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PROJECT TITLE: "DEVELOPMENT OF MOLECULAR GENETIC MARKERS IN *Phaseolus* AS BREEDING TOOLS: DISEASE RESISTANCE"

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INTRODUCTION

The main objective of our program is the construction of a saturation linkage map of DNA markers for *Phaseolus vulgaris*. Three basic steps have been taken to accomplish this objective:

1) We have obtained segregating progenies (BC_1 and F_2) between two distinct genotypes: 'Calima', a cultivar well adapted to Colombia and East Africa (obtained from Dr. W. Roca, CIAT), and 'XR-235-1', a *Xanthomonas campestris* resistant breeding line derived from the interspecific cross *P. vulgaris* x *P. coccineus* (Freytag *et al.*, 1982; seeds were obtained from Dr. M. J. Bassett, University of Florida).

   A backcross (BC_1) progeny ['Calima' x ('Calima' x 'XR-232-1')] of 50 individuals was grown and inoculated with a Florida isolate of *Xanthomonas campestris* pv. *phaseolii*. Inoculations were performed on the first true leaf with an air brush; the inoculum was at a concentration of 10^9 colony forming units per milliliter. The individals were evaluated for their response to the pathogen two and three weeks after inoculations. The results indicated that resistance to the pathogen is a monogenic trait with partial dominance. Leaf samples from these plants were taken and the tissues were immediately frozen in liquid nitrogen. DNA was extracted according to the methodology described below.

2) Genomic and cDNA libraries have been constructed and are being screened for clones that reveal DNA restriction fragment length polymorphisms between the parents. Screening is accomplished by preparing southern blots of parental DNA digests with a number of restriction enzymes, and probing the blots with individual clones from our libraries. This has allowed the identification of restriction enzyme/clone combinations revealing polymorphisms between the parents for 11 out of 22 clones tested so far (see Figure 1).

3) As appropriate enzyme/clone combinations are identified, southern blots are being prepared with digested DNA's from the progeny and probed with appropriate clones.

METHODOLOGY

Most of the the procedure used in this project are well known standard procedures. However, a procedure for the extraction of DNA had to be optimized for bean leaves.

DNA Isolation. After trying different protocols for DNA extraction, we have optimized a procedure that allows extraction of very large molecular weight leaf bean DNA. The procedure is as follows: young bean leaf samples are collected and immediately frozen in liquid nitrogen (-196°C). Samples can be stored at -70° C for and indefinite period of time. Extraction of DNA is started by grinding 10-40 g of sample with liquid nitrogen in a mortar. The

tissue powder is added to 4 volumes of chilled (4°C) extraction buffer (Tris.HCl pH 7.9, 0.1 M / Na_2EDTA 10 mM / Sucrose 0.5 M / B-mercaptoethanol 1%, added prior to use); the sample is further homogenized in a polytron homogenizer for 30-40 seconds at a medium setting. The homogenate is filtered through 4 layers of cheesecloth and one layer of miracloth. Triton X-100 is added to a final concentration of 0.5 %; this detergent disolves the organelles membranes, releasing their DNA while nuclear structures are not significantly disrupted. The homogenate is centrifuged at 4000 x g_{avg} for 15 minutes. The supernatant is discarded and the nuclear pellet is resuspended in 1 ml of resuspension buffer (Tris.HCl, pH 7.9, 0.1 M / Na_2EDTA 20 mM) for every 10 g of tissue. An equal volume of 2X lysis buffer (Tris.HCl pH 7.9, 0.1 M / NaCl 2 M / CTAB 2 %) is added to the resuspension and the mixture is incubated at 65° C for 30 minutes with gentle inversion of the tubes every 5 minutes. At the end of the incubation period, 1/2 volume of chloroform (Chloroform/Octanol, 24:1) is added, and tubes are inverted gently about 20 times. Phases are separated by centrifugation in a swinging bucket rotor at 4000 x g_{avg} for 20 min. The chloroform is in the lower phase containing all lipid compounds including chlorophyll. The interphase contains denatured proteins and starch. The upper aqueous phase, containing nucleic acids (DNA and RNA) and other water soluble compounds, is transferred to another tube and the nucleic acids are precipitated by addition of 2/3 volume of isopropanol (at -20° C). The precipitate is removed with a glass hook and immersed in ethanol (76%) / Na Acetate (0.2 M) for at least 30 min; it could also be stored in this solution for longer periods of time. After a short dip (20 sec) into ethanol (76%) / ammonium acetate (10 mM) the pellet is transferred to a new tube, air dried, and dissolved in TE buffer (Tris.HCl pH 8.0, 10 mM / EDTA 1 mM). The resuspended DNA occasionally contains some starch which can be removed by centrifugation. DNA prepared in this way is suitable for restriction digestions. Small amounts of RNA and other impurities can be removed by centrifugation in a cesium chloride gradient, however we don't find this step to be necessary. DNA concentrations are determined by absorbance at 260 nm (1 unit of A_{260} = 50 ug of DNA/ml). With this procedure we obtain high molecular weight DNA with yields varying from 7 to 20 ug / gr of fresh tissue. Higher yields are obtained with younger leaves.

Libraries

<u>Genomic Library</u>. A genomic library can be constructed by simply digesting nuclear DNA with an appropriate restriction enzyme, inserting the digested DNA into an appropriate vector and transforming a bacterial host. However, such a library will contain many clones of repetitive DNA sequences. Such clones give complex hybridization patterns which are difficult to score in segregating progenies. To be useful in mapping, a genomic library must be enriched for low copy number sequences. One strategy for enrichment is based upon the observation that DNA sequences which are expressed contain fewer methylated bases than sequences which are inactive, and that certain restriction enzymes will not cut at

their normal recognition sites if the sites contain methylated bases (Doefler, 1983). We took advantage of these observations to produce a genomic library enriched for expressed sequences of Phaseolus vulgaris.

Genomic DNA was isolated from the snap bean cultivar 'Sprite' by techniques described above. We digested 50 ug of DNA with the enzyme PstI, which does not cut methylated recognition sequences (Helentjaris et al., 1985). Digested DNA was layered onto a 10 ml, 10% - 30% sucrose gradient. Gradients were centrifuged in an SW41 rotor at 30,000 rpm for eight hours and fractionated by pumping from the bottom of the tube. Approximately 40 fractions of 250 ul were collected; 25 ul of each fraction was run on an agarose gel in parallel with molecular weight standards (bacteriophage lambda DNA digested with PstI). Gradient fractions containing DNA fragments of 0.5 - 2.5 kb were identified and pooled. DNA was recovered by ethanol precipitation, ligated with 0.5 ug of PstI digested plasmid vector (pTZ18R) (Pharnacia), and transformed into E. coli strain TB1.

cDNA library. A cDNA library was constructed following the procedure of Gubler and Hoffman (1983). However, this library is very incomplete and an attempt to construct another one will be repeated.

SUMMARY

We have produced segregating progenies between two distinct genotypes of Phaseolus vulgaris, and have generated cDNA and genomic clones suitable for the detection of RFLP's. To date, 22 cDNA and genomic clones have been hybridized to restriction digests of 'Calima' and 'XR-235-1'. Of these, 11 have revealed polymorphisms between the two lines. The process of evaluating potential probes is ongoing. The use of these two lines in our mapping effort will allow us to map the major gene for resistance to X. campestris pv. phaseoli; this gene is present in 'XR-235-1'. We have recently begun the process of probing the DNAs of an F_2 progeny which was scored for disease resistance.

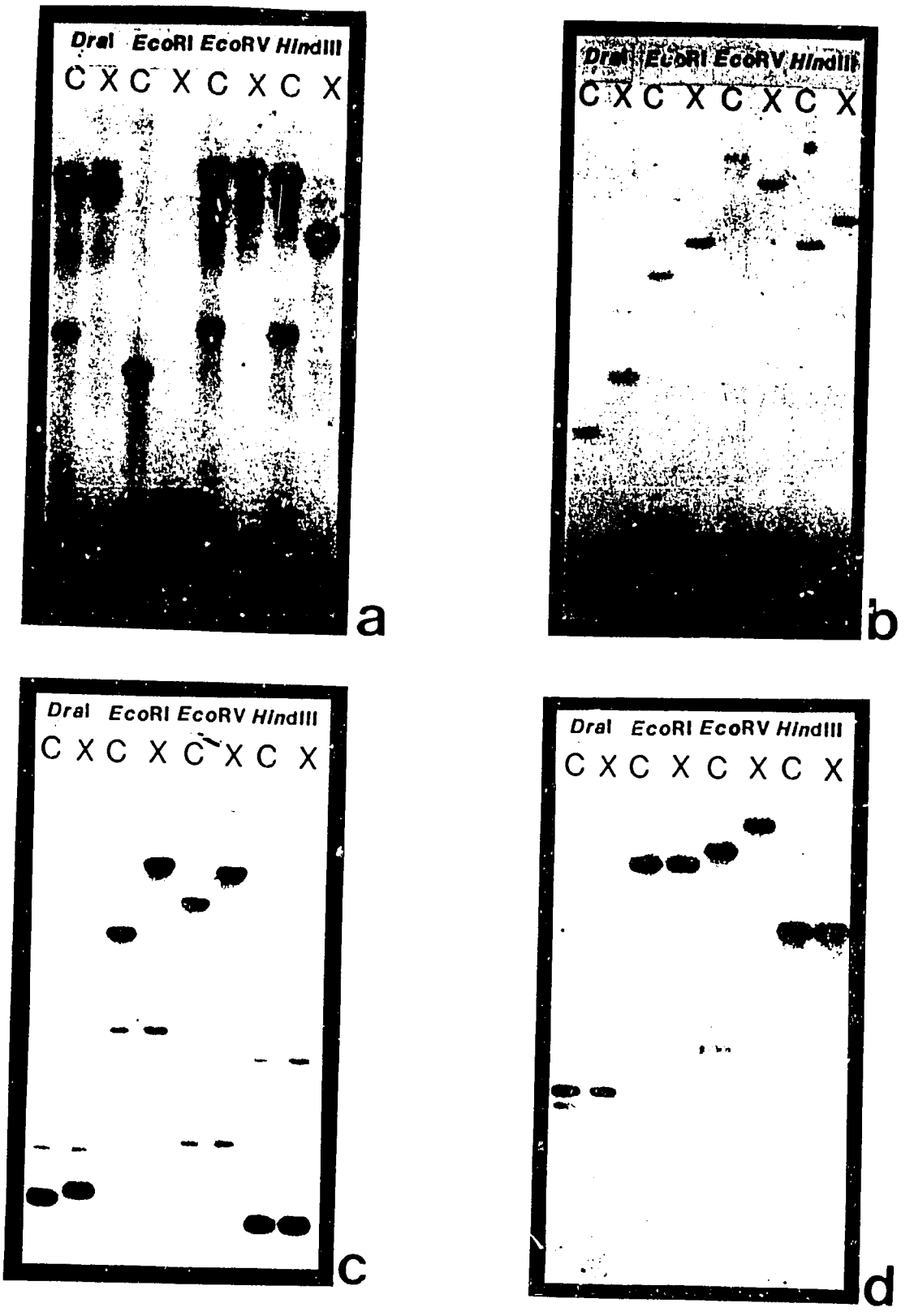

Figure 1. Autoradiograms of southern blots of parental DNA's hybridized with cDNA (a) and genomic (b, c, d) clones. DNA of the parental lines 'Calima' (C) and 'XR-235-1' (X) were digested with four restriction enzymes (DraI, EcoRI, EcoRV, and HindIII); DNA fragments were separated by electrophoresis in 1% agarose, and bloted on to nylon membranes prior to hybridizations.