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Assessment of Two Molecular Marker Techniques in Wheat (*Triticum aestivum* L.) Populations Segregating for Aluminum Resistance

by

Gregory Warren Peterson 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Molecular Biology and Genetics

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15 December 1997

Abstract

Aluminum (Al) becomes phytotoxic in acidic solutions producing symptoms of mineral deficiency and reducing root growth. The primary site of Al-toxicity is the root tip. Aluminum stress can be quantified by measuring root tip callose accumulation. The purpose of this thesis was to characterize the Al-response in segregating populations derived hexaploid wheat, Katepwa and Alikat, and completely homozygous Katepwa and Alikat lines, developed using the doubled-haploid technique. These populations were used to determine if randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) molecular markers co-segregate with Al-resistance. Doubled-haploid populations segregated as if controlled by a single dominant Al-resistance gene. The segregating population from Alikat and Katepwa appeared to be under multigenic control. RAPD primers were unable to generate polymorphisms linked to Al-resistance in the segregating populations. RFLP's identified by probes cdo938 and cdo1395 associated with parental genotypes, but technical difficulties prevented the mapping of these markers.

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Abbreviations

AFLP ..... amplified fragment length polymorphism

Al ..... aluminum

Al_T ..... total soluble aluminum

ANOVA ..... analysis of variance

BC_1 ..... first backcross generation

bp ..... base pair

cM ..... centimorgan

CTAB ..... hexadecyltrimethylammonium bromide

cv ..... cultivar

ddH_2O ..... double-distilled water

DGGE ..... denaturing gradient gel electrophoresis

DH ..... doubled-haploid

DNA ..... deoxyribonucleic acid

DMSO ..... dimethyl sulphoxide

dNTP ..... deoxy-"nucleic acid"-triphosphate

EDTA ..... ethylenediaminetetraacetic acid

F_1 ..... first filial generation

F_2 ..... second filial generation

F.M. ..... fresh mass

kb ..... kilobase

K_{sp} ..... solubility constant

LB ..... Laurier broth

MW ..... molecular weight

PAR ..... photosyntheticly active radiation

Pa ..... pascal

PCR ..... polymerase chain reaction

PE ..... pachyman equivalents

RAPD ..... randomly amplified polymorphic DNA

RFLP ..... restriction fragment length polymorphism

RH ..... relative humidity

RNA ..... ribonucleic acid

RNase ..... ribonucleic acid nuclease

SSC ..... NaCl (150 mM) sodium citrate (15 mM)

SDS ..... sodium dodecylsulphate

STS ..... sequence tagged site

t ..... Student t-test statistic

TBE ..... Tris-HCl (90 mM), borate (90 mM), EDTA (2 mM)

TE ..... Tris-HCl (10 mM), EDTA (1 mM)

TGE ..... Tris-HCl (25 mM), glucose (50 mM), EDTA (10 mM)

TNE ..... Tris-HCl (10 mM), NaCl (0.1 M), EDTA (1 mM)

Tris-HCl ..... tris(hydroxymethyl)aminomethane-HCl

wali ..... wheat aluminum induced

X^2 ..... chi-square statistic

Chapter 1. Introduction

1.1 Wheat as an Important Crop

Wheat is one of the most widely planted crops in the world and is most commonly represented by the two species: bread wheat, *Triticum aestivum* L. ($6x = 42$, *AABBDD*), and durum wheat, *Triticum turgidum* L. ($4x = 28$, *AABB*). World wheat production is about 590 million tonnes a year, making it the world's most productive crop. In comparison, 510 million tonnes of rice (*Oryza sativa* L.) are produced annually (Encarta, 1996). Wheat was planted on 3.1 million ha (33%) of Alberta's crop land in 1996, yielding 8.1 million tonnes of grain. One quarter of Canada's annual wheat production is grown in Alberta. Wheat accounts for 50% of Alberta's total grain production, adding nearly $1.7 billion to the Alberta economy. Barley (*Hordeum vulgare* L.) is the second most important crop in Alberta, representing 40% of Alberta's annual grain production and producing $1.13 billion annually. Canola (*Brassica* spp.) produced 20% of the annual yield of wheat and $625 million in 1996 (Alberta Agriculture, 1996). Wheat is a significant crop in Alberta and an important contributor to the Alberta and Canadian economies.

1.2 Acid Soils and Aluminum Phytotoxicity

Alberta has about 2.2 million ha of soil with pH below 6.0 and 300,000 ha of soil below pH 5.0. Areas most affected by acid soils are the Peace River Region, where over 30% of the soils are below pH 6.1, and the Thin Black and Dark Brown soils of the Bow River region, where about 28% and 20% of the soils, respectively, have a pH less then 6.1 (Penny *et al.*, 1977). Over 5% of Canadian soils are acidic with an additional 10-20% at risk due to nitrogen based fertilizers and acidic precipitation (Coote, 1981). Approximately 40% of the world's arable soils are acidic (Van Wambeke, 1976). At low pH, crystal lattice clay soils tend to disintegrate and release Al cations (Van Wambeke, 1976). Acidification also occurs through the nitrification of ammonium (NH_4^+) to nitrate (NO_3^-) with the accompanying release of hydronium ions (H_3O^+) (Coote, 1981).

Hydrated monomeric Al^{3+}, like other small, highly charged cations, behaves as a

Brønsted acid by donating a proton from the surrounding shell of coordinating water molecules, such that:

$$[Al(H_2O)_6]^{3+} + nH_2O \rightleftharpoons [Al(H_2O)_{6-n}(OH)_n]^{(3-n)+} + nH_3O^+. \quad (1)$$

Hydrolysis continues until $Al(OH)_3$ is formed. The anion, $Al(OH)_4^-$, is also formed, but lacks co-ordinating water molecules. Aluminum is only sparingly soluble at neutral pH values. The solubility constant (K_{sp}) of $Al(OH)_3$ is only 10^{-33}. As pH decreases, the solubility of Al increases as the hydroxide ions are removed in attempt to maintain equilibrium (Atkins, 1989). Although the toxic Al^{3+} anion is present at pH 6, at pH 4.9 nearly 80% of total soluble Al (Al_T) is the Al^{3+} anion. The extent of Al^{3+} formation is affected by the presence of other anions, ligands present in solution (Ritchie, 1989), and pH (Wright *et al.*, 1987). In general the concentration of Al_T in soil is between 10 and 350 μM (Ritchie, 1989), however it rarely exceeds 140 μM (Haug, 1984).

The phytotoxicity of Al in acid soils was identified early in the 1900's (Hartwell and Pember, 1918; Hoffer and Carr, 1923; Line, 1926). More recent studies confirmed the detrimental effects of Al on plants, but modern techniques are unable to determine the speciation of Al_T in acid soils. Thus, it has been difficult to determine the species of Al that are the most phytotoxic. Polynuclear Al_{13} (Parker *et al.*, 1987) and Al^{3+} (Kinraide, 1988) are believed to be the two primary toxic species of Al in aqueous growth media. Recent work has suggested that Al-hydroxy species may not be phytotoxic whereas Al-fluorine species are phytotoxic. (Kinraide, 1997). The Al-sulphate species are known to be nonphytotoxic (Kinraide and Parker, 1987; Kinraide, 1997). Depending on the nutrient solution and/or ligands present, Al^{3+} may only represent a fraction of a percent of Al_T (Kinraide and Parker, 1989), with other monomeric and polymeric species present. An aqueous growth media system was used in this study to carefully regulate pH and to control Al speciation, however, the microenvironment of the rhizosphere, cell surface, and the cytoplasm may be substantially different from the aqueous growth medium. Different Al species may be present even in an aqueous growth media.

Aluminum phytotoxicity can be alleviated either by increasing soil pH or by growing Al-resistant genotypes. Liming soils with $CaCO_3$ was suggested as early as 1925

by Harcourt, *et al.*, to increase wheat and clover yields in southern Ontario. Liming has been used successfully to ameliorate metal toxicity in land reclamation (Winterhalder, 1991), and to improve crop yield on acid soils (Dolling *et al.*, 1991a,b; McCray *et al.*, 1991). Use of lime is still advocated (Hoyt, 1979; Sparrow, 1984), however, regions like the Peace River of Alberta do not have nearby sources of limestone and liming would be expensive (Sparrow, 1984). Lime may not alleviate Al-toxicity in all cases. Lime may induce phosphorus and micronutrient deficiencies and remove soluble anions that complex with Al cations, thus enriching the Al_T with phytotoxic species of Al (Ritchie, 1989). Curtin and Smillie (1983) also found that NH_4^+ was released from organic matter in as little as 30 weeks after liming. Nitrification then releases hydronium ions, decreasing the pH, and increasing Al_T. For sustained growth on acid Al containing soils, cultivars of Al-resistant crops should be identified or bred (Foy, 1983).

1.3 Response of Plants to Aluminum

Symptoms of Al toxicity resemble those of nutrient deficiency and drought stress. Foliar symptoms resembling phosphate (PO_4^{3-}) deficiency include stunting, small dark green leaves, late maturity, purple stems and vascular tissue, and the yellowing or necrosis of leaf tips. Symptoms typical of Ca^{2+} deficiency include curling or rolling of young leaves, inhibited lateral branch growth and the collapse of petioles and apicies. Roots appear stubby, brittle, and lack fine branching (Foy, 1992). It has been hypothesized that Al can interfere with cell division in root apices, increase cell wall rigidity, reduce DNA replication by increasing the rigidity of the double helix, decrease root respiration, interfere with enzymes, and modify the structure and function of the cell membrane (Foy, 1992).

Taylor (1988) summarized the phytotoxic effects of Al into four categories: disruption of membrane structure and function; inhibition of DNA synthesis and mitosis by cross-linking DNA strands; inhibition of cell elongation; and disruption of mineral nutrition and metabolism. The primary site of Al toxicity is the terminal 2-3 cm of root tips (Ryan *et al*, 1993). Resistance mechanisms to these toxic effects can be summarized

into two main resistance strategies. The first strategy involves modification of the extracytosolic environment to render Al less toxic or creation of barriers against the entry of Al into the cytosol. The second strategy involves tolerating Al within the cytosol by detoxification or compartmentalization of Al (Taylor, 1988, 1991, 1995).

Several Al-resistance mechanisms are currently gaining general acceptance. One mechanism involves exudation of ligands to detoxify soluble Al (Basu *et al.*, 1994b; Basu, *et al.*, 1994c; Delhaize *et al.*, 1993; Lindburg, 1990; Pellet *et al.*, 1996). A second mechanism alleviates Al toxicity by use of the vacuole. Aluminum is either sequestered within the vacuole or a neutral cytosolic pH is maintained by means of pumping cytosolic protons into the vacuole (Matsumoto, 1991; Kasai *et al.*, 1992, 1993). A 51 kD protein associated with the tonoplast membrane in wheat has been found that may be involved with sequestering Al into the vacuole, however there is no evidence that this protein is associated with an Al or proton pump (Basu *et al.*, 1994a; Taylor *et al.*, 1997).

A number of ligands have been found to be exuded in response to Al-induced stress. Phosphate exudation was enhanced in *Beta vulgaris* (Lindberg, 1990) and *T. aestivum* (Pettersson and Strid, 1989) when exposed to Al. Phosphate is a strong ligand (Ritchie, 1989) that may reduce the activity of Al by forming a precipitate of $Al(OH)_2 \cdot H_2PO_4$ (Lindberg, 1990). Organic acid anions such as malate and citrate are also exuded. Delhazie *et al.*, (1993) found that a near-isogenic Al-resistant wheat genotype released more malate from the terminal 5 mm of root tip than an Al-sensitive near-isogenic line. Malate is produced *de novo* via the TCA cycle in Al resistant wheat cultivars Atlas 66 and Maringa when stressed by Al, but produced in lower concentrations in Al-sensitive cultivars Roblin and Katepwa (Basu *et al.*, 1994c). When Atlas 66 was compared with Al-sensitive Scout 66, phosphate and malate exudation were 4- and 11-fold greater in the Al-resistant cultivar than in the Al-sensitive cultivar. Aluminum resistant, ET3, produced 8-fold more malate than Al-sensitive, ES3, however ET3 produced less than a third of the phosphate as Atlas 66 (Pellet *et al.*, 1996). This suggests multiple Al-resistance mechanisms in the true-breeding cultivar Atlas 66, but a single Al-resistance mechanism in the backcross line ET3. Other external mechanisms

have also been suggested such as the immobilization of Al in mucilage around the root tip (Archambault *et al.*, 1996) and induction and efflux of polypeptides (Basu *et al.*, 1994b). These methods have not been examined to the same extent as other external methods.

Internal tolerance mechanisms have not been studied to the same extent as external mechanisms (Taylor, 1991). Basu *et al.*, (1994a) found two 51 kD proteins that were induced upon exposure to Al in the Al-resistant cultivar PT741. Both proteins were expressed in the tonoplast of 2 cm root tips within 24 hours, then disappeared within 72 hours after removal of Al. Incorporation of ^{35}S-methionine suggested that Al enhanced the synthesis of the 51 kD proteins in PT741 and not protein synthesis in general. In an F_2 segregating population arising from a cross between Al-sensitive Katepwa and Al-resistant PT741, the 51 kD proteins were induced within 6 hours after exposure to Al and co-segregated with the resistant phenotype (Taylor *et al.*, 1997). Matsumoto (1991) and Kasai *et al.* (1992, 1993) found that the activity of proton pumps in the tonoplast membrane increased in response to Al. However, Taylor *et al.* (1997) found that antibodies raised against known proton pump subunits did not cross-react with the 51 kD proteins and also suggested that the 51kD proteins are too small to be likely candidates. These proteins also appear to be distinct from the group of wheat Al-induced (*wali*) proteins (Snowden and Gardner, 1993; Richards *et al.*, 1994). Thus, these proteins may be part of a distinct resistance strategy (Taylor *et al.*, 1997).

Approaching the problem of understanding Al-resistance from a genetic rather than a physiological perspective may help to understand where the currently identified aluminum-induced proteins fit into an Al-resistance strategy and may help to identify the primary components of Al-resistance.

1.4 Genetics of Aluminum Resistance

As previously mentioned, Al-resistance differs from one cultivar to the next. European (Mesdag and Slootmaker, 1969; Rengel and Jurkic, 1992, 1993), North American (Mesdag and Slootmaker, 1969; Lafever *et al.*, 1977; Zale and Briggs, 1988; Briggs *et al.*, 1989) and Australian germplasm (Scott *et al.*, 1992) have shown a

continuum of Al-resistance from resistant to sensitive. Aluminum resistance in bread wheat appears to have originated mainly from Brazilian germplasm (Mesdag and Slootmaker, 1969; Zale and Briggs, 1988; Briggs *et al.*, 1989; Lagos *et al.*, 1991; Scott *et al.*, 1992). Lafever *et al.* (1977) and Foy *et al.* (1974) found evidence of unconscious selection for Al-resistance in eastern U.S. wheat cultivars developed on acid soils. Older cultivars, developed on unlimed soils in Ohio, U.S.A., were more Al-resistant than newer cultivars developed on limed soils (Foy *et al.*, 1974). Tetraploid durum wheat from central Europe and Australia show a continuum of Al-resistance, as well. However the range is smaller and durum genotypes are not as resistant as the Al-resistant bread wheat cultivar, Atlas 66.

Modern central European and Australian durum cultivars do not share a common ancestry and yet have the same level of Al-resistance (Cossic *et al.*, 1994), suggesting an ancient basis for Al-resistance. Since durum wheats contribute the A and B genomes to bread wheat, some Al-resistance from the A and/or B genomes contributes to overall Al-resistance (Cosic *et al.*, 1994). The multigene model for aluminum resistance in hexaploid wheat is supported by the evidence that different cultivars demonstrate a range of Al-resistance (Carver and Ownby, 1995). However, individual cultivars may only possess a single Al-resistance gene. Aluminum-resistant backcross line, Alikat, when crossed with the Al-sensitive cultivar, Katepwa, produces an F_2 population that segregated in a 3:1 (resistant: sensitive) ratio suggesting a single dominant Al-resitance gene in Alikat (Somers and Gustafson, 1995; Somers *et al.*, 1996).

One approach to determining the mechanisms of Al-resistance in wheat is to locate the genes responsible for Al-resistance. Polle *et al.* (1978) was one of the first to suggest a chromosome location for Al-resistance. Chromosome 4D from Al-sensitive cv. Thatcher was substituted for chromosome 4D in the moderately Al-resistant cv. Chinese Spring. The Al-resistance level of Chinese Spring decreased to that of Thatcher. Aniol (1990) screened ditelosomic lines of Chinese Spring to locate Al-resistance. Lines lacking chromosome arm 5AS lacked Al-resistance over a wide range of Al concentrations, however those lacking 2DL and 4DL lacked Al-resistance only at high Al concentrations.

When the lines lacking 5AS and 4DL were crossed with Al-resistant Brazilian cv., BH1146, twice as many F_2 plants lacked Al-resistance when compared to euploid Chinese Spring crossed with BH1146. In a separate experiment, plants lacking arm 5AS accumulated twice as much Al in roots at low Al concentrations compared with plants not lacking 5AS. Plants lacking arm 2DL accumulated less Al than plants with arm 2DL at all concentrations. Aniol (1990) suggested that genes on 5AS may be responsible for the control of Al uptake at low Al concentrations and genes on 2DL and 4DL may only control Al uptake at high concentrations. Genes on 5AS may play a role in Al detoxification, since all plants lacking arm 5AS were Al-sensitive (Aniol, 1990).

Lagos *et al.*, (1991) found that an F_2 population derived from a cross between Al-resistant cvs. BH1146 and Atlas 66 had a segregation ratio of 15:1 (resistant:sensitive), suggesting a combination of two different dominant resistance genes. A second cross between BH1146 and a sensitive cultivar CI 14124 had a segregation ratio of 13:3 (resistant:sensitive). This ratio is not the expected 15:1 (resistant:sensitive) segregation for two dominant genes, but it suggests that BH1146 may have two Al-resistance genes. Although not stated by Lagos *et al.* (1991), it is also possible that BH1146 and Atlas 66 have one Al-resistance gene in common, and two other genes that differ.

Chinese Spring behaves as an Al-sensitive cultivar at 300 μM Al (Lagos *et al.*, 1991). Monosomic Chinese Spring lines were crossed with the Al-resistant cultivar, BH1146, and all progeny were Al-resistant when BH1146 contributed chromosome 4D. Minor genes affecting Al-resistance were located on chromosomes 5D and 3B. These results differ somewhat from those reported by Aniol (1990) in that chromosome 5A had no influence on Al-resistance, and other minor genes were observed. A putative role for genes on chromosome 4D was further supported by Berzonsky (1992). When the *D*-genome of Atlas 66 was transferred to the Al-sensitive tetraploid, 4*x*-Canthatch, and selfed to the F_6 generation, fewer than 50% of the individuals in 11 different lines were sensitive and only four F_7 lines had more than 50% sensitive individuals. When F_6 lines were crossed with Atlas 66, the level of Al-resistance increased, whereas Al-resistance decreased when F_6 lines were crossed with Al-sensitive hexaploid, 6*x*-Canthatch. These

experiments suggested that not all the genes for Al-resistance were located in the *D*-genome.

Gustafson and Ross (1990) suggested the expression of Al-resistance can be influenced by genes located on different wheat genomes, supporting the multigene resistance model. As previously mentioned, two independent ligand exudation mechanisms have been observed in Atlas 66 (Pellet *et al.*, 1996), providing physiological support for the multigene resistance model. While all of the studies point to several different mechanisms of Al-resistance, it would be valuable to develop molecular markers specific to Al-resistance mechanisms in different cultivars to locate the genes responsible for Al-resistance in bread wheat.

1.5 Molecular Markers and Mapping

Mapping the location of genes within a genome is based on the concept of trait linkage and genetic recombination. Two phenotypic traits derived from genes on the same chromosome are considered to be linked if the chance of independent segregation is less than 50%. In general, the more often two traits occur together in a segregating population, the more tightly linked the two traits are said to be. If two traits in a segregating population are recombined 1% of the time, they are then said to be 1 centiMorgan (cM) away from each other (Tamarin, 1991). Molecular markers differ from phenotypic markers only in that molecular markers are based on a differing sequence of nucleotides in DNA or amino acids in a protein, rather than a differing morphological characteristic. Molecular markers can be used in the same way as phenotypic markers in breeding programs to select individuals with beneficial characteristics. Molecular DNA markers were used in this study and are referred to in the rest of the text simply as *molecular markers.*

To locate molecular markers linked to Al-resistance, the response of plants to Al must be measured and used to identify unique DNA sequences. A number of methods are currently used to measure Al-resistance in plants. One of the most popular methods is to measure root length or root mass of plants stressed by Al compared to unstressed plants

(Lafever *et al.*, 1977; Zale and Briggs, 1988; Briggs, *et al.*, 1989). The root length or mass of plants grown in high acid/high Al soil or aqueous growth media is compared to those not exposed to Al. The haematoxylin staining method differentiates between Al-resistant and Al-sensitive germplasm on the basis of the degree of staining.(Polle *et al.*, 1978) Haematoxylin has also been employed as a means of measuring root regrowth. Roots exposed to Al in an aqueous growth medium are stained with haematoxylin, then grown in an aqueous medium lacking Al (Riede and Anderson, 1996). Aluminum resistant plants will have a greater length between the stain and the root tip compared to Al-sensitive plants. A third method measures the amount of callose (1,3-ß-D glucan), a polysaccharide produced in response to root injury (Eschrich, 1975). Callose concentrations have been shown to be an accurate measure of Al-resistance and produce results similar to relative root length measurements (Schreiner *et al.*, 1994). Callose can accumulate to observable levels in as short as a few minutes in 75 μM Al solution (Zhang *et al.*, 1994).

Currently there are genetic maps for all 7 chromosome groups in wheat (Chao *et al.*, 1989; Gill *et al.*, 1991a; Anderson *et al.*, 1992; Devos *et al.*, 1992, 1993; Xie *et al.*, 1993; Hohmann *et al.*, 1994; Gale *et al.*, 1995; Mickelson-Young *et al.*, 1995; Nelson *et al.*, 1995; Van Deynze *et al.*, 1995b; Marino *et al.*, 1996), the related species *T. monococcum, Hordeum vulgare* (Dubcovsky *et al.*, 1996), *Zea mays, Oryza satvia, Avena sativa* (Van Deynze *et al.*, 1995a), *Aegilops squarrosa* (syn. *T. taushii*; Kerby and Kuspira, 1987) (Lagudah *et al.*, 1991; Gill *et al.*, 1991b), and the related wild diploid perennial Triticeae (Wei and Wang, 1995). The three genomes of bread wheat show an extreme degree of conservation in locus order (Gale et al., 1995). Most genetic maps are based on restriction fragment length polymorphism (RFLP) markers. This involves digesting genomic DNA using restriction endonucleases that recognize sequences six-nucleotides long. DNA fragments are separated on an agarose gel and transferred to a nitrocellulose or nylon membranes. Restriction fragment length polymorphism probes are derived from anonymous cDNA sequences or cDNA of known proteins that are labelled with ^{32}P. Labelled clones are hybridized to the digested genomic DNA on the membranes.

Recognized sequences are visualized on negative film as an autoradiogram (Botstein *et al.*, 1980). Polymorphisms arise when there is a change in base pair number between restriction sites, the loss or addition of a restriction site due to mutation, or chromosome rearrangement.

Another common molecular marker technique is randomly amplified polymorphic DNA (RAPD, Williams *et al.*, 1990). The RAPD technique is based on the amplification of genomic DNA sequences using random decamer primers or arbitrary sequences using the polymerase chain reaction (PCR). Polymorphisms arise when one genotype lacks a primer annealing site or the distance between primer annealing sites on opposite strands is too large to replicate a complete doubled stranded sequence. RAPDs allow the detection of polymorphisms in regions of repetitive sequences that are inaccessible to RFLP mapping. The usefulness of RAPD markers in wheat was demonstrated by Devos and Gale (1992) in large regions of repetitive sequences in wheat. RFLP probes cannot map in regions of highly repetitive DNA sequences because probes hybridize too many restriction fragments, making it impossible to resolve polymorphisms. RAPD primers will recognize a specific variation in the repetitive region and only amplify that specific region between two primers.

A number of molecular marker techniques have been developed based on PCR to overcome some of the limitations of RAPD and RFLP markers. Microsatellites are regions of genomic DNA that contain up to 40 tandem repeats of base pairs that are variable between species, cultivars and individuals. Regions of microsatellites are amplified using PCR primers that recognize the tandem repeat sequences. These tandem repeats are most likely caused by slippage errors during DNA replication (Tautz *et al.*, 1986). Röder *et al.*, (1995) evaluated the use of microsatellites in wheat and found that they were up to 10 times more variable than RFLP markers.

Another technique, sequence tagged site (STS)-PCR, uses PCR primers designed from RFLP probes to amplify genomic DNA. Sequence tagged site-PCR has been suggested as a non-radioactive alternative to RFLP (Talbert *et al.*, 1994). One drawback to STS-PCR is that the nucleotide sequence is required from RFLP probes before primers

can be designed (Talbert *et al.*, 1994). Amplified fragment length polymorphism (AFLP) is another combination of RFLP and RAPD-PCR techniques. Genomic DNA from a segregating population is digested as in the RFLP technique. Oligonucleotide adapters of a known sequence are added to the digested fragments. PCR probes that recognize these adapters and up to 3 nucleotides of the restricted fragment are used to amplify a subset of digested fragments. These amplifed fragments are then separated on a denaturing polyacrylamide gel. Typically 50 to 100 loci are amplified, making this technique more sensitive than RAPD and RFLP (Vos *et al.*, 1995). However AFLP has been optimized for genomes smaller than 6×10^9 base pairs (GibcoBRL, 1995) and the wheat genome is about 1.6×10^{10} base pairs (Arumuganathan and Earle, 1991), putting it outside the range of AFLP.

1.6 Objectives

One approach to understanding the biological basis of Al-resistance in wheat is to characterize the physiological response of wheat to Al. This is done by comparing the physiology and biochemistry of genotypes that differ in their response to Al. Another approach is to locate the genes that are responsible for Al-resistance in wheat, determine the proteins that are encoded, and place those proteins into a response mechanism model. This second method requires the development of molecular markers to locate Al-resistance loci. The technique of chromosome walking can then be used to locate the genes responsible for Al-resistance. Molecular markers can also aid in breeding for Al-resistance by providing a means of testing large numbers of individuals by collecting and testing DNA without having to expose these plants to Al or growing them under intensive laboratory conditions.

The objectives of this thesis were 1) to use segregating populations of hexaploid wheat (*Triticum aestivum* L.) and characterize the response of these populations to Al; and 2) to determine whether RAPD and RFLP techniques could identify molecular markers that co-segregate with the Al-resistance phenotype in these segregating populations. These two molecular marker methods were chosen to map the location of the

Al-resistance gene in wheat scored for Al-resistance using the callose method, since most molecular maps are based on RFLP markers and the RAPD technique is the most established PCR based marker method.

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Chapter 2. Aluminum-Induced Callose Accumulation in Segregating Populations

2.1 Introduction

Several methods can be used to measure the degree of Al stress perceived by roots. These include root elongation rates (Horst *et al.*, 1992; Llugany *et al.*, 1994), relative root length (Zale and Briggs, 1988; Briggs *et al.*, 1989; Hill *et al.*, 1989; Berzonsky, 1992; Schreiner *et al.*, 1994), root regrowth after Al exposure (Aniol, 1984; Gustafson and Ross, 1990; Rajaram and Villegas, 1990; Lagos *et al.*, 1991; Riede and Anderson, 1996), the degree and pattern of haematoxylin staining of Al containing tissues (Polle *et al*, 1978; Scott *et al.*, 1992; Luo and Dvořák, 1996), and the accumulation of callose (1,3-β-D glucans) in roots (Köhle *et al.*, 1985; Zhang *et al.*, 1994).

Accumulation of callose has been used to distinguish the response of different genotypic to Al in a number of species. Horst *et al.* (1992) used callose to evaluate soybean (*Glycine max*) cultivars for Al-resistance. Llugany *et al.* (1992) measured callose synthesis in 5 mm root tips of maize (*Zea mays*) cultivars exposed to Al. It has also been useful in ranking wheat genotypes for Al-resistance (Schreiner *et al.*, 1994; Zhang *et al.*, 1994). Accumulation of callose occurred within minutes of exposure to 75 μM of Al (Zhang *et al.*, 1994). Genotypes ranked on the basis of root length comparisons (Briggs *et al.*, 1989), were similar to those ranked on the basis of callose accumulation (Schreiner *et al.*, 1994). Taylor *et al.* (1997) found callose accumulation co-segregated with an Al-induced 51 kD membrane protein. This protein was associated with Al-resistance in a segregating F_2 population derived from the bread wheat parents, Katepwa (Al-sensitive) and PT741 (Al-resistant). Callose is believed to play a protective role under the conditions of stress by isolating cells that have been damaged by physical, chemical, or pathogenic disruption of the cell membrane (Škalamera and Heath, 1996). Accumulation of callose can be detected cytochemically using aniline blue or resorcin blue (Eschrich, 1975).

Aluminum-induced callose accumulation has not been used to locate co-segregating molecular markers in segregating populations; nor has it been used in genetic studies of the inheritance of Al-resistance. Recent reports of molecular markers linked to

Al-resistance have used the root regrowth (Riede and Anderson, 1996) and haematoxylin (Luo and Dvořák, 1996) methods. The objective of this study was to screen hexaploid wheat genotypes, Katepwa and Alikat, and doubled-haploid derived from these lines, using callose accumulation as a marker of perceived Al stress, and to characterize these populations to determine the genetics of Al-resistance inheritance. These mapping populations were then employed to locate molecular markers for Al-resistance (Chapter 3).

2.2 Materials and Methods

Three sources of germplasm were used to develop segregating populations for use in this study (Figure 2-1). The first segregating population consisted of F_2 individuals derived from a cross between a near-isogenic Al-resistant backcross line, Alikat (Katepwa*3/Maringa; Figure 2-1a) and a near-isogenic Al-sensitive cultivar, Katepwa (Figure 2-1b). The second and third segregating populations were derived from completely homogenous parent lines of Alikat and Katepwa developed using the doubled-haploid method to avoid the possibility of heterogeneity that may be present in Alikat and Katepwa parent populations. These segregating populations consisted of F_2 individuals derived from the cross between an Al-sensitive doubled-haploid line, DH Katepwa-W2 (DHK-W2), and two Al-resistant doubled-haploid lines, DHAlikat-33, (DHA-33) (Figure 2-1c) and DH Alikat-35 (DHA-35). Backcross (BC_1) populations were also developed from these crosses. Doubled-haploid lines were prepared by Dr. T. Aung at Agriculture and Agri-Food Canada, Winnipeg, from the near-isogenic Alikat and Katepwa. Doubled-haploid plants were made to have complete homogeneous material to avoid any complications that may be caused by possible heterogeneity in Alikat or Katepwa. Mapping populations consisted of 50 to 150 F_2 individuals from crosses between near-isogenic parents and doubled-haploid parents.

2.2.1 Screening Katepwa, Alikat, and Progeny

To screen for Al resistance, 600 F_2, 15 F_1, 100 Maringa, 100 Katepwa, and 100

A Katepwa x Maringa
↓
F_1 (Katepwa/Maringa) x Katepwa
↓ select Al-resistant lines
BC_1 (Katepwa*2/Maringa) x Katepwa
↓ select Al-resistant lines
BC_2 (Katepwa*3/Maringa)
↓ select Al-resistant lines = "Alikat"
Alikat (progeny tested)

B Alikat (and Katepwa)
↓ cross with maize (lose maize chromosomes)
↓ germinate haploid seeds
↓ treat with cholchicine (double chromosome number)
↓ harvest doulbed haploid seed
DHA-33, DHA-35 (and DHK-W2)

C Katepwa x Alikat
↓
F_1
↓ selfed
F_2 segregating population

D DHK-W2 x DHA-33 (or DHA-35)
↓
F_1
↓ selfed
F_2 segregating population

and

DHK-W2 x DHA-33 (or DHA-35)
↓
F_1 x DHK-W2
↓
BC_1 segregating population

Figure 2-1. The breeding strategies used to develop (A) Alikat, (B) the doubled-haploid parental lines, (C) the F_2 segregating population from Katepwa and Alikat, and (D) the F_2 and BC_1 segregating populations derived from doubled-haploid parents.

Alikat seeds were sterilized in 1% sodium hypochlorite for 20 minutes. Seeds were washed with distilled H_2O and germinated overnight in an aerated solution of 0.005 $g \cdot L^{-1}$ Vitavax fungicide (Uniroyal Chemical, Calgary, AB). Five clear Plexiglass aquaria were prepared containing 10 L of an aerated rooting solution of 1000 µM $Ca(NO_3)_2 \cdot 4H_2O$, 300 µM $Mg(NO_3)_2 \cdot 6H_2O$, and 300 µM NH_4NO_3 at pH 4.5. Seeds were plated on polyethylene mesh supported by 20 cm x 45 cm plastic grids with 1 cm^2 holes. Grids were suspended over the rooting solution to keep the seeds moist. Aquaria were covered with black plastic for 2 days to maintain high humidity. Aquaria were then uncovered and kept in a growth chamber for 1 day at 21°C, 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 16 hours day length, and 60% R.H.

Seedlings of uniform size were selected and transferred to five separate aquaria: three aquaria, each with 50 F_2 seedlings; one aquarium with 15 Alikat, 15 Maringa, 15 Katepwa, and 6 F_1; and one control aquarium with 10 F_2, 10 Alikat, 10 Maringa, and 10 Katepwa seedlings. Seedlings were placed in 1 cm^2 holes of the plastic support grid and held in place by a 1 cm diameter foam rubber test tube stopper that was cut longitudinally to the centre. The stopper was wrapped around the coleoptile so that the seed and roots were exposed below the stopper and the leaf blade was erect and not covered. The roots were immersed in the hydroponic solution. Seedlings were grown for 6 days suspended over 15 L of full nutrient solution at pH 4.5 (1000 µM Ca^{2+}, 300 µM Mg^{2+}, 800 µM K^+, 3300 µM NO_3^-, 300 µM NH_4^+, 100 PO_4^{3-}, 100.65 µM SO_4^{2-}, 34 µM Cl^-, 20.2 µM Na^+, 10 µM Fe^{3+}, 6 µM BO_3^{3+}, 2 µM Mn^{2+}, 0.5 µM Zn^{2+}, 0.15 µM Cu^{2+}, 0.1 µM Mo^{2+}, 10 µM $EDTA^{2+}$).

Nine-day-old seedlings were transferred to aquaria containing 1.0 mM $CaCl_2$, with or without 75 µM $AlCl_3$, for 24 hours. Three to five root tips were harvested from each plant, weighed, fixed in 95% ethanol, and stored for 16 hours at 4°C prior to determination of callose content. Seedlings were recovered, transferred to 15 L full nutrient solution at pH 6.0 for 5 days, then transplanted to a 1:1:1:1 (loam:sand:vermiculite:peat) soil mixuture, fertilized with 20:20:20 (nitrogen:phosphorus:potassium) slow release fertilizer. Leaves were harvested for DNA isolation and molecular marker analysis (Chapter 3) when 2 g fresh weight was available.

2.2.2 Screening Doubled-Haploid Parents and Progeny

A series of optimization experiments were done to increase the resolution between Al-resistant and sensitive genotypes by adjusting $CaCl_2$, $AlCl_3$, and seedling age at time of Al-exposure. This resulted in minor modifications to the screening method described in Section 2.2.1. To determine the aluminum resistance of DHA-33, DHK-W2, F_1, F_2, and BC_1 populations, seeds were sterilized and germinated as in the 12-day method (Section 2.2.1). Seedlings were grown in darkness in the rooting solution (pH 4.3) for 24 hours, transferred to the full nutrient solution (pH 4.3) for 24 hours of darkness, and then transferred to full light (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 16 hours day length) for 72 hours at 21°C and 60% R.H. Six-day-old seedlings of uniform size were removed from the polyethylene mesh, placed in 1 cm^2 grid using foam stoppers, and transferred to two separate aquaria for 24 hours at full light (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 16 hours day length), 21°C, and 60% R.H. Each aquarium contained 100 µM $AlCl_3$ and 500 µM $CaCl_2$ (pH 4.3). One aqarium held 5 DHK-W2, 5 DHA-33, 6 F_1, and 35 F_2; and the second held 5 DHK-W2, 5 DHA-33, 6 F_1, and 40 BC_1. Three to 5 root tips, 10 mm long, were harvested from each plant, weighed, and fixed in 95% ethanol for 16 hours prior to determination of callose content. The method was repeated for DHK-W2, DHA-35, F_1, F_2, and BC_1 populations, as well as the segregating populations of 50 F_2 plants from DHK-W2 x DHA-33 and DHK-W2 x DHA-35 crosses along with 10 of each respective parent. All populations were transferred, after sampling, to full nutrient solution at pH 6.0 for 3 days to allow roots to regrow. Seedlings were transplanted into a 1:1:1:1 (loam:sand:peat:vermiculite) soil mixture fertilized with 20:20:20 (nitrogen:phosphorus:potassium) slow release fertilizer and grown until 3-5 g of leaves could be harvested for DNA isolation and molecular marker analysis (Chapter 3).

2.2.3 Callose Determination

Callose accumulation was measured by the technique described by Zhang *et al.*, (1994). Ethanol was decanted from the preserved harvested root tips and 200 µl 1N NaOH was added to each Eppendorf tube. Excised roots were incubated at room

temperature for at least 5 minutes to soften tissue and homogenized for 15 to 20 seconds using a teflon mini-pestle (Mandel) attached to an electric hand drill. The pestle was rinsed with 800 µl 1N NaOH to bring the total volume to 1.0 ml. Homogenized root tissue was incubated at 80°C for 15 minutes to dissolve the callose. The homogenate was centrifuged (International Equipment Company Centra-M centrifuge) at full speed for 4 minutes and 400 µl of supernatant was transferred into 12 x 75 mm borosilicate disposable glass tubes (Fisher). Eight hundred microlitres of 1% (w/v) aniline blue (CI 42755, Polysciences, lot #60894), 42 µl of 1 N HCl, and 1.18 ml of 1 M glycine (pH 9.5) was added to each tube and incubated at 50°C for 20 minutes before cooling to room temperature.

Callose concentration was determined spectrofluorometrically using an Aminco-Bowman Series 2 Luminescence Spectrometer and SLM-Aminco Series 2 application software (SLM-Aminco, 1991). The excitation wavelength was 398 nm and the emission wavelength was 495 nm. Standards were prepared by diluting 2 mg pachyman (*Poria cocos*; Calbiochem, lot #B12389) in 100 ml 1 N NaOH and incubating at 80°C for 15 minutes. Pachyman was diluted to 0, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 µg Pachyman·ml^{-1}. Callose was expressed in units of µg Pachyman equivalents per g fresh mass (µg PE·g^{-1} F.M.)

2.2.4 Determination of Concentration of Aluminum in Solution

Samples of the Al/Ca hydroponic solutions were taken before exposure and after 24 hours exposure of the seedlings to Al. The concentration of soluble, inorganic Al was determined using the pyrocatechol violet method of Kerven *et al.* (1989) and Menzies *et al.* (1992). Standards were prepared by diluting 1.0 mM $AlCl_3$ in 1 mM HCl to a final concentration of 0, 0.1, 0.5, 1.0, 2.0, 5.0, 10, 15, 20, 25, 30, and 40 µM. Three ml of each sample or standard was transferred to a 10 ml glass tube and 500 µl of iron interference agent (50 mg of 1,10 phenanthroline (Sigma), 250 mg L-ascorbic acid, (Sigma), in 50 ml dd$H_2$0, prepared fresh) was added and the tube vortexed. After 1 minute, 200 µl pyrochatechol reagent (110 mg pyrocatechol violet (Sigma) in 100 ml dd$H_2$0) and 1.0 ml

imidazol buffer (1 M, pH 5.6) was added. Samples were incubated for 20 minutes and absorbance at 578 nm was measured using a Milton Roy Spectronic 1201 spectrophotometer.

2.2.5 Statistical Tests

A Student *t*-test was used to compare any two callose accumulation means and an analysis of variance (ANOVA) test was used to compare three or more means. The Bonferroni multiple comparison *t*-test was used to compare means if significant differences were observed in the ANOVA tests. The Bonferonni *t*-test resulted in comparison of parental genotype means that more closely resembled the actual distribution of genotypes when compared to the Student-Newman-Keuls (SNK) test, even though the Bonferonni *t*-test is less sensitive than the SNK test (Jandel Scientific, 1994). Backcross and F_2 population segregation ratios were tested against predicted segregation ratios for a single dominant Al-resistance gene using a chi-square (X^2) test. Statistical tests were calculated using SimgaStat 1.0 for Windows (Jandel Scientific, 1994) and were carried out at a probability level of 0.05 unless otherwise stated.

2.3 Results

Mean callose accumulation in the parental genotypes varied from one experiment to the next. Thus, a relative callose index (RCI) was developed to standardize the data and facilitate comparison of results. The mean callose concentration of Al-resistant parents was assigned an RCI value of 1.00. All data point values were divided by this mean to give an index theoretically ranging from zero (Al-resistant) to infinity (Al-sensitive), although relative callose index values typically fell in the range of 0.5 to 6.5. To separate Al-resistant and Al-sensitive genotypes, the mean callose concentration from Al-resistant and Al-sensitive parental genotypes was calculated and rounded to the nearest 0.25 RCI unit. The range of RCI values separating Al-resistant and Al-sensitive gentoypes was 1.75 to 2.25.

2.3.1 Response of Katepwa, Alikat, and Progeny

All genotypes had callose accumulation levels below 385 μg PE·g^{-1} F.M. (RCI = 1.1) in the absence of Al. In the presence of Al, the Al-resistant parent genotype, Alikat, had a mean callose concentration of 348 μg PE·g^{-1} F.M. (RCI = 1.00) and the Al-sensitive parent genotype, Katepwa, had a mean callose concentration of 1264 μg PE·g^{-1} F.M. (RCI = 3.63). Thus, Al-resistant and Al-sensitive plants were separated at 2.25 RCI units (810 μg PE·g^{-1} F.M.; Figure 2-2a). The mean RCI for the F_1 population was 2.08 (749 μg PE·g^{-1} F.M.), having a range of 1.25 to 3.75 RCI units. In the F_2 population (when all three aquaria were combined), Al-resistant F_2 plants had a mean RCI value of 1.08 (375 μg PE·g^{-1} F.M.) and Al-sensitive plants had a mean RCI value of 2.92 (1081 μg PE·g^{-1} F.M.).

Analysis of variance detected significant differences in mean callose accumulation between Katepwa, Alikat, and the F_1 generation with the F_1 generation showing callose accumulation intermediate between the Al-resistant and Al-sensitive parents (Appendix A.1; Table 2-1). The F_2 segregating population was not included in the ANOVA test because of the arbitrary separation of Al-resistant and Al-sensitive pools. The segregation of the F_2 population was also expected to have a wide range of values from Al-resistant to Al-sensitive because of the distribution observed by Somers and Gustafson (1995). Thus, the F_2 population was expected to show similarity to both parental populations. The distribution of callose accumulation values in the F_1 population suggested a segregation ratio of 2:1, indicating that the parent lines were not true-breeding for a single Al-resistance gene. The F_2 population did not segregate in a 3:1 (resistant:sensitive) ratio (X^2=9.45 $X^2_{0.05}$=3.841) (Figure 2-2c), which further suggests a genetic mechanism involving more than one gene. However when the three F_2 aquaria were examined separately, it appeared that the first two aquaria (Figure 2-3a, b) had a segregation ratio of 3:1 (resistant:sensitive; aquarium 1, X^2=0.007, $X^2_{0.05}$=3.841;aquarium 2, X^2=0.240, $X^2_{0.05}$=3.841), whereas the third aquarium (Figure 2-3c) had only Al-resistant individuals. If aluminum is not available, either in its absence, or rendered insoluble at pH 5 or greater, all plants would appear as Al-resistant. Over a 24 hour exposure period, the pH in all aquaria remained near 4.5, providing conditions for soluble Al (Appendix A.6). When

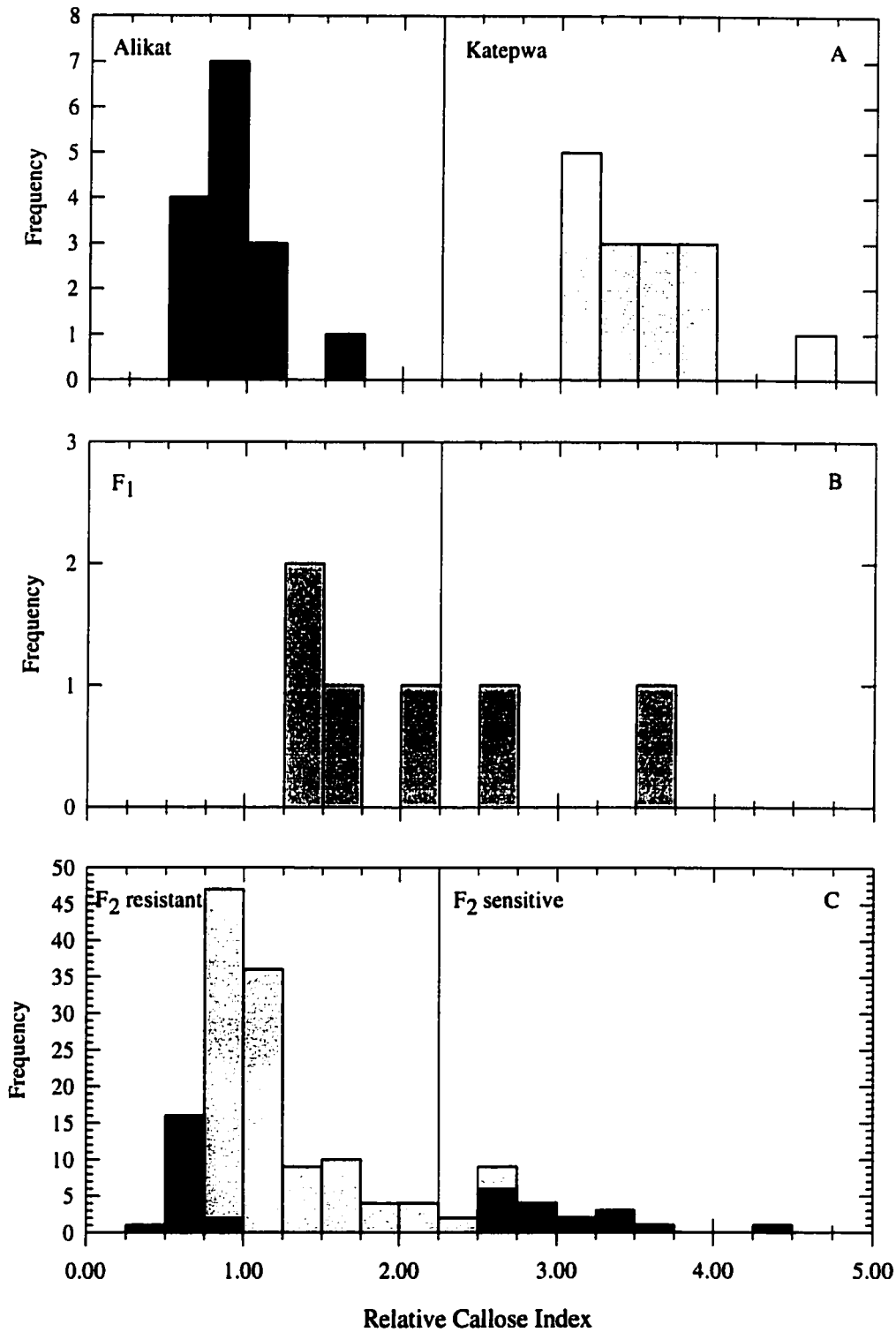

Figure 2-2. Frequency distribution of relative callose index (RCI) values of (A) near-isogenic parents Alikat and Katepwa, (B) F_1 population, and (C) F_2 segregating population (150 individuals) after exposure to 75 µM $AlCl_3$, 1 mM $CaCl_2$ (pH 4.4, 24 hours). The dark shading in (C) represents the individuals selected for bulked segregant analysis.

Table 2-1. Mean callose accumulation (μg PE·g^{-1} F.M.) ± standard error, and RCI value for near-isogenic genotypes. The F_2 populations were derived from aquaria 1, 2, and 3 or aquaria 1 and 2 only.

Genotype	Mean	RCI*
Alikat	348 ± 24	1.00[a]
Katepwa	1264 ± 37	3.63[b]
F_1	749 ± 125	2.15[c]
F_2-Resistant (1,2,3)	376 ± 11	1.08
F_2-Sensitive (1,2,3)	1081 ± 34	2.92
F_2-Resistant (1,2)	363 ± 10	1.04
F_2-Sensitive (1,2)	981 ± 34	2.92

*The letter following the RCI values indicates significant similarities as determined by a one-way ANOVA and Bonferroni's multiple comparison *t*-test The segregating populations were not tested.

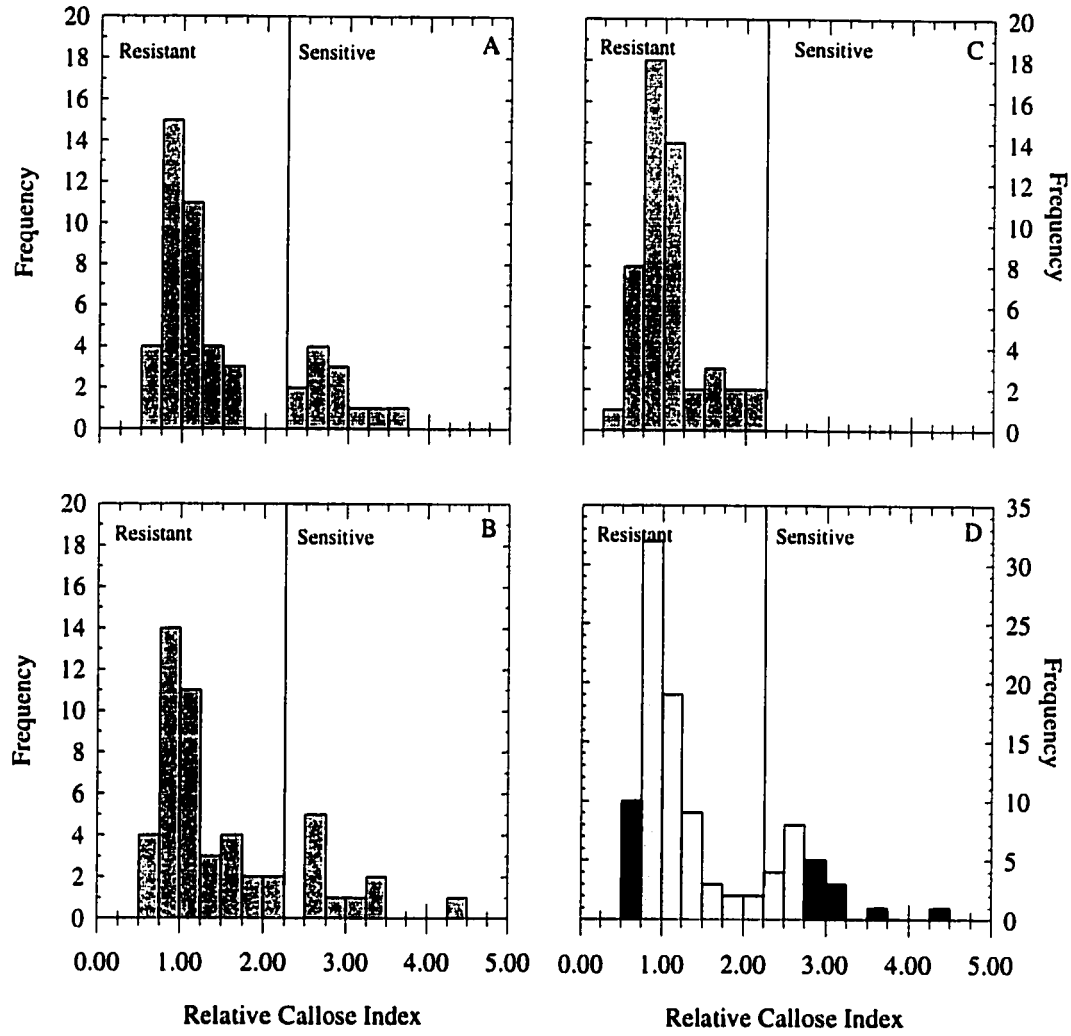

Figure 2-3. Frequency distribution of relative callose index (RCI) values of the F_2 progeny from a cross between Alikat and Katepwa. (A) Aquarium 1 (individuals 1-50), (B) aquarium 2 (individuals 51-100), (C) aquarium 3 (individuals 101-150), (D) aquaria 1 and 2 combined. Aquaria 1 and 2 segregate in a 3:1 ratio (tolerant:sensitive), aquarium 3 only has tolerant individuals. Aquaria 1 and 2 combined segregate in a 3:1 ratio. The dark shading in (D) represents individuals selected for bulked segregant analysis.

the final Al concentration in solution was measured, a significant decrease ($t=16.62$, $t_{0.05}=1.76$) was observed from the initial mean Al concentration of 74.6 μM to the final mean concentration of 59.7 μM in all three aquaria (Appendix A.6). Thus, a possible error could not be confirmed in aquarium 3. When only the first two aquaria were considered in the F_2 segregating population, the Al-resistant F_2 plants had an RCI value of 1.04 (363 μg PE·g^{-1} F.M.), Al-sensitive plants have an RCI value of 2.92 (1081 μg PE·g^{-1} F.M.) (Table 2-1), and the population segregated in a 3:1 (Al-resistant: Al-sensitive) ratio. These points raise questions about the availability of Al to the plants and the homogeneity of the parent plants used to develop the segregating population.

Bulked segregant analysis (Chapter 3) requires a clear segregation between two different genotypes. Given the concern about the reliability of data from aquarium 3, two bulks were made. In the first bulk, 19 of the most Al-resistant and 17 of the most Al-sensitive were chosen from aquaria 1, 2, and 3. In the second bulk, DNA from the 9 most Al-resistant and 9 most Al-sensitive individuals in aquaria 1 and 2 were pooled. Parental bulks were prepared consisting of three randomly chosen parents from each cultivar. The remaining 100 F_2 individuals from aquaria 1 and 2 were to be used for a mapping population once molecular markers were obtained (Chapter 3; Figure 2-3d).

2.3.2 Response of DHK-W2, DHA-33, and Progeny

Two different segregating populations, F_2 and BC_1, were tested (Table 2-2). In the BC_1 test, the Al-resistant parent, DHA-33, had a mean callose concentration of 586 μg PE·g^{-1} F.M. (RCI = 1.00) and the Al-sensitive parent, DHK-W2, had a mean callose concentration of 1368 μg PE·g^{-1} F.M. (RCI = 2.34; Figure 2-4a). The mean of these two values was 1.67 RCI units. In the F_2 test, DHA-33 and DHK-W2 had mean concentrations of 632 μg PE·g^{-1} F.M. (RCI = 1.00) and 1748 μg PE·g^{-1} F.M. (RCI = 2.76) respectively (Figure 2-4d). The mean of these two values was 1.88 RCI units. Thus, for sake of comparison between the BC_1 and F_2 populations, the RCI value separating Al-resistant and Al-sensitive genotypes was drawn at 1.75. In both cases, all F_1 individuals were found to be Al-resistant (Figure 2-4b, e). The F_1 individuals in the F_2 test had a

Table 2-2. Mean callose concentration (μg PE·g^{-1} F.M.) ± standard error and RCI value, for doubled-haploid populations: DHK-W2, DHA-33, F_1, BC_1, and F_2.

Genotype	BC_1 experiment		F_2 experiment	
	Mean	RCI*	Mean	RCI*
DHA-33	586 ± 31	1.00[a]	632 ± 24	1.00[a]
DHK-W2	1368 ± 75	2.34[b]	1748 ± 66	2.76[b]
F_1	715 ± 27	1.21[a]	821 ± 70	1.28[a]
Resistant[†]	856 ± 40	1.39	835 ± 31	1.33
Sensitive[†]	1800 ± 4	2.99	1862 ± 81	2.95

*The letter following the RCI values indicates significant similarities as determined by one-way ANOVA and Bonferroni multiple comparison *t*-tests. The segregating populations were not tested.
[†]BC_1 or F_2 segregating population.

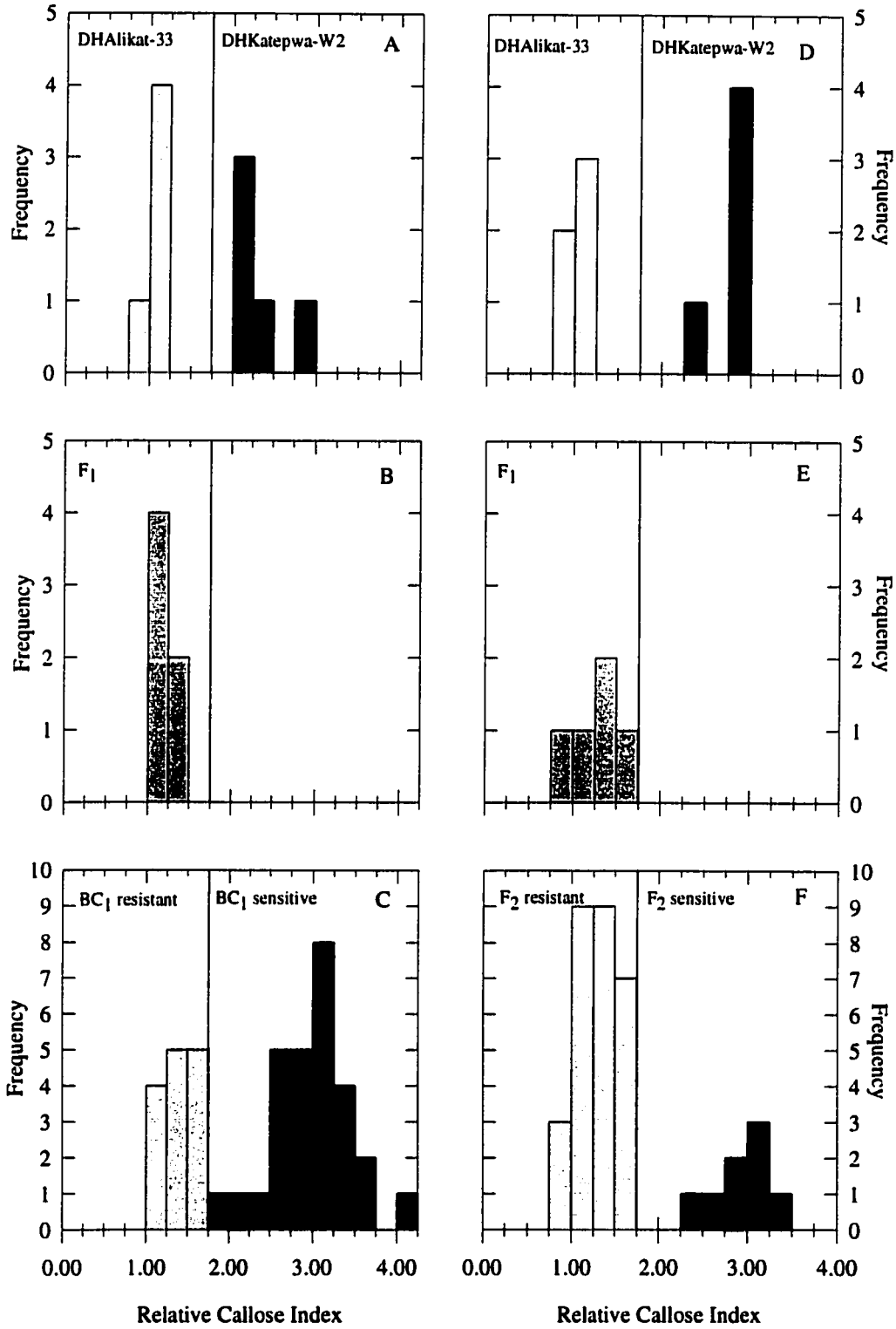

Figure 2-4. Frequency distribution of relative callose index (RCI) values of doubled-haploid lines (A,D) DHA-33, (A,D) DHK-W2, (B,E) F_1 population, (C) BC_1 segregating population, and (F) F_2 segregating population after exposure to 100 µM $AlCl_3$, 500 µM $CaCl_2$ (pH 4.3, 24 hours).

mean RCI value of 1.28 (821 μg PE·g^{-1} F.M.) and the F_1 individuals in the BC_1 test had a mean RCI value of 1.21 (714 μg PE·g^{-1} F.M.). Values were not significantly different from the Al-resistant parent, DHA-33 (Table 2-2). The BC_1 Al-resistant individuals had a mean RCI value of 1.39 (856 μg PE·g^{-1} F.M.) and the BC_1 Al-sensitive individuals had mean RCI value of 2.99 (1800 μg PE·g^{-1} F.M.). The F_2 Al-resistant and Al-sensitive individuals had mean RCI values of 1.33 (835 μg PE·g^{-1} F.M.) and 2.95 (1862 μg PE·g^{-1} F.M.) respectively (Table 2-2).

The BC_1 population did not segregrate for Al-resistance in the expected ratio of 1:1 at the probability level of 0.05. The F_2 population had a segregtation ratio of 3:1 (resistant: sensitive; (X^2=0.147, $X^2_{0.05}$=3.841; Figure 2-4f). The F_2 segregation ratio is consistent with the expected segregation of a single dominant gene. The BC_1 segregation ratio was not expected. This is most likely an artifact of the population size and random selection of seed. Significant differences were found between the parental and F_1 genotypes in both BC_1 and F_2 tests (Appendices A.2, A.3). The F_1 population and DHA-33 were significantly different from DHK-W2 in both tests (Table 2-2), as expected from a single dominant allele. The BC_1 and F_2 segregating populations were not included in the ANOVA tests because of the arbitrary separation of Al-resistant and Al-sensitive pools.

The F_2 mapping population derived from DHA-33 and DHK-W2 consisted of 50 individuals (Figure 2-5). The Al-resistant parent, DHA-33, had a mean callose concentration of 455 μg PE·g^{-1} F.M. (RCI = 1.00) and the Al-sensitive parent had a mean concentration of 1463 μg PE·g^{-1} F.M. (RCI = 3.22; Figure 2-5a). The mean RCI value was 2.1, thus Al-resistant and Al-sensitive genotypes were separated at 2.0 RCI units. The parental genotypes were significantly different (t=11.9, $t_{0.05}$=2.31). The F_2 mapping population segregated as a 3:1 ratio (X^2=0.240, $X^2_{0.05}$=3.841; Figure 2-5b). The 10 most Al-resistant and 10 most Al-sensitive individuals' DNA were pooled for bulked segregate analysis (Chapter 3).

2.3.3 Response of DHK-W2, DHA-35, and Progeny

Genoytpes were examined to determine the segreation ratio of BC_1 and F_2

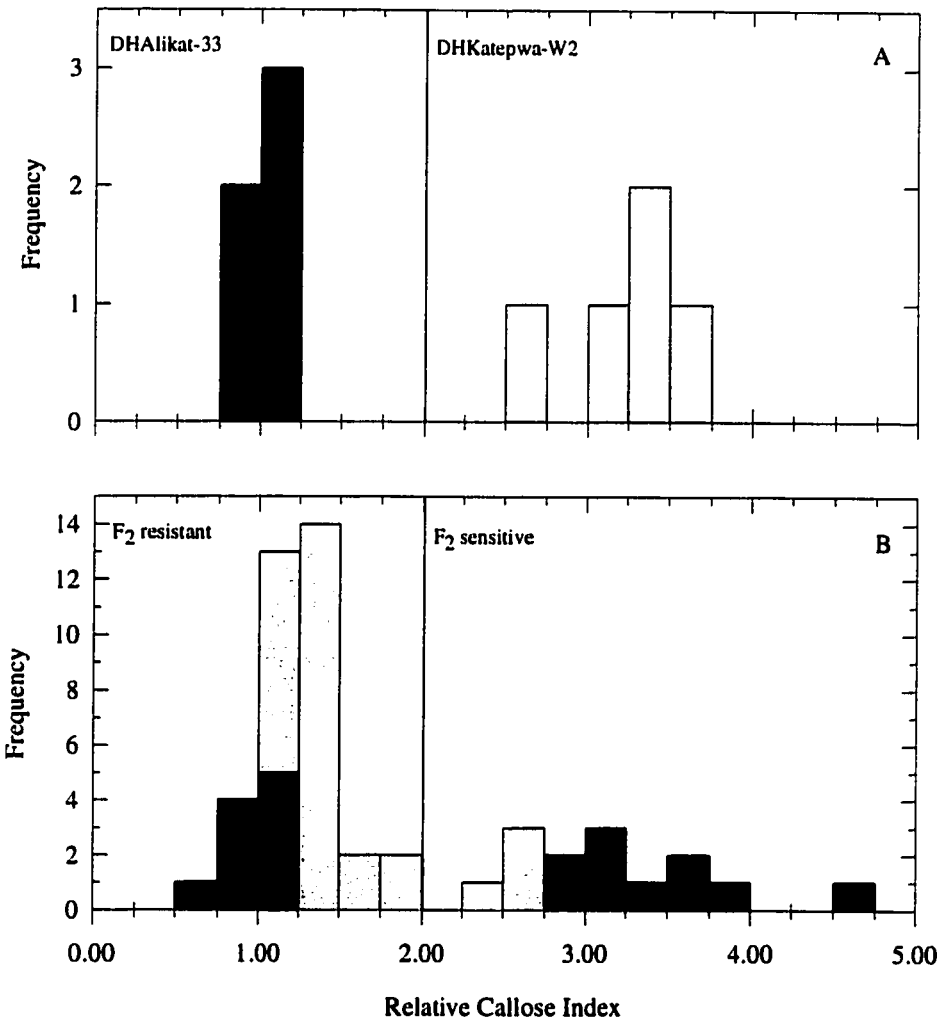

Figure 2-5. Frequency distribution of relative callose index (RCI) values of doubled-haploid (A) parent lines DHA-33 and DHK-W2, and (B) F_2 segregating mapping populations. The dark shading in (B) represents individuals that were selected for bulked segregrant analysis.

populations and to develop F_2 mapping populations for molecular markers. In the BC_1 test, the Al-resistant parent, DHA-35, had mean callose concentration of 635 μg PE·g^{-1} F.M. (RCI = 1.0) and the Al-sensitive parent, DHK-W2, had a mean callose concentration of 1711 μg PE·g^{-1} F.M. (RCI = 2.70; Figure 2-6a). The mean RCI value was 1.85. In the F_2 test, the Al-resistant and Al-sensitive parents had mean callose concentrations of 719 μg PE·g^{-1} F.M. (RCI = 1.0) and 1571 μg PE·g^{-1} F.M. (RCI = 2.19) respectively (Figure 2-6d). The mean value was 1.60 RCI units. Thus, the RCI value separating Al-resistant and Al-sensitive genotypes was 1.75. In both BC_1 and F_2 experiments, the F_1 individuals were Al-resistant as expected from a single dominant gene (Figure 2-6b, e). The F_1 individuals in the F_2 and BC_1 experiments had mean RCI values of 1.23 (881 μg PE·g^{-1} F.M.) and 1.06 (671 μg PE·g^{-1} F.M.) respectively. The Al-resistant and the Al-sensitive BC_1 individuals had mean RCI values of 1.18 (751μg PE·g^{-1} F.M.) and 2.53 (1608 μg PE·g^{-1} F.M.) respectively. Finally the Al-resistant and Al-sensitive F_2 individuals had mean RCI values of 1.11 (794 μg PE·g^{-1} F.M.) and 2.18 (1566 μg PE·g^{-1} F.M.) respectively (Table 2-3).

The BC_1 and F_2 segregating populations had Al-resistant to Al-sensitive segregation ratios of 1:1 (X^2=0.125, $X^2_{0.05}$=3.841) and 3:1 (X^2=0.690, $X^2_{0.05}$=3.841) respectively (Figure 2-6c, f), as expected from a single dominant gene. An ANOVA test found significant differences between the parental and F_1 genotypes in both experiments (Table 2-3; Appendices A.4, A.5). The DHA-35 and F_1 individuals had similar mean callose concentrations and were significantly different from the DHK-W2 parentals in the BC_1 experiment (Table 2-3). This was as expected from a single dominant gene. However, the parental and F_1 genotypes were all signficantly different in the F_2 experiment (Table 2-3). When the distribution of the individuals were examined (Figure 2-6e), they did not appear to be any different from the other doubled-haploid derived populations. The segregating populations were not tested as in previous sections (Sections 2.3.1, 2.3.2).

The F_2 mapping populations derived from DHA-35 and DHK-W2 consisted of 50 individuals (Figure 2-7). Aluminum-resistant and Al-sensitive parents had mean callose

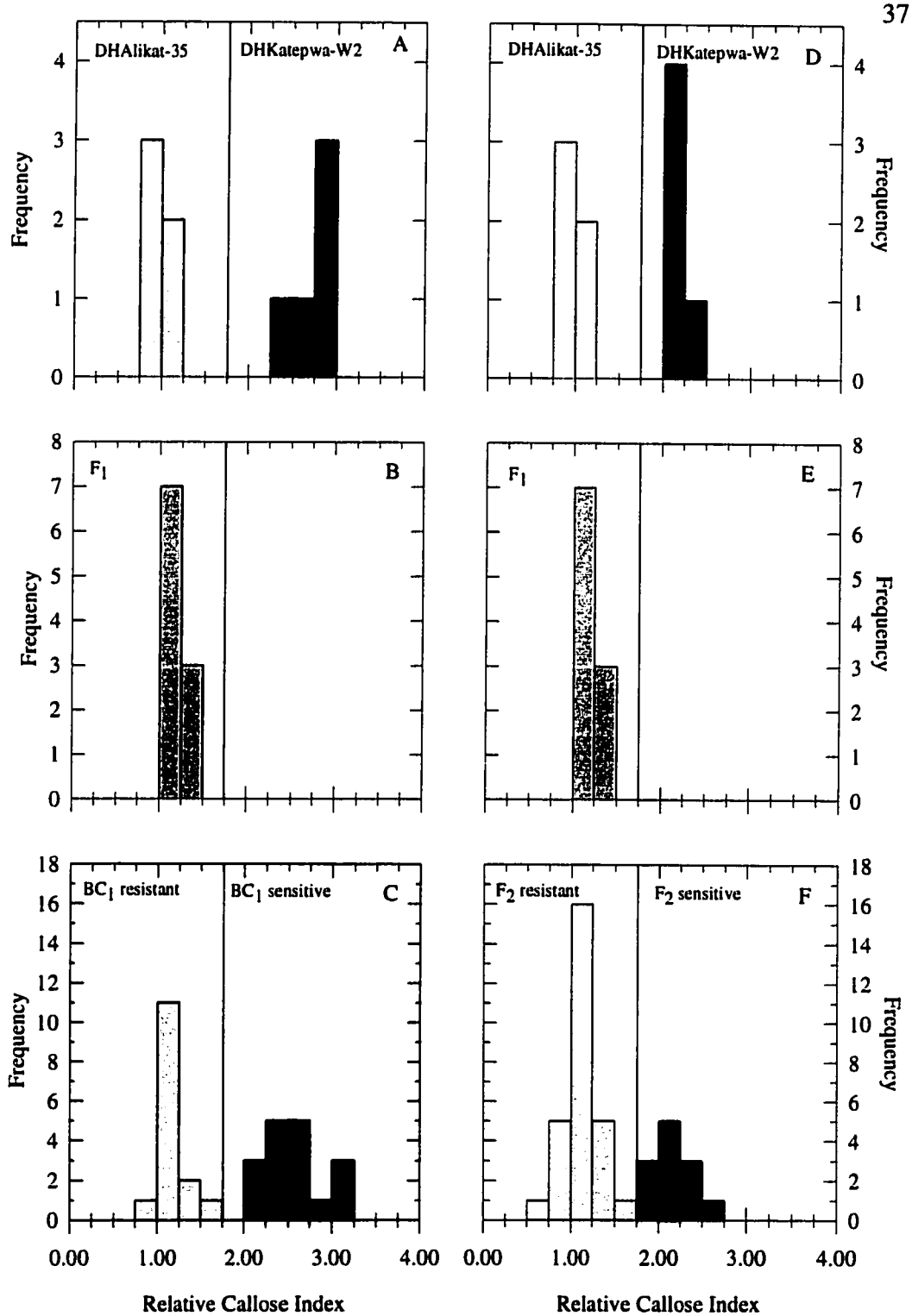

Figure 2-6. Frequency distribution of relative callose index (RCI) values of doubled-haploid (A,D) parent lines DHA-35 and DHK-W2, (B,E) F_1 population, (C) BC_1 segregating population, and (F) F_2 segregating population after exposure to 100 µM $AlCl_3$, 500 µM $CaCl_2$, (pH 4.3, 24 hours).

Table 2-3. Mean callose concentration (μg PE·g^{-1} F.M.) ± standard error, and RCI for doubled-haploid populations: DHK-W2, DHA-35, F_1, BC_1, and F_2.

Germplasm	BC_1 experiment		F_2 experiment	
	Mean	RCI*	Mean	RCI*
DHA-35	635 ± 41	1.00[a]	719 ± 10	1.00[a]
DHK-W2	1711 ± 43	2.70[b]	1571 ± 38	2.19[b]
F_1	671 ± 33	1.06[a]	881 ± 30	1.23[c]
Resistant[†]	751 ± 31	1.18	794 ± 24	1.11
Sensitive[†]	1608 ± 53	2.53	1566 ± 44	2.18

*The letter following the RCI values indicates significant similarities as determined by one-way ANOVA and Bonferroni multiple comparison *t*-tests. The segregating populations were not tested.
[†]BC_1 or F_2 segregating population.

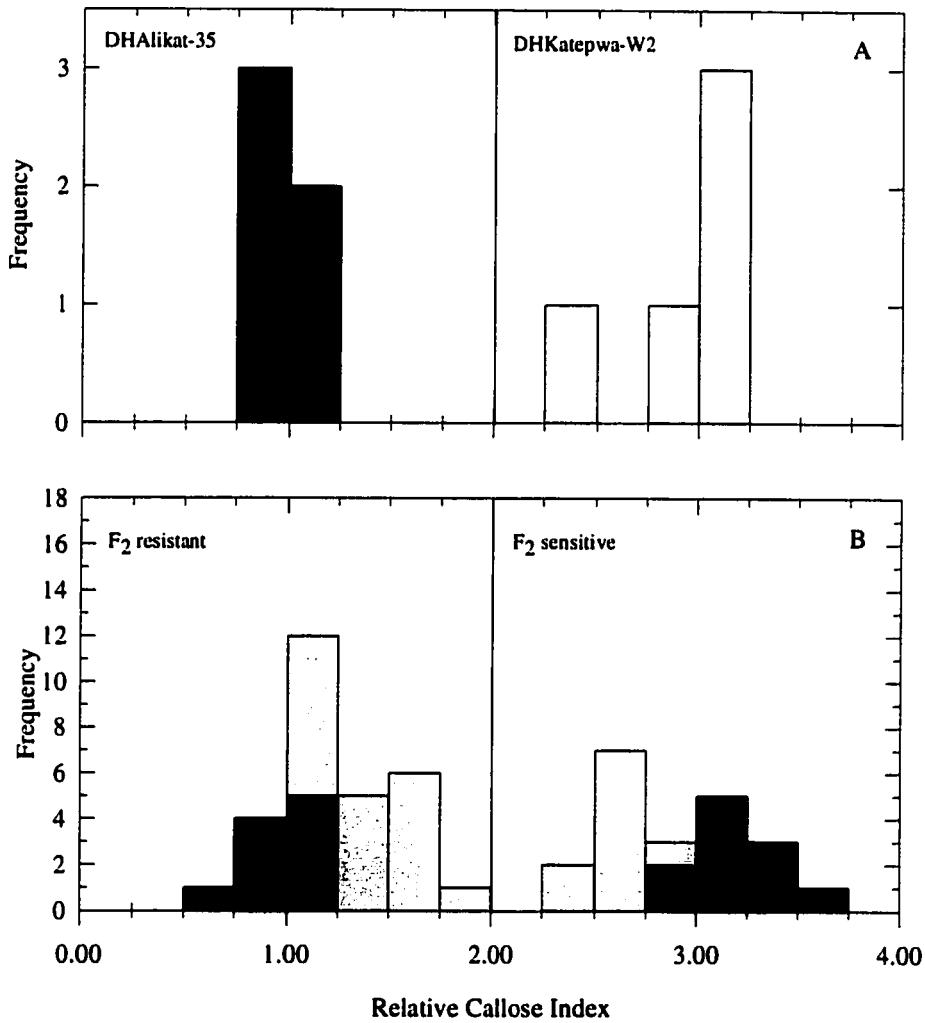

Figure 2-7. Frequency distribution of relative callose index (RCI) values of doubled-haploid (A) parent lines DHA-35 and DHK-W2, and (B) F_2 segregating mapping populations. The dark shading in (B) represent individuals that were selected for bulked segregrant analysis.

concentrations of 848 μg PE·g^{-1} F.M. (RCI =1.00) and 2462 μg PE·g^{-1} F.M. (RCI = 2.90) (Figure 2-7a) The mean parental RCI value was 1.95 thus the RCI value separating Al-resistant and Al-sensitive genotypes was determined to be 2.00. The parental lines were significantly different (t=12.65, $t_{0.05}$=2.31). Aluminum resistant F_2 individuals did not segregate in a 3:1, Al-resistant to Al-sensitive (X^2=7.707, $X^2_{0.05}$=3.841; Figure 2-7b) ratio as expected from a single dominant gene, and in fact appeared more like a 1:1 ratio

2.4 Discussion

The objective of this study was to screen near-isogenic and doubled-haploid genotypes and their progeny for their response to Al. Segregating F_2 populations were scored for their response to Al so that they could be used to locate molecular markers for Al- resistance (Chapter 3). Callose accumulation provided a reliable means of scoring plants for Al- resistance when expressed as a relative callose index (RCI) value that compared the callose accumulation of all genotypes relative to the callose accumulation of the Al-resistant parent of a segregating population. The use of the parental genotypes with a known response to Al served as a control in all cases, since it was important to determine the segregation of individuals experiencing Al stress relative to their parents rather than comparing the response of genotypes to varying concentrations of Al.

Cultivar Katepwa was 3.6 times more sensitive to Al than Alikat and DHK-W2 was 3.2 and 2.9 times more sensitive to Al than DHA-33 and DHA-35 respectively. These data are supported by work with other wheat cultivars. The Al-sensitive cultivar, Neepawa, produced 6.7 times more callose than the Al-resistant cultivar, Atlas 66, and the Al-sensitive cultivar, Scout 66, produced 3.2 times more callose than the Al-resistant cultivar, PT 741, after exposure to 75 μM Al for 24 hours (Zhang *et al*, 1994). Scout 66 also produced nearly 4 times more callose than the Al-resistant cultivar, Atlas 66, and Katepwa produced nearly twice the amount of callose as PT741 after 40 hours exposure to 400 μM Al (Schreiner *et al*, 1994). The near-isogenic lines of Alikat and Katepwa and their doubled-haploid counterparts accumulated the same relative amount of callose as other Al-resistant and Al-sensitive cultivar pairs.

The experiment with near-isogenic Alikat and Katepwa populations was designed such that the parental genotypes and the segregating F_2 populations were in separate aquaria to save space. The unexpected lack of segregation of the F_2 individuals in aquarium 3 (Figure 2-3c) illustrated the need to have control parental genotypes in the same aquarium as all the segregating individuals. Had Al-resistant and Al-sensitive parents been included in this aquarium, it would have been possible to diagnose the problems encountered. This design weakness was corrected in the doubled-haploid tests by including the parental genotypes in the same aquarium with the F_1 and the segregating populations.

Since the segregation of the F_2 population derived from Alikat and Katepwa was not 3:1 (resistant:sensitive) it suggests multigenic control of Al-resistance (Figure 2-3d). This in contrast to the 3:1 (resistant:sensitive) segregation observed in the same genotypes when tested using relative root length (Somers and Gustafson, 1995; Somers *et al.*, 1996). The segregation in this study could have been an artifact arising from the unusual behaviour of aquarium 3. However, determination of soluble monomeric Al by pyrocatechol violet (Kerven *et al*, 1989; Menzies *et al*, 1992) indicated that the concentration of monomeric Al in aquarium 3 was the same as that from aquaria 1 and 2. Thus, Al was available to all seedlings in all three aquaria and the same amount of aluminum was removed from solution after 24 hours exposure. The unexpected 2:1 ratio (Al-resistant:Al-sensitive) provided further support for a multigenic system of segregation of the F_1 population (Figure 2-2b). However, when the data from aquarium 3 was not included in the analysis, the expected 3:1 segregation ratio for a single dominant Al-resistance allele was observed (Figure 2-2c). Data from aquarium 3 could not be dismissed solely on the pH and Al concentration data alone. Nonetheless, these data remain suspect. Bulks for bulked segregant analysis (Chapter 3) were made from a pool consisting of aquaria 1 to 3 (Figure 2-2c) and from a pool of individuals from aquaria 1 and 2 (Figure 2-3d).

It is possible that a multigenic effect was observed, since multiple gene control of Al-resistance has been well documented in crosses of other wheat cultivars. Aluminum

resistance in the moderately Al-resistant cultivar, Chinese Spring, was controlled by genes located on chromosome arms 4DL and 2DL (Aniol, 1990) and Al-resistance in the Al-resistant cultivar, BH1146, was controlled primarily by genes on chromosome 4D and to a lesser extent on chromosome 2D (Lagos *et al*, 1991). Minor genes on chromosomes 5A (Aniol, 1990) and 5D (Lagos *et al*, 1991) appear to influence either the expression or dominance of the Al-resistant allele. Berzonsky (1992) predicted a multiple gene system. A synthetic hexaploid line, 6*x*-Canthatch, was derived from a cross between *T. turgidum* cv. Canthatch and Atlas 66. This synthetic hexaploid was crossed with Atlas 66 and found that the segregation ratios did not match a one or two gene system. This supported a polygenic system with major Al-resistance genes and supporting minor genes. In a cross between ditelosomic, Chinese Spring, and a rye (*Secale cereale*) cultivar, Blanco, Gustafson and Ross (1990) found that genes on nearly 20 different chromosome arms, including 2DL, 4DL, 5AS, 5DL, and 5DS, either enhanced or suppressed Al-resistance. The differing segregation observed between this study and those by Somers and Gustafson (1995) and Somers *et al.*, (1996) may be the result of the two different screening methods used. Progeny testing of the F_3 plants could reveal whether the 5:1 ratio observed was an accurate conclusion.

Analysis of variance and multiple comparison tests on the doubled-haploid parent and F_1 genotypes had shown that the F_1 population had the same callose accumulation as the Al-resistant parental line (Tables 2-3 and 2-4). Division of the BC_1 and F_2 segregating populations into Al-resistant and Al-sensitive populations on the basis of mean callose accumulation of the parental genotypes, occurred at the natural division between the distribution peaks (Figures 2-4 to 2-7). Statistical tests were not done to compare the segregating populations with the parental genotypes, because of the arbitrary division of the segregating population into Al-resistant and Al-sensitive phenotypes. The segregation trends were sufficient to be used as scoring data for the location of molecular markers (Chapter 3).

The doubled-haploid segregating populations BC_1 and F_2 from parental crosses, DHK-W2 x DHA-33 and DHK-W2 x DHA-35, segregated in 1:1 and 3:1 (Al-

resistant:Al-sensitive) ratios. In all cases, the F_1 lines were Al-resistant (Figures 2-4 and 2-6). This supports the single gene Al-resistance model. Segregating populations used for molecular marker mapping did not all reflect the expected 3:1 ratio (Figures 2-5 and 2-7). Confidence in the chi-square analyses could be improved by increasing the population sizes tested. An increase in the mapping population size should result in the expected 3:1 segregation ratio.

Segregation as a single, dominant, Al-resistant allele has been observed in a number of other segregating populations specifically established for mapping Al-resistance. Luo and Dvořák (1996) crossed an Al-resistant recombinant line of *T. turgidum* cv. Langdon (with chromosome 4B from Langdon replaced with chromosome 4D from Chinese Spring to confer resistance), with Al-sensitive *T. turgidum* cv. Capelli. The segregation ratio in this synthetic Al-resistant system was 3:1 (Al-resistant: Al-sensitive). Riede and Anderson (1996) found the expected backcross ratio of 1:1 (Al-resistant:Al-sensitive) in *T. aestivum* recombinant inbred lines of Al-resistant cultivar BH1146 x Al-sensitive cultivar Anahuac. It is likely that minor genes influencing Al-resistance are selected against in favour of less variable, monogenic systems in the process of developing specific lines for the purpose of mapping Al-resistance.

Doubled-haploid lines, DHA-33 and DHA-35, were derived from a single haploid seed and selected for mapping based on their low degree of variance in response to Al (J. Stevens, unpublished data). Alikat was developed as a backcross line from two common agricultural cultivars; Al-resistant Maringa and Al-sensitive Katepwa, as a means of introducing Al-resistance into Katepwa (Figure 2-1a). Selection for Al-resistance using haematoxylin staining and root length indices as an overall response to Al may have inadvertently allowed minor genes affecting Al-resistance to go undetected allowing for their random selection. The developmental stage of the plants and longer term exposure to Al may mask small variations due to minor Al-resistance genes. In contrast, doubled-haploid lines were selected using short-term responses of seedlings of the same age and size to controlled concentrations of Al in a controlled nutrient solution. This controlled, artificial system identified lines with lower variability in response to Al as parent lines for

mapping populations. The use of callose accumulation, as a sensitive means of measuring the root tip response to Al in a hydroponic solution, may have allowed the effect of minor genes to be observed in the near-isogenic population, whereas these genes may not have been detected using root growth or haematoxylin staining.

The doubled-haploid parents DHA-33, DHA-35 (Figure 2-1a) derived from Alikat, appear to have a single dominant allele that controls root tip response to Al. Alikat, may have a single major gene controlling root tip response to Al that is influenced by one or more minor genes already present in Katepwa or inherited from Maringa. Genetic systems, where Al-resistance has been introduced into a smaller genome (Luo and Dvořák, 1996) or genetically uniform lines such as isogenic or doubled-haploid lines, should be used to map the location of Al-resistance in wheat. Since it appears that the *D*-genome is the primary contributor of Al-resistance in wheat, *Aegilops squarrosa* should be evaluated for it's potential as a source of Al-resistance. Near-isogenic agricultural lines should then be used as a means of verification of markers for Al-resistance and the location of these genes.

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Chapter 3. Identification of Molecular Markers

3.1 Introduction

Aluminum is toxic to wheat, producing symptoms of calcium and phosphate deficiency and inhibition of root growth (Foy, 1992). Wheat cultivars vary in their response to Al from resistant to sensitive (Zale and Briggs, 1988; Briggs *et al.*, 1989), with the primary site of toxicity at the terminal 2-3 mm of root tips (Ryan *et al.*, 1993). Aluminum resistance occurs at the cellular level, however the exact mechanisms have yet to be determined (Taylor, 1995). Variables such as plant age and Al-dose, Al-concentration, and duration of exposure complicate the determination of the mechanism and genetic basis of Al-resistance (Carver and Ownby, 1995). As well, there is still discussion on which species of Al are phytotoxic (Kinrade, 1997). Aluminum resistance mechanisms that have been proposed are based on tolerance and detoxification of Al within the plant tissue, detoxification of Al in the apoplasm, and exclusion of Al from the roots (Taylor, 1988, 1991, 1995).

Chromosome 4D appears to be the primary contributor to Al-resistance in wheat. However, multigenic control of Al-resistance is supported by the identification of a number of other chromosomes contributing to the inheritance of Al-resistance (Polle *et al.*, 1978; Berzonsky and Kimber, 1986; Aniol, 1990; Lagos *et al.*, 1991; Berzonsky, 1992; Luo and Dvořák, 1996; Riede and Anderson, 1996). A multigenic basis for Al-resistance is indirectly supported by the number of mechanisms of Al-resistance currently proposed (Carver and Ownby, 1995).

Molecular markers such as restriction fragment length polymorphisms (RFLP; Bostein *et al.*, 1980), randomly amplified polymorphic DNA (RAPD; Williams *et al.*, 1990), microsatellites (Tautz *et al.*, 1986), and amplified fragment length polymorphisms (AFLP; Vos *et al.*, 1995) are means of attaching an easily identifiable tag linked to a trait which is not obvious, difficult to measure, or requires a long time to manifest itself. All these marker types are based on identification of variations in the DNA code in or near the gene of interest between two different genotypes. Molecular markers are phenotypic and differ from morphological markers only in that markers are based on a differing

sequence of nucleotides, rather than a differing morphological characteristic.

Two approaches to mapping Al-resistance were chosen for this work. The RFLP technique was selected because it is well established in the field of plant molecular mapping and because genetic maps are available for the wheat genome (Chao *et al.*, 1989; Gill *et al.*, 1991; Anderson *et al.*, 1992; Devos *et al.*, 1992; Xie *et al.*, 1993; Hohmann *et al.*, 1994; Mickelson-Young *et al.*, 1995; Nelson *et al.*, 1995a,b; Van Deynze *et al.*, 1995a; Marino *et al.*, 1996). The RAPD technique is not as well established as a reliable method in wheat, however it does have the advantages of being quick and not requiring the use of radioisotopes (Devos and Gale, 1992). Restriction fragment length polymorphism markers have been linked to Al-resistance in cultivars BH1146 (Riede and Anderson, 1996) and Chinese Spring (Luo and Dvořák, 1996). In each case, markers were identified on chromosome arm 4DL, but it has not yet been shown that the markers were linked to the same Al-resistance mechanism (Luo and Dvořák, 1996). Randomly amplified polymorphic markers have not been linked to Al-resistance.

Recent physiological evidence for multiple Al-resistance mechanisms has shown that the Al-resistant cultivar, Atlas 66, and the backcross line, ET3, have different physiological responses to Al-exposure (Pellet *et al.*, 1996). This raises the possibility that different cultivars may have different Al-resistant genes. Markers for Al-resistance in BH1146 and Chinese Spring may not necessarily be linked to the same Al-resistance genes identified in Alikat, DHA-33, and DHA-35. The purpose of this work was to determine whether RFLP and RAPD markers could be identified for Al-resistance in Alikat, DHA-33, and DHA-35. Three different genetic systems described in Chapter 2 (Figure 2-1) were chosen to locate RAPD and RFLP markers for Al-resistance in bread wheat. Markers identified by Riede and Anderson (1996) and Luo and Dvořák (1996) were tested to determine whether they were linked to Al-resistance in the near-isogenic and doubled-halploid genotypes being screened in this project.

3.2 Materials and Methods

3.2.1 DNA Isolation

Genomic DNA was isolated from leaf tissue using a method modified from Doyle and Doyle (1990). Fresh leaf tissue (2-5 g) was collected from individual wheat plants, stored in paper bags, wrapped in plastic, and frozen at -80°C until needed. Leaf tissue was lyophilized at room temperature and 4 Pa pressure for 24 hours using a Freezemobile 6 (Virtis Co., Gardiner, New York). Lyophilized tissue was ground to a fine powder using a commercial blender (Waring Blender 700 model 33BL79, New Hartford, Conneticut). To digest cell walls and remove polysaccharides and lipids, samples were transferred to 50 ml Oak Ridge tubes with 15 ml 2X CTAB solution (2% (w/v) hexadecyltrimethylammonium bromide *CTAB* (Sigma), 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 0.2% 2-mercaptoethanol), preheated to 60°C and incubated in a 60°C water bath for 30 minutes. The solution was extracted with 15 ml chloroform by gently rocking for 5 minutes and centrifuging at 9800 X g (Beckman JA-20 rotor, J2-21 centrifuge) for 10 minutes to remove proteins and hydrophobic compounds. The aqueous layer containing nucleic acids was transferred to a 50 ml Falcon tube, brought up to 15 ml with ddH_2O. Nine ml of 2-propanol was added to the sample to precipitate nucleic acids. The mixture were centrifuged at 3000 X g for 5 minutes at room temperature to pellet the nucleic acids. 2-Propanol was decanted and the nucleic acid pellet was washed with 70% ethanol (20 min. to overnight). Ethanol was subsequently decanted and the pellet dried at room temperature. The pellet was resuspended in 1 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0) and incubated for 30 minutes at 37°C with 100 ng RNase A (10 $\mu g \cdot ml^{-1}$) to remove RNA (ribonucleic acid). Two millilitres ddH_2O, and 1 ml 7.5 mM ammonium acetate were added to the solution and mixed by inversion. DNA was precipitated with 2.7 ml 2-propanol and pelletted by centrifugation at 3000 X g. The pellet was washed with 70% ethanol, resuspended as above, and stored at -20°C.

3.2.2 Quantification and Pooling of DNA

Genomic DNA was quantified fluorometrically by diluting 2 µl of dissolved DNA in 2 ml of 1X TNE dye solution (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA pH 8.0, 0.1 $\mu g \cdot ml^{-1}$ Hoechst Dye 33258). The DNA concentration was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm using a Sequoia Turner 450 fluorometer (R. Mayerhoffer, pers. com.). Quantities were determined using a standard curve of known concentration of plant genomic DNA.

DNA from near-isogenic and doubled-haploid Al-resistant and Al-sensitive pools of F_2 plants were combined for bulked segregrant analysis (Michelmore *et al.*, 1991). DNA from F_2 plants derived from Katepwa x Alikat, representing all three aquaria, was divided into one bulk of 19 resistant individuals and one bulk of 17 sensitive individuals. A second pair of bulks consisting of 9 individuals from each pool was made from F_2 plants from aquaria 1 and 2 (Section 2.3.1). DNA from F_2 plants derived from DHK-W2 x DHA-33 and DHK-W2 x DHA-35 were divided into bulks of 10 resistant and 10 sensitive individuals. In all cases the most resistant and most sensitive individuals were selected for the bulks. For the RAPD analysis, DNA from individual plants was diluted to 12 $ng \cdot \mu l^{-1}$ with TE and bulks were made by pooling 60 ng of DNA from each individual and diluting to 6 $ng \cdot \mu l^{-1}$ with TE. For the RFLP analysis, equal amounts of DNA from each individual were combined so that a total of 20 µg of DNA from each bulk was use for each restriction enzyme digestion.

3.2.3 Randomly Amplified Polymorphic DNA Amplification

The method for amplifying fragments of genomic DNA using random decamer primers was modified from Williams *et al.* (1990). The PCR reaction mixture consisted of 1.6 µl 10X Taq polymerase buffer (500 mM KCl, 100 mM Tris·HCl pH 8, 0.1% gelatin), 1.9 µl 25 mM $MgCl_2$, 0.3 µl of 10 mM dNTP's (2'-deoxyadenosine 5'-triphosphate, 2'-deoxythymidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate, 2'-deoxycytidine 5'-triphosphate; Pharmacia Biotech), 17.5 ng random decamer primer (*Operon primer*, Operon Technologies, Alameda, CA, U.S.A. or *UBC primer*, The

Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada), 2 µl 10 U·µl-1 *Taq* polymerase (courtesy Dr. M. Picard, University of Alberta) and 12 ng bulked or individual genomic DNA. The total volume was made up to 15.5 µl with sterile ddH_2O. A Perkin Elmer GeneAmp 9600/9700 thermocycler was used to provide the following temperature series: 96°C for 2 minutes to melt the initial template strands, 35 cycles of; 96°C for 5 seconds to melt template strands; 37°C for 30 seconds to anneal primer to template DNA; and 72°C for 1 minute to replicate the template strand, 72°C for 5 minutes to complete any incomplete fragments, and cooled to 4°C to stop the reaction. Completed reactions were either stored at -20°C or the amplification products were separated immediately on a 1.4% agarose gel in TBE tank buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0) with 0.1 $\mu g \cdot ml^{-1}$ ethidium bromide (Sigma) at 180 V for 2 hours. Agarose gels were photographed with Polaroid 667 black and white film, digitally imaged using The Imager version 2.04 (Appligene, Inc.), and stored in JPEG (joint photographers expert group) format. Amplified fragment sizes were calculated using *Bst*EII and *Hin*dIII endonuclease-digested lambda DNA markers separated on the same gel as RAPD fragments.

3.2.4 Probes for Restriction Fragment Length Polymorphism

An anchor kit of 150 oat (cdo), barley (bcd), rice (rz) cDNA and wheat (wg) genomic DNA clones in Bluescript SK- vectors covering all 7 wheat chromosome groups was provided by Mark Sorrells (Cornell University, Ithaca NY, U.S.A.; Van Deynze *et al.*, 1995b) and 3 wheat (psr 39, 914, 1051) cDNA clones were provided by Michael Gale (John Innes Centre, Norwich, U.K.). Plasmids were transformed into competent cells (*Escherichia coli* bacteria strain DH5αF') prepared following the method of Inoue *et al.* (1990). Transformed cells were selected on Laurier broth (LB) agar culture plates containing 50 $\mu g \cdot ml^{-1}$ ampicillin. Transformed colonies were selected and grown overnight in 3 ml of LB nutrient broth with 50 $\mu g \cdot ml^{-1}$ ampicillin to amplify the transformed plasmid. Half of the cells were removed (1.5 ml) to a 1.5 ml Eppendorff tube and 98 µl 100% DMSO (7% final concentration) was added. Cells were frozen in liquid

nitrogen and stored at -80°C. The remaining 1.5 ml of cells were transferred to a 1.5 ml Eppendorff tube and microcentrifuged at 13,200 rpm for 45 seconds. The nutrient broth was decanted, 100 µl of TGE (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) was added, and the pellet resuspended and left for 5 minutes at room temperature. Two-hundred µl of freshly made solution B (1% SDS, 1 N NaOH) was added to emulsify cell membranes and the tube was set on ice for 5 minutes, inverted twice. One-hundred and fifty µl of solution C (3 M potassium acetate, 11.5% (v/v) glacial acetic acid) was added, mixed by inversion, and kept on ice for 5 minutes to remove proteins and carbohydrates. The tube was microcentrifuged at 13,200 rpm for 15 minutes and the supernatant containing plasmid DNA was transferred to a fresh 1.5 ml Eppendorff tube. DNA was precipitated with 500 µl 100% 2-propanol, washed for 5 minutes with 70% ethanol, dried at room temperature, and resuspended in 25 µl TE. Plasmids were stored at -20°C until needed.

Clone size was confirmed and clones were radiolabelled with α^{32}P-dCTP using the polymerase chain reaction (PCR). Each amplification reaction had a total volume of 25 µl and contained 2 µl 5 ng·µl^{-1} plasmid containing the clone insert, 2.5 µl 10X *Taq* polymerase buffer (500 mM KCl, 100 mM Tris·HCl pH 8.0, 0.1% gelatin), 3.0 µl 25 mM $MgCl_2$, 1 µl AGT mix (0.5 mM of each dATP, dGTP, dTTP, 0.05 mM dCTP; Pharmacia Biotech), 1 µl (25 ng) of each T3 and T7 primer, 5.0 µl dCTP (0.5 mM) or *redi*vue α^{32}P-dCTP (0.37 MBq·µl^{-1}; Amersham Life Science), and 0.5 µl 10 U·µl^{-1} *Taq* polymerase (courtesy of Dr. M. Picard, University of Alberta). A Perkin Elmer GeneAmp 9600 thermocycler was programmed for 35 cycles of 96°C for 30 seconds, 55°C for 36 seconds, and 72°C for 84 seconds. The cycle was preceded by 94°C for 2 minutes to melt the template strands and concluded with 72°C for 5 minutes to complete any incomplete amplifications. Non-radiolabelled amplifications were separated on a 1.4% agarose gel in TBE with 0.1 µg·ml^{-1} ethidium bromide for 1 hour at 80 V and photographed as with RAPD's. Radiolabelled clones were isolated from unincorporated isotope and nucleotides on a Sephadex G50 DNA grade NICK columns (Pharmacia Biotech); yielding 400 µl of probe with 95% isotope incorporation. A probe solution was made of 400 µl probe, 900

µl ddH_2O, and 500 µg sheared salmon sperm DNA and boiled for 10 minutes to separate the DNA strands.

3.2.5 Digestion of Genomic DNA

Several concentrations of restriction endonucleases were tried on both the near-isogenic and doubled-haploid germplasm before the following concentrations were used. Parent DNA (10 µg) from Alikat and Katepwa was digested overnight at 37°C with 20 $U \cdot \mu g^{-1}$ *Eco*RI or *Hin*dIII concentrated endonuclease (Pharmacia Biotech) NEBuffer *RI* (5 mM NaCl, 10 mM Tris pH 7.5, 1 mM $MgCl_2$, 0.0025% Triton X-100; for *Eco*RI) or NEBuffer *2* (5 mM NaCl, 1 mM Tris, pH 8.0, 1 mM $MgCl_2$, 0.1 mM dithiothreitol; for *Hin*dIII) made up to a total volume of 47 µl with ddH_2O. Ten µg doubled-haploid DNA (DHA-33, DHA-35 and DHK-W2) was digested overnight at 37°C with 30 $U \cdot \mu g^{-1}$ *Eco*RI, *Hin*dIII (Pharmacia Biotech), or *Eco*RV (GibcoBRL Life Technologies) concentrated endonuclease; NEBuffer RI (*Eco*RI) or NEBuffer 2 (*Hin*DIII, *Eco*RV); and 100 $\mu g \cdot ml^{-1}$ bovine serum albumin fraction V (*Eco*RV only) made up to a total volume of 75 µl (*Eco*RI and *Hin*dIII digestions) or 85 µl (*Eco*RV digestion) with ddH_2O. Volumes of the doubled-haploid DNA digestions were adjusted to 85 µl with ddH_2O when the digestion was complete.

Doubled-haploid and near-isogenic DNA samples were concentrated to 20 µl using an ethanol/salt precipitation from Ausubel *et al.* (1989) to match the volume of the agarose gel wells. Volumes of solutions added to precipitate the DNA were measured based on the volume of the digestion reaction; ranging from 50 µl to 250 µl. First, 0.1 volume of 3 M sodium acetate ($CH_3COONa \cdot 3H_2O$) was added and mixed by inversion in 1.5 ml Eppendorff tubes. Then 2 resulting volumes of 95% ethanol (-20°C) were added and mixed by inversion. The samples were placed at -80°C for 60 minutes to precipitate DNA. DNA was microcentrifuged at 13,000 rpm at 4°C for 25 minutes to pellet. Ethanol was decanted, the DNA pellet was washed with 500 µl of 70% ethanol at -20°C for 5 minutes. The DNA pellet was microcentrifuged at 13,000 rpm at 4°C for 15 minutes. Ethanol was decanted, the DNA pellet dried for 2 to 3 hours at 60°C in an oven, and

resuspended in 20 µl TE.

Digestion of the near-isogenic and doubled-haploid parental DNA appeared to be inhibited by impurities such as proteins, carbohydrates, and residual CTAB present when the DNA was first isolated. Attempts were made to remove these impurities by re-isolating the DNA using a phenol/chloroform extraction followed by a high salt precipitation (Fang *et al.*, 1992). The re-isolated DNA was digested as above.

3.2.6 Genomic DNA Fragment Separation and Transfer to Nylon Membranes

Genomic DNA fragments were separated using agarose gel electrophoresis on 0.8% agarose gels in freshly made TBE, 0.1 $\mu g \cdot ml^{-1}$ ethidium bromide for 15 hours at 30 V. Agarose gels were photographed as in section 3.2.3. DNA fragments were transferred to GeneScreen Plus (DuPont) nylon membranes using a model 785 vacuum blotter (BioRad). A sheet of Whatman 3MM filter paper and nylon membrane were submersed in ddH_2O for 1 minute for each agarose gel. The filter paper was placed on the vacuum blotter first, followed by the membrane, and the agarose gel with wells facing upward. Wells were filled with agarose. Transfer solutions were pulled through the gel with 0.67 Pa vacuum. DNA was depurinated with 0.25 M HCl for 15 minutes and denatured with 0.5 M NaOH, 1.5 M NaCl for 20 minutes. Finally, the gel was neutralized with 0.5 M Tris pH 8.0, 3 M NaCl for 15 minutes. DNA was transferred to the nylon membrane with 10X SSC (1.5 M NaCl, 150 mM sodium citrate dihydrate) for 1 hour. To determine the transfer efficiency, the agarose gel was restained with ethidium bromide and photographed as above. The nylon membrane DNA blot was washed with 0.4 M NaOH for 1 minute and 0.2 M Tris pH 8.0, 2X SSC for 5 minutes to remove any agarose on the membrane. The blot was dried at room temperature overnight between two pieces of filter paper or at 80°C for 2 hours.

3.2.7 Southern Hybridization of RFLP Probes to Digested Genomic DNA

Hybridization was done according to the GeneScreen Plus protocol (DuPont, 1993). The blot was submersed in 2X SSC for 1 minute then placed in a hybridization

bottle with 10 ml hybridization buffer (1 g dextran sulphate MW 500,000, 1% SDS, 1 M NaCl) and 100 µl 100X Denhardt's solution (1g polyvinylpyrrolidone MW 40,000, 1 g bovine serum albumin fraction V (Sigma), 1 g Ficoll 400 in 50 ml ddH_2O) and prehybridized for 4 hours at 65°C with constant rotation in a Hybaid minidual 14 incubator. The prehybridization solution was decanted, replaced with an identical hybridization solution and the probe solution (see above), and hybridized for 16 to 18 hours at 65°C with constant agitation. Blots were washed (1) twice with excess 2X SSC at room temperature for 5 minutes, (2) twice with excess 2X SSC for 30 minutes at 60°C, and (3) twice with excess 0.1X SSC for 30 minutes at room temperature. Washed blots were wrapped securely in Saran Wrap to keep moist and exposed against Kodak X-OMAT AR autoradiogram negative film in an autoradiogram cassette with 2 intensifying screens (Fisher) at -80°C for 24 hours to 1 month. Autoradiograms were scanned using a flatbed scanner and stored in TIF (tagged image file) format.

3.3 Results

3.3.1 Randomly Amplified Polymorphic DNA

None of the 664 UBC primers used to screen the near-isogenic parents and F_2 bulks resulted in polymorphic bands (Figure 3-1). Only one Operon primer Q09 (GGCTAACCGA) differentiated between Al-resistant and Al-sensitive parental genotypes. A 790 bp band was present in the Al-resistant parent, Alikat, and in the Al-resistance donor, Maringa, but absent in the Al-sensitive parent, Katepwa (Figure 3-2). However, polymorphism was not observed for this marker in the F_2. The locus appeared to segregate randomly when the individuals comprising the bulks of individuals from all three aquaria were tested. Individuals composing the bulks from aquaria 1 and 2 were not tested with Q09 since most of these individuals were already represented in the bulks from all three aquaria. The near-isogenic parental lines and the two different sets of bulks were not used for further RAPD mapping because of the lack of identifiable polymorphims. Primer Q09 did not associate with the Al-resistance genotype when the doubled-haploid parent genotypes were tested (Figure 3-2).

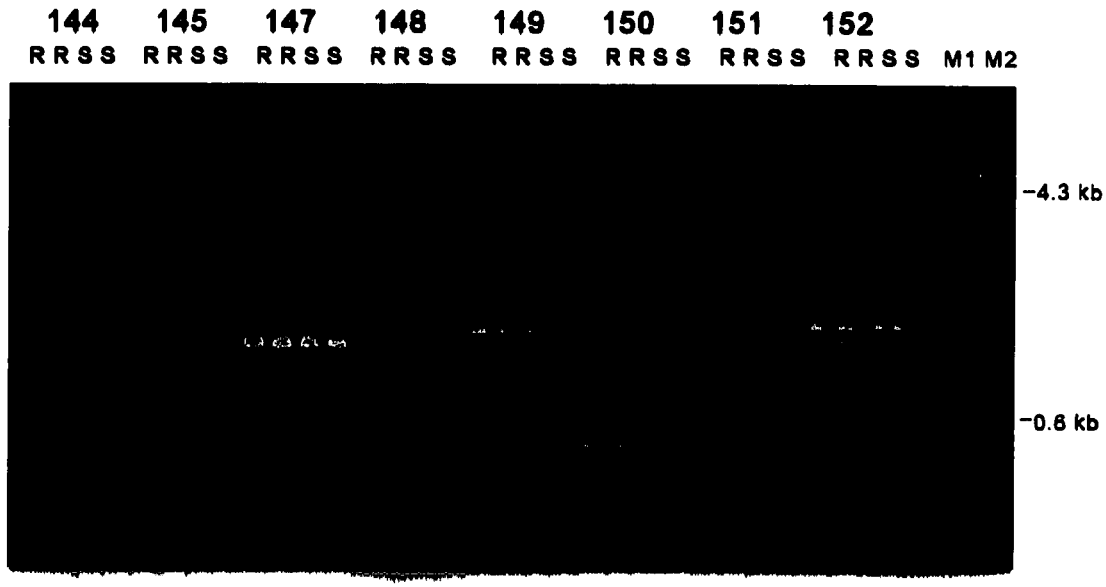

Figure 3-1. A typical agarose gel showing samples of F_2 bulked DNA amplfied using UBC primers. Fragment sizes ranged from 300 to 3000 bp. Numbers indicate UBC primer, R is the resistant F_2 bulk (replicated twice), S is the Al-sensitive F_2 bulk (replicated twice), M1 is *Bst*EII digested lambda DNA size markers, and M2 is *Hin*dIII digested lambda DNA size markers.

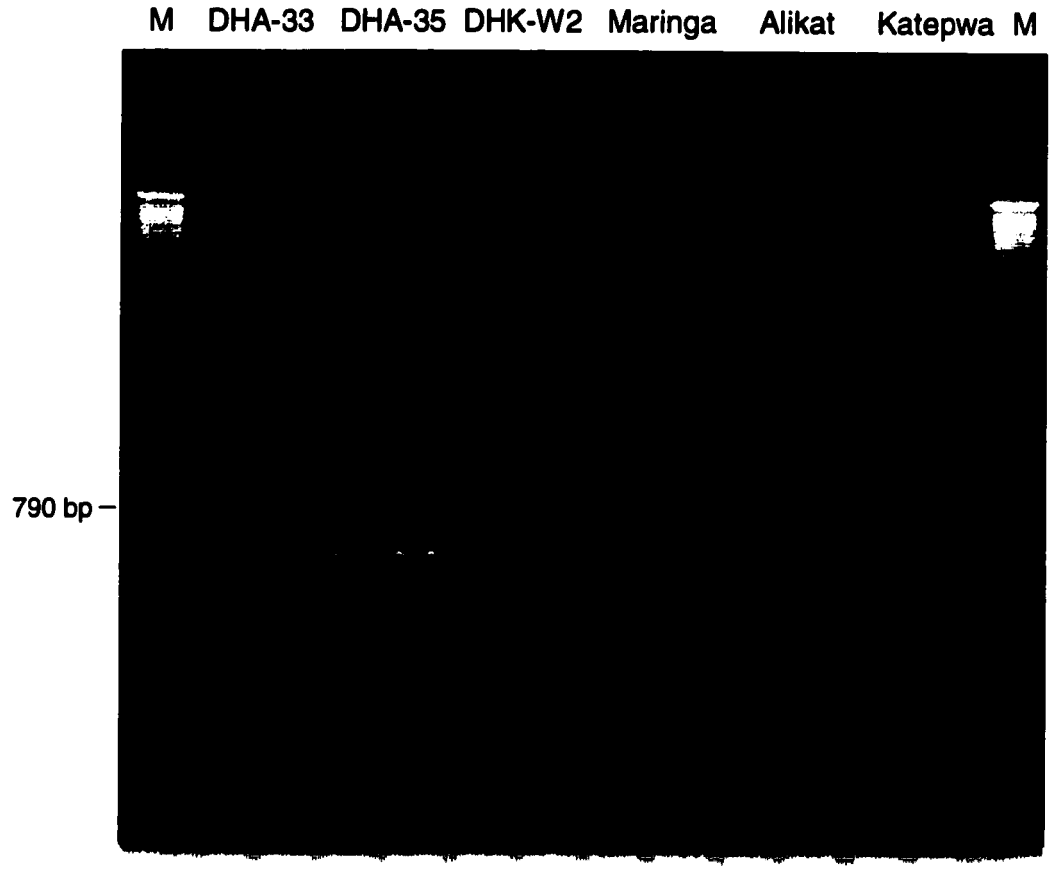

Figure 3-2. An agrose gel comparing near-isogenic and doubled-haploid cultivars amplified using Operon primer Q09. Note the presence of the 790 bp band in Maringa and Alikat, the weak presence in all doubled-haploid cultivars, and the absence of the band in Katepwa. Each parent cultivar was replicated twice. M is *Bst*EII digested lambda DNA size markers.

The performance of the UBC and Operon primers were compared (Table 3-1). The Operon primers had a higher probability of amplifying at least one locus, but yielded a similar number of loci per amplifying primer as the UBC primers. For these reasons the Operon primers were used to score the doubled-haploid material. Sixteen of 156 primers separated at least one Al-resistant parent from the Al-sensitive parent, but only 4 primers were consistent and reliable for both Al-resistant parents: Q10 (TGTGCCCGAA), 1013 bp; O19 (GGTGCACGTT), 786 and 836 bp; R03 (ACACAGAGGG), 934 bp; and R07 (ACTGGCCTGA), 608 bp (Figure 3-3). Primer Q10 amplified a locus in the Al-sensitive phenotype, O19 amplified a locus in the Al-resistant cultivars (836 bp) and one in the Al-sensitive cultivar (786 bp), and both R03 and R07 amplified a locus only in the Al-resistant parent cultivars. The F_2 bulks from each cross did not show polymorphisms (Figure 3-3) and a sample of F_2 individuals used in those bulks indicated a random segregation for the 4 primers.

3.3.2 Restriction Fragment Length Polymorphism

The near-isogenic parental genomic DNA did not digest completely over 24 hours with the standard enzyme concentration of 10 $U \cdot \mu g^{-1}$ genomic DNA (Figure 3-4), nor did the doubled-haploid parental genomic DNA digest at 12.5 $U \cdot \mu g^{-1}$. This suggested impurities such as proteins, polysaccharides, or restriction inhibitors such as methylated cytosine inhibited digestion. Addition of protein ameliorators, such as bovine serum albumin fraction V (BSA; Sigma) or spermidine (Ausubel *et al.*, 1989), did not improve digestion when *One Phor All Plus*® buffer (Pharmacia) was used, however BSA was necessary for certain New England Biolab buffer and enzyme combinations. Cleaning the DNA using phenol/chloroform extractions (Fang *et al.*, 1992) and/or high salt precipitations (Ausubel *et al.*, 1989; Fang *et al.*, 1992), did not improve the digestibility of the DNA (Figure 3-5). Increasing the restriction enzyme concentration to 30 $U \cdot \mu g^{-1}$ improved the digestion somewhat, but still did not appear to be fully effective (Figure 3-6). The enzyme *Eco*RI was able to digest better than other enzymes tested. Increasing the CTAB concentration from 2% to 4% to improve DNA purity resulted in an

Table 3-1. Comparison of the performance of University of British Columbia (UBC) and Operon decamer primers.

Germplasm and primers tested	Number of Primers		Mean number of bands per primer that amplified at least one locus (±S.D.)
	Total	Amplified at least one locus (% total)	
Near-isogenic F_2			
UBC	664	409 (62%)	6.1 ± 2.6
Operon	158	142 (90%)	7.0 ± 3.0
Doubled-haploid parents			
Operon	156	142 (91%)	5.8 ± 2.4

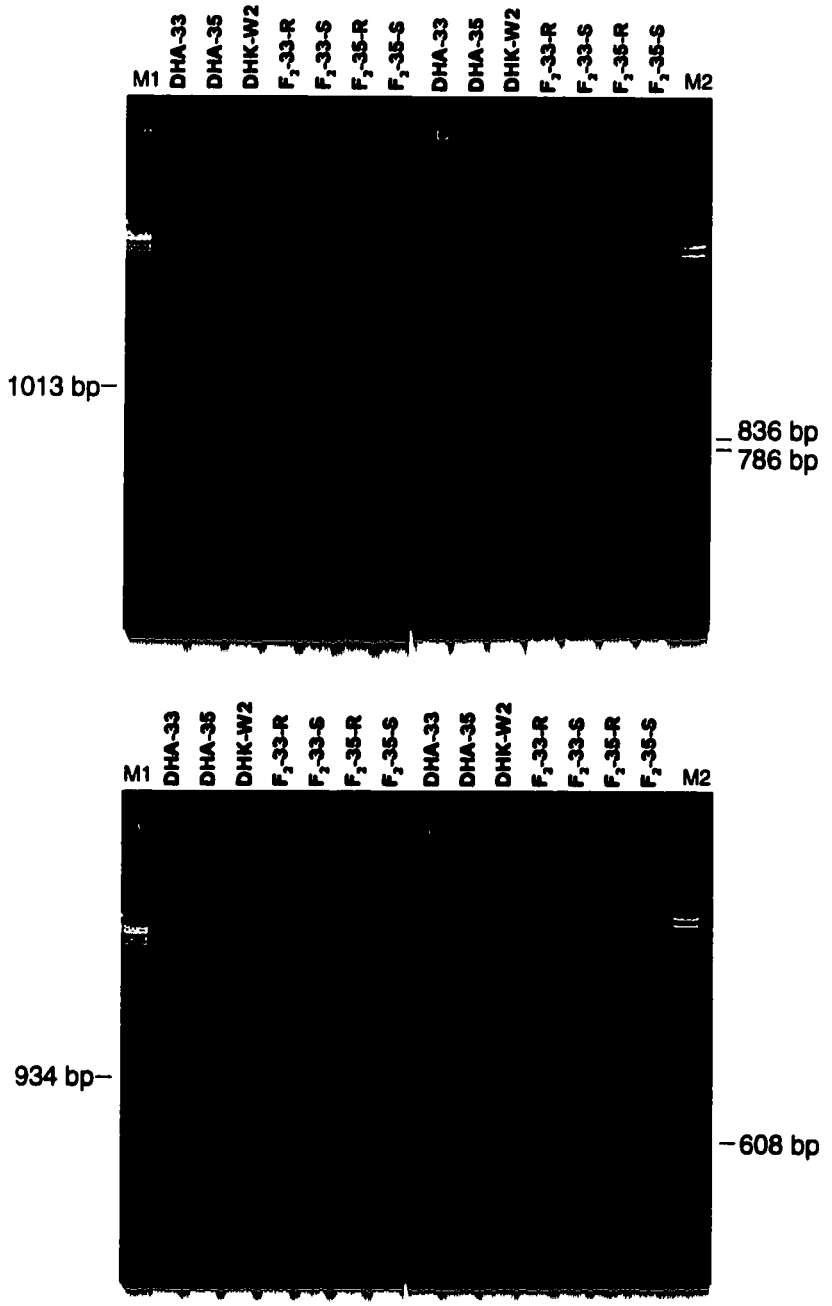

Figure 3-3. Agarose gels of doubled-haploid parents (DHA-33, DHA-35 and DHK-W2) and F_2 bulks. F_2-33 designates the F_2 progeny of DHK-W2 x DHA-33, F_2-35 designates F_2 progeny of DHK-W2 x DHA-35, R is the resistant F_2 bulk, and S is the sensitive F_2 bulk. M1 and M2 are *Bst*EII and *Hind*III digested lambda DNA size markers respectfully. (A) Amplified with Operon Q10. (B) Amplifed with Operon, O19, (C) Amplified with Operon R03, (D) amplified with Operon R07.

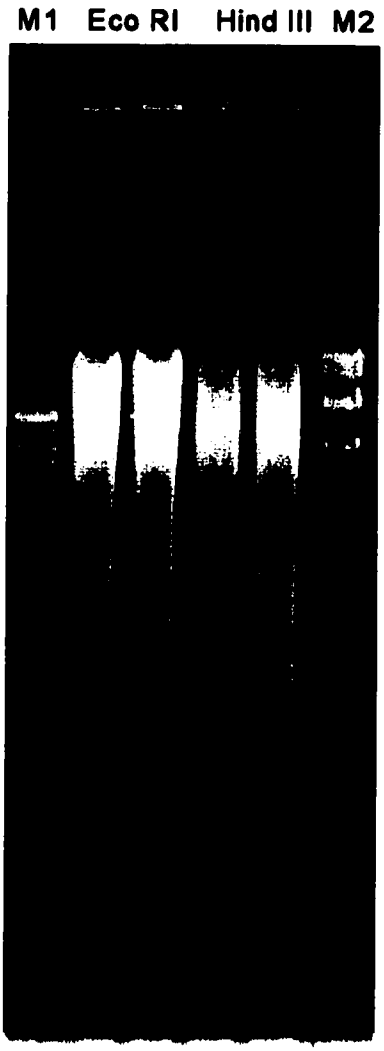

Figure 3-4. Agrose gel of the digestion of near-isogenic parental lines with 10 $U \cdot \mu g^{-1}$ *Eco*RI. and *Hin*dIII. Note the partially digested DNA at the top of each digestion. M1 is *Bst*EII digested lambda DNA size markers, and M2 is *Hin*dIII digested lambda DNA size markers.

Figure 3-5. An agarose gel of near-isogenic parental DNA (A is Alikat, K is Katepwa) digested with 15 $U \cdot \mu g^{-1}$ *Eco*RI. The high salt treatment involved precipitating genomic DNA with 2 M NaCl and resuspending in TE. The PCI/salt treatment involved a salt precipitation of the genomic DNA as in the high salt treatment. Then DNA was re-extracted three times with 1 volume of 24:24:1 phenol, chloroform, isoamyl alcohol. The uncleaned DNA is represented in the control lanes.

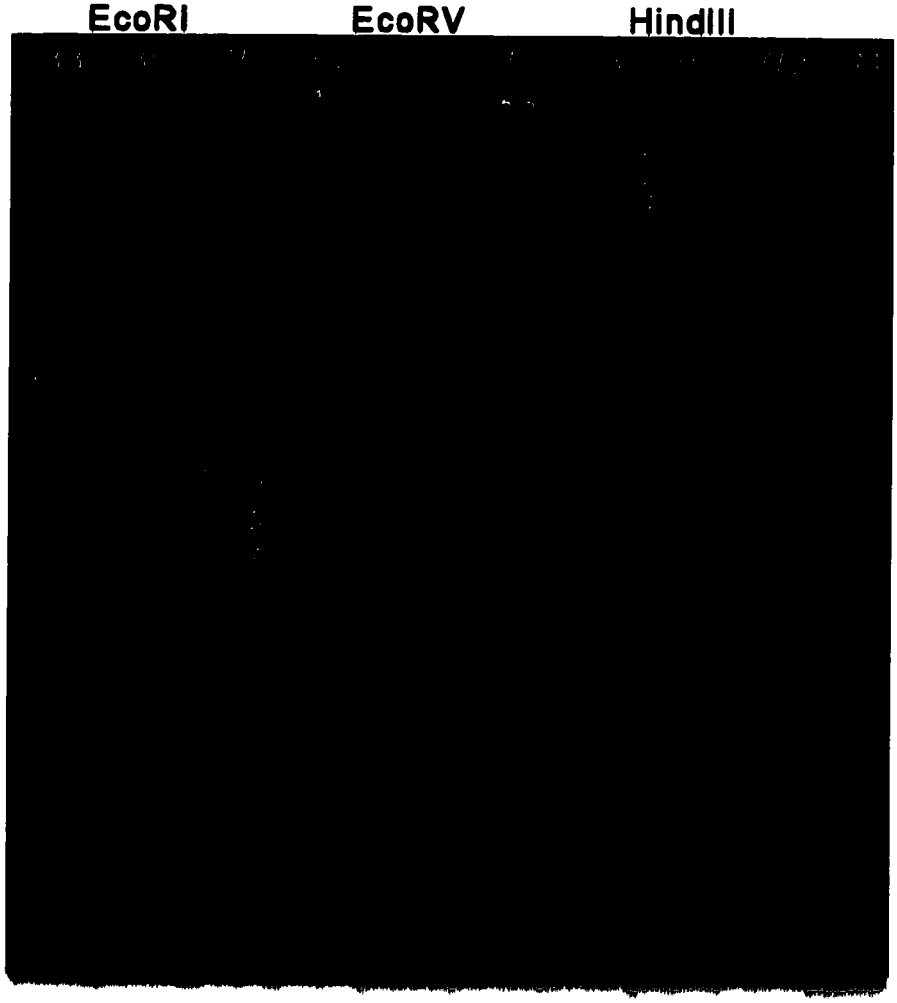

Figure 3-6. An agarose gel of double-haploid parental DNA (33 is DHA-33, 35 is DHA-35, and W2 is DHK-W2) digested with 30 $U \cdot \mu g^{-1}$ *Eco*RI, *Eco*RV, and *Hin*dIII. M is *Bst*EII digested lambda DNA size markers. *Eco*RI digested the DNA better than the other restriction endonucleases and these lanes were probed with cdo1395 (Figure 3-8).

exponential decrease in the yield of DNA. Doubling the CTAB concentration halved the final DNA concentration to unusable concentrations (data not shown).

When the near-isogenic parent cultivars, Alikat and Katepwa, were digested with *Eco*RI, clone cdo938 identified fragments that distinguished between the genotypes at two different loci (Figure 3-7). Alikat possessed two strong bands at 3.7 and 3.5 kb, whereas Katepwa only had one band at 3.6 kb, half way in size between the two Alikat bands. Clone cdo1395 identified fragments that distinguished between doubled-haploid parental genotypes, DHA-33 from DHK-W2; bands at 3.4 and 2.6 kb were present in DHA-33, but lacking in DHK-W2 (Figure 3-8). An identical banding pattern was produced by DHA-35 and DHK-W2 (Figure 3-8). Digestion using other enzymes (*Hin*dIII and *Eco*RV) and F_2 genomic DNA did not yield reproducible results. Re-isolating the F_1 and F_2 DNA from the cross between DHA-33 and DHK-W2 substantially improved the digestion of the DNA.

The clones associated with Al-resistance in Alikat and DHA-33 have been linked to Al-resistance in two different cultivars. Clone cdo1395 was mapped to chromosome arm 4DL, 11.1 cM away from the Al-resistance locus in cv. BH1146 (Riede and Anderson, 1996) and clone cdo938 mapped to chromosome group 4L in the Triticeae consensus genetic map developed by Van Deynze *et al.* (1995b). Clone cdo1395 appears to map to a second location on group 7S in the consensus map (Van Deynze *et al.*, 1995b), but there has been no evidence of Al-resistance controlled by group 7 chromosomes.

3.4 Discussion

3.4.1 Randomly Amplified Polymorphic DNA Markers

Genetic mapping of *T.aestivum* is difficult because of the low levels of polymorphism relative to the other *Triticum* and related species (Dubcovsky *et al.*, 1996). It has been suggested that the combination of the *T. turgidum* (*AABB*) genome with the *D*-genome from *Aegilops squarrosa* (*DD*; syn. *T. tauschii* [Kerby and Kuspira, 1987]) over 8000 years ago (Helbaek, 1959) occurred with few *D*-genotypes in a restricted area

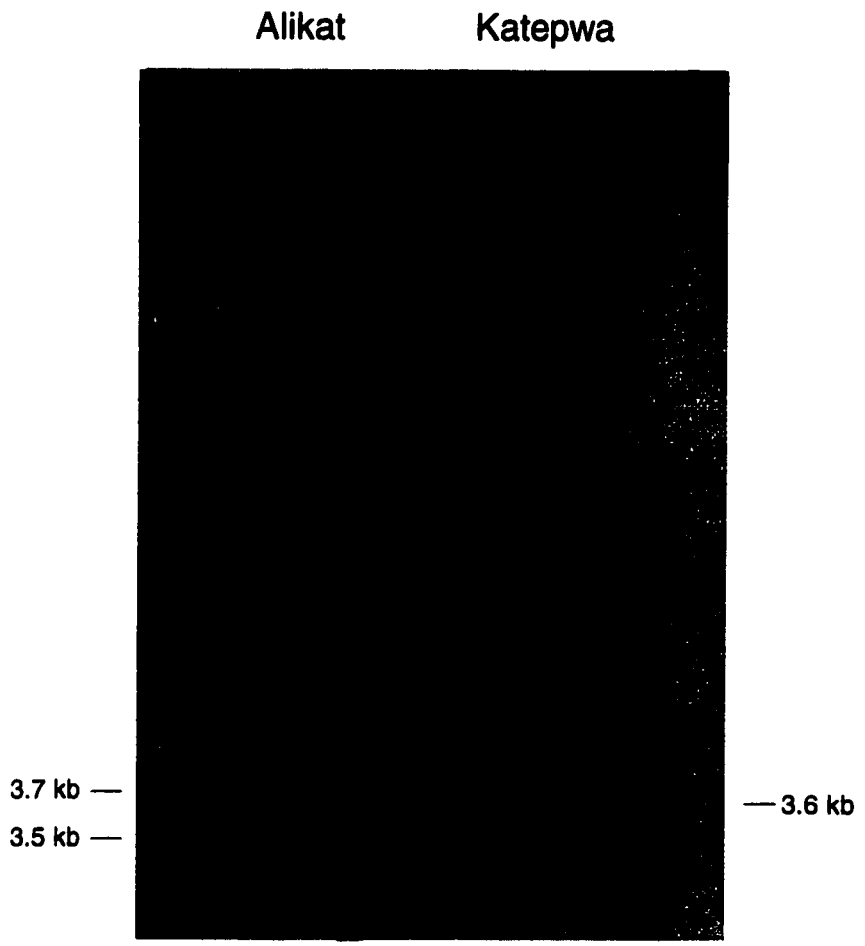

Figure 3-7. An autoradiogram of a Southern hybridization of clone cdo938 to DNA from near-isogenic lines Alikat and Katepwa digested with *Eco*RI. Exposure time was 24 hours.

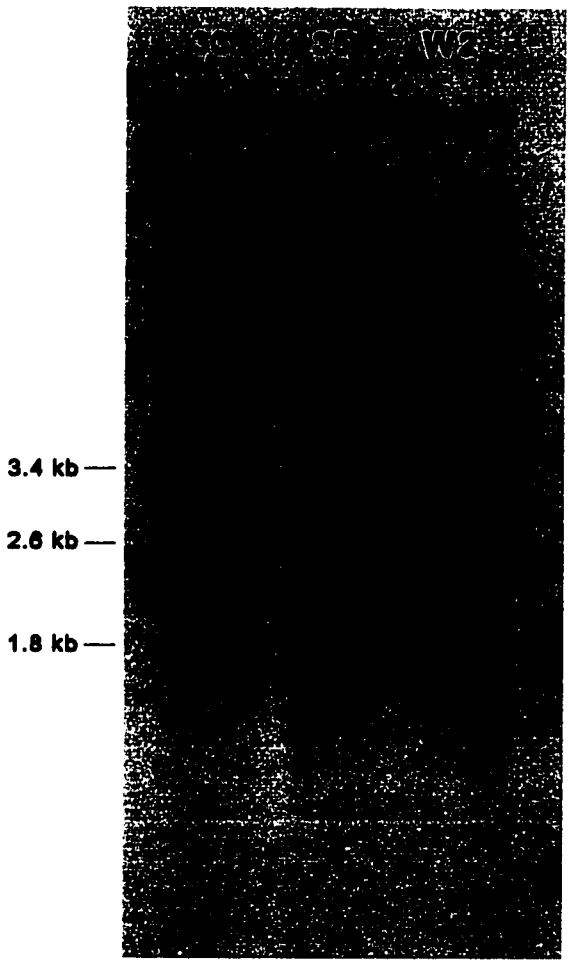

Figure 3-8. An autoradiogram of a Southern hybridization of clone cdo1395 to DNA from doubled-haploids DHA-33 (33), DHA-35 (35), and DHK-W2 (W2) digested with *Eco*RI. Exposure time was 1 month.

near the Caspian Sea, reducing the level of variation in the *D*-genome of *T. aestivum* (Lagudah *et al.*, 1991b). The *D*-genome has been identified as the primary genome conferring Al-resistance in bread wheat (Polle *et al.*, 1979; Aniol, 1990; Lagos *et al.*, 1991; Berzonsky, 1992; Luo and Dvořák, 1996; Riede and Anderson, 1996).

The use of RAPD markers as a means of mapping in hexaploid wheat was not effective in this study. A total of 822 decamer primers were used, but only 5 were able to differentiate between the parent phenotypes; one in the near-isogenic cultivars, and four in the doubled-haploid genotypes. None of the primers that separated the parent phenotypes co-segregated with Al-resistance in the F_2 generation. The RAPD technique was initially developed using human (*Homo sapiens*), soybean (*Glycine max*, *G. soja*), fungus (*Neurospora crassa*), and maize (*Zea mays*) DNA (Williams *et al.*, 1990). All these species possess a smaller genome than wheat and typically cross-breed or cross-pollinate to produce a genome with greater variation than self-pollinating wheat. Devos and Gale (1992) did not recommend RAPD's for wheat genetic mapping because the level of polymorphism was equivalent to RFLP's, but RAPD's lacked the co-dominant nature of RFLP's in a segregating population. They also expressed concern over the reliability of RAPD primers screened on different thermocyclers and found that it was difficult to assign RAPD polymorphisms to specific chromosomes, since amplification products of the same size from different cultivars were not necessarily amplifications of the same region of a chromosome. They did however recommend RAPD markers for use in recombination studies, following a particular region of an introduced chromosome or chromosome fragment and for use only with one genotype or population or in one laboratory. Randomly amplified polymorphic DNA polymorphisms have been successfully used to locate genome specific markers between cultivars (He *et al.*, 1992) and wheat relatives (Wei and Wang, 1995).

The primary factor for the inability to link RAPD markers to Al-resistance in wheat may be due to the lack of unique sequences in wheat (Devos and Gale, 1992). Nonetheless, RAPD's have been successfully used to locate markers for cadmium uptake in durum wheat (Penner *et al.*, 1995), daylight insensitivity (Wight *et al.*, 1994), and

crown rust resistance (Penner *et al.*, 1993) in oats (*Avena sativa*). In all cases, less than 400 primers were used to locate between 5 and 33 polymorphims in the initial screen and 1 or 2 markers linked within 20 cM of the target locus.

Several techniques have been used to improve the number of polymorphims detected by RAPD primers in cereals. He *et al.* (1992) found that primer pairs produced polymorphisms between cultivars in 38% of those tested, nearly doubling the number found by Devos and Gale (1992). Theoretically, paired primers rather than single primers would generate more amplified loci. Regions would be amplified by hybridization of two identical primers and of two different primers to the template DNA. These researchers also used a denaturing gradient gel electrophoresis (DGGE) system that separates DNA fragments based on fragment size and nucleotide composition, resolving bands of equal size. Penner *et al.* (1995) found that use of a denaturing gradient gel did not result in an increase of polymorphism significant enough to justify the use. They employed a technique of temperature sweep gel electrophoresis (Yoshino *et al.*, 1991; Penner and Bezte, 1994) that overcame some technical drawbacks of DGGE, doubling the amount of polymorphisms identified using single random primers compared to standard agarose gel electrophoresis. So far, there are no references in the literature to the use of this technique on hexaploid wheat.

Aegilops squarrosa has extensive intraspecific genetic variation (Lagudah *et al.*, 1991a) and may vary as much as 20% in the DNA content (Furuta *et al.*, 1975). Dinev and Netcheva (1995) identified *Ae. squarrosa* as an accumulator of Al when grown in soils with a pH near 4.0. The ability to accumulate metal suggests that *Ae. squarrosa* may have an internal means of tolerating high levels of Al. As well the *D*-genome has been implicated as the genome conveying Al-resistance in Atlas 66 (Berzonsky, 1992) and other cultivars and lines of bread wheat. The use of RAPD's as a means of identifying Al-resistance in hexaploid wheat using single arbitrary primers and agarose gel electrophoresis has not been successful in this study. If Al-resistant and Al-sensitive phenotypes of *Ae. squarrosa* could be identified, they may be good candidates for Al-resistance mapping using RAPD's and other molecular marker methods.

3.4.2 Restriction Fragment Length Polymorphism

The lack of variation between cultivars and the large amount of repetitive sequences in bread wheat also affects the number of RFLP polymorphisms that can be detected. A number of difficulties were encountered with the RFLP portion of this project that require additional investigation. As a result, only autoradiograms of near-isogenic (Alikat and Katepwa) and doubled-haploid (DHA-33, DHA-35 and DHK-W2) parental lines were completed. The primary problem encountered was unreliable digestion of the genomic DNA isolated from all genotypes. Increasing the concentration of restriction endonuclease partially improved the digestion of the genomic wheat.

Work with the near-isogenic and doubled-haploid parental genotypes did yield some encouraging results. Near-isogenic parentals, Alikat and Katepwa, differed in banding pattern when digested with *Eco*RI and probed with cdo938. Two fragments (3.5 kb and 3.7 kb) were identified in Alikat, but only one fragment (3.6 kb) was identified in Katepwa (Figure 3-7). Banding pattern differed between DHA-33, DHA-35 and DHK-W2 when digested with *Eco*RI and probed with clone cdo1395. Aluminum-resistant genotype, DHA-33, had two strong bands (2.6 kb and 3.4 kb) and one weak band (1.8 kb; Figure 3-8). However, cdo1395 did not identify the same fragments in DHA-35 that were found in DHA-33. The weak fragment (1.8 kb) was the only band identified in DHA-35 and DHK-W2 (Figure 3-8). Riede and Anderson (1996) found that clones cdo1395 and bcd1230 were 11.3 cM and 1.1 cM away from the Al-resistance locus on chromosome arm 4DL in Al-resistant cultivar BH1146. A segregating population of 80 recombinant inbred lines (F_6) advanced from the F_2 progeny from a cross between Al-resistant cultivar BH1146 and Al-sensitive cultivar Anahuac was used. Clone cdo938 is located on the long arm of homologous group 4 (Van Deynze *et al.*, 1995a), supporting the location of the primary Al-resistance locus on chromosome arm 4DL. Luo and Dvořák (1996) substituted chromosome 4B of the Al-sensitive tetraploid cultivar *T. turgidum* Langdon with chromosome 4D from the moderately Al-resistant cultivar Chinese Spring. An F_2 segregating population derived from *T. turgidum* cv. Langdon 4D/4B recombinant and Al-sensitive *T. turgidum* cv. Cappelli was used to map the location of the Al-resistant

locus. Wheat genomic clones psr914 and psr1051 were found to map to the same location as the Al-resistant locus on the 4B/4D *T. turgidum/T. aestivum* map and flanked a 4 cM region around the Al-resistance locus on the *T. aestivum* 4B-4D concensus map (Luo and Dvořák, 1996) further supporting the location of Al-resistance in another cultivar on chromosome 4D. The lack of hybridization of cdo1395 to DHA-35 could suggest that the Al-resistance mechanism in DHA-35 is not the same as DHA-33. It is possible that this may be an artifact of the restriction digest problems encountered, since DHA-33 and DHA-35 are sister doubled-haploid lines derived from Alikat. The RFLP results have not been replicated and repeating these experiments with re-isolated DNA may resolve whether the results observed are real or an artifact of the difficulties encountered. It is necessary to continue with the RFLP research to determine whether clones cdo938, cdo1395, and those identified by Luo and Dvořák (1996), are linked to Al-resistance in Alikat and DHA-33 F_2 mapping populations.

The technique of STS-PCR could be used to work around the DNA digestion difficulties. As with RAPD markers, STS-PCR markers are dominant and cannot identify heterozygotes. They are not a substitute for RFLP techniques, but may be used to identify useful RFLP markers. Riede and Anderson (1996) suggested that PCR primers based on clones bcd1230 and cdo1395 should be used to amplify the probe sequences in the wheat genomic DNA as a means of determining whether individuals or pools are Al-resistant. Talbert *et al.* (1994) determined 65% to 85% of PCR products from primers derived from RFLP clones mapped to the same chromosome as the original RFLP probe. Polymorphisms were also detected in the PCR products digested with four-base-pair-recognizing restriction enzymes when separated on a polyacrylamide gel. Primers for STS-PCR from wheat were able to amplify sequences in barley, however, it could not be assumed that the hybridization site of the primers and the resulting amplification product were of the same sequence in both species (Erpelding *et al.*, 1996). If primers can be transferred between species, then primers should be transferable between cultivars. It would be necessary to map the location of STS-PCR amplification products of intercultivar and interspecific transfer of primers to confirm that the site of interest was

correctly identified.

3.4.3 Other Molecular Marker Methods

Two other molecular marker methods could be used to locate the Al-resistance locus in wheat. Microsatellites are simple nucleotide sequence repeats such as $(GT)_n$ or $(CT)_n$ that are found in all eukaryotic genomes (Tautz and Renz, 1984) and can be amplified with flanking PCR primers (Ralfaski and Tingey, 1993). Recently Röder *et al.* (1995) evaluated $(GA)_n$ and $(GT)_n$ microsatellites in wheat. The two microsatellites occured every 271 kb along the genome on average. Four alleles per microsatellite were identifed on average; more than with conventional RFLP's. A second method, AFLP (amplified fragment length polymorphism; Vos *et al.*, 1995), is a combination of RFLP and RAPD-PCR techniques. Genomic DNA is digested and a short oligonucleotide is attached to each end of the fragments. A specific PCR primer is used to recognize the oligonucleotide extension and has 3 random nucleotides to recognize a subset of fragments with that specific sequence. Between 50 and 100 AFLP fragments are amplified per genome, increasing the number of loci recognized and thus the number of polymorphisms (Vos *et al.*, 1995). The primary limitation for the use of AFLP's in wheat is that available kits are optimized for plants with genomes smaller than wheat (GibcoBRL, 1995). Lin *et al.*, (1996) compared RFLP, RAPD, and AFLP techniques in soybean. Two-hundred RFLP probes, 200 RAPD primers, and 64 AFLP primers were used on separate 14 cultivars. Less than 50% of the RFLP probes and RAPD primers resulted in a polymorphism, whereas 94% of the AFLP primers resulted in a polymoprhism. Less than one polymorphism was identified per RFLP probe and RAPD primer, whereas nearly six polymorphic bands were identified per AFLP primer suggesting that AFLP is a more attractive method than RAPD and RFLP if it could be optimized for wheat. Genomic DNA digestion problems encountered may also result in problems with AFLP since this method requires completely digested DNA. A non-CTAB method of DNA isolation may reduce impurities and improve the digestibility of DNA. Marker systems based on PCR should be attempted in addition to RFLP's if alternate

isolation methods cannot sufficiently improve DNA digestion.

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Chapter 4. Discussion and Conclusions

The objectives of this work were to characterize segregating populations derived from near-isogenic Katepwa, Alikat, and doubled-haploid germplasm for Al resistance using callose accumulation in response to Al (Zhang *et al.*, 1994), to determine the basic genetics of Al-resistance in these lines, and to determine whether molecular markers can be identified for Al resistance in these near-isogenic and doubled-haploid lines. Molecular markers were sought to provide information on whether Al resistance observed in these study populations was linked to the same locus as BH1146 and Chinese Spring and to provide a means of locating Al resistance genes and identifying the method of Al resistance in wheat.

The near-isogenic genotypes, Katepwa and Alikat, provided genetic material for the doubled-haploid genetic systems. The Al-resistance of parent genotypes and their progeny was evident using Al-induced accumulation of callose as a marker of Al-injury. Callose proved to be a good method for scoring for Al-resistance in a segregating population for the purposes of mapping. The near-isogenic Al-sensitive parent, Katepwa, accumulated 3.6 times (Figure 2-2a) more callose than the Al-resistant parent, Alikat. The Al-sensitive doubled-haploid parent DHK-W2 accumulated between 3.2 (Figure 2-5) and 2.9 (Figure 2-7) times more callose than the Al-resistant doubled-haploid parents DHA-33 and DHA-35. Callose accumulation was similar to that of other wheat cultivars examined (Zhang *et al.*, 1994; Schreiner *et al.*, 1994). The basic genetics were identified for each parental pairing. Near-isogenic Alikat appears to have a multigenic control mechanism for Al-resistance as identified by callose accumulation in the F_2 segregating population. This is in contrast to the findings of Somer and Gustafson (1995) and Somers et al. (1996) who identified that Alikat may have a single dominant Al-resistance gene as identified using relative root length assays and may be the result of the differing methods of scoring the segregating populations. In this study the F_1 population were not all Al-resistant, as would be expected from a single dominant gene, and the F_2 individuals did not segregated in a 3:1 ratio (resistant:sensitive). Other near-isogenic systems have suggested a multigenic inheritance of Al-resistance (Aniol, 1990; Gustafson and Ross,

1990; Lagos *et al.*, 1991; and Berzonsky, 1992). The doubled-haploid F_1 and segregating populations suggested a single dominant Al-resistance gene (Figures 2-4, 2-6). This is not necessarily in contradiction to results from the near-isogenic cross. The doubled-haploid parents were selected based upon the consistency in the response to Al using the callose assay and lack heterogeneity that may have influenced the segregation of the cross between Katepwa and Alikat. Minor genes influencing Al-resistance in the doubled-haploids may have been unconsciously selected against. Other populations specifically designed for mapping Al-resistance also segregate as if controlled by a single dominant gene (Riede and Anderson, 1996; Luo and Dvořák, 1996). When parent cultivars were screened with UBC and Operon RAPD primers, only one Operon primer (OpQ09) was associated with the near-isogenic parent genotypes. None of the primers tested resulted in a polymorphism when bulks of individuals from the Al-resistant F_2 pool and Al-sensitive F_2 pool from Katepwa x Alikat where compared. The segregation of F_1, BC_1, and F_2 progeny from DHK-W2 x DHA-33 and DHK-W2 x DHA-35 suggested monogenic control of Al-resistance. Four primers, OpO19, Q10, R03, and R07, amplified loci associated with Al-resistance in the doubled-haploid parents. Again, none of these primers were polymorphic in the mapping F_2 populations. Primer OpQ09 amplified loci in both Al-resistant and Al-sensitive parents and segregating doubled-haploid populations.

Triticum aestivum is less polymorphic relative to other *Triticum* species (Dubcovsky *et al.*, 1996). This may reflect the hybridization between *T. turgidum* with the *D*-genome donor, *Aegilops squarrosa*, (syn. *T. tauschii* [Kerby and Kuspira, 1987]) 8,000 years ago (Helbaek, 1959), which occurred between a few *D*-genotypes individuals in a small restricted area near the Caspian Sea (Lagudah *et al.*, 1991b). Randomly amplified polymorphic DNA markers have been successfully used in species with smaller genomes than wheat such as humans, soybean, *Neurospora* sp., maize (Williams *et al.*, 1990) durum wheat (Penner *et al.*, 1995) and oats (Penner *et al.*, 1993; Wight *et al.*, 1994). Devos and Gale (1992) did not recommend RAPD markers in wheat because of the lack of unique sequences in wheat. Findings in this study are consistent with their

findings. The level of RAPD polymorphism in wheat was equivalent to RFLP, but lacked the co-dominance of RFLP makers (Devos and Gale, 1992).

A number of methods have been used to increase the sensitivity of RAPD makers. Amplification products have been separated on a denaturing gradient gel (Yoshino *et al.*, 1991; He *et al.*, 1992; Penner and Bezte, 1994) and RAPD primer pairs (He *et al.*, 1992) have been used to increase the number of amplification products observed, and thus increase the chance of locating a polymorphism. *Aegilops squarrosa* has been found to accumulate metals in the straw, including Al, as a possible avoidance mechanism to metal toxicity when grown in soil with a pH between 3.9 and 4.7 (Dinev and Netcheva, 1995). It is possible that *Ae. squarrosa* rather than *T. aestivum* could be used to map Al-resistance, since *Ae. squarrosa* is the *D*-genome donor. *Aegilops squarrosa* has a high level of genetic variation compared to other wheat relatives (Lagudah *et al.*, 1991a) and may vary as much as 20% in the DNA content between varieties (Furuta *et al.*, 1975).

It has been estimated that over 75% of the *T. aestivum* genome consists of repetitive DNA sequences. Restriction fragment length polymorphism probes that hybridize to these repetitive sequences cannot be resolved on an autoradiogram (Devos and Gale, 1992), reducing the number of potential polymorphisms that can be observed in bread wheat. Restriction fragment length polymorphism markers have been linked to Al resistance in cv. BH1146 (probes bcd1230; cdo1395; Riede and Anderson, 1996) and cv. Chinese Spring (probes psr39, psr914, and psr1051; Luo and Dvořák, 1996), located on chromosome arm 4DL, however a number of technical problems arose during the RFLP investigation of this project. Restriction endonucleases appeared to be inhibited by impurities in the isolated DNA. The addition of ameliorators such as bovine serum album or spermidine did not increase the digestibility of the isolated DNA. A second isolation improved the digestibility of the wheat DNA.

Preliminary work with the near-isogenic and doubled-haploid parents have suggested that probes cdo1395 (Riede and Anderson, 1996) and cdo938 are associated with the Al-resistance phenotype originating from the Brazilian cultivar, Maringa. Probe cdo1395 hybridized to produce polymorphisms between doubled-haploids DHA-33 and

DHK-W2 (Figure 3-8) and probe cdo938 (on the same chromosome arm as cdo1395) had shown a polymorphism between the near-isogenic lines Katepwa and Alikat. These results suggest a single common Al-resistance gene, however they do not suggest a monogenic system.

Cytogenetic and mapping studies have identified a number of chromosomes that may contribute to Al-resistance. Chromosome 4D has been the most commonly associated location for Al-resistance at average soil Al concentrations in a number of different cultivars and backcross lines (Polle *et al.*, 1978; Aniol, 1990; Lagos *et al.*, 1991; Riede and Anderson, 1996; Luo and Dvořák, 1996; Pellet *et al.*, 1996). Pellet *et al.*, (1996) suggested that two Al-resistance mechanisms are present in the Al-resistant cultivar Atlas-66, but only one mechanism is present in the backcross line ET3. Malate and phosphate were exuded in response to 20 μM Al in a $CaCl_2$ solution in Atlas 66, but only malate was exuded from ET3. Delhaize *et al.* (1993a,b) assigned malate exudation in response to Al in ET3 to a hypothetical gene, Alt_1. Lagos *et al.* (1991) observed that an F_2 population derived from Al-resistant cultivars BH1146 and Atlas 66 (each cultivar possesses a unique lineage) segregated in a 15:1 ratio (Al-resistant to Al-sensitive) at 150 μM Al. This implies that BH1146 must have two dominant Al-resistance genes; one in common with Atlas 66 and one unique. Each gene imparts Al-resistance to the progeny so that only when both genes are recessive do the progeny appear Al-sensitive, producing the two gene segregation ratio of 15:1 (Al-resistant to Al-sensitive).

Riede and Anderson (1996) and Luo and Dvořák (1996) have mapped Al-resistance to chromosome arm 4DL in BH1146 and Chinese Spring. These genes, Alt_{BH} and Alt_2, may represent the same Al-resistance gene. When BH1146 is crossed with aneuploid lines of Chinese Spring it restores Al-resistance in lines that lack chromosome arms 5AS and 4DL (Aniol, 1990) at 148 μM Al. Aniol (1990) suggested that chromosome arm 5AS is involved in Al-resistance at low Al (37 to 54 μM) concentrations and chromosomes 2DL and 4DL are involved in Al-resistance at high Al concentrations (>74 μM). When all these pieces of evidence are taken together, Atlas 66, BH1146, and Chinese Spring, ET3, and Carazinho may share a common gene for Al-

resistance that may code for the regulation for malate exudation. This gene may be located on chromosome arm 4DL. As well, Atlas 66 and BH1146 also appear to possess a unique gene for Al-resistance. Thus, there may be at least three different Al-resistance genes, supporting the multigenic model for Al-resistance. The following labelling system is used in this discussion: Alt_A, Atlas 66 Al-resistance; Alt_B, BH1146 Al-resistance; and Alt_C, Al-resistance gene common to Al-resistance cultivars; possibly located on chromosome arm 4DL

Alikat may also share the common Al-resistance mechanism (Alt_C) observed between Atlas 66, BH1146, Chinese Spring, ET3, and Carazinho. Maringa is the source of Al-resistance in Alikat (Figure 2-1). Basu *et al.* (1994) found that Maringa exudes four times more malate in response to 100 μM Al, compared to Katepwa. Preliminary evidence suggests that markers cdo938 and cdo1395 are associated with Al-resistance in Alikat and the doubled-haploid sister lines. Further investigation is necessary to determine whether the Al-resistance in Alikat is related to malate exudation. Restriction fragment length polymorphism mapping in Alikat needs to be completed for probes cdo1395 and psr1051 before the relationship between possible malate exudation, and the Alt_C Al-resistance gene on chromosome arm 4DL can be confirmed in Alikat.

To possibly remove the influence of modifying genes, more simple genetic systems should be examined, such as the *D*-genome donor, *Ae. squarrosa*, or a synthetic diploid derived from the *D*-genome of *T. aestivum.* This approach would be similar to that suggested by Taylor (1995). He recommended examination of the cellular basis of Al-resistance in the context of a suite of physiological adaptations. A simple Al-resistance mechanism most likely reflects a simple genetic mechanism. This, in turn, simplifies the identification of the gene responsible for imparting Al-resistance. Once dissection of a single resistance mechanism is complete, results then can be interpreted in a larger physiological context.

In conclusion, the basic RAPD method for mapping was not successful in identifying a polymorphism related to Al-resistance in near-isogenic and two doubled-haploid based segregating populations in this study. Several polymorphic bands were

identified that appeared to associate with Al-resistance in the parental genotypes, but these were not identified in the segregating populations. Mapping Al-resistance with the RAPD method may be more successful if the sensitivity of polymorphism identification can be increased, or a more simple and variable genetic system could be used. The RFLP method was hindered by a number of technical problems, primarily the inhibition of the restriction endonucleases. Re-isolation of the DNA improved the digestibility of the DNA. Preliminary results suggest that probes cdo1395 and cdo938 mapping to consensus chromosome arm 4L, may be linked to Al-resistance in Alikat, DHA-33, and DHA-35. This Al-resistance gene, Alt_C, may be common to Al-resistance genotypes from a variety of lineages, and may be associated with the control of the exudation of malate in response to Al as a means of detoxifying Al in the rhizosphere.

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Appendix A.1 One-Way ANOVA of Near-Isogenic Parental and F_1 Genotypes

Output from SigmaStat v. 1.00

Normality Test:	Passed	(P = 0.1010)
Equal Variance Test:	Failed	(P = 0.0071)

Group	N	Missing
Alikat	15	0
Katepwa	15	0
F_1	6	0

Group	Mean	Std Dev	SEM
Alikat	1.000	0.261	0.0674
Katepwa	3.631	0.415	0.1070
F_1	2.152	0.880	0.3591

Power of performed test with alpha = 0.0500: 1.0000

Source of Variance	DF	SS	MS
Between Treatments	2	52.06	26.032
Residual	33	7.23	0.219
Total	35	59.29	

Source of Variance	F	P
Between Treatments	118.8	<0.0001
Residual		
Total		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 8.33E-016).

All Pairwise Multiple Comparison Procedures (Bonferroni's method) :

Comparison	Diff of Means	t	P<0.05
Alikat vs F_1	-1.15	-5.09	Yes
Alikat vs Katepwa	-2.63	-15.40	Yes
Katepwa vs F_1	1.48	6.54	Yes

Appendix A.2 One-Way ANOVA of Doubled-Haploid Parents, DHK-W2 and DHA-33, and F_1 Genotypes from the BC_1 Test.

Output from SimgaStat v. 1.00

Normality Test:	Passed	(P = 0.1444)
Equal Variance Test:	Passed	(P = 0.5098)

Group	N	Missing
DHK-W2	5	0
DHA-33	5	0
F_1	6	0

Group	Mean	Std Dev	SEM
DHK-W2	2.336	0.287	0.1285
DHA-33	0.998	0.120	0.0539
F_1	1.220	0.113	0.0461

Power of performed test with alpha = 0.0500: 1.0000

Source of Variance	DF	SS	MS
Between Treatments	2	5.225	2.6124
Residual	13	0.452	0.0348
Total	15	5.677	

Source of Variance	F	P
Between Treatments	75.1	<0.0001
Residual		
Total		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.0000000719).

All Pairwise Multiple Comparison Procedures (Bonferroni's method) :

Comparison	Diff of Means	t	P<0.05
DHK-W2 vs F_1	1.116	9.88	Yes
DHK-W2 vs DHA-33	1.338	11.35	Yes
DHA-33 vs F_1	-0.222	-1.97	No

Appendix A.3 One-Way ANOVA of Doubled-Haploid Parents, DHK-W2 and DHA-33, and F_1 Genotypes from the F_2 Test

Output from SimgaStat v. 1.00

Normality Test:	Passed	(P = 0.5365)
Equal Variance Test:	Passed	(P = 0.2564)

Group	N	Missing
DHA-33	5	0
DHK-W2	5	0
F_1	5	0

Group	Mean	Std Dev	SEM
DHA-33	1.00	0.0853	0.0381
DHK-W2	2.76	0.2331	0.1042
F_1	1.29	0.2487	0.1112

Power of performed test with alpha = 0.0500: 1.0000

Source of Variance	DF	SS	MS
Between Treatments	2	8.950	4.4748
Residual	12	0.494	0.0411
Total	14	9.443	

Source of Variance	F	P
Between Treatments	108.8	<0.0001
Residual		
Total		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.0000000204).

All Pairwise Multiple Comparison Procedures (Bonferroni's method) :

Comparison	Diff of Means	t	P<0.05
DHA-33 vs F_1	-0.284	-2.21	No
DHA-33 vs DHK-W2	-1.762	-13.73	Yes
DHK-W2vs F_1	1.478	11.52	Yes

Appendix A.4 One-Way ANOVA of Doubled-Haploid Parents, DHK-W2 and DHA-35, and F_1 Genotypes from the BC_1 Test

Output from SimgaStat v. 1.00

Normality Test:	Passed	(P = 0.5262)
Equal Variance Test:	Passed	(P = 0.9478)

Group	N	Missing
DHK-W2	5	0
DHA-35	5	0
F_1	10	0

Group	Mean	Std Dev	SEM
DHK-W2	2.696	0.151	0.0674
DHA-35	1.000	0.143	0.0640
F_1	1.057	0.165	0.0522

Power of performed test with alpha = 0.0500: 1.0000

Source of Variance	DF	SS	MS
Between Treatments	2	10.319	5.1597
Residual	17	0.419	0.0246
Total	19	10.738	

Source of Variance	F	P
Between Treatments	209.6	<0.0001
Residual		
Total		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 1.05E-012).

All Pairwise Multiple Comparison Procedures (Bonferroni's method) :

Comparison	Diff of Means	t	P<0.05
DHK-W2 vs F_1	1.6390	19.071	Yes
DHK-W2 vs DHA-35	1.6960	17.091	Yes
DHA-35 vs F_1	-0.0570	-0.663	No

Appendix A.5 One-Way ANOVA of Doubled-Haploid Parents, DHK-W2 and DHA-35, and F_1 Genotypes from the F_2 Test

Output from SimgaStat v. 1.00

Normality Test:	Passed	(P = 0.1233)
Equal Variance Test:	Passed	(P = 0.1907)

Group	N	Missing
DHK-W2	5	0
DHA-35	5	0
F_1	10	0

Group	Mean	Std Dev	SEM
DHK-W2	2.19	0.1178	0.0527
DHA-35	1.00	0.0327	0.0146
F_1	1.23	0.1297	0.0410

Power of performed test with alpha = 0.0500: 1.0000

Source of Variance	DF	SS	MS
Between Treatments	2	4.197	2.0986
Residual	17	0.211	0.0124
Total	19	4.408	

Source of Variance	F	P
Between Treatments	168.9	<0.0001
Residual		
Total		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 6.07E-012).

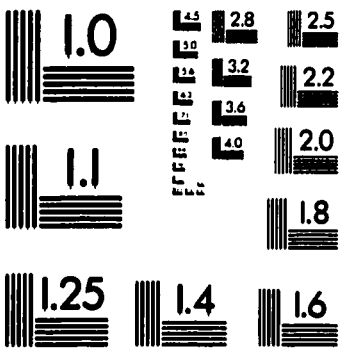
All Pairwise Multiple Comparison Procedures (Bonferroni's method) :

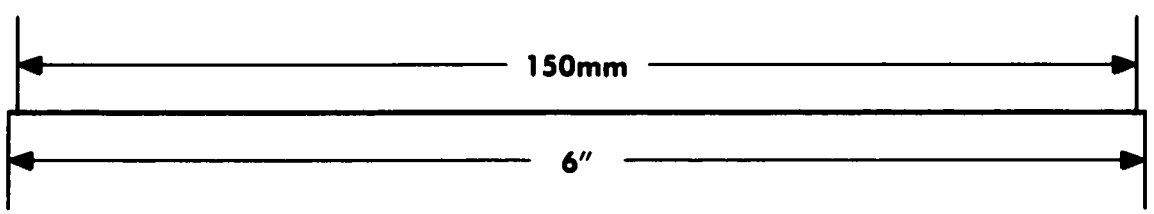
Comparison	Diff of Means	t	P<0.05
DHK-W2 vs F_1	0.962	15.76	Yes
DHK-W2 vs DHA-35	1.186	16.82	Yes
DHA-35 vs F_1	-0.224	-3.67	Yes

Appendix A.6 Free monomeric Al concentration (μM) and pH of Al/Ca solutions used to score the near-isogenic genotypes before and 24 hours after the addition of seedlings.

Aquarium	Concentration of Al (μM)		pH	
	0 hrs	24 hrs	0 hrs	24 hrs
1 (F_2 1-50)	75.7	62.2	4.40	4.51
2 (F_2 51-100)	68.8	58.7	4.40	4.53
3 (F_2 101-150)	68.6	58.5	4.40	4.52
4 (Parents and F_1)	69.4	59.3	4.40	4.52

TEST TARGET (QA-3)





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