Molecular genetics of Fusarium wilt resistance in flax

Wolfgang Spielmeyer

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry and Molecular Biology
Australian National University
Canberra, A.C.T.
Australia

November 1996

Acknowledgments

I would like to thank Dr. Neville Mendham, my co-supervisor at the University of Tasmania, for his support while I carried out studies in Tasmania.

I express my gratitude towards Dr. Allan Green who played a crucial role in formulating this project proposal. Dr. Green took me on as his student while I was still based in Tasmania and supported me throughout the course of the study. He made it possible for me to transfer my candidature from the University of Tasmania to the Australian National University half way through the project to continue research at CSIRO Plant Industry.

I am also grateful to Dr. Evans Lagudah for supervising the molecular aspects of this project and providing space in his laboratory during the last two years. Dr. Dennis Bittisnich kindly agreed to be my supervisor at the ANU providing support and advice on University matters.

The candidate was supported by a Junior Research Fellowship from the Grains Research and Development Corporation (GRDC) and a Travelling Scholarship from the Farrer Memorial Trust.

Certificate of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma at any tertiary institution and to the best of my knowledge, is original and contains no material previously published or written by another person, except where due reference is made.

Wolfgang Spielmeyer

Table of Contents

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarly-Primed Polymerase Chain Reaction
BSA	Bulked-segregant analysis
cDNA	complementary DNA
cM	centiMorgan, measure of genetic distance
CPI	Commonwealth Plant Introduction
CTAB	Hexadecyltrimethyl Ammonia Bromide
DNA	Deoxyribonucleic Acid
GC	Gas Chromatography
LOD	Log_{10} of the Odds Ratio
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate buffer
TAE	Tris-Acetate buffer
TBE	Tris-Borate buffer
UBC	University of British Colombia

Summary

Fusarium wilt is one of the major diseases threatening flax production worldwide. Because of the ubiquitous and highly persistent nature of the soil fungal pathogen (*Fusarium oxysporum* f.sp. *lini*) the employment of resistant cultivars is recommended in most flax growing areas. Selection based on phenotype for resistance to Fusarium wilt is labour intensive and often unreliable due to fluctuations in levels of fungal inoculum and temperature effects. Molecular marker-assisted selection is expected to support existing phenotypic selection schemes by providing rapid and reliable information regarding the resistance status of breeding lines.

As a first step towards developing a genotype-based selection method the mode of inheritance of Fusarium wilt resistance was elucidated using a recombinant doubled haploid population derived by polyembryony. It was found that most of the phenotypic variation was associated with the segregation of two major loci controlling resistance to flax wilt with minor loci likely to have also contributed to the resistance response. Apart from investigating resistance to flax wilt, the doubled haploid lines were used to study segregation of low linolenic acid determinants as well as seed colour and flower colour genes.

A two tier approach was adopted to target major genes conferring resistance to flax wilt. Firstly DNA clones from previously characterised plant disease resistance genes were used in DNA hybridisation experiments to target individual resistance loci directly. Secondly a random PCR-based technique was employed to assay the genome for markers linked to the trait. Neither approach identified markers tightly linked to major wilt resistance loci.

Quantitative trait analysis was used to identify chromosomal regions containing putative resistance loci. This approach required a genetic linkage map consisting of molecular markers spread over the entire genome. A

comprehensive linkage map was generated using the AFLP marker technique, a method well suited for generating a large number of reproducible marker loci. Interval mapping using this linkage map identified two chromosomal regions on linkage group 6 (LOD 7.3) and linkage group 10 (LOD 3.4) having a significant effect on the resistance to Fusarium wilt. Hence, results derived from molecular genetics using interval mapping corroborate the findings from classical genetics using segregation analysis in establishing that two loci account for most of the phenotypic variation observed for this trait. Studies such as these identify chromosomal regions associated with resistance to Fusarium wilt for the first time with sufficient accuracy to be of future use in targeting these regions.

Chapter One

Introduction

1.1 Linum usitatissimum

Background

Linum usitatissimum is an annual, self-fertilising crop plant with two distinct groups of cultivars. The fibre flax[1] cultivars are grown for the stems yielding strong, high quality fibres which are manufactured into linen cloth and twine. Linseed (seed flax) and Linola (low linolenic flax) cultivars produce seed oil. There are also dual-purpose cultivars grown for both fibre and seed oil production.

Oil seed cultivars have a shorter and stouter phenotype and are more branched than those sown for fibre. Their fibre yield and quality is reduced but stems have been used in North America in the manufacture of high-grade paper products. The fibre cultivars carry fewer flowers and yield less seed than oil seed types. Dense sowings induce slender growth and maximum stem height before branching occurs (Jackson and Jacob 1985).

Adaptation

The species is widely adapted to temperate climates of the world. Due to existing industries and 'market forces' fibre cultivars are mainly grown in the cool-temperate regions of the northern hemisphere especially Russia and northern Europe. The cultivation of oil seed cultivars predominates under less intensive farming practices. Argentina, India, and North America are the principal producers (Durrant 1976). Since the introduction of Linola cultivars in recent years, oil seed production in Australia has increased.

---

[1] flax is used in this thesis as a generic term to describe the species *Linum usitatissimum*

Cytotaxonomy

The genus *Linum* has approximately 200 species including outbreeding, self-pollinating, annual and perennial types (Durrant 1976). Most *Linum* spp. belong to two distinct groups based on their chromosome numbers 2n=2x=18 and 2n=2x=30, though other configurations have been reported (x= 8, 10, 12, 14, 16). Cytogenetic studies of hybrids among 6 taxa with a haploid chromosome number of 15 revealed that differentiation appeared to involve one or two translocation events. The pairing was normal between most of the chromosomes, indicating a high level of homology. Similar conclusions were reached for hybrids among taxa with 9 as the haploid set. Apart from one or two translocations, chromosomes formed normal bivalents indicating that they are largely homologous. On the basis of these hybridisation studies, it has been postulated that speciation has proceeded through chromosomal reorganisation (Gill and Yermanos 1967).

The cultivated species *Linum usitatissimum* belongs to the well-defined group of 2n=2x=30. The genome is small (1.5 pg/2C nucleus) in comparison to most crop plants (Timmis and Ingle 1973). Results from studies of DNA reassociation kinetics by Cullis (1981) estimated that one chromosomal complement (1C) consists of 3.5 $x10^8$ nucleotide pairs (0.38 pg of DNA) or approximately one quarter the amount of DNA present in a diploid nucleus. Cullis (1981) postulated that the species is probably a stable tetraploid (2n=4x=30). Durrant (1976) reported that in at least one haploid plant (n=15) bivalents were observed suggesting that some chromosomes contain regions of homology. Results from DNA hybridisation experiments using low copy DNA as probes on genomic DNA have repeatedly detected duplicate fragment patterns (Spielmeyer unpublished; Ellis and Lawrence pers. communication). These results suggest that some DNA probes are assaying 'homoeologous' sequences. In addition, apparent locus duplication in the case of rust resistance loci (Ellis et al. 1995) and qualitative traits, such as oil quality (Green 1986), are consistent with an ancient tetraploid origin of the species.

A current evolutionary model for the genus postulates that speciation occurred through chromosome doubling of a basic *Linum* genome (n=9) and subsequent reduction of chromosome number to give rise to tetraploid species with n=15. The above-mentioned studies and experimental observations suggest that during evolutionary history of the species similarities between some chromosomal regions remained; however through differentiation or other factors suppression of homoeologous pairing has occurred to allow formation of a stable 'diploidised' genome. A similar model was proposed for the evolution of the soybean genome. Because soybean possesses many examples of traits controlled by two loci and a high proportion of DNA clones detect independent duplicate loci, it has been suggested that soybean be regarded as a stable tetraploid with a 'diploidised' genome (Shoemaker et al. 1994).

Development of a low linolenic acid genotype

Linseed oil has long been regarded as a good drying oil used in the manufacture of paints, varnishes and linoleum. The high level of linolenic acid, a polyunsaturated fatty acid (Table 1.1), makes the oil susceptible to oxidation and hence improves its drying rate giving the oil its traditional industrial use. Although linolenic acid is an essential fatty acid in human nutrition the poor oxidative stability of linseed oil has precluded its use as an edible oil outside the relatively small 'health food market'. In 1979 researchers at CSIRO Plant Industry, Australia, conducted studies aimed at developing new crops as alternatives to cereal production. As part of this research effort seed of the Australian linseed cultivar 'Glenelg' (50% linolenic acid content) was subjected to the chemical mutagen ethyl methanesulphonate (EMS) followed by a large scale screening program aimed at isolating mutants with altered fatty acid profile. Two induced mutants were identified with reduced linolenic acid content (28-30%) (Green and Marshall 1984). These mutational events occurred at independent loci which were subsequently combined into one genotype (Zero) producing less than 2% of linolenic acid. The reduction of linolenic acid in the double

mutant genotype is accompanied by an equivalent increase in the level of linoleic acid while the proportions of other fatty acids remain unchanged (Table 1.1). Low linolenic flax having the 'Zero' genotype is named Linola™ (Green 1986). Rowland (1991) reported the isolation of a EMS-induced low linolenic acid mutant derived from the Canadian linseed cultivar 'McGregor'. This low linolenic acid phenotype is also controlled by two independent loci, apparently the result of a rare double mutation.

Table 1.1: Fatty acid comparison of Linola and traditional linseed to major oilseeds

Oilseeds	**Palmitic**	**Stearic**	**Oleic**	**Linoleic**	**Linolenic**
	%	%	%	%	%
Linola	6	4	16	72	2
Linseed	7	4	20	17	52
Sunflower	7	4	16	73	-
Canola	5	2	66	19	8
Soybean	10	3	24	55	8

(adapted from Green and Dribnenki 1994)

1.2 Linola breeding objectives

Fatty acid composition

The low linolenic acid phenotype is controlled by two independent major genes. The character is controlled by codominant alleles that act additively across loci, each mutant allele at either locus being responsible for an approximate reduction of 10% in linolenic acid content (Green 1986). The incorporation of these two independent loci is a fundamental step in the development of Linola cultivars.

Yellow seed colour marker

To distinguish the new, low linolenic seed from the traditional high-linolenic flax, a visual seed colour marker was incorporated. Since most flax cultivars are brown-seeded, it was decided to develop exclusively yellow-

™ Linola is a registered trademark of CSIRO

seeded Linola varieties. The yellow seed trait was derived from C.P.I. '84495', a flax line of unknown pedigree originally sourced from North America. In crosses with brown-seeded lines examined so far, the yellow seed trait appears to be consistently inherited as a single dominant gene (Green and Dribnenki 1995).

Fusarium wilt resistance

Fusarium oxysporum f. sp. lini, a soil-borne fungus, is the causal agent of a vascular wilt disease called Fusarium wilt. The fungus is limited in its host range to species belonging to the genus *Linum.* The pathogen occurs throughout production areas of the world, but is prevalent in warm temperate zones such as Australia, Argentina and southern Europe. The fungus, through the formation of resting spores (chlamydospores), can survive in the soil environment for decades. Temperature is the single most important environmental factor affecting the development of wilt symptoms. Kommendahl et al. (1970) showed that there was a direct relationship of the number of hours during the growing season that the soil temperature exceeded 21°C. and the percentage of plants that wilted. Once the fungus has penetrated the root surface, the infection spreads rapidly in the vascular tissues of susceptible plants. Wilt symptoms have been described throughout the developmental cycle of the plant, although early wilt occurring at the seedling stage is potentially the most destructive, since losses can be 100% of plants in a stand (Kommedahl et al. 1970) (Fig. 1.1).

Because of the ubiquitous and persistent nature of the fungal pathogen selection for resistance to Fusarium wilt is an important aspect in flax breeding around the world. In particular, countries with warm growing seasons such as Australia and Argentina constitute high risk environments for the development of the disease. The breeding for wilt resistance is therefore an integral part of the development of Linola cultivars.

Variety development

The Linola quality traits (low linolenic acid content and yellow seed colour) have been introgressed by backcrossing into a range of linseed varieties selected for their high yield potential from around the world (Green and

Fig. 1.1: Fusarium wilt symptoms on flax plants grown in the field nursery.
Top: Row of susceptible plants (arrows) in front of row of resistant plants.
Bottom: Typical wilt symptoms on seedlings in the upright position.

Dribnenki 1995). Linola segregants were recovered after one, two or three generations of backcrossing to the adapted variety. Backcross derived lines were evaluated for the level of resistance to Fusarium wilt in at least two field nursery screenings. Pedigree selection was carried out for seed yield, oil content and agronomic traits in selfed generations. The first Fusarium wilt resistant Linola varieties, 'Wallaga' and 'Argyle' were released in Australia in 1992 and 1995 respectively producing superior seed and oil yields than that of the recurrent flax parents. Parallel to the Australian breeding effort varieties 'Linola 947' and 'Linola 989' were released in Canada in 1993 and 1995 respectively. Recent efforts have focused on developing new Linola varieties from intercrosses between the best backcross derivatives and other high yielding flax germplasm (Green and Dribnenki 1995).

1.3 Potential for DNA marker technology in Linola breeding program

As Linola production expands worldwide into flax growing areas, the Linola quality traits need to be introgressed into locally adapted genetic backgrounds. In addition advanced breeding lines need to be screened for broad-based resistance to Fusarium wilt. DNA marker technology is expected to improve selection efficiencies by reducing the number of individuals and generations required to identify stable recombinants.

To assess the feasibility of marker-assisted selection as part of a backcross program, a comparison between a conventional, marker-unassisted process and a marker-assisted selection scheme is outlined (Fig. 1.2). The breeding objective is to transfer 5 unlinked loci from a Linola donor line to an adapted recurrent parent. The five genes include two independent loci *ln1* and *ln2* conferring the low linolenic acid phenotype, two loci, *R1* and *R2*, contributing largely to Fusarium wilt resistance and a single dominant locus *Y* conferring yellow seed colour.

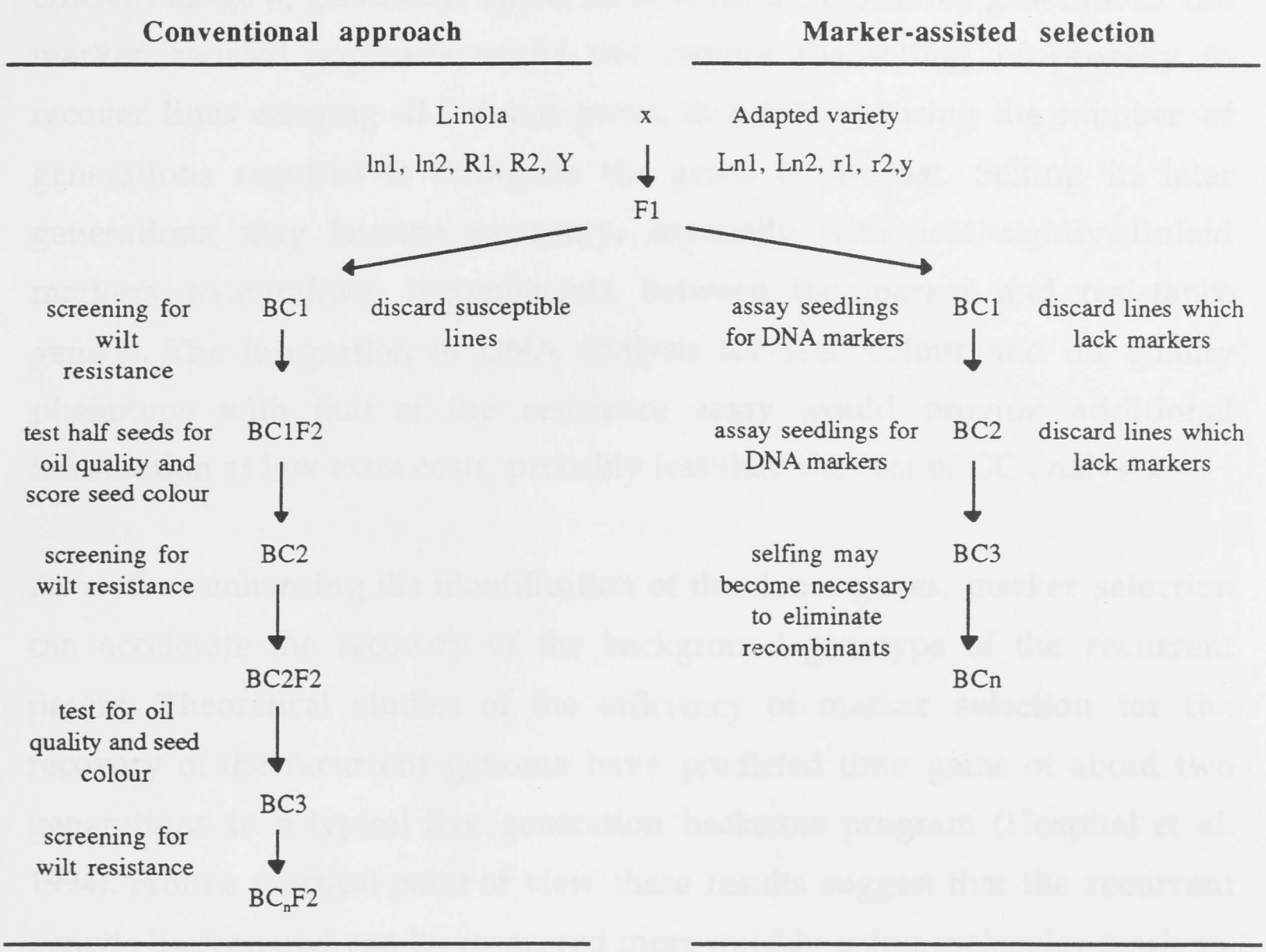


Fig 1.2: Comparison between conventional and marker-assisted selection in a backcross program for introgression of two linolenic acid mutations ln1 and ln2, two major Fusarium wilt resistance loci R1 and R2 and yellow seed colour locus Y.

Because the yellow seed colour and oil quality phenotype can be assayed visually and by gas chromatography (GC) respectively, these traits would probably not warrant the use of DNA markers by themselves. The main benefit is derived from using DNA markers to assist selecting for wilt resistance. Disease assays are currently carried out in field nurseries which introduce significant costs and restricts the timing of screening to within the growing season. Inherent difficulties associated with the field assay include unreliable natural inoculum levels and strong temperature effects on the infection rate and expression of disease symptoms. These limitations are expected to be overcome by screening of seedlings for DNA markers linked to the resistant phenotype. Using markers at a young growth stage allows for the early elimination of unwanted segregants, hence contributing to more

efficient usage of glasshouse space. At least for the first three generations the marker assisted approach would not require the selfing of progeny to recover lines carrying all 5 donor genes, therefore reducing the number of generations required to introgress the genes of interest. Selfing in later generations may become necessary, especially with less tightly linked markers, to eliminate recombinants between the marker and resistance gene(s). The integration of DNA analysis for seed colour and oil quality phenotype with that of the resistance assay would provide additional information at low extra costs, probably less than the cost of GC analysis.

As well as enhancing the identification of the donor genes, marker selection can accelerate the recovery of the background genotype of the recurrent parent. Theoretical studies of the efficiency of marker selection for the recovery of the recurrent genome have predicted time gains of about two generations in a typical five generation backcross program (Hospital *et al.* 1994). From a practical point of view these results suggest that the recurrent genetic background can be recovered more quickly using molecular markers. The recovery process of the genetic constitution of the recurrent parent can be accelerated further by exploring markers as tags for chromosome segments to select against the genetic background of the donor parent (Melchinger 1990).

1.4 Aim of Research

The aim of this research project was to study the molecular genetics of Fusarium wilt resistance in *L. usitatissimum* by establishing an appropriate segregating population, elucidating the mode of inheritance and utilising a range of DNA marker techniques to target genes contributing to the resistance response. This work is considered as a first step towards gaining an understanding of the molecular basis of resistance and developing a DNA marker based assay for one of the most important diseases affecting this crop species. The project was closely linked to the Linola breeding

program in progress at CSIRO Plant Industry in Canberra with a view of implementing marker-assisted selection during future development of Linola varieties.

The experimental approach was designed to provide detailed information on the following:

1. The genetic control of Linola quality traits
2. The mode of inheritance of resistance to Fusarium wilt in a cross between a resistant Linola type and a susceptible flax cultivar 'Glenelg'.
3. The targeting of Fusarium wilt resistance gene(s) by PCR using random primers and by DNA hybridisation employing homologous and heterologous DNA probes.
4. The generation of a comprehensive genetic linkage map of the species using a PCR-based DNA marker technique.
5. The location of quantitative trait loci which contribute significantly to the resistance response.

Chapter Two

Development of a recombinant doubled-haploid (DH) population in flax

2.1 Introduction

The study of both simple and more complex inherited characters requires a population segregating for the traits of interest. Homozygous segregants have long been regarded a useful tool in classical and molecular genetics. The reasons for this are threefold:

1. Homozygous lines constitute permanent populations due to the absence of segregation within lines.
2. Scoring of phenotypes is facilitated due to the absence of heterozygote genotypes (especially true for complex traits).
3. DNA marker techniques, which assay presence or absence of marker bands, yield fully informative marker loci, because heterozygotes do not have to be distinguished from homozygote genotypes.

The haploid genome is widely utilised as a source for rapid and complete homozygosity. Doubled-haploids derived from a haploid plant by doubling its chromosome number are an attractive choice in genetic analysis, because they allow for fixing of recombinant gametes as fertile homozygous lines in a single generation (Snape 1989). This form of 'instant' and complete homozygosity can be exploited to the advantage of molecular marker and mapping studies. Major benefits are derived from considerable time savings by reducing the number of generations required to obtain homozygosity. Furthermore, DH lines represent permanent populations, which can be replicated and used indefinitely for linkage studies. This aspect is particularly relevant for large scale, collaborative mapping projects. The same mapping population can be used by researchers at different

institutions to characterise molecular markers, traits controlled by major genes as well as quantitative trait loci to build up comprehensive linkage maps of species. Recombinant inbred lines display similar levels of homozygosity compared to DH lines and are generally more informative in regards to locating tightly linked markers due to more opportunity for recombination. However, recombinant inbred lines need considerably more time to develop, typically requiring five or six generations of inbreeding.

2.2 Production of haploids

Production of haploid plants has been reported in well over 200 species. A number of methods have been described by which female or male gametes yield haploid plants (Foroughi-Wehr and Wenzel 1993). These methods are summarised in Fig. 2.1 and can be grouped into two classes, namely the induced and the spontaneous systems of haploid production. Methods capable of producing DH lines on a large scale rely on the induction of haploid plant formation. This is achieved by either *in vitro* culture of male/female gametes or chromosome elimination through wide hybridisation.

The most widely used method of inducing haploid plants today is by *in vitro* androgenesis. It involves the culture of male gametes, either in the anther or as isolated microspores (Kasha et al. 1995). This technology is applied to a wide range of genera, but has been particularly successful in generating doubled haploids in *Brassica* spp. (Westecott and Huang 1995) and cereals such as wheat and barley (Kasha et al. 1995). *In vitro* androgenesis was applied successfully to regenerate flax plants using anther culture (Nichterlein et al. 1991) and from isolated microspores (Nichterlein and Friedt 1993), albeit at efficiencies considered too low for routine applications.

Wide hybridisation resulting in chromosome elimination has been used extensively in barley and wheat to generate haploid plants. Haploid production in barley has been achieved by crossing *Hordeum vulgare* to *Hordeum bulbosum* (Kasha and Kao 1970). During the early development of the hybrid embryo *H. bulbosum* chromosomes are eliminated yielding *H. vulgare* monoploids which are regenerated by rescuing the embryos on a nutrient medium. Haploids in wheat are produced through a similar mechanism of chromosome elimination by fertilising wheat with maize pollen (Laurie and Bennett 1988). This system is efficient enough for employment in commercial wheat breeding.

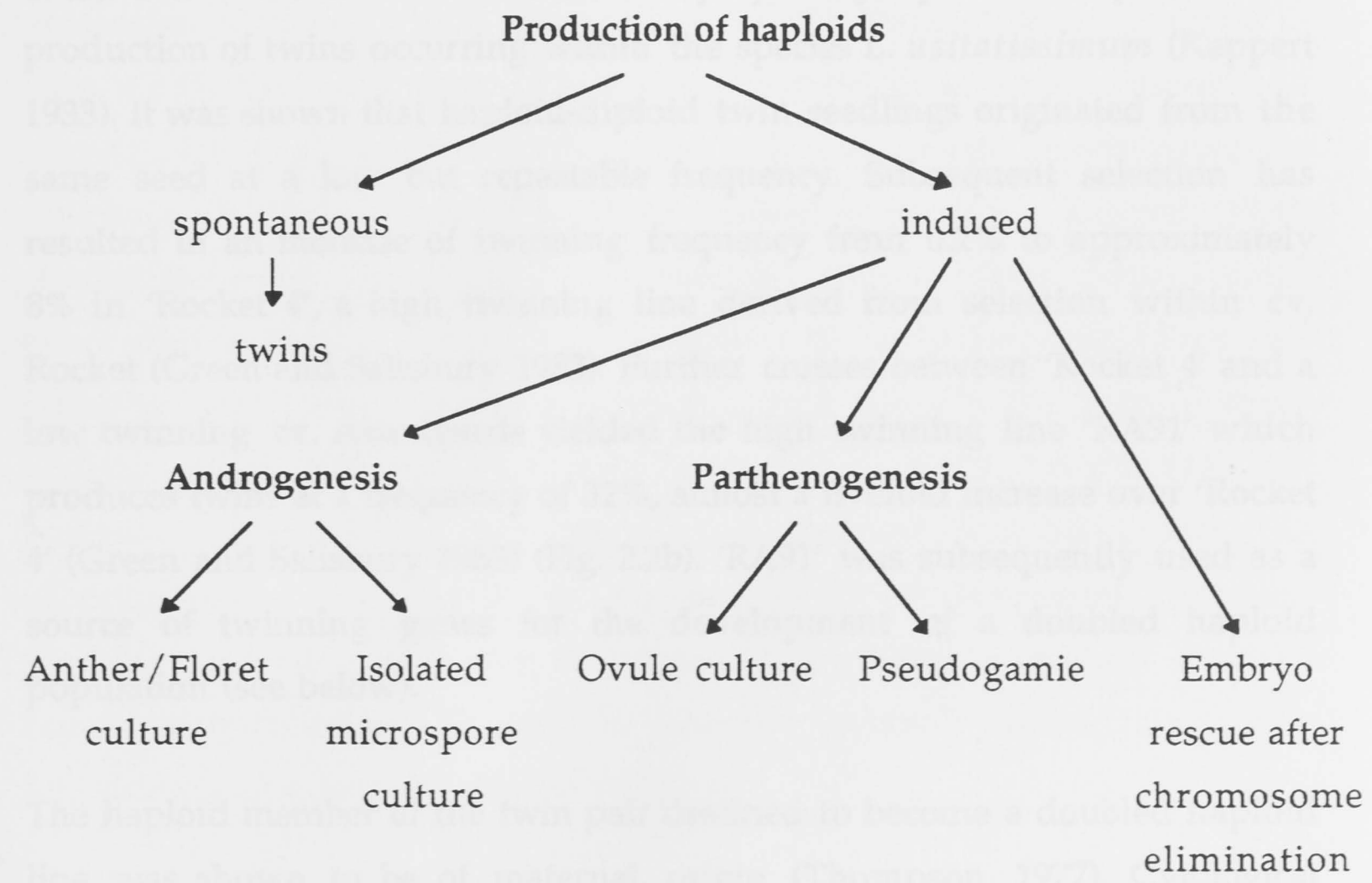


Fig. 2.1: Summary of procedures resulting in the production of haploid plants (adapted from Foroughi-Wehr and Wenzel 1993).

The application of *in vitro* production of haploid plants from unpollinated ovaries or ovules (parthenogenesis) has been limited by the low frequency of induction in most species. Plants originating from somatic tissue can also be a major problem and this has precluded wider application of this technique except in specialised systems such as potato (Foroughi-Wehr and Wenzel 1993).

2.3 Polyembryony-derived DH lines

The spontaneous production of haploid plants from polyembryonic seed has been reported widely in the plant kingdom, albeit only in a few species (eg. Citrus spp.) is polyembryony regarded as a regular phenomenon (Huyghe 1985). One of the earliest discoveries of polyembryony was the spontaneous production of twins occurring within the species *L. usitatissimum* (Kappert 1933). It was shown that haploid-diploid twin seedlings originated from the same seed at a low but repeatable frequency. Subsequent selection has resulted in an increase of twinning frequency from 0.2% to approximately 8% in 'Rocket 4', a high twinning line derived from selection within cv. Rocket (Green and Salisbury 1983). Further crosses between 'Rocket 4' and a low twinning cv. Avantgarde yielded the high twinning line 'RA91' which produces twins at a frequency of 32%, almost a fivefold increase over 'Rocket 4' (Green and Salisbury 1983) (Fig. 2.2b). 'RA91' was subsequently used as a source of twinning genes for the development of a doubled haploid population (see below).

The haploid member of the twin pair destined to become a doubled haploid line was shown to be of maternal origin (Thompson 1977). Cytological studies suggested that mitotic products from a single megaspore or egg-cell would be the most likely progenitors of haploid members (Huyghe 1985; Secor and Russell 1988). However, Murray (1985) studying the segregation of flower colour within twin pairs observed that in some cases the diploid plant had white petals (homozygous recessive) and its corresponding

haploid member blue petals. She postulated that a separate meiotic product from the one giving rise to the diploid member must constitute the embryological origin of the haploid component. In this present study the occurrence of white-flowered diploid plants in conjunction with blue-flowered haploid components suggests that respective twin members originated from separate meiotic products as reported by Murray (1985).

The inheritance of polyembryony was studied by Green and Salisbury (1983) and Huyghe (1985). The former authors showed that distribution of twinning percentage in hybrid generations were inconsistent with a simple genetic interpretation and concluded that production of polyembryonic seeds is controlled by multiple gene action. Huyghe, in contrast, proposed a two gene model for the control of twinning, one gene having gametophytic expression. Gametes which are favoured on the basis of their genotype to form viable haploid embryos could potentially result in a biased sample of gametes. Under these conditions markers linked to genetic determinants for twinning would be expected to show skewed segregation. Kappert (1933) studied the production of twinning seed on diploid plants derived from either diploid seed or the diploid component of haploid-diploid twins and found no significant difference between inbred sibs. On the basis of these findings in addition to the strong temperature effects he observed on twinning frequency within inbred lines Kappert concluded that the genotype of the plant on which the seed develops determines twinning frequency not the genotype of the gamete. It is therefore unlikely that the use of polyembryonic F2 seed to generate doubled haploid flax lines introduces a bias towards gamete selection.

2.4 The CRZY8/RA91 DH population

One primary aim of this project was to develop a segregating population for two Linola quality traits (ie. low linolenic acid content and yellow seed colour) and resistance to Fusarium wilt within the time constraints of my

candidature. For reasons outlined in Section 2.1 it was decided to establish a doubled haploid population. Polyembryony was used to generate DH lines, because efficient *in vitro* androgenesis in flax was not available at the time.

Production of doubled haploid lines

The low linolenic acid, yellow seeded and wilt resistant line 'CRZY8' was derived as outlined in Fig. 2.2a (Green, pers. communication). The twinning trait was incorporated by crossing 'CRZY8' with 'RA91', a high twinning flax line (Green and Salisbury 1983) (Fig. 2.2b). DH lines were derived from the F1 and screened in the field nursery for resistance to wilt (Green, pers communication). The doubled haploid line 'CRZY8/RA91' (wilt resistant, low linolenic acid, yellow seeded and blue flowered) was selected and crossed with the inbred Australian cultivar 'Glenelg' carrying the alternate alleles for all traits of interest (ie. wilt susceptible, high linolenic acid, brown seeded and white flowering). The parental lines, 'CRZY8/RA91' and 'Glenelg', are expected to share common chromosomal regions linked to the two independent fatty acid mutations, because the low linolenic acid mutant line 'Zero' was originally derived by mutagenesis of 'Glenelg'. The wilt resistance characteristic of the CRZY8 line was contributed by the resistant cultivar Croxton, which was a later selection from the same crossbred population that gave rise to Glenelg. The parental lines in the final cross are thus relatively closely related and are expected to have a high degree of genetic identity except for the genes controlling the selected characters and other closely linked genes.

To generate DH lines F2 seed was germinated on moistened paper sheets in polystyrene trays kept at 25°C and screened for the occurrence of twin seedlings after 3-4 days. Twin seedlings were transferred into perlite/vermiculite mix (50:50) and transplanted after 10 days into a soil medium (Fig. 2.3). The ploidy level of twin plants was determined at flowering on the basis of anther morphology and fertility. The stunted appearance of stamen and indehiscence of anthers proved to be a reliable

indicator of haploidy (Fig. 2.4). Diploid plants were discarded and fertility restored in the haploid component through colchicine treatment. Glass capillaries were attached to stems cut below the inflorescences to hold 0.05% colchicine solution (Fig. 2.5). The solution was absorbed into the stem tissues. As the solution moved down through the tissues, high colchicine concentrations usually resulted in the death of axillary buds near the top of the stem. Further down the stem colchicine concentrations became sufficiently diluted to cause chromosome doubling in axillary buds which grew out to form fertile shoots (Rajhathy 1976).

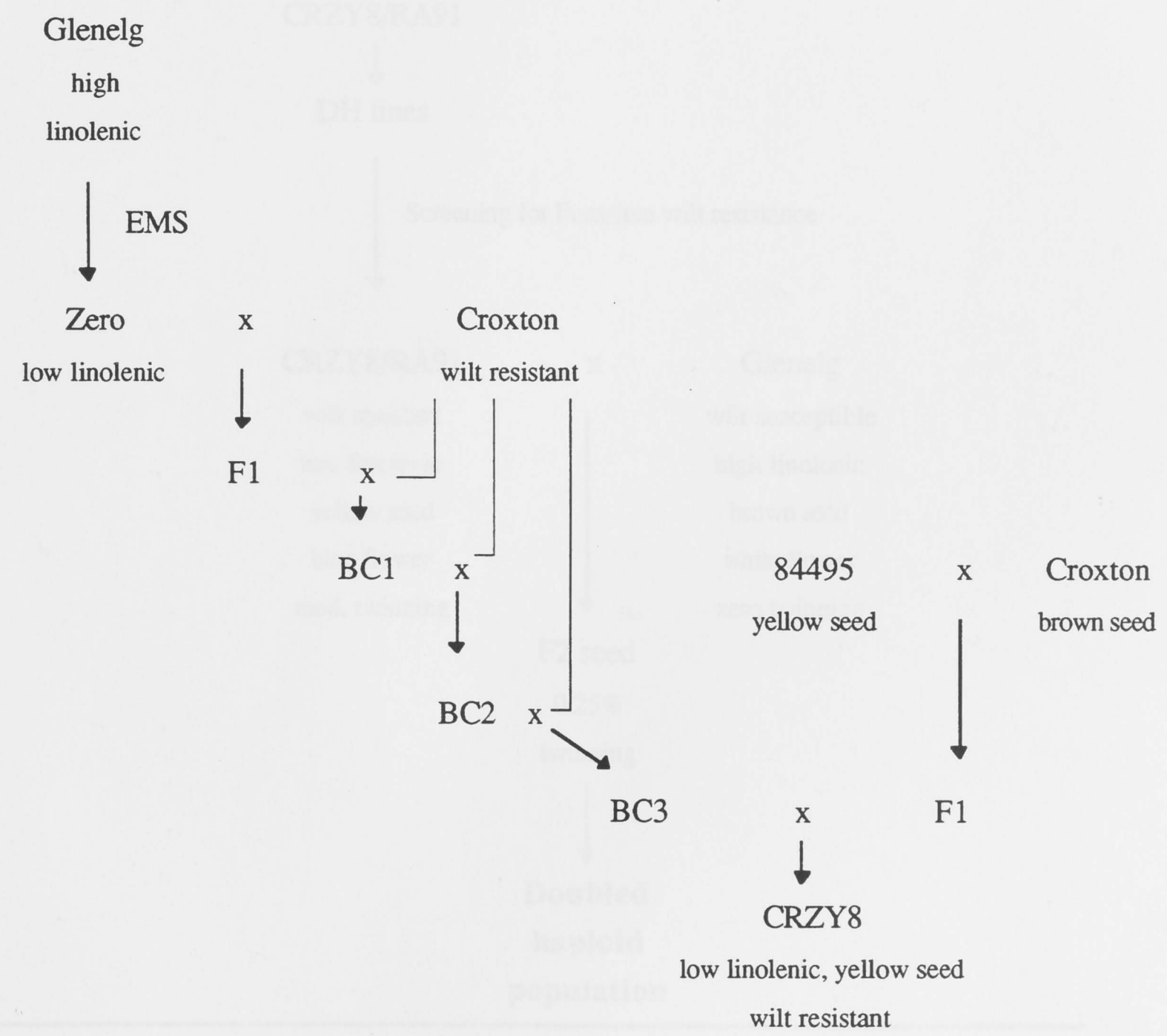


Fig. 2.2a: Pedigree of low linolenic acid, yellow seeded and wilt resistant 'CRZY8' line.

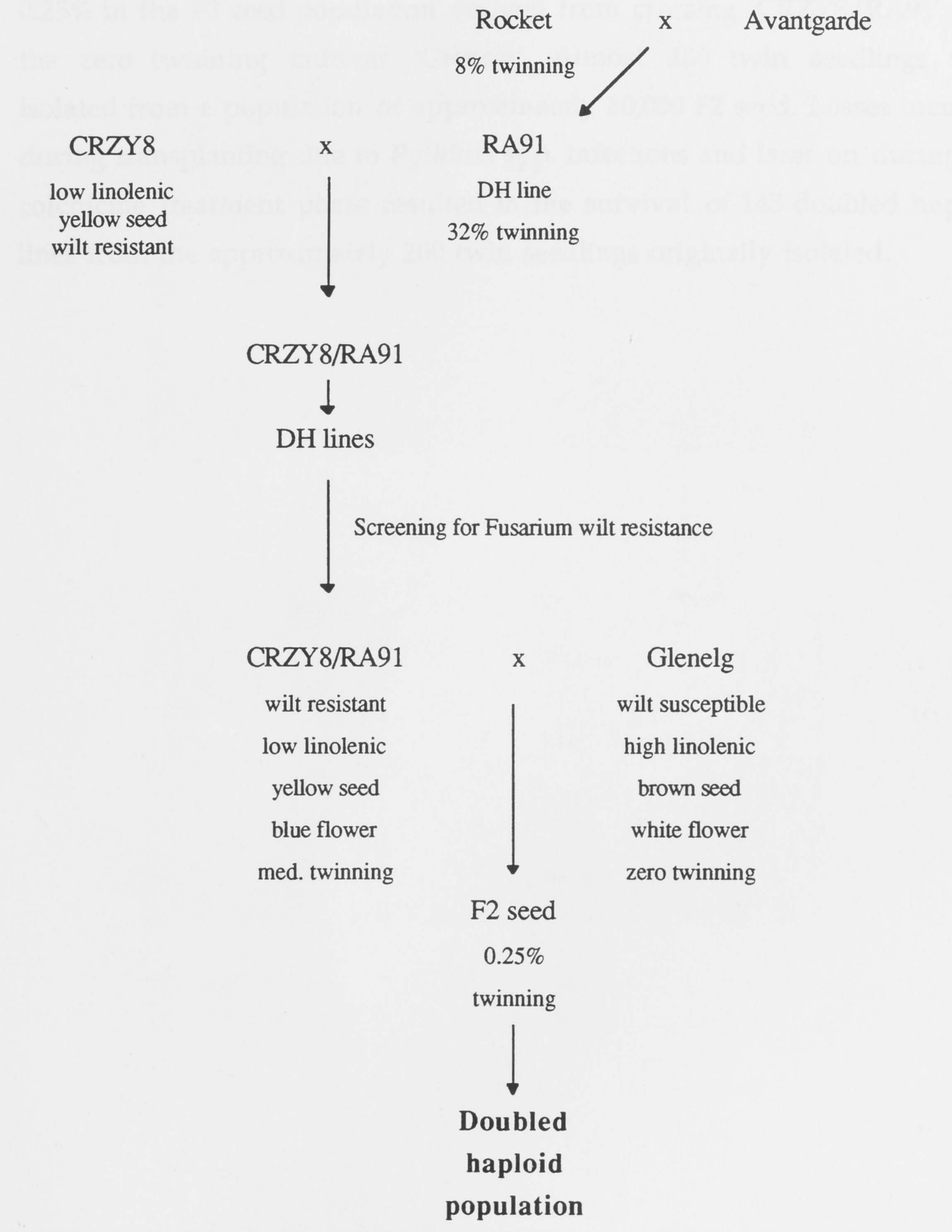


Fig. 2.2b: Pedigree of recombinant doubled haploid population.

Transmission rate of polyembryonic trait

The twinning frequency dropped markedly when the trait was introgressed from the high twinning 'RA91' line (32%) into the Linola parental line 'CRZY8' (approx. 5%). The twinning percentage was further reduced to 0.25% in the F2 seed population derived from crossing 'CRZY8/RA91' with the zero twinning cultivar 'Glenelg'. Almost 200 twin seedlings were isolated from a population of approximately 80,000 F2 seed. Losses incurred during transplanting due to *Pythium* spp. infections and later on during the colchicine treatment phase resulted in the survival of 143 doubled haploid lines from the approximately 200 twin seedlings originally isolated.

Fig. 2.3: (A) Twin seedlings (7 days old) consisting of haploid (small) and diploid member grown in vermiculite/perlite mix before transplanting into soil medium (2 weeks old) (B).

Fig. 2.4: (A) Fertile, white flower with yellow anthers on diploid flax plant. (B) Sterile, blue flower on haploid plant, stamen appear stunted. (C) Sterile, white flower with partially removed petals on haploid plant, stamen appear stunted.

Fig. 2.5: Colchicine treatment of haploid member. (A) Glass capillary was pushed onto cut stem and syringe fitted with long needle was used to fill the capillary with 0.05% colchicine solution from the bottom up to avoid the formation of air bubbles (B).

Chapter Three

Inheritance of seed colour, flower colour and low linolenic acid content in flax

3.1 Introduction

Flower colour

The petal colour of cultivated flax can be white (recessive) or blue. Within both categories different shades have been described ranging from white to dark pink and light to dark blue. Tammes (1928), conducting one of the first comprehensive genetic studies of morphological characters in flax, postulated the existence of three basal factors (B_1 , B_2 and C) which must be present for any colour to occur. She also described several other modifying factors (A, D, E and F) which influence the tint and intensity of the petal colour. Barnes et al. (1960) proposed the involvement of two basic factors (B_1 and D) together with at least two modifying factors (A and F). The assumption is that the dominant B_1 and D factors determine blue petal colour and homozygous recessive b_1b_1 produces white petals regardless of the rest of the genotype. By studying progeny from crosses involving a range of flower colour genotypes observed genetic ratios approximated the expected ratios for monogenic, digenic and trigenic patterns of inheritance (Barnes et al. 1960).

Seed colour

The seed colour of flax is usually referred to as being either yellow or brown. However, studies of seed colour are complicated by many shades occurring within a spectrum of light yellow to dark brown. In light coloured seed the lack of pigment within the testa exposes the yellow coloured cotyledons underneath. The deposition of varying amounts of pigment in the inner cell layer of the integument yield an array of darker seed colours (Beard and Beeson 1967). Tammes (1928) and Barnes et al. (1960) described three genes (B_1 , D and G) influencing seed colour, of which two (B_1 and D) appear to have pleiotropic effects on flower colour. Yellow seed colour was assumed

to be the result of the presence of one or more recessive allele in the homozygous state at one of the three loci (b, d and g). On the basis of segregation analysis of F2 and BC progeny, inheritance of yellow seed colour was controlled by two or three major genes (Barnes et al. 1960). Beard and Beeson (1967) presented evidence in support of monogenic inheritance, yellow seed colour being recessive. Green and Salisbury (1983) reported the segregation of two independent loci, yellow seed colour being the result of the presence of both genes in the homozygous recessive state. A single dominant gene for yellow seed colour derived from the line C.P.I. '84495' was reported by Green (pers. communication); line '84495' was used as the source for yellow seed colour in this present study. The dominant gene for yellow seed colour appears to be a separate determinant from previously reported recessive genes.

Deviations from the expected ratios have been reported in progeny segregating for both flower and seed colour. Deficiency of white flowers was repeatedly observed in F2 progeny derived from a cross between a white-flowered and blue-flowered parent (Kappert 1924; Tammes 1928). Kappert (1924) proposed a competition effect between male gametes leading to skewed segregation. Tammes (1928), however, attributed the shortage of white flowers to the presence of a semi-lethal factor and the homozygous genotype b_1b_1 resulting in reduced viability of white-flowered, yellow seeded zygotes. The effect of a semi-lethal factor associated with the same genotype was also proposed as reason for the apparent deficiency of white-flowered, yellow seeded segregants by Barnes et al. (1960). Two independent studies reported on the absence of blue flowered, yellow seeded segregants originating from a cross between blue-flowered, brown seeded and white-flowered, yellow seeded parents (Burnham 1932; Green and Salisbury 1983). The blue flower colour and brown seed colour appeared to be inherited through the action of one and two major genes respectively. This suggests that one of the two genes for seed colour is either tightly linked to the one

controlling flower colour or constitutes the same locus, as described by Tammes (1928) and Barnes et al. (1960).

Low linolenic acid trait

The inheritance of the low linolenic acid phenotype is determined by two independently-segregating major genes *Ln1* and *Ln2*. Gene action is additive between loci, each mutant allele at either locus contributing equally to the reduction in linolenic acid content (Green 1986). Linolenic acid content ranges from 50% in wild type to less than 2% in the double mutant genotype. Segregation analysis in an isogenic background resulted in an almost perfect fit of the observed to expected ratio for the segregation of two independent loci (Green 1986).

In the present study the segregations of flower colour, seed colour and fatty acid mutations were examined in a DH progeny derived from the cross between cv. 'Glenelg' (white flower, brown seed, high linolenic acid content) and the Linola line 'CRZY8/RA91' (blue flower, yellow seed, low linolenic acid content) as described in Chapter Two. Genetic ratios were tested against expected ratios for alternative genetic hypotheses.

3.2 Materials and Methods

A total of 143 DH lines were screened for flower and seed colour by growing 10 plants per line in the glasshouse. The true breeding characteristic of each DH line was confirmed by growing 100 plants in single row plots in a field nursery.

A 30-seed sample per line was taken at seed maturity and analysed for fatty acid composition as described by Green (1986).

3.3 Results

Segregation analysis of traits of interest

Table 3.1 summarises the frequencies of observed phenotypes for all three traits under study. The genetic ratio for flower colour segregants approximates the expected ratio of 1:1 indicative of monogenic inheritance of this trait. A deficiency, albeit not significant, of blue-coloured lines is evident. Seed colour phenotypes were grouped into yellow, yellow/brown and brown categories. The chi-square test of significance was applied to the expected ratio for the segregation of alternatively one or two major genes for seed colour by assigning the yellow/brown class to either the yellow or brown category respectively (Table 3.1). Under a monogenic model the observed data deviates significantly from the expected ratio due to a shortage of yellow seeded lines. When tested against the two gene model the data fit the expected 3:1 ratio.

Table 3.1: Chi-square test for observed frequencies of individual doubled haploid lines segregating for flower colour, seed colour and fatty acid composition.

	White	Blue	Tested ratio	Chi-square	P-value
Flower colour	84	63	1:1	2.6	0.25-0.1

	brown	y/b	yellow			
Seed colour	93	17	37			
	93		54	1:1	10.3*	<0.005
	110		37	3:1	0	

	high	inter-mediate	low			
Linolenic acid content	28	70	49	1:2:1	6.3*	0.05-0.025

* denotes significant deviation from expected ratio at P<0.05

Phenotypes for fatty acid composition were scored as Low containing linolenic acid levels of <2%, Intermediates (10-40%) and High (>40%). The respective genotypes represented within each category are assumed to

contain homozygous double mutants $Ln1^0Ln1^0$, $Ln2^0Ln2^0$, single mutants homozygous at each locus $Ln1^0Ln1^0$, $Ln2^1Ln2^1$ and $Ln1^1Ln1^1$, $Ln2^0Ln2^0$, and wild type $Ln1^1Ln1^1$, $Ln2^1Ln2^1$. The observed data deviates significantly from the expected two gene model through a deficiency of wild type genotypes (Fig. 3.1).

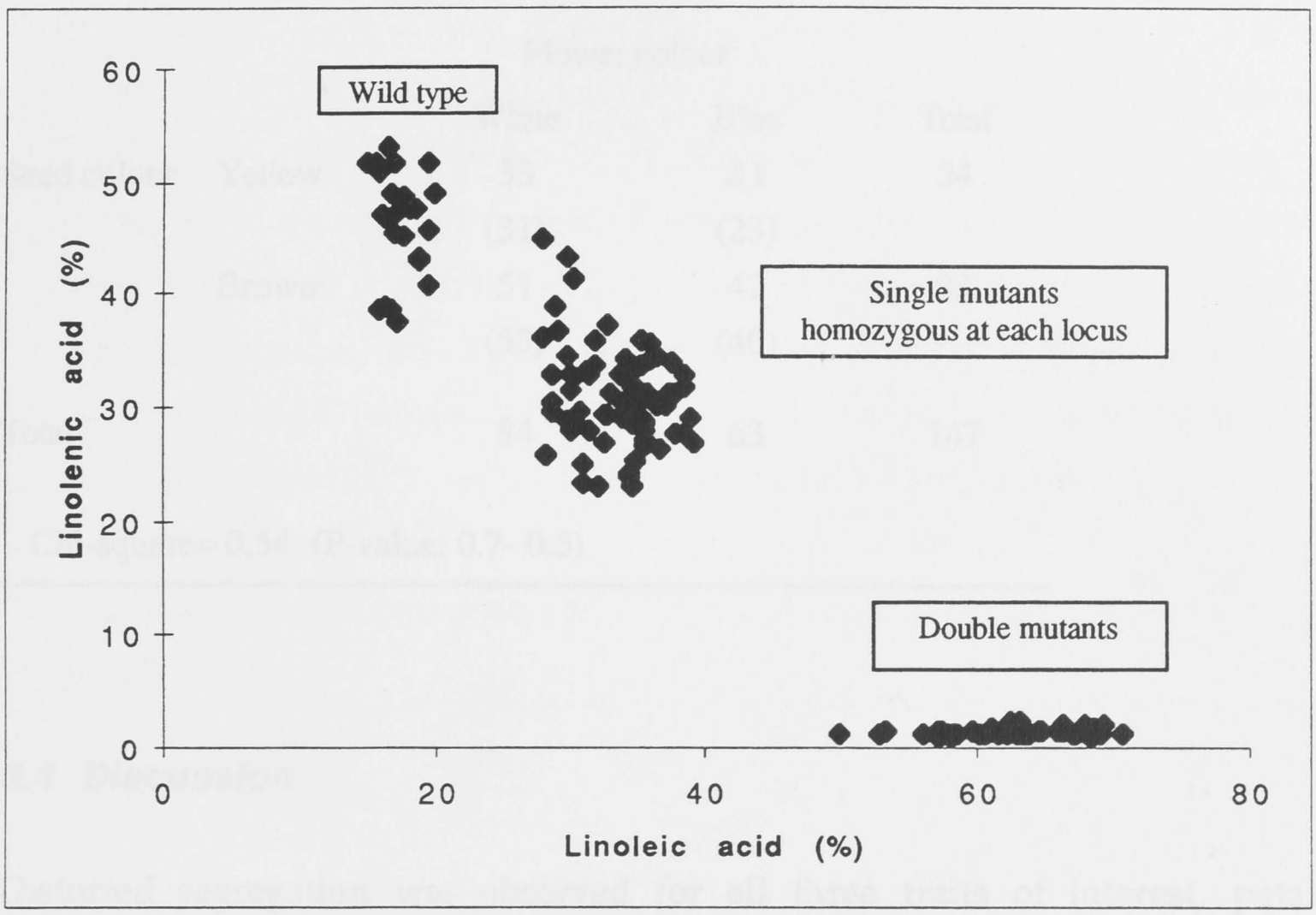


Fig. 3.1: Relationship between linoleic and linolenic acid contents in individual doubled haploid lines.

Independence of characters

To investigate linkage relationships between all three characters of interest, assuming monogenic inheritance of yellow seed colour, contingency table analysis was carried out (Table 3.2; contingency data for oil quality not shown). Observed frequencies for character contributions were tested against expected frequencies calculated by compounding the observed frequencies for the individual traits. Because no significant association of any two characters was detected, it is likely that loci controlling these traits belong to

separate linkage groups. Independent inheritance was subsequently confirmed by comprehensive linkage analysis in relation to more than 200 molecular marker loci (see Chapter Six).

Table 3.2: Contingency table analysis testing for independence of genetic determinants for seed and flower colour (expected frequencies shown in brackets).

		Flower colour		
		White	Blue	Total
Seed colour	Yellow	33	**21**	54
		(31)	(23)	
	Brown	51	42	93
		(53)	(40)	
Total		84	63	147

Chi-square= 0.54 (P-value: 0.7- 0.5)

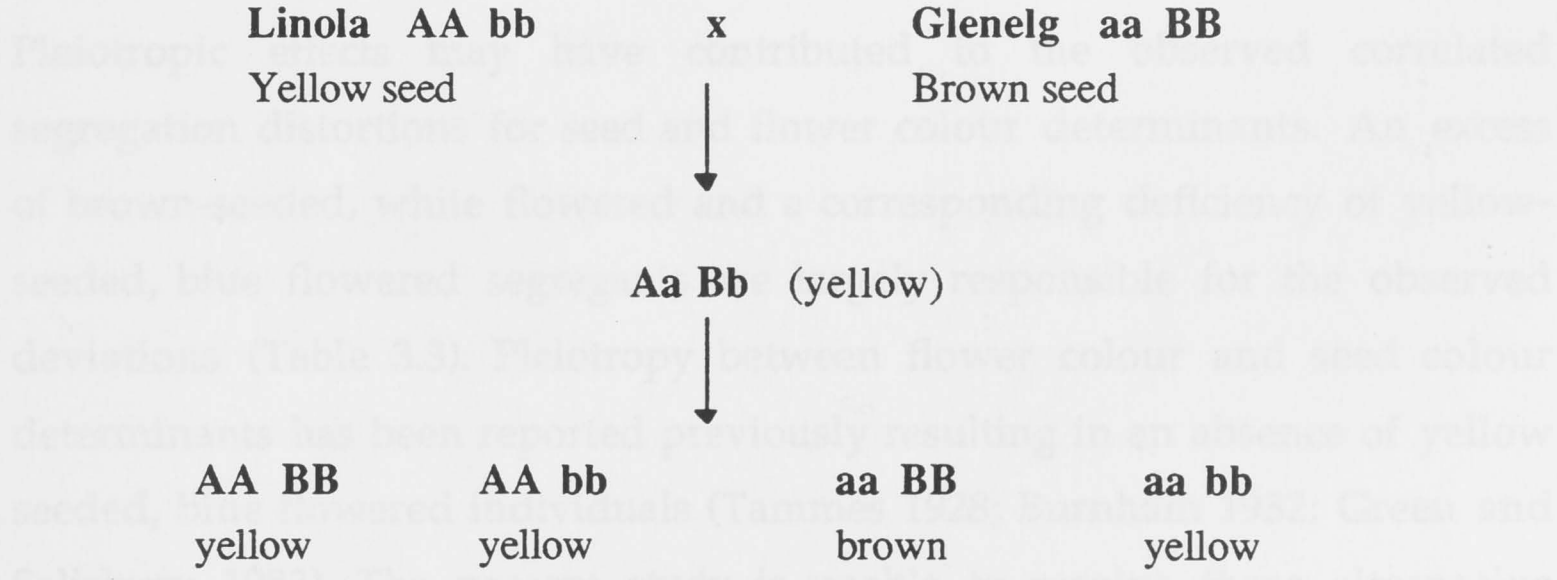
3.4 Discussion

Distorted segregation was observed for all three traits of interest, petal colour, seed colour and fatty acid composition. Altered segregation ratios can be attributed to preferential selection at the gametophyte and/or zygote level (Grant 1975; Zamir and Tadmor 1986). The non-random segregation of chromosomes during meiosis and the non-random selection of megaspores destined to become embryo sacs were proposed as possible determinants for aberrant segregation patterns at the level of sporogenesis (Grant 1975). Lethal or semi-lethal factors resulting in the preferential death of certain genotypes have been implicated at the level of spore function to cause distorted ratios at rust resistance loci in flax (Lawrence pers. comm.). Selective elimination of zygotes has been described as a possible factor distorting segregation of flower and seed colour determinants in flax (Tammes 1928). Post-germination selection at the juvenile or adult plant stage can also contribute

to altered Mendelian segregation. The results are discussed irrespective of the stage at which selection might have occurred.

The genetic ratio for segregation of blue flower colour approximates the expected dominant, monogenic inheritance pattern as described by Barnes et al. (1960) and Green and Salisbury (1983). One possible explanation for the observed genetic ratio of seed colour determinants is the segregation of two major genes (Fig. 3.2). The dominant factor *A* determines yellow seed colour with an epistatic effect on factor *B*, the determinant for brown seed colour if the recessive *aa* genotype is present. To reconcile a two gene model with the fact that this yellow seed trait behaved like a single dominant trait in other crosses, it is assumed that the recessive allele 'b' was introgressed from the brown-seeded high twinning line 'RA91'. Crosses between 'RA91' and the yellow seed parent C.P.I. '84495' have so far not been studied in detail.

Fig. 3.2: Model for two major genes determining seed colour phenotype in doubled haploid progeny.



An alternative model of seed colour inheritance for this particular cross is to accept a monogenic model despite a significant deviation from the expected genetic ratio. In crosses with brown-seeded flax lines studied so far, the yellow seed trait was shown to be consistently inherited as a single dominant gene (Green and Dribnenki 1995). Segregation analysis of molecular markers linked to the seed colour trait when scored as a single

locus also showed significant deviations from the Mendelian ratio (see Fig. 6.2). These marker loci together with the seed colour trait are skewed towards the 'Glenelg' parental alleles.

Table 3.3: Observed frequencies of segregants with deviations from the expected frequencies shown in brackets assuming monogenic inheritance for seed and flower colour.

		Flower colour	
		white	blue
Seed colour	yellow	33	21
		(-3)	(-15)
	brown	51	42
		(+15)	(+6)

Pleiotropic effects may have contributed to the observed correlated segregation distortions for seed and flower colour determinants. An excess of brown-seeded, white flowered and a corresponding deficiency of yellow-seeded, blue flowered segregants are largely responsible for the observed deviations (Table 3.3). Pleiotropy between flower colour and seed colour determinants has been reported previously resulting in an absence of yellow seeded, blue flowered individuals (Tammes 1928; Burnham 1932; Green and Salisbury 1983). The present study is unable to resolve these alternative explanations for seed colour inheritance.

The characters controlling fatty acid composition show significant distortion from the expected two gene model. Green (1986) studying the segregation of the low linolenic acid trait in a isogenic background firmly established that two independent loci control this trait without any segregation distortion. The observed distortion may be the result of the segregation of different

allelic backgrounds. A deficiency of wild type alleles is responsible for the aberrant ratio for fatty acid composition. If selection has occurred against wild type alleles, the selection process operated independently or with opposite effect on flower and seed colour determinants.

Assuming monogenic inheritance for seed colour, segregation distortion was observed for all three characters under investigation in this chapter.

Chapter Four

Inheritance of resistance to Fusarium wilt (*Fusarium oxysporum* f. sp. *lini*) in flax

4.1 Introduction

Fusarium wilt is caused by the ubiquitous and highly persistent soil fungal pathogen *Fusarium oxysporum* f.sp. lini and is potentially one of the most devastating diseases of flax. Although resistance to wilt is an essential selection criterion in breeding of flax and Linola cultivars, the genetic basis for resistance has not been definitively characterised and no major genes have so far been mapped to chromosomal locations. The identification of major genes and their linkage associations with DNA markers is a prerequisite for improved methods of selection for wilt resistance and for the eventual cloning and manipulation of such genes.

Several studies involving field assessment of resistance in segregating generations of crosses between resistant and susceptible parents failed to identify major gene effects and instead concluded that resistance was due to polygenic effects (Tisdale 1917; Burnham 1932; Kommedahl et al. 1970; Popescu and Schuster 1985). It is possible that major gene effects accounted for at least part of the resistance response in such studies but that conclusive identification was hindered by factors such as (a) the presence of a complex mixture of pathotypes of the wilt organism in field soil, (b) unreliable infection due to heterogeneity of field environments or sub-optimal infection conditions, (c) segregation of many genes affecting resistance due to choice of genetically diverse parental lines, and (d) difficulties in classification of resistance status in early generations (eg. F2 or F3) due to the presence of high levels of heterozygosity and genetic heterogeneity within families.

Inheritance studies that avoided some of these potentially complicating factors, frequently resulted in simpler patterns of inheritance. For example, Indian studies of mainly indigenous germplasm conducted in one field nursery postulated that resistance was determined by either one or two dominant genes (Jeswani and Joshi 1964; Jeswani and Upadhyaya 1970; Goray et al. 1987; Goray et al. 1988). Similarly, Kamthan et al. (1981) who studied crosses between Indian and North American germplasm in the glasshouse using a sterile soil medium which had been inoculated with a mixture of pathogenic fungal isolates also concluded that resistance was determined by a single dominant gene. Thus it is likely that major genes controlling wilt resistance in flax do exist and can be identified under experimental conditions optimal for their detection.

The relevance of major genes to breeding for durable resistance to flax wilt depends to a significant degree on the race specificity of the resistance phenotype. Some previous studies that have identified either monogenic or digenic inheritance of flax wilt resistance have also examined the race specificity of the response. Under conditions where plants were exposed to a mixture of pathotypes that probably exhibited only minor differences among races, resistance was considered to be race non-specific (Kommedahl et al. 1970). In contrast, Knowles and Houston (1955) and Knowles et al. (1956) demonstrated specific resistance to one particular isolate of flax wilt fungus when plants were grown in a sterile soil medium under glasshouse conditions. Absence of competition among isolates or races and a greater potential for infection in the glasshouse due to improved control over temperature and inoculum levels could explain the detection of race specific interactions in such studies. However, race specificity may also play a role under field conditions. For example, Kommedahl et al.(1970) reported that some varieties resistant at one field location proved susceptible when challenged with fungal populations at another location.

The present study was initiated to provide the genetic data required for bulked-segregant analysis aimed at identifying DNA markers tightly linked to wilt resistance genes in flax. The objective was to identify differences attributable to major gene effects between parents with contrasting resistance status. To maximise the opportunity to detect major gene effects the study analysed resistance under glasshouse conditions in a set of homozygous (doubled-haploid) recombinant lines grown in wilt nursery soil which had been super-inoculated with a mixture of fungal isolates. Field assessment of resistance was also undertaken to examine the value of glasshouse screening in predicting field response in the experimental population.

4.2 Materials and Methods

The recombinant doubled haploid population as described in Chapter Two was used for this study. The status of resistance to Fusarium wilt was determined for 143 DH lines under glasshouse and field conditions. The lines were screened under field conditions during the growing season of 1995/96 at a site near Warncourt (Victoria, Australia) that has been used for flax production since the 1940s and is heavily infested with the wilt pathogen. 100 seeds per DH line were sown in 2 m single-row plots with 2 replications and scored for resistance 3 times at regular intervals from the early seedling stage through to flowering. The level of field resistance was assessed by counting wilted plants up to a maximum of 10 plants per row and scoring the severity of chlorotic leaf symptoms. These observations were combined to classify each line into one of five categories. Upon completion of the trial the disease scores were averaged for each line. Scores from each replication were added to produce values ranging from 0 = resistant to 8 = susceptible. To allow for better comparison with glasshouse data, values were multiplied by 1.25 to convert them into final field scores ranging from 0= resistant to 10= susceptible.

The resistance status of DH and parental lines was also assessed under glasshouse conditions. Soil was used from the Warncourt wilt nursery, containing high levels of Fusarium inoculum, which had been super-inoculated with a mixture of fungal strains previously isolated from diseased plant stems. These isolates were recovered from diseased plant stems by placing 1 mm thick stem sections on potato-dextrose agar (half strength). All cultures were incubated in an alternating temperature regime, 25°C day/20°C night and 12 hr photoperiod (Burgess et al. 1988). After 3-4 days colonies of *F. oxysporum* were identified under the light microscope and agar plugs taken to inoculate 500 cm^3 of cereal straw (previously moistened with water and autoclaved for 20 min at 121°C). After 2 week incubation, the infected straw was dried to stimulate chlamydospore formation, mixed, ground up and used as inoculum. Approximately 30 plants per line were grown in single rows in 100 mm deep trays under constant temperature (25° C). At regular intervals the numbers of wilted plants were recorded and the diseased plants were removed from the trays. The final wilt score constituted the percentage of wilted individuals per line (0%= resistant to 100%= susceptible).

4.3 Results

Inheritance of Fusarium wilt resistance

Under glasshouse conditions the parental populations were clearly separated for levels of disease severity with 'Glenelg' exhibiting highly susceptible scores > 90%, while most of the scores for the resistant line CRZY8/RA91 were less than 40% (Fig. 4.1). The frequency distribution of disease severity amongst the DH lines suggests that three phenotypic classes exist, with a continuous spread of phenotypes evident within categories.

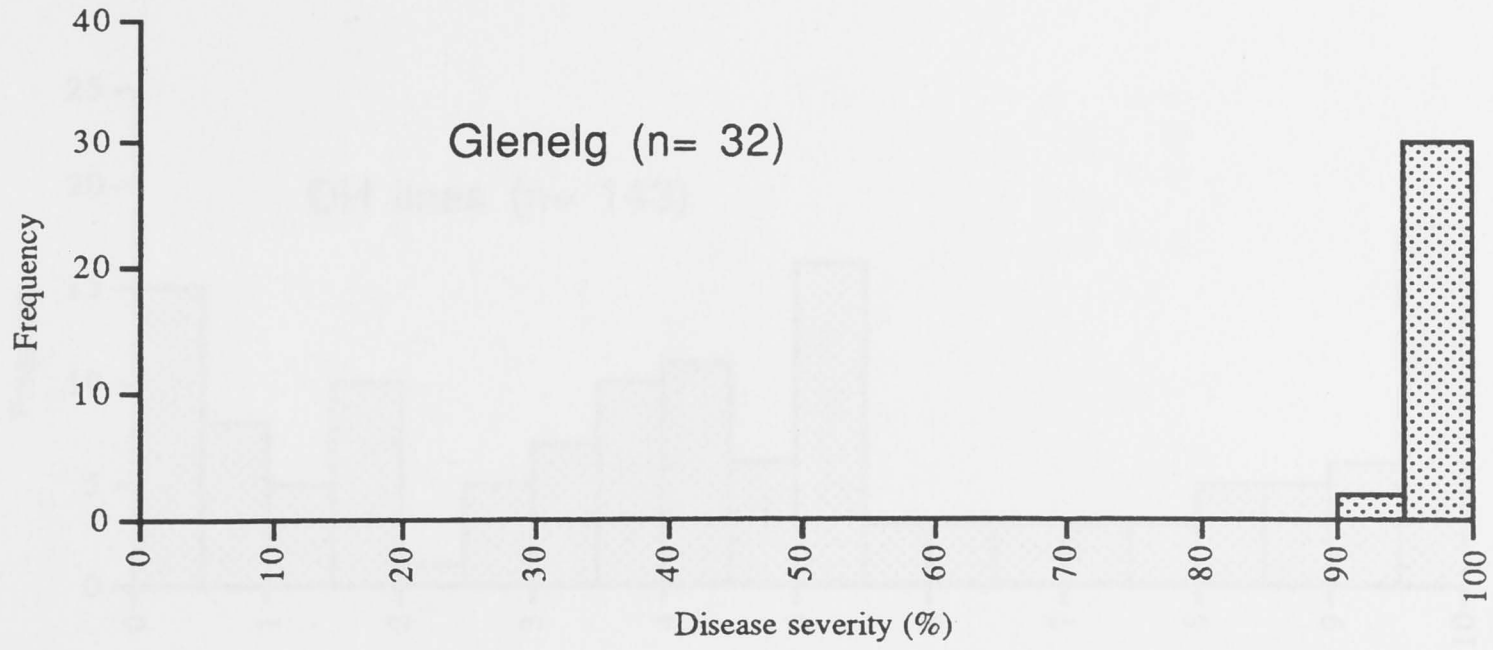
On the basis of the frequency distribution and the regression analysis DH lines that were grown in the glasshouse with disease scores of 0-29% were considered resistant, between 30% and 79% of intermediate phenotype,

while those in classes of 80% and greater were considered susceptible. The observed frequencies were tested against the expected ratio of 1:2:1, representing the resistant, intermediate and susceptible category respectively, for the segregation of two independent genes in homozygous F_2-derived recombinant lines. The segregation ratio indicated that Fusarium wilt resistance was likely to be determined by two major genes with additive effects (Table 4.1).

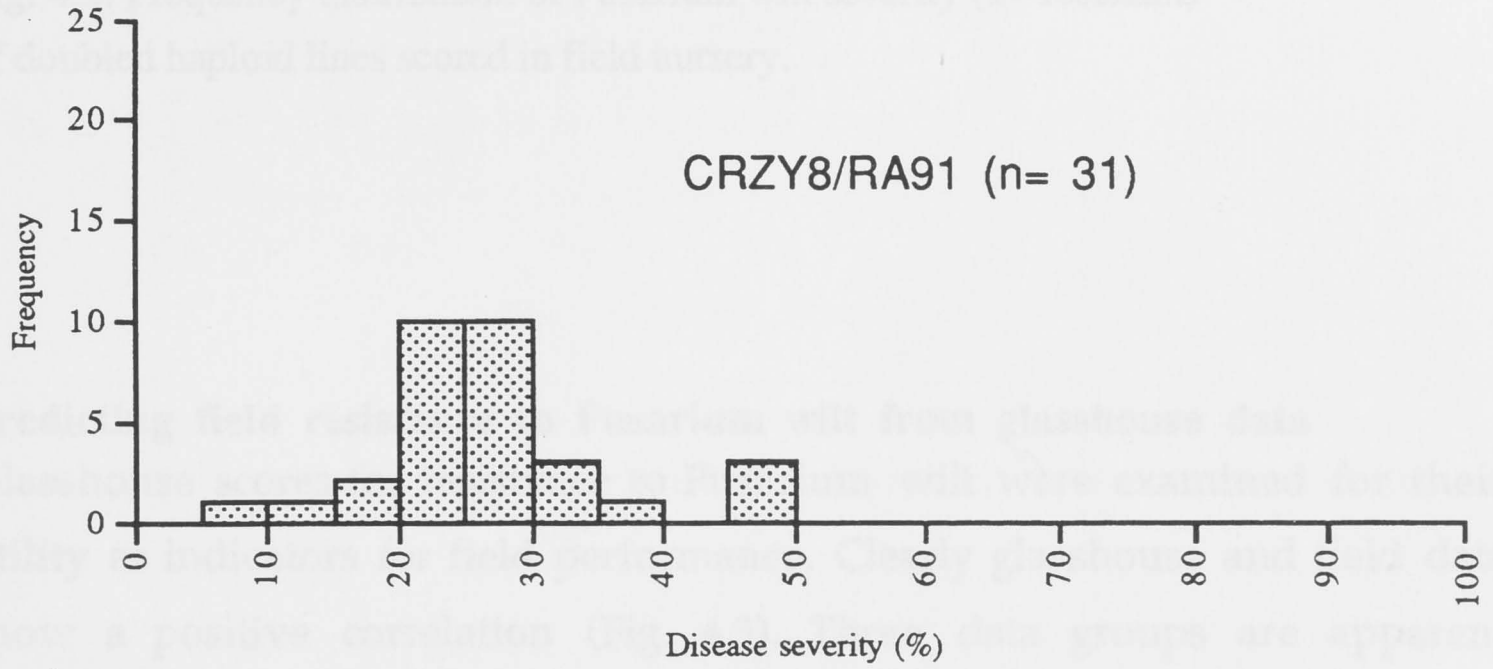
Table 4.1: Chi-square test of a two-gene hypothesis for segregation of Fusarium wilt resistance in doubled haploid progeny from a cross between 'CRZY8/RA91' and cv. 'Glenelg'.

		Observed frequencies					
	Location	resistant	intermed.	susceptible	tested ratio	Chi-squared	P-value
O_1	Glasshouse	31	66	46	1:2:1	3.9	0.25-0.1
O_2	Field	39	70	34	1:2:1	0.4	0.9-0.75
E		35.75	71.5	35.75			

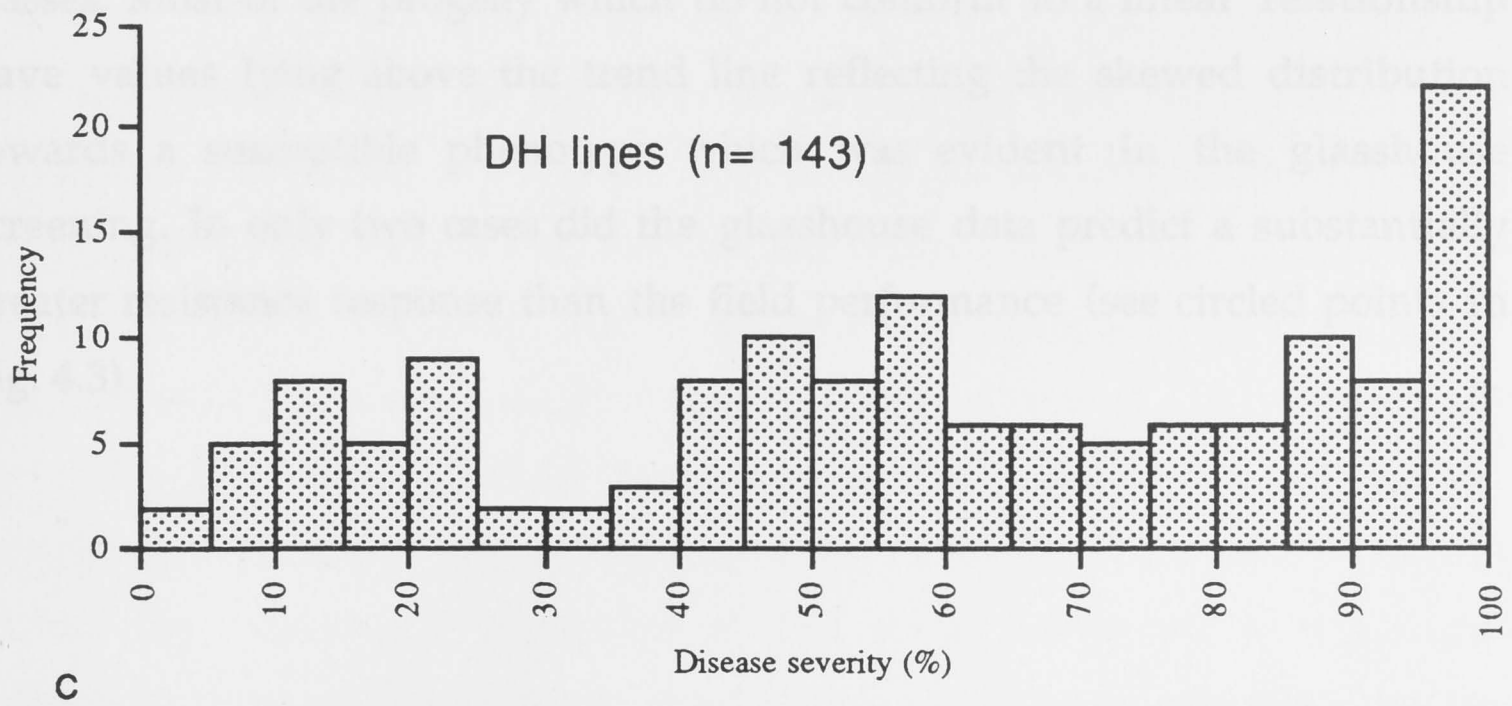
Analysis of Variance revealed that the average level of resistance displayed by the resistant DH lines was significantly ($P<0.05$) higher than that of the resistant parent indicating that additional resistance alleles from the susceptible parent might have contributed to the resistance response (Appendix 1). The wilt score data from the field nursery supported the hypothesis of a 1:2:1 segregation reflecting the glasshouse data in the appearance of three phenotypic classes (Fig. 4.2). Upon consideration of both the frequency distribution and the relationship of field data to the corresponding glasshouse scores DH lines exhibiting disease scores of less than 2.5 were considered resistant, between 2.5 and 7.9 of intermediate phenotype and plants with values of 8.0 and greater made up the susceptible category.



a



b



c

Fig. 4.1: Frequency distributions of Fusarium wilt severity (%) for parental (a+b) and doubled haploid lines (c) scored in super-inoculated soil in the glasshouse.

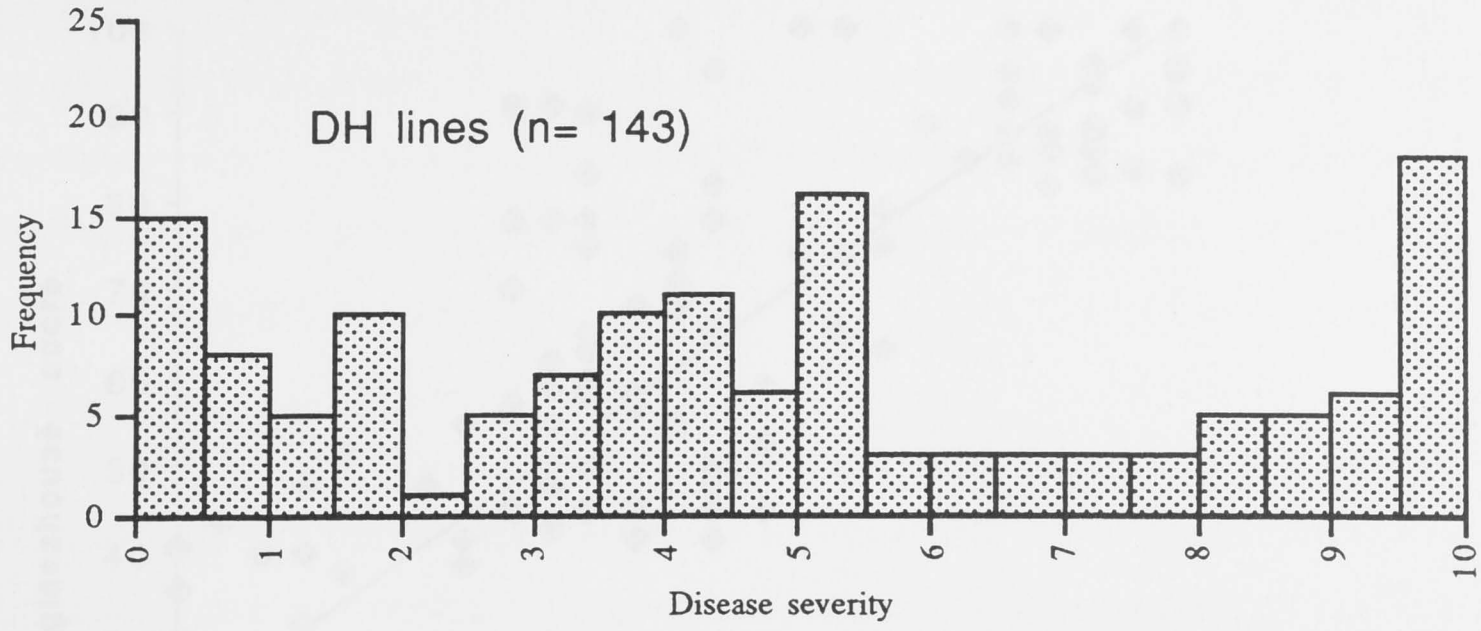


Fig. 4.2: Frequency distribution of Fusarium wilt severity (1= resistant) of doubled haploid lines scored in field nursery.

Predicting field resistance to Fusarium wilt from glasshouse data

Glasshouse scores for resistance to Fusarium wilt were examined for their utility as indicators for field performance. Clearly glasshouse and field data show a positive correlation (Fig. 4.3). Three data groups are apparent corresponding to the resistant, intermediate and susceptible phenotypic classes. Most of the progeny which do not conform to a linear relationship have values lying above the trend line reflecting the skewed distribution towards a susceptible phenotype which was evident in the glasshouse screening. In only two cases did the glasshouse data predict a substantially greater resistance response than the field performance (see circled points in Fig. 4.3).

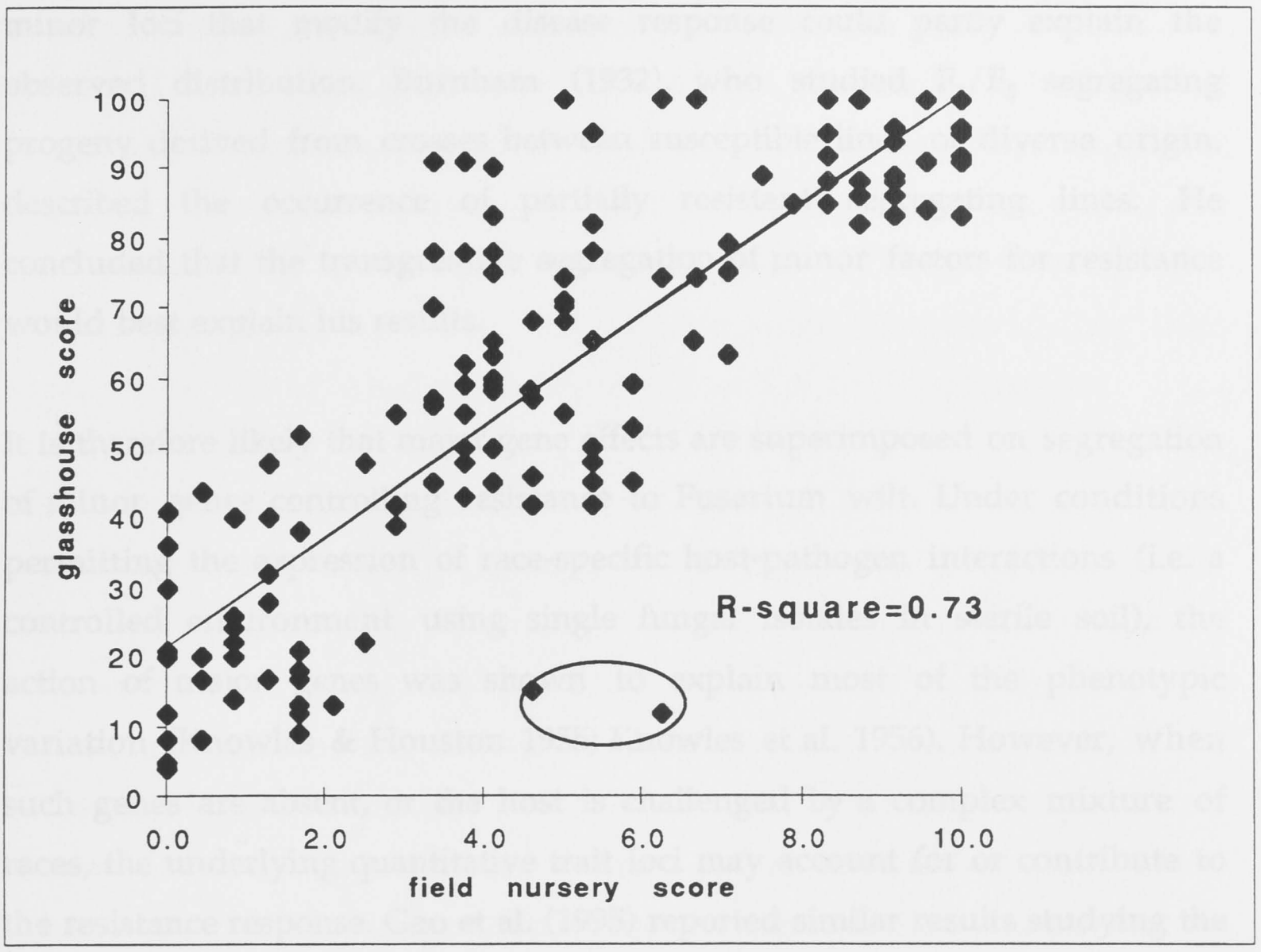


Fig. 4.3: Relationship of wilt scores between glasshouse and field nursery screenings.

4.4 Discussion

The segregation ratio in the DH lines suggests that resistance to the Warncourt population of Fusarium wilt in this particular cross between the Linola line CRZY8/RA91 and cv. Glenelg is predominantly controlled by two major genes. A similar level of variation within phenotypic categories was observed for glasshouse and field nursery screening. In view of the variability of reaction within the homozygous, resistant Linola parent, environmental effects apparently played a role in the observed variation within the phenotypic categories of DH lines. However, a significant number of resistant DH lines produced a more extreme phenotype than the resistant parent, suggesting that factors other than environmental influences contributed to the observed effect. The segregation of additional

minor loci that modify the disease response could partly explain the observed distribution. Burnham (1932), who studied F_2/F_3 segregating progeny derived from crosses between susceptible lines of diverse origin, described the occurrence of partially resistant segregating lines. He concluded that the transgressive segregation of minor factors for resistance would best explain his results.

It is therefore likely that major gene effects are superimposed on segregation of minor genes controlling resistance to Fusarium wilt. Under conditions permitting the expression of race-specific host-pathogen interactions (i.e. a controlled environment using single fungal isolates in sterile soil), the action of major genes was shown to explain most of the phenotypic variation (Knowles & Houston 1955; Knowles et al. 1956). However, when such genes are absent, or the host is challenged by a complex mixture of races, the underlying quantitative trait loci may account for or contribute to the resistance response. Gao et al. (1995) reported similar results studying the interaction of tomato and Fusarium wilt (*F. oxysporum* f.sp. *lycopersici*). They concluded that if a suitable single dominant gene for wilt resistance is absent, a polygenic complement would determine the response to infection by the pathogen.

The elimination of heterozygosity and hence genetic variation within individual recombinant lines through the use of doubled haploids assisted in elucidating the mode of inheritance to Fusarium wilt resistance. Studying F_2 segregating progeny would have introduced additional genotypic classes and hindered the dissection of phenotypic categories by obscuring the apparent differences between genotypes. In addition the disease reaction can be estimated with increased precision for each DH line compared with the accuracy of an individual estimate in F_2 progeny.

A reliable glasshouse screening method designed to predict field resistance to Fusarium wilt would be of considerable benefit to a flax or Linola

breeding program. The results in the present study indicate a strong positive correlation between the glasshouse and field data. In some cases lines grown in the glasshouse showed higher frequencies of diseased plants than they exhibited in the field. The increased infection frequencies in the glasshouse were probably due to the higher inoculum levels and higher average soil temperatures which favoured wilt development. Glasshouse data thus appear to be a reliable but conservative indicator of field performance.

This chapter concludes the first part of the thesis which was concerned with the development of a DH population, the inheritance study of Linola quality traits and resistance to Fusarium wilt. By elucidating the mode of inheritance, this study has provided the basis for investigating the molecular genetics of the corresponding wilt resistance mechanism. In the following chapters suitable molecular marker screening methods are employed to identify molecular markers linked to resistance loci. These markers constitute the first step in a series of strategies aimed at developing marker-assisted selection for a significant propotion of the resistance to an important fungal disease of this crop species.

Chapter Five

Targeting genomic regions containing Fusarium wilt resistance gene(s) in flax

5.1 Introduction

Homologous and heterologous DNA hybridisation

A growing number of plant resistance genes have been isolated from a wide range of crop species conferring resistance to a diverse spectrum of pathogens (Staskawicz et al. 1995). This class of resistance genes complies with the classical gene-for-gene concept originally proposed by Flor (1956) and provides the genetic basis for the specific recognition events involved in plant-pathogen interactions. For a host plant to resist an infection by a particular pathogen it must have a resistance gene that interacts, directly or indirectly, with a product of a corresponding avirulence gene from the pathogen (Lawrence et al. 1995). The subsequent resistance reaction nearly always is accompanied by an hypersensitive response (Keen 1990).

A comparison of amino acid sequences from the 'gene-for-gene' type of resistance genes which have so far been characterised revealed that they encode similar predicted protein products or contain conserved amino acid motifs. The following classes of resistance genes can be defined on the basis of their predicted products:

- serine-threonine protein kinase as in the *Pto* bacterial resistance gene of tomato (Martin et al. 1993),
- leucine-rich repeat protein as in the *Cf9* fungal resistance gene of tomato (Jones et al. 1994),
- proteins containing leucine-rich repeats and a nucleotide binding site as in the *RPS2* bacterial resistance gene of Arabidopsis (Bent et al. 1994; Mindrinos et al. 1994), mosaic virus resistance gene *N* of tobacco (Whitham et al. 1994), flax rust resistance gene *L6* (Lawrence et al. 1995) and Fusarium wilt resistance gene I_2 in tomato (Simons unpublished),

- proteins containing a serine-threonine protein kinase and leucine-rich repeats as in the *Xa-21* bacterial resistance gene of rice (Song et al. 1995).

It is thought that protein products encoded by resistance genes play either a direct role in the recognition of avirulence gene products or that they are part of the signal transduction pathway which is set in motion once binding of the appropriate elicitor molecule has occurred. Although conserved regions of gene products suggest a similar mode of action, their subcellular site of activity might differ (Lawrence et al. 1995).

Recent studies on the isolation of I_2, a resistance gene for Fusarium wilt in tomato (Simons, unpublished), showed that it contained similar sequence motifs (nucleotide binding site and leucine rich repeats) to the class of *L6*, *N* and *RPS2* resistance genes. Given the similarity of gene sequences for this class of resistance to diverse pathogens, this study investigated the possibility of using DNA clones derived from previously characterised resistance genes to detect loci that could potentially be involved in the resistance to Fusarium wilt in flax. DNA probes were evaluated which are associated with the *L6* rust resistance gene as well as heterologous DNA probes derived from resistance genes in *Triticum tauschii* and tomato.

PCR-based techniques

An alternative to DNA hybridisation is to adopt a PCR-based technique for targeting of individual loci containing flax wilt resistance genes. This approach makes no assumption about the nature of the fusarium wilt resistance gene(s) in flax. Without prior knowledge of relevant DNA sequence data or markers linked to the resistance trait, arbitrarily primed PCR (AP-PCR or RAPD) can be used to target specific genes of interest (Williams et al. 1990; Welsh and McClelland 1990). Such an approach necessitates the identification of a large number of marker loci. The difficulty this presents is one of scale; many markers need to be screened against many individual recombinant types derived from a segregating

population to achieve the objective (Hellens et al. 1993). Strategies have been developed to enhance the screening efficiency by reducing the number of assays required to locate additional and tightly linked marker loci. These methods include the construction of near-isogenic lines (Young et al. 1988; Martin et al. 1991; Paran et al. 1991) and the employment of DNA pooling strategies (Michelmore et al. 1991; Giovannoni et al. 1991; Hellens et al. 1993).

Near-isogenic lines can be used to quickly identify markers specific to target regions, but they constitute in themselves a highly specialised genetic population not suitable for deriving linkage estimates for the remainder of the genome. Since it was decided at an early stage of this project to generate a versatile segregating population which can be used to target several characters as well as provide linkage estimates, the development of near-isogenic lines was deemed inappropriate for this study.

An attractive alternative to using near-isogenic lines was first proposed by Michelmore et al. (1991) and named Bulked-segregant analysis (BSA) (Fig. 5.1). This technique involves combining DNA samples based on phenotype from resistant and susceptible segregants into separate DNA pools in equimolar amounts. Instead of screening individual segregants for marker loci, DNA pools are used as PCR templates containing allelic variants in target regions in an otherwise randomised heterogenous genetic background. Thus bulked DNA samples representing phenotypic extremes for the trait of interest can reduce the number of assays required to identify markers within the target region. This approach has successfully been applied to target major genes conferring various disease resistance in barley (Poulsen et al. 1995; Barua et al. 1993), *Triticum tauschii* (Eastwood et al. 1993), wheat (Hartl et al. 1995), oats (Penner et al. 1993), rice (Nair et al. 1995), sorghum (Oh et al. 1994), wild tomato species (Yaghoobi et al. 1995) and potato (Ballvora et al. 1995).

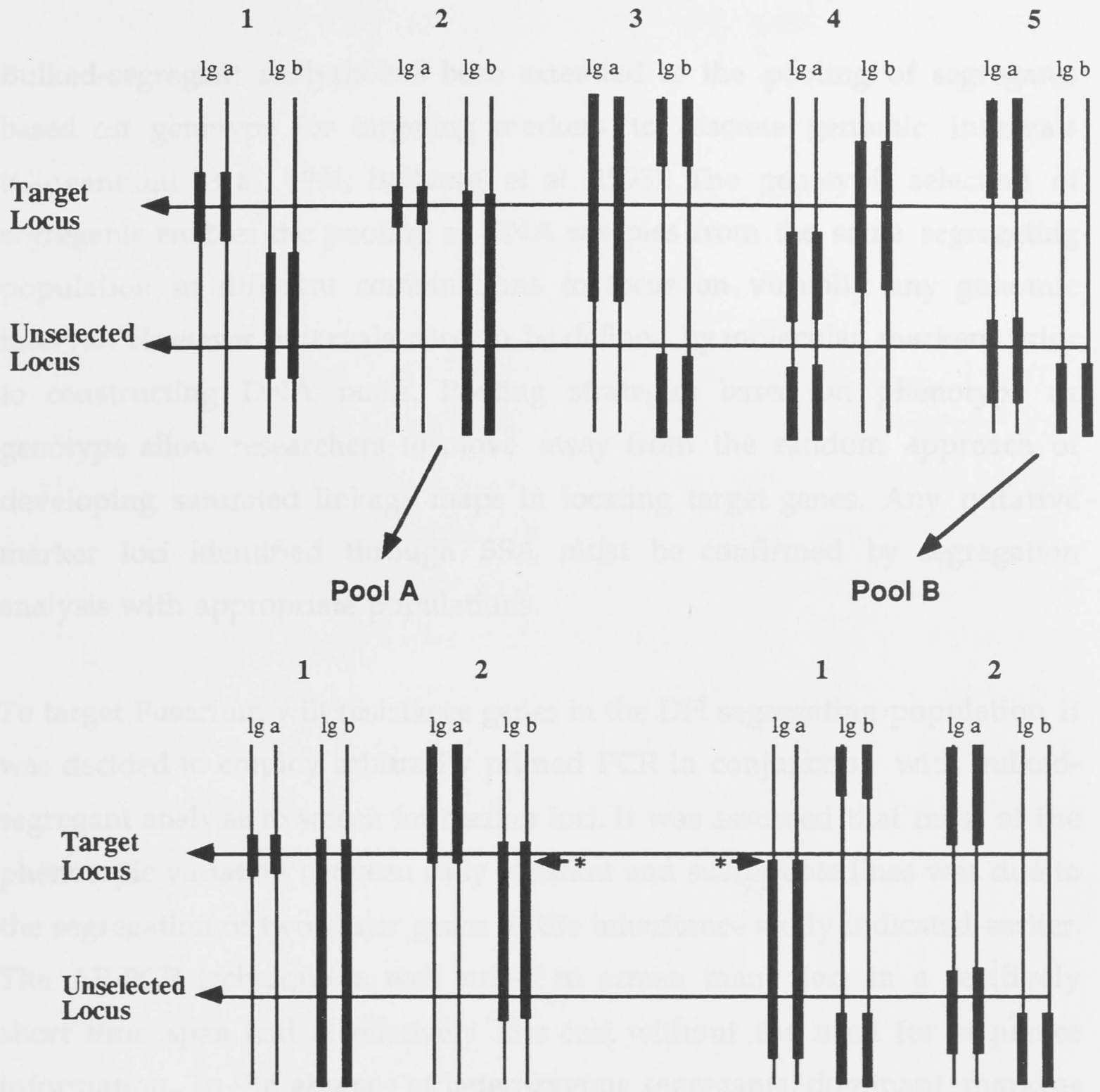


Fig. 5.1: Theoretical presentation of bulked-segregant analysis based on phenotype. The top row shows two linkage groups (lg a and lg b) containing one resistance locus per linkage group for five doubled haploid segregating lines. The black bars represent genomic regions inherited from the wilt resistant parent. Because pooling of DNA from segregating lines was done on the basis of extreme phenotype, it is assumed that only lines with similar allelic constitution at both target loci ended up in the resistant pool (A) and the susceptible pool (B). DH lines 1, 3 and 4 would have been scored as intermediates and excluded from DNA pools. Marker loci amplified from regions tightly linked to the target loci would be polymorphic between DNA pools provided sequence divergence exists between the resistant and the susceptible parent in the targeted region. However, recombination events close to the target loci can reduce the number of polymorphisms observed between DNA pools. Co-amplification from both linkage groups using the same primer due to sequence homology could further reduce the number of potential polymorphic markers (see asterisk).

Bulked-segregant analysis has been extended to the pooling of segregants based on genotype for targeting markers to discrete genomic intervals (Giovannoni et al. 1991; Ballvora et al. 1995). The genotypic selection of segregants enables the pooling of DNA samples from the same segregating population in different combinations to focus on virtually any genomic interval. However, intervals need to be defined by molecular markers prior to constructing DNA pools. Pooling strategies based on phenotype or genotype allow researchers to move away from the random approach of developing saturated linkage maps in locating target genes. Any putative marker loci identified through BSA must be confirmed by segregation analysis with appropriate populations.

To target Fusarium wilt resistance genes in the DH segregating population it was decided to employ arbitrarily primed PCR in conjunction with bulked-segregant analysis to screen for marker loci. It was assumed that most of the phenotypic variation between fully resistant and susceptible lines was due to the segregation of two major genes as the inheritance study indicated earlier. The AP-PCR technique is well suited to screen many loci in a relatively short time span and at relatively low cost without the need for sequence information. In the absence of heterozygous segregants, dominant markers (presence or absence) generated by AP-PCR become fully informative. DNA samples derived exclusively from homozygous lines reduce the chance of misclassified individuals occurring within DNA pools. Therefore, the use of doubled haploids, AP-PCR and bulked-segregant analysis complement each other in this marker study aimed at identifying linked markers rapidly and efficiently.

5.2 Materials and Methods

Plant material

A subset of 50 segregating DH lines from a total of 143 DH lines (as described in Chapter Two) were selected for marker analysis. The subset represented

individual lines showing phenotypic extremes with respect to their level of resistance to Fusarium wilt.

DNA extraction

Flax DNA was extracted from 2 week old leaf tissue using a CTAB extraction buffer as described by Ausubel et al. (1994).

DNA clones

Probe Lu-1 was derived from the promoter region of the rust resistance locus *L6* approximately 2 kb upstream of the start of the open reading frame (Fig. 5.2). Probes Lu-2 and Lu-3 were derived from within the open reading frame of *L6* and contain a nucleotide binding domain and a leucine-rich repeat region respectively. Probe cd15 was obtained from screening a cDNA library of *Triticum tauschii* and contains a leucine-rich repeat region with 87% identity to a genomic clone encoding a putative Cereal Cyst Nematode (CCN) resistance gene (Moullet et al. pers. comm.). The probe derived from the I_2 gene spans most of the open reading frame of the Fusarium wilt resistance in tomato.

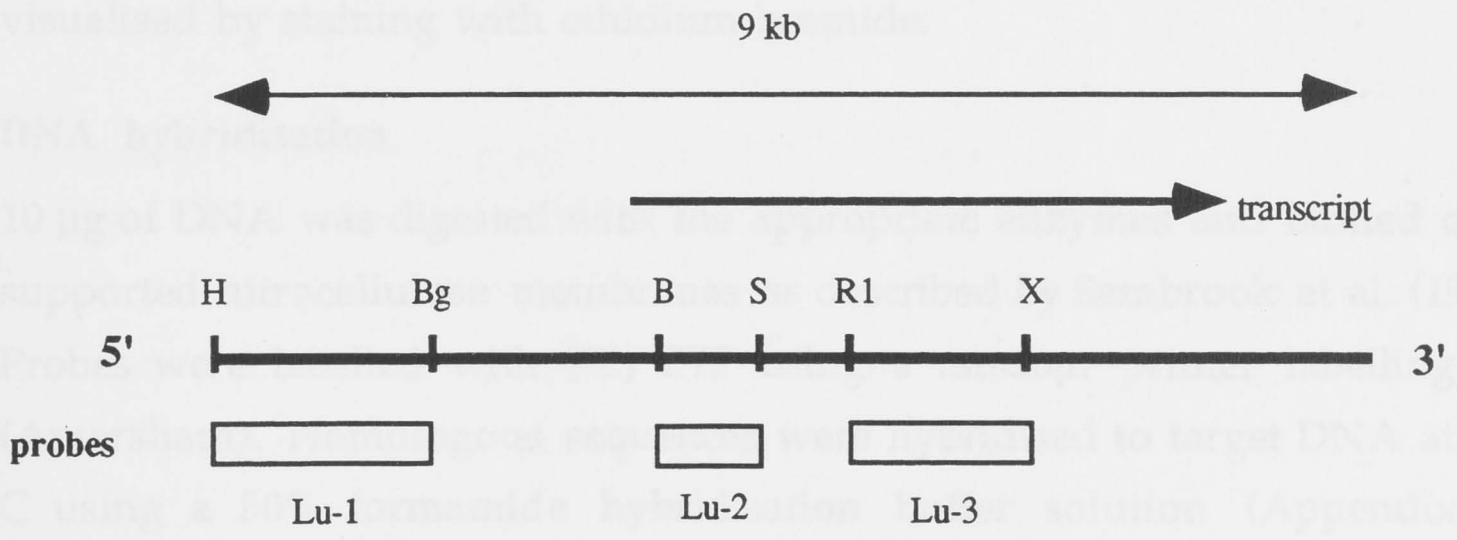


Fig. 5.2: Map of *L6* region and location of DNA probes. H, *HindIII*; Bg, *Bgl*, B, *BamHI*; S, *Sph I*, RI, *EcoRI*, X, *Xba I*. (adapted from Ellis et al. 1995).

DNA pools

DNA was extracted from approximately 1g of leaf tissue from 25 resistant and 25 susceptible DH lines. Two sets of one resistant and one susceptible

DNA pool were constructed from equimolar amounts of DNA samples derived from 10 resistant or 10 susceptible lines.

AP-PCR analysis

DNA polymorphisms between pooled samples were examined using 10mer random primers supplied by Operon Technology Inc. and University of British Colombia. More than 700 random primers were screened against DNA pools. Standard 10 µl reaction mixtures contained 50 ng of DNA template, 10 ng of primer, 2mM of dNTPs, 0.5 unit of Taq polymerase (Perkin Elmer), 10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin. The PCR amplifications were performed in microtitre plates using a Corbett Research Thermal Sequencer FTS-960. The following PCR protocol was used: Denaturation at 92°C for 2 min. (after cycle 1, 30 sec. of denaturation was used), annealing at 38°C for 30 sec. and extension at 72°C for 1 min. and a final extension time of 5 min. A total of 30 cycles were used under standard conditions.

The entire reaction mixtures were loaded on 1.5% agarose gels (TAE) and PCR products were resolved by electrophoresis in 1x TAE buffer and visualised by staining with ethidium bromide.

DNA hybridisation

10 µg of DNA was digested with the appropriate enzymes and blotted onto supported nitrocellulose membranes as described by Sambrook et al. (1989). Probes were labelled with [^{32}P] CTP using a random primer labelling kit (Amersham). Homologous sequences were hybridised to target DNA at 42° C using a 50% formamide hybridisation buffer solution (Appendix 2). Heterologous probing, when using the tomato and *Triticum tauschii* clones, was carried out under conditions of low stringency at 42°C with 25% formamide hybridisation buffer (Appendix 2). Membranes were washed twice with 2% SSC, 0.1% SDS for 30 min and once with 1% SSC, 0.1% SDS for 30 min at 65°C for standard conditions. Membranes hybridised under low stringency were washed at 58°C omitting the final 1% SSC, 0.1% SDS washing step. Filter membranes were exposed to X-ray film for 5 to 14 days.

Screening of genomic library

A genomic library of flax (Forge) was obtained from Dr. J. Finnegan CSIRO Plant Industry, Australia. The library was made from size-fractionated DNA partially digested with *Sau3A* and cloned into the bacteriophage λ vector EMBL4 (Lawrence et al. 1995). This library was screened under conditions of low stringency with probe cd15. Two lambda clones which hybridised to the probe were isolated and purified. Upon digestion with restriction enzyme *BamHI* both clones exhibited similar restriction patterns. The digested DNA was blotted on a membrane filter and hybridised to probe cd15 which detected a 4.4 kb DNA fragment. This 4.4 kb DNA fragment was ligated into Bluescript vector (Stratagene) using the *BamHI* cloning site and transformed into *E. coli*. Blue/white selection and colony blot hybridisation was used to recover the target DNA fragment. Purification of the 4.4 kb DNA insert provided the flax probe Ltt. Using primers for conserved motifs that are characteristic for a class of disease resistance genes containing nucleotide binding domains as well as leucine rich repeat regions, it was confirmed that the Ltt clone most likely contains a resistance gene-like sequence. When used as a probe Ltt detected RFLP loci *pr-1, pr-2, pr-3* and *pr-4*.

Heterologous probing using I_2 gene from tomato

Parental DNA digested with restriction enzymes *BamHI, DraI, HindIII, EcoRI, EcoRV* and *XbaI* was hybridised under low stringency with a DNA probe derived from the I_2 Fusarium wilt resistance gene isolated from tomato (Simons, pers. comm.).

5.3 Results

DNA hybridisation experiments

Probe Lu-1 derived from the promoter region of *L6* detects a single major fragment as was predicted from results obtained by Lawrence et al. (1995). An RFLP marker associated with this major band was detected in the present study and shown to be unlinked to wilt resistance. Probe Lu-3 derived from

the leucine rich repeat domain of *L6* hybridised with a complex fragment pattern (Fig. 5.3). Similar results obtained by Lawrence et al. (1995) prompted speculation that *L6* is a member of a multigene family. Subsequent RFLP analysis by Ellis et al. (1995) showed that most of the fragments detected by Lu-3 map to the unlinked and genetically complex rust resistance locus *M*. The RFLP markers detected by Lu-3 in this study were not linked to Fusarium wilt resistance when screened on DH lines (data not shown). Hybridisation of an additional probe Lu-2 containing a nucleotide binding domain of *L6* produced a less complex pattern than probe Lu-3. The polymorphic fragment detected by probe Lu-2 showed similar hybridisation strength and mobility as probe Lu-1 when analysed on parental lines and hence was not further characterised on segregating progeny.

The clone cd15 was isolated from a cDNA library of *Triticum tauschii* and is part of a putative resistance gene for CCN containing a leucine rich repeat region which is characteristic of resistance gene-like sequences (Moullet et al. pers.comm.). It hybridises under low stringency to flax DNA (Fig. 5.4), but when used on segregating progeny the hybridisation pattern was weak and ambiguous. Probe cd15 was used to screen a genomic library of flax with the aim of isolating related sequences which can be evaluated on recombinant DH lines. The library screen yielded one lambda clone from which a subclone of 4.4 kb was generated. The subclone, designated Ltt, was used as a hybridisation probe to the parental lines revealing RFLP patterns associated with the restriction enzymes *BamHI, DraI, EcoRI* and *EcoRV*. The RFLPs detected by the enzyme *EcoRI* were further characterised on 50 DH lines. Four independent RFLP markers (*pr1, pr2, pr3, pr4*) were detected, none of which show linkage to the wilt resistant phenotype (Fig. 5.5).

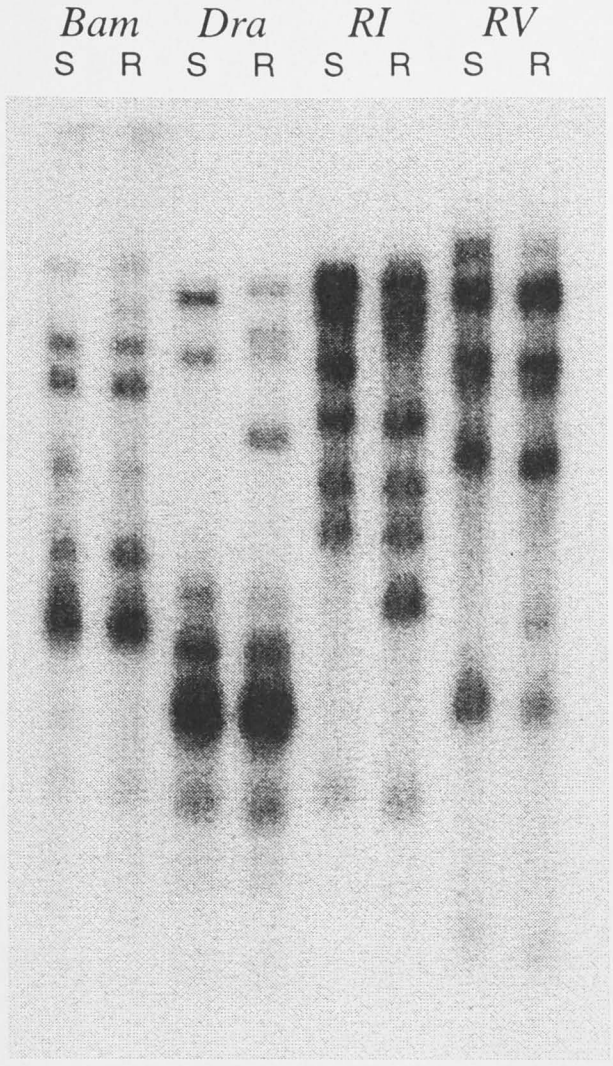


Fig. 5.3: DNA hybridisation of flax probe Lu-3 derived from the leucine rich repeat domain of L6 to parental lines (S= susceptible parent; R= resistant parent) digested with four restriction enzymes Bam= BamHI, Dra= DraI, RI= EcoRI, RV= EcoRV. RFLP markers detected with the EcoRI digest were unlinked to Fusarium wilt resistance.

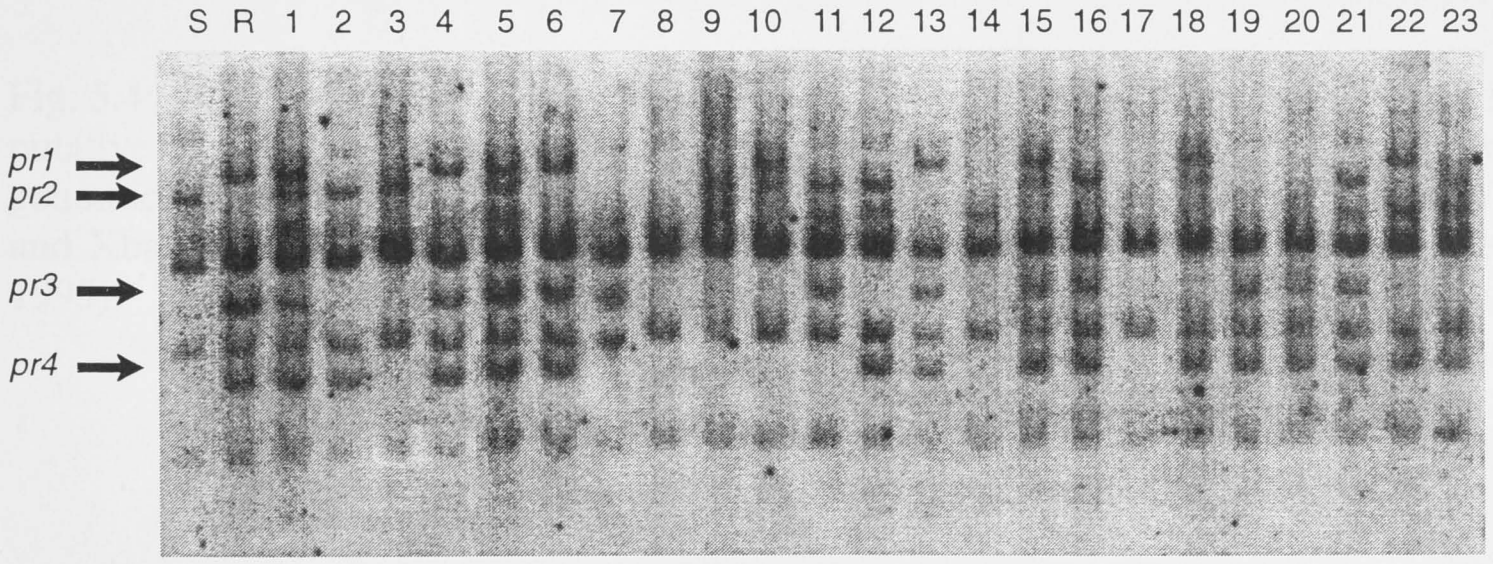


Fig. 5.5: DNA hybridisation of flax probe Ltt to parental lines (lane S= susceptible parent; lane R= resistant parent) and DH segregating lines (lane 1-23) detecting 4 RFLP markers *pr1*, *pr2*, *pr3* and *pr4*. DNA clone pr was isolated from a flax genomic library using probe cd15 from *Triticum tauschii* which contains conserved motifs characteristic for plant resistance gene sequences.

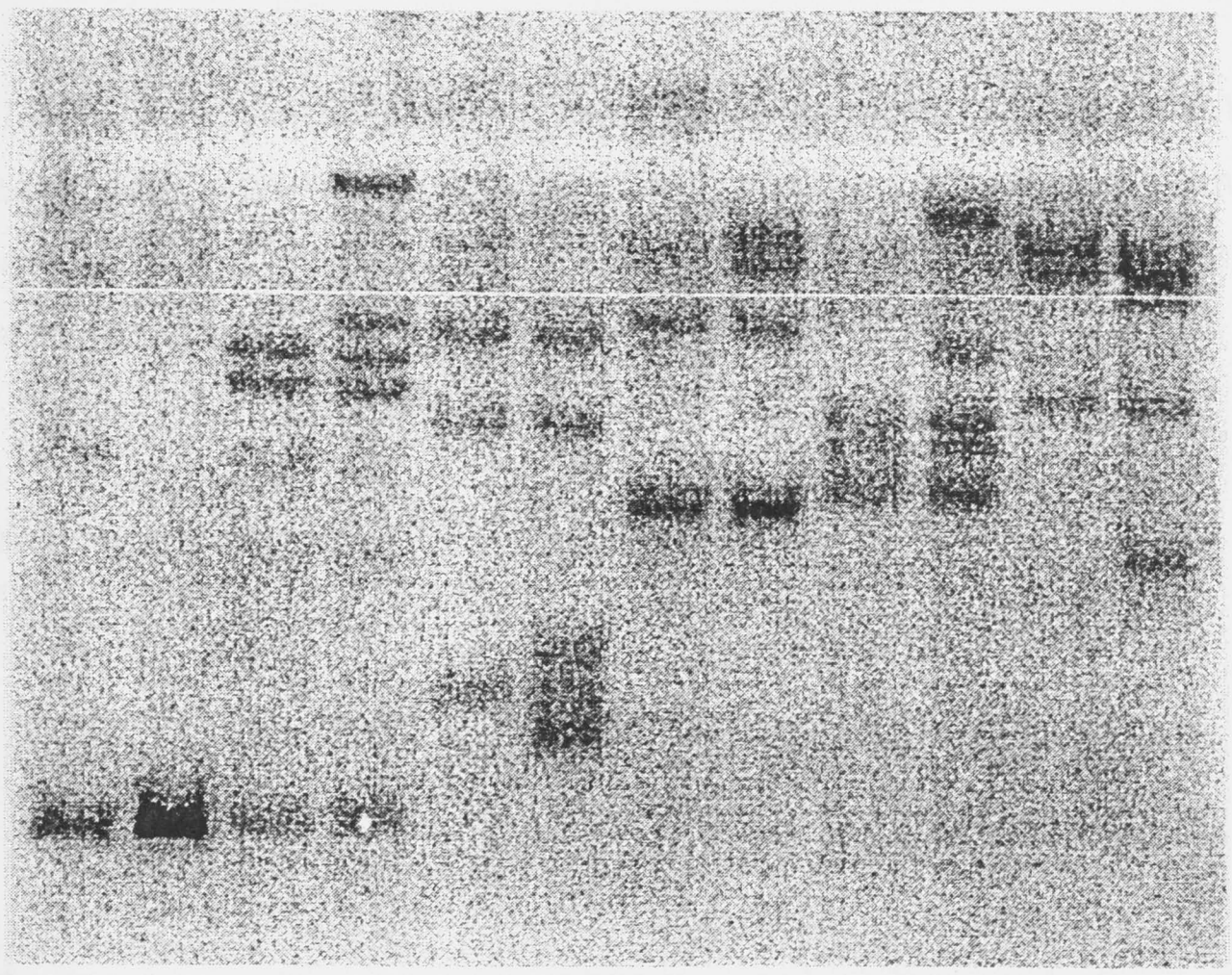


Fig. 5.4: Low stringency hybridisation of DNA probe *cd15*, a clone derived from a putative Cereal Cyst Nematode resistance gene isolated from *Triticum tauschii*, to flax genomic DNA digested with BamHI (B), DraI (D), HindIII (H), EcoRI (RI), EcoRV (RV), and XbaI (X). DNA from Forge (F) and Hosh (H) flax lines was used (Lawrence *et al.* 1995).

Heterologous probing using a clone derived from the I_2 gene conferring resistance to Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) in tomato failed to hybridise under low stringency to parental DNA. Since this work was carried out courtesy of Guus Simons at Keygene in Holland, it was not possible to independently verify these results. Therefore, it remains uncertain whether the outcome was due to experimental error or lack of sufficient homology.

PCR-based technique

The level of polymorphism between the two parents, 'CRZY8/RA91' and 'Glenelg', was assessed by screening DNA samples using approximately 60 random primers. Approximately 10% of primers detected polymorphisms between parents.

Table 5.1: Percent recombination between amplification products generated by 10mer random primers and wilt resistance tested on 20 DH lines.

primer		% recombination
U.B.C.	62	33
"	68	30
"	316	31
Operon Tech.	J13	50
"	M12	42
"	X20	31

Approximately 700 primers were used in amplification from DNA pools derived from resistant and susceptible DH lines. One random primer yielded on average 4.5 major amplification products and 2.5 minor products (Fig. 5.6). The fragment size of PCR products ranged from 0.5 to 2 kb and were considered to represent distinct genetic loci. Generally, major bands proved reproducible whereas weak bands were not. Hence scoring of polymorphisms was restricted to the presence or absence of major bands.

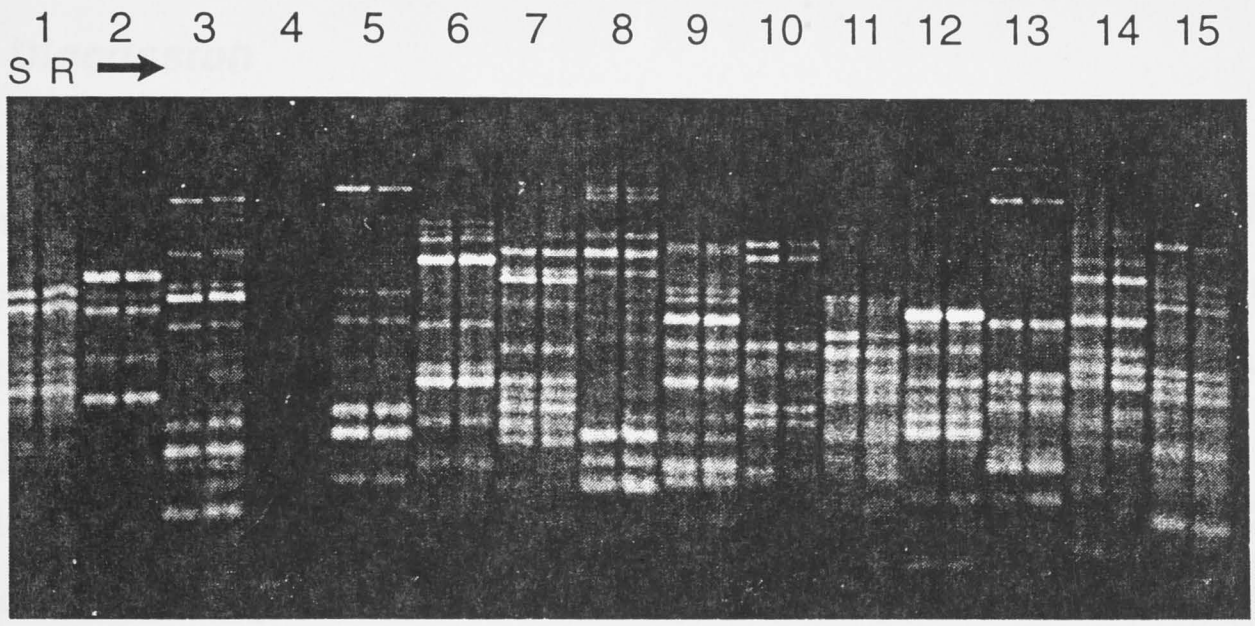


Fig. 5.6: Amplification profiles using 10mer random primers 1-15 (Operon Technologies Inc.) on DNA pools consisting of equimolar amounts of DNA from 10 susceptible doubled haploid lines (S) and 10 resistant lines (R).

Amplification of 6 polymorphic marker bands generated by 6 separate primers was reproducible between DNA pools representing phenotypic extremes. When these polymorphic markers were screened on individual DH lines present in the two bulks, marker loci were either not linked at all or showed only loose linkage to genes controlling wilt resistance. Skewed segregation accounted for the independent segregation patterns observed amongst individual DH lines of marker bands generated by primers OPM12 and OPX20 (Table 5.1). The remaining four unlinked marker loci were probably generated from common regions within pools other than the target loci.

5.4 Discussion

DNA hybridisation

None of the DNA probes associated with the rust resistance locus *L* detected genomic fragments linked to Fusarium wilt resistance. These findings do not come as a surprise. Although homology is high between *L* and another independent rust resistance locus *M*, reflecting perhaps a common ancestral origin, the same is not true for other rust loci such as *K* and *P* which are not detected with sequences derived from either *M* or *L* (Ellis et al. 1995). If lack of homology prevents the detection of additional loci conferring different rust specificities, then the chance of discovering unrelated resistance loci can be considered low.

The cross hybridisation of probe cd15 to genomic flax DNA raised the possibility of detecting related resistance genes in flax. Comparison of coding sequences of *L6* and putative CCN resistance gene yielded a maximum level of identity of 53% over a 130 base pair overlap. Even under low stringent conditions this level of sequence homology is unlikely to result in hybridisation, hence reducing the chances of targeting *L6* using probe cd15. This assumption was confirmed by the absence of linkage between the *L6* and *pr* RFLP markers detected by probe Ltt. Since RFLP markers associated

with probe Ltt were not linked to Fusarium wilt resistance, this clone was not further characterised.

Similar DNA hybridisation experiments carried out by other researchers have generally not led to the isolation of additional resistance loci within the same species or across species boundaries (Martin et al. 1993; Mindrinos et al. 1994). Sufficient sequence divergence even between genes encoding resistances to the same pathogen has prevented this approach from being an effective tool. However, an alternative approach using PCR or RT-PCR to amplify regions containing conserved motifs has yielded numerous DNA clones which are associated with resistance loci using appropriate segregating populations (Seah et al. pers. comm.; Gebhardt et al. unpublished; Kanazin et al. 1996; Yu et al. 1996). This method holds great promise for the future as a means to identify related genes within resistance gene families.

PCR-based approach

Although more than 700 random primers were screened on several pools made up of DNA from different combinations of resistant and susceptible lines, marker loci linked to the phenotypic trait could not be identified. Other studies which employed bulked-segregant analysis to target major resistance genes in more complex genomes than *L. usitatissimum* usually screened less than 700 primers to identify marker loci (Nair et al. 1995; Oh et al. 1994). It is therefore improbable that the number of primers used was a limiting factor during the screening process. The level of DNA polymorphism between parental lines was within expectations for a self-pollinating crop species. The number of segregating loci is unlikely to have contributed to the failure to identify linkage between potential markers and the trait.

The mode of inheritance of Fusarium wilt resistance was elucidated for this recombinant DH population. Most of the phenotypic variation was

explained by the action of two major genes (Chapter Four). In previous studies bulked-segregant analysis has been predominantly employed to target single major genes. However, in utilising fully homozygous lines in this study, it was assumed that DNA from each individual line represented in the resistant pool contained the resistant alleles at both major loci. This assumption was a reasonable one, since DNA was used exclusively from DH lines exhibiting extreme phenotypes. Failure to meet this criterion would have resulted in an increased likelihood that the two pools did not differ for alleles linked to the target regions. For instance, if the presence of resistance alleles at only one locus confers a resistant phenotype then DNA pools derived from 10 resistant lines are expected to contain a mixture of genotypes at both loci. This would eliminate the possibility of finding polymorphisms linked to target genes.

The occurrence of false positive PCR-derived markers indicated that pools shared areas of homozygosity other than the targeted regions. An increase in the number of pool members would have reduced the occurrence of these non-target PCR products, but would have also raised the probability of individuals being represented in DNA pools with double crossovers between gene and potential marker loci. These double crossover events could reduce or eliminate the chance of finding polymorphic markers in affected chromosomal regions (Giovannoni et al. 1991).

A possible explanation for failing to amplify linked marker loci may be related to the postulated tetraploid origin of the species. There is evidence to suggest that two chromosomal complements exist within the flax genome reflecting the ancient tetraploid origin of the species (Cullis 1981). If the two target loci are located at the corresponding positions on separate constituent genomes, regions flanking the two target genes would be expected to share some level of homology. This could result in the co-amplification of marker loci from both target regions using the same random primer potentially reducing the chance of identifying tightly linked marker loci (Fig. 5.1). DNA

sequence analysis by Ellis et al. (1995) of two independent rust resistance loci, *M* and *L*, showed that the coding sequences were highly conserved despite obvious differences in race specificity and gene structure. This finding prompted speculations about the origin of these genes, postulating that they are the result of a duplication event involving an ancient basic *Linum* genome. Putative duplicated loci have also been investigated within the soybean genome. Comparison of the DNA sequence of functional genes revealed a high level of sequence conservation within 1.5 kb of the duplicated loci followed by little or no homology outside this region (Polzin et al. 1994). This suggests that at least in the soybean genome the effect of co-amplification of PCR products from 'homoeologous' loci would be negligible.

Chapter Six

Construction of an AFLP linkage map of *Linum usitatissimum*

6.1 Introduction

Flax is a model organism in classical genetics to elucidate the basis for the specific recognition events involved in host-pathogen interactions between the flax host plant and the rust pathogen, *Melamspora lini* (Flor 1956). More recently this system is used in molecular genetics to isolate genes conferring resistance to flax rust (Lawrence et al. 1995). However, crop improvement of this species relies on an understanding of the genetic control of other important agronomic traits such as yield potential, resistance to lodging and to the soil-borne fungal disease Fusarium wilt (*Fusarium oxysporum* lini). Molecular marker technology enables the construction of comprehensive genetic linkage maps, thereby allowing the dissection of complex traits such as resistance to Fusarium wilt into their genetic components.

Genetic linkage maps of plant species constructed thus far using molecular markers have been based primarily on the RFLP technique (Helentjaris et al. 1986; McCouch et al. 1988; Graner et al. 1991; Gebhardt et al. 1991; Tanksley et al. 1992; Ferreira et al. 1994). The co-dominant mode of inheritance, reliability and locus specificity, are still the principal reasons behind the continued use of this class of marker in mapping studies. The advent of PCR-based marker systems provides new opportunities to identify a large number of DNA polymorphisms within genomes of autogamous species, such as *L. usitatissimum*. In particular, the recent introduction of the AFLP technique allows the identification of a sufficient number of markers to construct moderately saturated linkage maps in time spans of less than six months (Vos et al. 1995). This method relies on the enzymatic digestion of genomic DNA and ligation of appropriate adapters. The adapters serve as

priming sites for subsequent selective amplification of ligated DNA fragments with different primer combinations. The very stringent PCR conditions allow good reproducibility of amplification products. AFLP markers are transferable between mapping projects when locus specificity is assumed and mobility of DNA fragments accurately assessed with appropriate DNA markers (van Eck et al. 1995). The AFLP technique was used by Becker et al. (1995) together with RFLP markers to construct a linkage map of barley. AFLP markers were found to map throughout the barley genome filling in 'gaps' on several chromosomes.

The estimated genome size of *L. usitatissimum*, which is considered to be an ancient tetraploid (2n=30), is relatively small ($7.0x10^8$ bp/1C). Preliminary linkage mapping of the species has so far identified approximately 19 RFLP and 69 RAPD markers covering 15 expected linkage groups (Cullis et al. 1995). Other linkages have been reported, for example between rust resistance genes (Islam and Mayo 1990) and between isozyme markers and rust resistance loci (Cullis et al. 1992). The current study has generated a more comprehensive linkage map of the species *L. usitatissimum* by characterising AFLP and RFLP markers in a recombinant DH population. Employing a population comprised exclusively of homozygous genotypes together with a dominant marker system such as AFLP enabled those markers to be fully informative. In the previous chapter, molecular markers identified were limited to small portions of the genome and proved unsuccessful in detecting linkage to Fusarium wilt resistance. The mapping approach reported in this chapter represents part of a more concerted effort to identify chromosomal regions contributing to Fusarium wilt resistance in flax.

6.2 Materials and Methods

Plant material

A mapping population of 59 DH lines was selected on the basis of lines displaying either a resistant or susceptible phenotype in response to

Fusarium wilt. This number of DH lines was chosen, because the polyacrylamide gel apparatus used for the AFLP analysis could accommodate a maximum of 59 DNA samples. Doubled haploids were developed from polyembryonic F2 seed as described in Chapter Two.

Generation of AFLP markers

The method was based on the protocol published by Vos et al. (1995) with only minor modifications. The amount of genomic DNA used in the restriction digest was increased from approximately 0.5 µg to 2 µg and final PCR amplification conditions were altered slightly (see below).

Restriction enzymes, adapters and oligonucleotide primers

Two restriction enzyme combinations were used to generate separate fragment pools for analysis. Mapping of marker loci was initially performed using amplification products derived from EcoRI/Mse1 digested genomic DNA, these were later supplemented by markers derived from a PstI/MseI enzyme combination. Adapter and oligonucleotide sequences were synthesised at CSIRO Plant Industry, Australia. Sense and anti-sense oligonucleotide strands were annealed to form double stranded adapters by heat denaturing (95°C for 5 min.) the mixture followed by cooling to room temperature.

Adapter sequences are designed to ligate to sticky ends without restoring the enzyme restriction site. The structure of adapters are:

MseI adapter
```
5-GACGATGAGTCCTGAG
      TACTCAGGACTCAT-5
```

EcoRI adapter
```
5-CTCGTAGACTGCGTACC
     CATCTGACGCATGGTTAA-5
```

PstI adapter
```
5-CACGATGGATCCAGTGCA
GACGTGCTACCTAGGTC-5
```

AFLP primers are designed to anneal to adapter sequences by recognising the core sequence (Core), the enzyme specific sequence (Enz) and selective nucleotides (Ext) (Vos et al. 1995).

The structure of primers with three selective nucleotide attached are:

	Core	Enz	Ext
MseI	5-GATGAGTCCTGAG	TAA	NNN-3
EcoRI	5-GACTGCGTACC	AATTC	NNN-3
PstI	5-GATGGATCCAG	TGCAG	NNN-3

Nomenclature of AFLP marker loci

AFLP profiles were generated using primers with three selective nucleotides attached. AFLP marker loci were prefixed with 'af' and assigned capital letters encoding the specific primer combination (captial letter X denotes loci derived from Pst1/Mse1 enzyme combination) followed by a number describing the polymorphic marker band scored (Table 6.1). The lower case letter 's' as suffix denotes that these marker loci were not segregating according to the expected ratio ($P<0.05$).

Nomenclature of RFLP and morphological marker loci

Descriptions of marker loci without the prefix 'af' were used for loci generated by RFLP analysis with the exception of 'scol', 'fcol' and 'd15' which describe loci for seed colour, flower colour and Δ15 desaturase gene respectively.

Amplification of ligated DNA fragments

A two-step amplification method was used as described by Vos et al. (1995). During the pre-amplification step oligonucleotide primers were employed with one selective nucleotide attached to the 3' terminus. The PCR temperature profile was identical to conditions described by Vos et al. (1995). The pre-amplified DNA was diluted 1:10 with double-distilled water before being amplified a second time using oligonucleotide primers with three selective nucleotides attached (Table 6.1). During the second round of amplification the EcoRI or PstI primers were [^{33}P] ATP end-labelled. The 'touch down' PCR conditions consisted of a total of 36 cycles with constant

denaturation steps (30s, 94° C) and extension steps (60s, 72° C). The duration of annealing steps were kept constant at 60s. The annealing temperature for the first cycle was 65°C which was reduced to 64°C for cycle 2 and 3, to 63°C for cycle 4, to 62°C for cycle 5 and 6, to 61°C for cycle 7, to 60°C for cycle 8 and 9, to 59°C for cycle 10, to 58°C for cycle 11 and 12, and to 57°C for the remaining 24 cycles.

Gel analysis

The PCR products were separated on 5% denaturing polyacrylamide gels run in 1x TBE buffer pre-heated to 65°C at 25 W for 2.5 hours. The gels were fixed in 10% acetic acid/20% methanol for 30 min, dried at 65°C for 4 hours and exposed to X-ray film for 2 to 3 days.

RFLP analysis

DNA hybridisation was carried out as described in Chapter Five. Thirteen previously characterised genomic clones were supplied by Cullis (pers. comm.), one of which identified RFLP marker *cc94*, which maps to linkage group 7. A probe Δ15 containing the open reading frame of a flax Δ15 desaturase gene was provided by Singh (pers. comm.) and detected the RFLP marker Δ15 on linkage group 10. Two DNA fragments, (Lu-1) and (X-22), linked to flax rust resistance loci *L* and *M* respectively were obtained from J. G. Ellis (pers. communication). An additional clone from flax (Ltt) isolated by heterologous probing using a putative Cereal Cyst Nematode (CCN) resistance gene from *Triticum tauschii* detected RFLPs *pr1, pr3* and *pr4* (Moullet et al. pers. comm.).

Map construction

Linkage analysis was performed using 'MapMaker/EXP 3.0' software (Lincoln et al. 1993). Markers were grouped according to two-point analysis (LOD score > 4.0) and framework orders were established in most cases with LOD score > 3.0, except in linkage groups which comprised entirely of markers deviating significantly from the expected 1:1 ratio (LOD score > 2.5).

Table 6.1: Selective nucleotides of the 21 MseI/EcoRI primer combinations and 24 MseI/PstI primer combinations used to generate AFLP marker loci.

Code	MseI	EcoRI	Code	MseI	PstI
A	GCG	AGT	XA	GAC	AAG
B	GAG	AGT	XB	GAA	AGT
C	GCT	AGT	XC	GAC	AGT
D	GCA	AGA	XD	GAG	AAG
E	GAT	AGA	XE	GAG	ACA
F	GCT	AGA	XF	GAT	AAG
G	GAT	AGT	XG	GAA	ATC
H	GCA	AAC	XH	GAC	ATC
I	GAC	AAC	XI	GCC	ACA
J	GAC	ACT	XJ	GAT	AGT
K	GAT	ACA	XK	GAA	AAG
L	GCC	ACG	XL	GCG	AGT
M	GAG	ACC	XM	GCG	ACA
N	GAT	ATC	XN	GCT	ACA
O	GAA	ATC	XO	GAA	ACA
P	GAT	ACG	XP	GCG	AAG
Q	GCT	ACG	XQ	GTC	ACA
R	GCG	ATG	XR	GTT	ACA
S	GAG	ATC	XS	GTC	AGT
T	GCT	ATC	XT	GTG	AGT
U	GAA	ATG	XU	GTC	AAG
			XV	GTA	ATC
			XW	GTT	AGT
			XX	GTT	AAG

Additional markers were fitted into the reference frame with LOD scores > 2.0. Recombination values were converted into map distances (centiMorgan, cM) by applying the Kosambi function. The numbers denoting individual linkage groups were assigned arbitrarily.

6.3 Results

The two parents, 'CRZY8/RA91' and 'Glenelg', were screened for polymorphisms using 160 primer combinations (Fig. 6.1). A subset of 21 EcoRI/MseI and 24 PstI/MseI primer combinations was chosen for the mapping of marker loci on the basis of the high level of polymorphic bands identified between parents. Each primer combination generated up to 80 bands of which on average 5.7 were polymorphic. In general, marker loci derived from EcoRI/MseI and PstI/MseI fragments mapped with similar frequency across all linkage groups. In some cases PstI/MseI-derived marker loci showed a tendency to map towards the ends of individual linkage groups (see linkage groups 2, 3, 10 and 13, Fig. 6.2).

The linkage relationships of two morphological (seed and flower colour), eight RFLP (detected by Lu-1, X-22, Ltt and Δ15) and 213 AFLP marker loci were determined (Fig. 6.2). Approximately 1400 cM of the Linola genome was covered with an average spacing of 10 cM between loci comprising 18 linkage groups of three or more loci. Outside the 18 linkage groups 9 pairs of loci showed pairwise linkage or cosegregation. A further eight marker loci remained unassigned to linkage groups. Segregation of 60 AFLP markers (28%) deviated significantly ($P<0.05$) from the expected 1:1 ratio. Most of these marker loci clustered in five linkage groups three of which were composed entirely of marker loci showing significant deviations (Table 6.2). A considerable number of AFLP marker loci (35%) co-segregated with other AFLP or RFLP markers, therefore not improving the overall map resolution.

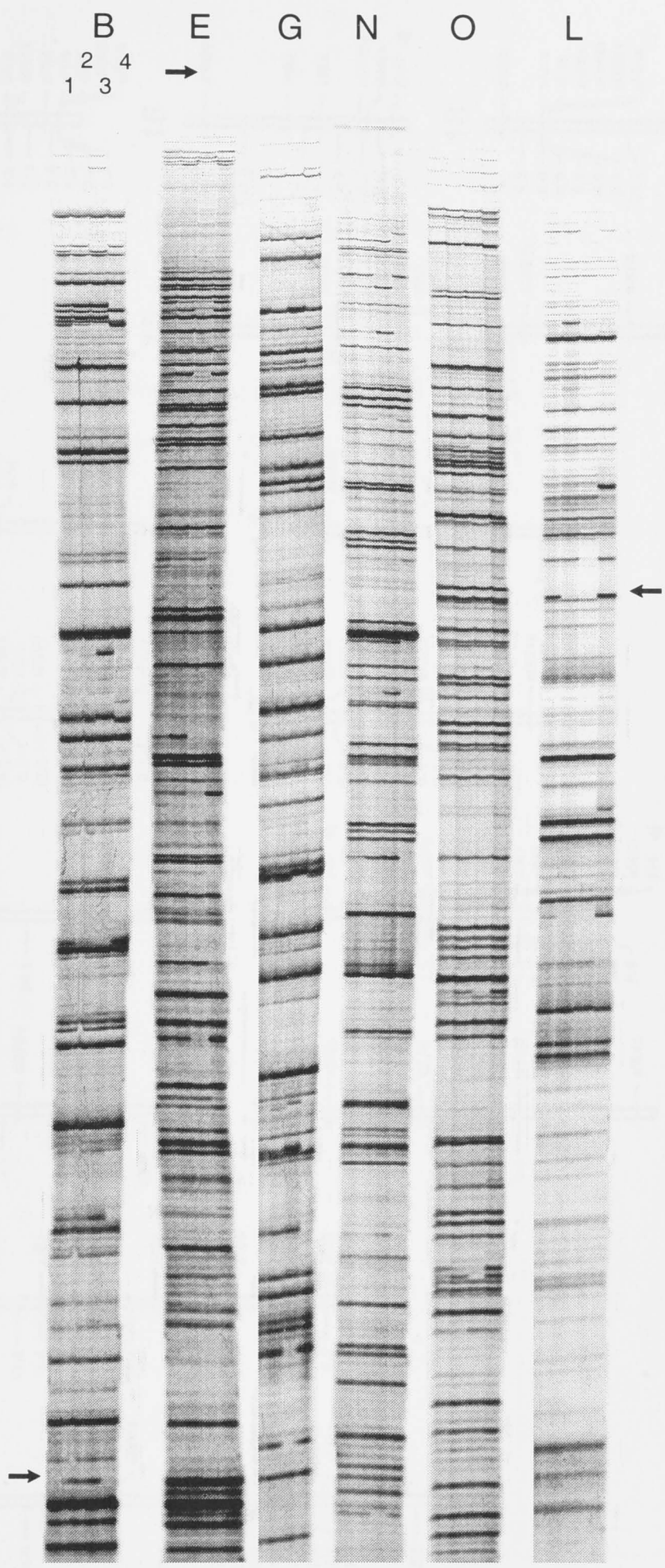


Fig. 6.1: AFLP profiles of DNA pools and parental lines using 6 primer combinations B, E, G, N, O and L (Table 6.1). Lane 1= Pool of DNA from 10 wilt resistant lines, Lane 2= Pool of DNA from 10 susceptible lines, Lane 3= susceptible parent 'Glenelg', Lane 4= resistant parent 'CRZY8/RA91'. Polymorphisms between pools and parental lines were detected with primer combination B and L (see arrows). The marker locus detected by primer combination B is linked to a QTL contributing to wilt resistance (Fig.7.2), polymorphism between pools and parental lines detected by L is due to distorted segregation at this marker locus.

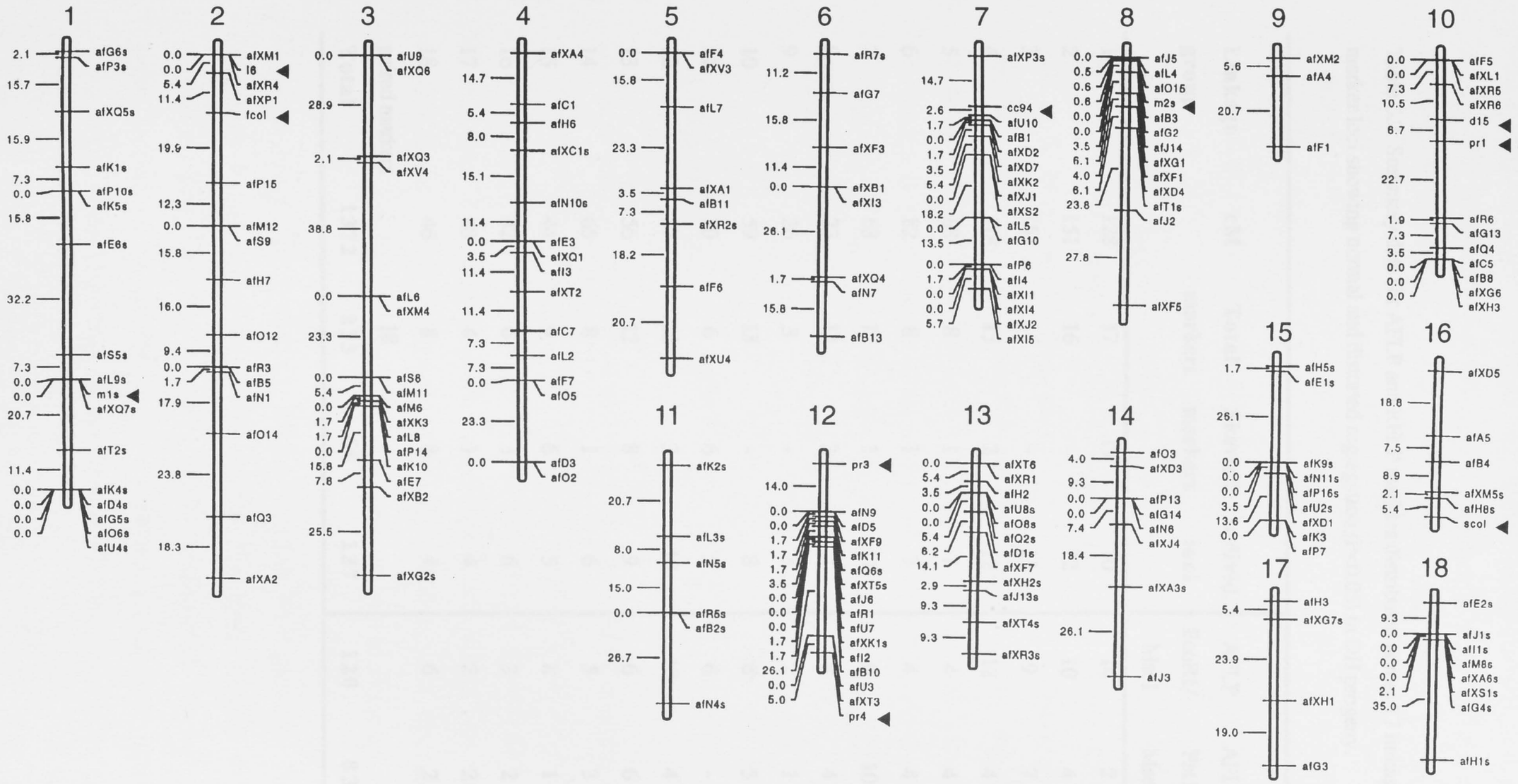


Fig. 6.2: Genetic linkage map of *Linum usitatissimum* consisting of AFLP, RFLP and morphological marker loci. AFLP marker loci were assigned the prefix 'af', a capital letter encoding the specific primer combination followed by a number describing the polymorphic marker band scored (see Table 6.1). The lower case letter 's' as suffix denotes that marker loci were not segregating according to the expected ratio ($P<0.05$). Marker loci without the prefix 'af' are RFLP markers (see text for details). Numbers on the left hand side of linkage groups denote genetic distances in centiMorgans.

Table 6.2: Summary data for AFLP and RFLP markers distributed across 17 linkage groups of 3 or more marker loci showing normal and distorted segregation (P<0.05) in DH progeny.

Linkage group	**cM**	**Total markers**	**Skewed markers**	**Individ. loci**	AFLP EcoR1/ Mse1	AFLP Pst1/ Mse1	RFLP markers	Morph. markers
1	128	17	17	10	14	2	1	-
2	151	16	-	12	10	4	1	1
3	151	16	-	11	9	7	-	-
4	118	15	2	12	11	4	-	-
5	88	8	1	7	4	4	-	-
6	82	8	1	7	4	4	-	-
7	68	17	1	11	6	10	1	-
8	73	13	2	7	8	4	1	-
9	26	3	-	3	2	1	-	-
10	59	13	-	8	6	5	2	-
11	70	6	6	5	6	-	-	-
12	57	16	3	10	10	4	2	-
13	56	12	8	9	6	6	-	-
14	65	8	1	6	5	3	-	-
15	44	9	6	5	8	1	-	-
16	42	6	3	6	3	2	-	1
17	48	4	1	4	2	2	-	-
18	46	8	8	4	6	2	-	-
paired markers		18						
Total	**1372**	**213**	**60**	**137**	**120**	**83**	**8**	**2**

AFLP amplification products segregated as dominant markers resulting in either the presence or absence of polymorphic products. However, 42 individual segregating bands (19%) were classified as putative co-dominant alleles, because two polymorphic amplification products originating from either parent and displaying only minor differences in mobility segregated as alternatives to each other in expected 1:1 ratios (see marker 9, Fig. 6.3). These putative allelic markers mapped randomly across linkage groups.

RFLP markers associated with putative disease resistance loci (*pr-1, m1, m 2* and *l6*) map independently to 4 linkage groups and two RFLP markers (*pr-3* and *pr-4)* both map to linkage group 12 (Fig. 6.2). The genomic clone Lu-1 detected RFLP *l6* which maps to linkage group 2. Lu-1 was previously shown to be tightly linked to the *L6* rust resistance locus. The clone X-22 linked to the complex rust resistance locus *M* detected RFLPs *m1* and *m2* which map to linkage group 1 and 8 respectively. Co-segregating AFLP markers were identified for RFLP markers *l6* and *m1*.

6.4 Discussion

In the present study, a total of 213 AFLP and 8 RFLP markers were identified to construct the first comprehensive linkage map of *L. usitatissimum* (n = 15). The expected number of 15 linkage groups is exceeded by three linkage groups and 9 additional paired markers, which could not be placed into any of the larger linkage groups with LOD values >2.0. Because some linkage groups are relatively small, the apparent excess of linkage groups is perhaps the result of incomplete coverage of the genome with marker loci. Alternatively, the common ancestry of the two parental lines (see Chapter 2.4) may have contributed genetically identical chromosome sections, for which no polymorphic markers could be detected. If these identical regions were sufficient in length, they would act to separate linkage groups that were located on the same chromosome (G. Lawrence pers. comm.).

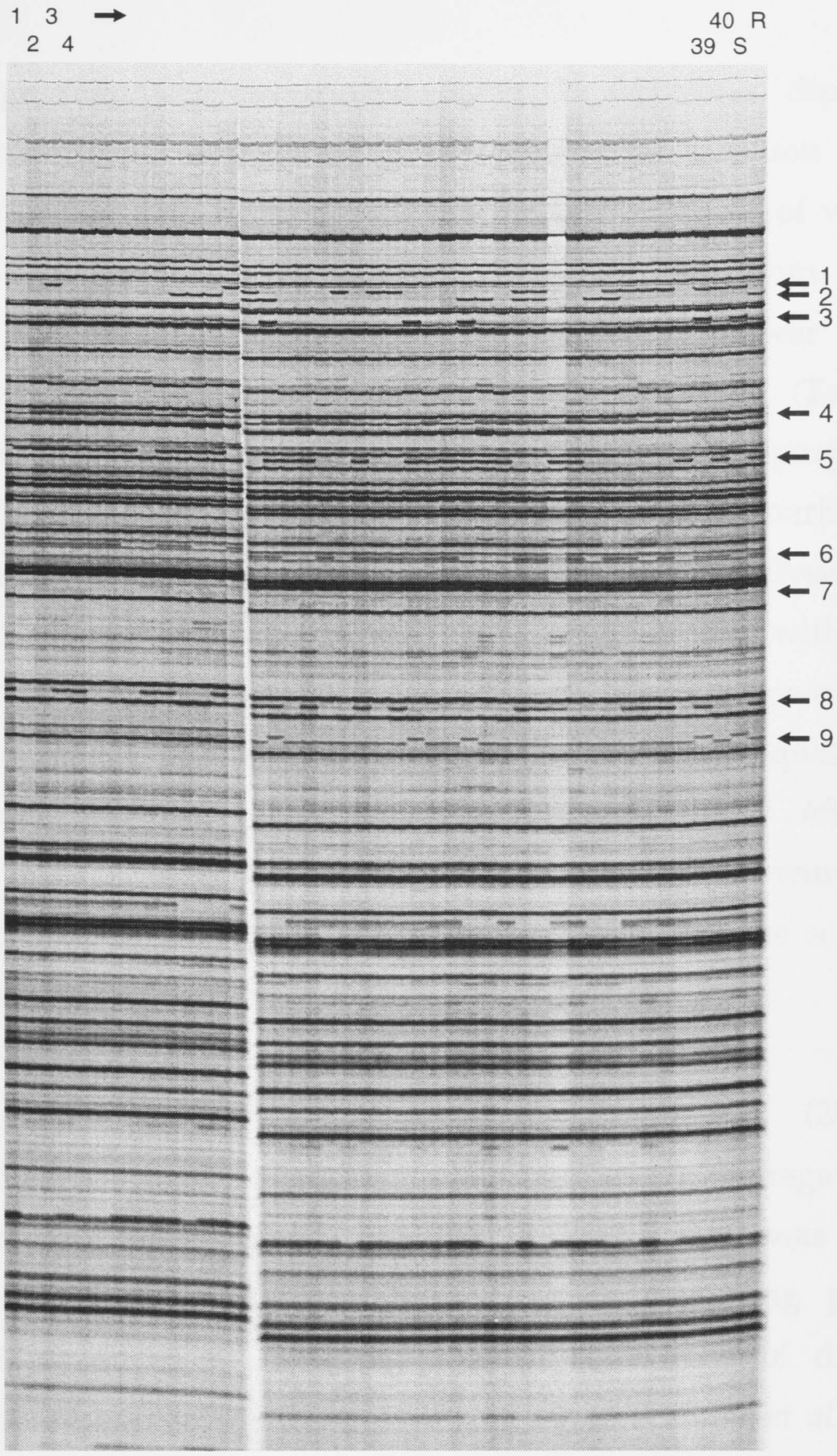


Fig. 6.3: AFLP profile of parental lines (lane S=susceptible, lane R=resistant) and doubled haploid segregating lines (1-40) using primer combination U (see Table 6.1). Nine amplification products were identified between parental lines and which segregated in progeny. Amplification products were scored as dominant markers (presence or absence) except for marker locus 9 which was scored as a putative co-dominant marker. The size of amplification products range from approximately 800 to 100 base pairs.

The linkage map was constructed using 59 DH lines diplaying either resistant or susceptible phenotypes in response to infection by Fusarium wilt. Because this map formed the basis for QTL analysis of wilt resistance (see following Chapter), a non-random set of DH lines from distributional extremes was chosen to enhance the statistical power of detecting quantitative trait loci that contribute to wilt resistance (Tanksley 1993). Selection within segregating populations to establish mapping lines carries the risk of introducing a genotypic bias directed towards marker loci linked to the selected trait. On the basis of the segregation analysis of over 200 marker loci the majority of loci possess a ratio compatible with the expected 1:1 ratio for this DH progeny. Although distorted segregation of markers was observed within several linkage groups, subsequent quantitative trait analysis revealed that two putative QTLs with major effects on wilt resistance are not associated with genomic regions showing segregation distortion (Chapter Seven). It is therefore unlikely that the selection of DH lines has biased segregation of marker loci in this study.

However, a substantial percentage of AFLP markers (28%) deviated significantly from the expected Mendelian ratio of 1:1. Segregation distortion has frequently been observed in mapping studies and was reported at a similar level amongst RFLP markers in a DH mapping population of rapeseed (Ferreira *et al.* 1994). Significant proportions of distorted RFLP markers were observed in DH progeny of barley (Graner *et al.* 1991) and in F2 mapping populations of sunflower (Berry et al. 1995), rice (McCouch et al. 1988) lettuce (Landry et al. 1987) and rapeseed (Landry et al. 1991). In the present study most of the distorted marker loci were confined to five linkage groups (linkage group 1, 11, 13, 15 and 18), three of which consisted entirely of such markers (linkage group 1, 11 and 18). The loci on linkage group 1 and 18 are skewed towards the 'Glenelg' parental alleles whereas the remaining linkage groups have a prevalence of the 'CRZY8/RA91' alleles. The degree of distortion of marker loci varied notably within linkage groups

1 and 11 (Fig. 6.4). Marker loci positioned at the ends of linkage groups deviated considerably less than more centrally located markers, perhaps indicating the presence of disturbance factors in proximal regions of linkage groups. Marker loci located furthest away from any hypothesised disturbance factors are expected to show less segregation distortion due to more opportunity for recombination.

Because this DH mapping family originated from polyembryonic F2 seed, such a selection may have introduced a bias towards marker loci linked to alleles that promote twinning. However, as discussed in Chapter 2.3 the genotype of the plant on which the seed developed was found to determine twinning frequency not the genotype of the gamete. It is therefore unlikely that the use of polyembryonic F2 seed biased the segregation of marker loci. Alternatively, disturbed segregation would occur for any marker band that represented the amplification products from two different polymorphic loci that, by chance, happened to be of the same size. The segregation of amplification products from two unlinked loci would result in a expected 3:1 ratio. Segregation patterns consistent with a 3:1 ratio were observed for several marker loci in this study.

A significant number of putative allelic AFLP marker loci were detected that segregated according to the expected ratio of 1:1. Although co-segregation of AFLP markers as alternatives to each other does not provide formal proof of allelism, these markers were probably derived from alleles that differ slightly in size because of a small addition or deletion between the primer sites in one of the alleles. Putative allelic AFLP markers were also detected in other species such as barley (Becker et al. 1995), potato (van Eck et al. 1996) and *Arabidopsis* (Decroocq-Ferrant pers. communication).

The apparent level of polymorphic bands generated per primer combination was similar to previously reported AFLP studies in soybean (Lin et al. 1996) and lower than in barley (Becker et al. 1995) and potato (van Eck et al. 1995).

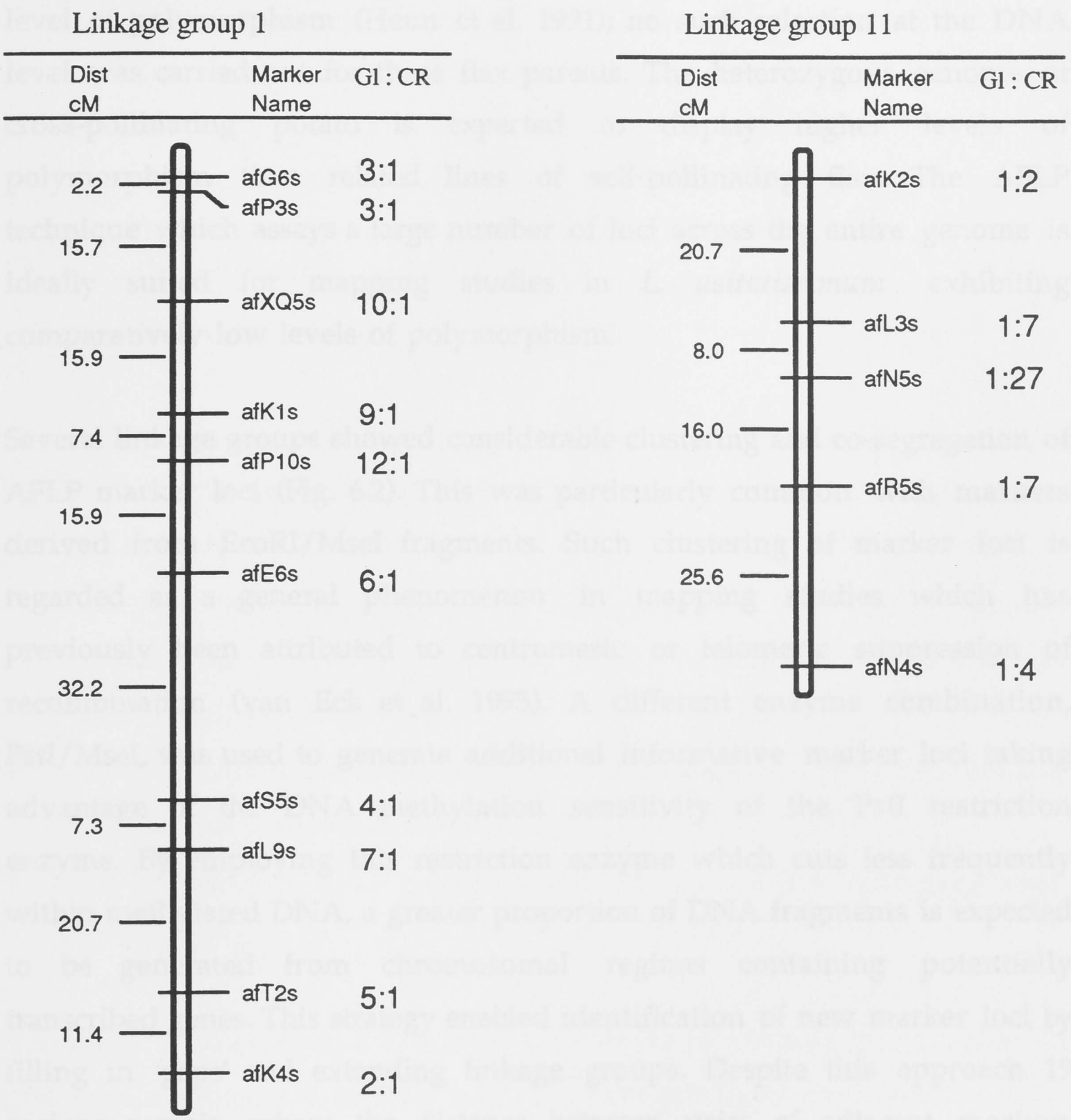


Fig. 6.4: Two linkage groups which consist entirely of marker loci showing distorted segregation ($P<0.05$). Loci on linkage group 1 are skewed towards the 'Glenelg' (Gl) parental alleles and loci on linkage group 11 are skewed towards the 'CRZY8/RA91' (CR) alleles.

The barley parents used for mapping were specifically selected for their high level of polymorphism (Heun et al. 1991); no such selection at the DNA level was carried out for these flax parents. The heterozygous genome of cross-pollinating potato is expected to display higher levels of polymorphism than related lines of self-pollinating flax. The AFLP technique which assays a large number of loci across the entire genome is ideally suited for mapping studies in *L. usitatissimum* exhibiting comparatively low levels of polymorphism.

Several linkage groups showed considerable clustering and co-segregation of AFLP marker loci (Fig. 6.2). This was particularly common with markers derived from EcoRI/MseI fragments. Such clustering of marker loci is regarded as a general phenomenon in mapping studies which has previously been attributed to centromeric or telomeric suppression of recombination (van Eck et al. 1995). A different enzyme combination, PstI/MseI, was used to generate additional informative marker loci taking advantage of the DNA methylation sensitivity of the PstI restriction enzyme. By employing this restriction enzyme which cuts less frequently within methylated DNA, a greater proportion of DNA fragments is expected to be generated from chromosomal regions containing potentially transcribed genes. This strategy enabled identification of new marker loci by filling in 'gaps' and extending linkage groups. Despite this approach 19 regions remain where the distance between pairs of adjacent markers exceeds 20 cM. Some of these regions devoid of marker loci may represent identical regions on homologous chromosomes due to the common ancestry of parental lines.

In addition to loci controlling seed and petal colour, a number of putative disease resistance loci were mapped with this DH population using probes linked to known resistance genes. The rust resistance locus *L* was mapped to linkage group 2 with probe Lu-1, a clone derived from the promoter region

of the *L6* allele, which detected a single fragment as RFLP marker *l6*. Probe X-22, which previously showed linkage (2 cM) to the *M* resistance cluster (Ellis et al. 1995), detected a strongly hybridising fragment and a minor fragment associated with RFLPs *m1* and *m2* respectively. The RFLP *m1* on linkage group 1 is likely to detect the homologous sequence linked to the rust resistance cluster *M*. RFLPs, *l6* and *m1*, map on independent linkage groups consistent with previous reports from classical linkage analysis of both loci (Lawrence et al. 1994). Additional disease resistance gene-like sequences were detected in linkage groups 10 and 12 using probe Ltt.

The AFLP technique presented a number of advantages over other PCR-based marker techniques such as arbitrarily primed PCR for the generation of a linkage map for flax, namely:

- High reproducibility of AFLP profiles generated with specific primer combinations provided for confident scoring even of minor amplification products.
- Marker loci are transferable to future mapping projects provided the size of amplification products are accurately assessed.
- High frequency of AFLP polymorphism allowed for the rapid generation of marker loci.

The AFLP approach requires technical expertise in pouring polyacrylamide gels and handling radioactive label. The cost per individual marker locus identified through AFLP is probably higher than for AP-PCR markers, although the costs associated with both marker systems have not been evaluated in detail. The potential for high frequency of identifiable polymorphism is particularly relevant in genomes with inherent low levels of genetic variation. High density mapping becomes feasible even for highly inbred species such as wheat as a means of generating additional marker loci in regions of interest on established RFLP maps.

The construction of genetic linkage maps within minor crop species such as flax, for which comprehensive RFLP linkage maps do not exist, is set to benefit greatly from the application of the AFLP technique. In the present study this technique allowed for the rapid generation of a reference linkage map, thereby enabling the identification of genomic regions contributing to Fusarium wilt resistance for the first time (Chapter Seven).

Chapter Seven

Targeting and mapping of quantitative trait loci (QTLs) controlling resistance to Fusarium wilt in flax

7.1 Introduction

Quantitative traits have been defined as characters which display continuous phenotypic variation and are controlled by the segregation of multiple loci, each with a small effect (Grant 1975; Tanksley 1993; Stuber 1995). However, as discussed by Griffiths et al. (1993), the critical difference between qualitative and quantitative traits is not the number of segregating loci but the level of phenotypic differences between genotypes in comparison to variation within genotypes. Some qualitative and quantitative traits might even share a common genetic basis and be controlled by the same loci (Tanksley 1993; Lee 1995). This notion rests on the hypothesis that qualitative variation is determined by mutant alleles which represent extremes in an allelic series at loci underlying quantitative variation (Robertson 1989). Recent results from numerous mapping studies have given credibility to this hypothesis by showing that genes contributing to qualitative and quantitative variation of one trait map to the same chromosomal region. Leonards-Schippers et al. (1994) found that the position of a QTL coincided with a major locus for resistance to *Phytophthora infestans* in potato. Saghai Maroof et al. (1994) reported similar findings for loci involved in resistance to powdery mildew in barley; Wang et al. (1994) for blast resistance in rice; and Dingerdissen et al. (1996) for northern leaf blight in maize.

To target quantitative trait loci (QTLs) Wang and Paterson (1994) assessed a DNA pooling strategy which bears strong resemblance to the bulked-segregant analysis originally proposed to target major genes (Michelmore et al. 1991). They evaluated the utility of DNA pools consisting of individuals

selected from phenotypic extremes to tag QTLs in different segregating populations. The success of this approach depends on minimising the rate of contamination of DNA pools by misclassified individual segregants. It is therefore important to be able to distinguish effects due to allelic differences from variation associated with environmental factors and genetic segregation due to the presence of heterozygotes. Wang and Paterson (1994) concluded that DH populations appear to be a good choice for tagging QTLs in DNA pools. Due to the absence of heterozygosity and fewer misclassified individuals in DNA pools, it was predicted that QTLs of large effect can be detected in a population size of 40 DH lines.

Instead of using bulked-segregant analysis to target individual loci linked to QTLs, interval analysis is commonly used to identify chromosomal regions harbouring QTLs (Lander and Botstein 1989). Interval mapping utilises existing molecular linkage maps to analyse linked markers simultaneously with regard to their contribution to phenotype. This interval approach can increase the likelihood of QTL detection by compensating for recombination events between marker loci and QTL and hence reducing the level of misclassified individual segregants (Tanksley 1993). QTLs contributing to disease resistance have been mapped successfully in a number of crop species using interval analysis (Heun 1992; Chen et al. 1994; Leonards-Schippers et al. 1994; Dirlewanger et al. 1994; Freymark et al. 1994; Jones et al. 1995; Dingerdissen et al. 1996).

Earlier studies in this thesis assuming major gene inheritance failed to identify linked markers to loci controlling resistance to Fusarium wilt. In this chapter quantitative inheritance was assumed by searching for loci with major and minor effects on resistance, not excluding the possibility that two loci, as discussed in Chapter Four, may explain most of the observed phenotypic variation. A two tier approach was adopted by firstly using DNA pooling of extreme phenotypes in conjunction with the AFLP technique to target marker loci linked to resistance. Secondly, the genetic linkage map

from Chapter Six was utilised for interval analysis to identify chromosomal regions containing loci underlying disease resistance. The characterisation of such regions of interest constitutes the first step in a series of strategies aimed at mapping genes contributing towards variation in complex traits with sufficient accuracy to be of use for plant breeding applications (Jones et al. 1995).

7.2 Materials and Methods

DNA Pooling

AFLP templates were prepared as described in Chapter Six and Vos et al. (1995). Following the ligation of adapter sequences, DNA from 10 resistant and 10 susceptible DH lines was bulked into separate pools. Resistant and susceptible DNA bulks in conjunction with AFLP templates from parental lines were subjected to a two-step PCR amplification using the same primer combinations as described previously. Visualisation of marker bands was achieved by ^{33}P end-labelling of primers prior to the final amplification step and separation of fragments on polyacrylamide gels.

QT analysis

The linkage map, described in Chapter Six, based on 59 DH lines consisting of 213 AFLP and RFLP marker loci covering approximately 1400 cM was used for interval mapping using Mapmaker/QTL (Lander and Botstein 1989). A QTL was declared for chromosomal regions which showed LOD scores greater than 2.0 indicating a significant association between molecular marker genotype and trait data. To examine possible environmental interactions QTL analysis was carried out using the resistance trait score from the glasshouse and field nursery screenings separately as described in Chapter Four.

Because the mapping population also segregated for two independent mutations responsible for the low linolenic phenotype (Chapter Three), the

trait was characterised by interval mapping and results were used to give some indication on the map's representative status.

7.3 Results

Fusarium wilt resistant and susceptible DNA pools together with parental lines were screened by evaluating approximately 10,000 marker loci detected by 160 AFLP primer combinations. 12 marker loci were scored as being polymorphic between the two DNA bulks and originating from one of the two parents. These polymorphisms were analysed on 59 DH lines of either resistant or susceptible phenotypes. The majority of polymorphic markers between DNA pools were the result of segregation distortion at these loci. However, one amplification product (*afB13*) showed association with the resistant phenotype when screened against 59 DH lines (Fig. 7.1). Subsequent interval mapping placed this marker locus into a chromosomal region containing a putative QTL for resistance to Fusarium wilt (Fig. 7.2).

The linkage map based on 59 DH lines was used for interval analysis. There was no evidence for significant genotype x environment interactions, as peaks of LOD profiles from the glasshouse data coincided with field nursery data (Fig. 7.3). Putative QTLs for resistance to Fusarium wilt on linkage group 6 (LOD 6.1) and 10 (LOD 3.6) contributed 38% and 26% to the phenotypic variation respectively (Table 7.1). To confirm these estimates a larger population of 110 DH lines was examined by random selection of 51 additional lines exhibiting an intermediate resistance response. A 'selective genotyping' approach was adopted whereby genotypic scores for the additional lines were entered as missing data (Lander and Botstein 1989). Table 7.1 summarises the proportions of explained phenotypic variation attributable to the major QTLs on linkage group 6 and 10. Analysing the larger population produced lower values for the explained variation for both QTLs compared to using 59 individuals. However, QTLs on linkage

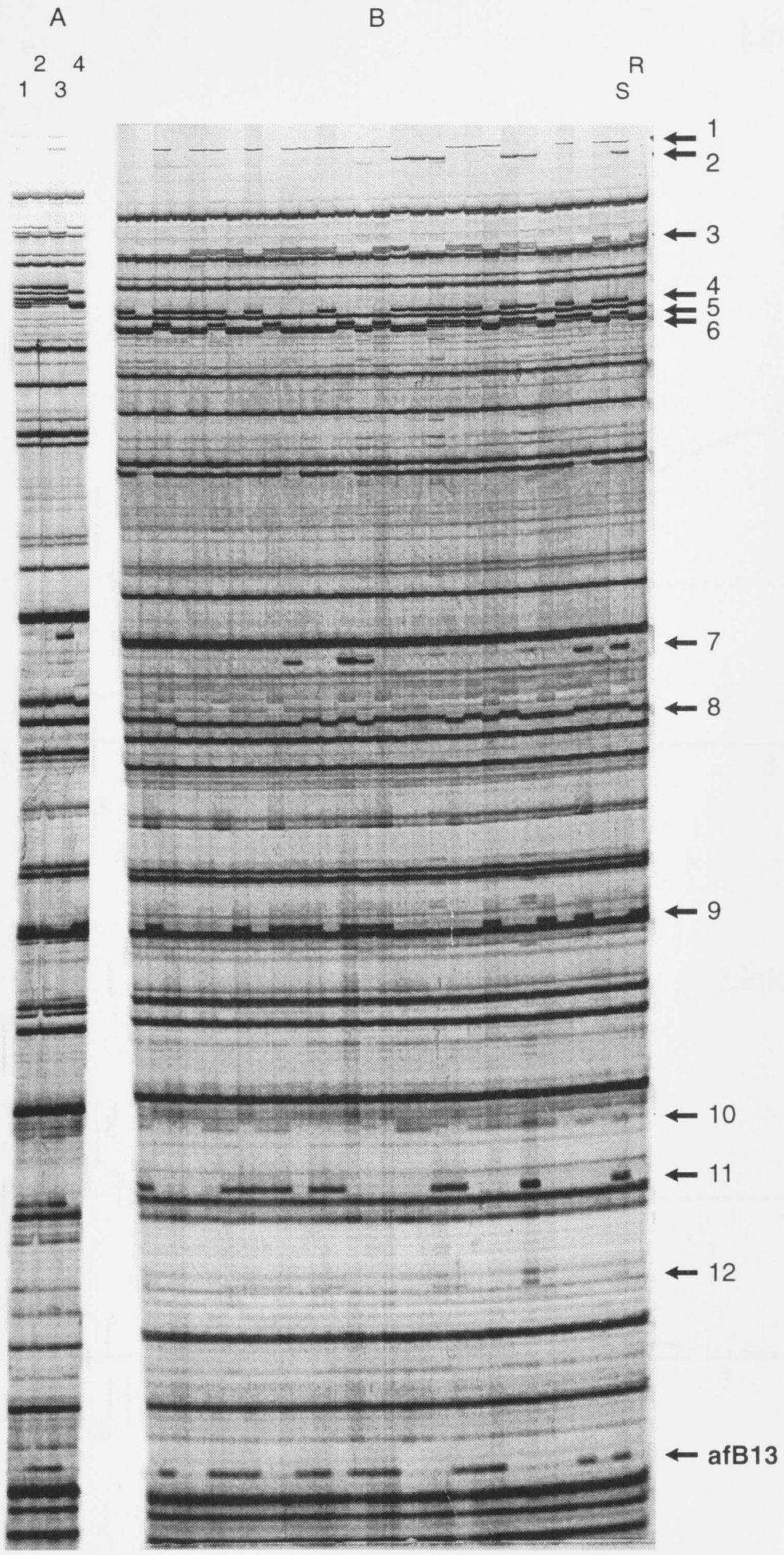


Fig. 7.1: AFLP profile using primer combination B (Table 6.1). A: Lane 1 = pool of DNA from 10 resistant lines, lane 2= pool of DNA from 10 susceptible lines, lane 3= susceptible parent, lane 4= resistant parent. Product 'afB13 amplified from the susceptible parent is polymorphic between DNA pools. B: Lane S= susceptible parent, lane R= resistant parent, Mendelian segregation of amplification product 'afB13' in doubled haploid lines. Subsequent QTL analysis showed that 'afB13' (approx. 80 - 100 bp) is linked to a genomic region containing a putative resistance gene to Fusarium wilt (Fig. 7.2).

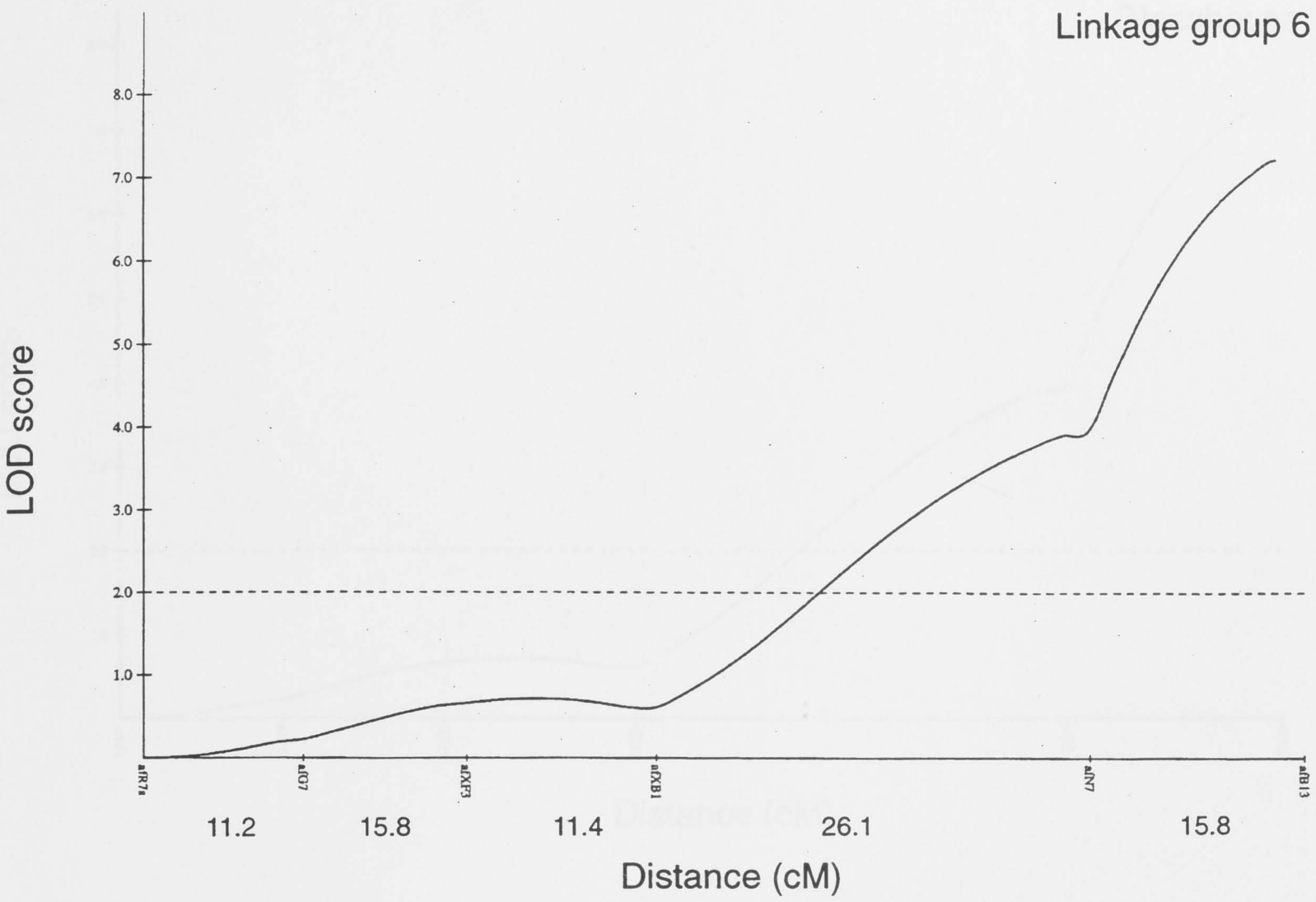
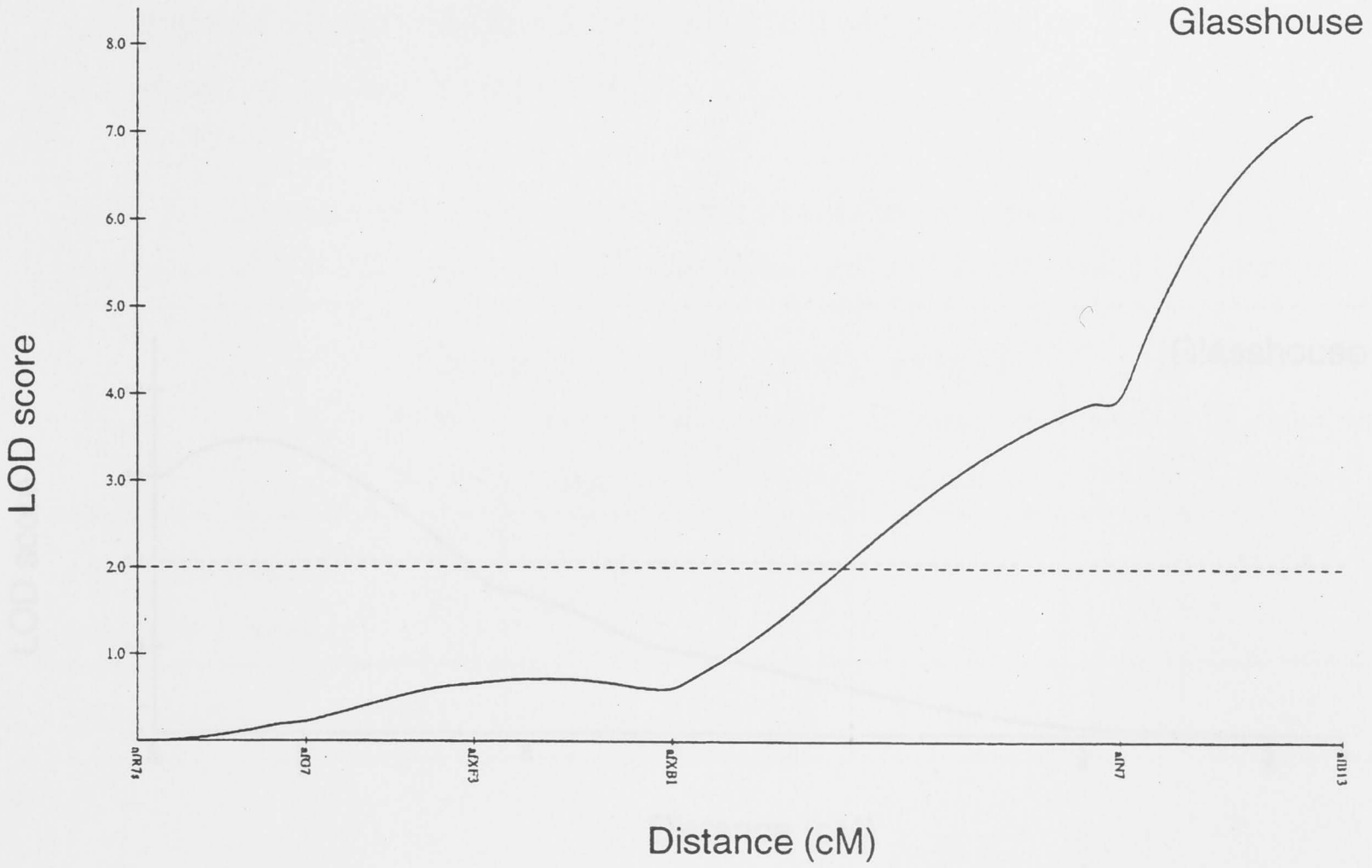


Fig. 7.2: QTL likelihood profiles of linkage group 6 (LOD 7.3) and 10 (LOD 3.4) contributing to Fusarium wilt resistance. The LOD significance threshold of 2.0 is marked (dotted line).



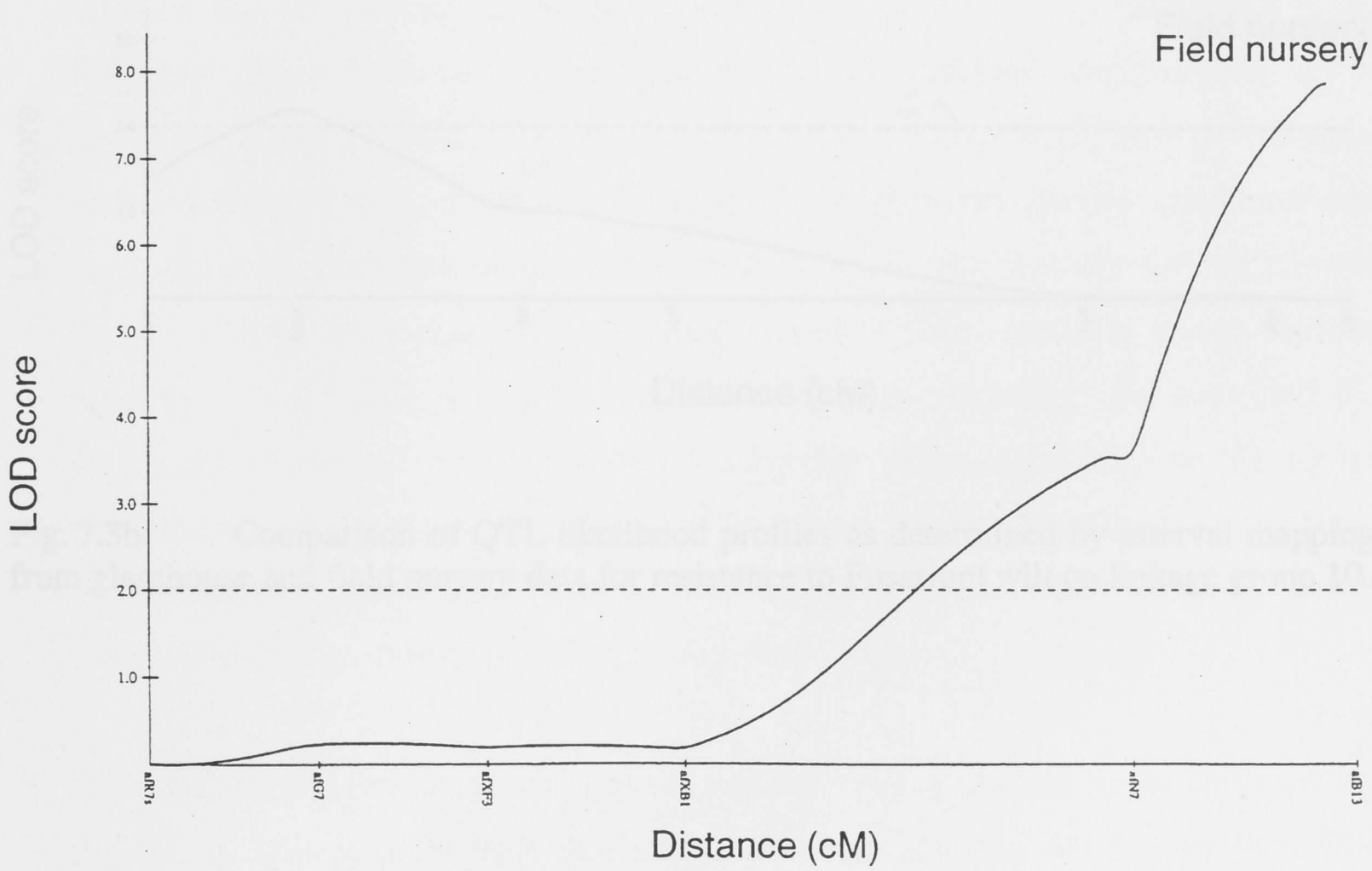
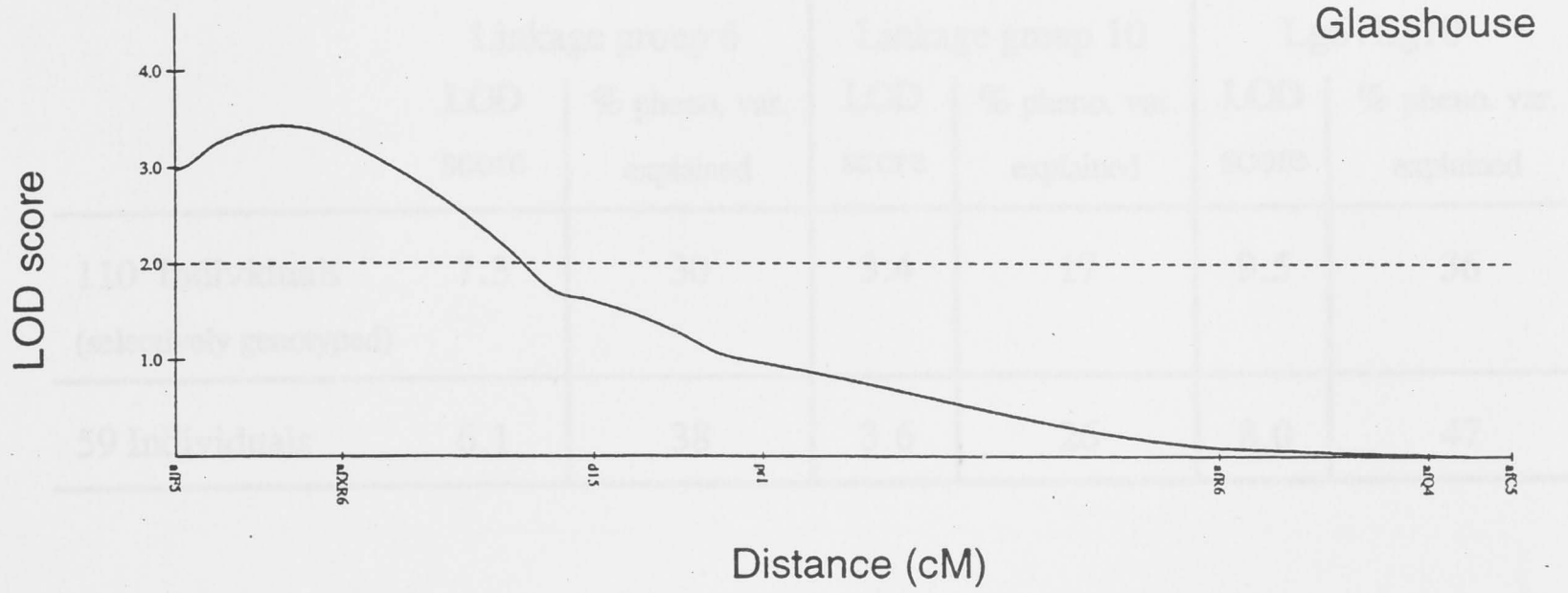


Fig. 7.3a: Comparison of QTL likelihood profiles as determined by interval mapping from glasshouse and field nursery data for resistance to Fusarium wilt on linkage group 6.



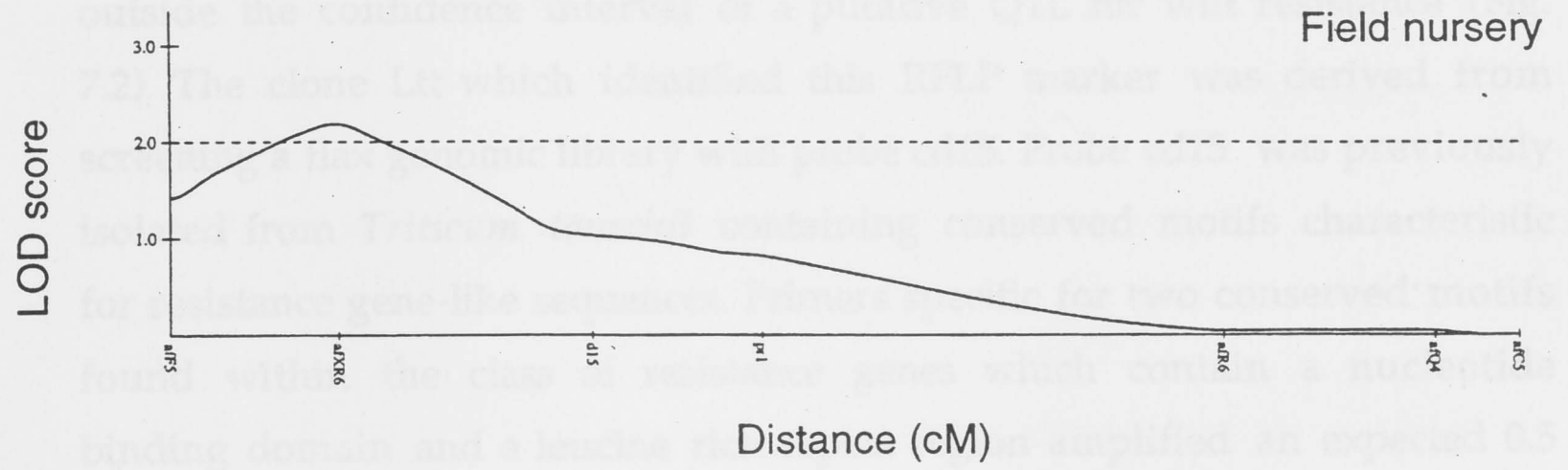


Fig. 7.3b: Comparison of QTL likelihood profiles as determined by interval mapping from glasshouse and field nursery data for resistance to Fusarium wilt on linkage group 10.

group 6 and 10 are still considered major loci accounting for 30% and 17% of the observed variation respectively.

Table 7.1: Summary data of putative resistance loci on linkage group 6 and 10 using a population of 110 DH lines (selectively genotyped) and 59 DH lines using QTL analysis.

	Linkage group 6		Linkage group 10		Lg6+Lg10	
	LOD score	% pheno. var. explained	LOD score	% pheno. var. explained	LOD score	% pheno. var. explained
110 Individuals (selectively genotyped)	7.3	30	3.4	17	9.5	36
59 Individuals	6.1	38	3.6	26	8.0	47

The RFLP marker *pr1* mapped to linkage group 10 approximately 12 cM outside the confidence interval of a putative QTL for wilt resistance (Fig. 7.2). The clone Ltt which identified this RFLP marker was derived from screening a flax genomic library with probe cd15. Probe cd15 was previously isolated from *Triticum tauschii* containing conserved motifs characteristic for resistance gene-like sequences. Primers specific for two conserved motifs found within the class of resistance genes which contain a nucleotide binding domain and a leucine rich repeat region amplified an expected 0.5 kb product from the Ltt clone. Without further characterisation of the clone the results so far suggest that the Ltt clone does contain motifs characteristic for resistance gene-like sequences and mapped to the same linkage group which is associated with a QTL for wilt resistance.

QT analysis identified two genomic regions with putative genes conferring the low linolenic acid trait. Earlier inheritance studies concluded that this trait is controlled by two independent loci (Green 1986). One major QTL on linkage group 3 explains 70% of the phenotypic variation (LOD 10.5) and a minor QTL located on linkage group 7 accounts for 17% of variation in the trait (LOD 2.4) (Fig. 7.4).

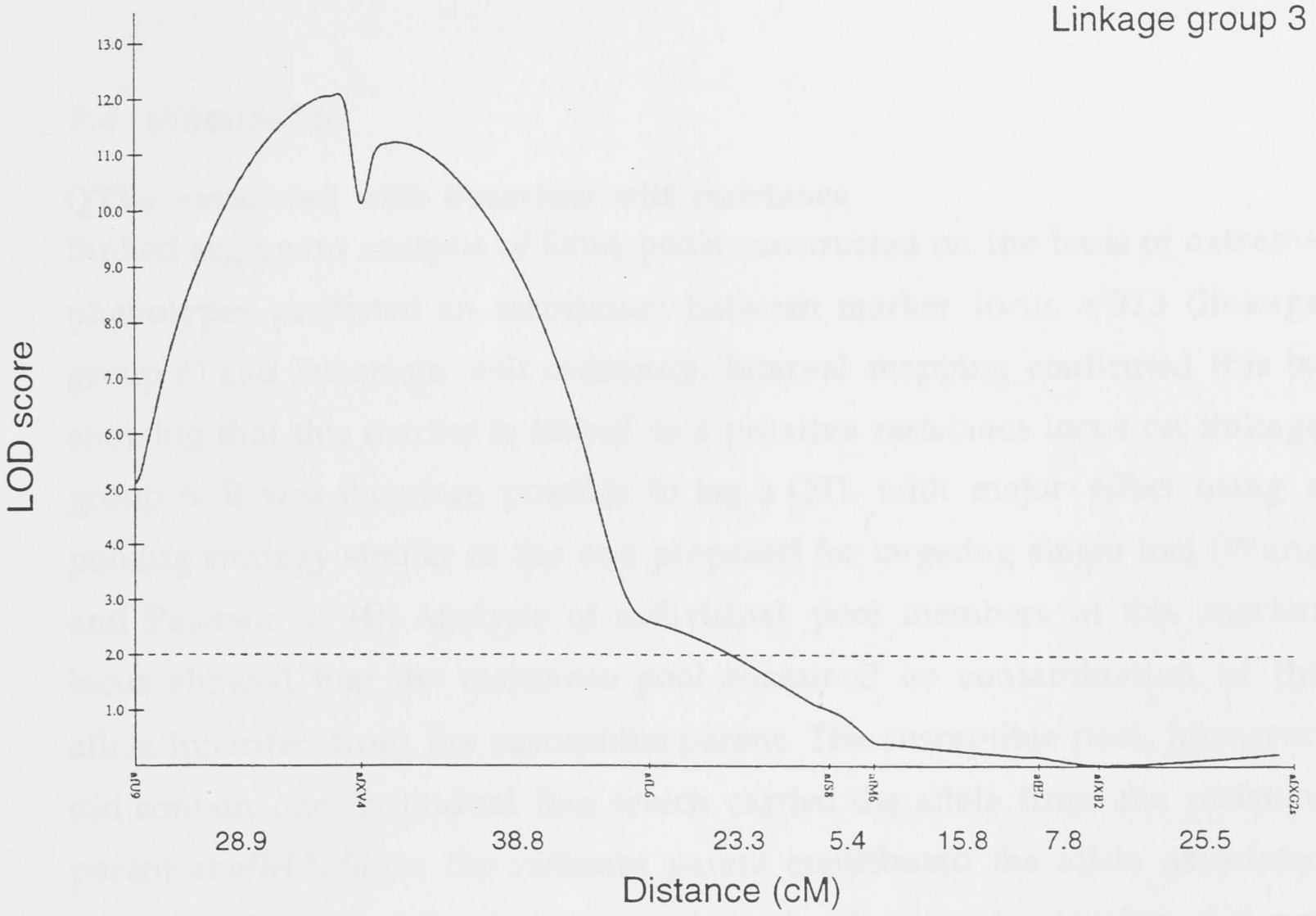


Fig. 7.4: QTL likelihood profiles of linkage group 3 (LOD 12.0) and 7 (LOD 2.4) contributing to the low linolenic acid phenotype of flax (Linola). The LOD significance threshold of 2.0 is marked (dotted line).

7.4 Discussion

QTLs associated with Fusarium wilt resistance

Bulked-segregant analysis of DNA pools constructed on the basis of extreme phenotypes predicted an association between marker locus *afB13* (linkage group 6) and Fusarium wilt resistance. Interval mapping confirmed this by showing that this marker is linked to a putative resistance locus on linkage group 6. It was therefore possible to tag a QTL with major effect using a pooling strategy similar to the one proposed for targeting single loci (Wang and Paterson 1994). Analysis of individual pool members at this marker locus showed that the resistance pool contained no contamination of the allele inherited from the susceptible parent. The susceptible pool, however, did contain one individual line which carried the allele from the resistant parent at *afB13*. Since the resistant parent contributed the allele associated with the absence of the marker band the level of contamination did not affect the results.

Quantitative trait analysis using interval mapping identified two regions located within two independent linkage groups that are associated with the resistance response. A population derived from distributional extremes was used to enhance the efficiency of detecting QTLs (Lander and Botstein 1989). While the statistical power of QTL mapping increases using extreme phenotypes, the accuracy of estimating QTL effects may be reduced. Tanksley (1993) explains: "Individuals in the extremes tend to have either a large number of positive or negative alleles at all QTL, depending on which extreme they represent. There is thus deficiency of individuals with a mixture of positive and negative alleles in the sub-populations being analysed, which confounds the ability to individually measure the effects of any specific QTL." Chen et al. (1994) used DH lines from distributional extremes to map RFLP markers and identify putative QTLs for stripe rust resistance in barley, before confirming the results with a larger mapping population. They found that the linkage map of markers using the subset

paralleled the profile of published maps and that the effect and position of major QTLs was preserved using the selected sub-population compared with results obtained for the full population. These results indicate that contributions of QTLs of major effect to phenotypic variation can be estimated using sample populations consisting of extreme phenotypes.

Since the percent phenotypic variance includes environmental variance, an estimate of the environmental influence is required to examine the overall contribution of resistance loci. Experimentation to provide such an estimate was not conducted specifically. However, regression analysis suggests that phenotypic scores of DH segregating lines from different environments are strongly associated (R^2= 0.73) (Fig. 4.2). It is, therefore, likely that heritability of the trait is high, since low heritability would have provided opportunity for greater environmental influences and hence weaker correlation between resistance scores from different environments. Other inheritance studies investigating the resistance to flax wilt estimated broad sense heritability to be high (H^2= 0.9) (Popescu and Schuster 1985). Therefore, the putative resistance loci on linkage group 6 and 10 are likely to constitute QTLs with major contributions to the genetic variation of resistance to Fusarium wilt.

QTLs associated with low linolenic acid trait

Results from QT analysis of the low linolenic acid trait corroborate previous inheritance studies by suggesting that the trait is controlled by two loci located on independent linkage groups (Fig. 7.4). A comparison of the relative contribution of each locus suggests that the two regions which control the low linolenic acid trait differ markedly in their effect on phenotype in this DH population. However, earlier studies investigating single homozygous mutants predicted that each locus contributed equally to phenotype (Green 1986). This discrepancy between relative effects of loci could be explained by the distribution of trait data input to Mapmaker/QTL software. The program presumed that data input approximates a normal distribution. Because segregants could only be grouped into three

phenotypic classes (low, intermediate, high) in relation to the linolenic acid content, the discrete nature and asymmetrical distribution of trait data is expected to influence the effect of individual QTLs.

Recent studies showed that a specific marker associated with the coding region of one Δ15 desaturase gene mapped to a genomic region containing a major QTL for the low linolenic acid trait in rapeseed (Jourdren et al. 1996). During the course of this study a RFLP marker (*d15*) detected by a probe derived from a Δ15 desaturase gene in flax mapped independently of both putative QTLs contributing to the low linolenic acid trait. Biochemical studies established a close association between desaturase enzyme activity and linolenic acid content (Stymne et al. 1992). The probe detected four fragments in total of which only one was polymorphic and mapped to linkage group 10 (Fig. 6.2). The detection of multiple copies of Δ15 desaturase homologs in the flax genome might reflect the presence of functionally redundant genes or specialised genes involved in desaturation of fatty acids in other tissues (eg. plastids). It is possible that one of the three remaining monomorphic bands could map in close proximity of a QTL. Because the Linola line was originally derived by mutating a 'Glenelg' genotype, the two parents are not expected to differ in the gene or the genomic regions surrounding the loci which control the low linolenic acid phenotype, except for the site of the mutation (Fig 2.2a). It is, therefore, unlikely that a RFLP marker closely linked to the trait can be found using the present parental material, until the site of mutation is identified within the locus.

There was some evidence for interaction occurring between putative QTLs for wilt resistance as well as low linolenic acid phenotype. In general, a reduction of LOD scores and percentage of explained variation was observed when pairs of QTLs were examined simultaneously compared to when they were analysed separately (Table 7.1). This reduction suggests that loci are not functioning additively and that effects due to epistasis or data error may be involved (Pecchioni et al. 1996).

Risk of spurious linkage between markers in QTL mapping

The linkage map used for interval mapping is based on the selection of phenotypes from distributional extremes. This non-random selection of phenotypes carries the risk of introducing a genotypic bias directed towards potential marker loci linked to characters controlling the trait. Martinez (1996) using simulated data sets demonstrated that incorrect estimation of linkage between independently inherited marker loci can occur when individuals are selected from the tails of the phenotypic distribution instead of obtaining a random sample. If such incorrect linkage relationships between marker loci are used as a framework for subsequent QTL analysis, the accuracy of mapping QTLs may also be compromised. Selective genotyped data sets were shown to reduce estimates of recombination frequency between QTLs when these were in the coupling phase (alleles which contribute positively to the trait are inherited from one of the two parents), and were shown to increase estimates of recombination frequency when alleles of QTLs were present in the repulsion phase (Lin and Ritland 1996). The magnitude of the potential bias of the linkage estimate between QTLs in selective genotyped data sets is more serious for QTLs of large effect (explain> 10% of phenotypic variance) than for minor loci (Lin and Ritland 1996).

In response to the concerns raised above, the possible occurrence of erroneous linkage relationships between marker loci (and by inference between putative QTLs contributing to wilt resistance) was investigated for this linkage map. On the basis of the segregation analysis of over 200 marker loci the majority of loci segregated according to the expected ratio. Although a significant number of marker loci showed distorted segregation patterns, most of these markers were confined to five linkage groups. If the selection of extreme phenotypes would have biased certain marker combinations, these marker loci would be expected to show a skewed segregation pattern as well as linkage to putative QTLs of large effects contributing to the selected

phenotype. However, none of these five linkage groups which consist entirely of distorted marker loci were associated with putative QTLs for wilt resistance. Assuming that the wilt resistant parent CRZY8/RA91 contributed both resistance alleles at the two putative QTLs, a biased linkage estimate introduced through selective genotyping would have resulted in the apparent linkage of putative QTLs for wilt resistance. Mapping results obtained from this study have shown that two putative QTLs of large effects contributing to wilt resistance map independently on separate linkage groups. In addition, the molecular mapping data corroborates early findings derived from classical genetical analysis which established that two independent loci account for most of the phenotypic variation observed for wilt resistance. Hence, there is no evidence to suggest that selective genotyping of recombinant DH lines has introduced biased linkage estimates for marker loci associated with genes controlling a significant proportion of the resistance to Fusarium wilt.

Chapter Eight

General Discussion

8.1 Inheritance of seed colour and low linolenic acid trait

The segregation of yellow seed colour derived from line '84495' was studied in a recombinant doubled haploid progeny. Although significant segregation distortion was observed for monogenic inheritance, the most plausible explanation for the inheritance of this trait is to accept a single dominant gene model as was observed in other crosses between '84495' and a range of brown-seeded germplasm (Green pers. comm.). The single dominant character for yellow seed colour is likely to constitute a separate genetic determinant from previously reported recessive genes (Barnes et al. 1960; Beard and Beeson 1967) and may act as a 'master' gene inhibiting the production of pigment in the seed coat. Similar genetic control has been proposed for seed colour in soybean with one dominant locus determining the presence/absence of pigment and an additional two loci controlling specific seed coat colour (Vodkin 1994).

Segregation analysis in an isogenic background by Green (1986) confirmed that mutations at two independent loci control the low linolenic acid trait. Green (1986) observed that genetic ratios accurately fit the expected frequencies when tested against a two gene model. This present study investigated the segregation of both loci in a heterogenous genetic background which probably contributed to the observed distortions from the expected ratio for two gene inheritance. Quantitative trait analysis identified two independent chromosomal regions having a significant effect on the low linolenic acid trait. Molecular markers which map to these linkage groups segregated according to the expected 1:1 ratio. Assuming that both mutations correspond to the QTLs, it is therefore unlikely that disturbance factors associated with either linkage group contributed to the observed

segregation distortion. The comparison between segregation of two genes controlling the low linolenic acid trait in a homogenous vs. a heterogenous background illustrates the potential role unlinked loci can play in influencing segregation patterns.

8.2 Inheritance of resistance to Fusarium wilt

Fusarium wilt is one of the major diseases of flax. Previous inheritance studies have produced equivocal results in relation to the likely number of genes controlling the trait. It was therefore necessary to elucidate the mode of inheritance in the genetic material used in this study, before deciding on a suitable molecular strategy to target genes contributing to the resistance response.

Most of the observed phenotypic variation was associated with the segregation of two major loci controlling resistance to Fusarium wilt. The resistant DH lines exhibited on average a more resistant phenotype than the resistant parental line indicating that additional minor loci are likely to have contributed to the resistance response. Apart from the genetic component environmental factors clearly influenced the capacity of the pathogen to infect and spread throughout the vascular tissues of the plant. Temperature was shown to be the single most important environmental factor (Kommendahl et al. 1970). The complexity of the soil environment no doubt contributed to the influence of non-genetic factors on host-pathogen interactions. This study did not attempt to quantify the relative contributions of minor genes and environmental factors upon phenotypic variation. However, a better understanding of the genetic control of wilt resistance necessitates a partitioning of variation into genetic and environmental components. The objective of partitioning of variation can be addressed in future experiments by scoring DH lines for resistance using a replicated experimental design.

8.3 Targeting major genes for resistance to flax wilt

Field or glasshouse screening for resistance to soil-borne diseases such as Fusarium wilt is labour intensive and often unreliable due to fluctuations in the levels of fungal inoculum and temperature effects. Environmental influences can be avoided by directly assaying the genotype. A genotype-based selection method can be developed by identifying molecular markers linked to the resistance phenotype or by targeting resistance genes directly.

A two tier approach was adopted by firstly utilising DNA clones from previously isolated plant resistance genes in DNA hybridisation experiments to target resistance loci. Secondly a random PCR-based technique was employed to assay the genome for markers linked to the trait. Neither approach identified markers tightly linked to major wilt resistance loci.

When DNA clones containing resistance gene-like sequences were hybridised to flax DNA, it was assumed that Fusarium wilt resistance genes in flax belong to the same class of resistance genes which have a nucleotide binding domain and leucine rich repeats (Staskawicz et al. 1995). These conserved motifs are present in I_2, a single dominant gene conferring race-specific resistance to Fusarium wilt in tomato (Simons pers. comm.). Race-specificity involving a single dominant gene could only be demonstrated in the flax wilt system under controlled conditions using single fungal isolates in a sterile soil medium. Thus, it remains uncertain whether the genetic determinants for resistance to flax wilt actually correspond to this class of resistance genes. Current knowledge of host-pathogen interactions and isolation of resistance genes is still in an early stage. It is therefore conceivable that future research into disease resistance, particularly into host-pathogen interactions which do not conform to the classic gene-for-gene concept, will describe additional classes of resistance genes.

8.4 Targeting quantitative trait loci associated with resistance to flax wilt

The targeting of quantitative trait loci contributing to wilt resistance required a change in focus. Using QTL analysis individual loci were no longer targeted but chromosomal regions containing putative resistance genes became the new focal points. Bulked-segregant analysis in conjunction with the recently developed AFLP marker technique (Vos et al. 1995) were used to identify one marker locus (*afB13*) associated with resistance. To estimate linkage relationships of marker *afB13* to putative resistance loci and to characterise additional QTLs, interval mapping and quantitative trait analysis was employed. This approach required a genetic linkage map consisting of molecular markers spread over the entire genome. A comprehensive linkage map was generated using the AFLP marker technique, a method well suited to generating a large number of reproducible marker loci. Interval mapping using this linkage map identified two chromosomal regions with significant effect on the resistance to flax wilt. Marker locus *afB13* showed linkage to one of the two target regions confirming that DNA pooling strategy can be used to tag QTLs of major effect. These results corroborate earlier findings which established that two loci account for most of the phenotypic variation observed in this trait. Hence, results derived from molecular genetics using interval mapping and QT analysis support the findings from classical genetics using segregation analysis. This study is not able to resolve the question of whether the two loci identified by segregation analysis are actually contained within the confidence intervals of the QTLs, although this scenario would be the most plausible one.

8.5 Potential for marker-assisted selection

One of the main research aims of this project was to identify molecular markers tightly linked to Fusarium wilt resistance for use in marker-assisted selection. Although oligogenic inheritance of this trait suggested that targeting individual resistance loci could yield markers linked to at least one of the target loci, no such linkage between marker loci and trait was detected. Interval mapping provided first estimates of chromosomal regions contributing to the resistance response. These regions can be targeted further in future experiments by bulked-segregant analysis. Screening for polymorphisms between DNA pools constructed on the basis of allelic variants at marker loci located closest to the QTLs should identify additional marker loci in the target regions. The saturation of these regions containing putative resistance loci with marker loci should enable the selection of flanking markers tightly linked to the resistance trait. For markers to be useful in routine breeding applications AFLP markers must be converted into robust PCR assays relying on colourimetric systems or separation on agarose gels for the detection of amplification products. Exploratory mapping projects such as the current one provide first estimates of target regions with sufficient accuracy to be of future use in more focused gene targeting experiments.

References

Ausubel FM, Brent R, Kingston, RE, Moore DD, Seidman JG, Smith JA and Struhl K (Eds.) (1994) Current Protocols in Molecular Biology, John Wiley & Sons Inc.

Ballvora A, Hesselbach J, Niewöhner J, Leister D, Salamini F and Gebhardt C (1995) Marker enrichment and high-resolution map of the segment of potato chromosome VII harbouring the nematode resistance gene *Gro1*. Mol. Gen. Genet. 249: 82-90.

Barua UM, Chalmers KJ, Hackett CA, Thomas WTB, Powell W and Waugh R (1993) Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. Heredity 71: 177-184.

Barnes DK, Culbertson JO and Lambert JW (1960) Inheritance of Seed and Flower Colors in Flax. Agron. Journal 52: 456-459.

Beard BH and Beeson A (1967) The inheritance of seed colour in the flax cross C.I. 479 x C.I. 2518 (*Linum usitatissimum*). Proc. 37th Annual Flax Institute of the United States, pp. 6-8.

Becker J, Vos P, Kuiper M, Salamini F and Heun M (1995) Combining mapping of AFLP and RFLP markers in barley. Molecular and General Genetics 249: 65-73.

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J and Staskawicz BJ (1994) *RPS2* of *Arabidopsis thaliana*: A Leucine-Rich Repeat Class of Plant Disease Resistance Genes. Science 265: 1856-1860.

Berry ST, Leon AJ, Hanfrey CC, Challis P, Burkholz A, Barnes SR, Rufener GK, Lee M and Caligari PDS (1995) Molecular marker analysis of

Helianthus annuus L., 2. Construction of an RFLP linkage map for cultivated sunflower. Theoretical and Applied Genetics 91: 195-199.

Borlaug NE and Christensen JJ (1941) Variations in *Fusarium oxysporum* f.sp. *lini*. Phytopathology 31: 4 (cited in Kommendahl et al. 1970).

Burgess LW, Liddell CM and Summerell BA (1988) Laboratory Manual for Fusarium Research, University of Sydney.

Burnham CR (1932) The inheritance of Fusarium wilt resistance in Flax. J. Amer. Soc. Agron. 24: 734-748.

Chen FQ, Prehn D, Hayes PM, Mulrooney D, Corey A, Vivar H (1994) Mapping genes for resistance to barley stripe rust (*Puccinia striiformis* f. sp. *hordei*). Theoretical and Applied Genetics 88: 215-219.

Cullis CA (1981) DNA Sequence organisation in the flax genome. Biochimica et Biophysica Acta 652: 1-15.

Cullis CA and Gorman MB (1992) Constructing a Genetic Map in Flax using Morphological, Biochemical and RFLP Markers. Proceedings of the 54th Flax Institute, pp. 119-125.

Cullis CA, Oh TJ, Gorman MB (1995) Genetic Mapping in flax (*Linum usitatissimum*). Proceedings of the Third meeting of the International Flax Breeding Research Group, St Valéry en caux, France, 7-8 November 1995.

Dingerdissen AL, Geiger HH, Lee M, Schechert A and Weiz HG (1996) Interval mapping of genes for quantitative resistance of maize to *Setosphaeria turcica*, cause of northern leaf blight, in a tropical environment. Molecular Breeding 2: 143-156.

Dirlewanger E, Isaac PG, Ranade S, Belajouza M, Cousin R and de Vienne D (1994) Restriction fragment length polymorphism analysis of loci

associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theoretical and Applied Genetics 88: 17-27.

Durrant A (1976) Flax and linseed. In: Evolution of Crop Plants (Ed. N.W.Simmonds) Longman, London, pp. 190-193.

Eastwood RF, Lagudah ES and Appels R (1993) A directed search for DNA sequences tightly linked to cereal cyst nematode resistance genes in *Triticum tauschii*. Genome 37: 311-319.

Ellis JG, Lawrence GJ, Finnegan EJ and Anderson PA (1995) Contrasting complexity of two rust resistance loci in flax. Proc. Natl. Acad. Sci. USA 92: 4185-4188.

Ferreira ME, Williams PH and Osborn TC (1994) RFLP mapping of *Brassica napus* using doubled haploid lines. Theoretical and Applied Genetics 89: 615-621.

Flor HH (1956) The complementary genetic systems in flax and flax rust. Adv. Genet. 8: 29-54.

Foroughi-Wehr B. and Wenzel G. (1993) Andro- and parthenogenesis. In: Plant Breeding: Principles and prospects (Eds.: Hayward MD, Bosemark NO and Romagosa I.) Chapman & Hall, London, pp. 261-277.

Freymark PJ, Lee M, Martinson CA and Woodman WL (1994) Molecular-marker-facilitated investigation of host-plant response to *Exserohilum turcicum* in maize (*Zea mays* L.): components of resistance. Theoretical and Applied Genetics 88: 305-313.

Gao H, Beckman CH and Mueller WC (1995) The rate of vascular colonization as a measure of the genotypic interaction between various cultivars of tomato and various formae or races of *Fusarium oxysporum*. Physiological and Molecular Plant Pathology 46: 29-43.

Gebhardt C, Ritter E, Barone A, Debener T, Walkemeier B, Schachtschabel U, Kaufmann H, Thompson RD, Bonierbale MW, Ganal MW, Tanksley SD and Salamini F (1991) RFLP maps of potato and their alignment with the homoeologous tomato genome. Theoretical and Applied Genetics 83: 49-57.

Gill KS and Yermanos DM (1967) Cytogenetic Studies on the Genus *Linum* 1. Hybrids Among Taxa with 15 as the Haploid Chromosome Number. Crop Science 7: 623-631.

Giovannoni JJ, Wing RA, Ganal MW and Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Research 19 (23): 6553-6558.

Goray SC, Khosla HK, Upadhyaya YM, Naik SL and Mandloi SC (1987) Inheritance of wilt resistance in linseed. Indian Journal of Agricultural Sciences 57 (9): 625-627.

Goray SC, Khosla HK, Upadhyaya YM, Nigam PK and Naik SL (1988) Genetic analysis of resistance to Linseed Wilt. Indian J. Genet. 48 (2): 247-249.

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G and Herrmann RG (1991) Construction of an RFLP map of barley. Theoretical and Applied Genetics 83: 250-256.

Grant V. (1975) Altered Segregation. In: Genetics of Flowering Plants, Columbia University Press, New York pp. 228-250.

Green AG and Salisbury PA (1983) Inheritance of polyembryony in flax (*Linum usitatissimum* L.), Canadian Journal of Genetics and Cytology 25 (2): 117-121.

Green AG and Marshall DR (1984) Isolation of induced mutants in linseed (*Linum usitatissimum*) having reduced linolenic acid content. Euphytica 33: 321-328.

Green AG (1986) Genetic control of polyunsaturated fatty acid biosynthesis in flax (*Linum usitatissimum*) seed oil. Theoretical and Applied Genetics 72: 654-661.

Green AG and Dribnenki JCP (1994) Linola- a new premium polyunsaturated oil. Lipid Technology, March/April: 29-33.

Green AG and Dribnenki JCP (1995) Breeding and development of Linola (low linolenic flax). Proceeding of the Third meeting of the International Flax Breeding Research Group (in print).

Griffiths AJF, Miller JH, Suzuki DT, Lewontin RC and Gelbart WM (Eds.) (1993) An Introduction to Genetic Analysis (5th Ed.), WH Freeman and Company, New York, pp. 715.

Hartl L, Weiss H, Stephan U, Zeller FJ and Jahoor A (1995) Molecular identification of powdery mildew resistace genes in common wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 90: 601-606.

Helentjaris T, Slocum M, Schaefer A and Nienhuis J (1986) Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theoretical and Applied Genetics 72: 761-769.

Hellens RP, Ellis THN, Lee D and Turner L (1993) Repeated sequences as genetic markers in pooled tissue samples. Plant Molecular Biology 22: 153-157.

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME and Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34: 437-447.

Heun M (1992) Mapping quantitative powdery mildew resistance of barley using a restriction fragment length polymorphism map. Genome 35: 1019-1025.

Hospital F, Chevalet C and Mulsant P (1994) Using Markers in Gene Introgression Breeding Programs. Genetics 132: 1199-1210.

Huyghe C (1985) Etude physiologique et génétique de la polyembryonie haploïde- diploïde chez le lin (*Linum usitatissimum* L.), PhD. Thesis, Ecole Nationale Superieure Agronomique de Rennes, Rennes, France.

Islam MR and Mayo GME (1990) A Compendium on Host Genes in Flax Conferring Resistance to Flax Rust. Plant Breeding 104: 89-100.

Jackson and Jacob (1985) Linaceae. In: Australian Agricultural Botany, Sydney University Press, Sydney pp. 189-191.

Jeswani, L.M. & A.B. Joshi (1964). Inheritance of resistance to wilt in linseed. Indian J. Genet. 24 (1): 92-94.

Jeswani, L.M. & Y.M. Upadhyaya (1970). Genetics of wilt-resistance in linseed. Indian J. Genet. 30 (1): 104-108.

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ and Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789-793.

Jones ES, Liu CJ, Gale MD, Hash CT and Witcombe JR (1995) Mapping quantitative trait loci for downy mildew resistance in pearl millet. Theoretical and Applied Genetics 91: 448-456.

Jourdren C, Barret P, Brunel D, Delourme R and Renard M (1996) Specific molecular marker of the genes controlling linolenic acid content in rapeseed. Theoretical and Applied Genetics 93: 512-518.

Kamthan KP, Misra DP and Shukla AK (1981) Independent genetic resistance to wilt and rust in linseed. Indian J. Agric. Sci. 51 (8): 556-558.

Kanazin V, Marek LF and Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc. Natl. Acad. Sci. USA 93: 11746-11750.

Kappert H (1924) Erblichkeitsuntersuchungen an weissblühenden Leinsippen. Bericht der Deutschen Botanischen Gesellschaft, 42: 434-441 (cited in Tammes T 1928).

Kappert H (1933) Erbliche Polyembronie bei *Linum usitatissimum*. Biologisches Zentralblatt 53: 276-307.

Kasha KJ and Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.), Nature 225: 874-876.

Kasha KJ, Yao Q, Simion E, Hu T, Oro R (1995) Production and application of doubled haploids in crops. In: Induced Mutations and Molecular Techniques for Crop Improvement, Proceedings of an International Symposium on the use of Induced Mutations and Molecular Techniques for Crop Improvement, International Atomic Energy Agency, Vienna.

Keen NT (1990) Gene-For-Gene Complementarity in Plant-Pathogen Interactions. Annual Review of Genetics 24: 447-463.

Knowles PF and Houston BR (1955) Inheritance of Resistance to Fusarium Wilt of Flax in Dakota Selection 48-94. Agron. J. 47: 131-135.

Knowles PF, Houston BR and McOnie JB (1956) Inheritance of Resistance to Fusarium Wilt of Flax in Punjab 53. Agron. J. 48: 135-137.

Kommendahl T, Christensen JJ and Frederiksen RA (1970) A Half Century of Research in Minnesota on Flax Wilt Caused by *Fusarium*

oxysporum. Agricultural Experiment Station, Technical Bulletin 273, University of Minnesota.

Lander ES and Botstein D (1989) Mapping Mendelian Factors Underlying Quantitative Traits Using RFLP Linkage Maps. Genetics 121: 185-199.

Landry BS, Kesseli RV, Farrara B and Michelmore R (1987) A genetic linkage map of lettuce *Lactuca sativa* L. with restriction fragment length polymporphism, isozyme, disease resistance and morphological markers. Genetics 116: 331-337.

Landry BS, Hubert N, Etoh T, Harada JJ and Lincoln SE (1991) A genetic linkage map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. Genomics 34: 543-552.

Laurie DA and Bennett MD (1988) The production of haploid wheat plants from wheat x maize crosses. Theoretical and Applied Genetics 76: 393-397.

Lawrence GJ, Shepherd KW, Mayo, GME and Islam MR (1994) Plant resistance to rusts and mildews: genetic control and possible mechanisms. Trends in Microbiology 2 (8): 263-270.

Lawrence GJ, Finnegan EJ, Ayliffe M A and Ellis JG (1995). The L6 Gene for Flax Rust Resistance is Related to the Arabidopsis Bacterial Resistance Gene *RPS2* and the Tobacco Viral Resistance Gene *N*. The Plant Cell 7: 1195-1206.

Lee M (1995) DNA Markers and Plant Breeding Programs. Advances in Agronomy 55: 265-344.

Leonards-Schippers C, Gieffers W, Schäfer-Pregel R, Ritter E, Knapp SJ, Salamini F and Gebhardt C (1994) Quantitative Resistance to *Phytophthora infestans* in Potato: A Case Study for QTL Mapping in an Allogamous Plant Species. Genetics 137: 67-77.

Lin J-Z and Ritland K (1996) The effects of selective genotyping on estimates of proportion of recombination between linked quantitative trait loci. Theor. Appl. Genet. 93: 1261-1266.

Lin J, Kuo J, Ma J, Saunders JA, Beard HS, MacDonald MH, Kenworthy W, Ude GN and Matthews BF (1996) Identification of Molecular Markers in Soybean Comparing RFLP, RAPD and AFLP DNA Mapping Techniques. Plant Molecular Biology Reporter 14 (2): 156-169.

Lincoln SE, Daly MJ and Lander ES (1993) Constructing Genetic Linkage Maps with MAPMAKER/EXP Version 3.0: A Tutorial and Reference Manual. A Whitehead Institute for Biomedical Research Technical Report.

Martin GB, Williams JGK and Tanksley SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc. Natl. Acad. Sci. USA 88: 2336-2340.

Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED and Tanksley SD (1993) Map-Based Cloning of a Protein Kinase Gene Conferring Disease Resistance in Tomato. Science 262: 1432-1436.

Martinez O (1996) Spurious linkage between markers in QTL mapping. Mol. Breed. 2: 351-358.

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR and Tanksley SD (1988) Molecular mapping of rice chromosomes. Theoretical and Applied Genetics 76: 815-829.

Melchinger AE (1990). Use of molecular markers in Breeding for Oligogenic Disease Resistance. Plant Breeding 104: 1-19.

Michelmore RW, Paran I and Kesseli RV (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proceedings Of The National Academy Of Science of USA 88: 9828-9832.

Mindrinos M, Katagiri F, Yu G, Ausubel FM (1994) The *A. thaliana* Disease Resistance Gene *RPS2* Encodes a Protein Containing a Nucleotide-Binding Site and Leucine-Rich Repeats. Cell 78: 1089-1099.

Murray BE (1985) Studies of haploid-diploid twins in flax (*Linum usitatissimum*), Can. J. Genet. Cytol. 27: 371-379.

Nair S, Bentur JS, Prasada Rao U and Mohan M (1995) DNA markers tightly linked to a gall midge resistane gene (*Gm2*) are potentially useful for marker-aided selection in rice breeding. Theoretical and Applied Genetics 91: 68-73.

Nichterlein K, Umbach H and Friedt W (1991) Genotypic and exogenous factors affecting shoot regeneration from anther callus of linseed (*Linum usitatissimum*). Euphytica 58: 157-164.

Nichterlein K and Friedt W (1993) Plant regeneration from isolated microspores of linseed (*Linum usitatissimum*). Plant Cell Reports 12: 426-430.

Oh BJ, Frederiksen RA and Magill CW (1994) Identification of Molecular Markers Linked to Head Smut Resistance Gene (*Shs*) in Sorghum by RFLP and RAPD Analyses. Phytopathology 84: 830-833.

Paran I, Kesseli R and Michelmore R (1991) Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines. Genome 34: 1021-1027.

Paterson AH, Tanksley SD and Sorrells ME (1991). DNA markers in plant improvement. Advances in Agronomy 46: 39-90.

Pecchioni N, Faccioli P, Toubia-Rahme H, Valè G and Terzi V (1996) Quantiative resistance to barley leaf stripe (*Pyrenophora graminea*) is dominated by one major locus. Theoretical and Applied Genetics 93: 97-101.

Penner GA, Chong J, Wight CP, Molnar SJ, Fedak G (1993) Identification of an RAPD marker for the crown rust resistance gene *Pc68* in oats. Genome 36: 818-820.

Polzin KM, Calvo ES, Olson TC and Shoemaker RC (1994) Duplicate Loci in Soybean: Characterisation of the *sle* and A071 Loci. In: Gresshoff, P.M. (Ed.) Plant Genome Analysis, CRC Press, pp. 135-145.

Popescu F and Schuster W (1985) Untersuchungen über die Vererbung der Resistenz des Leins gegen die Leinwelke (*Fusarium oxysporum* f.sp. *lini*). Probl. Genet. Teor. Aplic 17 (1): 1-12.

Poulsen DME, Henry RJ, Johnston RP, Irwin JAG and Rees RG (1995) The use of bulked-segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley. Theoretical and Applied Genetics 91: 270-273.

Rajhathy T (1976) Haploid flax revisited. Zeitschrift für Pflanzenzüchtung 76: 1-70.

Robertson DS (1989) Understanding the Relationship between Qualitative and Quantitative Genetics. In: Development and Application of Molecular Markers to Problems in Plant Genetics (Helentjaris T and Burr B, Eds.), Cold Spring Harbor Laboratory, pp. 81-87.

Rowland GG (1991) An EMS-induced low-linolenic acid mutant in McGregor flax (*Linum usitatissimum* L.). Can. J. Plant Sc. 71: 393-396.

Rozhmina TA, Rogash AR and Kulikova AE (1989) A Genetic Study of the Sources and Selection of the Donors of Resistance in Fibre-Flax to Fusarium Wilt and Rust. Sbornik-Nauchnykh-Trudov-po-Prikladnoi-Botanike,-Genetike-i-Selektsii 125: 69-74.

Saghai Maroof MA, Zhang Q and Biyashev RM (1994) Molecular marker analyses of powdery mildew resistance in barley. Theoretical and Applied Genetics 88: 733-740.

Secor DL and Russell SD (1988) Megagametophyte organization in a polyembryonic line of *Linum usitatissimum*, Amer. J. Bot. 75 (1): 114-122.

Shoemaker RC, Lorenzen LL, Diers BW and Olson TC (1994) Genome Mapping and Agriculture. In: Gresshoff, P.M. (Ed.) Plant Genome Analysis, CRC Press, pp. 1-18.

Snape JW (1989) Doubled haploid breeding: theoretical basis and practical applications. In: Review of Advances in Plant Biotechnology (Eds.: Mujeeb-Kazi and Sitch) International Maize and Wheat Improvement Centre. pp. 19-30.

Song W, Wang G, Chen L, Kim H, Pi L, Holsten T, Gardner J, Wang B, Zhai W, Zhu L, Fauquet C and Ronald P (1995) A Receptor Kinase-Like Protein Encoded by the Rice Disease Resistance Gene, *Xa21*. Science 270: 1804-1806.

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG and Jones DG (1995) Molecular Genetics of Plant Disease Resistance, Science 268: 661.

Stuber CW (1995) Mapping and manipulating quantitative traits in maize. Trends in Genetics 11 (12): 477-481.

Stymne S, Tonnet ML and Green AG (1992). Biosynthesis of Linolenate in Developing Embryos and Cell-Free Preparations of High-Linolenate

Linseed (*Linum usitatissimum* L.) and Low-Linolenate Mutants. Archives of Biochemistry and Biophysics 294 (2): 557-563.

Tammes T (1928) The Genetics of the Genus Linum. Bibliographia Genetica 4: 1-36.

Tanksley SD, Ganal MW, Prince JP, de Vincente MC, Bonierable MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Röder MS, Wing RA, Wu W, and Young ND (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 123: 1141-1160.

Tanksley SD (1993) Mapping Polygenes. Annual Review of Genetics 27: 205-233.

Thompson TE (1977) Haploid breeding techniques for flax. Crop Science 17: 767-760.

Timmis JN and Ingle J (1973) Nature New Biol. 244: 235-236. (cited in Cullis, 1981).

Tisdale WH (1917) Flaxwilt: A study of the nature and inheritance of wilt resistance. Journal of Agricultural Research 11 (11): 573-605.

Wang GL, Mackill DJ, Bonham M, McCouch SR, Champoux MC and Nelson RJ (1994) RFLP Mapping of Genes Conferring Complete and Partial Resistance to Blast in a Durably Resistant Rice Cultivar. Genetics 136: 1421-1434.

Wang GL and Paterson AH (1994) Assessment of DNA pooling strategies for mapping of QTLs. Theoretical and Applied Genetics 88: 355-361.

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18 (24): 7213-7218.

Westecott MB, Huang B (1995) Application of haploidy in genetic manipulation of Canola. In: Current Issues in Plant Molecular and Cellular Biology (Eds.: Terzi M, Cella R, Falavigna A), Kluver, Dordrecht, pp. 143-148.

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C and Baker B (1994) The Product of the Tobacco Mosaic Virus Resistance Gene *N*: Similarity to Toll and the Interleukin-1 Receptor. Cell 78: 1101-1115.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18 (22): 6531-6535.

van Eck HJ, van der Voort JR, Draaistra J, van Zandvoort P, van Enckevort E, Segers B, Peleman J, Jacobsen E, Helder J and Bakker J (1995) The inheritance and chromosomal localisation of AFLP markers in a non-inbred potato offspring. Molecular Breeding 1: 397-410.

Vodkin LO (1994) Molecular Exploitation of Soybean Genetic Resources. In: Gresshoff, P.M. (Ed.) Plant Genome Analysis, CRC Press, pp. 83-95.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23 (21): 4407-4414.

Yaghoobi J, Kaloshian I, Wen Y and Williamson VM (1995) Mapping a new nematode resistance locus in *Lycopersicon peruvianum*. Theoretical and Applied Genetics 91: 457-464.

Young ND, Zamir D, Ganal MW and Tanksley SD (1988) Use of Isogenic Lines and Simultaneous Probing to Identify DNA Markers Tightly Linked to the *Tm-2a* Gene in Tomato. Genetics 120: 579-585.

Yu YG, Buss GR and Saghai Maroof MA (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc. Natl. Sci. USA 93: 11751-11756.

Zamir D and Tadmor Y (1986) Unequal Segregation of Nuclear Genes in Plants. Bot. Gaz. 147: 355-358.

Appendix 1

Analysis of variance for distributions of wilt resistant parental lines 'CRZY8/RA91' and the resistant category of doubled haploid lines testing the null hypothesis. The P-value (0.0006) indicates that the two distributions are significantly different.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DH	42	771	18.357	100.63
CRZY8/RA91	31	818	26.387	72.845

ANOVA

Source of Var.	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1150	1	1150	12.938	0.0006	3.9
Within Groups	6311	71	88.887			
Total	7461	72				

Appendix 2

50% Hybridisation solution:

(100 ml)		
	Formamide	50 ml
	20x SSPE	30 ml
	20% SDS	2.5 ml
	50% Dextran sulphate	12 ml
	100x Denhardt	5 ml
	Denatured ssDNA	1.7 ml

25% Hybridisation solution:

	Formamide	25 ml
	as above	

100x Denhardt solution:

(100 ml)		
	Bovine serum albumin	2 g
	Ficoll 400	2 g
	Polyvinylpyrrolidine	2 g

20x SSPE:		
	NaCl	175.3 g
	$NaH_2PO_4.H_2O$	27.6 g
	EDTA	7.4 g
	pH 7.4 and make up to 1 litre	