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Identification of RAPD Markers in a Population of Common Bean (*Phaseolus vulgaris,* L.) Segregating for Iron Chlorosis Resistance

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ABSTRACT

A genetic map well saturated with genetic markers can provide the basis for a MAS breeding program. The use of RAPD technology along with DNA pools can accelerate the development of genetic linkage maps and the identification of marker loci tightly linked to a desirable gene(s). The main objective of this study was to identify RAPD markers in a population segregating for iron chlorosis resistance. Chlorotic and non-chlorotic bean plants were selected from an F_2 population of the cross between R-Ul-59 and S-Redkloud. Two contrasting DNA pools were formed and RAPD-screened with 300 random primers. A total of 28 RAPD markers were identified. MapMaker analysis assigned 11 RAPD markers to 5 linkage groups and 17 markers were unlinked. No association was found between RAPD markers and resistance to iron deficiency chlorosis. It is suggested that the number of random primers must be increased in order to increase the probability of finding RAPD markers tightly linked to the resistance gene(s) and also to assist in locating a linkage group for the 17 unlinked RAPD markers.

RESUMEN

Un mapa genético saturado con marcadores es escencial para un programa de selección basado en marcadores. El uso de la técnica RAPD junto con mezclas de DNA puede acelerar el desarrollo de mapas de ligamiento y la identificación de marcadores asociados a genes de interés. El principal objetivo del estudio fue la identificación de marcadores RAPD en una población segregante para resistencia a clorosis en frijol. Plantas cloróticas y no cloróticas fueron seleccionadas de una población F_2 de la cruza entre R-Ul-59 y S-Redkloud y utilizadas para formar 2 mezclas contrastantes de DNA. Las mezclas fueron utilizadas en reacciones RAPD y evaluadas con 300 iniciadores aleatorios. Un total de 28 marcadores RAPD fueron identificados. El análisis por MapMaker permitió asignar 11 marcadores RAPD en 5 grupos de ligamiento y 17 marcadores fueron independientes. No se detectó ligamiento entre los marcadores RAPD identificados y la resistencia a clorosis. Se sugiere incrementar el número de iniciadores aleatorios para aumentar la probabilidad de encontrar marcadores RAPD ligados a la resistencia a clorosis y también para encontrar un grupo de ligamiento para los marcadores independientes.

The methodology of marker-assisted selection (MAS) (Paterson et al., 1991) in plant breeding programs depends on the identification of a marker or two flanking markers tightly linked to the gene of interest (Melchinger, 1990). A well saturated genetic map can provide the basis for a MAS breeding program (Fatokun et al., 1992). Different genetic markers have been used to construct genetic maps, including morphological markers and molecular markers, such as isozymes, RFLPs and, more recently, RAPDs.

The RAPD assay was developed independently by two different laboratories (Williams et al., 1990; Welsh and McClelland, 1990). The RAPD technique makes use of the PCR (Mullis and Faloona, 1987) and a single random primer for amplification of DNA fragments. Polymorphisms are detected in electrophoresed agarose gels as the presence or absence of a particular amplification product. Most RAPD polymorphisms are the product of modification or elimination of priming sites as a result of base pair substitution, deletion or insertion in priming sites, or insertion between priming sites that either separate them to a non-amplifiable distance or result in two amplifiable (codominant) alleles (Williams et al., 1990).

RAPDs, combined with an appropriate genetic material, such as NILs (near-isogenic lines) favor the saturation of specific genetic regions, and allow the identification of genes of agronomic interest (Adam-Blondon et al., 1994). However, NILs are not available for most genes of interest and the development of NILs is time consuming (Michelmore et al., 1991).

An alternative strategy to identify linked markers in plants was developed by Michelmore et al. (1991) and Giovannoni et al. (1991). This strategy involves the formation of two genomic DNA pools which are then used as templates for random primer amplification. Polymorphisms identified by RAPD analysis between pools should represent markers located in the target segment because most genomic sequences are represented in the contrasting pools, excepting those selected for or against in the region of interest (Kochert,

Table 1. Chlorophyll readings for the resistant and susceptible parents and individual F_2 bean plants chosen for formation of DNA pools.

Resistant		Susceptible	
Plant[z]	Reading[y]	Plant	Reading
R-Ul-59	41	S-Redkloud	26
146-6	37	140-8	9
152-7	31	139-17	14
140-4	31	140-5	15
149-12	31	151-16	15
138-17	31	138-10	15
152-3	30	143-9	15

[z]Row-plant number.
[y]Readings by Minolta SPAD-502 chlorophyll meter.

1994).

Genetic differences for resistance to iron deficiency have been found in common beans (Zaiter et al., 1987) and it has been determined that susceptibility to iron deficiency chlorosis is controlled by 2 dominant genes (Cortinas-Escobar et al., 1995; Zaiter et al., 1988). The identification of molecular markers tightly linked to the genes controlling the response to iron deficiency can be beneficial in developing common bean cultivars which are more Fe-efficient or exhibit a greater iron stress response, because they allow for faster and more efficient selection of resistant genotypes. Also, the finding of new RAPD markers can contribute to saturate the genetic map of the common bean genome and provide the basis for a MAS breeding program.

The main objective of the present study was to identify RAPD markers in an F_2 population of common bean plants segregating for iron chlorosis resistance. Other objectives included the finding of RAPD markers linked to the resistance trait, and to establish linkage relationships between the markers.

MATERIALS AND METHODS

Plant material

The segregating population used in this study was derived from crosses between the cultivar R-Ul-59 (resistant) and S-Redkloud (susceptible). The selection of these cultivars as parents was based on the level of polymorphism between them and previous reports about the resistance/ susceptibility response (Zaiter et al., 1987).

Crosses between R-Ul-59 and S-Redkloud were made in the greenhouse at the Department of Horticultural Sciences, Texas A & M University, in the Fall, 1994, and F_2 seed was obtained in the Spring, 1995. Seeds of the parental and F_2, generations were planted in completely randomized blocks of 20 plants each during the Fall of 1995 at the Texas A&M University Agricultural Research and Extension Center at Temple, where environmental conditions are favorable for the expression of iron chlorosis. The soil at this location is classified as a calcareous Houston Black clay, fine, montmorillonic, thermic, Udic Pellustert soil, pH 8.2. Planting was on 1.5 m rows, no fertilizer was applied and weeds were controlled with 1.2 liters·ha^{-1} trifluralin, incorporated before planting.

Plants were allowed to grow for about 35 days, and color (greenness) of individual plants was measured using a chlorophyll meter (Minolta SPAD-502). The values determined for chlorophyll meter are indicators of the relative amount of chlorophyll present in the leaf. These values correspond to the amount of light transmitted by the leaf in 2 wavelength regions (red and infrared regions) in which the absorption of chlorophyll is different. The average of 4 readings from each of 362 F_2 plants and their parents was recorded and utilized to select resistant and susceptible plants to form DNA pools.

At the same time, a 200 mg tissue sample was collected from immature trifoliate leaves of each plant, placed in 1.5 ml microcentrifuge tubes, frozen immediately in liquid nitrogen, and placed in a cooler for transport to the lab. The samples were then stored at -80°C for subsequent DNA extraction.

The plants chosen for DNA pools are shown in Table 1. They represent the green and yellow extremes in the F_2 segregating population of the cross between R-UI-59 x S-Redkloud. A second group of 80 individual F_2 plants, which represents the 40 greenest and the 40 most chlorotic plants in the population, were selected as a mapping population for linkage analysis purposes.

DNA isolation

About 70 mg of tissue was used for organic DNA extraction as described by Fulton et al. (1995) with slight modifications. The tissue was placed in a 1.5 ml microcentrifuge tube and frozen in liquid nitrogen. The sample was then ground at room temperature with a plastic pestle mounted in a hand power drill, and 700 µl of isolation buffer was added. The isolation buffer contained 1 volume of DNA extraction buffer, 1 volume of nuclei lysis buffer, and 0.4 volume of 5% Sodium sarkosyl.

After grinding, the tissue was allowed to incubate for 30 min in a water bath at 65°C with occasional swirling. Following the addition of 700 µl of 24:1 chloroform-isoamyl alcohol the mixture was vigorously shaken for 20 sec to form an emulsion. The aqueous phase was separated by centrifugation at 13,000 rpm for 5 min at room temperature.

After organic and aqueous phases of the extraction mixture were separated, nucleic acids were precipitated by pipetting 1 volume (600 µl) of -20°C isopropanol into the aqueous phase. The mixture was gently inverted to allow DNA precip-

itation. After centrifuging at 13,000 rpm for 5 min, the aqueous phase was removed and the pellet was washed in 500 µl of 70% alcohol (- 20°C). The DNA was repelleted (1 min, 13,000 rpm) and allowed to dry for 1 h. The pellet was rehydrated by adding 100 µl of ddH_20 and placed in an oven for 15 min at 65°C. After re-hydration, a fraction of the DNA was diluted with ddH_20 to a working solution of 1:100 and stored at -20°C.

After DNA extraction, two DNA pools were constructed from the selected plants (Table 1). The DNA Pool Green (DP-G) was formed with approximately equal amounts of DNA from the following plants: 146-6, 152-7, 140-4, 149-12, 138-17, and 152-3, which showed the highest greenness score and were considered homozygous for the genomic region controlling iron chlorosis resistance. The contrasting DNA Pool Yellow (DP-Y) contained DNA from the most chlorotic plants in the segregating population: 140-8, 139-17, 140-5, 151-16, 138-16, and 143-9, which should be homozygous for the region that controls susceptibility to iron deficiency stress in bean plants. Note that the first number identifies the row and the second is the plant number on that specific row.

RAPD reactions

In order to identify RAPD markers linked to iron chlorosis resistance, the two contrasting DNA pools (DP-G and DP-Y) were screened with a total of 300 random decamer primers from Operon primer kits A through O (Operon Technologies Inc., Alameda, Calif.). Reactions were performed in a Perkin Elmer Cetus Thermal Cycler, model 480. The thermal cycler was programmed for a first step of denaturation at 94°C for 2 min; followed by 45 cycles of 94°C for 1 min for denaturation, 36°C for 1 min for annealing, and 72°C for 2 min for elongation. These 45 cycles were followed by a final cycle of 1 min for denaturation, 1 min for annealing, and 10 min for elongation (Giovannoni et al., 1991).

The 10X reaction buffer was composed of 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% (w/v) gelatin. The reactant concentrations were 120 µM dNTPs, 0.2 µM of RAPD primer, 5 ng of DNA template, 0.4 mM $MgCl_2$, 0.05 unit *Taq* DNA polymerase (Promega), and 1.25 µl 10X reaction buffer in a final reaction volume of 10 µl. The reaction was overlaid with 30 µl mineral oil before amplification.

The RAPD products were resolved by electrophoresis in 2.0% agarose gels containing 1X TBE buffer (10.8 g TRIS-base, 5.5 g Boric acid, and 4 ml 0.5 M EDTA, pH 8.0, in a volume of 1.0 liter) at 85 volts for 5 h, followed by staining with ethidium bromide. Subsequently, the stained gels were placed on a UV light trans-illuminator and a permanent photographic record was obtained on Polaroid 667 film.

RAPD and linkage analysis

Marker order and map distances were estimated using the MapMaker version 2.0 (Lander et al., 1987) computer program. The map order was based on maximum likelihood estimation. Two and three point analysis was used to determine genetic distances between markers. The RAPD markers were

Table 2. RAPD markers whose segregation was utilized for linkage analysis in common bean.

Marker[z]	Size (bp)[y]	Marker	Size (bp)
A-7	4000	H- 19	450
A-7	1430	I-1	1320
A-7	680	I-1	850
A-7	420	I-1	600
D-14	1600	I-4	200
G- 13	580	I-4	3000
G- 13	1510	I-4	2500
G- 13	770	J-4	1640
G- 13	640	J-4	620
G- 18	1640	J-4	344
G- 18	400	J-4	870
H-1	920	K-1	460
H- 19	1310	K-8	620
H- 19	680	L-9	950

[z]Markers were identified by the Operon random primer which produced the polymorphic band.
[y]Size of polymorphic band in base pairs (bp).

Table 3. Bean linkage groups formed by the analysis of segregation patterns by the MapMaker computer program.

Groups	Markers[z]
Group 1	$A7_{4000}$; $A7_{420}$
Group 2	$A7_{1430}$; $H1_{920}$; $K8_{620}$
Group 3	$G13_{770}$; $J4_{344}$
Group 4	$G13_{640}$; $K1_{460}$
Group 5	$H19_{450}$; $I4_{200}$

[z]Markers are identified by the Operon random primer which produced the polymorphic bands. The subscript identifies the size of the polymorphic band in base pairs (bp).

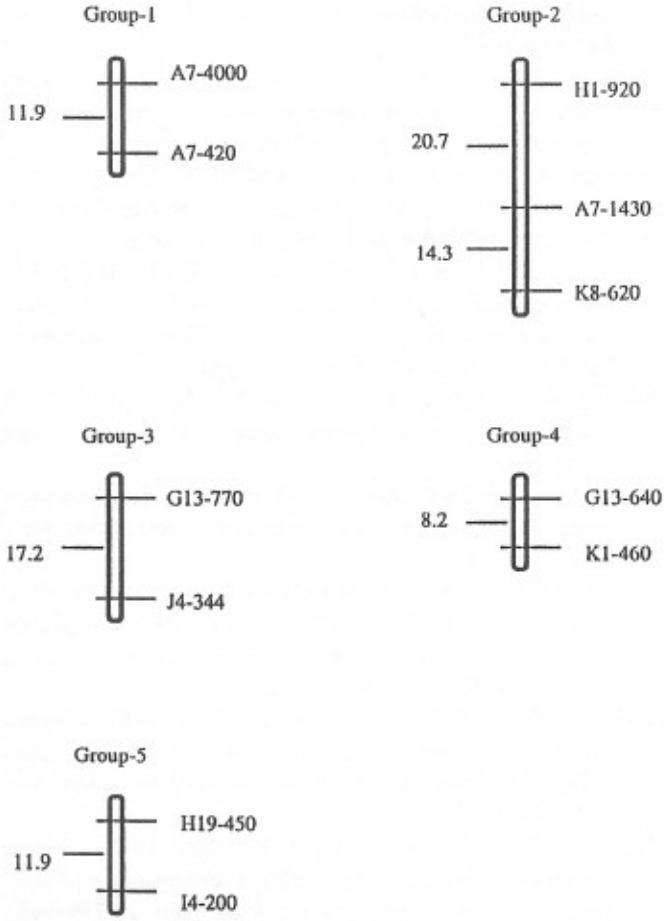

Fig. 1. Linkage groups comprising 11 RAPD markers identified in a F_2 population of 40 resistant and 40 iron chlorosis susceptible bean plants from the cross R-UI-59 x S-Redkloud. The genetic distances are shown at the left in cM. The markers are identified at the right by the Operon primer and the size of the polymorphic band in base pairs.

ordered with a LOD score equal to 2.5. The recombination fractions were transformed by the Kosambi map function (Kosambi, 1944) to estimate the map distance.

RESULTS AND DISCUSSION

The examination of 300 decamer primers with the DP-G (resistant) and DP-Y (susceptible) genomic DNA pools resulted in the amplification of 1872 discernible DNA fragments ranging from 150 to 4000 bp. The range of DNA fragments amplified per primer was from 0 to 11 with an average of 6.2. Some of the primers produced no DNA amplification or DNA smears and were not scored. Previous reports have suggested that DNA smears can be minimized by modifications of the DNA polymerase and/or genomic DNA concentration (Williams et al., 1990) or modifications in the amplification conditions (Haley et al., 1994). In the present study, an increased resolution of the bands resulted from modification in the DNA polymerase and DNA concentrations.

Initially, 52 primers identified polymorphisms between DP-G and DP-Y; however, most of them were eliminated when the polymorphic band was compared with individual DNA from the pools, or when the reaction was repeated. Analysis of the polymorphisms between the parents (R-Ul-59 and S-Redkloud) and the pools (DP-G and DP-Y) allowed the selection of 28 RAPD markers (Table 2) which were used to screen an F_2 population consisting of 40 resistant and 40 susceptible plants. The chi-square analysis (Weising et al., 1995) and the analysis of the segregation pattern of the 28 markers made by MapMaker indicated that none of the markers was associated with resistance to iron deficiency stress.

The lack of identification of tightly linked markers can be attributed to: 1) relatively low polymorphism between the parents of the cross resulting in reduced probability of identifying tightly linked markers, 2) inclusion of genomic DNA from mis-scored F_2 progeny in the DNA pools resulting in selection against tightly linked loci, and 3) inclusion of mis-scored F_2 progeny in the mapping population resulting in inaccurate linkage determinations. Concern 1 could be addressed via the analysis of additional RAPD primers. Concern 2 could be addressed through generation and simultaneous RAPD analysis of multiple sets of R and S genomic DNA pools. Finally, concern 3 could be addressed via generation of a much larger F_2 population such that inclusion of only the most extreme phenotypes in the mapping population would be ensured.

For MapMaker linkage analysis, a two-point group criterion (LOD=2.5) was initially used to assign markers to linkage groups. After that, a three-point criteria and multipoint-compare function were used to determine the likely order of the markers in each group. Finally, the multipoint-map function was used to obtain the best order and distances between markers. Map distances in centimorgans (cM) were obtained by using the Kosambi mapping function (Kosambi, 1944).

The linkage analysis resulted in the formation of 5 linkage groups (Table 3) which include 11 markers and 17 unlinked markers. The 5 linkage groups delimit 119 cM of the bean genome (Fig. 1). The longest group was 43 cM and the shortest group was 11.9 cM. The average distance between adjacent makers was 9.9 cM. A higher number of primers should be screened in order to increase the probability of finding associations between RAPD markers and resistance to iron chlorosis and also to assign the rest of the markers to linkage groups. In addition, these markers could be mapped in the population used by Vallejos (1994) to create a more detailed map of the common bean genome.

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