UNIVERSITY OF ALBERTA

IDENTIFICATION AND POTENTIAL APPLICATION OF RAPD MARKERS LINKED TO WESTERN GALL RUST RESISTANCE GENES IN JACK PINE

By

DONG HUA

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I give permission to use a diagram of the nuclear cycle of *Endocronartium harknessii* I published in Mycologia 35: 432-443 for M. Sc. thesis of Ms Dong Hua.

Sincerely,

Yasu Hiratsuka
Emeritus Research Scientist

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **IDENTIFICATION AND POTENTIAL APPLICATION OF RAPD MARKERS LINKED TO WESTERN GALL RUST RESISTANCE GENES IN JACK PINE** submitted by Dong Hua in partial fulfillment of the requirements for the degree of Master of Science in Plant Molecular Biology and Biotechnology.

Dr. A. Flanagan (Supervisor)

Dr. Y. Hiratsuka

Dr. F. Yeh

Date: Dec 1. 1995

To Kefei and Jason

ABSTRACT

Western gall rust (Endocronartium harknessii Y. Hiratsuka) is a widespread gall forming stem rust of hard pines in North America. Damage to pines occurs mainly as tree mortality. Studies of western gall rust resistance are advanced in jack pine (Pinus banksiana Lamb.). Open-pollinated families have been screened for resistance both in the field and under controlled inoculation conditions in the greenhouse. Two resistant families (M423, M116) and two susceptible families (M354, M154) formed the basis for selecting molecular markers for western gall rust resistance in jack pine in this research. DNA from haploid female gametophytes of selected families were subjected to Bulk Segregant Analysis using RAPD. 180 RAPD primers were screened on two bulked DNA samples consisting of DNAs extracted from individual megagametophyte of seed. One primer, UBC-382 appeared consistently with the resistant phenotype. Therefore, the potential RAPD marker was tested for segregation on the resistant x susceptible progeny. DNA was extracted from the megagametophyte isolated directly from the young seedling, while the resulting seedling was inoculated and assessed for relative resistance. The results indicate that primer UBC-382 is a marker for western gall rust resistance in jack pine.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
BR	Bulked resistant
BS	Bulked susceptible
BSA	Bulk segregate analysis
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)
E.	Endocrotartium
EDTA	Ethylene diaminetetraacetic acid, disodium salt
kb	Kilobase
M	Molar
MAS	Marker assisted selection
mg	Milligram
ml	Milliliter
mM	Millimolar
ng	Nanogram
NIL	Near-isogenic lines
P.	Pinus
PCR	Polymerase chain reaction
QTLs	Quantitative trait loci
R	Resistance
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA

S	Susceptible
SDS	Sodium dodecyl sulfate
1 x TBE	89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA
1 x TE	10 mM Tris-HCl, 1 mM EDTA
U	Unit
µg	Microgram
µl	Microliter
µM	Micromolar
WGR	Western gall rust

Chapter I

General Introduction

During the past few decades, a major fungal disease of pine in North America called western gall rust (WGR) (Endocronartium harknessii Y. Hiratsuka) prompted study of the genetics and cytology of both the fungus and the pine host. It is clear that resistance to WGR fungus differs among provenances (Martisson 1980), and/or among families within provenances of hard pines (Yanchuk et al. 1988). Similarly, different virulence of the pathogen leads to variable ability to infect different provenances and different families within a provenance (Van Der Kamp 1988). Therefore, the interaction between the spore sources and provenances or families within provenances of the pine host presents a challenge to tree breeders working to develop stable genetic resistance.

A further problem in selecting for resistance to WGR in pines is the long generation interval in trees. Genetic marker will assist tree breeders to select seedlings with combinations of desirable genes, and provide information for genomic mapping which enable us to identify and locate these genes. The plant selection by marker assisted selection (MAS), has the potential to become one of the most useful tree breediing tools ever developed (Yazdani, et al. 1995). Marker-aided selection may provide breeders with efficient and practical means to develop stable resistance. Markers are easy to detect when tightly linked to the resistant factors. Genetic markers such as isozymes and RFLP markers have been used but are laborious and, in the case of isozymes, limited in scope. Random amplified polymorphic DNA (RAPDs) have greatly changed the prospects for the application of molecular markers in breeding (Welsh and McClelland 1990; Williams et al. 1990; Rafalski et al. 1991; Williams et al., 1991). RAPDs are

based on amplification of genomic DNA in the PCR with a single primer of arbitrary sequence. RAPD markers are relatively easy to generate, require very small amounts of DNA. No prior knowledge of DNA sequences is needed.

Preliminary analysis, including field evaluation of test plantation and inoculation experiments of seedlings (Hiratsuka et al. 1992), initially identified one family (M423) of jack pine as an important source of genetic resistance in the Eastern Breeding District (Manitoba). In this thesis, four jack pine families (M423 and M116 for resistant families, M154 and M354 for susceptible families) were selected for study based on the report of field assessments (Klein et al. 1991) and a series of inoculation experiments of the family test plantation in the Eastern Breeding District.

I proposed that RAPD analysis could identify marker(s) that are linked to western gall rust resistance in jack pine. In order to test this I set the following objectives:

1. Identify RAPD markers linked or associated with western gall rust resistance genes in jack pine. The DNA bulked segregant analysis (BSA) strategy was used in this investigation to screen for RAPD markers in our primer prescreening work.

2. Screen megagametophytes of selected jack pine families for in vitro resistance against western gall rust, and verify the resistance in vivo by screening their corresponding seedlings for WGR infection using standard inoculation methods.

Chapter II

Literature Review

In this chapter, the biological events of jack pine, western gall rust of hard pines, molecular analysis with RAPD markers and genetic markers for resistance to western gall rust in hard pines are reviewed. Strategies employed in identification of genetic markers are also compared.

1. WESTERN GALL RUST OF HARD PINES

1.A. Jack pine

Forests occupy about 45% of Canada's landscape (Brand 1990). Jack pine (Pinus banksiana Lamb.) is a prime commercial tree species in Canada's boreal forest. Jack pine grows in pure, even aged stands but can be found in mixed hardwood/conifer stands (Hosie 1969). It is also a major component of the red pine, white pine and oak covertypes. Its scientific name honors the British naturalist, Sir Joseph Banks. Jack pine is also called scrub pine, gray pine, Banksian pine, black pine, and in Canada, princess pine and Hudson Bay pine (Rudloff 1958).

1.A.1. Morphology and distribution

Jack pine is a tree that can be up to 25 m high on productive sites (Rudolf 1958), but is usually smaller and can even be in the form of shrubs in Canada. The crown is broadly globose. The bark is dark brown or dark gray, slightly tinged with red, furrowed into narrow ridges and covered with scales. The branches are crooked and barely in whorls. Shoots develop two whorls in one vegetational period and are flexible, yellowish-green or reddish-brown. The buds are oblong-ovate, 8 mm long, pale brown, and very resinous. The needles are in 2's, rigid, twisted, 2-4 cm long, light green, and

persist on the tree for 2-4 years. The needles have resin ducts in the parenchyma. The leaf sheath is 3 mm long. The cones are 1-3 together, 3-5 cm long, 2-3 cm across, gray or yellowish-gray, glossy, oblong-ovate, usually asymmetrical or distinctly oblique. They persist on the tree for several years. The seed is minute, 3-4 mm long, black-brown, and winged. The somatic chromosome number in jack pine, as in other pines, is $2n = 24$. Trees 150 years old have been reported.

The northern boundary of the botanical range of jack pine extends west from Nova Scotia through central Quebec and Ontario, the northern part of Manitoba and Saskatchewan, and the southern part of the Northwest Territories as far as latitude 63° north. The southern boundary stretches west from central Maine through central Michigan, Wisconsin, and Minnesota, runs north to central Manitoba, and then directly west to British Columbia (Pratt and Littlefield 1938). In eastern Canada and on the borders of its range in the northeastern States, it usually grows in small, widely scattered colonies. It occurs sparsely on the isthmus connecting Nova Scotia to the mainland, but is virtually absent from the remainder of that province. It is more abundant in central Michigan and central Wisconsin at the south end of Lake Michigan. North and west of Lake Superior it is common and often grows to comparatively large sizes. It probably is most abundant and attains its greatest size and beauty west of Lake Winnipeg and north of the Saskatchewan River. It abounds in the valley of the Mackenzie River. The present distribution of the species results from reinvasion and migration over great distances in a short time (Rudolf 1958).

1.A.2. Reproduction and development

Jack pine usually flowers between age 5 and 10. Flowers appear in May to June (flowers of both sexes occur separately on the same tree), and female cones ripen in September of the second year. A crop of seed is produced every 3 to 4 years and germination rate is 60 - 70%. A well-developed, vigorous tree may produce from 1,000 to 1,200 cones in a year, although 300 to 500 probably is more common. Jack pine cones open best during dry weather, although many of them remain closed until they are exposed to fire or until the branches are subjected to higher temperatures near ground after wind breakage or logging. The cones are quite resistant to fire.

Vegetative propagation does not occur naturally. However, propagation by cuttings and grafting is successful. Positive results were obtained in propagation of the species by needle fascicles taken from young, 2-5 year old plants (Rudolph and Nienstadt 1964).

Jack pine seed occasionally exhibits some dormancy, but usually germinate to capacity within 15 to 60 days under favorable conditions. Germination may begin within 8 days after seeding. Seedling establishment generally is best where there is some shade and where mineral soil moisture is reasonably abundant. Most of the older jack pine stands appear to have been established following fires. Natural, spontaneous hybrids occur between jack pine and lodgepole pine (P. contorta Dougl. var. latifolia Engelm.).

1.B. Western gall rust

Western gall rust (WGR) is caused by the fungus Endocronartium harknessii (J. P. Moore) Y. Hiratsuka. It is the most common and destructive stem rust disease of hard pines in western Canada (Ziller 1974; Hiratsuka and Powell 1976). WGR is restricted to hard pine species. [Pine species belong to Subgenus Diploxylon are called hard pine (e.g. Lodgepole pine and Ponderosa pine. etc.). So called five needle pines such as P. strobus, P. monticola, etc. are soft pines belonging to the Subgenus Haploxylon]. Unlike other stem rusts of pines, WGR is capable of infecting directly from pine to pine (Hiratsuka 1987). Infection is detected as external globose galls produced on the stem or branch of seedling or young shoots of the host. In most cases, galls on branch of the host do not kill the host directly, especially when the host is mature. However, galls produced on the main stem often kill the host or make the stem of the host unsuitable for utilization.

1.B.1. The taxonomic status of Endocronartium harknessii

The taxonomic status WGR fungus in the fungi kingdom is showed as follows:

Species: harknessii

Genus: Endocronartium (J.P.Moore) Y. Hiratsuka.(1969)

Order: Uredinales

Class: Hemibasidiomycetes

Subdivision: Basidiomycotina

Division: Eumycetaces

Kingdom: Mycetae

1.B.2. The nuclear cycle of Endocronartium harknessii

Over the past 30 years, WGR fungus has been studied in great detail. In 1969, a new endocyclic genus Endocronartium, was established for autoecious pine stem rusts (Hiratsuka, Y.), based on the morphology and cytology of germinating spores. The nuclear cycle of E. harknessii is shown in figure II-1 (Hiratsuka 1973). In this nuclear cycle, hyphae in the gall are monokaryotic and likely haploid. Most young spores possess two nuclei, which means dikaryotization takes place at the base of the sorus. This is the same as in aeciospores of heteroecious species. However, upon germination, active nuclear divisions occur in the germ tubes, which divide eventually into two to five segments by septa. Each segment of a septate germ tube usually has one nucleus. Dikaryotization and de-dikaryotization is clearly taking place here. This is different from imperfect states of rusts in which dikaryotic spores, either aeciospores or urediniospores, germinate and two nuclei migrate into germ tubes without nuclear fusion or divisions. Germ tubes often have side branches. The side branches as well as tips of the germ tubes are capable of causing infection (Hopkin et al. 1989). Only one kind of spore, aeciospores, are produced on the woody galls from the end of May to July. They become airborne and infect the stem green tissues of elongating new young shoots of pine by direct penetration through the cuticle and epidermis. Small galls appear a few months after infection but do not produce spores until the year following infection. Galls grow each year and produce spores every spring for many years, unless the gall tissue dies with the stem or sori are inactivated by mycoparasites. This fungus has no alternative host; it can infect another pine directly by aeciospores. The life cycle of WGR is

remarkable for its simplicity, whereas many other stem rusts such as sweet fern blister rust, stalactiform blister rust, comandra blister rust, have alternate hosts.

1.B.3. Distribution

WGR is found across Canada, from Nova Scotia to the Yukon; in the east southward through New York, Pennsylvania, West Virginia, and Virginia; and in the west southward to Arizona and northern Mexico. In general, this disease is restricted to the North America continent. In Canada the major hosts of this rust are jack pine (P. banksiana Lamb.), lodgepole pine (P. contorta Dougl. var. latifolia Engelm.), and ponderosa pine (P. ponderosa Laws.).

1.C. Host-parasite interaction

At the early stage of infection, the typical external symptom is pigmentation of epidermal cells. Then conspicuous perennial globose galls are produced on the stems or branch of the host. Very young galls are sometimes spindle-shaped on the surface. During May to July, powdery, orange-yellow spores are produced on the surface of galls (Hiratsuka 1987).

Histological studies of the host-parasite interaction between E. harknessii and lodgepole pine have shown evidence of resistance (Allen et al. 1990). The resistant reaction involved death of infected cells, likely associated with the production of phenolic compounds, lignin, and a necrophylactic periderm.

Different E. harknessii isolates show different level of virulence. Furthermore, significant interactions between provenance and spore source has been found and this interaction suggests that different spore collections have varied ability to infect seedlings from different provenances or different families within a provenance of ponderosa pine (Van Der Kamp 1988). It has also been found that different WGR isolates from different host species show distinct RAPD patterns, this suggests the host-specificity may exist among the WGR population (Li, Yeh and Hiratsuka, in manuscript). The variation among isolates of the rust populations and the interaction between the spore sources and provenances or families of a host are very important considerations when screening resistance to WGR.

The search for genetic resistance in pine host has become a major area of the research. Studies of WGR resistance are advanced in jack pine. It has been found that one seed source has a high level of resistance, while other seed sources of jack pine are susceptible or moderately resistant (Burnes et al. 1988).

Evidence of resistance has also been reported in other pine stem rust systems. In lodgepole pine, resistance to WGR at the provenance and family level has been investigated in 214 open-pollinated families from 24 provenances. Resistance among provenances and among families within provenance differed significantly (Yanchuk et al. 1988). Other hard pines, such as ponderosa pine and Scots pine, etc, also showed provenance or family based differences in resistance to WGR (Hoff 1991; Van Der Kamp 1989).

Reliable inoculation techniques for greenhouse evaluation of WGR infection in hard pines have been developed (Myrholm and Hiratsuka 1993). Early symptoms of infection included general red stain, red flecks, or red streaks on the infected seedlings 2 to 8 weeks after inoculation. These, however, are not good markers of resistance. The frequency of early symptoms is poorly correlated with family resistance and the occurrence of early symptoms on individual seedlings did not give a good prediction of gall formation on them. Therefore, gall formation must be assessed in order to accurately identify resistant families or individuals (Kojwang and Van Der Kamp 1991).

2. MOLECULAR ANALYSIS WITH RAPD MARKERS

To be effective in MAS, we need a saturated map with many markers. The advantage of RAPDs is the ability to generate such maps in short time. There are other similar strategies such as arbitrarily primed PCR (AP-PCR) (Welsh and McClelland 1990), and DNA amplification fingerprinting (DAF) (Caetano-Anolles et al. 1991) that are used for targeting multiple annealing sites without knowing the template sequence. RAPD technology uses single short oligonucleotides of synthetic, arbitrary nucleotide sequences to search for variations in genomic DNA by polymerase chain reaction (PCR) amplification. The amplification reaction, catalyzed by DNA polymerase, results in amplification of the products corresponding to DNA between the sequences that bind the primers. PCR involves three basic steps: 1) Thermal denaturation of DNA; 2) annealing of oligonucleotide primers to the template DNA; and 3) primer extension by a DNA polymerase in the presence of

dNTPs. DNA fragments are amplified exponentially for 25 to 45 PCR cycles. The arbitrary primers used for the procedure are usually 9 to 10 bp in size; they have 50 to 80% C+G content and do not contain palindromic sequences. The number of DNA polymorphisms that are amplified is dependent on the primer and template DNA. RAPD markers may provide a convenient and rapid assessment of the differences in the genetic composition of related individuals (Halward et al. 1992). The polymorphisms can be identified as the presence or absence of particular amplification products, distinguished by their mobility on the ethidium bromide stained agarose gels. The variations in the length of DNA fragments obtained by PCR can be scored in a set of progeny from a genetic cross or among segregating megagametophytes in open-pollinated conifers and treated as a genetic marker. This study of haploid megagametophytes is useful because of dominance of RAPDs. In plants, DNA polymorphisms generated by RAPD method have been used for assessment of variation (Halward et al. 1991), fingerprinting (Caetano-Anolles et al., 1991), cultivar identification (Hu and Quiros 1991; Weining and Langridge 1991; Welsh et al. 1990), genetic mapping (Reiter et al. 1992; Williams et al. 1990), and extensive quantitative phylogenetic comparison of plant genotypes (Halward et al. 1992).

2.A. The RAPD assay

RAPD markers are very simple to use because they do not require DNA sequence information, cloning or synthesis of specific primers. Arbitrary primers as short as 5 nucleotides in length produce characteristic fingerprints (Caetano-Anolles et al. 1991). A limitation of RAPD markers is that they are

generally dominant, therefore, it is not possible to distinguish quantitatively whether an individual is heterozygous or homozygous on the amplified locus. In general, it is important to distinguish heterozygotes from homozygotes to find tightly linked RAPD markers. If an amplified DNA segment is homozygous, the RAPD fragment will either be present or absent. If it is heterozygous, statistically, the ratio of appearance of the specific amplified band should fit a 2:1 Mendelian segregation ratio. The use of paired dominant markers to detect heterozygotes for a different parental genotype requires twice as many markers as would be needed using co-dominant markers to asses the genotype. However, it has been shown by the genetic simulation analysis that dominant markers linked in coupling are as efficient for mapping as co-dominant markers, on a per-gamete basis (Tingey et al. 1992). Therefore, the dominance drawback of RAPDs is not a problem with haploid sample.

Linkages among RAPD markers and traits of interest can be determined by using a large numbers of linked markers. In open-pollinated trees, haploid or gametophytic tissue can be analysed as F_2 populations where RAPD markers that are amplified from a single parent can identify the investigated gene linkage and detect the genetic contribution of a single parent.

2.B. The RFLP assay

Restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1992) have been used as genetic markers for plant breeding purposes in many crop species. RFLP-based genetic linkage maps have been constructed

for maize (Helentjaris et al. 1986; Helentjaris 1987; Burr and Burr 1991; Beavis and Grant 1991), barley (Blake 1990; Nilan 1990), tomato (Bernatzky and Tanksley 1986), wheat (Kam-Morgan and Gill 1989), lettuce (Landry et al. 1987), rice (McCouch et al. 1988), Brassica oleraceae (Slocum et al. 1990), and Arabidopsis thaliana (Chang et al. 1988). They are used to study polymorphic differences in length or sequence in the genome by targeting specific, but often random, DNA segments. Since RFLPs directly reflect differences at the DNA level and they normally behave in a codominant manner, they can be determined at any developmental stage and in all tissues. The number of polymorphic RFLP markers is virtually limitless.

RFLPs are based on variation in the position of restriction endonuclease sites among genotypes. Polymorphisms in DNA fragment patterns of restriction digests occur as a result of changes in restriction enzyme recognition sites. These arise from genetic changes such as point mutations or rearrangements. They are detected by hybridizing radiolabeled DNA probes containing sequence homology to DNA regions of the digested fragments. Identical-size restriction fragments from different genotypes are interpreted as representing genetic similarities, whereas different-size fragments are interpreted as genetic differences. Genomic DNA or cDNA is cloned from a species of interest and used as a probe to follow the segregation of homologous regions of the genome in individuals from segregating populations. As fragments detected by a single probe can be assumed to share extensive sequence homology, genetic linkage maps can be constructed using a large number of markers. RFLP data for determining genetic relationships can be collected efficiently because Southern blots

containing DNA samples of interest can be probed in succession with many different cloned sequences. This allows one to survey regions throughout the entire genomes easily, and, if RFLPs for mapped loci are used to estimate genetic relationships, one can be certain of the accuracy of estimates.

2.C. Comparisons between RAPDs and RFLPs

Both RFLP and RAPD have their advantages and disadvantages. RAPD markers have a number of advantages over RFLPs: 1) a universal set of primers can be used for genomic analysis in all species; 2) it is more efficient at screening for nucleotide sequence-based polymorphisms; 3) there is no requirement for probe libraries or primer sequence information; 4) the PCR-based assay is relatively quick and easy to use; 5) there is no Southern transfer involved; 6) the procedure of amplification is automated; 7) only nanogram quantities of DNA are required. 8) RAPD markers are more suitable markers for relatively small- to medium- sample size; 9) they can provide information at many loci (Welsh et al. 1990); and 10) RAPD analysis is more amenable and has lower labor costs over the short term than does RFLP-based assays. RAPD markers also have some drawbacks. For example, there is some uncertainty about the genetic relationship of fragments from different genotypes and the genome origin of the fragments because the fragments are amplified based on homology to a very short, random DNA sequence. This could affect their utility in determining genetic relationships within some groups of germplasm.

Initially, RAPDs are faster and less expensive in relation to RFLPs. RFLPs involves a great deal of labor because of clone isolation and

maintenance, radiolabeling inserts from a library, producing Southern blots of both parental and F_2 DNA digested with different restriction endonucleases, and exposing the X-ray film. RFLPs also requires more, higher quality DNA. These are all time-consuming and afford many chances for errors. However, once set up, a great deal of information can be derived with little effort from RAPD.

RFLPs and RAPDs are different types of genetic markers and they provide different amounts of genetic information. RFLPs can provide genetic information at a single marker locus. They are codominant markers inherited in Mendelian fashion (Botstein et al. 1992). RFLP markers can show associations of germplasm that are in accordance with pedigree. For inbreeds connected by pedigree, RFLPs show associations that concur with F_1 yield and heterosis data. Therefore, RFLPs are powerful discriminatory tools. RAPDs are generally dominant markers, they are defined by only two alleles - presence and absence of a band (RFLP loci can have multiple, relatively distinguishable alleles). With RAPD markers, the genome location (nuclear or cytoplasmic) of the amplified fragments and the homology of identical-size fragments can not be determined unless each fragment is mapped by a separate segregation analysis.

2.D. Efficient tagging of specific genomic regions with RAPD markers

2.D.1. Increasing mapping efficiency by constructing near-isogenic line (NIL)

RAPDs are recognized as an advanced technique that allows identification of polymorphisms in specific regions by rapidly screening of a

large number of primers. Since the distribution of RAPDs on the linkage group is generally random, several methods can be used to improve the efficiency of mapping and aid the eventual cloning of genes (Michelmore et al. 1991). Near-isogenic lines (NILs; Young et al. 1988) have been developed in many crop species by introgression. There are screened in backcross programs to bring a line carrying a gene of interest into desired cultivars having otherwise desirable properties. After each cross, progeny are selected that possess the phenotype of the target gene. Pairs of near-isogenic lines result in a line that carries a gene segment of interest in a genetic background exclusively that of the recurrent cultivated line. Markers can then detect the divergence between the pair of NILs. There are likely be linked to the regions of interest in the genome (Paran et al. 1991). NILs are limited for some potential target intervals and are time-consuming to generate.

2.D.2. Bulk segregate analysis (BSA)

Although RAPD analysis can provide an efficient way to detect a particular region in the genome, its application to genetic studies of heterogeneous populations can be limited in cases where large number of individuals need to be examined. To efficiently target the specific regions of the genome, another method called Bulked Segregate Analysis (BSA) has been developed . This is a pooling procedure that greatly increased the efficiency of mapping (Kesseli et al. 1992). BSA involves segregating populations from the extremes of the target trait distribution in the population by artificially separating the DNA samples into two pools. Polymorphic RAPD markers between two bulked samples will be genetically linked to the

loci determining the trait. Therefore, BSA can only reveal markers linked to the trait that is used to segregate the population between the two pools. The optimum sample size of bulked DNA for RAPD survey is less than 20. Samples bulked from more than 20 may not necessarily increase the possibility of detecting more segregating markers (Yang and Quiros 1993).

Michelmore et al. (1991) used BSA to identified three RAPD markers linked to major genes using contrasting DNA-bulks composed of F_2 individuals of known genotype for the genes of interest. The bulked segregate method was also used in a backcross-derived population segregating for a major bean rust resistance gene (Miklas et al. 1993). BSA is recognized as an efficient approach for targeting single gene or multiple loci of the major quantitative trait in a population in which variability is high for all traits.

3. GENETIC MARKERS FOR RESISTANCE TO WESTERN GALL RUST IN HARD PINES

3.A. Genetic characteristics in conifers

3.A.1. Haploid genome of conifers

The estimates of physical genome size in Pinus are around 2,500 cM. On average there would be approximately 1.16×10^7 base pair per centimorgan (Neale and Williams 1991; Wakamiya et al. 1993). The chromosome number in Pinus species is 2N = 24.

A feature of gymnosperms is the haploid megagametophyte tissue in seeds. The megagametophyte serves as a nutritive tissue for the embryo in the germinating seed. The megagametophyte in conifers is 1N, and is a mitotic

derivative of a single haploid megaspore from the same megaspore that gives rise to the maternal gamete. Thus, embryo and the megagametophyte differ only in the paternal contribution to the zygote. Megagametophytes derived from seed of a single tree show 1:1 segregation at heterozygous loci and are equivalent to a testcross.

3.A.2. Half-sib mapping and analysis of QTLs within half-sibs

The mapping strategy applied in pine and spruce by using megagametophytes from the seed parent is half-sib mapping (Tulseriam et al. 1992). The half-sib mapping strategy is based on finding individual trees that are heterozygous for many loci by examining the genotypes of the haploid megagametophytes. QTLs within half-sib populations provide the genetic information of the maternal contribution to the zygotic embryo. The analysis of the maternal contribution to a quantitative trait may be carried out by determining the genotype of the megagametophyte and scoring the corresponding diploid progeny for quantitative traits. The genes contributed to the half-sib progeny by the donor male parent can be deduced from the progeny genotypes. The complete genetic information from the half-sib method for QTL analysis can be obtained by estimation of maternal contribution to quantitative traits.

3.B. Application of molecular markers in breeding for western gall rust resistance

Forest trees such as pine have long generation intervals. Therefore, marker assisted selection of heritable traits will greatly benefit forest tree

improvement programs. WGR (E. harknessii (J.P. Moore.) Y. Hiratsuka) is a widespread gall forming stem rust of hard pines in North America. Since WGR is an endocyclic, autoecious rust disease and capable of infecting directly from pine to pine (Hiratsuka 1987), the plant breeding efforts to control the disease must focus on the pine host (Allen et al. 1990). Seedlings and trees with potential resistance to the rust have been observed under field conditions (Merrill 1986). Evidence of resistance has been reported in several pine stem rust systems and histological resistance has been investigated in detail in P. contorta var. latifolia to E. harknessii (Allen et al. 1990). Currently, researchers and breeders are interested in reducing loss from this disease through the development of rust resistant pine families. The use of molecular markers to search for genetic resistance to the pathogen requires a reliable screening method for discriminating between resistant and susceptible trees. RAPD markers have been used to tag major-gene disease resistance in tomato (Klein-Lankhorst et al. 1991; Martin et al. 1990), common bean (Miklas et al. 1993), and lettuce [(Lactuca sativa L.), Michelmore et al. 1991; Paran et al. 1991], and to assist in the development of comprehensive genetic maps in several plant species (Williams et al. 1990; Klein-Lankhorst et al. 1991; Quiros et al. 1991; Echt et al. 1992; Reiter et al. 1992). Studies in tomato (Osborn et al. 1987; Nienhuis et al. 1987; Weller et al. 1988) and corn (Stuber 1987; Grant et al. 1988) have demonstrated that marker-assistant selection is not limited to qualitative traits that are controlled by one or a few genes.

3.B.1. Traditional methods of screening for western gall rust resistance

Resistance to WGR of hard pines shows considerable differences in susceptibility among provenances as well as families within a provenance (Martinsson 1980; Yanchuk et al. 1988; Kojwang and Van Der Kamp 1992). Many researchers have studied the resistance of the host pine to the pathogen and have screened for resistance to the disease on different levels.

Traditionally, screening for WGR resistance was limited to morphological assessments in the field (Yanchuk et al. 1988), or artificial inoculation of seedlings in greenhouse (Blenis and Hiratsuka 1986). However, the occurrence of early symptoms on individual seedlings may not be a good prediction of gall formation (Kojwang and Van Der Kamp 1992). Also, it is difficult to ensure uniform exposure to inoculum in the field and it is difficult to assess the response because the expression of WGR resistance genes can be affected strongly by environmental conditions. Further, field observations and greenhouse tests cannot be used as reliable predictors of resistance because both the frequency of early symptoms and resistance varied significantly among families (Kojwang and Van Der Kamp 1991). Field screening is convenient but the results are often unreliable because it is difficult to control the source and distribution of rusts under natural conditions (Blenis et al. 1993). Artificial inoculation can be a rapid and uniform test for WGR resistance. Myrholm and Hiratsuka (1993) developed a reliable inoculation technique called torn-needle method. It has been reported that the torn-needle method is more effective than the brush method (Table II-2.). The progression of WGR symptoms seemed to occur much more rapidly in seedlings inoculated with the " torn-needle" method.

3.B.2. Use of RAPD markers in pine breeding improvement program for western gall rust resistance

The utility of DNA-based markers in breeding for WGR is based on finding tight linkages between these markers and resistance genes. Once the markers have been selected, they will facilitate selection of individuals that are resistant to WGR fungus. In forest trees, biochemical methods such as isozyme analysis have been used in linkage studies (Conkle 1981). Isozyme analysis is based on differences that are determined by protein electrophoresis followed by some activity staining. This method is relatively simple and can be used on a large scale in certain aspects of plant breeding. However, the number of genetic markers provided by isozyme analysis is limited. Also different enzyme systems usually need specific laboratory procedures and the assay for some enzymes must be done at a specific developmental stage or in specific tissues. RFLP or RAPD analysis has advantages and disadvantages over isozyme analysis as described in this chapter.

The objective of the present study is to screen for RAPD markers that segregate for resistance to WGR jack pine (P. banksiana Lamb.). Family selection for this investigation is based on the preliminary segregation data from the results of field assessments of family test plantations in the Eastern Breeding District (Manitoba) and the results of a series of greenhouse inoculation experiments. Two WGR resistant half-sib families (M423 and M116) and two WGR susceptible half-sib families (M354 and M154) were selected (Table II-1.). DNAs extracted from female gametophytes will be screened for RAPDs using BSA. RAPD markers selected from BSA will be

investigated further by screening for the target RAPDs in megagametophytes whose corresponding embryos (seedlings) have been inoculated and assessed for relative resistance according to the torn-needle method (Myrholm and Hiratsuka 1993).

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FIGURES AND LEGENDS

Fig. II-1. The nuclear cycle of Endocronartium harknessii (Hiratsuka 1973) Most young spores possess two nuclei as aeciospores of heteroecious species. However, two nuclei of aeciospores fuse and divide meiotically into two to five segments in the germ tube during the germination of spores. Each segment of a septated germ tube usually has one nucleus. Dikaryotization and de-dikaryotization is clearly taking place, while the imperfect state of rusts germinate and two nuclei migrate into germ tubes without nuclear fusion or divisions.

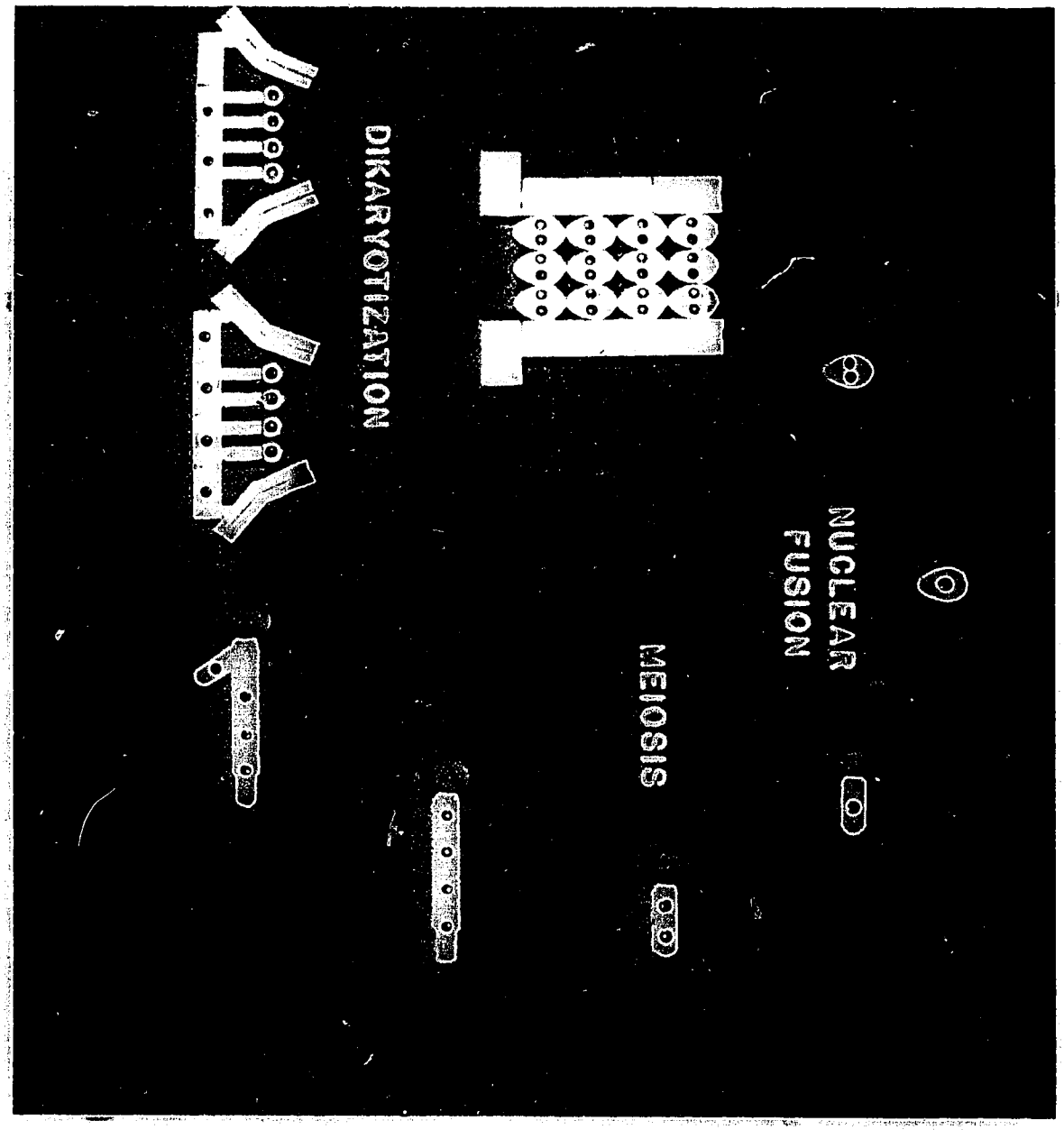

DIKARYOTIZATION
MEIOSIS
NUCLEAR FUSION

Table II-1. Results of 1990-1991 inoculation experiments with selected families (original and half-sib from genetic plantations, total 216) based on index values recorded in April 1991. Half-sib from original stored seeds and half-sib from plantation were seeded in 1990. Thirty seedlings from field collected seeds or half-sibs for 21 families were inoculated with spores from Manitoba. Based on the final assessment, the family M423 was found to be the most resistant, while M116 is highly resistant. M154 is the most susceptible, while M354 is highly susceptible.

Field Ranking	Family Number	Index Value		
		Original Seed	Half Sibs	Average
2	M423	0.20	0.57	0.38
15	M116	0.81	0.70	0.76
213	M354	1.73	1.22	1.47
216	M154	0.67	1.10	0.88

Table II-2. Disease ratings of four jack pine families 1, 3, and 5 months after inoculation with western gall rust (Myrholm and Hiratsuka 1993). Jack pine seedlings from four families of known relative resistance/ susceptibility to western gall rust were inoculated at 6, 8 and 10 weeks with the "brush" and "corn needle" methods. The symptoms were examined at 1, 3, and 5 months.

Method	Inoculation age (weeks)	Seedlings evaluated* after 1 month % rated +	3 months Index Value	3 months % rated ≥3	5 months Index Value	5 months % rated ≥3
Family M423						
Brush	All	8.9	0.04	0.0	0.07	0.0
Torn-needle	All	23.3	0.26	0.0	0.30	0.0
Family M212						
Brush	All	3.6	0.08	0.0	0.19	1.6
Torn-needle	All	21.3	0.52	2.5	0.92	9.9
Family M354						
Brush	All	14.9	0.20	0.0	0.65	9.1
Torn-needle	All	38.9	0.95	4.7	1.76	26.3
Family M154						
Brush	All	4.5	0.31	1.7	0.85	18.5
Torn-needle	All	42.0	1.39	29.4	2.28	62.1

*Disease evaluation:

+ = seedlings showing evidence of infection;

index value = mean of disease ratings (0-5);

≥3 = seedlings showing gall information

Chapter III

Identification and Potential Application of RAPD Markers Linked to Western Gall Rust Resistance Genes in Jack Pine

INTRODUCTION

Western gall rust (WGR), caused by Endocronartium harknessii (J. P. Moore) Y. Hiratsuka, is a major pine disease known to cause high mortality in jack pine (Pinus banksiana Lamb.) and other hard pines in Canada (Hiratsuka and Powell 1976). The fungus is an endocyclic, autoecious rust, possessing only one spore state and no alternate host. Therefore, efforts to control the disease must focus on the pine host (Allen et al. 1990). Rational development of effective, environmentally acceptable control and management strategies of western gall rust is being progressively investigated for successful cultivation of pines. In recent years, marker-aided selection has become an efficient method for the improvement of major-gene disease and insect pest resistance in crop plants (Melchinger 1990). A recent modification of PCR that rapidly generates and screens random DNA segments for polymorphisms between different genotypes has resulted in a type of molecular marker, the random amplified polymorphic DNA (RAPD) marker (Welsh and McClelland 1990; Williams et al. 1990). Polymorphic DNAs are detected as unique bands in agarose gels and can be screened for polymorphisms. Further, the DNA from segregating populations of near isogenic material can be bulked (DNA bulked segregant analysis strategy) to expedite selection and analysis (Michelmore et al. 1991). The objective of this research is to use molecular analysis by RAPDs to identify host (jack pine) loci involved in WGR resistance. Identification of a tightly linked RAPD markers for the broadly effective resistance of jack pine will enable the application of molecular genetic techniques to applied breeding efforts. The fundamental advantage of tightly linked markers for such resistance genes is the opportunity to

efficiently screen genotypes without reliance on test inoculation with the pathogen.

A program to develop genetically improved jack pine for Manitoba and Saskatchewan was initiated in 1967 by the Forestry Branch, Canada Department of Forestry and Rural Development, predecessor of Dr. J.I. Klein of Canadian Forest Service. Family tests consisting of replicated plantations of open-pollinated families were established in three areas in 1972, 1974, and 1976 to provide genetic quality information and breeding materials (Klein 1982). the test established in 1972 consisted of four plantations in eastern Manitoba. each plantation had the same 209 open-pollinated families derived from wild parent trees selected in eastern Manitoba south of 51°15'N, six families from trees selected in Saskatchewan, and one bulked control lot from southeastern Manitoba. Field survey results from the family test plantations and results of greenhouse inoculation of first-cousin families show a wide range of family means, indicating the possibility of substantial genetic variation and the apparent effectiveness of assessment procedures used in these trials (Klein *et al.* 1991).

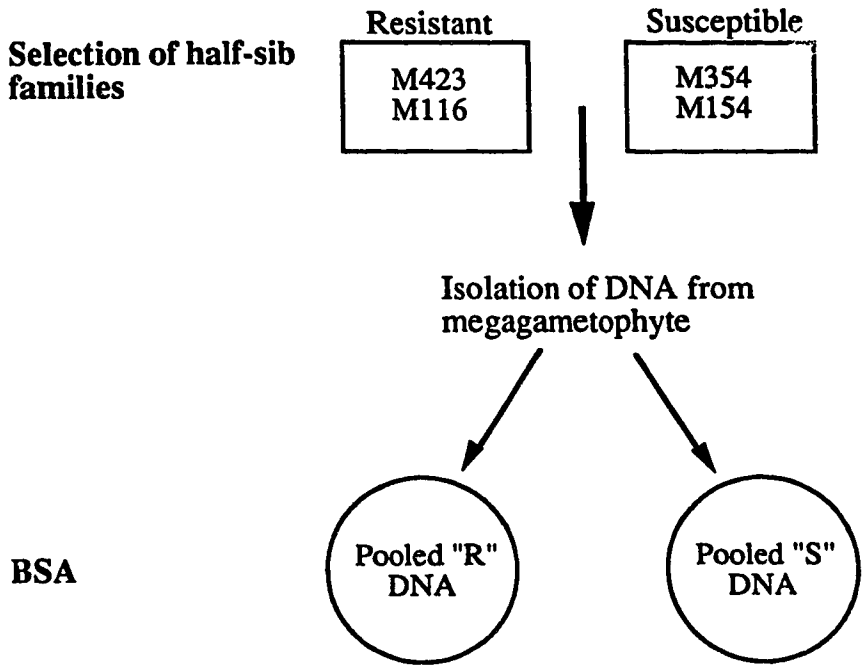
Half-sib approaches to quantitative trait dissections using molecular markers have been proposed for animal genetics (Dodds et al. 1992). A similar approach can be used in conifers by analyzing the haploid genotype of the female gametophyte. Four half-sib families was selected based on previous field testing and greenhouse examination: 2 R families (family M423 is the most resistant, family M116 is highly resistant), 2 S families (family M154 is susceptible, family M354 is highly susceptible).

BSA was used in this study because it is a efficient method that could link RAPD markers with a trait without constructing a whole map (Michelmore et al. 1991). The polymorphic DNA fragments generated were detected as unique bands that only appear in the resistant bulked sample and not in susceptible bulked sample on the ethidium bromide agarose gels. By using BSA, RAPD markers were obtained. Those that detected variation between the bulked genotypes were used to score for segregation of the polymorphic band with 20 individual seeds of each of four families. BSA was then carried out using megagametophytes isolated directly from young seedlings. The seedlings were grown in the greenhouse for inoculation.

MATERIALS AND METHODS

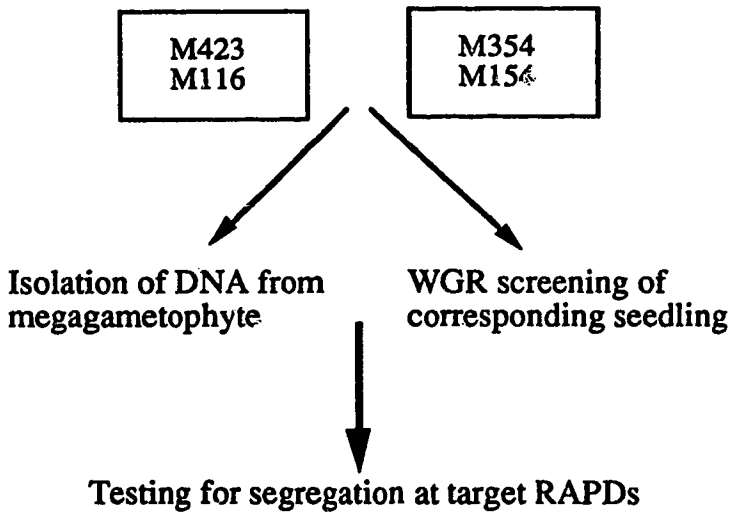
The strategy diagram

PHASE I



Identification of RAPDs

PHASE II



Phase I

Selection of half-sib families

Seeds from the Eastern Breeding District in Manitoba were collected from open-pollinated cones. Jack pine seeds for this study were from four half-sib families that were highly resistant and highly susceptible based on the preliminary segregation data.

In 1988, 5678 trees from 216 family lots were planted in Manitoba in 4 locations and each tree was examined individually for the presence and number of WGR infections (Nanka 1988; Hiratsuka et al. 1990, Klein et al. 1991). The average numbers of galls per tree for families ranged from 0 (M542, M423, and M152) to 3.37 (M354). The mean was 0.81 galls per tree. Percentages of infected trees for all four families; ranged from 0% (M542, M423, M152), to 79.2% (M154). On average, 24.7% of the trees in a family were infected. The genetic differences in the degree of resistance to western gall rust within jack pine populations in the " Eastern Breeding District" (south eastern Manitoba) are significant (Hiratsuka et al. 1995). This preliminary data strongly suggested the genetic variability in resistance to WGR exist among jack pine in Manitoba. Based on preliminary data of field survey, four half-sib families were chosen from among the polycross families available on the basis of large within family phenotypic variance. The jack pine seeds were collected from four half-sib families: 2 R (family M432 is resistant and family M116 is highly resistant), 2 S (family M154 is susceptible and family M354 is highly susceptible).

Isolation of DNA from megagametophyte of seed

Since it is not necessary for the extracted DNA to be high molecular weight in order to produce moderate-sized PCR products, the rapid procedure of Doyle and Doyle (1990) was used for the isolation of total DNA from a single megagametophyte. The seeds were placed on wet Watman paper and imbibed in distilled water overnight at room temperature. The seed coat and embryo were dissected from the seeds. DNA was extracted immediately from the remaining megagametophytic tissue. The procedure of extracting genomic DNA involves disruption of the megagametophyte to release the cellular constituents into the extraction buffer, and protection of the DNA from the activity of endogenous nucleases. The isolated megagametophytic tissue was ground in a microcentrifuge tube containing 150 µl extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 4% SDS, 0.1% ß-mercaptoethanol) using a motorized pestle grinder to homogenate, mixed by inversion and incubated at 80°C for 20 minutes. The homogenate was extracted with one volume phenol: chloroform: isoamyl alcohol (24:24:1) and the phases separated in a microcentrifuge run at maximum speed for 10 minutes. A second extraction with one volume chloroform: isoamyl alcohol (24:1) was then performed. DNA was precipitated by addition of 0.1 volumes 3M sodium acetate and 2.5 volumes ice cold 95% ethanol, and then incubated at -20°C for a minimum of 1 hour up to overnight. The DNA was pelleted by microcentrifugation at full speed for 15 minutes at 4°C, washed with 1 ml of 80% ethanol and dried briefly. The DNA pellet was resuspended in 1 X TE (10mM Tris-HCl, pH8.0, 0.1mM EDTA) to which DNase-free RNase was added to a final concentration of approximately 16-20 ng/µl, to a final volume of 50 µl.

Bulk segregate analysis (BSA)

Four families were divided into two pools (R and S) to make bulked genomic DNA samples. Each DNA sample contained 20 pooled DNA. Two sets of primers containing 180 random primers in total (#1-#100 and #321-#400) were screened. After primer prescreening, the selected primers were used to amplify DNA from 20 members of each family individually.

Another BSA was performed based on the seedling performance of western gall rust fungus infection in the greenhouse. One bulked DNA sample contained 20 individual DNAs corresponding to the seedlings in resistant families ("R"), and the other bulked DNA samples contained 20 individual DNAs corresponding to the seedlings with gall development in susceptible families ("S"). The BSA was carried out using megagametophyte corresponding to the final scoring of seedlings with different phenotype of WGR resistance. Two bulked samples "BR" and "BS" were run with the selected primer as above.

Identification of RAPDs linked to WGR

- Primers: The 10-meroligonucleotides decamers were synthesized at the University of British Columbia and are commercially available. The sequence of each primer was arbitrary and generated on a random basis within the constraints of a G + C content between 50 and 80%, and no palindromic sequences.

- DNA concentration and amplification: The initial concentration of DNA isolated from tissue was estimated by the size of the ethanol precipitate. Each pellet was dissolved in an appropriate amount of TE buffer to ensure

that the final DNA concentration of each sample was approximately equal. Then the final DNA concentration was determined by electrophoresis by loading 5 µl DNA solution from 50 µl on an 0.8% agarose gel (100 ml 1 x TBE buffer contains 0.8 gram agarose powder). The working range of template DNA concentration for obtaining identical RAPD phenotypes was between 5 - 10 ng.

The optimized PCR conditions for megagametophyte DNA was a modification of the procedure reported by Williams *et al.*, (1990). Amplification reactions were performed in 25 µl containing 10 mM Tris-HCl pH8, 2.3 mM $MgCl_2$, 200 µM dNTPs, and 0.3 µM random primer, 6 ng template DNA (in 5 µl TE) and 0.75 units of Taq polymerase (Boehringer Mannheim), overlaid with one drop (15 µl) of mineral oil. The master mix was made with common PCR components and then aliquoted to individual tubes containing the different template DNA. The tubes were mixed by tapping and microcentrifuged. Amplification was carried out using an thermo-cycler, programmed for 45 cycles with an initial denaturation step of 4 minutes at 94°C, then denaturation for one minute at 94°C, primer annealing for 1 minute at 36°C and primer extension for 2 minutes at 72°C. Amplification ended with a final incubation at 72°C for 10 minutes followed by a 4°C soak until recovery.

- RAPD product analysis: The PCR products were separated by loading 25 µl onto 1.4% agarose gel (100 ml of 1 x TBE buffer contains 1.4 gram of agarose powder, 40 ml in total volume for 12 x 6 cm^2 gel tray) in 1 x TBE running buffer. Ethidium bromide (10 mg/ml) was added to a final concentration of 50 µg/ml after cooling the melted agarose. The PCR

amplification products can be viewed on a UV transilluminator and photographed.

- Statistical analysis: Goodness-of-fit to a 1:1 Mendelian ration of segregating marker was tested by Chi square analysis at the 0.05 significance level. The chi-square goodness-of-fit test is used to analyze the approximate probability of obtaining deviations from expectancies based on previously known facts or principles. The formula for chi-square is

$$\chi^2 = \sum \frac{(Ob - Ex)^2}{Ex}$$

Ob is the observed value for each of two or more classes, and *Ex* is the corresponding expected value.

Phase II

Isolation of DNA from megagametophyte of seedling

In the experiment of progeny testing, megagametophytes had to be frozen prior to DNA extraction. The seeds were germinated in vermiculite without stratifying. The megagametophyte were isolated from the young seedlings after emergence from vermiculite and frozen at -80°C. The optimum stage for removing the megagametophyte from the seedlings was 10-14 days after sowing (Fig. III-6.). The best survival and least damage was obtained when the germinants were dissected by hand (no tool). Care was needed to avoid desiccation of the germinants during the dissecting procedure. The seedlings were watered as needed to keep moist. The extraction procedure is same as described above.

WGR screening of corresponding seedling

- Seedlings: Jack pine seeds were planted in Fives Rootrainers (Spencer-Lemaire, Ltd) containing peat moss. Four Rootrainer trays containing about 60 seed cavities each (55 cm^3/cavity) were seeded for each of the four families. Seedlings were grown in a greenhouse at 18-24°C under natural light and fertilized every two weeks with N-P-K (Carlson 1983). It has been concluded that seedling inoculation age didn't significantly affect infection or symptom development (Myrholm and Hiratsuka 1993; Blenis and Hiratsuka 1986). The inoculation was done at seedling age of 6-7 weeks.

- Spores: WGR spore were collected from areas in Manitoba where the original parent tree families were selected. Mature galls were air dried and stored at - 70°C until used. The spores were scraped off from the gall surface, passed through a sieve, and placed into glass vials. Before seedling inoculation, spore germination after 24 hours on 0.2% water agar at 25°C was examined. 50-90% germination rate was needed to inoculate successfully.

- Inoculation: The inoculation method described as the torn needle method is more efficient and reliable than the brush method (Myrholm and Hiratsuka 1993) (Table. II-2). It has been reported that seedlings inoculated with the torn-needle method show a greater percentage of infection and a more rapid progression of symptoms. The torn-needle method also preserves the relative order of resistance of families and shows greater sensitivity to differences in family resistance than the brush method (Blenis and Hiratsuka 1986). The inoculation procedure involves removing a single needle with a

downward pull and then applying the dry spores directly to the small scar with a fine paint brush immediately after the needle is removed. Inoculated seedlings were covered with wet paper towels to maintain high humidity for spore germination and the whole tray was wrapped in a black garbage bag to reduce the light. After 24 hours at approximately 20°C the seedlings were uncovered and the growing conditions returned to the regular growth conditions. Seedlings were examined carefully for the early symptoms at 1, 2, and 3 months after inoculation.

Testing for segregation at target RAPDs

Ten of seedlings without galls from resistant families and ten of seedlings with galls on them from susceptible families were selected and their megagametophyte DNAs were run PCR with primer #382. The correlation between the seedling performance and RAPD marker was tested.

RESULTS AND DISCUSSION

DNA yields of megagametophyte isolated from seeds and young seedlings

The extraction of DNA from megagametophyte tissue was a modification of a protocol following Doyle and Doyle (1990) for extraction of DNA from plant tissue. DNA yields per megagametophyte of jack pine seeds varied from 800 ng - 1200 ng (a single megagametophyte weighs ~ 2 mg). It was possible to run approximately 100 reactions per megagametophyte source. Extraction with phenol / chloroform improved the yield and purity of the genomic DNA (Fig. III-1.a.).

The amount of DNA extracted from the megagametophyte isolated from seedlings is approximately three times less than from the megagametophyte removed from the seed (Fig. III-1.b.). This could be caused by the presence of cellular components that interfere with extraction (phenolics & carbohydrates), or could reflect catabolism of the megagametophyte during seedling growth.

Identification of RAPD markers linked to western gall rust resistance

The generation and resolution of DNA amplification will be affected by many factors, such as the purity of the template DNA, and concentration of primer, magnesium, and deoxynucleotide triphosphate. Since the PCR process requires very small amounts of template DNA, the quality of isolated genomic DNA is more important than the quantity. DNA isolated with impurities such as polysaccharides or phenolics will inhibit the amplification reaction. Template DNA amounts between 3 to 20 ng will provide nearly identical RAPD products. The concentration of $MgCl_2$ in the reaction mix is relatively easy to control and should not represent a major source of variability if kept constant. Specificity can sometimes be achieved by lowering $MgCl_2$ concentrations below 1.5 mM. The amplification processes, conditions and cycle parameters can be modified with a wide range of reaction components for different plant species without seriously affecting the reproducibility of the technique.

Under constant conditions of amplification, 56 of 180 primers revealed polymorphisms in the primer prescreen, 17 of which revealed segregating bands among the two bulked samples, 3 primers generated polymorphic

fragments that were apparently linked to susceptibility [UBC-333 (-GAATGCGACG-), UBC-376 (-CAGGACATCCT-) and UBC-386 (-TGTAAGCTCG-).]. For the remaining 14 that segregated with resistance, 13 were transient, and one was stable and highly repeatable. These RAPDs were verified on the four segregating families with 20 individual megagametophytic DNAs per family. Again, one of the primers, UBC-382 (Fig. III-5), generated a polymorphic DNA fragment that was present in the R bulked group but absent in the S bulked group (Fig. III-2.). A 214 bp fragment generated by primer UBC-382 was reconfirmed against 20 individual megagametophytic DNAs from each of four families (Fig. III-3, III-4). The data for primer UBC-382 are summarized in Table III-1.

Seedling performance after inoculation

One month after seedling inoculation, the stem of inoculated seedlings showed red staining around the needle scar. The red stain faded away after several weeks in some seedlings and became darker in others. After three months, seedlings in family M154 and M354 began developing galls (Fig. III-7.). The percentage of seedlings in each family with early symptoms and galls 1, 2 and 3 months after inoculation is shown in Table III-2. The relative resistance in four jack pine families determined by this experiment corresponded with that of previous field ranking and greenhouse examination (M423 > M116 > M154 > M354).

RAPD marker and greenhouse examination

The DNA polymorphism between the two bulked DNA groups corresponded to the phenotypic variation in gall formation in the progeny (Fig. III-8.). The extra polymorphic fragment generated by primer UBC-382 was present in the "R" bulked DNA sample that consist of 20 individual megagametophyte isolated directly from 20 individual seedlings of two resistance families but not in the "S" bulked sample (the rate of gall formation on seedlings is: M423 - 0%; M116 - 3%; M154 - 25%; M354 - 28%). In order to verify this RAPD marker is heterozygous from open-pollinate allels, ten of resistant seedlings and ten of susceptible seedlings (with galls produced on them) were run PCR with primer UBC-382. The data (Table III-3) showed the expected correlation between the RAPD marker and progeny phenotype and both resistance families are heterozygous.

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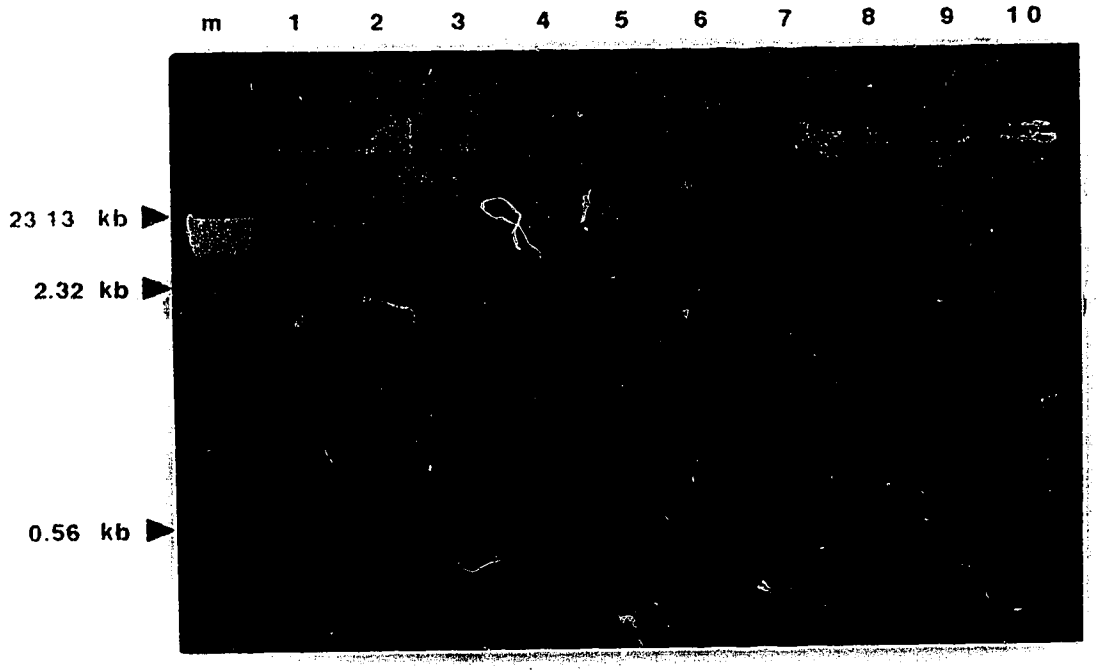
FIGURES AND LEGENDS

Fig. III-1.a. Genomic DNA extracted from megagametophyte of seed. DNA was extracted from megagametophyte of initiated seed. 5 µl from 50 µl genomic DNA solution in TE buffer was loaded on 0.8% agarose gel (30 ml of gel solution for mini-gel size) in 1 x TBE running buffer and electrophoresises at 60 volts for 45 minutes.

Fig. III-1.b. Genomic DNA extracted from megagametophyte of young seedling. DNA was extracted from megagametophyte that had been dissected from 10-14 days old seedlings. 5 µl from 50 µl genomic DNA solution in TE buffer was loaded on 0.8% agarose gel (30 ml of gel solution for mini-gel size) in 1 x TBE running buffer and electrophoresises at 60 volts for 45 minutes.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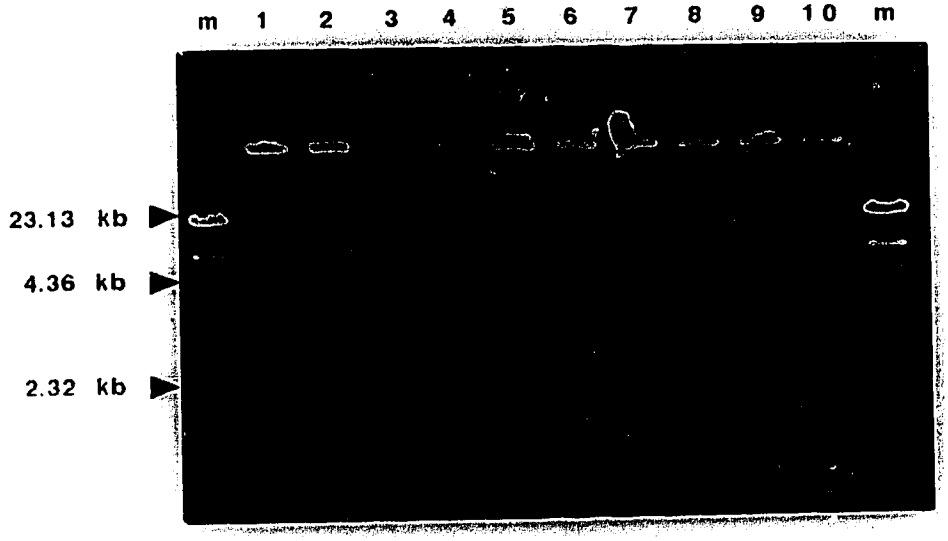
23 13 kb
2.32 kb
0.56 kb

a.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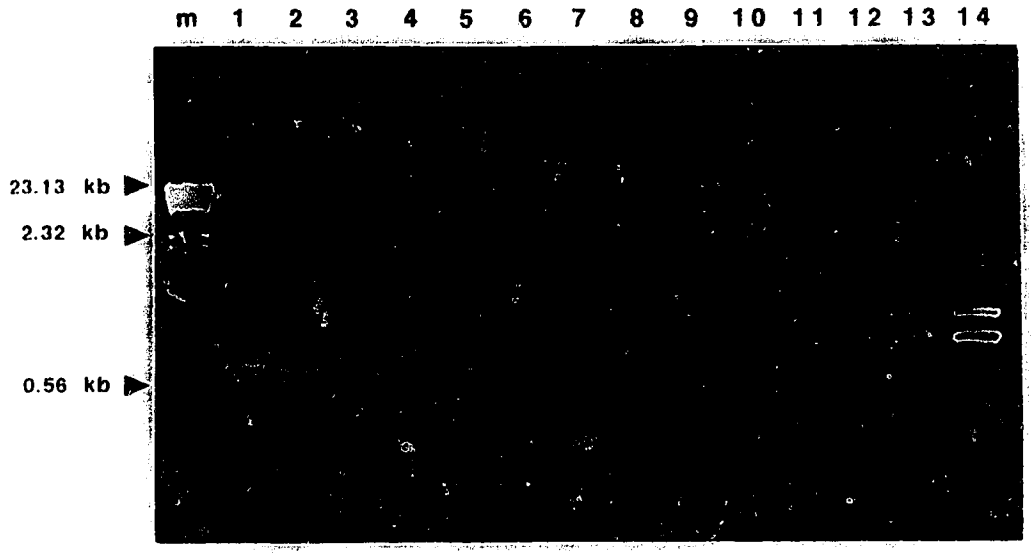
2.32 kb

b.

Fig. III-2. Amplification of genomic DNA from two bulked samples using primer UBC-329, -380, -382, -385, -386, -388, -389. Each set of 2 lanes results from PCR amplification with a different 10-mer oligonulceotide primers. In each set, the first lane contains bulked DNA from 20 homozygous susceptible individuals (BS), and the second lane contains bulked DNA from 20 homozygous resistant individuals (BR). Primer UBC-382 generated the polymorphisms distinguishing the bulks.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2.32 kb
0.56 kb

Fig. III-3. Ethidium bromide stained agarose gel of RAPD products from 40 megagametophyte DNAs of two susceptible families (M154 & M354) using primer UBC-382. Fragments were separated on a 1.4% agarose gel containing ethdium bromide (10 mg/ml) by electrophoresis in 1 x TBE running buffer for 1 hour at 100 volts and viewed on a UV transilluminator. The upper 1 - 20 lanes are RAPD pattern of megagametophyte from family M154 and the lower of 1 - 20 are from family M354. The ¢ X 174/ Hae III markers are shown at the extreme left and right lane of each panel (m).

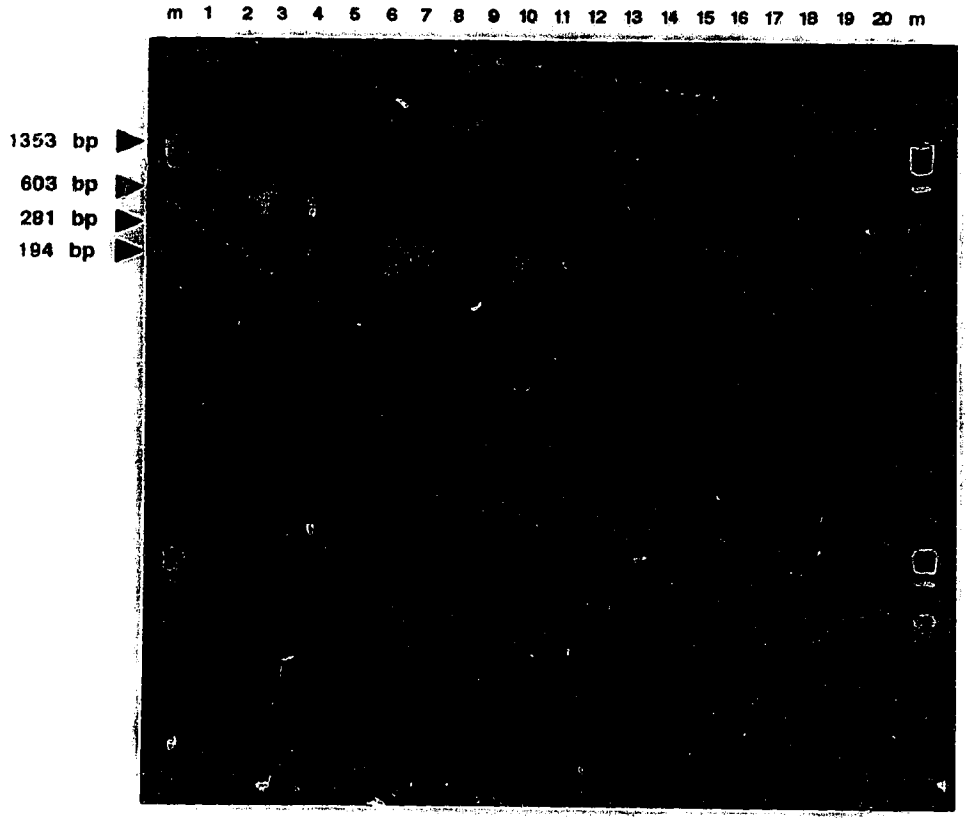

m 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 m
1353 bp
603 bp
281 bp
194 bp

Fig. III-4. Ethidium bromide stained agarose gel of RAPD products from 40 megagametophyte DNAs of two resistant families (M423 & M116) using primer UBC-382. Fragments were separated on a 1.4% agarose gel containing ethdium bromide (10 mg/ml) by electrophoresis in 1 x TBE running buffer for 1 hour at 100 volts and viewed on a UV transilluminator. The upper 1 - 20 lanes are RAPD pattern of megagametophyte from family M423. The lower of 1 - 20 lanes are from family M116. The 214 bp segregating band is indicated by a solid arrowhead. The ¢ X 174/ *Hae* III markers are shown at the extreme left and right lane of each panel (m).

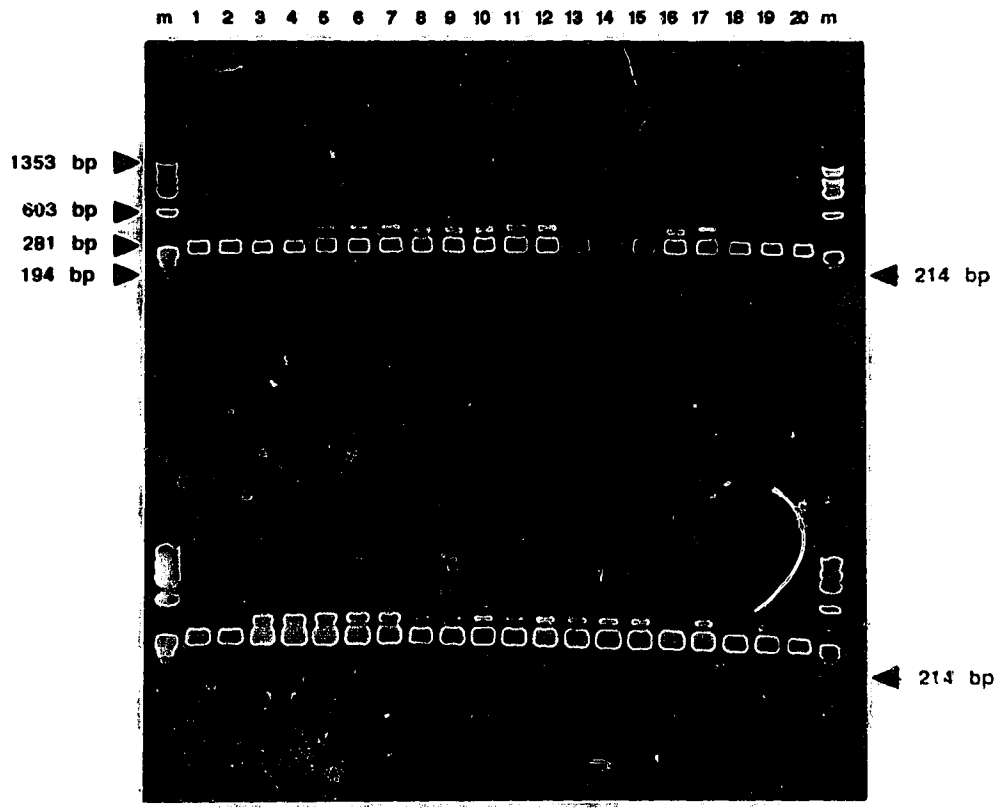

m 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 m
1353 bp
603 bp
281 bp
194 bp
214 bp
214 bp

Table III-1. Summary of RAPD marker data. Chi-square analysis for goodness of fit to a 1:1 of segregating PCR band in four jack pine families. 20 samples of each "R" family were expected to have 10:10 (present : absent) ratio. The observed data were tested by Chi square analysis at the 0.05 significant level.

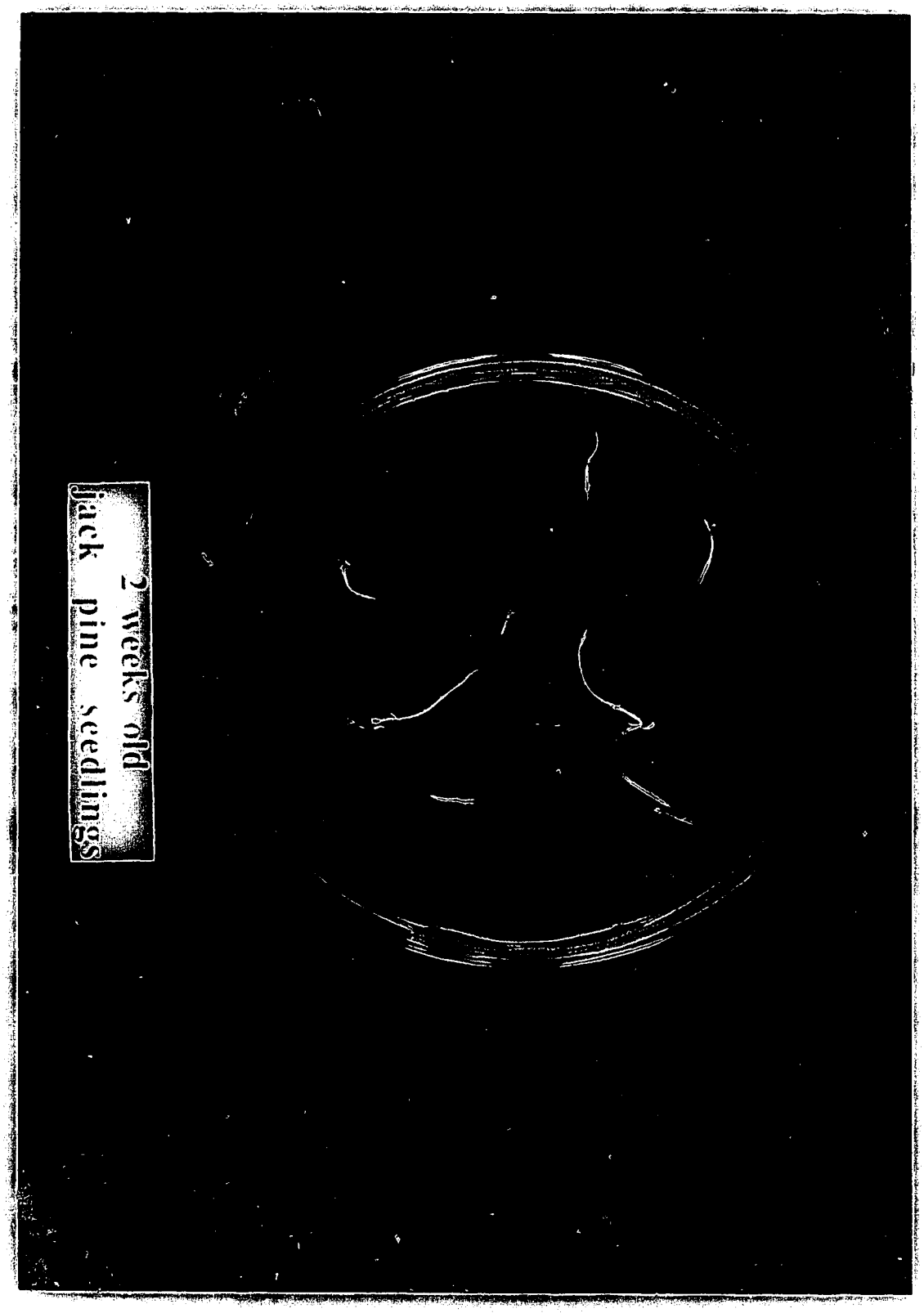
		Band present		Band absent		Chi
Family	Total	observed	expected	observed	expected	square
M423	20	12	10	8	10	0.8
M116	20	14	10	6	10	3.2
M354	20	0	0	20	20	
M154	20	0	0	20	20	

The value of Chi-square statistic for a = .05 and df = 1 is 3.841.

Fig. III-5. Nucleotide sequence of primer UBC-382. Artificially synthesized by the Biotechnology laboratory at the University of British Columbia.

5' - **ATACACCAGC** - 3'

Fig. III-6. 2-week-old seedlings with megagametophyte on the tip. The optimum developmental stage for isolating the megagametophyte from seedling is approximately 10-14 days after sowing.



2 weeks old
jack pine seedlings

Fig. III-7. 4-month-old seedlings of M154 and M354 with gall developed on the stem. The seedlings were examined four months after inoculation.

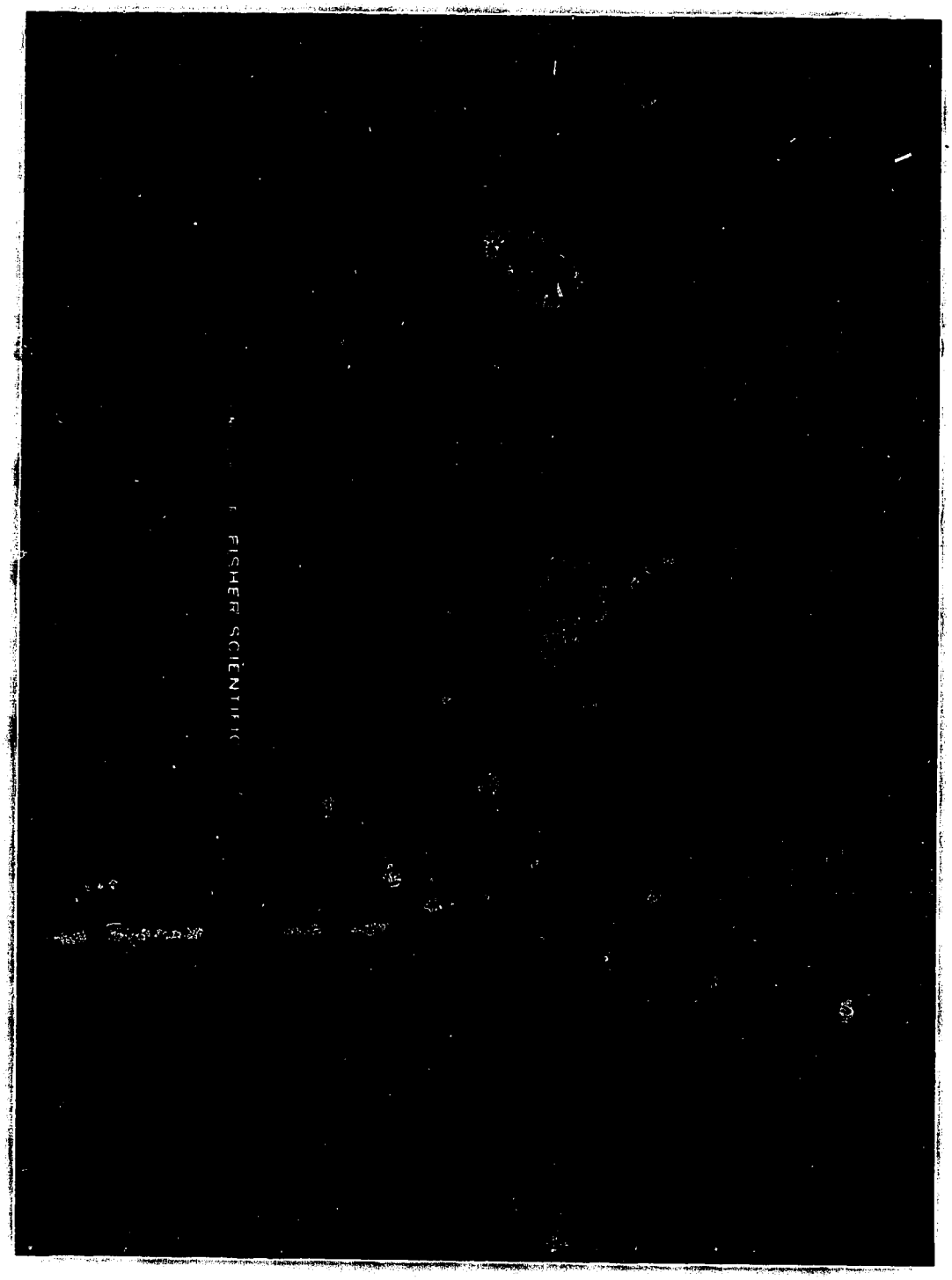

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Table III-2. The percentage of seedlings in four jack pine families with early symptoms and galls 1, 2, and 3 months after inoculation. The seeds of four families (M423, M116, M 354, M154) were planted peat moss and inoculated with the "torn needle" method approximately 7 weeks after sowing. The seedlings were examined for the early symptoms and gall development at 1, 2, and 3 months after inoculation.

Family	Total number of seedlings	months since inoculation	Symptom type		
			Red lesion	Swelling	Gall
M154	20	1	60%	0%	0%
		2	0%	50%	0%
		3	0%	35%	25%
M354	25	1	52%	0%	0%
		2	0%	36%	0%
		3	0%	48%	28%
M116	33	1	42.4%	0%	0%
		2	0%	15.2%	0%
		3	0%	6%	3%
M423	36	1	22.2%	0%	0%
		2	0%	5.6%	0%
		3	0%	5.6%	0%

Fig. III-8. RAPD marker detecting polymorphisms between two bulks made from megagametophyte DNAs of young seedlings. The first three lanes after the marker are repetitions of the bulked DNA from the 20 homozygous resistant individuals of two R families. Lanes 4, 5 and 6 are repetitions of the bulked DNA from the 20 homozygous susceptible individuals of two S families. The segregating band is indicated by a solid arrowhead.

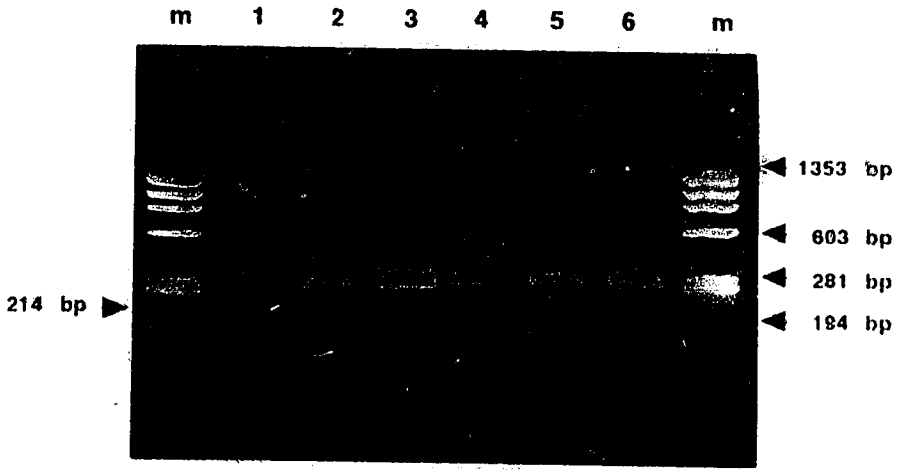
m
1
2
3
4
5
6
m
1353 bp
603 bp
281 bp
184 bp
214 bp

Table III-3. The megagametophyte RAPD haploid type and seedling phenotype. The megagametophytes corresponding with ten seedlings without galls from resistant families and ten seedlings with galls from susceptible families were selected to run PCR with primer UBC-382.

	Resistant seedling	Susceptible seedling
Seedling number	1, 2, 3, 4, 5, 6, 7, 8, 9, 10.	1, 2, 3, 4, 5, 6, 7, 8, 9, 10.
Seedling phenotype	R, R, R, R, R, R, R, R, R, R.	S, S, S, S, S, S, S, S, S, S.
Megagametophyte	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10
RAPD haploid type	- - + + - + - - + +	- - - - - - - - - -

Chapter IV

General Discussion and Future Perspective

GENERAL DISCUSSION

Western gall rust (WGR) is an important fungal disease of the hard pines in North America (Allen et al. 1990). Reports of damage in immature stands by this disease have been frequent (Hiratsuka and Powell 1976), resulting in great economic loss in growth vigor and wood value. Efforts to control WGR fungal infection have focused on either containing the fungus (Tsuneda and Hiratsuka 1979; Van Der Kemp and Spence 1987) or increasing genetic resistance in the host (Yanchuk et al. 1988). However, as management of pine plantations becomes intensive, effective manipulation of both fungus and hosts must be considered the prime objective.

Pines that confer resistance to WGR fungal infection have been observed both in the greenhouse and in the plantations. However, these studies were based on early morphological symptoms that could have poor correlation with the final gall formation (Kojwang and Van Der Kemp 1992). In addition, the problem of obtaining genetically defined patbogen and host populations have prohibited the use of routine genetic analysis that are common in crop plants. Consequently, the genetic basis for resistance in pines to WGR fungal infection remains largely unknown despite many years of continuous research. Data from both greenhouse and field studies were consistent with differing hypothesis regarding the mode of inheritance of resistance loci (Hoff 1985; Yanchuk et al. 1988).

The use of molecular markers such as RAPDs will enable us to identify, locate and study the mode of inheritance of target genes, and help tree breeders select seedlings with combinations of desirable genes (Yazdani et al. 1995). This type of plant selection, known as marker assisted selection

(MAS), has the potential to become one of the most useful tree breeding tools ever developed. The focus of my research was to identify RAPDs closely linked to gene(s) that confer resistnace to WGR fungal infection in jack pine. I selected four open-pollinated families, two resistant (Families M423 and M116) and two susceptible (Families M154 and M354) to WGR fungal infection in earlier greenhouse and field studies. A total of 180 RAPD primers was screened megagametophyte bulks (Michelmore et al. 1991) from the resistant and susceptible families in order to detect markers putatively associated with resistnace that were inherited from the maternal parent only. One candidate marker (UBC-382) exhibiting a large effect resistant gene was polymorphic, present in hte resistant bulk but absent in the susceptible bulk. Segregation analysis of megagameotphyte confirmed that the two susceptible families were homozygotes for the null while the two resistant families were heterozygotes at marker UBC-382 (Table III-1.).

To validate the hypothesis that a large effect resistance gene was segregating, I assessed for the presence/absence of this marker in megagametophyte and corresponding seedling that I screened for resistance to WGR fungal infection in the greenhouse. The degree of association was high (Table III-1) indicating that the probability of chance association was low. The magnitude of association between WGR resistance/susceptible phenotype and the marker is indicative of a large effect dominant resistance factor: of trees that were either homozygous or heterozygous for the marker, approximately 100% were gall free, and similarly, of trees without the UBC-382 marker (i.e., homozygous for the null), almost 50% had gall formation. These results suggest that marker UBC-382 is tightly linked to resistnace to

WGR fungal infection that might involve a gene of large effect, suggesting that the predominant architecture of resistance is more likely to be oligogenic than polygenic. This qualitative resistance to WGR fungal infection could be the result of a completely penetrant resistnat gene(s) with pathotype specificity such as gene-for-gene interaction (Thompson and Burdon 1992) or a threshold phenomenon due to the accumulation of incompletely penetrant gene(s) of large effect. A major dominant gene for resistance to Cronartium ribicola in Pinus lambertiana was also rapidly mapped using RAPDs and bulked segregant analysis (Devey 1994).

Deployment of marker UBC-382 could be accommodated as part of an operational breeding program in jack pine. Individuals carrying this marker could be selected and deployed in breeding and production populations. However, the long-term goal of genetic improvement in jack pine would be to confer broader resistance to WGR fungal resistance in the breeding and production population. Thus, it is necessary to identify other genes that confer resistance to WGR fungal infection using RAPDs and bulked segregant analysis to allow for the deployment of mixes of genotypes with different resistant genes. This would buffer from pathogen virulence and lead to more durable resistance by slowing the rate of increase in disease incidence.

To conclude, our investigation of identifying a RAPD marker linked to WGR resistance, suggests several applications with conifers: 1) useful for early selection in jack pine families; 2) providing the valuable information for genetic linkage mapping and genetic diversity studies; 3) generation of hybridization probes from total DNA for RFLP analysis; 4) generation of

single copy RAPD markers from cDNA; 5) determining the degree of heterozygosity within individual parent trees by amplifying DNA from single gametes. 6) demonstrating the feasibility of indirect selection at the seed stage and makes breeding options plausible for long-lived pine species.

FUTURE PERSPECTIVES

The future direction of WGR research will be establishment of gene location on a genetic map in pine by genetic linkage study. The quantitative trait loci of WGR resistance can be tracked by the half-sib approach coupled with BSA of megagametophytes and RAPD markers. The RAPD molecular markers available for WGR resistance from megagametophyte tissue can also be used to determine the exact contribution of the gametes. RAPD markers that are polymorphic can be also used as hybridization probes for cloning and expression study of WGR resistance genes.

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