔			↔		
Cre 4	*TATATGTAATTCCCC* ->								
	CAAATATATGTAATTCCCATAG	Susceptible	↔	—			↔		
	CAAATATATGTAATTCCCCTAG	Resistant	↔	↔		—			—
Cre 5	*AATATATGTAATTCCCC* ->								
	CAAATATATGTAATTCCCATAG	Susceptible	↔	—		—			
	CAAATATATGTAATTCCCCTAG	Resistant		↔		—		—	
Cre 1	*AAATATATGTAATTCCCC* ->								
	CAAATATATGTAATTCCCATAG	Susceptible		—		—		—	
	CAAATATATGTAATTCCCCTAG	Resistant		—	—	—			
Susc 3	*ATATATGTAATTCCCA* ->								
	CAAATATATGTAATTCCCATAG	Susceptible		↔	↔	—			
	CAAATATATGTAATTCCCCTAG	Resistant		—		—		—	
Susc 2	*AAATATATGTAATTCCCA* ->								
	CAAATATATGTAATTCCCATAG	Susceptible		—		—		—	

In an attempt to increase the reliability of the reactions, a dual PCR system was designed. The rationale behind this system was to use the consistent amplification with the AWP1 primer set to generate a good template for the second reaction with the allele-specific primers. For these assays, plant mini-prep DNA was used as a template for PCR with the AWP1 primer set and 1µl of 1:100 diluted AWP1 products used as template for a second amplification with the allele-specific primers.

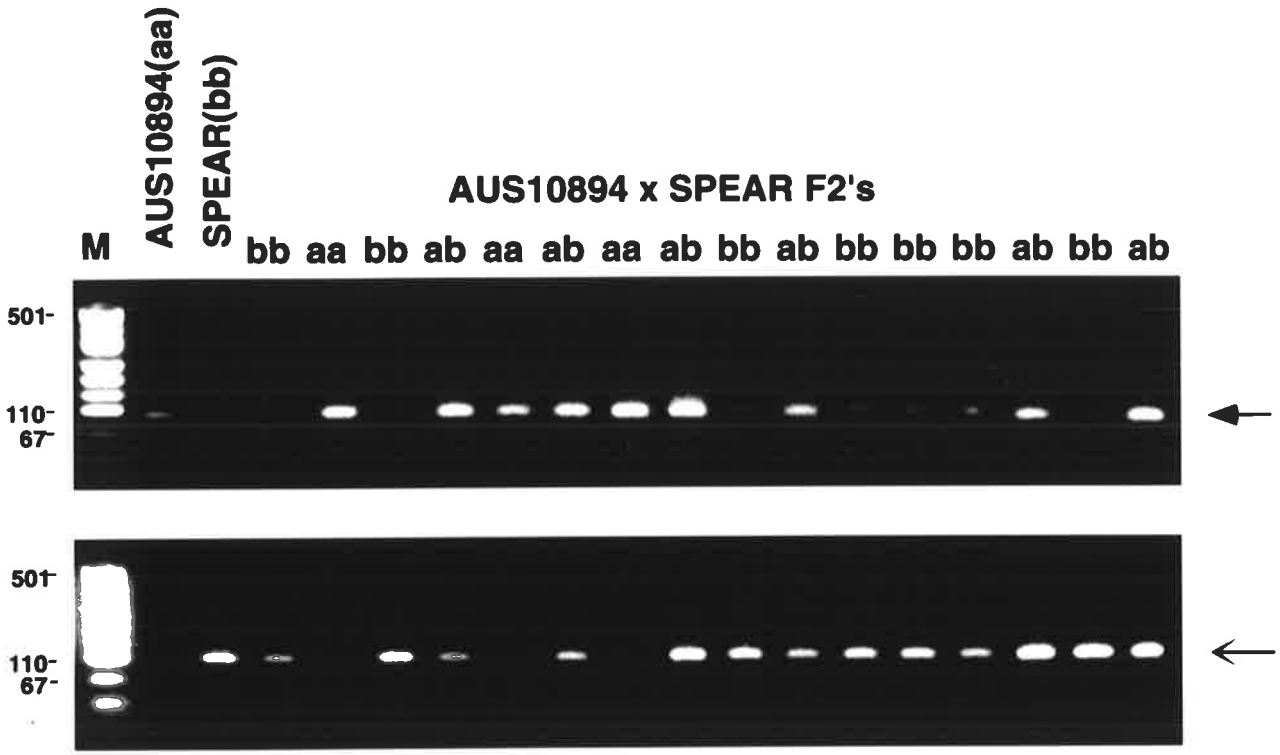
Using the dual-PCR system the 14mer resistance-allele specific lower primer Cre6 (re-designated AWP2-L) and the 16mer susceptible-allele specific lower primer Susc3 (re-designated AWP3-L) were able to differentially amplify DNA from resistant and susceptible genomic templates (Table 6.3).

Table 6.3 Dual PCR allele-specific amplification at increasing annealing temperatures. The first reaction used the AWP1 primer set and template DNA from AUS10894 (Resistant) and Prins (Susceptible) to produce AWP1 products. Diluted AWP1 products were used as the template for the second PCR reaction which was carried out with the upper primer AWP2/3 (5' TAC CCC AAA GTG TGT TAT 3') and the lower primers described below which had 3' bases corresponding to either the resistant allele sequence (*Cre 6*) or susceptible allele sequence (*Susc 3*). Shaded areas represent primer-temperature combinations that preferentially amplified the correct allele.

Allele-specific primers	Resistant template sequence *Primer Sequence* (5'-3') Susceptible template sequence	Template	Allele-specific PCR (↔ = correct product amplified) (— = no amplification) Annealing Temperature (°C)					
			45	47	50	51	52	53
	CAAATATATGTAATTCCCCTAG	Resistant	↔	↔	↔	↔	↔	—
Cre 6	*ATATGTAATTCCCC* ->							
	CAAATATATGTAATTCCCATAG	Susceptible	↔	↔	↔	↔	—	—
	CAAATATATGTAATTCCCCTAG	Resistant		↔	↔		—	—
Susc 3	*ATATATGTAATTCCCA* ->							
	CAAATATATGTAATTCCCATAG	Susceptible		↔	↔		↔	↔

The AWP2-L and AWP3-L primers were used in duplicate dual PCR reactions with the common upper primer 605R-U2 (re-designated AWP2/3-U) and genomic DNA templates

Figure 6.10 Allele-specific PCR assay. **(a)** AWP2 (*Cre*-linked) and **(b)** AWP3 (*cre*-linked) PCR products. DNA templates (as for Fig. 6.7) were amplified with AWP1. 1:100 diluted products from this reaction were amplified with allele-specific primers AWP2 or AWP3. The photograph shows alleles detected, matching those detected by RFLP analysis. Tag605 RFLP scoring of F2s is shown above lanes. → AUS10894 (*Aawp2-2Ba*) allele, → Spear (*Aawp3-2Bb*) allele. PCR products were fractionated on a 2.5 % agarose gel and stained with ethidium bromide.

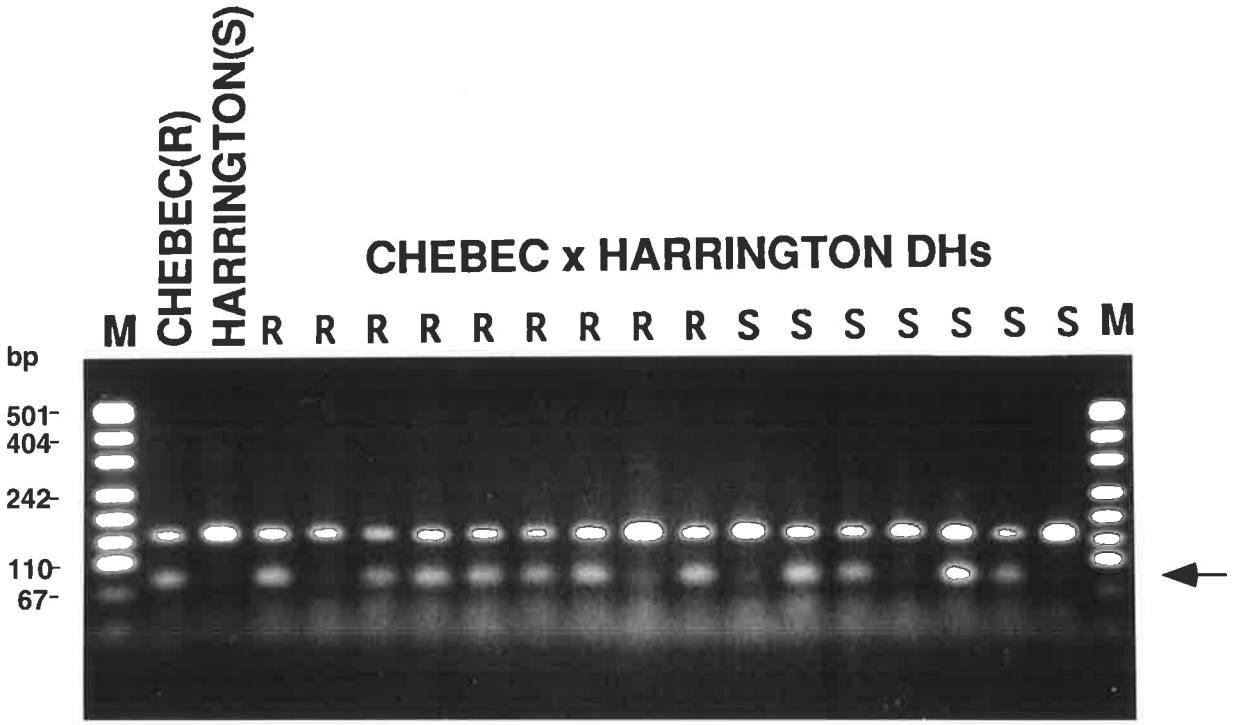

AUS10894(aa)
SPEAR(bb)
AUS10894 x SPEAR F2's
M
bb aa bb ab aa ab aa ab bb ab bb bb bb ab bb ab
501-
110-
67-
501-
110-
67-

producing a single 95 or 97 bp product (Fig 6.10). These primer sets were able to specifically amplify their respective alleles at an annealing temperature of 52°C (Fig 6.10, lanes 1,2). The accuracy of allele-specific amplification was verified using Tag605 RFLP-scored F2 individuals which were quickly scored for the presence or absence of each allele (Fig 6.10, lanes 3-16). PCR products from the reactions with each primer were run on separate gels which, in combination, can be scored for the presence or absence of each allele. The AWP2 primer set identifies the *Cre*-linked allele *Aawp2-2Ba* and the AWP3 primer set identifies the allele *Aawp3-2Bb*. The scores for these alleles matched the RFLP scores for each of the F2 individuals shown (Fig 6.10).

PCR marker for barley CCN resistance gene

The CCN resistance gene *Ha* is thought to be located on chromosome 2 of barley (Andersen and Andersen, 1973). The primer sets designed as part of the wheat PCR marker work were tested on barley lines resistant and susceptible to CCN to firstly determine if they would amplify barley DNA successfully, and secondly, to map any markers relative to the *Ha* gene. Only the AWP1 and 451R primer sets produced single bands of the expected size. These products were digested with panels of restriction enzymes in an attempt to reveal polymorphism. The 451R products were digested with *Alu*I, *Bfa*I, *Hae*III, *Hinf*1, *Mnl*I, *Sac*I, *Sau*3AI, *Sty*I. No polymorphism was detected. The AWP1 products were digested with *Alu*I, *Bfa*I, *Dde*I, *Hinf*1, *Mnl*I, *Sau*3AI, *Sty*I. The enzyme *Mnl*I detected a polymorphism between AWP1 products derived from the resistant barley cultivar Chebec and the susceptible cultivar Harrington (Fig 6.11, lanes 1,2). This marker, designated *Xawp1-Mnl1-2*, was mapped using double haploid F1 progeny of a cross between these two lines (Fig 6.11, lanes 3-18). Linkage analysis indicated that it mapped 20 cM from the Chebec CCN resistance gene *Ha* on chromosome 2.

Figure 6.11 AWP1 primer set used as a marker in barley. AWP1 PCR products digested with *Mnl*I. Templates were doubled haploid F1 progeny of the cross between the barley cultivars Chebec (CCN resistant) and Harrington (CCN susceptible). Arrow indicates the dominant marker band. DH - doubled haploid F1 individual, R - resistant, S - susceptible. The digested PCR products were fractionated on a 3 % agarose gel and stained with ethidium bromide.


CHEBEC x HARRINGTON DHs
M
CHEBEC(R)
HARRINGTON(S)
R R R R R R R R R S S S S S S S M
bp
501-
404-
242-
110-
67-

Allele-specific amplification using "PNA-clamp" method

An alternative method of achieving allele-specific amplification was investigated. It involved the use of a "Peptide Nucleic Acid" (PNA) to try to block the resistance-allele PCR primer binding site on the susceptible template. PNAs are oligonucleotide analogues with an achiral peptide backbone (Egholm *et al.*, 1992). Two of the properties of PNAs are their ability to form duplexes with DNA that are more stable and show greater base pair mismatch discrimination, than DNA-DNA duplexes (Egholm *et al.*, 1993). These features have been utilised in a PCR-clamp strategy to achieve discriminatory amplification of plasmids differing by only one base pair (Orum *et al.*, 1993). The strategy for using the PNA as a PCR-clamp involves designing a PNA homologous to the primer annealing site, which is added to the PCR reaction. A PNA binding step is added to the PCR cycle prior to the primer binding step. The PNA has a higher Tm than the primer, and should bind preferentially to the template, except where a mismatch exists. The PCR cycle and predicted annealing is expressed diagrammatically in Figure 6.12.

In the first experiment with the PNA, two types of PCR clamping were attempted. The resistance allele specific primer Cre1 (0.2 mM, Tm = 45°C) was used in reactions containing increasing concentrations of the PNA (Tm = 55°C) using genomic or AWP1 PCR product susceptible templates. No decrease in product amplification was observed with PNA concentrations up to 200 times greater than the primer concentration (Fig. 6.13, lanes 1-12). Another strategy was also attempted, where the PNA was annealed downstream of the AWP1-lower primer site to block *Taq* polymerase extension. Again, no effect on product formation was seen at any PNA concentration (Fig 6.13a, lanes 13-18).

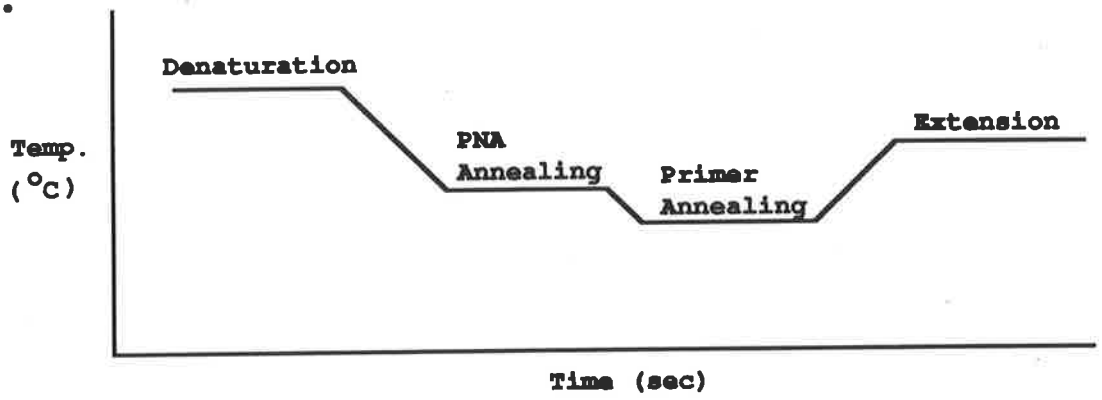
The experiment was repeated with shorter primers as they have lower Tm's giving the PNA a greater potential advantage at a given annealing temperature. Three primers were tested at two temperature regimes using AWP1 PCR products as templates. No differences in product amplification were observed (Fig 6.13b,c).

Figure 6.12

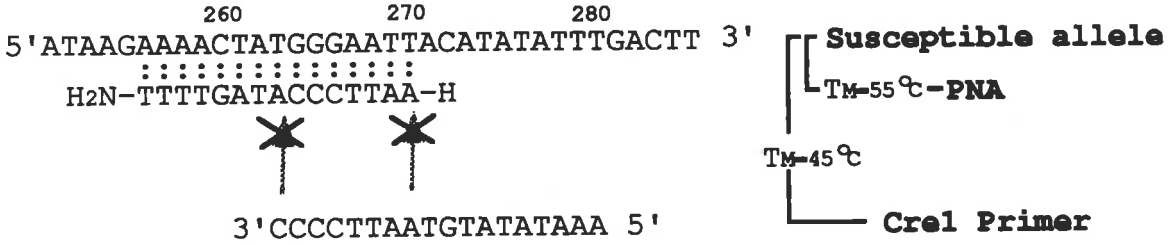
(**a.**) Diagrammatic representation of PCR cycle incorporating PNA annealing temperature prior to primer annealing (from Orum *et al.*, 1993).

(**b.**) Theoretical annealing at susceptible and resistant alleles. At the susceptible allele, the PNA should anneal preferentially to the template, preventing primer binding and product formation. At the resistant allele, the PNA's mismatch with the template (in shaded box) reduces it's Tm to below that of the primer, allowing the primer to bind preferentially, allowing amplification to proceed.

a.


Denaturation
PNA
Annealing
Primer
Annealing
Extension
Temp.
(°C)
Time (sec)

b.


260
270
280
5'ATAAGAAAACTATGGGAATTACATATATTTGACTT 3'
H2N-TTTTGATACCCTTAA-H
3'CCCCTTAATGTATATAAA 5'
Susceptible allele
TM-55°C-PNA
TM-45°C
Cre1 Primer

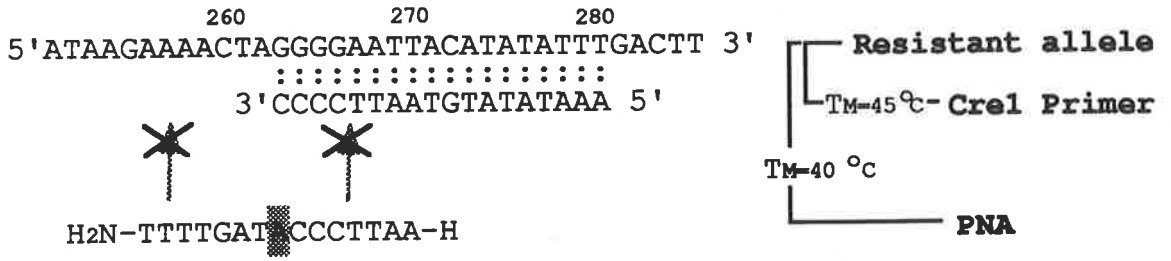

260
270
280
5'ATAAGAAAACTAGGGGAATTACATATATTTGACTT 3'
3'CCCCTTAATGTATATAAA 5'
H2N-TTTTGAT CCCTTAA-H
Resistant allele
TM-45°C- Cre1 Primer
TM-40 °C
PNA

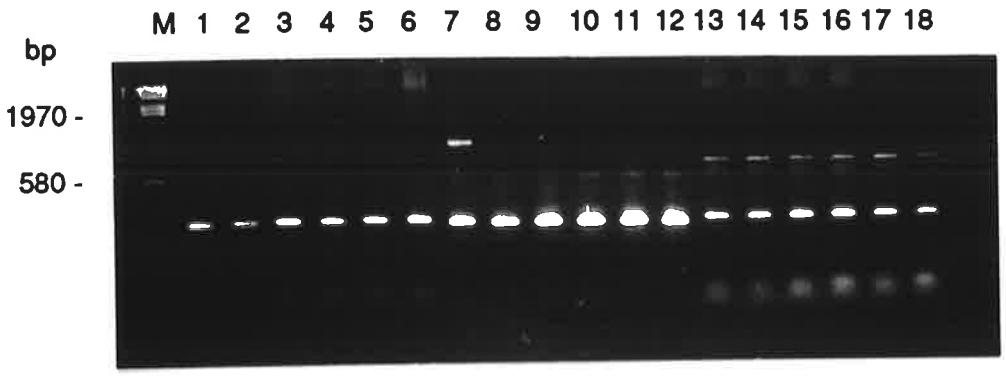
Figure 6.13

(a.) PCR using peptide nucleic acid "PCR-clamping". Lanes 1-6: Cre1 and 605R-U primers (0.2 μM) were used to amplify AUS10894 (lane 1) and Prins genomic templates. Lanes 7-12: Cre1 and 605R-U primers (0.2 μM) were used to amplify AWP1-AUS1O894 (lane 7) and AWP1-Prins PCR product DNA templates. Lanes 13-18: 605R U&L primers (0.2 μM) were used to amplify AUS10894 (lane 13) and Prins genomic templates. The PCR cycle for lanes 1-6, 13-18 was 30 sec at 94 °C; 120 sec at 50 °C; 30 sec at 45 °C; 60 sec at 72 °C. The cycle for lanes 7-12 was 30 sec at 94 °C; 120 sec at 53 °C; 30 sec at 48 °C; 60 sec at 72 °C. PNA was added to the reactions at the following rates: 0 μM (lanes 1,2,3,7,8,9,13,14,15); 8 μM (lanes 4, 10,16); 16 μM (lanes 5,11,17) and 24 μM (lanes 6,12,18).

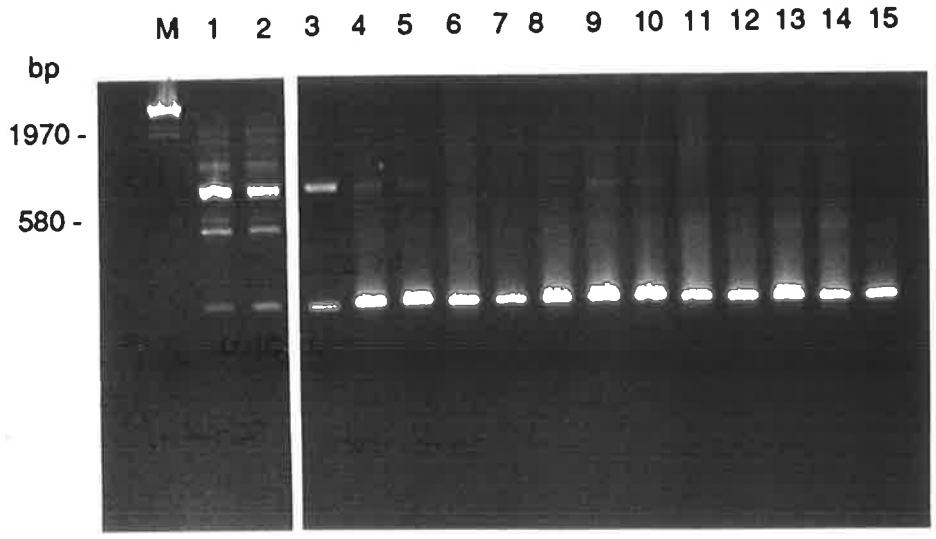
(b.) PCR using peptide nucleic acid "PCR-clamping". 605R-U&Cre3 primers (lanes 1-3), 605R-U&Cre4 primers (lanes 4-8) 605R-U&Susc2 primers (lanes 14,15) and 605R-U&Cre5 primers (lanes 9-13) were used in PCR reactions. Templates were AWP1-Prins PCR product (1,2,4,5,9,10,14); cloned AWP1-Prins PCR product (lanes 6,7,11,12); AWP1-AUS10894 PCR product (3,8,13,15). Reactions in lanes 2,5,7,10,12 contained 8 μM PNA. The PCR cycle was 30 sec at 94 °C; 120 sec at 50 °C; 30 sec at 45 °C; 60 sec at 72 °C.

(c.) PCR using peptide nucleic acid "PCR-clamping". Lanes as for (b.) (lanes 1-13). The PCR cycle was 30 sec at 94 °C; 120 sec at 48 °C; 30 sec at 43 °C; 60 sec at 72 °C.

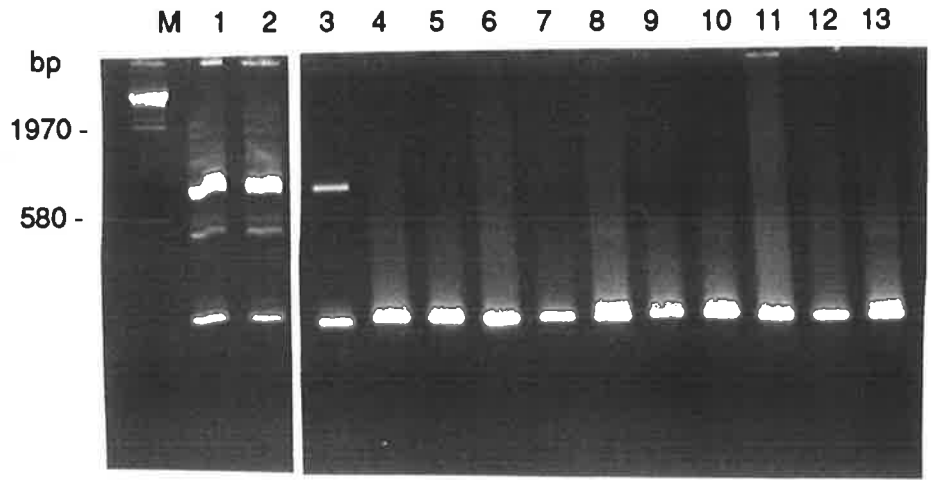
a.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
bp
1970 -
580 -

b.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
bp
1970 -
580 -

c.


M 1 2 3 4 5 6 7 8 9 10 11 12 13
bp
1970 -
580 -

Discussion

Several groups have identified RFLPs linked to agronomically important genes, including those for pathogen resistance (Kilian *et al.*, 1994; Hartl *et al.*, 1993). These RFLP markers could be used in marker assisted selection by breeders developing new lines. However, breeders screening large populations need fast and inexpensive assays. A study by Ragot and Hoisington (1993) showed that PCR markers are quicker, safer and cheaper to use than RFLP markers. Converting RFLPs into PCR-based markers uses the existing RFLP mapping information to its maximum potential and may produce the fastest outcome in terms of genetic improvement of cereals.

Three of the RFLP clones mapped in this study were chosen as candidates for development into PCR-based markers. The clones were sequenced and primers designed. Only primer sets from ABC451 and Tag605 amplified products of the expected size. It is difficult to gauge from the literature the success rate of this strategy in other laboratories. The only published evaluation of STS markers in wheat found that 23 out of 37 primer sets designed from RFLP clones amplified a product that mapped to the expected chromosomal location (Talbert *et al.*, 1994).

The identity of the ABC451 product was verified using Southern analysis. The product was not polymorphic between resistant and susceptible lines, even after restriction analysis. As ABC451 is a cDNA clone, it is not unexpected that the corresponding genes are highly conserved between varieties. Southern analysis and PCR also indicated extensive conservation of the ABC451 sequence between wheat genomes B and D. This may mean that any varietal differences in one genome may be masked by sequence conservation in the other, reducing the level of polymorphism revealed by PCR. Given this sequence conservation, cDNA clones may be unsuitable candidates for the development of PCR markers.

The AWP1 primer set designed from the wheat genomic clone Tag605 reliably amplified the expected product but there was no size polymorphism between products from susceptible and resistant varieties. The identity of the product was verified using nulli-tetrasomic and ditelosomic lines. Using the sequence data from the RFLP clone, it was possible to test for polymorphism with many restriction enzymes. A *Bfa*1 digest differentiated CCN resistant and susceptible varieties. This approach produces a dominant PCR-based marker.

It is common for STS markers developed from either RAPDs or RFLPs to involve a restriction stage to reveal polymorphism (Williamson *et al.*, 1994; Eastwood *et al.*, 1994). In their study with wheat STS markers, Talbert *et al.* (1994) found that 9 out of 16 primer sets revealed a polymorphism between 20 hexaploid wheat genotypes following restriction enzyme digestion.

The assay produced from *Bfa*1 digestion of the AWP1 product suffered from two major practical deficiencies: the polymorphism was only detectable using polyacrylamide gel electrophoresis and it was a dominant marker for the Spear/Schomburgk allele rather than AUS10894 allele. To develop a more informative assay, PCR products were cloned with a view to designing primers specific for the AUS10894 allele.

Double-stranded sequencing of AWP1 products from resistant and susceptible lines revealed only one sequence polymorphism, as expected in one of the *Bfa*1 sites. Primers were designed with 3' terminal bases matching the different alleles to determine whether the single mismatch with the different templates would be enough to prevent amplification. A potential problem with the strategy of allele-specific amplification was that a C:T mismatch has been found to be the mismatch which is the least likely to prevent extension by *Taq* polymerase (Huang *et al.*, 1992). This is the mismatch between the 3' end of the AUS10894 allele specific primer AWP2 and the template from other varieties.

The first attempts at PCR with allele-specific primers failed to achieve discriminatory amplification of the two alleles, so fourteen to eighteen base primers were tested with increasing annealing temperatures to determine if they could discriminate between the alleles present in resistant and susceptible lines. For most primers a discriminating annealing temperature was found. However, the allele-specificity was not reproducible when the analysis was extended to F2 individuals. This may have been due to variations in the concentration and quality of the F2 DNA. This could have been standardised but the extra manipulations required reduce the efficiency of PCR screening.

Peptide nucleic acids were investigated for their ability to facilitate allele-specific amplification by acting as "PCR clamps". This strategy was proposed by the designers of PNAs, who were successful in amplifying specific alleles with a model system using plasmid DNA templates (Orum *et al.*, 1993). In this study, a 15mer PNA homologous to the Spear allele failed to inhibit product extension with any annealing temperature regime or primer. To date, there have been no reports of the successful use of this strategy with eukaryotic DNA. This potentially useful technique may need to be developed further for use in practical applications.

Allele specific amplification with low quality DNA was finally achieved by using a dual PCR approach. In this system mini-prep DNA was amplified using the AWP1 primer set under stringent temperature conditions in the first reaction. Diluted products from this reaction were then used as a template for further amplification with the allele-specific primers. This approach appeared to overcome any problems associated with template concentration and quality. The use of allele-specific primers produces a marker which is as informative as the RFLP marker from which it was developed. Such co-dominant markers are usually only produced in PCR assays involving enzyme digestion to produce restriction fragments associated with each allele (Kilian *et al.*, 1994).

The limitations of this system lie with the single base difference upon which allele-specific amplification was designed. A very specific annealing temperature was required for allele-specific amplification. One degree below the optimum produced non-specific

amplification, while one degree above gave no amplification of either allele. The efficiency of the PCR makes suppressing amplification by the introduction of a single base mismatch very dependent on the control of temperature and the standardisation of all other reaction conditions. Practically, a different mutation or a larger sequence difference may need to be found to design a more robust assay.

The AWP1 primer set was tested for its ability to produce a molecular marker for the barley CCN resistance gene *Ha*. Digestion of the AWP1 product with *Mnl*1 produced a polymorphism between resistant and susceptible cultivars. The ability of primers designed from a wheat sequence to amplify barley DNA indicates considerable homology between the species at this locus. Recent investigations have shown that RFLP clones from one grass species can be used to map in others (Ahn *et al.*, 1993; Devos *et al.*, 1993), and this study shows that the potential exists to use primer sets for comparative mapping in cereals.

The *Xawp1-Mnl1-2* marker mapped 20 cM from the barley CCN resistance gene *Ha* , while CDO588 and WG996 were located 26 cM and 33 cM from the gene. This puts *Cre* and *Ha* in similar locations. The recent research on genomic synteny in the cereals (Ahn *et al.*, 1993, Devos *et al.*, 1993) has showed considerable conservation of gene order. It has been proposed by Bennetzen and Freeling (1993) that when traits are mapped to similar positions in different grass species they may be encoded by alleles of a single gene. *Cre* and *Ha* may be homologous CCN resistance genes in wheat and barley. If this is the case, it has serious implications for breeders working with these genes.

Chapter 7 General Discussion and Future Prospects

This study has increased our understanding of wheat resistance to the root pathogen *H. avenae*. As *Cre* is the only wheat CCN resistance gene found to date, our reliance on a single gene should be supported by a sound fundamental knowledge of the biology of compatible and incompatible interactions. This information can be integrated with studies on the genetics of resistance and used as a foundation for developing strategies to manage this pest.

The results of the morphological study of nematode-induced syncytia in roots of resistant and susceptible near-isogenic lines indicate that resistance to CCN conferred by *Cre* is expressed in cellular changes at about 15 days post-inoculation. This finding is similar to the reactions of other plant species resistant to cyst nematode genera.

The importance of determining the phenotypic expression of resistance genes lies in the application and use of the genes. Traditional plant breeding may require many years to produce nematode-resistant cultivars. If the goal of breeding programs is to pyramid resistance genes, they must have different modes of action. For example, the CCN resistance gene from *Ae. ventricosa* which has recently been transferred into wheat appears to give a similar morphological reaction to *Cre* (Delibes *et al.*, 1993). In contrast, the CCN resistance gene in the oat variety NZ Cape appears to have a very different action (Georgaras, 1990).

The rationale for investigating protein differences between resistant and susceptible lines changed during the course of this project. Initially, it was proposed to follow the strategy of Manners *et al.* (1985) who used 2-dimensional PAGE to identify *Mla*-linked proteins. However, when this approach failed to find a protein linked to the barley *Ha*-gene (J. Kretschmer, pers. comm.), it was decided to conduct a more fundamental study of the proteins in cells of syncytia from the near-isogenic lines. This type of investigation had

not been conducted for any nematode-host interaction. It was hoped to visualise some gene product differences which have been predicted by recent molecular and ultrastructural studies

It was found that some proteins did accumulate in greater or lesser quantities compared with sections from uninfected roots. This correlates with the apparent high metabolic activity of syncytia observed under the microscope and with the hypothesis that the nematode re-directs gene transcription of syncytial cells to its own benefit.

The only clear protein difference between syncytium-enriched sections from the NILs was the appearance of a protein of about 14 kD at 27-35 days in the susceptible line. This could be associated with a specific stage in the host-nematode interaction. If the experiment was scaled up significantly, it may be possible to purify enough of this putative syncytium-related protein to either raise antibodies or to obtain sequence information and use degenerate PCR primers to isolate the corresponding coding sequence.

Obtaining coding sequences from genes specifically expressed in syncytia could lead to the isolation of promoter elements conferring this specificity. An example of this strategy has recently been demonstrated by Opperman *et al.* (1994), who isolated syncytium-specific cis-acting sequences that mediate induction by *Meloidogyne* species in tobacco and have used them to construct transgenes that are expressed only in the syncytium. The potential exists to develop transgenes that are directly lethal to the nematode or which destroy its feeding site.

While these simple protein studies only give a "snapshot" of gene expression in syncytia, the results do allow some correlation of gene-product accumulation with the elegant molecular research being conducted in this area.

The major applied component of this project was the identification and development of *Cre*-linked molecular markers. RFLP markers were being used at the beginning of this study to construct the first comprehensive genetic maps of cereal species. The availability

of near-isogenic lines and the inferred chromosome 2B location of *Cre* meant that a directed search could be made for *Cre*-linked markers.

The proximal region in which *Cre* was mapped is a highly variable area of the wheat genome. Suppressed recombination and/or extremely localised crossing-over may affect genetic mapping in this region. With the resources available, the maximum numbers of resistance-tested F2s from two populations were used for linkage analysis. *Cre*-linked RFLP markers were, therefore, identified using NIL screening, F2 linkage mapping, and testing of breeders lines containing the *Cre* gene.

The necessity for replacing the existing bioassay was demonstrated many times during this study. Even on a small scale the assay was affected by equipment malfunction, nematode availability and other unknown influences. It often took over six months to determine the resistance status of the plants.

It was decided to convert the RFLP markers into PCR-based markers. The PCR has been used to produce RAPD and STS markers. In this study STS markers were developed for a *Cre*-linked RFLP marker. A PCR-enzyme digest marker was produced, which was refined into a co-dominant 2-step PCR marker.

The future prospects for improving crop resistance to *H.avenae* lie with the construction of synthetic resistance genes or the cloning of plant nematode resistance genes. A number of patents have been issued for gene constructs that have the potential to kill nematodes within the root (Sijmons *et al.*, 1993). It is likely that crops amenable to transformation will soon have engineered resistance to the major cyst nematode pathogens.

At the start of this project, resistance genes were the "Holy Grail" of plant pathology. Three years later the first one was cloned (Martin *et al.*, 1993) and others are near. While cloning the *Cre* gene of wheat remains difficult due to the nature of the wheat genome, several lateral strategies are available. It is possible, for example, that a homologue for *Cre* exists in rice, a species which offers much potential for map-based cloning. It is not known whether any other sources of resistance to CCN are available in the rice gene

pool. Another approach may be to use rice probes to establish much closer linkage to *Cre*, facilitating cloning in wheat.

In addition to the above applied goals, the molecular study of nematode-induced syncytia will yield much fundamental knowledge about a most complex interaction between a pathogen and its host.

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F2	No. of CCN	CCN	Status after		F3 families - No. of CCN on roots						
Plant	on roots	Status	F3 testing								
1	2	R	R	8	13	5	3	10	3	6	6
2	1	R	R	3	2	24	4	3	5	11	22
3	1	R	R	2	8	5	19	10	7	4	3
4	0	R*	S	15	9	32	18	13	23	12	12
5	1	R*	S	13	5	15	19	10	14	30	12
6	1	R	R	1	3	2	4	4	8	2	1
7	7	R	R	9	7	27	3	20	7	3	14
8	0	R	R	7	3	1	1	3	1	5	4
9	0	R*	S	7	10	15	8	31	47	11	26
10	2	R	R	10	6	6	15				
11	0	R	R	1	3	7	9	2	5	10	8
12	1	R	R	3	6	12	3	5	4	2	3
13	0	R	R	2	2	1	12	10	8	4	1
14	0	R	R	4	3	8	18	1	12	28	3
15	2	R	R	5	1	1	1	2	3		
16	0	R*	S	40	12	28					
17	5	R	R	5	8	10	5	12	2		
18	2	R	R	1	1	1	4	0	2		
19	1	R	R	6	2	14	4	2	6		
20	4	R	R	0	11	3	15	7	0		
21	0	R	R	8	39	23	4	4	6		
22	0	R	R	2	1	5	3	2			
23	1	R	R	4	4	8	2	12	6		
24	1	R	R	1	12	3	2	13	3		
25	1	R*	S	14	6	15	16	32	25		
26	2	R	R	0	0	5	0	0			
27	1	R	R	7	11	3	1	10	4		
28	9	S	S	31	8	25	5	23	49		
29			R	10	3	3	2	12	13		
30	1	R	R	7	1	3	5	4	1		
31	8	S	S	13	5	15	19	10	14		
32			S	23	20	37	39	37	18		
33			R	9	6	7	2	2	7		
34	6	R	R	3	9	32	2	10			
35	7	R	R	7	1	5	7				
36	8	S*	R	0	1	0	3	0			
37	2	R	R	1	1	0	6	1	6		
38	5	R*	S	25	36	18	33	29	73		
39	3	R	R	3	14	6	19	5			
40	4	R*	S	26	11	25	13	21	11		
41	7	R	R	3	3	3	26	10	12		
42	0	R	R	14	3	0	0	1	4		
43			R	3	2	12	5	5	7		
44	0	R	R	5	7	2	0	6	2		
45	1	R	R	0	7	4	0				
46	7	R	R	6	8	0	11	3	13		
47	13	S*	R	6	7	11	5				
48	10	S	S	73	67	45	3	18	23		
49	5	R	R	4	4	7	5	10	7		
50	1	R*	S	19	27	16					
51	7	R	R	17	10	4	25	2	14		
52	6	R	R	13	7	14	8	3	0		
53	12	S	S	17	30	25	24	39	40		
54	2	R	R	5	0	20	6	1	17		
55	2	R*	S	13	27	18	31				

No. of CCN on roots of AUS10894: number tested= 19, mean= 2.9, SD= 2.1, range= 0-8

No. of CCN on roots of Spear: number tested= 20, mean= 18.7, SD= 9.6, range= 4-42

Clones screened against NILs

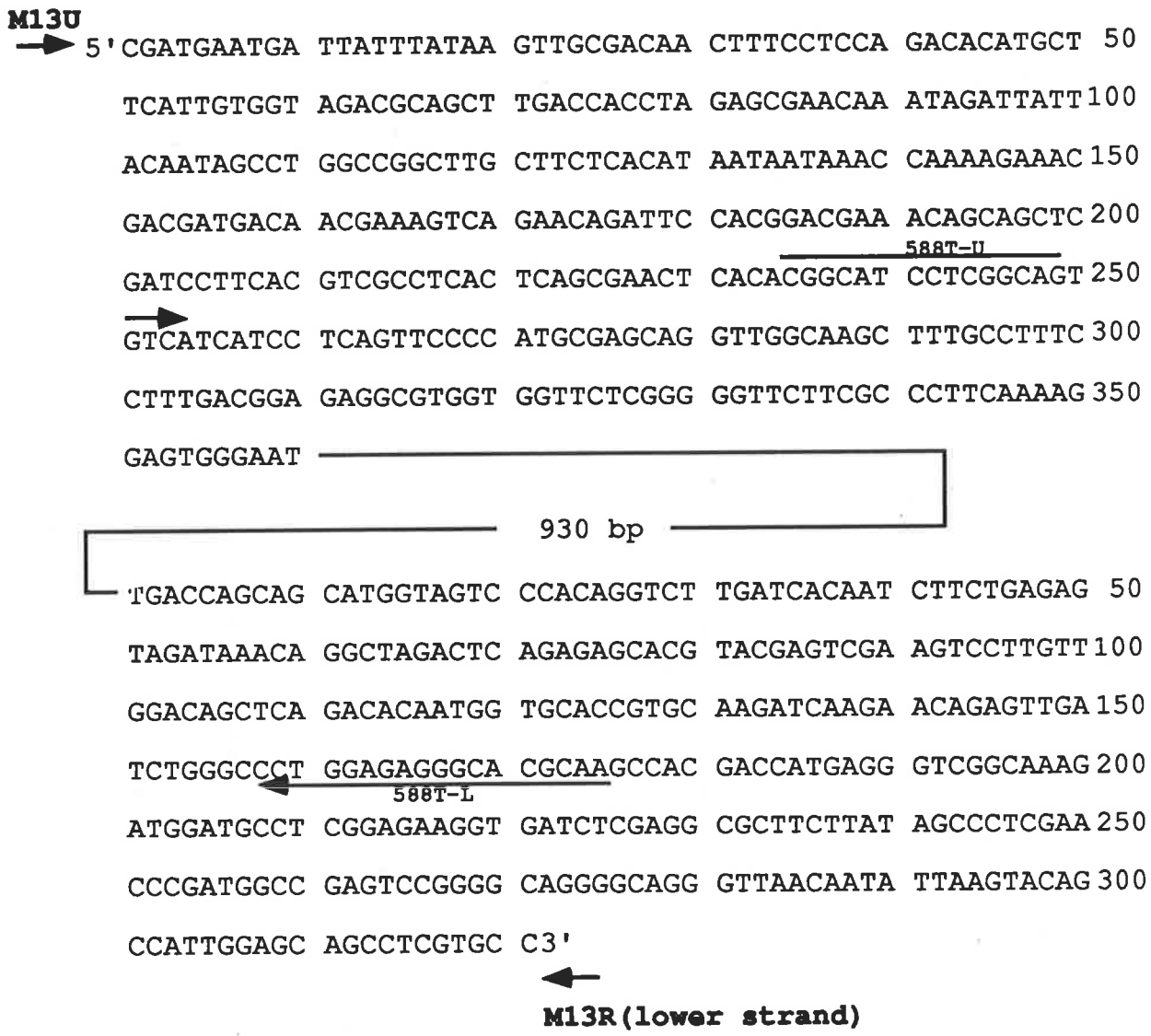
ABC 152	CDO 64	Tag 293*
ABC 153*	CDO366	Tag 302*
ABC 157	CDO370	Tag 331*
ABC 162	CDO373	Tag 370
ABC 165	CDO474	Tag 398
ABC 451	CDO 537	Tag 471*
ABC 468*	CDO 588*	Tag 529
ABG 356	CDO 668	Tag 539
BCD 111	CDO 678	Tag 592
BCD 120	CDO 680	Tag 594
BCD 135	CDO 770*	Tag 600
BCD 175	IPSR 135	Tag 605*
BCD 240	KSU D018	Tag 609
BCD 266	KSU D022	Tag 610*
BCD 334	KSU F015	Tag 618
BCD 410	KSU F041	Tag 632*
BCD 433	MWG 184	Tag 653
BCD 438	MWG 645	Tag 699
BG 123	MWG 996*	Tag 740

* CCN linkage tested on F2s

Clone reference:

ABC	Kleinhofs et al., 1993
ABG	Kleinhofs et al., 1993
BCD	Huen et al., 1991
BG	Kleinhofs et al., 1993
CDO	Huen et al., 1991
IPSR	Chao et al., 1988
KSU	Gill et al., 1991
Tag	Liu and Tsunewaki, 1991
MWG	Huen et al., 1991

Partial sequence of CDO588



Partial sequence of Tag699

M13U

5'CTGCAGTCGG ATAAGCGTTG GAACCTCAGT GTTGACTTTC CCTGAAACAA 50

GAGAAGACCA TGCCAAATCC TCCCCTGGTT TTCAGAGGGA GCCCATTACC100

CGGCATCGGT GGTTATCAGC CTTACCTGTG CACTCCCGGT TCACATCCTA150

CTCCCTCAAA GCTGCCTGCC TTTCCATCAT GACAAGATCT CTGTAGTAAG200

GGCCCGGACA CAGATTTCCA TTTCATGATG ACAAGGGCGC CGTAGTAAGG250

TGCATCATGG ACGCTGGGAT TAGTTTAGGG CTAGGGAGGG CCAGGCTAGA300

GAGATGCCTA CCCAAAAAAA ACTATGGGTT AACTTGGCCT TAAGCTTTTG350

AAAATTCCAA ATTTGAATAT TTGGAACAGT AGATAAATCC TAGGGAACAT400

GTTTTGGTTG AACTTAAACT TTTCCTGTAA AATATTTGAT GGCTTGGACT450

ACCTATAACC CGTGTTCCAT ACATGGAAAA ATGTGATATT CACTGCACTG500

GCTCATTCCA

2070 bp

699R-U

TTTGGGAATA AACAGGGCCT ATTCCCGCAA AAAAACAAGG AGATAGCAGG 50

TGGCCTACCC AAAAAAAACT ATGGGTTAAC TTGGCCTTAA GCTTTTGAAA100

ATTCCAAATT TGAATATTTG GAACAGTAGA TAAATCCTAG GGAACATGTT150

TTGGTTGAAC TTAAACTTTT CCTGTAAAAT ATTTGATGGC TTGGACTACC200

TATAACCCGT GTTCCATACA TGGAAAAATG TGATATTCAC TGCACTGGCT250

CATTCCAATT GATTAGGTGG ATTTGGCAAT TACTTGTTTG CCATGAGTGG300

AACCATCGCC AGCCAAGCAA CAGAGGCTAA CGATGCTCAG AATTTCAAGG350

ACCCCCACAT CGGGTGGATG ATAGGGTTCC TCTTCCTCGT TCAGTTTTCG400

TTGGAGCTCA TTTCGCTTCT TCGTGTCCCC TGCAGAAAGG TCAAATCTGT450

699R-L

CAGGC 3'

M13R(lower strand)

Partial sequence of WG996

M13U →

5'CATGTAGACC ACGACTTGCA CTCGGTACAA GGGGATCCTC TAGAGTCGAC 50

996F-U →

CTGCAGTGGC GTTTGAGTAG TGGGCAAGCA GGAGATGGGA GATAGGCCAG100

GATCCGCTAC AAAGCTTCAC TGACCCCTAA ATATAATAAT AATATATTAG150

CCCGACTTGG GCTTTCGGCA GTGTTCTACC ATTACAGCTA TCGATTCAGT200

CATACTCTTC GCCAGTGAAA GCCTCTGTGG ATTAGTACGC GGATTTGCCT250

TTTGTGCTTG TAACCATCAG ATTTGGGCAG TAAACCAAGG GCTAATCGTC300

ATCCTATCGG ATCGACCATG GAGCACTCGT GGANTCNTCG TCTTTCATAA350

GCAGCTCCTT TCCCGCGCAA AGACAAACAC TCGCTAATCC TTGA 3'

← 996F-L

Primer name	***Primer sequence (5' - 3')***
451-L	TCT TTG CTG ACC CTC CTG
451-U	CAA ACG GAA GTC AGA ACG
451R-L	CTT CAA CGA GGC ATC AAT CTT
451R-U	AAG ATG AAC ACC CAC GGA GTA
588-L	CTT GCG TGC CCT CTC CAG
588-U	GGC ATC CTC GGC AGT GTC
605F-L	TAG AGT GCC CTG CTG GAG
605F-U	AAA CCC CGA AAA AGC AAC
605R-L (AWP1-L)	GCT TCC AGT CAC CTT TTA
605R-U (AWP1-U)	CCA CCA CTG TCA ACT GTA
605R-U2 (AWP2/3-U)	TAC CCC AAA GTG TGT TAT
605T-L	TGA CAG TGG TGG GTT TAC
605T-U	AGG AGA GTT AGC ACA AGG
699R-L	TGA CAG ATT TGA CCT TTC
699R-U	AAA AAC AAG GAG ATA GCA
996F-L	CAA GGA TTA GCG AGT GTT
996F-U	AAG CAG GAG ATG GGA GAT
CRE-1	AAA TAT ATG TAA TTC CCC
CRE-2	AAA TCT ATG TCA TTC CCC
CRE-3	AAT TCC CCT AGT TTT
CRE-4	ATA TAT GTA ATT CCC
CRE-5	AAT ATA TGT AAT TCC CC
CRE-6 (AWP2-L)	ATA TGT AAT TCC CC
SUSC-1	AAA TCT ATG TAC TTC CCA
SUSC-2	AAA TAT ATG TAA TTC CCA
SUSC-3 (AWP3-L)	ATA TAT GTA ATT CCC A

Sequence homology between 3' ends of ABC451 and CDO588

```
ABC451 166 TGAGCAGAAG CATGGTAGTC CCACAGGTCT TGATCACAAT CTTCTGAGAG 216
       5'  :::*:::*:: :::::::::: :::::::::: :::::::::: ::::::::::
CDO588   1 TGACCAGCAG CATGGTAGTC CCACAGGTCT TGATCACAAT CTTCTGAGAG  50

           TAGATAAACA GGCTCGACTC AGATAGGACA TACGAGTCAA AGTCCTTGTT 266
           :::::::::: ::::*::::: :::*::*::* ::::::::*: :::::::::: 
           TAGATAAACA GGCTAGACTC AGAGAGCACG TACGAGTCGA AGTCCTTGTT 100

           GGAGAGCGTC GGACACAATG GTGCACCGTG CAAGATCAAG AACAGATGTC 316
           :::*:::*:: *:::::::::: :::::::::: :::::::::: ::::::*::* 
           GGACAGC-TC AGACACAATG GTGCACCGTG CAAGATCAAG AACAGA-GTT 148

           AATCTGGGCC CTACAGAGGG CGGTCAGGCC ACGACCAGGA GGGTCAGCAA 366
           *:::::::::: ::**:::::: :***::*::: ::::::::*:: :::::*:::: 
           GATCTGGGCC CTGGAGAGGG CACGCAAGCC ACGACCATGA GGGTCGGCAA 198

           AGATTGATGC CTCGTTGAAG GTGATCTCGA GGCGCTTGCT CGTAGCCCTC 416
           ::::*::::: ::::**:::: :::::::::: :::::::*:: **:::::::: 
           AGATGGATGC CTCGGAGAAG GTGATCTCGA GGCGCTT-CT TATAGCCCTC 247

           GAACCCGATG CGCAGAGGTC CGGGG--GGA 433 ABC451
           :::::::::: *::*:::*:: :::::**::* 3'
           GAACCCGATG -GCCGAG-TC CGGGGCAGGG 275 CDO588
```

: - conserved base
* - altered base

Williams, K. J. & Fisher, J. M. (1993). Development of Heterodera avenae Woll. and host cellular responses in susceptible and resistant wheat. *Fundamental & Applied Nematology, 16*(5), 417-423.