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รายงานวิจัยฉบับสมบูรณ์

โครงการ การทำแผนที่ยีนต้านทานโรคราแป้ง (*Erysiphe polygoni* DC) ในถั่วเขียว [*Vigna radiata* (L.) Wilczek]

โดย นางสาวบุบผา ใจเที่ยง

พฤษภาคม 2545

สัญญาเลขที่ BGJ/43/2543

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ผู้วิจัย สังกัด

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Abstract

Both restriction fragment length polymorphism (RFLP) and, using a pooled DNA approach, amplified fragment length polymorphism (AFLP) analyses were employed to map a new source of resistance to powdery mildew (*Erysiphe polygoni*) in mungbean (*Vigna radiata*). A RFLP linkage map was reconstructed with 98 probes across the 13 linkage groups of the current map using an F_2 mapping population derived from the cross between a moderately resistant breeding line (VC1210A) and a susceptible wild relative of mungbean (*V. radiata* var. *sublobata* accession TC1966). The disease score of the F_2 population showed continuous distribution and was treated as quantitative trait. QTL analysis base on the linkage map showed only marker that associated with resistance to powdery mildew. However, the direction of gene action and extent of genetic variation explained by the QTL does not completely explain the variation of the F_2 population. Consequently, using DNA pooled separately from the resistant and susceptible F_2 plants 100 AFLP primer combinations were tested for polymorphism associated with resistance. Out of more than 5700 bands 4 were associated with the resistance. The new RFLP loci detected by two cloned probes from the AFLP bands associated with resistance constitute a new linkage group. A major resistance QTL was found on a new linkage group that accounted for 64.9% of the total variation for plant reaction to powdery mildew. The resistant parent allele enhances powdery mildew resistance with partially dominant effect. One of the probes developed in this study has potential to assist in breeding for powdery mildew resistance in mungbean.

Key words AFLP, Molecular markers, Quantitative trait loci (QTL), RFLP

บทคัดย่อ

ในการศึกษาการทำแผนที่ยีนต้านทานโรคราแป้งตัวใหม่ในถั่วเขียวครั้งนี้ใช้ทั้ง Restriction fragment length polymorphism (RFLP) และ Amplified fragment length polymorphism: AFLP การทำแผนที่ยีนใหม่ของถั่วเขียวจากดีเอนเอติดตาม (probe) ทั้งหมด 98 ตัว โดยศึกษาจากแผนที่ยีนของถั่วเขียวในปัจจุบันที่มีจำนวน linkage group 13 กลุ่ม โดยใช้ดีเอนเอจากประชากรชั่วที่สอง (F_2) ที่เกิดจากการผสมระหว่างสายพันธุ์ต้านทาน (VC1210A) และ สายพันธุ์ที่อ่อนแอต่อโรค (TC1966) ในการประเมินการเกิดโรคพบว่าลักษณะการกระจายตัวของค่าคะแนนการเกิดโรคเป็นแบบต่อเนื่องซึ่งจัดเป็นลักษณะเชิงปริมาณ (quantitative trait loci : QTL)

ในการศึกษา QTL จากแผนที่ยีนเดิมพบว่ามี ดีเอนเอเครื่องหมาย(marker)หนึ่งตัวที่มีความสัมพันธ์กับลักษณะต้านทานโรคราแป้ง แต่ปฏิกิริยาของยีน (gene action) และ ความสัมพันธ์ของลักษณะการเกิดโรค (R^2) กับ QTL ยังไม่สามารถอธิบายความแปรปรวนของการเกิดโรคในประชากรชั่วที่สอง (F_2) ได้อย่างสมบูรณ์ ในขณะเดียวกันเทคนิค AFLP ถูกนำมาใช้เพื่อศึกษาความสัมพันธ์กับลักษณะต้านทานโรคราแป้ง โดยศึกษาในดีเอนเอที่เกิดจากการรวมภายในกลุ่มของลักษณะต้านทานและอ่อนแอต่อโรคที่สุดของประชากรชั่วที่สอง (F_2) โดยใช้ primer combination ทั้งหมด 100 คู่

จากการแยกความแตกต่างของชิ้นดีเอนเอที่เกิดจาก primer combination ทั้งหมดใน denaturing gel พบว่า มีจำนวนของแถบ (band) ดีเอนเอเกิดขึ้นมากกว่า 5700 แถบ แต่พบเพียงสี่แถบเท่านั้นที่มีความสัมพันธ์กับลักษณะการต้านทานโรค ดีเอนเอจากแถบทั้งสี่ได้รับการโคน (cloned) เพื่อใช้เป็นดีเอนเอติดตาม ซึ่งพบว่าดีเอนเอติดตามที่ได้จากเทคนิค AFLP สามารถตรวจสอบตำแหน่งของยีนต้านทานโรคราแป้งอยู่บน linkage group กลุ่มใหม่ และ QTL ที่ตรวจพบบน linkage group กลุ่มใหม่ นี้ มีความสัมพันธ์กับลักษณะการเกิดโรคราแป้ง (R^2) เท่ากับ 64.9 % ส่วนลักษณะการแสดงออกของยีนเป็นแบบ partial dominant และพบว่าหนึ่งในจำนวนดีเอนเอติดตามที่พัฒนาจากการศึกษาครั้งนี้ นี้มีศักยภาพในการนำมาใช้ในการปรับปรุงพันธุ์ถั่วเขียวเพื่อให้ต้านทานต่อโรคราแป้ง

คำสำคัญ: AFLP, Molecular markers, Quantitative trait loci (QTL), RFLP

Introduction

Breeding for disease resistant varieties depends on many factors such as plant type, species, growth stage, age of plant, pathogen, interaction between plant and pathogen, environment, and so fourth (Baird *et al.*, 1996). Backcrossing is the main breeding method for introduction of single major resistance genes. The plants are screened against pathogens in the field by natural infection or greenhouse by inoculation. The plants showing high resistance are selected and used for backcrossing into elite lines (cultivars) until resistant varieties are produced (Briggs and Knowles, 1967). Visual selection of individual plants is sometimes difficult due to low incidence of the disease. Breeders may depend on indirect selection by the application of closely linked genetic markers. The objectives of indirect selection via markers may be:

1) to identify the resistant individuals in the early growth stage and selection for resistant plant materials prior to flowering (e.g. backcross or population improvement program):
2) to correct inaccurate direct field selection of trait expression due to many loci involved (e.g. yield) or due to uneven inoculation / infection / infestation.

Indirect methods may use morphological markers (e.g. leaf color, hypocotyl color etc.), biochemical markers (e.g. isozymes), and molecular markers (e.g. restriction fragment length polymorphism: RFLP, random amplified polymorphic DNA: RAPD, DNA amplification fingerprinting: DAF, sequence characterized amplified region: SCAR, cleaved amplified polymorphic sequences: CAPS, simple sequence repeats : SSR or microsatellites and short tandem repeats and amplified fragment length polymorphism :AFLP).

The development of the molecular marker concept offers an opportunity to apply linkage or Mendelian genetic approach for the improvement of agriculturally important species. Isozymes were the first biochemical makers used in this context. There are some limitations to the number of informative loci within many species restricted their use, but initial results in many cases were quite promising and have served to maintain interest in this approach (Helenjaris, 1992). Recently, many molecular markers have been developed and used effectively in studying plant genetics and breeding.

Restriction fragment length polymorphism (RFLP) is a marker based approach to study the variation in length of DNA fragments obtained by digestion with restriction

endonucleases (Botstein *et al.*, 1980). The various lengths can be separated in agarose gels in an electric field and visualized by staining with ethidium bromide and observing with ultraviolet light. In some cases no distinct fragments can be visualized only a smear. Therefore, more complex techniques such as the use of cloned DNA probes and DNA hybridization are required to visualize differences in DNA. RFLP markers are co-dominant markers (Baird *et al.*, 1996). RFLP markers have been applied to find QTLs associated with useful traits in many crops, i.e. grain yield components in maize (Veldboom and Lee, 1994), resistance genes to cyst nematode, Javanese root knot nematode, Southern root knot nematode, and Phytophthora root and stem rot in soybean (Concibido *et al.*, 1997; Tamulonis *et al.*, 1997a, Tamulonis *et al.*, 1997b; Hegstad *et al.*, 1998), powdery mildew in wheat (Hartl *et al.*, 1993; Hartl *et al.*, 1995; Ma *et al.*, 1994; Liu *et al.*, 2001), common bacterial blight in common bean (Yu *et al.*, 1998; Correa *et al.*, 2000) and aphid in cowpea (Myers *et al.*, 1996). In addition RFLP markers have been used to analyze the size of chromosomal segments during backcross breeding in tomato (Young and Tanksley, 1989).

Menaceo-Hautea *et al.* (1992) developed a genetic linkage map of mungbean using RFLPs. This map has already been useful in the identification of RFLP markers associated with bruchid resistance gene that located on linkage group VIII (Young *et al.*,1992; Kaga and Ishimoto, 1998) and powdery mildew resistance genes located on linkage group III, VII and VIII (Young *et al.*, 1993).

There are limitations to the genetic diversity of many crops such as tomato (Rick, 1982 quoted in Weeden, 1991) and wheat (Chao *et al.*, 1990 quoted in Weeden, 1991). Despite the large number of DNA probes that can be generated from libraries, the identification of polymorphism can still be difficult. The other limitation of RFLP techniques is its high cost and complicated techniques involved. The technique requires several days and a skilled technician to obtain a marker (Weeden, 1991). So an attempt to overcome such limitations resulted in the development of other DNA markers based on polymerase chain reaction (PCR) such as RAPD, DAF , SCARs, CAPS, SSR, and AFLP.

Vos *et al.* (1995) has developed a new PCR – based method called amplified fragment length polymorphism (AFLP). This technique can enable a molecular linkage map to be developed and resolved by labeling with either radioisotope or non–

radioisotope. This technique produces a similar level of information to RAPD analysis in that random genomic DNA fragments are amplified and produced more polymorphisms per reaction than either RFLP and RAPD analysis, and generally amplifies smaller sized fragments. The polymorphism is due to presence/absence of a priming site, the relationship is dominance. However, AFLPs can be co-dominant markers when polymorphism is due to sequences within the amplified region. The AFLP technique is more technically complex than RAPD analysis, but fewer primers are needed to screen all possible sites (Melcher, 1999).

AFLP markers can reveal loci and alleles. They have been used to analyze genetic diversity in rice (Fuentes *et al.*, 1999; Garland *et al.*, 1999), wheat (Barrett and Kidwell, 1998), and azuki bean (Yee *et al.*, 1999; Xu *et al.*, 2000), to tag a major resistant gene to striga, a parasitic plant in cowpea (Ouedraogo *et al.*, 2001), to construct genetic linkage maps in many crops such as pines (Travis et al., 1998: Remington *et al.*, 1999), Eucalyptus (Marques *et al.*, 1998) and conifer (Nikaido *et al.*, 2000).

The molecular markers are being used to address many problems in plant breeding. DNA markers provide plant breeders and geneticists with new insights into the relationships among germplasm, DNA linked to important traits of interest to the plant breeding programs (Helenjaris, 1992). Molecular markers offer many other advantages over conventional phenotypic markers, because they are developmentally stable, detectable in all tissues, unaffected by environmental conditions, generally, insensitive to epistatic or pleiotropic effects, and provide a choice of co-dominant or dominant markers (Allen, 1994; Bostein *et al.*, 1980; Helenjaris *et al* ., 1985 quoted in Baird *et al.*, 1996; Williams *et al.*, 1990)

The utility and efficient application of molecular markers to identify the most appropriate traits relies on understanding the limitation of the system and correctly identifying the critical variables. The factors must be considered before using DNA markers such as trait heritability (usually low for important trait), minimum number of markers used, the density of markers on linkage map, markers located on each side of a QTL (flanking markers), choice of optimal sample size (e.g., number of lines and replication to evaluate), and program resources (e.g., cost of each trait or marker analysis in time and money, germplasm resources, etc.) (Baird *et al.*, 1996; Dudley, 1993; Young and Tanksley, 1989).

The objectives of this study were to determine the markers linked to powdery mildew resistant genes, to determine whether a breeding line, VC1210A, resistant to races of powdery mildew in Thailand represents a gene source at new map location and to determine the potential of using this resistant line in mungbean improvement.

Materials and Methods

Plant materials

Four mungbean lines obtained from and evaluated by AVRDC, VC1210A, VC1482A, VC2273, and VC3528A, found to be resistant to powdery mildew. They were re-evaluated in this study twice, on October 20, 1999 and on December 20, 1999 at Suranaree University of Technology experimental farm (SUT), Nakhon Ratchasima, Thailand. VC1210A was found to be highly resistant and was selected as the resistant parent in this study. TC1966, a wild relative (*Vigna radiata* var. *sublobata*) and highly susceptible to the disease was used in this study. These plants were crossed to produce F_1 and F_2 generations.

Disease assay

The two parents, F_1 and F_2 progenies were planted at SUT experimental farm, Nakhon Ratchasima, Thailand on October 30, 2000. Susceptible varieties, CN36, M5-5, and TC1966, were planted around the experiment and between the plots as a source of powdery mildew inoculum. Individual plants were scored for powdery mildew response at 55 days after germination using the scoring system described by Young *et al.* (1993) as follows: 1 (no visible mycelial growth), 2 (1-25% foliage area covered by fungus), 3 (26-50% foliage covered), 4 (51-75% foliage covered), and 5 (76-100% foliage covered). Broad sense heritability was estimated following Warner's method (1952)

$$\text{Broad-sense heritability } (h_b^2) = \frac{V_{F_2} - V_e}{V_{F_2}}$$

$$\text{The estimate of the environmental variance } (V_e) = \frac{(V_{P_1} + V_{P_2} + V_{F_1})}{3}$$

DNA isolation

DNA was isolated from young leaves using a procedure based on the CTAB method (Draper and Scott, 1988). In brief, young leaves were harvested from parents and F_2 individuals, frozen in liquid nitrogen and then stored at $-80^{\circ}C$ until beginning extraction procedures. The samples, around 0.3-0.5 g, were ground into powder in liquid nitrogen, dispersed into 15 ml tubes containing 6 ml of warmed-extraction buffer (1.5 % Cetyl-tri-methylammonium bromide: CTAB, 75 mM Tris-HCl pH 8.0, 15 mM EDTA pH 8.0, 1.05 M NaCl, 0.75 % 2-mercaptoethanol), and incubated at $65^{\circ}C$ for 20

min with continuous gentle shaking. 6 ml of chloroform/isoamylalcohol (24:1:v/v) was added. The solution was mixed by inversion for 20 min, and centrifuged at 2000 *g* for 20 min. The supernatant was transferred into a new tube. 0.6 ml of 10% CTAB as added and incubated in 65°C for 5 min. 6 ml of fresh chloroform/isoamylalcohol was added and mixed for 20 min, and centrifuged following the steps described above. The supernatant was transferred into a new tube, 9 ml of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), was added and mixed gently to precipitate DNA. The mixture was centrifuged at 500 g for 5 min at room temperature, supernatant discarded, 5 ml of 1 M NaCl and 4 µl of RNase A (10 mg/ml) were added. The mixture was incubated while shaking gently in a water bath at 65°C until the DNA pellet was completely dissolved. The solution was incubated at 37°C for 30 min. DNA was precipitated using 10 ml of ethanol and transferred into 1.5 ml tube containing 1 ml of 70% ethanol. After incubation at room temperature for 10 min, the tube was centrifuged briefly at high speed. The supernatant was discarded completely then 100-300 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) was added. The extracted DNA was stored at –20°C until use.

RFLP analysis

DNA from P_1, P_2 and F_2 individuals was digested with *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I restriction enzymes under conditions recommended by the manufacturer (New England BioLabs, UK). The digested DNA was separated on 1% agarose gel in 1×TAE buffer (40 mM Tris-HCl pH 8.0, 40 mM acetic acid, 1 mM EDTA pH 8.0) at 1 V/cm for 15 hr. The DNA was transferred onto Hybond N+ membrane (Amersham Pharmacia Biotech, UK) by alkaline solution (0.4 M NaOH, 1.5 M NaCl) and fixed by UV Crosslinker at 60 mJ/cm^2 (Amersham Pharmacia Biotech, UK). A RFLP linkage map for mungbean had been developed using the F_2 population of a cross between a cultivar 'VC3890' and the wild relative of mungbean 'TC1966' (Menancio-Hautea *et al.* 1992). A revision of this linkage map available on Beangenes Database (http://beangenes.cws.ndsu.nodak.edu) was used as the basis for probe positions. Ninety-eight probes for detecting RFLPs were from a mungbean library (University of Minnesota, USA), soybean library (Iowa State University, USA) and common bean library (CIAT, Colombia), respectively. Probe DNAs were amplified from plasmid DNA by PCR using universal M13 forward and reverse primer. The PCR was carried out in

a GeneAmp PCR system 9700 (Applied biosystems, USA) programmed for 60 sec at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 52°C and 60 sec at 72°C, and ending with 60 sec at 72°C. DNA hybridization was carried out by ECL direct nucleic acid labeling and detection systems according to manufacturers instructions (Amersham Pharmacia Biotech, UK). After detection, blots were placed against Fuji medical X-ray film at room temperature for 3-6 h to produce auto-radiographs. Autoradiogrammed X-ray film was used to score alleles in segregation F_2 population. A plant showing a single specific band to VC1210A and TC1966 was scored 'A' and 'B' respectively. Meanwhile, a plant showing both bands was scored 'H'.

AFLP analysis

AFLP analysis was performed according to Vos *et al.* (1995). Total genomic DNA from P_1, P_2, bulked resistance (six plants with the lowest disease score) and bulked susceptible F_2 plants (six plants with the highest disease score) were digested with *Eco*RI and *Mse*I for 3 hours at 37°C. The reaction volume was 40 µl containing 500 ng of genomic DNA, 5 unit of each restriction enzyme (New England BioLabs, UK) and 1x reaction buffer (10 mM Tris-Acetate pH7.5, 10 mM Magnesium acetate, 50 mM potassium acetate, 5 mM DTT and 2.5 µg BSA). Digested DNA fragments were ligated to *Eco*RI and *Mse*I adapters using T4 DNA ligase at 37°C, overnight. The reaction volume was 50 µl containing previous 40 µl reaction, 5 pmole *Eco*RI adapters, 50 pmole *Mse*I adapters and 1U of T4 DNA ligase (Roche Molecular Biochemicals, Germany). The restriction-ligation products were used as primary template DNA for the first PCR step (pre-amplification) with E_{oo} and M_{oo} primers with no selective nucleotides at the 3'end. The reaction volume was 20 µl containing 50 ng of restriction-ligation products, 30 ng each of E_{oo} and M_{oo} primers, 1x PCR buffer 0.2 mM dNTP, 0.25 unit ExTaq (TAKARA, Japan). PCR conditions are listed in Table 1. The PCR products were used in the second PCR (selective PCR) with 100 primer combinations (10 *Eco*RI primers and 10 *Mse*I primers) with 2 and 3 selective nucleotides at the 3'end respectively. The primer combinations are show in Table 2.

Five microliters of the PCR products were mixed with 5 µl of STR 3X loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF), denatured at 90°C 3 min and cooled on ice. Three microliters of denaturing products were run on 6% denaturing polyacrylamide gel (19:1) in 0.5x TBE buffer (44.5

Table 1. PCR conditions for AFLP analysis.

1. Pre-amplification.

94°C 60 sec

25 cycles of

denaturation	at 94°C	30 sec
annealing	at 56°C	60 sec
extension	at 72°C	60 sec
ending with	at 72°C	60 sec.

2. Selective-amplification

94°C 60 sec.

Step 1: 13 cycles

the first cycle denaturation	at 94°C	30 sec
annealing	at 65°C	30 sec
extension	at 72°C	60 sec

Subsequent cycles the annealing temperature is reduced by 0.7°C per cycle over 12 cycles.

Step 2: 23 cycles

denaturation	at 94°C	30 sec
annealing	at 56°C	30 sec
extension	at 72°C	60 sec

Table 2. AFLP primer combinations used in this study.

EcoRI/MseI	M-AAG	M-AAT	M-AGA	M-AGC	M-AGG	M-CAA	M-CCT	M-CGA	M-GAC	M-GTA
E-AC	17	18	19	20	21	22	23	24	25	26
E-AAC	28	29	30	31	32	33	34	35	36	37
E-AAG	39	40	41	42	43	44	45	46	47	48
E-AGA	50	51	1	52	53	54	55	56	57	58
E-AGT	110	59	60	61	62	63	64	65	66	67
E-ATC	111	68	69	70	71	72	73	74	75	76
E-CAA	112	77	109	78	79	80	81	82	83	84
E-CAC	113	85	2	86	87	88	89	90	91	92
E-CGT	114	93	3	94	95	96	97	98	99	100
E-CTG	115	101	4	102	103	104	105	106	107	108

The numbers in the table represent the name of primer combinations

mM Tris-HCl pH 8.0, 44.5 mM Boric acid, 1 mM EDTA pH 8.0). Electrophoresis was performed at constant 70 W for 1.5 h. The products were stained according to the Silver Sequence DNA sequencing system (Promega, USA). In order to clone some important polymorphic bands, the gel containing bands were excised and squashed in micro-centrifuge tubes containing 10 µl H_2O. The suspension was centrifuged at 12,000 g for 5 min at room temperature. Five microliters of supernatant was transferred to a new tube containing 20 µl of H_2O. This solution was used as template DNA in PCR. The selective PCR condition and primer combinations producing polymorphic bands were used to re-amplify the fragments. The PCR products were used directly to insert to pGEM®-T Easy plasmid vector according to protocol of the manufacturer (Promega, USA). The inserted plasmids were transformed into *E. coli* by electroporation (Sambrook and Russell 2001). The electroporation was performed using Gene pulser II (Bio-Rad, USA) to deliver an electrical pulse of 25 µF capacitance, 1.75 kV, and 200 ohm resistance. The plasmid DNA was extracted from *E. coli* by small-scale preparation method described in Maniatis *et al.* (1989) and used as template DNA for probe preparation by PCR. The PCR products were checked for the correct insert size by electrophoresis and used as probes.

Statistical analysis

One-way ANOVA was conducted to determine significant ($P < 0.05$) association between putative resistance- related markers and powdery mildew resistance. Chi-square tests were used to test Mendelian segregation ration (1 : 2 : 1) for codominant markers.

Linkage and QTL analysis

The genotypic data from RFLP analysis were analyzed with MAPMAKER/EXP version 3.0 program (Lander *et al.* 1987) to re-construct the RFLP linkage map of mungbean. A LOD score of 3.0 and Haldane function (Haldane and Waddington, 1931) were used. The positions of cloned fragments from AFLP analysis were determined by pairwise command against all of RFLP markers at a threshold of LOD 3.0. Then the position was determined using the compare command.

The mapping of QTLs was performed by the method of interval mapping (Lander and Botstein, 1989) using MAPMAKER/QTL version 1.1 (Lincoln *et al.*, 1992)

based on the phenotypic and linkage map data. Scan command at threshold of LOD 3.0 was used to identify putative QTLs in the linkage map. By fixing the strongest QTLs, others were searched. Try command was used to evaluate the genetic models. The fraction of the total phenotypic variation explained by an individual QTL was obtained by fitting the model to individual QTLs. One-way ANOVA was used to confirm the presence of QTL at the marker position.

Results

Evaluation

Of the four lines evaluated for resistance to powdery mildew, VC1210A was superior. This line exhibited rapid necrosis around a focus of powdery mildew infection that is indicative of a hypersensitive reaction. This line was selected as the resistant parent in this study. The average scores for powdery mildew resistance of P_1 (VC1210A), P_2(TC1966) , F_1 and F_2 are shown in Table 3. Broad sense heritability of this population is 81. The frequency distribution pattern of the F_2 population disease evaluation score is shown (Fig. 1). This frequency distribution suggests the involvement of a gene(s) with large effect(s).

QTL mapping of PM based on RFLP linkage map

P_1 and P_2 were analyzed for RFLP using three different sources of probes, mungbean, soybean, and common bean probes. Twenty-nine probes showed polymorphism and were used as probes to test for in polymorphism in 96 individuals from the F_2 population. Segregation of markers deviated significantly from the expected ratio 1 : 2 : 1 at $P < 0.05$ (Table 4.) A mungbean linkage map was reconstructed using segregation data from the 96 F_2 individuals. Of these only one genomic region from common bean probe, Bng065, located on linkage group 2 (Menacio-Hautea *et al.*, 1992), revealed statistically significance powdery mildew score at P = 0.009 by ANOVA. The average value of resistance to powdery mildew of homozygous, VC1210A and TC1966, from this marker was 2.1 and 2.88 respectively. The value of heterozygous plants was 2.79, nearly as susceptible to powdery mildew as TC1966 (Table 5.). No significant genomic region was observed by interval mapping in spite of a relatively simple segregation pattern. Consequently, a search for additional markers linked to the resistance gene was undertaken using AFLP analysis.

AFLP analysis

The initial results of screening DNA from P_1, P_2, and bulked DNA of 6 resistant and 6 susceptible F_2 plants with 100 primer combinations showed 5,734 and 5,729 polymorphic bands from bulked resistance and bulked susceptible F_2 DNA respectively. From this results, only four

Table 3. Reaction to powdery mildew of different populations derived from a cross between P_1 (VC1210A) and P_2 (TC1966).

Population	Number of plants	Disease reaction	σ^2
P_1 (VC1210A)	31	1.45 $\pm$ 0.09	0.256
F_1	7	2 $\pm$ 0	0.000
F_2	96	2.67 $\pm$ 0.1	0.899
P_2 (TC1966)	34	3.91 $\pm$ 0.09	0.265

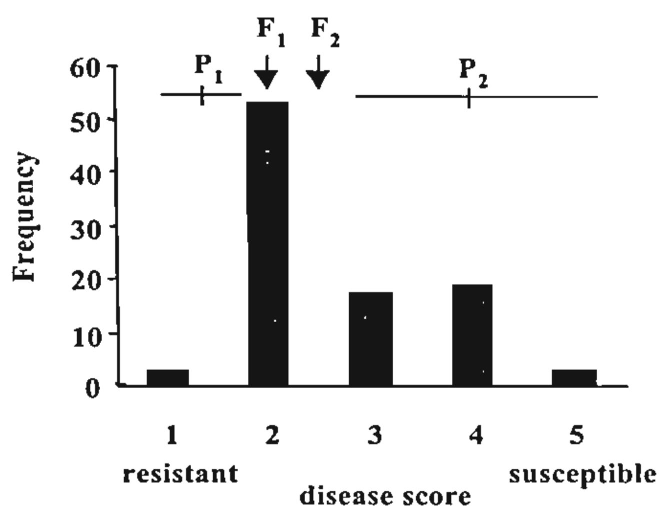

Fig. 1. Frequency distribution of F_2 population from the VC1210A × TC1966 for the disease score of powdery mildew resistance. Disease score means for parents, the F_1 hybrid and F_2 population designated as VC1210A(P_1), TC1966 (P_2), F_1 and F_2, respectively. Range for parents are indicated with bars.

Table 4. Segregations of RFLP markers in the F_2 population using mungbean, common bean, soybean and AFLP clone probes.

Marker	Restriction enzyme	No. of plants			Chi-square
		A (P_1)	H	B (P_2)	
Mungbean probes					
mc003	EcoRI	22	54	20	1.59
mc004	EcoRI	31	49	16	4.72
mgM208	BglII	18	49	19	1.74
mgM217	BglII	20	46	24	0.40
mgM244	XbaI	26	42	21	0.84
mgM247(1)	HindIII	13	60	12	14.44**
mgM247(2)	HindIII	22	47	20	0.373
mgM247(3)	HindIII	18	55	17	4.46
mgM307	HindIII	23	43	25	0.363
mgM339	XbaI	20	54	16	3.96
mgM392	EcoRV	21	53	16	3.39
mgM415	EcoRI	27	43	26	1.065
cgP137	XbaI	27	39	22	1.71
pQ062	EcoRI	24	54	18	2.25
mgQ117	EcoRI	24	41	31	3.06
Common bean probes					
Bng004	XbaI	17	57	15	7.11*
Bng025	EcoRI	38	15	43	12.07**
Bng031	XbaI	17	50	22	1.92
Bng065	HindIII	20	54	17	3.37
Bng107	EcoRI	30	47	19	2.56
Bng134	HindIII	30	34	27	6.01*

Table 4. (continued)

Marker	Restriction enzyme	No. of plants			Chi-square
		A (P_1)	H	B (P_2)	
Bng138	XbaI	23	51	16	2.69
Bng201	EcoRI	29	50	17	3.16
Soybean probe					
pA060	HindIII	22	51	16	2.71
pA106	HindIII	24	40	26	1.20
pA132	BglII	15	58	17	7.6*
pA315	EcoRI	25	50	21	0.50
pB032	BglII	23	46	23	0.31
pB069	EcoRV	18	55	17	4.46
Mungbean AFLP clone (Mac)					
Mac71(a)	XbaI	19	46	25	0.84
Mac71(b)	XbaI	17	36	33	8.03*
Mac86					
Mac95					
Mac114	BglII	19	46	25	0.84

A, B and H indicate homozygote genotype of VC1210A, TC1966 and heterozygote genotype respectively.

* and ** show markers deviated significantly from the expected ratio of 1 : 2 : 1 ($p < 0.05$)

Table 5. Significant association between marker segregation and average score of phenotype (disease resistance) from F_2 population.

Marker	Average score of phenotype			F (2, n-2)	P	Linkage group*
	A (P_1)	H	B (P_2)			
Mungbean probes						
mc003	2.25	2.296	2.325	0.034	0.966	6
mc004	2.209	2.265	2.531	0.645	0.527	7
mgM208	2.388	2.276	2.236	0.133	0.875	7
mgM217	2.450	2.141	2.438	1.138	0.325	3
mgM244	2.615	2.155	2.190	2.103	0.128	4
mgM247(1)	2.269	2.292	2.375	0.046	0.955	7
mgM247(2)	2.523	2.138	2.450	1.566	0.215	7
mgM247(3)	2.222	2.309	2.294	0.056	0.946	7
mgM307	2.565	2.127	2.360	1.661	0.196	3
mgM339	2.225	2.259	2.469	0.352	0.704	11
mgM392	2.190	2.368	2.156	0.445	0.641	6
mgM415	2.481	2.162	2.308	0.947	0.391	3
cgP137	2.259	2.269	2.432	0.248	0.781	4
pQ062	2.333	2.222	2.444	0.399	0.672	U
mgQ117	2.438	2.268	2.210	0.410	0.665	4
Common bean probes						
Bng004	1.882	2.368	2.500	2.167	0.121	U
Bng025	2.461	2.302	1.833	2.446	0.092	5
Bng031	2.529	2.190	2.364	0.872	0.422	6
Bng065	2.100	2.796	2.882	4.937	0.009*	2

Table 5. (continued).

Marker	Average score of phenotype			F (2, n-2)	P	Linkage group*
	A (P_1)	H	B (P_2)			
Bng107	2.083	2.436	2.263	1.295	0.279	9
Bng134	2.100	2.514	2.259	1.563	0.215	9
Bng138	2.500	2.156	2.235	0.800	0.453	2
Bng201	2.362	2.310	2.117	0.373	0.689	1
Soybean probes						
pA060	2.136	2.333	2.406	0.441	0.644	11
pA106	2.354	2.238	2.385	0.216	0.806	3
pA132	1.966	2.345	2.382	1.045	0.356	2
pA315	2.200	2.250	2.500	0.671	0.513	9
pB032	2.174	2.223	2.625	1.534	0.222	10
pB069	2.556	2.209	2.265	0.905	0.408	2
Mungbean AFLP clone (Mac)						
Mac71(a)	1.842	2.347	3.800	74.592	< 0.0001***	U*
Mac71(b)	1.882	2.388	3.424	31.474	< 0.0001***	U
Mac86						
Mac95						
Mac114	1.842	2.347	3.800	74.592	< 0.0001***	U

A, B and H indicate homozygote genotype of VC1210A, TC1966 and heterozygote genotype, respectively.

* and ***show significant level of F- test at 0.05 and 0.0001 propability levels, respectively.

U indicates unlinked marker.

There is no band was detected with Mac86 and Mac95 showed no polymorphism.

primer combinations, 71 (E-AGT/ M-AGG), 86 (E-CAC/M-AGC), 95 (E-CGT/M-AGG), and 114 (E-CGT/M-AAC), provided four polymorphic bands corresponding to powdery mildew resistance (Table 6). The approximate sizes of each band were 200bp, 100bp, 150bp, and 180bp, respectively. Three primer combinations, 71, 86, and 95, revealed bands in the resistant parent and resistant bulk but not the susceptible parent and susceptible bulk and one primer combination, 114, showed bands in the susceptible parent and susceptible bulk and not the resistant parent and resistant bulk. The example of AFLP band patterns and co–segregating bands corresponding to disease resistance are shown (Fig. 2.). Prior to cloning, the AFLP analysis of co-segregating bands corresponding to powdery mildew was re-confirmed using four primer combinations to amplify the DNA from P_1, P_2, six individual resistant plants, and six individual susceptible plants. The results of individual plant confirmed the result using bulked DNA of resistant and susceptible plants of co-segregating patterns for the four primer combinations. The three bands in the resistant parent and one in the susceptible parent were cloned and used as probes in RFLP analysis. These probes were named after their primer combinations (Table 2) as Mac71, Mac86, Mac95 and Mac114 (Mac-Mungbean AFLP clone). In the screening of parental polymorphism, Mac95 showed no polymorphism and no band was detected with Mac86. The Mac71 revealed multiple polymorphims. Single polymorphic band was with Mac114 (Fig.3. and Fig.4.). Four loci of Mac71 were designated as Mac71a, Mac71b, Mac71c, and Mac71d. Two of them, Mac71a and Mac71b, were co-dominant loci and the other two, Mac71c (detected in P_1) and Mac71d (detected in P_2), were dominant loci. There was no association of these loci to marker loci on the mungbean linkage map. However, linkage analysis revealed that these loci were tightly linked to each other. No recombination was found between Mac71a, Mac71d and Mac114 (Figure 5). The best order of the Mac probes was determined as Mac71b, Mac71a, Mac71d, Mac114 and Mac71d respectively by using the compare command of MAPMAKER/QTL. The distance between marker probes was calculated using the Map command.

QTL interval mapping revealed LOD score peaks at this new linkage group. A major resistance QTL was detected at marker Mac71a and Mac114 and had a LOD score of 20.22, additive value (a) = 0.98, dominant value(d) = - 0.50 and dominant to additive ratio = - 0.51. No other QTL was identified from re-scanning by fixing this QTL to the whole linkage map. (The peak of LOD was positioned on Mac71a and Mac114). This QTL accounts for 64.9 % of the

Table 6. AFLP primer combinations used, polymorphism between P_1 and P_2 and co-segregating bands corresponding to disease resistance revealed in this survey of resistance (P_1), susceptible (P_2), pooled DNA of resistance (R) and pooled DNA of susceptible (S).

Primer pair	Number of visible bands				Number of polymorphic bands	Number of co-segregating bands
	P_1	P_2	R	S		
EcoRI/MseI						
E-AC/M-AAC (17)	112	107	117	117	6	-
E-AC/M-AAT (18)	87	95	92	92	9	-
E-AC/M-AGA (19)	100	107	106	106	9	-
E-AC/M-AGC (20)	67	63	69	69	15	-
E-AC/M-AGG (21)	96	99	100	100	19	-
E-AC/M-CAA (22)	95	95	95	95	8	-
E-AC/M-CCT (23)	71	75	76	76	11	-
E-AC/M-CGA (24)	68	70	70	70	9	-
E-AC/M-GAC (25)	53	53	58	58	7	-
E-AC/M-GTA (26)	80	79	84	84	14	-
E-AAC/M-AAC (28)	105	105	114	114	10	-
E-AAC/M-AAT (29)	115	120	125	125	13	-
E-AAC/M-AGA (30)	53	59	58	60	5	-
E-AAC/M-AGC (31)	64	64	67	66	12	-
E-AAC/M-AGG (32)	76	86	85	85	9	-
E-AAC/M-CAA (33)	82	79	80	80	6	-
E-AAC/M-CCT (34)	57	62	63	63	13	-
E-AAC/M-CGA (35)	34	31	28	28	4	-

Table 6. (continued).

Primer pair	Number of visible bands				Number of polymorphic bands	Number of co-segregating bands
	P_1	P_2	R	S		
*Eco*RI/*Mse*I						
E-AAC/M-GAC (36)	42	42	43	43	10	-
E-AAC/M-GTA (37)	38	42	43	43	16	-
E-AAG/M-AAC (39)	80	87	90	90	11	-
E-AAG/M-AAT (40)	68	76	76	76	11	-
E-AAG/M-AGA (41)	55	54	58	58	10	-
E-AAG/M-AGC (42)	52	46	52	52	11	-
E-AAG/M-AGG (43)	56	64	60	60	14	-
E-AAG/M-CAA (44)	88	89	89	89	8	-
E-AAG/M-CCT (45)	77	82	87	87	11	-
E-AAG/M-CGA (46)	60	60	66	66	11	-
E-AAG/M-GAC (47)	51	57	58	58	7	-
E-AAG/M-GTA (48)	55	52	56	56	8	-
E-AGA/M-AAC (50)	68	74	77	77	11	-
E-AGA/M-AAT (51)	58	59	66	65	16	-
E-AGA/M-AGA (1)	41	48	48	48	5	-
E-AGA/M-AGC (52)	36	38	37	37	10	-
E-AGA/M-AGG (53)	63	53	61	61	9	-
E-AGA/M-CAA (54)	86	90	97	97	15	-
E-AGA/M-CCT (55)	56	56	64	64	13	-
E-AGA/M-CGA (56)	41	39	43	43	10	-
E-AGA/M-GAC (57)	48	45	48	48	12	-

Table 6. (continued).

Primer pair	Number of visible bands				Number of polymorphic bands	Number of co-segregating bands
	P_1	P_2	R	S		
*Eco*RI/*Mse*I						
E-AGA/M-GTA (58)	37	40	39	39	11	-
E-CAA/M-AAC (110)	92	88	87	87	18	-
E-CAA/M-AAT (59)	47	45	39	39	3	-
E-CAA/M-AGA (60)	57	65	68	68	14	-
E-CAA/M-AGC (61)	48	51	51	51	7	-
E-CAA/M-AGG (62)	80	88	87	87	13	-
E-CAA/M-CAA (63)	75	82	80	80	13	-
E-CAA/M-CCT (64)	58	59	61	61	14	-
E-CAA/M-CGA (65)	19	18	21	21	8	-
E-CAA/M-GAC (66)	51	51	52	52	8	-
E-CAA/M-GTA (67)	54	59	60	60	15	-
E-AGT/M-AAC (111)	74	82	86	86	15	-
E-AGT/M-AAT (68)	76	78	80	80	18	-
E-AGT/M-AGA (69)	50	47	48	48	5	-
E-AGT/M-AGC (70)	47	52	53	53	15	-
E-AGT/M-AGG (71)	48	46	49	48	15	1
E-AGT/M-CAA (72)	58	60	63	63	13	-
E-AGT/M-CCT (73)	33	28	27	27	10	-
E-AGT/M-CGA (74)	12	12	12	12	-	-
E-AGT/M-GAC (75)	17	19	17	17	3	-
E-AGT/M-GTA (76)	41	44	45	45	5	-

Table 6. (continued).

Primer pair	Number of visible bands				Number of polymorphic bands	Number of co-segregating bands
	P_1	P_2	R	S		
*Eco*RI/*Mse*I						
E-ATC/M-AAC (112)	83	84	87	87	13	-
E-ATC/M-AAT (77)	117	113	120	120	11	-
E-ATC/M-AGA (109)	87	92	101	101	17	-
E-ATC/M-AGC (78)	43	43	46	46	7	-
E-ATC/M-AGG (79)	66	78	78	78	12	-
E-ATC/M-CAA (80)	81	84	78	78	10	-
E-ATC/M-CCT (81)	58	64	63	63	6	-
E-ATC/M-CGA (82)	46	46	43	43	5	-
E-ATC/M-GAC (83)	40	43	41	41	3	-
E-ATC/M-GTA (84)	61	68	71	71	18	-
E-CAC/M-AAC(113)	43	53	52	52	10	-
E-CAC/M-AAT (85)	65	69	70	70	15	-
E-CAC/M-AGA (2)	50	50	53	53	3	-
E-CAC/M-AGC (86)	36	30	38	36	13	2
E-CAC/M-AGG (87)	57	66	65	65	12	-
E-CAC/M-CAA (88)	47	51	52	52	12	-
E-CAC/M-CCT (89)	36	47	43	44	10	-
E-CAC/M-CGA (90)	23	23	23	23	2	-
E-CAC/M-GAC (91)	32	36	37	37	12	-
E-CAC/M-GTA (92)	37	36	36	36	6	-
E-CGT/M-AAC (114)	71	68	69	70	6	1

Table 6. (continued).

Primer pair	Number of visible bands				Number of polymorphic bands	Number of co-segregating bands
	P_1	P_2	R	S		
EcoRI/MseI						
E-CGT/M-AAT (93)	12	12	12	12	-	-
E-CGT/M-AGA (3)	19	18	24	24	9	-
E-CGT/M-AGC (94)	12	8	8	8	5	-
E-CGT/M-AGG (95)	12	7	10	8	4	2
E-CGT/M-CAA (96)	20	19	22	22	6	-
E-CGT/M-CCT (97)	22	22	16	16	4	-
E-CGT/M-CGA (98)	21	32	35	35	12	-
E-CGT/M-GAC (99)	28	26	29	29	3	-
E-CGT/M-GTA (100)	33	27	36	34	7	-
E-CTG/M-AAC (115)	77	76	81	81	13	-
E-CTG/M-AAT (101)	43	44	47	47	10	-
E-CTG/M-AGA (4)	30	37	36	36	11	-
E-CTG/M-AGC (102)	17	17	16	16	4	-
E-CTG/M-AGG (103)	28	29	32	32	5	-
E-CTG/M-CAA (104)	30	30	35	35	13	-
E-CTG/M-CCT (105)	22	15	15	15	4	-
E-CTG/M-CGA (106)	17	27	26	26	3	-
E-CTG/M-GAC (107)	24	27	26	26	3	-
E-CTG/M-GTA (108)	32	33	31	31	13	-
Total	5386	5566	5734	5729		
Range	12-117	7-120	10-125	8-125		

Numbers in parentheses, ( ), indicated the primer combinations

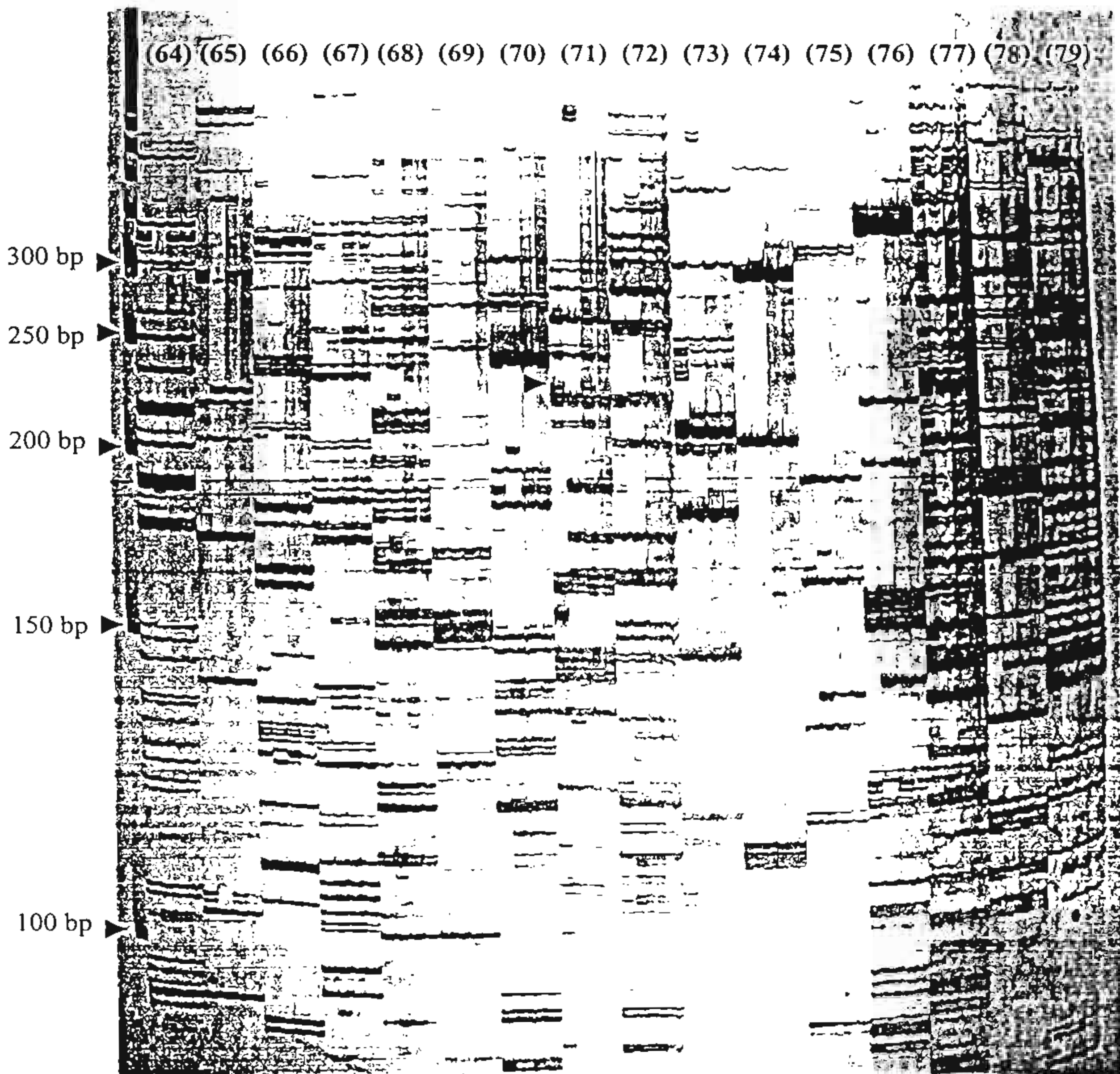

Fig. 2. A photograph of gel of AFLP bands from 15 primer combinations. Numbers in parentheses, ( ), refer to the name primer combinations as shown in table 2. The first lane from the left is 50 bp marker bands. All other lanes represent randomly selected bands for each primer pairs within samples of P1, P2 , R (bulked resistance) and S (bulked susceptible) respectively. Co-segregating bands corresponding to powdery mildew resistance are indicated by arrow.

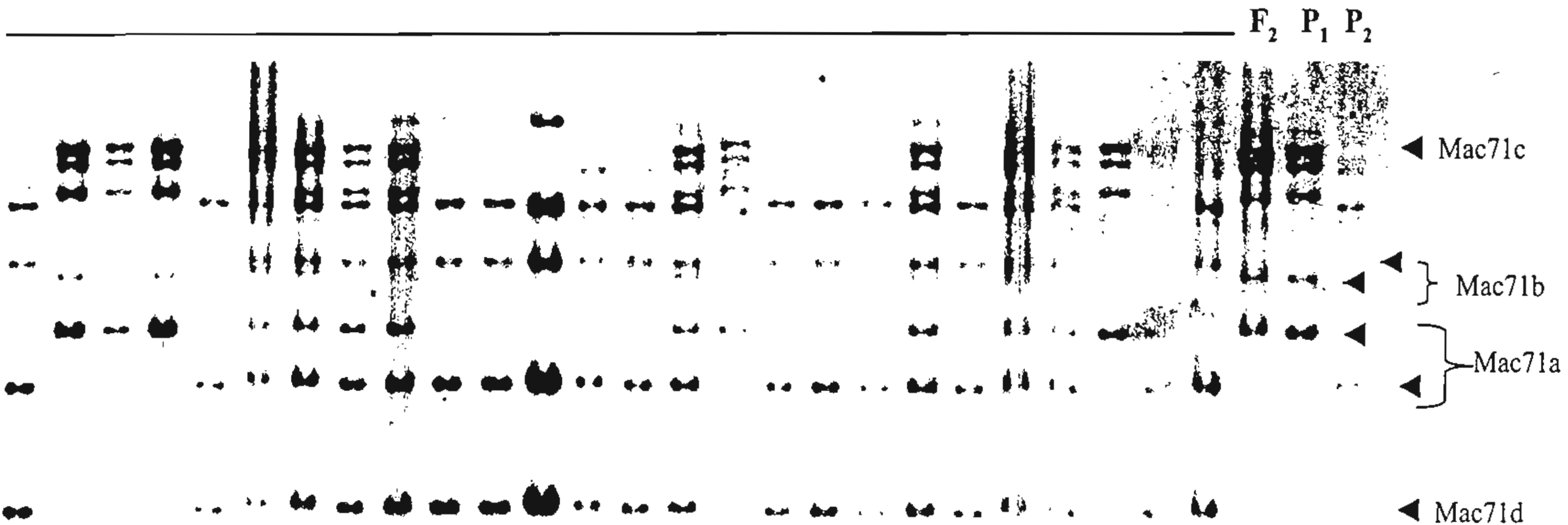

Fig. 3. Genomic DNA polymorphism among patents and F_2 population of VC1210A × TC1966. DNA was digested with *Xba* I restriction endonuclease and hybridized with Mungbean AFLP cloned probe : Mac71. P_1 and P_2 are VC1210A and TC1966. Polymorphic bands indicated by arrows.