Genetic analysis and molecular characterization of RFLP DNA markers in barley (Hordeum Vulgare L.)
by Jeong Sheop Shin

A thesis submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Crop and Soil Science
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Abstract:
Single or low copy number DNA clones from random genomic DNA libraries using the plasmid vector pBR322 and the phage EMBL4 were constructed using DNA from barley (Hordeum vulgare L.). This work was done to provide a relatively large number of genetic markers and to characterize the level of genetic variation in the barley genome. Selected genomic clones and cDNA clones were used to probe the barley genome for the presence of restriction fragment length polymorphisms (RFLPs). This methodology is based upon fragment size differences of defined length that are produced when DNA is cleaved by restriction endonucleases. A multiple recessive marker stock and a relatively distantly related cultivar 'Apex' were selected as parents in a cross to map the genomic location of seventeen RFLP loci. Nine genomic clones and seven cDNA clones produced clear polymorphisms using at least one restriction endonuclease. The majority of selected genomic clones showed polymorphisms using two or more restriction endonucleases. This suggests that the variation observed among barley lines is due to insertion/deletion or rearrangement events rather than point mutations. Utilizing selected single or low copy clones as probes, it was confirmed that polymorphisms are readily detectable among cultivars of barley.

Seventeen polymorphic DNA sequences were mapped relative to seventeen previously mapped marker loci. Genotypes of 34 loci in 100 mapping lines were characterized and described to simplify the mapping of additional RFLP loci. Twelve of seventeen RFLP loci showed codominant segregation. Four of the five loci which demonstrated dominance were from genomic clones which hybridized to several bands in each lane of the Southern blot. The probes and markers utilized in this mapping project span 680 recombination units of the barley genome, approximately 50 percent of its estimated recombinational length.

Detailed physical maps of fifteen polymorphic DNA fragments that were mapped in barley were developed using several restriction endonucleases. All fifteen DNA clones were well characterized by one or several restriction enzymes. In the Southern blot analysis of double digested genomic DNA probed with one of these clones, one allele was found to contain about 200 base pair inserted sequences compared with an alternate allele. The polymorphic region of this clone was sequenced using dideoxy chain termination reaction.

Polymorphic DNA markers were also utilized to identify barley cultivars. Some cultivars undifferentiated by hordeins were well discriminated using a subset of the DNA markers.

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Jeong Sheop Shin

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date Nov. 30, 1988

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ABSTRACT

Single or low copy number DNA clones from random genomic DNA libraries using the plasmid vector pBR322 and the phage EMBL4 were constructed using DNA from barley (Hordeum vulgare L.). This work was done to provide a relatively large number of genetic markers and to characterize the level of genetic variation in the barley genome. Selected genomic clones and cDNA clones were used to probe the barley genome for the presence of restriction fragment length polymorphisms (RFLPs). This methodology is based upon fragment size differences of defined length that are produced when DNA is cleaved by restriction endonucleases. A multiple recessive marker stock and a relatively distantly related cultivar 'Apex' were selected as parents in a cross to map the genomic location of seventeen RFLP loci. Nine genomic clones and seven cDNA clones produced clear polymorphisms using at least one restriction endonuclease. The majority of selected genomic clones showed polymorphisms using two or more restriction endonucleases. This suggests that the variation observed among barley lines is due to insertion/deletion or rearrangement events rather than point mutations. Utilizing selected single or low copy clones as probes, it was confirmed that polymorphisms are readily detectable among cultivars of barley.

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Detailed physical maps of fifteen polymorphic DNA fragments that were mapped in barley were developed using several restriction endonucleases. All fifteen DNA clones were well characterized by one or several restriction enzymes. In the Southern blot analysis of double digested genomic DNA probed with one of these clones, one allele was found to contain about 200 base pair inserted sequences compared with an alternate allele. The polymorphic region of this clone was sequenced using dideoxy chain termination reaction.

Polymorphic DNA markers were also utilized to identify barley cultivars. Some cultivars undifferentiated by hordeins were well discriminated using a subset of the DNA markers.

CHAPTER 1

INTRODUCTION

The lack of available genetic markers in cutivated genotypes has limited the development of saturated genetic linkage maps in plant species. Analysis of restriction fragment length polymorphisms (RFLPs) will provide a relatively unlimited number of genetic markers and permits the construction of detailed genetic linkage maps in eukaryotic species. The studies reported here focussed on the recombinational location of selected RFLP DNA markers relative to previously mapped marker loci in the nuclear genome of barley (Hordeum vulgare L.).

The goals of the first part of this investigation were to screen single or low copy number DNA probes selected from random genomic DNA libraries and to identify genomic RFLPs. The objectives of the second part of this study were to utilize the selected barley DNA clones as genetic markers and to locate them in barley chromosomes relative to previously mapped morphological and biochemical markers. In the third part of this study, the genotypes of thirty-four loci in 100 mapping lines were described and their application as a template to simplify the mapping of additional RFLP loci was discussed. Fifteen out of seventeen mapped DNA clones were also characterized by restriction mapping analysis and the polymorphic region of one interested polymorphic probe was sequenced. The objective of the final part of the study was to determine the relative utility of RFLP markers in cultivar identification.

CHAPTER 2

MOLECULAR CLONING AND EVALUATION OF BARLEY GENOMIC LOW COPY NUMBER DNA CLONES AS GENETIC MARKERS

Introduction

Restriction fragment length polymorphism (RFLP) analysis probes specific regions of the genome for the presence of variation at the DNA level (Grodzicker et al., 1974; Botstein et al., 1980). RFLPs were first identified in temperature sensitive mutations of adenoviruses (Grodzicker et al., 1974). This methodology is based on DNA fragment size differences of defined length that are produced by cleavage of DNA with restriction endonucleases and that are identified by Southern (1975) blot analysis. The use of RFLPs was proposed as a new source of genetic markers for the human genome in 1980 (Botstein et al., 1980; Bishop and Skolnick, 1980). These studies demonstrated the basic principle of using random single copy DNA probes to detect DNA sequence polymorphisms among different genotypes. Gusella et al. (1983) identified polymorphic DNA marker loci associated with the mutant allele causing Huntington's disease using this technology.

In basic plant genetics as well as in plant breeding, RFLPs have been suggested as potent tools (Tanksley, 1983; Beckman and Soller, 1983; Burr et al, 1983; Soller and Beckman, 1983; Evola et al., 1986; Helentjaris et al., 1985; Landry and Michelmore, 1987). The promising potential for this technology in plants is based on the practically unlimited amount of variability at the DNA level in their genomes.

Recently RFLPs were utilized to saturate the genetic linkage maps in maize and tomato (Helentjaris et al., 1986), in tomato (Bernatzky and Tanksley, 1986) and in lettuce (Landry et al., 1987).

The objectives of this study were to select single or low copy number DNA clones from barley random genomic DNA libraries using the plasmid vector pBR322 and the phage EMBL4, and to use these clones to identify RFLPs in the barley genome. In order to detect these RFLPs, selected single copy DNA probes were hybridized to Southern blots containing restriction endonuclease-digested barley DNA from a multiple recessive marker stock and the European 2-rowed cultivar 'Apex'.

Materials and Methods

Plant DNA Extraction

Leaf and stem tissues of barley seedlings were freeze-dried in a VirTis freezedryer for 3-4 days. Total plant DNA was extracted from the lyophilized tissue using modifications of the method of Murray and Thompson (1980) suggested by Saghai-Maroof et al. (1984).

Buffers and Abbreviations

Stock solutions and working solutions utilized in this study were prepared as followed:

20 x SSPE : 3.6 M NaCl, 0.2 M sodium phosphate (pH 7.0), 0.2 M EDTA

20 x SSC : 3 M NaCl, 0.3 M trisodium citrate

10 % Blotto : 10 % non-fat powdered milk, 0.2 % sodium azide

5 x TBE (Tris-Borate) Buffer : Tris base 54 g, boric acid 27.5 g, 0.5 M EDTA (pH 8.0) 20 ml, water up to 1 L

Gel loading Dye Solution : 0.25 % bromophenol blue, 0.25 % xylene cyanol, 40 % (w/v) sucrose

10 x Ligation Buffer : 0.66 M Tris-HCl (pH 7.5), 50 mM MgCl , 50 mM dithiothreitol, 10 mM ATP

10 x Nick-translation Buffer : 0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 ug/ml bovine serum albumin (BSA)

Prehybridization Solution (nitrocellulose) : 5 x SSC, 5 x Denhardt's reagent, 20 mM Na-phosphate (pH 6.5), 50 % formamide, 100 ug/ml denatured salmon sperm DNA

Hybridization Solution (nitrocellulose) : 5 x SSC, 1 x Denhardt's reagent, 20 mM Na-phosphate (pH 6.5), 50 % formamide, 100 ug/ml denatured salmon sperm DNA

1 x Denhardt's Solution : 0.02 % Ficoll 400, 0.02 % polyvinyl-pyrrolidone, 0.02 % BSA

Library Construction and Evaluation

Barley genomic DNA libraries were constructed in the phage vector EMBL4 (Frischauf et al., 1983) and the plasmid vector pBR322 using total DNA from the barley cultivars 'Betzes' and 'Traill', respectively. Phage and plasmid clones were randomly selected and amplified using the methods of Maniatis et al. (1982). Plasmid DNA was isolated from E. coli hosts using the mini-prep procedure of Birboim and Doly (1979). Phage clones were randomly picked from the library

and cloned DNAs were prepared following the procedure of Maniatis et al. (1982). Isolated phage DNA was digested with restriction endonucleases, Eco RI, Hind III, Bam HI or Sal I. For plasmid clones, prescreening was first performed by colony hybridization (Grunstein and Hogness, 1975). Selected plasmids were digested with Bam HI and electrophoresed in 0.8 %, 110 x 135 mm horizontal agarose gel using 1 x TBE buffer at 2 V/cm overnight.

Gels were then stained with ethidium bromide and photographed under UV light. Restriction fragments were transferred to either nitrocellulose (Southern, 1975) or Zeta-probe nylon membrane (Reed and Mann, 1985). Nitrocellulose filters were baked at 80 °C in a vacuum oven for 2 hours after transfer was completed. Filters were hybridized with total DNA from 'Betzes' barley which had been radioactively labeled by nick-translation (Rigby et al., 1977). Single and low copy number barley inserts were identified as those which bound low or undetectable amounts of the total barley DNA probe (Figures 1 and 2). Selected phage fragments were subcloned into the plasmid vector pBR322. Selected clones from the plasmid library were utilized directly.

Genomic Blot Preparation

The parents utilized in the mapping study were a multiple recessive marker stock (MMS) developed by Dr. R.W. Wolfe (1984) (discussed in Chapter 3) and the European 2-rowed cultivar 'Apex'. Isolated DNA from these lines was quantified by fluorometry using the DNA specific fluorescent dye Hoechst 33258. Fifteen ug aliquots of DNA were digested with the restriction endonucleases Bam HI, Hind III, Eco

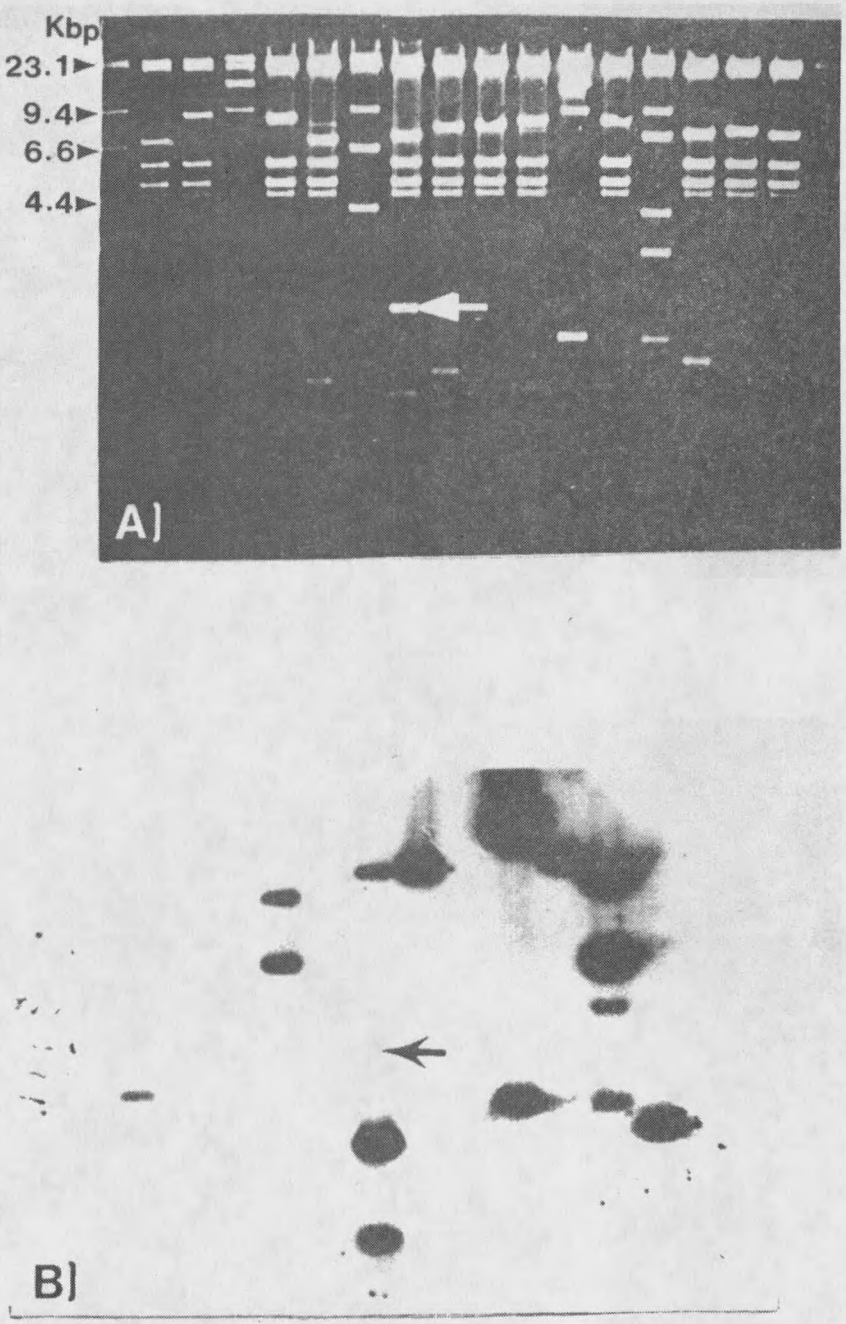
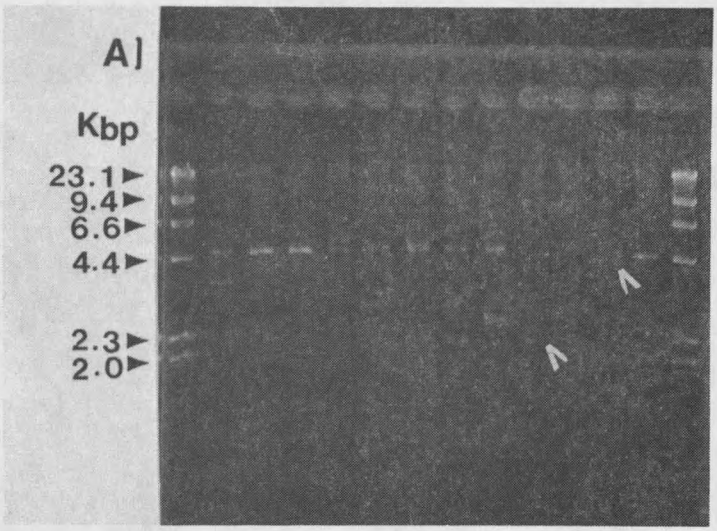


Figure 1. Identification of low copy number genomic clones using EMBL4. A: Gel of 14 EMBL4 clones containing random barley fragments digested with Hind III. B: Autoradiograph of blot of A probed with total nick-translated barley DNA.

Estimated molecular weights in kilobase pairs listed at left. Arrows indicate low copy number fragments tested for identification of polymorphisms between 'Apex' and 'MMS'.



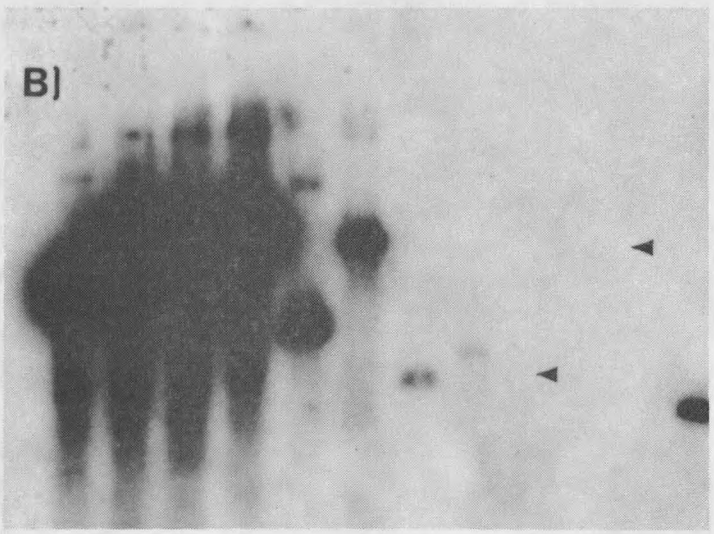


Figure 2. Identification of low copy number genomic clones using a plasmid vector. A: Gel of 12 plasmid vector pBR322 clones containing random barley DNA fragments digested with Bam HI. B: Autoradiograph of blot of A probed with nick-translated total barley genomic DNA.

Estimated molecular weights in kilobase pairs listed at left. Arrows indicate single or low copy number barley DNA fragments.

RI, Eco RV, and Dra I, separated by gel electrophoresis (Figure 3), and transferred to Zeta-probe nylon membrane as indicated above.

Probe Labeling

Approximately 0.1 ug of cloned DNA fragment or total barley DNA was labeled with ^{32}P dNTPs using nick-translation (Rigby et al., 1977). The labeled DNA probes were separated from unincorporated nucleotides using centrifuged Sephadex G-50 1 cc columns. Alternatively, agarose gel slices containing the fragment of interest were labeled by primer extension (Feinberg and Vogelstein, 1984) and utilized without removing the unincorporated nucleotides. Prior to hybridization, the labeled probes were mixed with 0.2 ml of 0.2 N NaOH and denatured by heating to 100°C for 10 minutes.

Hybridization

Nitrocellulose filters were prehybridized and hybridized at 42°C using 50 % formamide according to the method of Spruill et al. (1981). Zeta-probe nylon membrane was prehybridized and hybridized in 15-20 ml of 1.5 x SSPE, 1.0 % SDS and 0.5 % Blotto solution at 68°C in a water incubator with gentle shaking for 4-24 hours (Reed and Mann, 1985). The carrier salmon sperm DNA (5 mg) and radioactive-labeled probe were denatured immediately before adding it to the hybridization solution.

Washing and Autoradiography

Three washes for 15 minutes at room temperature in 300 ml of 2 x SSC/0.1 % SDS, 0.5 x SSC/0.1 % SDS and 0.1 x SSC/0.1 % SDS solutions